APRIL 10, 1959

THE JOURNAL® OF NUTRITION

VOLUME 67 NUMBER 4

HOWARD BISHOP LEWIS

GEORGE R. COWGILL, *Editor*
Yale Nutrition Laboratory, 333 Cedar Street
New Haven 11, Connecticut

EDITORIAL BOARD

JAMES S. DINNING
JAMES M. ORTEN
CARLETON R. TREADWELL
CLARENCE P. BERG
HERBERT R. BIRD
COSMO G. MACKENZIE
RUBEN W. ENGEL
PHILIP L. HARRIS
HOWARD A. SCHNEIDER
O. LEE KLINE
EDMUND S. NASSET
HERBERT POLLACK

Official organ of the American Institute of Nutrition

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA 4, PA.

Annual Subscription: $22.50, domestic; $24.00, foreign

Second class postage paid at Philadelphia, Pa.
Copyright 1959. The Wistar Institute of Anatomy and Biology. All rights reserved.

Publications of The Wistar Institute

THE JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the results of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE AMERICAN JOURNAL OF ANATOMY

Publishes the results of comprehensive investigations in vertebrate anatomy — descriptive, analytical, experimental.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

Publishes original articles on comparative human morphology and physiology as well as on the history of this branch of science and the techniques used therein. In addition, it gives comprehensive reviews of books and papers, a bibliography of current publications, abstracts and proceedings of the American Association of Physical Anthropologists, and informal communications.

Issued quarterly, 1 vol. annually: $10.00 Domestic, $11.00 Foreign, per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

THIRTY SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

well-balanced nutrients in moderate low-fat cereal and milk serving

Medical and nutrition authorities now include the cereal and milk serving when a moderate reduction of dietary fat is indicated. This dish is recommended because it is *moderately low in dietary fat*. Few foods can better its *well-balanced nutritional contribution* as shown in the table below. It furnishes approximately 10 per cent of the recommended daily allowances of protein, important B vitamins, essential minerals; and it also provides quick and lasting energy. Served with nonfat milk, the fat content is very low.*

nutritive composition of average cereal serving

	Cereal, 1 oz. Whole Milk, 4 oz. Sugar, 1 teaspoon	Cereal** 1 oz.	Whole Milk 4 oz.	Sugar 1 teaspoon
CALORIES	203	104	83	16
PROTEIN	7.3 gm.	3.1 gm.	4.2 gm.	
FAT	5.3 gm.	0.6 gm.	4.7 gm.*	
CARBOHYDRATE	32.2 gm.	22 gm.	6.0 gm.	4.2 gm.
CALCIUM	0.169 gm.	0.025 gm.	0.144 gm.	
IRON	1.5 mg.	1.4 mg.	0.1 mg.	
VITAMIN A	195 I. U.	—	195 I. U.	
THIAMINE	0.16 mg.	0.12 mg.	0.04 mg.	
RIBOFLAVIN	0.25 mg.	0.04 mg.	0.21 mg.	
NIACIN	1.4 mg.	1.3 mg.	0.1 mg.	
ASCORBIC ACID	1.5 mg.	—	1.5 mg.	
CHOLESTEROL	16.4 mg.	0	16.4 mg.*	

*Nonfat (skim) milk, 4 oz., reduces the Fat value to 0.1 gm. and the Cholesterol value to 0.35 mg.
**Based on composite average of breakfast cereals on dry weight basis.

Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1958.

Hayes, O. B., and Rose, G. K.: Supplementary Food Composition Table. J. Am. Dietet. A. 33:26, 1957.

Watt, B. K., and Merrill, A. L.: Composition of Foods-Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

CEREAL INSTITUTE, INC.
135 South La Salle Street, Chicago 3
A research and educational endeavor devoted to the betterment of national nutrition

THE BRITISH JOURNAL OF NUTRITION

Published for the Nutrition Society

SHORTENED VERSION OF TITLES OF ARTICLES IN VOL. 13, NO. 1

The diet of sledge dogs. By R. J. F. TAYLOR, A. N. WORDEN AND C. E. WATERHOUSE.

Water and electrolyte exchanges in obesity. By R. PASSMORE, J. A. STRONG AND FIONA J. RITCHIE.

Carotene metabolism of the goat. By E. C. OWEN, R. A. DARROCH AND R. PROUDFOOT.

Selenium, liver necrosis and tooth depigmentation in the rat. By K. ATERMAN.

Measurement of energy expenditure in man by indirect calorimetry. By J. V. G. A. DURNIN AND J. M. BROCKWAY.

Error in the provision of diets of known electrolyte content. By C. T. G. FLEAR, PAT HUGHES AND AILSA MCLELLAN.

Body composition of rats in B-vitamin deficiencies. By K GUGGENHEIM AND E. J. DIAMANT.

Surface area and body-weight in studies on human energy expenditure. By J. V. G. A. DURNIN.

Vitamin B_{12} in the developing chick embryo. By B. SKARŻYŃSKI, Z. ŻAK, W. OSTROWSKI, MARY K. DAVIES, MARGARET M. GREGORY AND S. K. KON.

Growth effects of penicillin in germ-free chicks and chicks with a defined intestinal flora. By M. LEV AND M. FORBES.

Dietary intake and activities of an Alaskan mountaineering expedition. By J. D. KINLOCH.

Cod-liver oil as both source and antagonist of vitamin E. By T. MOORE, I. M. SHARMAN AND R. J. WARD.

The conversion of 11-*cis* into all-*trans* vitamin A in the rat. By P. A. PLACK.

The combined subscription rate for the British Journal of Nutrition (4 issues) and the Proceedings of the Nutrition Society (2 issues) is $23.50.

Published by the

Cambridge University Press

32 East 57th Street, New York 22, N. Y.

Original first edition back volumes and numbers of

THE JOURNAL OF NUTRITION

Complete volumes	Year	Price per volume
1–7	1928–1929	$18.00
9–12	1935–1936	15.00
24	1942	12.00
26	1943	12.00
29–32	1945–1946	10.00
34–51	1947–1953	9.00
52–57	1954–1955	7.50
59–63	1956–1957	7.50
Index to volumes 1–15		.75
Index to volumes 16–36		2.25

Incomplete volumes	Year	Price per single number
16, nos. 1, 2, 4, 5, 6	1938	$4.00
18, nos. 3–6	1939	4.00
19, nos. 1, 2, 4	1940	3.00
20, nos. 2, 3, 6	1940	3.00
21, nos. 1, 4, 6	1941	3.00
22, nos. 5, 6	1941	3.00
23, nos. 2, 3, 5, 6	1942	3.00
25, nos. 2–6	1943	3.00
27, nos. 5, 6	1944	2.50
28, nos. 2–6	1944	2.50
33, nos. 2–6	1947	2.50
58, nos. 3–4	1956	2.50

Prices subject to change without notice. Availability depends upon prior sales

ALL UNLISTED VOLUMES AND NUMBERS PRIOR TO VOLUME 63 ARE OUT-OF-PRINT

Annual subscription $22.50 domestic; $24.00 foreign
Single copies $2.25 each

Send order with remittance to

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

Read in the May issue of . . .

THE JOURNAL OF NUTRITION

Biography with portrait of Walter Charles Russell. By M. Wright Taylor and James B. Allison.

Hydroxyanthranilic acid as a source of niacin in the diets of the chick, guinea pig and hamster. By R. H. Decker and L. M. Henderson.

The effect of dietary fat on the development of vitamin B_6 deficiency in the rat. By M. A. Williams, N. L. Cohen and B. Hata.

Biological and chemical studies on commercial frying oils. By K. W. Keane, G. A. Jacobson and C. H. Krieger.

Manganese deficiency in the guinea pig. By G. J. Everson, L. S. Hurley and J. F. Geiger.

The effect of protein and energy on the potassium requirement of the chick. By R. M. Leach, Jr., R. Dam, T. R. Zeigler and L. C. Norris.

The nutritional effect of polymers isolated from thermally oxidized corn oil. By E. G. Perkins and F. A. Kummerow.

The effect of high environmental temperature on basal metabolism and serum ascorbic acid concentration of women. By E. M. Thompson, M. G. Staley, M. A. Kight and M. E. Mayfield.

Effects of the prevention of coprophagy in the rat. By R. H. Barnes, S. Tuthill, E. Kwong and G. Fiala.

Metabolic studies on the sodium fluoride-fed rat. By Alice H. Sievert and Paul H. Phillips.

Comparative performance of baby pigs fed infant and baby pig diets. By F. Diaz, V. C. Speer, P. G. Homeyer, V. W. Hays and D. V. Catron.

The effect of the carbohydrate and fat content of diet upon the riboflavin requirement of the cat. By S. N. Gershoff, S. B. Andrus and D. M. Hegsted.

Methionine, homocystine, choline, folic acid and vitamin B_{12} in the nutrition of the mouse. By H. E. Sauberlich.

Effect of saturated fat upon essential fatty acid metabolism of the rat. By James J. Peifer and R. T. Holman.

Issued monthly — Three volumes of four numbers each — Twelve numbers

$22.50 annually in United States — $24.00 in all other countries

ALL SUBSCRIPTIONS PAYABLE IN ADVANCE

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
THIRTY-SIXTH STREET AT SPRUCE
PHILADELPHIA 4, PENNSYLVANIA

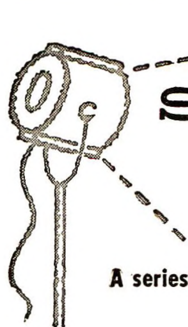
Enter my subscription to The Journal of Nutrition to begin with the current volume. I enclose $22.50 ($24.00 if mailing address is other than United States). Single copies, $2.25 each.

NAME

STREET

CITY ZONE STATE

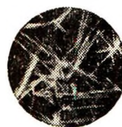
COUNTRY



SPOTLIGHT ON VITAMIN B_1

A series devoted to brief facts about the vital health-giving vitamins.

CHEMICAL AND PHYSICAL PROPERTIES. Vitamin B_1 (thiamine hydrochloride) is white, water soluble, with a nut-like, salty taste and yeast-like odor. Its empirical formula is: $C_{12}H_{17}ClN_4OS \cdot HCl$. Thiamine produced by synthesis is identical chemically and in biological activity with that obtained in pure form from nature.



DEFICIENCIES. A deficiency of thiamine is characterized by these symptoms: depression, irritability, fearfulness, lack of initiative and interest, loss of appetite. Symptoms vary since in usual practice deficiencies of other water-soluble vitamins occur. Medical treatment is simple: a sufficient amount of thiamine is administered to relieve symptoms quickly and the physician provides for a continuing adequate intake in combination with other members of the "B" complex.

A severe deficiency of thiamine leads to beriberi, a serious and sometimes fatal disease. While beriberi is almost a medical curiosity in the United States, it is common in countries in which polished white rice is a staple of the diet. Fortunately this dietary disease can be prevented through enrichment of milled white rice.

The paragraphs above have been taken from "The Vital Story of Vitamin B_1". If you would like the complete text of this, and all other stories in the series, please send your request to the Vitamin Division.

If you are engaged in the manufacture of foods and pharmaceuticals, our technical service is at your disposal . . . in confidence, of course.

VITAMIN DIVISION • **HOFFMANN-LA ROCHE INC.** • NUTLEY 10, N. J.

Roche Research and Roche Products Preserve and Protect the World's Health

ROCHE ROUND THE WORLD

AFFILIATED COMPANIES: BASEL • BOGOTA • BOMBAY • BRUSSELS • BUENOS AIRES • GRENZACH (BADEN) HAVANA • ISTANBUL • JOHANNESBURG • LONDON • MADRID • MEXICO CITY • MILAN • MONTEVIDEO • PARIS RIO DE JANEIRO • STOCKHOLM • SYDNEY • TOKYO • VIENNA • AGENCIES IN OTHER COUNTRIES

DIVALENT MINERALS AND PROTEOLYTIC ACTIVITY OF PANCREAS TISSUE FROM RATS AND CHICKS FED MANGANESE-DEFICIENT DIETS [1,2]

RONALD R. JOHNSON, ORVILLE G. BENTLEY [3] AND T. S. SUTTON
Ohio Agricultural Experiment Station, Wooster

(Received for publication September 6, 1958)

Divalent minerals are essential activators or co-factors for peptidases (Smith, '51a, '51b). Activating and stabilizing effects of rations on proteolytic enzymes have also been observed. The ion effect on the activation of chymotrypsinogen and trypsinogen was studied by Delezenne ('05) and re-investigated by McDonald and Kunitz ('41). Gorini ('50) concluded that bacterial proteinases needed calcium ions for both activation and for their stability. Bier and Nord ('51a, '51b) showed that calcium and manganese had a protective effect on the enzymatic activity of crystalline trypsin in alkaline solutions in which it is unstable. They stated that the "phenomenon" appears to be in clear contrast to the observed varying effect of different ions on the formation of trypsin from trypsinogen. In a later paper, Nord et al. ('56) presented additional evidence supporting the calcium stabilization of trypsin and investigated the kinetics of the reactions of trypsin, calcium-trypsin and acetyltrypsin. Kunitz and Northrop ('34) described the state of the proteolytic enzymes as being in

[1] Approved for publication as a journal article no. 86–59 by the Associate Director of the Ohio Agricultural Experiment Station.

[2] A preliminary report of this work is published in Federation Proceedings, *10*, 161, 1951 and in a Ph.D. Dissertation by Orville G. Bentley, University of Wisconsin, 1950.

[3] Present address: Dean of Agriculture, South Dakota State College, Brookings.

Copyright 1959
The Wistar Institute of Anatomy and Biology
All rights reserved

equilibrium as follows: native active form ⇌ denatured inactive form. The work of Gorini ('50, '51), Bier and Nord ('51a, '51b), Nord et al. ('56) and Green and Neurath ('53) on the activity of calcium and manganese would suggest that these ions cause a shift in the equilibrium to the native active form.

Burnett et al. ('52) found that radiomanganese appeared in pancreatic juice of dogs within one half hour after intravenous administration of the isotope. As pancreatic secretion increased, an increase in radiomanganese output was observed suggesting a relationship between manganese uptake by this organ and pancreatic secretions. Since our preliminary studies in 1951 [2] we have carried out experiments to determine the effect of feeding a manganese-deficient diet on the proteolytic activity of the pancreatic enzymes and their activation or stabilization or both by manganese or calcium as measured by the *in vitro* activity of the pancreas homogenates and the efficacy of protein digestion in rats fed a low-manganese ration. A summary of the enzymatic studies with divalent ions is also reported.

EXPERIMENTAL

Metabolism trials. Two nitrogen metabolism experiments were performed with rats. In the first experiment, weanling rats were divided into two groups. One group was fed a basal diet consisting of casein 18.0 gm, corn sugar [4] 72.1 gm, corn oil 5.0 gm, cystine 0.3 gm, Mn-deficient Salts IV [5] 4.0 gm, complete B vitamin mixture 0.4 gm, and choline 0.2 gm. The other group was fed the basal diet plus 5 mg of manganese per 100 gm of diet. The development of a manganese deficiency was accompanied by subnormal weight gains, e.g., 41 gm in two weeks in comparison with 53 gm for the manganese-supplemented rats. Also, manganese analysis showed that

[2] See footnote 2, page 513.

[4] Corn sugar or Cerelose — a sugar product prepared from corn by the Corn Products Refining Company, New York, N. Y.

[5] Phillips, P. H., and E. B. Hart J. Biol. Chem., *109:* 657 (1935).

the livers of the manganese-deficient and manganese-supplemented rats contained 0.9 and 3.6 μg Mn/gm wet weight, respectively.

When a deficiency developed in the group fed the manganese-deficient ration, three rats from each group were placed in metabolism cages. After a period of acclimation, two nitrogen metabolism trials were conducted for periods of 11 and 5 days using both diets which were fed ad libitum. In the second experiment weanling rats were placed on diets similar to that shown above and again placed in metabolism cages after a manganese deficiency had developed. Two 5-day metabolism trials were performed, similar to those in the previous experiment except that in this case the paired feeding technique was followed.

Chick feeding experiments. Six groups (15 each) of New Hampshire cockerel chicks were fed a practical type corn-soya ration with or without supplemental manganese for 6 weeks. Weight gains were comparable for all lots of chicks and perosis was not noted in the chicks fed the non-manganese supplemented rations. Liver manganese levels at the end of the experiment averaged 3.5 μg/gm, wet weight, for chicks on the low-manganese rations and 4.6 μg/gm for the chicks on the manganese-supplemented rations.

Enzyme studies. The enzymatic activity of the chick or rat pancreas was determined using homogenates prepared in water with a Waring blendor and filtered through glass wool. The filtered homogenates were diluted to a workable proteolytic activity range and portions thereof were added to equal volumes of 0.005 M divalent ion solutions prepared from $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, or $SrCl_2 \cdot 6H_2O$. These preparations or similar homogenates diluted with an equal amount of water, were either added directly to the casein-borate substrate or preincubated at 37°C for as long as 25 hrs. A drop of $CHCl_3$ and a crystal of thymol were added as preservatives. At various time intervals, 4-ml portions of the

preincubated homogenates were removed and placed in flasks containing 5 ml casein substrate (3% casein in 1% Na_2CO_3) and 1 ml pH 8.45 borate buffer (1M). The digestion mixtures were then incubated at 37°C for 0.5 to 4 hours (depending on the enzyme activity) after which time 5-ml aliquots were removed and placed in 5 ml of 10% trichloroacetic acid. After standing for 30 minutes to allow complete precipitation, the mixtures were filtered. The filtrates containing the non-protein nitrogen (NPN) released by proteolytic action were digested and analyzed for nitrogen by the micro-Kjeldahl method. Proteolytic activity was calculated as milligrams of NPN released per milligrams of homogenate nitrogen per hour.

RESULTS

Figure 1 illustrates the effect of preincubation with and without Ca^{++} or Mn^{++} ions on the proteolytic activity of chick and rat pancreatic homogenates.

Without prior preincubation, chick pancreas was practically inactive either with or without the addition of Ca^{++} or Mn^{++} to the casein-buffer substrate; however, preincubation increased the proteolytic activity of the homogenates markedly. The highest proteolytic activity was reached between 5 and 8 hours of preincubation after which time the activity dropped steadily. Although the sample incubated with no divalent ions had considerable activity following preincubation, the presence of Ca^{++} or Mn^{++} ions in the preincubation medium increased the activity over the control (water) almost two-fold.

Rat pancreas exhibited considerable initial activity which was not increased by the additions of Ca^{++} or Mn^{++} ions to the digestion mixture; however, as with chick pancreas homogenates, the additions of these ions to the preincubation mixture increased their activity somewhat. The highest activity was reached after two hours preincubation. The activity of the control sample (no Ca^{++} or Mn^{++}) was not increased by preincubation.

The activating effects of Zn^{++}, Fe^{++}, Mg^{++}, Co^{++}, Cu^{++}, and Sr^{++} ions were tested in a similar manner using chick pancreas. After 8 hours preincubation, only Mg^{++} and Sr^{++} showed slightly greater activity than the control (no divalent ion). After 24 hours preincubation, however, homogenates containing Zn^{++}, Mg^{++}, Sr^{++}, and, to a lesser extent, Co^{++} exhibited activity markedly greater than the control. In no case did any of these ions produce an activation as great as that produced by either Ca^{++} or Mn^{++}.

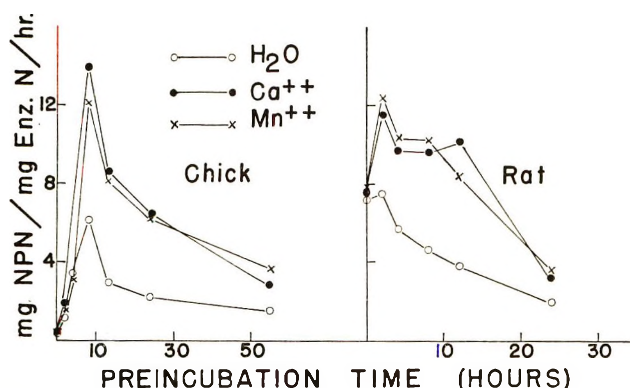


Fig. 1 The effect of preincubation in the presence of Ca^{++} and Mn^{++} ions on the proteolytic activity of chick and rat pancreatic homogenates.

The activation produced by preincubation with Ca^{++} exhibited a maximum at pH 5.9.

The marked effect of both Ca^{++} and Mn^{++} on the *in vitro* proteolytic activity of pancreatic homogenates suggested that the effect of these ions on the proteolytic activity of the pancreas from manganese-deficient and manganese-supplemented animals be investigated. Figure 2 illustrates this comparison with rat and chick tissues.

The initial proteolytic activity of the manganese-deficient rat pancreatic tissue was markedly lower than that of the manganese-supplemented pancreas. However, after sufficient preincubation in the presence of Ca^{++} or Mn^{++}, the deficient tissue exhibited as much activity as the pancreas from manganese-supplemented rats. The homogenates from the manga-

nese-deficient rats required 6 hours longer to reach their highest activity than the pancreas from the supplemented rats, probably because of the lower initial activity of the former. After 24 hours of preincubation, there was no difference in the activity of the two tissues.

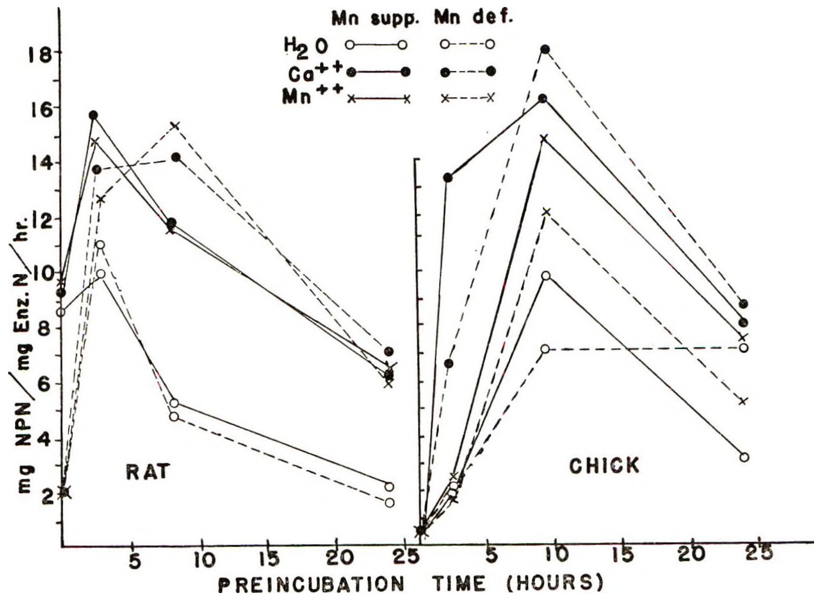


Fig. 2 The effect of preincubation in the presence of Ca^{++} and Mn^{++} ions on the proteolytic activity of pancreatic homogenates from manganese-deficient and supplemented rats and chicks. (Each point represents the value obtained with a homogenate of the pooled pancreas tissues from 6 rats and 6 chicks, respectively.)

The pancreatic tissues from chicks fed a ration to which no manganese was added and from manganese-supplemented chicks were also compared and the results are also shown in figure 2. Initially there was no difference — the activity of both tissues was low. After 8 hours preincubation, the activity of the manganese-supplemented pancreas was generally higher than that of the "deficient" pancreas although the differences were not large. Manganese-deficient pancreas preincubated 8 hours in the presence Ca^{++} was slightly more active than the supplemented pancreas.

Since the main proteolytic enzymes from the pancreas are trypsin and chymotrypsin, it was of interest to determine the effect of preincubation with Ca^{++} and Mn^{++} ions on the activity of these enzymes using our method for determining proteolytic activity. This comparison is shown in figure 3.

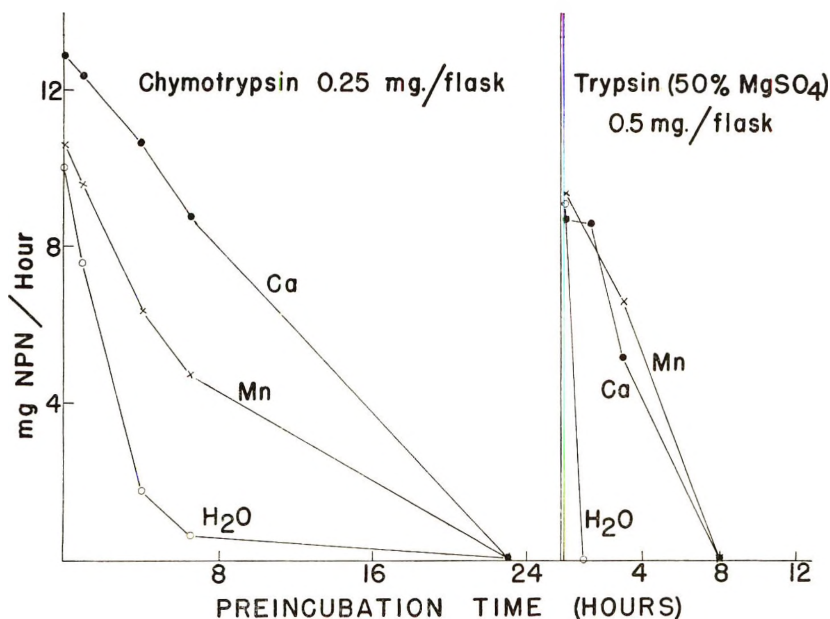


Fig. 3 The effect of preincubation in the presence of Ca^{++} and Mn^{++} ions on the proteolytic activity of crystalline chymotrypsin and trypsin.

Both chymotrypsin and trypsin[6] exhibited their maximum proteolytic activity at zero hours of preincubation. The activity of both enzymes decreased rapidly when incubated in water, presumably because of autodigestion. The addition of Ca^{++} or Mn^{++} ions to the preincubation medium slowed the rate of activity loss by the enzymes considerably but did little to increase the initial activity. This phenomenon appeared to be protection against autodigestion or increased stability of the metal-enzymes complex or both.

[6] Crystallized chymotrypsin and trypsin (bovine origin), Armour Laboratories, Chicago, Illinois.

The results of the metabolism experiments are shown in table 1. In the first experiment (ad libitum feeding) the manganese-deficient rats retained a greater percentage of their nitrogen intake than the supplemented rats. However, since the deficient rats ate considerably less, they actually retained less total nitrogen. In the paired feeding experiment, greater nitrogen retention occurred in the manganese-deficient group of the first trial, while in the second trial, the manganese-supplemented group retained the most nitrogen. In these experiments, no consistent relationship between nitrogen retention or utilization was found in animals fed the deficient or manganese-supplemented rations.

It is significant that the percentage of the total nitrogen intake found in the feces was relatively constant in both experiments. This would indicate that dietary protein was digested and adsorbed equally well by both the deficient and manganese-supplemented rats.

DISCUSSION

The proteolytic activity of both rat and chick pancreatic homogenates is markedly affected by preincubation of the homogenate in the absence of the substrate at a slightly acid pH. Fresh chick pancreatic tissue has little proteolytic activity at the alkaline pH of the substrate used herein. In contrast, fresh rat pancreas homogenates are active. The activity of both chick and rat pancreas was increased markedly by preincubation of the homogenates in the presence of Ca^{++} or Mn^{++} ions prior to their addition to the casein-borate buffer digestion mixture. From these data it cannot be determined whether the ion effect was due to an activation, greater enzyme stability or to protection of the enzyme against autodigestion.

A possible explanation might be drawn from the studies with crystalline trypsin and chymotrypsin. Here the ions appeared to protect the fully activated enzymes from loss of activity, presumably by autodigestion. The work of Bier and

TABLE 1

Nitrogen digestion and retention in rats on manganese-supplemented and manganese-deficient 18 per cent casein rations using ad libitum and paired feeding techniques

ITEM MEASURED	AD LIBITUM				PAIRED FEEDING			
	Trial 1	(11 days)	Trial 2	(5 days)	Trial 1	(11 days)	Trial 2	(5 days)
	+ Mn	− Mn	+ Mn	− Mn	+ Mn	− Mn	+ Mn	− Mn
Number animals	3	3	3	3	3	3	3	3
N intake, mg [1]	4352	3354	2236	1900	1716	1748	2777	2824
Fecal N, mg	236	199	128	110	122	101	224	191
Urine N, mg	2348	1520	1079	834	1452	1393	1502	1762
Retained N, mg	1762	1635	1029	956	143	254	1049	871
Per cent fecal N	5.42	5.93	5.72	5.79	7.1	5.8	8.1	6.8
Per cent urine N	53.95	45.32	48.26	43.89	84.6	79.7	54.1	62.4
Per cent retained N	40.62	48.75	46.02	50.32	8.3	14.5	37.8	30.8

[1] Figures given as total nitrogen intake, total fecal-N, etc., for all three animals in a given group.

Nord ('51a, '51b), Gorini ('50, '51), and Nord et al. ('53, '56) suggests that protection does occur. It is also known that calcium increases the yield of trypsin from trypsinogen *in vitro,* Delezenne ('05), McDonald and Kunitz ('41) and Pechére and Neurath ('57). Neither calcium nor manganese exerted an effect on the activity of the pancreatic enzymes when added to the casein-borate buffer substrate. Therefore, the role of the ions (Ca^{++} and Mn^{++}) did not appear to involve a direct contact or reaction with the pancreatic enzyme which is in agreement with the report of Green and Neurath ('53) showing that the active divalent ions are not involved in the trypsin-substrate complex. Perhaps, the explanation lies in the effect of calcium and manganese on the nature of the equilibrium between native active trypsin and denaturated trypsin. Nord and Bier ('53) found that calcium additions to trypsin solutions altered the ultra-centrifuge and electrophoretic patterns for the protein, indicating an intimate reaction between the enzyme and metal. Carr ('53) showed that trypsin will bind considerable quantities of calcium ions.

The pancreatic tissue from manganese-deficient rats apparently contained as much proteolytic activity as the pancreas from manganese-supplemented rats, as estimated *in vitro,* after preincubation in the presence of Ca^{++} or Mn^{++}. The activity of the unincubated fresh tissue, however, was considerably lower in the deficient group. This suggests that the enzymes were present but in an inactive form, possibly in the denatured form described by Kunitz and Northrop ('34), and that Ca^{++} or Mn^{++} supplied *in vitro* eventually enabled the enzymes to be activated. Another consideration is that even though the ration was low in manganese, the rats were receiving adequate calcium.

If this effect were manifest in pancreatic secretions *in vivo,* it is possible that protein digestibility would be reduced in a manganese deficient animal. The data did not suggest that feeding the manganese-deficient diet used altered protein digestion or nitrogen absorption. It appeared that differences noted in nitrogen retention were due to factors other than

lowered pancreatic enzyme activity. The protein, casein, used in these experiments is known to be readily digestible. Possibly a different result could be obtained using a less digestible protein in a low-manganese ration.

SUMMARY

Incubation of chick and rat pancreatic homogenates in the presence of Ca^{++} or Mn^{++} ions prior to combination with the substrate increased the proteolytic activity of these homogenates. Similar incubation of purified chymotrypsin or trypsin provided a protection against autodigestion but did not increase the enzyme activity.

The ions Zn^{++}, Mg^{++}, Sr^{++}, and Co^{++} exhibited some activation effect but were never as marked as Ca^{++} or Mn^{++}. The pH for the maximum effect of Ca^{++} was 5.9.

Pancreatic tissues from rats fed a manganese-deficient ration and chicks fed a practical corn soya ration without added manganese exhibited as much proteolytic activity as those of control animals. Homogenates prepared from pancreatic tissues from deficient animals had to be incubated longer with the divalent ions to obtain maximum proteolytic activity.

A dietary manganese deficiency in rats fed a purified type of diet did not lower the digestibility of the protein in the ration.

ACKNOWLEDGMENT

Portions of the nitrogen metabolism trials were performed by Mr. Robert Baab as part of an Independent Study project for the College of Wooster, Wooster, Ohio.

LITERATURE CITED

BIER, M., AND F. F. NORD 1951a On the mechanism of enzyme action. XLVI. The effect of certain ions on crystalline trypsin and re-investigation of its isoelectric point. Arch. Biochem. Biophys., *33:* 320.

——— 1951b The effect of certain ions and of radiation on crystalline trypsin. Ibid., *31:* 335.

BURNETT, W. T., JR., R. R. BIGELOW, A. W. KIMBALL AND C. W. SHEPPARD 1952 Radiomanganese studies on the mouse, rat and pancreatic fistula dog. Am. J. Physiol., *168:* 620.

Carr, C. W. 1953 Studies on the binding of small ions in protein solutions with the use of membrane electrodes. IV. The binding of calcium ions in solutions of various proteins. Arch. Biochem. Biophys., *46:* 424.

Delezenne, M. C. 1950 Activation du suc pancréatique par les sels de calcium. C. R. Soc. Biol., *59:* 476.

Gorini, L. 1950 Le role calcium daus l'activite et al stabilite de quelques proteinase bacteriennes. Biochim. Biophys. Acta, *6:* 237.

——— 1951 Role du calcium dans le septeme trypsine-serumalbumine. Ibid., *7:* 318.

Green, N. M., and H. Neurath 1953 The effects of divalent rations on trypsin. J. Biol. Chem., *204:* 379.

Kunitz, M., and S. H. Northrop 1934 Inactivation of crystalline trypsin. J. Gen. Physiol., *17:* 591.

McDonald, M. R., and M. Kunitz 1941 Effect of calcium and other ions on the autocatalytic formation of trypsin from trypsinogen. Ibid., *25:* 53.

Nord, F. F., and M. Bier 1953 Mechanism of enzyme action. LV. Interaction between calcium and trypsin. Biochem. Biophys. Acta, *12:* 56.

Nord, F. F., M. Bier and L. Terminiello 1956 On the mechanism of enzyme action. LXI. The self digestion of trypsin, calcium-trypsin and acetyltrypsin. Arch. Biochem. Biophys., *65:* 120.

Pechére, J. F., and H. Neurath 1957 Studies on the autocatalytic activation of trypsinogen. J. Biol. Chem., *229:* 389.

Smith, E. L. 1951a The Enzymes. J. B. Sumner and K. Myrback, eds., Academic Press, New York, *1:* 793.

——— 1951b Advances in Enzymology. Interscience Publishers, New York. *7:* 191.

PRODUCTION OF BIOTIN DEFICIENCY IN THE GUINEA PIG [1]

MACIE COLLINS COOTS, A. E. HARPER AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin
Madison

(Received for publication September 10, 1958)

INTRODUCTION

Signs of biotin deficiency have been described for many experimental animals including the rat, mouse, chick, turkey, pig and monkey; however, Reid ('54) reported that she could not produce signs of biotin deficiency in guinea pigs fed a biotin-free diet containing casein as the protein. As the biotin requirement of several species may be met through synthesis by the intestinal flora unless either avidin or sulfaguanidine is included in the diet, it seemed possible that this might also be true of the guinea pig.

The work reported in this paper was undertaken to determine the effect of feeding guinea pigs a diet containing raw egg white which should bind any biotin synthesized by the intestinal flora as the avidin-biotin complex, thus preventing it from becoming available to the animal.

EXPERIMENTAL

Guinea pigs [2] were housed in individual suspended cages with screen bottoms and were fed ad libitum a diet having the following percentage composition: dried raw egg white, 30;

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme Laboratories, Rahway, New Jersey.

[2] Both males and females, obtained from Zeimet-Bio Farms, Madison, Wisconsin.

แผนกห้องสมุด กรมวิทยาศาสตร์
กระทรวงอุตสาหกรรม

sucrose, 40; corn oil, 7.4; bulk,[3] 15; salts 4, 4 (Hegsted et al., '41); potassium acetate, 2.5; magnesium oxide, 0.5; vitamin mixture, 0.25; and choline chloride, 0.35. The vitamin mixture contained inositol, 200 gm; niacin, 20 gm; para-amino-benzoic acid, 10 gm; calcium pantothenate, 8 gm; riboflavin, 3 gm; thiamine·HCl, 2 gm; pyridoxine·HCl, 2 gm; folic acid, 1 gm; cyanocobalamin, 4 mg. The animals received daily a supplement of 5 mg of ascorbic acid/100 gm body weight. Each animal received weekly two drops of a fat-soluble vitamin concentrate containing haliver oil, 20 parts (vol.); α-tocopherol 7.2 parts (wt); menadione, 0.120 parts (wt); and corn oil to make 50 parts (vol.)

Experiment 1. Seven young adult guinea pigs weighing from 350 to 600 gm were given the diet described above and were weighed once a week. After the appearance of symptoms of biotin deficiency the animals were given a single injection of 80 µg of biotin (sodium salt) subcutaneously, and, thereafter, injections of 10 mg of biotin daily.

Experiment 2. This experiment was conducted in a manner identical to that of experiment 1 except that 12 adult guinea pigs weighing from 600 to 800 gm were used.

Experiment 3. Thirteen one- to two-day old guinea pigs were divided into two groups of 6 and 7, respectively. Group 1 received the raw egg white diet supplemented with biotin; group 2 received the biotin-deficient diet used in experiments 1 and 2.

RESULTS

The animals in experiment 1 as well as those in experiment 2 did not readily adapt to the diet containing raw egg white. This slow acceptance of the diet was at least partially responsible for the initial weight loss as shown in figure 1. After they had adapted to the diet the rate of weight loss decreased; however, due to biotin deficiency, the animals were unable to grow, and some weight loss continued to occur. During the 56-day depletion period in experiment 1 the

[3] Solkafloc, Brown Company, Berlin, New Hampshire.

average weight loss was 45 gm; in experiment 2 the depletion period was 70 days and the average weight loss was 123 gm.

The first symptom observed in addition to failure to grow was alopecia, which was usually confined to the dorsal posterior area of the pelage. This developed after approximately

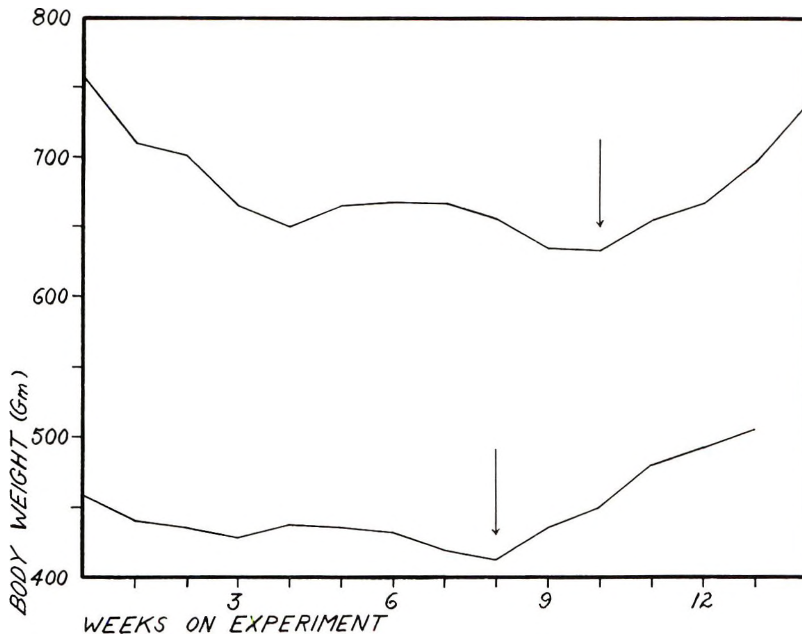


Fig. 1 Changes in body weight of guinea pigs during biotin depletion and repletion. The vertical arrows indicate the point at which biotin supplementation was begun. Lower curve, experiment 1, points represent the averages for 7 animals until 21 days, for 6 animals thereafter. Upper curve, experiment 2, points represent the averages for 12 animals until 28 days, 6 died between the 28th and 56th day, thereafter points represent the averages for 6 animals.

4 weeks. By the 8th week of the experiment a decoloration of the coat was noted in 5 of the 7 animals. The fur developed a coarse texture. This was particularly noticeable in the white guinea pigs. The two black animals became gray (see figure 2); the three red ones became blonde.

Half of the adult animals used in experiment 2 died before supplementation was begun but the survivors developed the

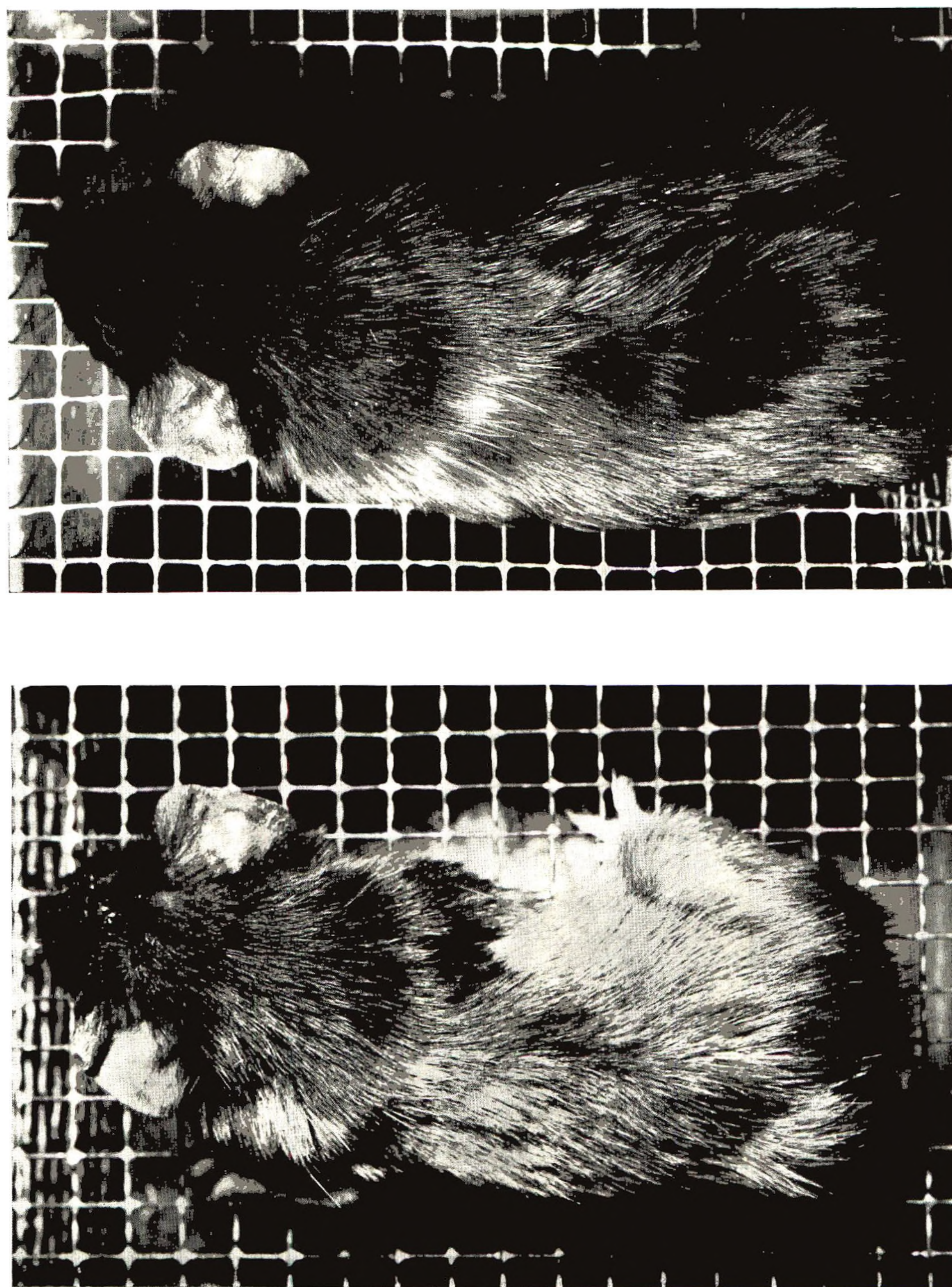


Fig. 2 Decoloration of fur of black guinea pigs as a result of biotin deficiency.
A, biotin depleted; B, after biotin supplementation.

same pattern of alopecia and coat decoloration as was seen in experiment 1.

The animals of both experiments 1 and 2 responded to biotin supplementation by an increase in weight (as shown in figure 1). The surviving animals in experiment 1 gained 85 gm in 28 days, those in experiment 2 gained 110 gm in 28 days. In addition, the pigmentation and texture of the hair coats returned to normal (see figure 2).

The infant animals of experiment 3 failed to adapt to the raw egg white-sucrose diet. Only one survived beyond 10 days. On autopsy renal hemorrhage occurring at the pelvis of the kidney was observed in 11 of the 13 animals.

DISCUSSION

The results reported above indicate that a biotin deficiency can be induced in the guinea pig by dietary means. However, it is probable that the biotin needs of this species can usually be met through intestinal synthesis by the microflora and that a dietary requirement can be demonstrated only by feeding avidin or possibly certain sulfa drugs. This would account for Reid's ('54) failure to observe signs of biotin deficiency since she merely omitted the biotin from an otherwise complete diet.

Lease et al. ('37), in comparing the effect of egg white injury in various species, did not obtain well-defined symptoms in the guinea pig as they did in other species. They also encountered difficulty in getting the animals to adapt to the diet containing egg white and, from the weight gains reported in their paper, it is evident that the diet was inadequate.

Several factors may be responsible for our success in producing a biotin deficiency: (1) any biotin arising through intestinal synthesis was made unavailable as the avidin-biotin complex; (2) an adequate purified diet was available which resulted in a weight gain of from 4 to 5 gm/day when all of the vitamins were supplied; (3) the young adult and adult animals were able to adapt fairly well to the diet containing egg white as the entire source of protein.

The loss of weight, alopecia and graying that occurred in guinea pigs fed this biotin-deficient diet have been observed in other species: e.g., in the rat (Boas, '27; Parsons et al., '37), the rabbit (Lease et al., '37), the monkey (Lease et al., '37; Waisman et al., '45) and the mouse (Wilson et al., '49).

In view of the evidence presented, it may be concluded that a dietary deficiency of biotin can be demonstrated in the guinea pig.

SUMMARY

Guinea pigs were fed a biotin-deficient diet containing raw egg white as the source of protein. The following symptoms were observed: loss of weight, alopecia, and decoloration of the fur. These symptoms were cured by biotin supplementation.

LITERATURE CITED

BOAS, M. E. 1927 The effect of desiccation upon the nutritive properties of egg white. Biochem. J., *21:* 713.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

LEASE, J. G., H. T. PARSONS AND E. KELLEY 1937 A comparison in five types of animals of the effects of dietary egg white and of a specific factor given orally or parenterally. Biochem. J., *31:* 432.

PARSONS, H. T., J. G. LEASE AND E. KELLEY 1937 The interrelationship between dietary egg white and the requirement for a protective factor in the cure of the nutritive disorder due to egg white. Ibid., *31:* 424.

REID, M. E. 1954 Nutritional studies with the guinea pig. B vitamins other than pantothenic acid. Proc. Soc. Exp. Biol. Med., *85:* 547.

WAISMAN, H. A., K. B. MCCALL AND C. A. ELVEHJEM 1945 Acute and chronic biotin deficiencies in the monkey. J. Nutrition, *29:* 1.

WILSON, J. M., E. H. LEDUC AND D. A. WINSTON 1949 The production of biotin deficiency in the mouse. Ibid., *38:* 73.

THE EFFECT OF ORALLY ADMINISTERED RUTIN ON THE ADRENAL ASCORBIC ACID LEVEL IN GUINEA PIGS [1]

CARL D. DOUGLASS AND GEORGE H. KAMP
Department of Biochemistry, University of Arkansas School of Medicine, Little Rock

(Received for publication September 8, 1958)

The biological relationship between ascorbic acid and the bioflavonoids has been the subject of much confusion due to the fact that contradictory results have been obtained by different investigators and on occasion by the same group of workers. This subject has been reviewed recently by Shils and Goodhart ('56). Pertinent to the experiment described here are several observations dating from the original report from Szent-Gyorgyi's laboratory (Bentsath et al., '36) in which it was reported that scorbutic guinea pigs lived longer when given an extract of citrus fruit containing flavonoids. A number of investigators failed to confirm this work (Shils and Goodhart, '56). Despite the lack of confirmatory evidence that these materials influence the severity of scurvy in experimental animals, there are reports indicating that flavonoids increase the biological value of a given quantity of ascorbic acid, aid in the maintenance of capillary integrity, and influence the tissue concentrations of ascorbic acid. Todhunter and associates ('40) found that pure ascorbic acid was biologically less effective than an equivalent amount of ascorbic acid administered as lemon juice. Cotereau and co-workers ('48) claimed that the administration of a flavonoid, catechin, increased the ability of the guinea pig to maintain high tissue

[1] This investigation was supported in part by a research grant from the National Vitamin Foundation, Inc.

levels of ascorbic acid. Papageorge and Mitchell ('49) reported that rutin (the 3-rutinoside of quercetin, which is 3, 5, 7, 3′, 4′-pentahydroxyflavone), when given to guinea pigs receiving adequate amounts of ascorbic acid, increased the levels of adrenal ascorbic acid but had no influence on plasma or liver levels. Papageorge and co-workers ('50) later reported their inability to confirm these observations. Ambrose and DeEds ('49) found that a combined supplement of a subminimal amount of ascorbic acid and rutin was apparently more effective in prolonging the lives of guinea pigs on a scorbutogenic diet than was either of the materials given separately. Using the odontoblast cell assay, Crampton and Lloyd ('50) noted that the biological value of a suboptimal amount of ascorbic acid was increased significantly by supplying rutin to the animals.

In none of the experiments mentioned above was a purified diet used, nor has anyone systematically studied the effects of a bioflavonoid on the tissue levels of ascorbic acid when the test animals were receiving graded doses of ascorbic acid. For these reasons we have fed guinea pigs a purified diet supplemented with varying amounts of ascorbic acid and with a constant amount of rutin and determined the levels of ascorbic acid in the adrenal glands and the liver.

EXPERIMENTAL

Young guinea pigs of both sexes weighing between 180 and 480 gm were fed a modification of the diet of Reid and Briggs ('53) as shown in table 1. It was found necessary to replace half the starch with sucrose in order to stimulate sufficient food consumption. The amounts of all the vitamins except tocopherol acetate were doubled because in preliminary experiments it was found that much better growth was obtained at these levels. Food and water were given ad libitum for three weeks. Animals were given daily oral supplements of zero, 0.5, or 1.0 mg of ascorbic acid per 100 gm body weight or 5 mg of rutin or both, as appropriate during this period.

The rutin was made up by grinding with an equal weight of starch and suspending in water to give a suitable volume for oral administration. Weights and any evidence of gross signs of scurvy were recorded biweekly. At the end of the feeding period the animals were killed by stunning and decapitation. The tissues were quickly dissected out, weighed and subjected to the ascorbic acid analysis of Roe and Oesterling ('44) as modified by Bolin and Book ('47).

TABLE 1

Composition of diet

INGREDIENTS	AMOUNT	INGREDIENTS	AMOUNT
	gm/kg		*mg/kg*
Casein	300	Thiamine·HCl	32
Cottonseed oil	43	Riboflavin	32
Cod liver oil	30	Pyridoxine·HCl	32
Sucrose	205	Calcium pantothenate	80
Cellu flour	150	Niacin	400
Corn starch	100	Biotin	1.2
Cerelose	78	Folic acid	20
Potassium acetate	25	Vitamin B_{12}	0.08
Magnesium oxide	5	α Tocopherol acetate	20
Salts [1]	60	Menadione	4
Choline chloride	2		
Inositol	2		

[1] Briggs et al. ('52).

RESULTS AND DISCUSSION

Table 2 shows the results of the ascorbic acid analyses of the liver and adrenal glands. Note that rutin exerted no influence on the liver ascorbate levels at any level of ascorbic acid supplementation. The adrenal concentrations were markedly increased by rutin at the supplementation levels of 0.5 and 1.0 mg per 100 gm body weight per day and rutin was without influence when no ascorbic acid was given to the animals. The adrenal ascorbic acid values obtained in the groups receiving rutin were compared statistically with those not receiving it by the Student "t" test. Values of P obtained indicated that the differences between the means of the groups

receiving supplements of 0.5 and 1.0 mg per 100 gm of body weight are highly significant (P values of < 0.01). This, taken with the fact that the response to the level of ascorbic acid administration was excellent, indicates that rutin acts to spare adrenal ascorbic acid at intake levels which are probably sufficient for growth but not for other functions (Reid, '58) while it is without effect on the liver levels. There were no differences in average growth rates between groups. The development of gross scurvy was not observed in the three-week feeding period in any of the groups receiving ascorbic

TABLE 2

Effect of rutin on liver and adrenal ascorbic acid levels of guinea pigs

SUPPLEMENTARY ORAL ASCORBIC ACID	LIVER ASCORBIC ACID (MG/100 GM WET TISSUE)		ADRENAL ASCORBIC ACID (MG/100 GM WET TISSUE)	
	+ Rutin	— Rutin	+ Rutin	— Rutin
(mg/day/100 gm body wt)	*(5 mg/day)*	*(5 mg/day)*	*(5 mg/day)*	*(5 mg/day)*
0	4.9 ± 1.0 [1] (7) [2]	4.8 ± 0.7 (7)	27.2 ± 1.4 (6)	28.5 ± 2.0 (6)
0.5	8.8 ± 2.0 (7)	8.3 ± 1.6 (6)	60.3 ± 4.3 (6)	44.8 ± 7.3 (6)
1.0	14.7 ± 2.6 (7)	13.5 ± 1.4 (6)	87.4 ± 5.6 (7)	79.3 ± 3.8 (6)

[1] Standard deviation of the mean.

[2] Number of animals.

acid. When signs of scurvy were seen in the groups receiving no ascorbic acid, there appeared to be no differences in time of onset or severity which were attributable to rutin administration. Only a few animals in either of the groups receiving no ascorbic acid developed such signs, however.

Douglass and Hogan ('58) found that flavonols are rapidly destroyed in liver tissue but are relatively stable in adrenal homogenates. Assuming that rutin is hydrolyzed in the gastrointestinal tract to its aglycone quercetin and that this is the form in which the flavonoid is absorbed and transported to the tissues, any protective effect which these materials may have on ascorbic acid would be expected to be apparent in

tissues which lack the ability to catabolize them. Hence, if a protective effect is to be expected, it should be seen in the adrenals and not in the liver.

SUMMARY

With the addition of rutin to diets of guinea pigs receiving less than optimal levels of ascorbic acid an increased adrenal ascorbic acid was observed. Liver ascorbic acid levels were not different from those of comparable animals without rutin supplementation.

LITERATURE CITED

AMBROSE, A. M., AND F. DEEDS 1949 The value of rutin and quercetin in scurvy. J. Nutrition, *38:* 305.

BENTSATH, A., S. RUSZNYAK AND A. SZENT-GYORGYI 1936 Vitamin nature of flavonones. Nature, *138:* 798.

BOLIN, D. W., AND L. BOOK 1947 Oxidation of ascorbic acid to dehydroascorbic acid. Science, *106:* 451.

BRIGGS, G. M., M. R. SPIVEY, J. C. KERESZTESY AND M. SILVERMAN 1952 Activity of citrovorum factor for the chick. Proc. Soc. Exp. Biol. Med., *81:* 113.

COTEREAU, H., M. GABE, E. GERO AND J. L. PARROT 1948 Influence of vitamin P (vitamin C_2) upon the amount of ascorbic acid in the organs of the guinea pig. Nature, *161:* 557.

CRAMPTON, E. W., AND L. E. LLOYD 1950 A quantitative estimation of the effect of rutin on the biological potency of vitamin C. J. Nutrition, *41:* 487.

DOUGLASS, C. D., AND R. HOGAN 1958 Formation of hydroxybenzoic acids from flavonols *in vitro.* Federation Proc., *17:* 213.

PAPAGEORGE, E., AND G. L. MITCHELL, JR. 1949 The effect of oral administration of rutin on blood, liver and adrenal ascorbic acid and on liver and adrenal cholesterol in guinea pigs. J. Nutrition, *37:* 531.

PAPAGEORGE, E., N. L. NOBLE AND J. R. AMERSON 1950 Rutin administration and adrenal ascorbic acid in the guinea pig. Absts. of Papers, 117th Meeting Am. Chem. Soc., Phila., p. 50C.

REID, M. E. 1958 The Guinea Pig in Research. Human Factors Research Bureau, Inc., Washington, D. C., p. 42.

REID, M. E., AND G. M. BRIGGS 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.

ROE, J. H., AND M. J. OESTERLING 1944 The determination of dehydroascorbic acid in plant tissues by the 2, 4-dinitrophenylhydrazine method. J. Biol. Chem., *152:* 511.

SHILS, M. E., AND R. S. GOODHART 1956 The Flavonoids in Biology and Medicine. The National Vitamin Foundation, Incorporated, New York, N. Y.

TODHUNTER, E. N., R. C. ROBBINS, G. IVEY AND W. BREWER 1940 A comparison of the utilization by guinea pigs of equivalent amounts of ascorbic acid (vitamin C) in lemon juice and in the crystalline form. J. Nutrition, *19:* 113.

THE DOMESTIC CAT AS A LABORATORY ANIMAL FOR EXPERIMENTAL NUTRITION STUDIES

VI. CHOLINE DEFICIENCY

ALBERTO CARVALHO DA SILVA, MARIO F. MANSUR GUERIOS [1] AND SYLVIO R. MONSAO [2]

Department of Physiology,[3] Faculdade de Medicina da Universidade de S. Paulo, S. Paulo, Brasil

(Received for publication September 16, 1958)

Fatty livers associated with choline deficiency have been described for the rat (Best and Huntsman, '32), mouse (Meader and Williams, '57), rabbit (Hove et al., '54), guinea pig (Casselman and Williams, '54), dog (Burns and McKibbin, '51), pig (Johnson and James, '48) and calf (Johnson et al., '51).

Best and Huntsman ('35) demonstrated that the choline requirements of the rat could be spared by raising the casein content of the ration to 20%, and Tucker and Eckstein ('37) explained this effect of casein by its methionine content. According to du Vigneaud et al. ('39) methionine acts as a methyl donor for tissue synthesis of choline. However, the tissues of the rat are able to synthesize the methyl group from glycine and serine if sufficient folic acid and vitamin B_{12} are available (Stekol et al., '52). In confirmation of this, Burns and McKibbin ('51) demonstrated that the fatty livers of dogs raised on choline-deficient diets could be corrected or pre-

[1] From the Department of Internal Medicine; head, Prof. A. B. Ulhoa Cintra. Hospital das Clínicas. Faculdade de Medicina da Universidade de S. Paulo.

[2] Assistant to Prof. Adriano Pondé, Faculdade de Medicina da Bahia, Brasil; on a fellowship at the Department of Gastroenterology; head, Dr. J. Fernandes Pontes. Hospital das Clínicas, Faculdade de Medicina da Universidade de S. Paulo; deceased, October 9, 1958.

[3] Head, Prof. Franklin A. de Moura Campos.

vented by vitamin B_{12}, and Young et al. ('55) observed that the choline requirements of chicks are one-tenth as much when a ration containing folic acid is fed.

Dragstedt and associates ('36) observed that the lipotropic effect of beef pancreas on pancreatectomized dogs could not be explained on the basis of its choline content and postulated the existence of a lipotropic hormone to which they gave the name "lipocaic." According to other investigators (Chaikoff and Entenman, '48), the lipotropic activity of beef pancreas on pancreatectomized dogs can be explained by its content of proteolytic enzymes.

Preliminary results (Carvalho da Silva et al., '58) indicated that the domestic cat develops high levels of liver fat when maintained on a purified diet containing 35% casein and supplemented with 300 mg of choline on alternate days. In this paper are presented data demonstrating that this species requires choline as a growth and a lipotropic factor when fed a diet containing 42% casein. The influence of supplements of raw beef liver and raw beef pancreas was also studied.

EXPERIMENTAL

Thirty young male and female growing cats were used in this study. They were housed separately in screen-bottom cages kept in a constant-temperature room (24 to 26°C). For a period of three weeks the animals were fed ad libitum a diet composed of equal parts by weight of ground raw beef heart and bread, supplemented with 2% cod liver oil. At the end of this period the cats were distributed among 6 groups of 5 animals each. Groups I to V inclusive were fed rations I to V (table 1). Group VI was fed a "natural ration" the composition of which is likewise shown in table 1.

The weights of the animals and their food consumption were recorded three times a week. After 8 weeks on experiment, blood counts were performed on samples collected from the saphenous vein (Carvalho da Silva et al., '55). The animals were deeply anesthetized [4] and the liver used for fat deter-

[4] Nembutal, Abbott, was used.

TABLE 1

Composition of rations used

Purified rations[1]						Natural ration	
Ingredient	I	II	III	IV	V	Ingredient	VI
	%	%	%	%	%		%
Casein, crude	42.0	42.0	42.0	42.0	42.0	Casein, crude	10
Sucrose	26.0	25.9	25.5	23.0	23.0	Beef, raw, lean, ground	20
Hydrogenated coconut fat	24.0	24.0	24.0	24.0	24.0	Hydrogenated coconut fat	20
Gelatin	2.5	2.5	2.5	2.5	2.5	Sardines, cooked eviscerated	20
Salt mixture[2]	5.5	5.5	5.5	5.5	5.5	Oats, cooked, compressed	15
Choline HCl	—	0.1	0.5	—	—	Potatoes, cooked, mashed	10
Beef liver, raw, ground	—	—	—	10.0	—	Cod liver oil	3
Beef pancreas, raw, ground	—	—	—	—	10.0	Bone meal	2

Vitamin mixture — when added to 100 gm of the "purified ration" furnished: 1 mg each of thiamine, riboflavin, vitamin K and para-aminobenzoic acid; 2 mg each of Ca-pantothenate and pyridoxine; 10 mg of niacin; 0.5 mg of folic acid; 0.02 mg d-biotin; 30 mg inositol. Each animal once a week received 2,500 I. U. of vitamin A, 500 I. U. of vitamin D and 15 mg of vitamin E dissolved in 1 ml of peanut oil.

Above ingredients, including cooking waters, mixed to form a soft paste

[1] When supplements are included, the equivalent amount of sucrose is deducted on a dry weight basis. The amount of sucrose deducted for groups IV and V corresponds to the approximate dry weight of liver and pancreas added.

[2] Phillips and Hart ('35).

minations and histologic sections. Tissue sections were stained with hematoxylin and eosin, sudan III and for reticulum by the Foot technic (Foot, '24).

Total liver fat was determined by the method of Van de Kamer and associates ('49); after saponification with 60% KOH, acidification and extraction with petroleum ether, the extract was titrated with 0.1 N KOH in isobutanol using a solution of thymol blue in isobutanol as indicator.

One animal in group VI died at the beginning of the experiment and was discarded; one cat in group II died during the 5th week and one in group V died during the 6th week; both were studied and their data included in the results.

RESULTS

Growth. The average and individual growth rates are presented in figure 1. The purified diet without choline (group I) did not support growth; when 0.1% choline was added (group II), there was a small weight gain during the first half of the experimental period; better and continuous growth was observed with the 0.5% choline supplement (group III). Growth rates of the same order as in groups II and III were observed when 10% liver (group IV) and 10% pancreas (group V) were added to the purified diet without choline. The "natural ration" (group VI) supported better growth, which accords with previous experiments (Carvalho da Silva, '50; Carvalho da Silva et al., '58).

Liver fat. Data on total liver fat in the 6 groups are presented in table 2. Higher values were obtained with the purified diet (groups I to V inclusive) than with the "natural diet" (group VI), but it is evident that with choline supplied at the level of 0.5% (group III) there was an appreciable reduction. Supplements of 0.1% choline, 10% liver and 10% pancreas were without effect.

Food consumption. The average food consumption for the 6 groups is presented in table 2. It is evident that the better growth with the "natural diet" (group VI) was associated with a greater food intake, and that the group that showed the

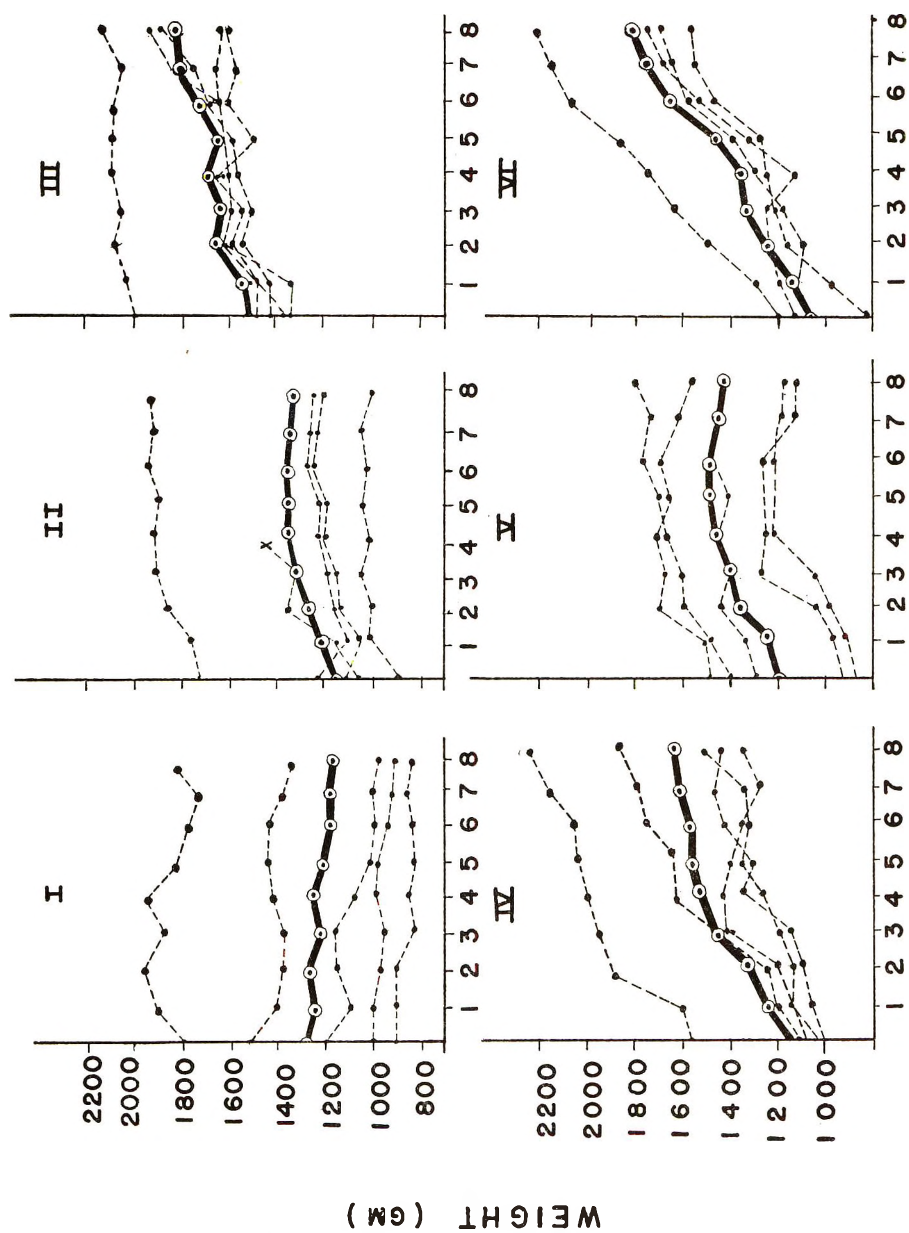


Fig. 1 Individual and average growth rates; I, without choline; II, with 0.1% choline; III, 0.5% choline; IV, with 10% raw beef liver; V, with 10% raw frozen beef pancreas; VI, fed a ''natural diet'' (table 1).

poorest growth, namely the one on the choline-deficient ration, ate the least. The food intakes for the other groups were intermediate. These results are what would be expected.

Hematology. The values for red cell counts, hematocrit, hemoglobin and white cell count were normal for cats of these ages (Carvalho da Silva, '50).

TABLE 2

Total liver fat and food consumption

GROUP	CHARACTERISTIC FEATURE OF THE DIET	TOTAL LIVER FAT		FOOD CONSUMPTION [1]	
		gm/100 gm wet tissue		*gm/day*	
I	Basal diet, no choline	21.1	2.75 [2]	28.8	0.53 [2]
II	Basal diet with 0.1% choline	22.4	4.00	37.5	1.19
III	Basal diet with 0.5% choline	11.5	1.00	46.1	1.72
IV	Basal diet with 10% liver	23.5	2.92	50.6	1.67
V	Basal diet with 10% pancreas	21.7	4.98	40.2	2.65
VI	''Natural ration''	5.8	0.62	62.0	4.66

[1] Average values are presented because the food intake was maintained practically constant during the experiment. The values for the ''natural ration'' were corrected for the amount of water added during the preparation.

[2] Standard error.

Histology of the liver. The histologic studies of the liver (plate 1) indicate a fatty infiltration as would be expected in view of the data presented in table 2. In group I (without choline) gross fatty deposition with confluent fatty cysts was present at the periphery of the lobules; the fatty infiltration of the centrolobular area was of mild degree. Pronounced perilobular fibrosis could be demonstrated by staining with hematoxylin and eosin and by the Foot method. In group II (0.1% choline) the periportal boundaries were heavily infiltrated with fat but the centrolobular areas were well preserved. Perilobular fibrosis was much less evident. In group III (0.5% choline) there was a mild perilobular infiltration of fat with normal centrolobular areas and without evidence of fibrosis. Group IV (without choline added but with 10% liver) and group V (without added choline but with 10%

pancreas) exhibited the same pattern as group I, the group that was on the basal choline-deficient diet.

DISCUSSION

The choline requirement of growing cats, as a growth and lipotropic factor, when fed a 42% casein diet, is difficult to explain. The choline requirement to prevent fatty livers in rats is approximately 5.5 mg daily (Griffith and Mulford, '41); 75 mg % of choline is sufficient to prevent and to correct fatty infiltration in puppies raised on a 19% casein diet (Burns and McKibbin, '51). Normal livers were observed in pigs (Johnson and James, '48) and calves (Johnson et al., '51) fed diets containing 0.2% choline. In contrast to these results, no complete protection was observed in growing cats, even when 0.5% choline and 42% casein were used, with an average choline intake of 250 mg per day. However, the livers were normal on the "natural ration" which furnished approximately 30 mg of choline daily, as calculated from the choline content of the natural foods used to make up the ration (Griffith and Nyc, '54).

As the mixture in the vitamin supplement used in our experiments did not contain vitamin B_{12} it would be reasonable to assume that the fatty livers were caused by a deficiency of B_{12}. Burns and McKibbin ('51) were able to correct the fatty livers of puppies raised on a purified diet without choline by the administration of vitamin B_{12}. However, no protection was afforded to our animals by supplementing the diet with 10% beef liver, which supplied approximately 0.5 μg of vitamin B_{12} per animal per day (Ungley, '55). According to Drill ('54), factors other than choline, vitamin B_{12} and folic acid are required to allow maintenance of normal liver fat in rats.

The possibility of a "lipocaic deficiency" in our experiments is excluded by the results obtained in group V which was fed the basal choline-deficient diet supplemented with 10% frozen raw beef pancreas.

Although the fatty infiltration in animals exhibiting experimental choline deficiency is described as being predominantly centrolobular in character, in our experiments the distribution was periportal. Periportal fatty infiltration has been related to protein deficiency in rats (Best et al., '56), and has also been described in kwashiorkor in children (Trowell et al., '54). In view of these observations it would be desirable to investigate in cats the effects of other levels and of other types of protein.

Further studies are necessary to disclose the mechanisms of fatty infiltration seen in the present experiments, but it seems obvious that the domestic cat offers an opportunity to investigate new aspects of this problem; and it is even possible that the transfer of methyl groups of methionine to choline does not occur in this species; according to Best et al. ('54) this finding would be of the utmost importance for investigative purposes.

SUMMARY

Fatty livers were produced in growing cats after 8 weeks of subsistence on a diet containing 42% casein and 24% hydrogenated coconut fat; the fatty infiltration was not influenced by feeding supplements of 10% raw beef liver or 10% raw beef pancreas. Choline to the extent of 0.1% was ineffective as a lipotropic agent but did produce a mild growth response; 0.5% choline caused an appreciable reduction in the fatty infiltration and improvement in the growth rate. The deposition of fat was principally in the periportal spaces and not around the centrolobular vein as usually described for choline deficiency in other species.

ACKNOWLEDGMENTS

We wish to thank Professor J. I. Lobo, of Laborterapica S. A., Sao Paulo, for the generous supply of frozen beef pancreas.

LITERATURE CITED

BEST, C. H., AND M. E. HUNTSMAN 1932 The effects of the components of lecithine upon deposition of fat in the liver. J. Physiol., 75: 339.

BEST, C. H., AND M. E. HUNTSMAN 1935 The effect of choline on the liver fat of rats in various states of nutrition. Ibid., *83:* 255.

BEST, C. H., C. C. LUCAS AND J. H. RIDOUT 1954 The lipotropic factors. In: Nutritional factors and liver diseases. Ann. N. Y. Acad. Sci., *57:* 646.

———— 1956 Vitamins and the protection of the liver. Brit. Med. Bull., *12:* 9.

BURNS, M. M., AND J. M. MCKIBBIN 1951 The lipotropic effect of vitamin B_{12} in the dog. J. Nutrition, *44:* 487.

CARVALHO DA SILVA, A. 1950 The domestic cat as a laboratory animal for experimental nutrition studies. II. Comparative growth and hematology on stock and purified ration. Acta Physiol. Lat. Amer., *1:* 26.

CARVALHO DA SILVA, A., R. C. DE ANGELIS, M. A. PONTES AND M. F. GUERIOS 1955 The domestic cat as a laboratory animal for experimental nutrition studies. IV. Folic acid deficiency. J. Nutrition, *56:* 199.

CARVALHO DA SILVA, A., N. PIMENTA, R. C. DE ANGELIS AND M. A. PONTES 1959 The domestic cat as a laboratory animal for experimental nutrition studies. V. Some data on nutritional requirements, biochemical and physiological values and organs weight. Acta Physiol. Lat. Amer., in press.

CASSELMAN, W. G. B., AND G. R. WILLIAMS 1954 Choline deficiency in the guinea pig. Nature, *173:* 210.

CHAIKOFF, I. L., AND C. ENTENMAN 1948 Antifatty-liver factor of the pancreas. Present status. Advances in Enzymology, Vol. VIII. Interscience, New York-London, p. 347.

DRAGSTEDT, L. R., J. VAN PROHASKA AND H. P. HARMS 1936 Observations on a substance in pancreas (a fat metabolizing hormone) which permits survival and prevents liver changes in depancreatized dogs. Am. J. Physiol., *117:* 175.

DRILL, V. A. 1954 Lipotropic effects of vitamin B_{12} and other factors. In: Nutritional factors and liver diseases. Ann. N. Y. Acad. Sci., *57:* 654.

DU VIGNEAUD, V., J. P. CHANDLER, H. W. MOYER AND D. M. KEPPEL 1939 The effect of choline on the ability of homocystine to replace methionine in the diet. J. Biol. Chem., *131:* 57.

FOOT, N. C. 1924 A technique for demonstrating reticulum fibers in Zenker-fixed paraffin sections. J. Lab. Clin. Med., *9:* 777.

GRIFFITH, W. H., AND D. J. MULFORD 1941 Choline metabolism. VI. Hemorrhagic degeneration and the labile methyl supply. J. Am. Chem. Soc., *63:* 929.

GRIFFITH, W. H., AND J. F. NYC 1954 Choline. In: The Vitamins — Chemistry, Physiology, Pathology, Vol. II. Academic Press, New York, p. 1.

HOVE, E. L., D. H. COPELAND AND W. D. SALMON 1954 Choline deficiency in the rabbit. J. Nutrition, *53:* 377.

JOHNSON, B. C., AND M. F. JAMES 1948 Choline deficiency in the baby pig. Ibid., *36:* 339.

JOHNSON, B. C., H. H. MITCHELL, J. A. PINKOS AND C. C. MORRILL 1951 Choline deficiency in the calf. Ibid., *43:* 37.

MEADER, R. D., AND W. L. WILLIAMS 1957 Choline deficiency in the mouse. Am. J. Anat., *100:* 167.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 157.

STEKOL, J. A., S. WEISS AND K. W. WEISS 1952 Vitamin B_{12} and folic acid in the synthesis of choline in the rat. Arch. Biochem. Biophys., *36:* 5.

TROWELL, H. C., J. P. N. DAVIES AND R. F. A. DEAN 1954 Kwashiorkor. Edward Arnold, London.

TUCKER, H. F., AND H. C. ECKSTEIN 1937 The effect of supplementary methionine and cystine on the production of fatty livers by diet. J. Biol. Chem., *121:* 479.

UNGLEY, C. C. 1955 The chemotherapeutic action of vitamin B_{12}. In: Vitamins and Hormones, Vol. XIII. Acad. Press, New York, p. 137.

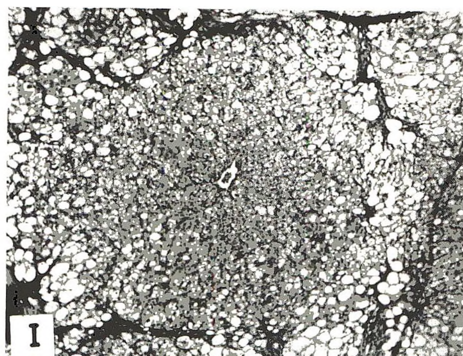
VAN DE KAMER, J. H., H. B. BOKKEL HUININK AND H. A. WEYERS 1949 Rapid method for the fat determination of fat in feces. J. Biol. Chem., *177:* 347.

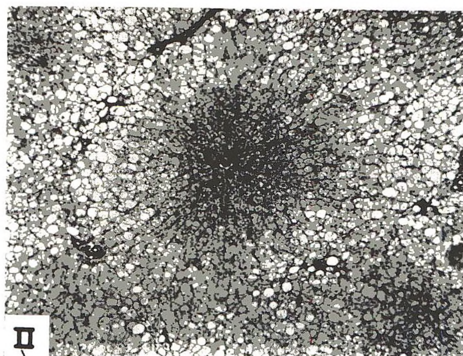
YOUNG, R. J., L. C. NORRIS AND G. F. HEUSER 1955 The chick's requirement for folic acid in the utilization of choline and its precursors betaine and methyl amino ethanol. J. Nutrition, *55:* 353.

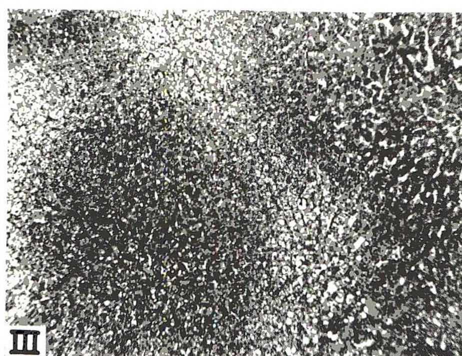
PLATE 1

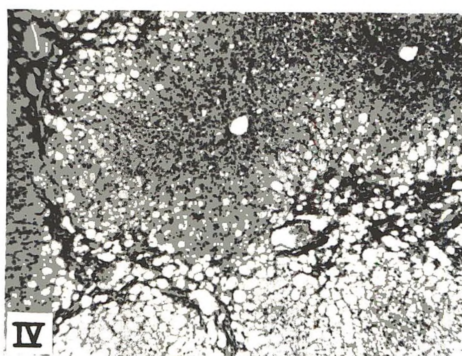
EXPLANATION OF FIGURES

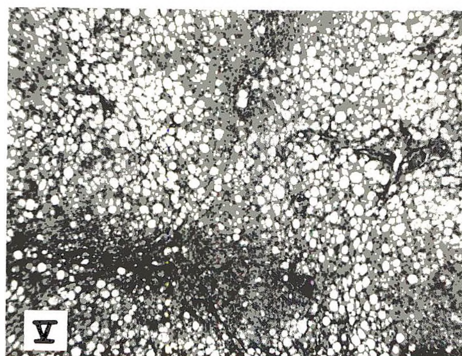
Histologic sections of liver, after 8 weeks on experiment; H. E. (× 100.) I to V, purified ration. I (without choline): gross periportal fatty infiltration with large confluent fatty cysts; perilobular fibrosis; mild fat infiltration of the centrolobular area. II (0.1% choline): gross periportal fat infiltration; centrolobular area well preserved; mild perilobular fibrosis. III (0.5% choline): mild periportal fat infiltration with normal centrolobular area. IV (without choline and with 10% liver) and V (without choline and with 10% pancreas) exhibit the same pattern as group I. VI ("natural ration"): control.

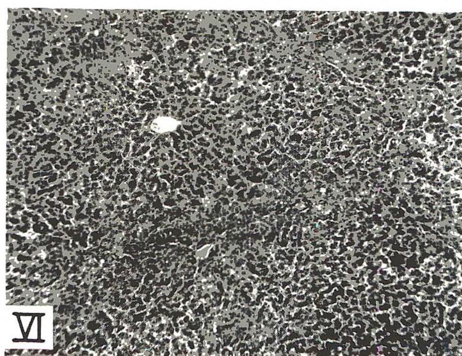

I


II


III


IV


V


VI

EFFECT OF LEVEL OF PROTEIN FEEDING UPON NUTRITIONAL VALUE OF LYSINE-FORTIFIED BREAD FLOUR [1]

ROBERT S. HARRIS AND DONALD A. BURRESS
Department of Food Technology, Massachusetts Institute of Technology, Cambridge

(Received for publication April 3, 1958)

Many investigators have confirmed the observation first made by Osborne and Mendel ('14) that the growth of rats is significantly improved by the addition of lysine to wheat protein. These investigators have generally reported, however, that lysine-fortified wheat protein is still considerably inferior to animal proteins.

Essentially all these comparisons have been made with diets containing only 12, 10, 8 or even 5% of total protein. Osborne et al. ('19) had demonstrated in growth experiments, and Mitchell ('23–'24) in nitrogen balance studies, that the utilization of protein is affected by its dietary level. Barnes et al. ('45) and others, have reported that the efficiencies of animal proteins are highest when fed at low levels (8 to 12%) in the diet of rats, whereas the efficiencies of cereal proteins improve as the level in the diet approaches 20%. Indeed, Barnes and Bosshardt ('46) commented that "the common practice of employing a 10% protein diet, regardless of the nature of the protein, will result in a considerable distortion of nutritive values, and the magnitude of the error will increase as the nutritive quality of the protein decreases."

Thus it may not be advisable to compare vegetable with animal proteins using diets containing low levels of protein.

[1] Presented at the 133rd national meeting of the American Chemical Society, April 14, 1958, at San Francisco. Contribution no. 365 from the Department of Food Technology, Massachusetts Institute of Technology, Cambridge.

Possibly the tradition for the use of low-protein diets developed because most vegetable foods are low in content of protein (i.e., rice 7.6%, corn meal 7.8%, potato flour 7.0%, banana 1.2%, farina 10.9%, squash 1.9%, sweet potato 1.8%, etc., Watt and Merrill, '50), for when a diet contains a maximum amount of one of these foods, the protein content cannot exceed 12%. It is necessary first to concentrate these food proteins in order to feed them at high levels in experimental diets.

Rats are truly in a state of protein starvation when they are maintained on diets containing as little as 12% protein. A level of at least 15% protein of high biological value is required in the diets of rats to produce satisfactory growth, and as much as 25% protein is required for the most rapid weight gain (Zucker and Zucker, '44). It may be questioned, therefore, whether comparisons of proteins in protein-starved rats are valid.

It is logical to suggest that when a food is evaluated for human use, the protein should be fed in the diet of experimental animals at a level approximating that of the dietaries of human beings. Dole ('57), using the Food and Agriculture Organization (Anon, '55) statistics of food, calculated that 31 of 32 nations consume in excess of 10% of protein calories and that 6 nations consume more than 13% of protein calories. From the data on food consumption in the United States according to different income groups (Anon, '49), Dole found that practically all groups consume 10 to 13% of protein calories. This is in good agreement with our calculation from recently published data (Anon, '57) that the average "consumption" in the U. S. in 1955 was about 11% protein calories.

All these data represent food disappearance rather than food consumption. For instance, the 1955 survey (Anon, '57) showed that an average of 3200 Cal. per capita were brought into the kitchen; however, food discarded during or after preparation or as plate waste was not deducted. Since it is customary to reject the carbohydrate and fat portions of food and to retain the protein portions, it is likely that the value for protein calories actually consumed was even higher than 11%.

The fat content of the average diet is not known exactly. It has been reported (Anon, '57) that the food entering kitchens of 5050 homes in the U. S. in 1955 contained 44% of fat calories. The Food and Nutrition Board of the National Research Council (Anon, '53) has advised 20% fat calories in the diet. The true intake in this country lies between 20 and 44%. Diets which contain 20 to 44% of fat calories and 11 to 13% of protein calories will contain 10 to 25% of fat and 12 to 15% of protein, dry basis. Thus, the diets of mankind appear to contain at least 12 to 15% of protein, and the average diet in the United States contains nearly 15% of protein.

These calculations indicate that protein evaluation studies would have more meaning in terms of human nutrition if the level of protein in the experimental diets approached 15% rather than 8%. One purpose of the research reported here was to evaluate the effects of lysine supplementation of flour protein when fed at 8% and 15% levels, in comparison with egg albumin at these levels.

A second purpose in this study was to evaluate the possible effects of imbalance when an excess of lysine is added to wheat flour. Berg ('53) indicated that the percentage of DL-lysine required to stop growth in rats was at least 8 times that needed for maximal gains in weight. Elvehjem and Harper ('55) have summarized reports that excess levels of certain amino acids may increase the requirements of other amino acids, may interfere with growth, or even be toxic. Though it is unlikely that flour will be fortified with excess lysine because of the high cost, it is important to determine whether the imbalance produced by adding a large excess of lysine is in any way harmful.

EXPERIMENTAL PROCEDURE

The diets used in this research are modifications of those used by Deshpande et al. ('55). The diets in series I (see table 1) contained 8% protein supplied by 70% extraction bread flour (diets A to D) or by egg albumin (diet E). Diets A to D were fortified with 0, 0.31, 0.62 and 3.48 gm of L-lysine·HCl per 100 gm of diet. The latter level of lysine is 5 to 8 times

that reported by Rosenberg and Rohdenberg ('52) and by Sure ('57) to be necessary to prevent lysine from being the most limiting amino acid in wheat flour. The diets in series II contained 15% protein supplied by bread flour (8%) and wheat gluten (7%) (diets F to I) or by egg albumin (diet J). Diets F to I were fortified with the same amounts of L-lysine·HCl per gram of protein as diets A to D, respectively.

The lysine content of each of the dietary constituents was estimated microbiologically (Barton-Wright, '52) using *B. mesentericus,* and from these data the lysine content of each diet was calculated (table 1). The amino acid content, and especially the lysine content, of wheat gluten (Pence et al., '50) is very similar to that of bread flour (Hepburn et al., '57).

The moisture and protein contents of the foods used in these diets were: wheat bread flour (10.6 and 12.8%), wheat gluten (6.0 and 79.3%), wheat starch (9.2 and 0.26%), egg albumin (7.9 and 80.2%), respectively. The protein content of the wheat products was calculated by the factor $N \times 5.7$; that of the egg albumin was calculated by the factor $N \times 6.25$.

Two hundred and fifty-two male weanling rats (Sprague-Dawley) were placed in individual metal cages ($12'' \times 3'' \times 5''$) with raised bottoms and offered diet A (table 1) for three days. They were then divided into 10 groups of 24 each and a half group of 12, with equal distribution according to weight and litter, and assigned to diets A to J (table 1). The diets and distilled water were offered ad libitum.

On day zero the 12 rats in the half-group (group K) were sacrificed by decapitation. The livers were pooled, weighed and preserved at −18°C until analyzed. The entrails and organs were removed, the carcasses were pooled, weighed, macerated and preserved at −18°C until analyzed.

The body weight of each surviving rat was recorded twice during the first week, then weekly until the end of the experiment. Records were made of the food intake on a group basis (table 4). At the end of 14 days, and again after 28 days, 12 rats from each group were sacrificed, and the pooled livers

TABLE 1

Composition of diets

CONSTITUENT	A	B	C	D	E	F	G	H	I	J
	%	%	%	%	%	%	%	%	%	%
Lysine · HCl		0.31	0.62	2.48			0.59	1.18	4.72	
Sucrose	28.0	27.69	27.38	25.52	26.7	19.2	18.61	18.02	14.48	21.58
Wheat starch					54.0					50.38
Bread flour, 70% extn.[1]	62.5	62.5	62.5	62.5		62.5	62.5	62.5	62.5	
Wheat gluten						8.8	8.8	8.8	8.8	
Egg albumin					9.8					18.54
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salts[2]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mixture[3]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total protein content	8.0	8.0	8.0	8.0	8.0	15.0	15.0	15.0	15.0	15.0
Total lysine content[4]	0.17	0.42	0.67	2.17	0.78	0.32	0.80	1.27	4.12	1.47

[1] KAK, Omaha Flour Mills.

[2] Hegsted et al. ('41).

[3] Vitamin mixture (mg/100 gm diet): thiamine·HCl 0.5, riboflavin 0.5, niacin, 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, folic acid 0.02, vitamin B_{12} 0.002, inositol 10.0 and choline chloride 150.0. Also, vitamin A (400 I.U.), vitamin D (4 I.U.), Menadione 0.04 mg, and alpha-tocopherol 4.0 mg, orally once each week.

[4] Calculated from data obtained by analysis of each dietary component for lysine content (egg albumin 7.9%, bread flour 0.27%, wheat gluten 1.73%, starch negative).

and carcasses were prepared and preserved as above until analyzed.

The water, alcohol-ether extract, nitrogen and ash contents of each of the pooled samples of liver and carcass were estimated according to AOAC ('55) procedures, and are reported in table 2. The increases in carcass nitrogen per gram of food nitrogen are presented in table 4.

RESULTS AND DISCUSSION

Series I. (8% protein diets)

Body weight gains. During the 28-day period, the weight gain of group A (5.9 gm) was significantly less than the gains of groups B to E, the body weight gain of group E (91.3 gm) was significantly greater than the gains of groups A to D, and the gains of groups B, C and D were the same (table 3). These data indicate that lysine was the limiting essential amino acid of the flour protein, that 0.62 gm of L-lysine·HCl per 8 gm of bread flour protein was sufficient to remove this limitation, and that the lysine-fortified protein was inferior to the egg albumin protein when fed in these diets at the 8% level. The high level of lysine in diet D had no adverse effect on body weight gain.

Protein efficiency (P.E.). The effects of differences in the amount of diet consumed by rats fed ad libitum may be corrected to a considerable extent by calculating the grams gain in body weight per gram of protein consumed (P.E.) These data are presented in table 3 on a weekly basis and on a 28-day basis. During each time interval, the group which received the egg albumin (E) showed a higher P.E. than the groups fed lysine-supplemented flour (B,C,D), and these in turn showed a higher P.E. value than the group fed the unsupplemented flour (A). Thus, the lysine supplementation improved the nutritional value of the wheat flour, but egg albumin was still significantly superior.

Liver composition. The livers of the rats fed bread flour (A) contained more ether-extract and less nitrogen than the

TABLE 2

Composition of carcasses and livers

GROUP	DIET CHARACTERISTICS	WEIGHTS		WATER	ETH.-ETHER EXTRACT	NITROGEN	ASH	LIVER		LIVER COMPOSITION			
		Body	Carcass					Wt.	Wt./gm body wt.	Water	Eth.-ether extract	Nitrogen	Ash
		gm	gm	%	%	%	%	gm	gm	%	%	%	%
				0 day autopsy									
K	0 day control	49.7	34.9	70.9	4.7	2.98	4.3	1.82	0.037	70.1	1.4	2.59	1.3
				Series A. 14-day autopsy									
A	8% flour protein	53.8	40.8	66.3	11.0	3.03	4.3	2.02	0.038	71.8	4.4	2.68	1.2
B	A + 0.31% L-lysine·HCl	60.1	46.2	66.9	8.4	3.14	4.3	2.16	0.036	71.2	2.5	2.94	1.4
C	A + 0.62% L-lysine·HCl	58.3	44.6	67.2	10.8	3.10	4.3	2.35	0.040	70.6	2.2	2.73	1.3
D	A + 2.48% L-lysine·HCl	55.8	44.5	67.5	9.2	3.22	4.2	2.00	0.036	70.8	2.0	2.76	1.3
E	8% egg albumin protein	97.5	76.5	66.9	9.5	3.05	3.9	4.32	0.044	69.6	3.0	2.58	1.2
				Series A. 28-day autopsy									
A	8% flour protein	57.9	45.5	63.4	10.4	3.12	5.3	2.06	0.036	70.4	5.8	2.46	1.2
B	A + 0.31% L-lysine·HCl	71.6	55.3	66.5	10.7	3.12	4.6	2.97	0.041	70.7	2.9	2.64	1.2
C	A + 0.62% L-lysine·HCl	71.2	55.3	66.5	9.4	3.05	4.6	2.88	0.040	71.6	2.6	2.76	1.3
D	A + 2.48% L-lysine·HCl	70.1	55.6	67.6	8.8	3.12	4.4	2.70	0.038	70.8	2.2	2.74	1.3
E	8% egg albumin protein	143.3	116.7	65.3	12.0	3.12	3.8	5.89	0.041	68.6	3.2	2.63	1.3
				Series B. 14-day autopsy									
F	8% flour + 7% gluten protein	63.5	49.4	66.4	10.1	3.09	4.7	2.26	0.036	72.8	2.3	2.86	1.3
G	F + 0.59% L-lysine·HCl	110.2	87.4	67.8	12.7	3.04	3.6	4.39	0.040	71.4	2.1	3.03	1.3
H	F + 1.18% L-lysine·HCl	106.3	77.3	67.4	11.6	2.96	3.6	4.00	0.038	70.2	1.7	2.98	1.4
I	F + 4.72% L-lysine·HCl	91.7	72.9	68.0	10.2	3.18	3.6	3.68	0.040	71.3	1.2	2.95	1.4
J	15% egg albumin protein	117.6	90.2	70.5	5.2	3.10	3.0	5.47	0.046	70.3	1.2	3.08	1.4
				Series B. 28-day autopsy									
F	8% fluor + 7% gluten protein	77.5	60.9	66.4	10.8	2.91	3.8	3.28	0.042	70.4	2.6	2.66	1.3
G	F + 0.59% L-lysine·HCl	163.2	130.9	65.7	11.4	3.10	3.5	6.60	0.040	70.2	2.3	3.16	1.3
H	F + 1.18% L-lysine·HCl	170.4	137.9	65.6	11.6	3.06	3.4	7.38	0.043	70.4	2.0	3.06	1.4
I	F + 4.72% L-lysine·HCl	154.3	121.1	66.3	9.6	3.26	3.3	6.69	0.043	70.0	1.2	3.08	1.3
J	15% egg albumin protein	162.3	130.3	67.9	7.2	3.26	3.0	6.82	0.042	68.0	1.4	3.30	1.4

TABLE 3

Body weight gains and protein efficiencies expressed on weekly and 4-weekly basis

GROUP	DIET CHARACTERISTIC	0–7 DAYS		7–14 DAYS		14–21 DAYS		21–28 DAYS		0–28 DAYS	
		gain /rat	P.E.[1]	gain /rat	P.E.	gain /rat	P.E.	gain /rat	P.E.	gain /rat	P.E.
		gm		*gm*		*gm*		*gm*		*gm*	
				Series I							
A	8% flour protein	1.0	0.16	0.1	0.03	3.0	0.56	1.8	0.4	5.9	0.25
B	A + 0.31% L-lysine·HCl	1.9	0.31	4.7	0.86	5.7	1.2	7.3	1.5	19.6	0.85
C	A + 0.62% L-lysine·HCl	1.3	0.22	3.8	0.84	6.8	1.8	7.3	1.8	19.2	0.95
D	A + 2.48% L-lysine·HCl	– 0.4	0.09	3.8	0.82	7.7	1.5	7.3	1.5	18.4	0.70
E	8% egg albumin protein	21.7	3.2	24.0	3.5	19.7	2.6	25.9	4.1	91.3	3.37
				Series II							
F	8% flour + 7% gluten protein	6.3	0.51	5.1	0.44	7.4	0.8	7.3	0.8	26.1	0.60
G	F + 0.59% L-lysine·HCl	23.3	1.8	31.0	2.4	20.3	1.7	36.7	2.6	111.3	3.00
H	F + 1.18% L-lysine·HCl	25.3	2.0	32.3	2.4	32.1	2.0	29.4	2.3	119.0	2.06
I	F + 4.72% L-lysine·HCl	17.3	1.5	25.3	2.2	30.3	2.1	29.4	2.5	102.3	1.6
J	15% egg albumin protein	29.5	2.9	35.5	3.1	23.1	1.8	22.1	1.9	110.2	2.35

[1] P.E. = Protein Efficiency (gm wt. gain/gm protein intake). In calculating the protein efficiencies the weights of the lysine supplements were included.

TABLE 3 (*Continued*)

Analysis of variance of the body weight data (0–28 days) in series I showed a between-sample variance estimate of 13763, based on 4 degrees of freedom, and a within-sample variance estimate of 51, based on 55 degrees of freedom, yielding an F ratio of 270 which is highly significant.

Internal comparisons were made between means according to Tukey ('57). The figures within parentheses are the D values (sample standard error of the mean × Q). When the D value is lower than the value above it, the difference is significant:

GROUP	$\bar{X}$	$\bar{X}$–70.8	$\bar{X}$–19.17	$\bar{X}$–20.08	$\bar{X}$–20.58
E	91.42	84.34 [2]	72.25 [2]	71.34 [2]	70.84 [2]
		(9.93)	(9.48)	(8.82)	(7.75)
B	20.58	13.50 [2]	1.41	0.50	
		(9.48)	(8.82)	(7.75)	
C	20.08	13.00 [2]	0.91		
		(8.82)	(7.75)		
D	19.17	12.09 [2]			
		(7.75)			
A	7.08				

[2] Significant difference between treatment means at $P = 0.01$ level. Thus, A < B, C, D, E; E > B, C, D.

A similar analysis of the body weight data (0–28 days) in series II showed a between-sample variance estimate of 17634, based on 4 degrees of freedom, and a within-sample variance estimate of 235, based on 55 degrees of freedom, yielding an F ratio of 75 which is highly significant.

Internal comparisons between means yielded the following:

GROUP	$\bar{X}$	$\bar{X}$–26.17	$\bar{X}$–102.6	$\bar{X}$–110.2	$\bar{X}$–111.8
H	118.9	92.73 [3]	16.3	8.7	7.1
		(21.3)	(20.3)	(18.9)	(16.6)
G	111.8	85.63 [3]	9.2	1.6	
		(20.3)	(18.9)	(16.6)	
J	110.2	84.03 [3]	7.6		
		(18.9)	(16.6)		
I	102.6	76.43 [3]			
		(16.6)			
F	26.17				

[3] Significant difference between treatment means at $P = 0.01$ level. Thus, F < G, H, I, J.

livers of rats fed lysine-supplemented bread flour (B,C,D). Lysine deficiency causes fatty livers in rats (Harper, '56), and is curable and preventable by lysine supplementation. The livers of the rats fed egg albumin (E) for 14 days were heavier and contained less water than the livers of rats fed lysine in the diet (B,C,D); in other respects they were similar. After 28 days, the livers of the rats fed egg albumin (E) contained slightly more fat and less nitrogen than the lysine-supplemented groups. The liver data do not show any inferiority in the lysine-supplemented groups as compared to the group fed egg albumin.

Carcass composition. The moisture, alcohol-ether extract and nitrogen contents of the carcasses of the 5 groups of rats (A to E) were not significantly different at the end of either 14 or 28 days. The ash content of the carcasses was lowest in the rats fed egg albumin (E), and highest in the group fed unsupplemented bread flour (A). This may be interpreted to mean that the lysine-deficient protein-starved rat deposits less body tissue per gram of skeleton.

The increases in carcass N per gram of dietary N, as a result of feeding diets A to E during days zero to 14, 14 to 28 and zero to 28, are presented in table 4. The amount of lysine fed in the diet of group D was excessively high, and undoubtedly a significant amount of it was not utilized in protein synthesis. Since supplementation of the flour with 0.62 gm of lysine per 100 gm of diet was ample, diet D contained 1.86 gm of lysine per 100 gm which was not biologically effective. If this excess is omitted in the calculation of the protein efficiency the "corrected" P.E. for group D is identical to that of group C. Therefore, the excess lysine had no effect on the efficiency of carcass protein synthesis.

The efficiency of carcass synthesis in the groups fed 8% egg albumin diets (table 4) was about three times better than that of any of the groups fed 8% wheat flour diets supplemented with lysine. It is clear that the lysine-fortified wheat flours were biologically inferior to egg albumin, when fed at 8% levels in the diet.

TABLE 4

Increase in carcass nitrogen in terms of food nitrogen intake

GROUP	DIET CHARACTERISTIC	AV. DIET INTAKE /RAT	NITROGEN /100 GM DIET	AV. DIET N INTAKE /RAT	AV. INCREASE CARCASS N/RAT	AV. INCREASE CARCASS N INCREASE /GM DIET N
		gm	*gm*	*gm*	*gm*	*gm*
		Series A.	*0 to 14 days*			
A	8% flour protein	155	1.4	2.18	0.196	0.090
B	A + 0.31% L-lysine·HCl	144	1.456	2.10	0.410	0.195
C	A + 0.62% L-lysine·HCl	128	1.503	1.92	0.342	0.178
D	A + 2.48% L-lysine·HCl	119	1.782	2.12 (1.79)	0.393	0.185 (0.220)
E	8% egg albumin protein	169	1.28	2.17	1.29	0.595
		Series A.	*14 to 28 days*			
A	8% flour protein	137	1.4	1.92	0.184	0.096
B	A + 0.31% L-lysine·HCl	133	1.456	1.94	0.275	0.142
C	A + 0.62% L-lysine·HCl	108	1.503	1.62	0.304	0.188
D	A + 2.48% L-lysine·HCl	133	1.782	2.37 (1.98)	0.301	0.127 (0.152)
E	8% egg albumin protein	170	1.28	2.18	1.31	0.601
		Series A.	*0 to 28 days*			
A	8% flour protein	290	1.4	4.10	0.380	0.093
B	A + 0.31% L-lysine·HCl	278	1.456	4.04	0.685	0.169
C	A + 0.62% L-lysine·HCl	235	1.503	3.54	0.646	0.183
D	A + 2.48% L-lysine·HCl	252	1.782	4.49 (3.77)	0.694	0.155 (0.183)
E	8% egg albumin protein	339	1.28	4.35	2.60	0.598
		Series B.	*0 to 14 days*			
F	8% flour + 7% gluten protein	159	2.63	4.17	0.486	0.117
G	F + 0.59% L-lysine·HCl	170	2.718	4.61	1.62	0.352
H	F + 1.18% L-lysine·HCl	171	2.807	4.81	1.25	0.260
I	F + 4.72% L-lysine·HCl	155	3.338	5.19 (4.36)	1.28	0.247 (0.294)
J	15% egg albumin protein	144	2.40	3.46	1.75	0.506
		Series B.	*14 to 28 days*			
F	8% flour + 7% gluten protein	129	2.63	3.40	0.245	0.072
G	F + 0.59% L-lysine·HCl	186	2.718	5.07	1.40	0.276
H	F + 1.18% L-lysine·HCl	186	2.807	5.21	1.93	0.370
I	F + 4.72% L-lysine·HCl	169	3.338	5.64 (4.75)	1.63	0.289 (0.343)
J	15% egg albumin protein	170	2.4	4.07	1.46	0.359
		Series B.	*0 to 28 days*			
F	8% flour + 7% gluten protein	288	2.63	7.57	0.731	0.097
G	F + 0.59% L-lysine·HCl	357	2.718	9.69	3.02	0.312
H	F + 1.18% L-lysine·HCl	358	2.807	10.02	3.18	0.317
I	F + 4.72% L-lysine·HCl	325	3.338	10.83 (9.11)	2.91	0.269 (0.319)
J	15% egg albumin protein	313	2.4	7.53	3.21	0.426

Note: values within parentheses were calculated on the basis that half the added lysine in diets D and I was not utilized in protein synthesis. This was done to determine whether the lower carcass N increase/gm diet N was due to poor N utilization, to lysine "toxicity," or both.

Series II. (15% protein diets)

Body weight gains. During the 28 days' feeding of 15% protein diets, the weight gain of group F (26.1 gm) was significantly less than the gains of groups G to J, and the body weight gain by group H (119.0 gm) was significantly higher than the gains by groups G, I and J, which were not significantly different (table 3). These data indicate that lysine was the most limiting amino acid of the flour protein, that this deficiency was not overcome by adding 0.59 gm of L-lysine·HCl (group G), but was overcome by adding 1.18 gm of L-lysine·HCl per 15 gm of wheat protein (group H), and that the body weight gains of the rats fed this lysine-fortified protein at the 15% level (H) were significantly higher than those of rats maintained on a similar diet which contained 15% of egg albumin.

Protein Efficiency (P.E.). At the 15% level of protein feeding, the addition of lysine (G,H,I) more than doubled the P.E. of bread flour protein (F).

The P.E. value of the group fed egg albumin was highest during the first two weeks, while that of the rats fed wheat protein supplemented with three levels of lysine (G,H,I) was approximately equal to that of rats fed egg albumin group (J). While the data for the total 28-day period indicate that the P.E. of the egg albumin diets was superior, the trend was in the opposite direction during the last week of the experiment.

The group fed 4.72% of L-lysine·HCl (I) showed a lower P.E. value than those (G,H) fed lower levels of lysine. This group was given an 5 to 8-fold excess of lysine in order to evaluate its effects. Since supplementation of the flour with 1.18% of L-lysine·HCl per 100 gm of diet is ample, diet I contained 3.54 gm (4.72 − 1.18 gm) of L-lysine·HCl per 100 gm of diet which was not biologically useful. If this excess is omitted from the calculation, the "corrected" P.E. value of diet I becomes 1.95. It is likely that the lower P.E. of diet I is due to

incomplete utilization of the excess lysine, rather than a manifestation of toxicity.

These P.E. data indicate that diets containing 15% of wheat protein supplemented with L-lysine·HCl may be satisfactory in the nutrition of the rat.

Liver composition. The liver weight per gram body weight was essentially the same in all groups at the end of 28 days. The fat content of the livers decreased as the lysine content of the diets was increased. The protein content of the livers at 28 days was highest in the group fed egg albumin.

Carcass composition. The additions of lysine (G,H,I) did not affect the amount of fat in the carcass of the rats fed wheat protein (F). The carcass fat in the rats fed egg albumin was definitely lower.

During 28 days the carcass ash content increased and the nitrogen content increased more or less according to the lysine content. Thus, the improvement in protein quality caused proportionally greater deposition of body tissue per gram of skeleton.

The efficiency of synthesis of carcass N by the rats on these 15% protein diets is shown in table 4. There is no evidence that the excess lysine interfered with the deposition of carcass N.

The three-fold increase in the synthesis of carcass protein resulting from the addition of lysine to the 15% wheat protein diets was nevertheless not as great as that produced by the 15% egg protein diets. Since the nutritional value of a food protein can be measured quite satisfactorily in terms of carcass protein synthesis, it is clear that the lysine-fortified wheat protein was still biologically inferior to egg albumin. This result is to be expected, for the total essential amino acid content of egg albumin is one and one-half times that of 70% extraction flour protein. One hundred grams of flour contain 28.4 gm of the essential amino acids required for nitrogen balance in man (Hepburn et al., '57). Even when 3.2 gm of L-lysine (= 4.0 gm of L-lysine·HCl) are added as in diet G, the content is still only 31.6 gm (table 5). On the other hand, egg

albumin contains 48.1 gm essential amino acids per 100 gm (Block and Weiss, '56). In spite of this, the amount of carcass protein synthesized "per gram of essential amino acids fed" was essentially the same in this experiment. A possible reason for this result is indicated in table 5, in which the proportions of each of 8 essential amino acids per 100 gm of total essential amino acids in lysine-fortified flour and in egg albumin proteins are presented. By fortification of the flour with lysine, the most important difference in the essential amino acid ratios of these foods is eliminated, and the ratios of essential amino acids in these two proteins are nearly the same except in methionine content. The lysine-fortified flour protein is inferior to egg albumin also because of a greater dilution by the non-essential amino acids, especially glutamic acid.

TABLE 5

Comparison of the essential amino acid composition of lysine-fortified flour and egg albumin

ESSENTIAL AMINO ACIDS FOR N BALANCE IN MAN	ESSENTIAL PER 16 GM N		GRAMS PER 100 GM ESSENTIAL AMINO ACID		RATIOS FOR N BALANCE IN MAN [3]
	lysine-fort. flour [1]	egg albumin [2]	lysine-fort. flour	egg albumin	
Isoleucine	4.26	6.8	13.5	14.1	11.0
Leucine	6.98	9.0	22.2	18.7	17.3
Lysine	5.28	6.5	16.8	13.6	12.6
Methionine	1.73	4.9	5.4	10.2	17.3
Phenylalanine	4.92	7.1	15.6	14.8	17.3
Threonine	2.82	4.2	8.9	8.7	7.9
Tryptophan	1.02	1.5	3.2	3.1	4.0
Valine	4.54	8.1	14.4	16.8	12.6
Totals	31.55	48.1	100.0	100.0	100.0

[1] Composition reported by Hepburn et al. ('57), plus 3.2 gm lysine.
[2] From Block and Weiss ('56).
[3] From Rose ('49).

The ratios of essential amino acids required for nitrogen balance in man are listed in table 5. It is evident that both lysine-fortified flour protein and egg albumin are deficient in

methionine, and to a lesser extent in tryptophan and phenylalanine.

The data on body weight gain and protein efficiency indicate that the protein of lysine-fortified wheat flour is nearly equal to that of egg albumin in the nutrition of the rat, when fed at the 15% level in the diet. However, the data on the amount of carcass protein synthesized demonstrate that the protein of egg albumin is superior. It is evident that the nutritional values of proteins can be compared properly only when the increases in the carcass protein resulting from feeding dietary proteins are measured.

Comparison between series I and II

Comparison of the data from series I and II indicates clearly that different results can be obtained on rats when nutritional evaluations of proteins are carried out with diets containing 15% protein rather than with 8% protein. Barnes et al. ('45) reported that the protein efficiency of whole egg protein was highest when fed for 42 days at a level of 10% in the diet of rats; the efficiency decreased when lower or higher levels of egg protein were fed. They reported also that the efficiency of wheat gluten improves as the level of protein in the diet is increased up to 20%. The results reported here conform with this finding.

These data also indicate that whereas lysine-supplemented wheat flour protein was quite inferior to egg albumin when fed at an 8% level, it was more nearly equivalent when fed at a 15% level in the diet. The amino acid requirements of the rats at these two levels of protein intake were not the same. This may explain why approximately 3.9 gm of L-lysine·HCl per 100 gm was sufficient to eliminate the deficiency of wheat protein in the 8% protein diets (group B), whereas approximately 7.9 gm per 100 gm were necessary in the 15% protein diets. Block and Weiss ('56) reported that the requirements of rats for lysine increase disproportionately as the protein content of the diet is raised.

It is generally agreed that a balanced intake of essential amino acids is especially important to those who are consuming diets low in total protein content. This can be achieved by a balanced intake of complementary food proteins or by the supplementation of unbalanced food proteins with the specific amino acids in which they are deficient. The present study, like others previously reported, indicates that lysine can significantly improve the nutritional value of wheat protein.

Since the average dietary level of protein intake of human beings approaches 15% (dry weight basis), and since the level of protein intake is of critical importance in the evaluation of proteins, it is suggested that proteins intended for human consumption should be evaluated in diets at the 15% level, whenever possible. The protein of those foods which are low in protein content may be concentrated by the removal of starch or fat, but this might affect the quality of the protein. An alternative method would be one in which all diets contain 5% standard animal protein (i.e., egg albumin), or 5% combined animal proteins (i.e., equal parts of egg albumin, liver and non-fat milk powder) in order to provide a background of good proteins in all diets. To these diets would be added 10% test protein or 10% animal protein. This would, in effect, be a measure of the animal protein replacement value of the test proteins, a technique similar to that first suggested by Murlin and Mattill ('38). These authors studied the milk replacement value of cereals by supplying 80% of the dietary nitrogen as milk, or as cereals, in alternate periods, and continually supplying 10% of the dietary nitrogen as milk and 10% as fruit.

In this research, rats received 5 to 8 times as much lysine as was necessary for bread flour fortification. The rats receiving this high level of lysine consumed slightly less diet and showed slightly lower protein efficiency than those receiving smaller amounts of lysine in the diet, yet the composition of the carcass or liver was the same. The lower protein efficiency can be accounted for entirely on the basis that the excess lysine was not utilized for body protein synthesis. There was no evidence

of any toxicity resulting from the high levels of lysine intake. This is to be expected, for lysine is unique among essential amino acids in that its excess does not exert antimetabolic effects upon other amino acids (Rauen, '56).

SUMMARY AND CONCLUSIONS

Four diets containing 8% wheat protein (70% extraction wheat flour) were supplemented with zero, 0.31, 0.62 and 2.48 gm of L-lysine·HCl per 100 gm. Four diets containing 15% wheat protein (8% wheat flour plus 7% wheat gluten) were supplemented with zero, 0.59, 1.18 and 4.72 gm of L-lysine·HCl per 100 gm. These 8 diets, plus two diets containing 8 and 15% egg albumin protein, were fed to weanling rats for 14 and 28 days. The carcasses and livers were then analyzed for content of water, fat, ash and nitrogen.

The lysine deficiency of the bread flour protein was overcome by including 0.31 and 1.18 gm of L-lysine·HCl per 100 gm of diets containing 8 and 15% wheat protein, respectively. The total lysine content of the protein in these diets was 5 and 7.8%, respectively.

When fed at the 8% level, lysine-fortified wheat protein was nutritionally inferior to egg albumin as measured by body weight increase, protein efficiency and carcass protein synthesis.

When fed at the 15% level, lysine-fortified wheat protein was nutritionally equal to egg albumin as measured by body weight increase and protein efficiency, but inferior in its ability to promote the synthesis of body protein. It is important to measure carcass protein synthesis when the nutritional values of proteins are evaluated.

The amount of carcass protein synthesized per gram of essential amino acids fed, whether as egg albumin or lysine-supplemented bread flour, was essentially the same. Thus the lysine-fortified bread flour protein was inferior in the rat because of dilution by glutamic acid and because of a poorer balance of essential amino acids.

"Fatty livers" were observed in the rats fed diets containing 8% of wheat protein, but not in those fed 15% of wheat protein. The "fatty liver" syndrome of lysine deficiency may not develop unless the intake of total protein is limited.

"Fatty livers" were prevented by the addition of lysine to the 8% wheat protein diets.

The nutritional evaluation of proteins intended for human use should be made by feeding experimental animals diets containing approximately 15% of protein, which corresponds to the level of protein intake by man.

A 5-fold excess of lysine produced no toxic effects.

LITERATURE CITED

ANON 1949 Nutritive value of diets of urban families of the United States, Spring 1948, and comparison with diets in 1942. 1948 Food Consumption Surveys Prelim. Report #12, U.S. Dept. Agric., Washington, D.C.

ANON 1953 Recommended Dietary Allowances, Revised 1953. Food and Nutrition Board, National Research Council, Washington, D.C.

ANON 1955 Food balance sheets. Second issue, Food and Agriculture Organization of the United Nations, Rome.

ANON 1957 Dietary levels of households in the United States, 1955. Household Food Consumption Survey, Report #6, U.S. Dept. Agric., Washington, D.C.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1955 Official and Tentative Methods of Analysis, 8th Ed., Washington, D.C.

BARNES, R. H., AND D. K. BOSSHARDT 1946 The evaluation of protein quality in the normal animal. Ann. N. Y. Acad. Sci., *47:* 273.

BARNES, R. H., J. E. MAACK, M. J. KNIGHTS AND G. O. BURR 1945 Measurement of the growth-promoting quality of dietary protein. Cereal Chem., *22:* 273.

BARTON-WRIGHT, E. C. 1952 Microbiological Assay of the Vitamin B Complex and Amino Acids. pp. 121–126. Pitman Pub. Corp., N. Y.

BERG, C. P. 1953 Physiology of the D-Amino Acids. Physiol. Rev., *33:* 145.

BLOCK, R. J., AND K. W. WEISS 1956 Amino Acid Handbook. C. C. Thomas, Springfield, Ill.

DESHPANDE, P. D., A. E. HARPER, F. QUIROS-PEREZ AND C. A. ELVEHJEM 1955 J. Nutrition, *57:* 415.

DOLE, V. P. 1957 A relation between dietary protein and total calorie intake of large population groups. J. Clin. Nutrition, *5:* 72.

ELVEHJEM, C. A., AND A. E. HARPER 1955 Importance of amino acid balance in nutrition, J. Am. Med. Assoc., *158:* 655.

HARPER, A. E. 1956 Amino acid imbalances, toxicities and antagonisms. Nutrition Reviews, *14:* 225.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459

HEPBURN, F. N., E. W. LEWIS, JR. AND C. A. ELVEHJEM 1957 The amino acid content of wheat, flour and bread. Cereal Chem., *34:* 312.

MITCHELL, H. H. 1923–24 The biological value of proteins at different levels of intake. J. Biol. Chem., *58:* 905.

MURLIN, J. R., AND H. A. MATTILL 1938 Digestibility and nutritive value of cereal proteins in the human subject. J. Nutrition, *16:* 15.

OSBORNE, T. B., AND L. B. MENDEL 1914 Amino acids in nutrition and growth. J. Biol. Chem., *17:* 325.

OSBORNE, T. B., L. B. MENDEL AND E. L. FERRY 1919 A method of expressing numerically the growth-promoting value of proteins. Ibid., *37:* 223.

PENCE, J. W., D. K. MECHAM, A. H. ELDER, J. C. LEWIS, N. S. SNELL AND H. S. OLCOTT 1950 Characterization of wheat gluten. II. Amino acid composition. Cereal Chem., *27:* 335–341.

RAUEN, H. M. 1956 Biochemisches Tachenbuch, page 1094. Springer-Verlag, Berlin.

ROSE, W. C. 1949 The amino acid requirements of man. Federation Proc., *8:* 546.

ROSENBERG, H. R., AND E. L. ROHDENBURG 1952 The fortification of bread with lysine. II. The nutritional value of fortified bread. Arch. Biochem. Biophys., *37:* 461.

SURE, B. 1957 Relative nutritive values of proteins in various foods at increasingly high levels of protein intake. Agric. Food Chem., *5:* 463.

TUKEY, J. W. 1957 Statistical Methods by Snedecor, 5th Ed., Iowa State College Press, p. 251.

WATT, B. K., AND A. B. MERRILL 1950 Composition of foods — raw, processed and prepared. Agric. Handbook #8, U.S. Dept. Agric., Washington, D.C.

ZUCKER, T. F., AND L. M. ZUCKER 1944 Significance of the protein level in synthetic diets. Proc. Soc. Exp. Biol. Med., *55:* 136.

FURTHER STUDIES ON THE DIFFERENCE IN CARIOGENICITY OF TWO DIETS COMPARABLE IN SUCROSE CONTENT

WINFREY WYNN, JOHN HALDI, KATHERINE D. BENTLEY AND MARY L. LAW

Department of Physiology, Emory University, Atlanta, Georgia

(Received for publication September 29, 1958)

A pronounced difference in cariogenicity has been noted in two highly purified diets each of which contained 64% sucrose (Wynn, Haldi, Shaw and Sognnaes, '53). These two diets, which have been employed routinely in the Harvard and Emory laboratories, respectively, in various studies on dental caries in the rat, have been referred to in previous publications as the Harvard and Emory diets. The Harvard diet has been found in a number of experiments conducted over a period of several years in our laboratories to be considerably more cariogenic than the Emory diet. In considering a possible explanation of the difference in their cariogenicity, it was noted that while both diets contained the same total amount (4%) of salt mixture and adequate amounts of all currently recognized essential minerals, there were several small but nevertheless possibly important differences in the relative amounts of different minerals.

In a recent study (Haldi, Wynn, Shaw and Sognnaes, '58), it was found that the cariogenicity of the Harvard diet was reduced by replacement of the Harvard salt mixture by the Emory salt mixture and conversely, the Emory diet was made more cariogenic by a corresponding interchange of salt mixtures. The present study was undertaken primarily to ascertain whether the effect obtained by the interchange of the salt mixtures of the diets could be related to the difference

in the concentration of certain specific ions in the two salt mixtures. The general procedure was to alter the concentration of a specific ion in the Harvard or Emory diet to determine whether the diet would thereby become more or less cariogenic.

It was noted that while the cariogenic properties of the two diets were modified by substituting in either diet the salt mixture ordinarily added to the other diet (Haldi, Wynn, Shaw and Sognnaes, '58), there still remained an appreciable difference in the cariogenicity of the diets after making this interchange of the salt mixtures. If the difference in cariogenicity of the two diets were due solely to the difference in composition of their salt mixtures, one might reasonably expect to find the two diets to be equally cariogenic when one salt mixture was substituted for the other. Since this was not the case, other factors apparently are involved besides the differences in the composition of the salt mixtures of the two diets.

In considering this problem it was noted that there was a difference in the relative amounts of the various vitamins added to the diets, although each vitamin mixture contained the different vitamins in amounts sufficient to meet currently recognized needs of the rat for adequate nutrition. Furthermore, yeast together with liver extract was added to the Emory diet whereas liver extract without yeast was added to the Harvard diet. In view of these considerations, it was decided that the scope of this investigation, which had been undertaken primarily to study the influence of the various ions on the cariogenic properties of the diet, should be enlarged to determine whether the difference in the cariogenicity of the diets may have been related to some extent to differences in the vitamin or the yeast content of the two diets.

EXPERIMENTAL

Two series of experiments were conducted in which variations were made (1) in the composition of the Harvard diet

and (2) in the composition of the Emory diet. The composition of the regular Harvard and Emory diets will be found in table 1. All experiments were done using the Emory-Wistar strain of albino rats. Since this strain is relatively caries-resistant, in order to accelerate the initiation and spread of dental caries, all the animals used in this study were desalivated at weaning by the technique described elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53). After desalivation, the animals were fed the experimental diets in littermate groups for a period of 70 to 80 days at which time the animals had developed moderate to severe caries. The animals were allowed to eat ad libitum. In the different experiments the animals were fed either the Harvard or Emory diet. If either of these diets were made more or less cariogenic by changes in the concentration of a specific ion, it might readily be assumed that a difference between the Emory and Harvard diets in the concentration of this ion was probably partially responsible for the difference in cariogenicity of the two diets.

At the end of the experimental feeding period, the animals were sacrificed and the mandibles and maxillae removed for examination of the teeth for the incidence and extent of caries. Examinations were made under a dissecting microscope and the teeth scored for caries according to the method described elsewhere (Haldi and Wynn, '52).

Series I—Harvard diet

In this series of experiments variations were made in the mineral components and in the vitamin components of the Harvard diet in the following manner:

Magnesium. The regular Harvard diet contains considerably less magnesium than the Emory diet: 0.033% as against 0.053%. Modified Harvard diets were prepared with twice the regular concentration of magnesium (0.066%), and 10 times the regular concentration (0.330%), by the addition of $MgSO_4$. The cariogenicity of each of these two diets was compared with that of the regular Harvard diet.

TABLE 1

Composition of the Emory and Harvard high-sucrose diets

CONSTITUENTS	EMORY DIET	HARVARD DIET
	%	%
Sucrose	64	64
Casein	20	23
Hydrogenated vegetable oil	8	—
Corn oil	—	5
Yeast and liver extract	4	—
Liver concentrate	—	4
Salt mixture	4	4

CONTRIBUTED BY SALT MIXTURE			VITAMINS		
	Emory	Harvard		Emory [1]	Harvard [2]
Potassium, %	0.72	0.45	Choline chloride, p.p.m.	3000	1000
Calcium, %	0.60	0.53	α-Tocopherol, p.p.m.	300	—
Sodium, %	0.40	0.44	Inositol, p.p.m.	130	1000
Phosphorus, %	0.38	0.55	Niacin, p.p.m.	32	25
Magnesium, %	0.053	0.033	Calcium pantothenate, p.p.m.	25	20
Chloride, %	0.18	0.76	Thiamine·HCl, p.p.m.	24	3.5
Iron, p.p.m.	480	150	Riboflavin, p.p.m.	24	3.5
Sulfur, p.p.m.	310	480	Pyridoxine·HCl, p.p.m.	10	3.5
Manganese, p.p.m.	78	50	β-Carotene, p.p.m.	3	30 (USPU/gm)
Copper, p.p.m.	10	15	Folic acid, p.p.m.	1	—
Zinc, p.p.m.	4	30	Biotin, p.p.m.	0.12	—
Iodide, p.p.m.	1.5	20	p-Aminobenzoic acid, p.p.m.	—	300
Aluminum, p.p.m.	0.16	—	Vitamin A concentrate, I.U./gm	10	—
Fluoride, p.p.m.	0.1	0	Viosterol, U.S.P. units/gm	3	1.5
Cobalt, p.p.m.	—	0.65	Linoleic acid, p.p.m.	500	—

[1] These values include the vitamins contained in the yeast and liver extract as supplied by the manufacturer.
[2] These values do not include the vitamins contained in the liver concentrate as these values were not available.

Manganese. The Harvard diet contains 50 p.p.m. of manganese as $MnSO_4$ which is approximately 30% less than that in the Emory diet. The regular Harvard diet and two modified Harvard diets containing 150 p.p.m. and 500 p.p.m. respectively, were prepared and fed to triplicate littermate groups of rats.

Brewers' Yeast. The Emory diet contains 4% yeast and liver extract while the Harvard diet contains 4% liver concentrate. An experiment was conducted in which 4% brewers' yeast was added to the regular Harvard diet and the cariogenicity of this diet was compared with that of the regular diet.

Yeast and liver extract. Another experiment was conducted in which litter mates were fed the Harvard diet and the same basic diet to which was added 4% yeast and liver extract.

Vitamin mixtures. Examination of the formulae of the Emory and Harvard diets in table 1 reveals several rather marked differences in the amounts of some of the added vitamins; for example, the Emory diet contains no *p*-aminobenzoic acid while the Harvard diet contains 300 p.p.m.; the Emory diet contains 300 p.p.m. of α-tocopherol, while the Harvard contains none; and the Emory diet has a concentration of 3000 p.p.m. of choline chloride and the Harvard only 1000 p.p.m. A study was conducted in which the Emory vitamin mixture replaced the Harvard vitamin mixture in the diet and the caries-conduciveness of this diet was compared with that of the regular Harvard diet.

Series II—Emory diet

In this series of experiments, variations were made in the mineral and vitamin components of the Emory diet, as follows:

Magnesium. Although the cariogenicity of the Harvard diet in the preceding experiments was not reduced by the addition of magnesium to the point where the concentration

was slightly higher than and also well in excess of that in the Emory diet, it was decided to conduct an experiment with the Emory diet to determine whether the cariogenicity of this diet would be affected by omitting magnesium from the salt mixture and also by adding twice the amount ordinarily added to the regular Emory diet. This procedure was suggested by the experiments of Klein, Orent and McCollum ('35) and of Irving ('40), which indicate that magnesium is essential for proper formation of both the incisor and molar teeth of the rat.

Sodium/potassium ratio. The amount of sodium in the Emory and Harvard diets is approximately the same while the Emory diet contains approximately 75% more potassium than the Harvard diet. This difference in potassium content makes the Na/K ratio equal to 1 : 1 in the Harvard and 1 : 2 in the Emory diet. The Emory diet was modified to give a Na/K ratio of 2 : 1 by increasing the sodium content to 0.56% and decreasing the potassium to 0.28%. Littermate pairs of rats were fed this modified Emory diet and the regular Emory diet to determine whether the Na/K ratio itself would affect the cariogenicity of a synthetic diet.

Iron. Another difference that may be seen in the composition of the Emory and Harvard diets is that the Emory diet contains 0.048% of iron and the Harvard diet contains 0.015%, both in the form of citrate. To determine whether a difference in iron concentration would have any effect on the cariogenicity of the Emory diet, 4 diets were prepared with (1) no added iron; (2) 0.024% iron; (3) 0.048% iron (regular Emory) and (4) 0.096% iron. The iron was added as ferric citrate. These diets were fed to quadruplicate littermate rats and their relative cariogenicity was determined.

Vitamin mixture. In the preceding series the Emory vitamin mixture was substituted for the Harvard vitamin mixture in the Harvard diet. In this experiment the Harvard vitamin mixture replaced the Emory vitamin mixture.

RESULTS AND DISCUSSION

Series I—Harvard diet

The caries incidence and the caries scores of the teeth of the animals fed the regular Harvard diet and the various modified Harvard diets are given in table 2. It may be seen from these data that increasing the amount of magnesium or of manganese in the Harvard diet had no significant effect on the number of carious lesions or the caries scores in the teeth of animals fed these diets. The results of these experiments, therefore, indicate that the concentrations of magnesium and manganese are not the factors responsible for the difference in cariogenicity of the Emory and Harvard diets.

As stated previously, the Harvard diet does not contain yeast while the Emory diet does. Furthermore, there are certain differences in the added vitamins in the two diets. It has been shown by Orland and co-workers ('54) that bacteria are necessary for the production of caries in the rat and that the vitamin content of the diet may affect the kind of oral flora (Orland, Hemmens and Harrison, '50) as well as the actual numbers of bacteria present (Orland, '46). These observations suggested to us that the difference in the cariogenicity of the Emory and Harvard diets may possibly be related to the vitamin mixtures added to the two diets. The experimental data in table 2 show that this is not the case. The cariogenicity of the Harvard diet, which is greater than that of the Emory diet, was not reduced by replacement of the Harvard vitamin mixture by the Emory vitamin mixture. On the contrary, it might appear from casual inspection of the data, that the Emory vitamin mixture may have increased the cariogenicity of the Harvard diet. The higher caries score, however, as compared with the score on the diet containing the Harvard vitamin mixture, was not statistically significant.

In view of the experiments reported by Dalderup and Jansen ('55) in which the addition of brewers' yeast to a high-sucrose diet reduced its cariogenicity, it was thought that the

TABLE 2

Dental caries in albino rats fed the Harvard diet with variations in mineral and vitamin components

VARIABLE FACTOR IN HARVARD DIET	NO. OF RATS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE CARIES SCORE	CRITICAL RATIO[1]
%				
Magnesium, 0.033	20	25 ± 0.4[2]	68 ± 5.3[2]	> 1.7
Magnesium, 0.066	20	25 ± 0.4	80 ± 5.1	> 1.3
Magnesium, 0.330	20	25 ± 0.4	74 ± 4.5	
Manganese, 0.005	25	23 ± 0.6	39 ± 2.4	> 1.8
Manganese, 0.015	25	24 ± 0.4	46 ± 2.8	> 0.9
Manganese, 0.050	25	24 ± 0.5	42 ± 2.7	
Brewers' yeast,[3] none added	20	22 ± 0.8	41 ± 4.2	> 0.5
Brewers' yeast,[3] 4.0	20	23 ± 0.8	44 ± 4.4	
Yeast and liver extract, none added	30	25 ± 0.4	66 ± 4.0	> 1.3
Yeast and liver extract, 4.0	30	26 ± 0.3	78 ± 4.7	
Harvard vitamin mixture	20	24 ± 0.6	60 ± 6.8	> 1.2
Emory vitamin mixture	20	24 ± 0.8	70 ± 5.3	

[1] The critical ratio (C.R.) is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is: less than 2.0, the difference between the means is considered to be statistically insignificant; 2.0 to 2.9, the difference is of borderline significance; 3.0 or more, the difference is highly significant. (Dunning, J. M., 1950, J. Dent. Res., *29*: 541.)

[2] Standard error of the mean.

[3] Fleischman.

yeast in the yeast and liver extract, which was added to the Emory but not to the Harvard diet, may perhaps have accounted, at least in part, for the difference in the cariogenicity of the two diets. However, the addition of yeast or of yeast and liver extract to the Harvard diet, as shown in table 2, did not reduce the caries-conduciveness of this diet. Our failure to obtain the same results as those obtained by Dalderup and Jansen may have been due to a difference in the American and European yeasts. We have been informed that the brew procedures in America and Europe are completely different and require different types of yeast. The results of our experiments are in agreement with those of Buttner and Muhler ('58) who found that the addition of 10% of irradiated yeast to their cariogenic diet did not bring about a reduction in caries.

Series II—Emory diet

The caries incidence and the caries scores of the teeth of animals fed the regular Emory diet and the modified Emory diets are presented in table 3. It may be seen from these data that (1) neither the omission of magnesium entirely from the salt mixture nor the addition of twice the amount present in the salt mixture of the regular Emory diet had any effect on the cariogenicity of the diet; (2) changing the Na/K ratio from 1 : 2 to 2 : 1 did not increase or decrease the relative caries-conduciveness of the diet; (3) increasing or decreasing the iron content of the Emory diet did not affect the caries incidence or the caries scores; (4) interchange of the Harvard and Emory vitamins was also without effect.

In connection with the experiments using various amounts of iron in the diets, it should be noted that Torell ('55) has concluded that the intake of ferric iron in high-sucrose diets may affect the cariogenicity of these diets. It has also been reported by McClure ('48) that the addition of 0.025% of iron as the chloride significantly reduced the cariogenicity of a diet while in a second experiment using 0.050% of iron

TABLE 3

Dental caries in albino rats fed the Emory diet with variations in mineral and vitamin components

VARIABLE FACTOR IN EMORY DIET	NO. OF RATS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE CARIES SCORE	CRITICAL RATIO [1]
%				
Magnesium, 0.053	20	20 ± 0.4 [2]	28 ± 1.5 [2]	> 2.0
Magnesium, none added	20	23 ± 0.4	33 ± 2.0	> 1.2
Magnesium, 0.106	20	21 ± 0.6	31 ± 2.0	
Sodium, 0.34/potassium, 0.71	25	20 ± 0.7	29 ± 1.9	> 0.9
Sodium, 0.56/potassium, 0.28	25	21 ± 0.6	27 ± 1.4	
Iron, 0.048	20	18 ± 1.0	24 ± 2.2	> 0.4
Iron, none added	20	18 ± 0.9	25 ± 1.8	> 0.0
Iron, 0.024	20	18 ± 1.0	24 ± 1.8	> 0.4
Iron, 0.096	20	18 ± 1.0	25 ± 1.8	
Emory vitamin mixture	20	19 ± 1.0	30 ± 3.1	> 1.8
Harvard vitamin mixture	20	18 ± 1.0	23 ± 2.1	

[1] The critical ratio (C.R.) is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is: less than 2.0, the difference between the means is considered to be statistically insignificant; 2.0 to 2.9, the difference is of borderline significance; 3.0 or more, the difference is highly significant. (Dunning, J. M., 1950, J. Dent. Res., *29*: 541.)

[2] Standard error of the mean.

as the citrate, there was no effect on the caries-conduciveness of the same diet. Our results are in agreement with those of McClure ('48) on the effect of the addition of ferric citrate to a cariogenic diet. As the account of Torell's investigations was available to us only in abstract form and did not state what iron compound was used in his diets, it is not possible to compare his results with those obtained in our laboratories.

Inasmuch as none of the factors under investigation in this study could account for the difference in the effect of the Harvard and the Emory diets, further investigation will be necessary to solve this perplexing problem.

SUMMARY AND CONCLUSIONS

These experimental studies were conducted in an attempt to determine what factor or factors are responsible for the marked difference in the cariogenicity of two high-sucrose diets, comparable in sucrose content but differing in the concentration of some of the vitamins and minerals.

The compositions of these diets have been varied by the addition, deletion and exchange of some of the various components which were in different concentrations in the two diets. The components studied were magnesium, manganese, iron, sodium, potassium, yeast, yeast and liver extract and the vitamin mixtures.

The results of these experiments indicate that variations in these components did not alter the cariogenicity of these two diets and that further investigation will be necessary to find the factor or factors which are responsible for the marked difference in their cariogenicity.

ACKNOWLEDGMENT

This investigation was carried out under Contract DA-49-007-MD-675 with the U. S. Army Medical Research and Development Command, Office of the Surgeon General of the U. S. Army.

LITERATURE CITED

BUTTNER, W., AND J. C. MUHLER 1958 The effect of a diet composed of barley and fish meal on dental caries in rats. J. Dent. Res., *37:* 418.

DALDERUP, L. M., AND B. C. P. JANSEN 1955 Nutrition and caries. Part VII. The influence of yeast on experimental dental caries in the rat. International Review of Vitamin-Research, *26:* 209.

HALDI, J., AND W. WYNN 1952 Cariogenicity of sugar containing diets. J. Nutrition, *46:* 425.

HALDI, J., W. WYNN, J. H. SHAW AND R. F. SOGNNAES 1953 The relative cariogenicity of sucrose when ingested in the solid form and in solution by the albino rat. Ibid., *49:* 295.

——— 1958 Influence of the salt mixture added to cariogenic diets of comparable sucrose content. Ibid., *66:* 333.

IRVING, J. T. 1940 The influence of diets low in magnesium upon the histological appearance of the incisor tooth of the rat. J. Physiol., *99:* 8.

KLEIN, H., E. R. ORENT AND E. V. MCCOLLUM 1935 The effects of magnesium deficiency on the teeth and their supporting structures in rats. Am. J. Physiol., *112:* 256.

MCCLURE, F. J. 1948 Observations on induced caries in rats. VI. Summary results of various modifications of food and drinking water. J. Dent. Res., *27:* 34.

ORLAND, F. J. 1946 The oral bacterial flora as related to dental caries in the Syrian hamster. Ibid., *25:* 455.

ORLAND, F. J., J. ROY BLAYNEY, R. W. HARRISON, J. A. REYNEIRS, P. C. TREXLER, MORRIS WAGNER, H. A. GORDON AND T. D. LUCKEY 1954 Use of the germ-free technic in the study of experimental dental caries. Ibid., *33:* 147.

ORLAND, F. J., E. S. HEMMENS AND R. W. HARRISON 1950 The effect of partly synthetic diets on the dental caries incidence in Syrian hamsters. Ibid., *29:* 512.

TORELL, P. 1955 The combination, iron and sugars, in relation to dental caries. Svensk. Tandlak. Takr., *48:* 249, (Dent. Abst., June, 1956).

WYNN, W., J. HALDI, J. H. SHAW AND R. F. SOGNNAES 1953 Difference in the caries-producing effects of two purified diets containing the same amount of sugar. J. Nutrition, *50:* 267.

THE EFFECTS OF LONG-TIME ADMINISTRATION OF SMALL AMOUNTS OF FLUORIDE IN FOOD OR WATER ON CARIES-SUSCEPTIBLE RATS[1]

R. E. WUTHIER AND P. H. PHILLIPS
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication September 29, 1958)

INTRODUCTION

There is a paucity of data on the long-term effects of the intake of small amounts of fluoride [F^-] on experimental animals, despite the consensus of opinion to the contrary. The only long-term studies on low-dose F^- that we are aware of are those on the rat by Auskaps and Shaw ('55) and Ramseyer et al. ('57). F^- storage in the rat from a lunch meat preparation containing about 4.6 p.p.m. F^- for periods up to 18 months has been reported by Jackson et al. ('50). Whereas Auskaps and Shaw reported no effects of F^- on weight, hemoglobin levels, thyroid glands, or reproduction in their 11-month study, Ramseyer et al. observed increases in dental caries, periodontal disease, and renal tubule hypertrophy from low levels of F^- at 520 days. In contrast, short-term studies by many workers (Hodge and Sognnaes, '46) have demonstrated that proper levels of F^- provide partial protection from dental caries in experimental animals. However, no demonstration of absolute essentiality of F^- has been made despite numerous attempts to do so (Maurer and Day, '57; Evans and Phillips, '39 among others). Further, long-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison. This work was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. We are indebted to Merck and Company for the vitamins used in these studies.

term study of F^- deposition in the skeleton is of interest because workers have long known that F^- accumulates in bone from either dietary or water-borne sources (National Research Council Div. Med. Sci. Publ., '51).

The purpose of this study was to determine the effects on the rat of prolonged exposure to low dietary levels of F^- *(fluoridation range)*. Comparative data on the rate and amount of skeletal storage of F^- from either food or water-borne sources of F^-, and the effects of F^- on dental health, reproduction and lactation, general health and well-being were sought.

EXPERIMENTAL

Seven groups of 25 litter-mate caries-susceptible weanling albino rats were fed a semi-natural cariogenic diet ad libitum continuously up to one year. The original plan was to remove three animals from each lot at three, 6, 12, and 18 months, and the balance at two years. However, because of the high susceptibility of this strain of rats to respiratory and glandular infections, death losses were such that the experiment was terminated at the end of one year. All groups received distilled water except lot 2 which received fluoridated city water. Graded increments of NaF were added either to the diet or to the drinking water. The experimental lots were as follows:

Lot 1 basal diet + distilled water [DW]; Lot 2 basal diet + city water (1.0 p.p.m. F)[2]; Lot 3 basal diet + DW + 1.2 p.p.m. F^-; Lot 4 basal diet + DW + 3.2 p.p.m. F^-; Lot 5 basal diet + 1.2 p.p.m. F^- + DW; Lot 6 basal diet + 3.2 p.p.m. F^- + DW and Lot 7 basal diet + 7.2 p.p.m. F^- + DW.

The percentage composition of the basal diet was as follows: Oat groats, finely ground, 25; soybean oil meal, solvent extracted, 25; sucrose, 23.65; dry skim milk powder, 20; corn oil, 5; $CaHPO_4 \cdot 2H_2O$, A.R., 0.85 and NaCl, iodized A.R., 0.50.

[2] City water was to have been fluoridated to maintain F levels at 1.2 p.p.m. F, however, analyses revealed only 1.0 p.p.m. F at the tap.

Halibut-liver oil was administered weekly as a source of the fat-soluble vitamins. Analysis of the diet indicated a F^- content of approximately 0.5 p.p.m.

The criteria sought were comparative F^- storage in the femur, dental caries incidence and severity, wear of the molars, evidence of periodontal effects, size, number, and weight of the litters and weanlings, growth rate, general health and well-being. Dental caries scores were determined by the method of Shaw et al. ('44). Femur F^- was analyzed by a modification of the Willard and Winter method ('33). Wear on the molars was assigned a numerical value from 1 to 5. Individual records were kept on health, reproduction, and body weights according to standard practice.

RESULTS

Progressive proportional F^- retention occurred from both food and water at all levels of administration, although at a decreasing rate with time (table 1). Both the concentration and the total quantity of F^- in the femur ash continued to increase throughout the 365-day study. When the accumulation of F^- from food and water was compared, it was evident that roughly twice as much F^- accrued from distilled water with added F^- as from an equivalent level in the food. Comparing the accumulation of F^- from different levels of F^- administered via both food and water, (assuming that added F^- was stored over and above that stored from the basal diet) it would appear that storage was roughly proportional to the level of F^- administered from either food or water, expressed either as concentration or total storage. Less F^- was deposited from city water (1.0 p.p.m.) than from distilled water which contained 1.2 p.p.m.

Attention is called to certain relationships in the retention of F^- in the rat femur. Water-borne F^- at levels of 1.2 and 3.2 p.p.m. in distilled water caused a retention of F^- in the femur ash of 8.1 to 16.8 times that of the controls, respectively. City water (1.0 p.p.m.) caused retention of about 5.9 times that of the controls. Food-borne F^-, on the other hand, in-

creased femur F^- retention from 4.1 to 20.0 times that of the control animals at levels from 1.2 to 7.2 p.p.m., respectively. These relationships displayed little change with time (from 90 to 360 days) expressed either as concentration in the femur ash or as total femur storage.

TABLE 1

The effect of time upon the F^- content of femur ash of the rat fed small amounts of NaF in the diet or in water

ADDITIONS TO BASAL DIET	F^- IN FEMUR ASH					
	90 days [1]		180 days		360 days	
	Conc.	Total	Conc.	Total	Conc.	Total
	p.p.m.	*μg*	*p.p.m.*	*μg*	*p.p.m.*	*μg*
None	23	8	38 ± 8 [2]	12 ± 3	54	18
City water	157	37	236 ± 71	72 ± 8	316	109
1.2 p.p.m. F^- in DW	178	67	308 ± 86	93 ± 24	467	155
3.2 p.p.m. F^- in DW	378	126	610 ± 148	214 ± 53	890	327
1.2 p.p.m. F^- in food	102	32	157 ± 37	49 ± 9	215	71
3.2 p.p.m. F^- in food	206	60	157 ± 78	104 ± 19	490	155
7.2 p.p.m. F^- in food	517	172	730 ± 97	262 ± 24	964	313

[1] Values at 90, 180, and 360 days were determined from the line of relationship between F^- concentration in the femur ash and time (Pearson and Bennett, '42). Correlation analyses of the data were used because they most accurately represented the relationships between F^- accretion and time. Correlation coefficients for the lines of relationship ranged between 0.96 and 0.99.

[2] Standard deviation about the line of relationship.

The severity of dental caries, molar wear, and periodontal effects increased progressively with time. Fluoridation of either water or food at these low levels did not alter the pattern of these changes (table 2). No significant protective effect against dental caries was observed from any of these F^- levels studied. Earlier work demonstrated that 40 to 50 p.p.m. F^- in the diet (Shaw et al., '45) or 20 p.p.m. F^- in water (Hodge and Sognnaes, '53) were necessary to give a measurable reduction of caries in the rat. Hence, the caries score was not expected to be affected in these studies. Apparent increases in periodontal cavitation in the higher F^- lots were not significant.

Growth rates, mature weights, reproduction, and lactation were all normal and reflected no effect due to F^-. After the

TABLE 2

The long-term effect of low doses of F^- on dental health

ADDITIONS TO BASAL DIET	AVERAGE TIME ON EXPERIMENT	NUMBER OF ANIMALS	DENTAL CARIES		MOLAR ATTRITION	PERIODONTAL CAVITATIONS	
			Incidence	Extent		Incidence	Extent
	days						
None	98	5	2.2	7.8	2.4	0.40	0.8
	186	6	2.5	10.0	2.7	0.67	2.5
	362	8	2.8	21.4	4.4	0.75	2.4
City water (1.0 p.p.m. F^-)	92	5	1.0	2.2	2.2	0.00	0.0
	185	4	2.2	12.2	3.0	0.75	1.2
	362	11	2.9	24.8	4.2	0.73	2.6
1.2 p.p.m. F^- in D.W.	87	4	2.2	7.8	2.0	0.25	0.2
	185	5	2.2	13.6	4.0	0.80	2.8
	366	7	3.6	32.6	4.9	0.86	4.1
3.2 p.p.m. F^- in D.W.	89	4	1.2	8.0	2.8	0.25	1.2
	180	4	1.0	5.8	4.0	0.75	2.8
	361	10	2.3	21.8	4.6	1.00	3.7
1.2 p.p.m. F^- in food	91	3	1.7	14.7	1.7	0.00	0.0
	185	4	2.2	5.0	4.0	0.50	1.0
	363	7	1.7	16.1	4.7	0.71	2.6
3.2 p.p.m. F^- in food	94	3	2.0	17.7	2.3	0.67	2.7
	178	5	1.6	4.2	3.8	0.40	1.0
	363	9	2.6	21.1	4.7	1.00	4.1
7.2 p.p.m. F^- in food	99	4	1.3	9.8	2.8	0.75	3.2
	180	4	2.2	15.2	4.0	1.00	2.2
	358	6	1.8	20.0	4.2	1.00	3.3

[1] These were bone cavitations in the mesial buccal gingival areas of the mandibular molars which were observed during dental scoring.

4th month the general health of this strain of rats was poor, but again, no effect attributable to F^- was indicated.

DISCUSSION

In contrast to data reported by Zipkin and McClure ('52), Zipkin et al. ('56) and Muhler ('54), the small amounts of added NaF and also the trace amount of F^- in the basal diet caused a continuous rise in the concentration and total quantity of F^- in the femurs of the experimental animals from the 90 to 365 days. Although the rate of accretion decreased with time, there was no indication that equilibrium was attained.

There was an apparently greater deposition (about twice) of F^- from water than from food, but when correction was made for total F^- consumption, no great difference in retention existed. However, Lawrenz et al. ('39), have shown by rigid separation of food and water intake that water-borne F^- is about 20% more available than food-borne F^-. Our data suggest that *per se* water-borne F^- was more available than food-borne F^-, but under *ad libitum* conditions where considerable mixing of food and water in the digestive tract was expected, little difference in availability was observed.

Accumulation of F^- appeared to be proportional to the concentration of NaF administered, in agreement with previous work here and that of Jackson et al. ('50).

Corroborating earlier short term studies (Hodge and Sognnaes, '53), and in contrast with the report of Ramseyer et al. ('57), F^- at these levels caused no effect on dental caries. Although there was an apparent increase in periodontal effects in the animals in the lots fed higher F^-, the differences were not significant. In view of these data and those of Ramseyer et al., more study is necessary before definite conclusions can be drawn concerning the effect of F^- on periodontal conditions.

That growth rate, mature weights, reproduction, lactation, and general health were unaffected by these amounts of F^- was to be expected since previous work had indicated that

no detectable effects would be expected until definitely toxic levels were reached.

Female rats accumulated a consistently higher concentration of F^- in the femur than the males in this study. When expressed as total femur storage, these differences were not observed. The higher concentration of F^- in the femurs of the females may be explained in part by the increased F^- consumption and the increased turnover rate of bone salts during the stress of lactation. Other factors may be involved.

SUMMARY

The long-term effects of low dosages of F^- (fluoridation range) in both food and water are reported. Fluoride accumulated in the femur from all dietary levels, including the controls (0.5 p.p.m. F^-). The quantity of F^- stored was proportional to the level given. At equivalent levels water-borne F^- storage was about double that from food-borne F^-, but when corrections were made for total F^- consumption, no great difference in retention was demonstrated. These concentrations of F^- did not alter the dental score of the exposed rats. Growth rate, mature weight, reproduction, lactation, and general health of the rats were normal and unaffected by these amounts of F^-. In this study the female rats consistently accumulated a higher concentration of femur F^- than the males.

LITERATURE CITED

AUSKAPS, A. M., AND J. H. SHAW 1955 Hemoglobin concentration, thyroid weight and growth rate in rats during minimum fluoride ingestion. J. Nutrition, *55:* 611.

EVANS, R. J., AND P. H. PHILLIPS 1939 A new low fluorine diet and its effect upon the rat. Ibid., *18:* 353.

HODGE, H. C., AND R. F. SOGNNAES 1946 Experimental caries and a discussion of the mechanism of caries inhibition by fluorine. In: Dental Caries and Fluorine, F. R. Moulton, editor. American Association for the Advancement of Science, Washington, D. C., p. 53.

JACKSON, S. H., F. F. TISDALL, T. G. H. DRAKE AND D. WIGHTMAN 1950 The retention of fluorine when fed as bone and as sodium fluoride. J. Nutrition, *40:* 515.

LAWRENZ, M., H. H. MITCHELL AND W. A. RUTH 1939 The comparative toxicity of fluorine in calcium fluoride and cryolite. Ibid., *18:* 115.

MAURER, R. L., AND H. G. DAY 1957 The non-essentiality of fluorine in nutrition. Ibid., *62:* 561.

MUHLER, J. C. 1954 Retention of fluorine in the skeleton of the rat receiving different levels of fluorine in the diet. Ibid., *54:* 481.

NATIONAL RESEARCH COUNCIL DIV. MED. SCI. (U. S.) Publ. No. 214 1951.

PEARSON, F. A., AND K. R. BENNETT 1942 Statistical Methods, John Wiley and Sons, New York.

RAMSEYER, W. F., C. A. H. SMITH AND C. M. MCCAY 1957 Effect of sodium fluoride administration on body changes in old rats. J. Gerontology, *12:* 14.

SHAW, J. H., B. S. SCHWEIGERT, J. M. MCINTIRE, C. A. ELVEHJEM AND P. H. PHILLIPS 1944 Dental caries in the cotton rat. I. Methods of study and preliminary nutritional experiments. J. Nutrition, *28:* 333.

SHAW, J. H., B. S. SCHWEIGERT, P. H. PHILLIPS AND C. A. ELVEHJEM 1945 Dental caries in the cotton rat. IV. Inhibitory effect of fluorine additions to the ration. Proc. Soc. Exp. Biol. Med., *59:* 89.

WILLARD, H. H., AND O. B. WINTER 1933 Volumetric method for determination of fluorine. Ind. Eng. Chemistry, Anal. Ed., *8:* 384.

ZIPKIN, I., AND F. J. MCCLURE 1952 Deposition of fluorine in the bones and teeth of the growing rat. J. Nutrition, *47:* 611.

——————— 1956 Urinary fluoride levels associated with the use of fluoridated waters. Pub. Health Rpts., *71:* 767.

GROWTH-PROMOTING ACTIVITY OF MEAT MEAL AND CERTAIN TISSUES IN GOITROGEN-FED RATS AND CHICKS [1]

C. J. ACKERMAN
Department of Biochemistry and Nutrition,
Virginia Polytechnic Institute,
Blacksburg

(Received for publication July 14, 1958)

In a previous report (Ackerman, '57) it was observed that the growth inhibition of rats fed 1% sulfaguanidine could be prevented or cured by the inclusion of 5% meat meal in the diet. The fortification of the basal diet, containing 1% sulfaguanidine, with all known dietary essentials failed to induce growth. Thyroid powder was effective in preventing this growth inhibition as reported by others (Mackenzie and Mackenzie, '43). Attempts to isolate thyroxine from meat meal were unsuccessful. That the active factor(s) in meat meal was not thyroxine was also supported by the observation that the growth-promoting property of meat meal was stable during short periods of acid hydrolysis but labile when hydrolyzed with 5% NaOH or concentrated NH_4OH. Thyroid powder was found to be stable to either acid or alkaline hydrolysis. Published reports (Gross and Pitt-Rivers, '53) indicate that triiodothyronine is also stable to alkaline hydrolysis and it is assumed that meat meal contains only negligible amounts of this substance. From these results the possibility is considered

[1] This work was supported by a grant from the Williams-Waterman fund of Research Corporation. Presented in part at the 21st annual meeting of the American Institute of Nutrition, Chicago, April 18, 1957. Federation Proceedings, *16*, part 1, p. 379 (1957). Also presented in part at the 10th Research Conference of the American Meat Institute, Chicago, February 28, 1958.

that the meat meal preparation contains an active factor(s) which effectively substitutes for the thyroid hormone.

Since the above observations suggested a relationship between thyroid function and sulfaguanidine stress the present work was conducted to determine the effect of sulfaguanidine and other sulfa drugs on growth and thyroid size in animals fed a diet with and without meat meal. In addition, further attempts have been made to establish the active tissue source in meat meal by assaying a number of tissue preparations for growth-promoting properties in sulfaguanidine-fed rats.

EXPERIMENTAL

Rat studies. Weanling rats (40 to 50 gm) of the Holtzman and Sprague-Dawley strains were housed individually in raised-screen wire cages and were given food and water ad libitum. The basal diet was similar to that used previously (diet A, Ackerman, '57) with the following changes: sulfaguanidine and biotin were omitted and 2.5% brewers' yeast was added at the expense of sucrose. With the exception of vitamin B_{12} and folacin, crystalline B vitamins were reduced by one-half.[2]

Various goitrogenic agents, meat meal, and soybean oil meal were added at the expense of the entire diet. Goitrogenic agents were added as shown in tables 1 and 2 and sucrose was added where necessary, so that the level of goitrogen plus sucrose was 1% of the diet. Meat meal was added at a level of 5% and an equivalent amount of soybean oil meal was added to the control diets. The animals were maintained for 6 or 7 weeks and then sacrificed. The thyroid glands were removed and weighed on a torsion balance. The results are

[2] Basal diet: ground wheat, 55; alfalfa leaf meal, 2.0; soybean oil meal, 13.0; crude casein, 6.5; brewers' yeast, 2.5; sucrose, 7.5; Crisco, 10.0; salts 5 (Salmon, '47), 2.5; and Viadex (4000 U. S. P. units vitamin A and 750 I. C. units vitamin D per gram, Nopco Chemical Co., Harrison, N. J.), 1.0; Crystalline vitamins in sucrose (milligrams per kilogram of diet): thiamine·HCl, 2; riboflavin, 2; niacin, 10; Ca-pantothenate, 5; pyridoxine·HCl, 1; inoistol, 100; menadione, 5; folacin, 4; vitamin B_{12}, 0.06. α-tocopherol (in hexane solution, 50 milligrams per kilograms of diet).

expressed in milligrams of fresh thyroid weight per 100 gm body weight.

Chick studies. One-day-old New Hampshire chicks of mixed sexes, or day-old DeKalb cockerels were housed in groups of 12 in thermostatically controlled chicken batteries. Food and water were supplied ad libitum. The basal diet consisted of the following: ground wheat, 40.0; wheat flour middlings, 9.0; soybean oil meal, 20.7; corn, 6.5; oats, 5.0; alfalfa, 3.0; Crisco, 9.0; limestone, 0.8; defluorinated rock phosphate, 2.5; iodized NaCl, 0.5; manganese sulfate, 0.025; Viadex, 1.0; sucrose, 1.975. Crystalline vitamins were added in sucrose as follows (milligrams per kilogram of diet): thiamine·HCl, 0.9; riboflavin, 0.9; pyridoxine·HCl, 0.48; calcium pantothenate, 2.4; niacin, 4.8; inositol, 48; menadion, 1.2; folacin, 4.0; vitamin B_{12}, 0.060; biotin, 1.0. Supplements were added at the expense of the entire diet as shown in tables 2 and 3. After 4 and 6 weeks, the chicks were sacrificed and the thyroid glands removed and weighed. Results are expressed as milligrams of fresh thyroid weight per 100 gm of body weight.

Growth-promoting properties of various tissues. Various samples of dried tissues obtained from different sources [3] were screened for growth-promoting activity by a curative-type growth assay as follows: weanling rats were fed the basal diet containing 1.1% sulfaguanidine. When the growth rate was less than 5 gm per week (4 to 5 weeks), the rats were divided into groups of three (two female, one male). The sample to be assayed was then added to the diet at a level of 5 or 10% at the expense of the entire diet. The rats were maintained on this diet for two weeks and then sacrificed. The thyroid glands were removed and weighed. These assays were not all conducted at the same time, but all results are summarized in table 4 for convenience. None of the assays are duplicated. Each assay is a test of a different lot of the tissue indicated.

[3] Kindly donated by Hudson Pharmacal Co., Union City, New Jersey; Armour and Co., Chicago, Illinois; The Wilson Laboratories, Chicago, Illinois; and Viobin Corp., Monticello, Illinois.

RESULTS AND DISCUSSION

The data in table 1 indicate that all of the compounds tested, with the exception of 0.3% sulfamethazine, depressed growth of rats when fed with the basal diet containing 5% soybean oil meal. When soybean oil meal was replaced by 5% meat meal, growth depression was less severe and approached normal levels in the groups receiving sulfaguanidine. The variation within groups is due to the normal disparity in weight of the males and females after 6 to 7 weeks. Normal growing males were more than 100 gm heavier than females in the same group.

At the dietary levels tested, all the sulfa drugs produced an enlargement of the thyroid gland, with sulfapyridine and sulfamethazine appearing to be the most active and sulfanilamide the least active. The goitrogenic activity of various sulfa drugs has been measured by others (Astwood et al., '43; Tabenkin et al., '53; Vanderlaan and Bissel, '46) but a rigid evaluation of the potencies of these drugs is difficult to make since thyroid enlargement would be the net result of the rate of intestinal absorption, rate of excretion, metabolism and mode of action. Nevertheless, if thyroid size be considered a reasonably reliable estimate of thyroid function, growth was not uniformly related to thyroid function. The goitrogenic activity of thiouracil was consistently greater than that of thiourea, yet thiourea appeared to exert a greater depressing effect on growth than did thiouracil. When sulfaguanidine plus meat meal was fed, body weight at 7 weeks was 226 gm as compared with 181 gm for rats in the group receiving 0.1% thiourea plus meat meal. However, thyroid gland weight of the sulfaguanidine, meat meal-fed rats was twice that of the thiourea, meat meal-fed rats.

It is generally accepted that an inhibition of thyroid hormone synthesis is associated with an increase in size of the thyroid gland and a decreased growth rate. Thyroid hyperplasia resulting from the stimulatory activity of the thyrotropic hormone is believed to be a compensatory reaction to the failure of thyroid hormone synthesis (Charipper and Gor-

TABLE 1

Effect of meat meal on growth and thyroid weights of goitrogen-fed rats[1]

DIET ADDITION	5% SOYBEAN OIL MEAL			5% MEAT MEAL		
	Av. body weight		Av. thyroid wt.	Av. body weight		Av. thyroid wt.
	gm	*gm*	*mg/100 gm B.W.*	*gm*	*gm*	*mg/100 gm B.W.*
Trial 1. Holtzman strain						
	5 wks.	7 wks.		5 wks.	7 wks.	
None	192	236 ± 59	5.3 ± 0.95	204	250 ± 45.2	4.8 ± 0.72
0.1% Thiourea	114	125 ± 12	11.8 ± 1.74	142	181 ± 41.6	7.8 ± 1.94
0.1% Thiouracil	119	128 ± 27	21.1 ± 3.53	166	205 ± 50.8	17.0 ± 4.37
1.0% Sulfaguanidine	135	136 ± 16	33.7 ± 12.6	207	226 ± 51.4	14.0 ± 5.56
1.0% Sulfathiazole	115	108 ± 17	29.6 ± 5.15	155	181 ± 48.1	27.0 ± 7.08
1.0% Sulfapyridine	85	86 ± 12	46.7 ± 14.6	119	150 ± 37.6	49.9 ± 14.4
1.0% Sulfanilamide	160	203 ± 55	10.3 ± 3.26	177	211 ± 65.5	8.5 ± 2.15
0.3% Sulfamethazine	181	242 ± 62	9.8 ± 1.15	196	236 ± 63.9	7.9 ± 2.39
Trial 2. Sprague-Dawley Strain						
	4 wks.	6 wks.		4 wks.	6 wks.	
None	147	210 ± 49	7.1 ± 2.67	164	219 ± 34	7.2 ± 1.12
0.1% Thiourea	97	94 ± 11	19.8 ± 6.34	110	136 ± 9.1	14.8 ± 8.61
0.1% Thiouracil	128	144 ± 20	26.9 ± 6.64	142	176 ± 42	21.2 ± 4.37
1.0% Sulfamethazine	109	108 ± 18	41.7 ± 8.30	158	179 ± 40	34.9 ± 16.2
1.0% Sulfaguanidine	109	125 ± 21	18.8 ± 1.71	178	203 ± 49	15.1 ± 6.34

[1] Four rats per treatment. Sucrose was added to the diet where necessary so that the level of goitrogen plus sucrose was 1.0%. Standard deviation is recorded for final body weight and thyroid weight.

don, '47; Higgins, '44). It would be expected then, that the degree of growth depression would be a reflection of thyroid enlargement, i.e., inhibition of thyroid hormone production. From the data presented here, it appears probable that goitrogenic agents inhibit growth by some mechanism other than by the inhibition of thyroid hormone synthesis or secretion.

From the results presented, it is not possible to define clearly the possible relationship between thyroid weight (as a measure of thyroid function) and the growth promoting properties of meat meal when rats are subjected to the stress of goitrogen ingestion. There is some suggestive evidence that the two measures of physiological response may be related. For example, considering all the data on goitrogen-fed rats, the mean difference between meat meal-fed and soybean oil meal-fed rats with regard to two-week body weight gains was 18.1 gm in favor of meat meal. The latter had thyroid gland weights approximately 20% smaller than those of the thyroid glands of soybean oil meal-fed rats. It is possible that this relationship could be demonstrated more clearly if the relative goitrogenic potencies of the various substances tested were to be more clearly established. Herein may lie the explanation of the variability in animal response.

The effect of sulfaguanidine and meat meal is not as pronounced on chick growth and thyroid weights as it is in rats (tables 2 and 3). Mackenzie and Mackenzie ('43) reported that thyroid glands of chicks fed 2% sulfaguanidine for 14 to 30 days were not enlarged. It was suggested that chicks were resistant to the goitrogenic effects of this drug. In the experiments reported here, meat meal did not appreciably stimulate growth of sulfaguanidine-fed chicks. Surprisingly, meat meal had a greater stimulatory effect on growth of cockerels fed 0.1% thiourea (table 3) although thyroid weight was approximately 13 times greater than in those birds fed sulfaguanidine plus meat meal. This is a more striking example of the lack of correlation between growth and thyroid weight.

The results of screening various tissue preparations to determine the source of activity of meat meal are shown in table

4. It appears that those tissues associated with digestion are active. This includes hog stomach, hog pancreas, and hog duodenum. Spleen and Tissue B were also active and mammary tissue had slight activity. Two out of 5 samples of hog stomach were inactive and only two out of 6 samples of fish

TABLE 2

Effect of meat meal on growth and thyroid weights of New Hampshire chickens fed 1% sulfaguanidine

DIET ADDITION	AV. 4-WEEK GAIN AND STANDARD DEVIATION		AV. THYROID WT. AND STANDARD DEVIATION [1]
	Female	Male	
	gm	*gm*	*mg/100 gm B.W.*
1% Sucrose, 5% SBOM [2]	277 ± 38 (7)[3]	343 ± 39 (5)	5.1 ± 1.32 (10)
1% Sucrose, 5% meat meal	267 ± 47 (6)	336 ± 56 (6)	4.4 ± 2.22 (6)
1% Sulfaguanidine, 5% SBOM	236 ± 26 (6)	248 ± 58 (6)	7.1 ± 1.63 (10)
1% Sulfaguanidine, 5% meat meal	270 ± 33 (5)	268 ± 40 (7)	5.4 ± 1.66 (10)

[1] No sex difference was observed. The data are averages of an equal number of males and females in each group.

[2] Soybean oil meal.

[3] Numbers within parentheses indicate the number of chicks.

TABLE 3

Effect of meat meal on growth and thyroid weights of sulfaguanidine and thiourea-fed cockerels [1]

DIET ADDITION [2]	AV. WT. AND STANDARD DEVIATION 6 WEEKS	AV. THYROID WT.[3] AND STANDARD DEVIATION
	gm	*mg/100 gm B.W.*
2% Sucrose	422 ± 59	4.7 ± 0.90
2% Sucrose; 10% meat meal	486 ± 68	4.3 ± 1.57
2% Sulfaguanidine	276 ± 14	7.8 ± 1.03
2% Sulfaguanidine; 10% meat meal	299 ± 74	8.3 ± 1.61
0.1% Thiourea	286 ± 42	122.3 ± 6.13
0.1% Thiourea; 10% meat meal	372 ± 36	113.4 ± 33.6

[1] Eighteen DeKalb hybrid cockerels were started on each treatment. After one week the three largest and three smallest birds in each group were discarded.

[2] Meat meal added at expense of entire diet.

[3] Average thyroid weights of the 6 birds in each treatment that were nearest the mean body weight of all birds in the group.

preparations were active. Liver, kidney, brain, and the pyloric section of hog stomach were inactive. It is not possible to compare rigidly the gain in grams of the various groups as a means of evaluating potency of the samples since these assays were conducted over a period of one year and the rats varied in age at the time of assay. In general, a gain of 10 gm or more over a two-week period is indicative of some growth promoting property of the sample tested. A gain of 45 gm or more is unusual and suggests that the sample tested is more active than meat meal.

TABLE 4

Effect of certain tissues on growth of sulfaguanidine-fed rats[1]

DIET ADDITIONS	AV. TWO-WEEK GAIN	AV. THYROID WT.
	gm	*mg/100 gm B.W.*
5% Soybean oil meal	6 (6, 6, 7)[2]	34.2 (30.9, 33.8, 38.1)
10% Hog stomach [3]	45 (43, 35, 57)	——
10% Hog stomach [3]	7 (14, 5, 3)	17.0 (22.6, 16.2, 12.3)
10% Hog stomach [4]	32 (24, 35, 38)	——
5% Hog stomach [4]	45 (24, 40, 73)	7.8 (8.8, 7.3, 7.3)
5% Hog stomach [6]	9 (7, 14, 7)	40.7 (40.0, 41.3, 40.8)
10% Hog pancreas [4]	41 (37, 38, 47)	——
5% Hog pancreas [5]	60 (58, 64, 59)	13.5 (16.9, 10.9, 13.8)
10% Hog duodenum [3]	32 (27, 23, 47)	17.6 (10.9, 24.6, 17.4)
5% Hog duodenum [5]	29 (28, 33, 28)	35.1 (38.9, 40.3, 26.0)
10% Hog intestinal ext.[4]	39 (34, 36, 47)	——
10% Hog pyloric section [3]	5 (4, 4, 8)	——
10% Ext. hog pylorus [4]	— 5 (— 4, — 8, — 3)	——
5% Liver residue [6]	9 (7, 5, 15)	40.1 (33.4, 45.6, 41.2)
5% Whole liver [5]	2 (9, — 3, 1)	40.8 (33.0, 40.2, 49.3)
5% Kidney [5]	5 (4, 1, 10)	48.9 (50.7, 45.2, 50.9)
5% Brain [5]	4 (2, 6, 3)	44.9 (65, 35.8, 33.9)
5% Mammary [5]	17 (9, 23, 20)	43.9 (49.9, 39.6, 42.2)
5% Spleen [5]	48 (41, 43, 59)	22.3 (22.0, 31.0, 13.9)
5% Tissue B [5]	49 (39, 42, 68)	17.1 (29.8, 13.4, 8.1)
10% Fish meal [6]	4 (— 1, 5, 8)	——
5% Fish meal [6]	31 (23, 21, 48)	24.3 (31.5, 24.1, 17.3)
5% Fish meal [6]	2 (3, — 1, 4)	21.9 (20.6, 24.1, 21.1)
10% Fish flour [6]	5 (3, 9, 4)	33.3 (35.5, 33.3, 31.2)
5% Fish flour [6]	37 (31, 38, 41)	22.7 (19.6, 23.0, 25.4)
10% Fish meal [7]	2 (0, 3, 2)	36.2 (36.8, 41.2, 30.8)

[1] Three rats per group fed 1% sulfaguanidine plus the sample to be assayed for a period of two weeks.

[2] The figures within parentheses are the values for the individual rats as follows: female, female, male. The same sequence of individual animals is used in both columns.

[3] Hudson.

[4] Armour.

[5] Wilson.

[6] Viobin.

[7] Redville.

This procedure was designed to screen various biological materials for growth-promoting activity in sulfaguanidine-fed rats (Ackerman, '57) and is used here to determine which tissues could be responsible for the activity of meat meal. A more rigid assay with more animals would be necessary to evaluate the potency of these tissues quantitatively. The data suggest that further studies on the nature of the growth-promoting principle may be more profitably investigated in hog stomach or hog pancreas rather than in meat meal.

Since whole stomach of hog promoted growth of sulfaguanidine-fed rats, and the pyloric section was inactive, it is possible that the activity is centered in the fundus, or secretory region of the stomach.

SUMMARY

The effect of meat meal on the growth of rats fed certain goitrogenic agents has been studied. Meat meal at a dietary level of 5% stimulated growth of rats fed thiourea, thiouracil, sulfaguanidine, sulfathiazole, sulfamethazine or sulfapyridine. However, growth approached normal levels only when sulfaguanidine plus meat meal was fed. The thyroid glands of rats fed goitrogen plus meat meal tended to weigh less than those from rats fed only the goitrogen. It appears that there is little correlation between the size of the thyroid gland and growth of the rats.

The growth stimulation from dietary meat meal in sulfaguanidine-fed chickens was not as pronounced as that observed in rats. One or two per cent sulfaguanidine markedly inhibited growth of chickens but thyroid glands were enlarged only slightly. Chicks fed 0.1% thiourea appeared to grow as well or better than those fed 2% sulfaguanidine but thyroid weights were approximately 16 times greater than in the latter.

Assays of various tissues revealed that three of 5 different hog stomach samples were active in promoting growth of rats fed sulfaguanidine. Hog pancreas and hog duodenum and spleen were also active. Two samples of fish preparations

stimulated growth but liver, kidney, brain, and hog pyloric section of the stomach were inactive.

ACKNOWLEDGMENTS

The author is indebted to Mrs. Helen Graham for her technical assistance in this work and to Merck and Co., Rahway, N. J. for the vitamins used in the diets.

LITERATURE CITED

ACKERMAN, C. J. 1957 Reversal of sulfaguanidine toxicity in the rat. J. Nutrition, *63:* 131.

ASTWOOD, E. B., J. SULLIVAN, A. BISSELL AND R. TYSLOWITZ 1943 Action of certain sulfonamides and of thiourea upon thyroid gland of the rat. Endocrinology, *32:* 210.

CHARIPPER, H. A., AND A. S. GORDON 1947 The biology of antithyroid agents. Vitamins and Hormones V: 274.

GROSS, J., AND R. PITT-RIVERS 1953 3 : 5 : 3′-Triiodothyronine 1. Isolation from thyroid gland and synthesis. Biochem. J., *53:* 645.

HIGGINS, G. M. 1944 A consideration of the physiological action of thiouracil and other goitrogens. Minnesota Med., *27:* 997.

MACKENZIE, C. G., AND J. B. MACKENZIE 1943 Effect of sulfonamides and thiourea on the thyroid gland and basal metabolism. Endocrinology, *32:* 185.

SALMON, W. D. 1947 Some physiological relationships of protein, fat, choline, methionine, cystine, nicotinic acid and tryptophan. J. Nutrition, *33:* 155.

TABENKIN, B., J. M. COOPERMAN, R. E. DRUCHER AND E. M. JENKENS 1953 Effect of feeding several sulfonamides and related compounds on growth of chicks. Proc. Soc. Exp. Biol. Med., *83:* 88.

VANDERLAAN, W. P., AND A. BISSELL 1946 The influence of selected goitrogenic compounds on the thyroid gland of the chick. Endocrinology, *38:* 308.

EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

IV. BIOTIN [1]

RICHARD H. BARNES, EVA KWONG AND GRACE FIALA
Graduate School of Nutrition, Cornell University, Ithaca, New York

(Received for publication October 9, 1958)

There are marked differences between animal species in their requirement for biotin in the diet. A complete lack of biotin in the diet does not interfere with the normal growth of the rat (Skeggs and Wright, '46). The weanling pig does not require biotin (Cunha et al., '46), but the young suckling pig does (Lehrer et al., '52). Certain strains of mice show the typical symptoms of biotin deficiency when this vitamin is excluded from the diet (Nielsen and Black, '44) and it is probable that all strains of chicks require a dietary source of biotin (Hegsted et al., '40, '42). It is probable that the intestinal flora of all species synthesize biotin and yet the differences in requirements are evident even though the animals are maintained on raised screen floors so as to minimize coprophagy. Barki et al. ('49) noted a growth depression in rats that were completely prevented from eating their feces when fed a biotin-free diet which suggests that the resistance of this species may be due to the practice of coprophagy.

Biotin deficiency is most commonly produced in the rat by feeding a source of avidin. Under such conditions it has been reported that cholesterol transport to the liver is blocked (Okey et al., '51), causing a low liver cholesterol. The present studies deal with the question of the role of coprophagy in the

[1] This research has been supported in part by funds provided by the State University of New York and by the National Science Foundation.

development of biotin deficiency and the relationship of this deficiency to cholesterol transport phenomena.

METHODS

The procedure for the prevention of coprophagy in the rat has been described previously (Barnes et al., '57). Male albino rats were obtained at weaning age and when received in the laboratory were maintained for three days on the control purified diet (diet A). They were divided into groups of 10 rats each in such a manner as to distribute body weight equally. They were housed in individual wire screen-bottom cages in a room maintained at 72 ± 2°C and fed ad libitum. Blood samples were taken by heart puncture under light ether anesthesia. Serum cholesterol was determined by the procedure of Abell et al. ('52). At the termination of experiments the livers were removed, blotted to remove blood and weighed. The excised liver was placed in a flask containing 20 ml of 70% ethyl alcohol and 1.5 gm KOH. The mixture was refluxed on a steam bath for 8 hours. The resulting brown liquid was transferred to a 100-ml volumetric flask using 95% alcohol. An aliquot was acidified with HCl, reduced to small volume and extracted with petroleum ether (30 to 60°C). The liver fat was determined gravimetrically after evaporation of the solvent. Liver cholesterol was determined in another aliquot by the method of Abell et al. ('52). The method of Wright and Skeggs ('44) was used for the microbiological determination of biotin in liver. The composition of the various diets is shown in table 1.

RESULTS

Experiment 1. Four groups of 10 rats each were set up so that two groups received the control diet (A) and two the same diet without biotin. One group on each of these diets was maintained with feces collection cups so as to prevent coprophagy. Body weights after 11 weeks are given in table 2. The characteristic decrease in weight gain due to the presence of the fecal collection cups can be noted. Lack of biotin in

the conventional rats had little if any effect, but a definite depression in growth was observed when coprophagy was prevented. At the end of the 11-week period 5 rats from each

TABLE 1

Diet composition

CONSTITUENT	DIET NO.[1]		
	A	B	F
	gm	*gm*	*gm*
Casein [2]	25.0	25.0	
Egg white [3]			25.0
Cerelose	53.0	58.0	58.0
Primex [4]	15.0		
Hydrol [5]		10.0	10.0
Salts [6]	4.0	4.0	4.0
Choline dihydrogen citrate	0.3	0.3	0.3
B vitamins in sucrose	2.0	2.0	2.0
Fat sol. vitamins in corn oil [7]	1.0	1.0	1.0

B VITAMINS IN 2.0 GM SUCROSE		FAT SOLUBLE VITAMINS IN 1.0 GM CORN OIL	
	mg		*mg*
Thiamine·HCl	0.40	Vitamin A acetate	0.31
Riboflavin	0.80	Vitamin D (calciferol)	0.0045
Pyridoxine·HCl	0.40	Alpha tocopherol	5.00
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		

[1] Diet C same as B with olive oil instead of Hydrol; diet D same as B with corn oil instead of Hydrol and diet E same as B with 1% cholesterol in place of 1% eerelose.

[2] Vitamin test, General Biochemicals, Inc.

[3] Fermented, spray dried, Swift and Company.

[4] Proctor and Gamble.

[5] Hydrogenated coconut oil made on special order without added lecithin, Durkee Division, Glidden Company.

[6] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

[7] Mazola, Corn Products Refining Company.

group were bled and their livers removed. Serum cholesterols appeared lower in the deficient group (coprophagy-prevented) but variability among rats necessitates larger groups in order

to establish the significance of any small deviation. Liver biotin values showed the small but consistent drop with increasing severity of the deficiency that was noted by Skeggs and Wright ('46). It is quite evident that the prevention of coprophagy resulted in a minimal biotin deficiency. There were no characteristic skin signs of deficiency, but most of the rats developed sores where the plastic feces collectors rubbed against the back. This was not noted in the animals on the control diet.

The 5 remaining rats from each diet were continued for three additional weeks. All rats received the control diet (containing biotin). No change in growth rate of the first three groups was noted, but the biotin-deficient rats in which coprophagy was prevented showed a definitely increased growth rate although not sufficient to reach the controls within this time.

TABLE 2

Body weight, serum cholesterol and liver biotin at the end of 11 weeks (experiment 1)

GROUP DESCRIPTION	DIET NO.	BODY WEIGHT		SERUM CHOLESTEROL [1]	LIVER BIOTIN [1]
		Start	Final		
		gm	*gm*	*mg/100 ml*	*µg/gm*
Control	A	55	397	77 ± 4.8	0.49 ± 0.24
Control + feces cups	A	55	347	75 ± 4.8	0.53 ± 0.33
Biotin-deficient	A [2]	55	380	82 ± 4.4	0.46 ± 0.37
Biotin-deficient + feces cups	A [2]	55	296	71 ± 3.4	0.30 ± 0.18

[1] With standard error of the mean.

[2] Diets without biotin.

Experiment 2. The second study was designed to evaluate the effects of different dietary fats on the development of biotin deficiency. Twelve groups of 10 rats each received the dietary treatments listed in table 3. Half of the groups were prevented from eating their feces. The two groups receiving egg white rapidly developed the severe skin signs of biotin deficiency including extensive alopecia, dermatitis, spectacled eyes and the typical spastic posture. At the end of 4 weeks these rats were bled by heart puncture and divided into subgroups for the study described under *experiment 3.*

TABLE 3

Body weight, serum and liver cholesterol in relation to fat (Experiment 2)

GROUP DESCRIPTION	DIET NO.	BODY WEIGHT			LIVER WEIGHT [1]	LIVER		SERUM CHOLESTEROL [1]	
		Start	6 wks.	11 wks.		Total fat [1]	Cholesterol [1]	4 wks.	11 wks.
			gm	*gm*	*gm*	*mg/gm*	*mg/gm*	*mg/100 ml*	*mg/100 ml*
Control (Hydrol)	B	53	285	365	10.78 ± 0.34	37.7 ± 1.7	2.03 ± 0.07	87 ± 1.4	83 ± 3.0
Control (Hydrol + feces cups)	B	53	248	327	9.68 ± 0.28	29.0 ± 2.9	1.89 ± 0.36	86 ± 1.6	84 ± 2.4
Biotin deficient (Hydrol)	B [2]	53	275	362	10.62 ± 0.38	27.9 ± 2.7	2.23 ± 0.07	90 ± 2.3	95 ± 2.8
Biotin deficient (Hydrol + feces cups)	B [2]	53	212	289	8.91 ± 0.35	33.2 ± 1.7	2.02 ± 0.11	87 ± 1.5	84 ± 1.4
Biotin deficient (Olive oil)	C [2]	53	281	380	11.47 ± 0.41	27.0 ± 1.6	2.32 ± 0.07	86 ± 2.9	89 ± 3.1
Biotin deficient (Olive oil + feces cups)	C [2]	54	179	232	6.47 ± 0.31	36.4 ± 2.7	2.54 ± 0.11	84 ± 1.7	78 ± 2.8
Biotin deficient (Corn oil)	D [2]	53	276	369	9.67 ± 0.38	42.4 ± 3.0	2.33 ± 0.16	94 ± 3.3	99 ± 4.3
Biotin deficient (Corn oil + feces cups)	D [2]	53	191	239	7.03 ± 0.33	36.4 ± 3.2	2.50 ± 0.14	92 ± 2.4	87 ± 3.0
Biotin deficient (Hydrol + cholesterol)	E [2]	53	279	360	11.57 ± 0.33	41.8 ± 2.4	12.85 ± 1.1	97 ± 2.4	101 ± 4.7
Biotin deficient (Hydrol — cholesterol + feces cups)	E [2]	53	215	273	9.48 ± 0.66	43.7 ± 5.2	18.69 ± 2.5	116 ± 9.9	111 ± 6.5
Biotin deficient (Egg white)	F [2]	53	146	—				84 ± 2.3	
Biotin deficient (Egg white + feces cups)	F [2]	54	119	—				89 ± 3.2	

[1] With standard error of the mean.

[2] Diets without biotin.

The usual depression in all groups with fecal collection cups was obtained. The conventional rats receiving as dietary fat hydrogenated coconut oil,[2] olive oil, corn oil [3] or Hydrol plus 1% cholesterol grew at approximately the same rate. There was no obvious effect due to the presence or absence of biotin in the diets. However, when coprophagy was prevented the rats receiving the biotin-deficient diets all grew at a lower rate and there was a definite difference between the growth of the rats receiving the saturated and unsaturated fats. Not only did the growth rate reflect a more marked biotin deficiency when olive oil or corn oil was in the diet, but the majority of rats from these two groups began to show the severe skin signs of alopecia, dermatitis and encrusted eyes by the 11th week post weaning. The coprophagy-prevented rats that received Hydrol showed no skin signs of the deficiency and weight gain was greater than the rats getting the unsaturated fats.

Total liver fat and total cholesterol did not show any consistent change due to the various treatments with the exception of the two groups that received 1% of cholesterol in the diet. In these latter two groups total liver fat appeared the same, but liver cholesterol was higher in the group in which coprophagy was prevented, giving evidence of a marginal deficiency of biotin.

In the groups that did not receive cholesterol, the serum cholesterol values appeared to be increased when coprophagy was permitted and a biotin-free diet was fed. The biotin-deficient groups (coprophagy-prevented) had values similar to the controls. This finding is difficult to explain and will bear further investigation. It is of interest that the change in serum cholesterol was in the same direction in both experiments 1 and 2. In the two groups fed cholesterol, the biotin-deficient groups (coprophagy-prevented) gave the higher value.

[2] Hydrol, made on special order without added lecithin, Durkee Division, Glidden Company.

[3] Mazola, Corn Products Refining Company.

TABLE 4

Serum and liver cholesterol of biotin-deficient rats receiving 25 % of egg white and 0.5% of cholesterol either with or without biotin supplement for two weeks

GROUP DESCRIPTION	NO. RATS	LIVER			SERUM CHOLESTEROL [1]	
		Weight [1]	Total fat [1]	Cholesterol [1]	Start	2 wks.
		gm	*mg/gm*	*mg/gm*		
Without biotin	7	4.71 ± 0.38	39.1 ± 2.2	3.00 ± 1.4	86 ± 3.6	100 ± 6.9
With biotin	9	7.30 ± 0.37	42.5 ± 2.4	3.97 ± 3.4	84 ± 3.1	88 ± 3.4

[1] With standard error of the mean.

Experiment 3. The two groups of rats that had received the egg white diets for 4 weeks were rearranged into two groups so that half of each of the new groups were animals in which coprophagy had been prevented and the fecal collection cups were maintained on these rats. All of these rats were continued on the egg white diets, but with a supplement of 0.5% of cholesterol. One of the new groups received 5 mg of biotin each day by stomach tube; the remaining group continued without biotin. The new regimen was followed for two weeks at which time the rats were bled and livers removed for total fat and total cholesterol determination. All rats that were given the biotin supplement showed an immediate spurt in growth and a dramatic induction of cure of signs of the deficiency. The lipid analyses are given in table 4. Liver fat did not change, but biotin therapy, even for the short two-week period, permitted an increase in liver cholesterol. The deficient group showed the higher serum cholesterol. The liver cholesterol change was the reverse of that found in experiment 2.

DISCUSSION

Development of biotin deficiency. It has already been mentioned that Barki et al. ('49) noted a depressed growth rate in rats on a biotin-deficient diet when coprophagy was totally prevented. The present results confirm and extend this observation to show that the complete picture of biotin deficiency can be obtained by coprophagy prevention when certain unsaturated fats are fed. The rats were about 15 weeks of age when the skin symptoms were well developed. This is about twice the age necessary for approximately equal deficiency to appear in the rats that were getting 25% of raw egg white in the diet. On the other hand the rats in which coprophagy was prevented developed the deficiency in about the same time period that was found necessary by Luckey et al. ('55a) in germ-free rats on a biotin-deficient diet. It is possible that some biotin synthesized in the intestine is absorbed directly, but it would seem more logical to conclude that the more severe deficiency produced by egg white is due in part, at least,

to effects in addition to the binding of newly synthesized biotin. For example, Luckey et al. ('55b) found rather high quantities of certain vitamins in the cecal contents of germ-free animals and this might reflect an intestinal secretion and reabsorption that would, of course, be blocked by a source of avidin.

The effect of the different dietary fats was completely unexpected. It is well known that oleic acid will replace the biotin requirement of certain microorganisms. Williams and Fieger ('46) and MacKay and Barnes ('41) have shown that highly unsaturated fats partially replace the biotin requirement in the egg white-induced deficiency in rats. This latter observation has not been studied by others. It would be of interest to repeat the study to see if under the same laboratory conditions there is a difference in the effect of fat on biotin deficiency developed by egg white as compared with a simple dietary exclusion of the vitamin. Salmon and Goodman ('34) reported that the skin lesions of "egg white syndrome" in rats were more severe on low-fat diets than on diets containing 18% of butterfat or hydrogenated cottonseed oil or 0.2 ml of linseed oil per rat per day. This finding relates fat to biotin deficiency, but is not comparable to the work of MacKay and Barnes ('41) or the present study. Many effects of dietary fat upon vitamin requirements have been traced to the influence of the fat on intestinal synthesis. The differences between the effects of the fats used in the present study probably are not explained on this basis, but must reflect some metabolic phenomenon in the tissues.

The differences in the susceptibility of various animal species to the development of biotin deficiency mentioned in the introduction may be related to their habits in practicing coprophagy. The chicken can become deficient in vitamin K, folic acid and biotin by simple exclusion of these vitamins from the diet, while most other monogastric animals require special treatment such as the use of antibacterial substances in order to develop these deficiencies. It has been shown here that dietary restriction with coprophagy prevention will lead to the development of biotin deficiency in the rat and in unpub-

lished observations the same has been found for vitamin K and folic acid. It is likely that the chicken that is maintained on raised wire screens does not eat its feces or at least to the same extent as the rat. Perhaps this is true also of certain strains of mice that readily develop biotin deficiency.

Liver and serum cholesterol. Okey et al. ('51) showed that when cholesterol is fed to rats receiving a diet containing avidin there is a block in the deposition of cholesterol in the liver. Although the biotin-deficient rats fed cholesterol consistently had lower liver cholesterol values than the controls no differences were found when cholesterol was not added to the diets. No serum cholesterol values were given in this study; in fact very little information is available on the serum cholesterol in biotin deficiency. Sydenstricker et al. ('42) found high serum cholesterols in 4 human subjects that ingested large quantities of egg white and developed some signs of biotin deficiency.

In the present studies when cholesterol was not being fed biotin deficiency was not associated with any consistent alteration of liver cholesterol. However, when 1.0% of cholesterol was included in the biotin-deficient diets there was an increased storage of liver cholesterol. Unlike the experiments of Okey et al. ('51) where biotin deficiency was induced by feeding a source of avidin, the biotin-deficient rats in which coprophagy was prevented showed a higher liver cholesterol than the controls. The rats in experiment 3 in which egg white and cholesterol were fed for two weeks showed the lowered liver cholesterol values as reported by Okey et al. Therefore, it would appear that the biotin deficiency *per se* may not be responsible for blocking the storage of cholesterol in the liver and the avidin-induced deficiency may have introduced a factor other than biotin deficiency that is involved in this storage.

The serum cholesterol in the biotin-deficient groups not receiving cholesterol (coprophagy prevention) was about 12% lower than in the corresponding controls (coprophagy permitted) but when cholesterol was fed the deficient rats had the higher values. The differences were not evident at the 4-week

period, but were established by the 11th week. When cholesterol was fed the rise in serum cholesterol in the egg white group appeared to be blocked by the administration of biotin. Probably these changes in cholesterol are not caused by effects of egg white or biotin upon cholesterol biosynthesis since several authors have found this to be practically unchanged in biotin deficiency (Curran, '50; Guggenheim and Olson, '52; Gram and Okey, '58). Furthermore, the only changes in liver cholesterol were found when exogenous cholesterol was supplied. Inanition could be responsible for the lowered serum cholesterol in the deficient animals not being fed cholesterol. However, severe inanition due to the removal of protein from the diet does not cause a lowering of the blood cholesterol.[4]

SUMMARY

Biotin deficiency can be induced in the rat by feeding a biotin-free diet and totally preventing coprophagy. The extent of the deficiency is dependent in part on the type of fat that is fed. Corn oil and olive oil in the diet caused a more severe deficiency to develop than hydrogenated coconut oil. Biotin deficiency developed much quicker when 25% of egg white was fed. However, the possibility exists that prevention of coprophagy may completely abolish the availability of biotin that is synthesized in the intestinal tract.

When cholesterol was fed to rats that were biotin-deficient due to feeding egg white inhibition of cholesterol storage in the liver was confirmed. This interference with storage was not observed in rats made mildly deficient by the prevention of coprophagy. Certain changes in serum cholesterol that are associated with biotin deficiency have been observed and their significance is discussed.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Lemuel D. Wright and Mrs. Jane Norton for the biotin analyses that are reported.

[4] Unpublished observations.

LITERATURE CITED

ABELL, L. L., B. B. LEVY, B. B. BRODIE AND F. E. KENDALL 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

BARKI, V. H., P. H. DERSE, R. A. COLLINS, E. B. HART AND C. A. ELVEHJEM 1949 The influence of coprophagy on the biotin and folic acid requirements of the rat. J. Nutrition, *37:* 443.

BARNES, R. H., G. FIALA, B. MCGEHEE AND A. BROWN 1957 Prevention of coprophagy in the rat. Ibid., *63:* 489.

CUNHA, T. J., D. C. LINDLEY AND M. E. ENSMINGER 1946 Biotin deficiency syndrome in pigs fed desiccated egg white. J. Animal Sci., *5:* 219.

CURRAN, G. L. 1950 Cholesterol and fatty acid synthesis in biotin-deficient rats. Proc. Soc. Exp. Biol. Med., *75:* 496.

GRAM, M. R., AND R. OKEY 1958 Incorporation of acetate-2-C^{14} into liver and carcass lipids and cholesterol in biotin-deficient rats. J. Nutrition, *64:* 217.

GUGGENHEIM, K., AND R. E. OLSON 1952 Studies of lipogenesis in certain B-vitamin deficiencies. Ibid., *48:* 345.

HEGSTED, D. M., J. J. OLESON, R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1940 Studies on a dermatitis in chicks distinct from pantothenic acid deficiency. Ibid., *20:* 599.

HEGSTED, D. M., R. C. MILLS, G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1942 Biotin in chick nutrition. Ibid., *23:* 175.

LEHRER, W. P. JR., A. C. WIESE AND P. R. MOORE 1952 Biotin deficiency in suckling pigs. Ibid., *47:* 203.

LUCKEY, T. D., J. R. PLEASANTS, M. WAGNER, H. A. GORDON AND J. A. REYNIERS 1955a Some observations on vitamin metabolism in germ-free rats. Ibid., *57:* 169.

LUCKEY, T. D., J. R. PLEASANTS AND J. A. REYNIERS 1955b Germ-free chicken nutrition. Ibid., *55:* 105.

MACKAY, E. M., AND R. H. BARNES 1941 Cure of signs of egg white disease by corn oil fatty acids and vitamin B_6. Proc. Soc. Exp. Biol. Med., *46:* 353.

NIELSEN, E., AND A. BLACK 1944 Biotin and folic acid deficiencies in the mouse. J. Nutrition, *28:* 203.

OKEY, R., R. PENCHARZ, S. LEPKOVSKY AND E. R. VERNON 1951 Dietary constituents which may influence the use of food cholesterol. I. Eggs: biotin and avidin. Ibid., *44:* 83.

SALMON, W. D., AND J. G. GOODMAN 1934 Studies of the raw egg white syndrome in rats. Ibid., *8:* 1.

SKEGGS, H. R., AND L. D. WRIGHT 1946 Vitamin B complex studies with diets differing in the carbohydrate component. Ibid., *32:* 375.

SYDENSTRICKER, V. P., S. A. SINGAL, A. P. BRIGGS, N. M. DE VAUGHN AND H. ISBELL 1942 Preliminary observations on "egg white injury" in man and its cure with a biotin concentrate. Science, *95:* 176.

WILLIAMS, V. R., AND E. A. FIEGER 1946 Oleic acid as a growth stimulant for *Lactobacillus casei.* J. Biol. Chem., *166:* 335.

WRIGHT, L. D., AND H. R. SKEGGS 1944 Determination of biotin with *Lactobacillus arabinosus.* Proc. Soc. Exp. Biol. Med., *56:* 95.

NUTRITIONAL STUDIES WITH THE GUINEA PIG

V. EFFECTS OF DEFICIENCY OF FAT OR UNSATURATED FATTY ACIDS

MARY ELIZABETH REID AND MARY G. MARTIN
Laboratory of Nutrition and Endocrinology,
National Institute of Arthritis and Metabolic Diseases,
Public Health Service,
U. S. Department of Health, Education, and Welfare,
Bethesda, Maryland

(Received for publication October 24, 1958)

The steadily increasing interest in the metabolic role played by dietary fats has stimulated studies with a wide variety of animals. A brief description of the fat-deficiency syndrome in the guinea pig was reported previously (Reid, '54). It was shown that the deficiency symptoms could be prevented or corrected by the inclusion in the diet of fat (corn oil) or linoleic acid. The present study deals with the effects of lack of dietary fat and of normal levels of fat on the intake of food and water, rate of growth and survival time, together with a detailed description of the fat-deficiency symptoms.

METHODS

Guinea pigs of the Hartley strain, weighing from 95 to 115 gm and two to 5 days of age, were fed the experimental diets. In the first experiment both male and female animals were employed but in all subsequent tests male animals only were used. For studying the effects of fat deficiency, fat was omitted from diet no. 13 (Reid and Briggs, '53) and an equal amount (7.3%) of glucose [1] substituted. Animals on the complete diet,

[1] Cerelose.

containing 7.3% of corn oil, served as controls. The fat-soluble vitamins were added to the diet in alcohol solution. The ration consisted of the following ingredients in percentage amounts: casein (vitamin-free) 30, sucrose 10.3, cellophane spangles 15, cornstarch 20, glucose 15.1, potassium acetate 2.5, magnesium oxide 0.5, salt mixture 6 (Briggs et al., '43), and 0.2 each of choline chloride, ascorbic acid, and inositol, with liberal amounts of the other known vitamins. The diets were refrigerated until used. As in previous studies, a few extra animals were placed on the deficient diet for use as substitutes. If any of the animals in the experimental groups failed to grow during the first 10 days, they were replaced from the substitute group. Since growth was good and the mortality rate was low, few substitutions were necessary. Seven tests with 6 to 10 animals each were conducted with both the fat-deficient and control diets.

RESULTS

The data in table 1 summarize the results obtained on food and water consumption with two groups of 6 guinea pigs each which had been fed the respective diets for periods ranging from approximately 4 to 25 weeks. Because of spillage, accurate measurements of the amounts of both food and water actually consumed by each of the two groups were difficult to obtain. Food consumption of the animals fed the diet lacking fat was slightly greater than that of the control group during the first three weeks on the diets, probably in part because of the difference in caloric value between the two diets. A difference in taste and texture may also have exerted an influence. The amount of food consumed by the control animals increased considerably as they continued to grow, whereas that of the deprived animals was less. Per 100 gm of body weight, however, the deprived group consumed slightly more than did the control group. Differences between the two groups in water consumption were slight, if any, but per unit of body weight the values obtained for the deficient group were higher.

TABLE 1

Average food and water consumption per guinea pig after 4 to 25 weeks on the diets (6 animals/group)

DAYS ON DIET	FAT-DEFICIENT				FAT-SUPPLEMENTED			
	Water		Food		Water		Food	
	Total	Per 100 gm body weight	Total	Per 100 gm body weight	Total	Per 100 gm body weight	Total	Per 100 gm body weight
	ml/day	*ml/day*	*gm/day*	*gm/day*	*ml/day*	*ml/day*	*gm/day*	*gm/day*
28–32	75 ± 25 [1]	32.5	18 ± 3	7.8	94 ± 27	38.5	21 ± 4	8.6
84–88	132 ± 35	33.0	34 ± 2	8.9	142 ± 60	24.9	39 ± 4	6.7
112–116	146 ± 31	32.6	37 ± 2	8.4	135 ± 28	19.7	43 ± 2	6.3
155–158	162 ± 37	31.4	40 ± 4	7.7	178 ± 34	23.2	48 ± 7	6.3
161–165	162 ± 37	30.6	38 ± 5	7.1	171 ± 44	21.6	50 ± 7	6.3
175–178	153 ± 38	28.1	38 ± 4	7.0	131 ± 56	16.1	52 ± 6	6.4

[1] Standard deviation.

A slight retardation in growth as a result of lack of fat in the diet was usually observed by the 5th week and became fairly marked by the 6th week as may be seen in the growth curves in figure 1. After 16 weeks on the diets the average percentage difference in weights between the two groups in 4 experiments was 27.2 ± 2.2; after 28 weeks the difference (two experiments) was 41.0 ± 3.0. In addition to retarded growth and in agreement with previously reported findings (Reid,

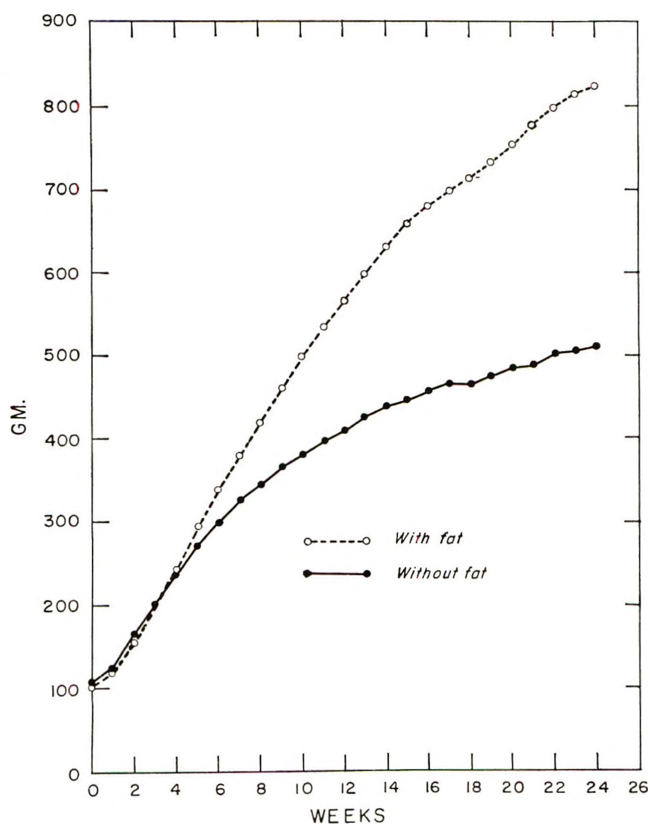


Fig. 1 Growth curves of guinea pigs reared with or without dietary fat. From 0 to 12 weeks, each point on the curve represents 56 animals in both the control and deficient groups. Thereafter the number per group became less as some of the experiments were terminated. At 16 weeks the average numbers were 40 and 38 and at 24 weeks they were 24 and 21 respectively in the control and deficient groups.

'54), other deficiency symptoms were observed. They included dermatitis, marked drying inside of the ears, loss of fur, and skin ulcers. About half of the male animals showed some degree of priapism after the deficiency became pronounced. A marked reddening of the muscles was observed in approximately one-third of the animals which had been fed a fat-free diet for a period of 140 to 196 days. In the fat-deficient groups the total number of fatalities in 76 weeks was 8 of a total of 58 as compared to two in a total of 53 in the control groups. The two deaths in the control group occurred in one experiment and were probably a result of an infection.

Considerable variation in the rate of development of deficiency symptoms was observed in different experiments. Because of the rather narrow range in humidity (40 to 50%) of the room in which the animals were kept, the variations herein described are not attributable to humidity changes. In a typical test, dermatitis was observed in some animals by the 44th day; 5 of the 8 guinea pigs in this test showed the change by the 63rd day and at 102 days all of the animals were affected, with severe symptoms in three. In most of the tests the symptoms were fairly severe by the 110th day. By the time the dermatitis was pronounced there was usually some loss of fur on the abdomen and inner surface of the legs. In some of the severely deficient animals the feet were swollen and in one animal a foot became cyanotic.

Histological examination of the skin of some of the experimental animals was made by Dr. G. L. Fite. The skin of the deficient animals appeared to be of normal thickness; the sebaceous glands were not abnormal in size or number and were generally not filled with sebaceous material to the expected degree. In the desquamatory areas the keratinized portion piled up into a thick area instead of being normally shed. A few animals showed alteration in the epidermis with edema and disruption in the basal layer suggesting some interference with the normal cycle of reproduction in the malpighian layer. Some of the animals also showed a suggestion of broad-

ening of the epidermal papillae. Inflammatory changes were trivial and presumably all secondary.

The effect of fat deficiency on the weight of some of the internal organs is shown in table 2. The data were obtained from 10 male animals which had been maintained on the diets for 201 days (5 with fat and 5 without). The average body weight of the deficient animals was 44 ± 0.54% less than that of the controls on the day previous to autopsy. On the day of

TABLE 2

Effect of fat deficiency on weights of body and organs of guinea pigs (5/group) kept on diets 201 days

	BODY WEIGHT	
	NO FAT	WITH FAT
	gm	*gm*
Before removal of food	488 ± 67 [1]	877 ± 32
16 hrs. after removal of food	441 ± 64	859 ± 32
	WEIGHT OF ORGANS PER 100 GM BODY WEIGHT	
	NO FAT	WITH FAT
	gm	*gm*
Liver	3.69 ± 0.18	2.54 ± 0.13
Kidneys	1.185 ± 0.11	0.586 ± 0.05
Spleen	0.101 ± 0.005	0.118 ± 0.003
Adrenals	0.091 ± 0.01	0.063 ± 0.01
Testes	0.186 ± 0.04	0.241 ± 0.01
Heart	0.313 ± 0.03	0.243 ± 0.01

[1] Standard deviation.

autopsy the difference was approximately 49 ± 0.65% because of the withholding of food for 16 hours. During this 16-hour period the control group lost only 2.0 ± 0.7% in weight as compared to a loss of 9.6 ± 1.0% in the deficient group. Removal of food produced a decreased intake of water which was greater in the deficient animals. In relation to body weight the weights of the kidneys, adrenals, and hearts of the deficient animals significantly were higher than those of the control animals. The reverse was true of the testes and to a lesser extent of the spleen. The gall bladders of the deficient animals were small and contained little bile. The cortex of the

adrenals appeared to be thickened. No fat or, in some cases, only slight traces were observed around the organs in the deficient animals.

The dry matter content of the blood was determined in the above-mentioned animals. The average values obtained were 18.1 ± 0.5% for the 5 deficient animals and 19.3 ± 0.3% for the 5 controls. Results of the determinations of the total dienoic, trienoic, and tetraenoic acid content of the serum of groups of fat-deficient and control animals are shown in table 3. Marked differences between the two groups were found in

TABLE 3

Fatty acids in serum[1]

TOTAL FATTY ACID	TOTAL DIENOIC, TRIENOIC, AND TETRAENOIC ACIDS	DIENOIC	TRIENOIC	TETRAENOIC
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Without fat in diet (8 guinea pigs)				
159 ± 20	5.36 ± 1.62[2]	5.05 ± 1.41	0.17 ± 0.05	0.15 ± 0.04
With fat in diet (7 guinea pigs)				
133.5 ± 10.6	62.32 ± 5.1	62.15 ± 5.1	0.0	0.16 ± 0.16

[1] Determined by Dr. J. G. Bieri and Mr. C. J. Pollard.

[2] Standard deviation.

dienoic acid but none in trienoic, tetraenoic, or in total fatty acids. Total fat and fatty acids were not determined on the entire carcass. The differences between the two groups in fat content must have been great considering the large amounts of fat stored in the fat reservoirs of the controls as compared to practically no visible fat in the deficient animals. Corroboration for this point came from moisture determinations of the carcasses which gave a value of 62.6% for the control animals and 74.0% for the deficient group. Since the water content of the fat-free body component is very close to 73 to 74%, the deficient animal must have had practically no fat in its body (Rathbun and Pace, '45; Morales, Rathbun and Pace, '45). Results of unsaturated fatty acid determinations of the

serum, erythrocytes, heart and abdominal muscles, liver, and kidney of fat-deficient and control animals have been reported previously (Bieri et al., '57). The dienoic acid decreased in all tissues on a fat-deficient diet whereas the tetraenoic acid showed no change in kidney, liver, abdominal muscle, and heart muscle as a result of the deficiency. The content of tetraenoic acid in the erythrocytes decreased to about one-third of that in the control animals.

Studies were also made of the effect of the presence or absence of dietary fat on the hemoglobin and hematocrit values and erythrocyte and leucocyte counts in a small group of animals in three experiments. Table 4 gives the average values found after different periods on the experimental diets. In general, slightly higher average values for hemoglobin and hematocrit were found in the control animals but there appeared to be no significant difference in the number of erythrocytes. The volume of the erythrocytes was slightly higher in the control groups and the number of granulocytes was higher in the deficient animals. In a few animals on the fat-deficient regime there was some deterioration in the red cell picture during the progress of the experiment and those animals which succumbed before the end of the experiment showed a lowering of the hemoglobin and hematocrit values previous to death. On the other hand, a few animals fed the fat-deficient diet showed a surprisingly constant red blood cell picture. In fact, a few individuals showed an increase in the hemoglobin and hematocrit values as the deficiency condition progressed. The highest values were in some cases found in those animals with the most severe dermatitis, redness of muscles, and hyperirritability. In taking blood samples from the latter animals the blood appeared to be of particularly low viscosity with a definite spreading character and little apparent tendency of the corpuscles to stick together. No readily noticeable difference in coagulability of the blood in any of the deficient and control animals was observed. If there were differences, they must have been slight.

TABLE 4

Effect of fat deficiency on the average blood picture after varying periods on the experimental diets

TIME ON DIET	NO. OF ANIMALS [1]	WEIGHT	HEMOGLOBIN	HEMATOCRIT	ERYTHROCYTES	MEAN VOL. OF ERYTHROCYTES	TOTAL LEUCOCYTES	GRANULOCYTES
days		*gm*	*gm/100 ml*	%	*cells × 10^6 mm³*	μ^3	*cells × 10^6 mm³*	*cells × 10^6 mm³*
				Without fat in diet				
57	10	342 ± 15 [2]	14.48 ± 0.33	43.3 ± 0.67	6.35 ± 0.18	68.5 ± 1.3	5650 ± 350	2905 ± 275
120	13	447 ± 30	14.19 ± 0.45	42.9 ± 1.0	6.32 ± 0.23	70.6 ± 2.5	5640 ± 525	3285 ± 375
193	8	536 ± 42	14.33 ± 0.47	42.0 ± 0.93	6.23 ± 0.28	67.9 ± 2.2	8675 ± 790	5400 ± 860
				With fat in diet				
57	10	452 ± 12	14.30 ± 0.31	43.8 ± 0.45	6.25 ± 0.25	72.4 ± 3.0	4600 ± 357	1535 ± 137
120	13	711 ± 25	15.10 ± 0.25	45.4 ± 0.69	6.22 ± 0.20	75.5 ± 1.6	5000 ± 225	2200 ± 270
193	8	884 ± 22	15.81 ± 0.30	45.0 ± 0.56	6.31 ± 0.17	72.5 ± 1.2	5660 ± 375	2720 ± 220

[1] These data were obtained from representative animals in three experiments. Blood studies were not made on all of the animals in each experiment.

[2] Standard deviation.

DISCUSSION

Fat deficiency in the rat increases water consumption to two or three times that of the control animals (Burr and Burr, 29). The guinea pig differs from the rat markedly in this respect, apparently a consequence of a less extensive and more superficial change in skin structure (Williamson, '41). Basnayake and Sinclair ('54) studied skin permeability in essential fatty acid deficiency in the rat and found that the skin was abnormally permeable, a condition which would account in part for the failure in water retention. Since the guinea pig's appetite for water is increased little, if any, by the deficiency, it may be assumed that the water loss by evaporation through the skin in this animal is much less than in the rat and differs only slightly, if any, from that of the control animal.

Little information is available in the literature on the effect of fat deficiency on the blood in different types of animals, doubtless because clear-cut effects have not been found. Witz and Beeson (51) studied the physiological effects of fat deficiency in swine and found no significant effect on the hemoglobin, cholesterol, or red and white blood cells. The total lipids in the plasma were significantly higher in the animals receiving dietary fat (5%). Swine fed the diet lacking fat had underdeveloped digestive systems, very small gall bladders, and retarded sexual maturity. These latter findings are in agreement with those herein described for the guinea pig. Although there is evidence in the literature (Josephs et al., 38; Loewy et al., '43) for a relationship between dietary fat and the rate of erythrocyte breakdown, no reports have been found on the effect of a complete lack of dietary fat on the rate of red cell breakdown in any type of animal. It seems possible that a retardation in the turnover rate of erythrocytes in fat-deficient animals in conjunction with a more rapid rate of production of red cells in the control animals may operate to maintain the number of red cells at about the same level in the two groups. Studies with radioactively-labeled cells should furnish an answer to the question of a possible

effect of dietary fat on the rate of red cell-turnover in the guinea pig.

SUMMARY

Purified diets containing all of the nutrients known to be required by the guinea pig except fat were fed to guinea pigs placed on the diet at two to 5 days of age. Comparable animals were given the same type of diet to which 7.3% of corn oil had been added. As previously found in this laboratory, lack of dietary fat caused retarded growth, dermatitis, skin ulcers, loss of fur, and some mortality. The following additional effects were also observed: priapism, reddening of the muscles, underdevelopment of the spleen, testes, and gall bladders, and, in relation to body weight, enlargment of the kidneys, liver, adrenals, and heart. There was also a lowering of the dry matter content of the blood, an apparent loss of blood viscosity, marked lowering of the dienoic acid content of the lipids of both the serum and erythrocytes. Deprivation of dietary fat produced, on an average, a mild microcytic anemia in the guinea pig; some animals, however, developed a marked anemia while some showed no alteration in the blood picture. No definite change was observed in the number of erythrocytes per unit of blood volume but an increase occurred in the number of granulocytes.

Skin changes in the guinea pig as a result of fat deficiency were found to be less extensive and were confined more to the surface layers than are those which occur in the rat. Fat deprivation caused little, if any, increase in water consumption in the guinea pig. Food intake, per unit of body weight, was higher in the deprived animals but the amount consumed per individual became less than that of the control animals as the deficiency became severe.

ACKNOWLEDGMENTS

The authors wish to thank Dr. G. M. Briggs and Dr. Olaf Mickelsen for helpful advice and criticism in the pursuance of these investigations; Dr. J. G. Bieri and C. J. Pollard for the fatty acid determinations; Dr. G. L. Fite for the histologi-

cal study of the skin; S. M. Takahashi for the dry weight determination of the entire animal and of the blood; Mrs. Helen Hood, Mrs. Ligia Ortiz, and Mrs. Esther Hurley for preparing the experimental diets; and Mrs. Mary K. Stull and Woodrow W. Duvall for assistance in the care of the animals.

LITERATURE CITED

BASNAYAKE, V., AND H. M. SINCLAIR 1954 Skin permeability in deficiency of essential fatty acids. J. Physiol. (London), *126:* 55.

BIERI, J. G., C. J. POLLARD AND M. E. REID 1957 Polyene fatty acids in guinea pig tissues. Biochim. et Biophys. Acta, *23:* 650.

BRIGGS, G. M., JR., T. D. LUCKEY, C. A. ELVEHJEM AND E. B. HART 1953 Studies on two chemically unidentified water-soluble vitamins necessary for the chick. J. Biol. Chem., *148:* 163.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. Ibid., *82:* 345.

JOSEPHS, H. W., L. E. HOLT, H. C. TIDWELL AND C. KAJDI 1938 The influence of fat upon the excretion of urobilin. J. Clin. Invest., *17:* 532.

LOEWY, A., L. W. FREEMAN, A. MARCHELLO AND V. JOHNSON 1943 Increased erythrocyte destruction on a high fat diet. Am. J. Physiol., *138:* 230.

MORALES, M. F., E. N. RATHBUN, R. E. SMITH AND N. PACE 1945 Studies on body composition. II. Theoretical considerations regarding the major body tissue components, with suggestions for application to man. J. Biol. Chem., *158:* 677.

RATHBUN, E. N., AND N. PACE 1945 Studies on body composition. I. The determination of total body fat by means of the body specific gravity. Ibid., *158:* 667.

REID, M. E. 1954 Production and counteraction of a fatty acid deficiency in the guinea pig. Proc. Soc. Exp. Biol. Med., *86:* 708.

REID, M. E., AND G. M. BRIGGS 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.

WILLIAMSON, R. 1941 A note on the epidermis of the rat on a fat-free diet. Biochem. J., *35:* 1003.

WITZ, W. M., AND W. M. BEESON 1951 The physiological effects of a fat-deficient diet on the pig. J. Animal Sci., *10:* 112.

STUDIES OF VITAMIN B_{12} IN TURNIP GREENS

LOUISE F. GRAY AND LOUISE J. DANIEL
U. S. Plant, Soil and Nutrition Laboratory, Agricultural Research Service, U.S.D.A., Ithaca, N. Y.; and the Department of Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

(Received for publication October 13, 1958)

Recent investigations of the relative nutritive value of turnip greens grown at two different locations in Georgia — Blairsville and Experiment — demonstrated the superiority of those from Blairsville (Gray et al., '57). Growth studies with the rat indicated that a part of this nutritional difference might be due to the presence of vitamin B_{12} or some other vitamin B_{12}-active substance in the Blairsville greens. Microbiological assays showed them to contain vitamin B_{12} activity for both *Lactobacillus leichmannii* and *Ochromonas malhamensis*.

The presence of vitamin B_{12} (cyanocobalamin) in the leaves of higher plants has not to date been conclusively demonstrated. This study was planned with two major objectives: first, to establish the presence or absence of vitamin B_{12} activity in the Blairsville turnip greens using vitamin B_{12}-deficient chicks as the test animals; and secondly, to ascertain whether or not this vitamin B_{12}-active substance was identical with cyanocobalamin, as determined by paper electrophoresis and chromatography.

EXPERIMENTAL PLAN

Turnip greens grown at Blairsville and Experiment in 1953 were used in these studies. They were washed and dried when harvested, and subsequently de-ribbed and pulverized.

Chick studies

Two chick experiments were conducted in which New Hampshire X Barred Plymouth Rock chicks from vitamin B_{12}-depleted hens were used. The composition of the basal diet and the Blairsville (B) and Experiment (E) turnip green diets is shown in table 1. All diets contained 20% of protein and 0.8% of sulfur amino acids. The B and E turnip green diets

TABLE 1

Composition of chick diets

CONSTITUENTS	BASAL DIET	TURNIP GREEN DIETS	
		E [1]	B
	%	%	%
Corn	40	6	6
Soybean oil meal [2]	30	6	6
Wheat	26	22	22
Turnip greens	—	24	20
DL-Methionine	0.05	0.15	0.14
Corn starch	—	36	40
Corn oil	—	2	2
Wheat germ oil	—	0.25	0.25
Minerals and vitamins [3]			

[1] E or B indicates the source of the turnip greens, Experiment (E) or Blairsville (B), Georgia.

[2] Obtained from Archer-Daniels-Midland Co., Minneapolis, Minn.; 50% protein, low-fiber, solvent-extracted soybean oil meal.

[3] Mineral supplements to all diets (grams per 100 gm): dicalcium phosphate 2, limestone 1, iodized sodium chloride 0.05, and manganous sulfate, monohydrate, 0.033. Vitamin supplements (milligrams per 100 gm): stable vitamin A (10,000 I.U. per gm) 100, activated animal sterol (3000 A.O.A.C. chick units of vitamin D per gm) 25, calcium pantothenate 0.441, riboflavin 0.31, and menadione 0.22.

were made to contain equal amounts of turnip green protein (7.6%), since it was recognized that the slightly higher level of protein (as well as sulfur amino acids) in the B greens [1] could have been a factor in their superior quality for the rat (Gray et al., '57). The importance of the choline content of the diet was also recognized, as choline and vitamin B_{12} have

[1] The protein (N × 6.25) and sulfur amino acid contents of the two turnip greens were: B — 38% protein, 448 mg% methionine, 240 mg% cystine; E — 32% protein, 338 mg% methionine, and 188 mg% cystine.

been shown to spare one another in the diets of both chicks and rats (Schaefer et al., '49a, b; Gillis and Norris, '49). The calculated choline contents (Engel, '43) of the diets used in experiment 1 were: basal, 0.12%; E turnip green, 0.13%; B turnip green, 0.12%. In experiment 2, all diets were made to contain 0.15% of choline (N.R.C. chick requirement, 0.13%) in order to eliminate this nutrient as a variable.

The modifications of the diets studied in both experiments are shown in table 2. In the first experiment, 6 levels of vitamin B_{12} were used, whereas only three were included in experiment 2 in order to increase the number of animals per treatment. At hatching, the chicks were uniformly distributed, according to sex and weight, among the treatments. Each lot was housed in a separate pen in an electrically-heated brooder. Feed and water were given ad libitum for the experimental period of 4 weeks. All chicks were fed the basal diet for three days prior to being fed the experimental diets.

Microbiological studies

The chick livers were assayed for their vitamin B_{12} content using *Lactobacillus leichmannii* (ATCC 4797) and the method of Peeler et al. ('49); all of these values were corrected for deoxyriboside activity (Hoffmann et al., '49). The samples were extracted, in preparation for assay, by steaming for 30 minutes in pH 5.0 acetate buffer containing 0.01% NaCN.

The turnip green samples and the fractions obtained in the course of the paper electrophoretic and chromatographic studies, were assayed with *Ochromonas malhamensis* by the method of Ford ('53). The extraction procedure is described below.

Paper electrophoretic and chromatographic studies

To facilitate the study of the vitamin B_{12}-active substance in the Blairsville turnip greens, it was necessary to purify and concentrate an extract of these greens. A 150-gm sample was placed in 1.5 liters of water, adjusted to pH 5.0 with HCl, and

extracted by steaming for 30 minutes. The filtrate from this suspension was stirred twice with activated charcoal.[2] The charcoal residues were combined, washed with 6% phenol, and stirred twice with hot 60% aqueous acetone. The combined acetone eluates were concentrated *in vacuo* to a small volume, and an aliquot of this concentrate was placed on an acid-washed aluminum oxide [3] column (21 cm × 2 cm). Distilled water was added to the column and the effluent was concentrated as before. This concentrate was the turnip green solution used in all electrophoretic and chromatographic studies subsequently described. All solvents contained 0.01% NaCN.

Two additional solutions were prepared for use in these studies. The first was an aqueous solution of crystalline vitamin B_{12}; the second was a solution containing turnip green extract to which crystalline vitamin B_{12} was added. The vitamin B_{12} contents of the three solutions (mμg/ml), as determined by *Ochromonas* assay, were: turnip green (T.G.), 35; vitamin B_{12} (B_{12}), 50; turnip green + B_{12} (T.G. + B_{12}), 76.

Paper electrophoresis. The hanging-strip type paper electrophoresis apparatus was used with Whatman No. 1 filter paper and with 0.5 N acetic acid (containing 0.01% NaCN) as the electrolyte. All runs were made at room temperature (approx. 22°C) for 16 hours with a constant current of 5 milliamperes per cell, following an equilibration period of one hour. The apparatus was shielded from direct light. Aliquots of the three solutions were pipetted onto filter paper strips (30 cm × 3 cm), each solution being placed on a separate strip. In order to have sufficient vitamin B_{12} activity for the subsequent assay of eluates from small portions of these strips, 6 5-μl aliquots were superimposed in a spot, with periods of drying between applications. After the completion of the run, the strips were dried at room temperature and cut crosswise at one-centimeter intervals. Each section (1 cm × 3 cm) was placed in a test tube and extracted by steaming for 30 minutes in 10 ml of water (containing 0.01% NaCN and adjusted to

[2] Darco G-60.

[3] Aluminum oxide, Merck. Suitable for chromatographic adsorption.

pH 5.0 with HCl). The vitamin B_{12} content of the eluate from each section was determined by assay with *Ochromonas*.

Paper chromatography. Ascending chromatography was performed in a dark, constant-temperature room (18.5°C) using a sheet of Whatman No. 1 paper (32 cm × 28 cm). The solvent system was *sec.* butanol-acetic acid-water-5% $NaCN_{aq}$ (100 : 1 : 50 : 0.25 by volume). Aliquots of each of the three solutions (T.G., B_{12}, and T.G. + B_{12}) were well spaced along a line about 4 cm from the lower edge of the paper. The vitamin B_{12} activity in each spot was increased by superimposing aliquots as previously described. When the spots were dry, the paper was supported above the solvent in the chromatography chamber for an equilibration period of 8 hours after which it was lowered into the solvent. After 18 to 19 hours, when the solvent front had advanced approximately 21 cm, the paper was removed from the chamber and dried at room temperature while protected from light. Three rectangular areas — each containing the chromatogram of one of the solutions — were cut from the sheet. Each rectangle was cut crosswise at one-centimeter intervals and the vitamin B_{12} content of each strip determined as described for the electrophoretic procedure.

Combined electrophoresis and chromatography. Investigators at the University of Reading (Kon, '55) reported that the best separation of a complex mixture of vitamin B_{12}-active compounds found in rumen and fecal contents was obtained by using a combination of paper electrophoresis and chromatography. The adaptation of this technique to the present work is described below.

The T.G. + B_{12} solution was applied to a sheet of Whatman No. 1 paper (32 cm × 28 cm) at a point which permitted the use of electrophoresis in the first direction and chromatography in the second. The procedures were the same as those described for each technique separately. After the completion of the chromatogram, it was divided into centimeter squares, each square was cut out, separately eluted, and its vitamin B_{12} content determined.

TABLE 2

Effect of turnip green supplements on growth and vitamin B_{12} liver-stores of vitamin B_{12}-deficient chicks

DIET	MEAN WEIGHT CHANGE [1]			MEAN VIT. B_{12} [2] IN LIVER (EXP. 1)
	Exp. 1	Exp. 2	Exp. 1 and 2 (combined data)	
	gm	*gm*	*gm*	*mμg/gm (dry wt.)*
Basal	224 (11)[3]	226 (13)	225 (24)	36.1
Basal + 50 mμg% B_{12}	256 (10)	260 (14)	259 (24)	49.9
Basal + 100 mμg% B_{12}	250 (10)	301 (16)	284 (26)	65.7
Basal + 150 mμg% B_{12}	322 (12)	282 (14)	301 (26)	101.1
Basal + 200 mμg% B_{12}	346 (10)			94.2
Basal + 400 mμg% B_{12}	328 (12)			182.5
Basal + 2 μg% B_{12}	373 (11)			690.2
E–T.G.[4]	203 (10)	194 (11)	198 (21)	30.3
B–T.G.	259 (13)	290 (15)	276 (28)	69.2

[1] Weighted means were calculated to eliminate the effect of unequal numbers of males and females. In experiment 1, 12 chicks were started on each diet except Basal (15) and E–T.G. and B–T.G. (14). In experiment 2, 17 chicks were placed on each treatment. The experimental period was 4 weeks.

[2] Determined with *Lactobacillus leichmannii* and corrected for deoxyriboside activity. The livers from all chicks in experiment 1 were assayed.

[3] Number within parentheses refers to the number of surviving chicks.

[4] E — T.G. and B — T.G. diets contained turnip greens from Experiment (E) or Blairsville (B) in amounts of 24% and 20% of the diets, respectively. (See table 1 for composition of diets.)

RESULTS AND DISCUSSION

All data obtained in the chick studies were subjected to an analysis of variance to determine the significance of the differences noted. The results of the two chick experiments are presented in table 2. The growth data for each experiment clearly indicate the presence of a vitamin B_{12}-active substance for the chick in the Blairsville turnip greens (Basal vs. B-T.G., $P < 0.01$). On the other hand, the chicks fed Experiment turnip greens grew no better than those on the basal diet. Thus, turnip greens grown at one location contained vitamin B_{12} activity for the chick while those from another did not.

The vitamin B_{12} contents of the livers of the chicks in experiment 1 are shown in table 2. These data likewise indicate that the Blairsville greens contained "vitamin B_{12}" (Basal vs. B- T.G., $P < 0.01$), while those from Experiment did not. It can be seen that the level of vitamin B_{12} in the diet was generally reflected by the amount found in the livers of the chicks.

Since but one level of Blairsville greens was fed in the chick experiments, only an estimate of their vitamin B_{12} content is possible from these results. No significant difference between the growth data for the two experiments was obtained, so these were combined (table 2) and used to plot the relationship between the vitamin B_{12} level of the diet and the weight change. When either the combined growth data or the data for the vitamin B_{12} contents of the livers were used for the purpose of estimation, the turnip greens contained 4 to 5 mμg vitamin B_{12}/gm. By *Ochromonas* assay, they were found to contain 7 mμg/gm. These values were for the greens as fed (moisture content = approximately 4%).

The finding of a vitamin B_{12}-active substance in turnip greens was of considerable interest since only two naturally-occurring compounds are now known which possess vitamin B_{12} activity for the chick and *Ochromonas,* i.e., cyanocobalamin and vitamin B_{12III} (Coates et al., '55, '56). However, most of the evidence to date supports the conclusion that vitamin B_{12} is not present in significant amounts (if at all) in the leaves of higher plants. Zucker and Zucker ('50) have written an excellent review of the findings relative to this question. In view of the above facts, further investigations of the nature of the vitamin B_{12}-active substance were deemed essential; and the results of the studies using paper electrophoresis and chromatography help to characterize this compound.

The distribution of vitamin B_{12} activity obtained by the examination of the three solutions by paper electrophoresis is shown in figure 1. It can be seen that the active material in the turnip greens moved at the same rate as crystalline vitamin B_{12}. The addition of vitamin B_{12} to the turnip green

extract increased the vitamin B_{12} activity only in those strips where it was noted for the unsupplemented turnip green solution.

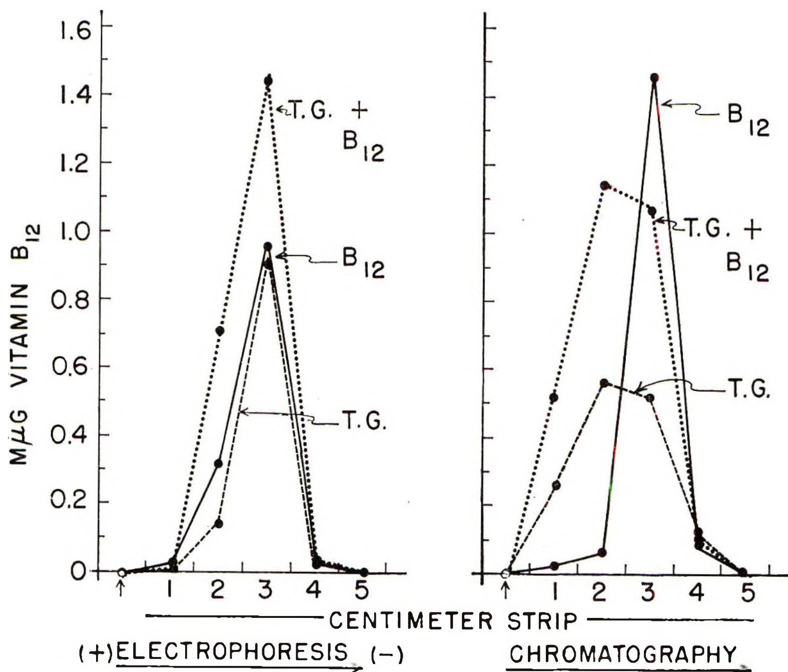


Fig. 1 Distribution of vitamin B_{12} activity following paper electrophoresis or chromatography of solutions of turnip green extract and crystalline vitamin B_{12}. The solutions studied were: turnip green (T.G.); crystalline vitamin B_{12} (B_{12}); and turnip green + vitamin B_{12} (T.G. + B_{12}). The arrow shows the point at which the solution was applied (origin). Each point on a curve represents the vitamin B_{12} activity found in the centimeter strip indicated.

The data obtained using paper chromatography are also presented in figure 1. In this instance, the activity in the turnip green solution was distributed among three strips with most in strips 2 and 3, while that in the vitamin B_{12} solution was located almost entirely in strip 3. However, when crystalline vitamin B_{12} was added to the turnip green solution, the distribution was essentially the same as that for the unsupplemented turnip green solution. It is evident that some extraneous material (s) in the turnip green solution affected the

movement of the added cyanocobalamin as it did that of the vitamin B_{12}-active substance in the greens.

The use of the combined techniques (electrophoresis in the first direction and chromatography in the second) produced the distribution of vitamin B_{12} activity shown in figure 2. A single elliptical spot of activity was the result. Thus, the presence of more than one vitamin B_{12}-active substance in the T.G. + B_{12} solution was not demonstrated, a fact corroborating the earlier findings with electrophoresis or chromatog-

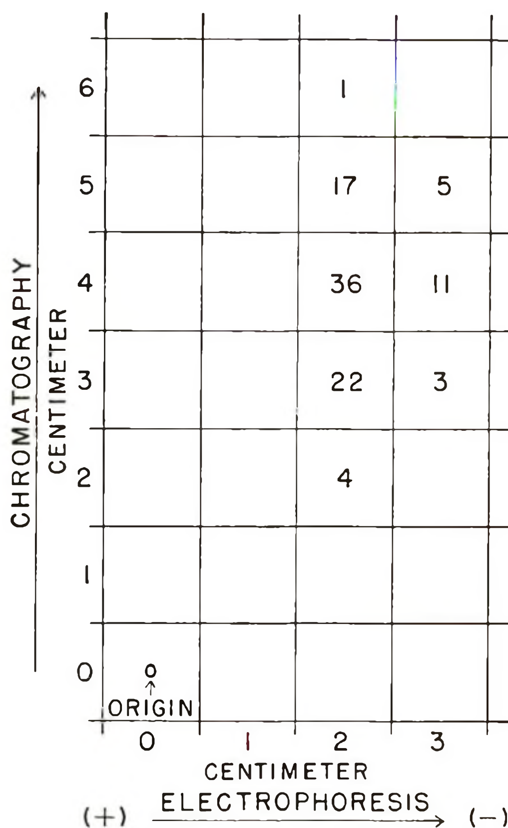


Fig. 2 Distribution of vitamin B_{12} activity after combined paper electrophoresis and chromatography of the turnip green + crystalline vitamin B_{12} solution (T.G. + B_{12}). Electrophoresis was employed in the first direction and chromatography in the second. The figures show the vitamin B_{12} activity (per cent of total) found in the centimeter squares indicated.

raphy singly. However, it is recognized that unequivocal proof of the identity of the vitamin B_{12}-active substance in the turnip greens with cyanocobalamin can only be obtained with its isolation in pure form followed by degradation studies.

The demonstration that vitamin B_{12} was present in the leaves of a higher plant in amounts of nutritional significance to the animal, and that its occurrence was in some way related to the plant's environment, posed the question of its origin. Was it elaborated by the turnip plant under certain environmental conditions? As mentioned earlier, there is no evidence supporting the synthesis of vitamin B_{12} by a higher plant. Was it produced by soil microorganisms and subsequently absorbed via the plant roots? There is ample proof of such synthetic activity by certain microorganisms (Lochhead and Thexton, '52; Burton and Lochhead, '52) and of vitamin B_{12} activity in soil (Stephenson et al., '48), but no evidence that the vitamin B_{12} molecule is absorbed by the plant from the soil. Could it have been produced by bacteria living epiphytically on the turnip leaf? Ericson and Lewis ('54) postulated such a relationship in their studies of vitamin B_{12} compounds found in certain algae. Further studies, some of which are now in progress, will have to be made before the answers to these questions are obtained.

SUMMARY

The presence in turnip greens of a substance with vitamin B_{12} activity for chicks and for *Ochromonas malhamensis* was demonstrated.

The possible identity of this active substance with vitamin B_{12} was investigated using paper electrophoresis and chromatography. By these criteria, there was no evidence that the substance in the turnip greens differed from cyanocobalamin.

The fact that the vitamin B_{12}-active material was found in turnip greens grown at one location but not in those from another raises the question of its origin. Possible sources of this vitamin B_{12} were discussed.

ACKNOWLEDGMENT

The authors are indebted to Drs. L. C. Norris, F. W. Hill and M. L. Scott of the Cornell University Poultry Department for their assistance in providing the vitamin B_{12}-deficient chicks and the facilities for conducting the chick experiments, and for their helpful suggestions during the course of the chick work. The assistance of Dr. G. Matrone, of North Carolina State University, in the statistical interpretation of the data is also gratefully acknowledged.

LITERATURE CITED

BURTON, M. O., AND A. G. LOCHHEAD 1952 Production of vitamin B_{12} by *Rhizobium* species. Canadian J. Bot., *30:* 521.

COATES, M. E., M. K. DAVIES, R. DAWSON, G. F. HARRISON, E. S. HOLDSWORTH, S. K. KON AND J. W. G. PORTER 1956 The activity for chicks of some vitamin B_{12}-like compounds. Biochem. J., *64:* 682.

COATES, M. E., AND J. E. FORD 1955 Methods of measurement of vitamin B_{12}. In: The Biochemistry of Vitamin B_{12}. Biochem. Society Symposia 13. Cambridge University Press, London, pp. 36–51.

ENGEL, R. W. 1943 The choline content of animal and plant products. J. Nutrition, *25:* 441.

ERICSON, L. E., AND L. LEWIS 1954 On the occurrence of vitamin B_{12}-factors in marine algae. Arkiv. f. Kemi, *6:* 427.

FORD, J. E. 1953 The microbiological assay of "vitamin B_{12}." The specificity of the requirement of *Ochromonas malhamensis* for cyanocobalamin. Brit. J. Nutrition, *7:* 299.

GILLIS, M. B., AND L. C. NORRIS 1949 Vitamin B_{12} and the requirement of the chick for methylating compounds. Poultry Sci., *28:* 749.

GRAY, L. F., M. SPEIRS AND G. MATRONE 1957 The nutritive value of several foods grown at different locations. J. Nutrition, *63:* 345.

HOFFMANN, C. E., E. L. R. STOKSTAD, B. L. HUTCHINGS, A. C. DORNBUSH AND T. H. JUKES 1949 The microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii.* J. Biol. Chem., *181:* 635.

KON, S. K. 1955 Other factors related to vitamin B_{12}. In: The Biochemistry of Vitamin B_{12}. Biochem. Society Symposia 13. Cambridge University Press, London, pp. 17–35.

LOCHHEAD, A. G., AND R. H. THEXTON 1952 Qualitative studies of soil microorganisms. X. Bacteria requiring vitamin B_{12} as growth factor. J. Bact., *63:* 219.

PEELER, H. T., H. YACOWITZ AND L. C. NORRIS 1949 A microbiological assay for vitamin B_{12} using *Lactobacillus leichmannii.* Proc. Soc. Exp. Biol. Med., *72:* 515.

SCHAEFER, A. E., W. D. SALMON AND D. R. STRENGTH 1949a Interrelationship of vitamin B_{12} and choline. I. Effect on hemorrhagic kidney syndrome in the rat. Ibid., *71:* 193.

——— 1949b Interrelationship of vitamin B_{12} and choline. II. Effect on growth of the chick. Ibid., *71:* 202.

STEPHENSON, E. L., J. MCGINNIS, W. D. GRAHAM AND J. S. CARVER 1948 Effect of soil on chick growth. Poultry Sci., *27:* 827.

ZUCKER, T. F., AND L. M. ZUCKER 1950 "Animal protein factor" and vitamin B_{12} in the nutrition of animals. Vitamins and Hormones, *8:* 1.

EFFECT OF DIETARY PROTEIN, VITAMIN E AND AGE ON THE LACTIC DEHYDROGENASE AND SUCCINOXIDASE OF THE HEARTS OF RATS

R. L. SHIRLEY AND G. K. DAVIS
Department of Animal Husbandry and Nutrition,
Agricultural Experiment Stations,
Gainesville, Florida

(Received for publication September 29, 1958)

Lactic dehydrogenase and succinoxidase are of interest in the metabolism of the heart because of their importance in the respiration of cardiac muscle. The heart depends largely on lactic acid as an energy metabolite. Wroblewski, Ruegsegger, and LaDue ('56) reported that lactic dehydrogenase is released into the blood serum during myocardial infarction. Govier, Yanz and Grelis ('46) studied *in vitro* the effect of α-tocopherol phosphate on the lactic dehydrogenase of guinea pig cardiac muscle.

Succinoxidase is more active in the heart than in any other tissue (Schneider and Potter, '43). Wainio et al. ('54) fed mature rats a protein-free diet for 7 weeks and found that succinic dehydrogenase was reduced proportionally to the decrease in protein in the heart. Bouman and Slater ('56) found that heart mitochondrial fragments contained α-tocopherol in amounts stoichiometric to some cytochromes, which suggested that it was a part of a respiratory chain. Houchin ('42) found that muscles of vitamin E-deprived rabbits, hamsters and rats had more succinoxidase activity than those fed the vitamin.

The present study was made to obtain information on lactic dehydrogenase and succinoxidase activity in the heart of rats,

as influenced by adequate and subadequate levels of dietary protein, each with and without vitamin E (*dl*, α-tocopherol acetate) at several different ages. The effect of sex was also studied.

EXPERIMENTAL

Twenty-four Long-Evans strain litters of 8 rats each (4 female and 4 male) were divided into 4 dietary groups when 12 days old. Up to this time the mothers were fed a commercial Purina pellet ration.[1] The diet containing the most protein was that of Mason and Harris ('47) and contained the following substances, in percentages: casein, 20; sucrose, 56; U. S. P. XIV salt mixture, 4 dried brewers' yeast, 10; lard, 10; 10 units of vitamin A and 1 unit of vitamin D per gram of diet. Four diets were prepared using 10, as well as 20%, of vitamin-free casein;[2] each protein level without and with added vitamin E (300 mg of *dl*, α-tocopherol acetate per kilogram of diet). The sucrose was varied inversely as the casein. The 10% of brewers' yeast contributed about 4.6% protein to each diet. By analysis the diets contained 14.3 and 23.7% of protein, respectively.

When the rats were 21 days old, the mothers were removed, the sexes separated, and the weanling rats continued on the diets in groups of three rats per wire cage. The rats were weighed approximately at weekly intervals. When mature, a random sample of the females fed the vitamin E-deprived diets produced litters only when supplemented with 2 to 4 mg of *dl*, α-tocopherol acetate per day for one week during early pregnancy. Some of the vitamin E-deprived rats were subjected to the dialuric acid hemolysis test (Rose and Gyorgy, '50) after they reached maturity and all gave a positive reaction.

At 65, 95, 185 and 365 days of age two rats of each sex, fed the high-protein diet, with and without vitamin E and three rats of each sex, fed the low-protein diet, with and without vita-

[1] Purina pellets.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

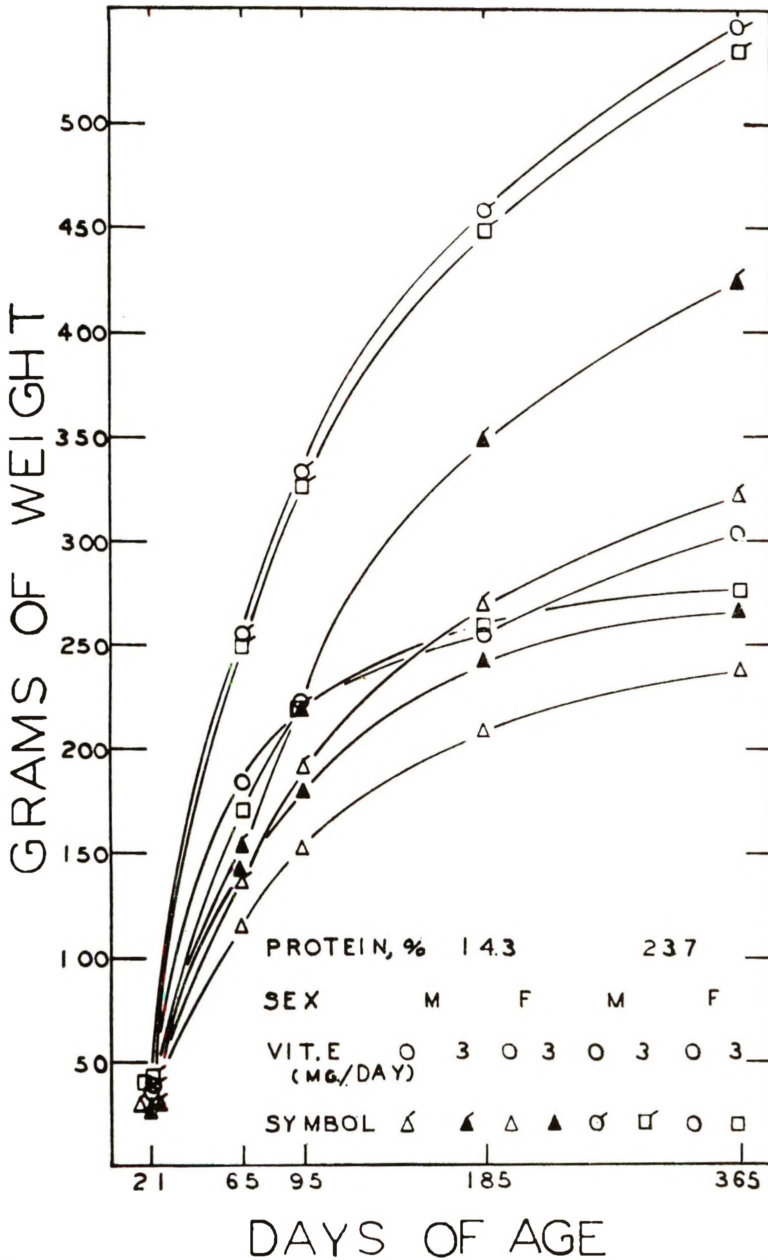


Fig. 1 Effect of two dietary levels of protein, with and without vitamin E, on the growth of rats fed the diets starting at 12 days of age. Significance: protein ($P < 0.01$); vitamin E with 14.3% protein for both sexes ($P < 0.01$).

min E were sacrificed by decapitation, and lactic dehydrogenase and succinoxidase were determined immediately on the ventricles of the heart. The lactic dehydrogenase was determined by the manometric method of Green and Brosteaux ('36), using adrenaline as the hydrogen carrier. The succinoxidase was determined by the manometric method of Schneider and Potter ('43) at 38°C. Total nitrogen in the homogenates was determined by the Kjeldahl method, and activities

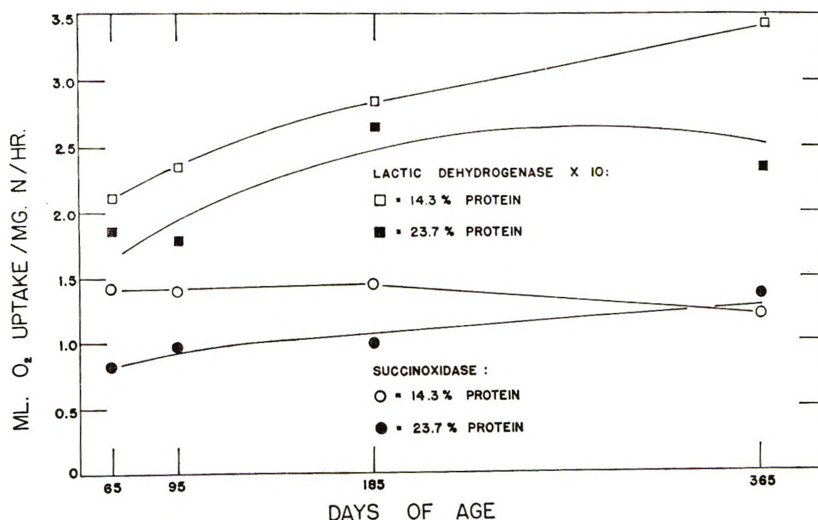


Fig. 2 Effect of two dietary levels of protein, and age, on the lactic dehydrogenase and succinoxidase of the hearts of rats. Each observation for the 14.3 and 23.7% protein diets is the mean of 12 and 18 rats, respectively. Significance: protein ($P < 0.01$) for both enzymes; age ($P < 0.01$) for the lactic dehydrogenase activity only.

of the enzymes were calculated as microliters of O_2 uptake per hour per milligram of nitrogen and per gram of wet weight. Statistical analysis of the data was made according to Snedecor ('46).

RESULTS AND DISCUSSION

As shown in figure 1 the vitamin E stimulated ($P < 0.01$) growth in both sexes of the rats fed the low-protein diet, the males more than the females. However, vitamin E had no significant effect on the growth of the rats fed the high protein

diet. Various investigators (Dam, '44; Hove, '46; Hove and Harris, '47) have reported that vitamin E stimulates weight gains on low-protein diets.

In figure 2 data are presented showing the effect of the two dietary levels of protein and of age on lactic dehydrogenase and succinoxidase activity in the ventricles of the rats. Sex had no significant effect on the enzyme activity, and so data for the individual sexes are combined. Each value in figure 2 for the 14.3 and 23.7% protein diets is the mean of 12 and 8 rats respectively. All values are expressed as microliters of oxygen uptake per hour per milligram of nitrogen in the homogenate. The lactic dehydrogenase values were multiplied by 10 before graphing. The rats fed the low-protein diet had higher ($P < 0.01$) values for both lactic dehyrogenase and succinoxidase activity. However, at 365 days of age the succinoxidase was not affected by the amount of dietary protein. On the basis of activity per gram of wet weight of the heart, the same effect of dietary protein was observed as graphed on the basis of per milligram of nitrogen.

Although the rats of the low-protein group received only about 66, 81 and 92% of their methionine, tryptophan, and phenylalanine requirements, respectively (Farris and Griffith, '49), they cannot be regarded as having a protein diet comparable to the protein-free diets of the mature rats of Wainio et al. ('54). These workers found a decrease of succinic dehydrogenase in the heart on the wet weight basis, but essentially no effect on the activity per milligram of nitrogen basis. The observations by Wainio et al. suggest that the effects of protein in the present study might be due to action on electron carriers associated with the cytochrome system, which was not involved in their study with succinic dehydrogenase. This dietary effect also occurred with the lactic dehydrogenase in the present study, which may involve cytochrome components even though adrenaline was used as an electron carrier. A simpler explanation may be that the rats on low dietary protein did not form as much non-enzyme protein in their tissues,

and thereby had greater enzymatic activity per milligram of total nitrogen.

As the rats grew older the lactic dehydrogenase activity increased ($P < 0.01$); age did not affect the succinoxidase activity. The influence of age on both enzymes was the same on the nitrogen and the wet weight basis.

In figure 3 data are graphed that show the effect of dietary vitamin E on the lactic dehydrogenase and succinoxidase activity of the ventricles of rats at 65, 95, 185 and 365 days of age. The values are reported as microliters of oxygen uptake per hour per milligram of nitrogen. The values obtained for the lactic dehydrogenase activity were multiplied by 10 before graphing. Each observation graphed is the mean of 10 rats. Vitamin E had no effect on the lactic dehydrogenase, either on the nitrogen or wet weight basis. With or without the vitamin E, the lactic dehydrogenase activity increased ($P < 0.01$) with age.

Statistically, the rats (fig. 3) that received the vitamin E had more succinoxidase activity in the ventricles on the nitrogen basis ($P < 0.05$) and on the wet weight basis ($P < 0.01$). However, the values obtained at 65 and 365 days of age were essentially the same for both of the groups fed vitamin E. These data appear to conflict with those of Houchin ('42), who observed more succinoxidase in muscles of vitamin E-deprived rats, hamsters and rabbits. The answer to the difference must lie in the observations of Jacobi et al. ('50), who found that a stimulation or an inhibition of succinoxidase activity could be demonstrated *in vitro* in homogenates of liver or muscle depending on the concentration of α-tocopherol phosphate present. Liver will store larger quantities of vitamin E than other tissues when animals are fed adequate diets (Mason, '42), and in the present study gave less ($P < 0.01$) succinoxidase activity on the wet weight basis for the group that received vitamin E. This same degree of inhibition was observed also for lactic dehydrogenase in the liver on the basis of activity per gram of wet weight. However, observations made on the skeletal muscles in this study showed slight-

ly more (not significant) activity of both enzymes in the group fed vitamin E. Apparently a stimulation or an inhibition of activity of both these enzymes may vary in different investigations, depending on the tissue studied and the dietary vitamin E treatment.

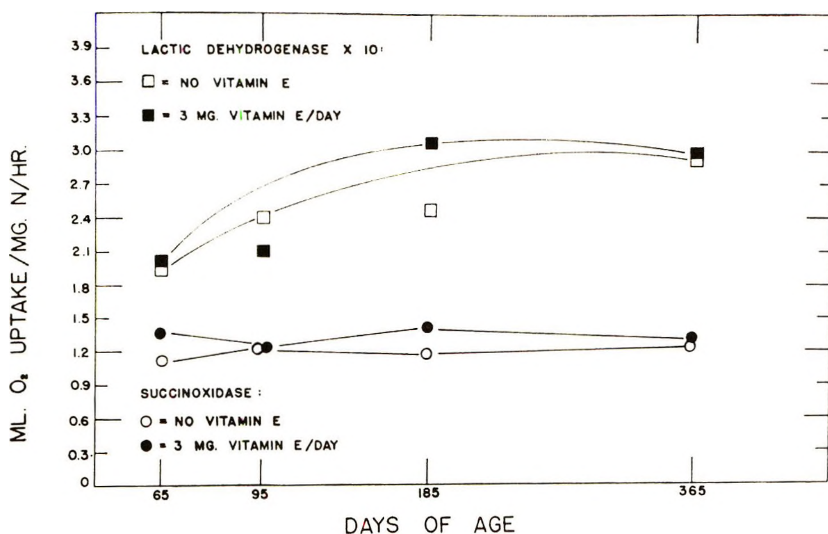


Fig. 3 Effect of vitamin E and age on the lactic dehydrogenase and succinoxidase activity of the hearts of rats. Each observation is the mean of 10 rats. Significance: vitamin E increased ($P < 0.05$) the succinoxidase, but not the lactic dehydrogenase; lactic dehydrogenase increased ($P < 0.05$) with age, but age had no effect on the succinoxidase.

SUMMARY

A study has been made of the effect of two dietary levels of protein (14.3 and 23.7%), each with and without vitamin E (*dl,* α-tocopherol acetate), upon the lactic dehydrogenase and succinoxidase activity of the heart ventricle of rats at 65, 95, 185 and 365 days of age. The animals were placed on the diets by litters at 12 days of age. Vitamin E aided ($P < 0.01$) the growth of the rats fed the low-protein diets. The groups fed the low dietary protein had more ($P < 0.01$) lactic dehydrogenase and succinoxidase activity in the heart. Those fed vitamin E had more ($P < 0.05$) succinoxidase, but a similar

amount of lactic dehydrogenase activity in the heart, compared to the rats deprived of vitamin E. As the rats grew older the lactic dehydrogenase increased ($P < 0.01$), but age had no effect on the succinoxidase activity. Sex did not affect the activity of either enzyme.

ACKNOWLEDGMENT

The writers wish to thank J. F. Easley and J. L. Evans for technical assistance in this study. The work was supported in part by Grants-in-Aid no. H-1318, National Heart Institute. Some of these data were presented at the American Chemical Society Meeting, Dallas, Texas, April 8–13–1956. Florida Agricultural Experiment Station Journal Series, no. 744.

LITERATURE CITED

BOUMAN, J., AND E. C. SLATER 1956 Tocopherol content of heart muscle preparations. Nature, *177:* 1181–1182.

DAM, H. 1944 Vitamin E and length of life of rats fed a diet with fatally low protein content. Proc. Soc. Exp. Biol. Med., *55:* 55–56.

FARRIS, E. J., AND J. Q. GRIFFITH, JR. 1949 Editors. The Rat in Laboratory Investigation. J. B. Lippincott Co., Phila., Pa.

GOVIER, W. M., N. YANZ AND M. E. GRELIS 1946 The effect of alpha tocopherol phosphate, digitoxin and certain compounds related to the latter on cardiac muscle metabolism *in vitro.* J. Pharm. Exp. Therapeutics, *88:* 373–381.

GREEN, D. E., AND J. BROSTEAUX 1936 The lactic dehydrogenase of animal tissues. Biochem. J., *30:* 1489–1508.

HOUCHIN, O. B. 1942 The *in vitro* effect of alpha tocopherol and its phosphate derivative on oxidation in muscle tissue. J. Biol. Chem., *146:* 313–321.

HOVE, E. L. 1946 Interrelation between alpha tocopherol and protein metabolism, body weight and tooth pigmentation. Proc. Soc. Exp. Biol. Med., *63:* 508–511.

HOVE, E. L., AND P. L. HARRIS 1947 Interrelation between alpha tocopherol and protein metabolism. II. The increased utilization of casein produced by alpha tocopherol, yeast digest and xanthine in the rat growth protein-quality test. J. Nutrition, *34:* 571–579.

JACOBI, H. P., S. ROSENBLATT, J. W. CHAPPELL AND S. MORGULIS 1950 The locus of action of alpha tocopherol phosphate in the succinoxidase system. Arch. Biochem., *27:* 9–18.

MASON, K. E. 1942 Distribution of vitamin E in the tissues of the rat. J. Nutrition, *23:* 71–81.

MASON, K. E., AND P. L. HARRIS 1947 Bioassay of vitamin E. Biol. Symposia, *12:* 459–483.

Rose, C. S., and P. Gyorgy 1950 Tocopherol requirements of rats studied by means of the (dialuric acid) hemolysis test. Proc. Soc. Exp. Biol. Med., *74:* 411–415.

Schneider, W. C., and V. R. Potter 1943 The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem., *149:* 217–227.

Snedecor, G. W. 1946 Statistical Methods. Iowa State College Press, Ames.

Wainio, W. W., J. B. Allison, B. Eichel, P. Person and G. R. Rowley 1954 Enzymes in protein depletion. II. Oxidative enzymes of heart ventricle. J. Nutrition, *52:* 565–573.

Wroblewski, F., P. Ruegsegger and J. S. LaDue 1956 Serum lactic dehydrogenase activity in acute transmural myocardial infarction Science, *123:* 1122–1123.

DENTAL CARIES IN THE ALBINO RAT IN RELATION TO THE CHEMICAL COMPOSITION OF THE TEETH AND OF THE DIET

IV. VARIATIONS IN THE CA/P RATIO OF THE DIET INDUCED BY CHANGING THE CALCIUM CONTENT

JOHN HALDI, WINFREY WYNN, KATHERINE D. BENTLEY AND MARY L. LAW
Department of Physiology, Emory University
Atlanta, Georgia

(Received for publication October 20, 1958)

A progressive decrease in the cariogenicity of a high sucrose synthetic diet has been observed when the Ca/P ratio was decreased from 1:0.5 to 1:1 and 1:2 by varying the phosphate content of the diet while maintaining the calcium at a constant 0.5% level (Wynn, Haldi, Bentley and Law, '56). The reduction in the incidence and extent of dental caries was not related to any detectable chemical changes in the composition of the enamel and dentin of the teeth.

The results of these experiments left unanswered the question whether the reduction in the cariogenicity of the diet should be attributed to changes in the Ca/P ratio *per se* or to an increase in the phosphorus concentration. The experiments reported in this paper have been conducted in an effort to throw some light on this problem, and at the same time to determine whether additional amounts of calcium in the diet would, like additional amounts of phosphorus, reduce its cariogenicity. Since the work of Sobel and Hanok ('48, '58) seems to indicate a possible interrelationship of the Ca/P ratio of the diet, dental caries and the chemical composition of the teeth, this study has been extended to include chemical analyses of both the molar and incisor teeth.

EXPERIMENTAL

Experiment on dental caries. Twenty quadruplicate groups of litter mate albino rats were selected at weaning and sialoadenectomized in the manner described previously (Haldi, Wynn, Shaw and Sognnaes, '53). They were then assigned to 4 experimental diets. The basic diet consisted of 64% sucrose, 20% casein, 8% fat, 4% yeast and liver extract, 4% salt mixture and vitamin supplements. The salt mixture was the same as that employed in our earlier experiments (Haldi, Wynn, Law and Bentley, '55) except that the calcium content was adjusted to give the desired Ca/P ratios for the different experimental diets. The phosphorus concentration of all 4 diets remained the same at the level of approximately 0.5%; available data indicate this level is sufficient to meet the nutritional requirements of the rat (Cox and Imboden, '36; Hubbell, Mendel and Wakeman, '37). Calcium, which was present in the amount of 0.29% in the diet with the Ca/P ratio of 1 : 2 was increased in the other three diets by the addition of calcium carbonate to give Ca/P ratios of 1 : 1, 1 : 0.5 and 1 : 0.3. All the diets were analyzed for their calcium and phosphorus content. The food intake of each quadruplicate group of animals fed the 4 diets was equalized so that each animal in a group would have the same phosphorus and caloric intake. At the end of a 10-day feeding period, the animals were sacrificed, the maxillae and mandibles removed and the teeth examined under a dissecting microscope and scored for dental caries by the method customarily employed in our laboratories (Haldi and Wynn, '52).

Experiment on tooth composition. Inasmuch as an accurate analysis of sound tooth substance cannot be obtained when the teeth are carious, another experiment was conducted in which the salivary glands were left intact and the animals fed the experimental diets 70 days as in the previous experiment on dental caries. In our laboratories dental caries does not develop in intact animals of our Wistar colony when fed our cariogenic diets for this length of time.

In this experiment only two diets were employed; namely, those with the lowest and highest Ca/P ratios of 1:2 and 1:0.3. The calcium concentration in the former was 0.29% and in the latter 1.57% with the phosphorus concentration 0.52% in each. The calcium and phosphorus contents of the experimental diets are given in table 1.

TABLE 1

Composition of diets with various Ca/P ratios and their effect on dental caries

Ca/P OF DIET	CA IN DIET	P IN DIET	NO. OF RATS	AVERAGE NO. OF LESIONS	CRITICAL RATIO [2]	AVERAGE CARIES SCORE	CRITICAL RATIO [2]
	%	%					
1: 2.0	0.29	0.52	20	23 ± 0.4 [1]		34 ± 1.5 [1]	
					> 3.0		> 3.5
1: 1.0	0.57	0.50	20	21 ± 0.5		27 ± 1.3	
					> 2.6		> 3.3
1: 0.5	1.01	0.49	20	19 ± 0.8		21 ± 1.2	
					> 1.9		> 0.6
1: 0.3	1.57	0.52	20	17 ± 0.8		20 ± 1.2	

[1] The ± values are the standard errors of the means (S.E.M.).

[2] The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is: less than 2.0, the difference between the means is considered to be statistically insignificant; 2.0 to 2.9, the difference is of borderline significance; 3.0 or more, the difference is highly significant (Dunning, J. M., 1950, J. Dent. Res., *29*: 541).

Forty pairs of littermates were selected at weaning and fed the two diets. As in the preceding experiment, the food intake of each pair of littermates was equalized. At the end of the 70-day feeding period, the animals were sacrificed and the incisors and molars removed, cleaned and dried. The two sets of teeth were ground separately fine enough to pass through a 100-mesh sieve. The ground material was dried in a vacuum over P_2O_5. The incisors from 5 animals were pooled in order to provide an adequate amount of tooth substance for chemical analyses. The same procedure was followed with the molars. As there were 40 pairs of animals, this gave 8 samples of enamel and the same number of dentin. Dentin was separated from enamel by the flotation method of Manly and Hodge ('39) as modified by Gilda ('51).

Analyses of the enamel and dentin were made by the following procedures: nitrogen, micro-Kjeldahl; calcium, Sobel, Rochenmacher and Kramer ('44); phosphorus, Fisk and Subbarow ('25); carbon dioxide, Van Slyke and Folch ('40) as modified by Sobel, Rochenmacher and Kramer ('44); magnesium, Young and Gill ('51). Replicate analyses of aliquots from the same sample gave the following degrees of precision in terms of per cent: $N_2 \pm 0.02$; Ca $\pm$ 0.07; P $\pm$ 0.07; $CO_2 \pm$ 0.024; Mg $\pm$ 0.005.

RESULTS

Dental caries. The number of carious lesions and the caries scores are presented in table 1. The addition of calcium to the diet in sufficient amounts to raise the Ca/P ratio from 1:2 to 1:1 resulted in a significant reduction in the number of carious lesions and in the caries score. This effect was accentuated by an additional increase in the calcium of the diet to give a ratio of 1:0.5. When more calcium was added to give a Ca/P ratio of 1:0.3 there was no further reduction in the number of carious lesions or in the caries score.

Casual inspection of these data would suggest that modification of the cariogenicity of the diet may have been due to changes in its Ca/P ratio. Comparison with data previously reported (Wynn, Haldi, Bentley and Law, '56), however, reveal that this was not the case. In the present experiments a progressive increase in the Ca/P ratio of the diet up to a certain point resulted in a progressive decrease in the number of carious lesions and in the caries score, whereas in our earlier experiments this relationship was reversed.

In the earlier, as in the later experiments, remarkably close quantitative results were obtained when the calcium and phosphorus content of the diet was the same. In the former, with both the calcium and phosphorus in the diet at approximately the 0.5% level (Ca/P = 1:1), the number of lesions was 18 and the average score 27; in the latter, under almost identical conditions, these values were 21 and 27, respectively.

It will be noted in comparing the present with the earlier experiments that a progressive increase in the calcium or phos-

phorus content of the diet up to a certain point, which may be regarded as the optimal level, resulted in a progressive decrease in its cariogenicity. It may therefore be concluded that the cariogenicity of the diet was related to its actual calcium and phosphorus content and not to the Ca/P ratio. It is of interest to note that Nizel, Keating, Sundstrom and Harris ('58) found that the addition of phosphoric acid to a cariogenic diet caused a marked reduction in dental caries in hamsters.

Tooth composition. The data obtained from analyses of the enamel and dentin of the molars and incisors are presented in table 2. There were no differences in the composition of enamel or dentin of either molars or incisors of rats fed diets with Ca/P ratios of 1:2 or 1:0.3.

Although the molars were almost completely calcified at the initiation of the experiment, it was thought that there might possibly have been an ionic exchange between the oral environment and the teeth, a possibility suggested by experiments of Armstrong and Barnum ('48), Volker and Sognnaes ('40), Sognnaes, Shaw and Bogoroch ('55) and others, working with radioisotopes. Obviously this did not take place, at least to the extent that could be detected by the microanalytical procedures that were employed. Modification of the cariogenicity of the diet by the addition of calcium could not therefore be attributed to any detectable changes in the composition of the teeth resulting therefrom.

Analyses were made on the incisors to determine whether the results of Sobel and Hanok ('48, '58) could be simulated under our experimental conditions. These investigators found that the composition of the teeth is related to the composition of the blood serum which, in turn, is related to the Ca/P ratio of the diet. In our experiments it was necessary to take into account the possibility that changes in the Ca/P ratio might affect the incisors and the molars differently. The first and second molars were calcified and erupted when the animals were placed on the experimental diets and the third molar erupted shortly thereafter, whereas the incisors grew con-

TABLE 2

Chemical analyses of tooth substance of albino rats fed diets with different Ca/P ratios [1,2]

Ca/P RATIO IN DIET	N	Mg	CO_2	Ca	P	Ca/P
	%	%	%	%	%	
			Dried enamel of molars			
1: 2.0	0.21 ± 0.06	0.18 ± 0.02	2.17 ± 0.03	34.2 ± 0.47	17.3 ± 0.18	1.98 ± 0.02
1: 0.3	0.20 ± 0.04	0.18 ± 0.03	2.20 ± 0.03	34.2 ± 0.54	17.3 ± 0.20	1.98 ± 0.03
			Dried dentin of molars			
1: 2.0	2.94 ± 0.11	0.31 ± 0.03	2.67 ± 0.02	26.7 ± 0.34	13.8 ± 0.41	1.95 ± 0.06
1: 0.3	2.90 ± 0.10	0.32 ± 0.04	2.67 ± 0.04	26.7 ± 0.15	13.8 ± 0.40	1.94 ± 0.05
			Dried enamel of incisors			
1: 2.0	0.22 ± 0.04	Trace	1.26 ± 0.07	34.7 ± 0.95	18.3 ± 0.47	1.90 ± 0.09
1: 0.3	0.24 ± 0.02	Trace	1.30 ± 0.05	35.0 ± 1.17	18.4 ± 0.28	1.90 ± 0.06
			Dried dentin of incisors			
1: 2.0	2.72 ± 0.03	1.59 ± 0.09	1.93 ± 0.05	25.6 ± 0.38	14.6 ± 0.58	1.75 ± 0.08
1: 0.3	2.69 ± 0.06	1.50 ± 0.14	2.02 ± 0.07	26.0 ± 0.56	15.2 ± 0.66	1.65 ± 0.15

[1] The calcium content of the first diet was 0.29% and of the second diet, 1.5%; the phosphorus content of both diets was 0.5%.

[2] Each value in the table is an average of 8 pooled samples. Each pooled sample was taken from the teeth of 5 animals. The (±) values are standard deviations.

tinuously. As the incisors are entirely replaced in about 40 days, the enamel and dentin of these teeth at the conclusion of the experiment were formed while the animals were on the experimental diets.

The data in table 2 show that there was no significant difference in the composition of either the enamel or the dentin of the incisors when the animals were fed diets with the widely divergent Ca/P ratios of 1:2 and 1:0.3. These results confirm the conclusion drawn from previous experimental data (Wynn, Haldi, Bentley and Law, '57) that if the hypothesis relating the inorganic composition of the incisor teeth to the Ca/P ratio of the diet is correct, it is applicable only under certain conditions which did not prevail in our experiments. The suggestion is offered that the results obtained by Sobel and Hanok may have been due to the very low calcium content of 0.10% in their low Ca- high P diet and to the phosphorus content of only 0.118% in their high Ca- low P diet.

SUMMARY AND CONCLUSIONS

Albino rats were fed synthetic cariogenic diets which differed from one another only in the calcium content which was changed by the addition of $CaCO_3$ to produce different Ca/P ratios. The phosphorus content of the diet was maintained at a constant level of 0.5%.

A progressive decrease in the cariogenicity of the diet occurred as its Ca/P ratio was increased from 1:2 to 1:1 and 1:0.5. There was no further reduction in cariogenicity when more calcium was added to give a Ca/P ratio of 1:0.3.

This modification in the cariogenicity of the diet by changing the Ca/P ratio did not produce any detectable changes in the composition of the molars. The composition of the incisor teeth which were formed during the feeding period was likewise the same on diets with the widely divergent ratios of 1:2.0 and 1:0.3.

From these observations considered along with others previously reported, it is concluded that changes in the cariogenicity of the diet induced by changing the Ca/P ratio in our

experiments must be attributed to the actual calcium and phosphorus content of the diet and not to its Ca/P ratio.

ACKNOWLEDGMENT

This investigation was carried out under Contract DA-49-007-MD-675 with the U. S. Army Medical Research and Development Command, Office of the Surgeon General of the U. S. Army.

LITERATURE CITED

ARMSTRONG, W. D., AND C. P. BARNUM 1948 Concurrent use of radioisotopes of calcium and phosphorus in the study of metabolism of calcified tissues. J. Biol. Chem., *172:* 199.

COX, W. M., JR., AND M. IMBODEN 1936 The role of calcium and phosphorus in determining reproductive success. J. Nutrition, *11:* 147.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 275.

GILDA, J. E. 1951 Studies on the physical properties of rodent enamel. I. The Manly-Hodge separation method as applied to the teeth of the rat and hamster. J. Dent. Res., *30:* 445.

HALDI, J., AND W. WYNN 1952 Cariogenicity of sugar containing diets. J. Nutrition, *46:* 425.

HALDI, J., W. WYNN, J. H. SHAW AND R. F. SOGNNAES 1953 The relative cariogenicity of sucrose when ingested in the solid form and in solution by the albino rat. Ibid., *49:* 295.

HALDI, J., W. WYNN, M. L. LAW AND K. D. BENTLEY 1955 Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. I. Effect of prenatal and postnatal feeding of high protein, high fat and high carbohydrate diets. Ibid., *57:* 215.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. Ibid., *14:* 273.

MANLY, R. S., AND H. C. HODGE 1939 Density and refractive index studies of dental hard tissues. I. Methods of separation and determination of purity. J. Dent. Res., *18:* 133.

NIZEL, A. E., N. KEATING, C. SUNDSTROM AND R. S. HARRIS 1958 Effect of phosphate supplement to diet on development of hamster caries. Ibid., *37:* 35.

SOBEL, A. E., M. ROCHENMACHER AND B. KRAMER 1944 Microestimation of the inorganic constituents of bone. J. Biol. Chem., *152:* 255.

SOBEL, A. E., AND ALBERT HANOK 1948 Calcification of teeth. I. Composition in relation to blood and diet. Ibid., *176:* 1103.

——— 1958 Calcification. XVI. Composition of bones and teeth in relation to blood and diet in the cotton rat. J. Dent. Res., *37:* 631.

SOGNNAES, R. F., J. H. SHAW AND R. BOGOROCH 1955 Radiotracer studies on bone, cementum, dentin and enamel of rhesus monkeys. Am. J. Physiol., *180:* 408.

VAN SLYKE, D. D., AND J. FOLCH 1940 Manometric carbon determination. J. Biol. Chem., *136:* 509.

VOLKER, J. F., AND R. F. SOGNNAES 1940 A study of the phosphorus metabolism in the dental tissues of the cat by the use of radioactive phosphorus. J. Dent. Res., *19:* 292.

WYNN, W., J. HALDI, K. D. BENTLEY AND M. L. LAW 1956 Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. II. Variations in the Ca/P ratio of the diet induced by changing the phosphorus content. J. Nutrition, *58:* 325.

——— 1957 Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. III. Composition of incisor teeth of animals fed diets with different Ca/P ratios. Ibid., *63:* 57.

YOUNG, H. Y., AND R. F. GILL 1951 Determination of magnesium in plant tissues with thiazole yellow. Anal. Chem., *23:* 751.

THE EFFECT OF ETHANOLAMINE AND ITS *N*-METHYL DERIVATIVES ON KIDNEY HEMORRHAGIC DEGENERATION IN RATS DUE TO 2-AMINO-2-METHYL-1-PROPANOL [1]

CHARLOTTE E. OUTLAND, EDWARD H. MEALEY,
WILLIAM J. LONGMORE AND DWIGHT J. MULFORD
Department of Biochemistry, University of Kansas, Lawrence

(Received for publication October 20, 1958)

Considerable evidence indicating that the *N*-methyl derivatives of ethanolamine prevent the effects of choline deficiency in animals has been recorded. It has been shown by duVigneaud, Chandler, Simmonds, Moyer and Cohn ('46) that dimethylethanolamine is effective in preventing kidney hemorrhagic degeneration in young rats deficient in choline. Young, Lucas, Patterson and Best ('57) have demonstrated that monomethylethanolamine also prevents kidney lesions in young rats on diets deficient in choline. Jukes and Oleson ('45) and Jukes, Oleson and Dornbush ('45) have shown that both dimethylethanolamine and choline prevent perosis in chicks. They found monomethylethanolamine to be effective but to a lesser extent. Ethanolamine was found to have no effect.

We have studied the effects of ethanolamine and its *N*-methyl derivatives on renal hemorrhagic degeneration in rats consuming diets containing 2-amino-2-methyl-1-propanol. Wells ('55a), using young rats and Mulford and Outland ('57), using adult rats reported that 2-amino-2-methyl-1-propanol markedly increases the severity of choline deficiency.

[1] This investigation was supported by a research grant (A-219) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

Both laboratories have shown further that betaine, methionine, β-dimethylpropiothetin and casein have essentially no effect in overcoming the anticholine action of this drug (Wells, '55b, '56; Mulford, '55). The present paper shows that monomethylethanolamine, dimethylethanolamine and choline protected against kidney hemorrhagic degeneration in young rats receiving low choline diets containing 2-amino-2-methyl-1-propanol while ethanolamine was without effect. The essence of these studies has been reported (Outland, Mealey, Longmore and Mulford, '58).

EXPERIMENTAL

Male rats of the Sprague-Dawley strain, 21 days of age and weighing between 40 and 50 gm, were placed in raised cages, 5 to a cage and fed experimental diets ad libitum for 6 days. Water was available to the animals at all times. Food consumption and body weights were recorded each day. At the end of the 6-day period the rats were sacrificed by decapitation and the kidneys examined for hemorrhages.

Two basal low-choline diets, A and B, were used in these experiments. Both diets contained dry brewers' yeast [2] 6, agar 2, salt mixture [3] 4, lard 19.9, fortified fish liver oil [4] 0.1, calcium carbonate 1, and L-cystine 0.3%. Diet A contained casein [5] 18 and cane sugar 48.7% and diet B contained casein 42 and cane sugar 24.7%.

2-Amino-2-methyl-1-propanol in diets A and B. The effect of the addition of 2-amino-2-methyl-1-propanol (2A2M1P) to diets A and B at a level of 5 mg per gram of food is shown in table 1. The table shows that when no 2A2M1P was added, each animal ate on the average 6 to 7 gm of food per day, grew at the rate of 4 gm per day and developed no hemorrhagic kidneys. When 5 mg of the drug were added to each diet per gram of food, 87 to 98% of the animals developed kidney lesions. Each ate on the average only 4.2 to 4.9 gm of

[2] Anheuser-Busch, Inc., strain G.

[3] General Biochemicals, Inc., Salt Mixture XIV.

[4] Parke-Davis and Company, Natola.

[5] General Biochemicals, Inc., vitamin-free.

TABLE 1

The effect of 2-amino-2-methyl-1-propanol (2A2M1P) in rats consuming diets low in choline

DIET AND NUMBER OF RATS	2A2M1P PER GRAM FOOD	RATS WITH KIDNEY LESIONS	AVERAGE WEIGHT INCREASE PER RAT PER DAY	AVERAGE FOOD INTAKE PER RAT PER DAY
	mg	%	*gm*	*gm*
A, 30	0	0	4.1	6.7
B, 30	0	0	3.6	6.0
A, 234	5	87 (1)[1]	1.9	4.9
B, 184	5	98 (3)	1.2	4.2

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

TABLE 2

The effect of ethanolamine or its N-methyl derivatives in rats consuming either diet A or B containing 5 mg of 2-amino-2-methyl-1-propanol per gram of food

SUPPLEMENT PER GRAM FOOD [1]	NUMBER OF RATS	RATS WITH KIDNEY LESIONS	AVERAGE WEIGHT INCREASE PER RAT PER DAY	AVERAGE FOOD INTAKE PER RAT PER DAY
$\times 10^{-3}$ *mM*		%	*gm*	*gm*
		Diet A		
5.4 EA	30	83	2.03	5.17
5.4 MME	30	37	2.71	5.33
5.4 DME	31	42	2.44	5.11
5.4 Choline	30	0	3.32	6.01
10.8 EA	30	50	1.70	4.79
10.8 MME	32	3	3.38	5.28
10.8 DME	31	13	2.68	5.72
10.8 Choline	30	0	3.31	5.70
		Diet B		
5.4 EA	15	100	1.38	4.06
5.4 MME	15	87	2.37	4.53
5.4 DME	15	100	1.64	4.16
5.4 Choline	15	73	2.06	4.42
10.8 EA	31	100	1.01	4.19
10.8 MME	32	0	2.76	5.15
10.8 DME	30	17	2.48	5.18
10.8 Choline	30	0	3.17	5.68

[1] EA = ethanolamine, MME = monomethylethanolamine, DME = dimethylethanolamine.

food per day and grew at a rate of 1.2 to 1.9 gm per day. Some of the animals in this group had ocular hemorrhages and a few died.

Effect of addition of either ethanolamine or one of its N-methyl derivatives to both diets containing 2-amino-2-methyl-1-propanol. The effect of adding ethanolamine or one of its *N*-methyl derivatives to diets A and B containing 5 mg 2A2M1P per gram of food is shown in table 2. Each supplement was fed at levels ranging from 1.8×10^{-3} to 10.8×10^{-3} mM per gram of food. Table 2 shows the results obtained when two levels (5.4×10^{-3} and 10.8×10^{-3} mM per gram) were fed.

TABLE 3

The effect of higher levels of ethanolamine in rats consuming either diet A or B containing 2-amino-2-methyl-1-propanol (2A2M1P)

DIET	2A2M1P PER GRAM FOOD	ETHANOLAMINE ADDED PER GRAM FOOD	NUMBER OF RATS	RATS WITH KIDNEY LESIONS
	mg	$\times 10^{-3}$ *mM*		%
B	5	16	15	100
B	5	49	15	100
A	3	82	15	100
B	3	82	15	80
A	3	164	15	100

While monomethylethanolamine, dimethylethanolamine and choline were effective in lowering the incidence of kidney lesions due to 2A2M1P, ethanolamine was quite ineffective. Ethanolamine was fed at levels higher than 10.8×10^{-3} mM per gram of food. Both diets A and B containing either 3 or 5 mg of 2A2M1P per gram of food were used. Table 3 shows that ethanolamine levels up to 164×10^{-3} mM per gram of food did not prevent kidney lesions in animals consuming 3 mg of 2A2M1P per gram of food. The average food intake per rat per day was between 3.8 and 4.7 gm and the average weight increase per rat per day was between 1.7 and 2.8 gm.

In another series of experiments ethanolamine or one of its *N*-methyl derivatives was administered to rats by one daily

intraperitoneal injection. 2-Amino-2-methyl-1-propanol was fed at a level of 5 mg per gram of food in both diets A and B. Table 4 shows that when 54×10^{-3} mM of either monomethylethanolamine, dimethylethanolamine or choline contained in 0.5 ml of normal saline was injected intraperitoneally, the percentage of rats with kidney lesions was markedly reduced. When ethanolamine was injected at the same level, the percentage was the same as that in the saline-injected control animals.

TABLE 4

The effect of a daily intraperitoneal injection of 54.0×10^{-3} mM of ethanolamine or its N-methyl derivatives in rats receiving 5 mg of 2-amino-2-methyl-1-propanol per gram of food

DERIVATIVE INJECTED [1]	NUMBER OF RATS	RATS WITH KIDNEY LESIONS
		%
Diet A		
None	15	80
Ethanolamine	15	87
Monomethylethanolamine	15	20
Dimethylethanolamine	15	0
Choline	15	0
Diet B		
None	15	100
Ethanolamine	15	100
Monomethylethanolamine	15	40
Dimethylethanolamine	15	0
Choline	15	13

[1] Each supplement injected was contained in 0.5 ml of normal saline. The pH was approximately 7.0 after adjustment.

In another series of experiments monomethylethanolamine and dimethylethanolamine were fed to rats in diet B at a level of 10.8×10^{-3} mM per gram of food. 2-Amino-2-methyl-1-propanol was administered by one daily intraperitoneal injection. Levels of 15, 20 and 25 mg each contained in 0.5 ml of normal saline were injected. Table 5 shows that while a large percentage of control animals had kidney lesions, only one animal receiving monomethylethanolamine and none of the animals receiving dimethylethanolamine had lesions.

TABLE 5

The effect of monomethylethanolamine (MME) and dimethylethanolamine (DME) in diet B on rats receiving one intraperitoneal injection of 2-amino-2-methyl-1-propanol (2A2M1P) daily

2A2M1P INJECTED DAILY [1]	SUPPLEMENT PER GRAM FOOD	NUMBER OF RATS	RATS WITH KIDNEY LESIONS
mg	$\times 10^{-3}$		%
15	0	19	58
15	10.8 MME	18	6
15	10.8 DME	10	0
20	0	24	88
20	10.8 MME	17	0
20	10.8 DME	20	0
25	0	24	96
25	10.8 MME	17	0
25	10.8 DME	20	0

[1] Each level of 2A2M1P injected was contained in 0.5 ml of normal saline. The pH was approximately 7.0 after adjustment.

DISCUSSION

The work of several investigators supports the hypothesis of Jukes, Dornbush and Oleson ('45) that, in the rat, choline is synthesized from ethanolamine by three successive steps each of which consists of the addition of a methyl group. Stetten ('41) found choline containing N^{15} in rats which had been fed N^{15} ethanolamine. duVigneaud, Chandler, Simmonds, Moyer and Cohn ('46) showed that dimethylethanolamine was effective in preventing kidney hemorrhagic degeneration in young rats even though it did not permit growth in the presence of homocysteine. On analysis of the kidney tissue of their rats they found that a considerable percentage of the methyl groups of choline was derived from dimethylethanolamine and monomethylethanolamine. Young, Lucas, Patterson and Best ('57) observed that monomethylethanolamine prevented the development of kidney lesions and reduced the liver fat in rats consuming diets low in both choline and methionine.

Evidence obtained on other biological systems gives support to the hypothesis that choline is synthesized from ethanolamine by three successive steps. Badger ('44a, b) found that

ethanolamine could support the growth of a strain of pneumococcus in the absence of choline. Another compound supporting growth was dimethylethanolamine. Horowitz, Bonner and Houlahan ('45) observed that both monomethylethanolamine and dimethylethanolamine were utilized by *Neurospora crassa*, strains 34486 and 47904. Horowitz ('46) found that *Neurospora crassa* 34486 could not synthesize monomethylethanolamine but could use an exogenous supply for choline synthesis, while strain 47904 could synthesize monomethylethanolamine but was unable to convert it to choline at a normal rate.

Jukes ('41) and Jukes, Oleson and Dornbush ('45) have shown that while ethanolamine was unable to prevent perosis in chicks, monomethylethanolamine and dimethylethanolamine, as well as choline, were effective. Schaeffer, Salmon and Strength ('50) observed that dimethylethanolamine was a replacement for choline in the prevention of perosis in chicks only when the diet contained vitamine B_{12}. Monomethylethanolamine was effective only when vitamine B_{12} and either methionine or betaine were present. Ethanolamine was ineffective under all conditions.

The data presented in this paper suggest that in the rat 2-amino-2-methyl-1-propanol probably interferes with the methylation of ethanolamine. Since monomethylethanolamine, dimethylethanolamine and choline protected against renal hemorrhagic degeneration both at high and low levels of casein and ethanolamine did not, it would appear that the drug prevented the incorporation of the first methyl group into ethanolamine. Investigations to ascertain the specific action of 2-amino-2-methyl-1-propanol are in progress in this laboratory.

SUMMARY

Two low-choline diets, one containing 18% casein (diet A) and the other 42% (diet B), were fed to weanling male rats for 6 days. None of the animals developed hemorrhagic kidneys. When 5 mg of 2-amino-2-methyl-1-propanol were added to these diets per gram of food, 87 and 98%, respectively, of

the animals developed hemorrhagic kidneys in 6 days. Some of the animals had ocular hemorrhages and some died. The addition of monomethylethanolamine, dimethylethanolamine and choline markedly reduced the percentage of animals having kidney lesions. Ethanolamine was quite ineffective at all levels studied. When injected intraperitoneally the *N*-methyl derivatives of ethanolamine reduced the incidence of lesions in animals consuming both diets containing 5 mg of 2-amino-2-methyl-1-propanol per gram of food. Ethanolamine had little effect. Both monomethylethanolamine and dimethylethanolamine at a level of 10.8×10^{-3} mM per gram of diet B prevented kidney lesions in animals receiving from 15 to 25 mg of 2-amino-2-methyl-1-propanol by one intraperitoneal injection daily for each of 6 days. Most of the saline-injected control animals at each level of dosage had lesions.

LITERATURE CITED

BADGER, E. 1944a The structural specificity of choline for the growth of Type III pneumococcus. J. Biol. Chem., *153:* 183.

———— 1944b The nutritional requirements of a strain of Type III pneumococcus. J. Bact., *47:* 509.

DUVIGNEAUD, V., J. P. CHANDLER, S. SIMMONDS, A. W. MOYER AND M. COHN 1946 The role of dimethyl- and monomethylaminoethanol in transmethylation reactions in vivo. J. Biol. Chem., *164:* 603.

HOROWITZ, N. H. 1946 The isolation and identification of a natural precursor of choline. Ibid., *162:* 413.

HOROWITZ, N. H., D. BONNER AND M. B. HOULAHAN 1945 The utilization of choline analogues by cholineless mutants of Neurospora. Ibid., *159:* 145.

JUKES, T. H. 1941 The effect of certain organic compounds and other dietary supplements on perosis. J. Nutrition, *22:* 315.

JUKES, T. H., A. C. DORNBUSH AND J. J. OLESON 1945 Some observations on nutritional effects of choline and related compounds. Federation Proc., *4:* 157.

JUKES, T. H., AND J. J. OLESON 1945 Choline-like nutritional effect of dimethylaminoethanol. J. Biol. Chem., *157:* 419.

JUKES, T. H., J. J. OLESON AND A. C. DORNBUSH 1945 Observations on monomethylaminoethanol and dimethylaminoethanol in the diet of chicks. J. Nutrition, *30:* 219.

MULFORD, D. J. 1955 Betaine and methionine on renal hemorrhagic degeneration in rats receiving 2-amino-2-methyl-propanol-1. Proc. Soc. Exp. Biol. Med., *89:* 471.

MULFORD, D. J., AND C. E. OUTLAND 1957 The effect of 2-amino-2-methyl-1-propanol on the incidence of kidney lesions in male rats of different ages fed diets low in choline. J. Nutrition, *61:* 381.

OUTLAND, C. E., E. H. MEALEY, W. J. LONGMORE AND D. J. MULFORD 1958 Ethanolamine and its *N*-methyl derivatives in rats receiving 2-amino-2-methyl-1-propanol. Federation Proc., *17:* 286.

STETTEN, D., JR. 1941 Biological relationships of choline, ethanolamine and related compounds. J. Biol. Chem., *140:* 143.

SCHAEFER, A. E., W. D. SALMON AND D. R. STRENGTH 1951 The influence of vitamine B_{12} on the utilization of choline precursors by the chick. J. Nutrition, *44:* 305.

WELLS, I. C. 1955a Antimetabolites of choline; studies in young rats. J. Biol. Chem., *217:* 631.

——— 1955b Choline antimetabolites; inhibition of choline synthesis. Federation Proc., *14:* 303.

——— 1956 Inhibition of choline synthesis in young rats by 2-amino-2-methylpropanol-1. J. Biol. Chem., *222:* 931.

YOUNG, R. J., C. C. LUCAS, J. M. PATTERSON AND C. H. BEST 1957 The role of dietary betaine and vitamin B_{12} in choline formation by the rat. Ibid., *224:* 341.

INDEX

AAES-JØRGENSEN, E., See Privett, O. S., 423.
AAES-JØRGENSEN, E., O. S. PRIVETT AND R. T. HOLMAN. Essential fatty acid activities of hydrocarbons and alcohols analogous to linoleate and linolenate, 413.
ACKERMAN, C. J. Growth-promoting activity of meat meal and certain tissues in goitrogen-fed rats and chicks, 589.
ALLISON, J. B. See Wainio, W. W., 197.
Amino acid(s), essential, biological availability to human subjects. I. Whole egg, pork and peanut butter, 483; II. Whole egg, milk and cottage cheese, 497.
— — of casein, sequence in which they become limiting for the growth of the rat, 109.
— —, urinary excretion of five essential by young women, 19.
2-Amino-2-methyl-1-propanol, effect of ethanolamine and its *N*-methyl derivatives on kidney hemorrhagic degeneration in rats, due to, 655.
beta-Aminopropionitrile-induced vascular hemorrhage in turkeys, effect of diet on the development of, 275.
ANDRUS, S. B. See Gershoff, S. N., 29.
Antibiotics, effect of on the weight of chicks and rats fed raw or heated soybean meal, 149.
Antioxidants, effect of on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken, 333.
Antithyrotoxic factor of liver. I. Method for assay, 397.
Antonowicz, I. See Gershoff, S. N., 29.
Aronoff, M. See Wainio, W. W., 197.
Arroyave, G. See Squibb, R. L., 351.
Ascorbic acid, adrenal level in guinea pigs, effect of orally administered rutin on, 531.

BABCOCK, M. J. Serum glutamic-oxalacetic transaminase activity of vitamin B_6-deficient rats, 205.
BARNES, R. H., E. KWONG AND G. FIALA. Effects of the prevention of coprophagy in the rat. IV. Biotin, 599.
BAUMANN, C. A. See Braham, J. E., 149; Kelleher, W. J., 433.
BEDRACK, E. See Shirley, R. L., 159.
BENDAÑA-BROWN, A., AND C. Y. LIM. Availability of calcium in some Philippine vegetables, 461.
BENTLEY, K. D. See Haldi, J., 569; Wynn, W., 569.
BENTLEY, O. G. See Johnson, R. R., 513.
BERNSTEIN, E. See Wainio, W. W., 197.
Biotin deficiency in the guinea pig, production of, 525.
—. Effects of the prevention of coprophagy in the rat. IV., 599.
BIRD, H. R. See Braham, J. E., 149.
BOOKER, L. K. See Watts, J. H., 483; 497.
BRADLEY, W. B. See Calhoun, W. K., 237.
BRAHAM, J. E. See Squibb, R. L., 351.
BRAHAM, J. E., H. R. BIRD AND C. A. BAUMANN. Effect of antibiotics on the weight of chicks and rats fed raw or heated soybean meal, 149.
Brain, enzymes of in protein depletion. III., 197.
BURRESS, D. A. See Harris, R. S., 549.

CALCIUM, availability of in some Philippine vegetables, 461.
— content of the diet, variations in the Ca/P ratio induced by changing. Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. IV., 645.
— oxalate excretion and hematuria in vitamin B_6-deficient rats fed phthalylsulfathiazole, 237.
— and phosphorus (fluoride), deposition in experimental low-phosphorus rickets, 59.
Ca^{45}, effect of high molybdenum intake on the distribution and excretion in the rabbit, 325.
CALHOUN, W. K., R. B. JENNINGS AND W. B. BRADLEY. Calcium oxalate excretion and hematuria in vitamin B_6-deficient rats fed phthalylsulfathiazole, 237.
Caries, dental, in the albino rat in relation to the chemical composition of the teeth and of the diet. IV. Variations in the Ca/P ratio of the diet induced by changing the calcium content, 645.
—, —, in caries-susceptible rats, studies on the relation of dairy products to, 253.
— -susceptible rats, effects of long-time administration of small amounts of fluoride in food or water on, 581.
CARVALHO DA SILVA, A., M. P. MANSUR GUERIOS AND S. R. MONSAO. The domestic cat as a laboratory animal for experimental nutrition studies. VI. Choline deficiency, 537.
Casein, amino acids of, sequence in which they become limiting for the growth of the rat, 109.
Cat, domestic, as a laboratory animal for experimental nutrition studies. VI. Choline deficiency, 537.
Chick(s), fed manganese-deficient diets, divalent minerals and proteolytic activity of pancreas tissue from rats and, 513.
—, germ-free and conventional, growth of, effect of diet, dietary penicillin and bacterial environment, 69.
—, goitrogen-fed, growth-promoting activity of meat meal and certain tissues in, 589
— and rats fed raw or heated soybean meal, effect of antibiotics on the weight of, 149.
Chicken(s), effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in, 333.
—, effect of methionine deficiency on nitrogen absorption from the intestinal tract of, 213.
Cholesteremia, diet and. I. Development of a diet for the study of nutritional factors affecting cholesteremia in the rat, 289.
Choline deficiency. The domestic cat as a laboratory animal for experimental nutrition studies. VI., 537.
CHRISTMAN, ADAM A. Howard Bishop Lewis (November 8, 1887—March 17, 1954), 7.

CLAWSON, A. J. See Matrone, G., 309.
COOTS, M. C., A. E. HARPER AND C. A. ELVEHJEM. Production of biotin deficiency in the guinea pig, 525.
Coprophagy in the rat, effect of the prevention of. IV. Biotin, 599.
Corn, raw and tortillas (lime-treated corn), comparison of the effect of with niacin, tryptophan or beans on the growth and muscle niacin of rats, 351.
Cottage cheese (Whole egg, milk and). Biological availability of essential amino acids of to human subjects. II., 497.
COUTINO-ABATH, E. See Gershoff, S. N., 29.

DANIEL, L. J. See Gray, L. F., 623.
DAVIS, G. K. See Feaster, J. P., 319, 325; Shirley, R. L., 159.
Dental caries (see Caries, dental).
Diet(s) and cholesteremia. I. Development of a diet for the study of nutritional factors affecting cholesteremia in the rat, 289.
— comparable in sucrose content, further studies on the difference in cariogenicity of two, 569.
—, effect of on the development of beta-aminopropionitrile-induced vascular hemorrhage in turkeys, 275.
—. high-fat, high-protein, some metabolic effects of during semistarvation under winter field conditions, 85
—, influence of upon the storage of vitamin B_{12} in liver and kidney, 185.
—, manganese-deficient, divalent minerals and proteolytic activity of pancreas tissue from rats and chicks fed, 513.
—, protein level of, influence on serum glycoprotein concentrations in the rat, 137.
—, purified-type, salt mixtures for. I. Effect of salt in accelerating oxidative rancidity, 123.
—, restricted, some biochemical effects of during successive field trials in winter, 99.
DOUGLASS, C. D., AND G. H. KAMP. The effect of orally administered rutin on the adrenal ascorbic acid level in guinea pigs, 531.
DRURY, H. F. See Vaughan, D. A., 99
DRURY, H. F., D A.. VAUGHAN AND J. P. HANNON. Some metabolic effects of a high-fat, high-protein diet during semistarvation under winter field conditions, 85.
DUMM, M. E. See Ralli, E. P., 41.
DUNCAN, B. J. See Ellis, L. N., 185.
Dystrophy, experimental muscular in the rat, effect of α-tocopherol, α-tocopherylhydroquinone and their esters on, 223.

EGG (whole), pork muscle and peanut butter. Biological availability of essential amino acids to human subjects. I., 483.
ELLIS, L. N., B. J. DUNCAN AND I. B. SNOW. The influence of diet upon the storage of vitamin B_{12} in liver and kidney, 185.
ELVEHJEM, C. A. See Coots, M. C., 525; Nath, N., 289.
ENSFIELD, B. I. See Shaw, J. H., 253.
Enzymes, oxidative, of the heart, muscle and liver of cattle, effect of dietary protein level on, 159.
— in protein depletion. III. Enzymes of brain, kidney, skeletal muscle and spleen, 197.
ERSHOFF, B. H., H. J. HERNANDEZ AND J. M. MUCKENTHALER. Beneficial effects of the plant residue factor on the survival of thyrotoxic rats, 381.

Ethanolamine and its *N*-methyl derivatives, effect of on kidney hemorrhagic degeneration in rats due to 2-amino-2-methyl-1-propanol, 655.
DL-Ethionine, effect of on skeletal growth in rats, 363.
EVERSON, G. J. See Hurley, L. S., 445.
Excretion, urinary, of five essential amino acids by young women, 19.

FAT(s) in the diet, effect on the inhibition of growth of an implanted fibrosarcoma in rats, studied with and without injections of guinea pig serum, 469.
—, effects in rats of vitamin B_{12}, with and without ethyl alcohol on, 41.
— or unsaturated fatty acids, effects of deficiency of. Nutritional studies with the guinea pig. V., 611.
Fatty acid(s), concentrates of polyunsaturated from tuna oil, effect of upon essential fatty acid deficiency, 423.
— —, essential, activities of hydrocarbons and alcohols analogous to linoleate and linolenate, 413.
— — — deficiency, effect of concentrates of polyunsaturated acids from tuna oil upon, 423.
— —, unsaturated, effects of deficiency of fat or. Nutritional studies with the guinea pig. V., 611.
FEASTER, J. P., AND G. K. DAVIS. Sulfate metabolism in rabbits on high molybdenum intake, 319.
———— Effect of high molybdenum intake on the distribution and excretion of Ca^{45} and P^{32} in the rabbit, 325.
FIALA, G. See Barnes, R. H., 599.
Fibrosarcoma, implanted, in rats, studies on the inhibition of growth of. The effect of fat in the diet with and without injections of guinea pig serum, 469.
Flour, bread, lysine fortified, effect of level of protein feeding upon nutritional value of, 549.
Fluoride, calcium and phosphorus, deposition of in experimental low-phosphorus rickets, 59.
—, effects of long-time administration in food or water on caries-susceptible rats, 581.
FORBES, M., AND J. T. PARK. Growth of germ-free and conventional chicks: Effect of diet, dietary penicillin and bacterial environment, 69.
FOX, M. R. S., AND O. MICKELSEN. Salt mixtures for purified-type diets. I. Effect of salts in accelerating oxidative rancidity, 123.
FREDERICKSON, R. L. See Overby, L. R., 397.
FROST, D. V. See Overby, L. R., 397.

GEIGER, J. F. See Hurley, L. S., 445.
GERSHOFF, S. N., E. COUTINO-ABATH, I. ANTONOWICZ, A. L. MEYER, G. S. H. SHEN AND S. B. ANDRUS. Some effects related to the potassium and lysine intake of rats, 29.
GITLER, C. See Kelleher, W. J., 433.
Glutamic-oxalacetic transaminase activity in serum of vitamin B_6-deficient rats, 205.
Glycoprotein, serum, concentrations in the rat, influence of protein level of the diet on, 137.
GORDON, R. S. See Machlin, L. J., 333.
GRAHAM, D. C. W. See Watts, J. H., 497.
GRAY, L. F., AND L. J. DANIEL. Studies of vitamin B_{12} in turnip greens, 623.

Growth, skeletal in rats, effect of DL-ethionine on, 363.
Guinea pig(s), nutritional studies with. V. Effects of deficiency of fat or unsaturated fatty acids, 611.
— —, effects of orally administered rutin on the adrenal ascorbic acid level in, 531.
— —, production of biotin deficiency in, 525.
— — serum, injections of, effect of fat with and without on the inhibition of growth of an implanted fibrosarcoma in rats, 469.

HALDI, J. See Wynn, W., 645.
HALDI, J., W. WYNN, K. D. BENTLEY AND M. L. LAW. Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. IV. Variations in the Ca/P ratio of the diet induced by changing the calcium content, 655.
HANNON, J. P. See Drury, H. F., 85; Vaughan, D. A., 99.
HARPER, A. E. Sequence in which the amino acids of casein become limiting for the growth of the rat, 109.
——— See Coots, M. C., 525; Nath, N., 289.
HARRIS, R. S., AND D. A. BURRESS. Effect of level of protein feeding upon nutritional value of lysine-fortified bread flour, 549.
HARTMAN, R. H. See Matrone, G., 309.
Heart(s) (cattle), effect of dietary protein level on several oxidative enzymes of, 159.
— of rats, effect of dietary protein, vitamin E and age on the lactic dehydrogenase and succinoxidase of, 635.
Hematuria in vitamin B_6-deficient rats fed phthalylsulfathiazole, 237.
Hemorrhage, vascular in turkey, induced by beta-aminopropionitrile, effect of diet on the development of, 275.
HENTGES, J. F., JR. See Shirley, R. L., 159.
HERNANDEZ, H. J. See Ershoff, B. H., 381.
HOLMAN, R. T. See Aaes-Jørgensen, E., 413; Privett, O. S., 423.
Human subjects, biological availability of essential amino acids to. I. Whole egg, pork muscle and peanut butter, 483; II. Whole egg, milk and cottage cheese, 497.
HURLEY, L. S., G. J. EVERSON AND J. F. GEIGER. Serum alkaline phosphatase activity in normal and manganese-deficient developing rats, 445
Hydrocarbons and alcohols analogous to linoleate and linolenate, essential fatty acid activities of, 413.

JAMESON, E., R. M. RYAN AND P. I. KRAMER. Studies on the inhibition of growth of an implanted fibrosarcoma in rats. The effect of fat in the diet with and without injections of guinea pig serum, 469.
JACKSON, C. D. See Mirone, L., 167.
JENNINGS, R. B. See Calhoun, W. K., 237.
JOHNSON, M. J. See Kelleher, W. J., 433.
JOHNSON, R. R., O. G. BENTLEY AND T. S. SUTTON. Divalent minerals and proteolytic activity of pancreas tissue from rats and chicks fed manganese-deficient diets, 513.
JONES, F., JR. See Watts, J. H., 483; 497.

KAMP, G. H. See Douglass, C. D., 531.
KAUFMAN, N. See Klavins, J. V., 363.
KELLEHER, W. J., C. GITLER, M. L. SUNDE, M. J. JOHNSON AND C. A. BAUMANN. Antinecrogenic property of torula yeast treated in various ways, 433.
Kidney, enzymes of in protein depletion. III., 197.
—, hemorrhagic degeneration of in rats, due to 2-amino-2-methyl-1-propanol, effect of ethanolamine and its N-methyl derivatives on, 655.
—, storage of vitamin B_{12} in, influence of diet upon, 185.
KINNEY, T. D. See Klavins, J. V., 363.
KLAVINS, J. V., T. D. KINNEY AND N. KAUFMAN. The effect of DL-ethionine on skeletal growth in rats, 363.
KRAMER, P. I., See Jameson, E., 469.
KREMZNER, L. T. See Wainio, W. W., 197.
KWONG, E. See Barnes, R. H., 599.

LACTIC dehydrogenase of the hearts of rats, effect of dietary protein, vitamin E and age on, 635.
LAKEN, B. See Ralli, E. P., 41.
LARSON, A. M. See Vaughan, D. A., 99.
LAW, M. L. See Haldi, J., 569; Wynn, W., 569.
LEVERTON, R. M., F. S. WADDILL AND M. SKELLENGER. The urinary excretion of five essential amino acids by young women, 19.
LEWIS, HOWARD BISHOP (November 8, 1887–March 17, 1954), 7.
LIKINS, R. C. See Zipkin, I., 59.
LIM, C. Y. See Beñdana-Brown, A. 461.
Linoleate and linolenate, hydrocarbons and alcohols analogous to, essential fatty acid activities of, 413.
Linolenate (linoleate and), hydrocarbon and alcohols analogous to, essential fatty acid activities of, 413.
Liver, antithyrotoxic factor of. I. Method for assay, 397.
— (cattle), effect of dietary protein level on several oxidative enzymes of, 159.
— nitrogen, effects in rats of vitamin B_{12}, with and without ethyl alcohol, on, 41.
— "peroxide" in the chicken, effect of antioxidants on vitamin E deficiency symptoms and production of, 333.
—, storage of vitamin B_{12} in, influence of diet upon, 185.
LONGMORE, W. J. See Outland, C. E., 655.
LUNDBERG, W. O. See Privett, O. S., 423.
LUSHBOUGH, C. H., J. M. WEICHMAN AND B. S. SCHWEIGERT. The retention of vitamin B_6 in meat during cooking, 451.
Lysine-fortified bread flour, effect of level of protein feeding upon nutritional value of, 549.
— (potassium and) intake of rats, some effects related to, 29.

MACHLIN, L. J., R. S. GORDON AND K. H. MEISKY. The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken, 333.
MACKENZIE, C. G. See Mackenzie, J. B., 223.
MACKENZIE, J. B., AND C. G. MACKENZIE. The effect of α-tocopherol, α-tocopherylhydroquinone and their esters on experimental muscular dystrophy in the rat, 223.
Manganese-deficient developing rats, serum alkaline phosphatase activity in normal and, 445.
— - — diets, divalent minerals and proteolytic activity of pancreas tissue from rats and chickens fed, 513.
— -iron antagonism, studies on in the nutrition of rabbits and baby pigs., 309.
MANSUR GUERIOS, M. F. See Carvalho da Silva, A., 537.
MARTIN, M. G. See Reid, M. E., 611.

MATRONE, G., R. H. HARTMAN AND A. J. CLAWSON. Studies of a manganese-iron antagonism in the nutrition of rabbits and baby pigs, 309.
MEYER, A. L. See Gershoff, S. N., 29.
MCAFEE, J. M. See Watts, J. H., 483; 497.
MCCLURE, F. J. See Zipkin, I. 59.
MEALEY, E. H. See Outland, C. E., 655.
Meat meal, growth-promoting activity of in goitrogen-fed rats and chicks, 589.
MEISKY, K. H. See Machlin, L. J., 333.
Methionine, effect of deficiency on nitrogen absorption from the intestinal tract of chickens, 213.
Mice, weanling, development and cure of pyridoxine deficiency symptoms in, 167.
MICKELSON, O. See Fox, M. R. S., 123.
Milk (Whole egg) and cottage cheese. Biological availability of essential amino acids to humans. II., 497.
Minerals, divalent, and proteolytic activity of pancreas tissue from rats and chicks fed manganese-deficient diets, 513.
MIRONE, L., AND C. D. JACKSON. The development and cure of pyridoxine deficiency symptoms in weanling mice, 167.
Molybdenum, high intake of, sulfate metabolism in rabbits on, 319.
—, — —, effect of on the distribution and excretion of Ca^{45} and P^{32} in the rabbit, 325.
MONSAO, S. R. See Carvalho da Silva, A., 537.
MUCKENTHALER, J. M. See Ershoff, B. H., 381.
MULFORD, D. J. See Outland, C. E., 655.
Muscle (cattle), effect of dietary protein level on several oxidative enzymes of, 159.
—, skeletal, enzymes of in protein depletion. III., 197.

NATH, N., R. WIENER, A. E. HARPER AND C. A. ELVEHJEM. Diet and cholesteremia. I. Development of a diet for the study of nutritional factors affecting cholesteremia in the rat, 289.
Niacin, muscle of rats, comparison of the effect of raw corn and tortillas (lime-treated corn) with niacin, tryptophan or beans on the growth and, 351.
—, tryptophan or beans, comparison with raw corn and tortillas (lime-treated corn) on the growth and muscle niacin of rats, 351.
NISHIHARA, H. See Weimer, H. E., 137.
Nitrogen absorption from the intestinal tract of chickens, effect of methionine deficiency on, 213.
— balance, effects in rats of vitamin B_{12} with and without ethyl alcohol on, 41.
Nutrition of rabbits and baby pigs, studies of a manganese-iron antagonism in, 309.
— studies, the domestic cat as a laboratory animal for experimental. VI. Choline deficiency, 537.

OUTLAND, C. E., E. H. MEALEY, W. J. LONGMORE AND D. J. MULFORD. The effect of ethanolamine and its *N*-methyl derivatives on kidney hemorrhagic degeneration in rats due to 2-amino-2-methyl-1-propanol, 655.
OVERBY, L. R., R. L. FREDERICKSON AND D. V. FROST. The antithyrotoxic factor of liver. I. Method for assay, 397.

PAINE, C. M. See Pisano, J. J., 213.

Pancreas tissue from rats and chicks fed manganese-deficient diets, bivalent minerals and proteolytic activity of, 513.
PARK, J. T. See Forbes, M., 69.
Peanut butter (Whole egg, pork muscle and). Biological availability of essential amino acids to human subjects. I., 483.
Penicillin, dietary, and bacterial environment, effect of on growth of germ-free and conventional chicks, 69.
Philippine vegetables, availability of calcium in, 461.
PHILLIPS, P. H. See Wuthier, R. E., 581.
Phosphatase, serum albumin, activity in normal and manganese-deficient developing rats, 445.
P^{32}, effect of high molybdenum intake on distribution in the rabbit, 325.
Phosphorus (fluoride, calcium and), deposition in experimental low-phosphorus rickets, 59.
Phthalylsulfathiazole, calcium oxalate excretion and hematuria in vitamin B_6-deficient rats fed, 237.
Pig(s), baby, study of a manganese-iron antagonism in the nutrition of, 309.
PISANO, J. J., C. M. PAINE AND M. W. TAYLOR. The effect of methionine deficiency on nitrogen absorption from the intestinal tract of chickens, 213.
Plant residue factor, beneficial effects on the survival of thyrotoxic rats, 381.
POMEROY, B. S. See Waibel, P. E., 275.
Pork muscle (Whole egg,) and peanut butter. Biological availability of essential availability of essential amino acids of to human subjects, 483.
Potassium and lysine intake of rats, some effects related to, 29.
PRIVETT, O. S. See Aaes-Jørgensen, E., 413.
PRIVETT, O. S., E. AAES-JØRGENSEN, R. T. HOLMAN AND W. O. LUNDBERG. The effect of concentrates of polyunsaturated acids from tuna oil upon essential fatty acid deficiency, 423.
Protein depletion, enzymes in. III. Enzymes of brain, kidney, skeletal muscle and spleen, 197.
—, dietary, effect on the lactic dehydrogenase and succinoxidase of the hearts of rats, 635.
— feeding, effect of level upon nutritional value of lysine-fortified bread flour, 549.
— level of the diet, influence on serum glycoprotein concentrations in the rat, 137.
— —, dietary, effect on several oxidative enzymes of the heart, muscle and liver of cattle, 159.
Pyridoxine deficiency symptoms in weanling mice, development and cure of, 167.

RABBIT(S), effect of high molybdenum intake on the distribution and excretion of Ca^{45} and P^{32} in, 325.
—, studies of a manganese-iron antagonism in the nutrition of, 309.
—, sulfate metabolism on high molybdenum intake, 319.
RALLI, E. P., M. E. DUMM AND B. LAKEN. The effects in rats of vitamin B_{12}, with and without ethyl alcohol, on nitrogen balance, serum albumin, liver nitrogen and fat, 41.
Rancidity, oxidative, effect of salts in accelerating. Salt mixtures for purified-type diets. I., 123.
Rat(s), albino, dental caries in relation to the chemical composition of the teeth and of the diet. IV. Variations in the Ca/P ratio of the diet induced by changing the calcium content, 645.

—, caries-susceptible, effects of long-time administration of small amounts of fluoride in food or water on, 581.
—, — - —, studies on the relation of dairy products to dental caries in, 253.
— (chicks and), fed raw or heated soybean meal, effect of antibiotics on the weight of, 149.
—, cholesteremia in, development of a diet for the study of nutritional factors affecting. Diet and cholesteremia. I., 289.
—, effects of the prevention of coprophagy in. IV. Biotin, 599.
—, effects of vitamin B_{12}, with and without ethyl alcohol, on nitrogen balance, serum albumin, liver nitrogen and fat, 41.
—, experimental muscular dystrophy in, effect of α-tocopherol, α-tocopherylhydroquinone and their esters on, 223.
— fed manganese-deficient diet, divalent minerals and proteolytic activity of pancreas tissue from, 513.
—, goitrogen-fed, growth-promoting activity of meat meal and certain tissues in, 589.
—, growth of, sequence in which the amino acids of casein become limiting for, 109.
—, heart, effect of dietary protein, vitamin E and age on the lactic dehydrogenase and succinoxidase of, 635.
—, normal and manganese-deficient developing, serum alkaline phosphatase activity in, 445.
—, potassium and lysine intake of, some effects related to, 29.
—, serum glycoprotein concentrations in, influence of the protein level of the diet on, 137.
—, skeletal growth in, effect of DL-ethionine on, 363.
—, studies on the inhibition of growth of an implanted fibrosarcoma in. The effect of fat in the diet with and without injections of guinea pig serum, 469.
—, thyrotoxic, beneficial effects of the plant residue factor on the survival of, 381.
—, vitamin B_6-deficient, fed phthalylsulfathiazole, calcium oxalate excretion and hematuria in, 237.
— — —, serum glutamic-oxalacetic transaminase activity of, 205.
REID, M. E., AND M. G. MARTIN. Nutritional studies with the guinea pig. V. Effects of deficiency of fat or unsaturated fatty acids, 611.
Rickets, experimental low-phosphorus, deposition of fluoride, calcium and phosphorus in, 59.
Rutin, orally administered, effect on the adrenal ascorbic acid level in guinea pigs, 531.
RYAN, R. M. See Jameson, E., 469.

SALT mixtures for purified-type diets. I. Effects of salts in accelerating oxidative rancidity, 123.
SCHWEIGERT, B. S. See Lushbough, C. H., 451.
SCRIMSHAW, N. S. See Squibb, R. L. ,351.
Serum albumin, effects in rats of vitamin B_{12} with and without ethyl alcohol on, 41.
—alkaline phosphatase activity in normal and manganese-deficient developing rats, 445.
— glutamic-oxalacetic transaminase activity of vitamin B_6-deficient rats, 205.
SHAW, J. H., B. I. ENSFIELD and D. H. WOLLMAN. Studies on the relation of dairy products to dental caries in caries-susceptible rats, 253.
SHEN, G. S. H. See Gershoff, S. N., 29.
SHIRLEY, R. L., E. BEDRAK, A. C. WARNICK, J. F. HENTGES, JR. AND G. K. DAVIS. Effect of dietary protein level on several oxidative enzymes of the heart, muscle and liver of cattle, 159.
SHIRLEY, R. L., AND G. K. DAVIS. Effect of dietary protein, vitamin E and age on the lactic dehydrogenase and succinoxidase of the hearts of rats, 635.
SKELLENGER, M. See Leverton, R. M., 19.
SNOW, I. B. See Ellis, L. N., 185.
Soybean meal, raw or heated, effect of antibiotics on the weight of chicks and rats fed, 149.
Spleen, enzymes of in protein depletion. III., 197.
SQUIBB, R. L., J. E. BRAHAM, G. ARROYAVE AND N. S. SCRIMSHAW. A comparison of the effect of raw corn and tortillas (lime-treated corn) with niacin, tryptophan or beans on the growth and muscle niacin of rats, 351.
Succinoxidase of the hearts of rats, effect of dietary protein, vitamin E and age on, 635.
Sulfate metabolism in rabbits on high molybdenum intake, 319.
SUNDE, M. L. See Kelleher, W. J., 433.
SUTTON, T. S. See Johnson, R. R., 513.

TAYLOR, M. W. See Pisano, J. J., 213.

α-Tocopherol, α-tocopherylhydroquinone and their esters, effect on experimental muscular dystrophy in the rat, 223.
Tortillas (lime-treated corn) and raw corn, comparison of the effect of with niacin, tryptophan or beans on the growth and muscle niacin of rats, 351.
Torula yeast, antinecrogenic property of when treated in various ways, 433.
Tuna oil, concentrates on polyunsaturated acids from, effect of upon essential fatty acid deficiency, 423.
Turkeys, beta-aminopropionitrile-induced vascular hemorrhage in, effect of diet on the development of, 275.
Turnip greens, studies of vitamin B_{12} in, 623.

VAUGHAN, D. A. See Drury, H. F., 85.

VAUGHAN, L. N. See Vaughan, D. A., 99.
VAUGHAN, D. A., H. F. DRURY, J. P. HANNON, L. N. VAUGHAN AND A. M. LARSON. Some biochemical effects of restricted diets during successive field trials in winter, 99.
Vitamin B_6-deficient rats fed phthalylsulfathiazole, calcium oxalate excretion and hematuria in, 237.
— — - — —, serum glutamic-oxalacetic transaminase activity of, 205.
— —, retention of in meat during cooking, 451.
— B_{12}, effect of in rats, with and without ethyl alcohol, on nitrogen balance, serum albumin, liver nitrogen and fat, 41.
— —, storage in liver and kidney, influence of diet upon, 185.
— —, studies of in turnip greens, 623.
— E deficiency symptoms in the chicken, effect of antioxidants on, 333.
— —, effect of on the lactic dehydrogenase and succinoxidase of the hearts of rats, 635.

WADDILL, F. S. See Leverton, R. M., 19.

WAIBEL, P. E., AND B. S. POMEROY. Effect of diet on the development of beta-aminoproprionitrile-induced vascular hemorrhage in turkeys, 275.

WAINIO, W. W., J. B. ALLISON, L. T. KREMZNER, E. BERNSTEIN AND M. ARONOFF. Enzymes in protein depletion. III. Enzymes of brain, kidney, skeletal muscle and spleen, 197.
WARNICK, A. C. See Shirley, R. L., 159.
WATTS, J. H., L. K. BOOKER, J. W. MCAFEE, D. C. W. GRAHAM AND F. JONES, JR. Biological availability of essential amino acids to human subjects. II. Whole egg, milk and cottage cheese, 497.
WATTS, J. H., L. K. BOOKER, J. M. MCAFEE, E. G. WILLIAMS, W. G. WRIGHT AND F. JONES, JR. Biological availability of essential amino acids to human subjects. I. Whole egg, pork muscle and peanut butter, 483.
WEIMER, H. E., AND H. NISHIHARA. The influence of the protein level of the diet on serum glycoprotein concentrations in the rat, 137.
WIENER, R. See Nath, N., 289.
WEICHMAN, J. M. See Lushbough, C. H., 451.
WILLIAMS, E. G. See Watts, J. H., 483.
WOLLMAN, D. H. See Shaw, J. H., 253.
Women, young, urinary excretion of five essential amino acids by, 19.
WRIGHT, W. G. See Watts, J. H., 483.
WUTHIER, R. E., AND P. H. PHILLIPS. The effects of long-time administration of small amounts of fluoride in food or water on caries-susceptible rats, 581.
WYNN, W. See Haldi, J., 645.
WYNN, W., J. HALDI, K. D. BENTLEY, AND M. L. LAW. Further studies on the difference in cariogenicity of two diets comparable in sucrose content,

YEAST, torula, antinecrogenic property of when treated in various ways, 433.

ZIPKIN, I., R. C. LIKINS AND F. J. MCCLURE. Deposition of fluoride, calcium and phosphorus in experimental low-phosphorus rickets, 59.

NOTICE TO CONTRIBUTORS

THE JOURNAL OF NUTRITION, a copyrighted periodical, appears monthly for the publication of original research bearing on the subject of nutrition and occasional reviews of the literature dealing with this subject.

THE JOURNAL OF NUTRITION is the official organ of the American Institute of Nutrition. The officers of the Institute for 1958–59 are: WILLIAM J. DARBY, *President;* D. WAYNE WOOLLEY, *President-elect;* GEORGE M. BRIGGS, *Secretary;* J. B. BROWN, *Treasurer;* E. W. MCHENRY, PAUL GYÖRGY, J. H. ROE, and R. R. WILLIAMS, *Councillors.*

Preliminary notices, or papers already published or in press elsewhere, will not be accepted. Unusually long papers that would take a disproportionate part of a single issue can be considered only if published as a supplement, the entire cost of which is assumed by the author.

Manuscripts and drawings should be sent by express prepaid or by registered mail to the Editor, Dr. George R. Cowgill, Yale University Nutrition Laboratory, 333 Cedar Street, New Haven 11, Connecticut. If two complete copies of the paper are submitted, consideration by the Journal can be expedited.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. *The Wistar Institute reserves the right to return to the author for revision material which is not in proper form for the printer.* **When the amount of tabular or illustrative material or both is judged to be excessive or unusually expensive, authors may be requested to pay the excess cost.**

Manuscripts should be typed in double spacing on one side of bond or heavy-bodied paper 8½ × 11 inches and should be sent flat. Page 1 should include, in the following order: COMPLETE TITLE, AUTHOR'S NAME, INSTITUTION FROM WHICH THE PAPER CAME, WITH CITY AND STATE, TOTAL NUMBER OF FIGURES, SHORTENED FORM OF TITLE **(not more than 35 letters and spaces)**, ADDRESS TO WHICH THE PROOF IS TO BE SENT. *The text starts on page 2.*

Tables, quotations (extracts of over 5 lines), and all other material usually set in type smaller than the text, should be typed each on a separate sheet. **Footnotes to the text should be numbered consecutively** (including those on page 1) and typed in order on a separate sheet. Explanations of figures should be treated in the same manner. Footnotes to a table should be made a part of the table, and should be typed directly beneath it. Citations of literature in the text should be made by author and by numerals to indicate the year of publication. **Authors' names (followed by year, title of paper, etc.) should be arranged alphabetically in a list at the end of the text.**

The original drawings, not photographs of drawings, should accompany the manuscript. When photographs are used for halftone reproduction, glossy prints should be sent. Authors should indicate on the manuscript the approximate position of text figures. If there are illustrations so large as to require mailing separately, reduced copies of them that can be mailed with the manuscript should be provided in order to expedite consideration of the paper.

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the cost of a more expensive form of illustration. Letters and figures should be uniform and large enough so that no character will be less than 2 mm high after reduction to at least the width of the page (4¼ inches). Double plates will not be accepted. Figures should be numbered consecutively beginning with the text figures and continuing through the plates. The reduction desired should be clearly indicated on the margin of the drawing.

Drawings intended for photographic reproduction should be made on white or blue-white paper or bristol board — **not** on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — **never** with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon. **For further details important for the preparation of a satisfactory manuscript, ask The Wistar Institute for a copy of the brochure, A GUIDE FOR AUTHORS.**

THE JOURNAL OF NUTRITION is a copyrighted scientific periodical and reprints are intended primarily for the author's use. Authors are furnished 50 reprints, with covers, gratis. Additional copies may be obtained according to the rates which will be sent the authors as soon as the manuscript has been examined at The Wistar Institute, after acceptance. Other parties ordering reprints must have their orders approved by the authors, and must give written assurance that distribution will be restricted to professional personnel and others interested in the science of nutrition, and that no advertising or imprinting will be attached to the reprints thus furnished.

THIS NUMBER COMPLETES VOLUME 67

THE JOURNAL® OF NUTRITION

VOL. 67 APRIL 1959 No. 4

CONTENTS

RONALD R. JOHNSON, ORVILLE G. BENTLEY AND T. S. SUTTON. Divalent minerals and proteolytic activity of pancreas tissue from rats and chicks fed manganese-deficient diets 513

MACIE COLLINS COOTS, A. E. HARPER AND C. A. ELVEHJEM. Production of biotin deficiency in the guinea pig 525

CARL D. DOUGLASS AND GEORGE H. KAMP. The effect of orally administered rutin on the adrenal ascorbic acid level in guinea pigs 531

ALBERTO CARVALHO DA SILVA, MARIO F. MANSUR GUERIOS AND SYLVIO R. MONSAO. The domestic cat as a laboratory animal for experimental nutrition studies. VI. Choline deficiency 537

ROBERT S. HARRIS AND DONALD A. BURRESS. Effect of level of protein feeding upon nutritional value of lysine-fortified bread flour 549

WINFREY WYNN, JOHN HALDI, KATHERINE D. BENTLEY AND MARY L. LAW. Further studies on the difference in cariogenicity of two diets comparable in sucrose content 569

R. E. WUTHIER AND P. H. PHILLIPS. The effects of long-time administration of small amounts of fluoride in food or water on caries-susceptible rats 581

C. J. ACKERMAN. Growth-promoting activity of meat meal and certain tissues in goitrogen-fed rats and chicks 589

RICHARD H. BARNES, EVA KWONG AND GRACE FIALA. Effects of the prevention of coprophagy in the rat. IV. Biotin 599

MARY ELIZABETH REID AND MARY G. MARTIN. Nutritional studies with the guinea pig. V. Effects of deficiency of fat or unsaturated fatty acids .. 611

LOUISE F. GRAY AND LOUISE J. DANIEL. Studies of vitamin B_{12} in turnip greens 623

R. L. SHIRLEY AND G. K. DAVIS. Effect of dietary protein, vitamin E and age on the lactic dehydrogenase and succinoxidase of the hearts of rats 635

JOHN HALDI, WINFREY WYNN, KATHERINE D. BENTLEY AND MARY L. LAW. Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. IV. Variations in the Ca/P ratio of the diet induced by changing the calcium content 645

CHARLOTTE E. OUTLAND, EDWARD H. MEALEY, WILLIAM J. LONGMORE AND DWIGHT J. MULFORD. The effect of ethanolamine and its *N*-methyl derivatives on kidney hemorrhagic degeneration in rats due to 2-amino-2-methyl-1-propanol 655

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America