CCXLII. VITAMIN B_4.

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(*Received October 29th, 1932.*)

In earlier publications [Reader, 1929; 1930, 1, 2] the evidence has been presented for the conclusion that crude watery or alcoholic extracts of yeast contain two heat- and alkali-labile factors necessary for the nutrition of the rat; in the literature these two factors are referred to as vitamin B_1 (the antineuritic vitamin) and vitamin B_4. A method of assay of vitamin B_4 has been described in detail by Reader [1930, 2] and has since been used with success by Halliday [1932]. As an appendix to the above paper [1930, 2] Reader described a method for preparing vitamin B_4 concentrates free from vitamin B_1, making use of the mercuric sulphate fraction obtained in the Kinnersley and Peters process for vitamin B_1. A stable concentrate (activity 0·6 mg. per rat day dose) was obtained, but further purification seemed impossible without loss of stability; consequently the method was abandoned for one in which the vitamin B_4 was removed from the yeast extracts by adsorption on charcoal at p_H 1·0 [*vide* Peters, 1931].

From these activated charcoals we have now prepared a white crystalline material of high vitamin B_4 activity and of apparently constant chemical composition. Essential steps in this new process are fractionation with sodium phosphotungstate [*cf.* Peters, 1930] and liberation of an actual base by hydrolysis of a base-pentose compound. We hesitate to describe our compound as vitamin B_4 itself, as we have certain indirect evidence as yet unpublished (Kinnersley and Peters), that a preparation of vitamin B_4 of higher activity can be obtained. Our crystals, stored in a desiccator over calcium chloride, have remained stable for 6 months and so form a useful standard for further work on vitamin B_4.

Experimental.

Testing. Throughout this work the adult rat curative method of assay [Reader, 1930, 2] was used to follow the vitamin B_4 activity. As previously mentioned our stock colony has been rigorously inbred in order to make the variation in response between the individual animals as small as possible. At least 5 animals have been used for testing the extracts after each stage in

[1] A preliminary account of this work has previously been published [Barnes, O'Brien and Reader, 1932]. Although the research is still incomplete we have decided to publish a detailed account, as one of us (V. R.) is unable to continue the work.

each preparation. After the phosphotungstate stage not more than 2 in any 20 animals gave results outside a $\pm$ 20 % range.

Plate IX shows (A) the condition of an animal when ready for test, and (B) the state of the same animal after 1 week's treatment with the active substance. When in condition (A), the animal is unable to walk without falling (usually to the left side), and cannot control the action of its head sufficiently to drink from the usual vertical bottle. Lack of co-ordination is the most marked symptom, but is frequently accompanied by a noticeable dilation of the capillaries between the digits of the paws.

Purification of active material. The preparation of vitamin B_4-active charcoals from yeast has been described elsewhere [Peters, 1931]. In this paper the technique used in preparing the crystalline material from these charcoals is described. The quantities of materials stated correspond to those required for the charcoals from one batch of 50 kg. of the original yeast. The p_H values were determined by the colorimetric method and are only approximate.

(*a*) *Extraction.* Immediately after the adsorption process, the charcoals were filtered on Büchner funnels, washed with dilute sulphuric acid (p_H 1) and then covered with 50 % ethyl alcohol to which was added sufficient concentrated hydrochloric acid to bring the fluid in contact with the charcoal to p_H 1·0. Four successive extractions, each with 800 cc. of fluid, were carried out by warming on the water-bath to 70° and filtering at the pump whilst hot. The total extraction fluids were mixed, sodium hydroxide was added to bring the p_H to 3·0, and the whole distilled *in vacuo* to remove alcohol. The residue, having a volume of approximately 1500 cc., was allowed to stand in cold store overnight.

(*b*) *Mercuric sulphate treatment.* The extract was again adjusted to p_H 3·0, and 350 cc. of Denigès's mercuric sulphate reagent added. After standing for 1 hour, the fluid was filtered, the precipitate being discarded: excess sulphate was removed from the filtrate by addition of hot saturated solution of baryta, until the p_H was 2·0. After standing another 2 hours, the fluid was filtered, treated with sodium hydroxide to bring it to p_H 4·0, and then with H_2S for 4 hours to remove mercury. After again filtering, the excess H_2S was removed *in vacuo*, the temperature not being allowed to exceed 30°, to prevent appreciable reduction of volume. The fluid, about 2000 cc., was allowed to stand in cold store overnight.

This treatment removes a certain amount of material without diminishing the activity of the extract. Such a result is in contrast with earlier experiments [*vide* Reader, 1930, 2] in which the active substance itself was precipitated by the mercuric sulphate reagent. There can be little doubt that the adsorption on charcoal, introduced into the present technique, is responsible for this altered behaviour.

(*c*) *Fractional precipitation with sodium phosphotungstate.* Several preliminary experiments were conducted, in which the merits of the 1:18 and the 1:24 phosphotungstates for fractional precipitation of the vitamin were

compared. A typical result is shown in Table I. To each of two flasks, each containing 20 cc. of the fluid (50 rat doses), 1 cc. of the appropriate phosphotungstate was added, and the p_H gradually changed from 6 to 1, precipitates being removed at the points indicated in Table I. Definite zones of precipitation were observed [*cf.* Kinnersley and Peters, 1930].

Table I.

	p_H at which precipitate removed	Approx. no. of vitamin doses recovered
Sodium salt of 1:18 acid	4·0	5
,,	2·5	10
,,	1·0	10
Sodium salt of 1:24 acid	4·0	0
,,	2·5	10
,,	1·0	40

From such results as are shown in Table I it was concluded that a more specific precipitation occurred with the 1:24 salt and that the maximum point for the vitamin was between p_H 2·5 and 1·0. Hence it was decided to use the 1:24 salt (prepared by the method of Wu [1920]) for all further work. Immediately before use 10 g. of the acid were dissolved in 50 cc. of distilled water, 20 % sodium hydroxide was added to bring the p_H to 6·0, and the whole made up to 100 cc.

The fluid from the mercury stage (*b*) was then brought up to p_H 6·0 and an excess of the 10 % phosphotungstate solution (about 50 cc.) added. 5 % sulphuric acid was gradually added to bring the whole to p_H 3·0. It was then allowed to stand in the cold store overnight. Next morning it was filtered and the precipitate discarded. 5 % sulphuric acid was again added to bring the filtrate to p_H 1·0 and the mixture allowed to stand in the cold store overnight. After filtration at the pump, the precipitate, which was straw-coloured, contained the activity.

(*d*) *Recrystallisation of the active phosphotungstate.* The above precipitate was washed through the filter-paper with the smallest possible quantity of hot 50 % ethyl alcohol (about 100 cc.) and allowed to stand in a cool place for 24 hours. Any precipitate which was not soluble in the hot alcohol was discarded.

(*e*) *Removal of phosphotungstic acid.* Next day the yellow deposit (usually, in the main, crystalline) was filtered from the alcohol, dissolved in 50 % acetone, and cold saturated baryta added until the solution turned phenolphthalein pink. It was then shaken well for 5 minutes and filtered as rapidly as possible. A few drops of dilute sulphuric acid were added to the filtrate immediately to remove any excess barium and to make the solution acid again (p_H 3). This filtrate, volume about 250 cc., and containing 30–40 % acetone, should contain 10–15 rat day doses of vitamin B_4 per cc. Test doses were always given to the animals before proceeding further. The acetone

present in 1/10 cc. of the solution was enjoyed by the animals and did not appear to do them any harm. The small deposit of barium sulphate was removed by decantation or filtration immediately before use. In all 19 lots, each representing 50 kg. of yeast were brought to this stage. All were free from vitamin B_1 and from the streptothrix factor [Reader, 1928] and have remained stable for many months.

(*f*) *Hydrolysis.* Acetone was removed *in vacuo* and the fluid concentrated to 50 cc. 5 cc. of concentrated hydrochloric acid were added and the whole was heated on the water-bath for 1–2 hours, or until a positive orcinol reaction for pentose was obtained. The time taken for this hydrolysis varied from 1 to 3 hours in the 17 lots tried, but the average time was 1 hour. It is useless to proceed to the next stage unless a positive orcinol reaction is obtained.

(*g*) *Crystallisation.* The 50 cc. fluid from the above were concentrated to 10 cc. on the water-bath and 20 cc. of acetone added. If any precipitate formed it was removed by filtration and discarded. (Last traces of barium sulphate and chloride were thus removed.) Then 70 cc. of ether were cautiously added and, after gentle stirring, the mixture was allowed to stand on ice for 12 hours or until crystals appeared. On two occasions the crystals appeared within 2 minutes from the time of addition of the ether. The product was recrystallised from a 1 part water–5 parts acetone-50 parts ether mixture. A photomicrograph of the final product is shown in Plate IX (C).

Results.

During the last year about a ton of yeast has been worked through the stages described above. The results from 12 successive lots, each of 50 kg., are recorded in Table II. The numbers represent the total number of rat day doses (curative) in the extracts.

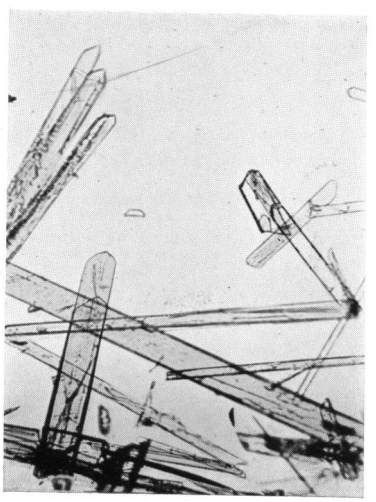
Table II.

Lot	Extract from charcoal	After mercury	After phospho-tungstic	After hydrolysis	Remarks
1	5000	5500	4000	4000	No pentose and no crystals
2	6000	5500	3000	Nil	—
3	6000	6000	5000	4000	30 mg. crystals
4	4500	4000	4000	3500	28 mg. crystals
5	5000	4500	4500	3000	15 mg. crystals
6	6000	5500	5500	3000	No pentose and no crystals
7	5500	5000	5000	4000	20 mg. crystals
8	4500	4200	4000	2500	10 mg. crystals
9	4800	4500	4500	Nil	—
10	6000	6000	5500	5000	5 mg. crystals
11	6000	5000	5500	4500	Nil
12	6500	6000	5000	1000	Nil

It will be seen that the method worked fairly constantly without great loss of activity up to the end of section (*e*), *i.e.* liberation from phosphotungstic acid: but that for the hydrolysis and crystallisation stages the conditions are not yet well defined.

(A) Animal suffering from deficiency of vitamin B_4.

(B) Same animal after one week's treatment with active material (0·01 mg. daily).



(C) Crystals with vitamin B_4 activity. ×100.

The improvement in purity as the preparation proceeds is shown in Table III. Lots (3), (4) and (5) from Table II are quoted as illustrations.

Table III.

Stage	Activity (organic solid weight required per unit dose (± 20 % variation)). mg. Lot 3	Lot 4	Lot 5
Extract from charcoal	10	9	10
After mercury	5	4	6
After phosphotungstic	0·5–0·2	0·5–0·3	1·5–0·5
Crystalline material	0·010	0·012	0·010

Analysis. Samples of crystals from lots 3 and 5 have been analysed by Dr Ing. Schoeller of Berlin. His findings are:

	C %	H %	N %	Cl %
Lot 3	35·5	3·5	38·03	21·6
Lot 5	34·8	3·4	37·70	22·2

The above figures were obtained after drying the substance at 80° in high vacuum over P_2O_5. The water content was 5·2 % and 5·4 % for the two samples respectively. The empirical formula is probably $C_4H_4N_4.HCl, \frac{1}{2}H_2O$.

Properties. Hydrochloride of a base: sublimes at 220°: very soluble in water: insoluble in absolute alcohol, ether or acetone: forms a crystalline picrate which melts at 278°: is adsorbed on charcoal at p_H 2·0: precipitated by sodium phosphotungstate (1:24) from a 1 in 500 solution at p_H 4·8: gives negative results for the following colour reactions: Pauly, Fehling, Millon, α-naphthol, and Tollens.

Stability to acid. The final product, a base forming salts with acids, is stable to acid treatment, even boiling with 20 % HCl for an hour. At first sight this does not harmonise with earlier results of Reader [1929], in which it was shown that after boiling a watery extract of yeast or marmite for an hour with 5 % HCl, a 50 % destruction of the vitamin had occurred. Again after liberation from phosphotungstic acid, the activity of the solution remains stable so long as acetone or alcohol is present but diminishes on storage, even at 0° in an aqueous solution. It would appear therefore that the stability of the product towards acid is variable and is influenced by the degree of purification achieved.

Stability to alkali. At all stages in the preparation the vitamin B_4 activity is lost on boiling with alkali. The final crystalline material is inactivated by heating on the water-bath with 10 % sodium hydroxide for 10 minutes. So far none of the crystalline base has ever been recovered after this treatment, but this may be due to the small amounts of material handled.

SUMMARY.

An account is given of an attempt to isolate vitamin B_4 from yeast. An unknown base, with a high and apparently constant vitamin content, has been isolated in the form of colourless crystals. Essential steps in its preparation are fractional precipitation with phosphotungstate, and removal of a pentose group from the molecule by acid-hydrolysis. Analyses suggest the formula $C_4H_4N_4.HCl, \frac{1}{2}H_2O$. Further work is required before it can be claimed that the product is identical with the vitamin.

In conclusion we wish to thank Prof. R. A. Peters for his advice and encouragement throughout the research, and to acknowledge gratefully a personal grant from the Medical Research Council to one of us (V. R.); also to thank Miss M. Kempson for her assistance and care with the animals.

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