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TRANSACTIONS

Studies on Vitamin B₂ Complex. VI

Rat-acrodynia and Fatty Acids.*

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In 1932, I reported that when young rats were maintained on the diets totally deprived of fats they developed characteristic symptoms accompanying impairment of growth, denuded area of skin, and "scaly" condition of feet, which were curable by the administration of either linoleic or linolenic acid.⁽¹⁾ Later, in the studies on vitamin B₂ deficiencies⁽²⁾⁽³⁾, it was found that rats suffering from lack of vitamin B₆ often developed symptoms similar to those of the fat deficiency mentioned above. However, when the diets contained moderate amounts of fats, for instance 10%, the symptoms were irregular and not so severe as with fat-free diets. Birch and György⁽⁴⁾ reported that certain fats had a sparing action on vitamin B₆, and they suggested that this action was due to the linoleic acid present in the fat. Salmon⁽⁵⁾ showed that oils alone or starch alone failed to cure or prevent acrodynia and that oils did not contain the entire dermatitis-preventing factor, but might contain an essential part of the factor which supplemented the heated yeast extract. More recently Birch⁽⁶⁾ reported that two factors were concerned in the cure of the acrodynia-like dermatitis. One was the water-soluble factor vitamin B₆; the other was fat-soluble and present in the fatty acid fraction of maize oil, which appeared to be similar to the "linoleic acid" of Burr and Burr.

The experiments presented in this paper are, therefore, concerned with the relation between vitamin B₆ and unsaturated fatty acids in the cure and production of acrodynia-like dermatitis of rats.

EXPERIMENTAL.

Methods.

In order to carry out this experiment, it was needed to prepare pure casein and vitamin B₆ extract free from fats. The following procedure, therefore, was adopted,

Purification of casein.— 2 kg of casein was stirred into 5 litres of water solution of 60 g of NaCl containing 6 cc of glacial acetic acid. After settling for several hours, the supernatant liquid was decanted off, and a similar treatment was repeated six times more. This was filtered on a large Buchner's funnel, washed free from acid, and then the casein was stirred into 4 litres of 95% alcohol. The alcohol was removed by filtration. This procedure was repeated once more, the casein was dried at about 50°C, and ground, then extracted with ether for 10 days to remove fatty materials completely.

Preparation of yeast extract.— 200 g of dried brewer's yeast was extracted with 800 cc of 75% alcohol by shaking for 2 hours at room temperature. It was filtered and reextracted as above, the process being repeated twice more. The combined extracts were then evaporated down to remove the alcohol, adjusted to pH 2 with HCl, and shaken with ether several times to remove all neutral fats and fatty acids. The solution was made to about pH 6 with NaOH, and concentrated in vacuo. 95% alcohol was added into the concentrate and the mixture was allowed to stand in the ice box until the inert materials had settled out, which were filtered off and the filtrate kept for assay (10 drops of this solution correspond to the yield from 0.8 g of the dried yeast).

A. Estimation of vitamin B₆ activity of the yeast extract. The basal diet used in this experiment had the following composition:

Diet I.

Purified casein	18%
Sucrose (<i>Pharmacopeia Japonica</i>)	67
Butter fat	9
McCullum's salt mixture	4
Agar-agar	2

This was supplemented with 10 γ B₁ hydrochloride, 20 γ riboflavin each rat daily, and 2 drops of biosterin** (45,000 I. U.) weekly as vitamins A and D.

The feeding technique employed in this work was for the most part similar to that described in the preceding paper.⁽⁷⁾

Fig. 1. Growth curves of rats on Diet I, supplemented daily by 5 or 10 drops of yeast extract, and B₆ alone or combination of B₆ and FF (filtrate factor), respectively. a acrodynia, c cured, FF (one drop=1.5 g of fresh beef liver).

From Fig. 1 (a and b), it may be noticed that the cure of acrodynia and

the response of growth were slow with 5 drops of the yeast extract, while more rapid cure and growth resulted with 10 drops of the extract. With 15 γ of crystalline B₆*** nearly the same result as with 10 drops of the yeast extract was obtained. By supplementing vitamin B₆ with 3 drops of filtrate factor, however, a very striking effect on growth was brought about as seen from Fig. 1 (c). This was in agreement with the previous experiments.⁽²⁾

Filtrate factor used throughout the experiments was prepared from beef liver by the method previously described.⁽⁸⁾

B. In consideration of the results obtained in the previous experiments⁽¹⁾⁽³⁾, the following attempts were made; to study firstly, the influence of vitamin B₆ on the production of the fat-deficient disease, and secondly, the influence of fats in vitamin B₆-free diets on the development of the acrodynia-like dermatitis. The basal diets used in these investigations are shown in Table I.

1. Experiments on fat-free diet. This was carried out by feeding rats on diets provided with different amounts of vitamin B₆. The basal fat-free diet is given in Table I.

TABLE I.
Composition of diets (per cent.)

Component \ Diet	Diet II	Diet III	Diet IV
Purified casein	20	20	20
Sucrose (<i>Pharmacopeia Japonica</i>)	73	70	70
McCullum's salt mixture	5	5	5
Agar-agar	2	2	2
Soy bean oil	—	3	—
Crisco	—	—	3

All the above diets were supplemented as in Diet I.

Group 1. The rats receiving 10 drops of the yeast extract grew well for 5 to 7 weeks, then the growth was retarded. At about 9 to 10 weeks the nose and mouth were inflamed, and scaly feet, dandruff and alopecia appeared, but no typical acrodynia was noticed. When this condition continued the rapid loss in weight occurred and death happened shortly unless fatty acid was fed. Administration daily of 5 to 10 drops of soy bean oil or 2 drops of linoleic acid brought about increase in weight and rapid cure, but crisco had apparently no such curing properties (Fig. 2).

Linoleic acid was prepared from linol-hydroxamic acid† $(C_{17}H_{31}-C \begin{smallmatrix} \nearrow NHO \\ \searrow OH \end{smallmatrix})-$
10 g of pure linol-hydroxamic acid (mp 41~42°C), which was separated from

cotton-seed oil, was added into a mixture of 100 g of 70% ethyl alcohol and 6 g of H_2SO_4 , and it was heated on a water bath under reflux condenser in the atmosphere of CO_2 until no more purple red colour reaction with FeCl_3 appeared; it took about three hours. The resulting solution was distilled in a reduced pressure to remove the alcohol, adding a small amount of water from time to time. The solution was now extracted with petroleum ether below bp 50°C , the extraction being repeated twice more. After removing the ether by distillation, the residual solution was saponified with alcoholic potassium hydroxide in order to remove the ethyl ester which might be present. This alkaline solution was now acidified with HCl , and again extracted with petroleum ether. After dehydrating with anhydrous Na_2SO_4 , the petroleum ether solution was evaporated as completely as possible in a high vacuum in CO_2 atmosphere. The yield of linoleic acid was nearly theoretical.

Group 2. The animals given 5 drops of the yeast extract grew for the first few weeks, but gradually declined in weight. They developed acrodynia-like dermatitis within 5 to 7 weeks, accompanying scurfy coat and denuded area on the skin. With 5 drops of soy bean oil, the improvement in weight and dermatitis was much slower than with 10 drops of the oil. Feeding 2 drops of linoleic acid brought about a prompt cure of dermatitis, whereas crisco was ineffective (Fig. 3).

Fig. 2. Growth curves of rats on fat-free diet (Diet II), supplemented daily by 10 drops of yeast extract.

d; fat-deficiency, S; soy bean oil, L; linoleic acid, H. C.; crisco, k; killed, c; cured, x; died.

Fig. 3. Growth curves of rats on fat-free diet (Diet II), supplemented daily by 5 drops of yeast extract.

a; acrodynia, d; fat-deficiency. The other abbreviations are the same as in Fig. 2.

These results indicate that rats may develop the acrodynia-like dermatitis if diet is free from fat even when moderately large amounts of vitamin B_6 are given. It would seem, therefore, that certain fats are necessary for normal growth of rats.

2. Experiments on vitamin B₆-free diets. This was carried out by feeding animals on diets containing varying amounts of fats to determine the time of the development of acrodynia-like dermatitis and the degree of the symptom. The diets used are shown in Table I.

Group 1. The rats fed on Diet II (fat-free diet) developed severe acrodynia in 3 to 4 weeks. Administration of 10 drops of the yeast extract caused some improvement on the symptom, but the rats declined in weight and death occurred among them. By the additional supplement, however, of 10 drops of soy bean oil or 2 drops of linoleic acid, there was an immediate resumption of growth and the symptoms cleared up within a few weeks. Cured animals maintained themselves free of all symptoms as long as the fatty acid and yeast extract were continued. If the oil or fatty acid was withheld, the rats declined in weight and developed acrodynia (Fig. 4).

Group 2. The acrodynia-like dermatitis appeared in about 6 to 7 weeks in the rats fed with Diet III (3% soy bean oil). By providing 5 or 10 drops of the yeast extract the acrodynia was quickly cured and growth restored. When 15 γ of B₆ was given the dermatitis was cured, but filtrate factor was needed to induce optimum growth in the rats. Soy bean oil alone did not prevent the acrodynia but did delay the onset of the symptom to some extent (Fig. 5).

Fig. 4. Growth curves of rats on vitamin B₆-free diet containing no fat. YE; yeast extract. The other abbreviations are the same as in Figs. 2 and 3.

Fig. 5. Growth curves of rats on vitamin B₆-free diet containing 3% soy bean oil. YF; filtrate factor. The other abbreviations are the same as in Figs. 3 and 4.

Group 3. Results obtained with Diet IV (3% crisco) were similar to those with Diet II (fat-free) except the delayed onset of the dermatitis, which was not

so severe as seen in the animals on Diet II. Without supplement of the yeast extract, the animals died with rapid loss of weight.

Fig. 6. Growth curves of rats on vitamin B₆-free diet containing 3% crisco.

The abbreviations are the same as in Figs. 3 and 4

nia develops very quickly, with subnormal weight and scaliness of hind feet. Feeding the yeast extract relieves the symptoms to some extent, but for complete cure and normal growth it is necessary also to administer certain fatty acids. It is seen that the effect of soy bean oil on cure of the dermatitis and promotion of the growth depends on its content of the essential fatty acid, which may perhaps be similar to linoleic acid.

5 drops of the yeast extract failed to cure the dermatitis, but 10 drops of the extract gave a slow improvement. Complete cure and normal growth were obtained by an additional supplement of either 5 drops of soy bean oil or 2 drops of linoleic acid (Fig. 6).

The results with 10% crisco diet were not shown in the figures but were similar to those observed with the 3% crisco diet. The development of acrodynia was irregular, and the onset was delayed, usually requiring 10 to 12 weeks.

These results show that when rats are fed on the basal fat-free diet without any addition of vitamin B₆, severe acrodynia develops very quickly, with subnormal weight and scaliness of hind feet.

The evidence indicated that two factors were concerned in the cure and production of acrodynia-like dermatitis; one water-soluble factor, *viz.*, vitamin B₆, and the other fat-soluble factor apparently similar to linoleic acid. On examining the results it was at once recognized that the close relationship between the amount of certain fatty acids in the diet and severity of the acrodynia-like dermatitis would indicate that vitamin B₆ was connected in some way with the metabolism of the fatty acids. Though the exact biological function between vitamin B₆ and the essential fatty acid has not yet been fully established, it seemed reasonable from the results to conclude that the presence of an adequate amount of vitamin B₆ and of the essential fatty acid in diet was necessary for normal health and growth of the animal. Birch⁽⁹⁾ found the unsaturated fatty acids of maize oil to be effective in relieving the symptoms of vitamin B₆ deficiency, and suggested this finding to be related to the observations of Burr and Burr⁽⁹⁾ concerning essential fatty acids, and perhaps also to the fat-soluble antidermatitis factor indicated by Hogan and Richardson.⁽¹⁰⁾ He could not find any evidence to indicate that the vitamin might exist in combination with lipoids, but he presented, instead, the evidence showing that there was a functional relationship between the unsaturated

Discussion.

fatty acids and the vitamin. Halliday⁽¹¹⁾ reported further evidence supporting this view, who observed that there was fatty liver in vitamin B₆-deficient animals and feeding choline remedied such condition to a large extent. Quackenbush and Steenbock⁽²²⁾ found that a B₆-deficient diet supplemented by unsaturated fatty acids, either as natural oils or as 10 mg per day of ethyl linoleate, protected rats from acrodynia and kept them in good health. Salmon⁽⁵⁾ also observed a relation between B₆ and fat metabolism. Such reports led to the conclusion that vitamin B₆ was connected in some way to fat metabolism.

SUMMARY.

Data are submitted which show that two factors are concerned in the production and cure of the acrodynia-like dermatitis. One is water-soluble factor, *viz.*, vitamin B₆; the other is fatty acid factor similar to linoleic acid.

The evidence suggests that vitamin B₆ is connected in some way with the metabolism of the fatty acids.

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