LV. ANTINEURITIC YEAST CONCENTRATES.

IV. THE FURTHER PURIFICATION OF YEAST VITAMIN B_1 (CURATIVE)[1].

BY HENRY WULFF KINNERSLEY AND RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

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THIS work is being communicated now for two reasons. Confirmation of a previous statement is needed that preparations of activity under 0·1 mg. per day pigeon dose can be obtained from yeast. Moreover the details of preparing these more active concentrates are required in explanation of an accompanying paper [Peters, Kinnersley, Orr-Ewing and Reader, 1928].

It must be stated at the outset that we are not yet in a position to say finally whether the curative substance in yeast is the same as the rice vitamin B_1 (protective), described by Jansen and Donath [1927]. Preparations of activity 0·027 mg. per day have been obtained in small amounts, but this does not yet reach the activity reported by the Dutch workers for the protection of their pigeons, namely 0·01 mg. per day. Owing to the heavy losses in the final stages, larger scale work is necessary for the solution of the problem.

Previous work. The principal points in this research up to the present may be briefly reviewed at this juncture. By the use of norite charcoal, following the removal of precipitates with lead acetate, barium hydroxide and mercuric sulphate, the curative factor in baker's yeast can be obtained in good yield at an activity of 0·5–1·0 mg. per day dose [Peters, 1924, Kinnersley and Peters, 1927] free from the thermostable vitamin B_2 for rat growth [Chick and Roscoe, 1927]. A highly active preparation of 0·08 mg. per day did not restore the loss of weight in pigeons fed upon polished rice [Kinnersley and Peters, 1925]. The successful preparation of these concentrates depends upon realising that the earlier stages are adsorption stages, an exact parallel to the case of the enzymes. (Some reflection upon the concentration of constituents present soon shows that this must be so.) The active principle can be obtained in solution in absolute alcohol[2] even at reactions not far removed from neutral, when it has been freed from some of the associated substances. This settles many of the discrepant statements in the literature as to solubility. It is to be noted that Jansen and Donath [1927] have found their rice vitamin B_1 soluble in

[1] The term "Yeast vitamin B_1 (curative)" is substituted for "torulin."

[2] Unless otherwise mentioned, alcohol means ethyl alcohol and "absolute" alcohol ordinary absolute alcohol throughout this paper.

absolute alcohol. The active principle is stable to the action of nitrous acid, but is destroyed by alkali, in confirmation of the work of others. The tedium of earlier methods led to a return to a careful study of pre-charcoal steps [Kinnersley and Peters, 1927], and to the introduction of two main improvements. These were the rapid removal of yeast gum by baryta in the presence of small amounts of lead and an accurate definition of the conditions for adsorption upon charcoal, p_H 7·0 being found to be the optimum under our conditions. In this manner it is now easy to concentrate quickly to an activity of 0·3–0·5 mg. per day. In fact this is the quickest way of obtaining material of this concentration which is known to us at present. The new technique eliminates the trouble which has formed the main difficulty of this work, namely the variability of different batches of yeast under the same treatment. Up to the stage 0·15–0·3 mg. the results can be considered to be reproducible. Beyond the stage of 0·1 mg., we have not defined the technique with the same success.

Present work. The results obtained in the present paper extend those which we have previously reported to a region of greater activity. We have also included certain experiments upon the stability of the active principle made at intervals during the last three years.

It is shown that though it was possible to obtain a concentration of part of the activity from material of 0·9 to 0·09 mg. per day by the further use of norite charcoal, the technique was uncertain. Further treatment with norite removed no more active substance from solution (*A* Exp. 1). It is therefore concluded that adsorption upon norite is due in the earlier stages to the presence of a co-adsorbent.

Recourse was therefore made to the previous technique of alcohol fractionation, by the use of which in an improved form it was found possible to work up all the aqueous HCl extracts of the charcoal to an activity which varied from 0·15–0·3 mg. per day. In order to eliminate loss, great care must be exercised in the removal of the last traces of metals and of sulphate. Further the solution must not be too acid for treatment with H_2S (*B* Exp. 2). The technique is reproducible down to this stage. It was stated in an earlier communication that no success had attended our attempts to apply the technique of the Dutch workers to our fractions. These workers employed a successive treatment with silver nitrate and baryta, working to a definite p_H, followed by fractionation with phosphotungstic acid and then by H_2PtCl_6 in alcohol. This was completed by a separation with the mixed solvents, alcohol and acetone, producing their crystalline vitamin B_1 of pigeon activity 0·01 mg. By translating their results for bondols to pigeons, upon the assumption that 5 bondol doses equal one pigeon dose, the average activity of their preparations at the various stages is as follows: after silver and baryta 0·4 mg., after phosphotungstic acid 0·150 mg. and after H_2PtCl_6 in alcohol 0·04 mg. per day. The material obtained by the alcohol fractionation of the $N/10$ HCl extracts is therefore about as concentrated as that which they obtained at the phosphotungstic acid stage. In the case of the yeast preparations, following the technique of

others [Seidell, 1922] we have often obtained fractionation by the use of silver nitrate, and in fact our most active preparation of 0·08 mg. per day was obtained with ammoniacal silver. It has also been known to us for some time that in many cases the greater part of the activity passed into the fraction precipitated between p_H 5·0 and 7·0 and was thereby concentrated (*C* Exp. 3). This result was not however always obtained, and we were led to abandon the technique for yeast, in the belief that we were dealing even in this case with an adsorption. A recent repetition of the use of silver (*C* Exp. 4) with material of activity 0·17 mg. has not led us to return to its use. Some of the activity is precipitated but the amount is variable.

We have been more successful with the use of phosphotungstic acid and of H_2PtCl_6. Making use of the charcoal purification step described in their latest description of the isolation of rice vitamin B_1 [Jansen and Donath, 1927] and modifying their technique of extraction, an increase of activity of 2–3 times to 0·08 mg. can be obtained with phosphotungstic acid, but at the expense of losses (*C* Exp. 5 and Table I). It would seem that precipitation by phosphotungstic acid is a genuine property of the active principle, because it has never been possible to regenerate any activity in the filtrates. There seems to be little doubt that there is a tendency to inactivate the preparations for the pigeons at this stage, especially where acetone is employed (Table I). The use of H_2PtCl_6 has been disappointing (Table II). Though in point of fact the most active preparations have actually been obtained by its use, the losses have been high and the yields variable. There has always been activity left behind in the filtrate. Several preparations of activity 0·04–0·05 mg. per day have been obtained in small amounts, and two of 0·027 mg.

Some properties of yeast vitamin B_1.

There have been included in this paper certain experiments which we have carried out during the course of the last two years upon the solubility and stability of the vitamin. It must be emphasised that the properties of the preparations change during the process of concentration. In a recent textbook [Pryde, 1928], there occurs the following statement, "Although both vitamins are soluble in water, B_2 is less soluble than B_1 in strong alcohol, acetone and benzene." This statement, in common with others of a similar type, in our opinion gives a wrong impression of the facts, and is really erroneous without some qualification as to the activity of the material to which it is applied. In the early stages of a fractionation, there is abundant evidence from the work of Seidell [1926] and others, that vitamin B_1 is thrown out upon alcohol precipitates together with the factors necessary for weight maintenance in the pigeon. At this stage, however, increasing acidification of the yeast extracts makes the factor more soluble in alcohol. At a later stage, after the removal of certain substances which are relatively insoluble in alcohol, vitamin B_1 is soluble in alcohol of a high concentration. We know of no evidence which shows that yeast vitamin B_1 at activity of 1·0 mg. is soluble in solvents other

than alcohol or water. The solubility in benzene at earlier stages, claimed by McCollum and Simmonds [1918], has been shown by Williams and Waterman [1926] to have been due to the presence of traces of alcohol and water. In our experience yeast vitamin B_1 of activity 1·0–0·03 mg. per day has never been found to be soluble in chloroform (acid or alkaline solution), in carbon tetrachloride, ether, acetone, or ethyl acetate. When fractionated from mixtures of benzene and alcohol or ether and alcohol in which the water was reduced to a minimum, these preparations are largely thrown out of solution. Much here also depends upon the acidity.

Stability to alkali. Alkali treatment has not given clear cut results. The general experience shows that vitamin B_1 is destroyed by alkali. Sherman and Burton [1926] have shown that the vitamin B (growth-promoting) in canned tomatoes at 100° suffers the following destruction in 1 hour: 90–100 % at p_H 10·9, 66 % at p_H 9·2, and 32 % at p_H 7·9. Our experiments show that one hour's heating (Table III) with $N/2$ NaOH completely destroys the activity of a preparation of 0·059 mg. activity. The vitamin is therefore unstable to alkali in the heat at this stage of activity. 10 minutes' heating with $N/10$ NaOH at an activity of 3·0 mg. does not interfere with the activity; one hour reduces it. Other factors appear to operate in rendering the factor more unstable to alkali. This was seen best in the case of the combined effect of alkali and alcohol. In one of the early experiments with an inactive preparation, making alkaline in the cold to thymolphthalein in alcoholic solution for a short time was sufficient to inactivate the whole of a preparation; with a purer concentrate of 1·0 mg. per day activity, no effect was produced by alkali at room temperature in alcoholic solution. In one case treatment with alkali and hydrogen peroxide caused complete inactivation. In acid solution hydrogen peroxide has no effect. It may be concluded that alkali is destructive to the vitamin, but that other factors may operate to accelerate the action. In practice it is advisable to carry out any alkaline stage at a low temperature in the absence of alcohol. The vitamin preparations can be best stored in 90–99 % alcoholic solution made more acid than p_H 2·0. One such preparation (of activity 1·0 mg.) has been kept under these conditions in 99 % alcoholic solution for a period of three years at room temperature, with no appreciable alteration in activity. The purest preparations, however, have been found to lose activity at room temperature in a few weeks under these conditions.

Oxidising agents. It is generally believed that vitamin B_1 is not susceptible to the action of atmospheric oxygen and is not affected by exposure to ultraviolet light or to ozone. This indicates a marked stability to oxidising agents.

It has been mentioned above that hydrogen peroxide in one experiment (Exp. 25) in alkaline solution and at room temperature produced rapid destruction. In acid solution, warming with hydrogen peroxide produced no decrease in activity. This was not surprising considering that treatment with permanganate in acid solution to a point at which no further reduction of the permanganate took place in one case produced no decrease in the activity of the

solution (Exp. 30). We can therefore confirm the great stability to oxidising agents. It was also found that heating with 5 % nitric acid produced no effect upon the activity (Exp. 31).

Reducing agents. This matter has not been thoroughly investigated. H_2S is a standard reagent in the course of the purification. It was found that the combined action of hot H_2S and alcohol was not destructive (Exp. 32), nor was treatment with tin and zinc (Exp. 33). In each case however only one experiment has been performed.

Comparison with rice vitamin B_1 (protective).

The rice preparation has been shown to be curative to pigeons in doses of 0·2 mg. by Eijkman [1927]. Our solubilities agree with the Dutch results, and the activity reached by the treatment with phosphotungstic acid and H_2PtCl_6 in alcohol is reasonably near their activity at a similar stage. In a previous paper, it was stated that the substances giving a reddish reaction by the Koessler and Hanke [1919] modification of the Pauly test did not fractionate with the curative activity. This is true but it is not entirely conclusive, as there is always considerable disappearance of activity produced by the use of these reagents. It is possible therefore that inactivated vitamin might be intensifying the colour given in the filtrates from the fractions. All the purer fractions contain small amounts of substances which give the Pauly reaction. An attempt was made to estimate the amount of substance producing a reddish colour still present in the most active fractions. This showed that if yeast vitamin B_1 is pure at 0·01 mg. per day (the rice figure), then the Pauly reaction which it gives is about one-fifth of the intensity of that given by an equal weight of histidine. More than this cannot be stated with certainty. The most active preparations when treated with mercuric sulphate give a slight precipitate. The Dutch preparations are stated to give a massive one.

Chemical nature.

Owing to the obvious impurity of the active preparations, not much attempt has been made to analyse them. Some of the fractions have been found to contain from 13–20 % nitrogen (Table IV).

The fractions show qualitative tests for sulphur, by fusion with sodium and treatment with sodium nitroprusside. Benedict's reaction is negative. The Molisch reaction is positive; judging approximately, a reaction was produced in the purest preparation equal to not more than one-tenth of its weight of cane sugar. The Pauly tests have already been discussed. The vitamin is much more stable to acid than is reported for co-zymase [Myrbäck and Euler, 1924], but it is interesting that co-zymase also is stable to treatment with permanganate [Euler, Myrbäck and Nillson, 1928]. When examined in ultra-violet light behind glass which excludes the visible rays, the less pure concentrates show a fine blue fluorescence. The purer concentrates[1], however, show

[1] We are indebted to Mr B. T. Squires for performing this test for us.

much less fluorescence than the filtrates from which they are separated, so that it is improbable that the active principle fluoresces under these conditions.

The possible existence of two forms of yeast vitamin B_1.

A number of conflicting results which we have obtained in the course of our work would be cleared up by the hypothesis that yeast extracts contain two forms of curative substance. The first example of diverse behaviour was a fractionation with neutral lead acetate following the charcoal stage. In material prepared commercially by the earlier methods, we always commenced with lead acetate. One of the functions of this step as we realise now was to remove the last traces of sulphate. It was always noticed that a certain proportion of activity, about 12 %, remained with the lead acetate precipitate, even upon a reprecipitation (Table V). The active substance so precipitated did not fractionate in quite the same manner as the remainder of the material. As the work has proceeded we have become aware of other cases, which could receive a similar explanation. The case of silver nitrate and baryta has already been discussed. Further, there is evidence to show that the active material extracted by 50 % acid alcohol from the charcoal is different in some way from that extracted by $N/10$ HCl. It is suggested that we are dealing with two forms of vitamin B_1, either differently combined or perhaps oxidised or reduced. We give the facts because possibly other workers in this field have had a similar experience.

Experimental.

Fractionation.

In practically all cases the material used for the further fractionation has been that obtained by extraction of the charcoal with $N/10$ HCl. The pigeon tests have been made by the method described elsewhere [Kinnersley, Peters and Reader, 1928]. The solid weights in all cases refer to organic solids.

Norite charcoal.

By the use of small amounts of charcoal upon the $N/10$ acid extracts after evaporating to a small volume, it was possible in some cases to adsorb part of the active substance at p_H 7·0, and to concentrate thereby 5–10 times. At one time it was hoped that this method would prove of value in the concentration, but later it was abandoned as the yields were poor and the steps impossible to define properly. The following experiment is illustrative of many carried out.

Exp. 1. $N/10$ HCl charcoal extract was concentrated to 50 cc. and found to contain about 5000 doses of activity 0·9 mg. NaOH was then added cautiously until the p_H was approximately 7·0. There was slight darkening of the fluid. 5 g. of norite charcoal were added and the whole was filtered. The charcoal was washed with a little water and extracted with $N/10$ HCl. The

extract was treated with alcohol for preservation. Solids, 0·129 g.; tests, pigeon doses, 1450; activity, 0·09 mg.

In this case about 30 % of the active material was separated by a very simple charcoal step giving a concentration of some 10 times. Further treatment of the filtrates from the second charcoal concentrates yielded hardly any more adsorption. It has been found to be an almost universal rule that the use of purified norite charcoal at a p_H acid to 4·0 clears the solutions of substances other than the vitamin. This holds down to an activity of 0·1 mg. per day. We thought that the rule was unbroken, but have recently used norite to clear a decomposed platinum precipitate (following Jansen and Donath). In this case much of the activity was picked out upon the charcoal, from which it could be recovered by warming with 50 % acid alcohol. The relations to charcoal of the highly active fractions (0·07 mg.) cannot therefore be said to have been defined with certainty.

The use of alcohol.

It is not possible to perform an alcohol fractionation upon the $N/10$ HCl extracts as they stand, because such trials are met with severe losses of activity. These have been found to be due to the combined effect of small traces of metals and of sulphuric acid. The losses can be avoided by treating with barium chloride in slight excess during concentration. The clearing of a charcoal extract from metals and the further fractionation with alcohol are carried out as follows. The details of an actual experiment are given together with the figures for the tests during the concentration. This is now a standard technique, and can be relied upon to give reproducible results.

Exp. 2. $N/10$ HCl extract of charcoal. The total volume of the extracting solution amounted to 800 cc. $BaCl_2$ (10 %) was added until no further precipitate appeared and the whole was concentrated to about 200 cc. More $BaCl_2$ was then added which brought down a further precipitate of sulphate. NaOH was then added cautiously until a p_H of 3·5 to 4·0 was reached. (This was roughly judged by the use of drops upon a plate with the indicator bromophenol blue.) H_2S was then passed for 2 hours. The large precipitate was removed by filtration. The filtrate was concentrated over the naked flame in a beaker to 30–40 cc. During the concentration, the H_2S is driven off and also much of the unpleasant mercaptan-like substance formed. To the small concentrate of some 30 cc., 80 cc. of 97 % alcohol were added. The whole was then subjected to the alcohol fractionation shown on p. 426.

The technique described can be relied upon to give material of activity about 0·15–0·3 mg. without loss of many doses. In this case it is further shown that charcoal at an acidity acid to Congo red does not extract the yeast vitamin B_1 (curative).

N.B. The alcohol precipitates are washed when they are flocculent. When gummy they are dissolved with minimal amounts of water and reprecipitated with alcohol.

Further fractionation of material of activity 0·1–0·3 mg. per day. Silver fractionation.

Work upon material of less activity than the above during intervals of the last 2 years has often given results in which the activity has fractionated as described by the Dutch authors. The results may be summarised by quoting the following experiment, illustrative of many.

Alcohol fractionation. Some 4000–5000 doses of activity (1·0 mg.).

N/10 extract concentrated over flame and finally *in vacuo* to about 20 cc., 140 cc. 97 % alcohol added.

Centrifugate. Concentrated to 8 cc. and 110 cc. alcohol added — 80/85 % alcohol precipitate

Centrifugate. Concentrated to 5 cc. and 115 cc. alcohol added. The precipitates were reworked with alcohol — 90 % alcohol precipitate

Centrifugate. Test dose. **4000.** Concentrated to 3 or 4 cc. +115 cc. absolute alcohol — 94 % alcohol precipitate

Centrifugate. Concentrated to 1 cc. + 120 cc. absolute alcohol — 96 % alcohol precipitate

Centrifugate. Test dose **4800** — 99 % alcohol precipitate
Taken to dryness—NaOH added to p_H 5·0
To the volume of 6 cc., 170 cc. alcohol added — (Small precipitate removed)

Centrifugate. Concentrated to dryness. 2 cc. water added to dissolve and 120 cc. alcohol — 98 % alcohol precipitate

Centrifugate. 120 cc. (1·485 g. solids) — 99 % alcohol precipitate
Concentrated to a light gum, 0·5 cc. H_2O added and 150 cc absolute alcohol

Centrifugate. 150 cc. — 99 % alcohol precipitate, etc.
Removed the alcohol and treated the residue with a little water and enough HCl to make acid to Congo red. A small insoluble precipitate was removed by the centrifuge. The filtrate was treated with 5 g. charcoal and washed well. NaOH was then added to the filtrate from the charcoal to about p_H 6·0. The whole was concentrated to a light oil, and taken up in 150 cc. absolute alcohol

Final centrifugate 150 cc. (1·02) — 99 % alcohol precipitate, etc.

Tests $\left\{\begin{matrix}2250\\4800\end{matrix}\right\}$ **3500** average.

Activity 0·29 mg.

Exp. 3. 1926. Yeast concentrate of about 2·0 mg. activity, which had been treated previously with silver nitrite. About 480 doses taken and treated with silver nitrate solution until a drop gave a brown stain with baryta.

Precipitate
Test under 50 doses

Filtrate (acid to Congo red) treated with dilute ammonia to p_H 7·0

Precipitate p_H 7·0 extracted with HCl and alcohol
Pigeon test $\left\{\begin{matrix}198\\99\end{matrix}\right\}$ doses
Activity 0·3 mg.

Filtrate treated with HCl to remove silver
Pigeon test $\left\{\begin{matrix}150\\150\end{matrix}\right\}$ doses
Activity 2·0 mg.

About one-third of the activity was here concentrated in the fraction at p_H 7·0 with an increase of some 6 times.

Though we had often obtained the above result, there were many experiments in which it could not be obtained, and in which no precipitation took place. These were thought to be due to the presence of salts, which were accordingly removed by alcohol in some experiments, without however getting over the difficulty. This suggested that precipitation by silver was due to the presence of some associated substance. Since the publication of the Dutch work we have returned to the matter, and have subjected material of activity 0·17 mg. to a silver fractionation. Exp. 4 gives the details of the work.

Exp. 4. *N*/10 HCl extracts of the charcoal. 0·240 g. was taken, containing about 1400 doses in solution in absolute alcohol. The alcohol was removed and the residue taken up in 20 cc. water, 2 drops of 50 % sulphuric acid being added to make faintly acid to Congo red. Hot silver sulphate solution was then added until a drop removed gave a brown stain with baryta, then baryta until a reaction of p_H 4·0 was reached. The mixture was then centrifuged.

Centrifugate. Treated with baryta to p_H 7·4 and centrifuged — Precipitate (discarded)

Centrifugate treated with one drop of H_2SO_4. Evaporated to half its volume at p_H 5·0 and then brought to p_H 7·0 with baryta — Precipitate p_H 7·4 Extracted with two drops HCl and 5 cc. of cold water. Then with acid warm water. The combined extracts were treated with $BaCl_2$ to free from H_2SO_4 and brought to p_H 4·5 with NaOH

Centrifugate freed from silver with HCl and from barium with H_2SO_4. Alcohol added to a concentration of 50 %
Pigeon test 630 doses
Activity 0·4 mg.

Second precipitate extracted as first
Pigeon test 160 doses
Activity 0·2 mg.

Centrifugate. Evaporated *in vacuo* and taken up in alcohol
Pigeon test 350 doses
Activity 0·16 mg.

Precipitates discarded

It will be noticed that the doses recovered are about 1150 out of 1400, which is reasonable considering the variation of the bird test. It is clear that the main bulk of the vitamin remains behind and is not precipitated by silver-baryta under these conditions. The point to be noted is that the activity is no greater before than after the precipitation for the *N*/10 HCl extract. There is some evidence to show that silver-baryta fractionation is more selective in the case of the material extracted from the charcoal by 50 % acid alcohol.

Use of phosphotungstic acid.

As was briefly mentioned in a previous paper, a repetition of the first description given by Jansen and Donath [1926] for the use of phosphotungstic acid upon material of activity 0·4 mg. gave a recovery of no activity. In their further description [1927] it was found that a charcoal adsorption had been inserted before the phosphotungstic acid stage. By using the charcoal step, it was found that much of the activity could be recovered after precipitation with phosphotungstic acid, provided that the preparations were first treated with charcoal, and that extraction was performed by the

classical method of grinding the precipitates with solid baryta. Using this technique, however, the losses with the small amounts employed by us in these tests have still amounted to between 25 and 66 %. No activity remains in the filtrates, so that we feel satisfied that even at this stage of activity the whole of the active principle is precipitated by the phosphotungstic acid. The activity reached by the use of phosphotungstic acid appears to be about 0·07–0·08 mg. per day (about twice the activity obtained for rice by the Dutch workers). It is noticeable about these preparations that after treatment with phosphotungstic acid, the activity for *S. corallinus* (see accompanying communication, p. 434) always remains, whereas that for the pigeon is apt to be reduced. Some of the more salient features of the protocols are recorded below.

It may be mentioned that similar results have been obtained with a Merck preparation of phosphotungstic acid, as well as with a Kahlbaum preparation repurified by Winsterstein's method.

One experiment (Exp. 5) is given in detail to show the method which we have found most effective. The remaining experiments are recorded in Table I.

Exp. 5. Preparation containing originally approximately 4500 doses. Activity 0·33 mg. Removed alcohol, added 85 cc. H_2O and HCl to bring to p_H 1·0. Treated with 1 g. norite charcoal, allowed to stand for 1½ hours and filtered on a Büchner funnel, the precipitate being washed with $N/10$ HCl. To the filtrate 20 cc. 10 % phosphotungstic acid in 5 % H_2SO_4 were added. The whole was allowed to stand 48 hours, then centrifuged and the precipitate washed once with water. The precipitate was ground up 4 times with solid baryta, and the filtrate collected by centrifuging and filtering. Excess barium was removed by sulphuric acid, and the whole acidified with HCl to p_H 2·0. A trace of $BaCl_2$ was left in excess. The whole was concentrated *in vacuo* and extracted with 50 cc. absolute alcohol. The average doses were 2250, and the organic solids 0·300 g. making an activity of 0·13 mg.

Where reprecipitation of the phosphotungstates from acetone solution was carried out, the usual technique for such treatment was followed. By reprecipitation, we have obtained a slight increase in activity, but with considerably more loss.

Table I. *Phosphotungstic acid.*

	Doses			Activity			Methods	
Exp.	Taken	Recovered	% recovery	Initial mg.	Final mg.	Increase in activity	Charcoal	Acetone reprecipitation
6	2500	1064	42	0·6	0·2	×3	+	+
7	3000	1500	50	0·6	0·25	×2·4	Nil	+
8	700	350	50	0·34	?	?	+	+
9	1700	380	22	0·3	0·16	×2	+	+
10	1700	580	30	0·3	0·087	×3·5	+	+
5	3000	2250	75	0·33	0·13	×2·5	+	Nil
11	240	168	74	0·19	0·074	×2·5	+	Nil

NOTE. *Exps.* 9 and 10 were made with a brewer's yeast preparation; yield here especially bad: the remainder with baker's yeast. The yield is higher in Exps. 5 and 11 in which no acetone was allowed to touch the preparation, but the final activity is not quite so good as in Exps. 6 and 10. In the case of Exp. 10, all filtrates and precipitates obtained were freed from phosphotungstic acid and tested. They were completely inactive.

Chloroplatinic acid.

According to the Dutch workers, using H_2PtCl_6 at an activity for the pigeon of 0·150 mg., they can concentrate to an activity of 0·04 mg. (some 4 times), without much loss of the active material.

Table II summarises most of the results which we have obtained. The procedure has been in practically all cases identical with that employed by the above workers. H_2PtCl_6 has been added to the solutions in absolute alcohol. The precipitate so formed has been removed by the centrifuge, taken up in water and treated with H_2S. It has then been left to stand, and the precipitate of sulphide removed by filtration. It was found impossible to remove this completely in one stage. After heating upon the water-bath, fresh separations of the sulphide appeared which were removed. Finally the whole was taken down to dryness, in many cases finishing in the vacuum desiccator, and taken up in absolute alcohol. The concentration obtained will be seen to vary. By the double treatment with phosphotungstic acid followed by H_2PtCl_6, the activity was increased to 0·04 mg. In two cases 0·025 mg. was reached. We made one attempt to clear the platinum sulphide filtrate by the use of a small amount of norite (as described by the Dutch workers). To our surprise the charcoal (even at an acid p_H) extracted much of the activity, and by a re-extraction of the charcoal with 50 % acid alcohol highly active material of 0·026 mg. per day was obtained, in yield of about one-third of the final activity recovered by precipitation.

Table II. H_2PtCl_6 *in alcohol.*

	Doses				Activity			
		Recovered						
Exp.	Taken	Precipitate	Filtrate	% in precipitate	Initial mg.	Final mg.	Increase in activity	Remarks
12	260	75	60	29	0·15	0·2	Nil	No previous treatment with phosphotungstic acid
13	630	470	60	74	0·25	0·037	×7	Filtrate from which a precipitate had been removed with Ag and Ba (see Exp. 4)
14	1500	678	200	45	0·25	0·087	—	After phosphotungstic acid (see Exp. 7)
15	1064	240	210	22	0·20	0·10	×2	After phosphotungstic acid (see Exp. 6)
16	350	200	Nil	57	Unknown	0·27	?	After phosphotungstic acid treatment (see Exp. 8). Concentrations: Exps. 8 and 16 = 10 times
17	700	290	80	41	0·32	0·03?	Under 10 times	Only *one* experiment. No previous phosphotungstic acid treatment
18	960	275	—	28	0·12	0·06	×2	After phosphotungstic acid treatment (Exps. 9 and 10). Loss by using charcoal
19	2250	500	—	22	0·13	0·05	×2·6	After phosphotungstic acid treatment (Exp. 5). Loss by using charcoal

The charcoal used to clear 18 and 19 was extracted with warm 50 % acid alcohol; 500 doses were recovered of activity 0·026 mg. per day.

Oxidising agents.

Permanganate. Several experiments were carried out. In most of these some inactivation occurred, but in the one described below the condition was realised in which extensive permanganate oxidation took place without vitamin destruction.

Table III. *Action of alkali.*

(*a*) *Alkali alone.*

Exp.	No. doses taken	Activity mg.	Treatment	Doses recovered	Inactivation
20	10	0·059	Boiled 1 hour *N*/2 NaOH	Nil	Complete
21	11	3·0	Boiled 10 mins. *N*/10 NaOH in nitrogen	8	Slight
22	11	3·0	Boiled 10 mins. *N*/10 NaOH in air	7	Slight
23	11	3·0	Boiled 60 mins. *N*/10 NaOH with oxygen passing	2	Marked
24	11	1·0	Heating with ammonia (0·550)	Nil	Complete

(*b*) *Alkali and hydrogen peroxide.*

25	4	1·0	Treated with 1 drop of 25 % NaOH in 1 cc. H_2O and 4 drops of H_2O_2. Made acid with HCl after standing 15 mins. Heated to remove H_2O_2.	Nil	Complete

(*c*) *Alkali and alcohol at room temperature.*

26	—	—	Very impure concentrate made alkaline to thymolphthalein (10–15 mins.) in presence of alcohol and found to be completely inactivated.	Nil	Complete
27	4	1·0	Made alkaline in 60 % alcohol to thymolphthalein at room temperature for 15 mins.	Nil	Complete

(*d*) *Alkali and alcohol* 50 % *at* p_H 5·5–7·4.
Control 5 days. Activity 1·0 mg.

Exp. 28. *Standing at room temperature*

p_H	Stood	Days recovered
5·5	6 days	2·5
6·0	6 days	3+
6·5	18 hours	4−
6·5	8 days	1−
6·5	8 days	Nil
7·2	11 days	3−

Exp. 29. *Heating.*

p_H	Days recovered	
6·0	5+	Evaporated 15 mins. in long tube
6·5	Nil	Evaporated 5 mins. in open tube
6·5	4	Boiled in long tube*
7·2	Nil	Heated 5 mins. on water-bath
7·4	2	Heated 1·5 hours on water-bath

* To avoid contact with air.

We are indebted to Miss Reader for carrying out the alkali treatment in Exps. 20–23.

Exp. 30. *Action of permanganate.* 300 pigeon doses of activity 1·0 mg. were treated in acid solution with 60 drops of strong potassium permanganate solution until no further reduction occurred. After this treatment the bird test gave 240 doses still present, an amount equal to the original within the limits of the test.

The action of nitric acid. Seidell [1921] makes the statement that the activity of some of his preparations diminished upon standing in the desiccator. This he attributed to the presence of nitric acid. The following experiment shows that we have not found the presence of dilute nitric acid dangerous to the activity. This would be anticipated from Tsukiye's [1922] results with a rice polishing concentrate.

Exp. 31. 0·2 cc. which contained about 5 day doses. Activity 1·5 mg. Samples of 0·2 cc. were concentrated to remove alcohol. Two drops of normal

HCl were added and 2 cc. of 5 % HNO_3. The whole was then heated on the water-bath for 10 minutes. The tests gave 8 and 10 days. If anything there was therefore increase in activity.

Reducing agents.

Exp. 32. H_2S *in alcoholic solution.* This experiment was done in order to find out whether addition of alcohol to an extract which still contained sulphuretted hydrogen was dangerous.

0·2 cc. containing 5 day doses in about 3 cc. of 75 % alcohol was treated with H_2S. The H_2S and alcohol were then boiled off and the whole concentrated to a small bulk. Two birds were tested and both gave 4 days, indicating that the loss was less than 30 %.

Exp. 33. *Reduction by zinc and tin in* HCl *solution.* After acting upon a preparation of 1·0 mg. with tin, followed by zinc in HCl solution, bringing to the neutral point and centrifuging off the large precipitate, the pigeon test showed the presence of the major part of the curative activity.

It may be concluded from the above that the material is fairly resistant to the action of reducing agents.

Pauly reaction [Koessler and Hanke, 1919]. This was performed as described by Koessler and Hanke, in small test-tubes. To 2·5 cc. of the sodium carbonate solution was added 1 cc. of nitrite reagent, the whole mixed and, one minute afterwards, this was poured into 0·5 cc. of the unknown solution and thoroughly mixed. The unknowns were compared against known amounts of histidine hydrochloride solution. 0·005 mg. (5 γ) gave a visible pink reaction, with 10γ the reaction was definite. The tests upon active preparations of yeast vitamin B_1 always showed much yellow. By adding small amounts of the preparations to known amounts of histidine hydrochloride solution, it was possible to show that the vitamin concentrates did not inhibit the production of the reddish coloration. They however masked it. Traces of a substance giving a reddish reaction were present in all the active samples. The amount present was roughly estimated to be equivalent to an amount of histidine hydrochloride equal to 2γ (0·002 mg.) per pigeon dose. If therefore yeast vitamin B_1 is the same as rice vitamin B_1 with a pigeon dose of 0·01 mg., then the reddish reaction given by vitamin B_1 must be about one-fifth of the intensity of that given by an equal weight of histidine.

Table IV. *Nitrogen.*

Source of material	Activity mg.	Total N %	N per dose mg.
Baker's yeast *N*/10 HCl extract of charcoal	0·09	20	0·018
„ „ „ „	0·026	13·5	0·0035
Brewer's „ „ „	0·31	19·0	0·059
Baker's „ 50 % alcohol extract of charcoal	0·10	12·7	0·013

Table V. *Charcoal concentrates prepared by earlier methods, showing constant precipitation of portion of activity by neutral lead acetate.*

Exp.	Doses taken	Doses recovered lead precipitate	Doses recovered lead filtrate	Notes
Q 1	2800	350	2000	The lead precipitate was extracted with HCl and alcohol, the alcohol removed, and reprecipitated
Q 3	3300	510	2750	No reprecipitation. Material had been treated with nitrite
S 1	—	450	3600	
S 5	—	300	2400	

In each case there is a definite precipitation by the neutral lead acetate, amounting to some 12 %. All precipitates were of course very thoroughly washed in each case.

The preliminary treatment consisted in removing alcohol from the 50 % acid alcohol extract of the charcoal, adding water and then treating with neutral lead acetate.

Note upon the behaviour of brewer's yeast.

We have recently had the opportunity of testing the behaviour of extracts of brewer's yeast to our latest methods of purification. The yields which we have obtained by our standard technique have been within the limits of those obtained with baker's yeast, but upon the low side. At present only the $N/10$ HCl extracts of the charcoal have been investigated.

Summary.

1. Preparations of yeast vitamin B_1 (curative) have been separated from the concentrates, which have reached in two cases an activity of 0·027 mg. per day (pigeon dose).

2. The technique for this separation can be considered to be reproducible down to an activity of 0·1 mg. per day.

3. Phosphotungstic acid and H_2PtCl_6 can be used for concentrating yeast vitamin B_1.

4. The solubility of vitamin B_1 in ethyl alcohol varies with the activity of the preparation and with the hydrogen ion concentration of the solution treated with the alcohol.

5. Vitamin B_1 so far as has been investigated is not soluble in chloroform, carbon tetrachloride, ether, acetone or ethyl acetate. This confirms the statements of others.

6. One hour's heating with 0·5 N NaOH destroys the curative powers of the vitamin completely at an activity of 0·06 mg. 10 minutes' heating with 0·1 N NaOH does not inactivate it appreciably. The vitamin may become more unstable to alkali in the presence of other substances.

7. The stability to oxidising agents is confirmed. Certain reducing agents do not destroy the activity.

8. It is suggested as a hypothesis that two forms of yeast vitamin B_1 occur in an aqueous extract of yeast.

9. Brewer's yeast can be used for obtaining the charcoal concentrates. The yields have not been quite as high as the best obtained from baker's yeast.

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