OCTOBER 10, 1958

THE JOURNAL® OF NUTRITION

VOLUME 66 NUMBER 2

HENRY ALBRIGHT MATTILL

Official organ of the American Institute of Nutrition

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

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

Second class postage paid at Philadelphia, Pa.

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

THE JOURNAL OF COMPARATIVE NEUROLOGY

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

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

THE AMERICAN JOURNAL OF ANATOMY

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

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

THE ANATOMICAL RECORD

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

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

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

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

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

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

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

Publishes original articles on comparative human morphology and physiology as well as on the history of this branch of science and the techniques used therein. In addition, it gives comprehensive reviews of books and papers, a bibiography 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

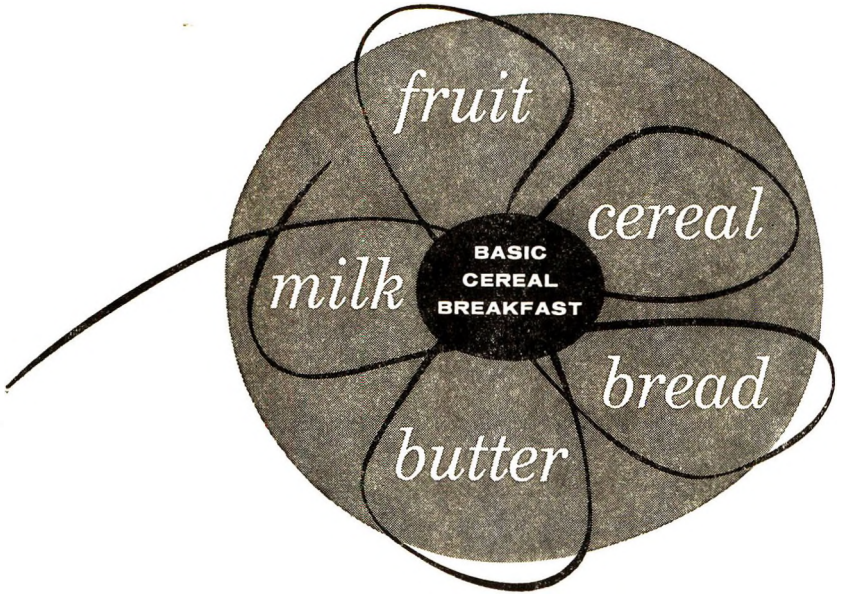
Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliography reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. The Advance Abstract Card Service is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



this moderately *low-fat* breakfast is a well-balanced meal

When a moderate reduction of the fat calories is to be recommended in the morning meal, the basic cereal and milk breakfast merits your consideration because it is moderately low fat and contributes well-balanced nourishment as shown in the table below.

The Iowa Medical College Breakfast Studies demonstrated that this basic cereal and milk breakfast, contributing about 20 gm. high quality protein, provided quick and lasting energy during the early and late morning hours and maintained mental and physical efficiency.

basic cereal 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	VITAMIN A	600 I.U.
PROTEIN	20.5 gm.	THIAMINE	0.46 mg.
FAT	11.6 gm.	RIBOFLAVIN	0.80 mg.
CARBOHYDRATE	80.7 gm.	NIACIN	3.0 mg.
CALCIUM	0.532 gm.	ASCORBIC ACID	65.5 mg.
IRON	2.7 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.: A Summary of the Iowa Breakfast Studies. Chicago: Cereal Institute, Inc., 1957.

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

THE BRITISH JOURNAL OF NUTRITION

Published for the Nutrition Society

SHORTENED VERSIONS OF TITLES OF ARTICLES IN VOL. 12, NO. 4

Health and nutritional status of children on poor vegetarian diets with rice or with tapioca macaroni. By V. SUBRAHMANYAN, R. K. BHAGAWAN, T. R. DORAISWAMY, KANTHA JOSEPH, G. S. BAINS, D. S. BHATIA, A. N. SANKARAN AND M. SWAMINATHAN.

The destruction of vitamin A by blood. By C. J. POLLARD AND J. G. BIERI.

Composition of sheep digesta. 1. By A. M. BADAWY, ROSA M. CAMPBELL, D. P. CUTHBERTSON, B. F. FELL AND W. S. MACKIE.

Composition of sheep digesta. 2. By A. M. BADAWY, ROSA M. CAMPBELL, D. P. CUTHBERTSON AND W. S. MACKIE.

Composition of sheep digesta. 3. By A. M. BADAWY, ROSA M. CAMPBELL, D. P. CUTHBERTSON AND W. S. MACKIE.

Digestion of foodstuffs in the stomach of the sheep. 1. By F. V. GRAY, A. F. PILGRIM AND R. A. WELLER.

Digestion of foodstuffs in the stomach of the sheep. 2. By F. V. GRAY, A. F. PILGRIM AND R. A. WELLER.

The conversion of plant nitrogen to microbial nitrogen in the rumen of the sheep. By R. A. WELLER, F. V. GRAY AND A. F. PILGRIM.

Metabolism of nitrogen, calcium and phosphorus in undernourished children. 4. Effect of replacing rice in the diet by tapioca macaroni. By KANTHA JOSEPH, M. NARAYANARAO, M. SWAMINATHAN AND V. SUBRAHMANYAN.

Magnesium in ruminant nutrition. By A. C. FIELD, JENNIE W. MCCALLUM AND E. J. BUTLER.

Diet in pregnancy. Dietary-survey technique and the nutritive value of diets taken by primigravidae. By A. M. THOMSON.

The vitamin D activity of pastures and hays. By KATHLEEN M. HENRY, S. K. KON, S. Y. THOMPSON, JENNIE W. MCCALLUM and the late J. STEWART.

Comparison of starch, lactose and hydrogenated palm oil with butterfat in milk diets for young calves. By A. M. RAVEN AND K. L. ROBINSON.

EFFECT OF ALKALI TREATMENT ON THE AVAILABILITY OF NIACIN AND AMINO ACIDS IN MAIZE [1]

A. E. HARPER, B. D. PUNEKAR AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication May 5, 1958)

In 1940 Kodicek observed that, although the free niacin of cereals was readily extractable with hot water, further material giving a positive reaction with the cyanogen bromide reagent was released upon treatment of cereal products with NaOH. Krehl and Strong ('44) found that this bound form of nicotinic acid was only partly available to *Lactobacillus casei* and *Lactobacillus arabinosus*. Subsequently the alkaline extracts were shown to improve the growth of rats consuming diets deficient in niacin (Chaudhuri and Kodicek, '50; Kodicek, '51) and it appeared evident that cereals, and maize in particular, contained a bound form of niacin which was not readily available to the rat. Laguna and Carpenter ('51) and Cravioto and associates ('52) also observed that the growth of rats fed a niacin-deficient diet was stimulated if the maize in their diet was treated with lime. Kodicek and associates ('56) have since demonstrated that the substitution of NaOH-treated maize for untreated maize exerts a curative effect in pigs fed a niacin-deficient diet. (Their paper also contains references to most of the previous studies.)

Since Cravioto et al. ('52) were unable to show that alkali treatment liberated niacin from maize they, suggested, in view

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board, Chicago, Ill. The crystalline vitamins were kindly provided by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

of the amino acid imbalance known to be produced by adding maize to a low-protein diet (Krehl et al., '45), that alkali treatment altered the amino acid composition of maize, thus correcting the amino acid imbalance. Others have since suggested that the beneficial effect of lime treatment of maize may be due to the correction of an amino acid imbalance rather than to the release of niacin from a bound form (Flodin, '53; Goldsmith '56). More recently, Cravioto and associates (Massieu et al., '56) have reported that the indispensable amino acid composition of maize is not affected by alkali treatment. They then suggested that this treatment may correct an imbalance among the dispensable amino acids.

The work reported in this paper, undertaken as a result of discussions of this problem with Dr. Kodicek,[2] represents an attempt to determine whether any relationship exists between amino acid imbalance (Harper, '57) and the beneficial effect of alkali treatment of maize (Kodicek et al., '56).

EXPERIMENTAL

Young male rats of the Sprague-Dawley strain weighing 40 to 60 gm were used in the experiments. They were depleted of niacin for two weeks on the basal diet (table 1, no. I) unless otherwise indicated. Then they were distributed, 5 rats per group, so that each group had almost the same average body weight. The rats were kept in individual suspended cages with screen bottoms and were fed ad libitum. The individual animals were weighed twice a week, both during depletion and when on experiment. The experiments were of two weeks duration. A few experiments were continued for 4 weeks but this was not done routinely because it provided no additional information.

The compositions of the basal diets are shown in table 1. All supplements were included at the expense of sucrose.

[2] Dr. E. Kodicek, Dunn Nutritional Laboratory, Cambridge, England, to whom one of us (A. E. H.) is indebted for a number of stimulating discussions and suggestions and who kindly made available to us a number of his unpublished observations.

Vitamins were added to provide in milligrams per 100 gm ration: thiamine·HCl, 0.5; riboflavin, 0.5; calcium pantothenate, 2.0: pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002; inositol, 10.0. The niacin-supplemented diet contained 2.5 mg niacin per 100 gm. Two drops of halibut liver oil fortified to furnish 1000 I. U. of vitamin A, 10 I.U. of vitamin D, 0.04 mg of 2-methyl-1, 4-napthoquinone and 0.8 mg of α-tocopherol were given weekly.

TABLE 1

Composition of basal diets

CONSTITUENTS	DIETS		
	I [1]	II [2]	III [3]
	%	%	%
Maize	40	40	89
Casein	3.5	9	—
L-Cystine	0.1	0.2	0.2
Sucrose	51	41.4	—
Salts 4 [4]	3	4	4
Corn oil	—	5	5
Cottonseed oil	2	—	—
Choline chloride	0.15	0.15	0.15
Vitamins (in sucrose)	0.25	0.25	0.25
Amino acids (with sucrose)	—	—	1.4

[1] Harris and Kodicek ('50).
[2] Krehl et al. ('46).
[3] Benton et al. ('55).
[4] Hegsted et al. ('41).

Maize was treated with lime as described by Laguna and Carpenter ('51) and Cravioto et al. ('52) and with 0.1 N NaOH as described by Kodicek ('51) except that the heating was done in an autoclave at 108° and the time was extended to one and one-half hours. The salt mixture was not altered to compensate for the addition of calcium phosphate or sodium chloride from the preparations.

RESULTS

Although Chaudhuri and Kodicek ('50), Laguna and Carpenter ('51) and Cravioto et al. ('52) observed that alkali

treatment of the maize in a diet deficient in niacin prevented the development of niacin deficiency in rats, Krehl et al. ('46) did not observe any alleviation of niacin deficiency in rats fed a diet in which tortillas were substituted for untreated maize. Since each of the above groups used a different basal diet and experimental procedure, the objective of the initial experiments was to determine whether results similar to those reported by Kodicek et al. ('56 for references) could be obtained using the procedure of Krehl et al. ('46).

TABLE 2

Growth of rats fed on niacin-deficient diets containing maize or lime-treated maize

EXPERIMENT NO.	TREATMENT OF MAIZE	NIACIN	AVERAGE GAIN	
			Depleted 2 wks. Diet I, 3.5% casein	Not depleted Diet II, 9% casein
			gm/wk	*gm/wk*
1 and 2	Untreated	—	5.5 ± 1.6 [1]	15.9 ± 1.9 [1]
	Untreated	+	20.9 ± 1.4	31.8 ± 1.8
	CaO (minus washings)	—	14.3 ± 1.4	24.8 ± 1.8
	CaO (minus washings)	+	16.4 ± 3.1	32.1 ± 3.0
	CaO (plus washings)	—	15.0 ± 1.6	33.8 ± 3.1
	CaO (plus washings)	+	14.0 ± 1.7	33.7 ± 1.6
3	Untreated	—	3.7 ± 1.1	
	Untreated	+	15.5 ± 1.8	
	Boiled 1.5 hr.	—	2.2 ± 0.9	
	Boiled 1.5 hr.	+	14.5 ± 1.2	

[1] Standard error of the mean.

It is evident from the results presented in table 2 that the absolute values are higher and the percentage responses smaller using the latter procedure (Krehl et al., '46). Nevertheless, the beneficial effect of lime treatment in preventing the development of niacin deficiency could be equally well demonstrated using either procedure. No growth response was obtained in this experiment with maize that had been heated in boiling water for one and one-half hours.

The results presented in table 3 are from an experiment in which all of the diets contained untreated maize. Equivalent quantities of maize were treated in the various ways indicated in the table; in each case the corn was filtered off,

then the filtrate was dried and added to the diet. No growth response was obtained with a water extract nor with such an extract subsequently treated with NaOH. The alkaline extracts, obtained after treatment with either CaO or NaOH, both stimulated growth but not to the same extent as a niacin supplement of 2.5 mg per 100 gm of diet.

In view of the preliminary observation that treatment with boiling water did not release sufficient niacin to stimulate the growth of rats receiving a niacin-deficient diet and the subsequent observations of Pearson et al. ('57), that boiling with

TABLE 3

Effect of extracts obtained by heating maize 1.5 hours with water, NaOH or CaO solutions on the growth of rats fed a diet containing 3.5% casein and untreated maize (diet I)

SUPPLEMENT	NIACIN	AVERAGE GAIN
		gm/wk
None	—	2.2 ± 0.6 [1]
None	+	20.4 ± 1.0
Water extract	—	3.3 ± 0.5
Water extract—NaOH treated	—	1.6 ± 0.6
Water extract	+	18.3 ± 0.9
CaO extract	—	13.1 ± 1.7
CaO extract	+	22.6 ± 3.1
NaOH extract	—	10.6 ± 1.8
NaOH extract	+	19.2 ± 2.8

[1] Standard error of the mean.

water did release niacin from corn, a series of experiments was performed in which the effects of the various preparative procedures used by Pearson et al. ('57) were examined under the experimental conditions used by Kodicek ('50). Maize was boiled for as long as 4 hours, was dried at 70° or at 100°, was ground before or after drying, was tested with and without the addition of calcium phosphate. In no instance did the response obtained with maize boiled in water approach that obtained with maize treated with alkali. These results are in substantial agreement with observations made at the Dunn Nutrition Institute.[3]

[3] E. Kodicek, Personal communications 1956–58.

The two major differences between the experiments of Pearson et al. ('57) and those of Kodicek ('51) and ourselves were that in one case, the rats were depleted of niacin prior to experiment; in the other, they were not; in one case, the diet contained 9% of casein and in the other, only 3.5%. Therefore, in subsequent experiments alkali-treated and boiled maize were compared using both experimental procedures. In neither case (table 4) did boiling the corn for one and one-half hours stimulate growth. Boiling for 4 hours produced a growth response in both cases, but the response, expressed as a percentage of the response to NaOH treatment (positive control), was considerably greater when the experimental procedure of Pearson et al. ('57) was used.

TABLE 4

Growth of rats fed different basal diets (diets I and II) containing untreated, NaOH-treated, or boiled corn

TREATMENT OF MAIZE	DEPLETED 2 WKS. 3.5% CASEIN, DIET I		NOT DEPLETED 9% CASEIN, DIET II		PEARSON ET AL.
	gm/wk	*% of positive control*	*gm/wk*	*% of positive control*	*gm/wk*
NaOH-treated [1]	13.5	100	33.0	100	25
Untreated	3.0	22	13.5	41	16
Boiled 1.5 hr.	4.0	30	13.0	40	
Boiled 4 hr.	7.5	55	26.5	80	23

[1] The group fed NaOH-treated maize was considered as the positive control group.

Analysis of the water and the alkaline extracts of maize for niacin revealed that the water extract (4 hours) contained only 0.63 μg per milliliter whereas the NaOH and CaO extracts contained 1.67 and 1.78 μg per milliliter respectively (10 ml of extract per gram of maize). These values are roughly proportional to the relative growth-promoting activity of the preparations.

The last experiment, the results of which are given in table 5, was designed more specifically to determine whether there was an increase in the availability of the amino acids of maize after alkali treatment. The procedure was similar to

that used previously by Sauberlich et al. ('53) and by Benton et al. ('55), to demonstrate that 5 amino acids are approximately equally limiting for growth when maize is the sole source of protein in a diet for rats. It is evident, from a comparison of groups 2 and 3, that this diet was primarily deficient in amino acids and, from a comparison of group 3 with groups 5 and 7, that if either tryptophan or isoleucine were omitted there was no growth response to a mixture of the other 4 amino acids. Treatment of the maize with NaOH did not increase the availability of either tryptophan or isoleucine sufficiently to stimulate growth.

TABLE 5

Effect of NaOH treatment on the growth of rats fed a diet containing 89% of maize (diet III) with or without various amino acid supplements

GROUP NO.	TREATMENT OF MAIZE	AMINO ACID SUPPLEMENT	NIACIN	AVERAGE GAIN
				gm/wk
1	None	None	+	5.4 ± 0.6 [2]
2	NaOH	None	+	2.9 ± 0.6
3	None	Essential amino acids[1]	+	20.4 ± 0.9
4	NaOH	Essential amino acids[1]	−	21.2 ± 0.5
5	None	Essential amino acids[1] — tryptophan	+	5.0 ± 0.9
6	NaOH	Essential amino acids[1] — tryptophan	+	1.6 ± 0.5
7	None	Essential amino acids[1] — isoleucine	+	8.4 ± 0.8
8	NaOH	Essential amino acids[1] — isoleucine	+	5.9 ± 0.7

[1] L-Lysine·HCl, 0.4%; DL-threonine, 0.3%; DL-valine, 0.2%; DL-isoleucine, 0.6%; DL-tryptophan, 0.1%.

[2] Standard error of the mean.

DISCUSSION

These results are in general agreement with those of other workers who have found that alkali treatment of the maize in a niacin-deficient diet alleviates or prevents the development of signs of niacin deficiency (Chaudhuri and Kodicek, '50; Laguna and Carpenter, '51; Cravioto et al., '52; Kodicek et al., '56; Pearson et al., '57).

The differences between the observations of Pearson et al. ('57) on the increased availability of niacin after boiling maize in contrast to the failure to demonstrate appreciable

release under these conditions by Kodicek [3] and by ourselves can be only partially explained. The higher protein level in the diet used by Pearson et al. apparently permits a greater growth response to such niacin as is released during boiling, and the extended period of boiling apparently releases more niacin than the shorter period (table 4). The growth results in both studies are in fairly close agreement except that alkali-treated corn supported a somewhat greater rate of growth in our experiments (table 4). The only striking discrepancy is the high value for water-extractable niacin reported by Pearson et al. This suggests that there may be differences in the ease with which bound niacin is released from different lots of corn.

With regard to the relationship between alkali treatment of maize and amino acid imbalance, Pearson et al. ('57) found that the amino acid composition of alkali-treated and untreated maize were similar. However, other workers (Bressani, '57) have reported that the amino acid composition of certain maize fractions is altered during such treatment. Unfortunately neither of the reports on the availability of tryptophan (Lushbough et al., '56; Gupta and Elvehjem, '56) contains information about maize. Nevertheless the observations on the availability of other amino acids from zein and maize (Geiger et al., '52; Benton et al., '55; Deshpande et al., '57) suggest that none of the amino acids are completely available and that amino acid analyses would not indicate whether there had been an increase in biological availability as a result of alkali treatment.

The growth experiments on rats fed untreated or alkali treated maize supplemented with various amino acid mixtures (table 5) indicate that the alkali treatment does not increase the availability of either tryptophan or isoleucine, specifically, nor of amino acids generally, in sufficient quantities to stimulate growth. Therefore, any effect of alkali treatment of corn in alleviating a niacin deficiency or in pre-

[3] See footnote 3, page 167.

venting an imbalance as described by Krehl et al. ('45), must be attributed directly to the release of niacin from an unavailable precursor as suggested by Kodicek ('51), and not to the destruction of a toxic factor or an amino acid present in excess, nor to an increase in the availability of amino acids that are limiting for growth.

SUMMARY

In confirmation of the results of other workers, it has been demonstrated that rats receiving a niacin-deficient diet in which untreated maize is replaced by alkali (NaOH or CaO)-treated maize, do not develop signs of niacin deficiency.

Although some niacin becomes available after prolonged boiling of maize in water more is released by a shorter alkali treatment. This could be demonstrated clearly, in growth experiments, only by using depleted rats fed on diets very low in tryptophan and explains, at least in part, some of the differences in the estimates reported by different workers for the ease of release of niacin from the bound form in maize. The growth results were supported by niacin analyses on some of the extracts.

The beneficial effect of the alkali treatment could not be attributed to the correction or prevention of an amino acid imbalance but only to the release of niacin from an unavailable form.

LITERATURE CITED

BENTON, D. A., A. E. HARPER AND C. A. ELVEHJEM 1955 Effect of isoleucine supplementation on the growth of rats fed zein or corn diets. Arch. Biochem. Biophys., *57:* 13.

BRESSANI, R. 1957 Effect of lime treatment on some characteristics of corn proteins. Federation Proc., *16:* 382.

CHAUDHURI, D. K., AND E. KODICEK 1950 The biological activity for the rat of a bound form of nicotinic acid present in bran. Biochem. J., *47:* xxxiv.

CRAVIOTO, R. O., G. H. MASSIEU, O. Y. CRAVIOTO AND F. DE M. FIGUEROD 1952 Effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet. J. Nutrition, *48:* 453.

DESHPANDE, P. D., A. E. HARPER, M. COLLINS AND C. A. ELVEHJEM 1957 Biological availability of isoleucine. Arch. Biochem. Biophys., *67:* 341.

FLODIN, N. W. 1953 Amino acids and proteins, their place in human nutrition. J. Agric. Food Chem., *1:* 222.

GEIGER, E., G. W. COURTNEY AND L. E. GEIGER 1952 The physiological availability of some essential amino acids present in zein, in the acid and in the enzymatic hydrolyzate of zein Arch. Biochem. Biophys., *41:* 74.

GOLDSMITH, G. A. 1956 Experimental niacin deficiency. J. Am. Dietet. Assoc., *32:* 305.

GUPTA, J. D., AND C. A. ELVEHJEM 1957 Biological availability of tryptophan. J. Nutrition, *62:* 313.

HARPER, A. E. 1957 Amino acid balance and imbalance. Annals. N. Y. Acad. Sci., *69:* 1025.

HARRIS, L. J., AND E. KODICEK 1950 Quantitative studies and dose response curves in nicotinamide deficiency in rats. Br. J. Nutrition, *4:* xiii.

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.

KODICEK, E. 1940 Estimation of nicotinic acid in animal tissues, blood and certain foodstuffs. Biochem. J., *34:* 724.

———— 1951 The biological activity for the rat of the bound form of nicotinic acid present in maize. Ibid., *48:* viii.

KODICEK, E., R. BRAUDE, S. K. KON AND K. G. MITCHELL 1956 The effect of alkaline hydrolysis of maize on the availability of its nicotinic acid to the pig. Br. J. Nutrition, *10:* 51.

KREHL, W. A., AND F. M. STRONG 1944 Studies on the distribution, properties and isolation of a naturally occurring precursor of nicotinic acid. Ibid., *156:* 1.

KREHL, W. A., L. J. TEPLY AND C. A. ELVEHJEM 1945 Corn as an etiological factor in the production of nicotinic acid deficiency in the rat. Science, *101:* 283.

KREHL, W. A., L. M. HENDERSON, J. DE LA HUERGA AND C. A. ELVEHJEM 1946 Relation of amino acid imbalance to niacin-tryptophan deficiency in growing rats. J. Biol. Chem., *166:* 531.

LAGUNA, J., AND K. J. CARPENTER 1951 Raw vs. processed corn in niacin deficient diets. J. Nutrition, *45:* 21.

LUSHBOUGH, C. H., T. PORTER AND B. S. SCHWEIGERT 1957 Utilization of amino acids from foods by the rat. IV. Tryptophan. Ibid., *62:* 513.

MASSIEU, H., O. Y. CRAVIOTO, R. O. CRAVIOTO, J. G. GUZMAN AND M. DE L. SUAREZ SOTO 1956 New data about the effect of corn and the tortilla on the growth of rats fed on diets low in tryptophan and niacin Ciencia (Mexico) *16:* 24. Chem. Abs., *51:* 8918.

PEARSON, W. N., S. J. STEMPFEL, J. S. VALENZUELA, M. H. UTLEY AND W. J. DARBY 1957 The influence of cooked vs. raw maize on the growth of rats receiving a 9% casein ration. J. Nutrition, *62:* 445.

SAUBERLICH, H. E., W.-Y. CHANG AND W. D. SALMON 1953 The comparative nutritive value of corn of high and low protein content for growth in the rat and chick. Ibid., *51:* 623.

THIAMINE REQUIREMENT OF EIGHT ADOLESCENT BOYS, AS ESTIMATED FROM URINARY THIAMINE EXCRETION [1,2]

EDNA C. DICK, SHIH DZUNG CHEN, MARK BERT [3]
AND JANICE M. SMITH
Department of Home Economics, College of Agriculture, University of Illinois, Urbana

(Received for publication August 15, 1957)

This study was undertaken to secure data which might lead to an estimation of thiamine requirements for boys in early adolescence (ages 14 to 17). Published data deal primarily with needs of adults and infants. Recently a report appeared concerning thiamine requirements of girls 16 to 18 years of age (Hart and Reynolds, '57), but published data on young teen-age boys are not currently available in the literature. Recommendations of the National Research Council ('53) on thiamine intakes for this age group have been inferred from values derived on adult and infant subjects. The rapid physical growth, sexual development, and mental and emotional changes occurring in adolescence are peculiar and intense enough to demand substantiation by controlled studies of the reliability of such interpolations.

The general method followed in the study herein reported was measurement of the urinary thiamine excretion of subjects as the thiamine intake gradually increased from low to higher levels. This was deemed the safest and most practical

[1] Supported in part by a United States Department of Agriculture contract sponsored by the Human Nutrition Research Division, Agricultural Research Service.

[2] A portion of these data was presented at the meetings of the American Institute of Nutrition, San Francisco, California, March 1955.

[3] Present Address: Wirthmore Research Laboratory, Waltham, Mass.

method of study for use with young subjects. In published studies on thiamine requirements a variety of methods and indices of adequacy as well as different definitions of the term requirement have been used. The authors believe that the most plausible definition is that of Holt et al. ('49) in the statement: "A minimum food requirement may be defined as that intake which will just protect against definite clinical symptoms or accepted laboratory criteria of deficiency." Holt further states: "It was not deemed justifiable to deplete infants to the point of clinical deficiency, and laboratory criteria were therefore used as an endpoint." As was the case with the infants in the work cited, it was likewise considered unjustifiable in the study herein reported to deplete growing boys of thiamine until actual clinical symptoms should appear. The validity of the use of a urinary excretion method is strengthened by its frequent use in deriving reasonable thiamine requirements for other age groups (Alexander et al., '46; Oldham et al., '46; Hathaway and Strom, '46; Holt et al., '49; Friedemann et al., '49; Louhi et al., '52; Hart and Reynolds, '57).

The results obtained in the present study by measurement of daily urinary excretion of thiamine were related to the changing thiamine intake by fitting of regression lines to these observations. The average minimum thiamine requirement of the boys on this study, calculated by this technique, was 1.41 mg/day. The requirement was further estimated on the basis of total daily excretion of thiamine, percentage of total thiamine intake excreted, percentage of increment of thiamine excreted, and the ratio of thiamine to creatinine in urine.

EXPERIMENTAL PROCEDURE

Subjects

Eight boys, aged 14 to 17 years, served as subjects. The boys were students in the University High School and were selected on the recommendation of the high school principal that they were able to get along in a group and to assume

responsibility. Each boy was certified by the family doctor as being healthy and able to participate. The age range to be used for study was selected by referring to the growth charts of Jackson and Kelly ('45) which show that the zone of rapid growth begins at ages 12 to 13 for boys and the "crest" of adolescent acceleration occurs around age 15. Although there is much individual variation in the age of onset of this growth, it was felt that an age range of 13 to 15 would be most likely to include boys in the rapid growth phase. It may be seen from the ages shown in table 1 that 4 subjects were 16 or older. It was considered desirable to include these 4

TABLE 1

Initial age, weight and height of subjects and weight changes during 69-day thiamine study

SUBJECT	AGE		WEIGHT		HEIGHT
			Initial	Gain	
	yrs.	*mos.*	*lbs.*	*lbs.*	*inches*
JW	14	2	142.5	0.5	67.8
SK	14	6	154.8	7.0	69.4
DRL	14	7	121.0	9.8	66.5
LH	15	11	158.5	2.5	66.1
DEL	16	1	130.2	3.8	69.2
RW	16	7	148.2	1.8	69.2
LM	16	10	176.4	8.1	70.1
DKS	17	6	237.2	6.3	73.8

older boys despite their age because of their experience as subjects in balance studies carried out in this laboratory in the previous year.

The subjects lived together in a home provided and maintained by the University as a laboratory for human nutrition research. They were under the general supervision of an adult male counselor, and pursued their own activities insofar as permitted by the additional obligations as experimental subjects.

Dietary plan

The basal diet served throughout the study was designed to be as low as possible in thiamine and completely adequate

in all other nutrients. Natural foods were used almost exclusively in order to increase the palatability of the meals and to maintain the interest and cooperation of the boys. It was possible to include milk in the diet by using florisil[4] adsorption (Adamson, '53) to reduce its thiamine content. Three menus were used in a constant 5-day rotation plan. The 5-day menus were calculated to contain a mean of 514 μg of thiamine (analyzed values in table 2). A typical day's meals included grapefruit juice, dry cereal, sugar, milk, casein bread, butter, jam, tuna casserole, celery, sliced peaches, casein cookies, macaroons, chicken breasts, beets, potatoes, lettuce, mayonnaise, apple crisp,[5] Hershey bar, and iced tea. In order to supply sufficient protein without adding thiamine, vitamin-free casein was added to bread and cookies. The mean protein content of 110 gm was planned to be considerably higher than the 85 to 100 gm listed in the 1953 revision of the National Research Council allowances since previous experience in this laboratory had indicated that adolescent boys retain additional nitrogen when the protein intake is raised from 85 to 100 gms or more. Details concerning recipes for the special high-protein foods and the menus mentioned above are the subject of another publication (Smith et al., '58).

The basal diet during the 5-day period was calculated to supply a mean of 3582 Cal./day. Individual Caloric needs were gauged by the boys' appetites. Subjects who desired more food ate recorded amounts of 25-gm packets of commercial chocolate creme cookies. These were calculated to supply 123 Cal./25 gm and negligible amounts of thiamine.

Vitamin and mineral supplements were added to the basal diet to provide generous intakes of nutrients other than thiamine. These additions were: 50 mg crystalline ascorbic acid, 500 mg calcium as calcium monophosphate, 5 mg iron as ferric citrate, and 3000 I. U. Vitamin A and 600 I. U. Vitamin D.[6] Riboflavin (1.50 mg/day) dissolved in distilled water was

[4] A synthetic adsorbent obtained from Floridin Co., Warren, Pa.

[5] A prepared dish containing applesauce, margarine, flour, spice, salt, and brown sugar.

[6] Natola oil, a Parke-Davis preparation.

added each day to the milk. These supplements, together with the nutrients provided by the basal diet, furnished daily a total of 112 mg ascorbic acid, 1.5 gm calcium, 15 mg iron, 10,700 I. U. vitamin A, 757 I. U. vitamin D, 2.5 mg riboflavin and 18 mg niacin.

TABLE 2

Thiamine increments and nitrogen intake for each period of feeding

PERIODS	LENGTH OF PERIOD	NITROGEN INTAKE	THIAMINE		
			Basal	Supplement	Total
	days	*gm*	*μg*	*μg*	*μg*
I	20	17.7	634	—	634
IIa	5	17.9	637	237	874
IIb	10	18.0	599	432	1031
III	10	17.8	596	902	1498
IV	10	18.0	621	1346	1967
V	10	17.6	621	1811	2432
VI	4	17.5	570	2136	2706

Plan of experiment

For 69 consecutive days, all subjects ate the basal diet intended to supply about 0.5 mg of thiamine daily. The first 10 days of experimental feeding were designed to equalize the thiamine nutriture of the subjects and to allow for gross Caloric and menu adjustments. The succeeding 10 days provided for thiamine excretion values typical of the basal level of feeding. After these 20 days on the unsupplemented basal diet, 440-μg increments of thiamine were to be added at 10-day intervals. The plan was to have 6 levels of thiamine intake increasing stepwise at regular intervals from the base value of 0.5 mg/day up to 2.7 mg/day (see table 2). When analysis of the thiamine supplement for the first period of supplementation (period IIa) showed a thiamine content of 237 μg rather than the intended 440 μg, this period was extended to 15 days. A new batch of thiamine supplement, shown by analysis to contain the calculated amount of thiamine (432 μg), was fed for the last 10 days (period IIb). The remaining periods continued as planned except for the last

period, VI, during which the highest level of thiamine was fed. Only 4 days remained for that period as a result of the extension of period II, mentioned above.

Methods of analysis

Throughout the study 24-hour urines were collected in brown bottles containing glacial acetic acid (60 ml). After being measured and made up to volume, the urines were analyzed daily for creatinine (Peters, '42) and for thiamine (Mickelsen et al., '45). Food was composited daily, preserved in 0.1N HCl and analyzed for thiamine by the thiochrome method (Association of Vitamin Chemists, '47). Milk was analyzed separately to maintain a check on the success of the thiamine-removal. Five-day pools of urine, stools and food were made in addition, to be analyzed for nitrogen, calcium and phosphorus.

RESULTS AND DISCUSSION

Creatinine excretion. The daily creatinine measurements were used as a means of checking the accuracy of the 24-hour urine collections. After the first few days of adjustment to the regime, creatinine values for each subject became reasonably constant and it is assumed therefore that urinary collections were generally complete. Occassionally an error such as loss or mixing of samples occurred with a resultant wide deviation from the average creatinine excretion. These mistakes occurred rarely and such samples were not included in the averages. The coefficients of variation on creatinine for all subjects for the entire experiment ranged from 4.7 to 11.6%, the mean being 5.2%. This degree of uniformity was considered adequate in view of the existence of several vaguely known or uncontrollable factors (e.g., emotions—Schottstaedt et al., '54; Schwartz and Shields, '54) said to contribute to variation in creatinine excretion of humans.

Urinary thiamine. Urinary thiamine excretion responded rapidly to changes in thiamine intake. On the first day of

the experiment urinary thiamine values ranged between 142 and 309 μg. After 5 days on the low-thiamine diet, 7 of the 8 boys were consistently excreting less than 100 μg/day. By the end of the 20-day low-thiamine period, the urinary thiamine values for these 7 subjects had reached 50 μg/day or less. A similar rapid drop in thiamine excretion to these low levels in response to limited dietary supply has been noted in studies with adults (Lowry, '52). Several occasions on which the lower levels of urinary thiamine were associated with clinical signs of deficiency are reviewed by Unglaub and Goldsmith ('54). In the present experiment however, no adverse signs of any kind were observed, doubtless because of the relatively short period of time on the thiamine-deficient diet.

When the thiamine intake was increased from 634 to 1031 μg, the urinary excretion again responded rapidly, changing from a mean of 47 μg/day for the last 5 days of period I to a mean of 102 μg/day for the last 5 days of period IIb (table 3). The next three increases in thiamine intake corresponded with much higher increases in thiamine excretion, until, on the final brief 4-day period, with the intake at 2.7 mg of thiamine (the highest intake of this study), the urinary excretion rose to a mean of 755 μg/day.

One subject, DRL, consistently excreted much more thiamine than any other subject, regardless of intake. This consistently high excretion rate could not be related to body size, age or other obvious factors. Thiamine excretion has been found previously to be highly characteristic of the individual (Mickelsen et al., '46).

Validity of 10-day periods. Periods of 10 days were selected on the basis of several publications (Wang and Yudkin, '40; Oldham et al., '46; Friedemann et al., '49) indicating a rapid response of urinary thiamine to changing intakes. The response in this experiment showed similar rapidity of response to each change in dietary thiamine level and relative stability of urinary excretion during the last 5 days of each period; therefore these days were the ones used to compile

averages and for statistical analyses. Actually the excretion levels reached in a 10-day period are likely to represent more nearly a dynamic type of equilibrium than a true equilibrium which would be maintained over a period of months or years. For example, Horwitt et al. ('48) found a significant drop in urinary thiamine excretion in the third year on a low-thiamine diet as opposed to the first two years. Likewise Daum et al. ('48) reported a progressively diminishing excretion of thiamine for 19 weeks for subjects receiving 200 μg of thiamine

TABLE 3

Average daily urinary thiamine excretions on 6 levels of thiamine intake for 8 boys 14 to 17 years of age

SUBJECT	MEAN URINARY EXCRETION [1]					
Period:	I	IIb	III	IV	V	VI
	μg	μg	μg	μg	μg	μg
JW	47	79	133	300	473	598
SK	47	117	185	400	621	816
DRL	94	199	344	570	860	1049
LH	39	104	209	452	677	891
DEL	34	78	181	384	573	766
RW	56	103	168	323	428	529
LM	25	63	118	288	473	590
DKS	31	71	148	339	577	803
Mean:	47	102	186	382	585	755
S. D.:	± 21.6	± 43.5	± 70.4	± 93.7	± 139.0	± 174.9

[1] Five-day means of days 16 to 20 for period I and days 6 to 10 for periods IIb to V; period VI, 4 days only. For DEL, period IIb, days 1 to 5 were used because of terramycin administration on days 6 to 10.

whereas in subjects receiving 600 μg there was a gradual decrease until the lowest level was reached at the third month. In the present study it was felt that the initial rapid adjustments in rates of urinary thiamine excretion were adequate indices for comparative purposes of the thiamine nutriture of these boys at the several different intakes of thiamine.

Statistical findings. The variances about each 5-day mean of urinary thiamine which appear in table 4 show a consistent increase with the magnitude of the urinary thiamine. The fact that the excretion during these 5-day intervals did not

always quite reach a plateau may be a factor in this phenomenon; however a similar dependence of variance on mean has been reported in the case of urinary thiamine excretions which had apparently reached a plateau (Mickelsen et al., '47) and can be observed in the data of Horwitt et al. ('48).

In view of this dependence of variance on the mean it was necessary to adopt a proper procedure before fitting, by the least-square method, regression functions. Bliss ('52) has suggested that unequal variation may be adjusted by the use of weighting coefficients, the weighting coefficient of a mean being, in this case, the reciprocal of the variance of this mean.

TABLE 4

Variance of urinary thiamine excretion at 5 different intakes of thiamine

	I	IIB	III	IV	V
Thiamine intake, μg	634	1031	1498	1967	2432
Thiamine excretion,[1] μg	47	102	186	382	585
Variance within subjects	55	108	374	944	1151

[1] Five-day mean: days 16 to 20 for period I and days 6 to 10 for other periods.

The regression of urinary thiamine excretion on thiamine intake was therefore established, using the weighted 5-day means of the excretion at each level of intake for each individual subject. A single straight line and both possible combinations of two straight lines were fitted to the weighted data by the method of least squares. In every case a system of *two* straight lines gave the best fit as measured by the residual variances.

To test further the hypothesis that a system of two straight lines is well suited to the data under consideration, the following functions were fitted to the unweighted means (therefore an inexact fitting) for a typical subject: (1) a single straight line, (2) a system of two straight lines similar to the best fitting system of two straight lines obtained on the weighted means, (3) a quadratic parabola, and (4) an exponential curve. The two straight lines system gave the best

fit of all 4, in general agreement with the results of the rigorous fitting on weighted means.

The calculated abscissa of the intersection of the two straight lines fitted to the observations on each subject as explained above, is thus taken as an index of thiamine requirement. The computed thiamine intakes corresponding to such abscissa for each adolescent boy varied from 0.99 to 1.68 mg of thiamine per day with 5 of the values falling between 1.41 and 1.46 mg (table 5).

TABLE 5

Individual thiamine requirements calculated from regression of excretion on intake at 5 levels of intake

SUBJECT	MEAN CALORIC INTAKE	THIAMINE REQUIREMENT	
		Per Person	Per 1000 Cal.
		mg	*mg*
JW	3651	1.44	0.39
SK	3797	1.55	0.41
DRL	3510	1.68	0.48
LH	3611	1.42	0.39
DEL	3663	1.33	0.36
RW	3652	0.99	0.27
LM	4077	1.46	0.36
DKS	3631	1.42	0.39
Mean	3700	1.41	0.38
S.D.	± 171.7	± 0.200	± 0.059
Coefficient of variation		14.2%	15.5%

A sharp change in slope in the thiamine excretion curve with increasing levels of thiamine intake has been observed by previous investigators (Wang and Yudkin, '40; Friedemann et al., '49). Such a break, beyond which the thiamine excretion suddenly rises rapidly, may also be approached from the opposite direction; the urinary thiamine then drops precipitously at the critical point as the level of thiamine intake decreases (Holt et al., '49; Melnick, '42). It is reasonable to suppose that the excretion rate below such a critical point corresponds to homeostatic conservation of thiamine (Melnick, '44), whereas the region of the curve above this point indi-

cates thiamine plethora (Holt et al., '49; Friedemann et al., '49).

Additional criteria of requirement. Urinary excretion data may be evaluated in ways other than the one just described. The most common methods are, (1) total urinary excretion, (2) percentage of total intake excreted, (3) percentage of increment in intake excreted and (4) ratio of thiamine to creatinine in the urine. These tests, when all are applied to the same data, often do not agree concerning the status of the subjects (Gifft and Hauck, '46; Louhi et al., '52; Hart and Reynolds, '57). One reason for this lack of agreement appears to be that some methods seem to be designed to assess the point of tissue saturation whereas others indicate more nearly a minimal requirement in the sense that a deficiency would probably begin at an intake not too much lower. The critical point, as described in this paper, does not necessarily indicate tissue saturation (Friedemann et al., '49).

Since "urinary excretion of 100 μg per day has been considered as the nutritional borderline by most workers" (NRC, '53), it is of interest that two of the adolescent subjects (SK and DRL) were consistently excreting over 100 μg of thiamine during the last 5 days of period IIb and the excretion levels of all 8 boys were well over 100 μg throughout period III. This implies that the level of thiamine intake during period III, namely, 1.50 mg, was adequate for all subjects, whereas the level fed during period IIb, namely, 1.03 mg, was inadequate for most of the subjects.

During this period III, which was more than adequate for all subjects by this first criterion, the subjects excreted between 54 (LM) and 183 (DRL) μg of thiamine per gram of creatinine. Excretion of 150 μg of thiamine per gram of creatinine is reported to be associated with good thiamine nutrition whereas less than 50 μg of thiamine per gram of creatinine has been considered inadequate (Adamson et al., '45).

Rather than absolute amount of thiamine excretion, the proportion of dietary thiamine excreted may be considered. In the present study, the mean percentage of the intake excreted during periods I, IIb, III, IV, V, and VI respectively was 7.4, 9.8, 12.4, 19.4, 24.1, and 27.9%. There is disagreement in the literature as to which percentage might be considered significant. A value of 13% has been suggested (Louhi et al., '52); all subjects in this study did not reach this percentage level until period IV (intake of 1.97 mg of thiamine per day). The figure of 20% has also been suggested (Benson et al., '42; Oldham et al., '44); even on the highest intake of the present study, 2.70 mg of thiamine per day, one subject (RW) did not quite excrete this percentage of his intake. This criterion does not appear to be an especially stringent one for the subjects of the present study.

The percentage of the increment excreted is a criterion which probably reflects the area of tissue saturation, since its basis is the assumption that when a large proportion of an increment is excreted, that increment is not needed. The subjects of this study excreted only 14 and 18% of the first two increments of thiamine in contrast to 42 and 44% of the next two. Therefore the "surplus area" was reached between 1.50 and 1.97 mg of thiamine.

The thiamine requirements reported by many investigators, together with the criteria employed, have been recently summarized (Meyer et al., '55). The levels considered adequate in these and other studies vary from 0.63 (Daum et al., '49) to 1.45 (Friedemann et al., '49) mg/day. Thiamine requirements are frequently expressed per 1000 Cal. On this basis requirements vary from 0.23 mg/1000 Cal. (Keys et al., '43) to 0.63 mg/1000 Cal. (Hart and Reynolds, '57). Expressed in terms of Calories, the mean thiamine requirement for the adolescent boys of this study is 0.38 ± 0.059 mg/1000 Cal., a value within the range cited for other age groups. The requirement figures for the 8 boys, when expressed in terms of body weight, surface area, or milligrams of creatinine excretion,

have coefficients of variation which are higher than that for the requirement expressed per 1000 Cal., namely, 27.5, 19.5, and 19.2% respectively vs. 15.5%.

Since there is some evidence that the amounts of fat vs. carbohydrate in the diet affect the thiamine requirement of humans (Holt and Snyderman, '55) it may be well to emphasize that dietary fat was constant at 36% of the Calories throughout this study. Since Williams et al. ('40) suggested a relationship of thiamine needs of humans to temperature and to degree of physical activity it may be pertinent to note that the maximum daily temperatures for the 69 days of this experiment ranged between 73 and 109°F., the mean being 98.9° with a standard error of ± 0.9. As noted earlier in this paper, no attempt was made to control the activity of these subjects.

SUMMARY

Eight healthy adolescent boys were maintained for a total of 69 days on a controlled diet at 6 different levels of thiamine intake ranging from 0.6 to 2.7 mg/day, for short periods of time. The urinary excretion of the thiamine was plotted as a function of the intake. A least-square system of two straight lines was fitted to weighted data for each subject. The abscissa of the intersection of the fitted lines was taken as the critical point or minimum requirement. The mean requirement for all subjects thus computed is 1.41 ± 0.200 mg of thiamine/day or 0.38 ± 0.059 mg/1000 Cal.

ACKNOWLEDGMENTS

The authors wish to acknowledge the contribution of Alice Forsythe Carver, Elizabeth Smith, Sandra Rosenthal, Martha Prather and Jane Kenney in making chemical analyses and preparing the weighed diets.

Appreciation is extended to Edward Adamek, subject supervisor, and to the 8 boys who made this study possible; Leonard Hays, Steve Knapp, David Lantz, Denis Larsen, Louis Mesker, David Simpson, John Williams and Royce Williams.

Assistance of the boys' parents and the members of the administrative staff of the University High School is acknowledged.

LITERATURE CITED

ADAMSON, J. D., N. JOLLIFFE, H. D. KRUSE, O. H. LOWRY, P. E. MOORE, B. S. PLATT, W. A. SEBRELL, I. W. TICE, F. F. TISDALL, R. M. WILDER AND P. C. ZAMECNIK 1945 Medical survey of nutrition in Newfoundland. Can. Med. Assoc. J., *52:* 227.

ADAMSON, L. F. 1953 Removal of thiamine and riboflavin from milk for dietary use. Science, *117:* 253.

ALEXANDER, B., G. LANDWEHR AND F. MITCHELL 1946 Studies of thiamine metabolism in man. II. Thiamine and pyrimidine excretion with special reference to relationship between injected and excreted thiamine in normal and abnormal subject. J. Clin. Invest., *25:* 294.

ASSOCIATION OF VITAMIN CHEMISTS, INC. 1947 Methods of vitamin assay. Interscience Publishers, Inc., New York, pp. 69-97.

BENSON, R. A., L. B. SLOBODY, C. M. WITZBERGER AND L. LEWIS 1942 Further studies on the urinary excretion of thiamine in children. J. Pediat., *20:* 454.

BLISS, C. I. 1952 The statistics of bioassay. Academic Press, Inc., N. Y., pp. 535–560.

DAUM, K., W. W. TUTTLE, M. WILSON AND H. RHODES 1948 Influence of various levels of thiamine intake no physiologic response. II. Urinary excretion of thiamine. J. Am. Dietet. Assoc. *24:* 1049.

DAUM, K., W. W. TUTTLE AND M. WILSON 1949 Influence of various levels of thiamine intake on physiologic response. VII. Thiamine requirements and their implications. Ibid., *25:* 398.

FRIEDEMANN, T. E., C. IVY, B. SHEFT AND V. MILLER KINNEY 1949 Work at high altitude. V. The relation between daily oral intake and excretion of thiamine and riboflavin. Quart. Bull. Northwestern Univ. Med. School, *23:* 438.

GIFFT, H. H., AND H. HAUCK 1946 A comparison of four methods for studying the urinary excretion of thiamine. J. Nutrition, *31:* 635.

HART, M., AND M. S. REYNOLDS 1957 Thiamine requirement of adolescent girls. J. Home Econ., *49:* 35.

HATHAWAY, M. L., AND J. E. STROM 1946 A comparison of thiamine synthesis and excretion in human subjects on synthetic and natural diets. J. Nutrition, *32:* 1.

HOLT, L. E. JR., R. L. NEMIR, S. E. SNYDERMAN, A. A. ALBANESE, K. C. KETRON, L. P. GUY AND R. CARRETERO 1949 The thiamine requirement of the normal infant. Ibid., *37:* 53.

HOLT, L. E. JR., AND S. E. SNYDERMAN 1955 The influence of dietary fat on thiamine loss from the body. Ibid., *56:* 495.

HORWITT, M. K., E. LEIBERT, O. KREISLER AND P. WITTMAN 1948 Investigations of human requirements for B-complex vitamins. Bulletin No. 116, National Research Council.

JACKSON, R. L., AND H. G. KELLY 1945 Growth charts for use in pediatric practice. J. Pediat., *27:* 215.

KEYS, A., A. F. HENSCHEL, O. MICKELSEN AND J. M. BROZEK 1943 The performance of normal young men on controlled thiamine intakes. J. Nutrition, *26:* 399.

LOUHI, H. A., H. YU, B. E. HAWTHORNE AND C. A. STORVICK 1952 Thiamine metabolism of women on controlled diets. I. Daily urinary thiamine excretion and its relation to creatinine excretion. Ibid., *48:* 297.

LOWRY, O. H. 1952 Biochemical evidence of nutritional status. Physiol. Rev., *32:* 431.

MELNICK, D. 1942 Vitamin B_1 (thiamine) Requirement of man. J. Nutrition, *24:* 139.

———— 1944 A critique of values suggested as the thiamine requirement of man. J. Am. Dietet. Assoc., *20:* 516.

MEYER, F. L., M. L. BROWN, H. J. WRIGHT AND M. L. HATHAWAY 1955 A standardized diet for metabolic studies, its development and application. U.S.D.A. Bull. No. 1126.

MICKELSEN, O., H. CONDIFF AND A. KEYS 1945 The determination of thiamine in urine by means of the thiochrome technique. J. Biol. Chem., *160:* 361.

MICKELSEN, O., W. O. CASTOR AND A. KEYS 1946 Urinary excretion of thiamine as a characteristic of the individual. Proc. Soc. Exp. Biol. Med., *62:* 254.

———— 1947 A statistical evaluation of the thiamine and pyramin excretions of normal young men on controlled intakes of thiamine. J. Biol. Chem., *168:* 415.

NATIONAL RESEARCH COUNCIL 1953 Recommended dietary allowances. National Academy of Sciences Publication 302, Washington, D. C.

OLDHAM, H., F. JOHNSTON, S. KLEIGER AND H. H. ARISMENDI 1944 A study of the riboflavin and thiamine requirements of children of pre-school age. J. Nutrition, *27:* 435.

OLDHAM, A. G., M. V. DAVIS AND L. J. ROBERTS 1946 Thiamine excretions and blood levels of young women on diets containing varying levels of the B vitamins, with some observations on niacin and pantothenic acid. Ibid., *32:* 163.

PETERS, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.

SCHOTTSTAEDT, W. W., W. J. GRACE AND H. G. WOLFF 1954 Life situations, emotions and renal excretion of fluid and electrolyte: variations in excretion of endogenous creatinine. Federation Proc., *13:* 130.

SCHWARTZ, T. B., AND D. R. SHIELDS 1954 Emotional tension and excretion of corticoids and creatinine. Am. J. Med., *16:* 608.

SMITH, E., A. F. CARVER, E. C. DICK AND J. M. SMITH 1958 Dietary aspects in a study of riboflavin and thiamine requirements of adolescents. J. Am. Dietet. Assoc., *34:* 823.

UNGLAUB, W. G., AND G. A. GOLDSMITH 1954 Evaluations of vitamin adequacy: Urinary excretion tests. Proceedings of Symposium, Methods for Evaluation of Nutritional Adequacy and Status, Quartermaster Research and Development.

WANG, Y. L., AND J. YUDKIN 1940 Assessment of the level of nutrition. Urinary excretion of aneurin at varying levels of intake. Biochem. J., *34:* 343.

WILLIAMS, R. D., A. L. MASON, R. M. WILDER AND B. F. SMITH 1940 Observations on induced thiamine (Vitamin B_1) deficiency in man. Arch. Int. Med., *66:* 785.

THE ROLE OF VITAMIN A IN THE OCCURRENCE OF GOITRE ON THE ISLAND OF KRK, YUGOSLAVIA

AGNES HORVAT AND HUBERT MAVER
Division of Nutrition, Central Institute of Hygiene, Zagreb, Yugoslavia

(Received for publication February 20, 1958)

A nutrition survey on the island of Krk, situated in the northern Adriatic, carried out in 1954 (Ferber and Buzina, '58) revealed that goitre is widespread on this island. By a systematic investigation of goitre in Croatia it was established that the percentage of goitre on the island of Krk was larger than on any other island or region along the coast and even larger than in some areas on the mainland. The fact that a large number of goitrous persons appeared on an island roused our interest and induced us to examine the factors which might play a role in the etiology of goitre on the island of Krk.

Most investigators adhere to the principle that endemic goitre is due to an absolute deficiency of exogenous iodine, whereas others believe there are other factors as well which may play a role in the genesis of goitre. Many goitrogenic substances are known but only a few of them have been associated with endemic goitre. It has been established that endemic goitre can be caused by goitrogens contained in vegetables from the family Brassicaceae (kale, cabbage, rutabaga, turnip). Astwood, Greer and Ettlinger ('49) isolated from rutabaga vinylthiooxazolidone which, in its goitrogenic action, is similar to thiouracil. Clements ('55), as well, ascribed the occurrence of goitre in Tasmania to the action of a goitrogen, probably vinylthiooxazolidone.

Another possible factor in the etiology of goitre is a deficiency of vitamin A. Haubold ('50) described an outbreak of goitre in Bavaria as a consequence of the deficiency of vitamin A and carotene in food. Eggenberger ('54) examined 59 pregnant women and found that those with normal dark adaptation had only 9.5% of children with enlarged thyroid glands, whereas in women with pathological adaptation there were 37% of children with goitre.

Hettche ('56) ascribed a strong goitrogenic action to urochrome, a product of the decomposition of hemoglobin found in water polluted with feces.

THE ISLAND OF KRK

The island of Krk is situated in the bay of Quarner in the northernmost part of the Adriatic Sea. On the northeast it is separated from the mainland by a channel a few kilometers in width and on the south, east and southeast it is surrounded by the islands of Cres, Plavnik and Rab. Krk is the largest island in the Adriatic. It is densely populated, with a large number of settlements both on the coast and in the interior. Most of its inhabitants, especially the males, are permanently employed in the nearby industrial centers (Rijeka, Kraljevica), while the remaining population is engaged in fishery and agriculture.

The western part of the island is very rich in vegetation while the eastern part is poorer and in some places there is but bare rocky soil. Those parts of the island that are not rocky are very fertile, particularly the valleys crossing the island from northwest to southeast.

As far as the geological formation is concerned there are no substantial differences either on the island itself or in comparison with the other islands in the northern Adriatic. The whole island is built from cretaceous formations with scattered layers of Eocene origin, consisting chiefly of carbonate and marl.

The climate on the island is that of the Mediterranean, as is the case on the other islands of the northern Adriatic. The

northern regions are recurrently exposed to a rather strong northeast wind (bora) blowing from the mountains on the mainland towards the sea. This wind carries drops of sea water a long way into the interior of the island. In all other regions of the island south (sirocco) and west (maestral) winds are most frequent.

GOITRE ON THE ISLAND OF KRK

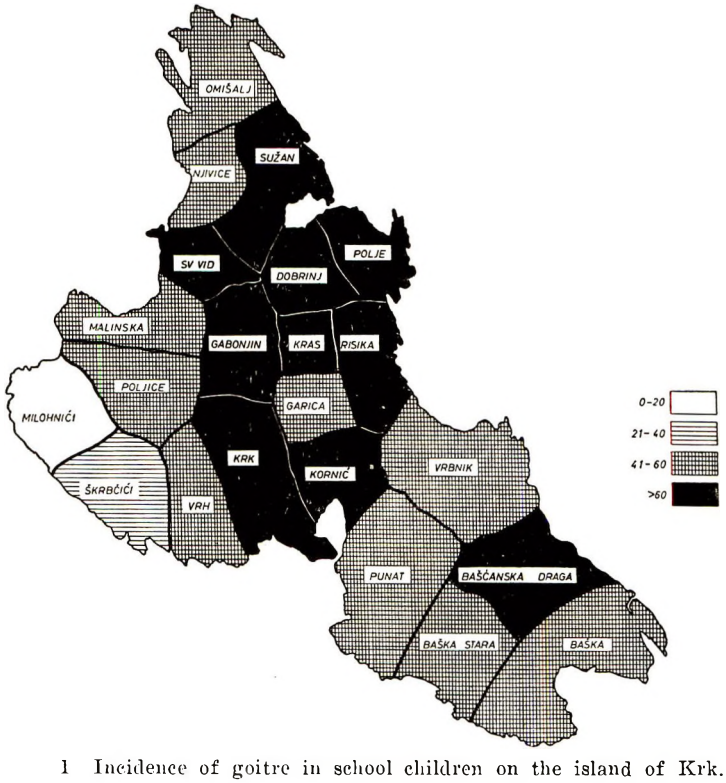
Goitre on the island of Krk was investigated by examinations of the adult population and school children. Particular attention was paid to school children, especially those 7 to 10 years old, since the increase of the thyroid glands in these children gives the best index of the incidence of goitre.

The children of all schools of the island were examined, a total of 1391, of whom 712 were boys and 679 girls. Goitre was determined according to the method described in the Bulletin of the World Health Organization ('53).

The average incidence of goitre on the whole island was found to be greater in girls (62%) than in boys (54%), a common picture in the distribution of this disease. Goitre proved to be more widespread among boys and girls of 11 to 15 years than among younger children. All the cases of goitre recorded in children proved to belong to the first stage. In some places, such as Risika and Polje, goitre is very frequent, even up to 80%. On the other hand there are places, such as Milohnić, where goitre is relatively rare—only 7% in boys and 21% in girls. The percentage of goitre is larger in the northern area than in the southern regions of the island (fig. 1).

Adults were examined only in those localities showing the largest differences in the percentage of goitre among school children, such as Risika and Polje with the largest and Milohnić with the smallest incidence of goitre. We were not in a position to examine all of the inhabitants of these villages, particularly the men. The examination of goitre in the adult population has confirmed the results obtained in the examination of the school children (table 1).

At the same time, school children on the other islands in the northern Adriatic were examined in order to find out whether goitre on those islands occurred to the same extent as on Krk. The results are shown in figure 2, from which it appears that, on all other islands, the occurrence of goitre is



1 Incidence of goitre in school children on the island of Krk.

very low, even lower than in the localities on Krk showing the lowest percentage of goitre. In 7- to 10-year-old children these values range between 0 and 29%. It appears there is a substantial difference in the percentage of goitre on the island of Krk in comparison with the other islands in the northern Adriatic, although the conditions of life appear to be nearly the same on all of these islands (fig. 2).

Anthropometrical measurements (height and weight) were carried out on children in order to establish whether there are some differences in these basic anthropometrical measures between the children of Krk and those on the other islands. The mean values for height and weight are within the range of those on the other islands.

TABLE 1

Goitre in adults in the villages of Risika, Polje and Milohnić

SEX	LOCALITY	WITHOUT GOITRE	DEGREE OF GOITRE			TOTAL GOITROUS
			I	II	III	
		%	%			%
Males	Risika	54	46			46
	Polje	65	35			35
	Milohnić	100				
Females	Risika	37	45	17	1	63
	Polje	43	47	9		57
	Milohnić	93	7			7

IODINE CONTENT IN WATER, AND NUTRITION OF THE POPULATION OF THE ISLAND OF KRK

The water supply on the island is of two kinds: on the one hand there are water plants drawing water from springs, and on the other hand most water is supplied by cisterns, the latter being used for irrigation as well. Because most foodstuffs are produced on the island, we examined the content of iodine in spring water as an indicator of the iodine supply of the population. In areas showing the largest and the smallest incidence of goitre on the island water is supplied from cisterns. The iodine content in the waters of the island varies between 0 and 5.0 μg/liter. Cistern water contains somewhat less iodine than spring water (fig. 3). There is no correlation between the iodine content of the water and the incidence of goitre.

According to Hettche ('56), and to our own experience in investigations of urochrome in other fields, water containing urochrome is of yellowish or brown color and foaming. The

waters we have examined from the island of Krk, mostly rain water from cisterns, proved absolutely clear and colorless and there was no indication that they should be checked for the presence of urochrome.

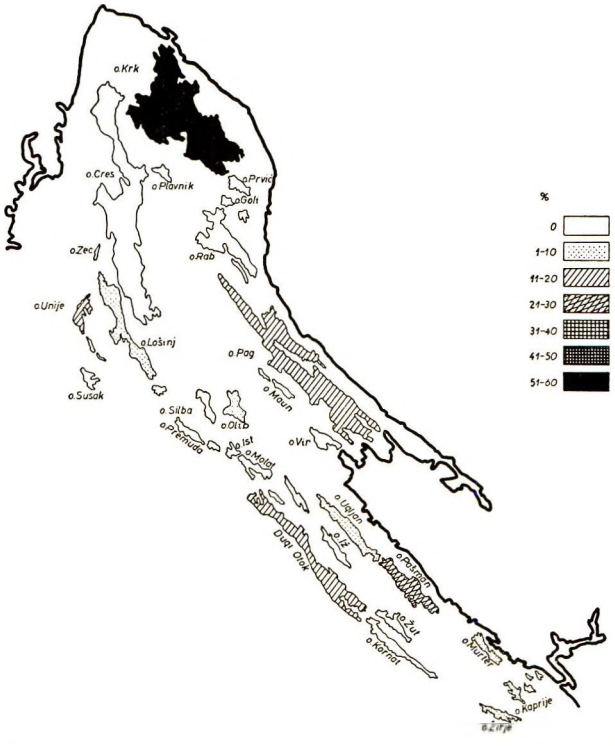


Fig. 2 Incidence of goitre in school children on the islands of the northern Adriatic.

During the nutrition survey (Ferber and Buzina, '54) some very interesting data were obtained. The nutrition survey was carried out in the localities with the highest and the lowest incidence of goitre, namely at Polje, Risika and Milohnić. It was run according to the food list method during a fortnight, once in the spring and again in the autumn, in 15 households of these villages. The results of this survey showed that food intake is quite uniform. The major part of

the food, especially in winter and in spring, consists of vegetables (chiefly cabbage) and maize meal. Fish is also frequently eaten, its quantity, however, depending on the amount caught by the single fishermen. This quantity is not large, but fish is a constant item of food.

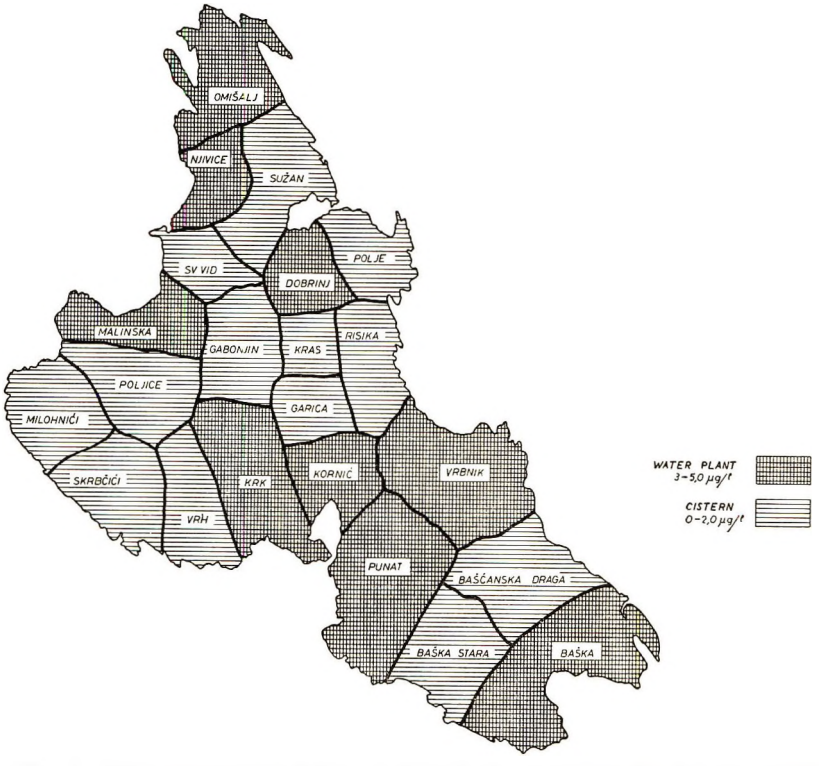


Fig. 3 Water supply and iodine content in the waters of the island of Krk.

Fruit is a seasonal food and eaten rarely because it is either marketed or processed into alcohol. Eggs, meat and milk are consumed in small quantities.

The daily consumption of fat, almost exclusively of vegetable origin, and of proteins is rather low both in localities with and without goitre. The differences in the consumption of fat and proteins at Polje, Risika and Milohnić are not statistically signifiant (table 2).

In analyzing the data on food intake we found the consumption of vitamin A to be very low and that there were differences between the localities with and without goitre. The differences in the amount of vitamin A between Milohnić (lowest incidence of goitre on the island) and Polje and Risika (highest incidence), in the spring, were found to be on the borderline of statistical significance ($t = 2.09$, $t_{0.05} = 2.201$). In the autumn these differences proved to be smaller (344 I.U.) and not significant statistically ($t = 1.067$, $t_{0.05} = 2.228$). The difference in the value of vitamin A in the food is rather high for both seasons combined (633 I.U.) without being statistically significant ($t = 1.675$, $t_{0.05} = 2.004$) (table 3).

TABLE 2

Daily consumption of fat and protein

(Expressed as grams per person)

	SPRING		AUTUMN		TOTAL	
	Risika	Milohnić	Risika	Milohnić	Risika	Milohnić
Fat	67	56	46	53	58	54
Protein	65	74	68	85	66	79

The difference in fish consumption between Risika and Polje on the one hand and Milohnić on the other is highly significant in the spring ($t = 3.910$, $t_{0.01} = 3.129$). A larger amount of fish is consumed by the inhabitants of the villages of Polje and Risika. In the autumn the difference was lower (8.04 gm) and not significant statistically ($t = 0.808$, $t_{0.05} = 2.30$). The average yearly difference in the consumption of fish is statistically significant ($t = 2.169$, $t_{0.05} = 2.13$) (table 3).

If it is assumed that seafish is rich in iodine, it might have been expected that Milohnić would show a larger percentage of goitre than Risika and Polje. The actual situation being quite the reverse, and Risika and Polje having been shown to have 5 times more goitre than Milohnić, we may assume that a lack of iodine in the food does not play the major role in the etiology of goitre on the island of Krk.

TABLE 3

Average daily consumption of vitamin A and fish

SEASON	VITAMIN A					FISH				
	Polje Risika		Milohnić		Difference	Polje Risika		Milohnić		Difference
	M ± S.E.	S.D.	M ± S.E.	S.D.		M ± S.E.	S.D.	M ± S.E.	S.D.	
	I.U.		*I.U.*		*I.U.*	*gm*		*gm*		*gm*
Spring	727 ± 104	294	1637 ± 432	965	910	19 ± 3	9	6 ± 1	2	13
Autumn	1829 ± 270	714	2174 ± 176	352	345	24 ± 6	15	16 ± 8	14	8
Total	1241 ± 197	765	1875 ± 322	968	634	21 ± 3	12	11 ± 4	9	10

In comparing other nutritional factors we found an actual difference only in the intake of vitamin A. The inhabitants of Milohnić consume a larger amount of vitamin A with their food than do those of Polje and Risika. In the spring this difference was at the borderline of statistical significance; the yearly difference appeared rather high (633 I.U.).

These results induced us to examine the influence of vitamin A on the occurrence of goitre on this island.

RESULTS OF THE ADDITION OF VITAMIN A TO THE DIET OF GOITROUS SCHOOL CHILDREN

For this purpose we chose the elementary school at the village of Krk, this school being the biggest on the island and having the best technical facilities for our work. Our research embraced 80 children, most of them from 7 to 10 years of age, with only a small number over 10 years.

The children were divided into two groups, an experimental group and a control one. All age groups and both sexes were equally represented in each group of 40 children. During the experiment two children in the experimental and 9 in the control group dropped out—either owing to illness or departure from Krk—so that at the end of our investigation there were 38 children in the former and 31 in the latter group.

The children were examined clinically, and special attention was paid to the symptoms of vitamin A deficiency. Dark adaptation was determined as well as the degree of goitre. As a measure of the status of saturation of the organism with vitamin A, carotene and vitamin A in the serum were determined in both groups.

The determination of vitamin A and carotene in the serum was carried out by the method of May, Blackfan, McCreary and Allen ('40). There being no facilities for laboratory work at Krk, the serum was separated there, and the determinations of vitamin A and carotene were carried out in Zagreb within three days after the blood had been collected.

Dark adaptation was measured with the Birch-Hirschfeld adaptometer. In this method adaptation cannot be expressed

in defined units and therefore the investigator's adaptation had to be taken as a basis of reference.

Clinical examinations of the children showed no symptoms of vitamin A deficiency. Dark adaptation was subnormal in 6 children only, one of them with anomalies of the eye, three with and two without goitre.

Children of the experimental group were given 3,000 I.U. of vitamin A[1] in form of chocolate sweets each day for three months to increase the intake of vitamin A to 4,000 I.U., as recommended by the National Research Council. The vitamin A sweets were distributed by teachers during the class and the children were required to consume their rations in the classrooms, in order to be sure that each child received his daily supplement of vitamin A.

Three months later all children were retested. In order to make the retesting objective the children were examined according to the school roll-call so that the examiners did not know whether a child had been given vitamin A or not. Vitamin A and carotene were determined again in the serum. The results of these determinations are presented in table 4.

Before the experiment was started the values of vitamin A in the serum were low in both groups (mean value 15.5 and 15.0 μg% respectively). May et al. ('40) give values of 49 to 164 I.U. % (14.7 to 49.2 μg%) for school children, whereas our investigations in adults in Zagreb showed a mean value of 23.6 μg% (Maver et al., '56). There is no doubt that these low values of vitamin A should be taken as indicating a pronounced vitamin A deficiency in the nutrition of the children at Krk and this was confirmed by the results of the nutrition survey as well. The mean values of carotene were rather low, 89.3 in the experimental group and 101.7 μg% in the control group.

At the time of the second determination of vitamin A and carotene in the serum after the intake of additional vitamin A, the mean value of vitamin A had increased to 22.3 μg% in

[1] The vitamin A was the synthetic vitamin A palmitate of Hoffmann La Roche, Basle, Switzerland. It was administered as a chocolate "sweet."

TABLE 4

Serum vitamin A and carotene in school children before and after treatment with vitamin A [1]

	VITAMIN A, µg %				CAROTENE, µg %			
	A[2]		B[2]		A		B	
	I	II	I	II	I	II	I	II
M ± S.E.	15.5 ± 0.68	22.3 ± 0.99	15.0 ± 0.64	15.4 ± 0.81	101.7 ± 0.81	119.2 ± 1.29	89.3 ± 1.96	104.8 ± 2.20
S.D.	4.12	6.04	3.50	4.50	7.19	7.94	10.90	12.06
Range	6.3 — 24.0	11.6 — 49.3	9.5 — 24.3	9.5 — 23.3	62.4 — 236.6	71.6 — 263.3	36.6 — 152.4	56.6 — 206.6

[1] Each child received 3000 I.U. of vitamin A each day for three months.

[2] A = experimental group, N = 38. B = control group, N = 31.

the experimental group, while there was no change in the control group (15.4 μg%). For the first determination the difference in the value of vitamin A between the experimental and the control group was slight ($\Delta = 0.5$, $t = 0.53$, $P > 0.1$), whereas for the second determination the difference proved statistically significant ($\Delta = 6.8$, $t = 5.39$, $P < 0.01$).

The mean values of carotene in the serum of both groups showed a minimum, quite negligible increase, an indication that the nutrition of the children underwent no change during the experiment. We may therefore assume that the increase of 6.8 μg% of vitamin A in the serum of the children in the experimental group was due to the intake of additional vitamin A.

The results of the testing for goitre showed, in the control group, but a slight change in the percentage, from 67 to 64%. In the experimental group goitre decreased by almost a half (from 66 to 37%) after the vitamin A intake had been increased. During the whole time both groups lived under the same conditions. There were no major changes in their food intake and this is also reflected in the slight and insignificant increase of carotene in the serum of both groups. The amount of iodine taken by the children was essentially unchanged during the experiment since the main sources of iodine supply, food and water, were the same. Accordingly, we may conclude that the addition of vitamin A to the diet was the main factor responsible for the decrease in the percentage of goitre in this group.

CONCLUSION

This investigation has shown that the deficiency in vitamin A on the island of Krk should be regarded as an important factor in the occurrence of goitre. Moreover, this contributes to the opinion that goitre cannot always and exclusively be attributed to absolute deficiency of exogenous iodine, but should be regarded as the consequence of a complex nutritional deficiency. The amount of vitamin A in food may be an important element in the genesis of goitre and in many cases it may serve as a therapeutic factor for its decrease.

SUMMARY

In investigating goitre in Croatia the authors met with a large percentage of goitre on the island of Krk in the northern Adriatic, where extensive investigations of the causes of this occurrence (clinical examinations of school children, biochemical analyses, examination of water for the content of iodine, nutrition survey) were carried out. The small quantity of vitamin A in the diet of the population induced the authors to investigate the influence of vitamin A on the occurrence of goitre. For this purpose an experiment was carried out at a school on the island of Krk. Children 7 to 10 years of age were given 3,000 I.U. of vitamin A daily for three months, and it was established that goitre in this group had decreased by 44%, whereas no change was noticed in a control group.

On the basis of this investigation the authors consider goitre to be the result of a complex nutritional deficiency and that in addition to lack of iodine, a deficiency of vitamin A plays an important role as well.

ACKNOWLEDGMENTS

The authors are indebted to the Commission for Scientific Medical Research in Belgrade for the financial support that enabled this field trial. Dr. Z. Panian contributed to this investigation by carrying out all the measurements on dark adaptation. The Institute of Hygiene in Rijeka, particularly its director Dr. M. Gradišnik, helped in overcoming the technical difficulties of the field trial. The vitamin A sweets were put at our disposal by Messrs. Jugodijetetika in Zagreb, and the crystalline beta carotene by Messrs. Hoffmann La Roche in Basle. To all our best thanks.

LITERATURE CITED

ASTWOOD, E. B., M. A. GREER AND M. G. ETTLINGER 1949 Antithyroid factor of yellow turnip. Science, *109:* 631.

CLEMENTS, F. W. 1955 A thyroid blocking agent as a cause of endemic goitre in Tasmania. Preliminary communication. Med. J. Australia, ii, 369.

EGGENBERGER, H. U. 1954 Vitamin A und Kropf. Inaugural Dissertation. Zürich, Schläpfer & Co.

FERBER, E., AND R. BUZINA 1958 Anketa prehrane seljačkog stanovništa NRH, KOMNIS, Beograd.

HAUBOLD, H. 1950 Die Bedeutung des Carotinoidmangels für die Entsstehung der neuen Kropfwelle. Münch. Med. Wchschrift, 10–12.

HETTCHE, H. O. 1956 Epidemiologie und Aetiologie der Struma in 100 Jahren Forschung. Archiv f. Hygiene und Bakteriologie, München, *140:* 79.

MAVER, H., A. HORVAT AND Z. PANIAN 1956 Vrijednost vitamina A i karotina u serumu i adaptacija na mrak. Liječnički vjesnik, *78:* 535.

MAY, C. D., K. D. BLACKFAN, J. F. MCCREARY AND F. H. ALLEN, JR. 1940 Clinical studies of vitamin A in infants and children. Am. J. Dis. Child., *59:* 1167.

STUDY GROUP ON ENDEMIC GOITRE. FINAL REPORT 1953 Bull. World Health Organization, *9:* 293.

HEMOLYSIS AND REAGENT PURITY AS FACTORS CAUSING ERRATIC RESULTS IN THE ESTIMATION OF VITAMIN A AND CAROTENE IN SERUM BY THE BESSEY-LOWRY METHOD [1]

MARGARET H. UTLEY, EILEEN R. BRODOVSKY
AND WILLIAM N. PEARSON
Division of Nutrition, Departments of Biochemistry and Medicine,
Vanderbilt University School of Medicine,
Nashville, Tennessee

(Received for publication March 12, 1958)

INTRODUCTION

The micro-method of Bessey et al. ('46) for the determination of vitamin A and carotene in small quantities of blood serum has been extensively used in nutritional surveys. Laboratories using this method have frequently experienced erratic results, yet its convenience has resulted in wide adoption for such studies.

Several reports are concerned with the reproducibility of results obtained with this method and some have recommended precautions necessary to obtain valid data (Sobel and Snow, '47; Bieri and Schultze, '51; Caster and Mickelsen, '55; Clayton et al., '54; Karmarkar and Rajagopal, '52). The effect of hemolysis has been mentioned by two groups of workers (Sobel and Snow, '47; Bieri and Schultze, '51), although no detailed study of it appears to have been made. Sobel and Snow ('47) stated that "hemolyzed blood has been found to give higher carotene values, and due to the high blanks after irradiation, the results tend to be unreliable." In the

[1] This research was supported by a grant from the Tennessee Valley Authority and augmented by the Williams-Waterman Fund.

presence of hemolysis they noted an increase in carotene values ranging from 23 to 117%. Although the increase in vitamin A caused by hemolysis was not mentioned, as much as 52% increase was recorded in their tabulated data.

Bieri and Schultze ('51) reported that the observation of certain precautions led to valid results. Each serum was examined against a white background and discarded if there was evidence of hemolysis. According to them "hemolyzed serums gave consistently high values for vitamin A, since the products of hemolysis are partially destroyed by ultraviolet irradiation."

The conditions of collecting, transporting and storing specimens in nutrition surveys often favor hemolysis; hence detailed understanding of the influence of hemolysis on this procedure is desirable.

In this laboratory, data obtained in nutrition surveys appeared to indicate that vitamin A levels of hemolyzed sera were consistently higher than those of non-hemolyzed sera. The present study confirms this observation, defines some factors responsible, and leads to the recommendation of precautions which avoid this source of error.

EXPERIMENTAL PROCEDURE

From each of 5 laboratory personnel, approximately 30 ml of blood were obtained and allowed to clot. After centrifugation, an aliquot (approximately 3 ml) of serum was removed. The remaining blood was vigorously stirred for several minutes and recentrifuged, so that slightly hemolyzed serum was obtained. More vigorous stirring for longer periods yielded further hemolysis; and freezing for several minutes was used to obtain extreme hemolysis. Varying degrees of hemolysis were thus obtained in the same serum sample and were coded "1" for the non-hemolyzed sample and "2", "3" and "4" for the samples with different degrees of hemolysis. The sera were analyzed for hemoglobin using the Bing and Baker method as modified by Ham ('50). Vitamin A and carotene determinations were performed on all serum aliquots by the

Carr-Price (antimony trichloride) method (Carr and Price, '26; Dann and Evelyn, '38) as well as by the micro-method of Bessey et al. ('46). The data, summarized in table 1, indicate a non-linear increase in apparent serum vitamin A corresponding to the increase in serum hemoglobin when the Bessey-Lowry method was used. Hemolysis did not have a consistent influence on the vitamin A levels as measured by

TABLE 1

Vitamin A and carotene values of sera exhibiting varying degrees of hemolysis

DONOR		LEVELS OF HEMOLYSIS OF EACH DONOR'S BLOOD			
		1	2	3	4
M	Serum hemoglobin[1]	0.00	0.02	0.08	0.28
	Vitamin A, micro[2]	216	277	312	366
	Vitamin A, Carr-Price[2]	145	154	153	140
	Carotene, micro[3]	190	190	190	188
	Carotene, Carr-Price[3]	169	181	188	178
F	Serum hemoglobin	0.00	0.07	0.24	0.43
	Vitamin A, micro	310	422	404	441
	Vitamin A, Carr-Price	164	229	187	190
	Carotene, micro	110	108	111	114
	Carotene, Carr-Price	97	108	97	94
T	Serum hemoglobin	0.00	—	0.22	0.59
	Vitamin A, micro	293	—	368	450
	Vitamin A, Carr-Price	213	—	217	217
	Carotene, micro	204	—	209	209
	Carotene, Carr-Price	188	—	206	204
K	Serum hemoglobin	—	0.01	0.22	0.78
	Vitamin A, micro	—	263	343	502
	Vitamin A, Carr-Price	—	192	174	168
	Carotene, micro	—	163	159	186
	Carotene, Carr-Price	—	151	164	145
H	Serum hemoglobin	0.00	0.03	0.10	0.27
	Vitamin A, micro	286	310	366	418
	Vitamin A, Carr-Price	149	—	158	142
	Carotene, micro	303	303	302	268
	Carotene, Carr-Price	255	271	286	308

[1] Serum hemoglobin, gm/100 ml serum.
[2] Vitamin A, I.U./100 ml serum.
[3] Carotene, μg/100 ml serum.

the Carr-Price reaction. Even in the absence of hemolysis, the vitamin A values obtained by the micro-method were higher in each instance than were those found by use of the Carr-Price procedure. Carotene values were unaffected by hemolysis, and the two methods of analysis were in relatively good agreement.

Washed erythrocytes were also analyzed by both the Carr-Price method and the micro-method. Blood samples from two laboratory workers were allowed to clot, and the sera separated by centrifugation. The red cells were then washed by centrifuging several times with 0.9% saline. Distilled water was added in an amount estimated to replace the serum and the mixture was stirred vigorously. Aliquots of this red cell hemolysate, as well as the collected sera, were analyzed by the two methods previously indicated. The results are recorded in table 2. The values obtained by the Carr-Price method were near zero for both vitamin A and carotene, but the micro procedure indicated very high apparent vitamin A and carotene levels. The Carr-Price and micro vitamin A and carotene analyses on serum agreed well when there was no hemolysis, but in the presence of slight hemolysis the vitamin A by micro-analysis was nearly three times greater, and the corresponding carotene was half that obtained by the macro-analysis.

In order to establish firmly that hemoglobin was the material responsible for the higher values obtained by the micro method, solutions of purified hemin and hemoglobin were studied. Equimolar solutions (approximately 0.0001 molar) of hemin [2] and hemoglobin [3] were used. Hemoglobin went into solution readily; the hemin was dissolved by the addition of dilute ammonia. This 0.0001 molar hemoglobin is equivalent to 0.67 gm per 100 ml which is a level that might be found in hemolyzed serum. It is obvious from table 3 that dilute solutions of both hemin and hemoglobin give vitamin A values that are falsely high when analyzed by the micro-method.

[2] Recrystallized, Eastman Organic Chemicals.

[3] Pure scales, Pfanstiehl.

Values obtained by the Carr-Price method are consistently low.

TABLE 2

Vitamin A and carotene values of washed red cells and serum

	SAMPLE	VITAMIN A		CAROTENE	
		Carr-Price	Micro	Carr-Price	Micro
		I.U./100 ml		*μg/100 ml*	
I	Red cell hemolysate	15	962	3	252
	Serum[1]	137	380	88	42
II	Red cell hemolysate	18	1658	9	318
	Serum	109	104	161	161

[1] Slightly hemolyzed.

TABLE 3

Vitamin A and carotene values of 0.0001 M solutions of hemin and hemoglobin

SAMPLE	VITAMIN A		CAROTENE	
	Carr-Price	Micro	Carr-Price	Micro
	I.U./100 ml		*μg/100 ml*	
0.0001 M Hemin	16	88	1	5
0.0001 M Hemoglobin	13	119	5	8

Correspondence with Dr. Guillermo Arroyave[4] revealed that hemolyzed samples analyzed by the Bessey-Lowry method at INCAP gave no indication of increased vitamin A value. Therefore, the possibility was examined that reagent differences might explain the discrepancy.

The reagents used in the micro-method are absolute ethyl alcohol (redistilled, stored in a ground-glass stoppered reagent bottle), 11 N KOH (kept in either a glass bottle with a rubber stopper or in a polyethylene bottle), and a 1 : 1 mixture of kerosene[5] and xylene,[6] stored in a reagent bottle with ground-glass stopper and not protected from light. When this

[4] Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala, C. A.

[5] Fisher odorless.

[6] Merck reagent.

study was undertaken, KOH and kerosene-xylene mixtures 6 months old were available; alcohol which had been redistilled 10½ months previously was also available. In addition to these "aged" solutions, fresh reagents were prepared. Kerosene and xylene were used without special purification and newly opened reagent grade alcohol was used without redistilling. All possible combinations of these three reagents (8 combinations, coded 1 to 8, described in table 4) were employed in the analyses of hemolyzed and non-hemolyzed aliquots of

TABLE 4

The effect of aged reagents on vitamin A and carotene values of serum before and after hemolysis

	REAGENT COMBINATION			VITAMIN A		CAROTENE	
				No hemolysis	Hemolysis	No hemolysis	Hemolysis
	Alcohol	*KOH*	*Kerosene-xylene*	*I.U./100 ml*	*I.U./100 ml*	*μg/100 ml*	*μg/100 ml*
1.	aged	aged	aged	186	586	120	6
2.	fresh	fresh	fresh	174	230	122	120
3.	aged	fresh	aged	169	955	119	6
4.	aged	fresh	fresh	186	235	124	120
5.	aged	aged	fresh	202	284	124	120
6.	fresh	aged	aged	211	772	124	4
7.	fresh	aged	fresh	197	279	125	124
8.	fresh	fresh	aged	178	762	122	6

one serum sample with the results which appear in table 4. Even with all freshly prepared reagents (combination 2), the vitamin A value of serum from hemolyzed blood was somewhat higher than the corresponding non-hemolyzed sample. However, in 4 cases (reagent combinations 1, 3, 6 and 8) vitamin A values were three to 5 times higher when the serum was hemolyzed; the carotene was reduced nearly to zero. This marked reduction in carotene is in contrast to the agreement on hemolyzed and non-hemolyzed samples shown in table 1. This discrepancy in carotene values is due to differences in the kerosene-xylene mixtures; the aged mixture described above was not the same batch as was used in obtaining the data in

table 1. It is evident that the high vitamin A and low carotene values on hemolyzed samples are due to the aged kerosene-xylene mixture, but that aged KOH (combinations 5 and 7) also gives a high vitamin A value.

To obtain further information concerning the effect of the ageing of kerosene-xylene mixtures on values for hemolyzed serum, data were obtained on hemolyzed and non-hemolyzed aliquots of the same serum using different kerosene-xylene mixtures which varied in age from freshly prepared to 38 weeks old. No purification of reagents was employed. The results are presented in table 5. In the presence of hemolysis

TABLE 5

The effect of the ageing of kerosene-xylene on vitamin A and carotene values of serum before and after hemolysis

AGE OF KEROSENE-XYLENE	VITAMIN A		CAROTENE	
	No hemolysis	Hemolysis	No hemolysis	Hemolysis
weeks	*I.U./100 ml*	*I.U./100 ml*	*μg/100 ml*	*μg/100 ml*
38	115	826	140	92
26	110	833	142	47
25	197	810	143	94
23	138	812	144	144
4	146	279	146	178
0	171	296	148	170

the older (23 to 38 weeks) kerosene-xylene mixtures caused a striking increase in apparent vitamin A content and in three of 4 cases an apparent reduction in carotene. A 4-week-old mixture gave similar results as did a freshly prepared one in non-hemolyzed blood, but both led to a nearly two-fold increase in apparent vitamin A content and slightly elevated carotene levels in hemolyzed samples. Since even a freshly prepared kerosene-xylene mixture proved unsuitable for use in the presence of hemolysis, purification of these reagents was studied. Changes in the spectral absorption characteristics of this reagent have been recorded by Bieri et al. ('51) who stated that the absorption of kerosene-xylene in the region of 310 to 400 mμ gradually increases after standing for about two

months in a glass-stoppered bottle unprotected from light. They suggest that errors due to changes in this solvent can be avoided by storing the kerosene-xylene mixture in the dark and by frequent checks of its absorption in the region of 310 to 400 mμ. Karmarkar et al. ('52) advise that the kerosene-xylene be refluxed over metallic sodium for several hours, followed by distillation of the xylene at 138°C and the kerosene at 180 to 195°C to eliminate impurities which interfere with the accuracy of the method. Arroyave [7] ('56) purifies the kerosene by shaking with activated charcoal and filtering through a fine sintered glass filter. The absorption spectrum of xylene as compared with water is tested when a new bottle is opened and it is redistilled when necessary.

A study of the influence of purification of xylene-kerosene on the phenomenon following hemolysis was made as follows: (1) Xylene was redistilled in an all glass still and the 134 to 137°C fraction collected; (2) Kerosene was shaken with a 5% solution of sodium bicarbonate, washed with distilled water, and stored overnight over 4-mesh calcium chloride. The kerosene (100 ml) was then shaken with approximately 3 gm of activated charcoal [8] and filtered through Whatman #1 filter paper; (3) The kerosene and xylene were mixed just before using and stored in a dark place. The purified kerosene and xylene were not stored for any length of time before mixing.

A venous blood sample was divided into two portions; one was allowed to clot before centrifugation and the serum removed and the second placed immediately in a freezer at −25°C for three hours. The hemoglobin concentration of the latter was determined and aliquots were added to known volumes of the non-hemolyzed serum to obtain increasing concentrations of hemoglobin up to 1.0 gm per 100 ml. These samples, along with a "completely" hemolyzed sample, were analyzed for vitamin A, using three batches of kerosene-xylene reagent as detailed in table 6. This experiment was repeated

[7] Arroyave, G., private communication.

[8] Darco G–60, activated charcoal obtained from Atlas Powder Company, Wilmington, Delaware.

TABLE 6

The effect of purification of kerosene-xylene on the reliability of data obtained on non-hemolyzed and hemolyzed aliquots of serum

		TRIALS													
		I [1]						II							
Reagents	Hemoglobin [2]	0.00	0.11	—	0.57	—	—	14.3	0.00	0.12	—	0.48	0.73	1.21	12.1
A Kerosene-xylene, freshly prepared and especially purified	Vitamin A [3]	129	140	—	149	—	—	432	134	154	—	186	157	138	401
	Carotene [4]	136	138	—	144	—	—	298	118	118	—	115	116	112	262
B Kerosene-xylene, 7 months old, not purified	Vitamin A	228	230	—	289	—	—	504	144	188	—	280	289	328	666
	Carotene	140	142	—	154	—	—	292	125	132	—	140	150	152	438
C Kerosene-xylene, 20 months old, not purified	Vitamin A	420	468	—	486	—	—	1060	138	478	—	447	512	552	1621
	Carotene	140	26	—	22	—	—	182	120	27	—	22	29	25	236
		III						IV							
Reagents	Hemoglobin [2]	0.00	0.13	0.26	0.52	0.78	1.29	12.9	0.00	0.12	0.24	0.48	0.72	1.17	11.7
A Kerosene-xylene, freshly prepared and especially purified	Vitamin A [3]	112	149	168	188	228	268	442	125	153	151	146	151	149	280
	Carotene [4]	115	116	123	122	136	159	294	118	118	116	116	113	111	182
B Kerosene-xylene, 7 months old, not purified	Vitamin A	182	206	234	250	219	254	754	168	188	219	258	241	219	508
	Carotene	124	128	118	123	126	126	346	124	124	122	119	121	121	251
C Kerosene-xylene, 20 months old, not purified	Vitamin A	99	635	522	523	537	526	1314	127	701	876	—	679	723	1411
	Carotene	121	28	12	14	17	12	188	121	68	23	12	12	12	210

[1] I, II, III and IV represent replicate experiments on serum samples from the same donor, but drawn on different days and analyzed at different times. Increasing degrees of hemolysis of each is indicated by the hemoglobin value.

[2] Hemoglobin, gm per 100 ml serum.

[3] Vitamin A, I.U. per 100 ml serum.

[4] Carotene, μg per 100 ml serum.

4 times on serum obtained from the same donor on different days. The kerosene used in reagent A was again treated with activated charcoal just prior to trial IV (this had not been done since approximately a week prior to trial I) but this apparently did not alter the results. The data summarized in table 6 show the varied results which may be obtained unless extreme care is taken in the purification of reagents. Considering trial IV, one may conclude that if the kerosene-xylene is especially purified and freshly prepared, the carotene value will not be affected by moderate hemolysis. Even the use of specially purified and freshly prepared kerosene-xylene mixture does not prevent a small increase in apparent vitamin A content in slightly hemolyzed samples. In the presence of a very old, unpurified kerosene-xylene mixture, the false increase in vitamin A content resulting from hemolysis is striking, and the carotene is reduced to a level approximating zero. It is also evident that unreliable data will result from non-hemolyzed samples if old or unpurified reagents are used.

The practical implications of this study are obvious since nutrition surveys which employ the micro-method of Bessey and Lowry are numerous. Under survey conditions, the samples must often be transported from field to laboratory as whole blood, and some hemolysis is not uncommon. If such hemolyzed samples are analyzed without the precaution of using a carefully purified kerosene-xylene mixture, false results will be obtained. The kerosene and xylene should be mixed immediately prior to using, and if it is necessary to keep this solvent for short periods of time, it must be protected from light.

SUMMARY

Hemolysis causes a false increase in serum vitamin A values when the micro-method of Bessey and Lowry is used. An especially purified and freshly prepared kerosene-xylene mixture minimizes the false increase in vitamin A caused by hemolysis and avoids errors in the carotene measurements. An unpurified kerosene-xylene mixture permitted to stand at

room temperature unprotected from light may cause as much as a 10-fold increase in the apparent vitamin A content of a hemolyzed blood sample and a definite diminution in the apparent content of carotene. In the absence of hemolysis, an aged, unpurified kerosene-xylene mixture resulted in a 4-fold increase in apparent vitamin A content but no change in the carotene level. Only freshly purified and mixed kerosene-xylene should be used in the Bessey-Lowry procedure for determining serum carotene and vitamin A. Serum samples showing evidence of more than a trace of hemolysis should be discarded. The Carr-Price reaction is relatively unaffected by hemolysis and is therefore the method of choice in circumstances where sufficient blood can be obtained.

LITERATURE CITED

BESSEY, O. A., O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ 1946 The determination of vitamin A and carotene in small quantities of blood serum. J. Biol. Chem., *166:* 177.

BIERI, J. G., AND M. O. SCHULTZE 1951 Microspectrophotometric determination of vitamin A and carotene in blood. Arch. Biochem. Biophys., *34:* 273.

CARR, F. H., AND E. A. PRICE 1926 Colour reactions attributed to vitamin A. Biochem. J., *20:* 497.

CASTER, W. O., AND O. MICKELSEN 1955 Serum vitamin A level: A critique of methods and significance. Am. J. Clin. Nutrition, *3:* 409.

CLAYTON, M. M., M. J. BABCOCK, W. D. FOSTER, S. STREGEVSKY, R. E. TUCKER, A. W. WERTZ AND H. H. WILLIAMS 1954 A referee blood experiment involving the use of microchemical methods. J. Nutrition, *52:* 383.

DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. Biochem. J., *32:* 1008.

HAM, T. H., Ed. 1950 A Syllabus of Laboratory Examinations in Clinical Diagnosis. Harvard University Press.

KARMARKAR, G., AND K. RAJAGOPAL 1952 Assay of vitamin A and carotene in blood serum. Current Sci., (India), *21:* 193.

SOBEL, A. E., AND S. D. SNOW 1947 The estimation of serum vitamin A with activated glycerol dichlorohydrin. J. Biol. Chem., *171:* 617.

EFFECT OF DIETARY LEVEL OF FAT AND TYPE OF CARBOHYDRATE ON GROWTH AND FOOD INTAKE [1]

A. YOSHIDA, A. E. HARPER AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication May 2, 1958)

In a previous study on the effect of the level of dietary fat on protein utilization it was observed that the growth rate of rats fed on diets containing different levels of fat but having the same protein per Calorie ratio increased when the level of dietary fat was raised from 1 to 30% (Yoshida, Harper and Elvehjem, '57). The gain per Calorie was also greater when the fat level was raised, the effect being particularly evident during the first week of the experiment. Nitrogen balance studies indicated that the greater rate of gain and improved Calorie utilization were not attributable to an improvement in protein utilization but rather to an increase in food consumption possibly due to the greater Caloric density of the high fat diet.

It was also observed that the greater rate of gain of rats fed a low-protein diet containing a complex carbohydrate (gelatinized starch) in place of a simple sugar (glucose, sucrose) could not be attributed to an improvement in protein utilization but appeared to be the result of a stimulation of food consumption (Spivey et al., '58). Both growth rate and food intake bore a direct relationship to the molecular weight of the dietary carbohydrate whereas the moisture content

[1] Published with the approval of the Director of the Wisconsin Agricultural Experimental Station. This work was supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. We are indebted to Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, for some of the crystalline vitamins.

and volume of the stomach contents were inversely related to the molecular weight of the dietary carbohydrate (Harper and Spivey, '58). It appeared, therefore, that the food intake of rats receiving low molecular weight carbohydrates that exert high osmotic pressure was limited because of the accumulation of a considerable amount of water in the stomach.

Inasmuch as the diets used in the previous study on the effect of the level of dietary fat contained sucrose (Yoshida, Harper and Elvehjem, '57) it seemed important to determine what interrelationships might exist among dietary level of fat, type of dietary carbohydrate, growth rate, food intake and the volume and moisture content of stomach contents. Several varieties of fats were also tested for their effects on the growth rates of rats receiving diets containing the same amount of protein per Calorie.

EXPERIMENTAL METHODS

Male weanling rats of the Sprague-Dawley strain weighing from 40 to 50 gm were used for the growth experiments and some of the stomach volume tests. Male adult rats were also used for stomach volume tests. The composition of the diets is summarized in table 1. All diets contained Salts 4 (Hegsted et al., '41), 4%; corn oil, 1%; and water soluble vitamins. Fat soluble vitamins were administered weekly. The diets (I–III) for the comparison of various kinds of fat contained 35 mg of protein per Calorie and those (diets IV–XII) for the other experiments 25 mg of protein per Calorie. Oleomargarine [2] was the fat source unless otherwise indicated.

The diets were supplied ad libitum for the growth experiment.

For the experiments on the effect of diet on stomach volume, mature rats were trained to eat 5 gm of diet within 30 minutes by feeding them diet VIII (containing 10% of fat and dextrin) for only two hours every morning. After two weeks they were divided into 4 groups and each group was

[2] Oleomargarine and butter were melted and the oils obtained by decantation were used in the diets.

TABLE 1

Composition of diets

DIETS	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
	%	%	%	%	%	%	%	%	%	%	%	%
Casein	15.5	17.4	21.9	11.0	12.5	15.6	11.4	12.8	15.8	9.7	11.3	14.8
Sucrose	79.5	68.6	44.1	84.0	73.5	50.4	—	—	—	—	—	—
Cerelose	—	—	—	—	—	—	—	—	—	85.3	74.7	51.2
Dextrin	—	—	—	—	—	—	83.6	73.2	50.2	—	—	—
Corn oil	1	1	1	1	1	1	1	1	1	1	1	1
Test fat	0	9	29	0	9	29	0	9	29	0	9	29
Salt IV	4	4	4	4	4	4	4	4	4	4	4	4
Vitamins [1] Prot./Cal., mg	35	35	35	25	25	25	25	25	25	25	25	25

[1] Vitamins (milligrams per kilogram of diet): thiamine·HCl, 16; riboflavin, 12; pyridoxine·HCl, 8; niacin, 100; Ca-pantothenate, 80; inositol, 400; biotin, 0.4; folic acid, 0.8; vitamin B_{12}, 0.08; choline·HCl, 400. Vitamin A, 1600 I. U.; D_2, 140 I.U.; tocopherol, 9.6 mg and menadione, 1.2 mg were administered per rat per week.

fed a different test diet (diets IV, VI, VII or IX) for three days. After this period the animals were fed 5 gm of test diet and 5 animals from each group were killed 1.5, 3 and 5 hours after the beginning of the feeding period. The stomachs were removed after ligation at the cardiac sphincter and at the pylorus. After weighing each stomach the contents were transferred to aluminum weighing dishes, dried to constant weight and the moisture content calculated.

For the stomach volume tests with young rats three groups of animals were fed a diet containing 1% of corn oil and sucrose (diet IV), 30% of corn oil and sucrose (diet VI) or 30% of butter fat and sucrose (diet VI) ad libitum. After one week they were fasted for 24 hours and again they were fed for one hour.

The weight and volume of the stomach contents were determined at the end of the feeding period and three hours after feeding.

RESULTS

Effect of type of fat. The weight gains and the Caloric intakes of the groups that were fed diets containing the different test fats are shown in table 2. All results are expressed as percentages of the value for the group fed the diet containing 1% of corn oil (negative control group). Every group that was fed a diet containing 30% of fat gained weight more rapidly than the negative control group, and in three cases increasing the level of fat in the diet to 10% caused a significant increase in the rate of gain. The differences between the groups receiving 30% of fat and those receiving 10% of fat were quite large when corn oil, butter fat or oleomargarine was the fat source but only small differences were observed with hydrogenated coconut oil, lard or olive oil as the fat source. Caloric intake showed the same trend as weight gain and there were no significant differences among the values for relative gain per Calorie.

Effect of type of carbohydrate. Groups of rats were fed diets containing sucrose, dextrin or cerelose as the carbo-

hydrate. Three dietary levels of fat were tested with each carbohydrate (diets IV–XII). The gains during the first week with dextrin as the carbohydrate were 36.9, 36.9 and 33.5 gm for the 1, 10 and 30% fat groups, respectively (fig. 1). In contrast to these results, the weight gains of the groups

TABLE 2

Effect of type of dietary fat on rate of gain and food intake [1]

TYPE AND LEVEL OF DIETARY FAT		RELATIVE GAIN/ 2 WEEKS	RELATIVE CAL. INTAKE/ 2 WEEKS	RELATIVE GAIN/CAL.
Corn oil	1%	100 ± 2.9 [2] (51 gm)	100 ± 2.2 [2] (460 Cal.)	100 ± 2.9 [2]
	10%	104 ± 5.7	107 ± 3.3	97 ± 5.3
	30%	126 ± 5.6 [3]	118 ± 3.9	106 ± 4.7
Butter	10%	119 ± 3.2 [3]	118 ± 3.1	102 ± 2.7
	30%	129 ± 4.7 [3]	123 ± 3.0	104 ± 3.8
Hydrogenated coconut oil	10%	116 ± 6.6 [4]	114 ± 4.4	102 ± 5.8
	30%	120 ± 3.4 [3]	116 ± 3.5	103 ± 2.9
Lard	10%	116 ± 4.8 [4]	114 ± 4.1	102 ± 4.2
	30%	119 ± 3.4 [3]	118 ± 2.0	101 ± 2.9
Olive oil	10%	110 ± 3.6	110 ± 2.4	100 ± 3.3
	30%	112 ± 3.4 [4]	107 ± 3.5	105 ± 3.2
Oleomargarine	10%	111 ± 5.8	107 ± 2.4	104 ± 5.4
	30%	120 ± 3.1 [3]	112 ± 2.0	107 ± 2.8

[1] All results are expressed as percentages of the value for the 1% corn oil group. The absolute values for this group are within parentheses.

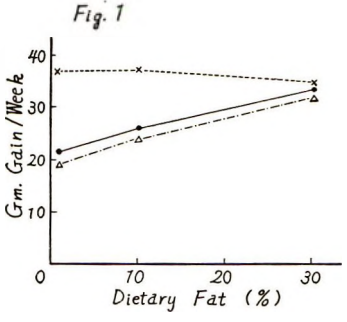
[2] Standard error of the mean.

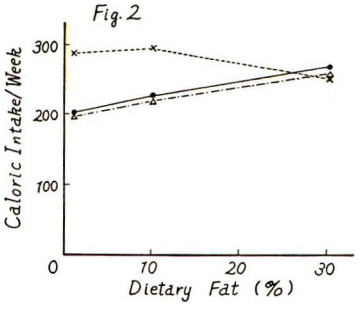
[3] Significantly different from negative control ($P < 0.01$).

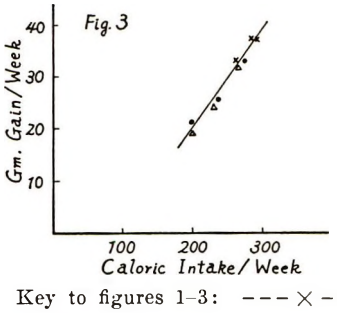
[4] Significantly different from negative control ($P < 0.05$).

receiving either sucrose or cerelose increased significantly with each increase in the fat content of the diets. With 1 or 10% of fat in the diet the difference between the weight gain of the dextrin group and that of either the sucrose or the cerelose group was statistically significant. With 30% of fat in the diet, the weight gains were almost the same regardless of the nature of the dietary carbohydrate.

Quite similar relationships were observed among the values for Caloric intake (fig. 2). For the dextrin series the Caloric intakes during the first week were 284, 291 and 262 for the 1, 10 and 30% fat groups, respectively. Caloric intakes of the sucrose and the cerelose groups increased with each increase in the dietary level of fat. Here too, with 1 or 10% of fat in the diets there were significant differences between the values for the dextrin groups and those for the sucrose or the cerelose groups.







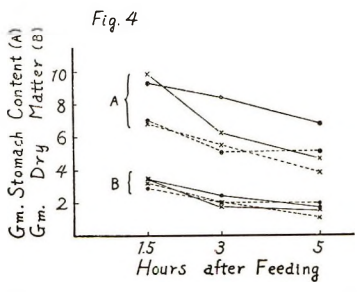
Key to figures 1–3: – – – × – – – dextrin; — ● — sucrose; — · — △ — · — cerelose.

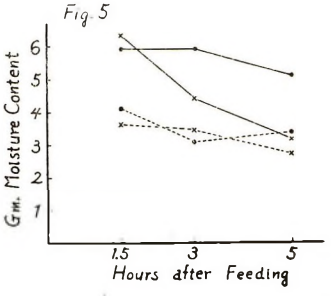
Fig. 1 Effect of type of carbohydrate and level of fat in the diet on the weight gain for the first week. The values for the dextrin groups were significantly different ($P < 0.05$) from those for the sucrose or the cerelose groups except when the diets contained 30% of fat.

Fig. 2 Effect of type of carbohydrate and level of fat in the diet on Caloric intake for the first week. The values for the dextrin groups were significantly different ($P < 0.01$) from those for the sucrose or the cerelose groups except when the diets contained 30% of fat.

Fig. 3 Relationship between Caloric intake and weight gain for first week.

The relationship between weight gain and Caloric intake for the first week is shown in figure 3. A straight line relationship was obtained over the range studied regardless of the type of carbohydrate or the level of fat in the diet. This relationship was the same at one and at three weeks.





Key to figures 4–5: —●— 1% fat and sucrose; —×— 30% fat and sucrose; ---●--- 1% fat and dextrin; ---×--- 30% fat and dextrin.

Fig. 4 Effect of type of carbohydrate and level of fat in the diet on wet weight and dry weight of stomach contents at intervals after feeding 5 gm of diets. The values for the stomach contents of the sucrose groups were significantly different from those for the dextrin groups 1.5 hours after feeding. ($P < 0.01$). The value for the group that was fed the diet containing 1% of fat and sucrose was significantly different from that for each of the other three groups, three hours and 5 hours after feeding ($P < 0.05$).

Fig. 5 Effect of type of carbohydrate and level of fat in the diet on the amount of moisture in the stomach contents at intervals after the feeding 5 gm of diet. The values for the sucrose groups were significantly different from those for the dextrin groups 1.5 hours after feeding ($P < 0.01$). The value for the group that was fed the diet containing 1% of fat and sucrose was significantly different from that for each of the other three groups, three hours after feeding ($P < 0.05$) and 5 hours after feeding ($P < 0.01$).

Influence of type of carbohydrate and level of fat on volume of stomach contents. The weights of the stomach contents of adult rats fed low or high fat diets containing dextrin or sucrose are shown in figure 4. One and one half hours after the ingestion of 5 gm of diet, approximately 9.5 and 6.9 gm of contents were recovered from the stomachs of the sucrose and the dextrin groups, respectively. The fat content of the diet did not appear to influence the volume of the stomach contents at 1.5 hours. However, three hours after feeding, the

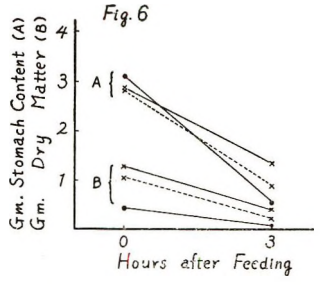
value for the group that received sucrose with 1% of fat was still about 8.4 gm while the value for the group fed a similar diet containing 30% of fat had dropped quite rapidly to about 6.2 gm. The latter value was not greatly different from those for the groups receiving dextrin. The same relationship was evident 5 hours after feeding.

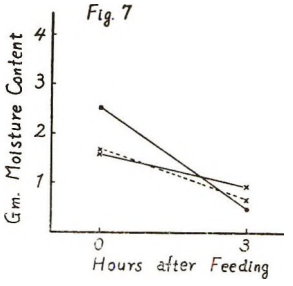
The values for moisture content of the stomach contents showed similar trends (fig. 5). One and one half hours after feeding there was about 6 gm of water in the stomachs of rats that consumed diets containing sucrose and only about 4 gm in the stomachs of those receiving dextrin. The amount of moisture in the stomach contents of rats that were given the diet containing 1% of fat and sucrose decreased slowly; the values for this group at 1.5 hours and three hours after feeding were almost the same and there was little decrease even after 5 hours. In contrast, the amount of moisture in the stomach contents of rats fed the diet containing 30% of fat and sucrose dropped rapidly. The initial values for rats receiving dextrin were low compared with those for groups receiving sucrose and the decrease over the 5-hour period was slow. Values for the dry weight of the stomach contents did not differ much from group to group (fig. 4).

Young rats, fasted for 24 hours then fed for one hour, consumed 1.2 gm of the sucrose diet containing 1% of corn oil and 2.2 gm of the diets containing 30% of corn oil or butter fat. In spite of this difference in food intake the weights of the stomach contents of the three groups were very little different just after feeding (fig. 6). After three hours, the values decreased to 0.6, 0.9 and 1.3 gm for 1% corn oil group, 30% butter group and 30% corn oil group, respectively. The amount of moisture in the stomach contents immediately after feeding was significantly higher when the diet contained 1% of corn oil than when it contained 30% of corn oil or butter fat (fig. 7) even though the average amount of dry matter in the stomachs of the rats fed the diet containing 1% of corn oil was significantly less than the amounts retained in the stomachs of the groups fed the diets that contained 30% of fat (fig. 6).

DISCUSSION

The observation that the substitution of 30% of oleomargarine for dietary sucrose improved the rate of growth of rats by stimulating food intake (Yoshida et al., '57) made it desirable to investigate the effects of other kinds of fats. The increase in growth rate that resulted from an increase in the level of fat was greatest when the diets contained 35 mg of protein/Cal., therefore the effects of various kinds of fat were compared using that protein/Cal. ratio in the diets.





Key to figures 6–7: — ● — 1% corn oil and sucrose; — × — 30% corn oil and sucrose; - - - × - - - 30% butter fat and sucrose.

Fig. 6 Effect of level of dietary fat on wet weight and dry weight of stomach contents of young rats fed for only one hour. The value for dry weight of stomach contents for the 1% fat group is significantly different from those for the 30% fat group ($P < 0.05$).

Fig. 7 Effect of level of dietary fat on the amount of moisture in the stomach contents of young rats fed for only one hour. The values for the 1% and 30% fat groups at zero time are significantly different ($P < 0.05$).

Although the kind of fat did affect the growth rate in some cases, the gains per Calorie were quite similar for all groups. This supports the conclusions of Deuel et al. ('47), and Thomasson ('55) who found that gain per Calorie was unaffected by the type of dietary fat.

The growth rates of rats fed on diets containing sucrose or cerelose were significantly improved when the level of fat in the diet was increased as had been observed previously (Yoshida et al., '57) but the growth rate was very little affected by the fat content of the diet when dextrin was the

dietary carbohydrate (fig. 1). There was however, a high positive correlation between growth and Caloric intake regardless of the carbohydrate source or the dietary level of fat in these experiments in which the protein to Calorie ratio was kept constant (fig. 3). The superior growth rates of dextrin groups as compared to those of sucrose or cerelose groups and the improvement in the growth rates of groups receiving sucrose or cerelose when these carbohydrates were partly replaced by fat, seems to be a result of the increased capacity of the animals to consume food under these conditions. This, in turn, appears to depend upon the capacity of the diet to exert osmotic pressure and, thereby affect the amount of water retained in the stomach.

The rate at which the moisture content of the stomach contents decreased was quite slow when the animals were fed a diet containing sucrose and 1% of fat whereas the moisture content of the stomach contents decreased very rapidly and reached almost the same level as that for dextrin groups 5 hours after feeding when the diet contained 30% of fat. The amount of moisture in the stomach contents of the 1% fat and 30% fat groups that received sucrose were the same 1.5 hours after feeding. At this point, just after feeding, the amount of dry matter in the stomach was high and adequate time had probably not been allowed for dilution of the stomach contents. The osmotic effect might, therefore, be evident only after a longer time interval and, indeed, the difference in moisture content was significant three and 5 hours after feeding. These osmotic differences could lead to differences in the capacity of the animals to consume food as was suggested for the effects of different types of carbohydrate (Harper and Spivey, '58). The small effect of the level of dietary fat on the growth of dextrin-fed animals can also be understood in terms of the smaller effect of the addition of fat on the osmotic capacity of a dextrin diet.

Young rats, fasted for 24 hours, then fed the diets containing 30% of corn oil or 30% of butter fat and sucrose for one hour ate twice as much as the group receiving 1% of corn oil

and sucrose. Nevertheless, the weights of the stomach contents just after feeding were about the same for the three groups. Therefore, it is considered probable that the animals ate the diets to the full extent of their stomach capacities and that the differences in the amounts of moisture that accumulated in the stomachs of these groups were responsible for differences in food intake (fig. 7).

In many instances it has been shown that fat is emptied from the stomach more slowly than either carbohydrate or protein (Thomas, '57). There was, however, no evidence of such an effect in these experiments with rats, an observation worthy of further study. The main effect appeared to be that of the lower osmotic pressure of the high-fat diets reducing the amount of water drawn into the stomach, thereby permiting greater food consumption. Since the high-fat diet also contains more Calories per gram the Caloric intake per gram of food would also be greater.

SUMMARY

The Caloric intake and the rate of gain of rats fed for 2 weeks on diets containing 35 mg of protein per Calorie increased when part of the dietary sucrose was replaced by fat, but there were no significant differences among the values for gain per Calorie with different dietary fats.

The Calorie intake and the rate of gain of rats fed on low-fat diets also increased when dietary sucrose or cerelose were replaced by dextrin. Dextrin was without effect when the diets contained 30% of fat.

The amount of water in the stomach contents of rats fed, in a single meal, 5 gm of a diet low in fat and containing sucrose was high and remained high for several hours thereafter. If a substantial part of the sugar in these diets was replaced by fat the moisture content fell quite rapidly after 1.5 hours. When dietary sucrose or cerelose was replaced by dextrin less water accumulated initially in the stomach and there was little effect of an increased level of fat.

It is suggested that, when a substantial part of a low molecular weight carbohydrate in a diet is replaced by fat or dextrin, the osmotic effect of the diet is reduced sufficiently to permit young rats to consume a greater quantity of the diet and, hence, gain weight more rapidly during the early stages of growth.

LITERATURE CITED

DEUEL, H. J., JR., E. R. MESERVE, E. STRAUB, C. HENDERICK AND B. T. LIBITUM 1947 The effect of fat level of the diet on general nutrition. I. Growth, reproduction and physical capacity of rats receiving diets containing various levels of cottonseed oil or margarine fat *ad libitum*. J. Nutrition, *33:* 569.

HARPER, A. E., AND H. E. SPIVEY 1958 Relationship between food intake and osmotic effect of dietary carbohydrate. Am. J. Physiol., *193:* 483.

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.

SPIVEY, H. E., M. C. KATAYAMA, M. YOSHIDA AND A. E. HARPER 1958 Significance of the "Protein-sparing" effect of dextrin. Am. J. Physiol., *193:* 479.

THOMAS, J. E. 1957 Mechanics and regulation of gastric emptying. Physiol. Rev., *37:* 453.

THOMASSON, H. J. 1955 The biological value of oils and fats. I. Growth and food intake on feeding with natural oils and fats. J. Nutrition, *56:* 455.

YOSHIDA, A., A. E. HARPER AND C. A. ELVEHJEM 1957 Effects of protein per calorie ratio and dietary level of fat on calorie and protein utilization. Ibid., *63:* 555.

EFFECT OF DIETARY LEVEL OF RAW SOYBEAN OIL MEAL ON THE GROWTH OF WEANLING RATS[1]

RAYMOND BORCHERS
Department of Biochemistry and Nutrition, University of Nebraska, Lincoln

(Received for publication May 5, 1958)

Raw soybean oil meal has been recognized as an inferior source of dietary protein for the rat as well as for other species. Numerous investigations, reviewed by Griswold ('51), Liener ('50), and Borchers et al. ('47), have failed to develop a satisfactory explanation for the slower rate of gain on raw soybean oil meal when compared with properly autoclaved soybean oil meal. The proposed explanations have frequently involved or suggested a heat-labile "toxic" factor in raw soybeans. In some cases, this "toxic" factor has been regarded as unidentified (Borchers and Ackerson, '51). In other publications, the "toxic" factor has been regarded as synonymous with the trypsin inhibitor of raw soybeans (Ham et al., '45; Klose et al., '46; Westfall and Hauge, '48; Almquist and Merritt, '51; Lyman and Lepkovsky, '57) or as "soyin" (Leiner, '53). Explanations, not involving a toxic factor, have suggested that amino acids in general or methionine in particular were not completely available or available at the right time from the protein of raw soybean oil meal (Hayward et al., '36b; Almquist et al., '42; Melnick et al., '46).

Pursuing the hypothesis that the effect of a toxic factor should be enhanced at higher levels of intake, the following

[1] Published with the approval of the Director as paper no. 873, Journal Series, Nebraska Agricultural Experiment Station.

investigations were undertaken to observe the effect of various dietary levels of raw soybean oil meal on the growth of weanling rats. Methionine (Almquist et al., '42) and antibiotics (Borchers et al., '57), previously shown to have a favorable response in raw soybean oil meal nutrition studies, were added in conjunction with various levels of raw soybean oil meal.

EXPERIMENTAL

Details concerning rations, animals and feeding procedures were described in a previous publication (Borchers et al., '57). Briefly, the rations consisted of raw or autoclaved soybean oil meal (46% protein, N × 6.25) at levels indicated, minerals, vitamins, 20% hydrogenated fat and starch to make 100%. Further additions replaced starch. Growth results were expressed in average grams of gain per day in a 20-day feeding period.

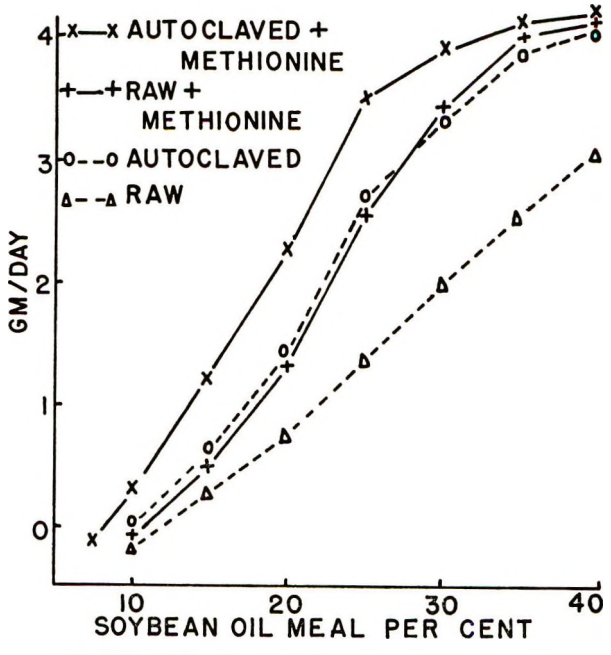


Fig. 1 Average daily gain of rats fed various levels of soybean oil meal and supplemented with 0.6% of DL-methionine. Each point represents the average gain of two groups of 8 weanling rats each for a 20-day feeding period.

Growth on various levels of soybean oil meal. Rations containing various levels of raw or autoclaved soybean oil meal with and without supplementary DL-methionine were fed as indicated in figure 1. An increase in growth rate was noted in each case as the protein level increased. In no case would the data support the hypothesis of a "toxic" factor exerting a greater effect as the level of feeding of the raw soybean

TABLE 1

Growth of rats fed autoclaved and raw soybean oil meal and supplemented with methionine and antibiotics

SOYBEAN [1] LEVEL	SUPPLEMENT	GROWTH RATE [2]	
		Autoclaved meal	Raw meal
%		*gm/day ± SE (gm/gm food)*	*gm/day ± SE (gm/gm food)*
25	None	2.72 ± 0.12 (0.33)	1.45 ± 0.19 (0.23)
25	Antibiotics [3]	2.90 ± 0.16 (0.40)	2.11 ± 0.13 (0.32)
25	Methionine [4]	3.43 ± 0.11 (0.41)	2.35 ± 0.17 (0.30)
25	Antibiotics + Methionine	3.47 ± 0.17 (0.40)	3.42 ± 0.20 (0.40)
40	None	4.09 ± 0.14 (0.46)	3.17 ± 0.14 (0.38)
40	Antibiotics	4.27 ± 0.18 (0.52)	4.26 ± 0.11 (0.50)
40	Methionine	4.11 ± 0.23 (0.49)	4.24 ± 0.14 (0.50)
40	Antibiotics + Methionine	4.19 ± 0.22 (0.50)	4.34 ± 0.14 (0.56)

[1] Autoclaved or raw soybean oil meal, 46% protein, N × 6.25.
[2] Average of 8 weanling rats for a 20-day feeding period.
[3] Streptomycin sulfate, 0.1% plus 0.1% procaine penicillin.
[4] DL-Methionine, 0.6%.

increased. The autoclaved soybean oil meal ration plus methionine supported the highest growth rate in this study, raw soybean oil meal without methionine the lowest. The autoclaved soybean ration without methionine and raw soybean with methionine supported an intermediate growth rate, the latter two being indistinguishable in growth-supporting value. The results are presented graphically in figure 1.

Effect of antibiotic. The effect of adding 0.1% procaine penicillin plus 0.1% streptomycin sulfate on the growth rate

of rats was compared at the 25 and 40% soybean levels both with and without additional methionine. The addition of antibiotic to the autoclaved soybean ration did not show a stimulatory effect in this study. Similar antibiotic addition to the raw soybean ration increased the growth rate at the 25% soybean level both with and without added methionine; at the 40% soybean level only in the absence of added methionine. Addition of methionine plus antibiotics was required to increase the growth rate on the raw soybean ration to that of the similarly supplemented autoclaved soybean ration at the 25% soy level; addition of either methionine or antibiotics resulted in equal gains at the 40% soy level. The growth rates are presented in table 1.

DISCUSSION

The results of this study, presented in figure 1, lend no support to a hypothesis that raw soybeans contain a "toxic" factor whose effect is enhanced by higher levels of raw soybean intake. However, the possibilities of a "toxic" factor or toxic effects which may reduce the availability of or increase the requirement for amino acids must be considered and is discussed below. The results, presented in figure 1 as curves of daily gain versus dietary soybean level, are typical for any protein, the position of the curve being characteristic for the essential amino acid array of the particular protein. The lower positions of the raw soybean curves in figure 1 strongly suggest that certain amino acids in addition to methionine are reduced in availability from raw soybeans or that the requirements for these amino acids are increased. Investigations attempting to identify the amino acids involved are being actively pursued in our laboratory.

Decreased amino acid availability has frequently been invoked as an explanation for the inferior value of raw soybeans, reduced availability being suggested as due either to the inherent nature of the raw soybean protein or to the trypsin inhibitor of raw soybeans. Publications opposing

the implication of availability may be summarized as follows: first, the digestibility of raw soybean protein is similar to that of autoclaved soybean protein (Hayward et al., '36a; Johnson et al., '39; Borchers et al., '47). Second, trypsin inhibitor concentrates do not adversely affect the growth rate (Borchers et al., '48). Third, soybean concentrates inhibit the growth rate with predigested proteins (Westfall et al., '48). Fourth, the effect of supplementary antibiotics, noted here and previously (Borchers et al., '57), are difficult to correlate with availability or with action of a trypsin inhibitor.

The possibility that antibiotics act by preventing undesirable facets of bacterial action in the intestinal tract may not be overlooked. Such undesirable bacterial action could involve the actual destruction in the gut of a portion of the necessary amino acids or the production of bacterial metabolites which, after absorption, increased the requirement for amino acids in detoxication reactions or in other ways.

The further possibility of a "toxic" factor acting *in vivo* to increase apparent amino acid requirements cannot be excluded. Such an increased requirement could be envisaged as resulting from a gamut of possibilities ranging from needs for detoxication of heat-labile soybean constitutents to the inhibition of particular synthetic reactions. However, again, the correlation of the effect of dietary antibiotics with such a postulate is difficult to rationalize.

SUMMARY

Data are presented comparing the rate of gain of weanling rats fed various levels of raw or autoclaved soybean oil meal with and without supplementary methionine and penicillin plus streptomycin. Raw soybean oil meal at the 40% level supplemented with either methionine or antibiotics supported the same rate of gain as autoclaved soybean oil meal. The results disprove the hypothesis that raw soybeans contain a "toxic" factor whose effect is enhanced by higher levels of raw soybean intake. The results, rather, support the con-

clusion that the availability of certain amino acids from raw soybean oil meal is less than that from autoclaved meal or that the requirement for certain amino acids is increased when raw soybean oil meal is fed. Arguments opposing the postulate of availability are reviewed. The postulate of increased requirement is discussed.

ACKNOWLEDGMENTS

The author acknowledges appreciation to the Dow Chemical Co. for DL-methionine, to Commercial Solvents Corp. for procaine penicillin, to Eli Lilly and Co. for streptomycin sulfate, and to Merck Sharp and Dohme for some of the vitamins used in this study.

LITERATURE CITED

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, *24:* 385–392.

ALMQUIST, H. J., AND J. B. MERRITT 1951 Effect of soybean antitrypsin on experimental amino acid deficiency in the chick. Arch. Biochem. Biophys., *31:* 450–453.

BORCHERS, R., W. E. HAM, R. M. SANDSTEDT, C. W. ACKERSON, R. H. THAYER AND F. E. MUSSEHL 1947 Trypsin inhibitor. Nebr. Agr. Expt. Sta., Res. Bull. No. 152: 1–15.

BORCHERS, R., C. W. ACKERSON AND F. E. MUSSEHL 1948 Growth inhibiting properties of a soybean trypsin inhibitor. Arch. Biochem. Biophys., *19:* 317–322.

BORCHERS, R., AND C. W. ACKERSON 1951 Counteracting the growth inhibitor of raw soybeans. Proc. Soc. Exp. Biol. Med., *78:* 81–83.

BORCHERS, R., D. MOHAMMAD-ABADI AND J. M. WEAVER 1957 Antibiotic growth stimulation of rats fed raw soybean oil meal. Ag. Food Chem., *5:* 371–373.

GRISWOLD, R. M. 1951 Effect of heat on the nutritive value of proteins. J. Am. Dietet. Assoc., *27:* 85–93.

HAM, W. E., R. M. SANDSTEDT AND F. E. MUSSEHL 1945 The proteolytic inhibiting substance in the extract from unheated soybean meal and its effect upon growth in chicks. J. Biol. Chem., *161:* 635–642.

HAYWARD, J. W., H. STEENBOCK AND G. BOHSTEDT 1936a The effect of heat as used in the extraction of soybean oil upon the nutritive value of the protein of soybean oil meal. J. Nutrition, *11:* 219–234.

———— 1936b The effect of cystine and casein supplements upon the nutritive value of the protein of raw and heated soybeans. Ibid., *12:* 275–283.

JOHNSON, L. M., H. T. PARSONS AND H. STEENBOCK 1939 The effect of heat and solvents on the nutritive value of soybean protein. Ibid., *18:* 423–434.

KLOSE, A. A., B. HILL AND H. L. FEVOLD 1946 Presence of a growth inhibiting substance in raw soybeans. Proc. Soc. Exp. Biol. Med., *62:* 10–12.

LIENER, I. E. 1950 The effect of heat processing and storage on the nutritive value of proteins of importance in the cereal industry. Trans. Amer. Assoc. Cer. Chem., *8:* 162–185.

———— 1953 Soyin, a toxic protein from the soybean. J. Nutrition, *49:* 527–539.

LYMAN, R. L., AND S. LEPKOVSKY 1957 The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. Ibid., *62:* 269–284.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymic digestion of proteins as a factor in nutrition. Science, *103:* 326–329.

WESTFALL, R. J., AND S. M. HAUGE 1948 The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. J. Nutrition, *35:* 379–389.

WESTFALL, R. J., D. K. BOSSHARDT AND R. H. BARNES 1948 Influence of crude trypsin inhibitor on utilization of hydrolyzed protein. Proc. Soc. Exp. Biol. Med., *68:* 498–500.

EFFECT OF SODIUM SALICYLATE ON THE FOOD AND OXYGEN CONSUMPTION OF RATS

EUGENE J. BECKER
Department of Physiological Sciences,
Dartmouth Medical School,
Hanover, N. H.

(Received for publication May 15, 1958)

In the normal metabolism of the animal body, oxidation of ingested food leads to the synthesis of nucleotide-bound polyphosphates, the so-called high-energy phosphate compounds, such as adenosinetriphosphate. These energy-rich phosphate compounds are believed to be the primary sources of energy required for the various functions of the cells. The healthy animal maintains a nice balance between oxidation and phosphorylation and is able to adjust the rates of these two coupled processes to the energy requirements of varying conditions. There are, however, substances which can disrupt this harmonious coordination of oxidation and synthesis of energy-rich phosphate bonds giving rise to the phenomenon termed "uncoupling" of oxidative phosphorylation. Many of these uncoupling agents when administered in certain concentrations seem to decrease the efficiency of phosphorylation and the cells respond to this impaired phosphate bond generation by increasing their rate of oxidation. This elevated oxygen consumption results, in the final analysis, in the burning of more organic matter, that is, the animal consumes more food than what it would require at the given set of conditions in the absence of the uncoupling agent.

Salicylates are known to be such uncoupling agents. *In vitro* experiments indicate that low concentrations of sodium salicylate increase the oxygen consumption of liver (Sproull, '54), brain (Fishgold et al., '51), and diaphragm (Smith and

Jeffrey, '56) slices. Recent experiments show that the decreased efficiency of oxidative phosphorylation can also be demonstrated in the mitochondria prepared from various tissues (Brody, '56). The over-all stimulating effect of salicylates on respiration has also been reported on the human (Cochran, '54) and animal (Meade, '54) organism. There are, however, no experimental data available about the quantitative relationship between increased oxygen consumption and food oxidation. In the study reported here an attempt was made to show that animals actually burn more food when salicylate is fed in a concentration which impairs the efficiency of phosphorylation.

EXPERIMENTAL

Young male rats weighing 30 to 35 gm were used. Six individually numbered animals in each group were placed in the same cage. The cages had raised wire bottoms and underneath removable pans. In the pans first a sheet of heavy aluminum foil and into this a double layer of heavy absorbent paper was placed; both were formed into rectangular low-walled containers tightly fitting into the heavy metal pans. The feces, urine, and wasted food were collected in these pans, air-dried, and weighed once a week. The difference between the weight of the food offered and the weight of air-dried feces, urine and wasted food was determined and accepted as the amount of food "oxidized."

Standard rat pellets were used as basal ration. The pellets were pulverized and calculated amounts of sodium salicylate, corresponding to 0.1, 0.25 and 0.5% of the diet were mixed with weighed portions of the basal diet. Tap water was supplied ad libitum.

All animals were weighed individually once a week and average growth curves were constructed for each group.

Oxygen consumption of each group was determined every week or 10 days using a modified closed circuit method originally described by Maclagan and Sheahan ('50). In preliminary experiments it was found that too much water

vapor and ammonia — evolved from urine coming in contact with the strong alkali which is put into the closed chamber to absorb carbon dioxide — made the animals uncomfortable and no uniform values for oxygen consumption could be obtained. By placing weighed amounts of anhydrous $CaCl_2$ and

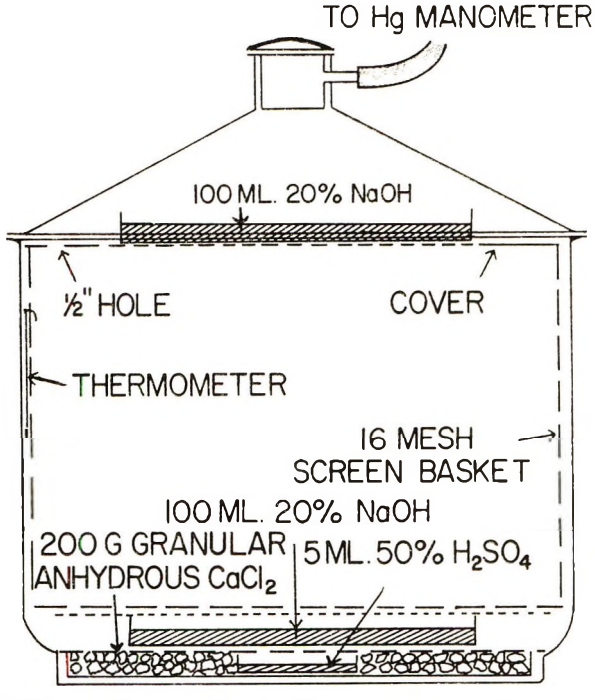


Fig. 1 Closed circuit apparatus for the measurement of oxygen consumption of rats.

50% H_2SO_4 into the desiccators, the amount of water vapor and ammonia can be greatly reduced, and after 15 to 20 minutes the animals will invariably quiet down and usually go to sleep for at least one and one-half to two hours. This is enough time to make three or 4 30-minute oxygen consumption readings on the manometers. Successive determinations usually agree within $\pm 5\%$ (fig. 1).

The calibration of the apparatus and the calculation of oxygen consumption were carried out as proposed by Mac-

lagan and Sheahan. For the calculation of the total gas space, all glass and metal parts were put into the desiccator and the volume of water needed to fill the desiccator was measured accurately. The volume of the reagents used should also be taken into account. The body volume of the animals was taken as milliliters per gram body weight. The volume of the top of the desiccator was determined again by filling it with water and the gas space of the tubings was simply calculated from the diameters and lengths.

Oxygen consumption was calculated by the formula:

$$O_2 \text{ consumption in ml/hr} = V \times \frac{p}{760} \times \frac{273}{273 + t} \times \frac{1}{T}$$ where

V = total gas volume in ml
p = observed fall in pressure, mm Hg
t = temperature inside of the apparatus
T = time of observation, hr.

At the termination of the experiment the animals were killed by asphyxiation. The thyroid glands were quickly dissected and weighed at once on a torsion balance. The glands were then dried at 105°C for 24 hours and weighed again. The weight of dry thyroid gland substance, in milligrams per gram of total body weight, was calculated for each animal.

RESULTS AND DISCUSSION

The curves in figure 2 show that 0.1 and 0.25% of sodium salicylate had no appreciable effect on the growth of the animals, but 0.5% of sodium salicylate definitely retarded growth. This growth retarding effect of larger doses of salicylates has already been observed in earlier experiments (Becker, '41) but at that time it was believed that the main reason for poor growth was the curbed intake of food. In the light of present day information on the metabolic effects of uncoupling agents, it seems to be more probable that retarded growth is primarily caused by the uncoupling effect of sodium salicylate.

As shown in figure 3 the measured oxygen consumption of animals on the diet containing 0.5% of salicylate was consis-

tently and significantly higher than that of animals on the basal diet or on diets with moderate amounts of sodium salicylate.

Figure 4 shows the average food consumption of the 4 groups of animals for the first 6 weeks of their growth period. As expected, the animals on the diet containing 0.5% of salicylate used about 50% more food for the same gain in body

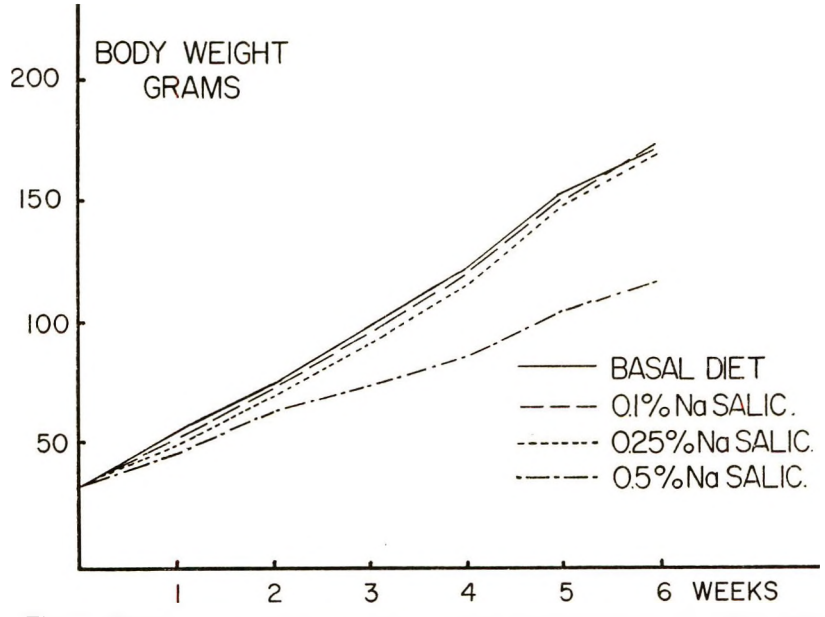


Fig. 2 Growth curves of rats on diets containing 0, 0.1, 0.25 and 0.5% sodium salicylate.

weight than animals on the basal or low salicylate diets.

In the mammalian body the thyroid hormone has a very important role in the regulation of the metabolic rate. Thyroxine and related compounds have been shown to have uncoupling actions (Lardy and Feldott, '51) and it was suggested (Brody, '55) that controlled dissipation of useful energy by the action of thyroxine may be part of the mechanism through which the thyroid gland exerts its profound effects. It was thought to be conceivable that high doses of sodium

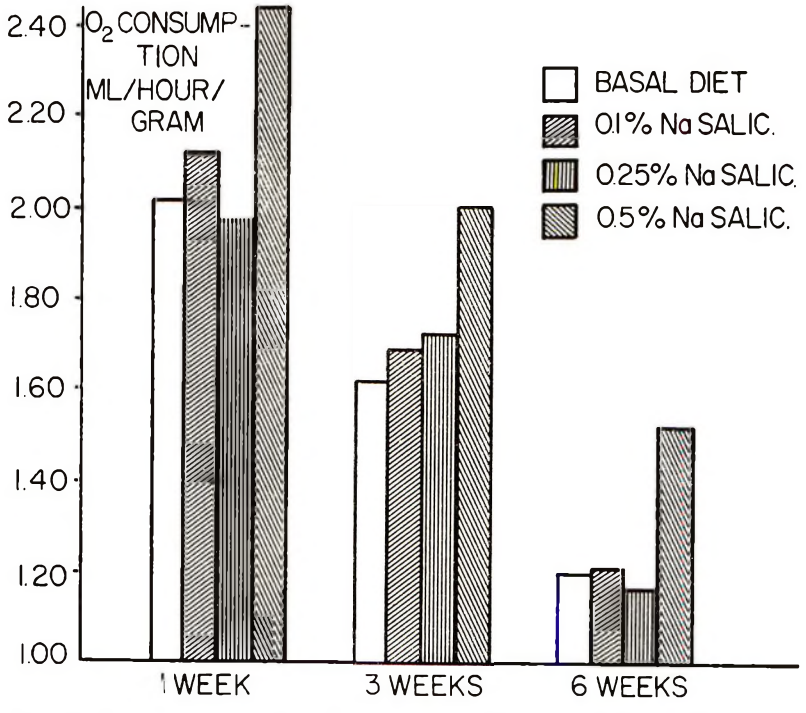


Fig. 3 Oxygen consumption of rats kept on diets containing 0, 0.1, 0.25 and 0.5% sodium salicylate.

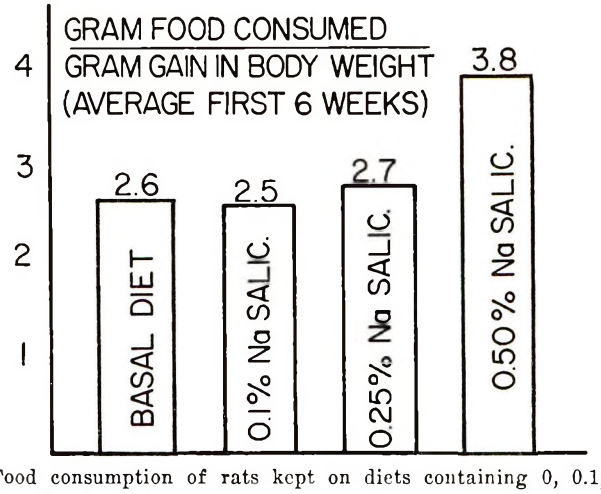


Fig. 4 Food consumption of rats kept on diets containing 0, 0.1, 0.25 and 0.5% sodium salicylate.

salicylate may affect the functions of the thyroid gland, at least to the extent that the amount of active gland tissue responsible for the production of the hormone may be reduced if salicylate is present in a high enough concentration to uncouple oxidative phosphorylation. From the figures presented in table 1 it seems that there was no direct relationship in these experiments between the weight of the thyroid glands and total body weights of animals on the basal diet and those on diets containing various amounts of sodium salicylate.

TABLE 1

Weight of dry thyroid gland tissue per gram body weight (average of all animals) in relation to intake of salicylate

CONTROL	SODIUM SALICYLATE ADDED		
	0.1%	0.25%	0.5%
mg	*mg*	*mg*	*mg*
0.52 ± 0.01 [1]	0.46 ± 0.03	0.52 ± 0.03	0.49 ± 0.01

[1] Standard error of the mean.

SUMMARY

The experimental data presented indicate that young rats when fed diets containing moderate amounts of sodium salicylate, grow normally and oxidize about the same amount of food as animals on a normal diet, containing no salicylate. Their oxygen consumption does not differ substantially from that of normal animals; the differences found between animals fed the basal diet and those which received 0.1%, and 0.25% of salicylate are probably within the errors of the techniques employed in the experiments. The amount of organic substance used up to produce 1 gm of body tissue is about the same for these three groups of animals. Apparently, these low concentrations of sodium salicylate, when administered through the diet, have no significant uncoupling action. The same amounts of sodium salicylate (about 10 and 25 mg/day/animal) administered intraperitoneally or intravenously (Tenney and Miller, '55) have been reported to stimulate oxygen consumption, indicating that uncoupling took place. The route of administration employed in the experiments reported

here may explain the negative findings at the lower salicylate levels.

The animals on the 0.5% salicylate diet were definitely retarded in their growth. On the average, they gained only about 85 gm during the 6 weeks of the feeding experiment as compared to 140 gm of average gain in body weight of the animals on the basal, 0.1%, and 0.25% salicylate diets. At the same time, their oxygen and food consumption was higher, indicating that they oxidized more organic matter to produce the same amount of new tissue. It is suggested that this excess oxidation may be the consequence of impaired phosphate-bond energy generation, caused by the uncoupling action of sodium salicylate.

ACKNOWLEDGMENT

The author is indebted to David G. Kamper and Thomas L. Watt for technical assistance.

LITERATURE CITED

BECKER, E. 1941 Die Einwirkung einiger Konzervierungsmittel auf den tierischen Organismus. Ber. ges. Physiol. u. exp. Pharmak., *126:* 477.

BRODY, T. M. 1955 The uncoupling of oxidative phosphorylation as a mechanism of drug action. Pharmac. Rev., *7:* 335.

———— 1956 Action of sodium salicylate and related compounds on tissue metabolism in vitro. J. Pharm. and Exp. Therap., *117:* 39.

COCHRAN, J. B. 1954 Further observations on the metabolic stimulating effect of salicylate. Brit. Med. J., *1:* 733.

FISHGOLD, J. T., J. FIELD AND V. E. HALL 1951 Effect of sodium salicylate and acetylsalicylate on metabolism of rat brain and liver in vitro. Am. J. Physiol., *164:* 727.

LARDY, H. A., AND G. FELDOTT 1951 Metabolic effects of thyroxine in vitro. Ann. New York Acad. Sci., *54:* 636.

MACLAGAN, N. F., AND M. M. SHEAHAN 1950 The measurement of oxygen consumption in small animals by a closed circuit method. J. Endocrinology, *6:* 456.

MEADE, B. W. 1954 Effect of certain hydroxybenzoic acids on the oxygen consumption of Wistar rats. Ann. Rheum. Dis., *13:* 60.

SMITH, M. J., AND S. W. JEFFREY 1956 The effects of salicylates on oxygen consumption and carbohydrate metabolism in the isolated rat diaphragm. Biochem. J., *63:* 524.

SPROULL, D. H. 1954 A peripheral action of sodium salicylate. Brit. J. Pharmacology, *9:* 262.

TENNEY, S. M., AND R. M. MILLER 1955 The respiratory and circulatory actions of salicylate. Am. J. Med., *19:* 498.

ESSENTIAL FATTY ACID DEFICIENCY

II. IN ADULT RATS [1]

ERIK AAES-JØRGENSEN,[2] E. E. LEPPIK, H. W. HAYES AND RALPH T. HOLMAN

The Hormel Institute and Department of Physiological Chemistry, University of Minnesota, Austin

(Received for publication May 12, 1958)

INTRODUCTION

The development of essential fatty acid deficiency in adult rats was reported by Barki, Nath, Hart and Elvehjem ('47), who first restricted caloric intake until the rats weighed one-half of their original weight and then fed the same fat-free diet ad libitum. During the period of re-growth, the animals temporarily developed the dermal symptoms characteristic of essential fatty acid (EFA) deficiency. To our knowledge, the development of EFA deficiency by feeding fat-free diets to adult rats has not been reported, with the above exception, which employed a stress condition. Experience in this laboratory has indicated that a supplement of cholesterol in otherwise fat-free diets may accelerate the appearance of EFA deficiency in young rats (Peifer and Holman, '55; Aaes-Jørgensen and Holman, '58). Dietary cholic acid is known to induce hypercholesterolemia when added to the diets of rats. The aim of the present study was to induce EFA deficiency in adult rats by feeding an EFA-free diet and to compare the effects of cholesterol and cholic acid, alone or

[1] Hormel Institute publication no. 173. Supported in part by grants from The Hormel Foundation, the National Live Stock and Meat Board, and the National Dairy Council.

[2] Fulbright Fellow, 1956-58. Permanent address: Department of Biochemistry and Nutrition, Polytechnic Institute, Østervoldgade 10L,[2] Copenhagen K, Denmark.

in combination, as causes of stress. The depletion of EFA was judged by observations of the growth rate, development of dermal symptoms, and histological and analytical studies of the testicular and epididymal tissues obtained by orchectomy of the right testis of representative animals at intervals during the nutritional study.

TABLE 1

Percentage composition of diets

DIET NO.	CONSTITUENT	AMOUNT
		%
	Vitamin test casein [1]	16
	Sucrose	73
1	α-Cellulose	4
	Vitamin mixture in vitamin test casein [2]	1
	Salt mixture [3]	4
	Choline chloride in vitamin test casein [2]	1
	Hydrogenated coconut oil [4]	1
2	As diet 1, but 1% of sucrose was replaced by 1% cholesterol [1]	
3	As diet 1, but 1.5% of sucrose was replaced by 1% cholesterol plus 0.5% of cholic acid [1]	
4	As diet 1, but 0.5% of sucrose was replaced by 0.5% cholic acid	
5	As diet 1, but 19% sucrose was replaced by 19% hydrogenated coconut oil	

[1] General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Aaes-Jørgensen and Holman ('58).
[3] Wesson.
[4] Hydrol.

EXPERIMENTAL

Part A. Forty-seven adult male rats having an average weight of 268 gm were divided into 4 groups of 10 animals each and a control group of 7 animals. All the groups were kept on a low-fat EFA-free diet (table 1). Groups 1 and 5 received no supplement. Groups 2, 3 and 4 received 1% cholesterol, 1% cholesterol plus 0.5% cholic acid, and 0.5% cholic acid, respectively, which replaced equal amounts of sucrose in the EFA-free diet. Diet and water were provided ad libitum, and each week the animals were weighed and in-

spected. The right testis and epididymis was removed from each of three rats in groups 1 through 4 at the end of 17, 22 and 27 weeks. After the 29th week, all except 4 rats in each of these groups were sacrificed by ether anaesthesia, and blood was drawn from the heart. Histological studies were performed on the heart, aorta, liver, kidney and the remaining left testis. The polyunsaturated acids in the lipides of the testis and the heart were measured by spectrophotometric analysis (Holman and Hayes, '58). Serum cholesterol determinations were made by the procedure of Abell, Levy, Brodie and Kendall ('52).

Part B. For the following 23 weeks, the 4 rats remaining in each of these groups 1 through 4 were fed diet no. 5 (table 1), which contained 20% hydrogenated coconut oil, but the supplements of cholesterol and cholic acids were discontinued. Group 5 continued to receive the low-fat EFA-free diet.

At the end of the 52nd week the remaining rats were killed by ether anaesthesia and the left testis, liver, heart, aorta and kidneys of each were taken for histological examination, and testis and heart tissues were analyzed for their content of polyunsaturated fatty acids.

RESULTS AND DISCUSSION

The growth data and general plan of the experiment are given in table 2. The growth rates of all groups decreased considerably after about 12 weeks of supplementation, resulting in a plateau in the growth curves for the following 17 weeks. The least growth was shown by the rats in group 4, whose diet was supplemented with cholic acid, and this may be explained partially by a slight, intermittent diarrhea which affected most of the rats in the group, especially at the start of the experiment. Orchectomy of the right testis did not affect the growth rate of the rats. Increasing the dietary fat content from 1 to 20% and omitting the previous supplements stimulated growth somewhat in all groups. The low-fat EFA-free

group lost weight in this phase of the experiment. Near the experiment's end (49th through 52nd weeks) weight loss became pronounced, and a few deaths occurred in all groups. Weight loss and death of the animals probably were related to the age of the rats, but also may have been influenced by their dietary history.

Dermal symptoms of EFA deficiency in the several groups are shown in table 3. The onset of symptoms in adult rats fed the EFA-free diet is late compared with the 9 to 12 weeks required by weanling rats fed the same diet. In the present experiments with adult rats, little or no hastening of dermal symptoms by dietary cholesterol was observed. Cholic acid supplementation hastened the appearance of dermal symptoms somewhat, but even in this case, only moderate symptoms occurred after 29 weeks. This is in contrast to the appearance of dermal symptoms within 4 to 6 weeks in weanling rats fed an EFA-free diet containing cholesterol (Peifer and Holman, '55; Aaes-Jørgensen and Holman, '58). The control rats (group 5), which were fed an unsupplemented, low-fat EFA-free diet throughout the entire experiment, developed mild dermal symptoms which reached a maximum around the 35th week and disappeared before the 52nd week. In groups 1 through 4, increasing the dietary fat level from 1 to 20% with hydrogenated coconut oil did not worsen the dermal symptoms.

Burr ('42) and Deuel et al. ('51) have suggested that increasing the dietary fat content increases the EFA requirement, but in the present experiments, adult rats fed the high-fat EFA-free diet exhibited spontaneous cures of the dermal disorder. This finding is in agreement with observations by Holman and Ener ('54) and by Aaes-Jørgensen et al. ('57), who found that, with weanling rats, dermal symptoms reached a maximum between the 13th and 15th weeks, followed by some spontaneous relief. There is no doubt that the dermal symptoms are related to EFA deficiency, and that the onset of the symptoms is, to a certain degree, related to other dietary components, such as cholesterol and hydrogenated fat. However, the fact that the syndrome also is affected by

TABLE 2

Experimental plan and weight record in grams

GROUP	DIET	0 WEEKS	12 WEEKS	17 WEEKS [1]	22 WEEKS [1]	27 WEEKS [1]	29 WEEKS [2]	DIET	39 WEEKS	49 WEEKS	52 WEEKS
1	Low-fat (LF)	268 [10]	439 [9]	451 [9]	458 [9]	460 [9]	459 [9]	HF	505 [4]	505 [4]	470 [2]
2	LF + cholesterol	268 [10]	446 [10]	461 [10]	465 [10]	466 [10]	470 [10]	HF	498 [4]	504 [4]	467 [3]
3	LF + cholesterol + cholic acid	268 [10]	430 [10]	449 [10]	453 [10]	455 [10]	454 [10]	HF	490 [4]	479 [4]	464 [3]
4	LF + cholic acid	268 [10]	414 [9]	423 [9]	430 [9]	436 [8]	441 [8]	HF	487 [3]	417 [2]	387 [2]
5	LF	283 [8]	430 [7]	445 [7]	455 [7]	458 [7]	459 [7]	LF	452 [7]	436 [7]	417 [5]

[1] Orchectomy of three rats from each of groups 1–4.

[2] At the end of the 29th week, groups 1–4 were reduced to 4 rats each, and diets were changed.

LF = Low-fat diet (1% hydrogenated coconut oil).

HF = High-fat diet (20% hydrogenated coconut oil).

Superscript numbers indicate the number of rats alive at each time period.

TABLE 3

Average skin scores[1] in experimental rats

GROUP	DIET CHARACTERISTICS	FIRST APPEARANCE OF SYMPTOMS (WEEKS)	SKIN SCORES				DIET CHARACTERISTICS	SKIN SCORES			
			17 wks.	22 wks.	27 wks.	29 wks.		35 wks.	41 wks.	46 wks.	52 wks.
1	Low-fat	ca 18 [9]	0 [9]	0.2 [9]	0.3 [9]	0.3 [9]	High-fat	0.1 [4]	0.1 [4]	0.1 [4]	0 [2]
2	Low-fat + cholesterol	ca 17 [10]	0.2 [10]	0.2 [10]	0.6 [10]	0.7 [10]	High-fat	0.6 [4]	0.1 [4]	0 [4]	0 [3]
3	Low-fat + cholesterol + cholic acid	ca 13–14 [10]	0.4 [10]	0.4 [10]	0.8 [19]	1.0 [10]	High-fat	0.3 [4]	0.1 [4]	0 [4]	0 [3]
4	Low-fat + cholic acid	ca 13–14 [10]	0.4 [9]	0.5 [9]	1.2 [8]	1.5 [8]	High-fat	1.0 [3]	0.4 [2]	0 [2]	0 [2]
5	Low-fat	ca 17–20 [7]	0.2 [7]	0.4 [7]	0.5 [7]	0.8 [7]	Low-fat	1.3 [7]	0.7 [7]	0.3 [7]	0 [5]

[1] Dermal symptoms were scored with a range of 0 to 3 each for tail, forelegs, hind legs, and dandruff, and the average of these 4 scores per animal was calculated for the group. This score multiplied by 3 approximately equals the score used by Holman and Ener, '54.

humidity or water intake, and undergoes spontaneous cure over a long period of time, indicates that dermal score alone cannot be used as an accurate indicator of the status of EFA deficiency.

The yellow-brown pigmentation on the skin of the backs of the rats was evident at the beginnning of the experiment (table 4). By the end of the 29th week, the normal pigment had almost disappeared from the rats in all groups as is characteristic of EFA deficiency. Feeding the high-fat diet caused the pigment to return, to a degree, for a time, and then to fade out again. These observations are similar to those made with weanling rats (Aaes-Jørgensen et al., '57).

Histological examinations and weights of the testes that were removed from some animals in each group at three different times in the first phase of the experiment showed that all except one appeared normal. The exception, a testis from an animal whose diet was supplemented with cholesterol, weighed only half that of the average normal testis of animals the same age, and histological inspection revealed severe degeneration of the spermatogenic tissue. The epididymis contained only a few degenerated cells. Histological examination of the left testes and epididymes taken at the end of the 29th week showed no disturbances in any case. Examination of the testes of the rats at the end of the 52nd week, after they had been fed the high-fat diet for 23 weeks, likewise revealed an apparently normal condition. Some degeneration of spermatogenic tissue, and a paucity of sperm in the epididymes, were observed in one or two rats of each group, but this might be expected in rats at least a year and a half old.

The kidneys of the rats killed after 29 weeks of experimentation showed no evidence of papillary degeneration, calculi, or other symptoms of EFA deficiency developed in rats fed a fat-free diet from the weanling age. All of the rats fed the high-fat EFA-free diet in the second phase of the experiment apparently had normal kidneys. A slight infiltration of fat in the liver was noticed in several cases among the high-fat groups.

TABLE 4

Yellow-brown pigmentation [1] *on the skin of the backs of animals*

GROUP	DIET CHARACTERISTICS	INITIAL	29 WKS.	DIET CHARACTERISTICS	35 WKS.	46 WKS.	52 WKS.
1	Low-fat	2.5 [10]	0.5 [9]	High-fat	1.3 [4]	0.6 [4]	0.7 [2]
2	Low-fat + cholesterol	2.5 [10]	0.1 [10]	High-fat	0.6 [4]	0.5 [4]	0.3 [3]
3	Low-fat + cholesterol + cholic acid	3 [10]	0 [10]	High-fat	1.3 [4]	0.4 [4]	0.5 [3]
4	Low-fat + cholic acid	2 [10]	0.3 [10]	High-fat	0.4 [3]	0.1 [2]	0.2 [2]
5	Low-fat	2.5 [7]	0 [7]	Low-fat	0 [7]	0 [7]	0 [7]

[1] Yellow-brown pigment was scored 0 to 3. Superscript numbers indicate the number of rats in the group.

Histological studies of the heart tissues by Dr. Stanley Hartroft, Washington University, St. Louis, revealed that lipide deposits were present in the coronary arteries of 4 out of 6 rats which had been fed the low-fat diet supplemented with cholesterol and cholic acid. It also is of interest that the plasma cholesterol concentration of this group was much higher (381 mg/100 ml) than that of the unsupplemented control (74 mg/100 ml) and also higher than that of cholesterol or cholic acid-supplemented groups (82 and 108 mg/100 ml, respectively).

Histological examination of hearts from the rats killed at the end of the 52nd week revealed no abnormalities in group 1. Striking sudanophilia of the serum within the coronary vessels was visible in the sections of three rats of group 2, in three rats of group 3, and in two rats of group 4. One animal of group 4 showed a very slight degree of abnormal accumulation of fat in myocardial cells. The rats of group 5 (EFA-free, low-fat) had none of these abnormalities.

The polyunsaturated acid content of testes was measured periodically during and at the end of the first phase of the experiment (table 5). Only small differences were noticed between the polyunsaturated acids of the testes of groups 1 through 4, and only small variations with time were observed. It should be pointed out that in all groups the content of trienoic acid was several times the content of dienoic acid at the end of the first phase of the experiment. At the end of the second phase, the testes of rats fed low-fat diets still had a low dienoic acid content, but they had an even larger trienoic acid content. The rats fed high-fat diets were found to have normal dienoic acid contents and high trienoic and pentaenoic acid contents in their testes. The contents of total polyunsaturated fatty acids of the testes of all groups are in the same range as those found in young rats fed a fat-free diet for 18 weeks, the testes of which appeared to be normal on histological examination (Aaes-Jørgensen and Holman, '58). Funch, Aaes-Jørgensen and Dam ('57) found severe degen-

eration of the spermatogenic epithelium in weanling rats fed a fat-free diet for 26 weeks. In the present experiment, beginning with adult rats, the microscopic anatomy of the testes appeared about normal after 52 weeks on an EFA-free diet, although the chemical analysis showed changes in composition indicative of EFA deficiency. This demonstrates that the age of the animal affects the development of the symptoms of EFA deficiency. This probably is related to the reserve of EFA at the beginning of the depletion period and to the requirement imposed by potential growth.

Table 6 shows the polyunsaturated acid contents of the hearts of animals sacrificed at the end of 29 and 52 weeks. Comparing the polyenoic acid contents of hearts of the rats fed low-fat EFA-free diets for 29 and 52 weeks reveals a decrease in dienoic acid content from 48 to 29 mg/100 gm of heart tissue. However, the trienoic acid content rose from 120 to 217 mg/100 gm of tissue in the same animals. Tetraenoic acid contents were 154 and 83 mg/100 gm, pentaenoic acid contents were 19 and 12, and hexaenoic acid contents were 22 and 20, respectively. The polyunsaturated acid pattern is similar to that seen in EFA deficiency induced in young rats (Aaes-Jørgensen and Holman, '58).

Supplements of cholesterol or cholic acid or both double the amount of trienoic acid of the heart. These supplements also increase the other polyunsaturated acids, but not so radically as the trienoic acids. Judged from trienoic acid contents, cholesterol and cholic acid stresses intensify EFA deficiency.

After 23 weeks on a high-fat, EFA-free diet, the dienoic acid contents of the hearts had increased in all groups except group 2. The content of trienoic acid in the hearts of rats in group 1 increased after the period of supplementation with a high level of saturated fat, suggesting that, although the dienoic acid content of the tissue increased, the full need of the animal was not met by the saturated fat. All the polyunsaturated acids except hexaenoic acid were higher in the hearts of these rats, and the total polyunsaturated acids were 526 mg/100 gm compared with 363 mg/100 gm found in the

TABLE 5

Average polyenoic acid contents of testis tissue from rats

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	WEEKS IN EXPERIMENT	POLYUNSATURATED ACIDS (MG/100 GM TISSUE)					
				Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic	Total
1	Low-fat	3	17	43	81	144	190	10	468
		3	22	26	66	96	144	5	337
		3	27	23	53	92	128	9	305
		5	29	12	74	117	122	12	337
2	Low-fat +	3	17	46	84	130	144	21	425
	cholesterol	3	22	22	59	84	123	4	292
		3	27	21	64	100	128	6	319
		6	29	15	55	104	119	11	304
3	Low-fat +	3	17	19	54	122	173	14	382
	cholesterol +	3	22	18	60	67	126	6	277
	cholic acid	3	27	23	104	117	137	7	488
		6	29	25	86	125	128	8	372
4	Low-fat +	3	17	5	26	60	80	14	185
	cholic acid	3	22	29	55	79	120	6	289
		3	27	19	74	105	132	8	338
		4	29	11	86	165	134	9	305
1	High-fat	2	52	94	88	111	140	12	445
2	High-fat	2	52	39	97	126	162	10	434
3	High-fat	2	52	36	77	108	140	7	368
4	High-fat	1	52	14	48	79	125	8	274
5	Low-fat	4	52	12	133	92	87	15	339

hearts of the low-fat group at the end of the first phase of the experiment. Withdrawal of the cholesterol and cholic acid supplements and increasing the saturated fat content of the diets to 20% (groups 2, 3 and 4) increased the dienoic acid contents of the hearts, but decreased the contents of each of the other polyunsaturated acids and the total polyunsaturated acids.

In a previous experiment (Aaes-Jørgensen and Holman, '58), weanling rats were found to contain 912 mg of total polyunsaturated fatty acids per 100 gm of heart tissue. Keeping these rats on a diet containing 5% of hydrogenated coconut oil and 1% of cholesterol for 18 weeks reduced the amount of polyenoic acids to 690 mg/100 gm of tissue. In the present experiment, beginning with adult rats, 29 and 52 weeks on a diet with 1% of hydrogenated coconut oil brought the total polyunsaturated fatty acids down to 363 and 351 mg/100 gm of tissue, respectively (table 6). In the first phase of the experiment, supplementation with cholesterol or cholic acid or both kept the total polyunsaturated acid contents high. Increasing the fat level to 20% and discontinuing the cholesterol and cholic acid supplementation slightly reduced the total polyunsaturated acid content. The high totals of these acids appeared to be due primarily to the high trienoic acid contents.

The rats of groups 1 to 4, which had undergone long periods of EFA deficiency with additional hypercholesterolemic stress followed by a high level of saturated fat, had a more normal pattern of polyunsaturated acids in the heart tissue than did rats of group 5, which had been kept on a low-fat EFA-free diet for the entire experimental period of one year. The hydrogenated coconut oil appeared to spare the polyunsaturated acid metabolism of the animals in some way. It is difficult to account for the sparing action on the basis of polyunsaturated acids present in a fat which has undergone hydrogenation and has an iodine value of 2. Hydrogenation is known to cause migration and isomerization of unsaturated linkages, and the true linoleic acid content of the preparation cannot

TABLE 6

Average polyenoic acid contents of heart tissue from rats

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	WEEKS IN EXPERIMENT	POLYUNSATURATED ACIDS (MG/100 GM TISSUE)					
				Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic	Total
1	Low-fat	5	29	48	120	154	19	22	363
2	Low-fat + cholesterol	6	29	75	228	262	32	31	628
3	Low-fat + cholesterol + cholic acid	6	29	43	238	202	27	28	538
4	Low-fat + cholic acid	4	29	29	241	255	21	30	576
1	High-fat	2	52	115	163	214	20	14	526
2	High-fat	3	52	79	180	121	15	11	406
3	High-fat	2	52	104	179	175	21	17	496
4	High-fat	2	52	140	166	121	16	7	450
5	Low-fat	5	52	29	217	83	12	20	351

be more than a trace. The high content of medium-chain fatty acids may provide an explanation, for these are known to have nutritional effects different from long-chain saturated acids (Kaunitz et al., '58; Funch, Aaes-Jørgensen and Dam, '57). For example, hydrogenated coconut oil in some instances has lowered the cholesterol content of plasma (Holman, Hayes, Malmros and Wigand, '57). In the present experiments, the medium-chain-length fatty acids may have substituted partially for the unsaturated acids in functions where similarities in physical properties permit substitution, sparing the polyunsaturated acids for more vital functions.

The overall conclusion of this study is that EFA deficiency as seen in young rats is difficult to induce in adult animals, although moderate deficiency was indicated from several points of view. It appears that the previous dietary history of an animal, and the age at which the animal is fed fat-free diets, influence the course of the deficiency, and that the increase of the trienoic acids in the tissues is, so far, the best evaluation of the EFA deficiency and status of the animals.

SUMMARY

Adult male rats were fed a low-fat (1%) EFA-free diet for 29 weeks. During this phase of the experiment, groups were supplemented with 1% of cholesterol or 0.5% of cholic acid or both. In the second phase (29th through 52nd week), all groups except low-fat controls were fed a diet containing 20% of hydrogenated coconut oil without the previous supplements.

Dermal symptoms of EFA deficiency developed very slowly in rats fed the low-fat, EFA-free diet, reaching a maximum at 35 weeks, followed by spontaneous curing. Supplementation with cholesterol or cholic acid or both had little effect upon the severity of the dermal symptoms or upon growth.

The use of the EFA-free diet with or without hypercholesterolemic stresses resulted in high contents of trienoic acid in heart and testis tissues and low contents of dienoic acid. Feeding 20% hydrogenated coconut oil resulted in increased

dienoic acid and somewhat decreased trienoic acid contents of heart tissue.

After 29 weeks of supplementation with cholesterol plus cholic acid, 4 of the 6 animals in this group were found to have lipide deposits in the coronary arteries. This group also had very high plasma cholesterol levels. At the end of the 52nd week, sudanophilia of the serum was visible in frozen sections of hearts from rats which had been fed 20% hydrogenated coconut oil during the second phase of the experiment (23rd week).

LITERATURE CITED

AAES-JØRGENSEN, E., J. P. FUNCH, P. F. ENGEL AND H. DAM 1956 The role of fat in the diet of rats. 10. Influence on reproduction of hydrogenated arachis oil as the sole dietary fat. Brit. J. Nutrition, *10:* 292.

AAES-JØRGENSEN, E., J. P. FUNCH AND H. DAM 1957 The role of fat in the diet of rats. 11. Influence of a small amount of ethyl linoleate on degeneration of spermatogenic tissue caused by hydrogenated arachis oil as the sole dietary fat. Ibid., *11:* 298.

AAES-JØRGENSEN, E., AND R. T. HOLMAN 1958 Essential fatty acid deficiency. I. Content of polyenoic acids in testis and heart as an indicator of EFA status. J. Nutrition, *65:* 633.

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

BARKI, V. H., H. NATH, E. B. HART AND C. A. ELVEHJEM 1947 Production of essential fatty acid deficiency symptoms in the mature rat. Proc. Soc. Exp. Biol. Med., *66:* 474.

BURR, G. O. 1942 Significance of the essential fatty acids. Federation Proc., *1:* 224.

CARROLL, K. K., AND R. L. NOBLE 1957 Influence of a dietary supplement of erucic acid and other fatty acids on fertility in the rat. Sterility caused by erucic acid. Can. J. Biochem. Physiol., *35:* 1093.

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

FUNCH, J. P., E. AAES-JØRGENSEN AND H. DAM 1957 The role of fat in the diet of rats. 12. Effect on rats of type and quantity of dietary fat with and without linoleate supplementation. Brit. J. Nutrition, *11:* 426.

HOLMAN, R. T., AND S. ENER 1954 Use of urea-inclusion compound containing essential fatty acid in an experimental diet. J. Nutrition, *53:* 461.

HOLMAN, R. T., H. HAYES, H. MALMROS AND G. WIGAND 1957 Effects of dietary fat upon plasma polyunsaturated acids. Proc. Soc. Exp. Biol. Med., *96:* 705.

HOLMAN, R. T., AND H. HAYES 1958 Determination of polyunsaturated acids in lipides of plasma and tissue. Anal. Chem., *30:* 1422.

KAUNITZ, H., C. A. SLANETZ, R. E. JOHNSON, V. K. BABOYAN AND G. BARSKY 1958 Nutritional properties of the triglycerides of saturated fatty acids of medium chain length. J. Am. Oil Chemists' Soc., *35:* 10.

PEIFER, J. J., AND R. T. HOLMAN 1955 Essential fatty acids, diabetes and cholesterol. Arch. Biochem. Biophys., *57:* 520.

THE PYRIDOXINE-DEFICIENT STATE IN TWO STRAINS OF INBRED MICE[1]

JOHN B. LYON, JR.,[2] HARRY L. WILLIAMS[3] AND EUGENE A. ARNOLD
WITH THE TECHNICAL ASSISTANCE OF DORIS A. BRADSHAW
Department of Biochemistry and Department of Pharmacology, Division of Basic Health Sciences, Emory University, Atlanta, Georgia

(Received for publication April 16, 1958)

Under the usual conditions of laboratory maintenance, mice of the I and C_{57} strains have almost identical body weights (Lyon, '57b). When they are fed high-carbohydrate, synthetic rations, they have almost identical growth rates (Fenton and Carr, '51a, b). The similarities, however, stop here. Diets rich in fat promote obesity in C_{57} strain mice, but promote even lower stores of body fat in I strain mice than are found when low-fat diets are fed (Fenton and Dowling, '53; Fenton, '56). Diets rich in protein induce lower growth rates in I strain mice than in C_{57} strain mice (Fenton and Carr, '51a). When high- or low-fat diets are fed, more muscle glycogen is found in I strain mice, but more liver glycogen is found in C_{57} strain mice (Lyon and Fenton, '56; Lyon, '57b). Similarly, the greater part of a test dose of glucose is deposited as muscle glycogen in I strain mice, and as liver glycogen in C_{57} strain mice (Lyon, '57a).

Some of these observations might be explained by hormonal differences, but it is not possible to account for all of the

[1] This investigation was supported in part by a research grant (A–1264) from the National Institute of Arthritis and Metabolic Diseases, Public Health Services.

[2] Life Insurance Medical Research Fellow (1954–56) and Lederle Medical Faculty Award (1956–59).

[3] Markle Scholar.

known metabolic differences in these terms (Lyon, '57b). It is also suggested that differential vitamin requirements might be involved. For example, the consistently low levels of body fat in the I strain (Fenton and Dowling, '53), and the poor growth rate of the I strain fed a 90% protein diet (Fenton and Carr, '51a), suggest the possibility of a higher requirement for pyridoxine (Sherman, '50; Miller and Baumann, '45). Since pyridoxal phosphate is now known to be the coenzyme of phosphorylase (Cori and Illingworth, '57), the unusual carbohydrate metabolism of the I strain might be related to pyridoxine as well. Further support for this idea comes from another observation. Female mice of the I strain, fed a commercial stock ration,[4] produced apparently healthy young, but the pups did not survive for more than three days. When this ration was supplemented with pyridoxine (200 mg/kg), they raised large, healthy litters. In contrast, mice of the C_{57} or the A strains produce healthy litters when fed only laboratory chow (Lyon, '55).[5]

However, neither the growth rate, nor the level of carcass fat could be increased in I strain mice by feeding diets (5 to 15% fat and 30% protein) containing 50 mg/kg,[6] or even 200 mg/kg of pyridoxine.[5] It is, therefore, difficult to classify these mice as animals with a higher requirement for pyridoxine in the usual meaning of the expression. In the hope of further clarification, we have compared the body weight gains, food intake and organ levels of pyridoxine for I and C_{57} strain mice fed complete or vitamin B_6-deficient diets. This comparison revealed that the I strain mouse is much more susceptible to a pyridoxine deficiency despite similar initial body weights and organ levels of pyridoxine.

[4] Purina Laboratory Chow. Our assay of this ration gave a concentration of 2.8 mg of pyridoxine per kilogram. The manufacturer estimates a content of 4.3 mg/kg.

[5] Unpublished data from this laboratory.

[6] Fenton, P. F., personal communication.

METHODS

Three strains of mice, the I, the C_{57} and the A, have been maintained in this laboratory since 1954.[7] The colony is quartered in an air-conditioned room, and it is maintained by brother and sister matings only. The breeding stock is fed a commercial mouse breeder diet.[8] At the time of pregnancy, the isolated females are fed a lactating diet,[9] which consists of 40% nonfat dry milk, 15% crude casein, 15% dextrin, 15% corn oil, 5% liver powder, 5% salts (Hubbell, Mendel and Wakeman, '37), and 5% roughage.

Male mice of the I and C_{57} strains were housed individually in screen-bottom, metal cages, and were fed vitamin B_6-deficient or complete diets. Food and water were supplied ad libitum. Over a two-year period, two separate experimental trials were made with weanling mice (initial age, 21 to 25 days), and three separate experimental trials were made with adolescent mice (initial age, 42 to 48 days). In each trial, 5 or more mice from each strain were fed the deficient diet, and 5 or more mice from each strain were fed the complete diet for periods of 5 to 7 weeks. The data were then pooled for each age group, and are presented as two experiments, (a) weanling mice, and (b) adolescent mice. Two other studies are also reported; (1) female mice from each strain were fed the vitamin B_6-deficient ration only from the time of weaning, and (2) male, adolescent mice from each strain were fed the vitamin B_6-deficient diet for 8 weeks, and then were fed the complete diet for one or two weeks. Body weights were measured (at the indicated intervals) for all mice, but food consumption, organ levels of pyridoxine, and total body fat were not determined for every animal.

[7] The stock was obtained from the colony of Dr. Paul F. Fenton, Brown University, Providence, R. I. In this laboratory the strains have been given the formal designations of I/FnLn, C_{57}BL/FnLn and A/FnLn. See Mouse News Letter, no. 17, July, 1957.

[8] J. W. Eshelman and Sons.

[9] Formulated by Dr. P. F. Fenton.

The pyridoxine-deficient diet was purchased from a commercial firm.[10] The complete ration was prepared by adding 20 mg/kg of pyridoxine hydrochloride. An analysis of 4 random samples of the complete ration gave a mean of 20.3 ± 0.7 (standard deviation) mg/kg, and an analysis of the deficient ration showed the presence of 0.1 mg/kg. In our early studies some of the C_{57} strain mice fed the complete ration lost weight and developed a moderately greasy coat, which was eliminated by injections of thiamine. Therefore, both the deficient and the complete ration were supplemented with 20 mg/kg of thiamine just prior to use. The symptoms described above were not seen again.

Total body fat was determined by the method outlined by Fenton ('56). Concentrations of pyridoxine in the brain, liver and kidney were measured by a modification of the method described by Atkins et al. ('43). Whole organs were removed from mice under anesthesia[11] or from mice killed by cervical dislocation. All of the mice were non-fasted, and the organs were removed at the same time of day, 3 to 5 P.M. The whole organs were weighed to the nearest 0.2 mg, then homogenized in 0.1 N sulfuric acid. After the final dilution, an aliquot was analyzed for nitrogen by a modification of the method of Johnson ('41). As an internal control, samples from the organs of both strains, and both control and experimental groups were assayed for pyridoxine at the same time. The concentrations of pyridoxine are expressed as micrograms per gram of wet weight of tissue, and as micrograms per gram of nitrogen.

RESULTS

A. Studies with weanling mice. The body weight change and the average daily food intake for the 5-week experimental period are given in figure 1. Within three days the I strain was affected by the lack of pyridoxine, whereas the body weight gains of the C_{57} strain, fed or deprived of pyridoxine,

[10] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[11] Nembutal, Abbott.

were equal for the first 6 days. The failure of the I strain to gain weight was due to a lower efficiency of utilization. The ratio of grams of body weight gained to grams of food consumed for the first 6 days was 0.28 ± 0.008 (standard error) and 0.22 ± 0.02 for 7 mice of the C_{57} and 5 of the I strain fed the deficient ration. The I strain fed the complete ration also showed a lower efficiency than the C_{57} strain: 0.26 ± 0.009 (6 mice) and 0.29 ± 0.02 (7 mice).

Except for the curve labeled I-a (I strain mice), the data given in figure 1 were taken from the second experimental

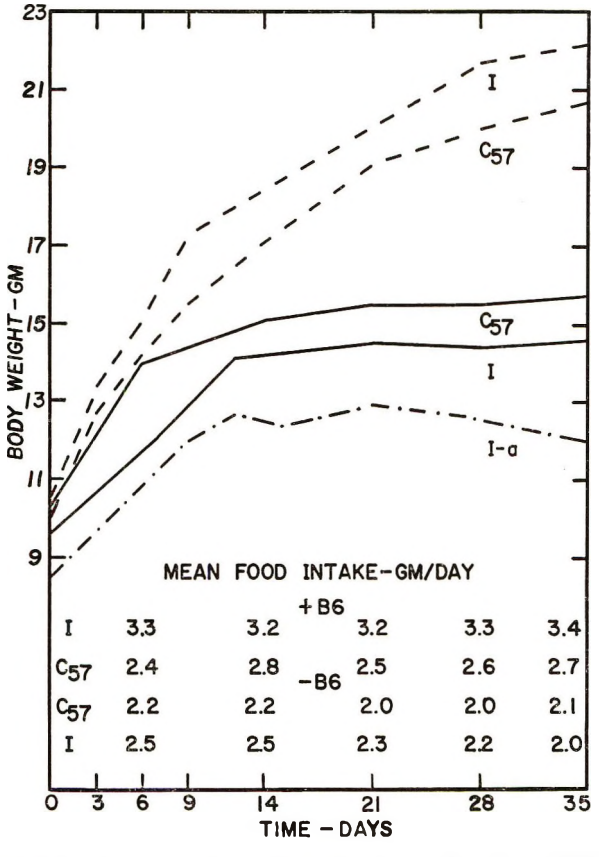


Fig. 1 Body weight gains and food intake of weanling C_{57} and I strain mice fed complete (dashed lines) or vitamin B_6-deficient (solid or dashed and dotted lines) diets. Each curve represents the average of 7 to 12 mice.

trial with this age group. In the first trial (curve I-a), the mice were younger (21 to 22 days of age), and the initial body weights were lower. Despite this, mice of the C_{57} strain were no more affected by the lack of pyridoxine, and for clarity, their body weight changes from the first study are not shown. At the end of the 5-week period, the average per cent body fat of 6 deprived I strain mice was 2.2 ± 0.04 (standard error), and that of 7 deprived C_{57} strain mice was 6.5 ± 1.1.

Some of the mice in both strains developed a greasy fur which was moderate to severe, but this did not affect the body weight gains. As the deficiency state progressed, the black coat of the C_{57} strain mouse became gray, and the paws almost white. The most striking symptom was the susceptibility of the I strain mouse to convulsions. Within 30 days of deprivation, running fits, clonic and tonic convulsions, and death could be induced by jangling keys near the cages. The C_{57} strain mouse never showed such a susceptibility.

At weaning the levels of pyridoxine in the kidneys and the livers of the two strains were similar (table 1, group I), and if the larger kidneys of the I strain are considered, 0.7 μg was present in the whole organ, as opposed to 0.5 μg in the kidneys of the C_{57} strain. Despite this initial similarity, the levels in both the kidneys and the livers of I strain mice, deprived of pyridoxine for 5 weeks, were about one-half ($P < 0.01$) of those found in C_{57} strain mice (table 1, group III).

A comparison of the concentrations of pyridoxine in the brains presents an entirely different picture from those in the livers or the kidneys. First, the concentrations in 60-day-old mice of both strains, fed the complete ration from the time of weaning, were 27 to 34% greater ($P < 0.01$) than those found in the weanling animals (groups I and II, table 1). This increase in concentration with age was not evident in the liver or the kidney. Secondly, in deprived mice of both strains, the levels in the brains were 44 to 46% lower than those of mice fed the complete ration, whereas the decrements in the livers and the kidneys ranged from 71 to 82% (groups

II and III, table 1). Consequently, the levels in the brains of the deprived mice were 1.4 to 2.2 times greater than those found in the livers if the reference is nitrogen content, and equal if the reference is wet weight of tissue. Finally, the concentrations in the brains of I strain mice fed the deficient ration were slightly, but not significantly, lower than those of C_{57} strain mice.

Female mice (5 I strain and 6 C_{57} strain), fed the deficient diet from the time of weaning, showed the same strain differences in body weight gains as found in the males (fig. 1).

TABLE 1

Pyridoxine content of brain, liver and kidney of weanling mice, and of 60-day-old mice fed vitamin B6-deficient or complete diets from weaning

STRAIN	GROUP [1]	DIET	NO. OF MICE	BODY WT.	ORGAN WT.	PYRIDOXINE CONTENT per gm tissue	PYRIDOXINE CONTENT per gm nitrogen
				gm	*gm*	*μg*	*μg*
				Brain			
C57	I		7	8.8	0.36	3.0 ± 0.1 [2]	166 ± 7
I	I		7	9.0	0.36	2.7 ± 0.2	158 ± 5
C57	II	+B6	8	19.2	0.37	4.0 ± 0.07	223 ± 6
I	II	+B6	4	20.7	0.35	3.8 ± 0.1	201 ± 2
C57	III	—B6	10	17.8	0.37	2.3 ± 0.1	124 ± 6
I	III	—B6	10	12.9	0.33	2.1 ± 0.1	109 ± 7
				Liver			
C57	I		6	8.5	0.49	9.0 ± 1.1	328 ± 44
I	I		7	9.0	0.51	9.4 ± 0.4	297 ± 9
C57	II	+B6	3	17.4	1.08	8.2 ± 0.6	300 ± 8
I	II	+B6	3	20.6	1.04	9.2 ± 0.2	278 ± 13
C57	III	—B6	8	16.2	0.90	2.7 ± 0.2	87 ± 9
I	III	—B6	10	12.0	0.67	1.7 ± 0.3	50 ± 7
				Kidney			
C57	I		7	8.8	0.12	4.3 ± 0.2	169 ± 9
I	I		7	9.0	0.15	4.7 ± 0.4	183 ± 6
C57	II	+B6	8	19.2	0.25	4.5 ± 0.3	157 ± 11
C57	III	—B6	10	17.8	0.22	1.2 ± 0.09	44 ± 4
I	III	—B6	7	12.8	0.24	0.7 ± 0.03	22 ±0.1

[1] Weanling mice, 21 to 25 days of age (I), and 60-day-old mice fed complete (II), or deficient (III) diets from the time of weaning.

[2] Standard error of the mean.

The concentrations of pyridoxine were also similar to those of the males of the respective strains (table 1, group III). One difference was noted; the average level in the livers of the I strain females was 83 ± 5 (standard error) μg/gm of nitrogen, which was higher ($P < 0.01$) than that found in I strain males.

B. Studies with adolescent mice. The same characteristic responses (body weight loss, fur changes and susceptibility to seizures) to the complete and deficient rations were found with the older group in each of three experimental trials. Furthermore, within each strain the body weights were similar in each trial. Since an analysis of variance of the body weights at zero, 3 and 6 weeks confirmed that the mice of each strain in the three trials were from the same population, the body weights were averaged. The summary is presented in figure 2. Within the first week the I strain was affected by the lack of pyridoxine, but the C_{57} strain continued to gain weight for three weeks. At the end of the experimental period, the differences in body weights of the two strains fed the deficient ration were small (10 to 11%), but significant (fig. 2, table 2). The effect of the vitamin deficiency on the body weights of C_{57} strain mice can be charged entirely to a loss in carcass fat since the fat-free weights of the control and the deprived animals were similar ($P = 0.17$). However, I strain mice showed losses in both carcass fat and fat-free weight ($P < 0.01$). Again, the greater body weight losses in I strain mice were due to a lower efficiency of utilization; I strain mice fed the deficient ration ate as much as C_{57} strain mice fed either ration (fig. 2).

The levels of pyridoxine in the brains of deprived I strain mice were only 15% lower than those of C_{57} strain mice, but this difference was significant (table 2). The variations encountered in this group were less than those found in the weanling mice (table 1). Because of the relatively small decreases, 36 to 41%, in the brain levels of deprived mice of both strains, the absolute brain levels were two to three times greater than those in the kidneys or the livers ($P < 0.01$), if

nitrogen is used as a reference. Older I strain mice deprived of pyridoxine also exhibited lower levels of pyridoxine in the kidneys and livers than C_{57} strain mice (table 2).

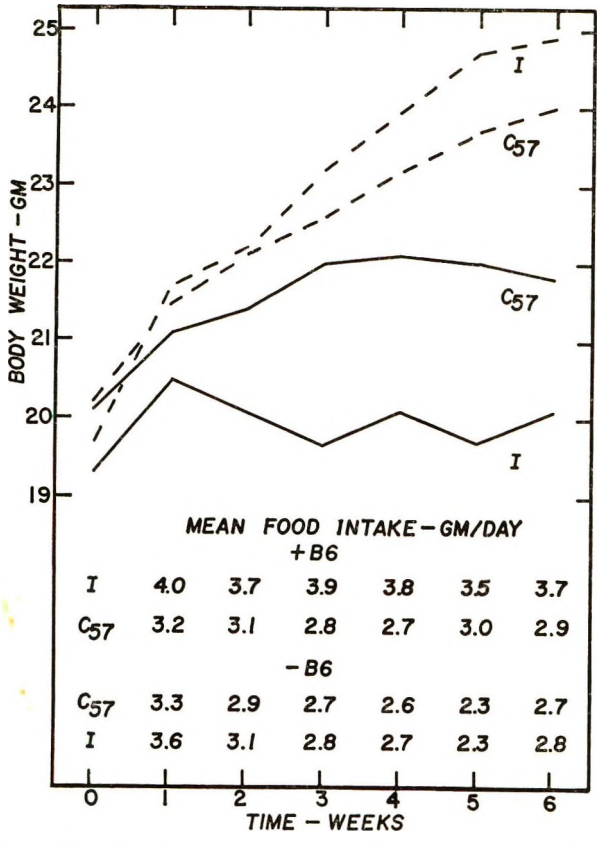


Fig. 2 Body weight gains and food intake of adolescent C_{57} and I strain mice fed complete (dashed lines) or vitamin B_6-deficient (solid lines) diets. Each curve represents the average of 18 to 20 mice.

A comparison of the organ concentrations in the three age groups (25, 60 and 100 days of age) fed the complete ration reveals another difference between the two strains (tables 1 and 2). In the C_{57} strain, the levels in the kidney and liver were relatively constant in the three age groups, but the levels in the brain increased 41% from weaning to 100 days of age.

On the other hand, the levels in the kidney and liver of the I strain decreased slightly (10%) from weaning to 100 days, and were considerably lower than those in 100-day-old mice of the C_{57} strain. Secondly, the levels in the brain of the I strain reached a maximum at 60 days of age, and then declined, so that the level in the 100-day-old animal was only 17% greater than that found in the weanling animal.

The relative kidney weights of the I strain mouse were consistently larger ($P < 0.01$) than those of the C_{57} strain mouse in all groups studied (tables 1 and 2). The kidney weights (grams per 100 gm of body weight) increased ($P < 0.01$) in the deprived C_{57} strain mouse, but decreased ($P = 0.03$) in the I strain mouse. No differences due either to strain or diet were found in the relative liver weights, but the relative brain weights were greater in the deprived than in the completely fed animals of both strains.

The data for I and C_{57} strain mice, fed the deficient diet and then fed the complete ration for one to two weeks, are summarized in table 3. After feeding the complete ration, the I strain gained 4½ times as much weight as the C_{57} strain. That this large weight gain merely reflected a retention of body water has been ruled out on the basis that the nitrogen content of the tissues was similar to that of animals fed the complete diet continuously. Even though the mice of the C_{57} strain failed to gain as much weight within one of two weeks, their tissue levels of pyridoxine were slightly higher than those found in the I strain, and similar to those found in the control animals (table 2).

DISCUSSION

In a total of 7 experimental trials in two age groups, the I strain mouse proved to be more susceptible to a pyridoxine deficiency than the C_{57} strain mouse. The initial failure in the I strain was a sudden and marked reduction in the efficiency of utilization of the food (figs. 1 and 2). This observation, the very rapid decline in the pyridoxine stores of the

TABLE 2

The body fat content, and the pyridoxine content of the brain, liver, and kidney of 100-day-old mice fed complete or vitamin B_6-deficient diets from 45 days of age

DETERMINATION	STRAIN		P [1]	STRAIN		P [1]
	C_{57}	I		C_{57}	I	
	Fed the complete diet			*Fed the vitamin B_6-deficient diet*		
Body weight, gm [2]	23.7 ± 1.3 [3]	25.2 ± 0.5	0.30	20.8 ± 0.6	18.7 ± 0.4	0.01
Body fat, % [2]	15.2 ± 2.5	13.0 ± 1.0	0.40	8.6 ± 0.4	6.9 ± 0.4	0.01
Organ levels of vitamin B_6, μg/gm nitrogen						
Brain	234 ± 9	185 ± 6	0.01	138 ± 3	119 ± 3	0.01
Liver	319 ± 9	266 ± 12	0.08	121 ± 5	80 ± 5	0.01
Kidney	186 ± 4	165 ± 5	0.01	60 ± 7	38 ± 1	0.01
μg/gm tissue [4]						
Brain	3.9 ± 0.2	3.6 ± 0.04	0.10	2.5 ± 0.04	2.2 ± 0.04	0.14
Liver	6.6 ± 0.7	7.9 ± 0.6	0.17	3.3 ± 0.2	2.7 ± 0.2	0.03
Kidney	5.4 ± 0.2	4.7 ± 0.1	0.01	1.5 ± 0.1	1.1 ± 0.02	0.03
Organ weight, gm [4]						
Brain	0.37	0.40		0.38	0.37	
Liver	1.13	1.28		1.07	0.90	
Kidney	0.25	0.48	0.01 [5]	0.25	0.31	0.01 [5]

[1] P value for the differences between the means of the two strains. A value of 0.01 indicates 0.01 or less.
[2] Nine determinations for deficient or control mice in all cases.
[3] Standard error of the mean.
[4] Six determinations in all cases for deficient mice, and 5 determinations in all cases for control mice.
[5] Calculated on the basis of relative tissue weight.

liver and kidney (tables 1 and 2), the loss of fat-free weight, and the early susceptibility to seizures, strongly suggest an unusual requirement for pyridoxine in this strain. As measured in terms of growth or carcass fat deposition, this requirement is satisfied if pyridoxine is available to the animal daily at concentrations in the diet of 2 mg/kg.

TABLE 3

Comparison of C_{57} and I strain mice (initial age 45 days) deprived of pyridoxine for 8 weeks, then fed the complete ration for 1 to 2 weeks.

STRAIN	C_{57}	I	P [2]
Number of mice	6	5	
Initial body weight, gm	20.8 ± 0.4 [1]	21.1 ± 1.4	
Body wt. changes, gm			
Minus vitamin B_6 for 8 weeks	— 0.6 ± 1.0	— 1.3 ± 0.7	0.036
Plus vitamin B_6 for 1 week	+ 1.0 ± 0.2	+ 4.5 ± 0.6	0.001
Pyridoxine levels, μg/gm nitrogen [3]			
Liver	299 ± 8	277 ± 26	0.12
Kidney	172 ± 7	162 ± 10	0.12
Brain	200 ± 11	188 ± 11	0.14

[1] Standard error of the mean.

[2] P value for comparison of the means of the two strains.

[3] Levels from mice fed the complete ration for 1 or 2 weeks. The determination from both time periods were averaged since no differences between them were found.

It is possible that the I strain mouse is unable to convert the dietary pyridoxine to pyridoxal or pyridoxamine phosphate at the same rate as the C_{57} strain mouse. We have made preliminary measurements of the relative concentrations of the phosphorylated and nonphosphorylated derivatives in brains, livers and kidneys of the two strains fed the stock ration. The levels of the nonphosphorylated compounds were similar in these organs of both strains, and were low, 1 to 2% of the total. Furthermore, the same relative concentrations of pyridoxal and pyridoxamine phosphate were present in the three organs of both strains. These separations were made with ion exchange resins [12] by the method developed

[12] Dowex 1 and 50.

by Wiegand ('56), and the concentrations were determined by the microbiological assay. In the deficiency state, a shift may occur in the concentrations of the members of the vitamin B_6-complex. This possibility is under investigation.

The observation that the I strain is susceptible to seizures so early in the deficiency state is surprising, particularly since the levels of pyridoxine in the brain were only slightly lower than those of C_{57} strain mice (tables 1 and 2). The changes in the pyridoxine concentration in the brains of either strain are unlike those described for thiamine in a thiamine deficiency. Salcedo et al. ('48) found that brain thiamine concentrations were conserved, but only for a few days longer than the stores in the liver, heart or kidney. We found that the pyridoxine stores of the brain are even greater than those of the liver or kidney (using nitrogen as a reference) after 6 to 7 weeks of the deficiency and even when the animal is susceptible to seizures. These observations would suggest that the absolute levels of total pyridoxine in the brain are not related to a susceptibility to seizures.

Davenport and Davenport ('48) demonstrated a lower electroshock threshold in the pyridoxine-deficient rat, which could not be related to partial starvation, or several other possible changes. They did find that tryptophan feeding lowered, and glutamic acid feeding increased, the threshold of the deficient rat. They suggested, therefore, that the change in electroshock threshold in the pyridoxine-deficient state was due to a greater competition for pyridoxine among the enzyme systems involved. An explanation of the early susceptibility to seizures by the deprived I strain mouse in these terms is reasonable. The observations made on the protein metabolism of this strain to date would lend further support to such an explanation. (Fenton and Carr, '51a; Fenton and Marsh, '56; Fenton, '57). It may be that the I strain has a greater metabolic turnover of pyridoxine, or that the metabolic pattern of this strain is such that certain pyridoxine-requiring enzymes are more active.

SUMMARY

Pyridoxine deficiency states were studied in weanling (21 to 25 days) and adolescent mice (42 to 48 days) of the C_{57} and I strains. In both age groups mice of the I strain exhibited the effects of the deficiency more readily and to a greater degree. Their food intake was similar, but they utilized it less efficiently. During the 5- to 8-week experimental periods they dissipated their stores of pyridoxine in the liver and kidney more rapidly. The I strain mice in pyridoxine deficiency developed a susceptibility to convulsions in both age groups, but the C_{57} strain mice did not. The levels of pyridoxine in the brain of the I strain mice were only slightly lower. In either strain the changes in pyridoxine concentrations in the brain were less than those of the liver or the kidney with respect to age or the deficiency state.

LITERATURE CITED

ATKINS, L., A. S. SCHULTZ, W. L. WILLIAMS AND C. N. FREY 1943 Yeast microbiological methods for determination of vitamins-Pyridoxine. Ind. Eng. Chem., Anal. Ed., *15:* 141.

CORI, C. F., AND B. ILLINGWORTH 1957 The prosthetic group of phosphorylase. Proc. Nat. Acad. Sci., *43:* 547.

DAVENPORT, V. D., AND H. W. DAVENPORT 1948 Brain excitability in pyridoxine-deficient rats. J. Nutrition, *36:* 263.

FENTON, P. F. 1956 Growth and fat deposition in the mouse. A definition of obesity. Am. J. Physiol., *184:* 52.

———— 1957 Hereditary factors in protein nutrition. Am. J. Clin. Nutrition, *5:* 663.

FENTON, P. F., AND C. J. CARR 1951a The nutrition of the mouse. X. Studies on the utilization of high and moderately low protein diets for growth in four strains of mice. J. Nutrition, *43:* 441.

———— 1951b The nutrition of the mouse. XI. Response of four strains to diets differing in fat content. Ibid., *45:* 225.

FENTON, P. F., AND M. T. DOWLING 1953 Studies on obesity. I. Nutritional obesity in mice. Ibid., *49:* 319.

FENTON, P. F., AND J. M. MARSH 1956 Inherited metabolic patterns in mice. Calorie requirements for protein utilization and determination of protein minima. Ibid., *60:* 465.

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

JOHNSON, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem., *137:* 575.

LYON, J. B., JR. 1957a Difference in glucose utilization among inbred strains of mice. Federation Proc., *16:* 215.

———— 1957b Muscle and liver glycogen levels in lean and obese strains of mice. Am. J. Physiol., *190:* 434.

LYON, J. B., JR., AND P. F. FENTON 1956 Muscle and liver glycogen levels of mouse strains susceptible or resistant to nutritionally induced obesity. Ibid., *187:* 415.

MILLER, E. C., AND C. A. BAUMANN 1945 Relative effects of casein and tryptophane on the health and xanthurenic acid excretion of pyridoxine-deficient mice. J. Biol. Chem., *157:* 551.

SALCEDO, J., JR., V. A. NAJJAR, L. E. HOLT, JR. AND E. W. HUTZLER 1948 The relation between urinary excretion and tissue concentrations of thiamine in rats. J. Nutrition, *36:* 307.

SHERMAN, H. 1950 Pyridoxine and fat metabolism. Vitamins and Hormones, VIII: 55, Academic Press, Inc., New York.

WIEGAND, R. G. 1956 The relation between pyridoxine analogues and hydrazide induced convulsions. Ph.D. Thesis, Emory University, Georgia.

THE INFLUENCE OF DIETARY FAT UPON THE NIACIN REQUIREMENT OF THE MOUSE [1]

WILLIAM N. PEARSON, J. SALVADOR VALENZUELA[2] AND JAN VAN EYS

Division of Nutrition, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

(Received for publication April 7, 1958)

It has become increasingly apparent that estimates of man's niacin requirement must take cognizance of the conversion of tryptophan to this vitamin. The revised National Research Council recommended dietary allowances ('58) recognize this and express the niacin allowances in "niacin equivalents" — 60 mg of tryptophan being considered the equivalent of 1 mg of niacin. This association of tryptophan and niacin has caused revision of concepts regarding the pathogenesis of pellagra and has led Horwitt et al. ('56) to express the opinion that the association of corn diets with pellagra is primarily a function of the low niacin — tryptophan content of this cereal. However, alternative explanations are possible. For example, Woolley ('46) observed that an alkaline-chloroform extract of ground whole corn depressed the growth of mice. The finding that the addition of niacin to the diet overcame this effect led him to suggest that the pellagragenic action of corn might be due to the presence of an alkali-extractable niacin anti-metabolite. Considerable purification of the "toxic" principle was attained, but no further reports on the nature of this substance have appeared. Lipke and Fraenkel ('55) found that Woolley's factor was not identical with the substance in corn that depresses the growth of *Tenebrio molitor*.

[1] This study was supported in part by a grant from the National Live Stock and Meat Board.

[2] Present address: Avenida Elena 12- 72, Guatemala City, Guatemala.

Interest in this problem has been heightened by the finding that the consumption of tortilla, a corn bread made in Latin America from alkali-treated corn, seems to be associated with a lower incidence of pellagra than would be expected. The evidence (Chaudhuri and Kodicek, '50; Kodicek et al., '56) that an alkali-labile precursor of niacin occurs in corn supports this view. Feeding experiments with rats (Cravioto et al., '52; Laguna and Carpenter, '51) established the nutritional superiority of lime-treated corn over raw corn, but recent work in this laboratory (Pearson et al., '57) demonstrated that boiling corn in water, even in the absence of alkali, is sufficient to enhance its nutritional value. Although the probable explanation for this effect is the hydrolysis of a bound form of niacin, it is conceivable that destruction or extraction of a niacin antimetabolite could account for the superiority of cooked corn over raw corn. In view of this possibility, we have attempted to confirm the findings of Woolley. This communication presents results of such experiments as well as of further studies of the niacin requirement of the mouse and the effect of the level of dietary fat on this requirement.

MATERIALS AND METHODS

Female weanling albino mice of the Swiss strain [3] were randomly divided into groups, and housed, three to 5, in screen-bottom cages. Water and diets were supplied ad libitum. Daily food intakes were recorded in some of the studies and the groups were weighed at regular intervals during the experiments which were usually of about three weeks duration.

Difficulty was experienced in interpreting the results of the preliminary studies because we had not recognized the requirement for niacin of the mouse fed a low-protein diet. In attempting to improve growth, the vitamin content of the original Woolley diet ('46) was doubled and the Phillips and Hart ('35) salt mixture was replaced by the Hubbell, Mendel

[3] Obtained from the following suppliers: Carworth Farms, Manor Farms, and Empire Farms.

and Wakeman ('37) salt mixture, since the former has been found to destroy thiamine in the diet (Rombouts, '53). In fact, in this laboratory mice have been found to develop thiamine deficiency on diets containing the Phillips and Hart salt mixture. The basal diet used is detailed below. It supports good growth only when supplemented with niacin — the extent of growth being similar to that reported by Bosshardt et al. ('46) who used a 8.5% casein diet. The diet consisted of 9% vitamin-free casein,[4] 0.4% L-cystine,[4] 83.6% sucrose, 5% salt mixture (Hubbell-Mendel-Wakeman, '37) and 1% Wesson oil fortified with Navitol and α-tocopherol acetate in amounts to supply approximately 20,000 I.U. of vitamin A, 4,000 I.U. of vitamin D, and 2.6 mg of α-tocopherol per kilogram of diet. In addition, vitamins were added in the following amounts (expressed as milligrams per kilogram of diet): thiamine hydrochloride, 4; riboflavin, 10; Ca pantothenate, 40; pyridoxine HCl, 4; 2-methyl-1, 4-naphthoquinone, 2; folic acid, 5.6; and biotin, 0.25. Choline chloride and inositol were added at a level of 2 gm/kg. All additions to this basal diet were made at the expense of sucrose.

An alkaline $CHCl_3$ extract of yellow corn[5] was prepared in the following manner: one kilogram of ground whole corn and 8 of 0.1 N NaOH were stirred at room temperature for one hour. Eight liters of $CHCl_3$ were then added and stirred for an additional 6 hours. The resulting emulsion was kept overnight at room temperature, and the solids then removed by filtration. These solids were dried under a fan and infra-red light and are referred to as "extracted corn. The emulsion of the filtrate was broken by gentle warming (35 to 40°C), the $CHCl_3$ layer siphoned off, and the $CHCl_3$ evaporated under reduced pressure at 40°C or lower. The distillation was stopped when it appeared that all of the $CHCl_3$ had been removed. This procedure usually yielded 18 to 22 gm of a heavy yellow oil with a suggestive corn odor, but in some early experiments a yield as high as 80 gm was

[4] National Biochemicals Corporation.

[5] A locally grown open-pollinated variety known as "Jarvis" was used.

recorded. The oil is referred to as "$CHCl_3$ corn extract." The opalescent NaOH layer was neutralized with H_3PO_4 syrup and evaporated to dryness under infra-red light and an electric fan.[6] This residue is referred to as "NaOH corn extract."

In one experiment a similar extract of zein was prepared. Removal of the $CHCl_3$ yielded 16 to 20 gm of a heavy pale yellow oil. Other fractions were not prepared from this batch.

The $CHCl_3$ extracts of corn and zein were taken up in ethyl ether, added to 100 gm of sucrose, and dried at room temperature for incorporation into the diets. The other fractions from the extraction procedures were dried and powered for feeding. A typical yield from 1 kg of corn was $CHCl_3$ extractives, 20 gm; NaOH extractives, 130 gm: extracted corn, 570 gm. Since the corn contained approximately 10% moisture, a recovery of about 80% of the starting material was realized. These and other supplements were added to the basal diet at the expense of sucrose to achieve the equivalence of 40% corn in the diet. The $CHCl_3$ extracts of corn and zein were added to each 100 gm of diet at levels equivalent to the yield from 100 gm of raw corn. The other supplements were added to each 100 gm of diet at levels corresponding to the expected yields from 40 gm of corn and are compared to a diet containing 40 gm of raw corn.

The corn preparations were analyzed microbiologically (*L. arabinosus*) for niacin and tryptophan. The medium of the Association of Official Agricultural Chemists ('55) was used. Samples for niacin assay were prepared by hydrolyzing in H_2SO_4 in an autoclave for 30 minutes. Some samples were also prepared for assay by hot-water extraction in an autoclave. The $Ba(OH)_2$ hydrolysis of Greene and Black ('44) was employed for tryptophan and some niacin assays.

[6] A neutral mixture of NaOH + H_3PO_4 was prepared, dried, and incorporated into the other diets at the levels necessary to equate them in this respect.

RESULTS AND DISCUSSION

The growth performance of the mouse on a 9% casein, niacin-free diet is not entirely predictable. For example, in 4 of 5 early experiments, mice failed to grow on the niacin-free diets — the mean growth response in two weeks ranging from −0.43 to +0.69 gm. However, the other group grew slowly on the niacin-free ration, gaining 4.7 gm in the two week period as compared to the usual response of around 9 gm when niacin was added. The reasons for such variability are not clear, but it is probable that coprophagy and refection are contributory. Also, the possibility of significant differences in the niacin or tryptophan content of various batches of "vitamin-free" casein cannot be disregarded.

In early experiments conflicting results were obtained when attempting to assess the effect of the alkaline-$CHCl_3$ extract on growth. Figure 1 depicts the results of a study that showed an apparent marked growth inhibition by this extract that was "reversed" by the addition of niacin to the diet. The yield of alkaline-$CHCl_3$ extract in this case was approximately 80 gm/kg of corn and, as mentioned previously, it was added in such a way that it contributed some 8% of fatty materials to the basal diet.

In another study, however, the alkaline-$CHCl_3$ extract enhanced rather than inhibited growth (table 1, group 3). In this case, the alkaline-$CHCl_3$ yield was only 20 gm/kg corn, thus increasing the diet content of fat by 2.0%. The addition of zein extract to the niacin-free diet (group 5) also permitted excellent growth. Those animals receiving the niacin-free basal diet (group 1) did not gain weight during this experiment and, because of their critically ill condition, were sacrificed on the 12th day of the study. This growth failure was of particular interest since the mouse has been assumed to resemble the rat in not requiring a dietary source of niacin (Woolley, '45). The addition to the basal diet of an amount (250 mg%) of niacin similar to that used by Woolley permitted excellent growth (group 2).

That the niacin and tryptophan contents of these oils were negligible and cannot account for the enhanced growth is shown by table 2. The addition of niacin (groups 4 and 6) enhanced growth only slightly over that obtained in its absence. However, the growth supported by the other supple-

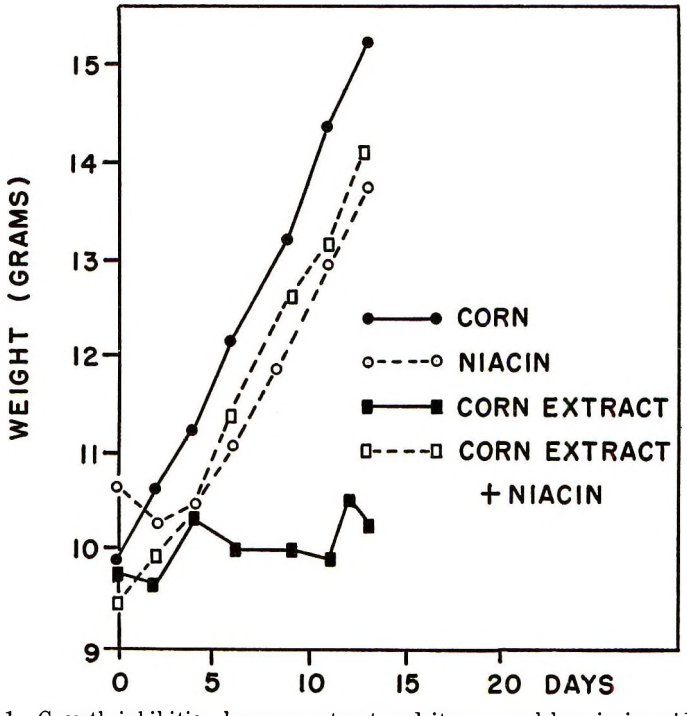


Fig. 1 Growth inhibition by corn extract and its reversal by niacinamide.

ments (groups 7 to 10) is proportional to the amounts of niacin and tryptophan in the preparations in question. The addition of niacin to these diets (groups 11 to 14) increased growth further.

Since these data suggested that the mouse required a dietary source of niacin when fed a 9% casein diet, experiments were designed to estimate the magnitude of this requirement. At the same time, the effects of various levels of dietary fat on this requirement were studied since the $CHCl_3$ extracts of

corn and zein were oils and the early experiments pointed to a relationship between level of dietary fat and growth on the niacin-free diet.

The growth data for groups 15, 16, 17 and 18, shown in table 3, confirmed the necessity for dietary niacin and suggested that supplementing the diet with an additional 5% of fat may slightly reduce this requirement.

TABLE 1

Mean weight gains of mice fed a 9% casein diet containing various corn extractives

GROUP NO. [1]	SUPPLEMENTS TO BASAL (9% CASEIN) DIET	MEAN TOTAL WEIGHT GAIN (20 DAYS)
		gm
1	None	0.18 [1]
2	0.25% Niacin	9.50
3	2.0% $CHCl_3$ corn extract	8.25
4	2.0% $CHCl_3$ corn extract + 0.25% niacin	10.27
5	2.0% $CHCl_3$ zein extract	6.00
6	2.0% $CHCl_3$ zein extract + 0.25% niacin	7.15
7	40% Ground whole corn	5.15
8	23% Extracted corn	2.42
9	5% NaOH corn extract	5.20
10	5% NaOH corn extract + 2.0% $CHCl_3$ corn extract + 23% extracted corn	6.65
11	40% Ground whole corn + 0.25% niacin	7.35
12	23% Extracted corn + 0.25 niacin	7.15
13	5% NaOH corn extract + 0.25% niacin	7.25
14	5% NaOH corn extract + 2.0% $CHCl_3$ corn extract + 0.25% niacin	6.80

[1] Each group consisted of 10 animals.

[2] This group was sacrificed on the 12th day of the experiment.

TABLE 2

Niacin and tryptophan contents of maize fractions

	FRACTION	NIACIN			TRYPTOPHAN	
		H_2SO_4 Extract	H_2O Extract	$Ba(OH)_2$ Extract	$Ba(OH)_2$ Extract	H_2O Extract
		μg/gm	*μg/gm*	*μg/gm*	*mg/gm*	*mg/gm*
1	NaOH extracted corn	2.5	1.3	—	314.4	15.2
2	NaOH extract of corn	95.34	88.6	—	1712	166
3	$CHCl_3$ corn extract	0.02	—	0.02	0.56	—
4	$CHCl_3$ zein extract	0.02	—	0.02	3.2	—

The remaining data in table 3 are taken from a separate experiment and it can be seen that the growth response on the niacin-free diet was better than in previous experiments but that addition of niacin increased growth substantially. Graded growth responses were not obtained by varying niacin between 0.5 and 2.0 mg%, suggesting that the amounts of niacin fed were optimum. Six-hundred milligrams per cent of DL-tryptophan (group 24), an amount probably far in excess of actual physiological needs, completely replaced this requirement indicating that the mouse can effect the tryptophan-niacin conversion.

TABLE 3

Mean weight gains of mice fed a 9% casein diet and the effect of fat, niacin and tryptophan supplementation

GROUP NO.	NO. MICE	SUPPLEMENTS TO BASAL (9% CASEIN) DIET	MEAN TOTAL WEIGHT GAIN
		%	*gm*
15	18	None	0.18 [1]
16	18	2 mg % niacin	6.50
17	18	250 mg % niacin	6.08
18	17	5% cottonseed oil	1.85
19	4	None	3.63 [2]
20	4	0.05 mg % niacin	7.25
21	4	0.10 mg % niacin	6.25
22	4	0.50 mg % niacin	9.37
23	4	2.00 mg % niacin	8.75
24	4	600 mg % DL-Tryptophan	9.75
25	4	1.8% Cottonseed oil	6.50
26	4	1.8% corn oil	6.04
27	4	1.8% Soybean oil	5.87
28	4	9% Cottonseed oil	1.50
29	4	1.8% Cottonseed oil + 2.0 mg % niacin	10.63
30	4	1.8% corn oil + 2.0 mg % niacin	10.25
31	4	1.8% Soybean oil + 2.0 mg % niacin	9.75
32	4	9% Cottonseed oil + 2.0 mg % niacin	9.13
33	6	20% Casein	9.30
34	6	20% Casein + 10% cottonseed oil	13.30
35	6	20% Casein + 2.0 mg % niacin	11.80
36	6	20% Casein + 10% cottonseed oil + 2.0 mg % niacin	13.30

[1] Groups 15 to 18 — experimental period = 18 days.

[2] Groups 19 to 36 — experimental period = 21 days.

Supplementing the basal diet with an additional 1.8% of fat (the approximate level present in diets containing $CHCl_3$ extract allowing arbitrarily about 0.2% for non-fatty substances) by adding cottonseed, corn, or soybean oil stimulated growth appreciably even on a niacin-free diet. Since the number of animals in each group was small it was not possible to determine whether the differences in response to the different oils were significant. Addition of niacin to the oil-containing diets also increased the growth response.

When 9% of additional fat was added to the niacin-free diet (group 28) — growth was considerably depressed and in this particular experiment was less than that obtained on the niacin-free diet. The mice fed this ration were remarkably unkempt and greasy in appearance. Initially, this condition was attributed to their habit of getting in the food cup. However, mice fed a similar diet supplemented with 2.0 mg% of niacin (group 32) had similar habits, but grew very well and were normal in appearance throughout the experiment. It appears that the unkempt appearance of the former group was due to the nutritional effect of the diet rather than to physical contact with it.

As might be expected, mice grow well on a niacin-free diet containing 20% of casein. However, they grow no better than those fed the 9% casein diet containing niacin. The addition of 2.0 mg% of niacin increased growth appreciably as did the addition of 10% of cottonseed oil. The performance on the 20% casein diet plus niacin was similar to that reported by Bosshardt et al. ('46).

Figure 2 presents the results of an experiment designed to determine if a graded growth response could be obtained by the administration of graded doses of niacin. In this particular experiment the animals receiving the niacin-free diet gained an average of 1.1 gm and as little as 12.5 μg% of niacin increased growth slightly. However, maximal growth occurred only at the 2000 μg% level. Estimates of the approximate daily requirements from this experiment assuming a generous daily intake of 4 gm of diet per mouse yields a value

of around 60 μg per day. In confirmation of earlier data, the addition of 2% of cottonseed oil to the niacin-free diet promoted growth although not as much as previously and a deleterious effect on growth of 9% cottonseed oil was again observed. In this instance 2000 μg% of niacin were necessary to overcome the growth inhibition induced by the fat.

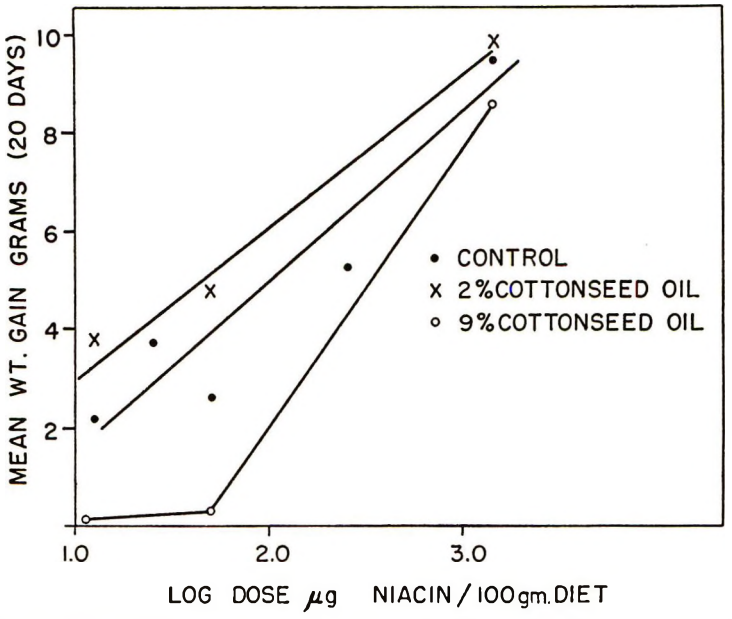


Fig. 2 Effect of dietary level of cottonseed oil on growth response to niacin.

DISCUSSION

The need for caution is recognized in interpreting results obtained on a "low" protein diet such as used in this study. The 9% casein diet is usually considered to be borderline for the rat — on such a diet these animals show fatty livers, apparently due to a suboptimal intake of threonine or lysine. Growth may be improved by the addition of niacin or tryptophan but the fatty livers remain unchanged (Singal et al., '53). However, this situation does not obtain in the Swiss albino mouse. This species grows nearly as well on a 9% casein diet as on a diet containing 40% casein — an optimum

protein-efficiency ratio being observed at a level of 10% (Boshardt et al., '46). Analyses performed in our laboratory reveal no signs of fatty liver in mice that have consumed a 9% casein diet for three weeks. For these reasons we presume that amino acid deficiencies (other than tryptophan) do not complicate our interpretation of results.

The literature regarding the nutritional requirements of the mouse is surprisingly small when one considers the number of these animals that are used experimentally. No report on the niacin requirement of this species seems to have been published. Woolley ('45) found that mice grew well on a 18% casein, niacin-free diet and concluded that they synthesize their own supply of this vitamin. In general, the consensus seems to be that the mouse resembles the rat and that niacin is "probably not needed unless (the) diet (is) deficient in tryptophan," (Cuthbertson, '57). The results persented here, while confirming the conclusion that niacin is unnecessary when adequate tryptophan is present, suggest that the mouse is not as efficient as the rat in making this conversion. Hundley ('47) found that the rat grew, although at a sub-optimum rate, on a 9% casein, niacin-free diet and this is in accord with our own observations (Pearson et al., '57). On the other hand, the mouse most frequently does not grow on this diet and may actually die of what appears to be a niacin deficiency. It is also pertinent to note that the presence of as much as 20% of fat in the 9% casein, niacin-free diet has no apparent deleterious effect on the growth of the rat.

The level of 10 mg of niacin per kilogram of diet suggested by Cuthbertson ('57) as optimal for the mouse would seem to be reasonable. In view of our finding that niacin increased growth even at a level of 20% of casein it would seem wise to include this vitamin in all purified diets irrespective of protein content.

Under our experimental conditions as specific growth-inhibiting factor was not detected in an alkaline-chloroform extract of corn or zein. Neither was evidence obtained for the presence of an antiniacin factor in the aqueous alkaline layer.

However, it is not possible to compare our extraction procedure with that of Woolley ('46) since he did not publish details of his method.

The growth inhibition noted when the alkaline-$CHCl_3$ extract was incorporated at a 9% level could be duplicated by the addition of 9% of cottonseed oil, suggesting that a nonspecific effect of fat was being observed. This deleterious effect of fat occurred only on a 9% casein, niacin-free ration and could be prevented by the addition of niacin or tryptophan. Incorporation of the extract at a level of 2 to 5% had a "niacin-sparing" effect, the magnitude of which varied considerably from experiment to experiment.

Numerous studies have been made on the influence of dietary fat upon the vitamin requirements of experimental animals. In particular, the "sparing" effect of fat upon the thiamine requirement has been known since the early report of Evans and Lepkovsky ('29). Since thiamine is essential for carbohydrate metabolism it has been assumed that fat acts merely by reducing the carbohydrate content of the diet. However, there is a difference of opinion concerning this explanation (Gershoff and Hegsted, '54; Holt et al., '55) and the exact mechanism is still in doubt.

The only report in the literature concerned with a niacin-fat relationship is that of Salmon ('47). This worker found that the addition of 30% of lard to a diet containing 40% of corn and no added niacin increased growth considerably. He concluded that fat "spares niacin and tryptophane" and that the supposition that the rat does not require a dietary source of niacin is false unless one presupposes a liberal supply of tryptophan or fat, or both in the diet.

The deleterious effect of a diet containing 25 to 40% fat on the growth of rats fed a riboflavin-low diet has been observed by Mannering et al. ('41) who suggest that this level of fat may alter the intestinal synthesis or destruction of riboflavin or that riboflavin may be directly concerned with fat metabolism. One may similarly envision that the latter mechanism may be operative in our study especially since diphospho-

pyridine nucleotide is required for the entry of acetyl coenzyme A into the tricarboxylic acid cycle. However, no experimental evidence for this or for any other mode of action has been obtained.

SUMMARY

The albino Swiss mouse requires a source of dietary niacin for growth when fed a 9% casein diet. This requirement may be partially spared by the addition of a small amount of fat (2 to 5%) to the diet, but the addition of 9% of cottonseed oil was deleterious and capable of reversion by 2.0 mg% of niacin.

Under our experimental conditions, it has not been possible to obtain evidence for the presence of an alkaline-$CHCl_3$ extractable antiniacin compound in corn. The apparent effects of such extracts upon the growth of mice is most likely a nonspecific effect probably related to their fatty nature.

LITERATURE CITED

Association of Official Agricultural Chemists 1955 Official and Tentative Methods of Analysis, 8th Ed., Washington, D. C.

Bosshardt, D. K., L. C. Ydse, M. M. Ayres and R. H. Barnes 1946 The use of mice for the measurement of the growth promoting quality of proteins. J. Nutrition, *31:* 23.

Chaudhuri, D. K., and E. Kodicek 1950 Purification of a precursor of nicotinic acid from wheat bran. Nature, *165:* 1022.

Cravioto, R. O., G. H. Massieu, O. Y. Cravioto and F. de M. Figueroa 1952 Effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet. J. Nutrition, *48:* 453.

Cuthbertson, W. F. J. 1957 Nutrient requirements of rats and mice. Proc. of the Nutrition Soc., *16:* 70.

Evans, H. M., and S. Lepkovsky 1929 Sparing effect of fat on the anti-neuritic vitamin B. J. Biol. Chem., *83:* 269.

Gershoff, S. N., and D. M. Hegsted 1954 The failure of thyroxine and high fat diets to modify the rate of thiamine loss from the body. J. Nutrition, *54:* 609.

Greene, R. D., and A. Black 1943 A microbiological method for the determination of tryptophan. Proc. Soc. Exp. Biol. Med., *54:* 322.

Holt, L. E., Jr., and S. E. Snydermann 1955 The influence of dietary fat on thiamine loss from the body. J. Nutrition, *56:* 495.

HORWITT, M. K., C. C. HARVEY, W. S. ROTHWELL, J. L. CUTTER AND D. HAFFRON 1956 Tryptophan-niacin relationships in man. J. Nutrition, *60:* Supplement 1.

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

HUNDLEY, J. M. 1947 Production of niacin deficiency in rats. Ibid., *34:* 253.

KODICEK, E., R. BRAUDE, S. K. KON AND K. G. MITCHELL 1956 The effect of alkaline hydrolysis of maize on the availability of its nicotinic acid to the pig. Brit. J. Nutrition, *10:* 51.

LAGUNA, J., AND K. J. CARPENTER 1951 Raw vs. processed corn in niacin deficient diets. J. Nutrition, *45:* 21.

LIPKE, H., AND G. FRAENKEL 1955 The toxicity of corn germ to the meal worm, *Tenebrio molitor*. Ibid., *55:* 165.

MANNERING, G. J., M. A. LIPTON AND C. A. ELVEHJEM 1941 Relation of dietary fat to riboflavin requirement of growing rats. Proc. Soc. Exp. Biol. Med., *46:* 100.

NATIONAL RESEARCH COUNCIL 1958 Recommended Dietary Allowances. Publication 589. Food and Nutrition Board, National Academy of Sciences, Washington. Publication.

PEARSON, W. N., S. J. STEMPFEL, J. S. VALENZUELA, M. H. UTLEY AND W. J. DARBY 1957 The influence of cooked vs. raw maize on the growth of rats receiving a 9% casein ration. J. Nutrition, *62:* 445.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluoride toxicosis in the rat. J. Biol., Chem., *109:* 657.

ROMBOUTS, J. E. 1953 The destruction of B vitamins by certain salt mixtures in purified rations. J. Nutrition, *50:* 255.

SALMON, W. D. 1947 Relation of corn products to the requirement of the rat for dietary nicotinic acid. Ibid., *33:* 169.

SINGAL, S. A., S. J. HAZAN, V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1953 The production of fatty livers in rats on threonine and lysine deficient diets. J. Biol. Chem., *200:* 867.

WOOLLEY, D. W. 1945 Production of nicotinic acid deficiency with 3 acetyl pyridine, the ketone analogue of nicotinic acid. Ibid., *157:* 455.

———— 1946 The occurrence of a "pellagragenic" agent in corn. Ibid., *163:* 773.

STUDIES ON GOITROGENIC AGENTS IN FOOD

III. GOITROGENIC ACTION OF SOME GLYCOSIDES ISOLATED FROM EDIBLE NUTS

N. R. MOUDGAL, E. RAGHUPATHY AND P. S. SARMA
University Biochemical Laboratory, Madras, India

(Received for publication April 24, 1958)

It has been shown in an earlier communication (Moudgal et al., '57) that the goitrogenic action of groundnut [1] is due to its glycoside (arachidoside) content. On the basis of increased excretion of phenols in the group of rats fed arachidoside, and of an increased iodine content in the phenolic fraction of the urine as compared with the control, it was suggested that this glycoside acted as an antithyroid compound by forming molecular compounds with elemental iodine in the gland. Confirmatory evidence for this hypothesis, obtained in studies using radioactive iodine (I^{131}) is presented in this communication. The study has been extended to cover other anthocyanin pigments isolated from the outer skin-covering of edible nuts similar in nature to groundnuts.

EXPERIMENTAL

Isolation of anthocyanin pigments. Red amorphous pigments were isolated from the outer red skin-covering of cashew nuts (*Anacardium occidentale*) and almonds (*Prunus amygdalus*) by the same method used earlier in the preparation of arachidoside from groundnuts (Moudgal et al., '57).

A red tannin pigment was also isolated from the common areca nut (*Areca catechu*) which is an article of human con-

[1] Peanut.

sumption in India. The commercially available nut is a processed variety containing only 15% of tannins whereas the original nut contains nearly 35% (The Wealth of India, '48). A 50% alcoholic extract of the processed areca nut was obtained by soaking the nuts overnight in alcoholic solution. The extract was filtered and the alcohol removed completely. The red amorphous powder thus obtained was purified by redissolving in a small amount of alcohol, followed by filtration and evaporation of the alcohol.

All 4 pigments are water-soluble. The pigment isolated from *Anacardium occidentale* has been named anacardioside, and this term has been used throughout in the present series of investigations.

Influence of arachidoside on the radioactive iodine (I^{131}) uptake and distribution in the thyroid glands of albino rats. Twelve albino rats weighing on an average 70 gm were divided into three groups of 4 each. Group I served as a control and was fed only the basal diet. Group II was the arachidoside-supplemented group, maintained on the basal diet plus a daily supplement of 25 mg of arachidoside per rat. Group III was similar to group II, except that a daily supplement of 10 μg of iodide as KI per rat was given in addition to arachidoside. The composition of the basal diet and the method of feeding etc., have been reported earlier (Srinivasan et al., '57). At the end of a feeding period of 7 weeks, each rat was given an intraperitoneal injection of 100 μc of carrier-free radioactive iodine. Four hours after injection, the rats were sacrificed under ether anesthesia, and the thyroids dissected out, quickly weighed on a torsion balance and transferred to test tubes containing 6 ml of 3.5 N NaOH. The samples were fractionated into inorganic iodine, diiodotyrosine iodine and thyroxine iodine fractions according to the method of Morton and Chaikoff ('43). Suitable aliquots of the various fractions were transferred to steel planchets for the measurement of radioactivity using a Geiger counter attached to a "Panax" scaler. The results of this investigation are presented in table 1.

TABLE 1

Distribution of radioactivity in thyroid gland 4 hours after an injection of radioactive iodine (I^{131})

	GROUP	PERCENTAGE OF ADMINISTERED RADIOACTIVITY RECOVERED IN DIFFERENT FRACTIONS OF THYROID HYDROLYSATE			
		Inorganic fraction	Diiodotyrosine fraction	Thyroxine fraction	Total iodine
I	Control	1.06 (0.82–1.20)[1]	6.32 (5.52–7.80)	2.34 (1.98–2.70)	9.72
II	Arachidoside, 25 mg per rat per day	1.48 (1.18–1.63)	2.00 (1.48–2.66)	1.22 (0.95–1.50)	4.70
III	Arachidoside, 25 mg per rat per day plus KI, 10 μg per rat per day	1.02 (0.76–1.20)	3.25 (2.62–4.81)	1.43 (1.30–1.73)	5.70

[1] Values within parentheses represent range.

Influence of anacardioside feeding on body weight, thyroid weight and thyroidal I^{131} uptake of albino rats. Fifteen albino rats weighing, on an average, 63 gm were divided into three groups of 5 each. Group I was maintained on the basal diet, whereas to group II a daily supplement of 20 mg of anacardioside per rat was given. Group III was given, in addition to anacardioside, a daily supplement of 15 μg of KI per rat. The composition of the basal diet and the feeding period were the same as those used in our earlier studies (Srinivasan et al., '57). At the end of the feeding period, 100 μc of carrier-free radioactive iodine was administered to each rat intraperitoneally, the rats transferred to individual metabolic cages and the urine collected for the subsequent 24 hours. The rats were then killed under ether anesthesia, the residuary urine in the bladder aspirated out as detailed earlier (Srinivasan et al., '57) and the thyroids dissected immediately and weighed on a torsion balance. The thyroid glands were then processed according to the method of Taurog and Chaikoff ('47), and the

radioactive iodine was fractionated into three portions — non-protein-bound iodine (NPBI), diiodotyrosine iodine and thyroxine iodine, according to the method of Taurog and Chaikoff ('46). The radioactivity in the various fractions was measured as in the earlier case using a Geiger counter attached to a "Panax" scaler. The results of these investigations are presented in tables 2 and 3. Thyroid glands from another set of rats grouped in the above manner were removed at the end of the experimental period for histological examination.

Influence of anthocyanin pigments and the areca catechin on the organic binding of I^{131} *by thyroid tissue slices.* The experimental techniques followed were essentially those of Franklin, Chaikoff and Lerner ('44). The reaction was carried out in 10-ml beakers in a Dubnoff metabolic shaking incubator. The composition of the reaction medium and the conditions of incubation are detailed in table 4. Each of these inhibitor compounds was tested in triplicate. In the control beakers, the inhibitor solution was replaced by an equal volume of Krebs, Ringer bicarbonate solution (KRB).

At the termination of the incubation period, the reaction media were decanted from the beakers and the slices immersed for 20 seconds in 3 ml of KRB solution. The solution was discarded and the operation repeated with a fresh 3-ml portion of KRB solution. The slices were then gently pressed between folds of a filter paper moistened with KRB and then ground well with 1.5 ml of 5% trichloroacetic acid and the ground mass transferred to centrifuge tubes. After centrifugation the residue was washed with 1 ml of 5% trichloroacetic acid, again centrifuged and the first and second supernatant solutions mixed and then made to a known volume. This represented the NPBI fraction.

The residue in the centrifuge tubes was hydrolysed for 8 hours over a steam bath with 6 ml of 2 N NaOH. The hydrolysates were then adjusted to pH 2.5 to 3.0 and repeatedly extracted with 10-ml portions of acidified n-butanol. The bu-

TABLE 2

Influence of anacardioside feeding on body weights and thyroid weights of albino rats

	GROUP	INITIAL WEIGHT	FINAL WEIGHT	GAIN IN WEIGHT	AVERAGE WEIGHT OF THYROID GLAND	RANGE OF THYROID WEIGHT
		gm	*gm*	*gm*	*mg*	*mg*
I	Control	64 (60–66)[1]	166 (157–171)	102	8.8	8.4– 9.2
II	Anacardioside, 20 mg per rat per day	63 (60–66)	143 (138–150)	80	16.4	13.0–21.0
III	Anacardioside, 20 mg per rat per day plus KI, 15 μg per rat per day	64 (61–67)	171 (160–182)	107	9.4	8.8–11.0

[1] Values within parentheses represent range.

TABLE 3

Distribution of radioactivity in thyroid gland 24 hours after an injection of radioactive iodine

	GROUP	PERCENTAGE OF ADMINISTERED ACTIVITY RECOVERED IN DIFFERENT FRACTIONS OF THYROID			PERCENTAGE OF ADMINISTERED RADIOACTIVE IODINE EXCRETED IN THE 24-HOUR URINE
		NPBI[1] fraction	Diiodotyrosine fraction	Thyroxine fraction	
I	Control	0.37	10.26	3.80	50 ± 2[2]
II	Anacardioside, 20 mg per rat per day	0.04	0.56	0.37	65 ± 1
III	Anacardioside, 20 mg per rat per day plus KI, 15 μg per rat per day	0.10	3.32	1.33	67 ± 3

[1] Non-protein-bound iodine.

[2] Standard deviation.

tanol extracts were pooled and made to a known volume. This represented the organic bound iodine fraction and included both thyroxine and diiodotyrosine fractions of Perlman et al. ('41). The radioactivity in the various fractions was measured as described earlier. The results are given in table 4.

"In vitro" formation of iododerivatives by the test compounds. It has been shown earlier (Moudgal et al., '57) that

TABLE 4

Influence of anthocyanin pigments and the areca catechin on the radioactive iodine (I^{131}) incorporating ability of surviving sheep thyroid slices

Three hundred milligrams of slices were incubated for three hours at 37°C in KRB [1] medium. Total volume was 3 ml. The inhibitors and I^{131} were added as KRB solutions; 25 μc of carrier-free I^{131} per beaker. No I^{127} was added.[2] Gas phase 95% O_2 and 5% CO_2.

TEST COMPOUND	CONCENTRATION	I^{131} OF RINGER'S SOLUTION RECOVERED IN SLICES AS		
		NPBI [3]	Organic iodine	Total iodine
	%	%	%	%
None	—	55.0	15.0	70
Arachidoside	0.0008	39.8	12.2	52
Anacardioside	0.0008	41.3	6.4	48
Almond anthocyanin	0.0008	41.0	5.4	46
Areca catechin	0.0008	38.5	7.3	46

[1] Krebs, Ringer bicarbonate.

[2] The only I^{127} contained in the reaction medium was that due to impurities in the reagent grade chemicals used in the preparation of the media.

[3] Non-protein-bound iodine.

arachidoside forms a stable iododerivative containing 19.8% iodine on treatment with iodine-potassium iodide solution at 37°C in phosphate buffer pH 5.8. Using a similar procedure, iododerivatives of the other three compounds were prepared. The iodine contents of these derivatives are shown in table 5.

Influence of the iododerivatives of the test compounds on the organic binding of I^{131} by thyroid tissue slices. The experimental technique adopted was the same as described earlier for uniodinated pigments. The concentration of iododerivatives in

the reaction medium, however, was of the order of 0.0016% (50 μg) in a final volume of 3 ml. The results of this study are presented in table 5.

Effect of test compounds on the monoiodotyrosine (MIT) synthesizing ability of sheep thyroid homogenates. The

TABLE 5

Influence of iodinated glycosides on the organic binding of radioactive iodine by surviving sheep thyroid tissue slices

The composition of the incubation medium and the conditions and duration of incubation are the same as detailed earlier in table 4.

IODINATED DERIVATIVE OF	IODINE CONTENT	CONCENTRATION OF IODINATED DERIVATIVE IN THE INCUBATION MEDIUM	I^{131} OF RINGER'S SOLUTION RECOVERED IN THE ORGANIC FRACTION
	%	%	%
None	—	—	22.5
Arachidoside	19.8	0.0016	19.3
Anacardioside	18.6	0.0016	18.0
Almond anthocyanin	27.0	0.0016	21.4
Areca catechin	25.8	0.0016	22.2

TABLE 6

Influence of anthocyanin pigments and areca catechin on the monoiodotyrosine (MIT) synthesis in cell-free preparations of sheep thyroid tissue

The experiment was carried out in test tubes containing the following reaction medium: 2 ml of 20% isotonic KCl homogenate, 0.5 ml of 0.1 M phosphate buffer pH 7.4, 0.2 ml of 0.04 M tyrosine, 0.2 ml of 0.04 M $CuCl_2$ solution, 0.05 ml of I^{131} solution containing 10 μc of I^{131} and 0.4 ml of inhibitor solution or distilled water, final volume 3.55 ml. Incubation for three hours at 37°C.

INHIBITOR	CONCENTRATION OF INHIBITOR	MIT SYNTHESIS [1]
	μg	
Arachidoside	25	+
	50	—
Anacardioside	25	++
	50	+
Almond anthocyanin	25	+
	50	+
Areca catechin	25	++
	50	—

[1] ++ No inhibition; + partial inhibition; — total or nearly total inhibition of MIT synthesis.

method followed for the preparation of the homogenate, incubation and the post-incubation treatment was essentially the same as that for the tyrosine iodinase system adopted by Fawcett and Kirkwood ('53). The composition of the incubation medium and the conditions of incubation are indicated in table 6. The radioautograph was developed according to the method of Fawcett and Kirkwood ('53). The results are presented in table 6.

RESULTS AND DISCUSSION

From the results presented in table 1, it can be seen that feeding arachidoside to albino rats brings about a considerable reduction in the I^{131} content of diiodotyrosine and thyroxine fractions of the thyroid gland. These results thus provide confirmatory evidence for the hypothesis that arachidoside is goitrogenic by virtue of its capacity to interfere with the organic binding of iodine. The slight compensatory increase in the radioactivity of the inorganic iodine fraction (table 1) however, is not equivalent to the observed decrease of radioactivity in the organic fraction. The total iodine of the thyroid gland of rats fed arachidoside is nearly 50% less than that present in the thyroid of control rats. This suggests that arachidoside, besides interfering with the organic binding of iodine, may directly inhibit the iodine uptake by the thyroid. This inhibitory effect on the iodine concentrating capacity of the thyroid gland could not be observed in the earlier study (Srinivasan et al., '57), presumably owing to the mildness of the goitrogenic effect of defatted groundnut cake. Small amounts of KI added as supplements to the arachidoside diet afford a partial protection against the goitrogenic action. This is in keeping with the results reported earlier with defatted groundnut cake (Srinivasan et al., '57).

The influence of anacardioside feeding on the body and thyroid weights of albino rats can be assessed from the data in table 2. Anacardioside, unlike arachidoside (Moudgal et al., '57), does not possess any growth-promoting property. On

the other hand, it can be seen that anacardioside supplementation inhibits the growth of rats. The thyroid weights of rats fed anacardioside are considerably increased. Supplementation of the diet with small amounts of KI reverses the inhibitory effect of anacardioside on growth and further helps to maintain the thyroid weight within the normal range.

The distribution of radioactivity in the various fractions of the thyroid, and the pattern of urinary excretion of radioactive iodine by rats fed anacardioside are presented in table 3. That anacardioside interferes with the normal uptake of I^{131} by thyroid is evident from the low radioactivity present in the NPBI fraction. It is also seen from the I^{131} content of the diiodotyrosine and thyroxine fractions, that anacardioside inhibits the organic binding of iodine as well. The increased urinary excretion of radioactivity in rats fed anacardioside is similar to the effect observed earlier with defatted groundnut cake (Srinivasan et al., '57).

Anacardioside supplementation thus results in an increase in thyroid weight of rats, a decrease in uptake as well as organic binding of iodine by the thyroid gland and an increase in the urinary excretion of radioactive iodine. These observations clearly point to this glycoside being a potent goitrogen.

The histological examination of thyroids of rats fed anacardioside showed increased colloid spaces filled with faintly staining colloid and thus provides further evidence for the goitrogenic action of this glycoside. The picture was that of a typical colloid goitre without any accompanying hyperplasia. Similar examination of thyroid slices of rats fed anacardioside and potassium iodide indicated a partial reversal of the goitre.

The results obtained with the above two anthocyanin pigments suggested a strong possibility that this goitrogenic action might be a property shared by other anthocyanin pigments and related compounds. Hence the *in vitro* effect of three such glycosides, as well as of a catechin, on the uptake and organic binding of I^{131} in thyroid tissue slices was stud-

ied. From table 4, it is clear that all of the 4 test compounds significantly inhibit the organic binding of iodine in surviving thyroid tissue slices. Unlike the thiocarbamides (Pitt-Rivers, '50), these compounds bring about a significant decrease in the iodine concentration of the non-protein fraction also.

Fawcett and Kirkwood ('53) from their *in vitro* experiments conclude that all aromatic compounds capable of forming substituted derivatives with iodine and possessing electron-donating groups are potential thyroid inhibitors. Anthocyanins and their closely related flavonol derivatives, like catechins, contain polyphenolic components in their moieties and one of the products of total breakdown of these compounds is phloroglucinol. The reactivity of these polyphenolic groups towards iodine is dependent upon the total number of the hydroxyl groups present in the molecule and the orientation of these hydroxyl groups with respect to each other. It can be seen from table 5, that all of the 4 compounds are capable of forming stable iododerivatives containing large amounts of iodine. This suggests a probable mechanism whereby these glycosides interfere with normal thyroid function. Thus they may act as competitors with tyrosine for elemental iodine in the thyroid gland. To test this possibility, the influence of the iododerivatives of these compounds on the organic binding of radioactive iodine in surviving thyroid tissue slices was studied. It is evident from table 5 that inclusion of these iododerivatives even at twice the concentration of the corresponding noniodinated analogues does not bring about any significant change in the organic binding of iodine.

Phloroglucinol, which is known to be the final breakdown product of many of these polyphenolic compounds, has been shown to be a powerful goitrogen (Arnot and Doniach, '52). The formation of iodophloroglucinol in *in vitro* systems has been observed by Fawcett and Kirkwood ('53). The fact that the test polyphenolic compounds form iododerivatives directly suggests that the formation of phloroglucinol from these com-

pounds in an *in vitro* system is not a prerequisite for these substances inhibiting normal thyroid function.

Fawcett and Kirkwood ('54) have reported the preparation of the enzyme tyrosine iodinase which catalyses the single-step iodination of tyrosine to monoiodotyrosine. Since the action of the enzyme is independent of the formation of elemental iodine from inorganic iodide (cupric ion is responsible for this oxidative conversion), the enzyme system offers itself as a convenient tool for studying the possible action of suspected goitrogenic agents at the stage of the synthesis of MIT. Hence, in the present case, the action of the above test compounds on the MIT synthesis by cell-free preparations of thyroid tissue was studied. The results are presented in table 6. Since the same volume of the incubation mixture (30 µl) was delivered on to the chromatogram paper in each case, the intensity of the MIT spot on the radioautogram gives an idea of the extent of inhibition brought about by a particular test compound. It can thus be seen that generally the above test compounds are inhibitory at the 50 µg level but have no action at the 25 µg level. Anacardioside even at the 50 µg level does not cause total inhibition of MIT synthesis. This observation, together with the fact that anacardioside feeding to rats results in a marked reduction of radioactivity, even in the NPBI fraction (table 3) suggests more than one mode of action for this compound, namely, that it may act both by inhibiting the uptake of inorganic iodine and by blocking the organic binding of iodine. It is also of interest to note that in experiments with iodinated derivatives of these anthocyanin pigments, the percentage of I^{131} recovered from the KRB medium is the least in the case of iodinated anacardioside (table 5). The fact that areca catechin totally inhibits MIT synthesis in the present case and loses its entire activity on iodination (table 5) shows that this compound mainly interferes with the organic binding of iodine. Arachidoside and the almond anthocyanin may have a mode of action very similar to anacardioside.

Thus the present investigation shows that all of the 4 compounds are goitrogenic by virtue of the fact that they compete with tyrosine for elemental iodine, forming stable iododerivatives at the gland site. Hence their goitrogenic activity can be partially overcome by supplementing the diet with small amounts of KI.

SUMMARY

The inclusion of arachidoside and anacardioside, the pigments isolated from the outer skin-covering of groundnuts and cashewnuts respectively, in the diet of rats, at a level of 20 mg per rat per day for 7 weeks results in goitre. Incorporation of potassium iodide in the diet counteracts partially the goitrogenic action of the anthocyanins. Results of studies on the distribution of radioactive iodine in the thyroid glands of the rats suggest that the pigments inhibit the organic binding of radioactive iodine.

An anthocyanin pigment and a catechin have been isolated from almonds and areca nuts respectively. *In vitro* experiments employing the tissue slice as well as homogenate technique reveal that all of the above 4 test compounds interfere with the organic binding of radioactive iodine. The test compounds form stable iododerivatives which, however, do not possess any significant inhibitory effect on the organic binding of radioactive iodine. The implications of the results are discussed.

ACKNOWLEDGMENT

The authors wish to thank the Council of Scientific and Industrial Research, (India), for financial assistance.

LITERATURE CITED

ARNOT, D. G., AND I. DONIACH 1952 The effect of compounds allied to resorcinol upon the uptake of radioactive iodine (I^{131}) by the thyroid of the rat. Biochem. J., *50:* 473.

FAWCETT, D. M., AND S. KIRKWOOD 1953 The mechanism of antithyroid action of iodide ion and of the "aromatic" thyroid inhibitors. J. Biol. Chem., *204:* 787.

———— 1954 Tyrosine iodinase. Ibid., *209:* 249.

FRANKLIN, A. L., I. L. CHAIKOFF AND S. R. LERNER 1944 The influence of goitrogenic substances on the conversion *in vitro* of inorganic iodine to thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. Ibid., *153:* 151.

MORTON, M. E., AND I. L. CHAIKOFF 1943 The formation *in vitro* of thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. Ibid., *147:* 1.

MOUDGAL, N. R., V. SRINIVASAN AND P. S. SARMA 1957 Studies on goitrogenic agents in food. II. Goitrogenic action of arachidoside. J. Nutrition, *61:* 97.

PERLMAN, I., M. E. MORTON AND I. L. CHAIKOFF 1941 Radioactive iodine as an indicator of the metabolism of iodine. II. The rates of formation of thyroxine and diiodotyrosine by the intact normal thyroid. J. Biol. Chem., *139:* 449.

PITT-RIVERS, R. 1950 Mode of action of antithyroid compounds. Physiol. Revs., *30:* 194.

SRINIVASAN, V., N. R. MOUDGAL AND P. S. SARMA 1957 Studies on goitrogenic agents in food. I. Goitrogenic action of groundnut. J. Nutrition, *61:* 87.

TAUROG, A., AND I. L. CHAIKOFF 1946 The determination of thyroxine in the thyroid gland of the rat. J. Biol. Chem., *163:* 323.

———— 1947 The metabolic interrelations of thyroxine and diiodotyrosine in the thyroid gland as shown by a study of their specific activity-time relations in rats injected with radioactive iodine. Ibid., *169:* 49.

WEALTH OF INDIA 1948 A dictionary of Indian raw materials and Industrial products. Published by Publications Division, Council of Scientific and Industrial Research, New Delhi (India), *1:* 110.

INVITATIONS FOR NOMINATIONS FOR 1959 AMERICAN INSTITUTE OF NUTRITION AWARDS

Nominations are requested for the 1959 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed and (2) a statement as convincing as possible as to the basis for the nomination, stating the eligibility of the candidate (this may include the pertinent bibliography of the most appropriate and significant recent papers on which the nomination is based, but such bibliography is not necessary unless later requested by the Nominating Committee). Reprints are not required, nor are seconding statements. *Five copies of all documents* must be sent to the chairman of the appropriate Nominating Committee *before December 1, 1958,* to be considered for the 1959 awards.

General Regulations for A.I.N. Awards. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award.[1] A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person but, if circumstances and justice so dictate, a Jury of Award may recommend that any par-

[1] Including recipients of the former Mead-Johnson award. These are listed at the end of this notice.

ticular award be divided between two or more collaborators in a given research.

Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age. Presentation of awards will be made at the annual dinner at the annual meeting and time will be allotted on the scientific program for a brief review by the recipient of the work upon which the award has been based.

1959 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 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. 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 — H. H. Mitchell; 1946 — P. C. Jeans and Genevieve Stearns; 1947 — L. A. Maynard; 1948 — C. A. Cary; 1949 — H. J. Deuel, Jr.; 1950 — H. C. Sherman; 1951 — P. György; 1952 — M. Kleiber; 1953 — H. H. Williams; 1954 — Agnes Fay Morgan and A. H. Smith; 1955 — A. G. Hogan; 1956 — F. M. Strong; 1957 — no award; 1958 — L. D. Wright.

Nominating Committee:

H. H. Williams, *Chairman*
C. A. Baumann
G. V. Mann

Send nominations to:

Dr. H. H. Williams
Cornell University, Savage Hall
Ithaca, New York

1959 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research 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 approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. 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.

Former recipients of this award are: 1949 — W. C. Rose; 1950 — C. A. Elvehjem; 1951 — E. E. Snell; 1952 — Icie Macy Hoobler; 1953 — V. du Vigneaud; 1954 — L. A. Maynard; 1955 — E. V. McCollum; 1956 — A. G. Hogan; 1957 — G. R. Cowgill; 1958 — P. György.

Nominating Committee:
ICIE MACY HOOBLER, *Chairman*
D. M. HEGSTED
J. S. DINNING

Send nominations to:
DR. ICIE MACY HOOBLER
Merrill-Palmer School
71 East Ferry Avenue
Detroit 2, Michigan

Former recipients of the Mead-Johnson Award presented by A.I.N. are: 1939 — C. A. Elvehjem; 1940 — W. H. Sebrell, Jr., J. C. Keresztesy, J. R. Stevens, S. A. Harris, E. T. Stiller, and K. Folkers; 1941 — R. J. Williams; 1942 — G. R. Cowgill; 1943 — V. du Vigneaud; 1944 — A. G. Hogan; 1945 — D. W. Woolley; 1946 — E. E. Snell; 1947 — W. J. Darby, P. L. Day, and E. L. R. Stokstad; 1948 — F. Lipmann; 1949 — Mary S. Shorb and K. A. Folkers; 1950 — W. B. Castle; 1951 — no award; 1952 — H. E. Sauberlich.

INVITATION FOR NOMINATIONS FOR FELLOWS

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows will be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society.

Nominations (in 5 copies) are due by January 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April. The following persons have been elected previously as Fellows of the Society:

Thorne M. Carpenter	Elmer V. McCollum
George R. Cowgill	Harold H. Mitchell
Eugene F. DuBois	John R. Murlin
Ernest B. Forbes	Harry Steenbock
Casimir Funk	Robert R. Williams

Fellows Committee:

T. H. JUKES, *Chairman*
PAUL GYÖRGY
COSMO MACKENZIE
W. D. SALMON
KARL FOLKERS

Send nominations to:

DR. T. H. JUKES
American Cyanamid Company
Research Division
Pearl River, N. Y.

NOTICE TO CONTRIBUTORS

THE JOURNAL OF NUTRITION, a copyrighted periodical, appears monthly for the publication of original research bearing on the subject of nutrition and occasional reviews of the literature dealing with this subject.

THE JOURNAL OF NUTRITION is the official organ of the American Institute of Nutrition. The officers of the Institute for 1958–59 are: WILLIAM J. DARBY, *President;* D. WAYNE WOOLLEY, *President-elect;* GEORGE M. BRIGGS, *Secretary;* J. B. BROWN, *Treasurer;* E. W. MCHENRY, PAUL GYÖRGY, J. H. ROE, and R. R. WILLIAMS, *Councillors.*

Preliminary notices, or papers already published or in press elsewhere, will not be accepted. Unusually long papers that would take a disproportionate part of a single issue can be considered only if published as a supplement, the entire cost of which is assumed by the author.

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, Connecticut. If two complete copies of the paper are submitted, consideration by the Journal can be expedited.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. *The Wistar Institute reserves the right to return to the author for revision material which is not in proper form for the printer.* **When the amount of tabular or illustrative material or both is judged to be excessive or unusually expensive, authors may be requested to pay the excess cost.**

Manuscripts should be typed in double spacing on one side of bond or heavy-bodied paper 8½ × 11 inches and should be **sent flat.** Page 1 should include, in the following order: COMPLETE TITLE, AUTHOR'S NAME, INSTITUTION FROM WHICH THE PAPER CAME, WITH CITY AND STATE, TOTAL NUMBER OF FIGURES, SHORTENED FORM OF TITLE **(not more than 35 letters and spaces),** ADDRESS TO WHICH THE PROOF IS TO BE SENT. *The text starts on page 2.*

Tables, quotations (extracts of over 3 lines), and all other material usually set in type smaller than the text, should be typed each on a separate sheet. **Footnotes to the text should be numbered consecutively (including those on page 1)** and typed in order on a separate sheet. Explanations of figures should be treated in the same manner. Footnotes to a table should be made a part of the table, and should be typed directly beneath it. Citations of literature in the text should be made by author and by numerals to indicate the year of publication. **Authors' names (followed by year, title of paper, etc.) should be arranged alphabetically in a list at the end of the text.**

The original drawings, not photographs of drawings, should accompany the manuscript. When photographs are used for halftone reproduction, glossy prints should be sent. Authors should indicate on the manuscript the approximate position of text figures. If there are illustrations so large as to require mailing separately, reduced copies of them that can be mailed with the manuscript should be provided in order to expedite consideration of the paper.

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the cost of a more expensive form of illustration. Letters and figures should be uniform and large enough so that no character will be less than 2 mm high after reduction to at least the width of the page (4¼ inches). Double plates will not be accepted. Figures should be numbered consecutively beginning with the text figures and continuing through the plates. The reduction desired should be clearly indicated on the margin of the drawing.

Drawings intended for photographic reproduction should be made on white or blue-white paper or bristol board — **not** on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — **never** with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon. **For further details important for the preparation of a satisfactory manuscript, ask The Wistar Institute for a copy of the brochure, A GUIDE FOR AUTHORS.**

THE JOURNAL OF NUTRITION is a copyrighted scientific periodical and reprints are intended primarily for the author's use. Authors are furnished 50 reprints, with covers, gratis. Additional copies may be obtained according to the rates which will be sent the authors as soon as the manuscript has been examined at The Wistar Institute, after acceptance. Other parties ordering reprints must have their orders approved by the authors, and must give written assurance that distribution will be restricted to professional personnel and others interested in the science of nutrition, and that **no advertising or imprinting** will be attached to the reprints thus furnished.

THE JOURNAL® OF NUTRITION

VOL. 66 OCTOBER 10, 1958 NO. 2

CONTENTS

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America