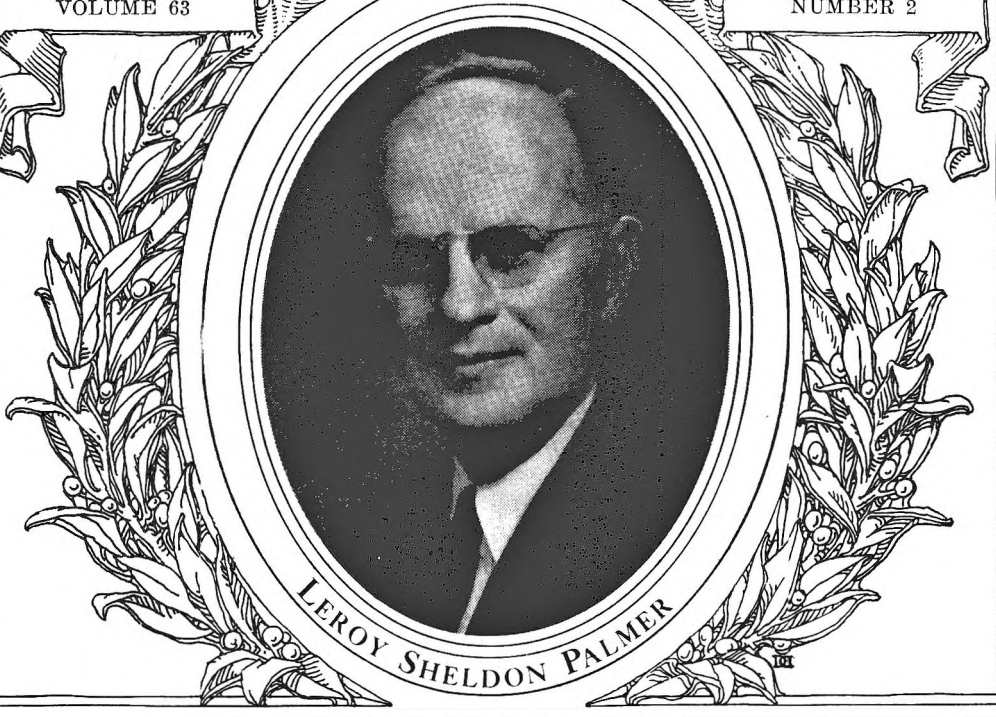
OCTOBER 10, 1957

THE JOURNAL OF NUTRITION

VOLUME 63 NUMBER 2



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

EDITORIAL BOARD

GRACE A. GOLDSMITH
W. D. SALMON
LEMUEL D. WRIGHT
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

Official organ of the American Institute of Nutrition

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

Price, $7.50 per volume, domestic; $8.00 per volume, foreign

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Copyright 1957. 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: $15.00 Domestic, $16.00 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.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliography reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. The Advance Abstract Card Service is issued promptly and in advance of the journal in which the paper is published.

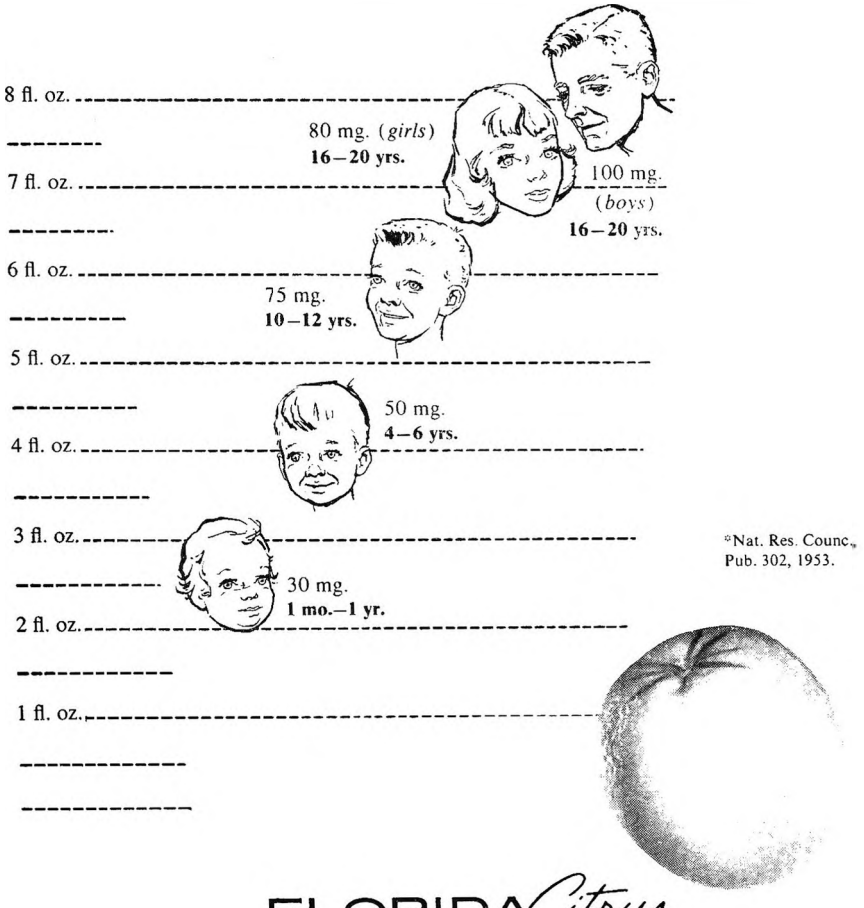
Issued monthly (approximately 500 abstracts annually). Single set subscriptions, $5.00; two sets to a single subscriber $8.00 our year

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.

for developmental years
orange juice
capably supplies recommended daily intakes of vitamin C*



*Nat. Res. Counc., Pub. 302, 1953.

FLORIDA *Citrus*
COMMISSION · LAKELAND, FLORIDA
ORANGES • GRAPEFRUIT • TANGERINES

Meat...

in the congestive phase of cardiac disease

Meat fits well into the moderate-protein, restricted-sodium, acid-ash diet currently recommended for many patients with congestive cardiac failure.[1]

The protein of meat—in the proportionate arrangement of its essential amino acids—closely approaches the quantitative proportions needed to promote human tissue synthesis and repair. For this reason lean meat proves important in maintaining positive nitrogen balance without excessive protein intake.

The sodium content of meat prepared without added salt is relatively low. Per 100 grams, beef muscle meat shows approximately 50 mg. of sodium, lamb 90 mg., pork 60 mg., and veal 50 mg.[2]

The acid ash of meat aids in the promotion of diuresis.

The easy digestibility of meat is a prime requisite of foods specified for the patient with congestive cardiac disease.

In addition to these important features, meat contributes other nutritional factors essential in any convalescence —the B vitamins thiamine, riboflavin, niacin, pantothenic acid, B_6, and B_{12}, and the minerals iron, phosphorus, potassium, and magnesium.

1. Odell, W. M.: Nutrition in Cardiovascular Disease, in Wohl, M. C., and Goodhart, R. S.: Modern Nutrition in Health and Disease, Philadelphia, Lea & Febiger, 1955, p. 699.
2. Bills, C. E.; McDonald, F. G.; Niedermeier, W., and Schwartz, M. C.: Sodium and Potassium in Foods and Waters, J. Am. Dietet. A. *25*:304 (Apr.) 1949.

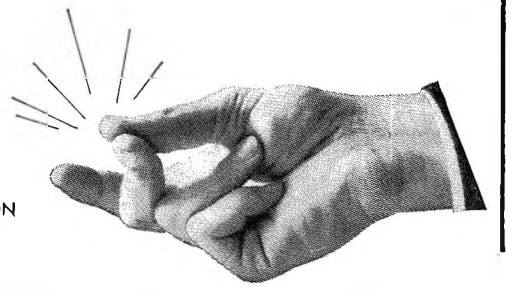
The nutritional statements made in this advertisement have been reviewed by the Council on Foods and Nutrition of the American Medical Association and found consistent with current authoritative medical opinion.

American Meat Institute
Main Office, Chicago...Members Throughout the United States

Your order will be shipped

. . . . THAT QUICK!

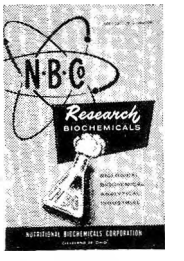
When it's a question of time . . . and it usually is . . . you can always rely upon NUTRITIONAL BIOCHEMICALS CORPORATION Most orders are shipped the same day that they are received.



A COMPLETE SELECTION OF MORE THAN 165 NUCLEOPROTEINS AND DERIVATIVES

Typical Derivatives

ADENOSINE TRIPHOSPHATE	COENZYME I, II, A	URIDINE
CYTIDINE	CYTOSINE	2, 6 DIAMINO PURINE SULFATE
COZYMASE	6 MERCAPTOPURINE	8 AZA GUANINE
	URIDYLIC ACID	



NUTRITIONAL BIOCHEMICALS CORPORATION

21010 Miles Avenue • Cleveland 28, Ohio

Write for New Catalog
October 1957
Over 1700 Items
Write Dept. 110

THE BRITISH JOURNAL OF NUTRITION

Published for the Nutrition Society

SHORTENED VERSION OF ARTICLES IN VOL. 11, NO. 4

Absorption of vitamin B_{12} in normal and gastrectomized rats. By C. G. CLAYTON, A. L. LATNER AND B. SCHOFIELD.

Food intakes, energy expenditures and faecal excretions of men on a polar expedition. By J. P. MASTERTON, H. E. LEWIS AND ELSIE M. WIDDOWSON.

Cereals as protein sources for growing chickens. By K. J. CARPENTER AND K. MARY CLEGG.

Penicillin and the gut flora of the chick. By M. LEV, C. A. E. BRIGGS AND MARIE E. COATES.

The "basal metabolism" of the newborn calf. By J. H. B. ROY, C. F. HUFFMAN AND E. P. REINEKE.

Food supplements and growth of Indian schoolchildren. By V. SUBRAHMANYAN, KANTHA JOSEPH, T. R. DORAISWAMY, M. NARAYANARAO, A. N. SANKARAN AND M. SWAMINATHAN.

Metabolism of nitrogen, calcium and phosphorus in undernourished children. Part 3. By KANTHA JOSEPH, M. NARAYANARAO, M. SWAMINATHAN AND V. SUBRAHMANYAN.

Heat increments of mixtures of volatile fatty acids in sheep. By D. G. ARMSTRONG, K. L. BLAXTER AND N. MCC. GRAHAM.

Vitamins and minerals in Israeli fruits and vegetables. By S. HALEVY, HANNAH KOTH AND K. GUGGENHEIM.

Utilization of volatile fatty acids by fattening sheep. By D. G. ARMSTRONG AND K. L. BLAXTER.

Role of fat in the diet of rats. Part 12. By J. P. FUNCH, E. AAES-JØRGENSEN AND H. DAM.

Pantothenol and the burning feet syndrome. By S. W. BIBILE, N. D. W. LIONEL AND GNANA PERERA.

Vitamin A and cerebrospinal-fluid pressures of hydrocephalic young rabbits. By J. W. MILLEN AND A. D. DICKSON.

Content of amino-acids in white flour and bread. By E. E. MCDERMOTT AND J. PACE.

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.

Read in the November issue of . . .

THE JOURNAL OF NUTRITION

Effect of ascorbic acid and of orange juice on calcium and phosphorus metabolism of women.

Assay of biologic value of milk proteins by liver xanthine oxidase determination I. Powdered products.

The nutritive value of several foods grown at different locations.

Excretion of certain nutrients by young college women consuming self-selected diets.

Effect of yeast and of yeast extracts on liver necrosis and hemolysis by dialuric acid of red blood cells of rats on a necrogenic diet.

Relative roles of niacin and tryptophan in maintaining blood pyridine nucleotides, nitrogen balance and growth in adult rats.

The effect of various fats upon experimental hypercholesteremia in the rat.

The role of lysine in the growth and feather pigmentation of turkey poults.

The addition of small amounts of defatted fish flour to whole yellow corn, whole wheat, whole and milled rye, grain sorghum and millet.

Vitamin E deficiency in the monkey. II. Tissue concentrations of nucleic acids and creatine.

Necrogenic potency of yeasts grown on various media.

Periodontal disease in the rice rat. III. Survey of dietary influences.

The effect of a low environmental temperature on the weight and food consumption of thiamine-deficient rats.

The improvement of the protein quality of white rice by lysine supplementation.

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

DE-VITAMINIZED CASEIN

(VITAMIN-FREE)

IN ECONOMICAL BULK QUANTITIES

Uniform high quality . . . highly concentrated animal source protein specially processed to effectively extract fat and water soluble vitamins. Essentially "vitamin-free," especially in regard to B-vitamin content. Each lot accurately tested.

For Biological and Microbiological Vitamin Tests

• Pharmaceutical Manufacturers • Laboratory Supply Houses • University Research Laboratories • Agricultural Experimental Stations • Medical School Research Laboratories • Governmental Research Laboratories and Institutes.

WRITE FOR SAMPLES AND DATA

SHEFFIELD CHEMICAL
NORWICH, NEW YORK

Leading Manufacturers of
ACID and ENZYME HYDROLYSATES CASEIN
SOY PROTEIN LACTALBUMIN

Also producers of
A.N.R.C. REFERENCE PROTEIN

DE-VITAMINIZED CASEIN

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
16–17	1938–1939	12.00
24	1942	12.00
28–32	1944–1946	10.00
34–51	1947–1953	9.00
52–60	1954–1956	7.50
Index to vols. 1–15		.75
Index to vols. 16–36		2.25

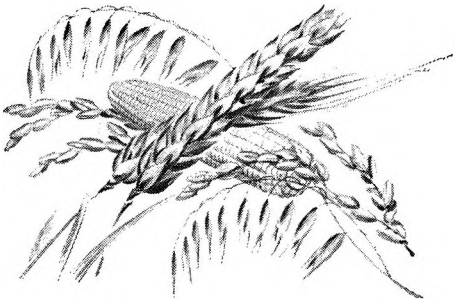
Incomplete volumes	*Year*	*Price per single number*
18, nos. 2–6	1939	$5.00
19, nos. 1–4	1940	5.00
20, nos. 2, 3, 4, 6	1940	5.00
21, nos. 1, 2, 4, 5, 6	1941	5.00
22, nos. 2, 5, 6	1941	4.00
23, nos. 2–6	1942	4.00
25, nos. 2–6	1943	4.00
27, nos. 2–6	1944	4.00
33, nos. 2–6	1947	4.00

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

ALL UNLISTED VOLUMES AND NUMBERS PRIOR TO VOLUME 61 ARE OUT-OF-PRINT

Send order with remittance to

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



You can have a Balanced Low-Fat Breakfast!

Recently scientific and medical authorities have stated that there will probably be a trend in this country to less rich diets which means less calories in the diet. Because fats are such a concentrated source of calories, a moderate reduction of fat intake will result in a generous reduction of calories.

Medical and nutrition authorities when recommending that the fat intake of the diet be lowered state that a low-fat breakfast should provide well-balanced nourishment. *A basic cereal breakfast pattern shown below has found wide endorsement because it makes a worth-while contribution of complete protein, essential B vitamins, and minerals to the daily diet and is low in fat.*

Basic Cereal Low-Fat Breakfast Pattern

Orange juice, fresh, ½ cup, Cereal, dry weight, 1 oz., with whole milk, ½ cup, and sugar, 1 tsp., Bread, white, 2 slices, with butter, 1 tsp., Milk, nonfat (skim), 1 cup, black coffee.

Nutritive Value of Basic Cereal Breakfast Pattern

Calories	**502**
Protein	**20.5 gm.**
Fat	**11.6 gm.**
Carbohydrate	**80.7 gm.**
Calcium	**0.532 gm.**
Iron	**2.7 mg.**
Vitamin A	**600 I. U.**
Thiamine	**0.46 mg.**
Riboflavin	**0.80 mg.**
Niacin	**3.0 mg.**
Ascorbic Acid	**65.5 mg.**
Cholesterol	**32.9 mg.**

Note: To further reduce fat and cholesterol use skim milk on cereal which reduces Fat Total to 7.0 gm. and Cholesterol Total to 16.8 mg. Preserves or honey as spread further reduces Fat and Cholesterol.

Bowes, A. deP., and Church, C. F.: Food Values of Portions Commonly Used. 8th ed. Philadelphia. A. deP. Bowes, 1956.
Cereal Institute, Inc.: The Nutritional Contribution of Breakfast Cereals. Chicago: Cereal Institute, Inc., 1956.
Hayes, O. B., and Rose, G. K.: Supplementary Food Composition Table. J. Am. Dietet. A. 33:26, 1957.

CEREAL INSTITUTE, Inc. • 135 South LaSalle Street, Chicago 3

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

Compatible...

with all other foods

For nutrition...protein...energy...
vitamins...minerals

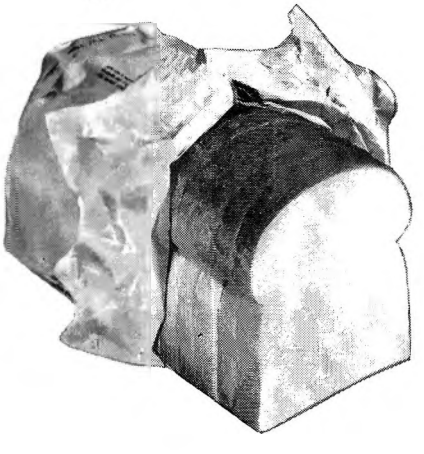
For digestibility...virtually free from residue

For texture...nonirritant...neither chemically
nor mechanically

For low fat content...when fat must be restricted

For taste...enhances and complements every
other food...

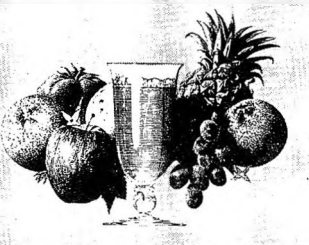
Enriched Bread



Regardless of the dietary adjustment indicated, Enriched Bread helps supply needed nutrients and is always compatible.

AMERICAN BAKERS ASSOCIATION
20 North Wacker Drive • Chicago 6, Illinois

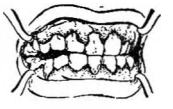
WHY SHOULD THE VITAMIN C CONTENT OF FRUIT AND VEGETABLE JUICES BE STANDARDIZED?



Because nutrition reports disclose that—

- **Vitamin C deficiencies exist throughout the country***

Proof of deficiencies among adults and children. You should know these facts!* Diet surveys from Maine and Vermont, New York, Illinois, New Mexico, Oregon, West Virginia, and Texas show a definite pattern of inadequate vitamin C intake. These studies covered grade school and high school children, college men and women.



Spongy, swollen gums symptomatic of scurvy.

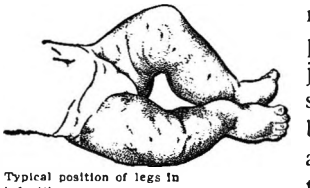
From these studies* of dietary habits and physical conditions there is considerable proof that many of the children and adults covered by the surveys have actual, physical symptoms of vitamin C deficiency.

Infantile scurvy. Scurvy* occurs, even today, among infants who are artificially fed. Fortunately the disease may be cured by the administration of vitamin C. How much more sensible it is to prevent it by supplying these bottle-fed infants with the daily allowance of 30 milligrams of vitamin C as recommended by the Food and Nutrition Board of the National Research Council for children under one year of age.

Symptoms of infantile scurvy are fretfulness and fear of being touched.

These bottle-fed infants, unless receiving a pediatric prescription containing vitamin C, are largely dependent on supplemental ascorbic acid which they receive from fruit or vegetable juices. Unless fortified with vitamin C many of the processed juices may not supply these babies with adequate quantities of this essential element.

- **Scurvy has not disappeared from the United States***



Typical position of legs in infantile scurvy.

What can be done? Because of the increasing evidence that American diets are deficient in vitamin C, leading nutrition experts believe that processed fruit and vegetable juices, on which the public is so reliant for its supply of this necessary vitamin, should contain the "Vital Ingredient."

The Vital Ingredient of any processed fruit or vegetable juice is a *dependable* and *adequate* supply of vitamin C. You can give your juice this ingredient by *standardizing its vitamin C content*. The processing is simple and the new low cost of ascorbic acid makes juice standardization more attractive than ever.

With ascorbic acid available *by the tons* at extremely modest cost fruit and vegetable juices can have a standardized vitamin C content for the better protection of the nation's health and well-being.

*Statements are based on documented reports. References on request.

Illustrations after photographs in The Vitamins in Medicine by Bicknell and Prescott, Third Edition, published by Grune & Stratton, Inc., New York.

ROCHE Pure, crystalline VITAMIN C (Ascorbic acid, U. S. P.)

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

In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon Street, St. Laurent, P. Q.

THE NET UTILIZATION OF NON-SPECIFIC NITROGEN SOURCES FOR THE SYNTHESIS OF NON-ESSENTIAL AMINO ACIDS

I. GROWTH AND NITROGEN UTILIZATION [1]

M. RECHCIGL, JR., J. K. LOOSLI AND H. H. WILLIAMS

Department of Animal Husbandry and Department of Biochemistry and Nutrition, Cornell University, Ithaca, New York

(Received for publication January 31, 1957)

INTRODUCTION

In order to reach maximum growth, monogastric animals require the essential amino acids and a source of nitrogen for the synthesis of the non-essential amino acids. Recent studies of Rose et al. ('49), Lardy and Feldott ('50), Rose and Smith ('50), Frost and Sandy ('51), Litwack, Williams and Elvehjem ('53) and Rose and Dekker ('56) demonstrated that the requirement for the latter was relatively non-specific. Various amino acids, ammonium salts and even urea could serve that purpose.

In the above experiments, however, some amino acids were supplied in the DL form. The presence of D-amino acids in the diet according to Frost and Sandy ('51), "creates variables both with regard to possible inhibitory effects and with regard to possible utilization of D-amino nitrogen."

In an attempt to find whether the inclusion of D-amino acids in the diet affects utilization, the present study was undertaken. This study differed from previous ones in that the essential amino acids were present exclusively in the L form, and the amounts furnished were based on requirements de-

[1] Supported in part by a grant to Cornell University from the Herman Frasch Foundation.

Copyright 1957
The Wistar Institute of Anatomy and Biology
All rights reserved

termined from carcass analyses (Williams et al., '54). The method of determining the essential amino acid requirements of a growing animal by amino acid assays of the entire carcass "may be as accurate as methods in current use" and "has the distinct advantage of avoiding the confusion concerning the utilization of the D-isomers that are commonly fed in racemic mixtures", according to Mitchell ('50).

Average gain and efficiency of food and nitrogen utilization were the criteria used for the evaluation of different diets. In addition, a modification of the Miller and Bender ('55) and Bender ('56) rapid procedure of estimating the net utilization of proteins was used for determining the net utilization of non-specific nitrogen sources. In this method the value of protein is determined by the increase in the body nitrogen which has been calculated from the moisture content of the carcasses. In a recent evaluation of this procedure, Forbes and Yohe ('55) found good agreement of the values for the biologic value of blood fibrin determined by this short procedure with those measured by the conventional Thomas Mitchell nitrogen balance method. A reason for selecting this method was the relatively short feeding period (10 days) required, which thus enabled us to use costly pure L-amino acids.

EXPERIMENTAL

Albino rats of the "Yale" strain, weaned at 21 days of age and fed for one week on stock diet, were used throughout. Each animal was housed in a separate cage and was permitted to consume food and water ad libitum.

In each experiment 4 litters of 6 or 8 rats each were divided into 6 or 8 groups consisting of two males and two females. Every group contained one rat from each litter and totalled the same weight at the onset of the treatments.

One of the groups was fed a nitrogen-free diet and each of the remaining groups was fed a separate experimental diet for 10 days. At the end of this period the animals were stunned by a blow on the head, decapitated and exsanguinated, care

being taken to save all of the blood. The livers were excised, placed immediately into cracked ice, and frozen.[2]

The hepatectomized carcasses were dried to constant weight at 105°C. and moisture determined. Body nitrogen was determined by the Kjeldahl method. In the earlier experiments the entire rat was digested in a Kjeldahl flask, the mixture was then diluted and aliquots taken for nitrogen determination. This method proved to be impractical because the digestion mixture frothed violently and required many hours for complete digestion as wall as large amounts of sulfuric acid. Therefore in later experiments the dried carcasses were fat-extracted in a Soxhlet apparatus, ground in a Wiley mill and an aliquot sample used for nitrogen analysis.

The basal diet contained the following, in grams: glucose,[3] 15.0; dextrin, 17.8; hydrogenated vegetable fat,[4] 14; cellulose [5] 2; salt mixture,[6] 4; and vitamin fortification mixture,[7] 2.2. Different experimental diets, shown in table 1, were prepared by mixing the above basal diet with one of the two amino acid mixtures, different sources of non-specific nitrogen and appropriate amounts of dextrin. All experimental diets with the exception of diet 1 furnished the same amount of nitrogen namely 2.11%. This figure was based on values obtained in carcass analysis (Williams et al., '54).

The composition of the two amino acid mixtures employed in the experiments is presented in table 2. Both mixtures contained cystine and tyrosine in addition to the 10 essential amino acids. The amount and ratio of the components of mixture AA-1 was also based on the values obtained in the amino acid assays of carcasses (Williams et al., '54).

Mixture AA-2 contained the same ratio of amino acids as mixture AA-1 but these were present in higher amounts.

[2] The authors are pleased to acknowledge the help of Mr. William G. Merrill in this part of the experiment.

[3] Cerelose

[4] Crisco

[5] Solka Floc, The Brown Company, Berlin, New Hampshire.

[6] Jones and Foster ('42).

[7] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Composition of experimental diets

DIET NO.	Basal	Dextrin	AMOUNT SUPPLIED BY: Amino acid mixture No.	Amino acid mixture %	Amino acid mixture % N	N supplement Source	N supplement %	N supplement % N	TOTAL N %
	%	%		%	% N		%	% N	%
1	55	45	None			None			..
2	55	29.57	AA-2	15.43	2.11	None			2.11
3	55	35.67	AA-1	6.80	0.93	Urea	2.53	1.18	2.11
4	55	28.68	AA-1	6.80	0.93	Diammonium citrate	9.52	1.18	2.11
5	55	25.81	AA-1	6.80	0.93	L-Glutamic acid	12.39	1.18	2.11
6	55	31.88	AA-1	6.80	0.93	Glycine	6.32	1.18	2.11
7	55	35.31	AA-1	6.80	0.93	Biuret	2.89	1.18	2.11
8	55	32.04	AA-1	6.80	0.93	L-Glutamine	6.16	1.18	2.11
9	55	26.98	AA-1	6.80	0.93	L-Aspartic acid	11.22	1.18	2.11
10	55	32.63	AA-1	6.80	0.93	L-Asparagine	5.57	1.18	2.11
11	55	30.69	AA-1	6.80	0.93	L-Alanine	7.51	1.18	2.11
12	55	29.35	AA-1	6.80	0.93	L-Serine	8.85	1.18	2.11
13	55	28.50	AA-1	6.80	0.93	L-Proline	9.70	1.18	2.11
14	55	27.15	AA-1	6.80	0.93	L-OH-Proline	11.05	1.18	2.11

Mixture AA-2 thus furnishes 1.18% more nitrogen than mixture AA-1. This figure was selected because it represented the amount of nitrogen supplied by the various non-specific nitrogen supplements.

RESULTS

Growth. Comparative growth effects of diets containing isonitrogenous levels of various nonspecific nitrogenous sources are summarized in table 3. The differences between the gains

TABLE 2

Composition of amino acid mixtures

	MIXTURE AA-1			MIXTURE AA-2		
	Form	%	% N	Form	%	% N
Arginine	L	0.77	0.25	L	1.75	0.56
Histidine	L	0.28	0.08	L	0.64	0.17
Isoleucine	L	0.46	0.05	L	1.04	0.11
Leucine	L	0.85	0.09	L	1.93	0.21
Lysine	L·HCl	1.25	0.19	L·HCl	2.84	0.44
Methionine	L	0.22	0.02	L	0.50	0.05
Phenylalanine	L	0.48	0.04	L	1.09	0.09
Threonine	L	0.51	0.06	L	1.16	0.14
Tryptophan	L	0.10	0.01	L	0.23	0.03
Valine	L	0.72	0.09	L	1.63	0.19
Tyrosine	L	0.38	0.03	L	0.86	0.07
Cystine	L	0.20	0.02	L	0.45	0.05
$NaHCO_3$		0.58			1.31	
Total		6.80	0.93		15.43	2.11

on experimental diets were analyzed by application of the multiple range test (Duncan, '53) and the results are shown in table 4.

In the first experiment (series I, table 3) the growth effect of essential L-amino acids, urea, diammonium citrate, L-glutamic acid and glycine added to the basal diet containing "adequate" amounts of essential L-amino acids, was studied. L-Glutamic acid gave the best growth response, closely followed by diet 2, in which the non-specific nitrogen was furnished by an excess of the essential L-amino acids. Glycine

and diammonium citrate gave the poorest growth response while urea was intermediate. By application of the multiple range test, the difference between glycine or diammonium citrate on one side and glutamic acid, excess of essential L-amino acids or urea on the other, was found to be highly

TABLE 3

Effect of various non-specific nitrogenous sources on the growth of rats

SERIES NO.[1]	DIET NO.	NON-SPECIFIC N SOURCE	AVERAGE GAIN	FOOD INTAKE	FOOD EFFICIENCY	N EFFICIENCY RATIO [2]
			gm	*gm*		
I	1	None	— 9 ± 1 [3]	41	— 0.22	..
	2	L-Essential A.A.	29 ± 2	72	0.40	19
	3	Urea	23 ± 3	82	0.28	13
	4	Diammonium citrate	13 ± 2	58	0.22	11
	5	L-Glutamic acid	32 ± 3	82	0.38	18
	6	Glycine	11 ± 2	52	0.21	10
II	1	None	— 10 ± 2	45	— 0.22	..
	7	Biuret	18 ± 2	77	0.23	11
	8	L-Glutamine	29 ± 1	87	0.34	16
	9	L-Aspartic acid	28 ± 1	83	0.34	16
	10	L-Asparagine	28 ± 3	84	0.33	16
III	1	None	— 12 ± 1	37	— 0.32	..
	3	Urea	20 ± 3	78	0.25	12
	4	Diammonium citrate	12 ± 1	59	0.20	10
	6	Glycine	5 ± 2	44	0.11	5
	11	L-Alanine	29 ± 5	85	0.34	16
	12	L-Serine	1 ± 1	36	0.03	1
	13	L-Proline	19 ± 1	64	0.30	12
	14	L-OH-Proline	— 14 ± 2	23	— 0.61	— 29

[1] Rats in series I had an average initial weight of 56.5 gm; in series II, 60.5 gm and series III, 47.6 gm.

[2] Average gain per gram nitrogen consumed.

[3] Standard error of the mean.

significant ($P<0.01$). There was also a significant difference between L-glutamic acid and urea ($P<0.05$).

The second experiment (series II, table 3) compared the growth effect of biuret, L-glutamine, L-asparagine and L-aspartic acid. The response to the last three compounds was almost identical and approached that of L-glutamic acid in

the first experiment. A highly significant difference ($P<0.01$) was shown between any of these three compounds on one side and biuret on the other.

In the third experiment (series III, table 3) the study of growth effects of urea, diammonium citrate and glycine was repeated and, in addition, the effects of L-alanine, L-serine, L-proline and L-hydroxyproline were compared. The responses to urea and to diammonium citrate were similar to that obtained in the first experiment, urea being again a better source of nitrogen ($P < 0.05$), while the response to glycine was poorer than observed previously. The response to L-serine

TABLE 4

Differences between the average gains on experimental diets as shown by the Duncan's multiple range test[1]

Series	I	Diet	I	6	4	3	2	5			
	II		1	7	10	9	8				
	III		14	1	12	6	4	13	3	11	

[1] Average gains on any two diets not underlined by the same line are significantly different ($P < 0.05$).

was even lower than that to glycine and the difference between the gains on L-serine and urea or diammonium citrate diets was highly significant ($P < 0.01$). L-Alanine gave the best growth response in this series and on the basis of food and nitrogen efficiency it was equal to L-glutamine, L-asparagine or L-aspartic acid. The response to L-proline was similar to that of urea but differed significantly from that of L-alanine ($P < 0.05$). L-Hydroxyproline was without any stimulatory effect, and in fact, the rats fed hydroxyproline lost more weight than rats on a nitrogen-free diet.

An experiment was also made in which the amino acid mixture AA-1 without supplementation with non-specific nitrogen was compared with the amino acid mixture AA-2.

At the end of 10 days the average gain of rats on the former diet was 12 gm and on the latter 23 gm and the total food consumption per rat was 75 and 71 gm, respectively.

Nitrogen utilization. Net nitrogen utilization of various non-specific nitrogenous sources, presented in table 5, was determined by the method of Miller and Bender ('55) and Bender ('56), with the modification that the nitrogen content

TABLE 5

Net nitrogen ratio (N.N.R.) and net nitrogen utilization (N.N.U.) of various non-specific nitrogen sources

NON-SPECIFIC N SOURCE	N.N.R.[1]	N.N.U.[2]
Essential L-amino acids	25	61
Urea	19,20	39,38
Diammonium citrate	18,19	48,43
L-Glutamic acid	24	57
Glycine	18,19	42,36
Biuret	17	43
L-Glutamine	21	51
L-Aspartic acid	22	49
L-Asparagine	22	50
L-Alanine	23	48
L-Serine	17	37
L-Proline	23	48
L-OH-Proline	— 4	— 9

[1] $\text{N.N.R.} = \dfrac{\text{gain of test group} + \text{loss of weight of N-free group}}{\text{N intake}}$.

[2] $\text{N.N.U.} = \dfrac{\text{body N of test group} - \text{body N of N-free group}}{\text{N consumed by test group}}$.

of the "nitrogen free" diets was considered to be zero and was omitted from the calculation. The values for the nitrogen content of the carcasses (minus liver) used in arriving at the net nitrogen utilization values (N.N.U.) were determined by a direct chemical analysis. N.N.U. values may be slightly lower in view of the fact that the liver nitrogen was not included in their calculation.

Figure 1 shows a comparison between the N.N.U. values (series II) determined by direct chemical analysis and by

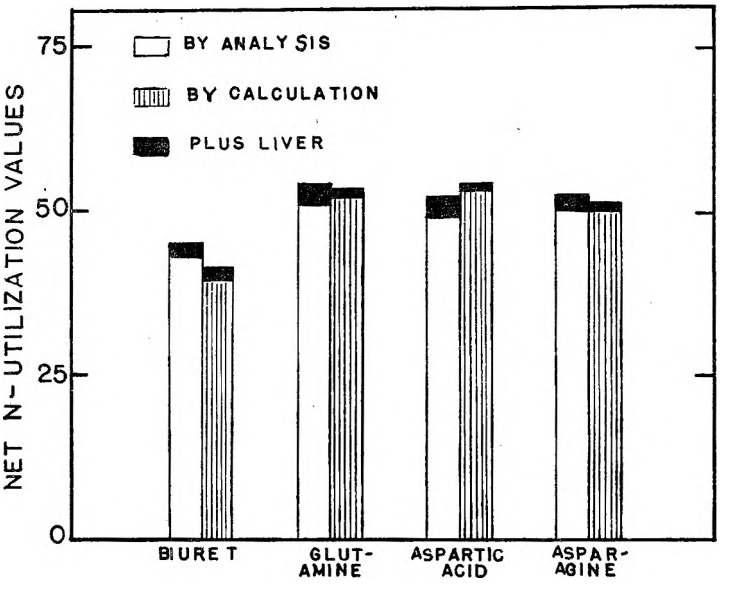


Fig. 1 A comparison of the net nitrogen utilization values (NNU) determined from carcas analyses and calculated from the water contant of the carcass.

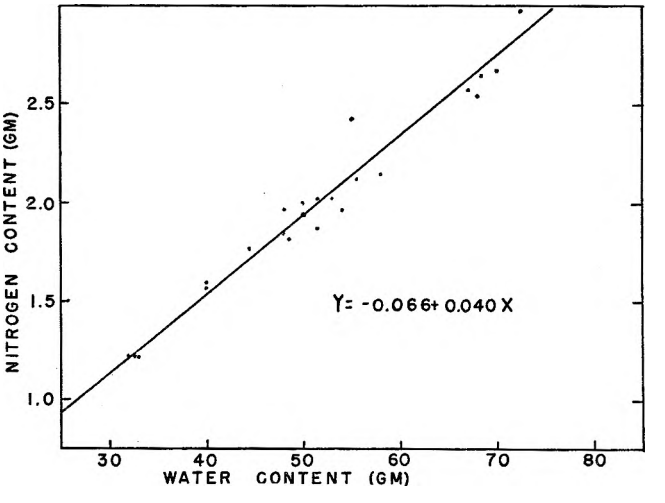


Fig. 2 Regression of water and nitrogen content of the rat carcasses; correlation = 0.98.

calculation from the N/H_20 ratio. The good agreement between the two procedures is apparent. The relationship between the water and nitrogen contents of the rat carcass is indicated in figure 2. The correlation between water and nitrogen was highly significant; $r = 0.982$; the regression equation being $y = -0.066 + 0.040X$. The correlation between body water and body nitrogen excluding the liver was also highly significant; $r = 0.980$, while the regression was $y = -0.056 + 0.040X$.

Net nitrogen ratio (N.N.R.), also shown in table 5, was determined by the method of Bender [8] and Doell ('57) with the modification that nitrogen intake instead of protein intake was used in the calulation.

Using the N.N.R. and N.N.U. values as the criteria, L-glutamic acid and an excess of essential L-amino acids appear to be the best nitrogen supplements. These are closely followed by alanine, aspartic acid, asparagine, proline and glutamine. Next in effectiveness come diammonium citrate, urea, biuret, glycine and serine. At the bottom of the list is L-hydroxyproline.

DISCUSSION

The results of the present study, in agreement with the experiments of Rose et al. ('49), Lardy and Feldott ('50) and Frost and Sandy ('51), demonstrate that the nitrogen of the non-essential amino acids may be derived from a variety of non-specific nitrogen sources including ammonium salts, urea and single non-essential amino acids. Different nitrogenous supplements, however, are not equally effective, which is well illustrated in our experiments by L-hydroxyproline and L-glutamic acid.

The effectiveness of a single nitrogenous compound as a nitrogen source for the synthesis of non-essential amino acids depends on its ability to supply NH_2 groups. Some nitrog-

[8] The authors are greatly indebted to Dr. A. E. Bender of the Research Dept., Bovril Ltd., London, for sending the senior author a copy of this manuscript before it was submitted for publication.

enous substances may exert additional stimulatory effects by being constituents of some vital compound or having some specific function in metabolism. Two other factors which are just as important or may be even more important are the palatability and the toxicity of the "ammonia" yielding compound.

The central role of glutamic acid in transamination reactions and metabolism of nervous tissue (Weil-Malherbe, '50) helps to explain its effectiveness as observed in ours as well as other laboratories (Rose et al., '49; Frost and Sandy '51). In addition, this amino acid is a constituent of glutathione and is involved in urea formation.

The similarity between the growth effects exerted by the L-isomers of different non-essential amino acids (alanine, aspartic acid, aspargine, proline and glutamine) suggests a similar pathway in their catabolism. This observation is in accord with the findings of Åqvist ('51). The present evidence in the literature indicates that L-amino acids are deaminated by transamination (Kamin and Handler, '51; Meister, '54, Braunstein, '47), probably via α-ketoglutarate.

The explanation for the relatively low effectiveness of glycine and serine when compared to other non-essential amino acids probably lies in their low palatability or in their "toxicity." This conclusion is in accord with the often observed growth depression on glycine diets (Arnstein, '54) and the well known interconversion between glycine and serine (Rose, Burr and Sallach, '52).

The detrimental effect of hydroxyproline on growth in our experiments agrees with similar findings of Womack and Rose ('47). This compound must be extremely unpalatable, or even toxic as is indicated by a low intake. That dietary hydroxyproline is not appreciably utilized for protein synthesis is also evident from Stetten's ('55) discussion.

The rats fed diets containing urea gained more weight and consumed more food than those on diammonium citrate diets. On the other hand, when the carcasses of these two groups of rats were analyzed for nitrogen and the N.N.U. value was

determined, diammonium citrate seemed to be a more efficient source of nitrogen. The higher efficiency of diammonium citrate compared to urea was also reported in growth studies of Rose et al. ('49) and Frost and Sandy ('51). The poorer growth on diammonium citrate in our studies can be explained by the difference in the amount fed. Thus, in our studies diammonium citrate was supplied at the level of 1.18 gm of nitrogen per 100 gm of diet, while it was supplied in the studies of Rose et al., at the level of 0.574 gm of nitrogen per 100 gm of diet, and in the studies of Frost and Sandy at the level of 32.2% of total nitrogen. This finding suggests the possibility that diammonium citrate may be toxic when fed at high levels. Similarly, Lardy and Feldott ('50) observed that increasing the amount of diammoninm citrate from 4 to 6% gave no further growth response when 8% of essential amino acids were fed, and it depressed growth when the diet contained only 4% of essential amino acids.

A greater effectiveness of glutamic acid or of a mixture of non-essential amino acids (alanine, aspartic acid, cystine, glutamic acid, tyrosine and glycine) than diammonium citrate is also indicated in the studies of Womack, Marshall and Parks ('53), Marshall and Womack ('54), and Schultze ('55, '56), and a higher toxicity of diammonium citrate than urea (at an isonitrogenous level) is apparent from the investigations of Fried and da Silva ('54a, b) and Finlayson and Baumann ('56).

Numerous experiments with C^{14} or N^{15} urea (Leifer, Roth and Hempelmann, '48; Hastings et al., '50; Von Korff, Ferguson and Glick, '51; Skipper et al., '51; Kornberg and Davies '52; Zbarsky and Wright, '53; Kornberg, Davies and Wood, '53) demonstrated that urea is not an inert substance but that it undergoes hydrolysis *in vivo* under the influence of the enzyme urease (Kornberg and Davies, '55), which was recently shown to be predominantly of bacterial origin (Chao and Tarver, '53; Dintzis and Hastings, '53; Kornberg, Davies and Wood, '54a, b; Cheosakul et al., '55). Following the hydrolysis of urea, the released ammonia may be used in the

biosynthesis of non-essential amino acids (Rose and Dekker, '56) or reconverted to urea by the liver. The resynthesis of urea by the liver and its subsequent excretion in the urine may account for the lower efficiency of this substance when compared to diammonium citrate. Another contributing factor may be the low urease activity in the rat stomach. (Chao and Tarver, '53; FitzGerald and Murphy, '50; Luck, '24; Weil, '44).

Biuret was almost as effective as urea in our studies. The metabolism of this compound within the body is still unknown. The proposed enzymatic hydrolysis of biuret by soybean or gastric urease has not been confirmed in recent studies (Kurzer, '56).

SUMMARY

The effectiveness of a mixture of essential L-amino acids, single non-essential amino acids, diammonium citrate, urea and biuret as a source of nitrogen for the biosynthesis of non-essential amino acids was investigated. These nitrogenous compounds were added at isonitrogenous levels to a mixture of essential amino acids, patterned after the composition of the rat carcass. The amino acids were present exclusively in the L form.

L-Glutamic acid and a mixture of L-essential amino acids were the most effective supplements when judged by such criteria as growth response, food efficiency and net nitrogen utilization. These were followed by alanine, aspartic acid, asparagine, proline, glutamine, diammonium citrate, urea, biuret, glycine and serine, all arranged in order of their effectiveness. Diets containing L-hydroxyproline caused depression in growth rate.

ADDENDUM

After the completion of these studies a letter to the editors by Greenstein, Birnbaum and Winitz ('56) bearing on this problem appeared. Although the experiments are not strictly comparable, their results are in agreement with those of the present study.

LITERATURE CITED

ÅQVIST, S. E. G. 1951 Metabolic interrelationships among amino acids studied with isotopic nitrogen. Acta Chem. Scand., *5:* 1046.

ARNSTEIN, H. R. V. 1954 The metabolism of glycine. Advances in Protein Chemistry, *9:* 1.

BENDER, A. E. 1956 Relation between protein efficiency and net protein utilization. Brit. J. Nutrition, *10:* 135.

BENDER, A. E., AND B. H. DOELL 1957 Biological evaluation of proteins: a new aspect. Typed manuscript.

BRAUNSTEIN, A. E. 1947 Transamination and the integrative functions of the dicarboxylic acids in nitrogen metabolism. Advances in Protein Chem., *3:* 1.

CHAO, F. C., AND H. TARVER 1953 Breakdown of urea in the rat. Proc. Soc. Exp. Biol. Med., *84:* 406.

CHEOSAKUL, P., L. W. BLIDE, M. VANKO AND A. KNUDSON 1955 Mechanism of urea metabolism. Fed. Proc., *14:* 191.

DINTZIS, R. Z., AND A. B. HASTINGS 1953 The effect of antibiotics on urea breakdown in mice. Proc. Natl. Acad. Sci., *39:* 571.

DUNCAN, D B. 1953 Multiple range and multiple F tests. Va. Agr. Exp. Sta. Tech. Report No. 6.

FINLAYSON, J. S., AND C. A. BAUMANN 1956 Responses of rats to urea and related substances. J. Nutrition, *59:* 211.

FITZGERALD, O., AND P. MURPHY 1950 Studies on the physiological chemistry and classical significance of urease and urea with special reference to the stomach. Irish J. Med. Sci., p. 97.

FORBES, R. M., AND M. YOHE 1955 Net protein value of blood fibrin for the albino rat: Evaluation of nitrogen balance and carcass analysis methods. J. Nutrition, *55:* 493.

FRIED, R., AND A. C. DA SILVA 1954a Some effects of nicotinamide, diammonium citrate and urea as supplements to low-protein diets. Intern. Ztschr. Vitaminforsch., *25:* 281.

———— 1954b Effects of nicotinamide and nitrogen supplements in low-protein diets containing sulfonamides. Ibid., *25:* 295.

FROST, D. V., AND H. R. SANDY 1951 Utilization of non-specific nitrogen sources by the adult protein-depleted rat. J. Biol. Chem., *189:* 249.

GREENSTEIN, J. P., L. BIRNBAUM AND M. WINITZ 1956 A water soluble synthetic diet. Arch. Biochem. and Biophys., *63:* 266.

HASTINGS, A. B., W. LANGHAM, R. E. CARTER AND L. J. ROTH 1950 Metabolism of C^{14}-labeled urea in mice. U. S. Atomic Energy Commission, Technical Information Division, AECU-859.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

KAMIN, H., AND P. HANDLER 1951 The metabolism of parenterally administered amino acids. III: Ammonia formation. J. Biol. Chem., *193:* 873.

KORNBERG, H. L., AND R. E. DAVIES 1952 The metabolism of subcutaneously injected N^{15} urea in the cat. Biochem. J., *52:* 345.

———— 1955 Gastric urease. Physiol. Rev., *35:* 169.

KORNBERG, H. L., R. E. DAVIES AND D. R. WOOD 1953 In vivo studies of the metabolism of C^{14}-urea and C^{14}-bicarbonate in the cat. Radioisotope Techniques (Proc. Isotope Techniques Conf., Oxford, July 1951), *1:* 130. London: H.M. Stationary Office.

———— 1954a The breakdown of urea in cats not secreting gastric juice. Biochem. J., *56:* 355.

———— 1954b The activity and function of gastric urease in the cat. Ibid., *56:* 363.

KURZER, F. 1956 Biuret and related compounds. Chem. Rev., *56:* 95.

LARDY, H. A., AND G. FELDOTT 1950 The net utilization of ammonium nitrogen by the growing rat. J. Biol. Chem., *186:* 85.

LEIFER, E., L., J. ROTH AND L. H. HEMPELMANN 1948 Metabolism of C^{14}-labeled urea. Science, *108:* 748.

LITWACK, G., J. N. WILLIAMS, JR. AND C. A. ELVEHJEM 1953 The roles of essential and non-essential amino acids in maintaining liver xanthine oxidase. J. Biol. Chem., *201:* 261.

LUCK, J. M. 1924 Ammonia production by animal tissues in vitro. II. The demonstration of urease in the animal body. Biochem. J., *18:* 825.

MARSHALL, M. W., AND M. WOMACK 1954 Influence of carbohydrate, nitrogen source and prior state of nutrition on nitrogen balance and liver composition in the adult rat. J. Nutrition. *52:* 51.

MEISTER, A. 1954 Enzymatic transfer of alpha-amino groups. Science, *120:* 43.

MILLER, D. S., AND A. E. BENDER 1955 The determination of the net utilization of proteins by a shortened method. Brit. J. Nutrition, *9:* 382.

MITCHELL, H. H. 1950 Some Species and Age Differences in Amino Acid Requirements. *In* Protein and Amino Acid Requirements of Mammals Ed. by A. A. Albanese. Academic Press, New York.

ROSE, W. C., W. W. BURR, JR. AND H. J. SALLACH 1952 Growth on diets devoid of glycine, serine, and cystine, and low in choline. J. Biol. Chem., *194:* 321.

ROSE, W. C., AND E. E. DEKKER 1956 Urea as a source of nitrogen for the biosynthesis of amino acids. Ibid., *223:* 107.

ROSE, W. C., L. C. SMITH, M. WOMACK AND M. SHANE 1949 The utilization of the nitrogen of ammonia salts, urea, and certain other compounds in the synthesis of non-essential amino acids in vivo. Ibid., *181:* 307.

ROSE, W. C., AND L. C. SMITH 1950 Role of alimentary microoorganisms in the synthesis of non-essential amino acids. Ibid., *187:* 687.

SCHULTZE, M. O. 1955 Concerning the alleged occurrence of an "animal protein factor" required for the survival of young rats. II. Reproduction of rats fed protein-free amino acid rations. J. Nutrition, *55:* 559.

———— 1956 Reproduction of rats fed protein-free amino acid rations. Ibid., *60:* 35.

SKIPPER, H. E., L. L. BENNET, JR., C. E. BRYAN, L. WHITE, JR., M. A. NEWTON AND L. SIMPSON 1951 Carbamates in the chemotherapy of leukemia. VIII. Overall tracer studies on carbonyl-labeled urethan, methylene-labeled urethan, and methylene-labeled ethyl alcohol. Cancer Res., *11:* 46.

STETTEN, M. R. 1955 Metabolic relationship between glutamic acid, proline, hydroxyproline, and ornithine. *In* Amino Acid Metabolism. Ed. by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, p. 277.

VON KORFF, R. W., D. J. FERGUSON AND D. GLICK 1951 Role of urease in the gastric mucosa. III. Plasma urea as source of ammonium ion in gastric juice of histamine-stimulated dog. Am J. Physiol., *165:* 695.

WEIL, L. 1944 Urease activity in mammals. J. Franklin Inst., *238:* 145.

WEIL-MALHERBE, H. 1950 Significance of glutamic acid for the metabolism of nervous tissue. Physiol. Rev., *30:* 549.

WILLIAMS, H. H., L. V. CURTIN, J. ABRAHAM, J. K. LOOSLI AND L. A. MAYNARD. 1954 Estimation of growth requirements for amino acids by assay of the carcass. J. Biol. Chem., *208:* 277.

WOMACK, M., M. W. MARSHALL AND A. B. PARKS 1953 Some factors affecting nitrogen balance in the adult rat. J. Nutrition, *51:* 117.

WOMACK, M., AND W. C. ROSE 1947 The role of proline, hydroxyproline, and glutamic acid in growth. J. Biol. Chem., *171:* 37.

ZBARSKY, S. H., AND W. D. WRIGHT 1953 The metabolism of C^{14}-urea in the rat. Canad. J. Med. Sci., *31:* 151.

GROWTH OF RABBITS ON PURIFIED DIETS [1]

E. L. HOVE [2] AND J. F. HERNDON [3]
Department of Animal Husbandry and Nutrition, Alabama Polytechnic Institute, Auburn

(Received for publication February 21, 1957)

The growth of rabbits fed casein-sucrose purified diets is usually less than optimum, and certain natural feedstuffs must be added for good gains. Hogan and Hamilton ('42) reported that rabbits grew normally on casein diets supplemented with yeast or liver extract. Kunkel, Simpson, Pearson, Olcese, and Schweigert ('48) also noted the beneficial effect of liver extract for rabbit growth and maintenance of the fur. Wooley ('54) reported that leafy material, such as kale, added to the casein diet greatly improved growth. Good growth was also obtained by readjusting the potassium level of the diet and increasing the casein level to 30%. The beneficial effect of soybean meal will be reported in the present paper. The rabbit has a high potassium requirement (Hove and Herndon, '55) and perhaps a high sodium and calcium requirement (Wooley and Mickelsen, '54); since little is known about the quantitative requirements of other nutrients it is hard to assess the reasons for the growth stimulation brought about by certain feed ingredients.

EXPERIMENTAL

The compositions of rabbit diets R43 and R14E are given in table 1. Variations in the level of protein source or of

[1] Supported in part by a grant-in-aid from the National Institute of Neurological Diseases and Blindness (Project B-430), and published with the approval of the Director of the Alabama Agricultural Experimental Station. Appreciation is expressed to Merck and Company, Lederle Laboratories, and A. E. Staley Manufacturing Company for vitamins used in these studies.

[2] Present address: National Institutes of Health, Bethesda, Md.

[3] Present address: Smith, Kline and French, Philadelphia, Pa.

supplements were compensated by adjustments in the carbohydrate level. Commercial, solvent-process soybean meal that was further subjected to a one- or 2-day continuous methanol extraction was used throughout (N $\times$ 6.25 = 51.3% protein). The casein was also methanol-extracted.

Rabbits of the New Zealand-white or the California-white strains were placed on the experimental diets at 4 weeks of

TABLE 1

Composition of diets

	R-43	R-14E
	%	%
Casein [1]	20	0
Soybean meal [1]	0	40
Sucrose	51	32
Cellulose [2]	10	10
Salt mixture no. 5 [3]	5	5
Potassium bicarbonate	1	0
Lard	6	6
Cod liver oil	2	2
Vitamin premix [4]	5	5

[1] Continuous one- or two-day methanol extracted.

[2] "Non-nutritive fiber" of General Biochemicals, Inc.

[3] W. D. Salmon, J. Nutrition, *33:* 155 (1947).

[4] Contributed per gram diet: thiamine, riboflavin, pyridoxine, 3 μg each; calcium pantothenate, 17 μg; choline chloride, 2,000 μg; i-inositol, 200 μg; niacin, 30 μg; methyl-1,4-naphthoquinone, 0.3 μg. (Vitamin B_{12}, 30 μg/kg, and folacin, 2 mg/kg, were added to some of the diets; exps. II, III and IV.) *dl*-α-Tocopherol acetate was given as 10 mg per week, orally.

age and at body weights usually of 400 to 500 gm. The animals were housed individually on half-inch mesh screens in an air-conditioned room. Water and feed were always available to the animals.

RESULTS

The normal rate of weight gain of young rabbits fed a ground, commercial rabbit feed [4] with 18% protein was about 35 gm/day (table 2). The simplified diet containing casein as the protein source, as used in the series I experiments, did

[4] Purina Rabbit Pellets.

not produce growth equivalent to that of the commercial feed even when the casein level was raised to 50%. The data in table 2 indicate that this high level of casein was superior to lower levels. However, the rate of gain was not maintained past 6 to 7 weeks on this diet. About one-third of the animals fed the casein diets showed marked loss of fur, regardless of

TABLE 2

Rabbit growth on purified diets

(Initial body weights 400 to 600 gm; time on diets usually 32 days, except for amino acid studies which were run 14 days)

EXPERIMENT SERIES NO.	LEVELS OF PROTEIN SOURCE AND ADDITIONS	NO. OF RABBITS	RATE OF GAIN
	%		*gm/day ± S.E.*
I	Casein, 10	4	4.8 ± 0.7
	Casein, 20	10	10.4 ± 3.2
	Casein, 33	4	12.4 ± 1.9
	Casein, 50	4	23.4 ± 2.2
	Soybean meal, 40	5	27.2 ± 2.7
	Casein, 20 + soybean meal, 15	5	26.6 ± 2.0
II	Casein, 20	12	14.2 ± 2.1
	Casein, 33	10	15.7 ± 2.5
	Soybean meal, 40	8	32.2 ± 2.3
	Casein, 20 + soybean meal, 25	6	35.3 ± 2.2
	Casein, 20 + water-insol. res. soybean meal, 25	2	31.5
	Casein, 20 + trypsin-digest of soybean meal, 16	5	19.1 ± 1.0
	Casein, 20 + ash of soybean meal, 4	4	15.6 ± 1.9
	Casein, 20 + dried brewers' yeast, 25	2	36.2
	Casein, 20 + arginine, 0.8, glycine, 0.8, methionine, 0.5, tryptophan, 0.1	4	23.6 ± 1.7
	Casein, 20 + arginine, methionine, tryptophan	2	18.0
	Casein, 20 + arginine, tryptophan	2	16.2
	Casein, 20 + glycine	2	12.0
III	Casein, 18	4	4.2 ± 1.2
	Enzymatic digest of casein, 18	4	15.3 ± 0.8
	Digest of casein, 18 + arginine, glycine, methionine	4	20.1 ± 1.9
	Soybean meal, 36	4	30.5 ± 3.5
IV	Casein-casein digest 1:1, 18	4	16.9 ± 2.4
	Casein-casein digest, 18 + arginine, glycine, methionine, tryptophan	7	20.8 ± 1.1
	Commercial rabbit feed (ground)	4	34.3 ± 1.5

casein level. Simplified diets containing soybean meal as the sole protein source, or added to the 20% casein diet, permitted growth in rabbits at a rate only slightly less than that of the commercial feed.

The growth-promoting properties of soybean meal could not be removed by water extraction (experiment series II, table 2). The water-soluble extract of the soy-bean meal was dried and added to the casein diet at a 3% level, but it did not improve growth (these data not shown). A water suspension of finely ground soybean meal was digested for 6 days with 1% trypsin (1–300). The filtrate was dried and fed at a 16% level; this gave a significant but not maximum growth stimulus. Dried brewers' yeast was as effective as soybean meal. The ash of soybean meal did not stimulate growth.

The additions of the amino acids arginine, glycine, methionine, and tryptophan to the 20% casein diet resulted in a significant growth increase. However, amino acid supplementation produced erratic results, and in a rather large number of other trials no benefit at all was apparent. This contrasts with the consistent and dramatic response to soybean meal.

In some experiments (as in III, table 2) an 18% casein level, with only 3% of cellulose, was used. The substitution of an enzymatic digest of casein [5] for the extracted casein improved growth significantly. The addition of the amino acids arginine, glycine, and methionine to this diet resulted in a further improvement of growth, but the growth rate did not equal that produced by the soybean meal diet. Reported growth factors, such as thioctic acid (10 mg/kg diet) and orotic acid (100 mg/kg diet), were included in the vitamin mixture used for experiment III; no benefit from these factors could be discerned.

The dried ceca (with contents) of rabbits fed the 20% casein diet were significantly heavier than those of the animals fed the soybean meal diet. Expressed as percentage of body weight, these values for 6 animals/group were 2.71 ± 0.15

[5] Nutritional Biochemicals, Inc., Chagrin Falls, Ohio.

and 1.73 ± 0.30, respectively. This may indicate a type of intestinal stasis with slower passage of ingesta through the tract.

The effect of the type of carbohydrate in the diet on the rate of body weight gain of rabbits is shown in table 3. All diets contained at least 5% sucrose, since this was the carrier for the vitamins. When corn starch was substituted for sucrose a slight but statistically insignificant improvement in growth resulted, regardless of whether the protein source was

TABLE 3

Effect of type of carbohydrate on rabbit growth

(4 rabbits per group with average initial weights of 350 to 410 gm; 40 days on diets)

PROTEIN SOURCE	CARBOHYDRATES ADDED TO DIET	RATE OF WEIGHT GAIN
	%	*gm/day ± S.E.*
20% Casein	Sucrose 51, cellulose 10	11.5 ± 0.93
	Cornstarch 51, cellulose 10	15.1 ± 1.88
	Cornstarch 61	13.7 ± 0.90
	Sucrose 31, cellulose 10, gum arabic 20	16.7 ± 1.28
	Sucrose 31, cellulose 10, agar 20	21.8[1] ± 1.33
	Sucrose 31, cellulose 10, triacetin 20	5.9
40% Soybean meal	Sucrose 32, cellulose 10	21.7 ± 1.88
	Cornstarch 32, cellulose 10	26.4 ± 1.44
	Cornstarch 42	25.2 ± 2.01

[1] Twenty-eight-day period.

casein or soybean meal. Omission of cellulose from either diet did not interfere with the growth rate. The addition of either gum arabic or agar, at a 20% level, resulted in significant improvement in growth rate in rabbits fed the casein diet. This is similar to the observation of Booth, Elvehjem, and Hart ('49) with guinea pigs. Triacetin-containing diets depressed rabbit growth.

The total carbohydrate of soybean is composed, according to Markley and Goss ('44), of about 4 to 6% each of cellulose, sucrose, stachyose, galactan and araban; only slight traces of starch or hemicelluloses are present. The inclusion of 25%

soybean meal in a 20% casein diet more than doubled the growth rate. This amount of soybean meal contributed about 10% of carbohydrate, 13% of protein, and 2% of ash. Since most of the carbohydrate consisted of substances known not to influence rabbit growth, such as sucrose, cellulose and stachyose, it is doubtful that the carbohydrate is a significant factor in the stimulation of growth. However, the galactans and arabans of soybean may have an effect on rabbit growth similar to that of gum arabic or agar.

DISCUSSION

The protein of soybean meal contains considerably more arginine and glycine than does casein. Supplements of these amino acids along with some methionine and tryptophan to the casein diet produced results that were erratic but positive enough to indicate that a partial deficiency of these amino acids contributed to the relatively poor growth on casein diets. Involvement of amino acids was further indicated by the better growth obtained when an enzymatic digest of casein replaced casein.

However, the erratic response would indicate that some nutrients other than the amino acids are limiting in the casein diet and are contributed by the soybean meal or yeast supplement. Almost nothing is known of the quantitative requirements of the rabbit for minerals or vitamins except for potassium (Hove and Herndon, '55); choline (Hove and others '55; pyridoxine (Hove and Herndon, '57) and niacin (Wooley, '47). Perhaps certain minerals or vitamins are supplied to the diet in insufficient or imbalanced amounts by the mineral and vitamin mixtures which, after all, are patterned upon the requirements for the growth of rats.

SUMMARY

For growth of rabbits fed a simple purified diet, defatted soybean meal was superior to casein as a protein source. With 20% casein the rate of growth was about 15 gm/day. Soybean meal as the sole source of protein at an equivalent level

permitted growth of about 30 gm/day. This compared favorably with growth of 35 gm/day for rabbits on a commercial feed. A supplement of arginine, glycine and tryptophan improved growth of rabbits fed the casein diet, and accounted for a part of the soybean meal effect. Other deficiencies in casein, as yet unidentified, were corrected by soybean meal or by dried brewers' yeast.

The type of carbohydrate in the diet had a minor effect on rabbit growth. Sucrose, starch, gum arabic, agar, cellulose and triacetin were compared. Gum arabic and agar influenced the growth rates most favorably.

LITERATURE CITED

BOOTH, A. N., C. A. ELVEHJEM AND E. B. HART 1949 The importance of bulk in the nutrition of the guinea pig. J. Nutrition, *37:* 263.

HOGAN, A. G., AND J. W. HAMILTON 1942 Adequacy of simplified diets for guinea pigs and rabbits. Ibid., *23:* 533.

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

HOVE, E. L., AND J. F. HERNDON 1955 Potassium deficiency in the rabbit as a cause of muscular dystrophy. Ibid., *55:* 363.

———— 1957 Vitamin B_6 deficiency in rabbits. Ibid., *61:* 127.

KUNKEL, H. O., R. E. SIMPSON, P. B. PEARSON, O. OLCESE AND B. S. SCHWEIGERT 1948 Effect of liver extract on growth of rabbits. Proc. Soc. Exp. Biol. Med. *63:* 122.

MARKLEY, K. S., AND W. H. GROSS 1944 "Soybean chemistry and technology" p. 24 Chemical Publishing Co., Inc., Brooklyn, N. Y.

WOOLEY, J. G. 1954 Growth of three- to four-week-old rabbits fed purified and stock rations. J. Nutrition, *52:* 39.

WOOLEY, J. G., AND O. MICKELSEN 1954 Effect of potassium, sodium or calcium on the growth of young rabbits fed purified diets containing different levels of fat and protein. Ibid., *52:* 591.

VITAMIN CONTENT OF FOODS EXPOSED TO IONIZING RADIATIONS

Z. Z. ZIPORIN, H. F. KRAYBILL AND H. J. THACH
U. S. Army Medical Nutrition Laboratory, Denver Colorado

(Received for publication April 15, 1957)

With developing knowledge in the field of irradiation sterilization of foods, it has become increasingly important to assess the effect of such treatment on the vitamin content of foods. Previous work on the radiosensitivity of niacin (Goldblith et al., '49; Proctor and Goldblith, '48, '49, '52,), ascorbic acid (Goldblith and Proctor, '49; Proctor and Goldblith, '48, '49; Proctor and O'Meara, '51), riboflavin (Goldblith and Proctor, '49; Proctor and Goldblith, '49), and thiamine (Dunlap and Robbins, '43) revealed a tendency for these substances to be destroyed, depending on the total dose of radiation, the type and rate at which it was delivered (Goldblith and Proctor, '49; Proctor and Goldblith, '48, '49), the constituents of the medium in which the vitamin was irradiated (Proctor and Goldblith, '48, '52), the concentration of the vitamin in the solution, and the physical state of the medium at the time of irradiation (Proctor and O'Meara, '51). As a result of these studies a sequence of increasing radiosensitivity for the vitamins could be set up, namely: niacin, riboflavin, and ascorbic acid. The only previous work on the radiosensitivity of thiamine was that of Dunlap and Robbins ('43), using *Phycomyces blakesleeanus* as the assay organism. This organism responds to intact thiamine as well as to the pyrimidine and thiazole moieties in solution. They reported that a solution of thiamine exposed to doses of 5,000 to 30,000 r of radiation from x-rays showed no loss of vitamin B_1 activity; on the

other hand, exposure of thiamine to 2650 millicurie-hours of radon in a glass capillary reduced the vitamin potency to 4% of that in the original solution. However, most of the previous work reported was done with solutions of the pure vitamins, or combinations of vitamins in solution. In work by Proctor and O'Meara ('51), using orange juice for the study of ascorbic acid destruction, and that of Proctor and Goldblith ('52), using ascorbic acid to prevent off-flavors of irradiated meat, vitamins were tested in natural foods. Since the radiosensitivity of these substances is altered by the constituents of the medium at the time of irradiation, it was important to determine vitamin retention in foods after exposure to sterilizing doses of radiation.

EXPERIMENTAL

Eight foods were selected for study. The foods were purchased and canned by the Quartermaster Food and Container Institute, Chicago, Illinois, so that each food item was uniformly distributed into no. 2 cans. After freezing, the samples were packed in dry ice and shipped to the radiation facility [1] where the radiation source consists of mixed fission products in spent fuel rods. The radiation may therefore be considered to contain essentially *gamma rays*. Each food was divided into three groups and treated as follows: one group was not irradiated and became the control; one group was irradiated with gamma rays, receiving 2.79×10^6 rad,[2] while the remainder was exposed to 5.58×10^6 rad. During irradiation the cans of frozen food were submerged in water and passed through an opening surrounded by fuel rods. The exposure was calculated at 2.79 megarad per hour, during which time the product was maintained at the ambient temperature of the water in the canal (24° C). Tests at the radiation facility have shown that the product thaws during irradiation. Following this treatment the cans were again packed in dry ice for ship-

[1] The Materials Testing Reactor, Idaho Falls, Idaho.

[2] Conversion factor – 1 rep = 93/100 rad.

ment to this laboratory. Vitamin analysis were performed on all samples.

Thiamine. The thiochrome method used for estimating thiamine was essentially that of the Association of Official Agricultural Chemists, ('55). Where the degradation products were assayed the procedure was that reported by Ziporin ('53), as a modification of the methods employed by Pavcek et al. ('37); van Lanen et al. ('42); and Obermeyer and Chen ('45).

In this procedure thiamine and its degradation products are extracted by autoclaving in an acid medium. An aliquot of this solution is incubated with the yeast *Saccharomyces cerevisiae* in a thiamine-free nutrient medium (Kline and Friedman, '47). The microorganism is capable of combining the pyrimidine and thiazole moieties of thiamine into the vitamin so that the latter substance may then be assayed by the thiochrome method. The pyrimidine moiety added to the nutrient medium was 2-methyl-5-ethoxymethyl-6-amino-pyrimidine while the thiazole portion was 4-methyl-5-β-hydroxyethyl thiazole.

Riboflavin. The fluorometric method as described in Association of Vitamin Chemists ('51), was used.

Niacin. This vitamin was assayed according to AOAC ('55) procedures, using cyanogen bromide with sulfanilic acid as the coupling agent.

Moisture was determined (60° C. 48 hours) on samples of each food taken at the same time as the material for vitamin assay.

RESULTS AND DISCUSSION

The data in table 1 indicate that thiamine is more radiosensitive than either niacin or riboflavin. There was a destruction of 70 to 95% of the thiamine in the foods tested, except powdered milk, with enhanced degradation occurring at the higher doses of radiation. From the data in table 2, using the values for foods on the "dry basis", it may be seen that in ham exposed to 2.79×10^6 and 5.58×10^6 rad the intact thiamine decreased to only 5% of the control value as shown

by the thiochrome test, while the yeast assay showed 50% destruction of thiazole at 2.79×10^6 rad and 60% destruction of this substance at 5.58×10^6 rad.

The fact that powdered milk showed no loss of any of the vitamins may be attributed to the lack of moisture and points up one of the factors that governs the radiosensitivity of these micronutrients. We cannot account for the loss of riboflavin in turkey. Since the peaches were a commercial grade product, it is possible that added ascorbic acid might

TABLE 1

Vitamin content of foods exposed to varying doses of gamma radiation

FOOD	THIAMINE			RIBOFLAVIN			NIACIN		
	0 [1]	2.79 [1]	5.58 [1]	0 [1]	2.79 [1]	5.58 [1]	0 [1]	2.79 [1]	5.58 [1]
	μg/gm	μg/gm	μg/gm	μg/gm	μg/gm	μg/gm	μg/gm	μg/gm	μg/gm
Haddock	0.11	0.035	0.026	0.71	0.76	0.68	19.16	16.90	17.53
Beef	0.24	0.057	0.037	1.86	1.76	1.79	29.57	28.90	29.28
Turkey	0.14	0.034	0.033	2.05	1.50	1.03	44.45	41.50	44.80
Ham (fresh)	8.15	1.03	0.31	1.65	1.86	1.62	33.70	33.00	32.90
Bacon	3.03	[2]	0.21	1.11	[2]	1.03	11.83	[2]	11.92
Peaches	0.79	0.045	0.017	0.26	0.32	0.27	7.62	4.00	3.36
Powdered milk	1.53	1.96	1.90	12.6	13.9	14.3	7.91	5.17	6.36
Beets	0.48	0.23	0.12	0.50	0.43	0.45	2.79	2.78	2.52

[1] Radiation dose in 10^6 rad.

[2] Sample not available for assay.

account for the destruction of niacin as suggested by the work of Proctor and Goldblith ('49).

The destruction of thiamine in all foods tested, except powdered milk, establishes the lability of this vitamin when irradiated in solution. Whether in animal or vegetable tissues, the extent of destruction increases with increasing doses of radiation. Turkey appears to be an exception. The degradation of thiamine may take place in many different ways; the simplest is the split between the pyrimidine and thiazole portions of the molecule. However, it is conceivable that thiamine potency may be lost by the rupture of either ring or by removal of substituent groups. At present there is no

knowledge of the mechanism for the destruction of thiamine when it is exposed to ionizing radiations.

The yeast assay method for thiamine split products has two requirements: (a) the presence of equimolar concentrations of the pyrimidine and thiazole components to form thiamine and; (b) a specific chemical structure for each component; the closer the structure is to that of thiamine, the more readily it is utilized for coupling with its counterpart to form thiamine. The thiochrome assay following the action of yeast adds a factor of specificity to the assay. Consequently, one may utilize these factors to establish the mechanism of destruction of the vitamin as a result of treatment with gamma rays.

From requirement (a) listed above, it is evident that the synthesis of one mole of thiamine requires one mole each of pyrimidine and thiazole. Thus, for example, a solution containing one mole of pyrimidine and one-half mole of thiazole would limit the yeast synthesis to one-half mole of thiamine. If an excess of thiazole were added, providing more than one mole of thiazole, the pyrimidine would then be the limiting factor in the solution and only one mole of thiamine could then be obtained. It is possible, then, that one moiety may be the limiting factor; this can be determined readily by adding that factor to the solution. An increase in thiamine synthesized would point to that substance as the one in lower concentration in the original solution. Thus, to determine the percentage destruction of thiazole, [column (7) of table 2], one must consider that the addition of excess pyrimidine reveals the thiazole as the limiting factor. In this instance, any decrease in the thiamine measured after re-synthesis by the yeast, as reported in column (4), must be due to destruction of thiazole by the radiation.

In table 2, using the values for foods on the "dry basis", it is seen that in the unirradiated control the addition of excess pyrimidine or thiazole gave thiamine values equal to that in which neither moiety had been added, indicating that no degradation products of thiamine may be found in the untreated food. When excess thiazole was added to the

TABLE 2

Recovery of thiamine degradation products in fresh ham following exposure to varying doses of gamma radiation

IRRADIATION DOSE (1)	THIAMINE BY THIOCHROME METHOD (2)	THIAMINE CONTENT FOLLOWING RE-SYNTHESIS BY YEAST			PER CENT DESTRUCTION OF THIAMINE [1] (6)	PER CENT DESTRUCTION OF THIAZOLE [2] (7)	PER CENT DESTRUCTION OF PYRIMIDINE (8)
		Yeast only (3)	Yeast and excess pyrimidine (4)	Yeast and excess thiazole (5)			
rad.		(μg/gm of food on "wet basis")					
0	8.15	5.47	5.55	5.62	..	..	..
2.79×10^6	1.03	2.20	2.13	3.85	87	62	0
5.58×10^6	0.31	1.73	1.77	4.15	96	68	0
		(μg/gm of food on "dry basis")					
0	15.26	10.24	10.39	10.52	..	..	..
2.79×10^6	2.58	5.51	5.34	9.65	83	49	0
5.58×10^6	0.79	4.42	4.53	10.61	95	56	0

[1] Using figures in column (2) calculated as: Thiamine content at $\frac{2.79 \times 10^6 \text{ or } 5.58 \times 10^6}{\text{Control}}$.

[2] Using figures in column (4) calculated as: Thiamine content at $\frac{2.79 \times 10^6 \text{ or } 5.58 \times 10^6}{\text{Control}}$.

irradiated ham, the thiamine recovered was equal to that in the unirradiated controls. If the pyrimidine, as well as the thiazole portion of the thiamine molecule, were destroyed by irradiation, then none of the values for treated ham as listed in columns (3), (4) and (5) would equal those for unirradiated ham as found in the same columns. In these experiments column (5) of table 2 shows that the thiamine values for treated ham are equal to those of untreated ham. One must conclude, then, that the thiamine is split by irradiation and that the thiazole portion of the molecule undergoes further degradative changes. This is found to occur most frequently in pharmaceutical products and points to the thiazole portion as more labile than the pyrimidine portion. This is in contradiction to the findings of Dunlap and Robbins ('43) who concluded that the inactivation of thiamine by irradiation was probably due to an attack on the thiamine molecule at some point other than the thiazole and pyrimidine linkage. No explanation is offered for the lower thiamine values reported for ham when incubated with yeast as compared with ham assayed by the thiochrome method. Ideally they should be equal. Attempts to resolve this difference revealed no satisfactory answer.

The radiosensitivity of niacin and riboflavin in the foods is considerably different from that in pure solution. Proctor and Goldblith ('48, '49), reported an effect of irradiation that varied with dose, concentration of the vitamin in solution, and characteristics of the radiation.

It has been shown (Proctor and O'Meara, '51) that equal doses of hard or soft x-rays destroyed greater amounts of vitamin in dilute solutions than in more concentrated ones. Also, hard x-rays were more destructive than soft x-rays. Thus, at a concentration of 10 μg/ml, 38% of the niacin was destroyed by exposure to 500,000 r of 50 kv x-rays while at 17 μg/ml 500,000 r of 3,000 kv x-rays caused destruction of 66% of the niacin. Goldblith et al. ('49), using niacin solutions, demonstrated that 28 % of the vitamin may be decarboxylated at doses of 170,000 rep (158,000 rad), and that of 660,000

rep (614,000 rad) the pyridine ring of niacin may be split. Since the chemical assay used in the studies reported here could not differentiate between intact or decarboxylated niacin, the question remains as to whether the niacin measured in the food is intact or has been altered. However, it is safe to say that 5.58×10^6 rad did not split the pyridine ring as reported by Goldblith et al. ('49) using 6.6×10^5 rep (6.14×10^5 rad). From the results reported here, niacin is less radiosensitive in food than in pure solution.

Riboflavin also displays differences in radiosensitivity dependent upon dose, concentration of the solution, and characteristics of the radiation. Goldblith and Proctor ('49) report that in a 50 μg/ml sample irradiated with 500,000 r of soft x-rays there was 68% destruction while 85% was destroyed when the solutions were exposed to 250,000 r of 3,000 kv x-rays. Values in table 1 indicate very little destruction in the foods tested except turkey, even though exposures of 5.58×10^6 rad were attained. This again points to the diminished radiosensitivity of the vitamin in food as compared with pure solutions.

Since the vitamin C determinations were made by use of the 2, 6-dichloroindophenol titration technique, the data for the three foods tested are not presented. It is known that irradiation of aqueous solutions may produce oxidizing substances. Their effect on the assay of vitamin C by the method used is not known. For this reason, it is felt that corroborative tests should be performed which are not so readily influenced by the presence of oxidizing substances.

SUMMARY

1. In 8 foods analyzed, namely, haddock, beef, turkey, ham (fresh), bacon, peaches, powdered milk and beets, turkey showed a significant decrease in riboflavin, while the niacin content was reduced in peaches. Thiamine was destroyed to the extent of 70 to 95 % in all foods tested except powdered milk.

2. Comparison of these results with results previously published indicates that vitamins in foods are less radiosensitive than those in pure solution.

3. The degradation of thiamine occurs by the splitting of the molecule into pyrimidine and thiazole moieties. The thiazole undergoes further degradative changes at higher doses of irradiation.

LITERATURE CITED

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

ASSOCIATION OF VITAMIN CHEMISTS 1951 Methods of Vitamin Assay, 2nd Ed. Interscience Pub., Inc., New York.

DUNLAP, C. E., AND F. C. ROBBINS 1943 The effect of roentgen rays, radon, and radioactive phosphorus on thiamine chloride. Am. J. Roentgenol. and Rad. Therap., *50:* 641–647.

GOLDBLITH, S. A., AND B. E. PROCTOR 1949 Effect of high-voltage x-rays and cathode rays on vitamins (riboflavin and carotene). Nucleonics, *5*, No. 2, 50–58.

GOLDBLITH, S. A., B. E. PROCTOR, J. R. HOGNESS AND W. H. LANGHAM 1949 The effect of cathode rays produced at 3,000 kilovolts on niacin tagged with C^{14}. J. Biol. Chem., *179:* 1163–1167.

KLINE, O. L., AND L. FRIEDMAN 1947 Microbiological Assay Methods for Thiamine. Biological Symposia *XII:* 65–85.

OBERMEYER, H. G., AND L. CHEN 1945 Biological estimation of the thiazole and pyrimidine moieties of vitamin B_1. J. Biol. Chem., *159:* 117–122.

PAVCEK, P. L., W. H. PETERSON AND C. A. ELVEHJEM 1937 Effect of growth conditions on yield and vitamin B_1 of yeast. Ind. Eng. Chem., *29:* 536–541.

PROCTOR, B. E., AND S. A. GOLDBLITH 1948 Effect of high-voltage x-rays and cathode rays on vitamins (niacin). Nucleonics, *3*, no. *2:* 32–43.

———— 1949 Effect of soft x-rays on vitamins (niacin, riboflavin, and ascorbic acid). Ibid., *5*, no. *3:* 56–62.

———— 1952 Prevention of side effects in sterilization of foods and drugs by ionizing radiations. Ibid., *10*, no. *4:* 64–65.

PROCTOR, B. E., AND J. P. O'MEARA 1951 Effect of high-voltage cathode rays on ascorbic acid. Ind. Eng. Chem., *43:* 718–721.

VAN LANEN, J. M., H. P. BROQUIST, M. J. JOHNSON, I. L. BALDWIN AND W. H. PETERSON 1942 Synthesis of Vitamin B_1 by Yeast. Ibid., *34:* 1244–1247.

ZIPORIN, Z. Z. 1953 The use of yeast to determine degradation products of thiamine. Ph. D. Thesis, Georgetown University, Washington, D. C.

STUDIES OF THE EFFECTS OF DIETARY NaF ON DAIRY COWS

I. THE PHYSIOLOGICAL EFFECTS AND THE DEVELOPMENTAL SYMPTOMS OF FLUOROSIS [1]

JOHN W. SUTTIE, RUSSELL F. MILLER [2]
AND PAUL H. PHILLIPS
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication April 18, 1957)

Fluorosis, a chronic fluorine toxicity, has been reported in the field or produced experimentally by many investigators. Among the developmental symptoms described have been mottling of the teeth, increased fluorine concentration in the urine and an increase with time of the fluorine concentration of the skeleton. More physiological in nature have been reports of a loss of appetite, an inability to maintain weight during lactation, and an onset of lameness and stiffness. Of lesser value in diagnosis, or of more doubtful occurrence, have been reports of effects on reproduction, transfer of fluorine into the milk, and across placental membranes. Since the subject of livestock fluorosis has been recently reviewed by Phillips et al. ('55) detailed references will not be listed here. Peirce ('40) drew attention to a latent period between the development of the dental and other physiological effects. Recent work has supported this premise (Phillips et al., '55).

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Aluminum Company of America, Pittsburgh, Pennsylvania, on behalf of itself and the Aluminum Laboratories Ltd., the American Smelting and Refining Co., the Kaiser Aluminum and Chemical Corporation, the Monsanto Chemical Co., the Reynolds Metal Co., the Tennessee Valley Authority, the U. S. Steel Corporation of Delaware, and Westvaco, Chemical Division of Food Machinery and Chemical Corp.

[2] Present address: Virginia Polytechnic Institute, Blacksburg, Virginia.

The toxicity of various fluorine compounds differs, depending for the most part on the solubility of the compound (Phillips et al., '55).

Most of the above work has dealt with fluorine in the form of fluorine-containing rock phosphate. More recently the contamination of forages with soluble fluorides from industrial processes has become a problem (Blakemore et al., '48; Agate et al., '49; Phillips, '52; Harris et al., '52; Spencer, '53; Hobbs et al., '54). An experiment was set up to study the effects of controlled levels of a soluble fluoride (NaF) on lactating dairy cattle. The objectives were: (a) to observe and catalogue the developmental symptoms of fluorosis; (b) to determine what level of fluorine fed as a sodium fluoride is associated with reduced milk production; and (c) to extend our knowledge of toxicological and biochemical effects of fluorine upon the animal body.

This paper deals with some of the physiological effects observed in the development of fluorine toxicosis in young dairy cows.

EXPERIMENTAL

Twenty-four bred grade Holstein heifers, one and one-half to two years old, ready to freshen in approximately 60 days were purchased from farmers in the vicinity of Madison. After selection, the cattle were twice tested and found negative for Brucellosis and were assembled at the University barn in December of 1950. In February, 1951, after 20 of these heifers had calved they were assigned to 6 groups of 4 each according to (a) dairy character, (b) milk production, (c) size, (d) general thrift, and (e) calving date. The basal ration fed all cows consisted of 30 to 40 lbs. of corn silage, 15 to 24 lbs. of alfalfa-brome grass, clover hay, and 1 lb. of the grain mixture per 4 lbs. of milk produced. The grain mixture contained; 700 lbs. ground oats, 700 lbs. ground corn, 400 lbs. wheat bran, 200 lbs. linseed oil meal, 20 lbs. trace mineralized salt, and 0.5 lb. irradiated yeast. Repeated analysis of this ration by the method of Remmert et al. ('53) for fluorine in plant

materials, indicated that it contained 3 to 5 p.p.m. of fluorine depending on the amount of grain fed, which contained less fluorine than the hay or silage. The lots and treatments were as follows.

Lot I	Basal ration only
Lot II	Basal + 20 p.p.m. of fluorine as NaF
Lot III	Basal + 30 p.p.m. of fluorine as NaF
Lot IV	Basal + 40 p.p.m. of fluorine as NaF
Lot V	Basal + 50 p.p.m. of fluorine as NaF
Lot VI	Basal + 50 p.p.m. of fluorine as NaF + 200 gm of $CaCO_3$ daily

Lot VI, with calcium carbonate, was included because excess dietary calcium has been shown to alleviate the toxicity of soluble fluorides (Phillips et al., '55).

The cattle were turned out for exercise daily, but were never allowed on pasture.

The hay and silage fed the cattle were weighed weekly and the amount of grain to be fed was calculated from the previous week's milk production. The amount of fluorine administered was then adjusted so that the necessary concentration of fluorine on a dry matter basis was maintained.

The correct amount of fluorine was originally administered by pouring the required amount of a solution containing 0.01 gm F/ml over the morning's feeding of grain and silage. Beginning with the second year, the concentration of the solution was halved and the administration was changed to twice daily. All cattle were weighed monthly. The cows were observed daily for signs of oestrus and bred artificially. At various times during the experiment, urine, fecal and milk samples were collected.

To measure the variation of skeletal fluorine concentration during the experiment, a rib sample was periodically obtained by biopsy. A five-eighth inch Galt's trephine or later a modification of this to fit a hand drill was used to obtain the sample of bone. The incisor teeth of all cows were periodically examined and classified according to the system of Hobbs et al. ('54).

The extent of fluorine transfer through the placental membrane was followed by determining the fluorine concentration

of the metacarpal bone of newborn calves dropped by the experimental cows.

Increased hoof length and diarrhea have been symptoms commonly attributed to fluorosis. Any occurrence of these conditions was noted and hoof length was measured after two years of fluorine feeding.

Unless otherwise indicated, all fluorine analysis reported were made by the Alcoa Research Laboratory modification ('47) of the Willard and Winter method ('33).

RESULTS AND DISCUSSION

Health. The chief health problem during these studies was mastitis. There was no correlation between the incidence of chronic mastitis and the fluorine intake. However, some animals seemed to be much more susceptible than others. Acute mastitis occurred with less frequency and was also equally prevalent among the cows of the various lots. The feeding of these levels of additional fluorine had no effect on the mortality rate. The causes of death of the 5 cows lost from the herd in the 5½-year period were: cow 1 (lot I) was slaughtered because of sterility after her first lactation; cow 9 (lot III) died of cardiac failure in her 4th lactation; cow 15 (lot IV) was slaughtered in her 6th lactation after an illness diagnosed as "Hardware", with the cause of illness still undetermined after post mortem examination; cow 18 (lot V) died after aborting her second calf; and cow 21 (lot VI) hemorrhaged at calving and died at the start of her 4th lactation.

Effects on body weight. Table 1 presents data on the minimum, maximum, and the average body weights of the cows during each year. These data would indicate that there was a slight depression in the attainment and maintenance of adult weight when the dietary fluorine level was raised to 50 p.p.m. This trend was more pronounced in the variations of the average yearly weight data, where it can be seen that the cows in lot V (50 p.p.m.) increased their body weight only 23% in 5 years compared to 38% for the control lot. Inspection of the monthly weight records revealed that the

cows with the higher fluorine levels (on the basis of body weight) tended to have an excessive and extended post partum and lactation weight loss. For example, cows 17 and 19 (lot V) experienced lactation weight losses of 450 and 350 lbs. respectively. No comparable extreme weight losses were observed in the cows of any other lot. This would explain the

TABLE 1

The effect of the level of fluorine fed as NaF on the body weight of dairy cows

LOT	F ADDED		WEIGHT				
			Year 1	Year 2	Year 3	Year 4	Year 5
	p.p.m.		*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
I	0	Av.	1035	1116	1319	1391	1431
		Max.	1145	1272	1475	1587	1611
		Min	944	1007	1040	1297	1283
II	20	Av.	1049	1144	1295	1301	1431
		Max.	1179	1301	1502	1454	1615
		Min.	952	1050	1152	1192	1287
III	30	Av.	1020	1092	1226	1327	1401
		Max.	1160	1261	1377	1509	1624
		Min.	918	998	1138	1235	1273
IV	40	Av.	1014	1137	1293	1373	1417
		Max.	1148	1269	1442	1513	1627
		Min.	919	1037	1192	1285	1221
V	50	Av.	1068	1145	1244	1324	1313
		Max.	1185	1297	1420	1490	1449
		Min.	1019	1031	1140	1138	1201
VI	50 + 20 gm $CaCO_3$	Av.	1045	1099	1221	1289	1337
		Max.	1144	1205	1352	1443	1522
		Min.	981	1020	1110	1207	1237

apparent depression in average yearly weight. Statistical analysis of variance showed no significant difference in percentage of body weight increase over 5 years between animals of the 6 lots.

Effects on teeth. One of the first observable physiologic effects of excess fluorine is the mottling of the growing tooth which is indicative of elevated fluorine ingestion during the

formative stage. Hobbs et al. ('54) used a scheme of classification which gives a numerical score to fluorotic incisors. A rating of IA to 2 is considered to be a normal tooth; a score of 2 indicates a questionable effect while a rating of 3 is considered the marginal zone. A score of 4 constitutes definite effects and a rating of 5 indicates severe effects due to fluorine. The latter two ratings are characterized by varying degrees of hypoplasia of the enamel and tooth.

TABLE 2

The effects of fluorine fed as NaF on various physiologic responses

LOT	F ADDED	AV. FECAL F (dry wt.)	AV. CALF BONE F (dry fat free wt.)	MILK F (whole milk)	AV. SCORE OF INCISORS		AV. NO. OF SERVICES/ COW/YEAR FOR CONCEPTION
					Pair 3	Pair 4	
	p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*			
I	0	14	11	0.16 (3)[1]	1 A	1 B	2.1
II	20	..	86	0.31 (5) 0.29 (6)	1 B	3	1.5
III	30	27	136	0.30 (11) 0.38 (12)	2	4	2.1
IV	40	30	104	.....	4	5 A	2.3
V	50	37	140	0.27 (19) 0.44 (20)	4	5 B	2.2
VI	50 + 200 gm $CaCO_3$ daily	48	130	0.32 (22) 0.51 (24)	4	5 A	1.5

[1] Cow number.

The cattle had two pairs of permanent incisors when they were placed on the experiment but the third and 4th pairs were still in the formative stage. The average ratings by lots, of these incisors, by the previously discussed classification scheme, are presented in table 2. This table represents observations made after 5½ years of fluorine ingestion.

The first and second pairs of permanent incisors erupted before exposure to fluorine and apparently remained normal. This was expected since the fully erupted tooth is thought

to undergo no gross change attributable to subsequent fluorine intake. Slight mottling of enamel and slight wear were evident in the 4th incisors of animals in lot II (20 p.p.m.) while the addition of 30 p.p.m. of fluorine to those of lot III caused mottling of the third pair as well as more severe effects on the 4th pair. Likewise, additional increments of fluorine affected all third and 4th pairs of incisors. When the data for individual cows were examined it was seen that the animals in group VI (50 p.p.m. + $CaCO_3$) behaved more like those in lot IV (40 p.p.m.) than like the lot V cows (50 p.p.m.) indicating an alleviating effect of about 10 p.p.m. due to the addition of 200 gm of $CaCO_3$ to the daily ration.

Urinary excretion and rib fluoride. Table 3 presents the data on the average urinary fluorine concentration of the cows at various times during the course of the experiment. The concentration of fluorine in the urine increased as the dietary fluorine level increased. Cows, fed the basal ration only, excreted less than 5 p.p.m. of fluorine in the urine (corrected to specific gravity of 1.04). The urinary fluorine levels ranged from approximately 15 p.p.m. for the 20 p.p.m. lot to about 38 p.p.m. for those fed 50 p.p.m.

The value of a single urinary fluorine determination was of qualitative significance only. A variation of as much as 100% between individuals of the same lot, and between different samples from the same cow was noted. However, average urinary fluorine concentrations found after repeated sampling were roughly proportional to the dietary fluorine concentration.

Studies of the diurnal variation of urinary fluorine concentration showed that the ingestion of a single large dose of fluorine caused a peak excretion in urinary fluorine concentration to occur in 6 to 12 hours, and a minimum urinary fluorine concentration immediately before the next ingestion of fluorine at 24 hours. Urinary fluorine concentrations as low as one-half of the peak concentration were occasionally noted immediately before the next ingestion period. Because of

TABLE 3

The effect of fluorine fed as NaF upon urinary F excretion

LOT	ADDED F	URINARY F (corrected to specific gravity of 1.04)					
		Jan. 1952	Sept. 1953	Aug. 1954	Jan. 1955	Oct. 1955	March 1956
	p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
I	0	*2.6* 2.0–3.0	*3.3* 2.3–3.7	*4.6* 1.8–8.6	*2.8* 1.5–4.5	*3.5* 2.2–4.3	*4.7* 1.4–7.2
II	20	*13.0* 9.5–14.3	*12.8* 9.9–14.1	*19.6* 14.1–28	*14.5* 10.5–20.4	*15.4* 12.1–20.3	*17.2* 15.2–20.4
III	30	*19.3* 16.2–21	*19.4* 15.1–24.8	*26.6* 17.5–44	*22.9* 16.9–34.4	*24.8* 19.6–30.8	*25.5* 16.1–42.4
IV	40	*30.0* 28–34.1	*33.9* 27.3–35.1	*32.2* 25–40	*23.1* 18.0–31.0	*33.3* 20.2–43.0	*29.2* 21.3–35.1
V	50	*39.0* 38–40	*34.3* 30–39.5	*42.2* 30–60	*36.7* 22.0–74.0	*38.1* 25.4–46.6	*38.4* 29.3–55.5
VI	50 + 200 gm $CaCO_3$ daily	*32.0* 26–40	*36.2* 29.4–42.5	*38.4* 26–52	*33.7* 25.0–47.0	*39.0* 21.6–56.4	*47.9* 31.2–59.0

TABLE 4

The storage of F in the 10th, 11th and 12th ribs of cows fed NaF

(Dry, fat-free weight)

LOT	F ADDED	MONTHS OF EXPOSURE TO FLUORINE						
		9	19	24	30	37	49	63
	p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
I	0	425 (3)[1]	360 (4)	400 (2)	498 (3)	512 (4)	495 (2)	590 (2)
II	20	1700 (6)	1940 (8)	1460 (5)	2240 (7)	2600 (6)	2635 (8)	3280 (6)
III	30	2200 (12)	2140 (11)	3250 (10)	2370 (9)	4280 (12)	3800 (11)	4600 (12)
IV	40	2200 (15)	2950 (16)	3750 (14)	3800 (13)	4440 (15)	3830 (16)	5450 (15)
V	50	1700 (20)	4280 (19)	6850 (17)	2310 (20)	3260 (19)	4160 (20)	4660 (20)
VI	50 + 200 gm $CaCO_3$ daily	2200 (24)	4470 (23)	3355 (21)	5080 (22)	4550 (24)	7800 (23)	6650 (22)

[1] Number within parentheses is the cow number.

this variation the fluorine was administered twice a day beginning with the second year.

In the light of these results, single urine samples cannot be considered clinically demonstrative of toxic levels of fluorine ingestion. Such factors as feeding schedule or grazing habits make a single determination of qualitative significance only.

Table 4 contains the data on the results of fluorine analysis of the rib biopsy samples. The fluorine concentration of the first rib biopsy samples, taken after 9 months' exposure to fluorine, varied with the individual and did not correlate well with the concentration of fluorine ingested. Thereafter, the fluorine concentration of the biopsy samples in general increased as the dietary fluorine level was increased, and it also increased progressively with age, independent of concentration. Any discrepancies can be explained on the basis of the variation that existed between animals in their total fluorine intake per unit of body weight. An example was cow 20 in lot V (50 p.p.m.) whose rib fluorine concentration was less than that of other cows in the lot. Her fluorine intake on a body weight basis was comparable to that of the animals in lot IV (40 p.p.m.) rather than to the other cows in lot V.

Effects on reproduction. Table 2 contains the data on the number of services per cow per year required for conception. The feeding of 50 p.p.m. of additional fluorine did not reduce the over-all reproductive performance of the cows. Observations of the weights of calves dropped by these cows indicated no differences due to added fluorine used in these studies and it would therefore appear that elevated dietary fluorine levels did not adversely effect conception or the *in utero* development of the calf.

The calves of all cows appeared to be normal. It was of interest to determine if there was placental transfer of fluorine. The third and 4th calves were sacrificed at birth and fluorine analysis was made on the metacarpal bone. The results of these analyses are presented in table 2. There was a definite increase in fluorine content of the metacarpal bone

as a result of the addition of fluorine to the ration of the dam, which was positive evidence of placental transfer of fluorine.

The analysis of milk fluorine with conventional equipment and methods was found to be subject to considerable error due to the large quantity of sample needed to obtain sufficient fluorine for an accurate determination. It was impossible to obtain reliable values by these methods.

It was demonstrated by Evans and Phillips ('39) that the fluorine in milk was in part deposited in the skeleton of the rat. Since the fluorine concentration of the skeleton increased, an attempt was made to demonstrate the presence of varying levels of fluorine in milk by a biological assay. To this end rats were maintained for 7 months exclusively on mineralized milk from cows in the experimental herd. At the end of this period the rats were sacrificed and their femurs removed for fluorine analysis. The fluorine concentration in the femurs of the rats fed milk from the control cows was 28 p.p.m. The fluorine concentration in the femurs from the rats fed milk from lot III (30 p.p.m.) was 56 p.p.m. and from lot V (50 p.p.m.) was 111 p.p.m.. It thus appears that the feeding of fluorine to lactating cows did increase the fluorine content of the milk which would confirm the results obtained by the analytical work of Smith et al. ('45) who demonstrated an increased fluorine content in milk from cows fed sodium fluoride.

Recently, a method was developed by Richter [3] which permits accurate determination of very low levels of fluorine. Analyses, by this method, of milk samples taken near the end of the experiment are presented in table 2. They demonstrate increased but minute quantities of fluorine in milk from cows fed added increments of NaF.

Analysis of the fluorine content of the feces during the third lactation indicated an increased fluorine concentration as a result of elevated fluorine levels in the diet (table 2). The feeding of 200 gm/day of $CaCO_3$ to each cow of lot VI receiving

[3] Richter, E., unpublished data.

50 p.p.m. of fluorine as NaF resulted in an increased fecal fluorine concentration. These data support the hypothesis that calcium salts alleviate fluorosis by converting soluble fluoride ions to CaF_2 in the gut.

Other physiologic effects. The concept that dietary fluorine increases the length of the hoof and results in excessive diarrhea was not confirmed in this study. Frequent observations were made of hoof length and incidence of diarrhea. There was no difference in hoof length among the cows of the various lots, and elongation of the hoof occurred with equal frequency in all cows under these experimental conditions. At no time during the course of the experiment was there an outbreak of diarrhea attributable to the added increments of NaF. Particular attention was paid to animals in the high fluorine lots during periods of heavy stress. Under the conditions of this experiment, diarrhea was not a symptom of fluorosis.

A refusal of feeds containing fluorine occurred as one of the symptoms of developing fluorine toxicosis. Under these experimental conditions, such a reaction to fluorine was observed during those periods when the fluorine ingestion by the animals had attained an intake level of 0.78 mg F/lb. of body weight (1.7 mg/kg) or more for a period in excess of a month. In lot V (50 p.p.m. F) cow 19 in her third lactation and cow 17 in her last three lactations showed a loss of appetite for feeds containing fluorine. Cows 13 and 16 in lot IV (40 p.p.m.) exhibited the same reaction to feed intakes when their ingestion levels were sustained at 0.64 mg F/lb. of body weight (1.4 mg/kg). This occurred during their 5th lactation.

The refusal of fluoride-supplemented rations occurred concurrently with the development of lameness and stiffness in the animal in most cases. Lameness also seems to have a "time lag" in its development. Cows 22 and 24 (50 p.p.m. + $CaCO_3$) were observed to be lame and somewhat stiff in their 5th lactation, when their intake had attained a level of 0.73 mg F/lb. of body weight (1.6 mg/kg) but their appetite was unimpaired at this time. Thus it appears that lameness may develop without the occurrence of the effect upon appetite.

These studies support the observation suggested by Peirce ('40) that there is a latent period between time of the appearance of the effects of fluorine upon the teeth and the subsequent development of other physiologic effects. In these studies the latent period extended from three to 5 years and its extent was dependent upon the dietary level fed.

These data were obtained with liberally fed Holstein dairy cows first exposed to NaF feeding at approximately two years of age. They depict the development of the fluorosis syndrome as it is affected by both dietary concentration, or intake and time.

In this study and others with laboratory animals the effect of dietary additions of fluorine as NaF had an interesting but variable effect upon appetite. Appetite for most feeds was apparently unimpaired since animals that became debilitated as the result of fluorine toxicosis readily ate feeds without added fluorine. Neither was there a strict reduction in appetite. There seemed to be a voluntary avoidance of fluorine-contaminated feed except for a meager intake in an effort to maintain life.

SUMMARY

The effects of dietary increments of fluorine fed as the soluble NaF salt to young adequately nurtured dairy cows for a period of 5½ years and through 5 lactations have been studied. The data obtained permit the classification of the evidence into three categories in relation to fluorine ingestion, and subsequent development of fluorine toxicosis.

1. This study indicates that there was no direct relationship between over-grown hooves, dew claws, or diarrhea in stall-fed dairy cows and the ingesting of as much as 50 p.p.m. of fluorine in the ration. Neither was there any interference with conception, gestation and parturition. There was a measurable fluorine transfer across the placental membranes and secretion of minute quantities of fluorine in the milk of NaF-fed cows.

2. The earliest observable measures of increased fluorine ingested by young cows were the physiologic effects upon the teeth and elevated concentrations of fluorine present in urine and rib bone biopsy samples. These data also indicate that the control-cow urine averaged under 5 p.p.m. and rib bone under 600 p.p.m. of fluorine.

3. After the early evidence of increased fluorine ingestion, no other untoward effects were observed except a steady rise in bone fluorine concentration until after a considerable period of time had elapsed. Depending upon intake level, and the duration or time interval, the latent period was followed by positive evidence of developing fluorosis. These were, in close sequence: a refusal of fluorine-supplemented feeds, accompanied by excessive loss in body weight and stiffness in the legs with lameness as the result thereof. These effects were severe enough to debilitate the animal within several weeks at sustained high intake levels.

The symptoms just enumerated were observed in two out of three animals fed 50 p.p.m. of fluorine during the third year on experiment. They were also observed in two out of three animals fed 50 p.p.m. fluorine with extra calcium and beginning in two out of 4 animals fed 40 p.p.m. during the 5th year. The inclusion of added calcium in the ration increased the tolerance of these cows to fluorine. Other criteria observed were fluorine concentrations in excess of 6,000 p.p.m. in rib biopsy and in urine levels above 30 p.p.m. Concurrently, teeth classifications were 5A or higher. From these results it would appear that lactating dairy cows tolerate 30 p.p.m. of fluorine fed as NaF with comparative safety, that 40 p.p.m. is near marginal tolerance level and 50 p.p.m. causes the development of fluorosis within three to 5½ years.

LITERATURE CITED

AGATE, J. N., G. H. BELL, G. F. BODDIE, R. G. BOWLER, M. BUCKNELL, E. A. CHEESEMAN, T. H. J. DOUGLAS, H. A. DRUETT, J. GARRAD, D. HUNTER, K. M. A. PERRY, J. D. RICHARDSON AND J. B. WEIR 1949 Industrial fluorosis. A study of the hazard to man and animals near Fort William, Scotland. Med Res. Council, Memorandum No. 22.

ALUMINUM COMPANY OF AMERICA 1947 Aluminum Research Laboratories Tech. Paper 914.

BLAKEMORE, F., T. J. BOSWORTH AND H. H. GREEN 1948 Industrial fluorosis of farm animals in England, attributable to the manufacture of bricks, the calcining of ironstone, and two enamelling processes. J. Comp. Path. Therap., *58:* 267.

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

HARRIS, L. E., G. T. BAIRD, G. Q. BATEMAN, W. BINNS, C. I. DRAPER, D. A. GREENWOOD, G. R. HENDERSON, W. R. JENKINS, L. H. RICH, D. W. THORN AND F. B. WANN 1952 Recommended practices to reduce fluorosis in livestock and poultry. Utah State Agr. Expt. Sta. Circular 130.

HOBBS, C. S., R. P. MOORMAN, J. M. GRIFFITH, J. L. WEST, J. M. MERRIMAN, S. L. HANSARD AND C. C. CHAMBERLAIN 1954 Fluorosis in cattle and sheep. Tenn. Agr. Expt. Sta. Bull. 235.

PEIRCE, A. W. 1940 Chronic fluorine intoxication in domestic animals. Nutr. Abst. Rev., *9:* 253.

PHILLIPS, P. H. 1952 The development of chronic fluorine toxicosis and its effect on cattle. Proc. 2nd Natl. Air Pollution Symposium. Pasadena, Calif.

PHILLIPS, P. H., D. A. GREENWOOD, C. S. HOBBS AND C. F. HUFFMAN 1955 The fluorosis problem in livestock production. National Research Council Publication 381.

REMMERT, L. F., T. D. PARKS, A. M. LAWRENCE AND E. H. MCBURNEY 1953 Determination of fluorine in plant materials. Anal. Chem., *25:* 450.

SMITH, H. V., M. C. SMITH AND M. VAVICH 1945 Fluorine in milk, plant foods, and foods cooked in fluorine containing water. Arizona Agr. Expt. Sta. Mimeographed report: 77.

SPENCER, G. R. 1953 Summary report of fluorosis investigations in the Sauvie Island area in Oregon from Dec. 1, 1951 to June 30, 1953. The State College of Washington and Oregon State College, December 1953.

WILLARD, H. H., AND O. B. WINTER 1933 Volumetric methods for determination of fluorine. Ind. Eng. Chem. Anal. Ed., *5:* 7.

METABOLISM OF PTEROYLGLUTAMIC ACID AND LIVER LEVELS OF NUCLEIC ACIDS

K. GUGGENHEIM AND S. HALEVY

Laboratory of Nutrition, Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(Received for publication April 12, 1957)

Several lines of investigation suggest that before pteroylglutamic acid (PGA) [1] can carry out its metabolic functions the organism must first convert it into the tetrahydro form and that citrovorum factor (CF), one of the metabolically active forms of folic acid (FA), is involved in some manner in the metabolism of a 1-carbon moiety. PGA may, therefore, be regarded as a provitamin which is transformed by the organism into a vitamin. In a previous paper (Guggenheim et al., '56) it was reported that rats maintained on a protein-free diet are less able to perform this conversion than are well-fed controls. In the present paper this phenomenon was further studied by the inclusion of various levels of dietary protein as well as proteins of different biological value.

Recent evidence indicates that PGA or its active form CF is involved in the metabolism of nucleic acids (Stokstad, '54). Since the liver is the main site of the conversion of PGA into CF (Nichol, '53), the effect on the level of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) exerted by diets shown to depress this conversion was investigated. Various procedures were followed in order to reduce liver CF, namely,

[1] As used in our previous publication (Guggenheim et al., '56) the term "pteroylglutamic acid" has been restricted to the synthetic compound and "citrovorum factor" to the substance(s) promoting growth of *Pediococcus cerevisiae* 8081 *(Leuconostoc citrovorum)*; the term "folic acid" has been considered a generic term applicable to substances which stimulate the growth of *Streptococcus faecalis R.*

diets deficient in protein as well as incorporation into the diet of either succinylsulfathiazol or 4-aminopterin. The first-mentioned substance is known to depress intestinal synthesis of FA (Mickelsen, '56), whereas 4-aminopterin interferes with both the conversion of PGA and CF and the subsequent utilization of CF (Nichol and Welch, '50; Nichol, '53; Reid and Couch, '55). Liver concentration of RNA and DNA has already been studied in rats suffering from deficiency of vitamin B_{12}, another vitamin which plays an important role

TABLE 1

Composition of diets[1]

	C 18	Z 18	Gl 18	C 12	C 6	PROTEIN-FREE
	%	%	%	%	%	%
Casein[2]	18	..	..	12	6	..
Zein[2]	..	18	..	..	..	..
Gluten[2]	..	..	18	..	..	..
Cornstarch	73	73	73	79	85	91
Vegetable oil	5	5	5	5	5	5
Salt mixture[3]	4	4	4	4	4	4

[1] The diets were supplemented with the following vitamins in milligrams per 100 gm ration: thiamine 0.2, riboflavin 0.3, pyridoxine 0.1, calcium pantothenate 1.6, niacin 5.0, and choline chloride, 100. Each rat received 100 I.U. vitamin A and 4 I.U. vitamin D twice weekly.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] No. 2, U.S.P. XIII.

in the formation of nucleic acids (Arnstein, '55). In those studies, both RNA and DNA were found to be significantly lower in livers of deficient animals than in controls (Rose and Schweigert, '52; Schweigert, Scheid and Downing, '54; Wong and Schweigert, '56).

METHODS

Young male rats, weighing about 60 gm, were fed one of the diets indicated in table 1 for three weeks, if not stated otherwise. At the end of this period they were sacrificed by decapitation and their livers examined for FA, CF, RNA, and DNA. For examination of FA and CF the livers were

homogenized and autolysed as described by Dietrich, Monson, Gwoh and Elvehjem ('52). FA was determined with *S. faecalis* (Association of Vitamin Chemists, '51) and CF with *Pediococcus cerevisiae* 8081 *(Ln. citrovorum)* according to Sauberlich and Baumann ('48). Nucleic acids were extracted according to the method of Schneider ('45). DNA was determined with the diphenylamine reaction as described by Seibert ('40). The color development was modified, however, by incubating the reagents at 37° for 20 hrs. RNA was determined with the orcinol reagent according to Albaum and Umbreit ('47), *d*-ribose being used as standard.

RESULTS

Protein nutrition and metabolism of PGA. Groups of young rats were kept on the various diets indicated in table 2. After three weeks about one-half of each group received an intraperitoneal injection of 1 mg PGA per 100 gm body weight. On the following day all the animals were killed and their livers examined for FA and CF. The results are shown in table 2.

Liver levels of both FA and CF decreased with diminishing casein content of the diet. Moreover, administration of PGA induced a smaller rise of both FA and CF in protein-deficient animals than in controls fed a full diet. It is noteworthy that the level of CF is more affected by protein deficiency than that of FA, in both PGA-treated and untreated animals. It may, therefore, be concluded that lack of dietary protein impairs both the ability of the liver to store FA as well as to convert it to CF.

Duration of protein deprivation appears equally to be of importance, since rats of group 6 maintained on a protein-free diet during three weeks exhibited a significantly lower concentration of both FA and CF in their livers than did those in group 6a, kept on the same diet for one week only.

The effect of protein deficiency on metabolism of PGA seems to be related specifically to lack of dietary protein and not to the accompanying caloric restriction. This con-

TABLE 2

Effect of dietary protein levels upon the liver content of folic acid and citrovorum factor

GROUP	DIET	NO. OF RATS	SUPPLEMENT PER KILO RATION	CHANGE IN WEIGHT DURING 3 WEEKS	PGA [1]	LIVER				CF AS PER CENT OF FA
						Folic acid		Citrovorum factor		
						Per 100 gm body weight	Increase per 100 gm body weight	Per 100 gm body weight	Increase per 100 gm body weight	
				gm		*μg*	*μg*	*μg*	*μg*	
1	C 18	18	..	+ 82 ± 5.6 [2]	—	5.9 ± 0.60		4.6 ± 0.43		78 ± 3.9
	C 18	14	..		+	15.8 ± 0.95	9.9 ± 1.12	13.2 ± 1.33	8.6 ± 1.40	
2	C 18 [3]	10	..	+ 35 ± 2.9	—	7.5 ± 0.76		5.6 ± 0.63		75 ± 7.5
	C 18	10	..		+	15.3 ± 1.50	7.8 ± 1.68	11.3 ± 1.70	5.7 ± 1.81	
3	C 12	10	..	+ 64 ± 3.5	—	6.8 ± 0.49		6.3 ± 0.58		85 ± 4.1
	C 12	8	..		+	12.0 ± 0.85	5.2 ± 0.98	10.0 ± 0.73	3.8 ± 0.93	
4	C 6	10	..	+ 23 ± 1.2	—	2.8 ± 0.46		1.3 ± 0.36		55 ± 8.6
	C 6	12	..		+	6.7 ± 0.78	3.9 ± 0.91	4.1 ± 0.88	2.8 ± 0.95	
5	C 6	10	60 μg vitamin B_{12}	+ 20 ± 1.8	—	3.7 ± 0.59		2.6 ± 0.53		69 ± 6.6
	C 6	10			+	7.1 ± 1.06	3.4 ± 1.21	4.4 ± 0.81	1.8 ± 0.97	
6	Protein-free	10	..	— 18 ± 0.8	—	1.1 ± 0.31		0.5 ± 0.11		51 ± 7.6
	Protein-free	10	..		+	1.9 ± 0.53	0.8 ± 0.62	1.1 ± 0.43	0.6 ± 0.44	
6a	Protein-free [4]	11	..	— 9 ± 0.3	—	4.2 ± 0.46		1.1 ± 0.26		26 ± 6.1
7	Gl 18	9	..	+ 27 ± 1.5	—	8.8 ± 0.92		3.4 ± 1.74		37 ± 5.8
	Gl 18	10	..		+	15.4 ± 1.69	6.6 ± 1.92	6.1 ± 1.50	2.7 ± 2.30	
8	Gl 18	12	9.0 gm lysine	+ 58 ± 3.3	—	10.4 ± 1.14		5.9 ± 0.98		57 ± 2.5
	Gl 18	12			+	16.8 ± 0.91	6.4 ± 1.46	10.6 ± 1.09	4.7 ± 1.47	
9	Z 18	10	..	— 14 ± 1.6	—	5.1 ± 0.39		2.3 ± 0.33		47 ± 6.4
	Z 18	11	..		+	8.1 ± 1.24	3.0 ± 1.30	3.9 ± 0.76	1.6 ± 0.83	
10	Z 18	12	9.0 gm lysine, 9.0 gm isoleucine, 1.8 gm tryptophan	+ 12 ± 1.1	—	2.1 ± 0.32		1.7 ± 0.32		76 ± 3.9
	Z 18	12			+	4.6 ± 0.57	2.5 ± 0.66	3.8 ± 0.51	1.1 ± 0.60	

[1] Plus sign indicates one intraperitoneal injection of 1 mg pteroylglutamic acid (PGA) per 100 gm body weight 24 hours before examination of liver; minus sign indicates no injection.

[2] Means and standard errors.

[3] Food intake restricted to 60% of normal.

[4] Animals were kept for one week only on protein-free diet.

clusion was reached from an experiment with rats offered a qualitatively full diet (C 18) but in amount restricted to 60% of normal (group 2). This was the approximate quantity of food consumed voluntarily by rats kept on a 6% casein diet (group 4). As can be seen, no impairment of either storage capacity of the liver for FA or of its ability to convert FA into CF was found under these conditions.

In view of some reports (Pfander et al., '52; Doctor et al., '53, '54) showing that vitamin B_{12} may, under certain conditions, improve the conversion of FA into CF, one group of rats was fed a 6% casein diet supplemented with 60 µg of vitamin B_{12} per kilogram ration (group 5). Examination of livers of both PGA-treated and untreated animals revealed no significant difference. Vitamin B_{12} seems, therefore, to be without effect in restoring to normal the depressed formation of CF from FA in protein deficiency.

Two experiments with proteins of low biological value, gluten and zein, were performed. Livers of rats fed a gluten diet (Gl 18, group 7) exhibited a significant impairment of conversion of FA into CF, which could be partially restored by supplementation of this diet with 9.0 gm DL-lysine monohydrochloride per kilogram (group 8). Rats fed a diet containing 18% zein (Z 18, group 9) had a significantly decreased FA concentration and a diminished formation of CF. Enrichment with 9.0 gm DL-lysine monohydrochloride, 9.0 gm DL-isoleucine, and 1.8 gm DL-tryptophan per kilogram diet significantly improved its nutritive value as indicated by the weight change during the observation period (group 10). The FA content of the liver remained, however, low in these rats, whereas the capacity of the liver to convert FA into CF showed a significant improvement.

Protein nutrition and levels of liver nucleic acids. Since various states of protein deficiency were found to affect both liver FA concentration and the capacity of this organ to convert it to CF, and in view of the fact that formation of nucleic acids is known to depend on the CF-mediated transfer of single carbon units, the amounts of nucleic acids in the

TABLE 3

Effect of dietary protein levels upon the liver content of ribonucleic and desoxyribonucleic acids (RNA and DNA)
Rats weighing about 60 gm were kept on specified diets for three weeks

GROUP	NO. OF RATS	DIET	WEIGHT		LIVER				RNA / DNA
				Weight	RNA		DNA		
					Per gram liver	Per 100 gm body weight	Per gram liver	Per 100 gm body weight	
			gm	*gm*	*mg*	*mg*	*mg*	*mg*	
1	20	C 18	135 ± 2.7 [1]	5.6 ± 0.22	7.91 ± 0.16	32.8 ± 0.67	3.43 ± 0.10	14.2 ± 0.42	2.31 ± 0.053
2	12	C 18 [2]	92 ± 2.8	3.7 ± 0.21	8.08 ± 0.10	32.5 ± 0.63	3.83 ± 0.09	15.4 ± 0.41	2.11 ± 0.028
3	12	C 12	124 ± 2.9	5.4 ± 0.20	7.65 ± 0.14	31.5 ± 0.72	3.88 ± 0.12	15.2 ± 0.45	2.11 ± 0.052
4	12	C 6	79 ± 2.5	3.4 ± 0.17	7.09 ± 0.10	30.5 ± 0.94	4.24 ± 0.13	18.2 ± 0.65	1.67 ± 0.046
5	12	C 6 + vit. B_{12} [3]	80 ± 2.2	3.3 ± 0.07	7.17 ± 0.14	29.6 ± 1.13	4.04 ± 0.11	16.7 ± 0.63	1.77 ± 0.069
6	10	Prot.-free	41 ± 1.2	1.8 ± 0.09	8.04 ± 0.44	34.5 ± 1.16	5.21 ± 0.14	22.3 ± 0.86	1.57 ± 0.093
7	11	Prot.-free [4]	49 ± 0.9	2.1 ± 0.11	7.17 ± 0.13	30.5 ± 0.57	4.80 ± 0.19	19.9 ± 0.78	1.51 ± 0.048
8	15	Gl 18	87 ± 2.6	3.8 ± 0.14	7.81 ± 0.72	34.0 ± 0.72	4.05 ± 0.10	17.6 ± 0.50	1.94 ± 0.050
9	14	Gl 18 suppl. [5]	118 ± 3.3	5.8 ± 0.34	7.55 ± 0.12	35.1 ± 0.76	3.55 ± 0.11	16.5 ± 0.53	2.14 ± 0.052
10	16	Z 18	46 ± 1.0	2.0 ± 0.13	7.58 ± 0.17	34.4 ± 0.65	5.45 ± 0.12	24.4 ± 0.60	1.42 ± 0.023
11	18	Z 18 + suppl. [5]	72 ± 2.2	3.2 ± 0.14	7.32 ± 0.16	32.7 ± 0.71	3.80 ± 0.11	16.9 ± 0.48	1.93 ± 0.019

[1] Means and standard errors.
[2] Food intake restricted to 60% of normal.
[3] Sixty micrograms per kilogram of ration.
[4] Animals were kept for one week only on protein-free diet.
[5] See table 2.

liver were studied under these conditions. Table 3 shows that restriction of dietary protein results in a decrease of RNA per gram liver as well as per unit of body weight. At the same time a rise of DNA content was observed. In severe protein deficiency, however, RNA rose again, whereas DNA was much increased (group 6). As a consequence the ratio RNA : DNA was significantly reduced in all states of protein deficiency. Supplementation of a 6% casein diet with 60 µg vitamin B_{12} per kilogram proved to be without influence upon RNA and DNA levels. This vitamin, which raises liver nucleic acids in vitamin B_{12}-deficient rats (Wong and Schweigert, '56), seems to be ineffective in restoring to normal the depressed level of RNA in protein-deficient animals.

Nucleic acid composition of the liver depends not only on the amount of protein in the diet but also on the duration of the period of feeding. This was borne out from an experiment with rats, which received the protein-free diet for one week only (group 7). Livers of these animals contained considerably less RNA and DNA than those of rats deprived of protein for three weeks. The RNA level in these rats was similar to that of rats fed a 6% casein diet for three weeks.

The changes in liver nucleic acids, which were observed in protein deficiency, are not the result of the diminished food intake accompanying the dietary lack of protein, as evinced from figures obtained with animals which received a qualitatively full but calorically restricted diet (group 2). Liver levels of both RNA and DNA were similar to those found in rats receiving the same diet ad libitum.

Feeding of diets containing qualitatively inferior proteins (gluten, zein; groups 8 and 10) had no marked influence on liver RNA, but significantly increased the DNA level, thus reducing the RNA : DNA ratio. Supplementation of these diets with the lacking amino acids diminished the concentration of DNA (groups 9 and 11).

It appears, therefore, that the level of nucleic acids in the liver is not necessarily dependent on PGA metabolism. Subsequent parts in our study will corroborate this conclusion.

TABLE 4

The effect of protein repletion following a period of depletion on the levels of folic acid, citrovorum factor and nucleic acids in the liver

	AT END OF ONE WEEK DEPLETION PERIOD	AFTER TWO DAYS REPLETION WITH A FULL DIET FOLLOWING A ONE WEEK DEPLETION PERIOD			
		Unsupplemented		Supplemented with 5 mg aminopterin per kilo	
No. of rats	11	11		11	
Weight change during depletion period, gm	— 9 ± 0.42 [1]	— 8 ± 0.64 [1]		— 11 ± 1.39 [1]	
Weight change during repletion period, gm		+ 6 ± 0.85		— 2 ± 0.77	0.001 [2]
Liver Weight, gm	2.1 ± 0.05	2.7 ± 0.12	0.001 [2]	2.5 ± 0.13	
Folic acid, μg per gm liver	0.99 ± 0.12	1.44 ± 0.16	0.05	1.22 ± 0.13	
per 100 gm body weight	4.23 ± 0.46	6.88 ± 0.68	0.001	6.65 ± 1.09	
Citrovorum factor, μg per gm liver	0.25 ± 0.05	0.44 ± 0.08	0.05	0.22 ± 0.06	0.05
per 100 gm body weight	1.08 ± 0.22	2.10 ± 0.42	0.05	1.18 ± 0.19	0.05
CF as per cent of FA	26 ± 6.1	27 ± 5.2		19 ± 3.2	
RNA, mg per gm liver	7.17 ± 0.13	8.37 ± 0.13	0.001	8.19 ± 0.10	
per 100 gm body weight	30.5 ± 0.57	39.9 ± 1.16	0.001	40.0 ± 1.18	
DNA, mg per gm liver	4.80 ± 0.19	3.96 ± 0.10	0.001	4.07 ± 0.10	
per 100 gm body weight	19.9 ± 0.78	19.0 ± 0.67		19.9 ± 0.60	
RNA:DNA	1.51 ± 0.048	2.10 ± 0.059	0.001	2.00 ± 0.060	

[1] Means and standard errors.

[2] Probability that the values of this column differ from those at its left.

The effect of protein repletion following a period of depletion. Three groups of rats were placed on a protein-free diet for one week. At the end of the depletion period the rats of the first group were killed and their livers examined for FA, CF, RNA and DNA. The remaining two groups were offered a full diet (C 18); the diet of group 3 was further supplemented with 5 mg of 4-aminopterin per kilogram. After two days on these repletion diets all rats were killed and their livers examined. From the results presented in table 4 it can be seen that two days repletion on a full diet induced an average weight increase of 6 gm. Furthermore, both FA and CF content of the liver increased significantly during these two days, the ratio CF : FA remaining, however, low. There was also a significant increase in RNA per gram liver as well as per unit of body weight, and a marked decrease of RNA, resulting probably from an increase of cytoplasm.

Incorporation of aminopterin into the repletion diet was associated with a definite weight loss suggestive of toxicity. Aminopterin did not prevent a significant increase of FA, whereas the level of CF remained rather low. No effect of aminopterin was observed on nucleic acids.

It follows, therefore, that aminopterin in spite of its effect on liver CF did not prevent the increase of RNA accompanying protein repletion, and it had no influence on DNA content.

Experiments with metabolic antagonists. In this part of our study, results obtained in experiments with metabolic antagonists on normal, well-fed animals will be presented. In the first experiment one group of rats was fed a full diet (C 18), in which 2.5 gm succinylsulfathiazol had been incorporated per kilogram of diet. The second group received the same diet, but further supplemented with 20 mg PGA per kilogram. After three weeks the rats were killed and their livers examined (table 5). As expected, the sulfa-treated rats had a very low liver FA concentration, it being similar to that in severely protein-depleted rats (table 2, group 6), whereas administration of PGA resulted in a very high con-

TABLE 5

The effect of metabolic antagonists on the levels of folic acid, citrovorum factor and nucleic acids in the liver

	SUPPLEMENTS PER KILO OF RATION				
	Succinyl-sulfathiazol, 2.5 gm	Succinylsulfathiazol, 2.5 gm, PGA, 20 mg		Aminopterin, 0.5 mg	Aminopterin, 0.5 mg, Leucovorin, 5 mg	
No. of rats	12	12		12	12	
Time on diet, weeks	3	3		2	2	
Weight increase, gm	53 ± 3.7 [1]	63 ± 3.2 [1]	0.05 [2]	27 ± 2.0 [1]	34 ± 2.3 [1]	0.02 [2]
Liver Weight, gm	4.8 ± 0.27	5.1 ± 0.33		4.4 ± 0.30	3.5 ± 0.17	0.02
Folic acid, μg per gm liver	0.36 ± 0.04	4.20 ± 0.51	0.001	1.38 ± 0.21	3.15 ± 0.26	0.001
per 100 gm body weight	1.49 ± 0.11	17.26 ± 1.89	0.001	7.08 ± 0.73	11.86 ± 0.76	0.001
Citrovorum factor, μg per gm liver				0.48 ± 0.12	1.90 ± 0.35	0.001
per 100 gm body weight				2.48 ± 0.40	7.15 ± 1.36	0.001
CF as per cent of FA				36 ± 4.3	48 ± 5.5	
RNA, mg per gm liver	7.93 ± 0.15	7.92 ± 0.11		8.34 ± 0.15	7.87 ± 0.18	0.01
per 100 gm body weight	32.7 ± 0.81	32.5 ± 0.80		43.3 ± 1.34	29.3 ± 0.81	0.001
DNA, mg per gm liver	3.51 ± 0.11	3.60 ± 0.08		3.69 ± 0.19	4.66 ± 0.17	0.001
per 100 gm body weight	15.0 ± 0.35	14.9 ± 0.47		19.0 ± 0.91	17.2 ± 0.60	
RNA:DNA	2.16 ± 0.031	2.18 ± 0.038		2.37 ± 0.140	1.68 ± 0.082	0.001

[1] Means and standard errors.

[2] Probability that the values of this column differ from those at its left.

centration of FA. But in spite of this large difference similar values for nucleic acids were found in both groups.

In a second series the experimental rats received a full diet (C 18) supplemented with 0.5 mg 4-aminopterin per kilogram, while the diet of the control animals contained both aminopterin and 5 mg leucovorin [2] per kilogram. After two weeks the animals were killed and their livers examined. It can be seen from table 5 that leucovorin partially abolished the depression of weight increase, which resulted from aminopterin treatment. It reduced also the enlargement of the liver, which was observed in this condition. Comparing aminopterin-treated rats with normal controls (table 2, group 1) no marked effect of the antagonist was noted on FA content of the liver. The CF level, however, was found to be reduced, thus leading to a lowered CF : FA ratio. Leucovorin significantly increased both FA and CF. Aminopterin-treated rats had more RNA and DNA per gram liver and a significantly heightened concentration of nucleic acids per unit of body weight. Leucovorin reduced RNA values and significantly increased DNA concentration per gram liver, thus leading to a decreased RNA : DNA ratio.

It follows, therefore, that aminopterin in spite of its depressing effect on liver CF does not lower the levels of nucleic acids; leucovorin, which reduces the enlarged liver and increases both FA and CF in livers of aminopterin-treated animals, does not increase the amounts of nucleic acids per unit of body weight.

DISCUSSION

In the first part of our study we were able to show that both quantitative and qualitative protein deficiency impair the ability of the liver to retain FA and to convert it to CF. This "biochemical lesion" of PGA metabolism may provide a partial explanation of the occurrence of macrocytic anemia seen in pregnant women and children suffering from deficiency of protein (Woodruff, '55).

[2] Citrovorum factor, Lederle Laboratories Division.

Lack of dietary protein affects the ability of the liver to store certain vitamins, e.g. riboflavin (Sarett and Perlzweig, '43; Czaczkes and Guggenheim, '45; Wright and Skeggs, '46) and pantothenic, nicotinic and folic acids (Wright and Skeggs, '46). Our study establishes an additional defect in PGA metabolism due to protein deficiency, i.e. an impairment in CF formation.

In the second part of our study it could be shown that hepatic levels of both RNA and DNA are to a certain degree independent of disturbances in PGA metabolism. This conclusion was arrived at on the basis of the results of three experiments: (1) different degrees of protein deficiency, leading to different contents of hepatic FA and CF, were not accompanied by corresponding changes in levels of RNA and DNA; (2) administration of aminopterin during protein repletion, although inhibiting an increase of liver CF, did neither inhibit a simultaneous increase of RNA nor produce a drop of DNA; (3) treatment of normal rats with metabolic antagonists affecting the level of either FA or CF in the liver did not depress the concentration of liver nucleic acids.

Moderate protein deficiency led to a progressive fall of liver RNA per gram of liver and a concomitant rise in DNA. This effect, which has already been observed by Campbell and Kosterlitz ('52) and Thomson, Heagy, Hutchison and Davidson ('53), is probably due to a decrease in cell volume and a loss of cytoplasm, the amount of DNA per cell remaining, however, remarkably constant. The phenomenon of RNA rising again in prolonged protein deficiency has been described by Mandel, Jacob and Mandel ('50) and by Thomson et al. ('53). The latter authors point out that the decrease in the liver RNA content in states of protein deficiency does not seem to be progressive like the changes of other constituents. From our data in table 3 (groups 6 and 7) it would follow that rats maintained on the protein-free diet for one and three weeks, respectively, have, in spite of their different body weights (49 and 41 gm) similar total amounts of RNA (15.0 and 14.4 mg) and DNA (10.1 and 9.4 mg) in their livers.

Protein repletion is, on the other hand, accompanied by a significant increase of RNA and a decrease of DNA per gram liver. These changes in hepatic nucleic acid concentrations are not affected by aminopterin. Administration of succinylsulfathiazol to normal rats likewise had no influence on the concentration of either RNA or DNA. Similarly, aminopterin treatment of normal rats did not diminish the content of nucleic acids in the liver.

From onr study it would follow that both RNA and DNA concentrations of rat liver are rather independent of PGA metabolism. This is at variance with the findings of Lowe and Barnum ('52), who reported a decrease of liver RNA in FA-deficient monkeys. In our experiments the addition of vitamin B_{12} to a low-protein diet did not raise the level of RNA or DNA in the liver. This is in contrast to the reports of Rose and Schweigert ('52) and Wong and Schweigert ('56), who found that a deficiency of this vitamin produced a considerable reduction in both RNA and DNA content of rat liver, the average RNA and DNA contents per cell remaining unchanged.

A certain independence of liver nucleic acids on PGA metabolism has already been reported. Whereas A-methopterin inhibits incorporation of formate-C^{14} into proteins of leucemic cells, no such effect of the antagonist was observed with normal mouse liver (Williams, Slater and Winzler, '55). Furthermore, Martin ('53) even found an increased uptake of formate in liver RNA in aminopterin-treated rats. It would appear, therefore, that formate incorporation proceeds by two different pathways, one sensitive and one resistant to PGA-antagonists. Our results support the view of the existence in rat liver of an alternative mechanism for the synthesis of nucleic acids independent of PGA.

SUMMARY

1. Young rats were offered diets differing in quality and quantity of protein. Feeding of low-protein or protein-free diets or of diets containing nutritionally inferior proteins

led to a decreased liver content of folic acid (FA) and citrovorum factor (CF), the level of CF being more depressed than that of FA. Intraperitoneal injection of pteroylglutamic acid 24 hours before examination caused a smaller rise of both FA and CF in deficient rats than in well-fed controls. Supplementation of a low-protein diet with vitamin B_{12} was without effect upon FA and CF. It is concluded that protein deficiency impairs the ability of the liver to store FA and to convert it into its biologically active form, CF.

2. Liver concentrations of RNA per unit body weight decreased, whereas DNA increased as a result of decreasing the dietary protein. Supplementation with vitamin B_{12} had no influence upon RNA and DNA levels. Diets containing proteins of low biological value increased liver DNA; supplementation of these diets with the lacking amino acids diminished DNA concentration. Comparison of livers from rats deprived of protein for one and three weeks, respectively, revealed that the latter contained less FA and CF but more nucleic acids per unit body weight.

3. Refeeding a full diet following a period of protein depletion increased liver FA, CF and RNA, the level of DNA remaining unchanged. Administration of aminopterin during the repletion period increased FA and RNA, but not CF and DNA.

4. Incorporation of succinylsulfathiazol into the diet of normal rats significantly depressed the liver concentration of FA. The addition of aminopterin induced a significant decrease of CF. Sulfa treatment had no effect on liver nucleic acids, whereas aminopterin raised the level of RNA per unit of body weight.

5. These results are consistent with the view of the existence in the rat liver of an alternative pathway of nucleic acid synthesis, independent of either FA or CF.

ACKNOWLEDGMENTS

The authors wish to acknowledge their indebtedness to Mrs. V. Usieli, Mr. Y. Fattal and Miss A. Eisen for their technical help.

LITERATURE CITED

ALBAUM, H. G., AND W. W. UMBREIT 1947 Differentiation between ribose-3-phosphate and ribose-5-phosphate by means of the orcinol pentose reaction. J. Biol. Chem., *167:* 369.

ARNSTEIN, H. R. V. 1955 The function of vitamin B_{12} in animal metabolism. Biochem. Soc. Symposia No. 13, University Press, Cambridge, 92.

ASSOCIATION OF VITAMIN CHEMISTS 1951 Methods of Vitamin Assay. New York, N. Y.

CAMPBELL, R., AND H. W. KOSTERLITZ 1952 The effects of dietary protein, fat and choline on the composition of the liver cell and the turnover of phospholipin and protein-bound phosphorus. Biochim. Biophys. Acta, *8:* 664.

CZACZKES, W., AND K. GUGGENHEIM 1946 The influence of diet on the riboflavin metabolism of the rat. J. Biol. Chem., *162:* 267.

DIETRICH, L. S., W. J. MONSON, H. GWOH AND C. A. ELVEHJEM 1952 Determination of folic acid and citrovorum factor in animal tissue. Ibid., *194:* 549.

DOCTOR, V. M., B. E. WELCH, R. W. PERRETT, C. L. BROWN, S. GABAY AND J. R. COUCH 1953 Metabolic interrelationship between folic acid, vitamin B_{12} and the citrovorum factor. Proc. Soc. Exp. Biol. Med., *84:* 29.

DOCTOR, V. M., J. F. ELAM, P. SPARKS, C. M. LYMAN AND J. R. COUCH 1954 Studies on the conversion of folic acid to citrovorum factor by avian liver homogenates. Arch. Biochem. Biophys., *48:* 249.

GUGGENHEIM, K., S. HALEVY, H. NEUMANN AND V. USIELI 1956 The metabolism of pteroylglutamic acid by the rat. Biochem. J., *62:* 281.

LOWE, C. U., AND C. P. BARNUM 1952 The synthesis of pentose nucleic acids in the liver of pteroylglutamic acid deficient megaloblastic monkeys. Arch. Biochem. Biophys., *38:* 335.

MANDEL, P., M. JACOB AND L. MANDEL 1950 Étude sur le métabolisme des acides nucléiques. I. Action du jeûne protéique prolongé sur les deux acides nucléiques du foie, du rein et du cerveau. Bull. Soc. Chim. Biol., *32:* 80.

MARTIN, L., quoted by TOTTER, J. R. 1955 Antimetabolite studies on bone marrow *in vitro,* in Rhoads, C. P., Antimetabolites and cancer, 153. Amer. Assoc. Advancement Sci., Washington, D. C.

MICKELSEN, O. 1956 Intestinal synthesis of vitamins in the nonruminant. Vitamins and Hormones, *14:* 1.

NICHOL, C. A. 1953 On the metabolic alteration of pteroylglutamic acid. Proc. Soc. Exp. Biol. Med., *83:* 167.

NICHOL, C. A., AND A. D. WELCH 1950 On the mechanism of action of aminopterin. Ibid., *74:* 403.

PFANDER, W. H., L. S. DIETRICH, W. J. MONSON, A. E. HARPER AND C. A. ELVEHJEM 1952 Citrovorum factor, vitamin B_{12}, and folic acid activity of whole blood of several species. Ibid., *79:* 219.

REID, B. L., AND J. R. COUCH 1955 Factors affecting *in vitro* conversion of folic acid to citrovorum factor. Arch. Biochem. Biophys., *56:* 388.

ROSE, J. A., AND B. S. SCHWEIGERT 1952 Effect of vitamin B_{12} on nucleic acid metabolism of the rat. Proc. Soc. Exp. Biol. Med., *79:* 541.

SARETT, H. P., AND W. PERLZWEIG 1943 The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats. J. Nutrition, *25:* 173.

SAUBERLICH, H. E., AND C. A. BAUMANN 1948 A factor required for the growth of *Leuconostoc citrovorum*. J. Biol. Chem., *176:* 165.

SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. 1. Extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Ibid., *161:* 293.

SCHWEIGERT, B. S., H. E. SCHEID AND M. DOWNING 1954 Liver changes in vitamin B_{12} deficient rats before and after partial hepatectomy. Am. J. Physiol., *178:* 338.

SEIBERT, F. B. 1940 Removal of impurities, nucleic acid and polysaccharide, from tuberculin protein. J. Biol. Chem., *133:* 539.

STOKSTAD, E. L. R. 1954 Pteroylglutamic acid. Biochemical systems, in Sebrell, W. H., and R. S. Harris, The Vitamins, *3:* 124. Acad. Press, Inc., New York.

THOMSON, R. Y., F. C. HEAGY, W. C. HUTCHISON AND J. N. DAVIDSON 1953 The deoxyribonucleic acid content of the rat cell nucleus and its use in expressing the results of tissue analysis, with particular reference to the composition of liver tissue. Biochem. J., *53:* 460.

WILLIAMS, A. D., G. G. SLATER AND R. J. WINZLER 1955 The effect of A-methopterin and formate-C^{14} incorporation by mouse leukemia *in vitro*. Cancer Res., *15:* 532.

WONG, W. T., AND B. S. SCHWEIGERT 1956 Role of vitamin B_{12} in nucleic acid metabolism. I. Hemoglobin and liver nucleic acid levels in the rat. J. Nutrition, *58:* 231.

WOODRUFF, A. W. 1955 The natural history of anaemia associated with protein malnutrition. Brit. Med. J., *I:* 1297.

WRIGHT, L. D., AND H. R. SKEGGS 1946 Vitamin B complex studies with diets differing in the level of protein. Proc. Soc. Exp. Biol. Med., *63:* 327.

NUTRITIVE VALUE AND SAFETY OF HYDROGENATED VEGETABLE FATS AS EVALUATED BY LONG-TERM FEEDING EXPERIMENTS WITH RATS [1,2,3]

ROSLYN B. ALFIN-SLATER, ARTHUR F. WELLS, LILLA AFTERGOOD AND HARRY J. DEUEL, JR.[4]

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

(Received for publication April 16, 1957)

INTRODUCTION

Two previous reports from this laboratory (Deuel et al., '45; Deuel et al., '50) have described the excellent nutritional condition of rats maintained for 25 generations on Sherman diet B modified by replacing the whole milk powder with skimmed milk powder plus a proportionate amount of an hydrogenated vegetable margarine fat. Comparisons between our study and an investigation carried out by Sherman and coworkers ('24, '29–'30, '49) have been made in the previous report from this laboratory (Deuel et al., '50). In view of the fact that hydrogenated fats are now suspect by some

[1] This work was supported by a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of the late Professor Anton J. Carlson of the University of Chicago, of Professor Arthur W. Thomas of Columbia University and of Dr. Chester M. Gooding, Dr. Daniel Melnick and Dr. Hans W. Vahlteich of The Best Foods, Inc. during the course of the experiments, and the helpful suggestions of Professor James B. Allison of Rutgers University and of Professor Herbert E. Longenecker of the University of Illinois in preparing the material for publication.

[2] Contribution 423 of the Department of Biochemistry and Nutrition, University of Southern California.

[3] Presented in part at the meetings of the Federation of Societies for Experimental Biology, April 1957.

[4] Deceased April, 1956.

investigators as being harmful *per se* and also as agents contributing to atherosclerotic disease because of their saturated, iso- or trans-fatty acid contents, it seemed desirable to report on the growth, longevity, reproduction and lactation performance, plasma and liver cholesterol levels, and liver lipid content of these animals which are now in the 46th generation.

EXPERIMENTAL

The procedures employed in this investigation have been reported earlier (Deuel et al., '45). The diet fed to the rats through the 26th generation consisted of ground whole wheat (66%), sodium chloride (1%), skimmed milk powder (23.8%) and margarine fat (9.2%). Beginning with the 27th generation, whole margarine (11.2%) was substituted for the margarine fat with slight concomitant decreases made in the concentrations of skimmed milk powder (23.3%) and ground whole wheat (64.4%). Once weekly, the rats (with the exception of the female animals during lactation) received a supplement of 5 gm of ground, lean horse meat and 5 gm of lettuce. The margarine oil in the margarine fed to the rats until the 44th generation was a commercial vegetable oil product, identified as Oil B in table 1, and contained 19% saturated fatty acids, 3.7% essential fatty acids (by biological assay) and 35.3% trans-fatty acids. A change in the formula in 1954, identified as Oil C in table 1, has since increased the essential fatty acid content to 6.9% and this product was used in generations 45 and 46.

The rats were bred at 13 weeks of age, using one male to two female rats. The onset of pregnancy was determined through the use of uterine smears. Litters were counted at birth and weighed at three days, at which time they were reduced to 7 rats each. The young rats were weaned at 21 days of age and were then housed in large cages bedded with pine shavings, with 4 rats to a cage. The animals were maintained in a thermostatically controlled room. The diet was prepared fresh twice weekly and kept under refrigeration.

TABLE 1

Average fatty acid compositions of fats pertinent to the present investigations

FAT	IODINE NO.	FATTY ACID COMPOSITION [1]				CHARACTERIZATION OF UNSATURATES	
		Saturated	Oleic	Linoleic	Linolenic	Essential [2]	Trans [3]
		%	%	%	%	%	%
Margarine oils [4]							
A. Obsolete; coconut oil type (calculated)	20	75.0	18.6	2.0	0.0	2.0 (max.)	2.0 (max.)
B. Straight hydrogenated domestic oil type	73	19.4	71.3	4.9	0.0	3.7	35.3
C. As B, but of the plastic type	80	16.4	69.5	9.7	0.0	6.9	35.0
Oils before hydrogenation							
D. Cottonseed oil	109	24.5	21.6	49.5	0.0	48.0	2.3
E. Soybean oil	135	11.7	26.2	50.7	7.2	59.1	2.6
F. 50:50 blend of D + E (calculated)	122	18.0	23.9	50.1	3.6	53.6	2.5
Reference oil							
G. Butter	41	54.9	37.2	2.3	1.2 [5]	3.0	5.0–9.5 [6]

[1] According to the spectrophotometric method (Official and Tentative Methods, A.O.C.S., '53) following alkali isomerization to convert the polyunsaturated fatty acids to their light-absorbing conjugated forms.

[2] Based upon biological assay and expressed as linoleic acid equivalent (Deuel and associates, '51; Alfin-Slater et al., unpublished data).

[3] According to infrared absorption (Swern and associates, '50).

[4] Composition of the margarine oils:

A. A blend of 78% of coconut oil, 9% oleo oil, 7% lard and 6% hydrogenated cottonseed oil; this blend simulates the earlier composition of margarine oil at about 1932 according to Stiebeling ('56).

B. A 50:50 blend of cottonseed-soybean oils selectively hydrogenated directly to the constants characteristic of margarine whole oils.

C. A 50:50 blend of cottonseed-soybean oils selectively hydrogenated, one portion to a degree in excess of that characteristic of margarine whole oils, and the other portion to a compensating lesser degree.

[5] Of this value, 0.2% is arachidonic acid (absolute basis).

[6] Samples no longer available for this test; figures listed are those reported by Cornwell and associates ('53).

The animals were given food and water ad libitum and were weighed weekly.

Bone length was determined on animals 90 days old by a modified procedure developed in this laboratory (Greenberg et al., '50).

Male and female rats of the 46th generation used in producing the 47th generation were sacrificed shortly after the young rats of the 47th generation were weaned. Free and total cholesterol determinations were performed on extracts of plasma and liver by a modified Sperry-Schoenheimer method described by Nieft and Deuel ('49). Total liver lipids were determined gravimetrically.

RESULTS

The rate of growth of generations 25 through 46 is shown in figures 1 and 2. The weights for the male rats are averages of the weights of 12 animals whereas, for the female rats, 20 animals are represented. The weights of the male rats, reported for 21, 30, 60, 90 and 120 days, are uniformly good although more fluctuations are noted in the more mature animals over the 21 generations than in the younger rats. The highest weaning weight for both male and female rats was achieved in the 40th generation (45.4 and 40.8 gm respectively). The highest adult weight in the case of the male rats was observed in the 44th generation where the 120-day weight reached 337.2 gm. The heaviest adult weight for the female rat was noted in the 30th generation where the 90-day weight was 206.0 gm. Since female rats were bred at this time, their weights were no longer followed.

Weight increments throughout the generations were extremely satisfactory. Tibia length measurements, selected as a further index of growth, performed on male rats of the 40th generation at 90 days of age compared favorably with those of male rats of the same age of the 20th generation (3.72 ± 0.07 cm and 3.52 ± 0.05 cm respectively).

In longevity experiments, where the hydrogenated vegetable oil margarine fat diet was fed to the rats over a prolonged

period of time, male animals of the 27th generation showed a 50% survival after 97 weeks. This should be compared with a value of 99 weeks obtained with animals of the same history subsisting on a stock diet.[5] Confirmatory data were obtained

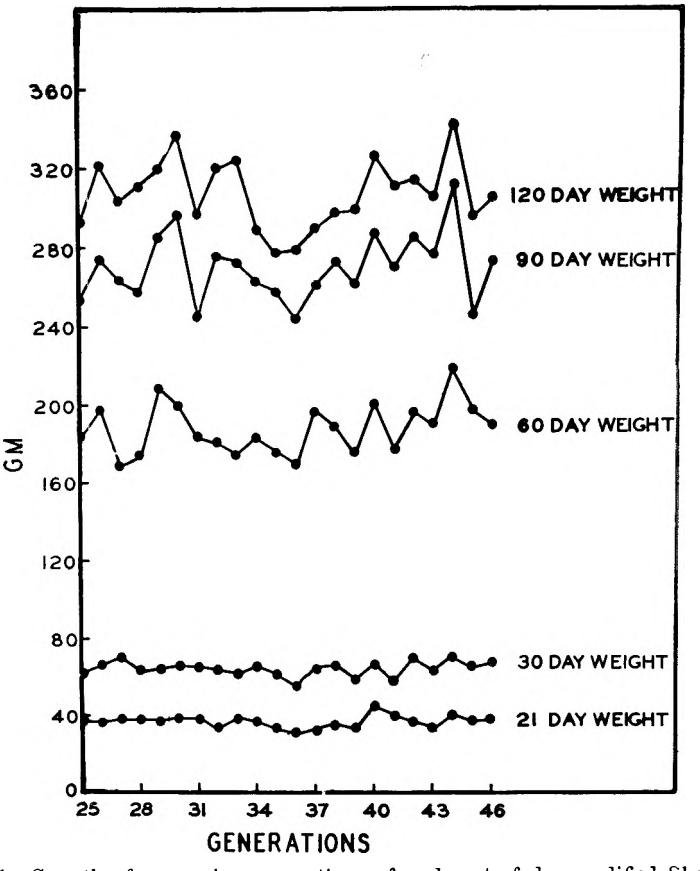


Fig. 1 Growth of successive generations of male rats fed a modified Sherman Diet containing hydrogenated vegetable margarine.

in a longevity test on rats fed a hydrogenated vegetable oil margarine fat at an average level of 12% of the diet; the results of this study were reported in an earlier paper (Deuel et al., '51b). In that test, at the end of two years, 50% of the 20 male rats and 50% of the 20 female rats still survived.

[6] Purina.

Histopathological sections of liver, kidney, stomach, small intestine, large intestine, spleen, heart, lung, pancreas, gonads, brain and adrenals of these survivors were examined and it was concluded that although the animals showed some diseased tissue attributable to old age, no changes traceable to the diet were observed.

A further longevity study was conducted with rats of the 34th generation. The results are shown in table 2 and indi-

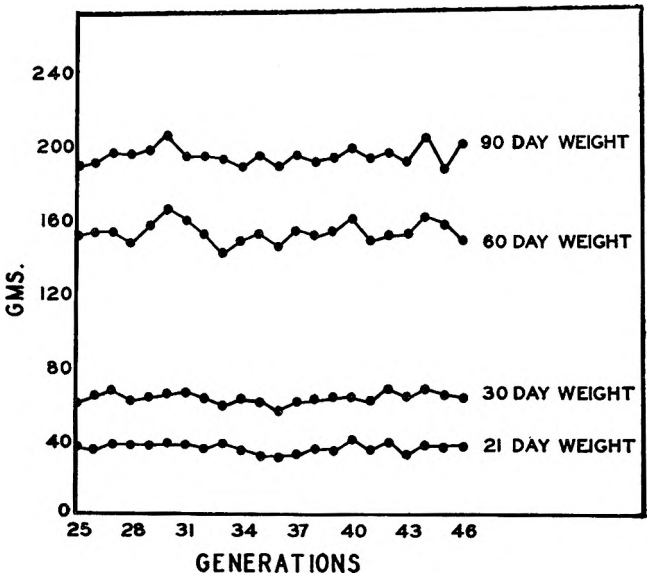


Fig. 2 Growth of successive generations of female rats fed a modified Sherman Diet containing hydrogenated vegetable margarine.

cate that good survival of these rats obtained in this generation as well.

In still another longevity study, the hydrogenated fat (margarine Oil C in table 1) was incorporated in the ration at graded levels and as the sole source of dietary fat. In one series cellulose flour was added to provide bulk so that the caloric density of the rations, containing the graded levels of hydrogenated fat, would be equal to that containing the lowest level of the hydrogenated fat. The control group was fed

the same ration but with unhydrogenated cottonseed oil replacing the hydrogenated fat at the lowest level. In all these rations, protein concentration (casein) was maintained constant in relation to calories and vitamin supplementation was liberal (table 3). Only the sucrose content of the diet was reduced with increasing additions of fat or of the cellulose flour. In table 4 are summarized the results of this study based upon ad libitum feedings to rats.

The survival of the animals in all groups is good. There is a suggestion that a fat intake of 18% of the diet was optimal for survival. Restricting the total caloric intake by reducing the caloric density of the diets seemed to be associated with

TABLE 2

Survival of male and female rats of the 34th generation fed the modified Sherman diet

SEX	NO. OF ANIMALS AT START	SURVIVAL			
		75%	50%	25%	0%
		weeks			
♂	22	55	80	106	120
♀	17	74	101	112	136

a small increase in survival time. Growth, as reflected by tibia length, was uniform for all the groups.

Within the week following the periods of 50% survival, the remaining animals in all groups were sacrificed. From each group three pairs of animals were selected on the basis of weight. Two animals approximated in weight the mean weight of the group, the second pair comprised the two heaviest animals, and the third pair included the two lightest animals in the group. Analyses were conducted in duplicate on the pooled carcasses of each pair of test animals. In table 5 are presented the average values for carcass composition of the rats surviving the longevity tests.

It will be noted that only the rats in the group fed ad libitum the ration containing 30% of the hydrogenated fat (about 50% of caloric intake) was there a marked increment in weight over that exhibited by the animals in the other groups. Tibia

TABLE 3

Diets employed in the third longevity experiment

FOOD COMPONENT	DIET 301	DIET 302	DIET 303	DIET 304	DIET 305	DIET 306	DIET 307	DIET 308
Commercial casein, %	20.0	21.5	23.6	26.6	20.0	20.0	20.0	20.0
Sucrose, %	72.67	64.84	53.26	37.65	59.1	38.7	11.5	72.67
Hydrogenated fat,[1,2] %	3.0	9.0	18.0	30.0	9.0	18.0	30.0	0.0
Cottonseed oil,[2] %	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0
Cellulose flour, %	0.0	0.0	0.0	0.0	7.57	18.97	34.17	0.0
Salt mixture (U.S.P. XIV), %	4.00	4.30	4.75	5.31	4.00	4.00	4.00	4.00
Water-soluble vitamin mixture,[3] %	0.33	0.36	0.39	0.44	0.33	0.33	0.33	0.33
Total calories per 100 gm	408	439	483	542	408	408	408	408
Calories from fat, % of total	6.8	19.1	34.7	51.5	20.5	41.0	68.4	6.8

[1] Hydrogenated margarine oil C in table 1.

[2] The following amounts of fat-soluble vitamins were added to the fat or oil used in the tests and the total made up to 100 gm with the fat or oil; vitamin A, 50,000 U.S.P. units; vitamin D, 5,000 U.S.P. units; α-tocopherol, 500 mg. This vitamin supplement comprised 1.0% of each diet with the total fat being that indicated under each diet number.

[3] Water-soluble vitamin mixture has the following percentage composition: choline, 36.7; inositol, 36.7; *p*-aminobenzoic acid, 18.35; thiamine chloride hydrochloride, 2.2; calcium pantothenate, 2.06; nicotinic acid, 1.83; riboflavin, 0.83; pyridoxine hydrochloride, 0.83; folic acid, 0.31; 2-methyl naphthoquinone, 0.15; biotin, 0.06; vitamin B_{12}, 0.00075.

TABLE 4

Growth and survival of rats on diets containing varying levels of fat

DIET VARIABLE	NO. OF RATS AT START		50% SURVIVAL			TIBIA LENGTH [1] OF SURVIVORS AFTER		
						42 weeks	110 weeks	
	Males	Females	Males	Females	Av. ♂ + ♀	Males [2]	Males	Females
%				*weeks*		*cm*	*cm*	*cm*
Hydrogenated fat, 3	24	23	112	97	105	4.1	4.1	3.6
Hydrogenated fat, 9	24	24	111	108	110	4.1	4.1	3.7
Hydrogenated fat, 18	24	24	109	108	109	4.1	4.1	3.7
Hydrogenated fat, 30	24	22	100	103	102	4.1	4.2	3.7
Hydrogenated fat, 9 + cellulose flour	24	24	121	112	117	4.0	4.1	3.7
Hydrogenated fat, 18 + cellulose flour	24	24	131	118	125	4.1	4.2	3.7
Hydrogenated fat, 30 + cellulose flour	24	24	106	110	108	4.0	4.1	3.6
Unhydrogenated, 3 cottonseed oil	24	24	116	91	104	4.1	4.2	3.7

[1] Standard error of mean = maximum of ± 0.02.

[2] At this time measurements were taken on males only.

TABLE 5

Carcass composition of the rats surviving the longevity tests[1]

DIET VARIABLE	AV. WEIGHT		MOISTURE		PROTEIN (N × 6.25)				FAT (ETHER EXTRACTED)				IODINE NO. OF FAT	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
	%	*gm*	*gm*	%	%	%	%	*gm/rat*		%	%	*gm/rat*			
Hydrogenated fat, 3	431	315	50.4	49.9	14.9	13.9	64.2	43.8	33.5	33.8	144	106	50.5	43.9
Hydrogenated fat, 9	405	358	51.8	45.9	15.1	12.5	61.2	44.8	27.7	36.1	112	129	51.5	55.9
Hydrogenated fat, 18	467	386	46.4	42.8	13.8	12.1	64.4	46.7	34.2	38.7	160	149	63.6	55.1
Hydrogenated fat, 30	647	406	41.8	41.3	11.6	11.5	75.1	46.7	37.0	44.5	239	181	48.6	51.9
Hydrogenated fat, 9 + cellulose flour	449	328	43.1	48.7	11.9	14.2	53.4	46.6	38.8	32.8	174	108	72.8	56.6
Hydrogenated fat, 18 + cellulose flour	349	300	58.9	54.1	17.6	15.7	61.4	47.1	19.0	26.2	66	79	50.4	58.2
Hydrogenated fat, 30 + cellulose flour	357	301	55.0	49.4	17.4	16.6	62.1	50.0	23.4	32.5	84	98	57.2	57.4
Unhydrogenated, 3 cottonseed oil	397	320	55.8	49.1	15.9	13.8	63.1	44.2	23.1	32.2	92	103	57.7	56.8

[1] Average of individual analyses conducted on three pairs of animals in each group.

length (see table 4) had shown that these animals were actually of the same size as the others. The carcass analyses revealed that this increment in weight was due in large part to fat deposition. However, the same ration reduced in caloric density by the addition of the cellulose flour produced a lean carcass, as low in fat content as that observed for the animals on the 3% cottonseed oil ration. Protein content, expressed as grams per rat, showed that all the test animals were comparable in this respect; possibly the largest animals in the series contained somewhat more protein. The iodine values of the carcass fat were also of comparable magnitude among the test groups. These rather low values indicated in every case that a rather saturated fat had been deposited. All in all, there were no consistent trends in biological responses, based upon growth, longevity and carcass composition, to indicate that feeding increasing concentrations of a selectively hydrogenated fat in the diet was in any way objectionable.

In table 6 is summarized the reproductive performance of the rats of generations 25 through 46, subsisting on the modified Sherman diet, containing the hydrogenated vegetable oil as practically the sole source of fat in the ration. The fertility of the animals has been maintained at a fairly constant level. Although in the 40th generation only 13 of 20 pregnancies were successful, in the 41st generation, 18 of 20 females delivered their young and the 42nd generation once again showed a 100% fertility. The lactation performance as judged by the 21-day weight of the weanling rats has also been fairly constant. A low of 26.3 gm was obtained in the 35th generation, but a return to 31.6 gm was achieved in generation 36, and a good weaning weight has been maintained since then (34.8 gm for the 40th generation; 34.9 gm for the 46th).

A multigeneration study is probably the most sensitive method for the evaluation of the nutritive value or safety of a particular foodstuff or dietary regime. Dietary deficiencies and toxicity often are not apparent until after several gen-

erations of animals on the test diet are examined. In a multigeneration experiment, many stresses are brought to bear upon the animals, i.e. growth, pregnancy and lactation. Diets which prove to be satisfactory for maintenance and even growth over a single generation may be quite unsatisfactory

TABLE 6

Pregnancy and lactation performance of rats fed the modified Sherman diet

GENERATION	BODY WT. OF FEMALES AT MATING	NO. FEMALES BRED	SUCCESSFUL PREGNANCIES	NO. RATS PER LITTER	WT. AT 3 DAYS		WT. AT 21 DAYS, PER RAT
					Litter	Rat	
					gm	*gm*	*gm* [1]
25	194	28	27	8.2	59.7	7.3	34.1
26	192	36	31	7.4	58.1	7.9	35.3
27	199	30	29	8.1	62.6	7.7	34.8
28	195	24	20	7.7	60.2	7.8	32.2
29	204	24	22	6.9	53.8	7.8	38.4
30	203	27	20	7.6	64.5	8.5	34.9
31	196	30	26	6.9	56.7	8.3	30.4
32	196	34	33	7.8	62.0	7.9	34.2
33	197	35	31	6.8	50.0	7.4	34.2
34	187	34	32	6.8	50.4	7.4	29.8
35	198	27	24	5.3	36.0	6.8	26.3
36	191	17	13	7.4	54.8	7.4	31.6
37	199	20	19	6.7	48.3	7.2	33.3
38	191	20	20	6.2	45.6	7.3	31.4
39	212	19	18	7.3	61.0	8.3	41.4
40	198	20	13	8.2	62.6	7.6	34.8
41	198	20	18	8.8	65.7	7.5	38.0
42	199	20	20	7.8	52.9	6.8	30.8
43	194	20	17	8.9	66.5	7.5	38.1
44	203	20	15	6.8	52.6	7.7	35.4
45	199	20	13	8.4	66.4	7.9	35.8
46	206	20	18	8.1	61.9	7.6	34.9

[1] Only litters with 7 young are included in this average.

for reproduction; a diet adequate for growth and reproduction may be entirely inadequate for lactation. The results of this study give positive evidence for the dietary safety of selectively hydrogenated margarine fats and show conclusively that the nutritional status and reproductive performance of rats may be maintained at a high level over 46 generations

TABLE 7

The plasma and liver cholesterol levels and total liver lipid concentration in male and female rats of the 46th generation on a modified Sherman diet containing hydrogenated fat as compared with rats fed a diet containing cottonseed oil

DIET	SEX	WT. AT SACRIFICE	WT. OF LIVER	LIVER CHOLESTEROL [1]			LIPIDS [1] IN LIVER	PLASMA CHOLESTEROL [1]		
				Free	Total	% Free		Free	Total	% Free
		gm	*gm*	*mg/gm*	*mg/gm*		*mg/gm*	*mg %*	*mg %*	
Modified Sherman B (Hydrogenated fat)	M (12)[2]	309	9.9	1.86 ± 0.03	2.03 ± 0.04	91.6	40.8 ± 0.9	16.7 ± 0.6	51.5 ± 2.0	32.5
	F (11)	228	8.3	1.92 ± 0.05	2.05 ± 0.08	93.6	36.3 ± 1.8	23.5 ± 1.2	69.9 ± 4.5	33.5
15% CSO	M (6)	336	10.2	2.12 ± 0.10	2.61 ± 0.13	81.2	54.5 ± 4.6	12.2 ± 0.5	50.2 ± 3.8	24.3
	F (9)	230	7.5	1.83 ± 0.06	2.16 ± 0.09	84.7	51.4 ± 4.4	29.2 ± 3.5	76.6 ± 5.8	38.1

[1] Including standard error of the mean.

[2] Numbers within parentheses = number of animals in each group.

when they are fed a diet containing hydrogenated vegetable margarine oils as practically the sole source of dietary fat.

The effect on plasma and liver cholesterol concentration and total lipid in liver of feeding the hydrogenated fat to animals over a period required to raise these rats to the 46th generation is reported in table 7. Values obtained on rats of our stock colony fed from weaning a diet containing 15% cottonseed oil have been included for comparison. It is obvious that the presence of the hydrogenated fat in the diet of rats has *not* led to an increase in either plasma or liver cholesterol concentrations over that observed when the limpid cottonseed oil is fed.

DISCUSSION

Recent reports (Ahrens et al., '55; Kinsell and Michaels, '55; Bronte Stewart et al., '56) that high blood cholesterol levels may be reduced by feeding fats containing a high proportion of unsaturated fatty acids have led these investigators to theorize that fats with higher proportions of saturated acids might be responsible for raising blood cholesterol values. Also, questions have been raised about the possible effects of hydrogenated fats because of the occurrence in them of fatty acid isomers not found in natural fats and oils (Mabrouk and Brown, '56; Sreenivasan and Brown, '56), and the lower percentage of essential fatty acids they contain.

Melnick and Deuel ('54) have reported on the significance of the changes which occur during the hydrogenation of oils. In the hydrogenation of an oil for margarine production under so-called selective hydrogenation, the concentration of the saturated fatty acids is not increased. However, there is a preferential hydrogenation of the polyunsaturated fatty acids to oleic acid, during which trans-isomers are produced. As can be seen in table 1, the change in the margarine fat formula from coconut oil to domestic oils has resulted in a marked decrease in saturated fatty acid content. The present margarine oil which consists of a blend of equal amounts of hydrogenated cottonseed oil and soybean oil has a saturated

fatty acid content no greater than that of the unhydrogenated oil blend and even less than that of the unhydrogenated cottonseed oil component. The firmness imparted to the oil on hydrogenation is due to the significantly higher melting point of the trans-oleic acids produced relative to that of the natural cis-oleic acid (viz. 43.5°C. for elaidic acid versus 13.0°C. for the natural cis-isomer).

Recently the essential fatty acid content of the hydrogenated margarine fat has again been increased as can be observed in table 1 when Oil C is compared with Oil B. Hydrogenated margarine oils, of course, do contain less essential fatty acids than do the original unhydrogenated oils. The data in table 1, however, demonstrate that butter oil contains less than one-half of the essential fatty acid concentration found in the hydrogenated margarine oil.

The long-term studies presented in this report have been conducted on rats subsisting for 46 generations on diets with selectively hydrogenated vegetable oils comprising practically the sole source of fat in the diet. The performance of the test animals as judged by gain in weight, tibia length, reproduction and lactation, longevity and histopathological examination of the tissues have shown that the hydrogenated fats are safe and have full nutritional value. The hydrogenated margarine oils (Oil B in table 1 of the current report) have Coefficients of Digestibility of 95% or greater (Calbert et al., '51) and the same results have been obtained in tests conducted on the present-day blended margarine oils (Oil C of table 1) (Alfin-Slater et al., '56).[6]

Carcass analyses conducted on the survivors of the longevity studies have also revealed nothing objectionable as a result of the presence of hydrogenated fats in the diet. It is interesting to note that a rather highly saturated fat had been deposited in all cases (table 5). Both the concentration and the absolute amount of fat in the carcass have been found to be independent of the percentage of fat in the ration. Only when there is excessive consumption of calories by animals

[6] Alfin-Slater, R. B., L. Aftergood and H. J. Deuel, Jr. Unpublished data.

subsisting on diets of high caloric density, is there an increase in the concentration and amount of deposited fat. However, the character of the obesity as revealed by the iodine value of the deposited fat is one of a quantitative rather than of a qualitative nature; the obese animals on the hydrogenated fat diets of high caloric density simply have more of the same type of fat deposited. The nature of the depot fat must be taken into consideration, if such animals were to be placed on a weight-loss regimen by feeding daily a limited quantity of a very low fat diet (e.g., 3% fat, containing preferentially a limpid oil to provide as much unsaturated fatty acids as possible). The depot fat will then be metabolized along with the dietary fat. Eliminating the hydrogenated fat from the ration even at the beginning would not change the picture. A high carbohydrate-protein diet can also promote fat deposition of the more saturated type when such a ration is consumed in excess of caloric requirements. This fact is substantiated by the results obtained with the 3% cottonseed oil ration in the present study. Since there is no difference between the saturated fatty acids ingested and those produced *in vivo* from other foodstuffs (Weinman et al., '51) the character of deposited fat is the same regardless of whether it is derived from hydrogenated fat or carbohydrate or protein. Recommendations to restrict fat in the diet to low levels with the idea of maintaining a "proper" ratio of saturated to unsaturated fat in the diet (May, '56) fail to take into account the full nutritional picture. In view of the evidence presented here, and also in consideration of the many non-caloric functions of fat in the diet (Deuel, '50; Barnes, '56), it would seem to be more realistic to ingest a diet rich in unsaturated fatty acids and, also, to restrict substantially the total caloric intake.

It has been shown in work reported from this laboratory (Alfin-Slater et al., '54) that the lack of essential fatty acids in the diet causes an accumulation of cholesterol in the liver. Since the liver cholesterol values reported here for rats of the 46th generation on a hydrogenated fat diet show no in-

creases over what is obtained with rats on diets containing unhydrogenated cottonseed oil, it can be concluded that the essential fatty acids supplied in the diet containing hydrogenated fat are sufficient for adequate control of cholesterol metabolism.

The diet in this still-continuing multigeneration study contains 9.24% hydrogenated fat which in turn contains 35% trans-fatty acids. If it is assumed that the rat eats an amount of ration approximating 10% of its body weight each day, then in terms of its body weight it ingests 0.92% of hydrogenated fat per day and 0.32% trans-fatty acids per day. If these figures are translated in terms of the human diet for a 70-kg man, the equivalent ingestion of hydrogenated fat would be 646 gm containing 225 gm of trans-fatty acids. However, in the daily average American diet of 3000 calories, 40% of which are derived from fat, there are only 133 gm of fat. Approximately half of this 133 gm — or 67 gm — is visible fat (Barnes, '56). If all of this visible fat were hydrogenated fat, it would then be only approximately one-tenth of the amount of hydrogenated fat — and one-tenth of the amount of the trans-fatty acid isomers — which had been ingested by the rats during the 46 generations encompassed by the investigation reported here.

In one of the three longevity studies included in the present investigation the hydrogenated fat comprised as much as 30% of two of the rations. Using the same method of calculation, these animals then ingested per unit of body weight about 30 times as much trans-fatty acid isomers as would be ingested by the human subsisting throughout the major part of his life span on a diet with all visible fat in the form of a selectively hydrogenated margarine oil.

Melnick and Deuel ('54) have shown by microbiological assay techniques that the iso-oleic acids formed during hydrogenation (studies conducted not only on the composites of fatty acids as found in hydrogenated oils but also on individual positional and stereoisomers) are not antimetabolites for natural oleic acid but are utilized as nutrients. Fatty acids with

conjugated double bonds (absent from the fats in table 1 of the current report but found as transitory products in lightly hydrogenated oils) were also shown by these investigators not to be antimetabolites for the essential fatty acids but to be readily metabolizable to carbon dioxide and water.

In addition, in the studies reported here, there was no evidence that the fatty acids produced on hydrogenation were antimetabolites for the essential fatty acids. Essential fatty acids have been shown to be required for adequate nutritional performance employing the same indices which were included in the present study, namely growth, reproduction, lactation, and survival (Deuel et al., '51a and '55). The level of linoleic acid in the hydrogenated fats in this investigation evidently was sufficient for adequate nutritional performances according to all indices in spite of the fact that an average ratio of 7:1 in trans-acid content to biologically active fatty acid content obtained (table 1).

SUMMARY

A multigeneration study in which rats have been fed a ration containing 9.24% of a selectively hydrogenated vegetable oil as practically the sole source of fat in the diet has now been carried through 46 generations. The performances of rats of generations 26 through 46 as judged by gain in weight, tibia length, reproduction, lactation, longevity and carcass analyses have shown that the hydrogenated fats are of full nutritional value. The presence of 6.9% essential fatty acids (expressed as linoleic acid) in the hydrogenated vegetable fat has been shown to be sufficient to maintain normal plasma and liver cholesterol levels and liver lipid levels in the rat. The test animals in the multigeneration studies ingested per unit of body weight about 10 times as much trans-fatty acid isomers as would be ingested by the human on a diet with all the visible fat in the form of the selectively hydrogenated vegetable oils. In the case of the longevity studies, this ratio was as high as 30:1. Hydrogenation of margarine fats does not produce excessive saturated fatty acids and indeed the essen-

tial fatty acid content of the hydrogenated margarine oil can be relatively high. Attention has been directed to the relatively saturated fat deposited in the body of animals on low fat diets, even those containing limpid vegetable oils rich in essential fatty acid content.

Previous experiments on generations one through 25 of this multigeneration study have yielded beneficial results on metabolism studies, digestibility studies and histopathological examination of the tissues in addition to the nutritional indices mentioned above. In other studies, the fatty acid isomers contained in the hydrogenated fats exhibited no measurable antimetabolite activity toward the essential fatty acids despite an average of about 7:1 in the ratio of trans-isomers to biologically active fatty acid content.

It is therefore concluded that selectively hydrogenated vegetable oils, such as are employed in margarine manufacture, containing positional and stereoisomers of the unsaturated fatty acids, are fully digestible, harmless, and of full nutritional value as determined by long-term studies conducted with rats. In all the multigeneration and longevity studies, herein reported, no deleterious effects were observed as a result of the ingestion of the small amounts of saturated fatty acids present in the hydrogenated fats.

LITERATURE CITED

AHRENS, E. H., JR., T. T. TSALTAS, J. HIRSCH AND W. INSULL, JR. 1955 Effect of dietary fats on the serum lipides of human subjects. J. Clin. Invest., *34:* 918.

ALFIN-SLATER, R. B., L. AFTERGOOD, A. F. WELLS AND H. J. DEUEL, JR. 1954 The effect of essential fatty acid deficiency on the distribution of endogenous cholesterol in the plasma and liver of the rat. Arch. Biochem. Biophys., *52:* 180.

BARNES, R. H. 1956 A review of some recent developments in fat nutrition, Proc. 1956 Cornell Nutrition Conference for Feed Manufacturers, Ithaca, N. Y.

BRONTE-STEWART, B., A. ANTONIS, L. EALES AND J. F. BROCK 1956 Effects of feeding different fats on serum cholesterol levels. Lancet, *1:* 521.

CALBERT, C. E., S. M. GREENBERG, G. KRYDER AND H. J. DEUEL, JR. 1951 The digestibility of stearyl alcohol, isopropyl citrates, and stearyl citrates, and the effect of these materials on the rate and degree of absorption of margarine fat. Food Res., *16:* 294.

CORNWELL, D. G., R. BLACKDERF, C. L. WILSON AND J. B. BROWN 1953 The trans-octadecenoic acid content of butterfat. Arch. Biochem. Biophys., *46:* 364.

DEUEL, H. J., JR. 1950 Non-caloric functions of fat in the diet. J. Amer. Diet Assoc., *26:* 255.

DEUEL, H. J., JR., L. F. HALLMAN AND E. MOVITT 1945 Studies on the comparative nutritive value of fats. VI. Growth and reproduction over 10 generations on Sherman diet B where butterfat was replaced by a margarine fat. J. Nutrition, *29:* 309.

DEUEL, H. J. JR., S. M. GREENBERG, E. E. SAVAGE AND L. A. BAVETTA 1950 Studies on the comparative nutritive value of fats. XIII. Growth and reproduction over 25 generations on Sherman diet B where butterfat was replaced by margarine fat, including a study of calcium metabolism. Ibid., *42:* 239.

DEUEL, H. J., JR., S. M. GREENBERG, L. ANISFELD AND D. MELNICK 1951a The effect of fat level of the diet on general nutrition. VIII. The essential fatty acid content of margarines, shortenings, butters, and cottonseed oil as determined by a new biological assay method. Ibid., *45:* 535.

DEUEL, H. J., JR., S. M. GREENBERG, C. E. CALBERT, R. BAKER AND H. R. FISHER 1951b Toxicological studies on isopropyl and stearyl citrates. Food Res., *16:* 258.

DEUEL, H. J., JR., C. R. MARTIN AND R. B. ALFIN-SLATER 1955 The effect of fat level of the diet on general nutrition. XVI. A comparison of linoleate and linolenate in satisfying the essential fatty acid requirement for pregnancy and lactation. J. Nutrition, *57:* 297.

GREENBERG, S. M., C. E. CALBERT, E. E. SAVAGE AND H. J. DEUEL, JR. 1950 The effect of fat level of the diet on general nutrition. VI. The interrelation of linoleate and linolenate in supplying the essential fatty acid requirement in the rat. Ibid., *41:* 473.

KINSELL, L. W., AND G. D. MICHAELS 1955 Hormonal-nutritional lipid relationships. Fed. Proceedings, *14:* 661.

MABROUK, A. F., AND J. B. BROWN 1956 The trans fatty acid content of margarines and shortenings. J. Amer. Oil Chem. Soc., *33:* 98.

MAY, C. D. 1956 Fat in the diet in relation to arteriosclerosis. J. Amer. Med. Assoc., *162:* 1468.

MELNICK, D., AND H. J. DEUEL, JR. 1954 Biological utilization of fatty acid isomers. J. Amer. Oil Chem. Soc., *31:* 63.

NIEFT, M. L., AND H. J. DEUEL, JR. 1949 Studies on cholesterol esterase. I. Enzyme systems in rat tissues. J. Biol. Chem., *177:* 143.

Official and Tentative Methods of the American Oil Chemists' Society, Polyunsaturated Acids, Cd 7–48, revised May, 1953.

SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. J. Biol. Chem., *60:* 5.

———— 1929–1930 Further experiments on the influence of food on longevity. J. Nutrition, *2:* 415.

SHERMAN, H. C., AND H. Y. TRUPP 1949 Further experiments with vitamin A in relation to aging and to length of life. Proc. Nat. Acad. Sci., *35:* 90.

SREENIVASAN, B, AND J. B. BROWN 1956 Octadecadienoic acids of shortenings and margarines. J. Amer. Oil Chem. Soc., *33:* 341.

STIEBELING, H. K. 1936 Food consumption of various income classes. Gordon Research Conferences, A.A.A.S., August 7.

SWERN, D., H. E. KNIGHT, O. D. SHREVE AND M. R. HEETHER 1950 Comparison of infrared spectrophotometric and lead salt-alcohol methods for the determination of trans octadecenoic acids and esters. J. Amer. Oil Chem. Soc., *27:* 17.

WEINMAN, E. O., I. L. CHAIKOFF, B. P. STEVENS AND G. W. DAUBER 1951 Conversion of the 1st and 6th carbons of stearic acid to CO_2 by rats. J. Biol. Chem., *191:* 523.

THE EFFECT OF FLUORIDE ADMINISTRATION ON FLUORIDE DEPOSITION IN TISSUES AND ON SERUM CHOLESTEROL IN THE RAT [1]

WOLFGANG BUTTNER [2] AND JOSEPH C. MUHLER
Department of Chemistry and School of Dentistry, Indiana University, Bloomington

(Received for publication April 4, 1957)

It has been mentioned that there is an increase in mortality from cardiovascular disease in people residing in fluoride areas over that for similar people who use a non-fluoride-containing communal water supply (Miller, '52). While epidemiological surveys conducted in such areas do not confirm this impression (Anonymous, '52; Galletti et al., '56), no laboratory evidence concerning a possible relationship of fluoride to the serum cholesterol content is available. It is well known that fluoride is retained in the skeleton (Muhler, '54), but little information is available concerning the storage of fluoride within the soft tissues. In order to provide experimental evidence in regard to this problem, the effect of varying concentrations of fluoride on the serum cholesterol of rats was studied, and the amount of fluoride retained in the skeleton, heart, and kidney was measured.

EXPERIMENTAL

This study was divided into three series, each varying in the duration of fluoride administration, the type of fluoride used, and the effect of different diets. In series I, 40 weanling

[1] This study was supported in part by a grant from the Procter and Gamble Company, Cincinnati, Ohio.

[2] Post-Doctorate Fellow. On leave of absence from the University of Mainz. Mainz, Germany.

male Sprague-Dawley strain rats received a stock corn diet (table 1) and redistilled drinking water ad libitum. According to their initial body weights the rats were equally divided into 4 groups. Groups 1, 2, and 3 received daily by stomach tube, 0.5, 1.0, or 2.0 mg of fluoride respectively as an aqueous solution of sodium fluoride. The 4th group received 1 ml distilled water daily by stomach tube and was used for control. All of the animals were weighed twice each week. After 4 weeks of fluoride administration, the rats were sacrificed with ether and fluoride was determined in the whole carcasses and

TABLE 1

Composition of diets

SERIES I AND III		SERIES II		
Stock corn diet		Semi-purified 5% fat		Semi-purified 15% fat
	%		%	%
Yellow corn grits	52.7	Cane sugar	60	50
Ground yellow corn	11.3	Casein	30	30
Powdered whole milk	30.0	Cottonseed oil [1]	5	15
Alfalfa	4.8	Salts [2]	4	4
Iodized NaCl	1.0	Vitamin mixture [2]	1	1
Irradiated yeast	0.2			

[1] Wesson.

[2] Muhler ('54).

femora according to methods already described (Muhler, Nebergall and Day, '54).

In series II, the same procedure was followed except that a semi-purified diet containing 5 or 15% fat (table 1) was used in place of the stock corn diet. Three groups of weanling male rats, each group consisting of 12 rats, received the semi-purified 5% diet. Another three groups of 12 rats each received the semi-purified 15% fat diet. In each of the two different groups of this series, two of each group received 1.0 or 2.0 mg fluoride daily by stomach tube. One group from each series was used for a control and received distilled water by stomach tube. After 8 weeks the animals were sacrificed as in series I and fluoride was determined in the whole carcasses, in the femora, and in the hearts and kidneys.

In series III, 24 weanling male rats were divided into 4 groups. Each received the same stock corn diet as used in series I, and the various fluorides were administered ad libitum in the drinking water all at a fluoride concentration of 30 p.p.m. All of the experimental diets contained less than 1 μg of fluoride per gram of diet. The fluorides which were used were sodium fluoride, stannous fluoride, and sodium fluorostannite. A 4th group received distilled water and served as a control. After 140 days of fluoride administration the animals were sacrificed and serum cholesterol was determined.

Once each week all animals were anesthesized [3] and 0.75 ml blood was removed by cardiac puncture. Serum cholesterol was determined in duplicate by direct colorimetry according to the method of Pearson, Stern and McGavack ('53), which requires 0.1 ml of serum per sample. The duplicates had an average deviation of less than 5%.

RESULTS

The data from these studies are shown in figures 1 and 2 and in tables 2 through 4. The growth curves of the animals in series I indicate a retarded growth in all of the fluoride groups when compared to the control. The weight gain in groups 1 and 2 which received 0.5 and 1.0 mg of fluoride daily by stomach tube, respectively, decreased 14% at the end of 4 weeks of fluoride administration when compared to controls. Group 3, which received 2.0 mg of fluoride daily by stomach tube, weighed 34% less than the control group at the end of 4 weeks. The level of serum cholesterol in these animals is seen in figure 1 and indicates a decrease of approximately 20% after two weeks in all fluoride groups, although these differences are not significant. After the 4th week of fluoride administration the serum cholesterol level of group 1, which received 0.5 mg of fluoride daily, returned to a level comparable to that of the control group, while the groups receiving 1.0 or 2.0 mg of fluoride showed an increase of serum

[3] Sodium nembutal was used.

cholesterol of about 20%. The control group had a steady decrease in serum cholesterol throughout the period of investigation. The fluoride storage in the skeleton from the series I animals is shown in table 2. One notes a steady fluoride uptake with increased fluoride concentration. Compared to the control group the fluoride storage in the whole carcass and femur of the group receiving 1.0 mg of fluoride daily is about 30 times higher while the group receiving 2.0 mg of fluoride

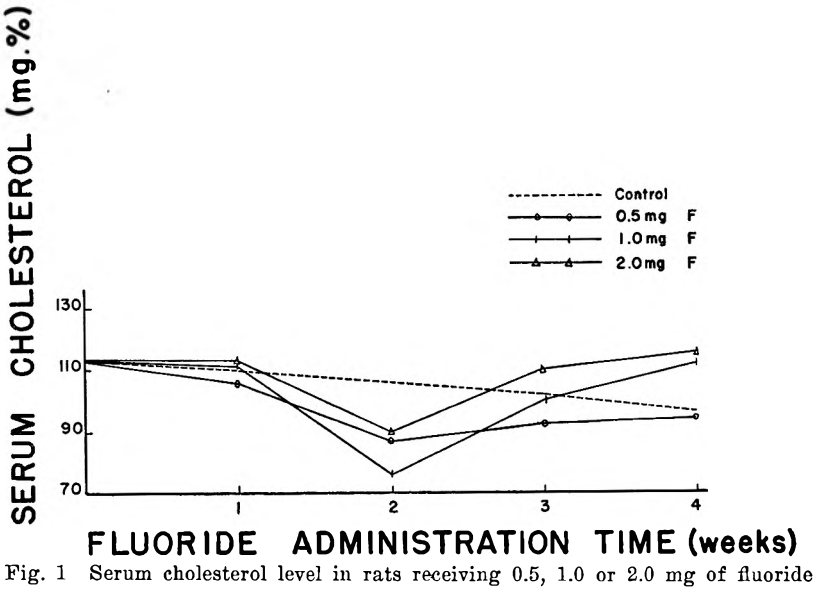


Fig. 1 Serum cholesterol level in rats receiving 0.5, 1.0 or 2.0 mg of fluoride daily by stomach tube over a period of 4 weeks.

is about 50 times higher. Considering this high uptake of fluoride, the 20% increase of serum cholesterol does not seem to be directly related to fluoride intake.

The apparent rise of cholesterol levels after 4 weeks of fluoride administration found in the first series, however, induced us to run the second series which lasted for a longer period. A semi-purified 15% fat diet was included in this portion of the experiment in order to confirm the observation of various other workers (Miller and Phillips, '55) who found an enhancement of the toxicity of sodium fluoride in rats

TABLE 2

The storage of fluorine in the femur and whole carcass of rats

DIET	DAILY FLUORIDE INTAKE	NO. OF RATS	FLUORIDE ANALYSIS			
			Femur		Whole carcass	
			Concentration	Total	Concentration	Total
	mg		*p.p.m.*	*mg*	*p.p.m.*	*mg*
			Series I			
Stock corn	0.5	8	2111 ± 100 [1]	0.45 ± 0.02 [1]	1430 ± 110 [1]	5.9 ± 0.3 [1]
	1.0	8	5295 ± 527	1.48 ± 0.06	3681 ± 215	17.0 ± 0.6
	2.0	4	9120 ± 710	2.03 ± 0.18	5650 ± 425	22.1 ± 1.3
	Control	10	168 ± 6	0.05 ± 0.002	123 ± 9	0.6 ± 0.04
			Series II			
Semi-purified 5% fat	Control	12	383 ± 57	0.12 ± 0.02	160 ± 17	1.46 ± 0.16
	1.0	12	1824 ± 165	0.59 ± 0.05	1650 ± 54	15.80 ± 0.90
	2.0	12	3565 ± 240	1.02 ± 0.05	2777 ± 280	22.50 ± 1.60
Semi-purified 15% fat	Control	12	253 ± 28	0.07 ± 0.01	164 ± 15	1.38 ± 0.13
	1.0	12	2260 ± 47	0.68 ± 0.03	1578 ± 106	13.60 ± 0.80
	2.0	12	3694 ± 225	1.12 ± 0.01	2488 ± 240	21.30 ± 1.55

[1] Standard deviation.

which received a diet containing 15% cottonseed oil. Furthermore, this portion of the experiment enabled us to correlate the effects of a semi-purified diet with and without fluoride with the serum cholesterol level. The growth curves of series II animals indicate no differences between any of the groups. Figure 2 shows the serum cholesterol levels of the 6 different groups of series II. Following the first two weeks of fluoride administration the serum cholesterol of the rats

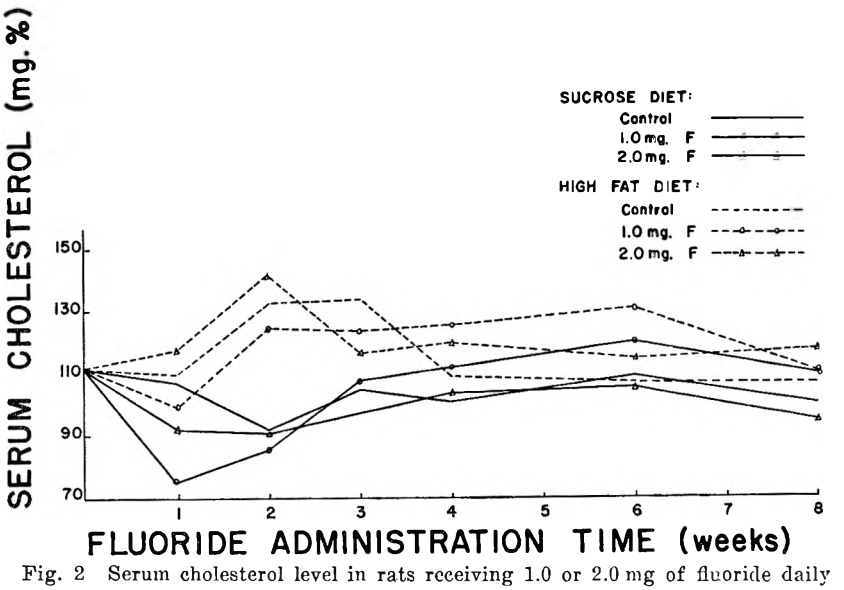


Fig. 2 Serum cholesterol level in rats receiving 1.0 or 2.0 mg of fluoride daily by stomach tube over a period of 8 weeks.

fed the semi-purified 5% fat diet decreased about 20% while the serum cholesterol of the rats on the 15% fat diet increased about 20%. There is, however, in both dietary groups no significant difference in serum cholesterol of the rats which received 1.0 or 2.0 mg of fluoride daily as compared to the controls. After 4 weeks these initial cholesterol changes in both dietary groups returned to the normal serum cholesterol of the controls which was between a level of approximately 100 or 120 mg %. This level remained consistent over a period of 4 additional weeks. According to these data, fluoride

administration in rats with a level as high as 1.0 or 2.0 mg daily over an 8-week period did not result in significant changes of the normal serum cholesterol level.

The data for skeletal fluoride storage in the series II animals are also shown in table 2. The data indicate that, when the total fluoride in the femur is used for comparative purposes, the animals receiving the 15% fat diet plus fluoride have a tendency to store more fluoride in the femur than similar animals receiving the same amount of fluoride but no added dietary fat. However, these differences are not significant. The data from the whole carcass do not indicate an increased skeletal retention in the animals receiving the 15% fat diet. The reason for these differences is not known at the present time.

Of considerable biological importance are the fluoride retention data obtained by analysing the hearts and kidneys from the animals receiving either the semi-purified 5% or 15% fat diets. These data are seen in table 3 and indicate that the animals which received the semi-purified 15% fat diet had more fluoride in the hearts. This is true in the control groups and those receiving 1 or 2 mg of fluoride daily. These differences are not statistically significant, however. Similarly, within each dietary group, more fluoride is found in the heart in the groups receiving fluoride than in those animals not receiving fluoride. The data for the kidneys similarly indicate an increased amount of fluoride in each dietary group when fluoride was received than in the control, but the kidneys of the control animals receiving the semi-purified 15% fat diet did not contain more fluoride than the kidneys from the animals receiving the semi-purified 5% fat diet, as did the hearts obtained from such animals. It is interesting to note that Muhler ('57) has also shown an increased fluoride retention in kidneys and hearts when guinea pigs are given vitamin C.

It is difficult, with our present state of information, to explain how soft tissues retain fluoride. More information is needed concerning the dynamic state of fluoride-skeletal

metabolism and the period of skeletal fluoride saturation where excess fluoride is retained in the soft tissues. The means of fluoride detoxification when the skeleton approaches saturation are interesting areas for future investigation.

Series III was conducted in order to obtain serum cholesterol data from rats which received various fluorides in the drinking

TABLE 3

Total fluoride in hearts and kidneys of series II rats

(Six rats/group)

GROUP	SEMI-PURIFIED 5% FAT DIET		SEMI-PURIFIED 15% FAT DIET	
	Heart	Kidneys	Heart	Kidneys
	μg F	*μg F*	*μg F*	*μg F*
Control	0.51 ± 0.02 [1]	0.39 ± 0.05 [1]	0.60 ± 0.06 [1]	0.32 ± 0.05 [1]
1.0 mg F daily	0.70 ± 0.05	0.59 ± 0.06	0.79 ± 0.06	0.46 ± 0.02
2.0 mg F daily	0.64 ± 0.09	0.58 ± 0.13	0.75 ± 0.13	0.47 ± 0.02

[1] Standard deviation.

TABLE 4

Serum cholesterol level in rats receiving 30 p.p.m. of fluoride in drinking water over 140 days

FLUORIDE USED	NO. OF RATS	SERUM CHOLESTEROL AFTER 140 DAYS
		mg %
NaF	6	147 ± 8 [1]
SnF_2	6	118 ± 4
Na_2SnF_3	6	134 ± 3
Control	6	134 ± 2

[1] Standard deviation.

water at a concentration of 30 p.p.m of fluoride over an experimental time of 4 months. The level of 30 p.p.m. is about the lowest possible concentration of fluoride that can be used to reduce experimental dental caries in rats significantly. To compare effects of chronic fluoride intoxication on the serum cholesterol level, the concentration of 30 p.p.m. of fluoride in drinking water administered to rats would be greatly in excess

of the 1 p.p.m. of fluoride used in an artificially fluoridated drinking water supply.

The data obtained from the rats receiving 30 p.p.m. of fluoride in drinking water did not indicate a significant increase in the serum cholesterol level at the end of an experimental period of 140 days. These data are seen in table 4. The level is greater when sodium fluoride was fed, and lower for stannous fluoride. The increase in serum cholesterol when sodium fluoride was fed is not significantly different from the controls. The reason for these differences between sodium fluoride and stannous fluoride is not known at this time.

The data obtained from all three series clearly indicate that the administration of fluoride by stomach tube or in the drinking water at high levels over periods of 4 weeks to 4 months may result in some initial changes in the serum cholesterol levels in the rat, but no permanent increase was found.

SUMMARY

The effect of fluoride administration in the rat on the serum cholesterol level was studied. Doses of 0.5, 1.0, or 2.0 mg of fluoride daily by stomach tube over an experimental time of 4 to 8 weeks did not result in an increase of serum cholesterol. A level of 30 p.p.m. of fluoride provided in the drinking water over a period of 4 months similarly did not increase the normal serum cholesterol level of rats. Fluoride doses as high as 2.0 mg daily in rats receiving a semi-purified 15% fat diet, which is reported to enhance the toxicity of fluoride, were not found to increase the serum cholesterol level. The femora of rats receiving 15% cottonseed oil contained more fluoride than similar rats not receiving the addded dietary fat, but whole carcass analysis did not confirm this observation. The hearts from animals receiving the 15% fat diet were shown to contain more fluoride than similar rats receiving no added dietary fat, while fluoride analysis of the kidneys did not show an increased fluoride retention in the animals receiving the semi-purified 15% fat diet.

LITERATURE CITED

ANONYMOUS 1952 Health Statistics Bulletin, special release No. 20. Springfield, Ill., Bureau of Statistics, Illinois State Department of Health, 1952.

GALLETTI, P., H. R. HELD, H. KORRODI AND T. WEGMANN 1956 Fluor und Schilddrüse. Zeitschrift für Präventivmedizin, Heft, *7:* 285.

MILLER, A. L. 1952 *Congressional Record,* 82 Congress, 2 session, 2805–6, Appendix, A2264–6, 2912.

MILLER, R. F., AND P. H. PHILLIPS 1955 The enhancement of the toxicity of sodium fluoride in the rat by high dietary fat. J. Nutrition, *56:* 447.

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

———— 1957 The effect of vitamin C on skeletal fluoride storage in the guinea pig. J. Am. Dental Assoc. (in press).

MUHLER, J. C., W. H. NEBERGALL AND H. G. DAY 1954 Studies on stannous fluoride and other fluorides in relation to the solubility of enamel in acid and the prevention of experimental dental caries. J. Dent. Res., *33:* 33.

PEARSON, S., S. STERN AND T. H. MCGAVACK 1953 A rapid accurate method for the determination of total cholesterol in serum. Anal. Chem., *25:* 813.

THE QUANTITATIVE EFFECTS OF CHOLESTEROL, CHOLIC ACID AND TYPE OF FAT ON SERUM CHOLESTEROL AND VASCULAR SUDANOPHILIA IN THE RAT[1]

D. M. HEGSTED, S. B. ANDRUS, A. GOTSIS AND O. W. PORTMAN
Department of Nutrition, Harvard School of Public Health, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts

(Received for publication May 13, 1957)

Of the many nutritional factors which have been implicated in the "atherosclerosis problem" perhaps most current interest is directed to the effect of the kind and amount of dietary fat. High-fat diets are believed to be associated with higher serum cholesterol levels and the development of atherosclerosis and coronary heart disease (Keys et al., '50; Keys, '56). Under comparable conditions the administration of certain liquid vegetable oils produces lower serum cholesterol levels than more solid fat, both in man (Groen et al., '52 Kinsell et al., '53; Ahrens et al., '54; Beveridge et al., '56; Bronte-Stewart et al., '56) and animals (Aftergood et al., '56; Portman et al., '56). It may be suspected upon this basis that either total unsaturation of the fatty acids, the "essential fatty acids" *per se*, or some relation between the latter and some other component of the fats, is the active factor in preventing hypercholesteremia. The length of the carbon chains, specific fatty acids, conjugation of double bonds, iso-

[1] Supported in part by grants-in-aid from the John A. Hartford Memorial Fund; the National Heart Institute (no. 136), National Institutes of Health, Bethesda, Maryland; Life Insurance Medical Research Fund, New York; Albert and Mary Lasker Foundation, New York; Nutrition Foundation, Inc., New York and the Fund for Research and Teaching, Harvard School of Public Health.

meric forms, and melting points of the fats are some of the factors which may be of importance in this relationship to cholesterol metabolism. The ratios of such factors rather than absolute amounts present in fat may also be of primary significance.

Rapid progress in this field may be aided by (a) the use of purified isolated or synthetic materials and (b) the development of a reasonably rapid and accurate assay for the active material. The whole history of nutrition research is replete with examples of the virtual necessity of finding quantitative bioassays for activity before substantial progress can be made in the isolation and identification of active components in biological materials. The cost in time and materials and the general inaccuracy of assays using human subjects are well known. Once the identification of the active material is achieved by animal assays, tests must be done with appropriate human subjects to assure that the bioassay does indeed measure the substance of interest.

Studies in experimental atherosclerosis have been curiously lacking in serious attempts to develop the kinds of assays so clearly needed. Even with the well known cholesterol-fed rabbit the quantitative response to different levels of cholesterol has received but little study. Recent work with the rabbit has done much to define the hypercholesteremic response in this species in terms of levels of dietary cholesterol as well as the individual variability and that due to sex (Fillios and Mann, '56). There is a need to pursue this type of standardization of technique.

The production of hypercholesteremia and atherosclerotic lesions in the rat has been reported from this laboratory by Fillios et al. ('56). The dietary regimens employed were rigorous, resulting in grossly demonstrable lesions in the aorta and heart valves of all animals. The need for less extreme conditions was appreciated at that time and the production of elevated serum cholesterol values and atherosclerotic lesions with lower dietary cholesterol levels and in

the absence of thiouracil has since been observed (Fillios and Andrus, '57).

This paper presents data upon which it is believed a rapid rat bioassay for the factor(s) in oils which affect serum cholesterol levels and the development of early vascular lesions may be based. Insofar as the data are available from preliminary tests, the activity of oils in this assay is similar to that observed in assays upon human beings, thus suggesting that the activity measured may have relevance in human nutrition. The amount of lipid deposition under the endothelium of the hearts and aortas of the animals tested also correlates closely with the levels of serum cholesterol produced.

EXPERIMENTAL

Three separate studies are reported. Male albino rats [2] weighing from 250 to 300 gm were used. They were housed in group cages and fed the experimental diets and water ad libitum. The basal diet contained 10% casein, 5% salt mixture (Hegsted et al., '41), 5% celluflour, 0.3% choline chloride, 59.6% glucose and 20% fat. Supplements of vitamins were added as previously described (Fillios and Mann, '54). Cholesterol [3] and cholic acid [4] were added in crystalline form in the amounts specified below. The animals were bled from the tail at intervals during the study and by cardiac puncture when the study was terminated. Serum cholesterol was determined by a modification (Carpenter et al., '57) of the method of Albers and Lowry ('55) which gives excellent agreement with the method of Abell et al. ('52) and in which only 0.02 ml of serum is required.

At the end of each experiment the hearts and aortas were stained for lipid and evaluated as previously described (Fillios et al., '56), without prior knowledge of the dietary treatment

[2] The rats were obtained from the Charles River Breeding Laboratories, Boston, Mass.

[3] Crystalline cholesterol was kindly furnished by Armour & Company.

[4] Cholic acid was furnished through the courtesy of Mr. Paul deHaen of the Miles Ames Research Division, Elkhart, Ind.

or serum cholesterol levels of the animals. For greater ease in handling the data statistically, the originally recorded degrees of Sudanophilia of 1 to 4 + were multiplied by a factor of 10. It should be emphasized that many normal rats will show traces of gross Sudanophilia in the region of the aortic valve and subjacent septum. Such spontaneous Sudanophilia in individual rats does not exceed 5. We have not seen spontaneous Sudanophilia in the aorta *per se* or in the coronary arteries.

RESULTS

In experiment I 8 different fats were tested using a diet containing 1% cholic acid and 3% cholesterol. Ten rats were included in each group and serum cholesterol determinations were made at two, 4, 8, and 12 weeks. Half of the animals from each group were sacrificed after 8 weeks on experiment and the remainder after 12 weeks. The various fats tested and their approximate iodine numbers together with the experimental data obtained are shown in table 1.

The oils may be divided into three broad groups either on the basis of serum cholesterol values or degree of endothelial Sudanophilia. Tung oil produced very high cholesterol values and marked Sudanophilia; sardine and cottonseed oil yielded low values, and the remaining oils fall in between these extremes. With the marked exception of tung oil, there is a tendency for the serum cholesterol values to be inversely related to the iodine numbers of the dietary fats (degree of unsaturation). However, there are other exceptions and the effect of tung oil makes it clear that unsaturation *per se* is not the factor controlling the serum cholesterol level. Tung oil, not an edible oil, was fed because of its high content of eleostearic acid, an acid containinig three conjugate double bonds (e.g., $-C=C-C=C-C=C-$).

When the oils are arranged in descending order of the serum cholesterol values produced, correlation analysis by ranks (Spearman's formulae, Snedecor, '37b) indicates that, in spite of obvious overlaps, the rankings by cholesterol values

and vascular response are similar to a significant degree ($p < 0.05$). When the degree of vascular Sudanophilia of individual animals is correlated with their mean serum cholesterol values, a product-moment correlation coefficient of 0.61 is obtained, $p < 0.01$ (Snedecor, '37a). Thus, it appears likely that, under a variety of conditions, the serum cholesterol values are causally related to the degree of vascular Sudano-

TABLE 1

Serum total cholesterol and endothelial Sudanophilia in rats fed different oils and fats at 20% by weight of the diet for 12 weeks. Cholesterol was fed at a 3% level and cholic acid at a 1% level

DIETARY FAT	I_2 [1] NO.	NUMBER OF ANIMALS	MEAN SERUM [2] TOTAL CHOLESTEROL	MEAN ENDOTHELIAL SUDANOPHILIA
			mg %	
Tung	190–194	8	1130	43
Coconut	10	10	553	13
Hydrogenated cottonseed	71 [3]	10	461	13
Butter	30	9	453	11
Linseed	180	9	419	13
Corn	123	10	392	16
Cottonseed	110	7	308	8
Sardine	160–190	9	299	6

[1] All I_2 numbers except that for hydrogenated cottonseed oil from Oil and Soap, *21:* 197, 1944 (Mattil, W. H., Fish oil in the protective coating field).

[2] Average of 8 and 12 weeks.

[3] Courtesy of the laboratories of Swift and Company.

philia produced. (Additional data of this nature are presented in experiments II and III.)

In experiment II the quantitative effects of cholic acid-cholesterol combinations were studied with two different oils, cottonseed oil and hydrogenated cottonseed oil. Three different levels of cholic acid and three different levels of cholesterol in all combinations were used. There were thus 9 groups of 6 animals which received each oil. The amounts of cholic acid and cholesterol used in the various groups together with the mean serum cholesterol values (averages of two, 4, 8, and

12 weeks' bleedings of 6 rats) and mean heart Sudanophilia are shown in table 2. The serum cholesterol values obtained at intervals are plotted in figures 1 and 2. From these figures the relatively greater effect of cholic acid compared to dietary cholesterol upon the serum cholesterol values is evident. It can also be seen that higher serum cholesterol values were obtained with the hydrogenated oil compared to the natural oil at equal levels of cholesterol and cholic acid. The peak in the serum cholesterol values obtained at 4 and 8 weeks in this experiment was not observed in a previous

TABLE 2

Mean serum cholesterol responses and mean endothelial Sudanophilia (see text) of rats fed different levels of cholesterol and cholic acid for 12 weeks

	DIETARY LEVEL OF CHOLESTEROL	DIETARY LEVEL OF CHOLIC ACID		
	%	0.15%	0.45%	1.35%
Hydrogenated cottonseed oil				
Mean serum cholesterol,[1] mg %	0.45	268	450	572
Mean endocardial Sudanophilia	0.45	6	9	10
Mean serum cholesterol, mg %	1.35	267	518	616
Mean endocardial Sudanophilia	1.35	7	13	13
Mean serum cholesterol, mg %	4.00	323	541	821
Mean endocardial Sudanophilia	4.00	7	8	14
Cottonseed oil				
Mean serum cholesterol, mg %	0.45	132	194	300
Mean endocardial Sudanophilia	0.45	3	7	8
Mean serum cholesterol, mg %	1.35	158	301	375
Mean endocardial Sudanophilia	1.35	6	8	9
Mean serum cholesterol, mg %	4.00	159	229	426
Mean endocardial Sudanophilia	4.00	6	7	10

[1] Each mean serum cholesterol represents the mean of two, 4, 8, and 12 weeks' serum total cholesterol values for 6 rats.

study (Fillios et al., '56). We have no explanation for this, although in earlier work the serum levels during the first few weeks of experiment have not been as extensively studied.

Since at each level of dietary cholesterol all three levels of cholic acid were fed, and, similarly, at each level of cholic acid all three levels of cholesterol were fed, it is possible to plot

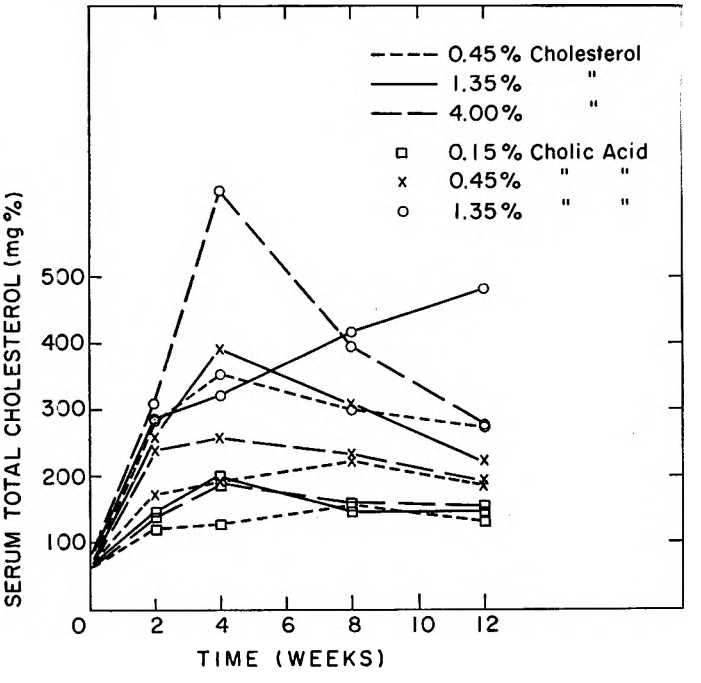


Fig. 1 The effect of different dietary levels of cholesterol and cholic acid on the serum cholesterol levels of rats. The diets contained 20% unhydrogenated cottonseed oil. Each point represents the mean of 6 determinations.

the average response of each of these hypercholesteremic factors as shown in figure 3. Essentially straight-line semilogarithmic plots are obtained, as in most biological assays, and an estimate of the relative potencies of the various materials tested can be made. The lines are not parallel, however, indicating a significant interaction of the cholesterol and cholic acid. This can also be shown by a variance analysis

(Snedecor, '37c) in which the degree of interaction of the cholesterol and cholic acid is found to be of the same order of magnitude for both the hydrogenated and unhydrogenated cottonseed oil. The interaction is to be expected, of course, and is explainable as either an effect of cholic acid upon cholesterol absorption, the demonstrated (Frederickson et al., '54) effect of cholic acid upon cholesterol metabolism, or an

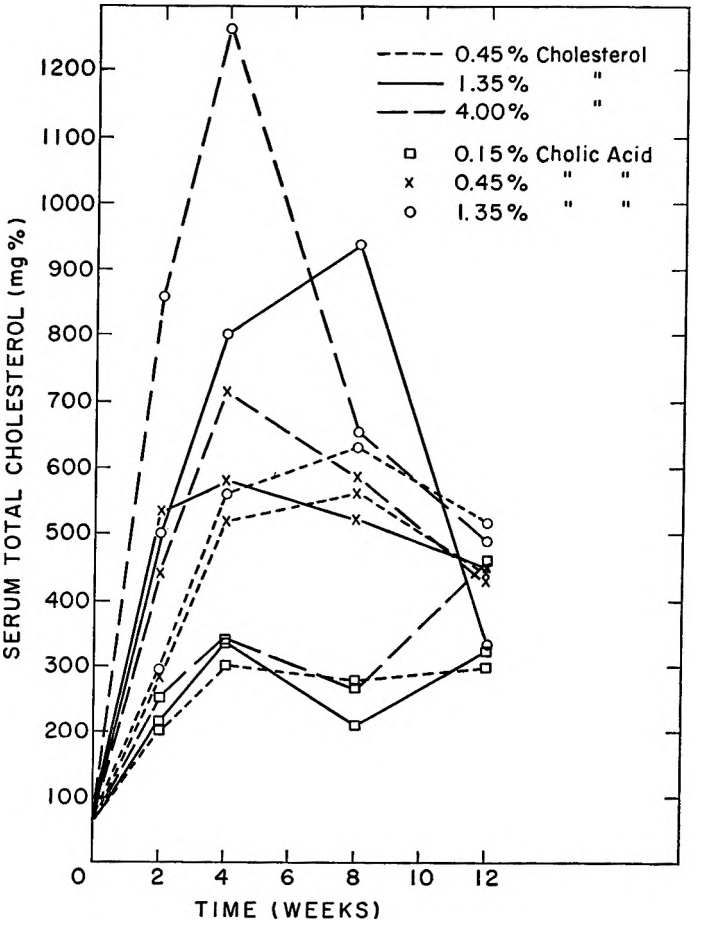


Fig. 2 The effect of dietary levels of cholesterol and cholic acid on the serum cholesterol of rats. The diets contained 20% of a commercial hydrogenated cottonseed oil. Each point represents the mean of 6 determinations.

effect of cholesterol upon cholic acid metabolism. It is possible that all of these phenomena occur.

Nevertheless the relative activities are of some interest. When one compares the unhydrogenated oil with the hydrogenated oil, it can be seen that 4 times as much cholic acid was required to produce a serum cholesterol level of 400

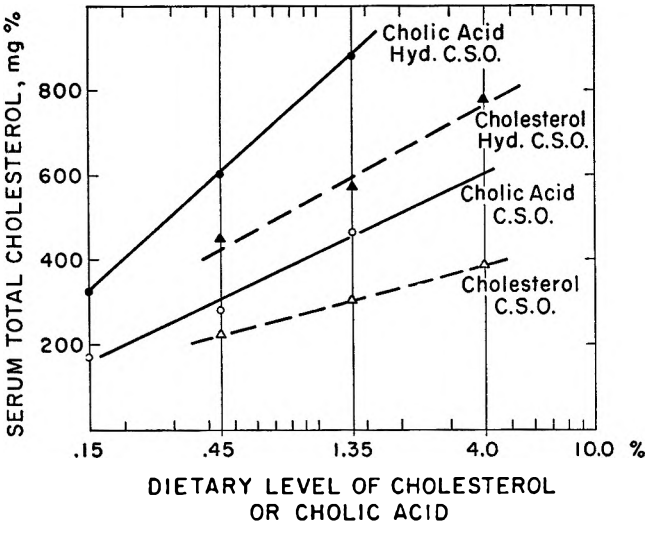


Fig. 3 An evaluation of the individual effects of cholic acid and cholesterol on the cholesteremic response of rats. Each point represents the mean serum total cholesterol of all rats fed the indicated level of cholesterol or cholic acid with all levels (see figs. 1 and 2) of the other component. For example, the upper line designated "cholic acid, Hyd. C.S.O." summarizes the dietary effect of the level of cholic acid on serum cholesterol response in a diet containing 20% hydrogenated cottonseed oil; each point represents a single level of cholic acid and three levels of cholesterol. Note that the dietary levels of cholic acid and cholesterol are plotted on a logarithmic scale.

mg % and about 8.5 times as much to produce a serum cholesterol of 600 mg %. Similarly, 13 times as much cholesterol was needed with the unhydrogenated oil as with the hydrogenated oil to produce a serum cholesterol of 400 mg %. Depending upon the level of serum cholesterol at which comparisons are made, from two to 5 times as much cholesterol are required as cholic acid with either fat tested. The differ-

ences between any two comparisons become increasingly larger as higher serum cholesterol levels are used for the comparison. It is of interest that the response lines in figure 3, if extended, converge at about 60 to 80 mg % of serum cholesterol, an approximately normal value for rats in our laboratory.

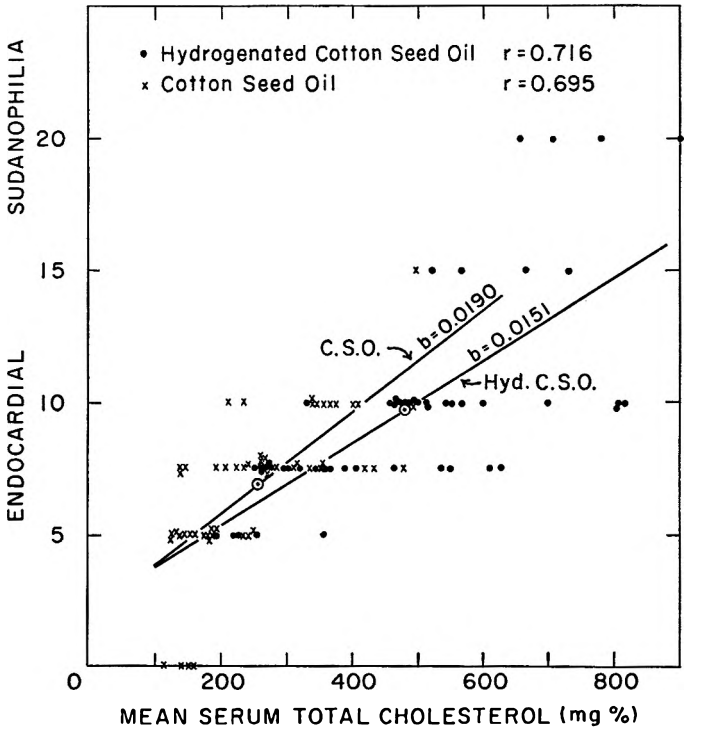


Fig. 4 The relationship between endocardial Sudanophilia (see text) and mean serum total cholesterol (mean of two, 4, 8, and 12 weeks' determinations) of 103 individual rats fed different levels of cholesterol and cholic acid and either hydrogenated or unhydrogenated cottonseed oil.

In comparing the relationship between the serum cholesterol level and the degree of Sudanophilia of the endothelium, the serum cholesterol levels at two, 4, 8, and 12 weeks were averaged for each rat. These averages are plotted against the independently determined vascular Sudanophilia in figure 4. It should be noted that in these animals, unlike

those of the other two experiments herein reported, gross Sudanophilia was limited to the valvular endocardium. The correlation coefficient for the animals fed the hydrogenated oil was 0.72 and for the unhydrogenated oil, 0.70. The slopes of the regression lines fitted by least squares are 0.015 and 0.019, respectively. Thus, it may again be concluded that the

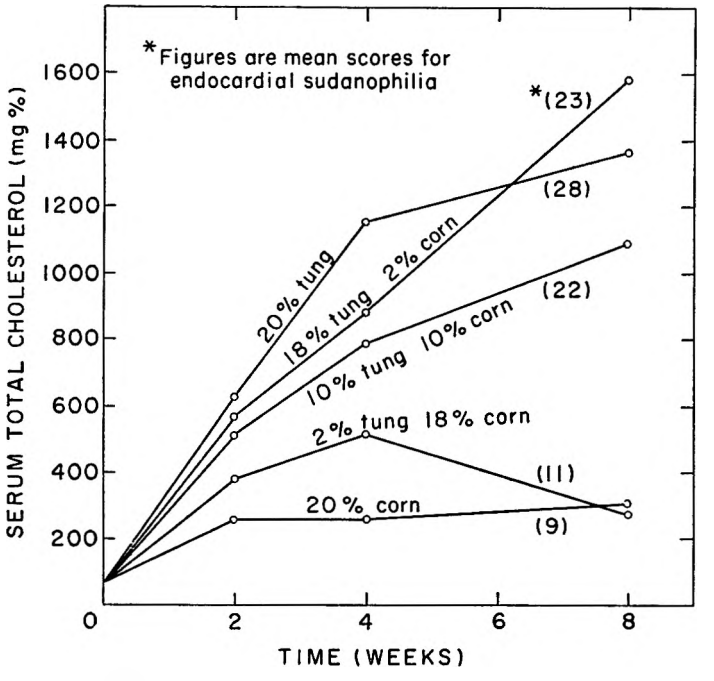


Fig. 5 The effect of graded dietary levels of corn and tung oils on the serum cholesterol response of rats fed diets including 1% cholic acid and 3% cholesterol. Each point represents the mean of 6 determinations.

vascular Sudanophilia is proportional to the serum cholesterol levels under the variety of conditions tested. There is no statistical indication that the two oils tested had any effect upon the lipid deposition directly, the differences being explained by the serum cholesterol levels.

The marked hypercholesteremia observed in experiment I when tung oil was fed was further studied in experiment III.

Graded mixtures of tung oil and corn oil were fed with diets containing 1% cholic acid and 3% cholesterol. There were 6 animals in each group. The oil combinations used, the serum cholesterol values at intervals, and the independently determined grading of endothelial Sudanophilia are indicated in figure 5.

The pronounced effect of relatively small amounts of tung oil in raising the serum cholesterol levels, or conversely, the effect of small amounts of corn oil in counteracting this activity of tung oil is evident. As in the previous experiment the serum cholesterol levels after 4 weeks of experiment appear to give a maximum differentiation between the oil mixtures used, and the results were less consistent at 8 weeks. As in the other experiments the cardiovascular Sudanophilia (8 weeks) correlates with the mean serum cholesterol levels ($r = 0.67$).

DISCUSSION

It is readily apparent from the data presented that the rat receiving a diet containing cholic acid and cholesterol can serve as a relatively sensitive test organism for estimating the hypercholesteremic action of various oils. Within the limits of the preliminary data the differences between oils appear roughly similar to those observed with human test subjects; that is, the unsaturated oils (tung oil excepted) produce lower serum cholesterol levels than do the more saturated oils. This suggests that the active components in the two tests may be the same, but obviously, insufficient data are available at this time to determine the worth of the rat assay in this regard. The mean serum cholesterol levels for each group at 4 weeks in experiment II where 6 animals were used, have a standard error of 3 to 5%. This is probably adequate for most assays but can be readily decreased, if desired, by the use of larger groups of animals. A 4-week test period, or perhaps less, appears optimal under the present conditions although distinctions can clearly be made after shorter intervals. As has been noted, the present animals were all male rats. That the factor of the sex of the assay

animal must be considered has been amply demonstrated (Moskowitz et al., '56; Fillios, '57).

The advantages of a rat assay are readily apparent from the standpoint of the time and work involved, the accuracy of the assay, and the amount of material needed. In these studies approximately 500 gm of oil are needed to feed a group of 6 animals over a 4-week period.

The peculiar effect of tung oil deserves further study. The major component of this oil is eleostearic acid which is characterized by conjugation of the three double bonds present. It is thus an isomer of linolenic acid. Although it may be premature to attribute the hypercholesteremic effect to this configuration, it appears to be the most likely explanation. From experiment III it is apparent that relatively small amounts of corn oil partially counteracted the action of tung oil. Assuming this action is typical of the oils which produce low serum cholesterol levels, another and perhaps more sensitive type of assay is suggested.

It should be noted that the dose-response lines in figure 3 are not parallel and the relative differences between oils depend upon the levels of serum cholesterol compared. This is a limitation of the assay which should be clearly recognized. However, the assay would appear adequate for present purposes, particularly if two levels of cholic acid were included with each oil to be compared.

The consistent relationship between the serum cholesterol levels and the cardiovascular lipid accumulation, as judged by gross Sudanophilia, emphasizes the significance of the serum cholesterol levels. Also, the similarity of this relationship in the presence of different oils indicates that the primary effect of the oil is upon the serum cholesterol level rather than upon the deposition of lipid or the integrity of the arterial wall. That such statements must be confined to the present experimental conditions is emphasized by the recent findings of Vitale et al. ('57). These workers in studying cardiovascular Sudanophilia in rats in relation to magnesium and cholesterol feeding, found no correlation between serum chol-

esterol and vascular Sudanophilia. There are, however, several broad differences between the two groups of experiments. The present animals were adult and while the fat composition of the diet was varied, the magnesium content was maintained constant at levels presumed normal (48 mg %). In contrast the animals in the magnesium studies were weanlings and received one type of dietary fat but with various levels of magnesium.

In the animals previously reported from this laboratory (Fillios et al., '56) the observed microscopic changes formed a spectrum ranging from early lesions evidenced solely by subendothelial Sudanophilia and increased metachromatic ground substance to the other extreme of full blown intimal plaques, characterized by cellular and stromal proliferation. In view of the milder hypercholesteremic stimulus and response of the present animals, as compared with those previously reported, and in view of the relatively short duration of the present experiments, it should be emphasized that most of the lesions herein reported probably represent early changes prior to the formation of intimal plaques. The gross Sudanophilia seen in these animals was generally limited to the aortic and mitral valves and the intervening portion of the septum. A small proportion of all of the animals studied, namely those with the most elevated serum cholesterol levels and the most marked endocardial Sudanophilia did demonstrate similar involvement of the aorta. No comprehensive microscopic study of these animals has been carried out, though a random sampling of the coronary arteries of the most Sudanophilic specimens has demonstrated true early intimal plaque formation. The relationships between serum cholesterol level, time and the development and progression of the aortic and coronary artery lesions is another area in which more quantitative data are needed.

SUMMARY

Marked differences in the effect of various oils on the serum cholesterol levels of rats fed diets containing cholic acid and

cholesterol are readily measured with considerable accuracy. In general, the effects of the oils tested tend to be similar to their reported effects upon serum cholesterol levels in human beings, suggesting that the active factors in the two species may be similar. The rat assay may thus be a useful, rapid, and relatively accurate method for determining the effects of the various constituents of fats upon serum cholesterol levels.

In general, the highly unsaturated oils result in lower serum cholesterol levels. Tung oil, rich in eleostearic acid, is a marked exception and produced the highest serum cholesterol levels of any oil tested. Admixture with corn oil counteracts this hypercholesteremic activity in proportion to the amount of corn oil added.

The effect of varying levels of cholic acid and cholesterol on serum cholesterol levels has been quantitated in two different oils.

With all oils and at various levels of cholic acid and cholesterol, the amount of Sudanophilic material under the endothelium was proportional to the mean serum cholesterol level. The "atherogenic" activity thus appears to be mediated through the serum cholesterol levels.

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.

AFTERGOOD, L., R. B. ALFIN-SLATER AND H. J. DEUEL, JR. 1956 Comparative effect of cottonseed oil and lard on cholestrol metabolism in rat. Federation Proc., *15:* 541.

AHRENS, E. H., D. H. BLANKENHORN AND T. T. TSALTAS 1954 Effect on human serum lipids of substituting plant for animal fat in the diet. Proc. Soc. Exp. Biol. Med., *86:* 872.

ALBERS, R. W., AND O. H. LOWRY 1955 Fluorometric determination of 0.1 to 10 micrograms of cholesterol. Anal. Chem., *27:* 1829.

BEVERIDGE, J. M. R., W. F. CONNELL AND G. A. MAYER 1956 Dietary factors affecting the level of plasma cholesterol in humans: the role of fat. Can. J. Biochem. Biophys., *34:* 441.

BRONTE-STEWART, B., A. ANTONIS, L. EALES AND J. F. BROCK 1956 Effects of feeding different fats on serum cholesterol level. Lancet, *270:* 6922.

CARPENTER, K. J., A. GOTSIS AND D. M. HEGSTED 1957 The estimation of total cholesterol in serum by a micro-method. Clin. Chem., in press.

FILLIOS, L. C. 1957 The gonadal regulation of cholesteremia in the rat. Endocrinology, *60:* 22.

FILLIOS, L. C., AND G. V. MANN 1954 Influence of sulfur amino acid deficiency on cholesterol metabolism. Metabolism, *3:* 16.

———— 1956 The importance of sex in the variability of the cholesteremic response of rabbits fed cholesterol. Circulation Res., *4:* 406.

FILLIOS, L. C., S. B. ANDRUS, G. V. MANN AND F. J. STARE 1956 Experimental production of gross atherosclerosis in the rat. J. Exp. Med., *104:* 539.

FILLIOS, L. C., AND S. B. ANDRUS 1957 Sex and incipient atherosclerosis in rats. Federation Proc., *16:* 356.

FREDERICKSON, D. S., A. V. LOUD, B. J. HINKELMAN, H. S. SCHNEIDER AND I. D. FRANTZ, JR. 1954 Effect of ligation of the common bile duct on cholesterol synthesis in the rat. J. Exp. Med., *99:* 43.

GROEN, J., B. K. TJIONG, C. E. KAMMINGA AND A. F. WILLEBRAND 1952 The influence of nutrition, individuality, and some other factors, including various forms of stress on the serum cholesterol; an experiment of 9 months duration in normal human volunteers. Voeding, *13:* 556.

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.

KEYS, A. 1956 The diet and the development of coronary heart disease. J. Chronic Dis., *4:* 364.

KEYS, A., O. MICKELSEN, E. V. O. MILLER AND C. B. CHAPMAN 1950 The relation in man between cholesterol levels in the diet and in the blood. Science, *112:* 79.

KINSELL, L. W., G. D. MICHAELS, J. W. PARTRIDGE, L. A. BOLING, H. E. BALCH AND G. C. COCHRANE 1953 Effect upon serum cholesterol and phospholipids of diets containing large amounts of vegetable fat. J. Clin. Nutrition, *1:* 224.

MOSKOWITZ, M. S., A. A. MOSKOWITZ, W. L. BRADFORD AND R. W. WISSLER 1956 Changes in serum lipids and coronary arteries of the rat in response to estrogens. Arch. Path., *61:* 245.

PORTMAN, O. W., D. M. HEGSTED, F. J. STARE, D. BRUNO, R. MURPHY AND L. SINISTERRA 1956 Effect of the level and type of dietary fat on the metabolism of cholesterol and beta lipoproteins in the cebus monkey. J. Exp. Med., *104:* 817.

SNEDECOR, G. W. 1937a Statistical Methods. Iowa State College Press, Ames, Iowa, p. 149.

———— 1937b *Ibid.*, p. 164.

———— 1937c *Ibid.*, p. 277.

VITALE, J. J., P. L. WHITE, M. NAKAMURA, D. M. HEGSTED, N. ZAMCHECK AND E. HELLERSTEIN 1957 The effect of feeding an atherogenic diet on magnesium metabolism. J. Expt. Med. (In press)

FURTHER STUDIES ON CHOLINE DEFICIENCY AND MUSCULAR DYSTROPHY IN RABBITS [1]

E. L. HOVE, D. H. COPELAND,[2] J. F. HERNDON AND W. D. SALMON

Department of Animal Husbandry and Nutrition, Agricultural Experiment Station, Alabama Polytechnic Institute, Auburn

(Received for publication April 26, 1957)

A detailed description of choline deficiency in rabbits was given by Hove, Copeland and Salmon ('54) and it was shown by Hove and Copeland ('54) that prolonged deficiency resulted eventually in creatinuria, diminished creatinine excretion, and some hyaline degeneration of striated muscle. Dietary supplements of choline prevented and cured this state of muscular dystrophy as well as other aspects of the deficiency. It is of interest to determine the effectiveness of betaine as a replacement for choline in the diet of rabbits, since the activity of this substance is known to vary with the species of animal under test. For the guinea pig betaine has little or no activity (Reid, '55), while, for lipotropic activity in rats, betaine is completely effective when included in the diet at three times the level of the choline requirement (Young, Lucas, Patterson and Best, '56).

The diet previously used for the production of choline deficiency in rabbits was based upon extracted peanut meal as the main protein source along with a low level of casein. Since methionine had only slight choline-like activity in rabbits (Hove et al., '54), and since Reid ('55) reported that

[1] Supported in part by a grant from the Institute of Neurological Diseases and Blindness (B-430), U. S. P. H. S.

Published with the approval of the Director, Alabama Agricultural Experiment Station. Vitamins used in this study were donated by Merck and Company, Lederle Laboratories, and A. E. Staley Manufacturing Company.

[2] Deceased, June 28, 1957.

guinea pigs fed a 20% casein diet developed a choline deficiency characterized by the absence of fatty livers, it was of interest to feed rabbits a casein diet deficient in choline.

The results of these experiments are reported in this paper, together with some data on the effect of choline deficiency on the blood-clotting time and on the quantitative requirement for vitamin E in rabbits. The use of oxidized casein for the production of a methionine deficiency in rabbits is briefly noted.

EXPERIMENTAL

Three choline-deficient diets have been used. These differed in the source of protein and in the level of fat. Diet R36 contained 36% methanol-extracted peanut meal and 6% extracted casein, with 19% lard. Diet R43-C contained 20% extracted casein, with 9% lard. Diet R14E-C contained 40% methanol-extracted soybean meal, with 6% lard. The balance of these diets was composed of 5% salt mixture,[2] 1% potassium bicarbonate, 10% cellulose (non-nutritive fiber), 1% cod-liver oil, and sucrose to 100%. To this was added 0.01% *dl*,α-tocopheryl acetate and the following pure vitamins as micrograms per gram of diet: thiamine, 5; riboflavin, 5; pyridoxine, 5; calcium pantothenate, 25; i-inositol, 200; niacin, 40; methyl, 1–4, naphthoquinone, 0.3; folacin, 2; and vitamin B_{12}, 0.03. The control diets contained 0.12 or 0.20% added choline chloride.

Weanling 4-week-old California-white rabbits were distributed into groups, housed individually in an air-conditioned room on raised, half-inch screens and fed an appropriate diet. They were weighed thrice weekly and the urinary creatine and creatinine were determined three times a week by previously described methods (Hove and Copeland, '54). At death the animals were examined, tissues saved for histological examination by fixing in Bouin's solution and usually stained with hematoxylin-eosin, and the fat content of the oven-dry liver determined by ether extraction overnight.

[2] W. D. Salmon, J. Nutrition, *33:* 155 (1947).

Clotting time of whole blood taken from the marginal ear vein was determined by noting the number of seconds for a drop of blood on a watch glass to develop strands. In most cases this time was determined with and without the addition of a source of "tissue-factor." All determinations were run in triplicate and at 78°F. The source of "tissue-factor" was a 9 : 1 water-extract of normal rabbit minced lung.

TABLE 1

The effects of betaine and choline on rabbits receiving peanut meal-casein diet

RABBIT NO.	INITIAL BODY WEIGHT	RATE OF GAIN	SURVIVAL TIME	LIVER FAT (dry)	LIVER CIRRHOSIS	URINARY		MUSCLE LESIONS [1]
						Creatine	Creatinine	
	gm	*gm/day*	*days*	*%*	*rating*	*mg/kg/day*	*mg/kg/day*	
(Without supplement)[2]								
122	440	3.4	120	57.3	4	41.0	33.8	mild
123	370	0.6	147	40.0	4	67.8	24.4	severe
125	440	2.8	140	37.7	2	26.0	31.0	mild
220	430	2.7	142 [3]	49.1	4	40.3	26.0	moderate
222	480	4.3	142 [3]	54.8	4	32.4	24.3	..
Av.:		2.8		47.7		41.5	27.9	
(With 0.3% betaine hydrochloride)[2]								
218	450	14.1	129 [3]	31.8	2	16.2	40.8	trace
219	530	11.5	129 [3]	21.7	3	25.4	36.2	none
221	480	13.4	134 [3]	40.9	2	22.2	40.9	trace
223	410	15.4	125 [3]	24.0	2	15.0	39.7	none
Av.:		13.6		32.1		19.7	39.3	
(With 0.12 choline chloride)								
126	460	21.0	100 [3]	7.9	0	8.6	43.2	none
121	510	21.3	100 [3]	12.8	0	7.0	46.0	none
217	750	22.0	114 [3]	7.9	0	11.2	38.8	none
Av.:		21.4		9.4		8.9	42.7	

[1] Muscle lesions refer to hyaline degeneration in the *femoris triceps.*

[2] All animals on this treatment showed moderate to severe myocardial and endocardial degeneration. Little or no ceroid was present in the cirrhotic livers, and only mildly diffuse bile duct proliferation was seen.

[3] Killed.

RESULTS AND DISCUSSION

Rabbits fed diet R36 (peanut meal + casein), without choline for long periods of time showed poor growth, fatty and cirrhotic livers, high urinary creatine, low urinary creatinine, and histologically defined hyaline degeneration of striated muscle (table 1). Rabbits fed this diet supplemented with 0.3% betaine hydrochloride grew much better, although not at the normal rate, and had normal creatinine and only slightly elevated urinary creatine. On gross inspection the striated muscles of these animals were full-bodied, but had a glassy, translucent, pale greenish-white cast that appeared abnormal. However, no clear evidence of degeneration or abnormality was noted upon histological examination of these tissues. The livers were fatty and cirrhotic; all of the animals in this group had marked cardiac damage characterized as myocardial degeneration, fat in the endocardium, and some valvular degeneration with calcification in a few cases. In spite of the obvious damage to the hearts none of these rabbits died spontaneously.

With 0.12% choline chloride added to the above diet, all animals were normal. The data in table 1 indicate that 0.3% betaine hydrochloride in the diet was too low a level to replace choline completely. Betaine gave effective growth responses and protected striated muscle against histopathology, but it was much less effective than choline in preventing damage to the liver and heart.

Rabbits fed diet R43-C (20% casein) readily developed a choline deficiency (table 2). Only two of the 7 animals fed this diet failed to develop clinical muscular dystrophy (or paralysis), and these two rabbits had died at a relatively early age with marked hydrothorax, ascites, and edema. It is of interest to note that these two rabbits had essentially normal fat in the liver. This is reminiscent of the response of guinea pigs to a similar choline-deficient diet (Reid, '55). Two others of the 7 rabbits had severely fatty livers and cirrhosis; the remainder had moderate cirrhosis and only slightly elevated liver fat. Little correlation existed between the

TABLE 2

Choline deficiency in rabbits fed the 20% casein diet R43-C

RABBIT NO.	INITIAL BODY WEIGHT	RATE OF GAIN	SURVIVAL TIME	GROSS MUSCLE ABNORMALITIES	LIVER FAT (dry basis)	HISTOPATHOLOGY [1]		
						Liver cirrhosis	Muscle lesions	Remarks
	gm	*gm/day*	*days*	*ruling*	%			
(Without choline)								
264	260	4.7	71	0	11.5	moderate	trace	edema, jaundice, ascites, hydrothorax
265	510	2.3	143 [2]	3	16.9	moderate	mild	incoordination, head retraction
266	310	5.2	170	3	43.1	severe	mild	total hindquarter paralysis, edema
267	390	2.8	60	0	11.2	none	none	edema, ascites, hydrothorax
270	320	4.1	133 [2]	2	20.7	moderate	none	no edema
271	580	3.7	133 [2]	3	50.1	severe	moderate	no edema
274	490	2.6	128 [2]	4	14.6	moderate	none	total paralysis, no edema
(With 0.20% choline chloride)								
268	410	11.3	133 [2]	0	12.3	some atypical fatty changes in liver with occasional zonal necrosis; normal muscle		
269	380	18.2	133 [2]	0	8.9			
272	510	7.9	133 [2]	0	14.8			

[1] Extensive bile duct proliferation accompanied the liver cirrhosis but little or no ceroid was noted. The muscle lesions were hyaline degeneration in the *femoris triceps*.

[2] Killed.

clinical state of the muscular dystrophy or other gross muscle abnormalities, and the histologically demonstrable lesions in the striated muscle. As an example, rabbit 274 was unable to move or to right itself and was classified as grade 4 dystrophy; yet no evidence of hyaline degeneration was noted on histological examination. Urinary creatine was not determined on these animals. Obviously the explanation must be that

TABLE 3

Prolonged blood clotting time in choline-deficient rabbits fed 40% soybean meal diet R14 E-C

RABBIT NO.	INITIAL BODY WEIGHT [1]	BLOOD CLOTTING TIME					LIVER FAT (dry basis) (98 days)
		0 days	21 days	28 days	95 days		
					Direct	With "tissue-factor"	
	kg	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*%*
291	2.25	45	31	37	70	17	18.0
292	2.43	17	33	165	120	16	31.4
293	2.59	34	21	78	125	17	29.2
298	1.31	22	24	93 [2]	30	11	28.0
300	1.65	24	75	120 [2]	20	11	31.4
302	1.89	24	144	137 [2]	44	13	28.0
372 [3]	2.12	18	..	14	13	9	7.8
373 [3]	1.76	20	..	17	18	13	6.4

[1] These rabbits had previously been fed a complete casein-basal diet for 66 days from weaning.

[2] Choline supplements of 50 mg/day by dropper started at this time.

[3] Control rabbits fed diet R14E, with choline added at 0.12%.

the paralysis was neurologically induced. However, the brain acetylcholine in such rabbits was lowered to only a slight, and statistically insignificant, extent.[3] On the other hand, the acetylcholine content of brain of rabbits paralyzed due to a vitamin E deficiency was depressed to a significant degree.

It is apparent from the data in table 2 that even with supplementation of choline, the 20% casein diet was not completely adequate for the rabbit; the growth rate of the con-

[3] Hove, E. L., and J. F. Herndon, unpublished data.

trols was not at a maximum level, and some minor liver changes were seen.

Effect of choline deficiency on blood clotting time. A group of 8 control rabbits that had been fed the theoretically com-

TABLE 4

Increased vitamin E requirement in choline-deficient rabbits fed 40% soybean meal diet R14-C or R14

(Initial body weight was 500 to 620 gm)

RABBIT NO.	CHOLINE CHLORIDE IN DIET	TIME TO INITIAL DYSTROPHY	DOSE OF DL, α-TOCOPHERYL ACETATE [1]	BODY WEIGHT AT TIME OF DOSAGE	DAYS CURED OF CREATINURIA	CALCULATED REQUIREMENT FOR VITAMIN E
	%	*days*	*mg*	*kg*	*days*	*mg/kg/day*
1075	0.12	45	20	1.42	19	0.74
			20	1.85	25	0.43
			20	2.13	22	0.43
1073	0.12	26	20	1.44	47	0.30
			20	2.37	27	0.31
1072	0.12	33	20	1.35	35	0.42
			30	1.97	34	0.45
	Av.:					0.44
		35				
1071	0	29	20	0.82	17	1.44
			20	1.01	11	1.80
			20	1.12	13	1.37
			20	1.29	10	1.55 (Died)
1069	0	44	20	0.92	21	1.03
			20	0.99	19	1.06
			20	1.13	18	0.99
1068	0	45	20	1.25	26	0.62
			20	1.46	14	0.98
			20	1.62	16	0.77
						1.16
	Av.:	39				

[1] These doses were administered when the creatine excretion had reached 80 mg/day.

plete diet R43 (20% casein diet with added choline at 0.2%) was transferred in early adulthood to the 40% soybean meal diet without choline, R14E-C, or to this diet supplemented with choline chloride, diet R14E. Blood clotting time was determined at this point and at intervals thereafter (table 3). After 28 days on the diets, three of the deficient rabbits were supplemented with oral doses of 50 mg choline chloride/day.

Gradual development of a prolonged blood clotting time is apparent from the data in table 3. The clotting time returned nearly to normal in the three rabbits supplemented with choline, even though their livers were still fatty and cirrhotic. The addition of a source of "tissue-factor" resulted in essentially normal clotting times regardless of the severity of the bleeding tendency. These observations indicate that the prolonged clotting time in choline deficiency can not be explained as inadequate absorption of vitamin K from the intestines. Perhaps the explanation may be found in the inadequate formation of the choline-containing phospholipids that make up the "tissue-factor" required in the blood clotting mechanism.

The effect of choline deficiency on the vitamin E requirement. A group of 6 weanling rabbits were fed 40% soybean meal diets with or without choline, diets R14 or R14-C. Both of these diets were deficient in vitamin E, while only one was deficient in choline. The development of muscular dystrophy was followed by measuring the daily creatine excretion. When this had increased to 80 mg/kg body weight, the animals were adjudged dystrophic and were dosed orally with a standard solution of vitamin E (20 mg of *dl*,α-tocopheryl acetate in olive oil was the usual dose). From the body weight at dosage and from the number of days cured of the creatinuria associated with the muscular dystrophy, the values for the vitamin E requirement could be calculated. These are given in table 4. Seven determinations on the three rabbits receiving choline showed an average need of 0.44 mg of *dl*,α-tocopheryl acetate/day/kg body weight. When the rabbits were deficient

in choline, the vitamin E requirement was increased to 1.16 mg of the tocopheryl acetate/day/kg body weight. The increased requirement for vitamin E by choline-deficient rabbits was mentioned by Hove and Copeland ('54).

None of the rabbits on the soybean meal basal diet developed obvious clinical muscle abnormalities due to the prolonged simple choline deficiency. In general, this diet produced less severe choline deficiencies than either of the other two diets used. This was indicated by better growth rates and the absence of spontaneous deaths, as well as by less severe lesions in liver and heart. Perhaps the presence of a better balance of amino acids, such as arginine and glycine or of other less well defined growth factors in the soybean meal, lowered the choline requirement of the rabbit.

Methionine deficiency in rabbits. A methionine deficiency in rabbits was produced by feeding a diet similar to the casein diet R43, except that it contained casein oxidized by the method of Toennies ('42) and supplemented with 0.3% DL-tryptophan. Young rabbits of 400 to 500 gm body weight were used. Two rabbits fed the oxidized-casein diet maintained their body weight for about 50 days after which gradual weight loss began. When these animals died at 68 and 99 days on experiment, their average weight loss was 120 gm. During the last two weeks of life these rabbits developed a paralysis accompained by a creatine excretion as high as 40 mg/day/kg body weight (during the first 50 days the creatine excretion had averaged 5 mg/day). The creatinine excretion averaged 21.0 mg/day/kg. Histologic sections revealed severe hyaline degeneration of striated muscle.

Two other rabbits were fed this diet to which 0.6% methionine had been added. In 83 days these animals had gained 730 gm and were without gross pathology or symptoms when killed. Similarly, two more rabbits were fed the basal oxidized-casein diet to which 0.6% homocystine had been added. This material effectively substituted for methionine, since the average weight gain was 655 gm.

SUMMARY

Choline deficiency in rabbits fed the peanut meal-casein basal diet was characterized by poor growth, early death, fatty and cirrhotic liver, badly damaged heart muscle and valves, high creatine and low creatinine excretion, and hyaline degeneration of striated muscle. Betaine hydrochloride added to the diet at the 0.3% level markedly improved growth, prevented deaths and the muscle damage, but did not prevent heart and liver damage at the level fed. Choline chloride at 0.12% gave complete protection. This diet contained an adequate level of α-tocopherol.

Choline deficiency in rabbits fed the 20% casein basal diet was characterized by edema, hydrothorax, ascites, and early death. About one-half of the cases had approximately normal liver fat and only mild to moderate liver cirrhosis. Incoordination, paralysis, and head retractions were common, but they did not necessarily correlate with the severity of the histopathology of striated muscle.

Choline-deficient rabbits had a prolonged blood clotting time. Choline-deficient rabbits required 1.16 mg *dl*,α-tocopheryl acetate/day/kg, as compared with choline supplemented controls which required 0.44 mg/day/kg. As previously shown, however, 10-fold the normal level of vitamin E will not protect against the muscular dystrophy and creatinuria produced by choline deficiency.

Methionine deficiency in rabbits resulted in moderate weight-loss, death, creatinuria, paralysis and severe hyaline degeneration of striated muscle. Supplements of methionine or of homocystine were equally effective in preventing these deficiency symptoms.

LITERATURE CITED

HOVE, E. L., AND D. H. COPELAND 1954 Progressive muscular dystrophy in rabbits as a result of chronic choline deficiency. J. Ntutrition, *53:* 391.

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

HOVE, E. L., AND J. F. HERNDON 1956 Acetylcholine concentration in tissues of rabbits with nutritional muscular dystrophy. Unpublished data.

REID, M. E. 1955 Nutritional studies with the guinea pig. III. Choline. Ibid., *56:* 215.

TOENNIES, G. 1942 Oxidative conversion of casein into protein free of methionine and tryptophane. J. Biol. Chem., *145:* 667.

YOUNG, R. J., C. C. LUCAS, J. M. PATTERSON AND C. H. BEST 1956 Lipotrophic dose-response studies in rats: comparison of choline, betaine, and methionine. Canadian J. Biochem. Physiol., *34:* 713.

DIETARY COPPER SALTS AND AZO DYE CARCINOGENESIS [1]

HENRY J. KING, J. D. SPAIN [2] AND C. C. CLAYTON
Department of Biochemistry, Medical College of Virginia, Richmond

(Received for publication April 29, 1957)

INTRODUCTION

Sharpless ('46) reported that increasing the copper levels in the diet of rats fed the carcinogen, 4-dimethylaminoazobenzene, resulted in an increase in the induction time for liver tumor formation. Pedredo and Kozelka ('51), using the more potent carcinogen, 3'-methyl-4-dimethylanimoazobenzene, confirmed his results.

In the experiments to be described the method of measurement of carcinogenicity was different in that all the animals were fed the carcinogen for the same length of time and the percentage of rats that had developed liver tumors at the conclusion of the experiment was ascertained. Analyses of the liver were also made to note the effect of dietary copper salts upon liver azo dye and liver riboflavin.

During the course of these studies it was found that the increased level of copper salts in the diet resulted in destruction of azo dye in the diet. Certain procedures to minimize or to circumvent this dye destruction are described.

[1] A portion of a thesis submitted by H. J. K. in partial fulfillment of the requirements for the M.S. degree. Presented in part at the annual meeting of American Society of Biological Chemists, Chicago, Illinois. Federation Proc., *12:* 190 (1953).

Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and by research grant C-1541 of the National Cancer Institute of the National Institutes of Health, Public Health Service.

[2] Present address: Department of Chemistry, Michigan College of Mining and Technology, Houghton.

METHODS

Male rats [3] weighing approximately 200 gm were fed the carcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-m-DAB) at a dietary level of 0.064% for 8 weeks and then fed the basal diet dye-free for an additional 8 weeks after which time the animals were killed and the incidence of liver tumors was determined grossly or microscopically or both. In some experiments the 8-week dye-feeding period was interrupted at 4 weeks for a 4-week period on the dye-free diet with varying levels of copper to determine the effect of the metal salt between two dye feeding periods (Clayton and Baumann, '49). The basal diet was similar to that used previously (Clayton and Baumann, '49; Rusch et al., '45) and had the following composition; glucose monohydrate [4] 79, vitamin-free casein 12, Wesson's salts ('32) 4, and corn oil 5%. Vitamins were added such that each kilogram of diet contained 3 mg thiamine hydrochloride, 7.5 mg calcium pantothenate, 2.5 mg pyridoxine, 2 mg riboflavin, and 1 gm choline chloride. Halibut liver oil was given every two weeks by dropper. Distilled water was supplied for drinking. The Wesson's salts were compounded from analytical grade components to reduce copper contamination from this source. Three different levels of copper were fed. The low-copper diet had no copper salts added (omitted from Wesson's salts) and the diet was found to contain 1 mg of copper per kilogram of ration.[5] The normal diet was that with the amount of copper usually added in the Wesson's salts ($CuSO_4$ equivalent to 3.9 mg of copper per kilogram of diet). The high-copper diet had cupric sulfate added equivalent to a level of 300 mg of copper per kilo of diet. The animals were usually housed 5 to a cage in cages constructed of stainless steel with no. 2-mesh wire bottoms.

Analyses were made of livers of rats fed the diets with the three different amounts of copper, with and without the azo

[3] Holtzman.

[4] Cerelose.

[5] Analysis of diet for copper by Crippen and Erlich Laboratories, Inc., Baltimore 2, Maryland.

dye, after varying lengths of time on the diets. Riboflavin was determined fluorometrically and the total and bound liver azo dye by the method of Spain and Clayton ('55).

RESULTS

Tumor experiments. The results confirmed those of Sharpless ('46) and of Pedredo and Kozelka ('51) in that the high level of copper salts in the diet inhibited the development of liver tumors. Thus, in one experiment, the percentage of tumors was reduced from 84% of the animals on the normal-copper diet to 22% of the animals on the high-copper diet. In another experiment the incidence was reduced from 50% to 0. There was no appreciable or consistent difference in tumor incidence between the low and normal-copper diets. Changing the level of dietary copper in the final 8 weeks during the period when the dye was not fed (and thus eliminating the effect of copper on destruction of the dye) indicated that the amount of copper at that time was ineffective in altering the incidence of liver tumors and only the amount of copper present during the dye-feeding period had any effect (table 1, groups 1 to 4). When the dietary level of copper was low during the dye-feeding period, the incidence was 83% whether the final 8 weeks was high or low in copper and the incidence was markedly reduced if the diet was high in copper during the dye-feeding period irrespective of the copper level in the final 8 weeks.

Similarly when the dye-feeding period was interrupted for a 4-week period to feed a diet either high or low in copper, the final incidence of liver tumors was altered only by the level of copper during the dye-feeding period (table 1, groups 5 to 10). When the low level of copper was fed during the two separated dye-feeding periods, the incidence of tumors was 36 and 29% when the basal period between was low and high in copper respectively. With the high-copper diets during the dye-feeding period in this experiment, the incidence was low in all groups, but there was no evidence of a decreased percentage of animals having tumors with the higher level of

copper during the dye-free period. Two other experiments of this type produced comparable results.

Another method of feeding a diet high in copper with a minimum of dye destruction in the diet was to compound fresh diet daily. The diet was prepared in two halves such

TABLE 1

Effect of level of dietary copper during periods when dye[1] is not fed upon incidence of liver tumors

GROUP	REGIMEN (diet and weeks)		SURVIVAL[2]	TUMORS
				%
1	Low Cu[3] + dye (8)[4]	Low Cu basal (8)	11/15	83
2	High Cu[5] + dye (8)	High Cu basal (8)	11/15	9
3	Low Cu + dye (8)	High Cu basal (8)	12/15	83
4	High Cu + dye (8)	Low Cu basal (8)	13/15	23
5	Low Cu + dye (8)	Low Cu basal (8)	13/15	77
6[6]	Low Cu + dye (4)	Low Cu basal (4)		
	Low Cu + dye (4)	Low Cu basal (8)	11/15	36
7	Low Cu + dye (4)	High Cu basal (4)		
	Low Cu + dye (4)	Low Cu basal (8)	14/15	29
8	High Cu + dye (8)	High Cu basal (8)	14/15	7
9	High Cu + dye (4)	Low Cu basal (4)		
	High Cu + dye (4)	High Cu basal (8)	12/15	0
10	High Cu + dye (4)	High Cu basal (4)		
	High Cu + dye (4)	High Cu basal (8)	13/15	15

[1] Dye = 3′-methyl-4-dimethylaminoazobenzene.

[2] Survival is number alive at conclusion of experiment over number at start.

[3] Low copper diet contained 1 p.p.m. of copper.

[4] Weeks of feeding in ( ).

[5] High copper diet contained 300 p.p.m. of copper.

[6] In groups 6, 7, 9 and 10 the 8-week dye feeding period was interrupted at 4 weeks for a 4-week period on the dye-free diet which was high or low in copper content.

that one half contained the salts and glucose and the other half contained the oil, dye, vitamins, casein and the balance of the glucose. Sufficient amounts of these two halves were mixed and placed in clean food cups daily. Even with this precaution, the tumor incidence was still markedly reduced by the high level of dietary copper; i.e. 8 of 14 rats on the diet of normal copper content developed liver tumors while only

one rat of 12 developed liver neoplasia on the high level of dietary copper. Food consumption was comparable between individual groups of a series in all studies.

Analytical results. In a study of the change of total liver azo dye with increasing time of dye feeding, it was found that the liver azo dye of animals on the high-copper diet deviated markedly from that found previously (Miller and Miller, '47;

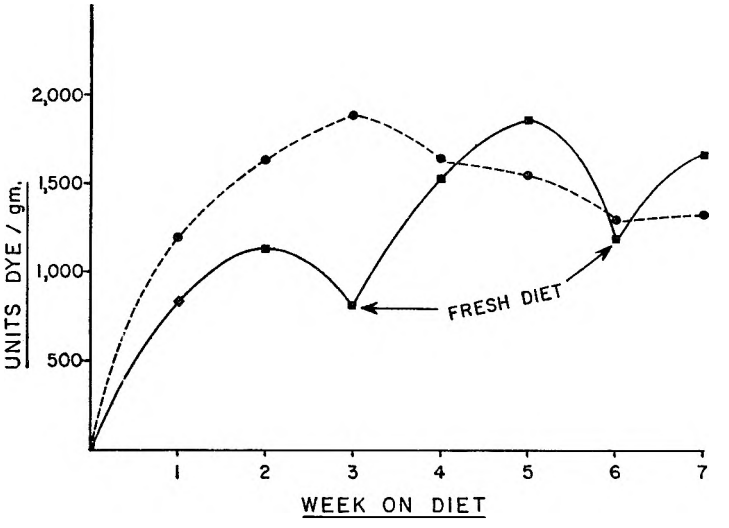


Fig. 1 Change in concentration of total liver azo dye with time.

■———■ Diet high in copper (300 p.p.m. Cu)

●- - - - -● Diet low in copper (1 p.p.m. Cu)

Units of dye per gram fresh weight liver. A unit of dye is equal to the optical density $\times 10^4$.

Miller et al., '49), and also from the findings in this study when the diet was low in copper. The amount of liver azo dye increased for the first two weeks on the high-copper diet, but not as rapidly as that observed in rats fed the lower level of copper (fig. 1). With the diet high in copper there was a decline in the concentration in the liver at three weeks and 6 weeks which rose again sharply the following week. This rise after the decline could be accounted for by the prepa-

ration of fresh diet at the third and 6th week and was the first indication of the dye destruction.

Analysis of the dye in various diets by the method used for analysis of dye in the liver (Spain and Clayton, '55) showed that the effect of high copper was observed most markedly when fat was present and that storage of the diet high in copper at 10°C. markedly delayed the dye destruction as compared to that observed at room temperature (table 2). However, it was also found that a diet of high copper content (and 5% fat) when stored for 7 days at 10°C. (no loss of dye

TABLE 2

Effect of temperature and fat content upon the rate of dye destruction in a diet high in copper salts (300 p.p.m. Cu)

FAT	TEMP.	DAYS AFTER DIET PREPARATION					
		3	7	10	17	24	94
%	°C.	*% change in azo dye content*					
0	10	0	0	0	+ 3	+ 9	— 6
0	20–25	+ 3	+ 3	0	..	— 3	— 3
5	10	+ 6	+ 3	+ 6	+ 3	— 9	— 50
5	20–25	0	— 23	— 44	— 56	— 56	— 62

Dye = 3'-methyl-4-dimethylaminoazobenzene (3'-m-DAB).
Average dye content at start of experiment = 34,000 units/gm.
Units of dye found from optical density: 1 μg 3'-m-DAB = 50 units.

apparent at the time) and then fed to the rats, had a marked loss in dye after being in the food cup for 24 hours.

The total and bound liver azo dye and liver riboflavin concentrations were determined at weekly intervals when the rats were fed the high-copper diet prepared fresh daily as described in the tumor experiments. Even with this precaution to prevent dye destruction, it was found that the high level of copper in the diet did not result in as great a concentration of total or bound dye in the liver and that the riboflavin concentration did not decrease as markedly as when the diet was low in this element (fig. 2). The concentration of liver riboflavin and of total and bound liver azo dye was comparable between the groups on the low and normal copper diets.

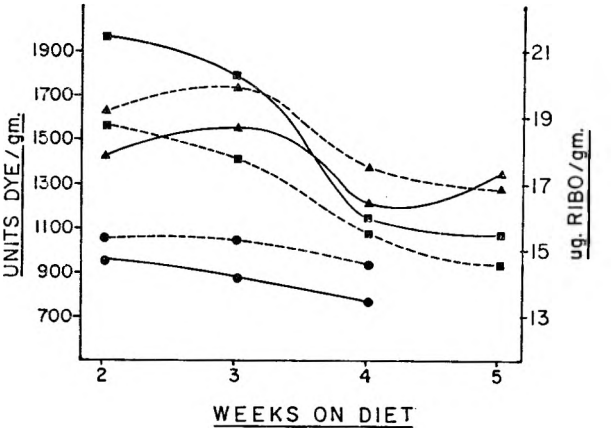


Fig. 2 Changes in concentration of liver total and bound azo dye and liver riboflavin with time when diet is prepared fresh daily.

● bound azo dye ▲ total azo dye ■ riboflavin

– – – – – – – – low-copper diet ———— high-copper diet

DISCUSSION

The effect of dietary copper upon the incidence of liver tumors produced by azo dyes is difficult to evaluate at the present time. It would seem that much of the effect is due to destruction of the dye in the diet. Even when the diet is prepared fresh daily, there is evidence of dye destruction in the food cup and further destruction of the dye in the gut of the rat before absorption is possible. The decreased effect of the azo dye upon liver tumor formation and upon liver azo dye and riboflavin concentration would be compatible with lowered dye intake.

It has been reported that diets containing crude linoleic acid cause a rapid disappearance of azo dye *in vitro* (Gyorgy et al., '42). However, the azo dye in a diet of normal copper content as used in some of the present experiments has been found to be stable for at least 30 days when stored at room temperature (Miller et al., '44).

Copper salts have been reported to hasten the oxidation of fats in the diet, especially in the presence of choline (White et al., '53). Rancidity of the high-copper diet was observed in the present experiments and could be detected by the odor

and consistency of the diet. The rancidity was not noted until after a part of the dye in the diet was destroyed.

It would be premature to state definitely that increased dietary copper does not have an effect upon liver tumor formation. Enzymes which have copper as a component may be involved in carcinogenesis or the higher tissue copper concentration achieved with feeding more copper (Boyden et al., '38) may alter some enzymatic activity associated with neoplasia. The low-copper diet used in this study contained 1 p.p.m. of copper and with a food consumption of 10 gm per day per rat, this would mean a daily intake of copper of 0.01 mg. Since this amount of copper has been found adequate for normal hemoglobin formation in the rat (Schultze et al., '34), it might be that lower levels of the element could influence carcinogenesis.

Since the incidence of tumors induced by azo dyes can be altered to some extent by diet (Baumann, '48), the use of these carcinogens to study the effect of copper upon liver tumor formation appears rational. However until a means is obtained to administer the dye in the presence of high levels of copper without dye destruction, conclusive results cannot be obtained on the effect of dietary copper.

SUMMARY

The incidence of liver tumors in rats resulting from the feeding of 3′-methyl-4-dimethylaminoazobenzene was markedly lowered by the addition of high levels of copper salts (300 p.p.m. of Cu) to the diet. This could be attributed largely to the destruction of azo dye catalyzed by the copper, resulting in actual feeding of a lower dye concentration. Analyses for liver azo dye and riboflavin of rats fed the dye in the presence of high dietary copper showed decreased concentration of the dye and a slower rate of decrease in riboflavin concentration. These results are compatible with decreased dye intake. A diet low in copper (1 p.p.m.) had no effect upon azo dye carcinogenesis or upon liver azo dye or liver riboflavin when compared to a normal diet containing 4 p.p.m. of copper.

ACKNOWLEDGMENTS

We wish to thank Dr. G. Z. Williams and Dr. G. R. Hennigar for the microscopic examination of the livers.

LITERATURE CITED

BAUMANN, C. A. 1948 Diet and tumor development. J. Am. Dietet. Assoc., *7:* 573.

BOYDEN, R., V. R. POTTER AND C. A. ELVEHJEM 1938 Effect of feeding high levels of copper to albino rats. J. Nutrition, *15:* 397.

CLAYTON, C. C., AND C. A. BAUMANN 1949 Diet and azo dye tumors: Effect of diet during a period when the dye is not fed. Cancer Research, *9:* 575.

GYORGY, P., R. TOMARELLI, R. P. OSTERGARD AND J. B. BROWN 1942 Unsaturated fatty acids in the dietary destruction of N,N-dimethylaminoazobenzene (Butter Yellow) and in the production of anemia in rats. Jour. Exptl. Med., *76:* 413.

MILLER, J. A., B. E. KLINE, H. P. RUSCH AND C. A. BAUMANN 1944 The carcinogenicity of *p*-dimethylaminoazobenzene in diets containing hydrogenated coconut oil. Cancer Research, *4:* 153.

MILLER, E. C., AND J. A. MILLER 1947 The presence and significance of bound aminoazo dyes in the livers of rats fed *p*-dimethylaminoazobenzene. Ibid., *7:* 468.

MILLER, E. C., J. A. MILLER, R. W. SAPP AND G. M. WEBER 1949 Studies on the protein-bound aminoazo dyes formed in vivo from 4-dimethylaminoazobenzene and its C-monomethyl derivatives. Ibid., *9:* 336.

PEDRERO, E., AND F. L. KOZELKA 1951 Effect of copper on hepatic tumors produced by 3'-methyl-4-dimethylamino azo benzene. A.M.A. Arch. Path., *52:* 455.

RUSCH, H. P., C. A. BAUMANN, J. A. MILLER AND B. E. KLINE 1945 Experimental liver tumors. A.A.A.S. Research Conf. on Cancer. Science Press, Lancaster, Pa., 267.

SCHULTZE, M. O., C. A. ELVEHJEM AND E. B. HART 1934 The availability of copper in various compounds as a supplement to iron in hemoglobin formation. J. Biol. Chem., *106:* 735.

SHARPLESS, G. R. 1946 The effects of copper on liver tumor induction by *p*-dimethylaminoazobenzene. Federation Proc., *5:* 239.

SPAIN, J. D., AND C. C. CLAYTON 1955 A simplified method for the determination of azo dyes in tissues, urine and feces. Virginia J. Sci., *6:* 88.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

WHITE, P. L., D. M. HEGSTED AND J. MAYER 1953 Two complex salts of choline and copper chloride and their activity as catalysts of fat oxidation. J. Am. Chem. Soc., *75:* 2352.

THE EFFECTS OF VITAMIN DEFICIENCY ON SOME PHYSIOLOGICAL FACTORS OF IMPORTANCE IN RESISTANCE TO INFECTION

IV. RIBOFLAVIN DEFICIENCY

K. F. WERTMAN,[1] R. J. LYNN AND D. T. DISQUE
Department of Biological Science, University of Pittsburgh, Pittsburgh, Pennsylvania

(Received for publication May 25, 1957)

The effects of pyridoxine, niacin-tryptophan, folic acid and vitamin B_{12} deficiencies in white rats on: (1) cellular composition of blood; (2) complement activity; (3) the quantitative and qualitative cellular migration in inflammation; (4) capillary permeability, and (5) the cellular composition of bone marrow have been previously reported (Wertman et al., '53, '54, '55, '56).

The purpose of the present investigation was to perform similar studies in white rats maintained on a well-defined diet that was deficient in riboflavin.

EXPERIMENTAL

Male weanling albino rats of the Sprague-Dawley strain, approximately 21 days old, were employed in this investigation. All animals were housed individually in wide-mesh screen-bottom metal cages provided with glass water bottles and food dishes which were replenished daily. The animals were divided into three groups: ad libitum control, inanition control and riboflavin deficient.

[1] Present address: Department of Bacteriology, University of Arizona, Tucson.

The basal diet was a modification of that employed by Wertman and Sarandria ('51). The ingredients used in the preparation of the basal diet were obtained from commercial sources [2] and were of the highest purity available. The basal diet had the following percentage composition: casein (vitamin-free), 25.00; sucrose, 58.75; hydrogenated vegetable oil, 10; salt mixture no. 2,[3] 4; corn oil,[4] 2; choline chloride, 0.20; i-inositol, 0.03; *p*-amino-benzoic acid, 0.01; *d*-α-tocopherol acetate, 0.01; and 2-methyl-1, 4 naphthoquinone, 0.001.

Each animal was fed one vitamin pill daily prior to receiving the daily ration, which was withheld until the pill was consumed. The pills prepared for the control animals contained the following vitamins in micrograms (Griffith and Farris, '49): thiamine, 40; pyridoxine, 50; calcium pantothenate, 150; niacin, 150; biotin, 1; folic acid, 1; and riboflavin, 60. Riboflavin was omitted from the pills prepared for the riboflavin-deficient animals. Lactose was used as the binder in the preparation of the vitamin supplement.

In addition to the vitamins supplied in the basal diet and supplementary pills, each animal was administered by mouth two drops of haliver oil [5] weekly which supplied 300 U.S.P. units of vitamin A and 24 U.S.P. units of vitamin D.

The animals were maintained on the basal diet and vitamin preparations until weights of approximately 45 gm were attained. Thereafter, the animals in the deficient group received vitamin pills lacking riboflavin. Ad libitum and deficient animals received the diet ad libitum while inanition control animals were fed enough of the basal diet to maintain their weights equal to those of the deficient animals with which they were paired. This approximated the amount consumed by the vitamin-deficient animals. The rats were maintained on the basal diet and vitamin preparations for a period of 4 weeks. At this time, the deficient animals showed the expected signs

[2] General Biochemicals Company, Inc., Chagrin Falls, Ohio, National Biochemicals Company, Cleveland, Ohio.

[3] Nutritional Biochemicals Corp., Cleveland, Ohio

[4] Mazola

[5] Abbott's

of deficiency. Initial and final mean weights for each group appear in table 1.

The day before the rats were to be sacrificed, blood samples for complete blood counts were obtained from all animals by tail bleeding. Standard hematological techniques were employed for these counts, the results of which are recorded in table 2. Following the tail bleedings, each rat was injected intraperitoneally with 10 ml of an inflammation-inciting fluid.

TABLE 1

Body weight changes of rats during riboflavin deficiency

GROUP	NUMBER OF RATS	MEAN WEIGHTS	
		Initial	Final
		gm	*gm*
Ad libitum control	10	42.0	151.8
Inanition control	13	47.8	60.8
Riboflavin-deficient	40	45.8	57.6

TABLE 2

Total red and white cell and differential white cell counts of peripheral blood in riboflavin-deficient and control rats

BLOOD CELLS		DIET		
Type	Count	Ad libitum control (10)[1]	Inanition control (13)	Riboflavin-deficient (40)
		Total count		
Red,	Median	750	800	840
cells $10^4/mm^3$	Range	590–990	620–1,000	570–1,070
White,	Median	18,100	6,100	6,700
cells/mm^3	Range	13,200–20,400	2,300–12,200	3,300–14,100
		Differential count[2]		
		%	%	%
Neutrophiles,	Median	10	13	13
segmented	Range	7–15	11–17	10–24
Lymphocytes,	Median	80	79	72
large and	Range	73–83	71–84	63–79
small	Median	8	8	9
Monocytes	Range	5–14	4–16	4–26

[1] Number of rats given within parentheses.

[2] Eosinophile, basophile and blast cell enumeration were also conducted but did not exceed 1% in any instance and are not included in the table.

This fluid was a mixture of Locke's solution and double strength nutrient broth in a ratio of 85 to 15 on a volume basis.

Twelve hours after the intraperitoneal injections, the rats were anesthetized with ether and exsanguinated by the cardiac puncture technique (Farris and Griffith, '49). The blood specimens so obtained were permitted to clot and the sera were collected, pooled and utilized for the determination of complement activity as described in a previous paper (Wertman et. al., '54). The results of these determinations are recorded in table 3 and stated as the volume of a 1: 6 dilution of pooled sera representing one exact unit of complement.

TABLE 3

Complement activity of sera of riboflavin-deficient and control rats

GROUP	SERUM POOL	COMPLEMENT ACTIVITY E. U.[1]
Ad libitum	1	0.12 ml of 1:6 dilution
	2	0.15 ml of 1:6 dilution
	3–4	0.12 ml of 1:6 dilution
Inanition control	1–4	0.18 ml of 1:6 dilution
Riboflavin-deficient	1–6	0.18 ml of 1:6 dilution
	7	0.24 ml of 1:6 dilution
	8	0.21 ml of 1:6 dilution

[1] One exact unit.

Immediately following the cardiac bleeding, the peritoneal exudates were collected from the anesthetized animals by washing the peritoneal cavities with heparinized Locke's solution (Wertman et al., '54). The exudates plus washings were centrifuged at low speed to remove all the cellular elements which were then resuspended in 2 ml of Locke's solution for the purpose of performing total and differential leucocyte counts. Standard research techniques were employed for the total counts. The differential counts were performed on smear preparations of the cell suspensions which were stained by Wright's method. Two hundred cells were counted on each slide using the method of Menkin ('40, '50) and Maximow and Bloom ('44) for classification. The

results of the total and differential counts of the exudate cells obtained from the inflamed areas appear in table 4.

Samples of each cell-free exudate supernatant were concentrated ten fold by the evaporation of water from dialysis bags as described in a previous paper (Wertman et al., '54) and pooled in groups. In an effort to determine the presence and activity of "leukotaxine" in the concentrated exudate supernatant, Menkin's intradermal dye-accumulation technique was employed (Menkin, '40). Two tenths milliliter of

TABLE 4

Total and differential leucocyte counts of inflammatory exudate in riboflavin-deficient and control rats

EXUDATE CELLS		DIET		
		Ad libitum control (9)[1]	Inanition control (13)	Riboflavin-deficient (40)
Total leucocytes, cells/mm^3 ($\times 10^3$)	Median	18.2	2.6	1.8
	Range	11.2–21.0	1.4–4.2	0.7–6.1
Granulocytes, %	Median	38.5	39.0	44.0
	Range	29–49	29–46	33–53
Lymphocytes, %	Median	20.0	24.5	28.0
	Range	15–26	17–30	19–34
Monocytes, %	Median	41.8	36	29.5
	Range	32–50	37–45	20–35

[1] Number of rats.

pooled exudate concentrate was injected intradermally into rabbit's shaved abdomen along with control injections of saline, heparinized Locke's solution and the inciting fluid. Ten milliliters of sterile 1% trypan blue dye were injected into each rabbit's marginal ear vein immediately after the intradermal injections were completed. The sites of the intradermal injections were observed and measurements of the areas of dye-accumulation made after a period of 15 and 30 minutes.

Following the cardiac bleedings and removal of peritoneal exudates, bone marrow specimens were taken from each rat

by cutting through the proximal end of the tibia and removing a portion of the marrow so exposed. The marrow was mixed with a drop of normal rabbit serum and smeared across a slide. The resultant films were stained with Wright's stain and differential counts made. Three hundred cells were ob-

TABLE 5

Cellular composition of the bone marrow in riboflavin-deficient and control rats

BONE MARROW CELLS		DIET		
		Ad libitum control (9)[1]	Inanition control (13)	Riboflavin-deficient (40)
		%	%	%
Nucleated red cells	Median	63.2	40.7	57.0
	Range	55–70	30–51	44–67
Total granulocytes	Median	24	49.3	34.0
	Range	20–36	36–54	28–41
Metamyelocytes	Median	9.5	29.0	17.5
and segmenters	Range	6–17	16–30	11–27
Myelocytes and	Median	11.5	16.3	15.0
premyelocytes	Range	7–16	11–26	10–25
Lymphocytes	Median	10.5	1.7	2.0
	Range	5–14	0.7–5	0.7–5
Blast cells	Median	1.2	1.3	0.7
	Range	0.3–2.3	0–1.7	0.3–2.7
Monocytes	Median	0.5	0.7	0.8
	Range	0.3–1.7	0.3–2	0.3–1.7
Eosinophiles	Median	3.0	4	1.5
	Range	1–4.7	1–6.3	0–4.3
Plasma cells	Median	0	0	0
	Range	0	0–1	0–2
Mast cells	Median	0.3	1.0	1.0
	Range	0–1	0–2.3	0–2
Unclassified	Median	0.5	2.0	1.0
	Range	0–1.7	0.7–3.3	0.3–3.7

[1] Number of rats.

served on each slide and classified using a modification of the method of Endicott and Ott ('45). The results of these counts appear in table 5.

RESULTS AND DISCUSSION

The results of this investigation indicated no alteration of the peripheral erythrocyte count due to either inanition or

vitamin deficiency. Riboflavin deficiency and inanition resulted in a reduction of the total number of leucocytes in the peripheral blood from a normal value of 18,000/mm^3 to 6,000/mm^3. The total and differential counts are recorded in table 2.

The pooled sera of the riboflavin-deficient animals and the inanition controls demonstrated the same degree of reduction in complement activity. The exact unit of the ad libitum controls was 0.12 ml of a 1: 6 dilution and that of the deficient and inanition animals was 0.18 ml of 1: 6 dilution.

The total leucocyte counts of the inflammatory exudates removed from the inanition and riboflavin-deficient animals demonstrated a marked reduction in number over the ad libitum controls. A median count of approximately 18,000 cells/mm^3 was recorded for the ad libitum animals, while median counts of 1,800 cells/mm^3 and 2,600 cells/mm^3 were noted for the riboflavin-deficient and inanition-control groups respectively. The fact that both riboflavin deficiency and inanition produced approximately the same level of response suggests that the decrease in total leucocytes might be due to the reduced nutritional intake rather than the specific riboflavin deficiency.

Differential cell counts of the peritoneal exudate from the riboflavin-deficient animals demonstrated a relative increase of polymorphonuclear leucocytes and lymphocytes. The median percentage of lymphocytes was 20 for the ad libitum and 28 for the deficient group. Mononuclear leucocytes were reduced in both the inanition and the riboflavin-deficient groups. The median percentages were: ad libitum 41.8, inanition 36.0 and vitamin deficient 29.5.

Experiments were performed to determine whether or not "leukotaxine" activity was present in those animals which demonstrated a reduction in cellular migration to an inflamed area. No alteration in capillary permeability could be detected by the Menkin dye-accumulation technique. In every instance, the cell-free exudate from all groups of animals produced the same type and degree of reaction in the skin of the rabbit after the injection of the dye. It was evident that the reduc-

tion in cellular response was not due to the inability of the rats to produce the material and reaction described by Menkin ('40).

In the bone marrow study, the inanition-control and riboflavin-deficient groups showed a relative increase in total granulocytes. The increase was greater in the inanition-control animals. The median percentages were 24.0, 49.3 and 34.0 for the ad libitum, inanition, and deficient groups respectively. There was noted a relative decrease in lymphocytes in both deficient and inanition-control groups. The median percentages of lymphocyte cells were 10.5, 1.7 and 2.0 for ad libitum, inanition and riboflavin-deficient animals.

SUMMARY

Male white rats were maintained on a well defined diet deficient in riboflavin, and various physiological factors of importance in resistance to infection were studied. Adequate numbers of inanition and ad libitum control animals were included. The following physiological factors were studied: (1) the cellular composition of the peripheral blood; (2) complement activity; (3) cellular migration in inflammation; (4) the cellular composition of the exudate in inflammation; (5) "leukotaxine" activity, and (6) the cellular composition of bone marrow.

The following observations were made from these studies:

1 No change was noted in the total erythrocyte count in the peripheral blood. A leucopenia was evident in the riboflavin-deficient and inanition-control animals. Differential cell counts of the blood leucocytes of the riboflavin-deficient rats and their inanition controls showed a slight percentage increase in polymorphonuclear neutrophiles.

2 Complement activity of the sera of riboflavin-deficient and the inanition-control rats was decreased to the same degree.

3 There was a marked decrease in the total number of leucocytes in the peritoneal exudates of the riboflavin-deficient and inanition-control groups.

4 Peritoneal exudates of riboflavin-deficient rats demonstrated a decrease in the relative percentage of mononuclear leucocytes and a relative increase in lymphocytes.

5 No alteration in capillary permeability, as measured by the Menkin dye-accumulation technique, was noted in any group.

6 Relative granulocytoses and lymphopenia were observed in the bone marrow of inanition controls as compared with ad libitum controls. Bone marrow of the riboflavin-deficient rats demonstrated a less severe granulocytosis than that of the inanition controls. The degree of lymphopenia between the two groups was approximately identical.

LITERATURE CITED

ENDICOTT, K. M., AND M. OTT 1945 The normal myelogram in albino rats. Anat. Rec., *92:* 61.

FARRIS, E., AND J. Q. GRIFFITH 1949 The Rat in Laboratory Investigations. J. B. Lippincott Co., Philadelphia, Pa., 2nd Ed.

MAXIMOW, A. A., AND W. A. BLOOM 1944 Textbook of Histology. 4th Ed. W. B. Saunders Co., Philadelphia, Pa.

MENKIN, V. 1940 Dynamics of Inflammation. Macmillan Co., New York, N.Y.

———— 1950 Newer Concepts of Inflammation. Charles C. Thomas Co., Springfield, Illinois.

WERTMAN, D., AND J. L. SARANDRIA 1951 Complement-fixing murine typhus antibodies in vitamin deficiency states. Proc. Soc. Exp. Biol. Med., *78:* 332.

WERTMAN, K., R. ROTUNDO AND R. YEE 1953 Blood and bone marrow study of vitamin deficient rats. J. Nutrition, *50:* 479.

WERTMAN, K., L. W. SMITH AND W. M. O'LEARY 1954 The effects of vitamin deficiencies on some physiological factors of importance in resistance to infection. I. Niacin-tryptophan deficiency. J. Immunology, *72:* 196.

WERTMAN, K., W. M. O'LEARY AND L. W. SMITH 1955 The effects of pyridoxine deficiency on some physiological factors of importance in resistance to infection. J. Nutrition, *57:* 203.

WERTMAN, K., P. J. LYNN, D. T. DISQUE, G. W. KOHR AND M. E. CARROLL 1956 The effects of vitamin deficiency on some physiological factors of importance in resistance to infection. III. Vitamin B_{12} and folic acid deficiencies. Ibid., *60:* 473.

INVITATION FOR NOMINATIONS FOR 1958 AMERICAN INSTITUTE OF NUTRITION AWARDS

Nominations are invited for the 1958 annual awards administered by the American Institute of Nutrition. Nominations may be made by anyone, including all members of the Nominating Committees. The following information must be submitted: Name of the award for which the candidate is proposed and as convincing a statement as possible as to the basis for the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the appropriate nominating committee before January 1, 1958, to be considered for the 1958 award.

1958 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1,000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or any of its components.

The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another

administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are: 1944 — E. V. McCollum; 1945 — Harold H. Mitchell; 1946 — Philip C. Jeans and Genevieve Stearns; 1947 — Leonard A. Maynard; 1948 — Charles A. Cary; 1949 — Harry J. Deuel, Jr.; 1950 — Henry C. Sherman; 1951 — Paul György; 1952 — Max Kleiber; 1953 — Harold H. Williams; 1954 — Agnes Fay Morgan and Arthur H. Smith; 1955 — A. G. Hogan; 1956 — Frank M. Strong; 1957 — no award.

Chairman, Nominating Committee:
DR. W. C. ROSE
Department of Biochemistry
University of Illinois
Urbana, Illinois

1958 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 has been established by the Nutrition Foundation, Inc., for the recognition of outstanding basic research accomplishments in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Former recipients of this award are: 1949 — William C. Rose; 1950 — Conrad A. Elvehjem; 1951 — Esmond E. Snell; 1952 — Icie Macy Hoobler; 1953 — Vincent du Vigneaud; 1954 — L. A. Maynard; 1955 — E. V. McCollum; 1956 — A. G. Hogan; 1957 — G. R. Cowgill.

Chairman, Nominating Committee:
DR. ICIE MACY HOOBLER
Merrill-Palmer School
71 East Ferry Avenue
Detroit 2, Michigan

Your key to current biological research literature in

- ANATOMY
- NEUROLOGY
- ANTHROPOLOGY
- NUTRITION
- MORPHOLOGY
- PHYSIOLOGY
- ZOOLOGY

ADVANCE ABSTRACT CARD SERVICE

- Issued promptly and in advance of publication of manuscripts appearing in the biological science journals of The Wistar Institute.
- Contributed to and edited by outstanding scientists. Serves the needs of librarians and investigators.

A quick method of keeping informed of the trend of current biological literature

Your name and address on the coupon below, mailed with your remittance brings these important science abstracts to you regularly.

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

Date _____

Send to _____

Address _____

_____

ADVANCE ABSTRACT CARD SERVICE

Issued monthly — Over 500 abstracts annually

Permanent Library Card, 75 × 125 mm, center hole to fit library card files.

Postage paid in United States or abroad.

☐ Single set subscriptions $5.00
☐ Two sets to a single subscriber 8.00
☐ 1955 INDEX (published April 1956 following the December issue of Advance Abstract Card Service) 2.50

Begin my subscription with January ☐; July ☐ 19

(Please check one) *(year)*

Amount enclosed $.....

1956 INDEX

OF THE

ADVANCE ABSTRACT CARD SERVICE

for abstracts in the disciplines of

• MORPHOLOGY • NEUROLOGY • ANATOMY • ZOOLOGY

• ANTHROPOLOGY • PHYSIOLOGY • NUTRITION

For all Papers Published in Journals of
The Wistar Institute of Anatomy and Biology
January to December 1956

To those who received the Advance Abstract Card Service, this Index is invaluable.

Non-subscribers to the Advance Abstract Card Service will find the Index of real merit.

Now in preparation for abstract published in 1956

Price $2.50

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

Send ..... copies of the 1956 INDEX of the Advance Abstract Card Service.

NAME

STREET

CITY ZONE STATE

☐ I enclose $2.50 for each copy. ☐ Please bill me.

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 1957–58 are: R. R. Williams, President; William J. Darby, Vice-President; George M. Briggs, Secretary; J. B. Brown, Treasurer; L. A. Maynard, E. W. McHenry, Paul Gyorgy, 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.

The paper must be accompanied by an author's abstract not to exceed 225 words, which will be published in The Wistar Institute Advance Abstract Card Service.

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, Conn. 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 its leaflet entitled: 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.