

CCXI. THE RELATION OF HYDROGEN ION CONCENTRATION TO THE PRECIPITATION OF PURIFIED TORULIN (YEAST VITAMIN B₁) BY PHOSPHOTUNGSTIC ACID.

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IN a previous communication [Kinnersley and Peters, 1927] we described methods by which torulin (yeast vitamin B₁) could be concentrated to an activity in our terms of 0.025 mg. *per diem* (pigeon dose). The object of this paper¹ is to describe certain improvements in obtaining material of this activity, especially the use of new methods of phosphotungstic acid fractionation. At the same time, we propose to mention some of our experiences with other methods of fractionation. It must be stated at once that we have not yet reached an activity greater than 0.01 mg. *per diem* as the average dose for several pigeons, but we believe that the methods described here are in some respects simpler than others, and may be capable of application in other fields. A specimen of the crystals of the Jansen and Donath vitamin [1926] from rice polishings which we have recently had the opportunity of testing [Jansen, Kinnersley, Peters and Reader, 1930] was 40 % more active than the preparations described here.

EXPERIMENTAL.

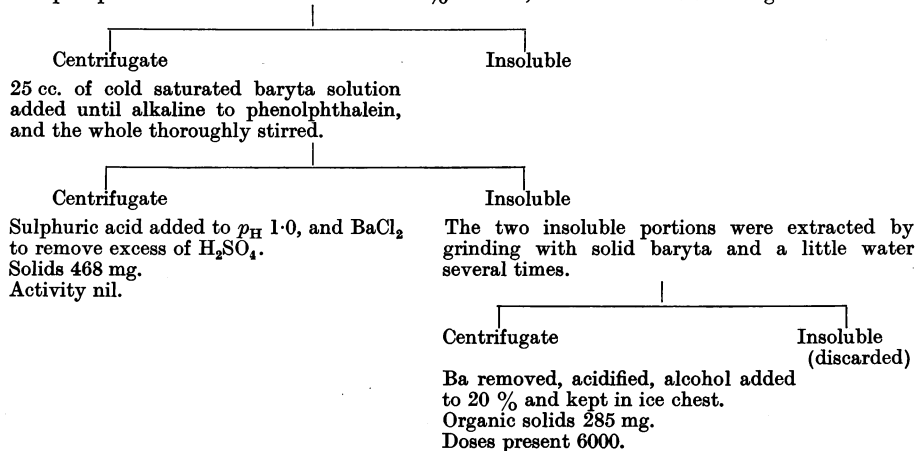
The method previously described by us involved adsorption upon charcoal by a technique which is now standardised, subsequent treatment of the charcoal extracts with sulphuretted hydrogen to remove metals, and fractionation with alcohol with gradual change in p_H of the extract. After this the active material was obtained by the use of phosphotungstic acid and platonic chloride, following the Jansen and Donath method in the main, with the exception that the decomposition of the phosphotungstate was carried out by the classical method of grinding with solid baryta. The behaviour of these yeast extracts to phosphotungstic acid is a very curious one. At an earlier stage in our procedures, before the baryta treatment had been introduced, we found that it was impossible to recover the vitamin from the phosphotungstates, owing we now believe to its precipitation with traces of gum. Later in the preparation, after removal of these impurities, the active substance can be

¹ A preliminary account has appeared, *Chem. Ind.* (1930) *Proc. Biochem. Soc.* p. 517.

recovered from a phosphotungstic acid precipitate; but we have never been able to recover it by the use of acetone and baryta, as described by Jansen and Donath for the rice vitamin, and also confirmed by us [Peters, 1930, 1]. We do not understand why this is so, but it forms an admirable illustration of the manner in which the apparent properties of these more active biological substances may change during the course of a purification. It is safe to say that at each stage of purification it is wise to treat the material present as a fresh chemical problem and not to assume that a process rejected at an earlier stage may not be applied with success at a later. We give below an experiment illustrating that the baryta-acetone method cannot be applied to the yeast vitamin, even when this has reached the purity of 0.8 mg. *per diem*.

Exp. 1. Decomposition of phosphotungstate by baryta-acetone.

A phosphotungstate precipitate of torulin was made by adding to a torulin solution (475 cc.) 30 cc. of 10 % phosphotungstic acid neutralised to p_H 6.0 together with sulphuric acid to p_H 4.5. The precipitate was extracted twice with 50 % acetone, and the extract centrifuged.



The activity in the final centrifugate corresponded very closely to the number of doses originally present, showing that none of the activity had passed into solution by decomposition with baryta-acetone. It will be recalled that the Dutch workers employ baryta-acetone for the decomposition of their phosphotungstates from rice polishings. It is curious that the method should not be applicable in the purification of torulin.

Among the attempts made by us to eliminate the use of metals in the later stages of our fractionation was an extensive use of mixed solvents, especially ether, alcohol and acetone. It does not seem to be worth while to give in detail the many experiments made: one of them has been presented elsewhere [Peters, 1930, 1]. A marked feature of the experiments, which were carried out with material of activity of 0.08 mg., was the disappearance of activity whenever the conditions were those of dry ether-alcohol. It was found that this disappearance of activity was especially marked when the initial p_H of the substance for fractionation was less acid than p_H 4.5. Quite small traces of water influenced the result as to whether the substance was thrown out by

alcohol-ether or not. For maximal removal of inactive substances, conditions must be kept at as alkaline a point and as anhydrous as possible. Unfortunately these conditions are just those which are found to lead to instability. We have excluded from our experiments the possible inactivating effect of impurities in the solvents by careful purification, and are therefore satisfied that the effects are not due to adventitious impurities. It is quite remarkable to find how unstable torulin is to alkali in alcoholic solution. We have several experiments upon this point, but think that only one is worth quoting. Exp. 2 shows the loss of activity when changing the p_H in alcohol by the addition of aqueous NaOH.

Exp. 2. Effect of NaOH in the cold on alcoholic solution of torulin.

The torulin solution used contained 4.060 g. of organic solids and 10150 doses.

The 98 % alcoholic solution was treated at room temperature with 2.5 cc. of 20 % NaOH in 5 cc. of alcohol, making the final p_H about 5.2. The precipitate so formed was removed upon the centrifuge.

Centrifugate Doses 3000 (2) ¹ . The precipitate and filtrates were then combined and concentrated, subsequent tests giving 3700 doses present (2).	Precipitate The precipitate and filtrates were then combined and concentrated, subsequent tests giving 3700 doses present (2).
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It must be concluded that though small amounts of impurities can be removed from material at p_H values on the acid side of 4.0 by the use of mixed solvents, the method is not reliable.

Adsorption by barium phosphotungstate.

The occasional losses experienced especially in the less pure concentrate led us to try whether the base was adsorbed by barium phosphotungstate *per se*. The following experiment shows that it is not.

Exp. 3. 80 doses of torulin of activity 0.12 mg. in solution in 3.0 cc. of water were treated with a precipitate of barium phosphotungstate made by adding to 1.4 cc. of 10 % phosphotungstic acid 2-3 cc. of hot baryta together with some solid baryta: the precipitate was centrifuged and ground with more solid baryta. The combined filtrates were brought to p_H 1.0 with sulphuric acid.

The tests showed 80 (3) doses meaning that the activity was still present, and therefore that any losses of torulin at a phosphotungstic stage are not likely to be due to adsorption by the barium phosphotungstate.

It will be observed that no regeneration of activity occurred in Exp. 2 upon adding together the two fractions. We have often tried to obtain this effect upon a sudden loss of activity but have never observed it, and cannot therefore confirm the experience of Guha and Drummond [1929].

Improvements in the use of phosphotungstic acid.

In the attempt to reduce the laborious operations involved in working up the charcoal concentrates by our original technique, we noticed that the

¹ Average of tests given throughout, the number of tests upon which it is based being added in brackets.

vitamin was very largely precipitated even when a marked defect of phosphotungstic acid was present. This led us to develop some observations made previously upon the effect of p_H upon the precipitation of bases by phosphotungstic acid [Peters, 1930, 2]. In brief, it is found that if neutralised phosphotungstic acid (p_H 6.0) is added to solutions of bases initially at p_H 7.0 followed by sulphuric acid, precipitation of certain bases seems to take place rather sharply at a p_H dependent upon the nature of the base. In the case of the vitamin, we brought the material for fractionation to p_H 7.0 approximately, added 10 % phosphotungstate (p_H 6.0) and sulphuric acid alternately drop by drop until commencing precipitation as indicated by opalescence appeared. After standing, the various fractions were removed at various hydrogen ion concentrations. Some substances present precipitate sharply at p_H 7.0: others as the p_H is changed to 5.0. Exp. 4 illustrates in detail one of the earlier experiments bearing upon this, and shows the rough grouping of the precipitates between p_H 7.0 and 1.0 approximately.

Exp. 4. Fractional precipitation at different p_H values.

The torulin used for the experiment was obtained from the decomposed phosphotungstate obtained from a charcoal concentrate fractionated first with alcohol and later with phosphotungstic acid at an acid p_H .

Organic solids 36 mg., containing approximately 170 doses.

The alcohol was removed after addition of a little water. At a volume of 6.0 cc. previously brought to p_H 8.0, the neutralised phosphotungstate was added until no further precipitate appeared. A series of precipitates was obtained, keeping the phosphotungstic acid in slight excess at each stage, and gradually increasing the hydrogen ion concentration by the addition of sulphuric acid. The precipitates so obtained were decomposed by grinding with solid baryta several times in a small centrifuge tube and removing the barium phosphotungstates etc. by centrifuge and filtration. The excess Ba was removed with addition of sulphuric acid, and each acidified to Congo red with HCl where necessary. 10 % alcohol was added for preservation.

	Precipitates p_H	Nature	Corresponds to fraction	Organic solids mg.	Bird test
1	8.0-8.5	Bulky yellow	Guanidine	5.2	Less than 20 doses
2	8.0-5.5	Small "	Histamine	3.3	18
3	5.5-4.6	" "	—	2.8	37
4	4.6-3.5	" "	—	?	130
5	3.5-2.0	Large white	Choline	15.6	Nil
6	More acid than 2.0	" "	Ammonium salts, etc.	—	?
			Total	26.9	

Though the above tests were not made upon many birds, they leave no doubt in the mind that the activity lies between p_H 8.0 and 3.5 under these conditions. It will be seen that the total amount of solids recovered is satisfactory. The details of column 4 are given to indicate to some extent the substances which might be expected to come down at these p_H values.

It will be noted that the precipitation of the vitamin appears to start at p_H 7.0-7.5.

Further experiments upon these lines and upon a rather small scale showed that if the precipitate which came down between p_H 7.0-5.0 was gradually defined and precipitated upon successive occasions in smaller volume, it was possible to attain a high degree of concentration by using these methods. It

was however found best to insert an alcohol precipitation stage between successive fractionations. In one case by this method, we obtained in two stages from torulin of activity 0.1 mg. a small amount of torulin of activity 0.012 mg. We give below the details of a larger scale experiment, which illustrates as clearly as possible the best method known to us of carrying out this fractionation.

Without giving the experiments in detail, we may say that we have not found the methods applicable to torulin before the charcoal adsorption stage. Immediately after this stage however fractionation can be applied with a considerable degree of success, and we have been able to eliminate the metals present without H_2S treatment. This is however somewhat uncertain so that we believe that it is advisable to remove metals by our customary technique.

Immediately after this removal and elimination of the H_2S fractional precipitation can be applied. The limits of the precipitation are less sharp in the early stages, so that it is necessary to collect the precipitate over the range p_H 5.0-2.0 in order to include the greater bulk of the vitamin. This precipitate is usually known as the first phosphotungstic precipitate.

The material is worked up by repeated extraction with solid baryta and minimal amounts of water, the process being repeated five times and in some cases more.

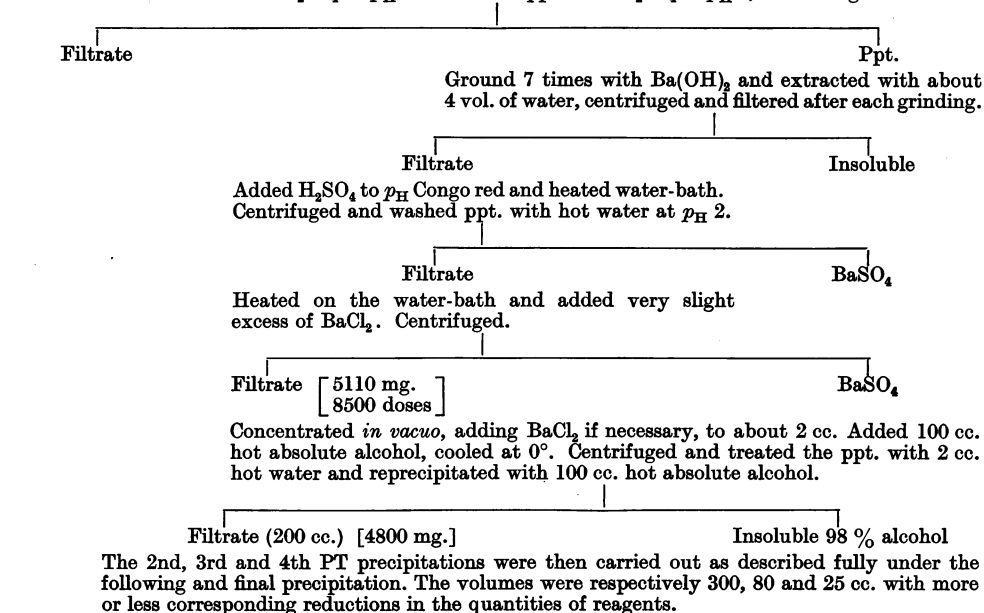
After grinding in the centrifuge tube with the moistened baryta, the precipitate is centrifuged, and the cloudy supernatant fluid filtered through a small paper into another centrifuge tube. This is necessary to remove the last traces of phosphotungstate. The combined filtrates are treated with sulphuric acid to remove excess of baryta, and with HCl or H_2SO_4 until acid to Congo red. The precipitate is centrifuged, and extracted with warm acid (approx. $N/100$). If this is not done, some of the bases may adhere to the precipitate. A second or further phosphotungstate precipitation is carried out by bringing the solution to p_H approx. 7.0, and then adding as before alternately sulphuric acid and phosphotungstate, separating off the precipitates as they appear. It will be found that substances can be defined to some extent by the separation of groups of precipitate. If the precipitate is colloidal, sodium sulphate can be added to ensure complete precipitation. It is also well to cool in ice water. A faint opalescence will usually be found to yield a good precipitate when the solution is allowed to remain for 2 hours at 0° . Each subsequent step of phosphotungstate precipitation is carried out in a smaller volume than the one preceding. Before describing this experiment, we wish to draw attention to a point which may influence results obtained with the pigeon tests. As was stated in one of our earlier papers, it has been our custom to work with slight excess of barium chloride in our solutions owing to the destructive effect of traces of sulphuric acid at any stage approaching dryness. Extraction of dried vitamin by absolute alcohol removes mere traces of such barium chloride together with the vitamin; but these traces have been sufficient to interfere with the test and in some cases to inhibit the effect altogether. We have not

Table I. *Exp. 6.*

Original organic solids 46,000 mg.

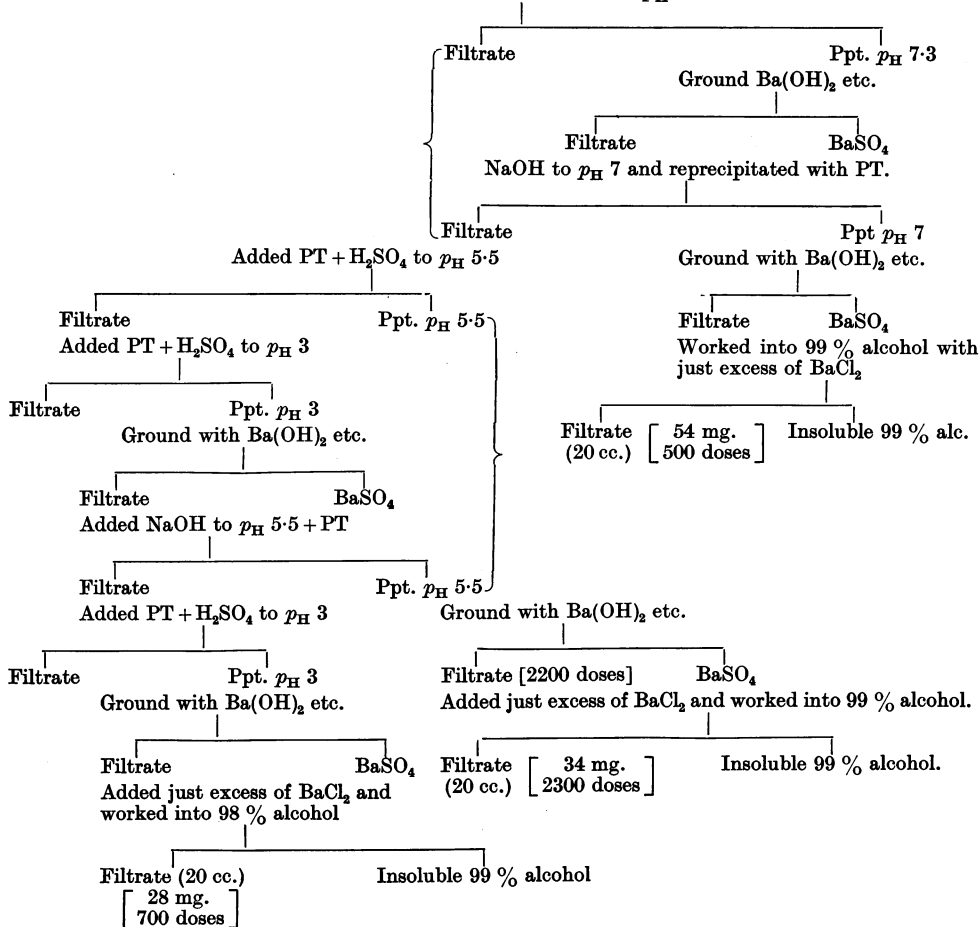
1st PT precipitation

V=1100 PT added 350 cc. H_2SO_4 to p_H 2. Washed ppt. with H_2SO_4 at p_H 2, containing a little PT.



5th PT precipitation.

Removed alcohol. Added water to about 6 cc. NaOH to p_H 7.3 + PT.



had the opportunity to investigate this matter. It would seem to be some chemical effect, as the amount of barium present would not be enough to be toxic: in practice we add sulphuric acid to each test (one drop of $N/10$ H_2SO_4) to ensure the absence of barium. A few experiments have indicated that the vitamin is soluble in absolute alcohol even in the presence of barium chloride both at p_H 2.0 and 5.0, but that it is no longer soluble at p_H 5.0 in strong alcohol if it is converted to the sulphate.

We record the full details of an experiment in which a sample of vitamin was carried through 5 phosphotungstate precipitations without much loss.

Exp. 5. The starting material was a 50 % acid alcoholic extract of vitamin adsorbed upon norite charcoal, worked up commercially to this stage by Messrs Burroughs Wellcome and Co. (to whom our best thanks are due). Organic solids present 45.7 g. The alcohol was removed upon the water-bath, and metals by treatment with H_2S at p_H 3.5–4.0 as usual. After concentration at p_H 3.0, water was added to a volume of 1100 cc., followed by 350 cc. of neutralised phosphotungstic acid (10 % Merck at p_H 6.0) then sulphuric acid to p_H 2.0. The precipitate was centrifuged and washed once. The subsequent treatment is given in Table I. All phosphotungstate precipitates unless otherwise stated were ground with solid baryta, and extracted with a little water (about 3–4 times the volume of the precipitate), centrifuged and the supernatant fluid filtered. This process was repeated with each precipitate some five times. The combined filtrates were treated with H_2SO_4 to remove the greater part of the barium, and with HCl until acid to Congo red, leaving a very slight excess of $BaCl_2$. The precipitate was re-extracted with approx. $N/100$ acid. In the following description, Vol = volume, PT = phosphotungstate solution of p_H 6.0, and AA = absolute alcohol.

Table II summarises the experiment. Volume refers to volume at the stage of phosphotungstate precipitation.

Table II.

Stage	Volume cc.	Org. solids g.	Total doses	Activity mg.
1st phosphot. ppt. p_H 7.0–2.0	1100	5.110	? 8000	? 0.6 probably 1.0
2nd phosphot. ppt. p_H 6.0–5.0	300	0.653	3500	0.5
3rd phosphot. ppt. p_H 7.0–5.0	80	0.265	4200	0.06
5th phosphot. ppt. p_H 7.0–5.5	25	0.050	1760	0.035
Final ppts.* p_H to 7.4	—	0.054	500	0.1
p_H 7.4–5.5	—	0.034	2300	0.012
p_H 5.5–3.0	—	0.028	700	0.04
		Total	3500	

* Collected precipitates.

It will be noticed that, considering all the operations and the degree of concentration achieved, the recovery in the last stages is good. It is probably much better than this all through the experiment because from another experiment carried out on the same material it appears that the first two birds must have given abnormally high tests. There would seem to be little doubt

that the important fraction is that precipitating between the limits of p_H 7.4–5.5 and that this will prove to be a property of pure torulin. By the use of phosphotungstic acid alone, the activity has been increased from 1.0 mg. approx. per day to 0.012 mg., or some 100 times. The final material is mainly crystalline and has a little more than half the activity of the rice vitamin crystals of the Dutch workers. The experiment lends additional support to the view that we are dealing with a basic substance.

We have tried to apply this method to the direct $N/10$ HCl extracts of norite charcoal in preparations starting initially from baker's yeast, but find that it does not succeed unless there is a preliminary stage of concentration and removal of metals by H_2S . The concentration can readily be effected upon the open water-bath, at p_H 2.0 approximately, without damage to the activity. After the collection of the first phosphotungstic precipitate, the second treatment with phosphotungstate does not succeed well unless an alcohol stage is inserted, as in Exp. 5. Preparations worked up from a British Drug Houses concentrate have however been treated successfully with phosphotungstate direct, without preliminary removal of metals. We believe however that the H_2S stage is worth the time which it occupies¹. When applied to a $N/10$ HCl extract of the norite charcoal of initial activity approximately 0.5 mg., one may expect to reach 0.3 mg. or under at the first phosphotungstate stage, and 0.06–0.07 mg. in the precipitate at p_H 7.0–5.0 at the second stage. It is to be noted that the amounts of phosphotungstic acid required for this method of fractionation are comparatively small, thereby effecting economy in the use of a rather expensive reagent.

SUMMARY.

A method is described of preparing a torulin (yeast vitamin B_1) concentrate of activity 0.012 mg. by the use of fractional precipitation by phosphotungstic acid at varying p_H values.

Certain further experiments upon torulin are described.

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¹ See accompanying paper for details of the method of handling the British Drug Houses concentrate [Carter, Kinnersley and Peters, 1930].