XXXV. CRYSTALLINE PREPARATIONS OF VITAMIN B_1 FROM BAKER'S YEAST.

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Recently [1932, 1, 2] we have referred to the preparation of crystals of high vitamin B_1 activity, which by comparative tests are more potent than those of Jansen and Donath [1926] and of Windaus *et al.* [1931; 1932]. The work of the latter authors, together with that of Ohdake [1931; 1932] appeared during the progress of our work [see also Van Ween, 1931; 1932, 1, 2]. In our belief, the claim that vitamin B_1 itself has been prepared requires re-examination. We here describe our methods of obtaining these crystalline plates from "active" charcoals. The average activity in terms of day doses for our pigeons by the mouth[1] has varied from 1·6 to 2·8 γ in the most recent experiments, 1·6 γ representing the greatest activity reached.

In all, we have a yield of some 500 mg. of crystals from 2000 kg. of baker's yeast. A special feature of our method is the use of sodium phosphotungstate at two stages and of alcohol at one to concentrate from an activity of approximately 0·3 mg. to 0·01 mg. No benzoylation is needed, and H_2S can be avoided if desired down to an activity of 0·005–0·01 mg.

Use is made in the separation of the following properties of vitamin B_1 alone; precipitation by sodium phosphotungstate at p_H 4–5·5, solubility in alcohol at p_H 3·0 and insolubility of the gold salt in aqueous solution at p_H 2·5. The description applies to aqueous HCl extracts of the "active" charcoals. The cruder 50 % alcohol extracts are not so suitable and have not been much studied.

Preparation.

All volumes recorded are approximate and are apt to vary slightly in different preparations.

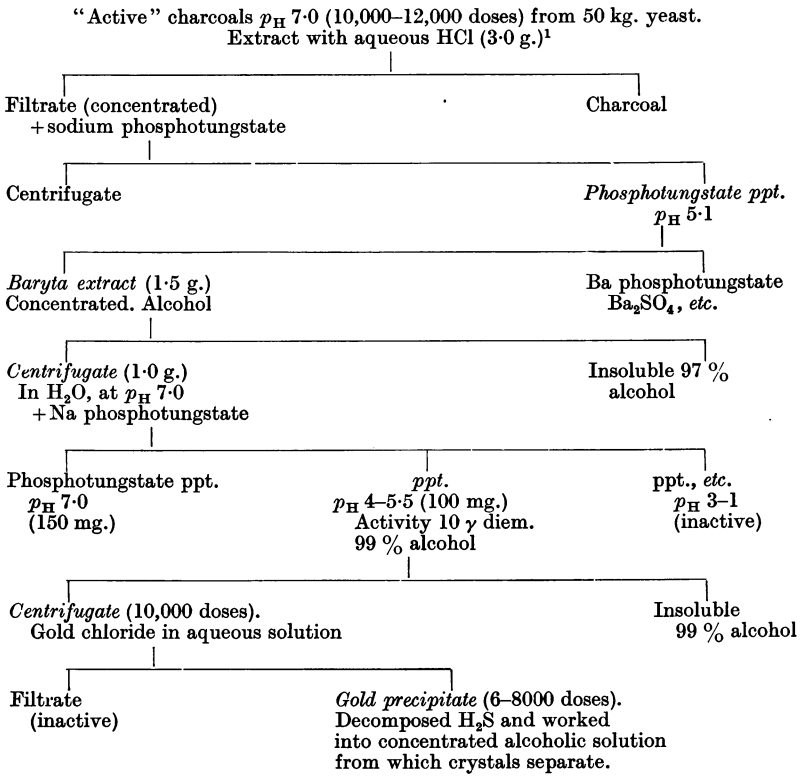
The stages in preparation of "active" charcoals are fully described in an accompanying paper [Kinnersley, O'Brien, Peters and Reader, 1933]. The "p_H 7·0" charcoals containing vitamin B_1 are extracted with $N/2$ HCl and $N/10$ HCl in succession. The combined and nearly colourless filtrates from 50·5 kg. (1 cwt.) of baker's yeast have a volume of about 1500 cc. After adding NaOH to p_H 2·0, they are concentrated on the water-bath or with naked flame to 750 cc., and cooled.

20 % NaOH is added cautiously to bring to p_H 5·0. Any precipitate which appears during this process is removed by centrifuge and washed with water. (The washings sometimes require addition of sodium sulphate to flocculate for filtration or centrifuging.) To the combined centrifugate and washings, a clear fluid of volume 750–1000 cc., are added 250 cc. of 10 % Merck's best phospho-

[1] One day dose is about one international vitamin B_1 unit.

Plan of preparation.

The reagents required are alcohol, phosphotungstic acid, gold chloride, baryta, and acid and alkali.



tungstic acid (previously brought to p_H 5·0 with NaOH)[2]. The phosphotungstate is added in slight excess and then 20 % H_2SO_4 and phosphotungstate until p_H 1–2·0 is reached. The precipitate so formed is allowed to stand overnight and settles to the bottom of the beaker. The supernatant fluid is often coloured. This has no effect upon the activity; practically all the vitamin B_1 present in the aqueous HCl extract (some 10,000–12,000 day doses) passes into the precipitate.

It is to be noted that the phosphotungstates thus precipitated are only a small proportion of the total bases precipitable by phosphotungstic acid in 5 % H_2SO_4, as will be readily seen by adding this reagent to the filtrate from the above.

The phosphotungstate precipitate p_H 1–5 is collected in two or four 100 cc. centrifuge-tubes, according to the quantity of precipitate. An amount of powdered baryta rather larger in volume than the precipitate is added to each tube, and after well grinding with a large glass rod in the shape of a pestle, the grinding is continued with the addition of 25 cc. H_2O, the mass centrifuged and the supernatant fluid filtered; the filtrate is kept acid to Congo red by additions of sulphuric acid during the filtration. The grindings with water are continued until the extracts are almost colourless (4–6 as a rule). Addition of H_2SO_4 is so arranged

[1] Organic solids. [2] 10 g. phosphotungstic acid need approximately 4·5 cc. 5*N* NaOH.

that the final p_H is just acid to Congo red. The combined filtrates (250 cc.) are then heated on the water-bath and centrifuged, the $BaSO_4$ being washed with $N/1000$ H_2SO_4.

One author [Guha, 1931] has criticised our use of solid baryta (rather than acetone) at this stage. In our opinion, the older technique amply repays the extra labour by the consistency and excellence of the yields [see Kinnersley and Peters, 1928].

Alcohol. The extract (250 cc.) is concentrated on the water-bath at p_H 3·0 with addition of 25 cc. 10 % $BaCl_2$ drop by drop to very slight excess, finally *in vacuo* to 1–2 cc. 100 cc. absolute alcohol are then added, and the whole is allowed to cool thoroughly at 0°; the inactive insoluble precipitate is then separated by the centrifuge.

Second phosphotungstate precipitation. Alcohol is removed *in vacuo* after addition of 10 cc. water, and the volume brought to 100 cc. with more H_2O. $5N$ NaOH is added to p_H 7·0[1] and then 10 cc. of pure 10 % sodium 1 : 24-phosphotungstate [see Barnes and Peters, 1932].

The p_H *7·0 precipitate* is allowed to settle for not more than 30 minutes, and extracted with $Ba(OH)_2$ as above. The filtrate from this p_H 7·0 precipitate is reprecipitated by bringing to p_H 7·0 with NaOH and adding 10 cc. Na phosphotungstate as before. The combined filtrates from the two p_H 7·0 precipitates, which contain 95 % or more of the vitamin B_1 present, volume 200–250 cc., are treated gradually with approximately 20 cc. $N/10$ H_2SO_4 from a burette until the p_H is about 4·0. Fresh precipitation of phosphotungstate commences at approximately p_H 5·5[2], after addition of 10 cc. H_2SO_4. More phosphotungstate is added, if necessary to maximum precipitation.

The precipitate at p_H *5·5–4·0* is allowed to settle for several hours (best overnight) and contains 95 % at least of the vitamin B_1 in the original HCl extract. Generally no loss is detectable. It is extracted by grinding with baryta, and freed from $BaSO_4$ as before; the filtrate is adjusted to p_H 3·0 with $N/10$ $Ba(OH)_2$ and is concentrated upon the water-bath with addition of $BaCl_2$ in slight excess; finally it is taken to dryness *in vacuo* and extracted with 100 cc. absolute alcohol. After standing 7 hours at 0°, the slight insoluble and inactive precipitate is removed by centrifuging.

Gold precipitation. Alcohol is removed *in vacuo* at 40° and water added to 15 cc., any insoluble matter being removed by centrifuge. At p_H 3·0 ± 0·2, 2·5 cc. 10 % aqueous gold chloride are added, and the whole is allowed to stand for 1 hour in the ice-chest. It is then centrifuged and decanted. The centrifugate can be discarded. The gold precipitate is suspended in 15 cc. $N/200$ HCl and treated with H_2S for 1–2 hours. The sulphide is removed by a small filter, and nitrogen passed to remove H_2S. About 5 cc. $N/10$ $Ba(OH)_2$ are then added to p_H 3·5, followed by one drop 10 % $BaCl_2$ and the whole is concentrated on the water-bath to 0·3–0·5 cc. 5 cc. absolute alcohol are then added, the mixture warmed and washed quantitatively into a small 7–8 cc. test-tube, cooled, and centrifuged. The supernatant fluid is decanted, and the insoluble substances heated with 3 drops H_2O and 2 cc. absolute alcohol and cooled.

For maintenance of full activity, it is important to keep barium chloride in slight excess to minimise chances of excess of H_2SO_4, which is very dangerous to the activity. After this stage, the vitamin itself becomes somewhat insoluble in absolute alcohol [*cf.* Van Ween, 1932, 1, 2]. Barium chloride can be separated because of its greater insolubility. It is impossible to crystallise without

[1] Important not to make more alkaline.

[2] p_H 5 judged by plate, using the smallest possible drops.

the use of gold chloride. It will be noted that the description for the gold stage is quite different from that of Windaus *et al.* [1932]. Our preparation must evidently be purer before this treatment is started. The gold salts can be recrystallised, but it is safer to reserve such treatments for the hydrochloride. It seems impossible to avoid some loss of activity at the gold stage; the reason for this is not yet clear. We always employ glass-distilled water and redistilled alcohol.

Preparation of crystals. The combined extracts containing 70–80 % of the activity are added in portions of about 1 cc. at a time to another tube and concentrated after each addition until the final volume is about 2 cc. By this means and by addition of more absolute alcohol one or more small inactive precipitates may be obtained. The bulk of the water is removed in this way, but since there is a very small margin between removal of the last trace of $BaCl_2$ and appearance of active crystals this step requires practice. After removal of the last trace of $BaCl_2$, the crystals should appear as flat plates from a warm 98 % alcohol solution in half an hour, but it is advisable to leave in the ice-chest overnight. Decant, wash and centrifuge. 15–20 mg. of crystals (hydrochloride) are obtained of activity about $2{\cdot}3\gamma$ containing 6–8000 day doses of vitamin B_1. They can be recrystallised by adding one drop of H_2O, in which 15–20 mg. crystals will dissolve and making up to 2 cc. with absolute alcohol made acid with conc. HCl to p_H 3·0 (theoretical). The object of the acid is to inhibit hydrolysis and inactivation of the vitamin. When dried, the plates are apt to shrivel.

From more dilute solutions in absolute alcohol, crystallisation can often be induced by a layer of light petroleum; ethyl ether will not do. In this case, needles appear, which become plates upon recrystallisation.

The above technique has been applied to a large number of 50 kg. batches with uniform success and also to aqueous HCl extracts of "active" charcoals worked up by the older technique involving a mercuric sulphate stage, instead of the acid charcoal. The yield in this case has varied from 15 to 17 mg. per 50 kg. It is of course both possible and advantageous to work batches of 100–200 kg. through certain of these stages.

Stability and test solutions.

So far as is known, the crystals retain their stability in a desiccator over calcium chloride at room temperature for 2–3 months.

For purposes of test, solutions should be made up in absolute alcohol containing HCl (0·1 cc. of conc. HCl in 100 cc.), or in $N/1000$ HCl containing 20 % alcohol by volume for preservation. Such solutions keep well in cold store. Solutions tested at stages in fractionation when excess of barium is present, must be treated with H_2SO_4 in suitable strength to remove barium before dosing.

Estimation of activity.

The curative pigeon test has been used throughout, doses being given by mouth and reckoned as day doses for cure and protection. Occasional tests by injection indicate that a given sample of vitamin B_1 is about 30 % more active by injection than by mouth. In favour of the oral route, we have the better approach to physiological normality and the lessened risk of liberating tissue vitamin by trauma. For soluble preparations the standard deviation (σ) is 44 as a maximum, reckoning the mean as 100. In a recent test upon 10 birds for instance, the average day dose was 5·4 and the standard deviation[1] (σ) 2·36. From this can be calculated the standard error of the mean[2] (ϵ) with different

[1] $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ d = difference from mean; n = number of observations.

[2] $\epsilon = \frac{\sigma}{\sqrt{n}}$.

numbers of animals upon test; in the Tables are given the values of the mean and of 2ϵ; 21/22 of such tests should fall within the range $\pm 2\epsilon$. This is probably a severe estimate of the probable accuracy. We have noticed that some preparations give results with much smaller standard deviation, but have not enough evidence to decide whether this is significant. With solids as source of vitamin B_1, larger variations are often found, probably related to the removal of vitamin from the solid in the gut. In the latter case the relation between time of cure and dose is much obscured. This has led to a revision of the figure given for the day dose [Peters *et al.*, 1931] in terms of the International B_1 Unit. In tests not yet complete, a revised figure indicates one pigeon day dose (under our conditions) to be approximately equivalent to 1 International Unit (instead of 1·5). Pending a settlement, we have adhered here to the day dose unit.

It will be noticed that many of the tests in Table I give an average cure of 5–6 days, and are therefore tested at points of equal biological effect.

Table I. *Vitamin B_1 potency of crystals (hydrochloride).*

Prep. no.	Melting-point (4)	Dose γ	Average day cure	No. of birds	Day dose γ	2ϵ (1)	Remarks
25.11	230°	17·0	6·5	4	2·6	±1·14	"Acid" charcoal method
28.4		12·0	5·2	6	2·3	±0·78	,, ,,
53.A.4		13·8	5·5	10	2·5	±0·70	,, ,,
56.20		14·0	5·0	5	2·8	±1·09	,, ,,
57.7	222°	11·5	5·0	4	2·3	±1·01	,, ,,
43.17		10·0	6·8	6	1·5	±0·51	,, ,,
63.A.9	221–3°	14·0	6·1	4	2·3	±1·01	Mercuric sulphate instead of "acid" charcoal
64.19	225–30°	14·6	6·6	4	2·2	±0·97	Mercuric sulphate instead of "acid" charcoal
42.A	221–50°	9·0	4·5	7	2·0	±0·66	Refractionated with Na phosphotungstate
61.A.15	221°	9·0	5·6	9	1·6	±0·46	Refractionated with Na phosphotungstate
G. (2) (Windaus *et al.*)		14·2	3·3	10	4·31	±1·21	For the 18 tests
		23–28	5·6	8	4·58	±1·42	=4·45 ±0·93.
J. (3) (Jansen)		22·0	3·7	4	6·0	±2·8	

(1) 21/22 tests would fall within 2ϵ.
(2) Preparation kindly supplied by Dr Tschesche (method of Windaus *et al.*).
(3) Preparation kindly supplied by Dr Jansen (method of Jansen and Donath).
(4) M.P. quite sharp in case of 57.7, 61.A.15, 63.A.9. Remainder indefinite.

Comparison of activities.

Table I gives the estimated potency of various samples of crystals. An average of all our tests, which were oral, gives $2{\cdot}17\gamma \pm 0{\cdot}11$. It is certainly a striking circumstance that our values should be so near those for tests "by injection" given by Windaus *et al.* [1932] as $2{\cdot}4\gamma$, and by Ohdake [1932] as of "order of $2{\cdot}5\gamma$." Nevertheless we consider this to be coincidence. Firstly, oral tests usually give a lower response than tests by injection. Our results by injection would give an average under 2γ. Secondly, direct comparison by simultaneous tests upon our pigeons indicated a much higher potency for 53.A.4 than for G. As calculated above, we have assumed a maximum standard deviation of 44 % for G; actually these tests gave a smaller standard deviation by direct calculation. The difference between 53.A.4 and G is significant. A few tests upon

a new preparation made by Jansen gave a higher potency than previously [Jansen *et al.*, 1930], but slightly lower than G. Thirdly, in more than one case we have ourselves reached crystals of greater potency than 2γ. 61.A.15 for instance gives an average of $1{\cdot}6\gamma \pm 0{\cdot}46$, the difference between this value and that of 53.A.4 is again significant. Hence, even if we decided to set aside the cogent evidence from direct comparison, we still have to account for the greater activity of 61.A.15. This leads to the conclusion that crystals more potent than the alleged vitamin B_1 can be obtained by the charcoal-sodium phosphotungstate technique.

Properties of crystals.

Precipitation and colour reactions. Table II shows that these follow those described by others. It is interesting to note that, in confirmation of earlier assertions by two of us, there is now general agreement that the present vitamin B_1 preparations do not give precipitates with mercuric sulphate in acid solution. In Table II the limits for the reaction have been added, where these are known.

Table II.

Precipitating substance	Van Ween	Ohdake	Windaus *et al.*	Present crystals	Remarks
Phosphotungstic acid	1/10,000	+	+	+	
Na phosphotungstate p_H 5·0	.	.	.	+	1/100,000–1/1,000,000
$AgNO_3 + Ba(OH)_2$	.	+	+	.	
$HgCl_2$ (aqueous)	.	+	+	+	In presence of Na acetate 1/10,000. Redissolves immediately in absence
$HgSO_4$ (acid)	−	−	−	−	
Picric acid (aqueous)	.	−	−	−	
Picrolonic acid	.	+	+	+	
Gold chloride (aqueous)	1/10.000	+	+	+	
Platinic chloride (alcoholic)	+	+	+	+	
Flavianic acid	.	−	−	−	
Rufianic acid	.	.	+	.	
Iodine KI	.	+	.	±	Rather indefinite ppt.
Dragendorff	.	+	.	+	
			Colour reactions.		
Pauly (diazo)	Yellow-brown	+[1]	Nil	Yellow	Technique of Koessler and Hanke [1919]. 10γ used.
Hunter (ergothioneine)	.	.	.	−	22γ used
Ninhydrin	.	−	.	−	In p_H 7·0 phosphate buffer; 20γ alanine good reaction; 44γ vitamin B_1 yellowish-green only. 0·05 cc. 0·1 % ninhydrin, 0·6 cc. solution
Bial (pentose)	.	.	.	−	132γ in 0·1 cc. + 0·2 cc. Bial's reagent. 10γ arabinose good reaction under same conditions
Sakaguchi (arginine)	.	−	−	±	44γ in 0·1 cc. + 1·0 cc. $N/10$ NaOH + 0·05 cc. 0·15 % naphthol, 0·05 cc. 2 % hypochlorite; faint reaction less than $2{\cdot}5\gamma$ arginine nitrate
Sulphur−SH (direct)	.	−	−	−	
Sulphur−SH (after heating with alkali)	.	+	+	+	10γ used

[1] Differed from histidine.

Sulphur reactions. The nitroprusside S reaction is given by addition of ammonium sulphate, ammonia and sodium nitroprusside after some 20 minutes' heating on the water-bath with 20 % alkali, or after fusion with sodium, but not well by rapid boiling with NaOH. In this, it differs from cystine. Some preparations showed the reaction much less intensely than others. Preliminary heating with ferric chloride did not restore the Pauly reaction (difference from ergothioneine). It may be noted that by reduction of scale, 0·1 cc. of solution *plus* one drop of 40 % NaOH, the nitroprusside reaction can be detected in 10γ

or less of vitamin B_1 crystals and about 2γ of cystine. After heating and cooling, a few crystals of ammonium sulphate are added and then dilute nitroprusside.

Sakaguchi reaction. Some preparations gave negative results; where present it is probably due to traces of impurity carried over from earlier stages, which give the reaction intensely.

Properties of crystals. When precipitated from dilute alcoholic solutions with light petroleum, the crystals are needle-shaped. From acid alcohol however, by the technique here given, the crystals always form beautiful plates, strikingly like the photographs of Ohdake [1932], and give an impression of purity. With the exception of Ohdake's Pauly reaction, their behaviour to precipitants and general reactions appears similar to those of others. Yet we have evidence that they are not pure. Their biological potency has been shown to vary. The melting-point of the recrystallised dihydrochloride never reaches 250°, that given by others. We have obtained crystals with quite sharp melting-points of 221°. The M.P. of recrystallised picrolonates has varied, values of 165°, 221° and 229° having been observed. In face of the greater activity, this cannot mean that we merely have impure vitamin B_1. It might be due to slight differences in hydrochloride composition, as in the well-known case of histidine; but there is other evidence of difference. Preparation G is stated to have an absorption band at 260 $m\mu$ [Windaus *et al.*, 1932]. In unpublished work in 1927 (upon impure specimens) Dr C. W. Carter in this laboratory found absorption at 247·5 $m\mu$ which did not change upon heating with alkali, although all vitamin B_1 activity had disappeared. Recently Mr Philpot with our crystalline preparations has found maximum absorption at 248/9 $m\mu$. Therefore the absorption spectrum of our crystals is different from that described by others [*cf.* Heyroth and Loufbarrow, 1932; Guha, 1931].

Further, preliminary experiments show that some crystals of vitamin B_1 are still contaminated with vitamin B_4[1] which is stated to contain no S [*cf.* Barnes, O'Brien and Reader, 1932]. Hence vitamin B_1 itself must be part only of our preparations, *i.e.* potent in doses of 1γ or under. This leads again to the unfortunate conclusion that neither ourselves nor others have yet reached pure vitamin B_1. Elaborate analyses are of little value, until consistency of biological and chemical composition has been proved. For comparison we quote without comment and with no emphasis the following analyses by Dr Ing Schoeller made upon various samples of crystals dried *in vacuo* at 80°. Weight lost at 80° approximately 3·0 %. There was a residue of $1{\cdot}0 \pm 0{\cdot}5$ % (compare Van Ween [1932, 2, p. 127] as to difficulty of freeing from traces of residue).

Table III.

	C	H	N	S	Cl
Present crystals	42·6	5·53	14·5	9·45	20·92
	41·8	5·82	15·2	9·36	—
	—	—	15·1	9·81	—
Average	42·2	5·7	14·9	9·5	—
Ohdake (Average)	40·87	5·53	15·94	9·26	20·3
Van Ween (Average)	40·31	6·32	15·71	8·71	19·77
Windaus *et al.*	40·52	5·68	14·96	8·92	19·78

It will be seen that there is a general agreement, but variations from other figures large enough to be of consequence. There is a distinct tendency for higher carbon and sulphur values.

[1] Personal communication by Mrs Walker.

By several lines of evidence, the conclusion is reached that the crystals made by the methods described in this paper are more potent than and different from those of others. Three possibilities remain, and are now receiving attention.

(1) All crystals hitherto made are a mixture of active and inactive vitamin B_1. This is hard to reconcile with the conflicting position of the ultra-violet bands, presuming these to represent part of the vitamin molecule.

(2) The crystals contain vitamin B_1 as impurity. In this case the activity of the true vitamin could be at least $0{\cdot}1\gamma$ in our terms.

(3) More than one compound can function as vitamin B_1. In this event, we might have either a base able to exist free or in combination with other substance, or different bases with identical biological functions.

SUMMARY.

Methods of making crystalline preparations of high vitamin B_1 activity are described in detail, based upon the principle of separation by sodium phosphotungstate at p_H 4·0–5·0 followed by gold chloride and crystallisation from alcohol. Comparatively few stages are needed to concentrate from the activated charcoal stage. Approximately 500 mg. have been made from 2000 kg. baker's yeast. The crystals vary in activity, the highest potency being $1{\cdot}6\gamma \pm 0{\cdot}4$ for a day dose, orally administered to pigeons. This is greater than the activity of other crystalline preparations previously described.

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