CCVII. THE ASSAY OF VITAMIN B_4.

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In earlier publications [Reader, 1929, 1930] evidence was presented for the division of the vitamin B complex into three components all necessary for the nutrition of the rat[1]. It was shown that two of these (B_1 and B_4) were alkali-labile while B_2 was not appreciably destroyed by autoclaving a crude yeast extract solution at p_H 9 for one hour at 120° [see Reader, 1930, p. 79].

The work reported here deals with the methods of assay used during the concentration and purification of the second alkali-labile factor B_4. In the earlier papers the restoration of growth of young rats, which had come to constant weight on a basal diet [Reader, 1929] plus excess of vitamins A, B_1, B_2 and D, was used as a criterion of the presence or absence of vitamin B_4 in a given extract. As there is a prolonged storage period for vitamin B_4 a certain amount of difficulty was experienced in keeping the animal colony sufficiently deficient in this vitamin, while, at the same time, supplying essential natural products (milk, etc.) to the breeding rats to get adequate lactation. Consequently a new "adult-curative" method was devised. Use of this method has led to the surprising discovery that the symptoms usually considered to be typical of polyneuritis in rats are really a mixture of symptoms due to lack of vitamins B_1 and B_4. By eliminating the vitamin B_1 deficiency at the critical stage the true clinical picture of vitamin B_4 deficiency is revealed and can be used in a curative test.

The main features of the adult-curative test are as follows. The animals, grown to maximum weight with adequate, but not excess, vitamin B_4 for normal growth, were completely deprived of both the alkali-labile vitamins B_1 and B_4 until polyneuritis occurred (3 to 4 weeks). At this stage it was found that vitamin B_1 cures the typical polyneuritis symptoms [see Kinnersley, Peters and Reader, 1930] but it does not restore weight or ameliorate all the symptoms, even when given in large excess doses daily, up to 12 pigeon doses per day. Instead the animal remains in a weak condition with swollen red paws, spastic gait, loss of co-ordination, and other symptoms apparently due to the specific lack of vitamin B_4.

Now if a positive test dose of vitamin B_4, proved free from vitamin B_1,

[1] The term B_4 is used throughout this paper to denote the third rat "B" factor, and is equivalent to B_3 in the earlier papers by the author. Thus it is hoped to avoid confusion with the Williams and Waterman thermolabile pigeon factor which has been termed B_3.

is given daily, the weight is immediately increased (20 g. per week) and at the end of 3 weeks all the above symptoms have disappeared (Fig. 1). If the substance contains no vitamin B_4, then the animal gets generally weaker and usually dies within 10 days, without any further marked loss of weight (Fig. 2).

Using this method it has been possible to concentrate vitamin B_4 from a 50 % alcohol extract of distillery yeast (British Drug Houses product) from 500 mg. to 0·4 mg. per daily rat dose, and to decide how far any given vitamin B_1 preparation is free from vitamin B_4.

Experimental.

Albino rats were brought to approximately maximum weight by feeding an approved basal diet [Reader, 1930] plus vitamins B_1, B_2 and B_4. It has been my experience throughout this work that it is necessary to give 2–3 pigeon day-doses of vitamin B_1 to each rat per day to get the normal growth rate (20 g./wk.). This is very interesting since it has been shown by Kinnersley, Peters and Reader that the curative dose for the polyneuritic rat is approximately the same as that for the pigeon. The vitamin B_2 was supplied as yeast extract autoclaved under the conditions previously described by the author [Reader, 1930]. The vitamin B_4 used for this earlier part of the experiment was always a stock preparation made from the decomposed mercuric sulphate precipitates obtained in the Kinnersley and Peters process for torulin (vitamin B_1). Thus the animals were brought to maximum weight under carefully controlled laboratory conditions.

These full-grown animals were deprived of the two alkali-labile factors (B_1 and B_4) for a period of about 4 weeks, or until polyneuritis occurred. During this period each animal loses nearly half its weight. Rather suddenly typical polyneuritis convulsions occur, accompanied by great weakness and ataxia. The paws are often oedematous and very red between the digits, and there is partial paralysis mainly manifested in the hind legs. Now the animal is given a dose of vitamin B_1 and the convulsions and paralysis disappear within a few hours. If daily doses of vitamin B_1 (2–3 curative doses daily) are now instituted, the animal can be maintained in this condition, *i.e.* with a general muscular weakness, spastic gait, swollen red paws, and a tendency to sit in a hunched position, for about 10 days. The weight, which has been falling rapidly up to this point, now ceases to fall, and maintains a steady level. If food and water are put at the far end of the cage, the rat can be persuaded to move, but frequently walks with a rolling movement and generally shows a lack of co-ordination. Such an animal is now ready for the vitamin B_4 test (see Fig. 1). The increase in weight is the first obvious sign that the sample under test contains B_4, but within 2 days the animal becomes much more active. At the end of a week the gait is apparently normal. If the sample tested does not contain vitamin B_4, the symptoms get rapidly worse and death occurs in 10–14 days.

When an animal has been completely restored to its original weight, it

may be used again for the same type of test. Frequently the same animal has been used four or five times, and it has been noted that for any given animal polyneuritis occurs each time at the same weight (± 5 g.) (see Fig. 2).

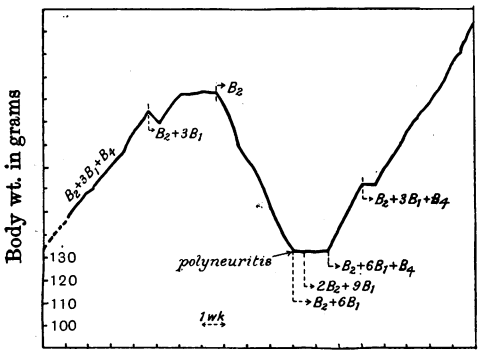

Fig. 1.

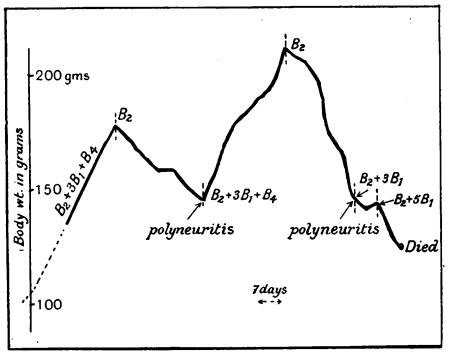

Fig. 2.

Figs. 1 and 2. Showing typical curves obtained when using adult rats for assay of B_4.

Discussion.

The technique elaborated above has one outstanding advantage that each animal is grown to maximum weight under carefully controlled conditions, and receives daily allowances of stock laboratory preparations of all three "B" factors. Thus any variation in the requirements of individual animals can be recorded and taken into consideration in the interpretation of later results. Although all the animals used in this work were bred in this department from an inbred Wistar albino strain, it is impossible to avoid occasional irregular results; probably the variations observed are not as great as with a mixed colony. An interesting observation is that an anomalous rat very seldom, if ever, requires excess of all three "B" factors, but rather appears to need a slightly larger dose of one of the several factors.

The symptoms described above have been frequently described, wholly or in part, in the literature. Originally they were thought to be due to a deficiency of vitamin B, then of B_1, and only now has it become evident that they are complex and due to a dual-deficiency of vitamins B_1 and B_4. Most workers have used young animals, and death may often have occurred before the complete development of all the symptoms reported in this paper.

In an accompanying paper [Kinnersley, Peters and Reader, 1930] it is shown how adult polyneuritic rats can be used for curative tests for B_1 and reference is made in this connection to Smith [1930]. In his table on p. 127, Smith shows a marked disparity in the ratio pigeon dose/rat dose for two preparations. From this he concludes that the maintenance test for pigeons (Seidell) is not a test for vitamin B_1 alone. However correct this conclusion may be, and there is evidence elsewhere to support it [Williams, Waterman and Gurin, 1929; Carter, Kinnersley and Peters, 1930], the experiments

described in this paper provide an alternative explanation of Smith's discrepancies. Either some of his litters may have been more deficient in vitamin B_4 than others; or, assuming that his rats were all equally deficient in vitamin B_4, the preparations under test may have varied in vitamin B_4 content. Until information is available upon these points, it cannot be decided whether his conclusions were justified. Since his preliminary period of depletion of 6–10 weeks is greater than the usual storage period for vitamin B_4, probably the problem of uneven storage does not arise. This 6–10 week period is worthy of comment since, with the adult albino rats used in this department, polyneuritis is always obtained in 21 to 28 days. It is still an open question how far the dual-deficiency of two vitamins hastens or hinders the onset of symptoms due to either one.

Sandels [1930] describes two types of polyneuritis, "acute" and "subacute or chronic" depending on whether the deficiency in the antineuritic vitamin is complete or only partial. Perhaps those of his animals which were subjected to a complete deficiency of antineuritic vitamin and died without showing noticeable symptoms of polyneuritis were suffering from a marked lack of vitamin B_4 also, especially as this often manifests itself in young animals as muscular weakness, followed by inanition and death. That his diets were lacking in vitamin B_4 may be judged from his description of symptoms, which include "cartwheel turning, spastic gait, and frantic circling when there was movement or noise in the laboratory." These I would not consider to be true polyneuritic symptoms, since it has been shown in the above experiments that they can be cured when, and only when, vitamin B_4 is supplied as a separate entity or in the vitamin B_1 extract used.

In conclusion, it must be emphasised that an animal will die from lack of vitamin B_4 even though a highly potent antineuritic concentrate (vitamin B_1) has been supplied daily in large excess for the 14 days preceding death (see Fig. 2). It is of interest, and perhaps of great importance, that all the concentrates of vitamin B_1, which have been obtained free from vitamin B_4, have been prepared from the $N/10$ HCl extracts of norite in the Kinnersley and Peters process for concentration of vitamin B_1. So far all the 50 % alcohol extracts from the same norite have contained abundant vitamin B_4.

At frequent intervals during the last 8 months parallel tests have been made on vitamin B_4 extracts by the adult-curative tests and the growth tests formerly described [Reader, 1930] and in all cases equivalent results have been obtained. The adult-curative method is now preferred since it is easier to control and gives a quicker result.

Appendix.

The following method for the concentration of vitamin B_4 has been used with much success when the precipitate to be decomposed is obtained by the application of the Kinnersley and Peters process to a B. D. H. product which we believe to be a 50 % alcohol extract of distillery yeast. So far the yield

has always been poor with the precipitate from the baker's yeast usually used in this Department. The reason for this is not yet clear.

Concentration of vitamin B_4. Wash the mercuric sulphate precipitate from 40 lbs. distillery yeast with distilled water until the washings give a blue colour with bromophenol blue. Suspend the precipitate in 6 litres water, pass H_2S for 4 hours, with occasional stirring. Filter, remove excess H_2S from the filtrate by distillation *in vacuo* (inside temp. 40–60°). At this stage there is danger of skin irritation due to unidentified volatile sulphides. Add NaOH to p_H 3, concentrate the whole *in vacuo* to 1 litre, adjust the p_H to 4·5 and add alcohol to 50 %. Allow to stand in cold store overnight, filter off any solid matter, remove alcohol *in vacuo* and concentrate to 400 cc. Add acetone to 80 %. Stir well and allow to stand at room temperature overnight. Decant next morning and remove acetone and some water *in vacuo* to 200 cc. (inside temp. never above 60°). Allow to stand in cold store for 2 days and filter.

The filtrate is now ready for dosing. The dose varies in different preparations from 1/20 to 1/4 cc.

To preserve, adjust the p_H to 4 and add alcohol to 20 %.

Summary.

An adult-curative method for the assay of vitamin B_4 is described.

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