CXIV. FURTHER EVIDENCE FOR THE EXISTENCE OF VITAMIN B_4.[1]

BY ORAL LEE KLINE, CONRAD ARNOLD ELVEHJEM AND EDWIN BRET HART.

From the Department of Agricultural Chemistry, University of Wisconsin, Madison.

(*Received March 25th, 1936.*)

THE existence of a water-soluble thermo-labile nutritional factor distinct from vitamin B_1 or B_2 was first described by Reader [1929]. This substance was designated vitamin B_4 [Reader, 1930, 1], indicating a similarity in respect to solubility to other factors in the vitamin B complex. In a series of publications [Reader, 1930, 2; Barnes *et al.*, 1932; Kinnersley *et al.*, 1933] a method of producing vitamin B_4 deficiency in the rat, a method for assay of this factor and a scheme for concentrating the substance were described.

More recently there has appeared expression of doubt of the existence of vitamin B_4 as a separate entity. Interpretations of feeding experiments have resulted in an opinion that vitamin B_4 deficiency is a chronic phase of vitamin B_1 deficiency and may be cured by excessive doses of vitamin B_1 [see Harris, 1935; O'Brien, 1934; Birch *et al.*, 1935].

In opposition to this denial of the reality of vitamin B_4, we wish to express belief in its existence, with a precise function in the metabolism of at least two species of animals.

Keenan *et al.* [1933] demonstrated the necessity of this factor for growth and normal behaviour of the chick in this laboratory. Concentrates of vitamin B_4, prepared according to the method of Barnes *et al.* [1932], prevented the development of paralytic symptoms in the chick, a syndrome which developed in the animal receiving a diet containing adequate amounts of vitamin B_1. In this species the vitamin B_4 deficiency is characterised by a complete loss of coordination, a tendency to lie on the side and turn cartwheels and finally general prostration and death. A degeneration in the cerebellum of the vitamin B_4-deficient chick was found, similar to the encephalomalacia reported by Pappenheimer and Goettsch [1931]. Improvements in technique and further purification of the constituents of the chick ration [Kline *et al.*, 1936] have given us a valuable method for assay and study of vitamin B_4.

For further proof of identity of the antiparalytic factor with the vitamin B_4 of Reader and associates, it was necessary to produce the deficiency in the rat and to cure it with a vitamin B_4 concentrate. We felt that this was not impossible, because symptoms of vitamin B_4 deficiency have been observed in our vitamin B_1 studies in rats fed diets low in both vitamins B_1 and B_4, as well as in experiments on other factors of the vitamin B complex.

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

A synthetic diet, designed after that used in the chick experiments but more highly purified, was used. Diet 44_{2A} consisted of:

Dextrin	63·5	
Purified caseinogen	24·0	
Salts I	4·0	
Lard	6·0	
Autoclaved liver residue ...	2·0	
Vitamin B_2 concentrate ...	$\cong$ 2 %	liver extract powder
Cod liver oil	1·0	

The dextrin was heated for 24 hours at 120°. Caseinogen, prepared from skim milk, was reprecipitated from dilute NH_4OH solution with dilute HCl at p_H 4·6, then washed with distilled water. This treatment was repeated three times. The salt mixture used has been described previously [Kline *et al.*, 1936]. A commercial, water-extracted liver residue, autoclaved 10 hours at 120° to destroy any residual vitamin B_4, was used to supply the essential liver growth factor [Kline *et al.*, 1934]. A vitamin B_2 concentrate, prepared according to the method of Elvehjem and Koehn [1935], was added to the diet equivalent to 2 % liver extract powder.

In our first experiments the technique for producing the deficiency described by Reader was employed. Diet 44_{2A} was made available to suckling rats 10 days old. The mother was removed to a separate cage for several hours daily and fed the stock ration. The young were weaned at 23–25 days of age, with a beginning weight of 40–45 g., and placed in individual screen-bottomed cages. At the first indication of polyneuritis, which occurred usually during the fifth week, a vitamin B_1 supplement was given.

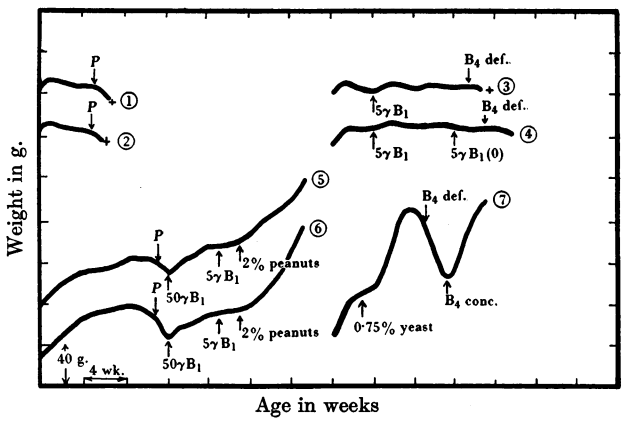

Fig. 1. Growth curves of rats which received basal diet 44_{2A} with the various supplements indicated. *P* denotes the onset of polyneuritis; B_4 def. indicates onset of symptoms of vitamin B_4 deficiency. The vitamin B_1 used was Merck's crystalline B_1 in all cases except curve 4, 5 γ B_1 (O), which was Ohdake's crystalline preparation. The vitamin B_4 concentrate was prepared from peanuts.

The growth curves presented in Fig. 1 are typical records and illustrate the points we wish to bring out. Records of two control animals (curves 1 and 2), which received the basal diet without vitamin B_1 supplement, indicate that polyneuritis was followed by death in the fifth week. Curve 3 demonstrates a

lack of growth response to vitamin B_1 (5γ daily of Merck's crystalline preparation), with the onset of vitamin B_4 deficiency during the twelfth week, followed by death of the animal. Curve 4 is the record of an animal which received 5γ daily of Merck's crystalline vitamin B_1 beginning on the 28th day of the experiment, replaced by 5γ daily of Ohdake's crystalline vitamin B_1 in the twelfth week. Onset of vitamin B_4 deficiency was observed during the fifteenth week. It is apparent that after a very brief rise in weight vitamin B_1 had no further effect on weight increase.

Curves 5 and 6 are records of rats which developed a severe polyneuritis during the eleventh week of experiment. The basal diet was supplemented with 50γ daily of Merck's crystalline vitamin B_1 beginning at the twelfth week. Polyneuritis was completely cured, but only a slight growth response was obtained in a 5-week period. No change in rate of weight increase was noted when the dose of vitamin B_1 was reduced to 5γ daily for a 2-week period. Growth was resumed, however, after the nineteenth week of the experiment when 2% of peanuts, as a source of vitamin B_4, were added to the basal diet and the 5γ daily dose of B_1 continued. In chick assays, peanuts have been found to be an excellent source of this factor. For the rat represented by curve 7, 0·75% brewer's yeast was used as a source of vitamin B_1, added at the beginning of the third week, before polyneuritis had developed. Rapid growth until the eighth week was followed by marked loss in weight with the onset of vitamin B_4 deficiency occurring during the ninth week. Administration of a vitamin B_4 concentrate prepared from peanuts was begun during the eleventh week, allowing an increase in weight of 75 g. in 4 weeks, when the animal was killed for histological study.

Typical symptoms of vitamin B_4 deficiency, similar to those reported by Reader, were observed in these animals. The rats were inactive, sat in an extremely hunched position, and when walking retained the characteristic humped back, "walking high" on the hind legs. As the condition developed there was a noticeable loss of coordination. These animals failed to show the swollen red paws which Reader described.

In a subsequent experiment it was found necessary to use crude liver extract powder (1 : 20) to furnish vitamin B_2 in place of the vitamin B_2 concentrate

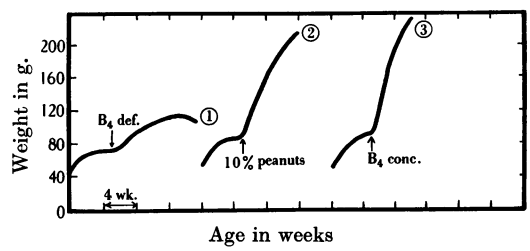

Fig. 2. Growth curves of rats fed basal diet 44_{2H} *plus* 5γ daily of vitamin B_1 concentrate.[1] Vitamin B_4 deficiency symptoms were observed at ↓. Indicated supplements added at ↑.

This diet is designated 44_{2H}. The liver extract powder was aerated in water solution at 100° for 24 hours to decrease the small amount of vitamin B_4 it might contain. The practice of first producing polyneuritis in the rats was found

[1] Vitamin B_1 concentrate was obtained from Sankyo Company, Tokyo, Japan. 1 ml. contains 500γ of crystalline vitamin B_1, of which 1·5γ is a rat day dose equivalent to 1 I.U. [Ohdake, 1935].

unnecessary, and from the tenth day the suckling rats had access to diet 44_{2H} containing 0·5γ of vitamin B_1 per g. of diet. After weaning on the twenty-fifth day the animals received 5γ of vitamin B_1 daily by dropper.

When a decline in growth rate was noted and symptoms of vitamin B_4 deficiency appeared, supplements were made to diet 44_{2H} *plus* vitamin B_1. These are indicated in Fig. 2. An increase of 25 g. in a 5-week period on the basal diet may be compared with an increase of 139 g. when vitamin B_4 concentrate was used, and 105 g. when 10% of peanuts was included in the diet. The marked growth increase was accompanied by complete cure of the paralytic symptoms. This response we believe is the result of supplementing the diet with a single factor, vitamin B_4, and bears no relation to vitamin B_1, which at the level of 5γ daily we have shown to be adequate on a diet low in vitamin B_1. Ohdake [1935] found 1γ per day to be sufficient for a rat on a diet containing arachis oil which was probably furnishing a considerable amount of vitamin B_4.

Discussion.

If vitamin B_4 is a factor distinct from vitamin B_1 in both constitution and function, it should be unnecessary to produce polyneuritis in the rat prior to the development of vitamin B_4 deficiency symptoms. It is possible that diets which have been used by other workers have not been sufficiently low in vitamin B_4 to limit the intake of this factor to such an extent that symptoms could appear without first lowering the food intake by avitaminosis-B_1. This may explain the apparent vitamin B_4 potency of vitamin B_1 preparations. This relationship has been discussed by Elvehjem and Arnold [1936].

Success in producing vitamin B_4 deficiency in the rat depends to a very large extent upon purification of the various constituents of the diet. We feel that the reprecipitation of the caseinogen is highly essential, and the purification of the dextrin or use of sucrose may aid materially in reducing vitamin B_4 intake. The use of more concentrated supplements furnishing the necessary dietary constituents other than vitamin B_4, as for example the use of crystalline vitamin B_1 in place of yeast and highly potent liver fractions as a source of vitamin B_2, flavin, and other necessary factors, was essential to our success. In continued studies the use of more highly purified concentrates of the B-vitamins will undoubtedly improve our method.

Summary.

Further experimental evidence is offered for the existence of vitamin B_4 and its necessity in the normal nutrition of the rat. Our work confirms the earlier observations of Reader.

The authors are indebted to Prof. S. Ohdake for a generous supply of crystalline vitamin B_1, and to Dr David Klein of Wilson Laboratories for the liver fractions used.

REFERENCES.

Barnes, O'Brien and Reader (1932). *Biochem. J.* **26**, 2035.
Birch, György and Harris (1935). *Biochem. J.* **29**, 2830.
Elvehjem and Arnold (1936). *Nature*, **137**, 109.
—— and Koehn (1935). *J. Biol. Chem.* **108**, 709.
Harris (1935). *Ann. Rev. Biochem.* **4**, 331. (Stanford Univ. Press.)
Keenan, Kline, Elvehjem and Hart (1933). *J. Biol. Chem.* **103**, 671.
Kinnersley, O'Brien, Peters and Reader (1933). *Biochem. J.* **27**, 225.
Kline, Bird, Elvehjem and Hart (1936). *J. Nutrition* (in press).
—— Keenan, Elvehjem and Hart (1934). *J. Biol. Chem.* **107**, 107.
O'Brien (1934). *Chem. Ind.* **53**, 452.
Ohdake (1935). *Bull. Agric. Chem. Soc. Japan*, **11**, 1.
Pappenheimer and Goettsch (1931). *J. Exp. Med.* **53**, 11.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.