CXXXII. VITAMIN B₃.

By JOHN RICHARD O'BRIEN.

From the Department of Biochemistry, Oxford.

(Received May 2nd, 1934.)

Of the components of the vitamin B complex, three have been considered necessary for the nutrition of the pigeon: vitamins B₁, B₃ and B₅. Williams and Waterman [1928] reported that pigeons on a diet deficient in the vitamin B complex required for weight recovery a water-soluble, heat-labile factor. This factor differed from the antineuritic vitamin B₁ and the alkali-stable rat factor B₂. Their belief in the existence of this factor was based on the observation that the addition of a special activated fullers' earth preparation of vitamin B₁ cured polyneuritis and checked the decline in weight of birds on deficient diets but failed to restore weight even when large doses were administered. Autoclaved yeast given as a further supplement had no effect, whilst dried yeast produced a gain in weight. Substitution of the synthetic diet for whole wheat also caused a similar increase. The factor postulated to explain these results was designated vitamin B₃.

Confirmation of the views of Williams and Waterman came from several sources. Randoin and Lecoq [1926] had previously stated that pigeons required in addition to vitamin B₁ a growth-promoting factor which was destroyed by autoclaving in an alkaline medium. In discussing vitamin B₃, Lecoq [1931] expressed the opinion that it was identical with the nutritional factor of Randoin and Lecoq. Carter *et al.* [1930] produced evidence which suggested that the pigeon needed, besides vitamin B₁ and B₃, a third alkali-stable factor, vitamin B₅. At the same time, they supported the claims of Williams and Waterman by showing that amounts of vitamin B₁ as large as 20 pigeon day doses caused no gain in weight in birds on polished rice. The possible existence of some such factor as that postulated by these workers needs consideration since it offers an explanation of the fact that quite large doses of concentrates of vitamin B₁ are required together with vitamin B₃ to cause weight increase. Further, Eddy *et al.* [1930] thought that the factor B₃ was essential for the normal growth of the chick as well as the pigeon. They also demonstrated the extreme lability of vitamin B₃ to heat and alkali.

Williams and Eddy [1931] jointly stated that vitamin B₃ was nothing more than an abundant supply of vitamin B₁, ten to twenty times the antineuritic dose. Nevertheless, they could not be certain that their purest preparation of vitamin B₁ was free from B₃. Their opinion was supported by the work of Morris [1933]. She showed that the properties of vitamin B₃ were very similar to those of vitamin B₁ and that pigeons on a diet of autoclaved whole wheat gained in weight when given large doses of vitamin B₁.

In view of the statement of Williams and Eddy, it was considered desirable to report such evidence as we had obtained in an attempt to elucidate the problem of vitamin B₃. The results suggest that pigeons utilise in addition to the vitamin B₁ a factor for weight restoration. We found that supplements of large

doses of vitamin B₁ concentrate given to birds on a polished rice or autoclaved whole wheat diet had no effect upon their weight. Moreover, it was possible to prepare a concentrate of vitamin B₃ which retained its antineuritic curative properties when its weight-restorative potency had been destroyed. Preliminary experiments tend to indicate that the vitamin B₃ effect is not to be attributed to an insufficiency of protein in the basal diet.

Method of assay.

The adult pigeons, used in this investigation, were fed on polished rice, autoclaved at 15 lbs. for 3 hours to destroy completely vitamin B₁. They were weighed every alternate day at 4 p.m. Twice weekly, they were given by mouth 8–10 drops of cod-liver oil as a supply of vitamins A and D. It has been stated that pigeons do not require vitamin C [Sugiura and Benedict, 1923], which is supported by the fact that vitamin C concentrates prepared from lemon juice as a source of vitamin B₃ had little effect. On the diet, the birds dropped to 60–70 % of their maximum weight in a space of 14–21 days which approximates to the length of the storage period of the B₃ factor. To ensure the depletion of the factor, the birds were taken to maximum weight on whole wheat or by additions of 1 g. dried yeast or 3 g. wheat germ daily and then dropped a second time on polished rice. They were now given by mouth 3 doses of vitamin B₁ daily in the form of an alcoholic concentrate prepared according to the method of Kinnersley and Peters. This alcoholic concentrate is considered to contain, together with the antineuritic vitamin B₁, the factor B₅, the rat factor B₄, and possibly also the factor Y of Chick and Copping [1930]. Throughout the present communication, when speaking of vitamin B₁, we refer to the impure alcoholic concentrate. Under these conditions birds can be maintained at surprisingly constant weight far below their maximum for periods as long as 6 months, the fluctuation in weight being never greater than ± 5 g. This weight maintenance has been observed with 60 birds and amply supports the findings of Carter *et al.* [1930]. Under such treatment the birds develop no symptoms peculiar to vitamin B₃ deficiency. The only ill effects seen so far have been occasional cases of birds showing ruffled feathers, lassitude and loss of appetite.

Concentrates and foodstuffs to be tested can now be given into the crop by pipette. Groups of 5 birds were employed to test any preparation. It is to be noted that although three doses of vitamin B₁ suffice for weight maintenance, experiment shows that at least six are necessary in conjunction with a vitamin B₃ preparation to promote weight recovery.

RESULTS.

The richest sources of the factor B₃ proved to be wheat germ and dried yeast. The effect of these supplements on weight is illustrated in Fig. 1 where it is seen that doses of 3 g. wheat germ and 2 g. dried yeast produce a weight recovery practically equal to that obtained on replacing by whole wheat a diet of polished rice supplemented by vitamin B₁ (Fig. 2). Some gain in weight was caused by egg-yolk and to a smaller extent by malt extract and marmite. No effect was seen with egg-white in doses equal to 1 egg per day, liver powder or milk (50 ml./day) even when the dosage of vitamin B₁ was increased to ten times the antineuritic dose.

The restorative effects on weight of the wheat germ and yeast could not be reproduced by supplying the birds with large doses of the concentrate of Kinnersley and Peters. Doses of vitamin B₁ of forty times the curative amount

have had no influence upon weight. Such doses would presumably contain adequate supplies of the other factors present. A concentrate of vitamin B₂ was prepared from aqueous extract of yeast by lead precipitation. The precipitate was decomposed by warming with sulphuric acid, and the last traces of

Fig. 1.

Fig. 2.

Fig. 1. Weight recovery with wheat germ and dried yeast. Typical curves obtained on giving dried yeast (curve 1 A) or wheat germ (curve 2 B) to birds on polished rice + 6 doses vitamin B₁.

Fig. 2. Weight recovery with whole wheat. Curve 1 shows drop in weight of bird on polished rice. At A, 3 doses B₁ are given. Curve 2 shows a similar case. At B, a whole wheat diet is substituted for polished rice + 3 B₁.

lead were removed by H₂S. The solution obtained was inactive as a source of vitamin B₃ in doses four to five times the amount required to cure young rats of dermatitis and to promote growth of 15–20 g./week. From these experiments, it was concluded that none of the factors investigated was responsible for the vitamin B₃ effect.

Preparation of vitamin B₃ extracts.

Attempts were made to prepare extracts of vitamin B₃ from foodstuffs normal to pigeons' diets—maize, corn, wheat. Aqueous and alcoholic extracts were inactive as supplements. Similar extracts of yeast were likewise without activity. It was found that yeast extracted with 97 % alcohol at 40° retained only 50 % of the original vitamin B₃ activity. Slight activity was shown by extracts of grains prepared by means of acidic solvents. These observations led to the conclusion that vitamin B₃ was being held in some form of chemical combination insoluble in the usual solvents. More drastic chemical treatment was therefore attempted to liberate the factor, the most successful being acid hydrolysis. Potent extracts were obtained by hydrolysis of wheat germ and dried yeast.

The wheat germ extract was prepared in the following manner. Wheat germ was extracted three times at 60° with 97 % alcohol and dried in air. Batches of 75 g. were hydrolysed with 300 ml. N H₂SO₄ by vigorous boiling under reflux for 6 hours. The mixture was filtered while hot and cooled. The main bulk of the sulphuric acid was removed by the addition of baryta or barium carbonate until the *p*_H was about 2. Before dosing, these concentrates were brought with NaOH to *p*_H 6.8. Given in doses equivalent to 3 g. of wheat germ, the hydrolysate caused an immediate increase in weight in pigeons (Fig. 3).

The properties of the extracts are as follows: There is present little or no protein matter, since the usual protein reagents, trichloroacetic acid, alcohol, sodium tungstate, ammonium sulphate and sodium sulphate caused negligible precipitation under varied conditions. Amino-acids are present together with some reducing substances. The extracts contain vitamin B₁. Tests on two groups of 5 birds in head retraction showed that on an average, there are 4–5 doses of B₁ in one dose of hydrolysed wheat germ. The peculiar property of the extracts is the rapid loss in vitamin B₃ activity on standing at room temperature or in cold store for 6–8 days. In Fig. 3, it is seen that a rapid rise in weight occurs with a freshly prepared hydrolysate followed by decline or maintenance in weight. The addition of another fresh extract renews the weight increase.

It seemed probable that the inactivation of vitamin B₃ arose from oxidation. Such a view was supported by the fact that when air was drawn through the hydrolysed wheat germ preparations at p_H 2 for 1 hour almost complete loss of activity was observed. Similarly, after treatment with hydrogen peroxide under the same conditions, the extracts produced no effect on weight. Further, the loss in activity can, to some extent, be inhibited by bubbling nitrogen through the mixture during hydrolysis and storing the preparations in nitrogen or *in vacuo*. By such procedure, the activity can be preserved for 10–12 days. Other attempts at preservation with carbon dioxide atmospheres, however, caused inactivation. Little success was obtained with the addition of reducing substances such as sulphite as preservatives. The vitamin B₁ content is unimpaired by oxidation with air; tests on a group of 5 polyneuritic birds revealed that the original 4–5 doses of vitamin B₁ per dose of hydrolysate were retained.

Fig. 3. Weight recovery with hydrolysed wheat germ. Curve 1, control bird on polished rice + 6 doses B₁. Curve 2, bird receiving in addition dose of hydrolysed wheat germ $\equiv$ 3 g. wheat germ daily. The arrows indicate fresh preparations of hydrolysate.

Attempts at fractionation of vitamin B₃ preparations.

The very labile character of vitamin B₃ has rendered difficult attempts to prepare more potent extracts. As mentioned above, protein precipitants are useless. Fractionation with phosphotungstic acid and mercury salts have also proved unsatisfactory. With lead acetate, on some occasions, at least 50 % of the activity has been precipitated but the results have been so variable that it was impossible to use this reagent. It can be concluded from these experiments that the factor B₃ behaves quite differently from vitamin B₁.

Behaviour of vitamin B₃ towards heat.

Eddy *et al.* [1930] considered that vitamin B₃ was extremely heat-labile, being destroyed at temperatures above 60°. On the other hand, Carter *et al.* [1930] state that the vitamin B₃ potency of whole wheat is not completely destroyed after 5 hours' steaming. The conclusions reached by these two groups of workers are directly contradictory. From our experiments, in its behaviour

towards heat the factor B_3 is somewhat similar to vitamin B_1 in that it is more susceptible to moist than to dry heat. We have shown that vitamin B_3 resisted boiling with $N H_2SO_4$ for 6 hours, which indicates a stability greater than would be expected from the results of the American workers. We have also observed that complete destruction of the factor occurred on autoclaving whole wheat, wheat germ and yeast at 120° for 3 hours while heating these materials at 120 – 160° for 2 hours hardly impaired the activity. The divergent results of different investigators suggest that the stability of vitamin B_3 is probably dependent upon its state of combination in the foodstuff.

The relation of protein to the vitamin B_3 effect.

Considerable criticism has been levelled at the postulation of factors other than vitamin B_1 being essential for maintenance nutrition of pigeons, on the basis that the experimental diets were not always adequate in respect to other nutrients. The use of polished rice as a basal diet has been particularly condemned. The question of an insufficiency of mineral salts and other vitamins does not arise in our experiments. Further, the caloric requirements are satisfied. Food intakes were measured, and it was found that a pigeon on a polished rice diet receiving 6 doses daily of vitamin B_1 ingested 20 g. daily, the caloric value of which is 71. On a whole wheat diet, which supplies all the essentials for pigeon nutrition, a bird will consume 25 ± 5 g. daily; caloric value 86 ± 17 . Sugiura and Benedict [1923] found that pigeons could be maintained on 20 g. of a synthetic diet of a caloric value of 70. The discrepancies in the caloric values of the amounts of different diets consumed daily are hardly sufficient to explain the failure of vitamin B_1 concentrates to restore weight in pigeons on polished rice.

Since the low protein content of polished rice might be responsible for the failure, experiments were made in which protein was given to birds. 2 g. of caseinogen were given daily to birds on rice supplemented by 6 doses of vitamin B_1 . No response was observed. When, however, the dosage of vitamin B_1 was doubled, rapid increase in weight was seen in the first 4–7 days of the experiment followed by large fluctuations in the next 7 days. Two groups of 5 birds were used in the experiments and in no case was a rise to maximum weight obtained. In another series of experiments, 2 g. caseinogen were given to birds receiving hydrolysed wheat germ equivalent to 3 g. wheat germ daily in addition to the vitamin B_1 supplement. Complete recovery to maximum weight was obtained (Fig. 4). In Fig. 4, it is shown that the weight increase ceased when the vitamin B_3 concentrate lost its activity.

Morris [1933] has tried to overcome the difficulty of the insufficiency of protein by the use of autoclaved whole wheat as a basal diet. She obtained recovery in weight by giving doses of vitamin B_1 roughly six to twelve times the dose curative of polyneuritis, and concluded that

Fig. 4. Caseinogen supplements. Curve 1, control bird on polished rice + 6 doses B_1 daily. At A, 2 g. caseinogen given daily. Curve 2, birds on polished rice + 6 doses B_1 + hydrolysed wheat germ $\equiv$ 3 g. wheat germ + 2 g. caseinogen daily. The arrows indicate fresh preparations of hydrolysate given when decrease in weight occurred with loss in activity of vitamin B_3 .

the vitamin B₃ effect was probably a function of a large quantity of vitamin B₁. Our experience differed from that of Morris. A group of 10 birds was placed on whole wheat autoclaved at 120° for 3 hours, and when they had dropped to 60–70 % of their maximum weight, they were given 6 of doses vitamin B₁ daily. After slight fluctuations, the weights of the birds became constant. No recovery in weight was observed on increasing the amount of vitamin B₁ to 30 pigeon day doses. The disagreement of the results of our experiments on caseinogen supplements with those of Carter [1934] and the failure to confirm Morris on autoclaved wheat are possibly connected with fuller depletion of factors in the preparation of the birds used here, but the matter requires further investigation.

DISCUSSION.

From the experiments so far made, it appears that some factor is required for weight restoration by a pigeon on a diet of polished rice supplemented by an alcoholic concentrate of vitamin B₁. The nature of this factor is doubtful. Our results certainly fail to substantiate the statement of Williams and Eddy [1931] and of Morris that vitamin B₃ is merely an abundance of vitamin B₁, many times the dose curative of polyneuritis. The fact that pigeons rise rapidly in weight when removed from polished rice to whole wheat is difficult to reconcile with this view. The daily intake of a bird on wheat would scarcely provide it with such large doses of vitamin B₁. Hydrolysed wheat germ contains a factor which can be distinguished from vitamin B₁ by its instability to oxidation. The mode of preparation of the extract together with its properties suggest that the factor may be an amino-acid. It may, in fact, be one or more of the known amino-acids which, owing to the nature of the extract, break down readily on oxidation. But no evidence has yet been obtained by us in experiments in which known amino-acids such as histidine (20 mg. given daily to 2 birds), tryptophan (20 mg. daily to 2 birds), or cystine (50 mg. daily to 3 birds), present in negligible amounts in polished rice, have been administered together with vitamin B₁ that such a view is correct. In fact, the experiments with caseinogen supplements suggest that the vitamin B₃ effect does not arise from a faulty basal diet.

SUMMARY.

1. Supplements of 20–30 doses of vitamin B₁ fail to restore the weight of pigeons on a polished rice or autoclaved whole wheat diet.
2. Extracts can be prepared from wheat germ which restore the weight of pigeons on polished rice supplemented by vitamin B₁. These extracts are inactivated by oxidation.
3. Birds on polished rice + 6–12 doses daily of vitamin B₁ fail to recover weight when given 2 g. caseinogen daily.
4. It is concluded that the pigeon requires in addition to vitamin B₁ a factor to restore weight.

I wish to express my thanks to Prof. Peters for his advice and criticism throughout this work and to the Department of Scientific and Industrial Research and the Medical Research Council for grants.

REFERENCES.

- Carter (1934). *Biochem. J.* **28**, 933.
—— Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832, 1844.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Eddy, Gurin and Keresztesy (1930). *J. Biol. Chem.* **87**, 729.
Lecoq (1931). *J. Biol. Chem.* **91**, 671.
Morris (1933). Dissertation. New York.
Randoin and Lecoq (1926). *Compt. Rend. Acad. Sci.* **182**, 1408.
Sugiura and Benedict (1923). *J. Biol. Chem.* **55**, 33.
Williams and Eddy (1931). Carnegie Year Book.
—— and Waterman (1928). *J. Biol. Chem.* **78**, 311.