MARCH 10, 1959

THE JOURNAL® OF NUTRITION

VOLUME 67 NUMBER 3

HOWARD BISHOP LEWIS

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

THE JOURNAL OF COMPARATIVE NEUROLOGY

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

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

THE AMERICAN JOURNAL OF ANATOMY

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

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

THE ANATOMICAL RECORD

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

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

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

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

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

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

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

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

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

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

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

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

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

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

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

THE AMERICAN ANATOMICAL MEMOIRS

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

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

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

THIRTY SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

A COMPARISON OF THE EFFECT OF RAW CORN AND TORTILLAS (LIME-TREATED CORN) WITH NIACIN, TRYPTOPHAN OR BEANS ON THE GROWTH AND MUSCLE NIACIN OF RATS [1,2]

ROBERT L. SQUIBB,[3] J. EDGAR BRAHAM, GUILLERMO ARROYAVE AND NEVIN S. SCRIMSHAW
Institute of Nutrition of Central America and Panama (INCAP), Guatemala, C. A.

(Received for publication August 15, 1958)

Pellagra, although traditionally associated with a high dietary intake of corn (*Zea mays*), is rare in Central America and Mexico where this cereal may contribute as much as 80% of the Calories of the rural diet (Anderson et al., '48; Sogandares et al., '53; Flores and Reh, '55). Evidently in these areas the combined niacin and tryptophan activity of the diets is sufficient to prevent the appearance of clinical pellagra. This may be due in part to the fact that beans (*Phaseolus vulgaris*) make up the second most common ingredient in the diet and are a good source of niacin (Bressani et al., '54). Moreover, it has been shown that rats fed lime-treated corn grow better than those fed raw corn (Cravioto et al., '52; Laguna and Carpenter, '51). Pearson et al. ('57) conclude that the conversion of "bound" niacin to free niacin by this process would appear to explain the superiority of tortillas over raw corn in the diet of the rat.

[1] A portion of the information in this publication has been presented in abstract form in Federation Proc., *14*: 451, 1955.

[2] Supported in part by the Williams Waterman Fund of the Research Corporation. INCAP Publication 1–116.

[3] Present address, Department of Poultry Science, Rutgers University, New Brunswick, New Jersey.

The present rat growth experiments were designed to study the nutritive value of corn and beans in Central American diets. Lime-treated corn in the form of ground tortillas with and without beans was substituted for ground raw corn in a niacin-free, low-tryptophan diet. The effects on growth and skin condition, as well as on the protein and niacin content of blood serum, liver and muscle were measured. Niacin-deficient animals were also irradiated in an effort to induce pellagroid skin lesions of the type which occur in niacin-deficient humans upon exposure to sunlight.

MATERIALS AND METHODS

Weanling rats of the Wistar strain were housed in individual all-wire cages with raised screen bottoms in a room at approximately 24°C. Food and water were provided ad libitum. For each experiment, all rats were depleted for a 21-day period on the following niacin-deficient, low-tryptophan basal diet: vitamin test casein, 7; zein, 14; sucrose, 74.15; L-lysine, 0.55; U.S.P. XII mineral mixture, 4; and cod liver oil, 0.30. The following nutrients were added to supply the specified amounts per 100 gm of diet: choline chloride, 345 mg; thiamine chloride, 0.52 mg; pyridoxine, 0.52 mg; riboflavin, 0.52 mg; folacin, 0.52 mg; calcium pantothenate, 3.45 mg; and biotin, 0.017 mg. This basal ration contained approximately 18.5% of crude protein and 0.16% of tryptophan. Under the conditions of this laboratory, rats fed this basal diet did not gain more than an average of 5 gm during the 21-day depletion period and developed an appreciable alopecia. White corn (*Zea mays*) of known moisture and nitrogen content was ground finely to facilitate its incorporation into the diet. Tortillas were prepared from the same corn by cooking the whole grain in water containing about 0.43% calcium oxide at 90°C. for one hour and then letting it stand in this solution at room temperature overnight. The water was discarded with most of the episperm, but without removing the germ. The material so treated was then ground, prepared and cooked as flat circular cakes (tortillas) for one to two

minutes on a hot plate. The procedure commonly used for the preparation of tortillas and the corresponding nutrient changes have been described in detail (Bressani et al., '58; Bressani and Scrimshaw, '58). The resulting tortillas were dried to about 10% moisture and finally ground. Corn or tortillas, when fed, replaced 48% of the zein and 92% of the sucrose of the basal ration. This rate of substitution gave the various test diets a uniform crude protein content. Black beans (*Phaseolus vulgaris*) were cooked in water, dried, ground, and included in the diet at a 15% level, equivalent in relative quantity to that consumed by some rural populations of Guatemala. In order to equalize the crude protein content of the diets, the beans replaced 27% of the zein and 20% of tortillas of the basal diet. When niacin was added, one mg was given orally every day to each rat. Tryptophan, when required, was added at the rate of 0.24%, which increased the total content of the diet to 0.40%. At the end of a depletion period the rats were stratified by clinical appearance, weight and sex, and then distributed equally among the experimental groups. At the end of the experimental period the rats were sacrificed and the blood sera, livers and leg muscles analyzed for proteins and niacin. Protein in the tissue was determined by the Kjeldahl method and niacin by the micro-biological method described in the U.S. Pharmacopoeia, XIV edition ('50). For these analyses the animals in each group were autopsied and the livers as well as the muscles of the right hind leg pooled to give single samples.

Experiment 1

Supplementation of niacin-deficient, low-tryptophan diets with corn or tortillas with and without niacin

Trial 1. Corn and tortillas were compared as sources of niacin. Forty-eight rats were matched for weight and distributed among 6 experimental groups, each containing 4 males and 4 females. The 6 groups received the basal ration with one of the following supplements: group 1, none; group

2, niacin; group 3, corn; group 4, tortillas; group 5, corn + niacin; and group 6, tortillas + niacin. At the end of 20 days on the basal diet alone it was apparent that the rats would soon die. To test their recuperative ability, the diets of groups 1 and 2 were reversed. The other 4 groups remained on the same diets for 30 additional days, making a total of 50 days on trial for all 6 groups.

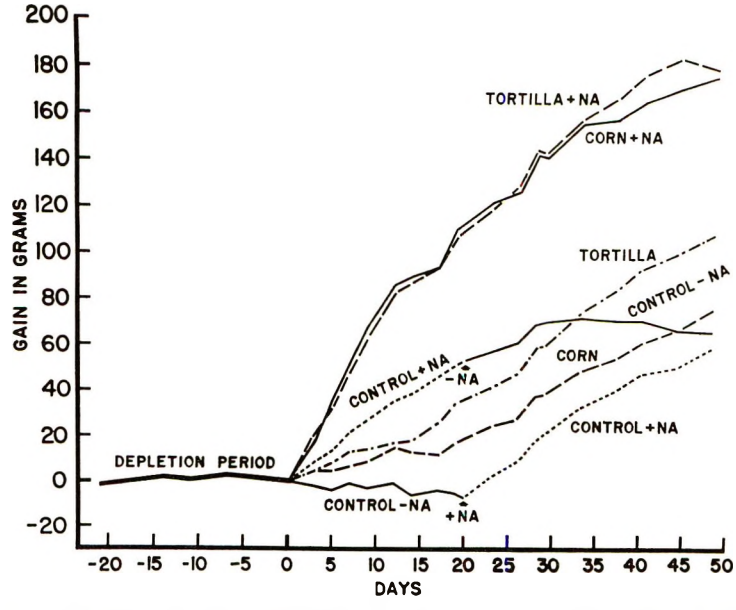

Fig. 1 Weight gain of rats fed diets based on corn or tortilla with and without the addition of niacin. The diets of the groups initially receiving the basal diet alone and the basal + niacin were reversed after 20 days.

The growth of the rats receiving the different treatments is shown in figure 1. Those consuming the basal ration lost weight steadily and showed severe alopecia over their entire bodies; the addition of niacin increased growth without appreciably affecting the alopecia. The alopecia that was present after 20 days on the unsupplemented basal diet disappeared, however, in the groups receiving corn or tortillas. When the diets of groups 1 and 2 were interchanged at the end of 20 days, the growth of the rats showed a cross-over

effect. The increase in weight compared with that of rats on the control diet was highly significant with both the corn and tortilla diets although the latter caused the greater response. Niacin, when added to either the basal diet or the diets containing corn or tortillas, produced a further significant increase in the growth of the rats. In this case niacin supplementation obscured or eliminated the "tortilla effect" on rat growth. The efficiencies of food utilization, calculated at the end of 20 days on trial before the cross-over was initiated, showed that to produce 1 gm gain in weight the rats required 7.77 gm of the diet containing corn, 4.43 gm for tortillas, 3.55 gm for corn + niacin, and 3.21 gm when the diet contained tortillas + niacin. The corresponding absolute gains in weight at the end of 50 days were 77 gm for the group receiving corn, 110 gm for that fed tortillas, and 179 and 182 for the groups receiving corn + niacin and for tortillas with added niacin, respectively.

Trial 2. This was a replicate of trial 1 except that the groups fed the basal ration and the basal ration + niacin were not included. Thirty-two depleted rats were distributed among the 4 experimental groups, each containing 4 males and 4 females. At the end of 50 days on these diets the results were closely similar to those of trial 1.

Experiment 2

Supplementation of raw corn and tortilla diets with niacin, tryptophan and beans

In this trial, 66 depleted rats were distributed among 6 experimental groups, matched for weight, each containing 5 males and 6 females. The substitutions in the basal diet were as follows: group 1, corn; group 2, tortillas; group 3, tortillas + niacin; group 4, tortillas + tryptophan; group 5, tortillas + beans; and group 6, tortillas + beans + tryptophan.

As may be seen in figure 2, tortillas again significantly increased the growth rate of the rats over those fed corn. While the supplementation of the tortilla diet with beans, trypto-

phan and niacin increased the rate of growth of the rats over those fed tortillas, these gains were not significant.

The data of table 1 show no significant differences in serum proteins among rats fed corn or tortillas or when the latter was supplemented with tryptophan or niacin. Nor was there

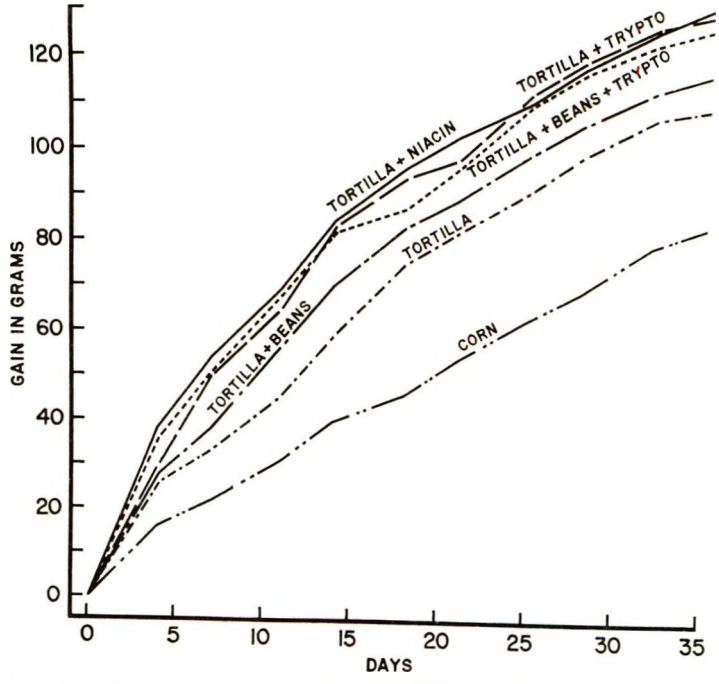

Fig. 2 Weight gains of rats fed corn, tortilla or tortillas supplemented with combinations of beans, niacin and tryptophan.

TABLE 1

Protein and niacin content of rat sera, livers and muscle

GROUP	SERUM PROTEINS	LIVER PROTEINS	MUSCLE PROTEINS	MUSCLE NIACIN
	gm %	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Corn	5.77	19.5	22.3	3.52
Tortilla	5.87	20.6	23.3	4.30
Tortilla + niacin	6.31	19.8	22.4	7.74
Tortilla + tryptophan	6.43	21.1	24.2	8.22
Tortilla + beans	5.91	21.3	23.0	6.30
Tortilla + beans + tryptophan	6.11	22.3	23.9	8.51

any appreciable influence of the various diets on liver or muscle protein. The substitution of tortillas for corn resulted in a slight increase in muscle niacin which became more marked with the addition of beans, niacin or tryptophan to the tortilla diet.

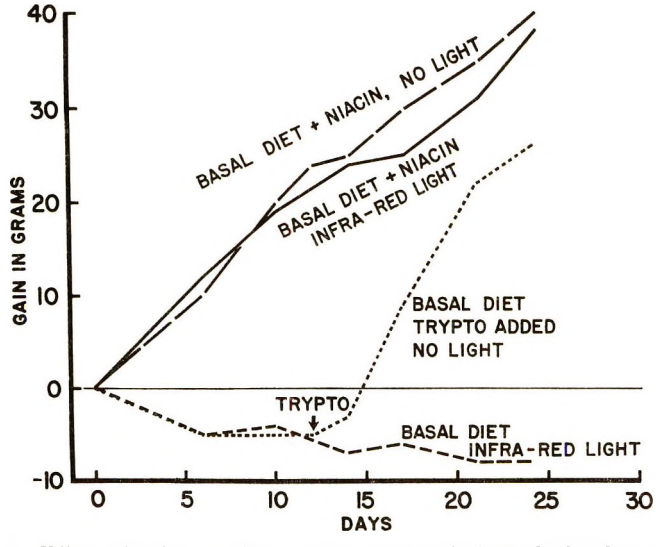

Fig. 3 Effect of infra-red light on the growth of niacin-depleted rats receiving the basal diet with and without added tryptophan or niacin.

Experiment 3

Effect of infra-red light and of supplementation with niacin and tryptophan on niacin-depleted rats

Forty depleted rats with medium-to-acute alopecia were shaved clean over an area 1-inch square on their backs and then distributed among 4 experimental groups of 5 males and 5 females each matched for weight and severity of alopecia. The 4 groups were treated during a 24-day period as follows: group 1, housed under normal rat colony conditions (24°C) and fed the basal diet; group 2, same treatment as group 1 but with supplementary niacin; group 3, same diet as group 1 but subjected to continuous infra-red light which raised the temperature within the cages to 31°C; and group 4

received the same treatment as group 3 but was given supplementary niacin similar to group 2.

There was no effect of light on the rate of growth of rats (fig. 3), although niacin supplementation again significantly increased the growth rate over the corresponding control groups. Tryptophan supplementation of the depleted rats in group 1, at the end of 12 days, significantly increased their growth rate, but neither niacin nor tryptophan supplementation had an apparent effect on the alopecia. Furthermore, no morphological changes were observed in the depleted rats as a result of exposure to light. The rats deficient in niacin, however, showed a tendency to huddle as if they had become light-sensitive. Huddling was not observed in the irradiated group which was supplemented with niacin. Similar results were observed with ultra-violet light.

DISCUSSION

A niacin-deficient, low-tryptophan basal ration containing 18.5% of crude protein has proved useful for studying niacin deficiency in rats. Weanling rats placed on this diet failed to grow and developed a mild-to-acute alopecia within 21 days. The alopecia observed in rats fed the basal ration, while not affected by the addition of niacin, was improved by the addition of raw or lime-treated corn. The alopecia observed may have been related to the content and availability of the amino acids of the diet.

It was apparent from the data of these trials that supplementation of a niacin-deficient, low-tryptophan basal diet with raw corn, tortillas or niacin increased the growth rate and muscle niacin of the rats. The greater growth of the rats fed tortillas over those fed raw corn was apparently due to increase in the availability of niacin, a result of the lime treatment of the corn used in their preparation. This is substantiated by the lack of significant differences in the growth of rats fed raw as compared with lime-treated corn (tortilla) when both diets were supplemented with niacin. Kodicek ('56), Pearson et al. ('57) and more recently McDaniel and

Hundley ('58) have presented evidence that alkaline hydrolysis of corn increases the availability of a "bound niacin." While niacin supplementation of raw corn and tortilla-containing diets apparently eliminated the "tortilla growth effect" observed by Cravioto et al. ('52) and Laguna and Carpenter ('51) and shown also in the present study, the rats fed the basal control diet supplemented with 1 mg of niacin per rat per day did not grow as well as when either raw corn or tortillas were present in the diet. This may have been due to insufficient niacin supplementation but was more likely the result of an imbalance of the amino acids of the basal ration which contained in addition to 7% casein only the zein fraction of the protein of corn.

Under the conditions of these experiments it was not possible to induce skin lesions in the niacin-deficient rats. A continuous source of infra-red or ultra-violet light had no apparent effect on bare skin areas of niacin-depleted or supplemented rats. The tendency of the depleted rats to huddle under light treatment, however, was lessened by niacin supplementation. This indicates that even though no morphological changes in the skin areas were apparent, a niacin deficiency does increase light sensitivity in rats.

The lime treatment of corn for the preparation of tortillas apparently increases the availability of niacin. However, the relatively large effect of niacin supplementation on the growth of the rats fed either raw corn or tortillas, would indicate that the quantity of niacin released by lime treatment is small. While lime treatment of corn may still be a minor contributing factor to the absence of pellagra among Central American and Mexican rural populations consuming high corn diets, the rat studies reported here suggest that the relatively high consumption of beans is a factor of greater significance. The higher levels of niacin observed in the muscle tissue of the bean-supplemented group support this conclusion. It has also become evident that coffee may play a role as a source of dietary niacin and its contribution in Central America has recently been demonstrated by Bressani and Navarrete ('59).

SUMMARY

A niacin-deficient, low-tryptophan basal ration containing 18.5% of crude protein has proved useful for studying niacin deficiencies in rats. Weanling rats of this laboratory placed on such a diet failed to grow and developed mild-to-acute alopecia within 21 days. Daily oral administration of 1 mg of niacin per rat or increasing the tryptophan content of the basal ration to 0.40% restored the growth of the rats but had no influence on the alopecia. When either raw or lime-treated corn was substituted for the zein and sucrose of the basal ration the rate of growth was significantly improved and the growth of hair returned.

The results confirm the increased growth rate of rats fed lime-treated corn (tortillas) compared with those fed raw whole ground corn. Beans fed at a level calculated to be equivalent to that consumed by part of the rural population of Guatemala produced a slight further improvement in growth and significantly increased the muscle niacin of depleted rats. Continuous infra-red or ultra-violet light had no apparent effect on the skin of shaved areas of niacin-depleted or supplemented rats. The addition of niacin, however, lessened the sensitivity of the depleted rats to the light as manifested by a tendency to huddle.

LITERATURE CITED

ANDERSON, R. K., J. CALVO, W. D. ROBINSON, G. SERRANO AND G. C. PAYNE 1948 Nutrition appraisals in Mexico. Am. J. Public Health, *38:* 1126.

BRESSANI, R., E. MARCUCCI, C. E. ROBLES AND N. S. SCRIMSHAW 1954 Nutritive value of Central American beans. I. Variation in the nitrogen, tryptophan, and niacin content of ten Guatemalan black beans (*Phaseolus vulgaris*, L.) and the retention of the niacin after cooking. Food Research, *19:* 263.

BRESSANI, R., AND D. A. NAVARRETE 1959 Niacin content of coffee in Central America. Ibid., in press.

BRESSANI, R., AND N. S. SCRIMSHAW 1958 Effect of lime treatment on the *in vitro* availability of essential amino acids and solubility of protein fractions in corn. J. Agr. Food Chem., *6:* 774.

BRESSANI, R., R. PAZ Y PAZ AND N. S. SCRIMSHAW 1958 Chemical changes in corn during preparation of tortillas. Ibid., *6:* 770.

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.

FLORES, M., AND E. REH 1955 Estudios de hábitos dietéticos en poblaciones de Guatemala. I. Magdalena Milpas Altas. Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 90.

KODICEK, E. 1956 The effect of alkaline hydrolysis of maize on the availability of its nicotinic acid to the pig. British J. Nutrition, *10:* 51.

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

MCDANIEL, E. G., AND J. M. HUNDLEY 1958 Alkali-treated corn and niacin deficiency. Federation Proc., *17:* 484.

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.

PHARMACOPOEIA OF THE UNITED STATES 1950 XIV Edition.

SOGANDARES, L., A. P. DE GALINDO AND H. P. MEJÍA 1953 Estudios Dietéticos de grupos urbanos y rurales de la República de El Salvador. Suplemento No. 1 del Boletín de la Oficina Sanitaria Panamericana "Publicaciones Científicas del Instituto de Nutricíon de Centro América y Panamá," p. 27.

THE EFFECT OF DL-ETHIONINE ON SKELETAL GROWTH IN RATS[1]

JANIS V. KLAVINS, THOMAS D. KINNEY AND NATHAN KAUFMAN

Department of Pathology, Western Reserve University School of Medicine at Cleveland Metropolitan General Hospital, Cleveland, Ohio

(Received for publication August 14, 1958)

In the course of a series of experiments having to do with the administration of ethionine under different conditions, it was observed that certain definite changes took place in the bones of the experimental animals (Kinney et al., '55, a, b; Klavins et al., '55; Kaufman et al., '56). In order to determine if the changes observed were valid, the present experiments were designed to determine the effects of DL-ethionine on skeletal growth. As far as can be determined, this represents the first description of the bone changes in animals fed this antimetabolite of methionine.

MATERIALS AND METHODS

Part I. This section of the experiment was designed to compare the changes in the bones of animals fed ethionine with the bones of animals in which the weights were reduced comparatively by means of a restricted basal diet. Young albino male rats of the Sprague-Dawley strain, weighing approximately 220 gm each were used. They were divided into three groups and housed 4 to a cage with the exception of the animals in group III, which were housed individually. Eight rats in group I were fed ad libitum a basic diet of the following composition: casein 18 gm, glucose 67 gm, corn oil 11 gm

[1] This work was supported by United States Public Health Service Grant no. A–1388.

(containing 0.001 ml of Haliver oil [2]) and salt mixture 4 gm (Hegsted et al., '41). Crystalline vitamins were added in the following amounts per 100 gm of diet: thiamine chloride 400 µg, riboflavin 800 µg, pyridoxine hydrochloride 400 µg, calcium pantothenate 1.5 mg and nicotinic acid 2.5 mg. The 16 animals composing group II received the same basic diet as the group I rats but supplemented with 0.5% of DL-ethionine. The 15 animals making up group III were fed the basic diet in limited amounts to match the weight loss in group II.

All the animals had free access to fresh water. Nineteen animals (4 from group I, 8 from group II and 7 from group III) were killed after one month and the remaining rats were killed one month later. The proximal end of a tibia from each animal was fixed in 10% formalin, decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Part II. This section of the experiment was set up so that a comparison could be made between animals fed ethionine and animals on an isocaloric normal diet. The rats were divided into two groups and housed individually. Twelve animals comprising group IV were fed ad libitum the basal diet supplemented with 0.5% of ethionine. (This diet was identical to that fed to group II, in part I of this experiment.) The 12 animals in group V were pair-fed controls for the animals in group IV and received an isocaloric normal diet identical in composition to that fed groups I and III. Twelve animals (6 from each group) were killed after one month and the remaining rats were killed one month later. The thorax was opened under ether anesthesia and with the heart still beating, the ascending aorta was cannulated via the left ventricle. The vascular system was perfused with approximately 50 ml of 10% buffered formalin. This procedure produced rapid fixation of tissues and as a result the tibias of the animals in this part of the experiment showed much better preservation of cytologic detail and less shrinkage of cartilage.

[2] Parke-Davis Haliver oil containing no more than 60,000 U.S.P. units of vitamin A and no more than 1,000 U.S.P. units of vitamin D per gram.

RESULTS

Part I. At the end of one month the tibias of the rats in group I, fed the basic diet ad libitum, showed normal growth. The epiphyseal plates were thick and the bony trabeculae were long and delicate (fig. 1). In contrast there was reduction of bone growth in all the group II animals fed ethionine. There was a decrease in thickness of the epiphyseal plate of cartilage. There was no change in the zone of resting cartilage but the zones of proliferating, maturing and calcifying cartilage were markedly narrowed. There was some irregularity in the arrangement of cells in the vertical column in the zones of proliferating and maturing cartilage. The most prominent changes were noted in the diaphyseal side of the epiphyseal plate where the bony trabeculae were blunted, shortened and occasionally absent (fig. 2). The cartilaginous cores of the trabeculae were thickened and the continuity with the cartilaginous intercellular substance of the epiphyseal plate was well preserved.

Changes were also present in the bones of the group III animals fed the basic diet in limited amounts. Here the changes were similar to, but less marked than, those in the animals fed ethionine. The epiphyseal plates were slightly thicker than in the ethionine-fed rats and the usual regular vertical columnar arrangement of cells was present in the zones of proliferating and maturing cartilage. Also, the bony trabeculae were somewhat longer and more delicate (fig. 3) and the cartilaginous cores were narrower. Further, the tips of the tunnels where the osteogenic cells and capillaries invaded the areas of disintegrating chondrocytes and cartilaginous matrix were wider than in the animals fed ethionine.

After two months on their respective diets, the differences between the appearance of the bones of the three groups of rats were even more marked (figs. 4, 5 and 6). This was particularly true for the bones of the rats in groups II and III. The epiphyseal plates were thinner, the bony trabeculae

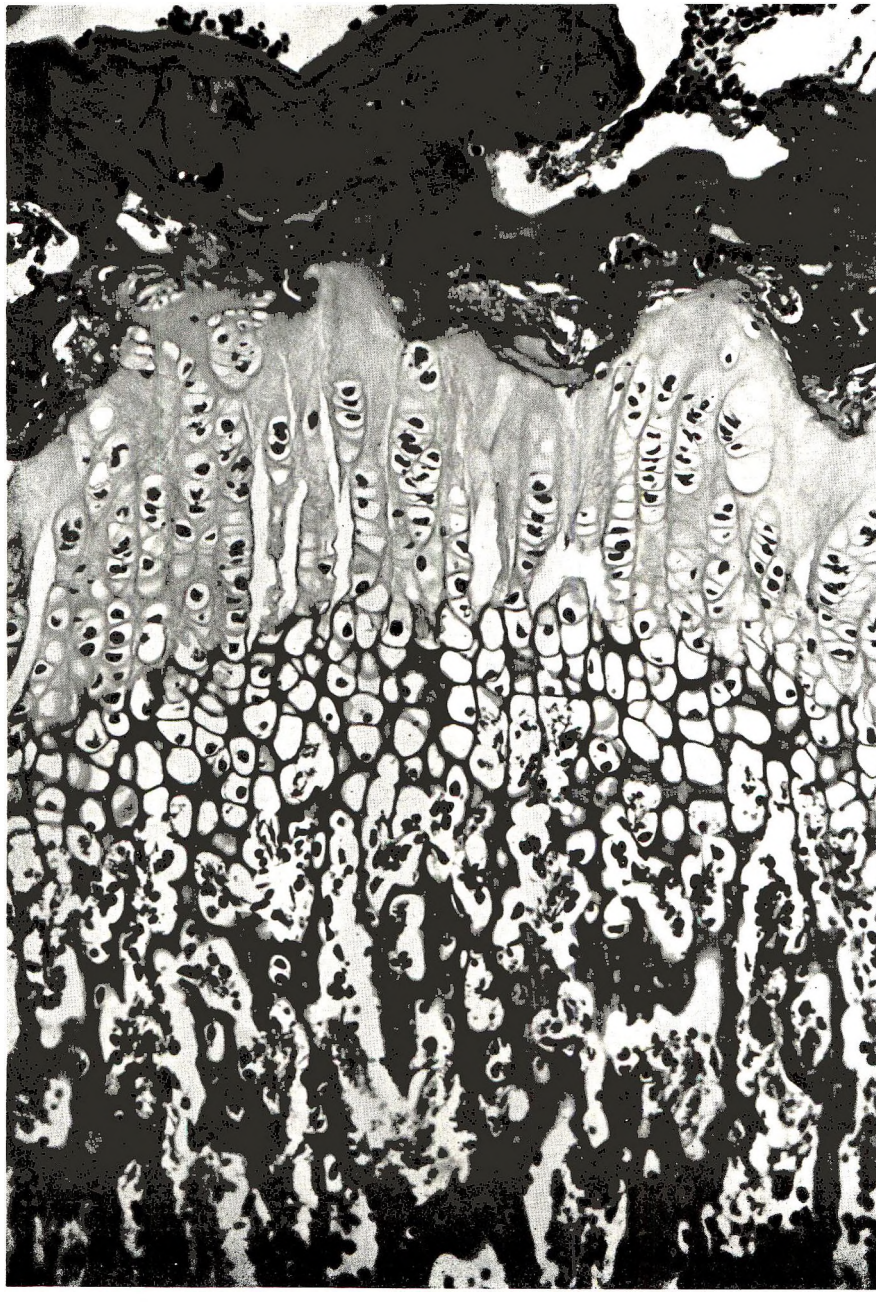

Fig. 1 Tibial epiphysis of rat fed the basal diet ad libitum for one month (group I). Hematoxylin and eosin. × 180.

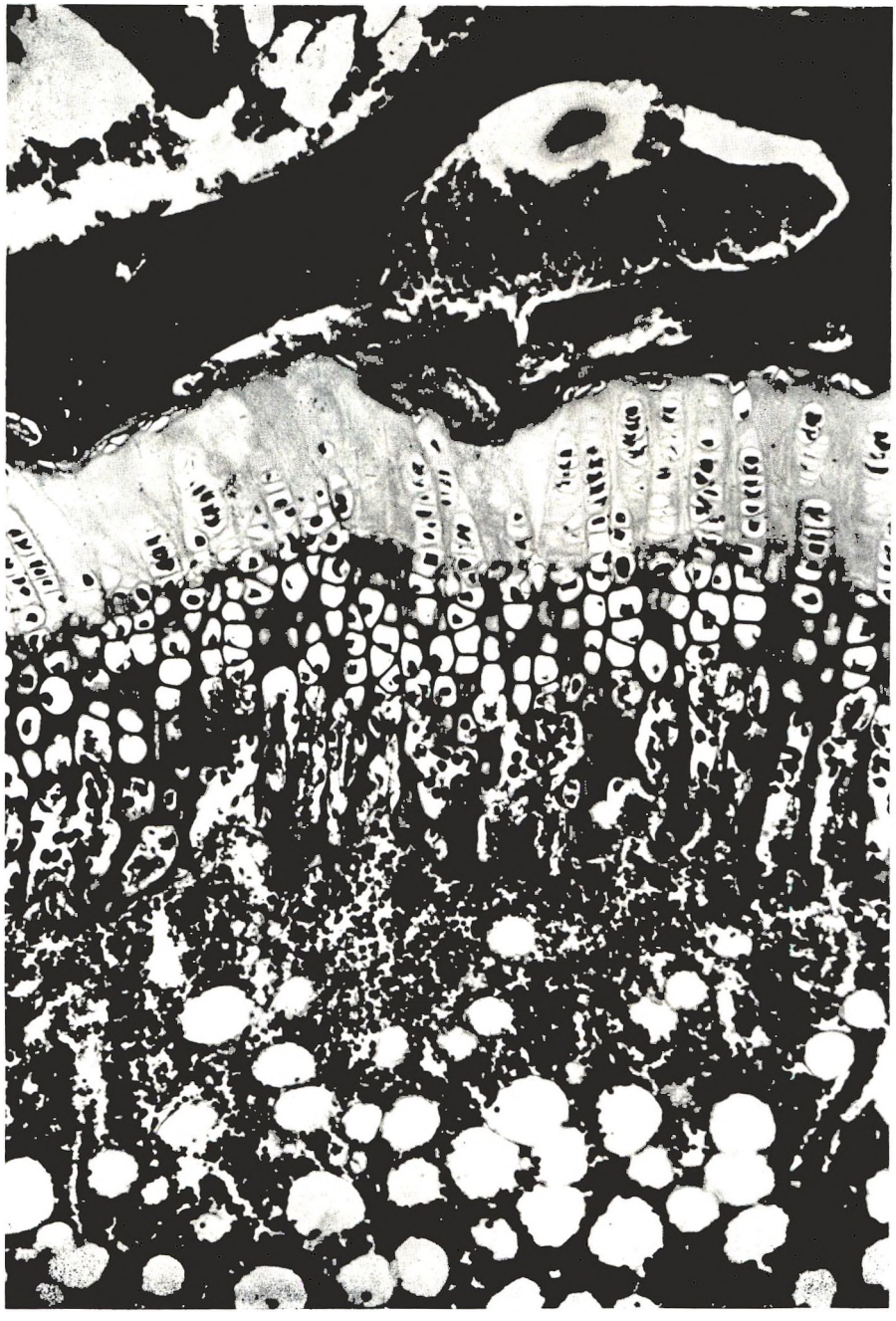

Fig. 2 Tibial epiphysis of rat fed ethionine-containing diet for one month (group II). The bony trabeculae are more blunted and shorter than in weight control animal (compare with fig. 3). Hematoxylin and eosin. × 180.

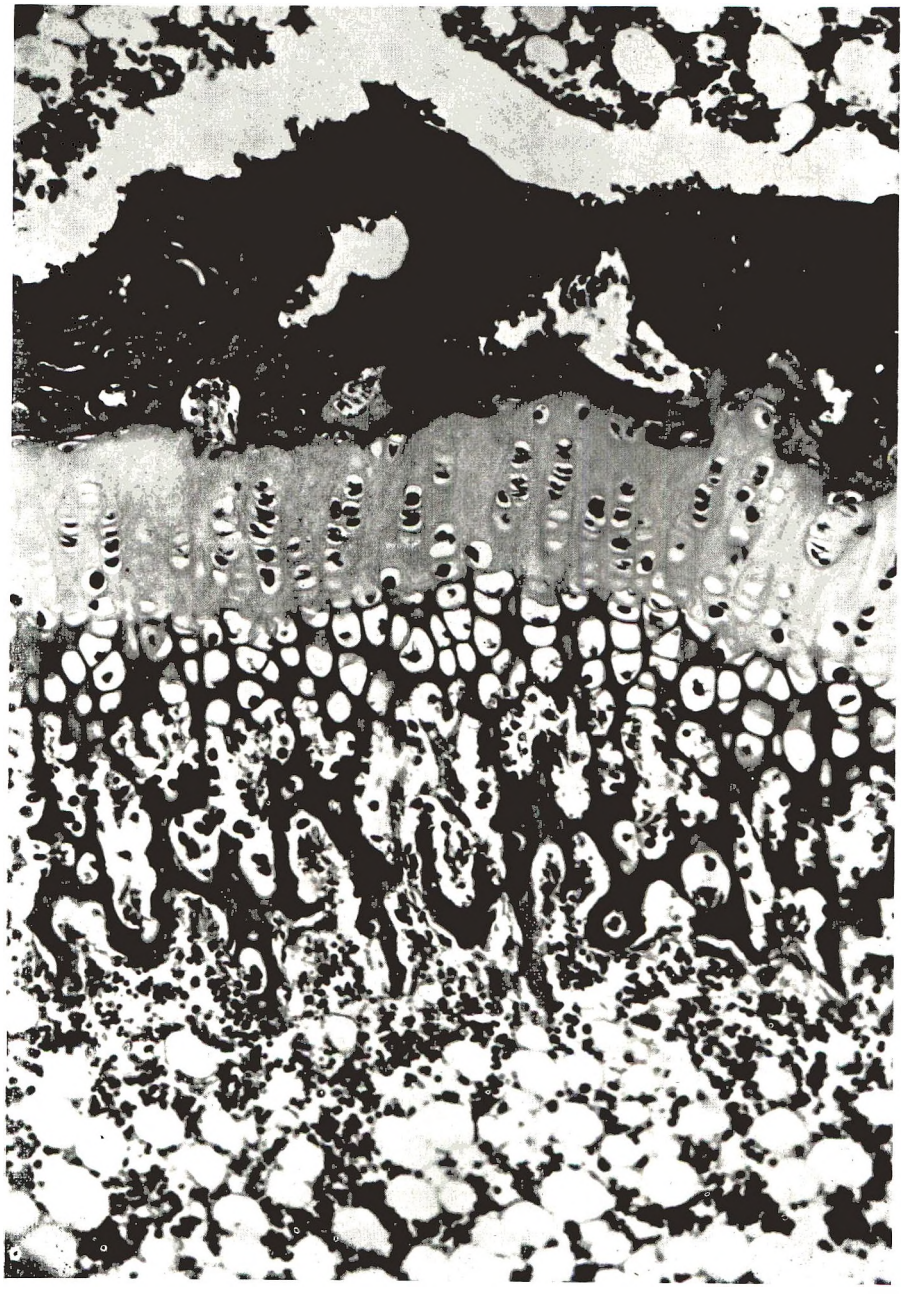

Fig. 3 Tibial epiphysis of weight control rat fed the basal diet in limited amounts for one month (group III). Hematoxylin and eosin. × 180.

were more blunted and the cartilaginous cores were thicker in the group II animals fed ethionine than in the group III animals on the weight control diets.

Part II. After one month the changes in the rats of group IV were similar to those described for the animals of group II, as was to be expected, since both groups were fed identical diets for the same period of time. When the tibias of group IV were compared with those of the isocaloric controls, group V, the differences were more striking than those found when the group II animals were compared with the weight controlled rats of group III. In particular, the bony trabeculae of the tibias of the rats in group IV were considerably shorter, more blunted and fewer in number (fig. 7) than in the bones of the group V rats (fig. 8).

At the end of two months the epiphyseal plates were thinner and the bony trabeculae were shorter in both the experimental and pair-fed controls (figs. 9 and 10). When the two groups were compared, the cartilaginous plates of the ethionine-fed animals were thinner. Also, the trabeculae were short and blunt, whereas in the pair-fed controls, the trabeculae, although shorter than at one month, retained some of their delicate characteristics.

In addition to the retardation in skeletal growth in the animals of groups II and IV fed ethionine, there was less weight gain, greater loss of subcutaneous fat and wasting of the skeletal muscles. For example, following an initial weight loss during the first week the group IV rats gained weight slowly and were far behind the weight gain of pair-fed controls at the end of the experiment (fig. 11). The animals consumed an average of 13 gm of food during the first 24 hours. Thereafter, the food consumption dropped to 3.6, 2.8, 7.2 and 7.8 gm on the second, third, 4th and 5th days of ethionine administration respectively. After the 6th day the food consumption varied from 12 to 13 gm per rat per day.

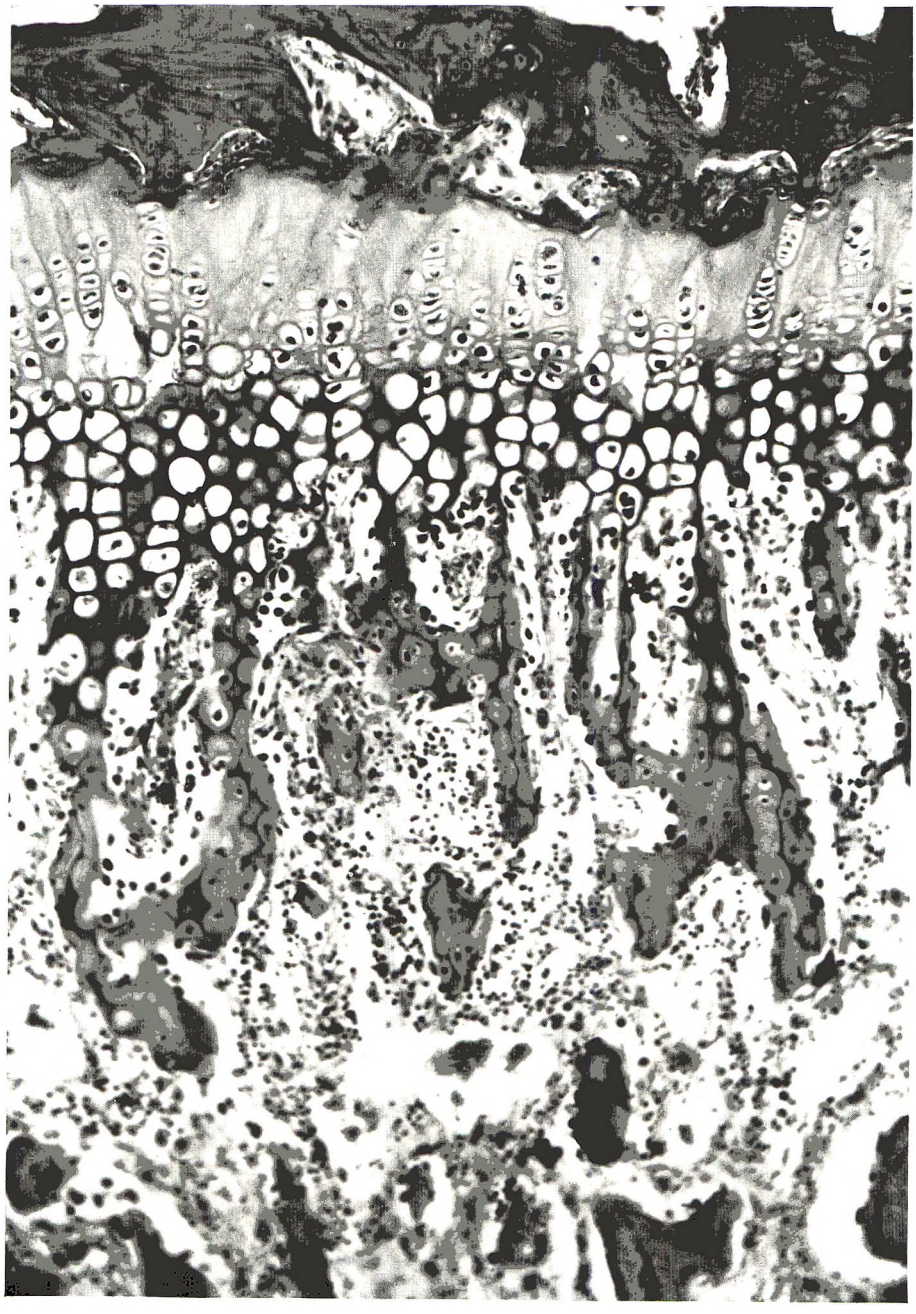

Fig. 4 Tibial epiphysis of rat fed the basal diet ad libitum for two months (group I). Hematoxylin and eosin. × 180.

Fig. 5 Tibial epiphysis of rat fed ethionine-containing diet for two months (group II). The epiphyseal plate is thinner, the bony trabeculae shorter, more blunted, and the cartilaginous cores thicker than the weight control (compare with fig. 6). Hematoxylin and eosin. × 180.

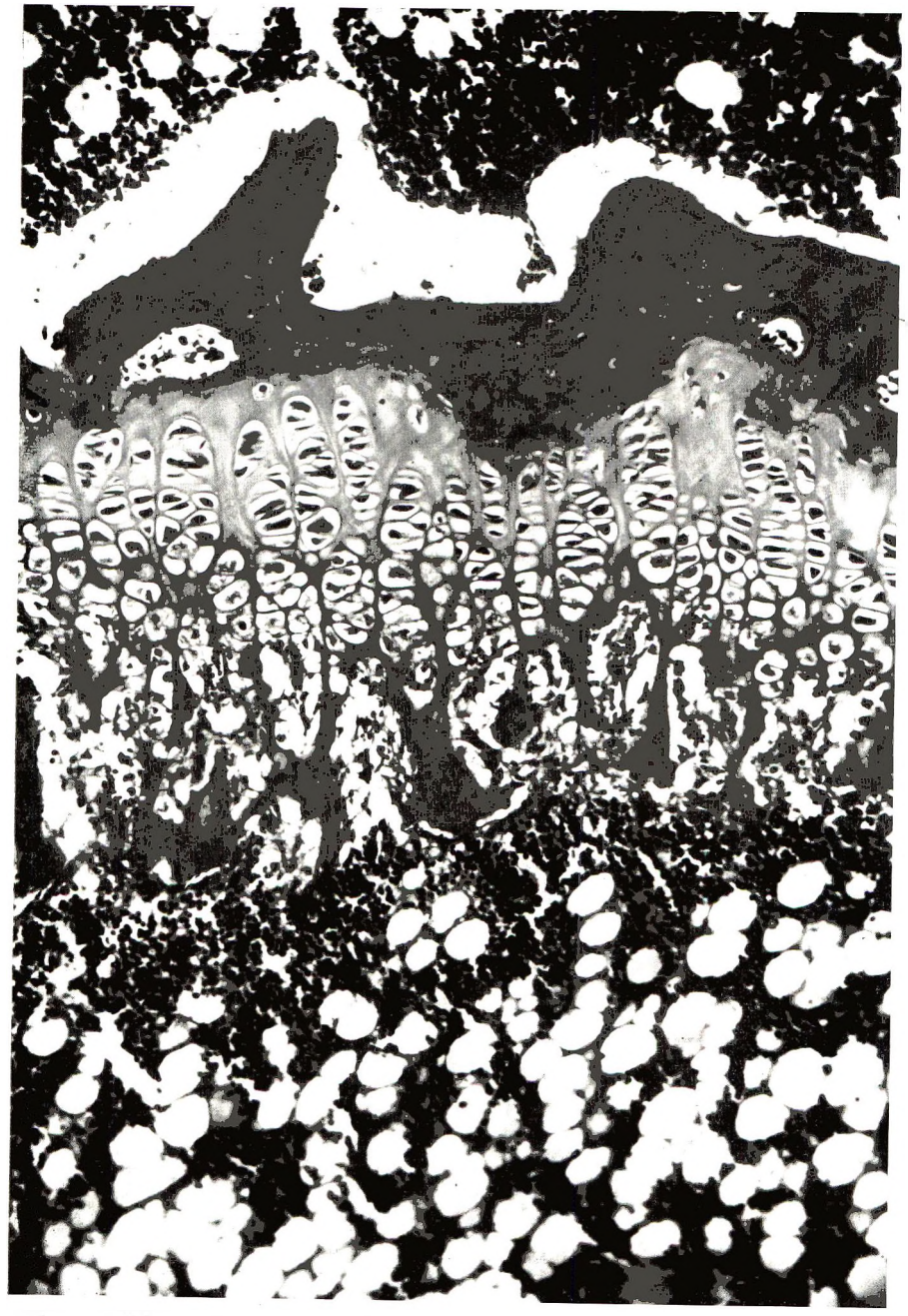

Fig. 6 Tibial epiphysis of weight control rat fed the basal diet in limited amounts for two months (group III). Hematoxylin and eosin. × 180.

Fig. 7 Section through tibial epiphysis after one month of ethionine administration (group IV). The bony trabeculae are shorter and fewer in number than in the pair fed control (compare with fig. 8). Hematoxylin and eosin. × 180.

Fig. 8 Section through tibial epiphysis of a pair-fed control rat after one month. Hematoxylin and eosin. × 180.

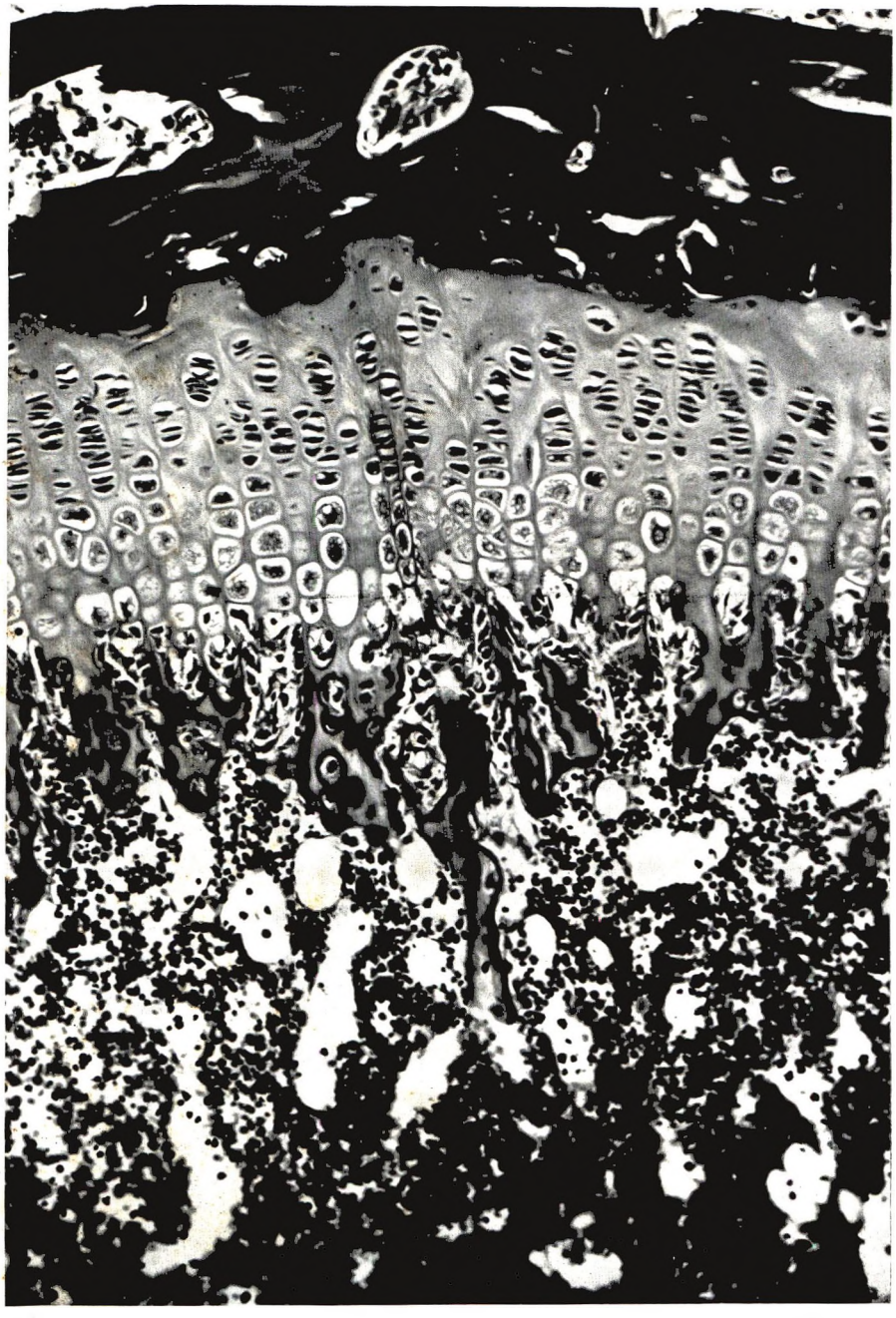

Fig. 9 Section through tibial epiphysis after two months of ethionine administration (group IV). The epiphyseal plate is thinner and the bony trabeculae are more blunted and shorter than in pair fed control (compare with fig. 10). Hematoxylin and eosin. × 180.

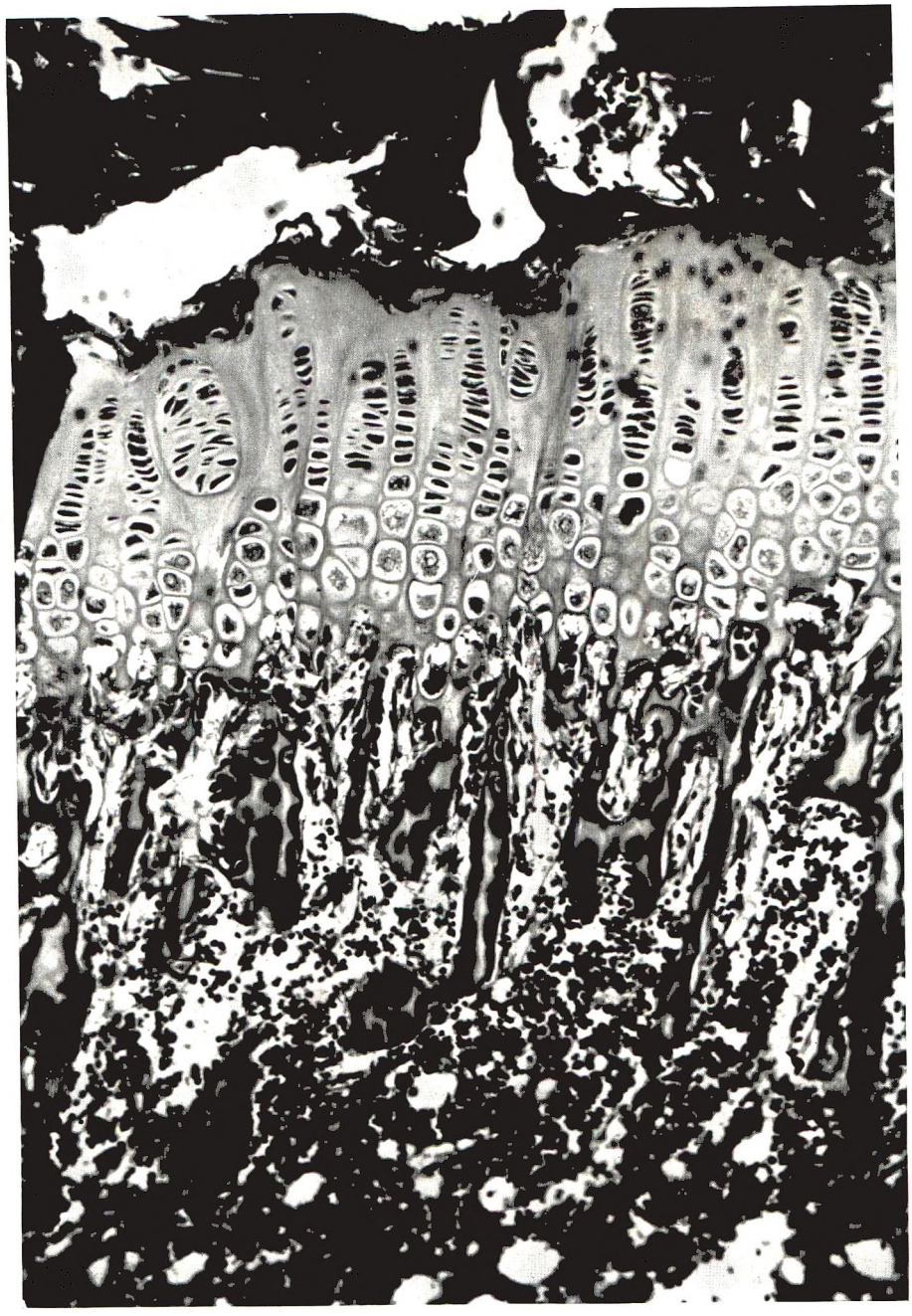

Fig. 10 Section through tibial epiphysis of a pair fed control rat after two months. Hematoxylin and eosin. × 180.

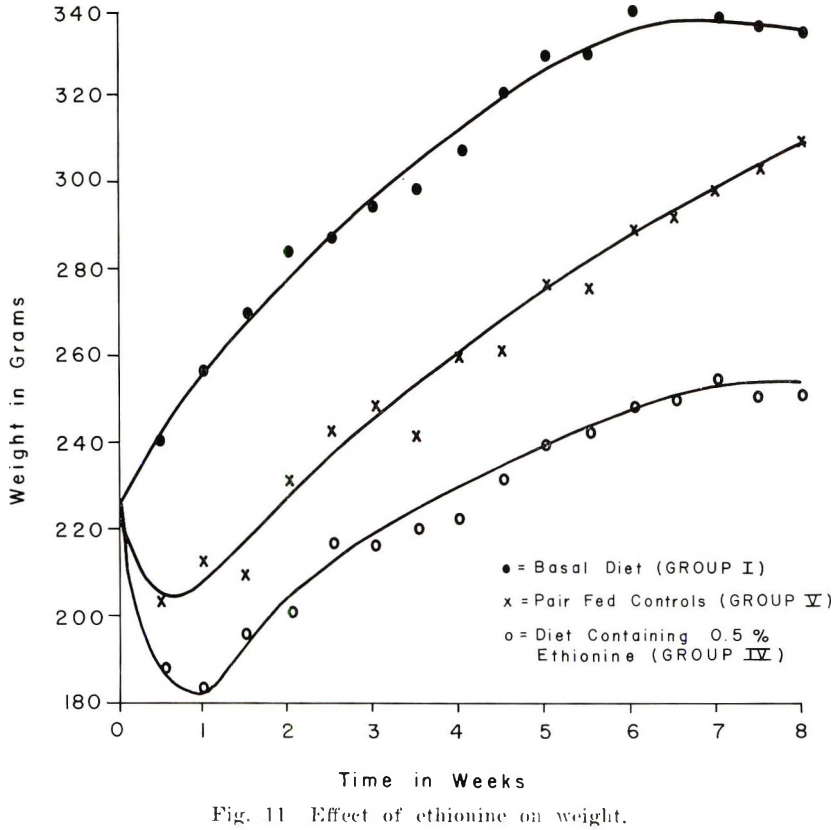

Fig. 11 Effect of ethionine on weight.

DISCUSSION

In these experiments it was evident that ethionine causes retardation of skeletal growth. Decrease in thickness of the epiphyseal cartilage of the tibia, persistent thickness of the cartilaginous cores of the bony trabeculae with shortening, blunting and decreased number of the bony trabeculae indicate partial failure of endochondral ossification and retardation of skeletal growth. These changes were similar to those seen in lysine (Harris et al., '43; Haggar et al., '55), phenylalanine (Maun et al., '45a; Schwartz et al., '51); threonine (Scott and Schwartz, '53); histidine (Scott, '54; Maun et al., '46); leu-

cine (Maun et al., '45b) and tryptophan (Scott, '55) deficiencies as well as protein-deficient diets (Follis, '56). It appears that there is little or no change in growing bone which can be attributed to lack of a single specific amino acid. Rather, the response on the part of the bone appears to be the same for all the amino acids on which studies have been reported.

Methionine is necessary for proper protein synthesis. When its antagonist, ethionine, is administered, there is interference with the incorporation of methionine into the protein (Simpson et al., '50), interference with lipid metabolism (Farber et al., '50), and inhibition of growth (Stekol and Weiss, '49, '51). However, when methionine is administered in large enough amounts the inhibition of growth is alleviated (Stekol and Weiss, '49, '51) and protein synthesis and lipid metabolism proceeds as before (Simpson et al., '50; Farber et al., '50). It is presumed that retardation of skeletal growth in these experiments is a result of the inhibition of methionine and therefore, interference with normal protein metabolism.

The weight changes during ethionine administration were similar to those previously reported (Kinney et al., '55b). Since during the first 24 hours the animals consumed a considerable amount of ethionine and thereafter the food intake was markedly decreased, it appears that the initial decrease of weight was attributed to a combination of decreased intake of food and the effect of ethionine. Pair-fed animals on the basal diet also lost weight, but not to the same extent as the rats fed ethionine (fig. 11).

SUMMARY

Retardation of skeletal growth was observed in rats when the diet contained 0.5% of ethionine. The animals were maintained on this diet for periods of one to two months. The changes in the tibial epiphyses consisted of decrease in thickness of the cartilage and blunting, shortening and decrease in number of bony trabeculae.

LITERATURE CITED

FARBER, E., M. V. SIMPSON AND H. TARVER 1950 Studies on ethionine. II. The interference with lipid metabolism. J. Biol. Chem., *182:* 91.

FOLLIS, R. H., JR. 1956 Some observations on experimental bone disease. Ciba Foundation Symposium on Bone Structure and Metabolism, p. 249.

HAGGAR, R., T. D. KINNEY AND N. KAUFMAN 1955 Bone healing in lysine deficient rats. J. Nutrition, *57:* 305.

HARRIS, H. A., A. NEUBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Biochem. J., *37:* 508.

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

KAUFMAN, N., J. V. KLAVINS AND T. D. KINNEY 1956 Testicular damage following ethionine administration. Am. J. Path., *32:* 105.

KINNEY, T. D., N. KAUFMAN AND J. V. KLAVINS 1955a Effect of ethionine-induced pancreatic damage on iron absorption. J. Exp. Med., *102:* 151.

———— 1955b Regeneration of pancreatic acini during ethionine administration. Arch. Path., *60:* 639.

KLAVINS, J. V., T. D. KINNEY AND N. KAUFMAN 1955 Hepatic nodules resembling tumors in rats after administration and withdrawal of ethionine. Proc. Soc. Exp. Biol. Med., *89:* 540.

MAUN, M. E., W. M. CAHILL AND R. M. DAVIS 1945a Morphologic studies of rats deprived of essential amino acids. I. Phenylalanine. Arch. Path., *39:* 294.

———— 1945b Morphologic studies in rats deprived of essential amino acids. II. Leucine. Ibid., *40:* 173.

———— 1946 Morphologic studies of rats deprived of essential amino acids. III. Histidine. Ibid., *41:* 25.

SCHWARTZ, C., E. B. SCOTT AND R. L. FERGUSON 1951 Histopathology of amino acid deficiencies. I. Phenylalanine. Anat. Rec., *110:* 313.

SCOTT, E. B. 1954 Histopathology of amino acid deficiencies. III. Histidine. Arch. Path., *58:* 129.

———— 1955 Histopathology of amino acid deficiencies. IV. Tryptophan. Am. J. Path., *31:* 1111.

SCOTT, E. B., AND C. SCHWARTZ 1953 Histopathology of amino acid deficiencies. II. Threonine. Proc. Soc. Exp. Biol. Med., *84:* 271.

SIMPSON, M. V., E. FARBER AND H. TARVER 1950 Studies on ethionine. I. Inhibition of protein synthesis in intact animals. J. Biol. Chem., *182:* 81.

STEKOL, J. A., AND K. WEISS 1949 A study in growth inhibition by D-, L-, and DL-ethionine in the rat and its alleviation by the sulfur-containing amino acids and choline. Ibid., *179:* 1049.

STEKOL, J. A., AND K. W. WEISS 1951 Inhibition of growth of C3H mice by ethionine and its alleviation by methionine or choline. Proc. Soc. Exp. Biol. Med., *77:* 213.

BENEFICIAL EFFECTS OF THE PLANT RESIDUE FACTOR ON THE SURVIVAL OF THYROTOXIC RATS [1]

B. H. ERSHOFF, H. J. HERNANDEZ AND JOAN M. MUCKENTHALER
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles, and Nutrilite Products, Inc., Buena Park, California

(Received for publication August 8, 1958)

Available data indicate that, in addition to the known nutrients, substances are present in natural foodstuffs which are required in increased amounts during various conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small that they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other stressor agents may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. Alfalfa meal is a potent source of at least one such factor. Thus alfalfa meal, or fractions therof, has been shown to (1) increase significantly the average survival time of immature rats fed toxic doses of iodinated casein (Tappan et al., '53), (2) counteract growth retardation (and other manifestations of toxicity) in immature mice (Ershoff, '54) and rats (Ershoff, '57a) fed massive doses of glucoascorbic acid, (3) partially counteract the inhibition of ovarian development of immature rats fed massive doses of alpha-estradiol (Ershoff et al., '56)

[1] Communication no. 460 from the Department of Biochemistry and Nutrition, University of Southern California.

and (4) counteract symptoms of mineral oil toxicity in rats and mice fed a low-fat ration (Ershoff and Hernandez, '58). The protective factor (or factors) in each of the experiments indicated above appeared to be distinct from any of the known nutrients. In the present communication further data are presented on the effects of alfalfa and fractions thereof on symptoms of thyrotoxicity in the immature rat.

TABLE 1

Composition of experimental rations

DIETARY COMPONENTS [1]	DIET A	DIET B
L-Cystine	0.2	
DL-Methionine		0.75
Salt mixture [2]	5.0	5.0
Corn oil	5.0	5.0
Casein [3]	24.0	
Soy protein [4]		24.0
Sucrose	65.8	65.25

[1] To each kilogram of the above rations were added the following vitamins: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-napthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose.

[2] Hubbell, Mendel and Wakeman Salt Mixture, General Bochemicals, Inc., Chagrin Falls, Ohio.

[3] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Drackett Assay Protein C-1, The Drackett Products Co., Cincinnati, Ohio.

PROCEDURE

A series of experiments was designed to study the effects of alfalfa and fractions thereof on the growth and length of survival of thyrotoxic rats. Two basal rations were employed: diet A and diet B. Diet A was a purified ration containing casein supplemented with cystine as the source of dietary protein; diet B was similar in composition but contained soy protein supplemented with methionine as the source of protein (table 1). Desiccated thyroid [2] or iodinated casein [3] was

[2] Thyroid Powder, U.S.P. Armour and Co., Chicago, Ill.

[3] Protamone, Cerophyl Laboratories, Kansas City, Missouri.

incorporated in each of these diets, replacing an equal amount of sucrose. The alfalfa meal and other supplements tested were incorporated in the basal diets in place of an equal amount of sucrose. The rats were housed in metal cages with raised screen bottoms (two or three animals per cage). Diets were made up bi-weekly and stored under refrigeration when not in use. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Studies were conducted with both male and female rats. All experiments were conducted with animals of the Long-Evans strain except experiment 4 in which rats of the Holtzman strain were employed. In each of the experiments rats were selected at 21 to 24 days of age and at a body weight between 38 and 50 gm and were fed ad libitum the various supplements indicated in tables 2 to 6. Feeding was continued for 100 days or until death whichever occurred sooner except for experiment 1 (a) which was of 35 days duration.

Experiment 1

Comparative effects of rations containing casein or soy protein as the source of dietary protein on the survival of thyrotoxic rats

a. In agreement with earlier findings (Ershoff and Hershberg, '45; Ershoff, '47; Ershoff and McWilliams, '48) rats fed purified rations containing casein as the dietary protein and sucrose as the dietary carbohydrate were highly susceptible to the ingestion of desiccated thyroid or iodinated casein as evidenced by a high and early mortality rate apparently due to cardiac failure. In contrast to the results obtained with the casein-containing ration (diet A), rats fed comparable amounts of thyroid or iodinated casein in conjunction with the soy protein-containing ration (diet B) exhibited a significant increase in percentage survival. Growth was retarded with both the casein and soy protein-containing rations when the

TABLE 2

Comparative effects of rations containing casein or soy protein as the source of dietary protein on the survival time of thyrotoxic rats [1]

DIETARY GROUP	THYROACTIVE SUBSTANCE IN DIET	MALES		FEMALES	
		Per cent survival	Average survival time [2,3]	Per cent survival	Average survival time [2,3]
			days		*days*
		Experiment 1a			
A	None	100	35	100	35
A	0.5% thyroid	0	19	33	24
A	0.5% iodinated casein	0	20	17	18
B	None	100	35	100	35
B	0.5% thyroid	80	29	60	25
B	0.5% iodinated casein	80	33	80	31
		Experiment 1b			
A	None	100	100	100	100 ± 0.0
A	0.5% thyroid	0	18 ± 1.5	11	30 ± 5.2
½A + ½B	0.5% thyroid	0	23 ± 1.95	22	52 ± 11.0
B	0.5% thyroid	0	41 ± 3.96	67	78 ± 10.6

[1] Six male and 6 female rats per group were employed in experiment 1a; 9 male and 9 female rats per group in experiment 1b.

[2] Experiment 1a was of 35 days duration; experiment 1b, 100 days duration. Average survival time was computed on the basis of a 35- and 100-day survival respectively for animals alive at the termination of experiments 1a and 1b.

[3] Including standard error of the mean calculated as follows: $\sqrt{(\Sigma d^2)}/n/\sqrt{n}$ Where "d" is the deviation from the mean and "n" is the number of observations.

diets contained thyroid or iodinated casein. The soy protein-containing ration, although effective in prolonging survival of thyrotoxic rats, was without effect in counteracting the attendant retardation in growth. Similar results were obtained with male and female rats.

b. The increased survival of thyrotoxic rats fed the soy protein-containing ration (diet B) over that of those fed the casein-containing ration (diet A) was confirmed. The average survival time of thyrotoxic rats fed a diet in which half the protein was provided in the form of casein and cystine and half as soy protein and methionine was intermediate between the two groups. The results are summarized in table 2.

Experiment 2

Comparative effects of alfalfa meal, alfalfa juice and alfalfa residue on the survival time of thyrotoxic rats fed purified rations containing casein or soy protein as the source of dietary protein

Tests were conducted to determine the comparative effects of alfalfa meal, alfalfa juice and alfalfa residue (the water-washed alfalfa pulp remaining after the extraction of the juice) on the survival time of thyrotoxic rats fed purified rations containing either casein or soy protein as the source of dietary protein. Results are summarized in table 3. In agreement with the findings of Tappan et al. ('53), a supplement of 20% alfalfa meal significantly increased the average survival time of thyrotoxic rats fed a purified casein-containing ration. The alfalfa meal supplement also increased the average survival time of thyrotoxic rats fed the diet containing soy protein. A significant difference was observed, however, between the casein and soy protein-containing rations in the effects obtained with alfalfa fractions. Whereas alfalfa residue increased the survival of thyrotoxic rats fed both the casein and soy protein-containing diets, dried alfalfa juice was without significant effect on the survival time of thyrotoxic rats

fed the diet containing casein whereas it increased the survival of such rats on the ration containing soy protein. With neither the casein nor soy protein-containing ration, however, did alfalfa meal or any of its fractions counteract the growth retardation which occurred in all rats fed the diets containing thyroid or iodinated casein.

TABLE 3

Comparative effects of alfalfa meal, alfalfa juice and alfalfa residue on the survival time of thyrotoxic rats fed purified rations containing either casein or soy protein as the source of dietary protein [1]

(10 male rats per group)

DIETARY GROUP	THYROACTIVE SUBSTANCE IN DIET	PER CENT SURVIVAL	AVERAGE SURVIVAL TIME [2,3]
A	None	100	100 ± 0
A	0.5% thyroid	10	39 ± 6.9
A + 20% alfalfa meal	0.5% thyroid	70	88 ± 5.7
A + 5% dried alfalfa juice	0.5% thyroid	0	37 ± 3.2
A + 15% alfalfa residue	0.5% thyroid	30	66 ± 8.6
B	None	100	100 ± 0
B	0.5% iodinated casein	10	52 ± 5.2
B + 20% alfalfa meal	0.5% iodinated casein	60	77 ± 8.6
B + 5% dried alfalfa juice	0.5% iodinated casein	50	81 ± 6.4
B + 15% alfalfa residue	0.5% iodinated casein	80	88 ± 5.9

[1] The alfalfa samples were kindly provided by the Research and Development Division of Nutrilite Products, Inc., Buena Park, California.

[2] Experimental period — 100 days. Averages were computed on the basis of a 100-day survival time for animals alive at the termination of the experiment.

[3] See footnote 3, table 2.

Experiment 3

Effects of graded levels of alfalfa meal and the variation in response to different lots of alfalfa meal on the survival of thyrotoxic rats fed a purified casein-containing ration

The findings indicate that the effect of alfalfa meal on the survival of thyrotoxic rats is dependent on the amount fed. A supplement of 5 or 10% alfalfa meal had little if any effect on length of survival. When the ration contained 20% alfalfa meal, however, the average survival time of thyrotoxic rats was significantly longer than that of rats fed the unsupple-

mented diet. Considerable variation was observed in the activity of different lots of alfalfa meal. Of 6 different batches of oven-dried alfalfa meal which were presumably processed by identical procedures, two were highly active, one had marked activity and the remaining three were only moderately active in prolonging the survival of thyrotoxic rats. The results are summarized in table 4.

TABLE 4

Effects of graded levels of alfalfa meal and the variation in response to different lots of alfalfa meal on the survival of thyrotoxic rats fed a purified casein-containg ration [1]

(10 male rats per group)

DIETARY GROUP	THYROACTIVE SUBSTANCE IN DIET	PER CENT SURVIVAL	AVERAGE SURVIVAL TIME [2,3]
			days
A	None	100	100 ±
A	0.5% thyroid	0	26 ± 3.7
A + 5% alfalfa meal no. 1	0.5% thyroid	0	30 ± 2.3
A + 10% alfalfa meal no. 1	0.5% thyroid	20	38 ± 12.0
A + 20% alfalfa meal no. 1	0.5% thyroid	60	76 ± 6.7
A + 20% alfalfa meal no. 2	0.5% thyroid	20	49 ± 10.9
A + 20% alfalfa meal no. 3	0.5% thyroid	20	57 ± 8.9
A + 20% alfalfa meal no. 4	0.5% thyroid	0	47 ± 8.7
A + 20% alfalfa meal no. 5	0.5% thyroid	10	45 ± 8.0
A + 20% alfalfa meal no. 6	0.5% thyroid	50	77 ± 9.6

[1] See footnote 1, table 3.
[2] See footnote 2, table 3.
[3] See footnote 3, table 2.

Experiment 4

Comparative effects of alfalfa meal, alfalfa fractions and supplements of the known nutrients on the survival time of thyrotoxic rats fed a purified casein-containing diet

In agreement with previous findings alfalfa meal when fed at a 20% level in the diet significantly increased the average survival time of thyrotoxic rats fed a purified casein-containing diet. The active factor (or factors) was retained in the alfalfa residue fraction (the water-washed pulp remaining after extraction of the juice). Dried alfalfa juice, when fed

at a level corresponding to the amount present in 20% alfalfa meal or a 5% supplement of the water-soluble extract of alfalfa meal, had little if any activity. Alfalfa ash, when fed at a level corresponding to the amount provided by a 20% alfalfa meal supplement and alfalfa lipids at a 2% level in the diet, were without significant effect. Cellulose, when fed at a 5% or 10% level in the diet or additional protein in the form of a 10% casein supplement, also had no significant activity nor did a supplement of 2.5% Hubbell, Mendel and Wakeman ('37) salt mixture or the known vitamins in amounts equal to or exceeding the amounts of such nutrients in a 20% alfalfa meal supplement. In agreement with previous findings (Ershoff, '53) a cottonseed oil supplement prolonged the average survival time of thyrotoxic female rats fed the casein-containing ration, although to a smaller extent than did alfalfa meal. The cottonseed oil supplement had less activity in the male. The effects of alfalfa meal on the survival of thyrotoxic rats were as marked in the male, however, as in the female. Inasmuch as alfalfa lipids, when fed at a 2% level in the diet (which exceeded the amount of lipids present in a 20% alfalfa meal supplement), had no significant effect on survival, it would appear that the active factor in alfalfa which prolonged survival of thyrotoxic rats was non-lipid in nature. A high correlation was observed between the activity of alfalfa samples in prolonging survival and their plant residue (PR) factor activity.[4] Thus, those samples of alfalfa meal or alfalfa residue which were most potent in PR factor activity were also most active in prolonging survival in the thyrotoxic rat. The results are summarized in table 5.

Experiment 5

Beneficial effect of alfalfa meal on the survival of rats administered daily intraperitoneal injections of L-*thyroxine*

Inasmuch as the beneficial effects of alfalfa meal on the survival of thyrotoxic rats may conceivably have been due to

[4] Plant residue (PR) factor activity was assayed by the activity of the test samples in counteracting symptoms of glucoascorbic acid toxicity in the rat (Ershoff, '57a, '58).

TABLE 5

Comparative effects of alfalfa meal, alfalfa fractions and supplements of the known nutrients on the survival time of thyrotoxic rats fed a purified casein-containing diet [1]

(10 male and 10 female rats per group)

DIETARY GROUP	THYROACTIVE SUBSTANCE IN DIET	MALES		FEMALES	
		Per cent survival	Average survival time [2,3]	Per cent survival	Average survival time [2,3]
			days		*days*
A	None	100	100 ± 0	100	100 ± 0
A	0.5% thyroid	0	22 ± 1.6	0	23 ± 1.5
A + 20% alfalfa meal no. 7 [4]	0.5% thyroid	20	49 ± 8.9	20	46 ± 8.0
A + 20% alfalfa meal no. 8 [4]	0.5% thyroid	40	69 ± 10.1	20	66 ± 12.6
A + 5% dried alfalfa juice	0.5% thyroid	0	26 ± 2.5	10	32 ± 8.7
A + 15% alfalfa residue no. 1	0.5% thyroid	10	42 ± 6.3	20	45 ± 8.1
A + 15% alfalfa residue no. 2	0.5% thyroid	40	66 ± 8.6	40	67 ± 10.2
A + 2.5% alfalfa ash	0.5% thyroid	0	24 ± 1.7	0	31 ± 4.0
A + 5% water-soluble extract of alfalfa	0.5% thyroid	0	25 ± 2.7	10	30 ± 8.4
A + 2% alfalfa lipids	0.5% thyroid	0	26 ± 2.1	0	30 ± 4.4
A + 5% cellulose [5]	0.5% thyroid	0	28 ± 1.6	0	31 ± 4.0
A + 10% cellulose [5]	0.5% thyroid	0	26 ± 1.9	0	29 ± 2.4
A + 2.5% salt mixture [6]	0.5% thyroid	0	24 ± 1.7	0	32 ± 3.9
A + 10% casein [7]	0.5% thyroid	0	23 ± 2.0	0	28 ± 1.2
B vitamins, C and K [8]	0.5% thyroid	0	26 ± 1.4	10	36 ± 6.3
Vitamins A, D and E [9]	0.5% thyroid	0	23 ± 1.4	0	31 ± 5.5
5% cottonseed oil	0.5% thyroid	0	27 ± 1.1	0	41 ± 5.3
10% cottonseed oil	0.5% thyroid	0	29 ± 0.8	10	40 ± 8.7

[1] See footnote 1, table 3.

[2] See footnote 2, table 3.

[3] See footnote 3, table 2.

[4] Alfalfa meal no. 7 and alfalfa residue no. 1 assayed 1 to 1.5 PR factor units per gram and alfalfa meal no. 8 and alfalfa residue no. 2 assayed 3.5 to 4 PR factor units per gram in counteracting symptoms of glucoascorbic acid toxicity in the rat (Ershoff, '57a, '58).

[5] Solka-floc, Brown and Co., Boston, Mass.

[6] Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[7] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[8] The following vitamins were added per kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; and choline chloride 2 gm.

[9] Five thousand U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D_2 and 100 mg alpha-tocopherol acetate per kilogram of diet.

an impaired absorption of thyroid or iodinated casein in the alfalfa-fed animals, experiments were conducted to determine the effects of alfalfa supplementation on the survival of rats administered daily intraperitoneal injections of 100 μg L-thyroxine.[5] The basal ration employed in these studies was diet A. Tests were also conducted with rats fed graded levels of desiccated thyroid in an effort to determine what level of thyroid feeding would be comparable, insofar as growth and survival were concerned, to the effects obtained with the thyroxine injections. The findings indicate that, on the basis of growth retardation, percentage survival and average length of survival, the effects of daily injections of 100 μg L-thyroxine were intermediate between those obtained with 0.1 and 0.25% desiccated thyroid supplements in the diet. Alfalfa meal when fed at a 20% level in the diet significantly increased the survival of thyroxine-injected rats over that of similarly treated animals fed the unsupplemented basal ration. No significant differences were observed, however, in the weight increments of the two groups. The results are summarized in table 6. The findings indicate that alfalfa meal was effective in prolonging the survival of thyrotoxic rats not only under conditions where toxic doses of iodinated casein or desiccated thyroid were fed in the diet, as reported previously by Tappan et al. ('53) and confirmed in the present experiment, but following the parenteral administration of thyroxin as well. It would appear, therefore, that the beneficial effect of alfalfa meal on the survival of thyroid-fed or iodinated casein-fed rats was due to some mechanism other than one which decreased the absorption of these materials from the intestinal tract.

DISCUSSION

The present findings confirm the observations of Tappan et al. ('53) that supplements of alfalfa meal prolonged signi-

[5] L-Thyroxine (sodium), Nutritional Biochemicals, Inc., Cleveland, Ohio, was dissolved in alkaline alcohol solutions; the pH was adjusted to 8.5, and the solution diluted to a volume containing 100 μg per milliliter.

TABLE 6

Beneficial effects of alfalfa meal on the survival of rats administered daily intraperitoneal injections of l-thyroxine
(10 male rats per group)

DIETARY GROUP	THYROACTIVE SUBSTANCE	AVERAGE GAIN IN BODY WT. AFTER 100 DAYS ON EXPERIMENT	PER CENT SURVIVAL	AVERAGE SURVIVAL TIME [2,3]
		gm		*days*
A	None	342	100	100 ± 0
A	0.125% thyroid in diet	306	60	88 ± 5.0
A	0.25% thyroid in diet	199	20	49 ± 8.4
A	0.5% thyroid in diet		0	25 ± 2.3
A	100 μg one-thyroxine daily per rat	215	30	67 ± 2.3
A + 20% alfalfa meal no. 1	100 μg one-thyroxine daily per rat	237	90	99 ± 0.1

[1] See footnote 2, table 3.

[2] See footnote 3, table 2.

ficantly the average survival time of thyrotoxic rats fed a purified casein-containing diet. In addition, data obtained in the present study indicate (1) that the protective factor in alfalfa meal is distinct from any of the known nutrients, (2) that supplements of dried alfalfa juice, the water-soluble extract of alfalfa, alfalfa ash or alfalfa lipids had little if any protective effect, and (3) that alfalfa residue (the water-washed pulp remaining after extraction of the juice) was a potent source of the active factor. The latter is the same alfalfa fraction that partially counteracted the inhibitory effects of massive doses of estradiol on ovarian development in the immature rat (Ershoff et al., '56), prolonged the survival of immature hamsters fed highly purified diets (Ershoff, '56), promoted growth of immature guinea pigs fed a mineralized dried milk ration (Ershoff, '57b), counteracted the toxic effects of massive doses of glucoascorbic acid in the rat (Ershoff, '57a) and counteracted symptoms of mineral oil toxicity in rats and mice fed a low-fat ration (Ershoff and Hernandez, '58). The term "plant residue (PR) factor" has been suggested as a generic term for the substance (or substances) in alfalfa residue (and other succulent plants) responsible for the effects indicated above (Ershoff, '58). A rapid bioassay procedure for measuring PR factor activity has been described employing toxic doses of glucoascorbic acid as a stressor agent (Ershoff, '57a, '58). The present findings indicate that a high correlation exists between the PR factor activity of alfalfa samples as determined by the above assay procedure and the activity of the same alfalfa samples in prolonging survival of the thyrotoxic rat.

The studies of Tappan et al. ('53) were conducted with thyrotoxic rats fed a basal ration containing casein as the sole source of dietary protein. The present investigation was conducted with thyrotoxic rats fed a soy protein-containing diet as well. The findings indicate that, although alfalfa meal was active in prolonging the survival of thyrotoxic rats on both the casein and soy protein-containing diets, different factors

were responsible for the increased survival on the two diets. Thus, dried alfalfa juice when fed at a 5% level in the diet was without significant effect on the survival time of thyrotoxic rats fed the casein-containing diet but significantly prolonged the average survival time of thyrotoxic rats fed the soy protein-containing ration.

In addition to alfalfa meal, a number of other materials of both plant and animal origin have been found to be active in prolonging the survival of hyperthyroid rats fed a purified casein-containing ration. Among the materials active in this regard were yeast (Ershoff and Hershberg, '45; Ershoff, '47), desiccated whole liver (Ershoff, '47), defatted liver residue and liver fat (Ershoff, '48), dried penicillin mycelia and aureomycin mash (Ershoff, '50), whole egg (Graham et al., '53), dried and defatted pork and dried and defatted mutton (Tappan et al., '53), cholesterol (Marx et al., '48; Ershoff and Marx, '48), xanthine (Ershoff, '48) and cottonseed oil (Ershoff, '53). More recently, Boldt et al. ('53) reported that methionine and betaine were also active in prolonging the survival of hyperthyroid rats. In the present experiment data were obtained showing that soy protein supplemented with methionine was also active in prolonging survival of hyperthyroid rats. It is apparent that the survival of hyperthyroid rats can be prolonged by a number of substances that are unrelated chemically to one another. It is not known to what extent the protective factor in alfalfa meal or alfalfa residue may be identical to that present in one or more of the foodstuffs indicated above. Since the alfalfa lipid fraction had little if any activity in prolonging survival, it would appear that the protective factor was nonlipid in nature. Since alfalfa ash when fed in an amount comparable to that present in a 20% alfalfa meal supplement was inactive, the protective factor would appear to be organic. Cellulose when added to the basal casein-containing ration at a 5 or 10% level gave no protection, a finding previously reported by Tappan et al. ('53). The beneficial effect of the alfalfa meal supplement does not appear

to be due to its methionine content. This is indicated by the fact that a supplement of 20% alfalfa meal increased the methionine content of the basal ration by only 0.09% whereas a supplement of 10% casein which provided almost three times as much methionine (0.26%) had no significant effect on length of survival. Additional protein *per se* provided by the alfalfa meal supplement does not appear to be involved since the 10% casein supplement supplied more than twice as much protein as the 20% alfalfa meal supplement. Since alfalfa meal prolonged the survival of rats administered thyroxine intraperitoneally as well as those administered desiccated thyroid or iodinated casein orally, it would appear that the protective factor in alfalfa meal exerts its effect on the survival of thyrotoxic rats fed a casein-containing diet by some mechanism other than decreasing the absorption of thyroid or iodinated casein from the intestinal tract.

SUMMARY

Immature rats were fed purified rations containing either casein supplemented with cystine or soy protein supplemented with methionine as the source of dietary protein. Survival was impaired with both rations following the concurrent administration of massive doses of desiccated thyroid or iodinated casein. The average survival time of hyperthyroid rats was significantly longer with the soy protein than the casein-containing ration.

Alfalfa meal, when fed at a 20% level in the diet, significantly increased the average survival time of hyperthyroid rats fed both the soy protein and casein-containing rations. When fed with the soy protein-containing diet both alfalfa residue (the water-washed pulp remaining after the extraction of the juice) and dried alfalfa juice were active in prolonging survival of thyrotoxic rats. When fed with the casein-containing ration, however, dried alfalfa juice had little if any activity whereas alfalfa residue significantly increased the average survival time of hyperthyroid rats.

The protective factor (or factors) in alfalfa residue which increased the survival of hyperthyroid rats fed the casein-containing ration was distinct from any of the known nutrients. A high correlation was observed between the activity of alfalfa samples in prolonging survival of hyperthyroid rats and their plant residue (PR) factor activity as judged by their effect in counteracting symptoms of glucoascorbic acid toxicity in the rat.

LITERATURE CITED

BOLDT, R. E., A. E. HARPER AND C. A. ELVEHJEM 1958 The effect of amino acids on the survival of the hyperthyroid rat. Arch. Biochem. Biophys., *74:* 437.

ERSHOFF, B. H. 1947 Comparative effect of liver and yeast on growth and length of survival of the immature thyroid-fed rat. Arch. Biochem., *15:* 365.

———— 1948 Further studies on the antithyrotoxic factor of liver. Exp. Med. Surg., *6:* 438.

———— 1950 Prolonged survival of hyperthyroid rats fed penicillin and aureomycin residues. Arch. Biochem., *28:* 359.

———— 1953 Effects of the fat content of the ration on the antithyrotoxic properties of B vitamins and liver residue in the hyperthyroid rat. Metabolism, *2:* 175.

———— 1954 Protective effects of alfalfa in immature mice fed toxic doses of glucoascorbic acid. Proc. Soc. Exp. Biol. Med., *87:* 134.

———— 1956 Beneficial effects of alfalfa, aureomycin and cornstarch on the growth and survival of hamsters fed highly purified rations. J. Nutrition, *59:* 579.

———— 1957a Beneficial effects of alfalfa and other succulent plants on glucoascorbic acid toxicity in the rat. Proc. Soc. Exp. Biol. Med., *95:* 656.

———— 1957b Beneficial effects of alfalfa and other succulent plants on the growth of immature guinea pigs fed a mineralized dried milk ration. J. Nutrition, *62:* 295.

———— 1958 Unidentified nutritional factors and resistance to stress. Proc. Amer. Pharm. Manufacturers' Assoc., Western Section, 12th Annual Conference, p. 283.

ERSHOFF, B. H., AND H. J. HERNANDEZ 1958 An unidentified factor in alfalfa which counteracts mineral oil toxicity in the rat and mouse. J. Nutrition, *65:* 575.

ERSHOFF, B. H., H. J. HERNANDEZ AND J. H. MATTHEWS 1956 Beneficial effects of alfalfa on the ovarian development of immature rats fed massive doses of alpha-estradiol. Ibid., *59:* 147.

ERSHOFF, B. H., AND D. HERSHBERG 1945 The beneficial effects of yeast on the cardiac failure of hyperthyroid rats. Am. J. Physiol., *145:* 16.

ERSHOFF, B. H., AND W. MARX 1948 Effect of dietary cholesterol on the length of survival of hyperthyroid rats. Exp. Med. Surg., *6:* 145.

ERSHOFF, B. H., AND H. B. MCWILLIAMS 1948 Effects of the antithyrotoxic factor of liver on growth and survival of immature rats fed massive doses of thyroactive materials. Science, *108:* 632.

GRAHAM, C. E., I. P. REICHSTEIN, W. J. WATSON AND S. W. HIER 1953 Effects of proteins on body and organ weights in thyroid-fed rats. Proc. Soc. Exp. Biol. Med., *82:* 7.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 275.

MARX, W., E. R. MESERVE AND H. J. DEUEL, JR. 1948 Protective action of dietary cholesterol in experimental thyrotoxicosis. Proc. Soc. Exp. Biol. Med., *67:* 385.

TAPPAN, D. V., R. E. BOLDT AND C. A. ELVEHJEM 1953 Unidentified factors capable of reducing stress in iodinated protein-fed rats. Ibid., *83:* 135.

THE ANTITHYROTOXIC FACTOR OF LIVER

I. METHOD FOR ASSAY

L. R. OVERBY, R. L. FREDRICKSON AND D. V. FROST

Nutrition Research Department, Abbott Laboratories, North Chicago, Illinois

(Received for publication August 23, 1958)

This is the first of a series of reports on the antithyrotoxic properties of liver residue in rats. These studies were carried out over a period of 6 years and involved about 20,000 rats. Initial work (Overby et al., '53) led to a test procedure which distinguished the antithyrotoxic activity of liver from that of known nutrients. We report here studies to develop a purified diet for rats (1) to give maximum growth; (2) to give controlled inhibition of growth and survival in thyrotoxic rats; and (3) to restore growth and survival by dietary means.

The nutrition of thyrotoxic animals has been studied extensively for 20 years, but is still a little understood problem. The reader is referred to the publications of Ershoff ('52, '55), Betheil et al. ('47), O'Dell et al. ('55), Stevens and Henderson ('58) and Tappan et al. ('53) for background on the antithyrotoxic factor.

The intriguing theory has been suggested that manifestations of thyrotoxicosis may be similar to those observed during chronic biochemical or environmental stresses. A study of the nutritional requirements of thyrotoxic animals might thus lead to understanding of the requirements for adaptation to non-specific stress. Ershoff ('47) has summarized this idea as follows:

"Available data indicate that, in addition to the major nutrients, substances are present in our diet, which may be required in increased

amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animal's own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifested by retarded growth or tissue pathology and preventable by the administration in appropriate amounts of the missing nutrient."

Many investigations have been directed toward testing Ershoff's premise and isolating the antithyrotoxic principles of liver. This also is the objective of the present series of studies.

As shown by Maley and Lardy ('55) excess thyroxine disrupts the oxidative phosphorylation system. The animal obviously "spins its motor" to no purpose. The interrelationships between nutritional status and metabolism are, however, not yet clear. Cardiac failure appears as the cause of death, but the mechanism is again not entirely clear.

EXPERIMENTAL

Our experiments were run with groups of 60 or 120 21-day old weanling male rats, weighing 50 to 55 gm. In early experiments rats from the Abbott colony (originally Sprague-Dawley) were used. These were later replaced by animals purchased from Sprague-Dawley, because rats from the Abbott colony were more susceptible to respiratory infections. This made it difficult to assess experiments where deaths might be due to infection rather than thyrotoxicosis. In parallel experiments it was shown that Abbott and Sprague-Dawley rats had nearly the same growth potential on identical diets. Therefore, experiments using each strain of animal may be compared.

The animals were divided equally as to weight into groups of 10. They were housed in individual screen-bottom cages and consumed diet and water ad libitum. The room was air-conditioned with controlled temperature (75°F.) but no humidity control. The animals were weighed weekly and the experiments terminated at 4 to 5 weeks. Significant differ-

ences in weight gain were often observed as early as one week; however, the total 4-or 5-week gains and survival rates were used most reliably to measure the antithyrotoxic activity of test materials.

Three control groups were run in each experiment: (1) the basal diet, (2) the basal diet plus iodinated casein [1] and (3) the basal diet plus iodinated casein plus defatted liver residue.[2] The remaining groups, usually 9 in number, were used for testing fractions of liver residue or other materials.

We felt that a minimum requirement for the proposed studies was a purified diet that would give maximum growth of the rats under "non-stress" conditions. Furthermore, the addition of more of the same nutrients should not improve the thyrotoxic animal's growth and survival.

The basic ingredients of the diets are shown in table 1. Casein varied from 20 to 30%. Cottonseed oil, corn oil and hydrogenated coconut oil were used with the level of fat varying from 0 to 10%. Vitamins were 10 to 100 times the estimated normal requirements for the growing rat. Additions to the diet were made at the expense of carbohydrate unless the additive was protein or fat, in which cases the additive was substituted for an equal quantity of casein or fat. Defatted liver residue ($N = 13\%$) was substituted for an equal weight of casein.

Weight gains of the surviving animals were taken as criteria of response. When only two or three animals survived from a group of 10 no statistical treatment of weight gain was possible. Both maximum growth and survival were consistently dependent on the dietary adequacy of the active principle in liver residue. As described in later publications, certain types of fat and bile acids also counteracted thyrotoxicosis.

[1] Protamone, Cerophyl Laboratories, Kansas City, Mo., represented to contain 1% thyroxine equivalent.

[2] This is the water insoluble portion remaining from the preparation of aqueous extracts of pork liver. From 1800 pounds of fresh liver about 440 pounds of dried residue is produced. This is ground to 20 mesh and extracted continuously with petroleum ether, yielding about 375 pounds of defatted residue.

TABLE 1

Composition of diets used for antithyrotoxic factor assay

INGREDIENT	DIET NO.								
	1–2	3–4	5–6	7 [1]	8–9	W [2]	10–13	12	14
	%	%	%	%	%	%	%	%	%
Casein (vitamin free)	22	22	22	20	30	20	30	30	30
Sucrose	69	69	67	—	59	70	57.5	57.5	57.5
Glucose	—	—	—	20	—	—	—	—	—
Dextrin	—	—	—	47.5	—	—	—	—	—
Salts [3]	4	4	4	4	4	4	4	4	4
Celluflour	2	2	2	2	2	—	2	2	2
Agar	1.4	1.4	1.4	—	1.2	—	1.25	1.25	1.25
Cod liver oil	1	1	1	1	1	—	—	—	—
Choline chloride	0.1	0.1	0.1	0.2	0.1	0.5	0.1	0.1	0.1
Cystine	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.3
Cottonseed oil	—	—	2	—	2	—	5	—	5
Corn oil	—	—	—	5	—	5	—	—	—
Hydrol [4]	—	—	—	—	—	—	—	5	—
	mg/100 gm								
Riboflavin	0.3	0.3	2.5	1.6	2.5	2.5	2.5	2.5	2.5
Thiamine·HCl	3	3	5.0	0.8	5.0	1.5	5.0	5.0	5.0
Niacin	5	5	10.0	4.0	10.0	10.0	10.0	10.0	10.0
Pyridoxine·HCl	3	3	1.5	0.8	1.5	1.0	1.5	1.5	1.5
Folic acid	0.5	0.5	0.5	0.2	0.5	0.125	0.5	0.5	0.5
Biotin	0.02	0.02	0.05	0.02	0.05	0.05	0.05	0.05	0.05
Ca-DL-pantothenate	5	5	20.0	4.4	20.0	10.0	20.0	20.0	20.0
Inositol	20	20	50.0	20.0	50.0	50.0	50.0	50.0	50.0
p-Amino benzoic acid	1	1	10.0	4.0	10.0	62.5	10.0	10.0	10.0
2-Methyl, 1,4-naphthoquinone	0.5	0.5	1.0	1.0	0.1 [5]	— [6]	0.1 [5]	0.1 [5]	0.1 [5]
alpha-Tocopherol	10	10	15.0	15.0	15.0	— [6]	15.0	15.0	15.0
Vitamin B_{12}	—	0.0015	0.010	0.0003	0.010	0.010	0.010	0.010	0.010
Vitamin A, crystalline	—	—	—	—	—	— [6]	4000 [7]	4000 [7]	4000 [7]
Vitamin D, crystalline	—	—	—	—	—	— [6]	400 [7]	400 [7]	400 [7]
Procaine penicillin G	—	—	—	—	—	—	—	—	5

[1] Bosshardt, D. K., and J. W. Huff, J. Nutrition, *50:* 117 (1953).
[2] Tappan, D. V., R. E. Boldt and C. A. Elvehjem, Proc. Soc. Exp. Biol. Med., *83:* 135 (1953).
[3] Jones, J. H., and C. Foster, J. Nutrition, *24:* 245 (1942).
[4] Hydrogenated coconut oil, Procter and Gamble, Cincinnati, Ohio.
[5] Na_2SO_3 addition compound.
[6] Four drops weekly 1:1 haliver oil-corn oil containing 1 mg menadione and 2.5 mg alpha-tocopherol per milliliter.
[7] International units.

RESULTS

Growth on the basal diet. In table 2 the data from the three control groups are summarized for 9 variations of the basal diet. Three diets were tested in only one experiment. The other 6 were tested from 10 to 62 times, with 10 animals in each group. The diets are listed in chronological order of their use, and each change was made to improve the basal growth of the animals.

In diet 1-2 the only source of fat was 1% of cod liver oil. Casein and vitamins were at presumed adequate levels in these early studies, except for vitamin B_{12}. In 10 experiments growth at 4 weeks was well below the potential for the animals. The addition of vitamin B_{12}, which had just become available (diet 3–4), gave no enhancement of growth. However, after the addition of 2% of cottonseed oil and increased vitamins (diet 5–6) the 4-week weight gain was 127 gm. The growth potential probably was not realized in these experiments because of chronic respiratory infections. This is evidenced by only 67% survival on the basal diet during this period.

Increasing casein to 30% or changing fat to 5% gave no increased growth (diets 7, 8–9, W, 10–13 and 12). The inclusion of procaine penicillin (50 mg/kg) in the 30% casein, 5% cottonseed oil ration appeared to give optimum growth. Growth with diet 14 was equivalent to that shown by rats receiving two practical rations. In a series of 4 experiments the average 4-week weight gains were: diet 14, 174 ± 5.0 gm, commercial [3] rat diet, 179 ± 5.6 gm and Abbott stock colony diet,[4] 147 ± 3.2 gm.

Results in table 2, diet 14, represent 62 consecutive tests on this diet. The data shown graphically in figure 1 illustrate the weekly and seasonal variations in growth. Peak growth was realized during the spring and early summer.

[3] Rockland.

[4] Wheat meal, entire kernel (30%); yellow corn meal, entire kernel (34%); whole milk powder (21%); old process linseed oil meal (7%); alfalfa leaf flour (2%); calcium carbonate (0.5%); sodium chloride (0.5%); liver, vacuum dried (1%); salts, Jones and Foster (0.5%); Primex (3%); brewers' yeast (0.5%).

TABLE 2

Growth of rats on synthetic rations with and without liver residue

(4-week results)

DIET NO.	MAIN CHARACTERISTICS	NUMBER OF EXPS.[1]	BASAL Av. gain and survival Standard error [2]		BASAL + IODINATED CASEIN Av. gain and survival Standard error [2]		BASAL + IODINATED CASEIN + 10% LIVER RESIDUE Av. gain and survival Standard error [2]	
			gm	%	*gm*	%	*gm*	%
1–2	22% Casein 1% cod liver oil as only fat	10	91 ± 4.8	100	40 ± 5.3	56 ± 5.8	68 ± 3.4	76 ± 4.7
3–4	Vitamin B_{12} added (1.5 μg/100 gm)	24	91 ± 5.6	100	32 ± 4.2	47 ± 5.1	69 ± 3.3	80 ± 2.9
5–6	2% Cottonseed oil and additional vitamins	24	127 ± 5.7	67 ± 6.2	63 ± 2.5	43 ± 4.7	80 ± 3.1	65 ± 3.4
7	Glucose and dextrin substituted for sucrose; 20% casein and 5% corn oil	1	131 ± 6.1	100	92 ± 5.0	60	112 ± 5.8	90
8–9	Casein increased to 30%, Cod liver oil substituted by vit. A & D crystalets, 2% cottonseed oil	29	128 ± 2.0	82 ± 3.3	62 ± 5.2	29 ± 4.4	99 ± 2.6	80 ± 2.8
W	20% casein, 5% corn oil	1	153 ± 2.8	100	101 ± 3.0	40	109 ± 9.4	90
10–13	5% cottonseed oil 30% casein	43	130 ± 2.1	95 ± 1.9	84 ± 1.9	67 ± 4.3	112 ± 2.2	93 ± 1.6
12	5% Hydrol, only source of fat	1	124 ± 3.5	100	65 ± 2.5	80	91 ± 4.5	100
14	30% casein, 5% cottonseed oil, 50 mg procaine penicillin per kg diet	62	168 ± 2.0	100	114 ± 2.8	62 ± 3.6	150 ± 1.9	95 ± 0.93

[1] Ten rats per group in each experiment.

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

Response to liver residue. Ten per cent defatted liver residue used in the diet as positive control, uniformly promoted growth and survival of thyrotoxic rats. An almost equal absolute response was obtained whether the casein was 20 or 30% (table 2). The response was roughly similar when the source of fat was cod liver oil (1%), corn oil (5%), cottonseed oil (5%) or Hydrol [5] (5%). On diet W (20% casein, 5% corn oil) liver residue appeared to enhance survival

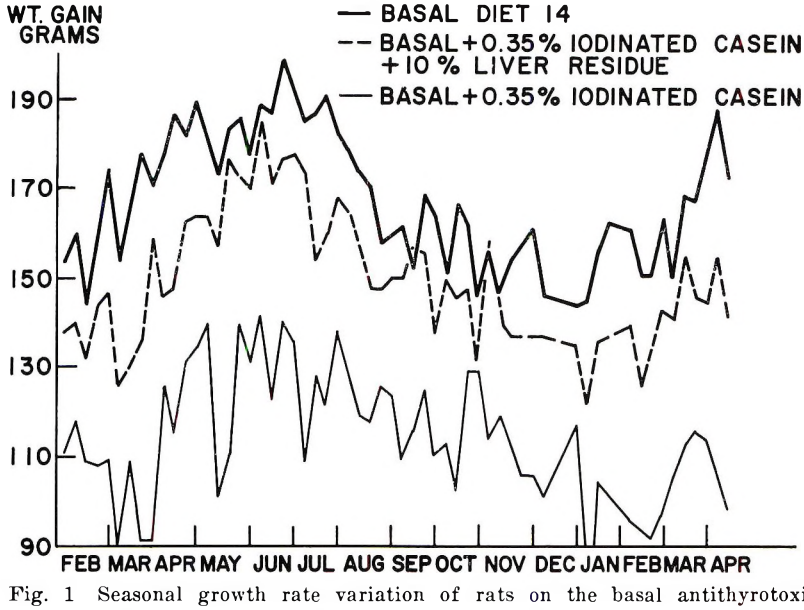

Fig. 1 Seasonal growth rate variation of rats on the basal antithyrotoxic assay diet with and without iodinated casein and with added liver residue.

rather than growth. Figure 2 shows the quantitative aspects of the assay, in which 1, 5 and 10% of liver residue were compared. There was a graded response in growth and survival at the 5-week period. All animals survived on 10% liver residue but only 50 and 30% at the 5 and 1% levels.

Growth inhibition by iodinated casein. It was expected that the protection by liver residue would vary with the degree of thyrotoxicosis. In the experiments recorded in table 2

[5] Hydrogenated coconut oil, Procter and Gamble Company, Cincinnati, Ohio.

the level of iodinated casein varied from 0.2 to 0.35%. Table 3 summarizes three experiments in which iodinated casein was used at 0.1, 0.2, 0.3 and 0.4%. Liver residue did not improve the growth of the rats on the basal diet alone, but

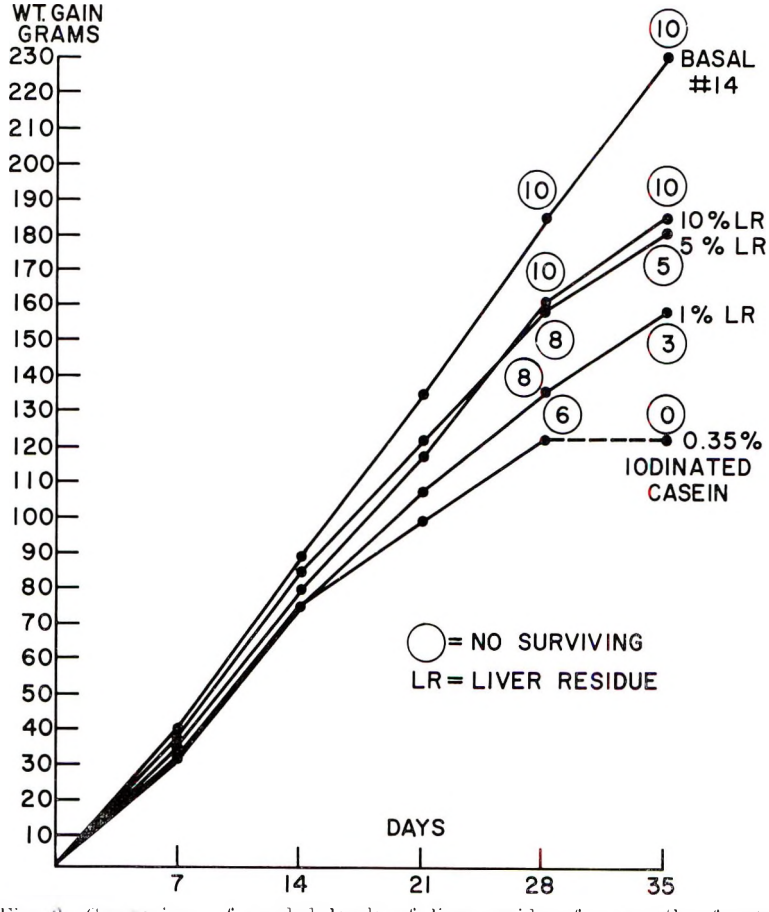

Fig. 2 Comparison of graded levels of liver residue for growth of rats in antithyrotoxic factor assay.

growth and survival were improved at all levels of thyrotoxicosis.

The effects of vitamins. Table 4 summarizes the response of liver residue when excess vitamins were added. It is ob-

TABLE 3

Effect of graded levels of iodinated casein on weight gain and mortality in rats with and without liver residue

(Average 4-week results of three experiments)

DIET		% IODINATED CASEIN				
		0	0.1	0.2	0.3	0.4
Basal 10–13 (table 1)	Survival, %	100	80	50	40	40
	gain, gm	144 ± 6.7 [1]	124 ± 14.7	65 ± 5.8	59 ± 3.7	50 ± 5.8
Basal + 10% liver residue	Survival, %	100	100	100	80	90
	gain, gm	147 ± 5.1	135 ± 6.3	115 ± 6.5	100 ± 5.5	102 ± 2.9

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

vious in this experiment that the response to liver residue is not duplicated by excess amounts of known vitamins. In many other experiments, too numerous to record in detail, we were unable to alter the response to liver residue with excess vitamins.

The effects of inorganic substances. Several inorganic substances were compared with liver residue. Table 4 shows the results found with selenium, magnesium, bromide, the ash

TABLE 4

Effects of added vitamins and inorganic substances on weight gain and survival of thyrotoxic rats

(Comparison with liver residue)

SUPPLEMENT	5-WEEK RESULTS	
	Gain and S.E. [1]	Survival
	gm	%
None (basal 14)	204 ± 6.5	100
0.3% iodinated casein [2]	136 ± 10.8	60
10% liver residue	190 ± 4.5	100
Vitamins [3]	126 ± 6.4	30
Vitamins [3] + 10% liver residue	181 ± 5.9	80
SeO_2, 3 mg/kg	131 ± 6.0	60
MgO, 0.21%	134 ± 5.8	70
NaBr, 0.01%	134 ± 8.3	40
Salts (Jones and Foster), 2.5%	76	20
Liver residue ash, 1.3%	122 ± 18.5	30

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

[2] All remaining groups also contained 0.3% iodinated casein.

[3] All vitamins used at three times the level recorded for diet 14 in table 1.

prepared by direct heating of liver residue, or additional 2.5% of Jones and Foster ('42) salt mixture. None of these materials showed activity comparable to that of liver residue.

The effects of inert ingredients. Inert ingredients and bulk quality of a ration might alter the animals' intestinal flora, irreversibly adsorb nutrients or Protamone, or change the capacity of the animal to absorb nutrients. The substitution of Hyflo, Celluflour or Darco G-60,[6] at the 10% level, for an equal quantity of sucrose in the ration did not favorably affect thy-

[6] Atlas Powder Company, Wilmington, Delaware.

rotoxic rats or alter the protective action of liver residue. The Darco appeared to inhibit growth even on the basal diet.

The effects of antibiotics. The results of three typical experiments are shown in table 5. Low levels of procaine penicillin (30 mg/kg) appeared to stimulate growth on the basal diet alone, with iodinated casein and with the latter plus liver residue. The nature of the antibiotic growth stimulation is still not known. Presumably antibiotics alter intestinal flora, thereby improving nutrition of the host. These studies were the basis for incorporating procaine penicillin in diet 14 for routine use. The antibiotic appeared to improve the rate and uniformity of growth throughout.

DISCUSSION

Liver residue has been shown consistently to protect growing rats against death and growth inhibition caused by thyroxine feeding. The nature of the protective factors has been elusive. Exact definition of the problem is difficult from the nutritive standpoint. Because of the acute hormone imbalance, requirement for most essential nutrients is altered. The extent of this increase over normal requirements is not precisely known but must be taken into account for any level of thyroxine feeding.

The animal may vary from mild hyperthyroidism to fatal thyrotoxicosis as the amount of thyroid material in the diet is increased. If a basal diet were marginal in any one nutrient a mildly hyperthyroid animal might well respond favorably to increased amounts of the nutrient. Animals made thyrotoxic in the presence of excessive amounts of all known required nutrients should respond specifically to antithyrotoxic substances. We have endeavored to produce a true, deep-seated thyrotoxicosis in rats dissociated from known dietary deficiency. The known antithyrotoxic effects of certain lipides must always be considered.

The purified diet employed in most of these studies (diet 14) contained adequate quantities of all nutrients to support excellent growth of rats under normal laboratory conditions.

TABLE 5

Antibiotics and the response of thyrotoxic rats to liver residue

(5-week growth and survival)

ADDITIVE	BASAL (10–13)		BASAL + 0.3% IODINATED CASEIN		BASAL + 0.3% IODINATED CASEIN + 10% LIVER RESIDUE	
	Gain, S.E. [1]	Survival	Gain, S.E. [1]	Survival	Gain, S.E. [1]	Survival
	gm	%	*gm*	%	*gm*	%
None	183 ± 6.6	100	122 ± 5.8	70	144 ± 4.6	80
Procaine penicillin, 200 mg/kg	177 ± 6.7	100	130 ± 7.5	100	169 ± 4.8	100
None	181 ± 4.8	100	112 ± 5.3	30	144 ± 4.9	100
Procaine penicillin, 30 mg/kg	200 ± 7.4	100	152 ± 3.9	100	194 ± 4.0	100
None	185 ± 6.1	100	124 ± 2.6	40	145 ± 2.7	100
Combination of procaine penicillin, bacitracin, and terramycin, each at 15 mg/kg	198 ± 9.1	100	132 ± 3.2	40	156 ± 5.8	100

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

In 62 consecutive weekly tests animals averaged 220 gm at 7 weeks of age (range 200 to 250 gm). When 0.3% of Protamone was included, the average weight at 7 weeks was 166 gm and survival was only 60% (curve C, figure 1). This ration does not promote "normal" growth and survival at this level of thyrotoxicosis. When liver residue was substituted for an equal weight of casein, the average weight was 202 gm and 95% of the animals survived the 4-week test period.

The response to liver residue was also observed in 8 other diets despite variation in levels of vitamins, minerals, fats and casein. In experiments to be reported in a later publication certain unsaturated fats at 10 to 20% of the diet, or bile acids at 0.1 to 0.2% afforded partial protection against thyrotoxicosis. These results are similar to the findings of Greenberg ('52) for dietary fat and Page et al. ('56) for bile acids. In our experiments, the effect of lipids appeared to be separate and distinct from that of liver residue. The effect of bile acids appeared supplementary to that of liver residue.

Ershoff ('50, '50a) reported that supplements of dried penicillin mycelia or aureomycin mash, but not crystalline aureomycin hydrochloride, prolonged the survival of thyrotoxic rats. Meites and Ogle ('55) reported that crystalline penicillin, neomycin or streptomycin counteracted growth retardation in rats receiving 0.16% of Protamone. In our experiments penicillin tended to stimulate growth on the basal diet alone and increased survival and growth of the thyrotoxic animals. However, the activity of liver residue was even more pronounced in the presence of the antibiotic.

Tappan et al. ('53) found that cellulose added to the diet gave insignificant protection to hyperthyroid rats. Our results with Darco, Hyflo and Celluflour also indicate that bulk content of the diet does not alter the response of the animals to liver residue.

Liver residue used was found to be about 14% ash. Spectrographic analyses showed the ash to have the following heavy metal composition: Pb (0.013%), Cu (0.071%), Fe (1.3%), Al (1.4%), Ca (0.16%), Zn (0.18%), Ni (0.007%), Mg

(0.23%), Cr (0.0018%), Ti (0.013%), Mo (0.009%), Mn (0.022%) and V (0.005%). The ash had no antithyrotoxic activity. Increasing the salt mixture to 6.5% did not favorably affect the thyrotoxic animals. Other investigators (Morrison and associates, '56, Dannenburg et al., '55) have reported evidence that the chick requires unidentified minerals. Selenium has been shown by Schwarz ('57) to play an integral part in protection against dietary necrotic liver degeneration in rats. Huff et al., ('56) found that the growth retardation in mice fed iodinated casein was reversed by sodium bromide. Bromide or selenium showed no activity in our assay.

Vitale and associates ('57) found that rats receiving no thyroxine grew maximally on a diet containing 20 mg % of magnesium. The growth inhibition produced by thyroxine feeding was partially overcome by extra supplements of magnesium, up to 160 mg %. Our diet 14 contained 47 mg % of magnesium. Supplementing this with an additional 60 mg % gave no significant growth increment as shown in table 4 [127 gm (5 survivors) vs. 134 gm (7 survivors)]. Liver residue (10%) supplied only an additional 3 mg % of magnesium and the weight gain was 189 gm (9 survivors). The marked protective action of liver residue did not appear due to this extra magnesium.

SUMMARY

The antithyrotoxic activity of liver residue was studied for growth of rats on a purified diet. The experimental diets contained varied levels of vitamins, fats, casein, minerals, carbohydrates, antibiotics and inert ingredients. A diet was developed which gave uniform growth and low mortality only with liver residue. The activity in liver residue appears distinct from known dietary essentials.

LITERATURE CITED

BETHEIL, J. J., V. D. WIEBELHAUS AND H. A. LARDY 1947 Studies of thyroid toxicity. I. A nutritional factor which alleviates the toxicity of ingested thyroid substances. J. Nutrition, *34:* 431.

BOSSHARDT, D. K., AND J. W. HUFF 1953 Nutritional deficiencies in mice caused by stress agents. Ibid., *50:* 117.

DANNENBURG, W. N., B. L. REID, E. E. ROZACKY AND J. R. COUCH 1955 An inorganic chick growth response. Poultry Sci., *34:* 1023.

ERSHOFF, B. H. 1947 Comparative effects of liver and yeast on growth and length of survival of the immature thyroid-fed rat. Arch. Biochem., *15:* 365.

———— 1950 Prolonged survival of hyperthyroid rats fed penicillin and aureomycin residues. Ibid., *28:* 359.

———— 1950a Distribution of an unidentified antithyrotoxic factor in materials of plant and animal origin. Proc. Soc. Exp. Biol. Med., *74:* 391.

———— 1952 Increased survival of liver fed rats administered multiple sublethal doses of x-irradiation. J. Nutrition, *47:* 289.

———— 1955 Nutrition and stress. Nutrition Reviews, *13:* 33.

GREENBERG, S. M. 1952 The protective effect of dietary fat on immature rats fed thyroid. J. Nutrition, *47:* 31.

HUFF, J. W., D. K. BOSSHARDT, O. P. MILLER AND R. H. BARNES 1956 A nutritional requirement for bromine. Proc. Soc. Exp. Biol. Med., *92:* 216.

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

MALEY, G. F., AND H. A. LARDY 1955 Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rats. J. Biol. Chem., *215:* 377.

MEITES, J., AND R. C. OGLE 1951 Antithyrotoxic effects of antibiotics in rats. Proc. Soc. Exp. Biol. Med., *77:* 758.

MORRISON, A. B., R. DAM, L. C. NORRIS AND M. L. SCOTT 1956 Further evidence on the requirement of the chick for unidentified minerals. J. Nutrition, *60:* 283.

O'DELL, B. L., S. J. STOLZENBERG, J. H. BRUEMMER AND A. G. HOGAN 1955 The antithyrotoxic factor: Its solubilization and relation to intestinal xanthine oxidase. Arch. Biochem. Biophys., *54:* 232.

OVERBY, L. R., R. L. FREDRICKSON AND D. V. FROST 1953 The antithyrotoxic factor. Federation Proc., *12:* 425.

PAGE, A. C., JR., F. R. KONIUSZY, D. E. WOLF, P. ALDRICH AND K. FOLKERS 1956 Factors in liver reversing thyroid stress in rats. Ibid., *15:* 568.

SCHWARZ, K., AND C. M. FOLTZ 1957 Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., *79:* 3292.

STEVENS, C. O., AND L. M. HENDERSON 1958 Nutritional studies with the hyperthyroid rat. J. Nutrition, *64:* 67.

TAPPAN, D. V., R. E. BOLDT AND C. A. ELVEHJEM 1953 Unidentified factors capable of reducing stress in iodinated protein-fed rats. Proc. Soc. Exp. Biol. Med., *83:* 135.

VITALE, J. J., D. M. HEGSTED, M. NAKAMURA AND P. CONNORS 1957 The effect of thyroxine on magnesium requirement. J. Biol. Chem., *226:* 597.

ESSENTIAL FATTY ACID ACTIVITIES OF HYDROCARBONS AND ALCOHOLS ANALOGOUS TO LINOLEATE AND LINOLENATE [1]

ERIK AAES-JØRGENSEN,[2] ORVILLE S. PRIVETT AND RALPH T. HOLMAN

The Hormel Institute, University of Minnesota Austin

(Received for publication September 2, 1958)

Thus far, the only substances which have been found to exhibit full activity as essential fatty acids (EFA) are linoleic acid and its esters, linoleyl alcohol, and arachidonic acid and its esters. These substances are related in that, counting from the terminal methyl groups, all have their first double bonds between carbons 6 and 7 and a second double bond between 9 and 10. (Thomasson, '53). Those polyunsaturated acids which have their first double bonds at the third carbon atom and are related to linolenic acid, stimulate growth, but do not maintain dermal integrity as does the linoleic acid family of substances. The present study was undertaken to test the EFA activity of hydrocarbons and alcohols analogous to linoleate and linolenate to learn if the carboxyl function is necessary for activity.

EXPERIMENTAL

Ethyl linoleate was prepared from the fatty acids of safflower oil by urea crystallization and repeated low temperature

[1] Hormel Institute publication no. 182. Supported in part by grants from The Hormel Foundation, Office of Naval Research (Contract no. N8onr66218, 101-344) and the National Dairy Council.

[2] Fulbright Scholar 1956–58. Permanent address: Department of Biochemistry and Nutrition, Polytechnic Institute, Østervoldgade 10I.,[2] Copenhagen K, Denmark.

crystallization of the acids and subsequent fractional distillation of the ethyl esters in a Podbielniak Hyper-cal column. The infrared curve of the ethyl linoleate revealed no *trans* or conjugated isomers. The preparation had an iodine value of 160.5 (theory 165).

Linoleyl alcohol was prepared by lithium aluminum hydride reduction of pure methyl linoleate according to the method of Lightelm et al. ('50). Its infrared spectrum showed no evidence of unreacted carbonyl substances. Diene conjugation was very low. The iodine value was 186.6 (theory 190.7) and $n_D^{30} = 1.4676$.

Linolenyl alcohol was prepared from methyl linolenate which had been isolated from linseed oil via bromination-debromination. The methyl linolenate had an iodine value of 259.4 (theory 260.4) and its infrared spectrum revealed the presence of about 5% of isolated *trans* unsaturation. Reduction of the methyl linolenate (Lightelm et al., '50) yielded the alcohol which had an iodine value of 288 (theory 288), and $n_D^{30} = 1.4778$. Infrared spectra showed that the alcohol contained no residual carboxyl substances, and that the content of diene conjugated substances was low.

6,9-Octadecadiene was prepared from linoleyl alcohol via the alkyl bromide and the Grignard reagent according to the procedure of Deatherage and Olcott ('39). The octadecadiene contained a negligible amount of diene conjugation but about 10% isolated *trans* unsaturation. The Beilstein test for halides was negative. The iodine value was 201 (theory 203) and $n_D^{30} = 1.4495$.

3,6,9-Octadecatriene was prepared from linolenyl alcohol by the method of Deatherage and Olcott ('39). It was found to contain negligible amounts of conjugated isomers but 40% of isolated *trans* unsaturation. The preparation gave a negative Beilstein test, had an iodine value of 300 (theory 306) and $n_D^{31.5} = 1.4615$.

The biological experiment was carried out using 6 groups of 8 weanling male rats which were fed a fat-free diet for 11

weeks. The fat-free diet consisted of 16% vitamin-test casein, 4% α-cellulose, 74% sucrose, 4% Wesson salt mixture, 1% of a mixture of vitamins in casein, and 1% of a mixture of choline chloride in casein. (Aaes-Jørgensen and Holman, '58). Diet and water were given ad libitum, and the rats were weighed and inspected weekly. Definite dermal symptoms of EFA deficiency appeared within 11 weeks (table 2). At that time the rats were divided into 6 equal groups with similar average weights. All groups were fed the same fat-free diet, but each of 5 groups was fed one of the supplements at the rate of 200 mg per rat thrice weekly. The 6th group received no supplement.

During the experiment irritation was noticed around the mouths of rats in some groups. To test for a possible irritant effect 0.2 ml of each supplement was applied to the backs of the rats three times during one week. At the end of the experiment, the rats were killed by ether anesthesia. Autopsy examination was made on all animals and the heart and testes were taken for analysis. Rats of each group were chosen at random and polyunsaturated fatty acid analyses were carried out on the lipides extracted from the hearts and testes by the method of Holman and Hayes ('58).

RESULTS AND DISCUSSION

Feeding the fat-free diet to weanling rats induced distinct dermal symptoms of EFA deficiency within 11 weeks (table 2). By that time the growth curve was approaching a plateau. During the period of supplementation the fat-free group gained 31 gm and the group fed linoleate gained 61 gm. The groups fed the hydrocarbons gained less than those fed no supplement, whereas those fed the alcohols gained more weight than the fat-free control group.

The dermal symptoms of rats fed linoleate or linoleyl alcohol decreased markedly, whereas those of the other groups remained about the same (fig. 1 and table 1). The observation that linoleyl alcohol has EFA activity confirms the report of

TABLE 1

Average growth and gross symptoms from curative experiment

SUPPLEMENT	AVERAGE WEIGHT		AVERAGE DERMAL SCORE [1]			
	Initial	Final	Initial	2 weeks	4 weeks	6 weeks
Ethyl linoleate	235 ± 9.5 [2]	296 ± 8.2	2.0	1.4	0.4	0.4
Linoleyl alcohol	235 ± 9.5	284 ± 9.0	1.7	1.2	0.5	0.6
Linolenyl alcohol	231 ± 11.1	279 ± 8.2	1.6	1.3	1.3	1.3
Octadecadiene	231 ± 9.3	242 ± 10.2	1.6	1.3	1.6	1.5
Octadecatriene	231 ± 12.0	253 ± 11.1	1.8	1.2	1.1	1.2
Fat-free	231 ± 6.3	262 ± 6.6	1.8	1.7	1.9	2.0

[1] Average of scores of tail and fore-and-hind legs zero to 3.
[2] Standard error of the mean.

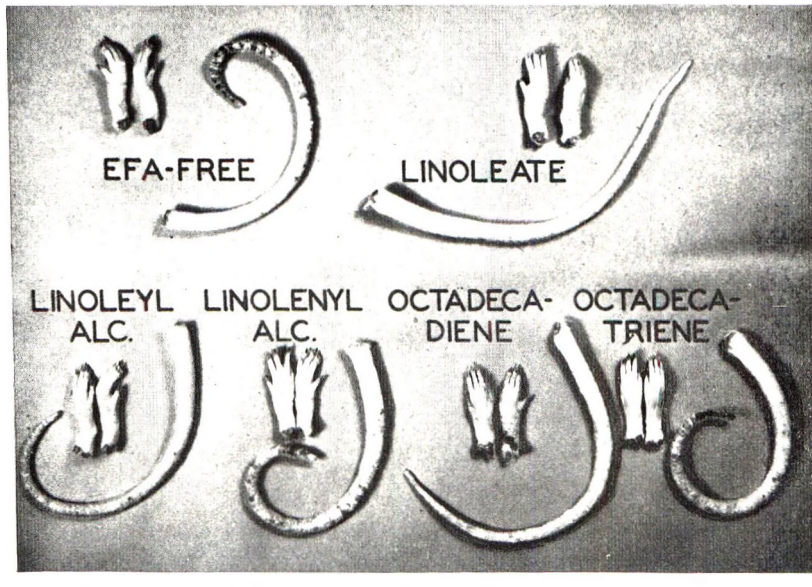

Fig. 1 Tails and feet from typical rats of each experimental group.

Turpeinen ('38). Rats fed linoleyl and linolenyl alcohols developed a rough coat, and the area around the mouth became red and irritated and lost its hair. However, application of the alcohol to the rats' backs did not cause irritation. Rats which were fed octadecadiene and octadecatriene also developed severe skin irritation around the mouth. Application of the hydrocarbons to the backs of the rats caused loss of hair

Fig. 2 The effect of topical application of octadecadiene to the back of a rat.

and severe irritation. The irritant effect was so great that the rats became morbid after the third application (fig. 2). Therefore the patch test was stopped and the lesions healed within 5 to 12 days.

Autopsy revealed fatty livers in the rats fed octadecadiene and octadecatriene. A few animals scattered among all groups had relatively small testes. Aside from these observations, no gross abnormalities were observed.

The analyses of the heart tissues of representative rats from each group for their content of polyunsaturated fatty acids are given in table 2. These data indicate that all groups except those fed linoleate and linoleyl alcohol had low dienoic acid content indicative of EFA deficiency. The high trienoic acid content characteristic of EFA deficiency occurred in all groups except those fed linoleate and linoleyl alcohol. Octadecadiene supplementation induced a lesser content of trienoic acids and a greater content of tetraenoic acid in heart tissue than found in the group fed the fat-free diet. The tetraenoic acid content of the heart tissue was highest in the rats fed linoleate and linoleyl alcohol, suggesting that linoleyl alcohol may also be used as a precursor of arachidonate. The groups fed linoleyl alcohol or ethyl linoleate had considerably more pentaenoic acid in heart tissue than did the fat-free control group. This was true likewise for the groups fed octadecatriene and linolenyl alcohol. These latter two supplements also stimulated the accumulation of very high contents of hexaenoic acids in the heart lipides as does linolenic acid (Widmer and Holman, '50). Thus the hydrocarbons must have been absorbed and affected polyunsaturated acid metabolism to some extent. However, octadecadiene was not effective as a substitute for the EFA activity of linoleate, and octadecatriene was not the equivalent of linolenate.

Linoleyl alcohol and linolenyl alcohol caused changes in polyunsaturated fatty acid contents of the heart similar to changes caused by linoleic and linolenic acids. This suggests that the alcohols are metabolized and are the approximate metabolic equivalent of the corresponding acids. Thus, for EFA activity, the unsaturation must be present in a hydrocarbon chain with a terminal functional group convertible to a carboxyl group through which lengthening of the chain may take place.

TABLE 2

Polyunsaturated acids in heart and testis tissue from rats fed various supplements and the standard errors of the means

SUPPLEMENT	ACID, MG/100 GM TISSUE					
	HEXAENOIC	PENTAENOIC	TETRAENOIC	TRIENOIC	DIENOIC	TOTAL
Heart tissue						
None	16.8 ± 1.3 [1]	7.8 ± 1.8	62.5 ± 13.2	286 ± 44.5	28.2 ± 24.3	403 ± 48
Ethyl linoleate	16.0 ± 1.4	43.0 ± 4.6	257.0 ± 35	62.0 ± 5.9	169 ± 19.2	548 ± 19
Linoleyl alcohol	27.7 ± 12.4	40.0 ± 8.7	186.5 ± 45.8	74.8 ± 9.8	99.5 ± 31.0	428 ± 78
Octadecadiene	24.8 ± 7.8	9.0 ± 4.1	84.8 ± 8.2	228.3 ± 34.5	24.3 ± 3.4	371 ± 39
Linolenyl alcohol	95.5 ± 9.1	52.5 ± 5.0	55.8 ± 6.7	126.3 ± 20.5	− 0.2 ± 9.7	330 ± 36
Octadecatriene	57.0 ± 7.3	25.5 ± 2.0	90.0 ± 9.8	289.5 ± 49.2	1.8 ± 4.4	464 ± 68
Testis tissue						
None	7.3 ± 1.2	56.3 ± 9.2	107.0 ± 38.6	164.3 ± 6.4	15.7 ± 3.2	351 ± 52
Ethyl linoleate	8.5 ± 2.4	88.3 ± 34.1	79.5 ± 27.8	52.3 ± 16.3	26.0 ± 6.7	255 ± 51
Linoleyl alcohol	9.0 ± 0.2	102.8 ± 29.4	92.5 ± 25.9	35.0 ± 14.2	13.0 ± 2.2	252 ± 72
Octadecadiene	5.8 ± 1.3	65.8 ± 6.1	113.0 ± 9.3	111.8 ± 12.5	12.5 ± 7.2	309 ± 31
Linolenyl alcohol	27.5 ± 10.8	38.3 ± 15.2	84.3 ± 24.6	60.8 ± 20.4	16.0 ± 14.8	227 ± 62
Octadecatriene	18.8 ± 3.0	59.8 ± 9.1	104.3 ± 15.1	118.0 ± 23.4	9.5 ± 5.9	310 ± 49

[1] Standard error of the mean.

Analyses of testes from each group of rats revealed high levels of trienoic acid in the deficient group, and in the group fed octadecatriene (table 2). The variability of trienoic acid in the other groups does not allow interpretation. Octadecatriene and linolenyl alcohol supplements induced the appearance of much more hexaenoic acid in testes than did the supplements having two double bonds. This indicates that the hydrocarbon octadecatriene was metabolized to some extent.

If the hydrocarbons undergo ω-oxidation, octadecadiene should yield either 9, 12-octadecadienoic acid (linoleic acid) or 6, 9-octadecadienoic acid depending upon the end of the molecule oxidized. If these substances were formed from octadecadiene, growth should be stimulated in either case because the 6, 9-isomer is related to linolenic acid and the 9, 12-isomer to linoleic acid which both induce growth in EFA deficient animals. ω-Oxidation of octadecatriene should produce linolenic acid or its isomer 3, 6, 9-octadecatrienoic acid or both. The former, at least, should induce growth. Unfortunately, the irritant effects of the hydrocarbons obscured any EFA or growth activity of their metabolic products. It may be that any acids formed by this mechanism are partially metabolized farther for energy and that little appears in the tissue as polyunsaturated acids. Nevertheless, it is clear from their effects upon polyunsaturated fatty acid contents of heart and testis lipide that the hydrocarbons are metabolized to more unsaturated acids to some extent.

SUMMARY

The effect of linoleyl and linolenyl alcohols, octadecadiene and octadecatriene upon EFA-deficient male rats has been tested. Only linoleyl alcohol relieved the symptoms of EFA deficiency.

Octadecadiene and octadecatriene, hydrocarbon analogs of linoleic and linolenic acids, acted as severe skin irritants and induced fatty livers in the rats. These compounds also caused

changes in the polyunsaturated fatty acid pattern of the heart lipides indicating that they were absorbed and metabolized to some extent.

Linoleyl and linolenyl alcohols stimulated growth and induced changes in the polyunsaturated fatty acid content of heart tissue similar to those induced by the corresponding acids.

ACKNOWLEDGMENT

The authors are grateful to F. J. Pusch, Pendra Rusley and Arlen E. Viste for technical assistance and to W. O. Lundberg for consultation.

LITERATURE CITED

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.

DEATHERAGE, F. E., AND H. S. OLCOTT 1939 Synthesis of *cis*-9-octadecene, 9-octadecyne and 9, 10-octadecane-diol. J. Am. Chem. Soc., *61:* 630.

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

LIGHTELM, S. P., E. VON RUDLOFF AND D. A. SUTTON 1950 Preparation of unsaturated long chain alcohols by means of lithium aluminum hydride: Some typical members of the series. J. Chem. Soc., *1950:* 3187.

THOMASSON, H. J. 1953 Biological standardization of essential fatty acids (a new method). Intern. Rev. Vitamin Res., *35:* 62.

TURPEINEN, O. 1938 Further studies on the unsaturated fat acids essential in nutrition. J. Nutrition, *15:* 351.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

WIDMER, C., AND R. T. HOLMAN 1950 Polyethenoid fatty acid metabolism II. Deposition of polyunsaturated fatty acids in fat-deficient rats upon single fatty acid supplementation. Arch. Biochem., *25:* 1.

THE EFFECT OF CONCENTRATES OF POLYUNSATURATED ACIDS FROM TUNA OIL UPON ESSENTIAL FATTY ACID DEFICIENCY[1]

ORVILLE S. PRIVETT, ERIK AAES-JØRGENSEN,[2]
RALPH T. HOLMAN AND WALTER O. LUNDBERG
Hormel Institute, University of Minnesota, Austin

(Received for publication August 23, 1958)

INTRODUCTION

Marine oils are known to contain large proportions of highly unsaturated acids, which are generally regarded to have low potency in the relief of dermal symptoms of essential fatty acid (EFA) deficiency. Recently accumulated evidence suggests that the polyunsaturated fatty acids may be grouped into a few classes depending upon the location of the first double bond from the methyl end of the fatty acid molecule (Thomasson, '53). Thus far, only those acids which have a terminal structure like that of linoleic acid have been found to exhibit all the activities of an essential fatty acid. That is, only linoleic and arachidonic acids, having their first double bond between the 6th and 7th carbon atoms from the methyl group, and a second double bond between the 9th and 10th carbon atoms are able to mantain normal skin and stimulate growth of rats when added to an otherwise fat-free diet. It is not known with certainty whether fish or marine oils contain sufficient acids of the linoleic acid type to meet the needs

[1] Hormel Institute publication no. 181. Supported in part by grants from the Hormel Foundation, Fish and Wild Life Service of the Department of Interior and the Office of Naval Research, contract N8onr66218, 101–344.

[2] Fulbright scholar 1956–58. Permanent address: Department of Biochemistry and Nutrition, Polytechnic Institute, Østervoldgade 10 I.², Copenhagen K, Denmark.

for essential fatty acids. It was therefore of interest to test a typical marine oil for its EFA activity. In order that the information be more precise, the individual chain-length fractions from tuna oil unsaturated methyl esters were tested in rats for their effects upon growth, dermal symptoms and polyunsaturated acid contents of representative tissues.

PREPARATION OF MATERIALS

Tuna oil was saponified and the unsaponifiable matter was removed by extraction of the soaps. The fatty acids were liberated with acid and the free acids were subjected to a

TABLE 1

Characterization of the ester supplements

SAMPLE	UNSATURATED COMPONENTS BY PAPER CHROMATOGRAPHY		IODINE VALUE	MOST UNSATURATED COMPONENT BY ALKALINE ISOMERIZATION
	Major	Minor		
C_{16}	2	2	154.5	tetraenoic acid
C_{18}	2	2	200.5	tetraenoic acid
C_{20}	1 (pentaenoic acid)	2	352	pentaenoic acid
C_{22}	1 (hexaenoic acid)	1	414	hexaenoic acid
Ethyl linoleate	1	0	160.5	dienoic-acid

scheme of low temperature crystallization in light petroleum ether (Skelly F). The fraction which was soluble at −70°C at a 10:1 ratio of solvent to solute was recovered and further fractionated by urea crystallization. Most of the less unsaturated acids formed urea-inclusion compounds at −15°C, and the highly unsaturated acids which comprised the bulk of the sample remained in the filtrate. This fraction was esterified with methanol and then was fractionated according to chain length by distillation through a Podbielniak whirling band column. The chemical characterization of the 4 fractions fed in this study are given in table 1. It should be emphasized that

the fractions fed represent concentrates of the highly unsaturated acids from tuna oil and do not represent the total fatty acids of tuna oil fractionated according to chain length. The C_{22} fraction represents a high concentrate of methyl docosahexaenoate having only one unsaturated component detectable by the paper chromatography (Schlenk et al., '57) although its iodine value, 414, is less than the theoretical 445. The ultraviolet absorption spectra after alkaline isomerization of the 4 fractions indicated that the most highly unsaturated components were tetraene for C_{16}, tetraene for C_{18}, pentaene for C_{20} and hexaene for C_{22}. The hexaenoic acid present in the C_{22} portion was found to be 4, 7, 10, 13, 16, 19-docosahexaenoic acid by the method of von Rudloff ('56).

NUTRITIONAL EXPERIMENT

A fat-free diet was fed ad libitum to 48 male weanling rats of the Sprague-Dawley strain. The diet consisted of 16% vitamin-test casein, 4% α-cellulose, 74% sucrose, 4% Wesson salt mixture ('32), 1% of a mixture of vitamins in casein and 1% of a mixture of choline chloride in casein (Aaes-Jørgensen and Holman, '58). After 11 weeks, the growth rate was rather slow, and distinct dermal symptoms of essential fatty acid deficiency had appeared in all animals. The rats were then divided into 6 equal groups and continued on the same fat-free diet during the period of supplementation. Group 1 received no supplement, group 2 received the C_{16} fraction, group 3 the C_{18} fraction, group 4 the C_{20} fraction, group 5 the C_{22} fraction and group 6 ethyl linoleate. All supplements were administered in doses of 200 mg thrice weekly. The rats were weighed and inspected each week. After 6 weeks of supplementation the rats were killed by ether anaesthesia and the hearts and testes were taken for analysis. Polyunsaturated acid contents were determined using the method of Holman and Hayes ('58).

RESULTS AND DISCUSSION

Biological effects. The effects of the various fatty acid supplements upon the EFA-deficient rats is shown in table 2,

and the condition of the hind feet and tail of representative rats from each group is shown in figure 1. None of the supplements except linoleate was able to cure the dermal scaliness or stimulate the development of the normal yellow-brown skin pigment. The rats from all groups except those fed linoleate had stiff, extremely scaly tails. In figure 1 it will be noticed that the tail from the linoleate animal was relatively straight, whereas the remainder had curled tails which the rats could

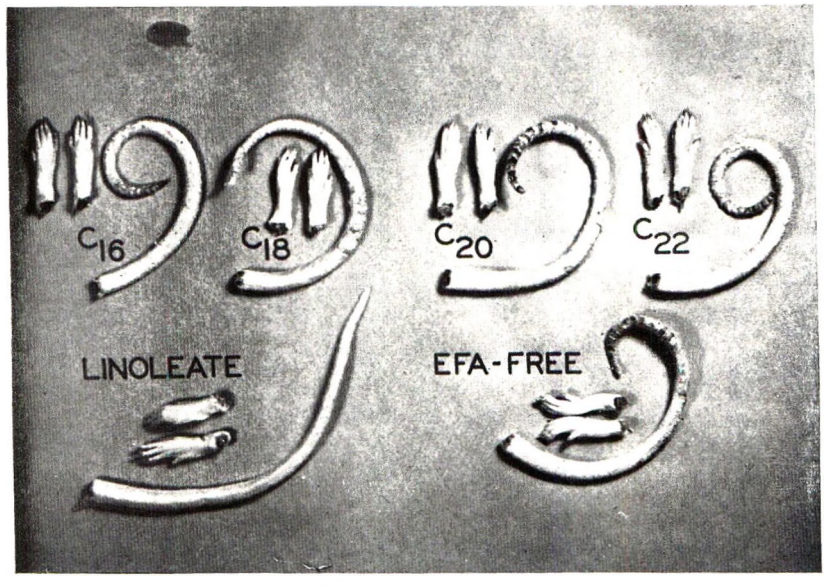

Fig. 1 Tails and feet from representative rats of each group.

not straighten. From these observations it may be concluded that none of the fractions of highly unsaturated fatty acid esters from tuna oil contained sufficient EFA to effect even a partial dermal cure in 6 weeks. Eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid have been shown here to stimulate growth but not to cure the dermal symptoms of EFA deficiency. These observations strengthen the concept that unsaturated fatty acids which have identical terminal structures have similar biological activities. Since all the fractions

from tuna oil stimulated growth except the C_{16} fraction, and none affected the dermal symptoms, it appears that the major polyunsaturated acids in tuna oil resemble linolenic acid rather than linoleic acid in their biological activities.

Fatty acids of heart tissue. The hearts of rats chosen at random from each experimental group were analyzed for their contents of polyunsaturated fatty acids. The data, shown in table 3, indicate a general uniformity in the amount of total polyunsaturated acids in the heart tissue of the several groups.

TABLE 2

Effects of supplements upon EFA-deficient rats

SUPPLEMENT	AV. WT.		AV. DERMAL SCORE [1]		SKIN PIGMENT [2]	CAUDAL NECROSIS [3]
	Initial	Final	Initial	Final		
	gm	*gm*				
None	231 ± 6.3 [4]	262 ± 6.6	1.8	2.0	0	1
C_{16}	231 ± 13.9	265 ± 10.7	1.8	1.8	0	1
C_{18}	232 ± 12.9	291 ± 13.6	1.5	1.2	0	3
C_{20}	231 ± 9.4	305 ± 8.9	1.5	1.8	0	1
C_{22}	231 ± 6.1	287 ± 8.0	1.5	2.0	0	2
Linoleate	235 ± 9.5	296 ± 8.2	2.0	0.4	8	0

[1] Average scores for tail, forelegs and hind legs, each evaluated on a scale of 0 to 3.

[2] Number of rats having skin pigment.

[3] Number of rats having necrosis for at least 1 cm of the tail.

[4] Standard error of the mean.

The group fed ethyl linoleate had somewhat more total polyunsaturated acids in the heart tissue than did the others.

As was expected from previous studies (Rieckehoff, Holman and Burr, '49; Aaes-Jørgensen and Holman, '58) the proportion of dienoic acids was low in the fat-free group and high in the linoleate-fed group, whereas trienoic acid was very high in the fat-free group and low in the linoleate-fed group.

Supplementation with the esters of the unsaturated C_{16} acids resulted in higher contents of pentaenoic and hexaenoic acids than found in the rats fed the fat-free diet. However, the dienoic acid contents of hearts of the C_{16} group remained low and the trienoic acid contents remained high, comparable

to the condition found in EFA-deficient rats. Although pentaenoic and hexaenoic acids were synthesized when the C_{16} fraction was fed, neither the supplement nor the metabolites produced were adequate to meet the need for EFA.

TABLE 3

Polyunsaturated fatty acid content of hearts from rats fed various fatty acid esters

SUPPLEMENT	ACID					
	Hexa-enoic	Penta-enoic	Tetra-enoic	Trienoic	Dienoic	Total
	mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
None	18	6	46	240	29	339
	19	5	44	224	96	388
	13	7	59	261	2	342
	17	13	101	417	— 14	543
C_{16}	55	23	71	288	4	441
	61	27	96	284	34	502
	18	10	44	121	— 23	170
	44	28	74	190	63	399
C_{18}	132	33	67	58	37	327
	85	40	101	109	69	404
	61	39	136	123	14	373
	57	23	61	126	88	355
C_{20} (largely pentaenoic)	115	63	135	54	17	384
	73	61	115	33	11	293
	110	97	198	51	10	466
	102	79	180	59	5	425
C_{22} (largely hexaenoic)	147	46	53	0	5	251
	153	52	57	38	7	307
	248	69	73	106	— 5	491
	140	61	95	131	142	569
Ethyl linoleate	14	49	249	55	158	525
	14	30	162	76	226	508
	16	43	311	68	149	587
	20	59	307	50	143	570

Supplementation of a fat-free diet with the C_{18} fraction induced increased deposition of pentaenoic and hexaenoic acids in the hearts of the rats. However the contents of dienoic acids remained low, suggesting that the dienoic acid of the sample was not incorporated in the tissue and that reduction

of the more highly unsaturated acids of the sample to dienoic did not take place to any great extent. Trienoic acid appeared in the heart tissues in amounts intermediate to those obtained in deficient rats and rats fed linoleate. The hexaenoic and perhaps the pentaenoic acids of the hearts apparently arose from less unsaturated precursors in the supplement fed.

Feeding the C_{20} fraction, which was largely pentaenoic acids, resulted in a pattern of polyunsaturated acids similar to that found in hearts of rats fed the C_{18} fraction. That is, the dienoic content was low, the trienoic acid content was equivalent to that found in linoleate-fed rats, and the tetraenoic, pentaenoic and hexaenoic acids were present in high proportions. Therefore, it may be concluded that the hexaenoic acid of the heart tissue was synthesized from the pentaenoic acid fed, for the supplement contained no hexaenoic acid.

The C_{22} supplement, which was mostly hexaenoic acid, caused the deposition of a high proportion of hexaenoic acid in the hearts of rats. The pentaenoic acid content of the hearts was one magnitude greater than that found in rats fed fat-free diet. However, the tetraenoic acid content was about the same as in the deficient rats, suggesting that hexaenoic and pentaenoic acids were not reduced to provide tetraenoic acid. The trienoic acid content was found to be low, suggesting that abnormal synthesis of trienoic acid did not take place when the highly unsaturated acids were provided, even though these did not relieve the external symptoms of EFA deficiency.

Fatty acids of testis tissue. The testes of EFA-deficient rats were found to contain more trienoic acid and less dienoic acid than those from the rats fed linoleate (table 4). None of the supplements of esters from tuna oil increased the dienoic acid content of testes significantly. The trienoic acid content of testis tissue was found to be abnormally high only in the group fed the C_{16} fraction. All fractions of unsaturated esters increased the hexaenoic acid content of the testis when fed as a supplement to the fat-free diet, suggesting that all contained significant proportions of acids which are precursors of hexaenoic acid and are thus presumably of the linolenic acid family.

TABLE 4

Polyunsaturated fatty acid contents of testes from rats fed various fatty acid esters

SUPPLEMENT	ACID					
	Hexa-enoic	Penta-enoic	Tetra-enoic	Trienoic	Dienoic	Total
	mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
None	5	38	30	152	21	246
	8	63	148	174	10	403
	9	68	143	167	16	403
C_{16}	17	74	108	109	4	312
	15	46	86	101	7	255
	17	45	83	102	4	251
	21	66	90	125	56	358
C_{18}	34	75	108	81	19	317
	48	77	140	113	35	413
	77	41	69	60	5	252
	30	46	87	86	6	255
C_{20}	42	67	90	55	8	262
	36	70	97	65	17	285
	43	102	146	85	12	388
	63	160	193	134	46	596
C_{22}	28	29	51	53	4	165
	55	53	85	91	17	301
	31	35	50	37	5	158
Ethyl linoleate	3	52	49	30	16	150
	15	12	20	101	46	194
	7	128	105	39	20	299
	9	161	144	39	22	375

CONCLUSIONS

From this study it appears that the concentrated highly unsaturated fatty acids of tuna oil do not show complete EFA activity when fed at the level of 600 mg per week. None of the fractions, which were essentially single chain lengths, relieved dermal symptoms of EFA deficiency. None induced the deposition of dienoic acid in heart tissue such as is observed when linoleate is fed. However, only the C_{16} fraction allowed the deposition of abnormally high trienoic acid in heart tissue, such as is observed in EFA deficiency. None of the supple-

ments allowed as great a deposition of total polyunsaturates in heart tissue as did an equal dosage of linoleate. The polyunsaturated acids present in the 4 fractions of tuna oil esters were not the metabolic equivalent of linoleate. With the exception of the C_{16} fraction, all stimulated growth as do both linoleate and linolenate. The lack of growth stimulus, and the high proportion of trienoic acid deposited in the hearts of rats fed the C_{16} fraction suggest that this fraction has a nutritional and metabolic activity less than or different from the other groups of unsaturated acids tested.

No evidence was found in this investigation that any portion of esters of unsaturated acids from tuna oil contained essential fatty acids (linoleic acid family) in detectable amounts. Marked growth-promoting activity was present in the polyunsaturated acids of the 18-, 20- and 22-carbon fractions, suggesting that their acids were of the linolenic acid family.

SUMMARY

Highly unsaturated acids from tuna oil were concentrated and their esters were separated according to chain length by fractional distillation. Each chain-length fraction was fed as a supplement to fat-deficient rats and the effects were compared with those induced by ethyl linoleate.

None of the fractions containing fatty acids of 16, 18, 20 or 22 carbon atoms in length relieved the dermal symptoms of EFA deficiency. However, all the fractions except the 16-carbon fraction showed marked stimulation of growth. Only the 16-carbon fraction allowed the deposition of abnormally high amounts of trienoic acid in heart and testis tissue.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of F. J. Pusch, P. A. Rusley and Arlen E. Viste.

LITERATURE CITED

Aaes-Jørgensen, E., and R. T. Holman 1958 Essential fatty acid activity. I. Content of polyenoic acids in testes and heart as an indicator of EFA status. J. Nutrition, *65:* 633.

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

RIECKEHOFF, I. G., R. T. HOLMAN AND G. O. BURR 1949 Polyethenoid fatty acid metabolism. Effect of dietary fat on polyethenoid fatty acids of rat tissues. Archives Biochem., *20:* 331.

SCHLENK, H., J. L. GELLERMAN, J. A. TILLOTSON AND H. K. MANGOLD 1957 Paper chromatography of lipides. J. Am. Oil Chem. Soc., *34:* 377.

THOMASSON, H. J. 1953 Biological standardization of essential fatty acids (a new method). Int. Rev. Vitamin Res., *35:* 62.

VON RUDLOFF, E. 1956 Periodate-permanganate oxidation. IV. Determination of the position of double bonds in unsaturated fatty acids and esters. J. Am. Oil Chem. Soc., *33:* 126.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

ANTINECROGENIC PROPERTY OF TORULA YEAST TREATED IN VARIOUS WAYS[1]

W. J. KELLEHER, C. GITLER,[2] M. L. SUNDE, M. J. JOHNSON AND C. A. BAUMANN

Departments of Biochemistry and Poultry Husbandry, University of Wisconsin, Madison

(Received for publication August 14, 1958)

In a previous study (Gitler et al., '57), vitamin E-deficient diets containing either *Saccharomyces cerevisiae* or *Torulopsis utilis* grown on a synthetic medium were necrogenic when fed to rats, whereas these yeasts prevented hepatic necrosis when grown or incubated in the presence of beer wort. The present study deals with the antinecrogenic properties of torula yeast grown or treated with a variety of natural materials under well-defined conditions. While these studies were in progress, Schwarz and Foltz ('57) reported that selenium prevents necrosis in rats, and Schwarz et al. ('57) and Patterson et al. ('57) reported that it prevents exudative diathesis in chicks. As a result, our studies were extended to the incorporation of selenium by *T. utilis* and to a preliminary evaluation of the relative effectiveness of inorganic selenite and the selenium bound in yeast.

FERMENTATION METHODS

Torulopsis utilis was used in all experiments. The basal synthetic medium consisted of the following in grams per liter:

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Sulphite Pulp Manufacturer's Research League, Appleton, Wisconsin.

[2] Present address: Instituto Nacional de la Nutricion, Mexico D. F., Mexico.

glucose (Cerelose) 50; dibasic ammonium citrate, 20; dibasic potassium phosphate, 2; and magnesium sulfate·$7H_2O$, 1.25. Trace metals added in micrograms per liter were iron (ferrous ammonium citrate), 100; and zinc (sulfate), 20. The pH was adjusted to 6.0 with potassium hydroxide. General Electric silicone Antifoam 60 was added at a level of 50 p.p.m. The glucose and the salt mixture were sterilized separately at 120°C for 30 minutes, and the temperature was controlled at 30°C for all fermentations.

An inoculum of approximately 2% of the expected yield was grown in 500 ml Erlenmeyer flasks, each containing 20 ml of the basal medium. These were shaken at 253 r.p.m. on a Gump rotary shaker that described a circle 2¼ inches in diameter. The yeast was then grown in 30-liter fermentors similar to those described by Hosler and Johnson ('53). The agitator speed was 520 r.p.m., and air was supplied through a sparger at 1.4 volumes per volume of medium per minute.

For larger amounts of yeast, the contents of a 30-liter fermentor was used as an inoculum (in 10% of the expected yield) for fermentations in a 50 gallon glass-lined fermentor (Buelow and Johnson, '52), which contained an additional 5″ × 10″ stainless steel baffle directly opposite the original baffle to prevent vortexing of the medium. The agitator was driven at 200 r.p.m. and air was supplied through the sparger at 1.6 volumes of air per volume of medium per minute.

Samples taken from the fermentations were analyzed for glucose by the method of Shaffer and Somogyi ('33) and for ethanol by the method of Maxon and Johnson ('53). Ethanol determinations were discontinued after it was apparent that no ethanol was produced under the conditions employed. The pH of each sample was measured and cell yield was determined turbidimetrically at 630 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer.

Harvesting procedures were not begun until glucose analyses showed complete exhaustion of glucose from the medium. Yields of yeast on the synthetic medium were regularly over

50%, based on the weight of glucose utilized. The yeast cells were separated from the fermentation broth, the various treatment media, or the wash water by centrifugation at 15,000 r.p.m. with a no. 16 Sharples super-centrifuge. The yeast was dried from a final slurry on a Buflovak laboratory vacuum double drum drier at atmospheric pressure. The drums were heated to 142°C and were rotated at a speed of 10 r.p.m. Acetone drying was also employed for some samples.

The treatment media described in table 1 were prepared by combining the indicated materials, adjusting the pH to 4.0 with either potassium hydroxide or phosphoric acid, and removing the bulk of insoluble solids by passage through a Sharples centrifuge. Yeast grown on the synthetic medium was harvested, added to the treatment media contained in 30-liter fermentors, and incubated either aerobically or anaerobically. One lot of yeast was grown on the synthetic medium to which corn steep liquor had been added.

For the incorporation of selenium into the yeast, the concentration of $MgSO_4 \cdot 7H_2O$ in the basal medium was reduced to 0.25 gm/liter and selenate was added at a concentration of 4 mg of Se per liter. This modification was deemed desirable because sulfate inhibits selenium toxicity in *S. cerevisiae* (Fels and Cheldelin, '49). The acetone-dried yeast crop was analyzed for selenium by the method of Horn ('34).

Se^{75} was incorporated into yeast by adding radioactive selenite, together with carrier, to the low-sulfate basal medium at a total concentration of 7.15 mg of Se per liter; the addition was made only two hours before the expected end of the fermentation in an attempt to minimize reduction of the selenite to elemental selenium. Removal of any elemental selenium that might have been formed was accomplished by adjusting the pH of the medium to 10 with potassium hydroxide, adding sodium sulfite to approximately 1%, and agitating the contents of the fermentor for three minutes prior to harvesting. The harvested yeast crop was washed 5 times with 5-liter portions of distilled water. Part of the final washed

product was drum dried and part was dried with acetone. Samples were taken before and after all these operations to determine the radioactivity present.

Radioactivity determinations were made with a thin window counter. Aliquots of samples diluted with known amounts of gelatin solutions (1% gelatin in 0.1 N NaOH) were pipetted onto 5 cm^2 copper planchets and dried at about 40°C. Corrections were made for self-absorption.

BIOASSAY

The procedure used in the necrosis assay was modified from that previously described (Gitler et al., '57) in order to decrease the amount of test material used per trial. Weanling rats were depleted of vitamin E on a casein-containing diet[3] for one week (permitting growth), then on a deficient diet[4] containing 40% of torula-SSL[5] yeast for two weeks, this procedure bringing the rats to within an average of 10 days from death. At this point, the rats were placed on experimental diets in which the yeast or other materials under test replaced part of the torula-SSL.

Corn steep solids were obtained from corn steep liquor by drying in a vacuum oven or by forming a slurry with dextrin and drying at 50°C in a constant temperature drier. Barley malt was prepared for feeding by finely grinding commercial malt in a hammer mill and extracting for 24 hours with petroleum ether (Skellysolve B) in a Soxhlet apparatus.

EXPERIMENTAL

Sources of protective factor. Table 1 shows the survival of rats fed the low-vitamin E diet containing the various batches

[3] The depletion diet consisted of the following in grams per kilogram: casein 200; cooked starch ("dextrin") 630; Wesson salts 40; lard 90; codliver oil 10; and cellulose 30. The vitamins in milligrams per kilogram were: choline 2000; inositol 1000; calcium pantothenate 20; niacin 10; menadione 4; riboflavin 3; thiamine 2; pyridoxine 2.5; biotin 0.1; folic acid 0.2; and vitamin B_{12} 0.001.

[4] Torula-SSL replaced casein at an equivalent level of protein ($N \times 6.25$). Dextrin was appropriately adjusted to bring the total weight to one kilogram.

[5] Torula-SSL yeast (*Torulopsis utilis*) was feed grade yeast grown aerobically on spent sulfite liquor. Obtained through the courtesy of Dr. P. L. Pavcek, Lake States Yeast Corp., Inc., Rhinelander, Wisconsin.

TABLE 1

Effect of medium on the anti-necrogenic properties of T. utilis

LINE NO.	PRODUCTION OF YEAST			NECROSIS ASSAY			
	Growth medium [1]	Subsequent treatment [2]	Duration of treatment	Wet weight of treated yeast [3]	Exp. no.	Test yeast in diet [4]	Survival
			min.	*gm*		*gm/kg*	
1	SSL [5]	—	—	—	II–V	400	0/20
2	Synthetic [6]	—	—	—	III	400	0/5
3	Synthetic	8.5 l beer wort [7] (aerobic)	275	750	III	100	2/5
					III	50	0/5
4	Synthetic	1.5 l beer wort water to 8.5 l	335	750	III	70	2/5
5	Synthetic	1 kg whey solids [8] 300 gm glucose water to 10 l	310	500	III	70	1/5
6	Synthetic	30 gm whey solids, 300 gm glucose water to 10 l	330	500	III	70	2/5
7	Synthetic	2 l corn steep liquor, 300 gm glucose water to 10 l	240	500	III	70	3/5
8	Synthetic	60 ml corn steep liquor 300 gm glucose water to 10 l	280	500	III	50	2/5
					V	100	1/5
9	Synthetic	180 ml corn steep liquor 900 gm glucose water to 15 l (aerobic)	240	2000	IV	50	0/5
					IV	100	1/5
					V	100	3/5
10	Synthetic containing 15 ml corn steep liquor per liter	none	—	—	IV	25	0/5
					IV	50	1/5
					IV	100	0/5
					V	100	1/5
11	Beer wort medium [9]	none	—	—	II	50	2/5

[1] Aerobic conditions were used for all growth media.
[2] All treatments were anaerobic except where otherwise indicated.
[3] Approximately 75% water.
[4] Dry yeast added at the expense of the torula-SSL in the basal diet.
[5] Commercial torula yeast grown on spent sulfite liquor.
[6] For composition, see text under methods.
[7] Obtained from the Barley and Malt Lab., U.S. Department of Agriculture, Madison, Wisconsn.
[8] Sweet dairy whey (granular, spray process, U.S. extra crude) produced by Western Condensing Co., Appleton, Wisconsin.
[9] *T. utilis* grown aerobically on beer wort (Gitler et al., '57).

of treated torula yeast. Survival was no better on diets containing the yeast grown on the synthetic medium than with commerical torula-SSL grown on spent sulfite liquor (table 1, lines 1 and 2) or the samples of torula grown by others on synthetic media (Gitler et al., '57).

Table 1 also shows the results obtained when beer wort, whey solids, or corn steep liquor were added to the medium either during growth of the yeast or during a subsequent treatment under aerobic or anaerobic conditions. The yeasts resulting from such treatments exerted some protection against necrosis when incorporated into the necrogenic basal diets (table 1, lines 3 to 10), but protection was never 100% at the low levels fed.

The protective activity of yeast treated with, or grown in the presence of beer wort (table 1, lines 3, 4, and 11) was not significantly different from that of yeast treated with, or grown in the presence of other natural materials (table 1, lines 5 to 10). Variation of the concentration of the treatment media or the condition of aeration showed no marked effect on the antinecrogenic property of the yeasts. None of these mild experimental treatments produced a yeast with protective activity comparable to that of commercial brewers' yeast.

The materials that conveyed protection when added to the fermentation media also proved to be protective when added directly to the necrogenic basal diet. Barley malt and whey solids (table 2, lines 2 and 4) were found to be protective at levels of 5% in the diet; corn steep solids, prepared as described under "methods," were protective in amounts as low as 3% of the diet (table 2, lines 5, 6, and 7). Barley malt, extracted with petroleum ether (Skellysolve B) was equivalent in its protective property to untreated barley malt (table 2, line 3). The alkaline ash prepared from corn steep solids or brewers' yeast was protective in both cases but less so than an equivalent amount of the natural material (table 2, lines 8 and 11).

Action of selenium. In agreement with Schwarz and Foltz ('57), it was found that the feeding of low levels of sodium selenite markedly prolonged the lives of rats fed a necrogenic diet devoid of vitamin E (table 3, lines 12 and 13). The addition of selenate to the low-sulfate basal medium for the growth of torula yeast resulted in a yeast that was also highly active

TABLE 2

The prevention of liver necrosis by various natural materials

LINE NO.	EXP. NO.	ADDITIONS TO THE TORULA-SSL BASAL DIET [1]	LEVEL IN THE DIET	SURVIVAL
			gm/kg	
1	I–VI	None	—	0/30
2	I	Barley malt	50	2/5
3	II	Barley malt [2]	50	1/5
4	III	Whey solids	50	1/5
5	III	Corn steep solids [3]	50	4/5
6	IV	Corn steep solids [3]	30	1/5
7	V	Corn steep solids [3]	30	2/5
8	V	Alkaline ash of corn steep solids [4]	—	3/5
9	II–VI	Brewers' by-product yeast [5]	50	5/5
10	II	Brewers' by-product yeast [6]	25	2/5
11	V	Alkaline ash of brewers' by-product yeast [6]	—	3/5

[1] For composition see text footnote 2. Additions were made at the expense of the torula-SSL yeast.

[2] Extracted for 24 hours with petroleum ether (Skellysolve B) in a Soxhlet extractor.

[3] For preparation see text under methods.

[4] Added at a level equivalent to 50 gm corn steep solids.

[5] Brewers' by-product yeast (*Saccharomyces cerevisiae*) obtained from the Jos. Schlitz Brewing Co., and from the Pabst Laboratories, Milwaukee, Wis.

[6] Added at a level equivalent to 100 gm of brewers' by-product yeast.

in prolonging the life of rats on a necrogenic diet (table 3, lines 6 to 9). The results do not, however, indicate whether the activity of such yeasts was due to organic selenium (factor 3?) or to inorganic selenium that resisted efforts to remove it by washing. The selenium content of the yeast washed 4 times was 68.7% of that of the yeast washed only twice. Hence, there is doubt as to the effectiveness of the removal of inor-

TABLE 3

The prevention of liver necrosis by various forms of selenium

LINE NO.	ADDITIONS TO THE TORULA BASAL DIET [1]	Se ADDED OVER THAT IN BASAL DIET	SURVIVAL
		μg/kg	
1	None	0	0/20
2	1 mg Se [2] — 5 mg As [3]	1000	5/5
3	1 mg Se [2] — 10 mg As [3]	1000	5/5
4	50 gm brewers' yeast [4] — 5 mg As [3]	(600) [5]	5/5
5	50 gm brewers' yeast [4] — 10 mg As [3]	(600)	5/5
6	5 gm torula-Se-2X [6]	(161)	5/5
7	10 gm torula-Se-2X [6]	(323)	5/5
8	5 gm torula-Se-4X [7]	(111)	5/5
9	10 gm torula-Se-4X [7]	(222)	5/5
10	0.166 gm torula-Se^{75} [8]	30	5/5
11	0.083 gm torula-Se^{75} [8]	15	3/5
12	Sodium selenite	31	4/5
13	Sodium selenite	15	5/6
14	15 mg α-tocopherol	0	5/5
15	7 mg α-tocopherol	0	5/5

[1] Additions were made at the expense of torula-SSL. For composition of the basal diet, see text footnote 2.

[2] Added as sodium selenite.

[3] Added as sodium arsenite.

[4] Commercial brewers' by-product yeast.

[5] The values within parentheses were obtained by the method of Horn ('34). The results are probably too high since the reagent responds to other elements.

[6] *T. utilis* grown on the modified synthetic medium containing selenate and washed twice with water.

[7] Same as 6, but washed 4 times.

[8] *T. utilis* grown on the modified synthetic medium containing radioactive selenite, treated with sodium sulfite at alkaline pH, and washed 5 times with distilled water.

ganic selenium, whether as the soluble selenate inside the cell or as insoluble elemental selenium inside or outside the cell.

Table 4 shows that the addition of radioactive selenite to the low-sulfate synthetic medium yielded a yeast of substantial selenium content even after treatment with alkaline sodium sulfite solution and repeated washing with water. No selenium could be detected in the final wash water (table 4, line 6). Yeast that was washed only with water had a significantly higher selenium content (table 4, line 2). Variations in the method of drying (oven, acetone, or drum drying) were without

effect on the selenium content of the product (table 4, lines 3 to 5).

Radioactive torula yeast fed at a level equivalent to 30 μg of selenium per kilogram of basal diet, imparted complete protection (table 3, line 10), while at the 15 μg equivalent, protection was only partial (table 3, line 11). Within the limitations imposed by the small number of animals used, the

TABLE 4

Incorporation of Se^{75} into T. utilis [1]

LINE NO.	SAMPLE [2]	RADIOACTIVITY OF SAMPLE [3]	Se CONTENT OF YEAST
		c.p.m./gm	*μg/gm*
1	50 μg selenium [4]	8.72×10^7	—
2	12.5 mg yeast [5] washed 4 times with dist. water	2.56×10^4	294
3	24.2 mg of product,[6] oven dried at 105°C	1.56×10^4	179
4	29.0 mg drum dried product [6]	1.56×10^4	179
5	31.8 mg acetone dried product [6]	1.58×10^4	182
6	0.66 ml final wash water from sulfite treated yeast	0	0

[1] Radioactive Se (7.15 mg) as sodium selenite added per liter of medium two hours before harvesting.

[2] Weights and volumes corrected for added NaOH and gelatin.

[3] Corrected for background and self absorption.

[4] Aliquot taken from the radioactive selenite solution added to the fermentation.

[5] Grown in the presence of Se^{76} and untreated with sodium sulfite.

[6] *T. utilis* grown in the presence of Se^{75}, treated with sodium sulfite, and washed with distilled water.

activity of the selenized yeast appeared to be very similar to that of an equivalent amount of selenium as sodium selenite (table 3, lines 10 to 13).

The addition of 5 or 10 mg of arsenic (as sodium arsenite) per kilogram, to diets containing protective levels of brewers' yeast or selenite failed to prevent protection in all cases (table 3, lines 2 to 5). The inclusion of 7 or 15 mg of α-tocopherol per kilogram of basal diet conferred complete protection at both levels (table 3, lines 14 and 15).

DISCUSSION

These experiments show that a nonprotective torula yeast can be rendered protective against dietary liver necrosis by treatment with, or growth in the presence of beer wort, corn steep liquor, or whey solids. Since these materials were also protective when added directly to a necrogenic diet, it follows that an active substance was present in these materials and that the living yeast cell is not necessary for its synthesis, a conclusion also implicit in the observation that inorganic selenium added directly to the diet of rats prevents hepatic necrosis (table 3; Schwarz and Foltz, '57).

The decrease in activity resulting from the alkaline ashing of corn steep solids or of brewers' yeast suggests either that selenium was being lost in the ashing process or that it was being converted to a less active form. The lack of a reliable method for the determination of selenium at the very low levels encountered in the natural materials used precluded a direct approach to this problem. Torula yeast containing unlabeled selenium was produced, and was found to be completely protective when fed to rats at low levels in the basal diet. But any attempt to compare the effectiveness of selenium as it occurs in such yeast with inorganic selenium would have been complicated by the possibility that elemental selenium, formed by reduction of the selenate, was present in or on the harvested yeast cells.

Radioactive selenite was utilized to produce a yeast that was highly active in prolonging the lives of rats when added to a necrogenic basal diet, and the selenium content of this yeast could be estimated readily by a radioactivity determination. Residual selenite and any red elemental selenium that might have been formed was removed by treatment with alkaline sodium sulfite solution followed by several washings with distilled water. Gray elemental selenium would not be removed by this treatment but other experiments have shown that it is not formed under the conditions existing in the fermentation medium. The protective activity of this yeast

was similar to that of sodium selenite fed at equivalent levels of selenium.

SUMMARY

1. *Torulopsis utilis* was grown on a synthetic medium and treated with various materials either during growth or during a subsequent incubation period. The washed and dried yeasts were then added to a low-vitamin E diet and fed to rats.

2. When the yeast was grown on the unsupplemented medium, all rats died of hepatic necrosis. Substantial protection was conferred by yeasts grown or incubated in the presence of beer wort, corn steep liquor, or whey solids. These materials were also protective when added directly to the necrosis-producing diet.

3. Survival was also prolonged by feeding selenium or the alkaline ash of corn steep liquor or of brewers' yeast. The ash of brewers' yeast, however, appeared to be somewhat less active than the original yeast.

4. Torula yeast grown in the presence of Se^{75} and treated with sodium sulfite at an alkaline pH prior to washing, protected rats to approximately the same degree as sodium selenite fed at equivalent levels of selenium.

LITERATURE CITED

BUELOW, G. H., AND M. J. JOHNSON 1952 Effect of aeration on citric acid production in 50-gallon tanks. Ind. Eng. Chem., *44:* 2945.

FELS, I. G., AND V. H. CHELDELIN 1949 Selenate inhibition studies III. The role of sulfate in selenate toxicity in yeast. Arch. Biochem., *22:* 402.

GITLER, C., M. L. SUNDE AND C. A. BAUMANN 1957 Necrogenic potency of yeasts grown on various media. J. Nutrition, *63:* 399.

HORN, M. J. 1934 Determination of selenium using codeine. Ind. Eng. Chem. Anal. Ed., *6:* 34.

HOSLER, P., AND M. J. JOHNSON 1953 Penicillin from chemically defined media. Ind. Eng. Chem., *45:* 871.

MAXON, W. D., AND M. J. JOHNSON 1953 Aeration studies on propagation of baker's yeast. Ind. Eng. Chem., *45:* 2554.

PATTERSON, E. L., R. MILSTREY AND E. L. R. STOKSTAD 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., *95:* 617.

SCHWARZ, K., AND C. M. FOLTZ 1957 Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., *79:* 3292.

SCHWARZ, K., J. G. BIERI, G. M. BRIGGS AND M. L. SCOTT 1957 Prevention of exudative diathesis in chicks by Factor 3 and selenium. Proc. Soc. Exp. Biol. Med., *95:* 621.

SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determinations. J. Biol. Chem., *100:* 695.

SERUM ALKALINE PHOSPHATASE ACTIVITY IN NORMAL AND MANGANESE-DEFICIENT DEVELOPING RATS [1,2]

LUCILLE S. HURLEY, GLADYS J. EVERSON AND JEAN F. GEIGER

Department of Home Economics, University of California, Davis

(Received for publication August 22, 1958)

INTRODUCTION

The effect of manganese deficiency upon the alkaline phosphatase activity of several tissues has been studied in a number of species. Wiese et al. ('39) observed lower phosphatase activity in both the plasma and the bone of perotic chicks than in their manganese-supplemented controls. Combs, Norris and Heuser ('42) also reported a decrease in bone phosphatase in manganese-deficient chicks. In the rat, however, Wachtel and his co-workers ('43) found no difference in bone phosphatase due to manganese deficiency, but did observe a two to threefold increase in the activity of this enzyme in the blood serum. Conflicting results with regard to bone were reported by Amdur, Norris and Heuser ('45), who concluded that manganese deficiency in the rat produced a decreased phosphatase activity in this tissue. Studies of manganese deficiency in the rabbit showed a lowered alkaline phosphatase activity of the ulna, but no change in the blood serum (Ellis et al., '47). In the duck, however, Bernard and Demer ('52) found that the alkaline phosphatase activity of serum was reduced by manganese deficiency, and Van Reen and Pearson ('55) observed a reduced activity in the plasma, liver, kidney and heart as well.

[1] This investigation was supported in part by research grant No. A-1340 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

[2] Presented in part at the 22nd annual meeting of the American Institute of Nutrition, April, 1958 (Hurley, Everson and Geiger, '58a).

In none of the investigations cited was alkaline phosphatase activity studied in the offspring of manganese-deficient animals. In this laboratory, the congenital nature of ataxia in the young of female rats deficient in manganese has recently been studied (Hurley, Everson and Geiger, '58b). During the course of these investigations, it became of interest to determine the serum level of alkaline phosphatase in the congenitally deficient animals as well as to investigate the correlation between ataxia and alkaline phosphatase activity.

Serum alkaline phosphatase activity was therefore measured in the young of manganese-deficient and -supplemented rats, from the 18th [3] day of gestation to 28 days after birth.

EXPERIMENTAL

Weanling female rats of the Sprague-Dawley strain were purchased from commercial sources and maintained on a manganese-deficient fresh milk ration fortified with minerals and vitamins, as described previously (Hurley, Everson and Geiger, '58b). After a body weight of 180 gm was reached, the animals were mated with normal stock males. The serum alkaline phosphatase activity of the resulting young was compared with that of young produced by females receiving a similar diet containing added manganese.

Blood samples were obtained in some cases by bleeding from the tail; in most cases, however, the animals were sacrificed by decapitation, and blood from the neck was used. For samples of fetal blood, the mother was killed by decapitation, the young were removed quickly from the uterus, and blood was obtained from the umbilical cord.

Serum alkaline phosphatase was determined by the method of Bessey, Lowry and Brock ('46).

RESULTS

The results are summarized in figure 1. The data include samples from both males and females, since there was no ap-

[3] The day of finding sperm in the vaginal smear was considered the first day of gestation.

parent sex difference. The lines were drawn by inspection of the means, merely to indicate the trend. No significant differences were observed between the alkaline phosphatase activities of the manganese-supplemented as contrasted with the deficient rats at any of the ages examined, although in the newborn, the enzyme activity appeared to be slightly but

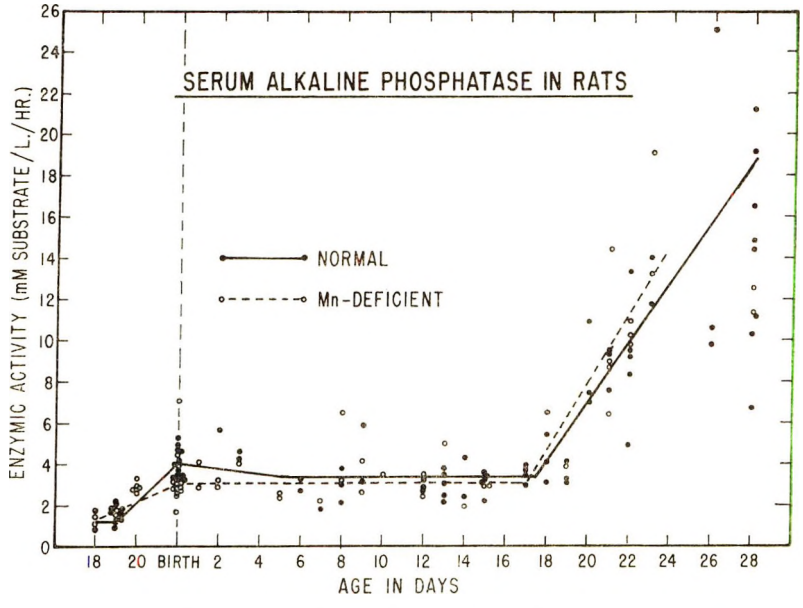

Fig. 1 Serum alkaline phosphatase in normal and manganese-deficient rats. Alkaline phosphatase activity is expressed as millimoles of p-nitrophenyl phosphate hydrolyzed per liter of serum per hour.

insignificantly ($P > 0.02$)[4] lower in the deficient animals. Neither was there any correlation between ataxia and the extreme values. Both groups, however, showed an increase in enzyme activity from the 18th day of gestation to birth, a plateau, and a sharp rise beginning about the 18th day of age.

DISCUSSION

The data clearly indicate that under the present conditions manganese deficiency in the maternal diet had no influence on

[4] P value estimated by Student's "t" test. Fisher, Statistical Methods for Research Workers.

serum alkaline phosphatase activity in the young. This finding is in accordance with observations in guinea pig young, which also showed no change in serum alkaline phosphatase as a result of manganese deficiency in the maternal diet (Everson and Hurley, '58). A comparison of these observations with those previously cited shows that these data, with respect to the influence of manganese deficiency, agree only with the findings of Ellis et al. ('47).

The changes reported here in serum alkaline phosphatase activity in normal young as a function of age are in general agreement with the findings of Weil ('41), who determined plasma phosphatase activity in three litters of young rats from two to 40 days of age. No change in activity was noted until the animals were about 30 days old. In the present report, however, phosphatase activity increased sharply at 18 days of age. No explanation for this difference is at hand. Since Weil also fed cow's milk, the difference in results cannot be attributed to dietary effects. It is possible that the discrepancy may be due to a difference in the rates of maturation of two strains of animals, but since Weil does not state the strain used, this possibility cannot be evaluated.

Serum phosphatase activity appears to follow a different course in the developing rat from that observed in other species during the first weeks of life. In chicks, plasma phosphatase fell during the first 13 days after hatching (Wiese et al., '39).

In the suckling pig, like the chick, serum alkaline phosphatase activity was high at birth and fell considerably thereafter (Young and Underdahl, '48). In nursing puppies, on the other hand, Bodansky ('34) observed that serum phosphatase rose during the first 24 hours after birth, then decreased rapidly for a few days subsequent to a slower drop to two weeks of age. Stearns and Warweg ('33), studying human infants, reported similar findings. Serum phosphatase was low at birth, but rose abruptly to a maximum during the first month of life and remained fairly high until during the second year. Barnes and Munks ('40) also noted a rise in the serum phosphatase

activity of human infants from a low level at birth. In the young guinea pig, on the other hand, serum alkaline phosphatase was somewhat lower at 4 and at 21 days of age than at birth (Everson and Hurley, '58).

The origin of the serum alkaline phosphatase remains obscure (Moog, '46). In this connection it is of interest to note, with due regard to the difficulties of inter-species comparisons, that the changes in serum phosphatase activities with age reported in the present publication follow a pattern resembling in many respects that observed for intestinal alkaline phosphatase in the mouse (Moog, '51).

SUMMARY

Serum alkaline phosphatase activity was determined in the young of normal and manganese-deficient female rats maintained on fortified fresh milk diets, from the 18th day of gestation to 28 days after birth.

Manganese deficiency had no significant effect upon the serum phosphatase at any of the ages examined. Both manganese-supplemented and deficient young exhibited a rise in alkaline phosphatase activity from the 18th day of gestation to birth followed by a plateau to 18 days, when a sharp rise began which continued to 28 days of age.

LITERATURE CITED

AMDUR, M. O., L. C. NORRIS AND G. F. HEUSER 1945 The need for manganese in bone development by the rat. Proc. Soc. Exp. Biol. Med., *59:* 254.

BARNES, D. J., AND B. MUNKS 1940 Serum phosphatase, calcium and phosphorus values in infancy. Ibid., *44:* 327.

BERNARD, R., AND J. M. DEMER 1952 Le manganèse dans le nutrition du caneton. Rev. can. biol., *11:* 147.

BESSEY, O. A., O. H. LOWRY AND M. J. BROCK 1946 A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem., *164:* 321.

BODANSKY, A. 1934 Phosphatase studies. VII. Inorganic phosphorus and phosphatase of the serum in new born puppies. Ibid., *104:* 717.

COMBS, G. F., L. C. NORRIS AND G. F. HEUSER 1942 The interrelationship of manganese, phosphatase and vitamin D in bone development. J. Nutrition, *23:* 131.

ELLIS, G. H., S. E. SMITH AND E. M. GATES 1947 Further studies of manganese deficiency in the rabbit. Ibid., *34:* 21.

EVERSON, G. J., AND L. S. HURLEY 1958 Importance of manganese for normal embryonic development of guinea pigs. Federation Proc., *17:* 476.

HURLEY, L. S., G. J. EVERSON AND J. F. GEIGER 1958a Teratogenic aspects of manganese deficiency in rats. Ibid., *17:* 480.

———— 1958b Manganese deficiency in rats: Congenital nature of ataxia. J. Nutrition, *66:* 309.

MOOG, F. 1946 The physiological significance of the phosphomonoesterases. Biol. Rev., *21:* 41.

———— 1951 The functional differentiation of the small intestine. II. The differentiation of alkaline phosphomonoesterase in the duodenum of the mouse. J. Exp. Zool., *118:* 187.

STEARNS, G., AND E. WARWEG 1933 Studies of phosphorus of blood. I. The partition of phosphorus in whole blood and serum, the serum calcium and plasma phosphatase from birth to maturity. J. Biol. Chem., *102:* 749.

VAN REEN, R., AND P. B. PEARSON 1955 Manganese deficiency in the duck. J. Nutrition, *55:* 225.

WACHTEL, L. W., C. A. ELVEHJEM AND E. B. HART 1943 Studies on the physiology of manganese in the rat. Amer. J. Physiol., *140:* 72.

WEIL, L. 1941 Studies on plasma phosphatase activity during embryonic and tumor growth. J. Biol. Chem., *138:* 375.

WIESE, A. C., B. C. JOHNSON, C. A. ELVEHJEM, E. B. HART AND J. G. HALPIN 1939 A study of blood and bone phosphatase in chick perosis. Ibid., *127:* 411.

YOUNG, G. A., JR., AND N. R. UNDERDAHL 1948 Phosphatase activity in suckling pigs. Ibid., *172:* 759.

THE RETENTION OF VITAMIN B_6 IN MEAT DURING COOKING [1]

C. H. LUSHBOUGH, JEAN M. WEICHMAN AND B. S. SCHWEIGERT
Division of Biochemistry and Nutrition, American Meat Institute Foundation, and Department of Biochemistry, The University of Chicago, Chicago, Illinois

(Received for publication August 25, 1958)

The nutritional importance of vitamin B_6 has become increasingly evident during recent years. Certain anemias, sensory neuritis, skin lesions, and convulsions in infants may develop when the dietary intake of vitamin B_6 is inadequate (Coursin, '54; Tower, '56). Since meat is one of the major sources of vitamin B_6 in the diet, the apparent low retention of this vitamin in cooked meat (15 to 40%) reported earlier (McIntire et al., '44) appeared to merit further investigation.

This study was undertaken to re-evaluate the vitamin B_6 content of fresh muscle and organ meats and the retention of vitamin B_6 in cooked and processed meats. The assay of natural foods for vitamin B_6 is complicated by the occurrence of the vitamin in several natural forms (pyridoxine, pyridoxal and pyridoxamine) which have varying biological activities for different experimental animals and microorganisms. Both a microbiological yeast assay and a rat bioassay were employed in this research, and fresh and cooked samples of beef, lamb, veal and pork, and several processed meat products were tested for vitamin B_6 content.

EXPERIMENTAL METHODS

Paired cuts of meat from the same carcass were selected, and one of each pair was roasted until well-done (oven temper-

[1] This work was supported in part by a grant-in-aid from the National Live Stock and Meat Board; Journal Paper no. 166, American Meat Institute Foundation.

ature, 325°F.; internal temperatures: beef, 170°F.; veal and lamb, 180°F.; pork, 185°F.). The fresh and cooked cuts were then boned and all visible fat removed. The meat was ground and proximate analyses performed. Several organ meats and processed meats were also included in these studies.

The yeast assay employed was that of Atkin et al. ('43) using *Saccharomyces carlsbergensis* (ATCC 4228) as the test organism. The stock culture medium employed consisted of 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% glucose and 1.5% agar. The yeast was serially transferred twice in this medium and incubated 24 hrs. at 24°C. On the third day, the inoculum was prepared by adding three wire loopfulls of the rapidly growing yeast to 10 ml of sterile saline solution. One drop of this inoculum was then added to each 50 ml Erlenmeyer assay flask. The standard growth curve was obtained using 0.5 ml increments (from 0 to 4 ml) of standard pyridoxine·HCl solution (0.01 μg/ml). Duplicate flasks of standards and samples were incubated in the dark, without shaking, and the 24 hr. growth response was measured turbidimetrically at 660 mμ. Use of the 24 hr. period gave improved growth response and a more sensitive standard curve than that obtained using the 18 hr. period.

The rat bioassay methods employed for vitamin B_6 were described previously by Sarma et al. ('46). Using 7 male weanling rats per group, a two-week depletion period was found adequate, and this was followed by the three-week experimental period. "Vitamin-free" casein [2] was used as the source of protein in the basal ration, and 25 μg of folic acid and 400 mg of L-cystine were added per 100 gm of ration. Corn oil (2.82%) and fish liver oil (0.18%; 2,250 I.U. vitamin A, 300 I.U. vitamin D/gm) were added directly to the ration, to make 3% of added fat. The standard growth curve was obtained using 0, 15, 30, 45, 60, 75, 120, and 240 μg of pyridoxine·HCl/100 gm of ration. The pyridoxine·HCl was dissolved in 70% ethanol (100 μg/ml) and added to the animal

[2] Nutritional Biochemicals Corporation.

rations in appropriate amounts. Each meat sample tested was added to the rations at two levels approximating 30 and 60 μg of vitamin B_6/100 gm as determined by yeast assay.

RESULTS AND DISCUSSION

A typical standard growth curve obtained with the *S. carlsbergensis* assay is shown in figure 1. Duplicate hydrolysates of samples were tested on the same day and again the following day, and samples within an assay were tested at each

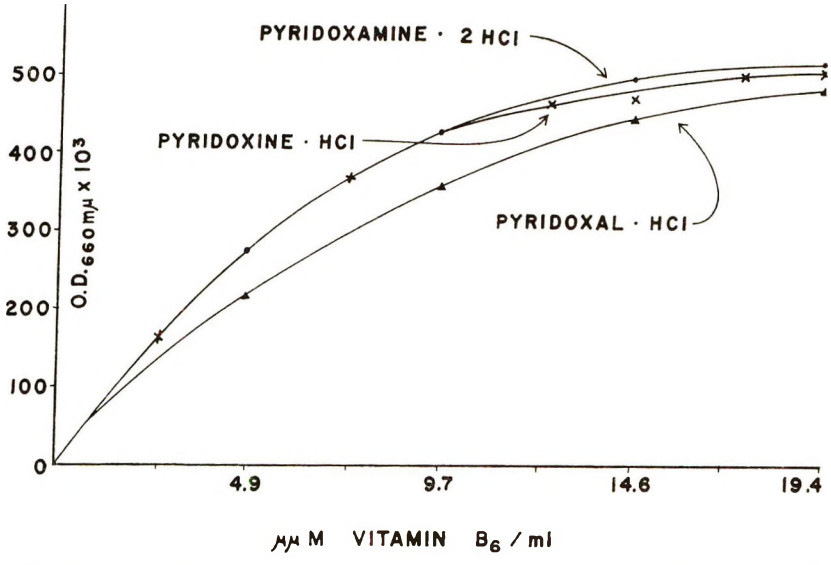

Fig. 1 Twenty-four-hour growth response of *S. carlsbergensis* to pyridoxine·HCl, pyridoxal·HCl and pyridoxamine·2HCl.

of 4 levels in duplicate flasks. Values for the vitamin B_6 content of a given sample were consistent throughout an assay, and the results obtained on duplicate hydrolysates differed by not more than ± 5% from the mean value in nearly all cases. Recovery of standard pyridoxine·HCl solution added to test samples ranged from 97 to 103%. When the growth responses to standard equimolar solutions of pyridoxine·HCl, pyridoxal·HCl and pyridoxamine·$(HCl)_2$ were compared, the

curves for pyridoxine·HCl and pyridoxamine·$(HCl)_2$ were substantially identical up to the level of 9.7 μμM of vitamin B_6/ml, while the pyridoxal·HCl curve was approximately 20% lower at this point (fig. 1).

In order to determine whether the autoclaving of the standard and sample solutions prior to inoculation destroyed vitamin B_6, standard pyridoxine·HCl and pyridoxal·HCl solutions, a sample of cooked lamb leg and the basal medium were individually sterilized by filtration through an ultra-fine sintered glass filter. The filtered pyridoxine·HCl solution gave values approximately 7% higher, while the filtered pyridoxal·HCl solution gave values 50% higher than the respective autoclaved controls at the level of 7.3 μμM of vitamin B_6/ml. Filtration of the basal medium resulted in slight increases in the vitamin B_6 values observed, while small decreases were noted for the lamb leg sample, when compared to the autoclaved controls. Thus, while autoclaving appeared to destroy approximately one-third of the pyridoxal·HCl, it was concluded that autoclaving was without significant effect on the vitamin B_6 activity of the pyridoxine·HCl standard solution, the basal medium, and the meat samples tested in these experiments, when compared to sterilization by microfiltration.

In order to determine whether the drippings from cooked meat contained significant amounts of vitamin B_6, the drippings from 4 of the cooked roasts were assayed, using the *S. carlsbergensis* method. The percentage of vitamin B_6 originally present in the fresh meat which was found in the meat drippings varied from 1% for fresh ham to 13% for lamb leg. At most, approximately one-fifth of the observed loss of vitamin B_6 was accounted for in this way (lamb leg), and it appeared that the remaining loss, as measured by the yeast method, was due to destruction of the vitamin in the meat and drippings during cooking. It was concluded that the vitamin B_6 present in the drippings from cooked meats represents a relatively small proportion of the total.

In addition to the work done on the meats prepared in these laboratories, *S. carlsbergensis* assays for vitamin B_6 were also performed on several cooked meat samples provided by Dr. R. M. Leverton, Agricultural Experiment Station, Oklahoma State University, in conjunction with studies on the proximate composition, and the protein, vitamin, mineral and Caloric content of these meats. These results have been published elsewhere (Leverton and Odell, '58).

In using the rat bioassay for vitamin B_6, several pilot experiments were performed to determine whether a depletion period was necessary, and whether blood fibrin was preferable to casein in the basal ration. Using fibrin and no depletion period, the total experimental period was reduced to 4 weeks, but good agreement was not observed between values for samples tested at each of two levels in the same assay, or for the same sample tested in independent experiments. The average growth response to graded levels of pyridoxine·HCl ranged from 20 to 125 gm. When "vitamin-free" casein was used without a depletion period, total average growth response to graded levels of pyridoxine·HCl ranged from 45 to 115 gm for the 4 wk. test period, and values for meat samples tested at each of two levels were in good agreement.

Improved results were obtained with the "vitamin-free" casein basal ration, when a two-week depletion period was used preceding the three-week assay period. Average growth response to graded levels of pyridoxine·HCl ranged from 15 to 105 gm (fig. 2), and the vitamin B_6 values for meat samples tested at each of two levels in a single experiment, and in independent assays were in good agreement, differing by not more than $\pm$ 10% from the mean in nearly every case. This assay method was used for testing all of the meat samples included in these studies. The response of the vitamin B_6-depleted rat to equimolar amounts of pyridoxine·HCl, pyridoxal·HCl and pyridoxamine·$(HCl)_2$ was found to be approximately equal, in confirmation of earlier studies.

The results obtained for the vitamin B_6 content of the fresh meats, and for the retention of vitamin B_6 in the cooked meats are given in table 1. The retention values were computed as the ratio of the vitamin B_6 content of the cooked, boned, trimmed meat, corrected for weight loss during cooking, to the vitamin B_6 content of the fresh, boned, trimmed meat. The vitamin B_6 content of the organ meats and processed meat products as determined with each of the assay methods employed is given in table 2.

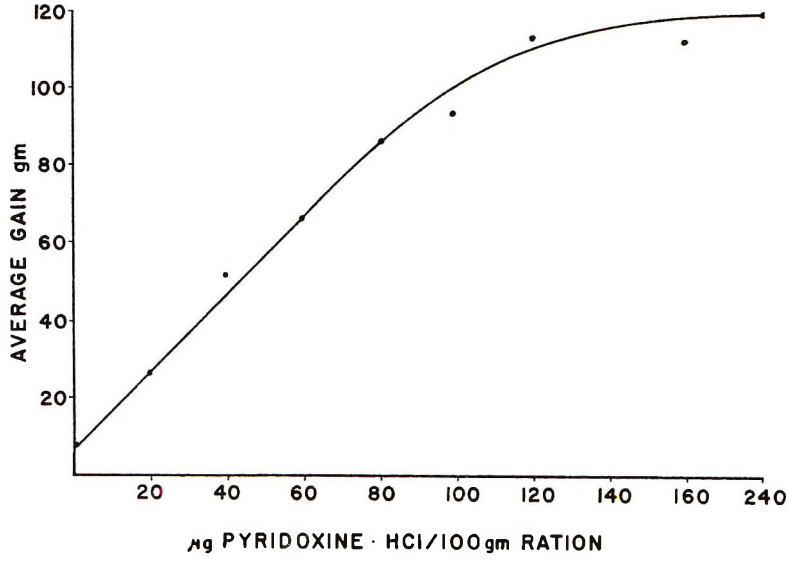

Fig. 2 Rates of gain of vitamin B_6-depleted male rats fed the casein basal ration plus graded levels of pyridoxine·HCl for 21 days.

From the results in tables 1 and 2, it will be noted that the vitamin B_6 values for meats observed with the *S. carlsbergensis* assay method were approximately half as large as those observed with the rat bioassay. The rat bioassay values are significantly higher than those which have been reported in earlier studies (Sarma et al., '46). They have been observed repeatedly, however, and it would appear that the values shown represent the vitamin B_6 activity for the rat, of the

TABLE 1

Vitamin B_6 content of fresh and cooked meats

MEAT TESTED	PROTEIN [1]		RAT BIOASSAY			*S. Carlsbergensis* ASSAY		
	Fresh	Cooked	Fresh	Cooked	Retention	Fresh	Cooked	Retention
	%	%	*mg/100 gm*	*mg/100 gm*	%	*mg/100 gm*	*mg/100 gm*	%
Beef, standing rib roast	17.0	23.3	0.59	0.40	44	0.32	0.28	56
Beef, Boston cut	19.5	25.8	0.54	0.46	56	0.38	0.25	42
Pork, ham, uncured	19.1	30.1	0.70	0.59	50	0.42	0.40	57
Lamb, leg	21.8	27.7	0.52	0.43	63	0.26	0.16	43
Veal, leg	22.1	31.9	0.57	0.44	65	0.33	0.29	67

[1] N × 6.25.

TABLE 2

Vitamin B_6 content of organ meats and processed meats

MEAT TESTED	PROTEIN [1]	RAT BIOASSAY	*S. Carlsbergensis* ASSAY
	%	*mg/100 gm*	*mg/100 gm*
Bologna	13.9	0.28	0.12
Corned beef	13.4	0.30	0.15
Frankfurters	13.9	0.31	0.15
Ham, cured, fully cooked	21.8	0.70	0.39
Liver, beef, fresh	24.3	1.42	0.74
Liver, pork, fresh	24.2	1.11	0.51
Liver sausage	14.2	0.45	0.23
Processed pork, canned	13.6	0.30	0.18
Strained beef	14.4	0.10	0.05
Summer sausage, semi-dry	22.5	0.43	0.24

[1] N × 6.25.

fresh, cooked and processed meat samples tested. Thus, the divergence in values observed with the *S. carlsbergensis* and rat bioassay methods may be due to a species difference in the ability to utilize the vitamin B_6 present in fresh, cooked and processed meats.

The values obtained for the retention of vitamin B_6 in cooked meats were significantly higher than those which have been reported previously. In the present studies, percentage retention values ranging from 42 to 67% and averaging approximately 54% have been observed. Earlier studies had indicated vitamin B_6 retention values of approximately 15 to 40% (McIntire et al., '44). Although the values for the vitamin B_6 content of the fresh and cooked meats differed, using the rat bioassay and *S. carlsbergensis* methods, the percentage retention values observed with each of the assay methods were in good agreement for the cooked meat samples tested (table 1).

SUMMARY

Several fresh, cooked, and processed meats have been assayed for vitamin B_6 content, using *S. carlsbergensis* microbiological yeast assay and rat bioassay methods. Values obtained for the vitamin B_6 content of fresh meats, using the *S. carlsbergensis* method, were consistent with earlier work, while the vitamin B_6 values observed with the rat bioassay were significantly higher, approximating levels twice as great. These differences are thought to be due to species differences in the ability to utilize the vitamin B_6 present in fresh, cooked and processed meat and meat products. The retention of vitamin B_6 in cooked meats averaged 54%, a value significantly greater than those reported previously.

LITERATURE CITED

ATKIN, 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.

COURSIN, D. B. 1954 Convulsive seizures in infants with pyridoxine deficient diet. J. Am. Med. Assoc., *154:* 406–408.

LEVERTON, R. M., AND G. V. ODELL 1958 The nutritive value of cooked meat. Oklahoma Agricultural Experiment Station, Publication MP–49.

MCINTIRE, J. M., B. S. SCHWEIGERT AND C. A. ELVEHJEM 1944 The choline and pyridoxine content of meats. J. Nutrition, *28:* 219–223.

SARMA, P. S., E. E. SNELL AND C. A. ELVEHJEM 1946 The vitamin B_6 group. VIII. Biological assay of pyridoxal, pyridoxamine, and pyridoxine. J. Biol. Chem., *165:* 55–63.

TOWER, D. B. 1956 Nutrition Symposium Series No. 12. National Vitamin Foundation, New York, N. Y.

AVAILABILITY OF CALCIUM IN SOME PHILIPPINE VEGETABLES[1]

ADELAIDA BENDAÑA-BROWN AND CLARA Y. LIM
Chemistry Department, State University of the Philippines, Diliman, Quezon City

(Received for publication June 3, 1958)

Previous studies by Miller, Louis and Ross ('47), Sur and Subrahmanyan ('52), Devadetta and Appana ('54), and Bendaña-Brown and Lim ('57) have shown that on the dry basis some commonly used vegetables are superior to milk as gross sources of calcium. On the other hand, it has also been reported by Majundar and De ('38), Kohman ('39), Miller et al. ('47), Armstrong et al. ('53), Devadetta and Appana ('54), and Bendaña-Brown and Lim ('57) that a number of green leafy vegetables contain unusual amounts of oxalates. Lovelace, Lui and McKay ('50) observed that soluble oxalates when present in chemical equivalent amounts were 100% effective in immobilizing calcium in rats which were 50 days old. Imada et al. ('54), using calcium^{45} oxalate mixed with calcium lactate, phosphate or carbonate in the diet of young albino rats, indicated that only 20 to 44% of the calcium of the diet was retained. Iwao ('51–'52) found that calcification of bone in rats was decreased in proportion to the oxalic acid content of the diet. He observed the same reduction in calcium utilization when K, Na and NH_4 oxalates were used. Interference with calcium utilization by oxalic acid was also reported by Iwao et al. ('53) when three healthy men were given 1.57 gm of oxalic acid per day. Studies have been made on the possible bearing of the oxalate content of vegetables on the utilization of calcium. In general, the availability of vegetable calcium

[1] Partly supported by a research grant from the National Research Council of the Philippines.

has been shown to be inferior to that of milk by Blatherwick and Long ('22), McLaughlin ('27), Fincke and Sherman ('35), Fairbanks and Mitchell ('38), Kung et al. ('38), Shields et al. ('40), Bendaña-Brown and Brown ('47), and Talapatra et al. ('48). Although an inverse relationship between calcium retention and oxalate content of plant foods has been reported, it has been claimed by Devadetta and Appana ('54) that the calcium of *Amaranthus gangeticus* was as well utilized by rats as the calcium of milk in spite of its high oxalate content. If this claim is correct, it is evident that the whole question of the relation of oxalates to dietary calcium should be reexamined.

That vegetables vary among themselves in calcium availability has been shown by the studies of Fincke and Sherman ('35), Speirs ('39), Bendaña-Brown and Brown ('47), Iwao ('51–'52), Johnstone et al. ('52), Armstrong et al. ('53), and Devadetta and Appana ('54). It has been reported by Talapatra et al. ('48) that calcium from spinach was unavailable to rats, humans and ruminants.

Several factors, like dietary fats, proteins, lactose, dextrin, etc., affecting the interference of oxalic acid on the retention of calcium by rats and humans have been investigated by Iwao ('51–'52), Iwao, Takai and Monya ('54) and Fournier ('55).

In the Philippines, work on the availability of calcium in the vegetables was started by Bendaña-Brown and Geronimo.[2] The purpose of this paper is to record results showing how well mice utilize the calcium of some Philippine vegetables.

EXPERIMENTAL

Table 2 shows the composition of the experimental diets. The basal diet was patterned after the type that was successfully employed by Bendaña-Brown and Brown ('47). Calcium of the basal diet was supplied principally by powdered whole milk while that of the vegetable diet was supplied by milk and the vegetable tested. The vegetables employed (table 1) were

[2] Unpublished data, Bendaña-Brown, A., and F. Geronimo.

dried and powdered before they were used in the preparation of the diets. The amount of the vegetable that could be tolerated by the mice in each case was determined by preliminary feeding experiments. The dietary calcium was determined by the rapid digestion method of Bolin and Stamberg ('47) while the dietary oxalates were determined by an analytical method standardized by Bendaña-Brown and Lim ('57).

TABLE 1

Vegetables analysed [1]

COMMON NAME	SCIENTIFIC NAME	EDIBLE PART USED
Alugbati	*Basella rubra* L.	tops and leaves
Amargoso	*Momordica charantia* L.	tops and leaves
Cabbage	*Brassica oleracea* L.	leaves
Calabasa	*Cucurbita maxima* Duchno	tops and leaves
Colitis	*Amaranthus viridis* L.	leaves
Lettuce	*Lactuca sativa* L.	leaves
Malungay	*Moringa oleifera* Lam.	leaves
Pechay	*Brassica juncea* L.	leaves
Pepper	*Capsicum frutescens* L.	leaves
Okra	*Hibiscus esculentus* L.	pods
Onion	*Allium cepa* L.	leaves
Saluyot	*Corchorus olitorius* L.	leaves
Talinum	*Talinum triangulare*	tops and leaves
Ulasiman	*Portulaca oleracea* L.	tops and leaves

[1] The vegetable parts employed are those that are commonly included in the diet of the Philippine population.

Weanling albino mice of both sexes from one stock colony were used in this work. Their initial weights ranged from 5 to 8 gm. Throughout the studies the experimental mice were housed in individual cages. At the start of the feeding experiments two representative mice from each group were sacrificed for calcium determination. Food in paste form was given at suboptimal levels to insure maximum utilization. Distilled water was given ad libitum. When the animals had doubled their weights, they were killed with chloroform, the gastrointestinal tracts were removed and the bodies analyzed for calcium.

TABLE 2

Composition and calcium content of experimental diets

TYPE OF DIET	CONSTITUENTS								CALCIUM
	Rice [1]	Milk	Vegetable [2]	Corn oil	Cod liver oil	NaCl	Beef liver	Dry yeast	
	%	%	%	%	%	%	%	%	%
Basal 1	61.5	23.5	—	9	2	1.5	0.5	2	0.282
Basal 2	65.0	20.0	—	9	2	1.5	0.5	2	0.240
Alugbati	62.0	18.0	5.0	9	2	1.5	0.5	2	0.274
Amargoso	69.0	11.0	5.0	9	2	1.5	0.5	2	0.280
Cabbage	60.0	15.0	10.0	9	2	1.5	0.5	2	0.245
Calabasa	66.0	15.0	4.0	9	2	1.5	0.5	2	0.286
Colitis	67.7	12.3	5.0	9	2	1.5	0.5	2	0.285
Lettuce	64.5	15.5	5.0	9	2	1.5	0.5	2	0.274
Malungay	71.7	9.5	4.0	9	2	1.5	0.5	2	0.290
Pechay	65.5	15.0	5.0	9	2	1.5	0.5	2	0.280
Pepper	64.6	15.4	5.0	9	2	1.5	0.5	2	0.290
Okra	65.0	10.0	10.0	9	2	1.5	0.5	2	0.235
Onion	69.5	11.5	4.0	9	2	1.5	0.5	2	0.230
Saluyot	66.0	14.0	5.0	9	2	1.5	0.5	2	0.290
Talinum	64.4	16.6	4.0	9	2	1.5	0.5	2	0.270
Ulasiman	65.0	15.0	5.0	9	2	1.5	0.5	2	0.280

[1] Powdered polished rice was used.

[2] Each vegetable was dehydrated and powdered.

TABLE 3

The effect of dietary oxalates on the retention of dietary calcium by albino mice

VEGETABLE IN DIET	NO. OF MICE	DIETARY CALCIUM	DIETARY OXALATES	DIETARY CALCIUM RETAINED
		mg	*mg*	*%*
None	7	282.1 ± 0.12 [1]	——	51.93 ± 0.54
Cabbage	11	244.9 ± 0.10	8.1 ± 0.18	48.45 ± 0.37
Okra	11	234.9 ± 0.08	8.2 ± 0.14	47.02 ± 0.83
Amargoso	13	280.3 ± 0.09	9.9 ± 0.20	43.44 ± 0.50
Lettuce	12	274.2 ± 0.15	12.2 ± 0.08	40.35 ± 0.85
Malungay	13	290.2 ± 0.16	15.1 ± 0.21	38.43 ± 0.37
Pechay	12	281.8 ± 0.08	14.9 ± 0.09	37.95 ± 0.51
Calabasa	12	285.9 ± 0.07	21.9 ± 0.19	35.23 ± 0.65
Onion	12	229.7 ± 0.14	25.8 ± 0.31	32.04 ± 0.52
Saluyot	13	210.2 ± 0.17	51.3 ± 0.07	28.79 ± 0.59
Pepper	12	290.1 ± 0.21	419.1 ± 0.26	16.32 ± 0.69
Alugbati	13	273.8 ± 0.22	478.9 ± 0.19	16.37 ± 0.37
Ulasiman	12	282.1 ± 0.17	525.9 ± 0.27	14.97 ± 0.39
Colitis	12	285.1 ± 0.09	550.8 ± 0.10	14.86 ± 0.45
Talinum	8	270.1 ± 0.31	1016.1 ± 0.38	5.28 ± 0.38

[1] Standard deviation from the mean.

DISCUSSION

Table 3 shows the effect of dietary oxalates on dietary calcium retention. A marked reduction in the retention of calcium was observed with the diets containing vegetables as compared with the diet containing milk as the principal source of calcium. It is also apparent from the results that the capacity of the mice to retain calcium from the different vegetable diets was affected by the amount of dietary oxalates. The lowest retention of calcium was shown by mice fed the talinum diet which had the highest oxalate content. In general, the results recorded indicate an inverse relationship between dietary oxalates and dietary calcium retained.

The data in table 4 can be interpreted to mean that oxalates in the diet appear to render unavailable not only a portion of the calcium from milk but also the calcium from the vegetable. Calcium from talinum alugbati, colitis, ulasiman, Pepper, Saluyot and onion seems to be unavailable to the mice. These vegetables were shown to have very high oxalate content

TABLE 4

Retention of vegetable calcium by albino mice

VEGETABLE IN DIET	NO. OF MICE	CALCIUM INTAKE		CALCIUM RETENTION		CALCIUM RETENTION FROM VEGETABLE		
		Vegetable	Milk	Total	Possible from milk			
		mg	*mg*	*mg*	*mg*	*mg*	%	
Okra	11	111.9	123.2	110.4	70.4	40.4	35.71	0.96
Amargoso	13	145.2	134.8	121.6	70.0	51.6	35.54	0.67
Cabbage	11	65.2	180.0	118.4	102.8	15.6	23.93	0.42
Lettuce	12	86.4	187.6	110.0	97.6	12.4	14.35	0.75
Pechay	12	97.2	181.6	107.2	94.4	12.8	13.17	0.57
Calabasa	12	106.0	180.0	100.8	93.6	7.2	6.79	0.43
Malungay	13	80.4	212.0	111.6	110.0	1.6	1.99	0.29
Onion	12	87.6	142.4	53.6	80.8			
Saluyot	13	77.6	212.4	83.6	110.4			
Pepper	12	107.2	182.8	47.2	94.8			
Alugbati	13	47.2	226.8	44.8	118.0			
Ulasiman	12	99.6	180.4	42.0	93.6			
Colitis	12	137.6	147.6	42.4	77.8			
Talinum	8	68.8	201.2	14.4	104.4			

(Bendaña-Brown and Lim, '57). It is worthwhile to note that talinum was shown to contain the highest amount of oxalic acid, namely, 24.82% on the dry basis. In preliminary feeding experiments, when the talinum diet contained twice as much of the vegetable as the amount indicated in table 2, the experimental mice died after two weeks of feeding.

SUMMARY

1. In each case the calcium from the 14 vegetables tested was found to be less well utilized than the calcium from milk.

2. The calcium from *Talinum triangulare, Amaranthus viridis, Basella rubra, Portulaca oleracea, Capsicum frutescens, Corchorus olitorius*, and *Allium cepa* appeared completely unavailable to mice. *Talinum triagulare* seemed to be the poorest source of calcium.

3. Calcium from *Hibiscus esculentus, Momordica charantia, Brassica oleracea, Lactuca sativa, Brassica juncea, Cucurbita maxima* and *Moringa oleifera* was available to mice to a certain extent. Calcium from *Hibiscus esculentus* (okra) was utilized best by mice.

4. In general, the vegetables containing appreciable amounts of oxalates were the ones whose calcium appeared to be unavailable. Those which contained small amounts of oxalates appeared to have a certain amount of available calcium.

LITERATURE CITED

ARMSTRONG, R. H., T. BYNMOR AND K. HORNER 1953 Availability of calcium in three herbs of Grassland. J. Agri. Sci., *43:* 337–342.

BENDAÑA-BROWN, A. M., AND B. H. BROWN 1947 The use of albino mice to determine the utilization of calcium of dehydrated cabbage and carrots. J. Nutrition, *33:* 195–207.

BENDAÑA-BROWN, A. M., AND C. Y. LIM 1957 Determination of calcium and oxalic acid and oxalates in some Philippine plant foods. J. Phil. Pharm. Assoc., Vol. *44:* 45.

BLATHERWICK, N. R., AND L. L. LONG 1922 Utilization of calcium and phosphorus of vegetables. J. Biol. Chem., *52:* 125.

BOLIN, D. W., AND A. E. STAMBERG 1947 Rapid digestion method for the determination of calcium. Anal. Chem., *19:* 85–86.

DEVADETTA, S. C., AND A. C. APPANA 1954 Availability of calcium of some leafy vegetables. Proc. Indian Acad. Sci., *39B:* 236–242.

FAIRBANKS, B. W., AND H. H. MITCHELL 1938 The availability of calcium in spinach, skim milk and in calcium oxalate. J. Nutrition, *16:* 179.

FINCKE, M. L., AND H. C. SHERMAN 1935 The availability of calcium from some typical foods. J. Biol. Chem., *110:* 421.

FOURNIER, P. 1955 Osteogenic action of glycolic acid and its effects. Compt. rend., *240:* 25553–25557. C.A. *49:* 14952 i.

IMADA, S., AND OTHERS 1954 Utilization of calcium[45] oxalate. Repts. Sci. Living (Osaka City), *2:* 25–30. C.A. *49:* 14989 g.

IWAO, H. 1951-52 Utilization of calcium in green leaves. Ann. Rept. Natl. Inst. Nutrition (Japan), pp. 34–39.

IWAO, H., T. HASHIMOTO, K. SUZUKI, T. MONYA AND R. MATSUMOTO 1953 Effect of calcium oxalate and of oxalic acid in the body. Ann. Rept. Natl. Inst. Nutrition (Japan), pp. 50–51.

———— 1953-54 Availability of calcium. Japan J. Nutrition, *11:* 117–137: 152–165.

———— 1954-55 Availability of calcium. Ibid., *12:* 1–19: 82–86.

IWAO, H., Y. TAKAI AND T. MONYA 1954 Availability of calcium. XIII. Prevention of influence of oxalic acid on calcium. Ann. Rept. Natl. Inst. Nutrition (Japan), pp. 1–19.

JOHNSTONE, A. A., T. J. MCMILLAN AND G. D. FALCONER 1952 Calcium retained by women after adding spinach to the diet. J. Am. Dietet. Assoc., *28:* 933–938.

KELLY, J. J. 1943 The availability of calcium of some New Zealand vegetables. J. Nutrition, *25:* 303–308.

KOHMAN, E. F. 1939 Oxalic acid in foods and its behavior and fate in the diet. Ibid., *18:* 233–246.

KUNG, L. G., H. L. YEK AND W. H. ADOLPH 1938 The availability of calcium in some vegetable food materials. Chinese J. Physiol., *13:* 307.

LOVELACE, F. E., C. H. LIU AND C. M. MCKAY 1950 Age of animals in relation to the utilization of calcium and magnesium in the presence of oxalates. Arch. Biochem., *27:* 48–56.

MAJUNDAR, B. M., AND N. K. DE 1938 The oxalic acid content of some Indian foodstuffs. Indian J. Med. Res., *25:* 671–675.

MCLAUGHLIN, L. 1927 The utilization of calcium in spinach. J. Biol. Chem., *74:* 455.

MILLER, C. L. LOUIS AND W. ROSS 1947 Hawaiian green vegetables proximate composition. Hawaiian Agri. Experiment. Sta. Bull. Tech., *5:* 45 pages.

SHIELDS, J. B., B. W. FAIRBANKS, G. H. BERRYMAN AND H. H. MITCHELL 1940 The utilization of calcium in carrots, lettuce, string beans in comparison with calcium in milk. J. Nutrition, *20:* 263.

SPEIRS, M. 1949 The utilization of calcium in various greens. *17:* 557.

SUR, B. K., AND V. SUBRAHMANYAN 1952 Availability of calcium in Lucerne and its value in nutrition. Indian J. Med. Research, *40:* 481–486.

TALAPATRA, S. K., S. C. RAY AND K. C. KEN 1948 A new method of estimation of oxalic acid in biological materials and oxalic acid content of Indian feeding stuffs. Indian J. Vet. Sci., *18:* 99–108.

STUDIES ON THE INHIBITION OF GROWTH OF AN IMPLANTED FIBROSARCOMA IN RATS

THE EFFECT OF FAT IN THE DIET WITH AND WITHOUT INJECTIONS OF GUINEA PIG SERUM

ELOISE JAMESON, R. MARK RYAN AND PHILIP I. KRAMER
Department of Medicine, University of Southern California, Los Angeles

(Received for publication June 12, 1958)

INTRODUCTION

A number of review articles have been written on the effect of various diets on the initiation and growth of malignant tumors, as for example that by Tannenbaum and Silverstone ('53). Tannenbaum ('42) reported that fats enhanced the growth of carcinomas, although he included one experiment which showed that fat increased the resistance of mice to an implanted sarcoma. Greenstein ('54) and others state that a starvation level of calories in the diet increases the resistance of the animal to the development of tumors.

In a series of mortality experiments Cheng et al. ('54) found that rats fed a synthetic diet containing 15% cottonseed oil, or only the ester of a single essential fatty acid (Deuel et al., '53), were protected in part from the effect of x-irradiation. Following Cheng's procedures and using electrophoresis, we found that fat in the diet protected the gamma-1 globulin during x-ray, suggesting that this protection against x-ray afforded rats may have been due to the protection of antibodies in the gamma globulin or of antibody producing systems.[1]

[1] Jameson, E., R. J. Martinez and R. M. Ryan, unpublished data.

It had been found previously that injections of guinea pig serum alone were instrumental in controlling initiation, development and growth of the fibrosarcoma $ACMCA_2$ (Jameson et al., '56) and Murphy-Sturm lymphosarcoma (Jameson et al., '58) in rats. Kidd ('53) injected guinea pig serum into mice to inhibit and control growth of neoplasms, particularly lymphomas and related tumors.

The experiments reported herein were planned to determine whether the resistance of rats to an implanted fibrosarcoma could be increased by a treatment which protected the gamma-1 globulin, such as fat in the diet, both with and without injections of guinea pig serum.

The present report summarizes 5 experiments on the tumor-inhibiting effects of two factors in rats: (a) adequate fat diet, and (b) injections of guinea pig serum.

METHODS

AXC9935 Irish gray rats [2] were used in these investigations. Randomly bred rats were utilized in the first three experiments; and inbred rats, in experiments 4 and 5. In all experiments litter mates were distributed throughout the experimental and control groups, and all groups were equalized as to age, weight, and sex. The rats were from 75 to 125 days old at the beginning of the diet experiments.

Except where otherwise specified, the diets were fed ad libitum for three weeks prior to tumor implantation. All rats were implanted subcutaneously in the back by the trocar method with an $ACMCA_2$ fibrosarcoma.[2] Implants were made with sections of the same size from the tumor of a single donor bearing only one tumor. Rats were continued on the various diets until termination of the experiment. Three milliliters of guinea pig serum [3] were injected intraperitoneally daily, starting at implantation, for three days, followed by

[2] Obtained through the courtesy of Dr. Barrett of the National Institutes of Health, Bethesda, Maryland.

[3] The guinea pig serum used in these experiments was secured under asceptic conditions from the Capital S Caviary, Buena Park, California, and was tested for and kept free of contamination by the methods described in Ainis, H. ('58).

a three-day rest. The number of series administered in each experiment is specified in table 2.

All rats were observed daily and the time noted when the tumor could first be detected. The period from implantation until the tumors became palpable is termed the "latent period." Thereafter the size of the developing tumors was measured and recorded daily. Measurements of growing tumors were taken with calipers in three dimensions from the time tumors were sufficiently large to measure.

The 40th day after tumor implantation was selected for testing the significance of the treatments, and making comparisons between paired groups in the 5 different experiments to determine whether the effects of the treatments were consistent. The 40th day was selected because by then the effects were quite distinct, and death had not yet taken a significant toll of the untreated rats.

In selecting a suitable statistic for comparisons, there were several factors to consider: (1) that under some treatments the tumors did not develop at all; (2) under others, they developed relatively slowly; (3) in some cases they regressed; (4) while in others, they developed very rapidly. A nominal value was assigned to the tumors of negative animals as some developed later. Tumors which killed the animals were carried on as the volume measurement at death. Other statistical methods might have given them a higher value, but this was the conservative estimate. From volume measurements of the tumors, which had been made every day, the growth was found to be clearly exponential where the inhibition was not strong. In the following formula: $x = 16 + 9 \log_{10} y$, where $y =$ tumor volume in milliliters, x corresponds well with the number of days after tumor implantation for the fastest growing tumors (apparently the least inhibited). Actually, the relation breaks down as the tumor becomes large with respect to the host, and death nears. It does, however, seem to present a reasonable measure of the disease progress amenable to statistical treatment (Bliss, '52; Wallis and Roberts, '56). Death occurs when x is near 34 days after implantation.

RESULTS

Statistical results

In table 1 the 5 experiments at the 40th day after implantation are compared.

Fat diet alone. The effects of fat in the diet on the development of the implanted fibrosarcoma (on rats not injected with guinea pig serum) are that (1) there is no significant difference between the effects of the 4 and 15% levels of cottonseed oil in the diet, and (2) there is no significant difference between the effects of methyl linoleate and 15% cottonseed oil in the diet. In experiment 1, 0% fat is significantly different in effect from the standard 15% cottonseed oil diet. In experiments 2 and 5 there are significant differences between the effects of the stock diet and the 15% cottonseed oil diet, but not in experiment 4, in which the rate of tumor growth was too rapid for the diet to be effective.

Because one of the above differences (in experiment 4) was not significant, comparisons were made to check the overall differences in the rats' response to the implanted tumor between (1) those animals fed the fat deficient diets or stock diet, and (2) those fed the standard 15% fat diet. Factorial design tests were used to combine the results of experiments 1, 2, 4 and 5. Significance was found at the 5% level.

Four per cent butter in the diet is significantly less effective than 4% cottonseed oil, but 15% butter does not differ significantly from either 4 or 15% cottonseed oil.

Inasmuch as a small dose of methyl linoleate (100 mg per day per rat) is sufficient to give almost the full effect of the 15% cottonseed oil diet, while 4% butter is insufficient, the inference may be drawn that the tumor inhibitory effect is due to essential fatty acids.

Guinea pig serum injections. The effect of guinea pig serum is significant at all tested levels of fat in the diet, as can be seen by comparing (1) the means obtained from the growth of tumors in rats injected with guinea pig serum with (2) the means on similar diets where no guinea pig serum is

TABLE 1

Statistical analysis of experiments at the 40th day after tumor implantation

EXP.[1]	RATS PER GROUP	RINGER'S SOLUTION INJECTED					GUINEA PIG SERUM INJECTED		
		Type of diet					Type of diet		
		0% fat	Stock	0% fat plus linoleate	0% fat plus 4% CO [2]	0% fat plus 15% CO	Stock	0% fat plus 4% CO	0% fat plus 15% CO
					Randomly-bred rats				
1	38	*20.76*				*16.26*			
	or	17.72				13.28			
	41	*14.68*				*10.30*			
		p = 0.041				Standard			
2	15		*29.66*	*22.08*		*20.58*	*26.75*		
	or		23.87	13.72		15.48	21.96		
	16		*18.08*	*5.36*		*10.38*	*17.17*		
			p = 0.0351	p = No sig.		Standard	p = No sig.[4]		
3	22				*16.89*	*12.89*		*7.24*	*3.09*
	or				12.70	9.10		4.79	1.84
	24				*8.58*	*5.31*		*2.34*	*0.59*
					p = 0.11	Standard		p = 0.04	Standard
					Inbred rats				
4	20		*33.17*		*32.28*	*33.00*	*19.93*	*4.01*	*3.53*
	or		32.03		31.23	31.18	19.30	2.42	2.46
	26		*30.89*		*30.18*	*29.36*	*18.67*	*0.93*	*1.39*
			p = No sig.		p = No sig.	Standard	p = 0.001	p = No sig.	Standard
5	15		*28.68*		*24.27*	*24.27*	*6.66*	*1.00*	*1.09*
	or		26.83		21.31	20.85	4.15	1.00	1.67
	16		*24.93*		*17.43*	*17.43*	*1.68*	*1.00*	*1.05*
			p = 0.025		p = No sig.	Standard	p = 0.01	p = No sig.	Standard
			28.68		Butter	Butter	*6.66*	Butter	Butter
			26.83		*28.59*	*25.95*	4.15	*1.00*	*1.00*
			24.93		26.72	21.25	*1.68*	1.00	1.00
			p = 0.025		*24.86*	*16.25*	p = 0.01	*1.00*	*1.00*
					p = 0.02	Standard		p = No sig.	Standard

[1] For design of experiment, see table 2.

[2] Cottonseed oil.

[3] The squares filled in on the table indicate the composition of the experiments. The groups of columns on the left indicate various diets, with no guinea pig serum injections, while the ones on the right indicate the diets with additional treatment of guinea pig serum injection. The rows indicate the different experiments. The first column on the left shows the number of animals used in each group. The number in the center of each square is the mean ($\bar{x}$), the measure of the disease progress for the group at the 40th day; the numbers in italics above and below it are the 5% confidence limits of this mean. A 15% fat diet is used as a standard because it appeared in each experiment and was adequate in all known requirements. The mean of each of the other groups in each experiment on the left is compared with the mean of the group receiving no guinea pig serum injections and a 15% fat diet. On the right the mean of each group is compared with the mean of the group receiving 15% fat diet plus injections of guinea pig serum. P equals the number giving the single tail probability that the mean for the group could differ by chance from the standard 15% fat diet groups (Wallis and Roberts, '56).

[4] Not significantly different from stock diet without guinea pig serum.

injected. The only exception is in experiment 2, in which only one series of injections was given, ending on the third day after implantation. In experiments 3, 4 and 5, the injections were continued until the 14th, 52nd and 74th days respectively.

The importance of fat in the diet is demonstrated in experiments 4 and 5, where a highly significant difference occurs between the means representing an adequate fat diet with means representing an inadequate fat diet or a stock diet.

The question arises as to whether there is an interaction between guinea pig serum and fat in the diet. An interaction is an effect demonstrated over and above the total effects of the two factors combined.

In experiment 4 there is a strong interaction as shown by the F values in a factorial analysis: effect of guinea pig serum, $F = 294$; effect of fat, $F = 53$; and interaction, $F = 44$ (with $n_1 = 1$ and $n_2 = 54$). In experiment 5, since the mean (1.00) indicates no tumor appearance at all, there is no possibility of further improvement and consequently no opportunity for an interaction to be demonstrated.

Descriptive results

As shown in table 2, the latent period was not greatly lengthened in experiment 1 by addition of fat to the diet (fig. 1). The principal difference was in a reduced number of tumor "takes." Since there were 38 and 41 rats, respectively, in the two groups, application of a K value showed a statistical significance of fat in the diet.

The primary differences in experiment 2 were in the slowed growth of tumors in the fat fed and methyl linoleate dosed groups over those of the controls (fig. 2).

In experiment 3 (fig. 3) the dietary fat of group 2 (15%) as compared to group 1 (4%) resulted in prolonged latent periods, reduced number of "takes," and added regressions. These effects were further enhanced by the injections of guinea pig serum into rats in groups 3 and 4.

Experiment 4 (fig. 4) demonstrated a definite up-swing in the number of "takes" in the group injected with guinea pig

TABLE 2

Effect of diet and injections of guinea pig serum on rats implanted with a fibrosarcoma [1]

EXP. NO.	DIET	NO. OF RATS IN GROUP	TUMOR "TAKES"	COMPLETE REGRESSION	PARTIAL REGRESSION	NEGATIVES	REMARKS
			%	%	%	%	
				Randomly bred			
1	0% CO [2] (On diet from weaning)	38	79	2.63		21	3 regressed and reappeared
	15% CO	41	61	2.44		39	6 regressed and reappeared
2	Stock diet [3]	15	100			0	
	Stock diet + GPS [4]	15	100			0	1 series of injs.
	Methyl linoleate [5]	11	100			0	
	15% CO	13	100			0	
3	4% CO	23	65.2	13.0	34.8	34.8	
	15% CO	22	40.9	4.55	18.2	59.1	
	4% CO + GPS	24	20.8	8.33	12.5	79.2	3 series of injs.
	15% CO + GPS	23	8.7		8.7	91.3	3 series of injs.
				Inbred			
4	Stock diet	20	100			0	
	Stock diet + GPS	26	100			0	9 series of injs.
	4% CO	20	100			0	
	4% CO + GPS	26	73.1			26.9	9 series of injs.
	15% CO	20	95			5	
	15% CO + GPS	26	73.1			26.9	9 series of injs.
5	Stock diet	15	100			0	
	4% CO	16	100			0	
	15% CO	15	100			0	
	4% Butter	16	100			0	
	15% Butter	15	87.5	12.5		12.5	
	Stock diet + GPS	16	62.5	6.25		37.5	13 series of injs.
	4% CO + GPS	15	0			100	13 series of injs.
	15% CO + GPS	16	13.3			86.7	13 series of injs.
	4% Butter + GPS	16	0			100	13 series of injs.
	15% Butter + GPS	15	6.7			93.3	13 series of injs.

[1] Experiments 1, 2 and 3 of 100 days duration, experiments 4 and 5 of 75 days duration.
[2] Cottonseed oil.
[3] Rockland Complete Rat Diet.
[4] Guinea pig serum.
[5] Supplied at 100 mg per rat per day.

serum after cessation of injections on the 52nd day, but a still very significant proportion of animals remained negative. In fact, the significance of differences between the groups was much the same at 20, 30, 40, 55, and 96 days after tumor implantation.

Experiment 5 added comparisons with an additional dietary fat (table 3). The necessity was observed of supplying a greater concentration of butter than of cottonseed oil to give

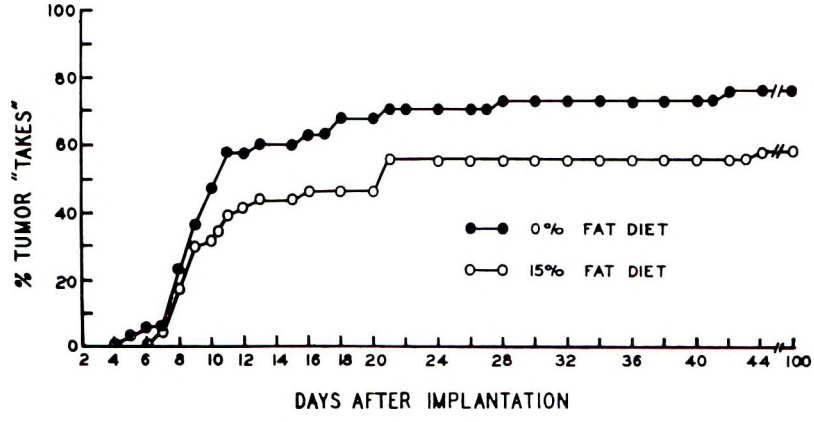

Fig. 1 Comparison of 0% fat diet and 15% fat diet on "takes" of fibrosarcoma in rats.

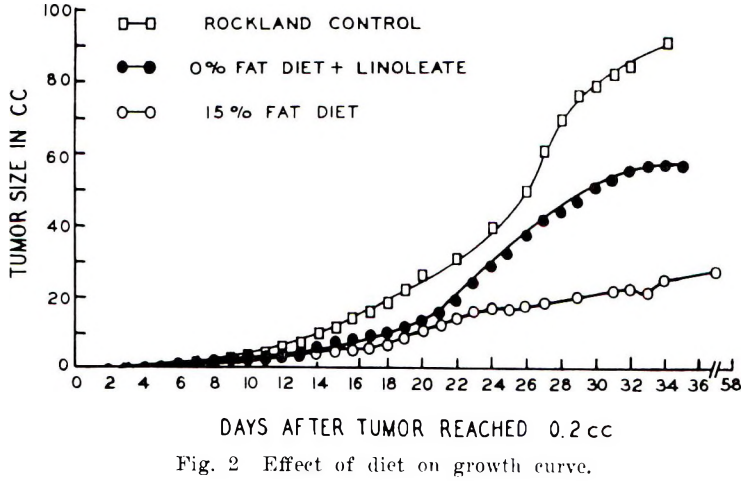

Fig. 2 Effect of diet on growth curve.

TABLE 3

Per cent "takes" of fibrosarcoma in inbred rats

Experiment 5

TREATMENT	DAYS AFTER IMPLANTATION					
	15	30	45	60	75 [1]	100
	%	%	%	%	%	%
			Ringer's solution injected			
Stock diet [2]	100	100	100	100	100	100
4% Cottonseed oil	100	100	100	100	100	100
15% Cottonseed oil [2]	93.3	93.3	100	100	100	93.3
4% Butter	100	100	100	100	100	100
15% Butter	100	100	93.8	87.5 [3]	87.5	87.5
			Guinea pig serum injected			
Stock diet	6.3	37.5	43.8	56.3	62.5	88
4% Cottonseed oil	0	0	0	0	0	13.3
15% Cottonseed oil [2]	0	0	6.7	13.3	13.3	40
4% Butter	0	0	0	0	0	31.3
15% Butter	0	0	0	0	6.7	31.3

[1] Guinea pig serum injections ended.

[2] These groups consisted of 15 rats each; all other groups contain 16 animals each.

[3] Two regressions.

the desired protection of rats against tumors. Some tumors remained viable even after the 74-day periods during which guinea pig serum was injected. Tumors appeared in 4 rats in the group fed 15% cottonseed oil, 5 each in the groups fed 15

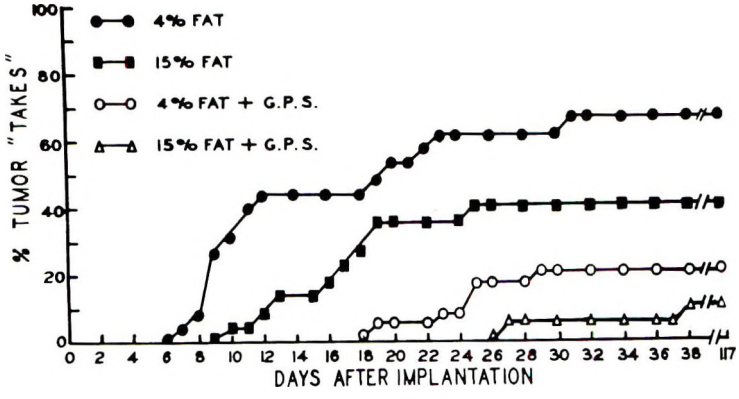

Fig. 3 Effect of diet alone and diet + guinea pig serum on per cent "takes" of fibrosarcoma in gray rats and on the latent period.

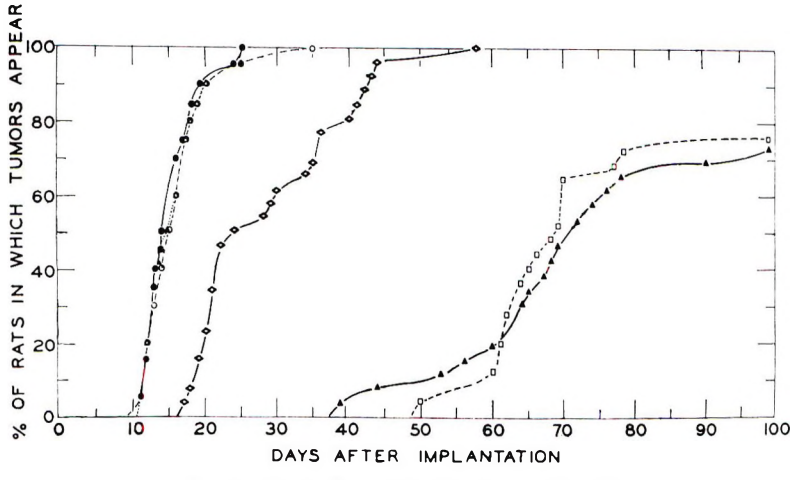

Fig. 4 Days for tumor to reach 0.5 cm³.

●, Stock diet + Ringer's solution injection.
○, Synthetic diet with 4% cottonseed oil + Ringer's solution injection.
△, Synthetic diet with 15% cottonseed oil + Ringer's solution injection.
▲, Synthetic diet with 4% cottonseed oil + guinea pig serum injection.
□, Synthetic diet with 15% cottonseed oil + guinea pig serum injection.
◇, Stock diet + guinea pig serum injection.

and 4% butter, three tumors in rats fed 4% cottonseed oil, and 4 in animals fed stock diet. After this group of tumors appeared no further tumors developed to the present (172nd) day. Again the significant differences between groups did not vary greatly during the length of the experiment.

DISCUSSION

Our experiments have differed from some reported in the literature, in that we have maintained physiological conditions using diets of low and normal levels of fat. Of course, the 0% fat diet is sub-normal, and for this reason the stock diet was substituted in the later experiments. Except in experiment 5, the fat consisted of cottonseed oil, which is largely unsaturated and contains a considerable proportion of essential fatty acids.

We found that in using Cheng's basic synthetic diet ('54), fat increased the resistance of rats to the implanted fibrosarcoma. Fat in the diet led to a lengthening of the latent period before tumor growth began, and either slowed the growth of the tumor, or suppressed it altogether, in a significant number of the rats tested.

Our results, tested statistically, show that a dietary supplement of methyl linoleate alone has almost as great an effect as the 15% cottonseed oil diet. Four per cent butter in the diet is inadequate, but a 15% level is sufficient to give maximum effect. Essential fatty acids, therefore, may be important to inhibit tumor growth (Sinclair, '56).

We had found that injections of guinea pig serum have a very significant effect in inhibiting the fibrosarcoma implanted into rats (Jameson et al., '57). The effect is greatly increased by the addition of adequate fat to the diet. Continuing injections for 72 days into inbred rats eliminated the greater proportion of tumors completely and held others latent. Less than 30% developed after cessation of injections. In general it may be stated that guinea pig serum injected into inbred rats acts primarily against the initial development of the tumor, although tumor growth is also slowed. Apparently its effect

is to hold the tumor inactive for a period of time sufficient for mobilization of tumor-destroying resistance factors (Ainis et al., '58).

The rats tested have some natural immunity to the implanted fibrosarcoma. The randomly bred animals have significantly more immunity than do the inbred rats, as the latter have 100% tumor "takes." The former are genetically further removed from the tumor and show more regressions (experiment 3). In the inbred rats, which probably actively form less tumor inhibitory substance, fat alone has little effect and guinea pig serum injections must be added for effective control.

Application of statistics substantiates these conclusions, and also shows that there is a definite interaction between the treatments over and above the additive effect of the two. The mode of action by which dietary fat and injections of guinea pig serum independently and together exert their tumor inhibitory effect is still under investigation.

SUMMARY AND CONCLUSIONS

Five experiments were conducted to study the effects of inhibiting growth of an implanted fibrosarcoma in randomly bred and inbred Irish gray rats by (1) adding essential fats to otherwise ineffective diets, and by (2) giving intraperitoneal injections of guinea pig serum.

Statistical analysis revealed that cottonseed oil in the diet, without guinea pig serum injections, had significant effects in (1) reducing the number of tumor "takes," (2) slowing tumor growth or (3) bringing about regressions, unless the tumor was too rapidly growing to allow sufficient time for the production of possible immunologic inhibitory agents. Considering all experiments together the results were significant at the 5% level.

Guinea pig serum injections greatly enhanced the effect of fat in the diet, statistically significant at less than 0.01 or less than 0.001 level, except in experiment 2, in which only one series of injections was given.

Fat in the diet increased the activity of guinea pig serum injections significantly. The final conclusion must be reached that essential fatty acids are necessary for the fullest resistance of rats to this tumor.

ACKNOWLEDGMENTS

We wish to acknowledge the work of Mr. Clement J. Todd and Miss Beverly Gore in preparing the statistics, and the advice of Dr. R. B. Alfin-Slater for assistance in selection and preparation of diets. Our research was supported in part by Mr. and Mrs. John J. Elmore, the Strick Foundation, and the University of Southern California, to all of whom grateful acknowledgment is extended. We are indebted to the Hancock Foundation for laboratory space and facilities.

LITERATURE CITED

AINIS, H. 1958 Studies on the inhibition of some transplantable rat tumors by normal guinea pig serum. Doctoral Thesis, University of Southern California.

AINIS, H., H. M. KURTZ, P. I. KRAMER, H. E. WEIMER, R. M. RYAN AND E. JAMESON 1958 In-vivo and in-vitro studies of the action of guinea pig serum against the ascites form of the Murphy-Sturm lymphosarcoma. Cancer Research, *18:* 1309.

BLISS, C. I. 1952 Statistics of Bioassay with special reference to the Vitamins. Academic Press, New York.

CHENG, A. L. S., R. B. ALFIN-SLATER AND H. J. DEUEL, JR. 1954 The effect of fat level of the diet on general nutrition XIII. The effect of increasing dosages of x-irradiation on the protective action of fat on radiation injury. J. Nutrition, *54:* 201.

DEUEL, H. J., JR., A. L. S. CHENG, G. D. KRYDER AND M. E. BINGEMAN 1953 The protective effect against x-irradiation of methyl linoleate in the rat. Science, *117:* 254.

GREENSTEIN, J. P. 1954 Biochemistry of Cancer, 2nd edition. Academic Press Inc., New York, p. 239.

JAMESON, E., H. AINIS AND R. M. RYAN 1956 Action of guinea pig serum and human gamma globulin on the growth of a rat tumor. Science, *124:* 980.

———— 1957 Further studies on the inhibitory action of guinea pig serum on transplantable tumors. Proc. Am. Assoc. Cancer Res., *2:* 218.

———— 1958 The inhibition by guinea pig serum of the growth of the Murphy-Sturm lymphosarcoma. Cancer Res., *18:* 866.

KIDD, J. G. 1953 Regressions of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse or rabbit serum. J. Exp. Med., *98:* 565.

SINCLAIR, H. 1956 Deficiency of essential fatty acids and atherosclerosis. Lancet, *1:* 381.

TANNENBAUM, A. 1942 The genesis and growth of tumors. III. Effects of a high-fat diet. Cancer Res., *2:* 468.

TANNENBAUM, A., AND H. SILVERSTONE 1953 Nutrition in relation to Cancer. Advances in Cancer Research, *1:* 451. Academic Press, New York.

WALLIS, W. A., AND H. V. ROBERTS 1956 Statistics: A New Approach. Free Press, Glencoe, Illinois.

BIOLOGICAL AVAILABILITY OF ESSENTIAL AMINO ACIDS TO HUMAN SUBJECTS

I. WHOLE EGG, PORK MUSCLE AND PEANUT BUTTER [1,2]

JOHNNIE H. WATTS, LOVIE K. BOOKER,[3] JANET W. McAFEE,[4] E. GERALDINE WILLIAMS,[5] WALTER G. WRIGHT[6] AND FRED JONES, JR.

The Carver Foundation, Tuskegee Institute, Alabama

(Received for publication August 30, 1958)

INTRODUCTION

The nutritional value of a protein is dependent not only upon its content of essential amino acids, but also upon the biological availability of these amino acids (Rose, '38; Kuiken and Lyman, '48). Since synthesis occurs only when a complete mixture of the essential amino acids is present at one time, consideration must be given to the effective composition of a food protein in preference to the total composition (Elman, '39; Melnick, Oser and Weiss, '46; Geiger, '47). The effective composition is influenced by the completeness of digestion of the protein, the absorption of essential amino acids, and the simultaneous appearance in the blood stream of the amino acids in suitable proportions. It has been demonstrated that

[1] A preliminary report was presented before The American Institute of Nutrition, Chicago, Ill., April, 1956 (Federation Proc., *16:* 461, 1956).

[2] This project was supported by a U. S. Dept. of Agriculture Contract, sponsored by the Human Nutrition Research Division, Agricultural Research Service.

[3] Present address: Parkdale, Arkansas.

[4] Present address: Dept. of Home Economics, University of California, Berkeley, California.

[5] Present address: Dept. of Zoology, University of California, Los Angeles, California.

[6] Present address: Dept. of Chemistry, A. & T. College of North Carolina, Greensboro, North Carolina.

amino acids are liberated from proteins at different rates, characteristic of the amino acid or its linkage in the protein (Mitchell and Hamilton, '29; Jones and Gersdorff, '33; Melnick, Oser and Weiss, '46), and that specific amino acids are absorbed into the blood at different rates (Chase and Lewis, '34).

Kuiken and Lyman ('48) determined the availability of the 10 essential amino acids in roast beef, cottonseed flour, peanut flour, and wheat flour. Their calculations were based on the determination of amino acids in the food and feces of rats. Later, Kuiken ('52) used the same technique and observed differences in the availability of essential amino acids in cottonseed meal that had been processed in various ways. Deshpande et al. ('57) determined the biological availability of isoleucine in several proteins, using growth response in young rats as the criterion for availability. They reported that isoleucine in pork, beef and egg albumin was available to the extent of 80 to 90%. Several investigators have determined availability of amino acids on the basis of both growth response and measurements in food and feces of rats (lysine, Guthneck et al., '53, and Gupta et al., '58; tryptophan, Gupta and Elvehjem, '57; methionine, Schweigert and Guthneck, '54). Availabilities determined by the two methods checked reasonably well.

To our knowledge, the investigation reported here is the first attempt to determine the availability of essential amino acids, using human subjects. In addition to the 8 amino acids essential for the human adult, availabilities of cystine and tyrosine have been determined because of the sparing effects of these amino acids on methionine and phenylalanine, respectively.

Pork muscle and peanut butter were selected as the test foods. Because egg protein has been reported to have a high biological value (Bricker and Mitchell, '47; Hoagland et al., '47; Sumner and Murlin, '38), it has been included as a reference protein for comparison of the values from test foods with

those from egg. Diets and feces have been analyzed for essential amino acid content.

EXPERIMENTAL PROCEDURE

Subjects. Six male students in the School of Veterinary Medicine at Tuskegee Institute were subjects in this investigation. Regular class schedules were carried during the term of these experiments. The subjects were between 22 and 28 years of age. A physical examination was given to each subject, including complete hemogram, total proteins, blood sugar, urinalysis and albumin-globulin ratio. All subjects were considered to be healthy by the physician.

Experimental periods. Caloric intakes needed to maintain body weight and protein intakes necessary for nitrogen equilibrium were established in preliminary periods.

Each subject participated during 4 experimental periods: (1) a period of low-N intake for 7 days during which a basal diet, consisting chiefly of fruits and vegetables and containing 0.8 gm N, was fed along with an amount of a N-free Calorie adjustor that provided a total intake of 48 Cal./kg/day for the three lighter subjects and 45 Cal./kg/day for the three heavier subjects; (2) an egg period of 10 days during which each subject received the same basal diet, whole egg in amounts to supply one gm of protein/kg/day, and sufficient amounts of the Calorie adjustor to make this diet isocaloric with the low-N diet; (3) a pork period of 10 days during which the same basal diet was fed along with amounts of pork muscle and the Calorie adjustor to make this diet isonitrogenous and isocaloric, respectively, with the egg diet; (4) a peanut butter period of 10 days during which each subject received the same basal diet, peanut butter in amounts to make the total intake isonitrogenous with the egg and pork diets, and the Calorie adjustor in amounts to make this diet isocaloric with the preceding ones. The subjects were weighed each morning before breakfast.

For the egg, pork and peanut butter periods, urinary and fecal collections were made during the 6 days immediately following a 4-day adjustment period. For the low-N period, collections were made during the 4 days immediately following a three-day adjustment period. Carmine (Alum Lake) was used as the fecal marker.

Foods. In planning the intakes, the Calorie/protein ratio and the carbohydrate/fat distribution for each subject were kept as nearly constant as possible in the 4 diets. The test food of highest fat content was peanut butter; butterfat in addition to the 78 gm included in the basal diet was used to adjust the fat content of other diets to that of the peanut

TABLE 1

The basal diet

Breakfast:	*Dinner:*
150 ml Orange juice	75 gm Glazed carrots
2 Peach halves	75 gm Harvard beets
Hot tea	Celery-Pineapple salad
Lunch:	*Plus:*
Lettuce salad	50 gm Cornstarch wafers [1]
Baked apple	78 gm Butterfat
Iced tea	

[1] Consisting of 200 gm starch, 8 gm baking powder, 120 gm sucrose, 120 gm butter, 12 gm agar, 4 gm salt and 122 ml distilled water. Energy value: 469 Cal./100 gm.

butter diet. Adjustments in the amount of carbohydrate were made with mint wafers, which consisted of sucrose, water, food coloring and peppermint flavoring. Adjustments in Caloric intakes, due to differences in initial body weights of the subjects, were made also with the mint wafers.

Meals were prepared and served in the Diet Kitchen of the School of Home Economics. The composition of the basal diet is shown in table 1. By analysis, this diet consisted of 4.8 gm protein, 112.3 gm fat, 5.1 gm ash and 232.6 gm carbohydrate (by difference). Cornstarch wafers [7] were prepared every

[7] The cornstarch was a gift from A. E. Staley Manufacturing Company, Decatur, Illinois.

other day. Eggs, produced by a closed flock of hens, were obtained from the Experimental Farm at Tuskegee Institute. Pork loins and tenderloin tips were stripped of as much connective tissue and fat as possible and ground. Individual weighed patties of the ground pork muscle were frozen for use later. Peanut butter [8] was obtained in a single batch; its only constituents were roasted Spanish peanuts and salt.

The amounts of the various test foods that were consumed daily ranged between 470 to 600 gm of egg (9 to 12 eggs), 260 to 335 gm of pork muscle and 185 to 240 gm (6 to 8 oz.) of peanut butter per day, depending upon individual body weights.

A homogenate of the basal diet was prepared during each dietary period. Basal diet homogenates, samples of the ground pork, and homogenized whole eggs were freeze-dried[9] at a pressure of 200 μ or less. The powders obtained on freeze-drying were used in making the various analyses.

Supplements. The mineral and vitamin contents of the 4 diets were estimated, using food composition tables. In order to make them conform to N.R.C. allowances for vitamins and minerals, supplements in the form of dicalcium phosphate,[10] riboflavin,[11] nicotinic acid,[11] and thiamine chloride[11] were given.

Collection and handling of excreta. Twenty-four-hour urine samples were collected in bottles which contained 25 ml of 2% acetic acid. Analyses to be done on urines were made on the individual 24-hour collections. Fecal specimens were stored daily in a freezer and a composite of the 6-day collections was made for each period. The composite was homogenized and autoclaved at 15 lbs. pressure for 20 min. except for the first 6 composites, which were dried by infrared lamps,

[8] A gift from the Tom Huston Peanut Company, Columbus, Georgia.

[9] A Holzman Lyophil Apparatus, made by Greiner Glassblowing Laboratory, Los Angeles, California, was used. Specifications published in Science, *111*: 550 (1950), fig. 1.

[10] Abbott.

[11] Lilly.

homogenized feces were freeze-dried at a pressure of 200 μ or less.

Analytical procedures. To determine the total moisture content of the original food and fecal samples, the freeze-dried samples were subsequently dried in a vacuum oven at 70°C and 30 mm pressure to remove residual moisture. Total N in the diets, urines and feces was determined by the Kjeldahl procedure. Daily creatinine determinations in urine were made according to the method of Clark and Thompson ('49). Food and fecal samples were assayed microbiologically for amino acids; procedures for tryptophan, methionine, threonine, lysine, phenylalanine and tyrosine were those of Barton-Wright ('52); leucine, isoleucine and valine according to Horn, Jones and Blum ('50); and cystine, Horn and Blum ('56).

RESULTS

Nitrogen balances. Nitrogen balances are shown in table 2. As expected, all N balances during the low-N period were negative. The most positive average nitrogen balance occurred during the pork period and the least positive during the peanut butter period, although the dietary N was 0.43 gm less in the pork diet than in the egg diet. Dietary N was approximately the same during the peanut butter and egg periods, but both fecal and urinary N excretions were highest during the peanut butter period, indicating lower degrees of digestibility and utilization, respectively, of N during the peanut butter period.

Urinary creatinine. The average urinary creatinine excretion (table 2) was the same for the low-N and egg periods, 1.66 gm/day. By analysis of variance, the increase in urinary creatinine during the pork period was shown to be significantly higher than during the low-N and egg periods, while the increase during the peanut butter period was not significant.

Dietary amino acids. The average amino acid intakes of all subjects during the 4 dietary periods are presented in

table 3. Intakes of most of the essential amino acids were lower in the peanut butter diet than in the pork and egg diets. Rose has defined the safe intake of an amino acid as twice the minimal level required for a slightly positive N balance.

TABLE 2

Creatinine and nitrogen data

DIETARY PERIOD	SUBJECT	URINARY CREATININE	DIETARY NITROGEN	URINARY NITROGEN	FECAL NITROGEN	NITROGEN BALANCES
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*
Low-nitrogen	O. J.	1.94	0.77	2.24	1.16	— 2.63
	E. P.	1.73	0.77	2.02	0.80	— 2.05
	E. H.	1.56	0.77	1.94	0.55	— 1.72
	D. R.	1.76	0.77	2.03	1.20	— 2.46
	L. S.	1.51	0.77	2.40	0.75	— 2.38
	T. R.	1.48	0.77	1.93	0.81	— 1.97
	Average	1.66	0.77	2.09	0.88	— 2.20
Egg	O. J.	2.00	12.50	7.78	1.42	+ 3.30
	E. P.	1.87	11.99	8.33	1.29	+ 2.37
	E. H.	1.54	10.45	7.08	0.93	+ 2.44
	D. R.	1.54	10.45	7.23	1.02	+ 2.20
	L. S.	1.70	9.95	8.02	1.12	+ 0.81
	T. R.	1.31	9.95	7.88	1.45	+ 0.62
	Average	1.66	10.88	7.72	1.20	+ 1.96
Pork	O. J.	2.31	12.10	8.07	0.99	+ 3.04
	E. P.	2.20	11.43	8.58	1.39	+ 1.46
	E. H.	1.95	10.08	7.99	0.83	+ 1.26
	D. R.	1.82	10.08	5.47	1.15	+ 3.46
	L. S.	1.89	9.35	6.87	0.91	+ 1.77
	T. R.	1.68	9.47	7.35	1.04	+ 1.08
	Average	1.98	10.45	7.39	1.05	+ 2.02
Peanut butter	O. J.	2.00	12.36	8.44	1.87	+ 2.05
	E. P.	2.01	11.70	9.11	1.22	+ 0.37
	E. H.	1.78	10.33	8.22	1.66	+ 0.45
	D. R.	1.75	10.33	8.19	1.22	+ 0.92
	L. S.	1.52	9.80	7.12	1.30	+ 1.38
	T. R.	1.45	9.71	8.51	1.38	— 0.18
	Average	1.75	10.70	8.26	1.44	+ 0.83

Except in the case of the sulfur-containing amino acid content of the peanut butter diet, the amounts of the amino acids provided in the three diets which contained a protein food were in excess of the safe intakes recommended by Rose ('49).

Amino acids in feces. The average fecal excretions of the 10 amino acids (table 4) during the low-N period were appreciably in excess of amino acid intakes. Thus, it would seem that a large part of the fecal amino acid excretion was either endogenous or bacterial in origin. Variation in amino acid excretions among individuals is shown by the relatively high fecal amino acid excretions for O. J. and D. R. during this period when N intakes were the same for all subjects. The average fecal amino acid excretions were highest during the peanut butter period.

TABLE 3

Average amino acid intakes of total diets

AMINO ACIDS	DIETARY PERIODS				SAFE INTAKES [1]
	Low N	Egg	Pork	Peanut butter	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*
Isoleucine	172	4223	3696	2494	1400
Leucine	217	6286	4997	3940	2200
Lysine	201	5279	5324	2225	1600
Methionine	34	2254	1407	614	2200
Cystine	10	949	681	642	—
Phenylalanine	109	3470	2539	3044	2200
Tyrosine	114	2272	1949	2286	—
Threonine	158	3825	4467	2433	1000
Tryptophan	31	818	685	629	500
Valine	185	4397	3243	2586	1600

[1] Rose, W. C. Current Research in The Science of Nutrition, March, 1955. Publication of the Nutrition Foundation, Inc. For purposes of comparison, the data are expressed in this paper as milligrams instead of grams.

Availability of amino acids. The per cent availability of amino acids in the total diets, making no allowance for fecal amino acids of endogenous or bacterial origin, and the per cent availability of amino acids in each of the test foods were calculated as follows:

$$\%\ \text{Availability in total diet}\ [12] = \frac{\text{Intake from total diet} - \text{Amount excreted in feces}}{\text{Intake from total diet}} \times 100$$

$$\%\ \text{Availability in test food} = \frac{\text{Intake from test food} - (\text{Fecal excretion, test food period} - \text{Fecal excretion, low-N period})}{\text{Intake from test food}} \times 100$$

[12] Total diet includes basal diet + Calorie adjuster + test food.

TABLE 4

Amino acids in feces

DIETARY PERIOD	SUBJECTS	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	CYSTINE	PHENYLALANINE	TYROSINE	THREONINE	TRYPTOPHAN	VALINE
		mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
Low-nitrogen	O. J.	296	443	424	128	115	244	200	354	112	468
	E. P.	242	345	218	103	74	150	194	248	61	282
	E. H.	160	191	216	58	52	120	141	184	46	198
	D. R.	332	414	386	94	86	192	165	416	76	243
	L. S.	218	290	328	100	45	154	154	295	66	297
	T. R.	208	283	266	76	62	154	138	331	74	286
	Average	243	328	306	93	72	169	165	305	72	296
Egg	O. J.	314	372	513	91	94	228	240	354	118	582
	E. P.	276	363	418	88	100	198	264	284	107	348
	E. H.	193	206	304	78	59	162	184	272	62	284
	D. R.	297	340	344	118	70	184	192	306	70	308
	L. S.	264	380	436	109	70	332	157	350	84	342
	T. R.	397	475	562	118	90	272	298	512	148	480
	Average	290	356	430	100	80	229	222	346	98	391
Pork	O. J.	296	349	391	113	83	200	244	226	89	427
	E. P.	406	378	515	144	110	224	336	498	120	448
	E. H.	214	279	309	91	76	132	178	260	70	294
	D. R.	300	412	261	136	82	204	245	346	86	353
	L. S.	218	333	367	96	87	172	162	266	100	330
	T. R.	258	204	308	60	84	104	216	335	86	240
	Average	282	326	358	107	87	173	230	322	92	349
Peanut butter	O. J.	412	492	690	171	99	359	268	442	123	642
	E. P.	300	282	489	140	86	280	204	347	84	336
	E. H.	420	512	550	150	138	260	307	480	120	630
	D. R.	367	276	460	228	94	272	244	353	84	361
	L. S.	249	380	492	104	83	214	204	450	86	399
	T. R.	374	442	478	150	116	224	259	398	102	438
	Average	354	391	526	157	103	268	248	412	100	468

Availability data calculated for the total diets (table 5) were similar for the egg and pork diets. Availabilities in the egg diet ranged from 88.2% for tryptophan to 95.4% for methionine and were over 90% for all but tryptophan; availabilities in the pork diet ranged from 86.6% for tyrosine and tryptophan to 93.4% for leucine and were over 90% for all but tyrosine, tryptophan and cystine. In contrast, the values for the peanut butter diet ranged from 74.3% for methionine to 91.2% for phenylalanine and were below 90% for all but phenylalanine. Except for the availability of tyrosine, the availabilities of the various amino acids in the peanut butter diet were consistently lower than corresponding values in the egg and pork diets.

In making calculations for availability in test foods alone, the assumption was made that the amount of each nutrient excreted from the basal diet was the same whether fed in the presence of large or small amounts of Calorie adjustor and whether fed in the presence of large amounts of the test food or in the absence of a test food. Several availability values obtained in this way exceeded 100% because fecal excretions of amino acids were less during a period when protein was fed than during the low-N period (table 4). These results indicate that the amount of metabolic nutrient excreted varies with the type of diet and that calculation of the availability of test foods alone is without significance.

DISCUSSION

In addition to the low methionine content of the peanut butter diet, mentioned previously, the availability of methionine was low, namely 74%, while corresponding values were 92% in the pork diet and 95% in the egg diet. The amounts of available methionine in the egg, pork and peanut butter diets were 2150, 1301 and 456 mg, respectively, whereas, the safe intake of methionine is 2200 mg. Rose and Wixom ('55) have shown that 80 to 89% of the methionine requirement can be spared by L-cystine in normal young men. Since in all cases

TABLE 5

Availability of amino acid in total diets

DIET	SUBJECTS	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	CYSTINE	PHENYLALANINE	TYROSINE	THREONINE	TRYPTOPHAN	VALINE
		%	%	%	%	%	%	%	%	%	%
Egg	O. J.	93.6	94.9	91.6	96.5	91.4	94.3	90.8	92.0	87.7	88.5
	E. P.	94.1	94.8	92.8	96.5	90.5	94.8	89.5	93.3	88.4	92.8
	E. H.	95.2	96.6	94.0	96.4	93.5	95.1	91.6	92.5	92.2	93.2
	D. R.	92.7	94.4	93.2	94.5	92.3	94.5	91.2	91.7	91.3	92.7
	L. S.	93.1	93.4	90.9	94.7	91.9	89.5	92.4	90.0	89.0	91.5
	T. R.	89.7	91.7	88.3	94.2	89.6	91.4	85.6	85.3	80.6	88.0
	Average	93.1	94.3	91.8	95.4	91.5	93.3	90.2	90.8	88.2	91.1
Pork	O. J.	93.1	94.0	93.7	93.1	89.5	93.2	89.2	95.7	88.8	88.6
	E. P.	90.0	93.1	91.2	90.7	85.3	92.0	84.4	89.8	84.0	87.4
	E.H.	94.0	94.2	94.0	93.3	88.4	94.6	90.5	94.0	89.4	90.6
	D. R.	91.6	91.4	94.9	90.0	87.5	91.6	86.9	92.0	87.0	88.7
	L. S.	93.5	92.7	92.4	92.5	87.4	92.6	90.9	93.5	84.0	88.8
	T. R.	92.3	95.5	93.6	95.3	86.3	95.5	87.7	91.7	86.1	91.8
	Average	92.4	93.4	93.3	92.5	87.4	93.2	86.6	92.8	86.6	89.3
Peanut butter	O. J.	85.7	89.2	73.1	75.9	86.7	89.8	89.9	84.3	83.1	78.5
	E. P.	87.9	93.4	79.8	79.2	87.8	91.6	91.8	87.0	87.8	88.0
	E. H.	82.6	86.5	74.4	74.7	77.7	91.1	86.1	79.6	80.2	74.8
	D. R.	84.8	92.7	78.6	61.5	84.8	90.7	88.9	85.0	86.2	85.4
	L. S.	89.1	89.4	75.9	81.5	85.8	92.3	90.2	79.8	85.0	83.1
	T. R.	83.4	87.6	76.3	73.0	79.9	91.8	87.5	81.9	82.1	81.3
	Average	85.6	89.8	76.4	74.3	83.8	91.2	89.1	82.9	84.1	81.8

the amounts of cystine were less than 80% of the total methionine + cystine content of the diets, all of the cystine present can be considered interchangeably with methionine. The amounts of methionine + cystine present in the egg, pork and peanut butter diets were 3203, 2088 and 1256 mg, respectively, while amounts available in the same order were 3018, 1896 and 994 mg. Thus, the differences in N balances obtained may have been due to the differences in methionine + cystine values or to the different assortments of amino acids in these diets.

The interpretation of any study involving the amino acid content of feces is limited by one's ability to measure the extent of degradation and synthesis of amino acids by intestinal bacteria. Kuiken ('52) pointed out that where relatively large amounts of nondigestible proteins are present, intestinal microorganisms may alter the amino acid distribution of food residues as they pass through the intestine. However, under the experimental conditions used by Kuiken, the effect of intestinal bacteria in rats was negligible. Further research is needed to determine whether the differences observed in apparent availability of amino acids in these diets are due chiefly to differences in the rate of liberation of the amino acids from the protein moieties, to bacterial flora, or to other factors.

SUMMARY

Urinary and fecal excretions of total N and fecal excretions of the 8 essential amino acids, and of cystine and tyrosine, were determined for 6 healthy young men on a low-protein diet and on diets in which the chief source of N was (1) egg, (2) pork and (3) peanut butter.

Average values for the availability of amino acids and for nitrogen balances were similar for the egg and pork diets. With the exception of tyrosine, the average values for the availability of the amino acids in the peanut butter diet were consistently lower than those for the egg and pork diets. The average nitrogen balance also was lower for the peanut butter

diet, due to higher excretions of both fecal and urinary N during this period. Limitation of the method used with regard to the calculation of availability for an individual food, rather than the total diet, was shown.

LITERATURE CITED

BARTON-WRIGHT, E. C. 1952 Microbiological Assay of the Vitamin B-complex and Amino Acids. Pitman Publishing Corporation, N. Y., N. Y.

BRICKER, M. L., AND H. H. MITCHELL 1947 The protein requirements of the adult rat in terms of the protein contained in egg, milk and soy flour. J. Nutrition, *34:* 491.

CHASE, B. W., AND H. B. LEWIS 1934 Comparative studies of the metabolism of amino acids. J. Biol. Chem., *106:* 315.

CLARK, L. C., JR., AND H. L. THOMPSON 1949 Determination of creatine and creatinine in urine. Anal. Chem., *21:* 1218.

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

ELMAN, R. 1939 Time factor in retention of nitrogen after intravenous injection of a mixture of amino acids. Proc. Soc. Exp. Biol. Med., *40:* 484.

GEIGER, E. 1947 Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, *34:* 96.

GUPTA, J. D., A. M. DAKROURY, A. E. HARPER AND C. A. ELVEHJEM 1958 Biological availability of lysine. Ibid., *64:* 259.

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

GUTHNECK, B. T., B. A. BENNETT AND B. S. SCHWEIGERT 1953 Utilization of amino acids from foods by the rat. II. Lysine. Ibid., *49:* 289.

HOAGLAND, R., N. R. ELLIS, O. G. HANKINS AND G. G. SNIDER 1947 Supplemental relationship between pork protein and egg protein. Ibid., *34:* 43.

HORN, M. J., AND A. E. BLUM 1956 A microbiological method for determination of cystine in foods. Cereal Chem., *33:* 18.

HORN, M. J., D. B. JONES AND A. E. BLUM 1950 Method for microbiological and chemical determinations of essential amino acids in proteins and foods. U. S. Department of Agriculture, Miscellaneous Publication No. 696.

JONES, D. B., AND C. E. F. GERSDORFF 1933 Studies on digestibility of proteins *in vitro*. Rate of liberation of cystine on hydrolysis of casein. Some observations on colorimetric tests for cystine when applied to peptic and acid digests of casein. J. Biol. Chem., *101:* 657.

KUIKEN, K. A. 1952 Availability of the essential amino acids in cottonseed meal. J. Nutrition, *46:* 13.

KUIKEN, K. A., AND C. M. LYMAN 1948 Availability of amino acids in some foods. Ibid., *36:* 359.

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

MITCHELL, H. H., AND T. S. HAMILTON 1929 The Biochemistry of the Amino Acids. Chemical Catalog Company, Inc., New York, N. Y.

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

———— 1938 The nutritive significance of the amino acids. Physiol. Revs., *18:* 109.

ROSE, W. C., AND R. L. WIXOM 1955 The amino acid requirements of man. XIII. The sparing effect of cystine on the methionine requirement. J. Biol. Chem., *216:* 763.

SCHWEIGERT, B. S., AND B. T. GUTHNECK 1954 Utilization of amino acids from foods by the rat. III. Methionine. J. Nutrition, *54:* 333.

SUMNER, E. E., AND J. R. MURLIN 1938 The biological value of milk and egg proteins in human subjects. Ibid., *16:* 141.

BIOLOGICAL AVAILABILITY OF ESSENTIAL AMINO ACIDS TO HUMAN SUBJECTS

II. WHOLE EGG, MILK AND COTTAGE CHEESE[1]

JOHNNIE H. WATTS, LOVIE K. BOOKER,[2] JANET W. McAFEE,[3]
DONALD C. W. GRAHAM AND FRED JONES, JR.
The Carver Foundation, Tuskegee Institute, Alabama

(Received for publication August 30, 1958)

INTRODUCTION

While the essential amino acid composition of a protein is paramount in determining the quality of a protein, other factors must be considered in the assessment of its biological value. The extent to which the constituent amino acids are available is one important determinant. The degree of availability of amino acids in different proteins is influenced by several factors, among which are resistance to hydrolysis by digestive enzymes, amino acid antagonism, absorption rates of the individual amino acids, and, in some cases, solubility.

Mauron et al. ('55) used *in vitro* digestion techniques to study the alteration in availability of tyrosine and the heat labile essential amino acids—lysine, methionine and, in some instances, tryptophan—in milk manufactured in various ways. Both destruction and inactivation were determined in boiled milk, spray-dried milk, sweetened condensed milk, evaporated milk, and two types of roller-dried milk. They found no destruction of tryptophan, tyrosine or methionine in any milk. Lysine destruction varied from zero in the boiled milk to 26.6% in one of the roller-dried milks. There was no inacti-

[1] Supported by a grant from the National Dairy Council.

[2] Present address: Parkdale, Arkansas.

[3] Present address: Department of Home Economics, University of California, Berkeley, California.

vation of tryptophan in any milk. Methionine was slightly inactivated in the roller-dried milks. Lysine was inactivated in evaporated milk (11.2%), roller milk A (20%), and roller milk B (45.8%).

In the investigation reported here, adult males have been used to observe biological availabilities for the 8 essential amino acids, and for cystine and tyrosine in milk and cottage cheese. Cystine and tyrosine are included because of the sparing effects of these amino acids on methionine and phenylalanine, respectively. Because egg protein has been reported to have a high biological value (Bricker and Mitchell, '47; Hoagland et al., '47; Sumner and Murlin, '38), it has been used as a reference protein for comparison of the values from the dairy proteins with those from egg.

EXPERIMENTAL PROCEDURE

Planning of dietary intakes, food components of the basal diet, mineral and vitamin supplements, collection and handling of excreta and procedures for chemical analyses were the same as reported previously (Watts et al., '59).

Subjects. Eight male students in the School of Veterinary Medicine at Tuskegee Institute were subjects. Their ages were between 24 and 28 years. Each subject was given a physical examination and was considered by the physician to be in good health. Initial body weights ranged between 137 and 170 lbs.

Experimental periods. Caloric intakes needed to maintain body weights were established in a preliminary period.

One subject, E. B., participated during two dietary periods only, the milk and the egg periods. The other 7 participated during 4 experimental periods of 10 days each: (1) a period of low-N intake during which a basal diet, consisting chiefly of fruits and vegetables and containing 0.9 gm N was fed along with an amount of sucrose that provided a total daily intake of 43 Cal./kg for R. J. and C. W., 45 Cal./kg for L. C., A. C., E. B., and O. C., and 47 Cal./kg for D. H. and R. D.; (2) an

egg period during which each subject received the same basal diet, whole egg in amounts to supply 1 gm protein/kg/day and sufficient amounts of sucrose to make this diet isocaloric with the low-N diet; (3-a) a milk period for three subjects, during which the same basal diet was fed along with amounts of milk and sucrose to make this diet isonitrogenous and isocaloric, respectively, with the egg diet; or (3-b) a milk + egg period for the other 5 subjects, during which the same basal diet was fed along with milk and egg in amounts such that each food provided 0.5 gm protein/kg/day, and sucrose in amounts to make this diet isocaloric with the preceding ones; (4) a cottage cheese period during which each subject received the same basal diet along with amounts of cottage cheese and sucrose to make the total intakes isonitrogenous and isocaloric, respectively, with the egg diet. Sucrose, the Calorie adjustor, was used to adjust for differences in both Caloric content of the test foods, and Calorie requirements of the individual subjects as determined by their initial body weights.

All subjects received the egg diet during the first 10-day period. During the second 10-day period, three subjects received the milk diet, three the cottage cheese diet, and two the low-N diet. Because the first three subjects to receive the milk diet had diarrhea, the remaining 5 were given ½ their N intakes as milk and ½ as whole egg. Stools were normal for the 5 subjects fed the milk + egg regime.

Urinary and fecal collections were made during the 6 days immediately following the 4-day adjustment period. Carmine (Alum Lake) was the fecal marker.

Foods. The basal diet, by analysis, contained 5.6 gm protein, 116.2 gm fat, 9.3 gm ash, 309.1 gm carbohydrate (by difference), and 2,322 Cal.

Eggs were produced by a closed flock of hens on the Experimental Farm at Tuskegee Institute. On analysis, they were found to contain 12.8% protein, 10.4% fat, 0.9% ash, 1.32% carbohydrate (by difference), and 171 Cal./100 gm.

Cow's homogenized (vitamin D-fortified) milk and cream-style cottage cheese were obtained from one of the Montgomery dairies. One shipment of milk for each of the three 10-day periods and 5 shipments of cottage cheese during the three 10-day periods were received. Whereas, under proper refrigeration, milk kept well for a 10-day period, cottage cheese was purchased to last a maximum of 6 days. Each shipment of milk and cottage cheese consisted of consecutively filled cartons from a single batch of the particular food. The milk contained 3.5% protein, 4.2% fat, 0.7% ash and 4.4% carbohydrate (by difference). Cottage cheese contained 16% milk solids and 11.1% protein, 4.7% fat, 1.4% ash, and 3.6% carbohydrate (by difference). Energy values per 100 gm were 74 Cal. for milk and 119 Cal. for cottage cheese.

The amounts of the test foods consumed daily, depending upon body weights, were between 500 and 659 gm of whole egg (approximately 12 eggs); approximately 2 qts. of milk for the milk period, or 1 qt. milk + 6 eggs for the milk + egg period; and 450 to 600 gm (approximately 1 pt.) of cottage cheese.

RESULTS

Urinary creatinine and nitrogen balances. Urinary creatinine group averages (table 1) varied by less than 0.1 gm creatinine/day and were considered to be constant during all periods.

The average variation in urinary nitrogen was between 1.3 and 1.7 gm/24 hrs. for the protein periods and was 0.6 gm for the low-N period. Except during the low-N period, the average N balance (table 1) was most positive during the milk + egg period, +2.82, and least positive during the cottage cheese period, + 1.10. It should be noted, however, that dietary N was 1.81 gm lower during the cottage cheese period than during the milk + egg period. While dietary N was 15% lower, urinary N was 10% higher, and fecal N 13% lower during the cottage cheese period than during the milk + egg period. These results seem to indicate that cottage cheese

TABLE 1

Creatinine and nitrogen data

DIETARY PERIODS	SUBJECTS	URINARY CREATININE	DIETARY NITROGEN	URINARY NITROGEN	FECAL NITROGEN	NITROGEN BALANCES
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*
Low nitrogen	R. J.	1.97	0.90	2.90	1.11	— 3.11
	C. W.	2.03	0.90	2.80	1.02	— 2.92
	D. H.	1.88	0.90	2.74	0.70	— 2.54
	R. D.	1.59	0.90	2.61	0.91	— 2.62
	L. C.	1.97	0.90	3.60	1.15	— 3.85
	A. C.	1.67	0.90	2.64	0.76	— 2.50
	O. C.	1.50	0.90	2.19	1.02	— 2.31
	Average	1.80	0.90	2.78	0.95	— 2.84
Egg	R. J.	2.03	13.89	9.94	1.20	+ 2.75
	C. W.	2.01	13.51	9.05	1.31	+ 3.15
	R. D.	1.78	12.37	9.33	1.55	+ 1.49
	L. C.	2.03	12.22	8.38	1.28	+ 2.56
	A. C.	1.73	12.45	8.43	1.43	+ 2.59
	E. B.	1.68	12.57	8.93	1.38	+ 2.26
	O. C.	1.67	10.94	6.34	2.09	+ 2.51
	Average	1.85	12.56	8.63	1.46	+ 2.47
Milk	D. H.	1.95	12.61	7.89	1.84	+ 2.88
	A. C.	1.76	10.90	8.28	1.74	+ 0.88
	E. B.	1.51	11.02	7.72	2.19	+ 1.11
	Average	1.74	11.51	7.96	1.92	+ 1.62
Milk, with egg supplement	R. J.	1.86	13.55	8.80	1.50	+ 3.25
	C. W.	2.02	12.38	9.55	1.25	+ 1.58
	R. D.	1.70	12.08	7.05	0.90	+ 4.13
	L. C.	1.97	11.95	8.74	1.19	+ 2.00
	O. C.	1.64	10.69	6.10	1.46	+ 3.13
	Average	1.84	12.13	8.05	1.26	+ 2.82
Cottage cheese	R. J.	2.08	11.36	10.34	1.11	— 0.09
	C. W.	2.09	11.07	9.97	1.06	+ 0.04
	D. H.	1.81	10.89	7.37	0.94	+ 2.58
	R. D.	1.91	10.14	8.71	1.16	+ 0.27
	L. C.	2.07	10.02	7.98	1.06	+ 0.98
	A. C.	1.64	10.21	7.74	1.15	+ 1.32
	O. C.	1.61	9.00	7.07	1.24	+ 0.69
	Average	1.89	10.38	8.45	1.10	+ 0.83

protein is highly digestible and that the resulting amino acids are highly absorbed, but that the absorbed N is less well utilized from cottage cheese than from a combination of milk + egg.

N absorption figures for the milk, egg, milk + egg, and cottage cheese diets were 83, 88, 90, and 94%, respectively. The relatively high average fecal N during the milk period was doubtless related to the fluid nature of the stools.

Differences in dietary N among periods were due to differences in assay values obtained for test foods during a prelim-

TABLE 2

Average amino acid intakes in total diets

AMINO ACIDS	DIETARY PERIODS					SAFE INTAKES [1]
	Low N	Egg	Milk	Milk with egg	Cottage cheese	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
Isoleucine	108	3623	2881	3219	2739	1400
Leucine	236	5844	5231	5461	5192	2200
Lysine	165	4727	4650	4639	4155	1600
Methionine	52	2220	1499	1842	1444	2200
Cystine	10	1050	316	678	185	—
Phenylalanine	113	3318	2294	2778	2122	2200
Tyrosine	114	2513	3376	2911	2685	—
Threonine	114	2710	2622	2638	1822	1000
Tryptophan	28	849	721	777	705	500
Valine	152	4024	3699	3822	3225	1600

[1] W. C. Rose. Current Research in the Science of Nutrition, March, 1955. Publication of the Nutrition Foundation, Inc. For purposes of comparison, the data are expressed in this paper as milligrams instead of grams.

inary period and, later, during the experimental periods. Intakes were based upon preliminary values.

Dietary amino acids. The average amino acid intakes and the safe intakes of the essential amino acids are shown in table 2. The safe intake of an amino acid has been defined by Rose ('55) as twice the minimal level required for a slightly positive N balance. Except for the sulfur-containing amino acid content of the cottage cheese and milk diets, the amounts of the individual amino acids provided in the total diets which

TABLE 3

Amino acids in feces

DIETARY PERIOD	SUBJECTS	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	CYSTINE	PHENYLALANINE	TYROSINE	THREONINE	TRYPTOPHAN	VALINE
		mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
Low nitrogen	R. J.	252	342	364	72	98	200	237	297	103	259
	C. W.	282	382	374	114	91	244	308	298	100	282
	D. H.	168	244	196	76	58	128	156	160	47	184
	R. D.	170	199	267	83	86	125	232	229	67	208
	L. C.	256	378	314	95	102	193	312	286	106	320
	A. C.	178	198	184	70	65	121	215	180	85	238
	O. C.	280	302	352	102	104	252	309	200	76	286
	Average	226	292	293	87	86	180	253	236	83	254
Egg	R. J.	255	346	428	104	91	186	309	256	89	244
	C. W.	364	484	462	139	100	222	350	346	92	340
	R. D.	354	572	545	231	124	296	319	421	121	390
	L. C.	334	468	402	148	118	230	462	313	151	364
	A. C.	282	362	415	172	112	232	342	348	108	346
	E. B.	332	388	520	196	95	270	358	373	86	357
	O. C.	612	654	704	288	185	363	510	615	106	602
	Average	362	468	496	182	118	257	378	382	108	378
Milk	D. H.	575	639	594	196	98	276	488	500	106	529
	A. C.	290	579	493	144	112	194	447	433	131	360
	E. B.	556	548	686	226	105	302	348	686	132	548
	Average	474	589	591	189	105	257	428	540	123	479
Milk, with egg supplement	R. J.	396	504	490	156	114	282	417	328	94	443
	C. W.	366	498	435	123	86	186	405	266	108	404
	R. D.	276	362	366	93	52	164	232	210	76	281
	L. C.	340	459	412	126	84	220	368	260	122	372
	O. C.	384	310	654	186	122	245	258	404	95	484
	Average	352	427	471	137	92	219	336	294	99	397
Cottage cheese	R. J.	288	242	373	113	46	184	368	278	106	464
	C. W.	280	365	392	118	37	158	308	207	112	319
	D. H.	256	338	390	118	32	146	243	226	69	310
	R. D.	310	408	402	122	54	202	326	310	93	317
	L. C.	254	408	382	94	39	155	316	262	127	366
	A. C.	284	416	426	124	36	190	385	308	128	377
	O. C.	368	496	522	164	72	220	441	362	101	383
	Average	291	382	412	122	45	179	341	279	105	362

contained a protein food were in excess of the safe intakes recommended by Rose ('49).

Amino acids in feces. The average fecal excretions for the 10 amino acids (table 3) were highest during the milk period and lowest, among the protein periods, during the cottage cheese period. This corroborates the values obtained for total N in feces. Except for cystine and phenylalanine excretions in feces during the cottage cheese period, the average fecal amino acid excretions during the low-N period were less than during the protein periods. The 48% lower excretion of cystine during the cottage cheese period than during the low-N period was anomalous but interesting.

Availability of amino acids. The methods for calculation of the per cent availability of amino acid in both the test foods and the total diets were the same as reported previously (Watts et al., '59).

The highest values for the availabilities of amino acids computed for test foods alone were obtained for the milk + egg and cottage cheese. Because the average fecal excretion of cystine was higher during the low-N period than during the cottage cheese period, the calculated availabilities for cottage cheese alone were greater than 100%. Availabilities for amino acids in all test foods were greater than 90%, except for threonine in milk, 88%, and cystine in milk, 86%. Limitations of the method for the determination of availability in test foods alone have been discussed (Watts et al., '59).

Availabilities for the total diets (table 4) were in all cases lower than values for the corresponding test foods. The lowest availabilities in total diets were obtained for the milk diet. While availabilities for most of the 10 amino acids were less than 5% lower in the milk diet than corresponding values in the other diets, cystine, in some instances, was as much as 25% lower. Amino acids in the cottage cheese diet showed higher availabilities than corresponding values in the milk diet, but slightly lower, except in the cases of isoleucine, leucine and lysine, than those for the milk+egg diet. With one

TABLE 4

Availabilities of amino acids in total diets

DIET	SUBJECTS	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	CYSTINE	PHENYLALANINE	TYROSINE	THREONINE	TRYPTOPHAN	VALINE
		%	%	%	%	%	%	%	%	%	%
Egg	R. J.	93.6	94.6	91.8	95.7	92.1	94.9	88.8	91.4	90.5	94.5
	C. W.	90.6	92.2	90.9	94.2	91.1	93.7	87.0	88.1	89.9	92.1
	R. D.	90.0	90.0	88.2	89.3	87.9	90.8	87.0	84.1	85.4	90.1
	L. C.	90.4	91.7	91.2	93.1	88.4	92.8	81.0	88.0	81.6	90.6
	A. C.	92.1	93.7	91.1	92.1	89.1	92.9	86.2	86.9	87.0	91.2
	E. B.	90.8	93.3	88.9	91.1	90.9	91.8	85.6	86.1	89.8	91.1
	O. C.	80.3	87.0	82.7	84.9	79.4	87.3	76.4	73.6	85.4	82.6
	Average	89.7	91.8	89.2	91.5	88.4	92.0	84.6	85.4	87.1	90.3
Milk	D. H.	80.9	88.3	87.8	87.5	70.3	88.5	86.1	81.7	85.9	86.3
	A. C.	89.6	88.6	89.1	90.1	63.5	91.3	86.4	83.0	81.3	90.0
	E. B.	80.4	89.4	85.0	84.7	66.2	86.0	89.5	73.4	81.4	84.9
	Average	83.6	88.8	87.3	87.4	66.7	88.8	87.3	79.4	82.9	87.1
Milk, with egg supplement	R. J.	88.9	91.6	90.4	92.3	84.8	90.8	87.0	88.8	89.1	89.5
	C. W.	89.4	91.6	91.3	93.8	88.2	93.8	87.1	90.6	87.1	90.2
	R. D.	91.3	93.1	92.0	94.8	92.2	94.0	91.9	91.9	90.0	92.5
	L. C.	89.1	91.4	90.8	93.0	87.2	91.8	87.0	89.8	83.8	90.0
	O. C.	86.2	93.5	83.7	88.3	79.1	89.8	89.8	82.3	85.9	85.4
	Average	89.0	92.2	89.6	92.4	86.3	92.0	88.6	88.7	87.2	89.6
Cottage cheese	R. J.	90.4	95.8	91.8	92.9	77.3	92.1	87.5	86.1	86.3	86.9
	C. W.	90.4	93.4	91.2	92.4	81.2	93.0	89.3	89.4	85.1	90.7
	D. H.	91.1	92.8	91.1	92.2	83.5	93.4	91.4	88.2	90.7	90.8
	R. D.	88.4	91.9	90.1	91.3	70.0	90.2	87.6	82.6	86.5	89.9
	L. C.	90.4	91.8	90.4	93.2	78.1	92.4	87.8	85.1	81.3	88.2
	A. C.	89.4	91.8	89.6	91.3	80.2	90.9	85.4	82.8	81.5	88.1
	O. C.	84.4	88.9	85.4	86.8	54.7	88.0	80.9	77.0	83.3	86.2
	Average	89.2	92.5	89.9	91.4	75.0	91.4	87.1	84.4	85.0	88.7

exception, availabilities of the amino acids in the egg, milk + egg, and cottage cheese diets did not show appreciable variation — the availability of cystine was approximately 10% lower in the cottage cheese diet than in the egg or milk + egg diet.

In the egg diet, availabilities ranged from 85% for tyrosine and threonine to 92% for phenylalanine, methionine and leucine; in the milk diet, from 67% for cystine to 89% for phenylalanine and leucine; in the milk + egg diet, from 86% for cystine to 92% for methionine, leucine, and phenylalanine; and in the cottage cheese diet, from 75% for cystine to 92% for leucine.

DISCUSSION

The sulfur-containing amino acid content of the cottage cheese diet was 1629 mg and of the milk diet, 1815 mg, both of which are less than the safe intake, 2200 mg. It has been shown that 80 to 89% of the methionine requirement can be spared by L-cystine (Rose and Wixom, '55). In the milk diet, 87.4% of the methionine and 66.7% of the cystine were available, or 1521 mg of the two sulfur-containing amino acids. In the cottage cheese diet, 91.4% of the methionine and 66.7% of the cystine were available, a total of 1459 mg of the two amino acids. Nitrogen balances were less positive during the milk and cottage cheese periods than during the other two protein periods. The lower N intakes during the milk and cottage cheese periods are partially responsible for this. In the case of the milk diet, the high fecal excretion is also a contributing factor to lower N retention. Possibly in the case of both the milk and the cottage cheese diets the low amounts of available cystine as well as the presence of cystine + methionine in amounts below the safe intake, are partly responsible for the less positive N balances obtained during these periods. The higher urinary excretion of N, despite the lower N intake, during the cottage cheese period seems to indicate that a nutrient is present in a limiting amount.

Although the availabilities of the essential amino acids and cystine were lowest in the milk diet, this does not necessarily indicate that the amino acids in milk are less available than those in the other test foods. The amount of milk consumed during the milk period was in excess of a normal intake and, therefore, the data should be interpreted to indicate that availabilities of the amino acids in milk are lower when intakes of milk are excessive than when moderate. The similarly high values obtained for the milk + egg and the egg diets substantiate this interpretation.

The availability of isoleucine in the egg diet was 1% higher than the corresponding figure for the milk + egg diet. The availability of the 9 other amino acids was either higher in the milk + egg diet than in the egg diet, or the same in both diets. The tendency toward superiority of the milk + egg diet when compared with the milk or the egg diet indicates that either protein is an excellent adjunct to the other and emphasizes the value of variety in the diet.

SUMMARY

Four diets containing generous amounts of egg, milk, milk + egg, and cottage cheese, singly, were fed along with the same low-N basal diet. The availabilities of the 8 essential amino acids, and of cystine and tyrosine were determined using 8 healthy young men as subjects. Egg was included as a reference protein, for comparison of availabilities from dairy proteins with those from egg.

Except for isoleucine, the availabilities of the essential and sparing amino acids in the milk + egg diet were equal to or slightly higher than corresponding values in the egg diet. The lowest availabilities were obtained for the milk diet. Since availabilities for the egg and the milk + egg diets were quite similar and the milk intake more nearly approximated a normal milk consumption in the latter diet, the abnormal high intake of milk is a likely explanation for the low availabilities obtained during the milk period. Availabilities for amino acids

in the cottage cheese diet were higher than corresponding values in the milk diet, but lower, except for isoleucine, leucine and lysine, than those in the milk + egg diet.

LITERATURE CITED

BRICKER, M. L., AND H. H. MITCHELL 1947 The protein requirements of the adult rat in terms of the protein contained in egg, milk and soy flour. J. Nutrition, *34:* 491.

HOAGLAND, R., N. R. ELLIS, O. G. HANKINS AND G. G. SNIDER 1947 Supplemental relationship between pork protein and egg protein. Ibid., *34:* 43.

MAURON, J., F. MOTTU, E. BUJARD AND R. H. EGLI 1955 The availability of lysine, methionine and tryptophan in condensed milk and milk powder. *In vitro* digestion studies. Arch. Biochem. Biophys., *59:* 433.

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

———— 1955 Current Research in the Science of Nutrition, Publication of the Nutrition Foundation, Inc.

ROSE, W. C., AND R. L. WIXOM 1955 The amino acid requirements of man. XIII. The sparing effect of cystine on the methionine requirement. J. Biol. Chem., *216:* 763.

SUMNER, E. E., AND J. R. MURLIN 1938 The biological value of milk and egg proteins in human subjects. J. Nutrition, *16:* 141.

WATTS, J. H., L. K. BOOKER, J. W. MCAFEE, E. G. WILLIAMS, W. G. WRIGHT AND F. JONES, JR. 1959 Biological availability of essential amino acids to human subjects. I. Whole egg, pork muscle and peanut butter. J. Nutrition, *67:* 483.

NOTICE TO CONTRIBUTORS

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

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

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

Manuscripts and drawings should be sent by express prepaid or by registered mail to the Editor, Dr. George R. Cowgill, Yale University Nutrition Laboratory, 333 Cedar Street, New Haven 11, Connecticut. If two complete copies of the paper are submitted, consideration by the Journal can be expedited.

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

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

Tables, quotations (extracts of over 5 lines), and all other material usually set in type smaller than the text, should be typed each on a separate sheet. **Footnotes to the text should be numbered consecutively (including those on page 1)** and typed in order on a separate sheet. Explanations of figures should be treated in the same manner. Footnotes to a table should be made a part of the table, and should be typed directly beneath it. Citations of literature in the text should be made by author and by numerals to indicate the year of publication. **Authors' names (followed by year, title of paper, etc.) should be arranged alphabetically in a list at the end of the text.**

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

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

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

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

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

THE JOURNAL® OF NUTRITION

VOL. 67 MARCH 1959 No. 3

CONTENTS

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America