XXXIV. LARGE SCALE PREPARATIONS OF VITAMIN B_1 AND VITAMIN B_4 CONCENTRATES.

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In this communication, we are recording the details of a large scale process for preparing vitamin B_1 and B_4 concentrates, which has stood the test of over a year's working in this laboratory. It is based upon the original Kinnersley and Peters procedure, and has been briefly described [Peters *et al.*, 1931].

We shall describe the technique upon the basis of 100 kg. (2 cwt.)[1] of yeast, but it will be realised that economy in time results from running several processes together. We ourselves have worked up to the charcoal stage in the laboratory as much as 300 kg. (6 cwt.) of yeast in a week. 200 kg. is however more convenient with the staff and apparatus available. Limitation of scale depends more upon apparatus than increase of staff.

Baker's yeast (supplied by D.C.L. Company) has been used throughout and can be relied upon to give standard results. It is kept at room temperature for 1–2 days before use in the 7 lb. bags as supplied. Examination of our schedule of time and labour shows that our methods compare favourably with those of others, and we can see no inherent objection, apart from the expense, to working upon the 1000 kg. scale[2].

It may be stated that we have tried several variations on the procedures described. In our experience rather trifling changes, to which no theoretical objection can be seen, usually diminish the yield, and should not be embarked upon lightly. This is probably due to the complexity of the mixtures from which adsorption takes place.

Reagents required (100 kg. scale).

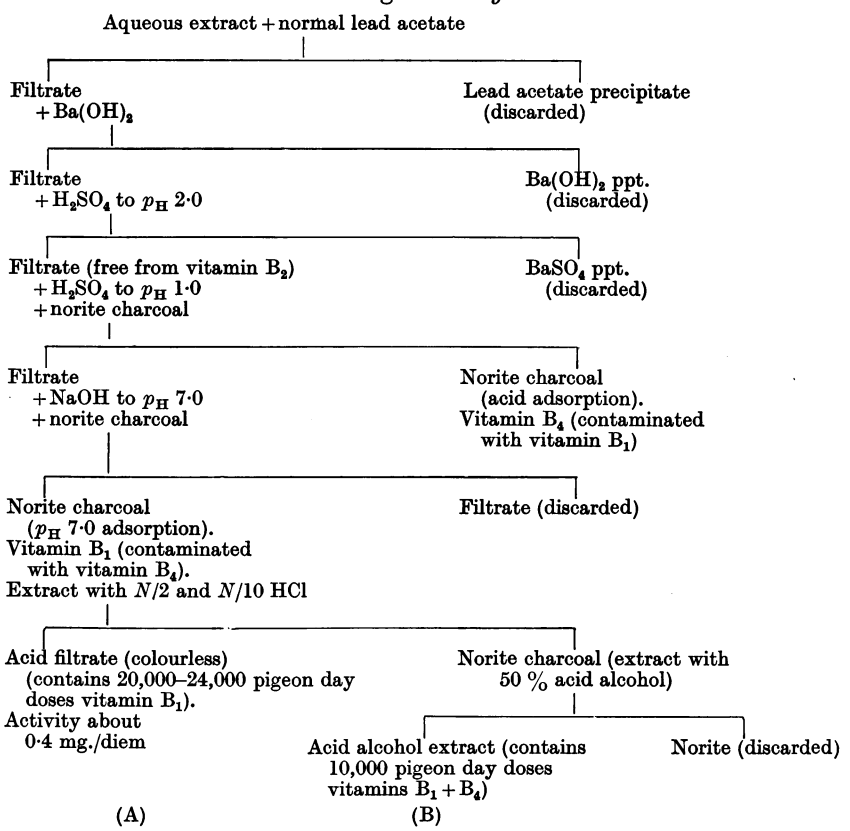
Lead acetate (normal)	2250 g.
$Ba(OH)_2, 8H_2O$	9–10 kg.
NaOH	1500 g.
H_2SO_4 (conc.)	1500–2000 cc.
Norite charcoal	2000 g.
HCl (conc.)	350–500 cc.
Alcohol	6–8 litres.

The yields are expressed in pigeon day doses. One such dose is approximately equivalent to 1 unit of vitamin B_1. (1 pigeon dose $\equiv$ 10 mg. Jansen acid clay.) Previously, we have stated 1 pigeon dose was equivalent to 15 mg. Jansen clay. Further work with doses of 50 mg. instead of 100 mg. has led to the revised figure.

[1] 2 cwt. = 101·5 kg. Round figures used in text for convenience.

[2] The general speed can be judged from the fact that vitamin B_1 crystals have been reached in 14 days from the initial boiling.

Plan. 100 kg. *baker's yeast.*



(A) Can be used to obtain crystalline preparations of vitamin B_1 as described in a separate communication (Kinnersley *et al.*, 1932; 1933).

(B) Is available as a basal source of vitamins B_1 and B_4 for various rat and pigeon tests or can be used for clinical purposes.

Preparation of aqueous yeast extract. 20 litres of tap-water are heated to boiling in a steam-heated pan (steam pressure 10 lb.). During the heating, 42 lb. (19 kg.) of yeast are crumbled by hand and stirred gradually into the water as it boils. The whole is made cool by the addition of the yeast and is raised to the boil again. It is then transferred by enamel pail to a sedimentation centrifuge. The one used for this purpose was supplied by Messrs Broadbent and has a rustless steel basket of diameter 20–22 in. Centrifuging for 15 minutes is usually sufficient, after which the clear yellow layer is removed by the skimmer: final temperature 50–60°. The moisture of the yeast residue varies somewhat at different times of the year. The volume of extract obtained from 100 kg. of yeast is 124–7 litres. In order to economise time, a second batch of water is heated during the centrifuging, which is then ready immediately for the second batch of 19 kg. of yeast. In all the extraction of the 100 kg. is complete in 5 batches, and occupies the time of one man for 7–8 hours. The extracts are allowed to cool to a temperature of 18° or under.

The extract (p_H 6·5–6·8) so obtained is allowed to stand overnight after addition of 16 cc. 25 % normal lead acetate per litre; making the p_H 5·0–6·0.

Notes. (1) Acetic acid (1 %) has been often recommended for the extraction, and is considered ideal upon theoretical grounds, in order to ensure coagulation of proteins, *etc.* We have found no improvement from its use.

(2) The "standing" period overnight seems to be essential; earlier attempts to dispense with it gave poor yields of vitamin B_1. It is not clear what happens, but some change (possibly autolytic) must liberate the vitamins in suitable form for adsorption.

(3) Originally two extracts of the yeast were made, but we now dispense with the second extraction. The slight gain in yield does not compensate for the time lost in making it.

Lead acetate precipitation. After standing for 17–20 hours, the yeast extract is treated with 25 % normal lead acetate until maximum precipitation has occurred. The tendency for fresh additions to produce a faint cloud marks the completion of this process; about 2000–2500 cc. are needed for the aqueous extract from 100 kg. The extracts are then run through a Sharples supercentrifuge (size No. 5 long boss bowl, revs. 16,000). The barrel requires one cleaning during this process, each 50 kg. equivalent running through in 10–15 minutes; nitrogen is passed through the centrifuge and solutions to minimise oxidation. As soon as the filtrate from the first 50 kg. is ready, it is treated with baryta, so that the processes run concurrently. The weight of wet lead acetate precipitate so obtained is approximately 5000–6000 g. It may be dried and stored if required. The lead acetate stage occupies 1–1½ hours.

Note. In the absence of a Sharples centrifuge, the lead precipitate is best filtered upon funnels with folded paper, and the filtration occupies some 24 hours.

Baryta precipitation. During the centrifuging or filtering of the lead precipitate, 1500 cc. beakers (or flasks) are set up each containing 200–300 cc. H_2O. Solid baryta is added until the mixture occupies about 2/3 the volume of the flask. The mixture is heated vigorously to boiling, and more baryta added until a thick suspension is obtained consisting of about 1200 g. baryta in 300 cc. H_2O.

To each 32 litres of filtrate from lead precipitate, 1000–1500 cc. of hot "baryta" are added with vigorous stirring; the final temperature should not exceed 25°. The vessels are allowed to stand for 3–5 minutes. If flocculation does not occur immediately, a few cc. of lead acetate solution may be added and the solution agitated. The mixture is filtered at once and should filter rapidly. A number of 6-inch funnels has been lately used complete with folded filter-papers. The funnels discharge into two rectangular gutters which collect the filtrate into a receptacle. As soon as possible, sulphuric acid is added to the filtrate until the reaction is acid to Congo red (or purple to thymol blue; about p_H 1·0). The latter acidity induces the more rapid sedimentation of the barium sulphate, and inhibits to some extent the frothing caused by the centrifuging. If the filtrate from baryta is not quickly acidified, considerable darkening in colour may take place; there is also risk of destruction of the vitamins. The lead acetate filtrate from 100 kg. can be taken through the baryta stage with the funnels in 4 hours by one worker.

Notes. Practice may be required to adjust the flocculation stage. If successful the filtration should be very rapid indeed (almost like water). Fractious extracts may be cured by a small addition (1–2 cc./litre) of lead acetate before addition of baryta.

Removal of $BaSO_4$. This can be allowed to settle, or is preferably removed by the Sharples centrifuge. The latter process only occupies 2 hours. It is usually run in 3 batches of 40–60 litres each.

Charcoal adsorptions.

Adjust p_H of the filtrate from $BaSO_4$ to 1·0 (slight purple to thymol blue).

Acid charcoal adsorption. To each batch of 32 litres[1], add 180 g. dry norite charcoal, purified previously by boiling with conc. HCl and washing till free from acid[2]. Allow to stand for 20 minutes, stirring frequently, before commencing filtration. Filter through 10 Büchner funnels (6 inch). Add the suspension continuously, collecting the filtrate as it appears and never letting the charcoal on the funnels become completely dry. The time of filtration varies, but usually one worker can filter the extract from 100 kg. (120 litres) in 2 hours through one set of filter-papers. The charcoals (700 g.) are washed with $N/10$ H_2SO_4, 200 cc. per Büchner funnel. The filter-papers and charcoals are transferred to a large beaker (4 litre) and treated with 800 cc. 50 % alcohol (by volume) and enough conc. HCl to bring to p_H 1·0. At this stage they may be left overnight. It is somewhat easier to handle the charcoal, if the filter-papers are extracted separately, but this is not essential.

Note. This step was introduced by one of us (V.R.) as a modification of the original shortened process of Kinnersley and Peters. The extra acidity is found to cause adsorption of much of the vitamin B_4 in a form suitable for subsequent separation from vitamin B_1 [see Barnes, O'Brien and Reader, 1932].

Charcoal adsorption at p_H *7·0.* To the charcoal filtrates from "acid charcoal" add 20 % NaOH, until the p_H is 7·0 ± 0·04. The amount varies to some extent, but usually lies between 1000 and 1200 cc./32 litres. It is important not to make much more alkaline than p_H 7·0. Even a temporary sojourn at p_H 7·6–8·0 is apt to effect some change and it is advisable to approach p_H 7·0 with caution. A precipitate sometimes appears, which is not removed separately. To each batch of 32 litres at p_H 7·0, add 320 g. norite charcoal, and stir well. Adsorption is rapid; we allow to stand 10 minutes, and then commence to filter through the 10 Büchner funnels, as before. The filtration is now more rapid and the filtrate practically colourless. The charcoal on the funnels is thoroughly washed with distilled water 3–4 times, some 1500 cc. to each Büchner funnel.

The 1200 g. charcoal (p_H 7·0) (with or without the filter-papers) is placed in a large vessel (6 litre), and 2 litres of cold $N/2$ HCl added. After thoroughly stirring, the p_H is tested with Congo red. If not acid, conc. HCl is added until the mixture turns Congo red blue. It may now be left overnight.

The stages from lead acetate to this point for a 100 kg. batch can be carried through in one working day by two workers, if care is taken to organise the work. As here arranged, with use of the supercentrifuge, parts of the following stages can be run concurrently, (1) lead acetate removal and filtration of baryta flocculate, (2) the latter and removal of $BaSO_4$, (3) removal of $BaSO_4$ and acid charcoal filtration.

Note. In the initial work care was taken to use charcoal which had been purified by extraction with conc. HCl, and subsequent washing until free from acid. Owing to the considerable labour involved, we have recently substituted commercial norite. The metals (mainly Zn and Fe) so introduced can be removed later if necessary (from the charcoal extracts) with much less labour than is involved in purifying the charcoal, and there has seemed to be no substantial loss of activity for vitamin B_1, in the 400 kg. tried. The fate of the vitamin B_4 has not yet been tested.

[1] The volume suitable for our receptacles.

[2] See note p. 230.

Elution of vitamin from charcoals.

"Acid" charcoal (vitamin B_4). The acid alcohol containing the charcoal is heated on the water-bath for 30 minutes, the temperature gradually rising to 70°. The mixture is then filtered through a Büchner funnel. The charcoal is re-extracted 3 times with acid alcohol. The filtrate is transferred to a 2 litre distilling flask and alcohol removed *in vacuo* at 45°. (The alcohol in the distillate is 75 % approximately and can be used for subsequent extractions.) To the final volume of 3 litres, NaOH is added to p_H 3·0 and the solution kept in cold store. The extract should contain about 18,000 day doses of vitamin B_4 together with 3000 day doses of vitamin B_1.

"p_H 7·0" charcoal (vitamin B_1). The 1200 g. charcoal, obtained at p_H 7·0, are extracted with $N/2$ HCl in four 1500 cc. beakers. We have found it best to use the steam-heated boiler as a water-bath for this purpose and to extract at 80° for 10–15 minutes, with constant stirring. The contents are sucked off on Büchner funnels; 3 suffice for 1200 g. Two further extracts are made with 2000 cc. of $N/10$ HCl. The combined filtrates (volume 6 litres approx.) are brought to p_H 3·0 ± 0·2 (bromophenol blue) and concentrated to 1500 cc. over a naked flame or on the water-bath in basins. 10 % barium chloride should be added before and during the process of concentration to remove sulphate as it appears. The extract contains 20,000–24,000 pigeon day doses of vitamin B_1, together with some vitamin B_4. It is only faintly coloured and may be concentrated to a convenient volume. Neutralisation to p_H 5·5 sometimes causes the formation of a rather large precipitate, consisting in part of Mg compounds. The amount varies and seems to depend especially upon the length of time during which the extract stands at p_H 1·0 before charcoal adsorption. The precipitate can be readily removed by centrifuge and washed without loss of activity. After this, the extract may be either (1) used directly to prepare crystals of high vitamin B_1 activity [Kinnersley, O'Brien and Peters, 1932], or (2) stored after addition of acid to p_H 3·5 and alcohol to 75 %, under which conditions it keeps well.

Before administration to animals (or patients) it is advisable to test for metals with H_2S at p_H 4·0. If present, they should be removed by treatment with H_2S at p_H 4·0 and filtration. The extract can be reacidified to p_H 3·0 with HCl and the H_2S removed either by passing nitrogen or by boiling. Removal *in vacuo* is apt to lead to troublesome frothing. The theoretical objection to boiling with H_2S present is of no consequence in this connection, as we have repeatedly found no loss of activity by boiling such solutions for an hour or more at p_H 3·0–4·0.

The $N/10$ HCl extract is the purer source of vitamin B_1 at this stage; 100 pigeon doses contain 25–50 mg. organic solids. It cannot be dried, but solids can be estimated by drying at 105° at which temperature the vitamin is not volatile.

A further supply of more impure vitamin B_1 is obtained together with vitamin B_4 by a further extraction with 50 % acid alcohol.

Extraction of p_H 7·0 "charcoal" by 50 % acid alcohol. The charcoals are extracted 4 times with 1400 cc. 50 % (by vol.) alcohol containing $N/10$ HCl, the extractions being made, as before, for 15 minutes at 80°. The combined filtrates are concentrated on the water-bath to avoid the frothing usually experienced *in vacuo*. The p_H is brought to 3·0, and removal of metals carried out if necessary as previously advised for $N/10$ HCl extracts.

This extract contains 10,000–12,000 doses vitamin B_1 together with vitamin B_4. It is very useful as a cruder source of vitamin B_1, either for administration to patients, or as a basal source of vitamin B_1 in rat and pigeon experiments. In the latter cases, the vitamin B_4 (and B_5), *etc.* present are often of definite value.

Summary of the labour required.

The extraction of 2 cwt. (100 kg.) yeast takes the time of one worker for one day. The subsequent processes from lead acetate to charcoal adsorption, two workers for one day; the extraction of the charcoal one worker for ½ day. It is an advantage to run continuously for 3 days (300 kg.) in all. In this case the extraction of the 2nd and 3rd 100 kg. on the second and third days needs one worker, making 3 in all for 3 days.

Summary.

Stage	100 kg. yeast: Volume litres	No. of workers	Days
A. Aqueous extraction of yeast	124–7	1	1
B. Lead precipitation and removal	124	.	.
C. Baryta clearance	120	.	.
D. $BaSO_4$	120–2	2	1
E. Acid charcoal	120–2	.	.
F. p_H 7·0 charcoal	122	.	.
G. Extraction of charcoals	.	1	½

Discussion.

1. The process outlined here, has been tested upon about 2500 kg. of yeast. Using the standard baker's yeast, we have never had a failure. Some workers use brewer's yeast for this preparation. In a modification for instance of our process by Chick and Roscoe [1929], adopted recently by Heyroth [1932], brewer's yeast is washed, and the extract is freed from metals before adsorption upon charcoal by treatment with H_2S. Theoretically perhaps this sounds an ideal procedure; in practice, we believe that the gain is illusory, at the expense of the very considerable labour. By this modification, 120 litres of solution require treatment with H_2S, instead of some 3–4 litres, which is the volume after adsorption. In the preparation of crystalline vitamin B_1, the H_2S stage may often be entirely eliminated by the use of sodium phosphotungstate [see Kinnersley, O'Brien and Peters, 1932].

2. The method of preparation here given leads to a vitamin B_1 concentrate still contaminated with vitamin B_4. If freedom from this is desired, the mercury stage (previously described) [Kinnersley and Peters, 1927], must be substituted instead of the acid charcoal. The resulting liquid filters quite well through the battery of funnels. Mercury present after adsorption is removed by treatment with H_2S. The mercuric sulphate precipitate can be used for the preparation of vitamin B_4 concentrates [*cf.* Reader, 1930].

3. For the preparation of concentrates of vitamin $B_1 + B_4$ for rat work upon vitamin B_2, *etc.*, it is best to use the original Kinnersley and Peters shortened process. The "acid" charcoal adsorption is performed at p_H 2·5 instead of p_H 1·0. Subsequent elution is made with acid 50 % alcohol (until acid to Congo red). The remainder of the procedure is as in the text for 50 % alcohol concentrates. Some 40,000 pigeon doses of B_1 may be obtained in this way[1].

This concentrate can also be used for clinical purposes.

4. Adsorption upon fuller's earth (Seidell) has been used, following a lead acetate precipitation [*cf.* Guha, 1931]. In our hands, however, there was no gain in time as compared with the charcoal adsorption technique.

[1] Probably use could be made of the lead acetate precipitate for the preparation of glutathione or vitamin B_2 [see Narayanan and Drummond, 1930; Chick and Roscoe, 1929].

SUMMARY.

The complete details are given for obtaining rapidly concentrations upon a large scale of vitamin B_1 and B_4 by the charcoal adsorption method from baker's yeast. Modifications suitable for various phases of work are also described.

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