

LXXVIII. A SECOND THERMOLABILE WATER-SOLUBLE ACCESSORY FACTOR NECESSARY FOR THE NUTRITION OF THE RAT.

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DURING the last two years numerous papers have been published confirming the work of Goldberger *et al.* [1926] on the division of the water-soluble vitamin B into at least two components. That further factors may be present has been suggested by Kennedy and Palmer [1928], Hunt [1928] and others. The experiments to be recorded below deal exclusively with the more labile factors concerned. The work was undertaken in order to find the daily requirements of a rat of the antineuritic vitamin in terms of the pigeon day-dose, the method employed in this laboratory for standardising this vitamin. A preliminary account of the work has already appeared [Reader, 1928].

The nomenclature used throughout is that provisionally adopted by the Biochemical Society and defined in detail by Chick and Roscoe [1928]. Briefly, B₁ signifies the antineuritic, and more heat-labile factor; B₂, the more heat-stable factor.

Although control animals on our basal diet + crude marmite grew to adult size at the same rate as those on normal food (corn, bread, lettuce, etc.), repeated attempts to get growth to adult size on basal diet + marmite which has been subjected to alkaline hydrolysis for one hour at 120° + purified extracts of vitamin B₁ have failed. Thus it would seem that at least two factors necessary for rat nutrition had been destroyed by this treatment. Whether or not this second thermolabile substance is identical with that described by Williams and Waterman [1928], as associated with the maintenance of weight and general conditions of adult pigeons, will be discussed later.

It is proposed to call this second thermolabile rat factor, provisionally, vitamin B₃.

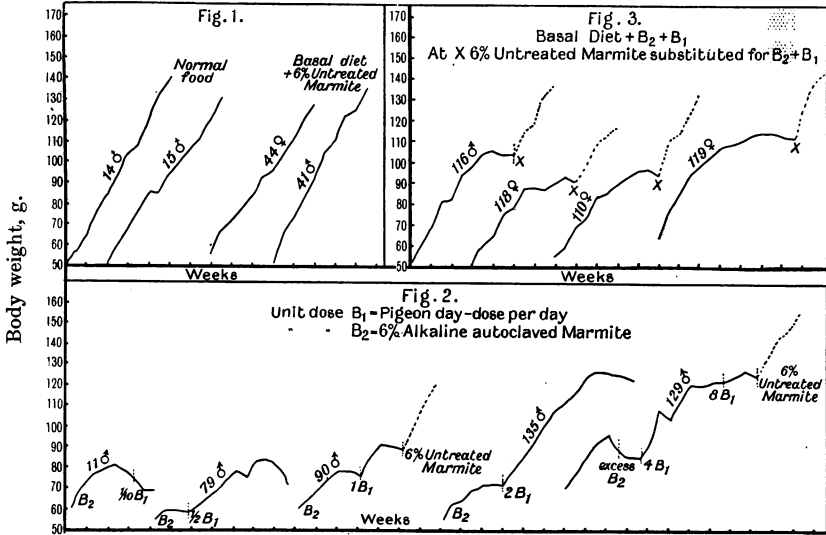
EXPERIMENTAL.

Albino rats, bred in this department from the Wistar strain supplied by Glaxo Limited, were used throughout. In all routine experiments animals of 50-60 g. were used for these tests. However, in order to compare these experiments with those reported by the Lister Institute, one set of animals of about 40 g. was put on to the experimental diet (see Fig. 5).

The basal diet used was:

Glaxo caseinogen (free from vitamins A, D, B ₁ and B ₂) ...	20 %
Rice starch	70
Agar-agar	2
McCollum's salt mixture	5
Cod-liver oil	3
Water <i>ad lib.</i>	

This diet was freshly prepared daily, and was tested to show that, when supplemented with 6% marmite as source of B₁ and B₂, it gave growth equal to that obtained with normal food, including corn, "Hovis" bread, lettuce, cod-liver oil, and occasionally milk. The curves in Fig. 1 are selected from groups of 20 animals and are typical of the group. The daily food intake gradually rose from 10 to 15 g. as the body-weight increased from 60 to 120 g., so that in all each animal was having daily B₁ and B₂ equivalent to 0.6-1.0 g. marmite.



In all further experiments the thermostable factor or factors (B₂) were supplied by adding 6% autoclaved marmite solution (120° for one hour at p_H 9.0) to the basal diet. Curative tests on pigeons showed that this product did not now contain any B₁, the antineuritic factor. That it did contain one factor necessary for the rat (presumably B₂) was shown by the method of Chick and Roscoe [1928] for the assay of B₂. Also that 6% was sufficient for the daily requirements was demonstrated by doubling this quantity after growth ceased in one group of rats (*e.g.* rat 129, Fig. 2) and noting that no further growth occurred.

Two types of experiments were designed. In (A) the rats were fed on the basal diet + B₂ till constant weight was obtained for three consecutive

weighings (three-day intervals). Then varying doses of several B₁ concentrates were administered daily, in aqueous solution adjusted to p_H 6.5. The doses were given by the mouth from a small pipette. The concentrates used were prepared by Mr H. W. Kinnersley¹. Unless otherwise stated they were the 50 % alcohol extracts from the charcoal. Daily doses were varied from 1/10 to 8 pigeon day-doses. Typical results from a large number of experiments are shown in Fig. 2. From these it may be seen that even eight pigeon day-doses per day do not give growth at a normal rate to adult size.

In (B) (Fig. 3) there was no preliminary lag period to constant weight as the animals were fed from the start on basal diet + B₁ + B₂. It will be seen from Fig. 3 that in every case growth ceased or proceeded at a very subnormal rate after 4 or 5 weeks. Immediately untreated marmite was substituted as source of B₁ and B₂ growth was resumed to maximum weight.

Thus it would seem clear from Figs. 1, 2 and 3 that a second factor, other than B₁, had been destroyed by the alkaline hydrolysis of the marmite.

0.1 N HCl extracts of B₁.

Experiments of type (A) above were arranged with B₁ concentrates prepared by extraction with 0.1 N HCl from charcoal in place of the 50 % alcohol extracts used above. Results were similar to those in Fig. 2, but slightly more marked, so presumably these extracts were more free from the second thermolabile factor than the 50 % alcohol extracts (see Fig. 4).

Use of younger animals.

As four weeks' feeding was needed in many of the above experiments before the absence of the second thermolabile substance became evident, it was now decided to carry out a set of experiments using younger animals (40 g. instead of 60 g.), in order to compare these results with those reported from the Lister Institute. As was expected, these animals were more sensitive to the lack of B₃; in fact, some of them died within a week of the time they began to drop in weight. However, if carefully watched and fed with untreated marmite they completely recovered (Fig. 5).

Properties of second thermolabile substance.

(a) It is generally agreed that alkaline hydrolysis for one hour at p_H 9 at 120° destroys the polyneuritis-curative properties of an aqueous suspension of marmite, yeast, etc. If this heating is carried out at a more acid reaction (p_H 6)² destruction of vitamin B₁ does not occur, but from the

¹ Note by H. W. Kinnersley. The concentrates used in the above experiments were the 0.1 N HCl or 50 % alcohol extracts from charcoal. In each case, after removal of metallic ions by H₂S, they were fractionated into 99 % alcohol [see Kinnersley and Peters, 1928, pp. 425, 426].

² I am indebted to Prof. R. A. Peters for testing these solutions. He has found that they retain at least 50 % of their curative properties when autoclaved under these conditions. The matter is still under investigation.

curves in Fig. 6 (rats 167 and 104) it would appear that there has been more than 50% destruction of B_3 . As would be expected, upon the view that B_1 is not destroyed, the early part of the curves is similar to those obtained with B_2 + the less impure extracts of B_1 . Additional indirect proof was obtained by adding B_1 at the point where growth first ceased and noting that no increase in weight was observed (see Fig. 6, rat 104).

(b) From the above experiments it seemed clear that B_3 is more thermolabile than B_1 . Further proof of this was obtained by boiling marmite in an open vessel on a water-bath for 2 hours (the actual temperature of the marmite was $95^\circ-97^\circ$). More than 50% destruction occurred. 12% marmite in the diet did not give growth equal to that previously obtained with 6% (Fig. 6, rat 152).

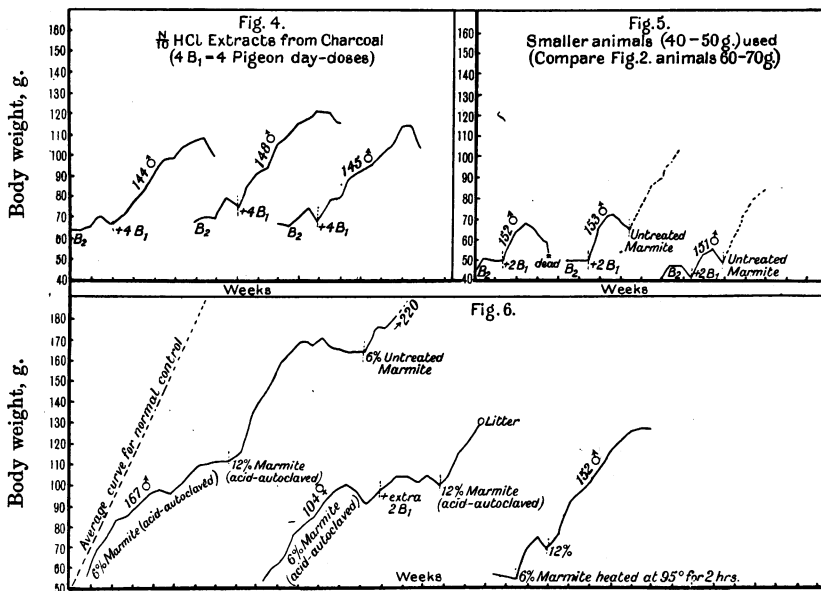


Fig. 6. Partial destruction of second thermolabile factor

- (a) by acid hydrolysis at 120° for 1 hour;
 (b) by heating at 95° for 2 hours.

(c) *Solubility in ether.* Six hours' extraction with ether in a Hurtley extractor removes most of the B_3 . However, if the ether is now distilled off, the substance cannot be redissolved in fresh ether. Thus it does not seem to be a true solubility of the substance itself, but rather dependent on adsorption upon associated substances. Further work upon this point is being carried out in the hope of getting a supply of B_3 free from B_1 .

DISCUSSION.

From the above experiments it would seem established that when marmite is subjected to alkaline hydrolysis at 120° for one hour at least two factors necessary for rat nutrition are inactivated. One of these is the antineuritic vitamin B₁. It is suggested that the other is a second thermolabile factor B₃. Evidently B₁ concentrates can be prepared free from this second factor by the extended method of Kinnersley and Peters [1928], and it is due to the advantage of having these more pure concentrates at my disposal that I have been able to demonstrate the absence of the second thermolabile factor from diets complete in other respects. However, so far a sample of B₃ has not been prepared free from B₁: therefore it cannot be definitely proved that the lack of growth is not due to the racemisation of some amino-acid or similar substance. In fact this view is supported by the fact that acid hydrolysis at 95° and at 120° apparently cause a certain definite loss of efficiency (> 50 %) whether the heating be for one hour or two: but absolute destruction of both B₁ and B₃ occurs on alkaline hydrolysis at 120° for one hour.

Williams and Waterman [1928] have recently reported the presence of a very thermolabile factor in yeast necessary for the maintenance of the weight and general condition of pigeons. However, they were unable to show its necessity for the rat. Whether they observed complete destruction of their factor by heating is not stated. As their preparation of B₁, made by the fuller's earth method, was not heated in the process, the B₃ reported in my experiments was probably still present associated with their B₁; hence they would not get a cessation of growth due to lack of this factor. Moreover, certain preliminary experiments carried out in this department (Peters, private communication), indicate that the second rat factor does not restore the weight of pigeons; thus the evidence seems to be in favour of two further factors—one, reported by Williams and Waterman, for pigeons: the other, reported in this paper, for rats.

At first sight these results seem to be contradictory to the results of Chick and Roscoe [1928]. In their work on the assay of B₂, they used B₁ concentrates prepared by the method of Kinnersley and Peters [1927] but not purified by alcohol fractionation, so it is possible that traces of B₃ were still present. However, it must be emphasised that in their actually recorded experiments they assessed their results from the growth obtained in the first 2 weeks after addition of B₂ to diets apparently complete in other respects. This procedure employs the justifiable assumption that if a diet is deficient in more than one factor, an immediate response is obtained by addition of any one of these. If, however, they had continued their experiments for 4 or 5 weeks, the picture presented might have been a different one.

SUMMARY.

Evidence is presented for the destruction of at least two factors, necessary for the nutrition of the rat, by alkaline hydrolysis of yeast extract at p_{H} 9 at 120° for one hour.

The conclusion is therefore drawn that a second thermolabile factor (B_3) is present in yeast extract.

My thanks are due to Professor R. A. Peters for his interest and criticism throughout the work, to Mr Kinnersley for the supply of B_1 concentrates, and to the Medical Research Council for a grant.

REFERENCES.

- Chick and Roscoe (1928). *Biochem. J.* **22**, 790.
Goldberger, Wheeler, Lillie and Rogers (1926). *U.S. Pub. Health Rep.* **41**, 297.
Hunt (1928). *J. Biol. Chem.* **79**, 723.
Kennedy and Palmer (1928). *J. Biol. Chem.* **76**, 591.
Kinnersley and Peters (1927). *Biochem. J.* **21**, 777.
—— — (1928). *Biochem. J.* **22**, 419.
Reader (1928). *Chem. Ind.* **47**, 1247.
Williams and Waterman (1928). *J. Biol. Chem.* **78**, 311.