

XCVII. OBSERVATIONS ON THE CONCENTRATION OF VITAMIN B₁.

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INTRODUCTION.

VITAMIN B, as required for the normal nutrition of rats, has been demonstrated in recent years to consist of at least two factors. Vitamin B₁ is the factor, a deficiency of which is apparently related specifically to the typical convulsive symptoms associated with vitamin B-deficiency. It is sharply differentiated from vitamin B₂ by its relative instability to heat and alkali. Obviously the recent discovery of its complex nature necessitates a repetition and revision of much of the earlier work on vitamin B.

The present paper records the results of certain attempts to concentrate vitamin B₁ which have been made during the last two and a half years. The following table summarises the more important progress in the chemical isolation of vitamin B that has been recorded.

Investigators	Source of vitamin	Dose	Remarks
Suzuki <i>et al.</i> [1912]	Rice-polishings	5 mg. (by mouth) curative for pigeons	The dose was given daily for 3 days. Afforded protection for more than 10 days
Edie <i>et al.</i> [1912]	Yeast	3-6 mg. (by mouth) curative for pigeons	6 mg. administered on the 1st day, 3 mg. on the 3rd and 9th days. Protected for 15 days
Funk [1913]	Rice-polishings Yeast	4-8 mg. (by injection) curative for pigeons	Protected for 4-6 days
Aberhalden and Schaumann [1918]	Yeast	5 mg. (by injection) curative for pigeons	Protected for 19 days (only one pigeon tested)
Hofmeister [1920]	Rice-polishings	5-10 mg. curative for pigeons	Protected for 8-10 days
Seidell [1924]	Yeast	2 mg. fed daily to pigeons	Prevented loss of weight for 8-14 days
Bowman and Yee [1925]	Mung bean	3 mg. (by injection) curative for pigeons	No mention as to how many days' protection afforded
Jansen and Donath [1926]	Rice-polishings	0.012 mg. fed daily to pigeons	Protected for more than 4 weeks
Kinnersley and Peters [1928]	Yeast	0.027 mg. (by mouth) curative for pigeons	Day-dose*
Levene [1928]	Yeast	0.07 mg. fed daily to rats in addition to a B ₂ -preparation	Produced good growth for a period of 3 weeks

* Day-dose = $\frac{\text{curative dose}}{\text{number of days for which protection is afforded}}$.

Although a strict comparison of the potency of different preparations obtained by various investigators is impossible in view of variations in the method of testing, it is clear that the products obtained by Jansen and Donath, Kinnersley and Peters, and Levene are much more potent than the earlier preparations. Of these recent attempts to concentrate and isolate vitamin B₁ most importance is to be attached to the claims of Jansen and Donath. These investigators used an extract of rice-polishings as the starting material. Their method involved adsorption by a kind of "acid-clay," resembling fuller's earth in its selective powers of adsorption [Seidell, 1916]. This was combined with modifications of the usual methods for the isolation of simple natural bases. The active substance was obtained in the form of a crystalline hydrochloride, which protected pigeons from "polyneuritis" in daily doses of 0.012 mg. for over 4 weeks. Rice-birds (*Munia maja*), which were employed as the chief test-animals, were protected by the same material in daily doses of 0.002 mg. for more than 3 weeks. If left on the basal diet without the vitamin preparation, the pigeons and rice-birds were stated to exhibit symptoms generally within 3 weeks and 13 days respectively. The activity of the substance was confirmed by Eijkman [1927], who effected a cure by administering 0.2 mg. by mouth to a pigeon in severe convulsions. Smaller doses were not tried. Jansen and Donath suggested from its behaviour towards silver nitrate and baryta and from the fact that it gave a strong Pauly reaction that the "vitamin hydrochloride" obtained was a glyoxaline derivative.

EXPERIMENTAL.

Biological technique.

In view of the fact that both rats and pigeons have been extensively employed in researches on vitamin B₁, it was thought advisable to study the activity of the preparations made during the present work by tests on both species. Rats were employed as test-animals throughout the experiments and both rats and pigeons in the more important stages of fractionation.

Rat tests were carried out by studying the influence of the supplement on the growth of young rats whose development had been inhibited by a diet deficient in vitamin B₁. The rats, averaging 50 g. in weight, were kept in separate cages with screened bottoms and were fed on an artificial basal diet consisting of 75 % rice-starch, 21 % commercial "casein" and 4 % salt-mixture (McCollum). Each rat received in addition one drop of cod-liver oil of proved potency and 1 cc. of a 50 % solution of marmite, autoclaved for 3 hours at 14-15 lb. pressure in an alkaline medium. Such treatment is known to destroy all the vitamin B₁ in marmite, while the amount of vitamin B₂ in it is not seriously reduced [Hassan and Drummond, 1927]. An adequate supply of vitamins A, D and B₂ was thus ensured. All these supplements, as well as the B₁ preparation to be tested, were supplied daily in a separate dish

so that consumption could be checked. The standard of good growth in these experiments was a weekly gain in weight of 10-12 g. for a period covering 2-4 weeks. It was considered that a more prolonged test might be unsafe in view of the evidence which has been accumulating lately regarding the existence of a third yeast factor necessary for the normal development of rats. In all cases the vitamin B₁ supplements were given when the growth-curve was either flat or falling, showing a depletion of the vitamin B₁ reserves of the body. In order to obviate complication by refection it was always ensured that the withdrawal of an active supplement was followed by a decline in weight. In the course of this work a few cases of refection were, in fact, encountered. In a number of the fractions the curative property was also investigated by subcutaneous injection into rats exhibiting typical beriberi convulsions as a result of prolonged deprivation of vitamin B₁.

In the case of the pigeon tests the curative technique was adopted. The pigeons were fed on polished rice *ad libitum*. This technique, as a method for the quantitative assay of vitamin B₁, has been much improved in recent years [Kinnersley, Peters and Reader, 1928]. In our experiments the pigeons were not fed on a standard complete diet before being placed on the polished rice dietary. Kinnersley, Peters and Reader's observations indicate, however, that such treatment only increases the proportion of pigeons which exhibit typical symptoms within 30 days of feeding on polished rice, but does not appear to reduce appreciably the variability in response of different birds to the same dosage. It is clear that under these circumstances a mean may be taken only when the different doses do not vary greatly from one another. It was necessary, therefore, to leave out all abnormal cases, which were fortunately few in number. In our experiments the possibility of spontaneous cures has been excluded by using polished rice as the basal diet [Kon and Drummond, 1927; Kon, 1927], whilst heat-cures [Peters, 1924; Roche, 1925] were guarded against by keeping the pigeons in a comparatively warm room. Under these conditions 50-60% of the pigeons exhibited the classical symptoms, the majority doing so within 30 days. They were treated by injection within 5 hours after their condition had been noticed, and tests were considered positive only when the cure lasted for 3-13 days. Usually the pigeons after being treated with an active preparation were afforded protection for periods considerably more than 4 days. When the preparations found active were tried at the second or even at the third onset of convulsions, cure was effected provided the birds had sufficient strength to go through the tests. Injection was preferred to administration by mouth as in the latter case there is some risk of the dose being rejected or not being absorbed [Funk, 1911]. If the preparation was active, the symptoms of head-retraction usually cleared up within 2-5 hours after treatment and the paresis of the legs usually improved. A few cases of emprostotonos [McCarrison, 1921] were encountered, which also responded to the treatment with vitamin B₁ extracts, though less uniformly than the typical cases of opisthotonos. Such cases were, therefore,

not usually considered. The day-dose was calculated as the curative dose divided by the number of days for which protection was afforded on continued feeding on polished rice.

Testing for vitamin B₁ with known substances.

Before starting experiments on the concentration of vitamin B₁ it was considered advisable to re-test certain substances, which have at one time or another been claimed to possess antineuritic or growth-promoting properties, since most of the experiments on which these claims were based were made before the multiple nature of vitamin B was recognised. Of these substances, yeast nucleic acid [Schaumann, 1909; Ganassini, 1924], nicotinic acid [Funk, 1913], betaine [Williams, 1916, 1917], and the substance of m.p. 234–5° [Drummond and Funk, 1914] gave negative results when tested in the present investigation.

The fact that vitamin B₁ preparations are rendered inactive by heating with alkali led us to test the activity of the volatile bases liberated from marmite by boiling it with 20 % sodium hydroxide, again with a negative result.

Attempts at the isolation of vitamin B₁.

In these experiments we selected wheat embryo as the starting material in view of the fact that it is very rich in vitamin B₁ and relatively poor in B₂. A concentrate was finally obtained which cured pigeons in a day-dose of 0.005 mg., and produced good growth in rats in a daily dose of 0.015 mg. Evidence was also obtained which suggests that the action of vitamin B₁ itself is probably to be ascribed to more than one chemical individual, a point to which we shall again refer.

Extraction of wheat embryo. Wheat embryo was best extracted for our purposes with 50 % alcohol, containing a very small quantity of hydrochloric acid. 101.8 kg. of "Bemax" wheat embryo were, therefore, extracted twice with 254.5 litres of 50 % alcohol (by volume) containing 0.5 % concentrated hydrochloric acid, each extraction being carried out for 3–4 hours between 60° and 70°. This was filtered under pressure. The filtrate on concentration *in vacuo* yielded a yellow, viscous mass weighing 20.15 kg. In this condition the active substance can be kept without appreciable loss for considerable periods (10 months at least), stored at 0° in the dark in well-closed vessels. The amount of total solids was 15.59 kg., of which 14.96 kg. were organic. It produced good growth in rats in doses containing 77.2 mg. solids, of which 73.5 mg. were organic, and equivalent to 0.5 g. embryo. This extract formed the starting material for two different processes of fractionation.

First method of fractionation.

Stage 1. Fractionation by lead acetate. An amount of the above extract equivalent to 49 kg. embryo was dissolved in 22 litres of distilled water. The opalescent fluid was treated with 1.2 kg. lead acetate in 50 % solution, which

represented a slight excess over that required for complete precipitation. After allowing the precipitate to settle overnight, about 12 litres of the supernatant liquid were syphoned off and the bottom layer was filtered through a Büchner funnel. The lead precipitate was finally washed with a little water. To the total filtrate and washings, approximately 25 litres, concentrated hydrochloric acid (*ca.* 310 cc.) was added to make the solution just acid to Congo red. The resultant precipitate was allowed to settle and then removed. The filtrate was freed from lead by treatment with hydrogen sulphide. Subsequent removal of the hydrogen sulphide gave a solution which was active in doses equivalent to 0.5–0.75 g. embryo and contained 48–72 mg. organic matter of which 2.3 % was nitrogen (with reference to the organic matter present).

Stage 2. Charcoal adsorption at various hydrogen ion concentrations. The p_H of the solution at this stage was usually 2.8–3, but, if not, it was adjusted to this value and the solution (24 litres) stirred for two periods of 20 min. with 500 g. norite each time. After removal of the charcoal the p_H of the filtrate was raised in stages to 4, 5, 6 and 7 by carefully adding 2 *N* NaOH. At each of these stages it was similarly treated with norite. Each lot of 1 kg. norite was extracted by refluxing twice successively with 5 litres of 50 % alcohol containing 1 % HCl, each extraction lasting half an hour and being carried out at 60°. All the extracts were subsequently concentrated *in vacuo* to remove alcohol and then tested on rats. The active material was preferentially adsorbed under our conditions at p_H 4 and 5¹.

	Total organic matter (g.)	
p_H 3	94.5	N=7.3 %. Very slightly active in doses equivalent to 4 g. embryo and containing 7.9 mg. organic matter
p_H 4	90	N=9.6 %. Very active in doses equivalent to 2 g. embryo and containing 3.8 mg. organic matter
p_H 5	90.2	N=9.6 %. Very active in doses equivalent to 2 g. embryo and containing 3.8 mg. organic matter
p_H 6	24.8	N=17.4 %. Very slightly active in equivalents of 6 g. embryo and containing 3.0 mg. organic matter
p_H 7	30.6	N=5.0 %. Inactive in equivalents of 6 g. embryo and containing 3.7 mg. organic matter
Final filtrate	4185	N=1.8 %. Inactive in equivalents of 6 g. embryo, and containing 0.524 g. organic matter

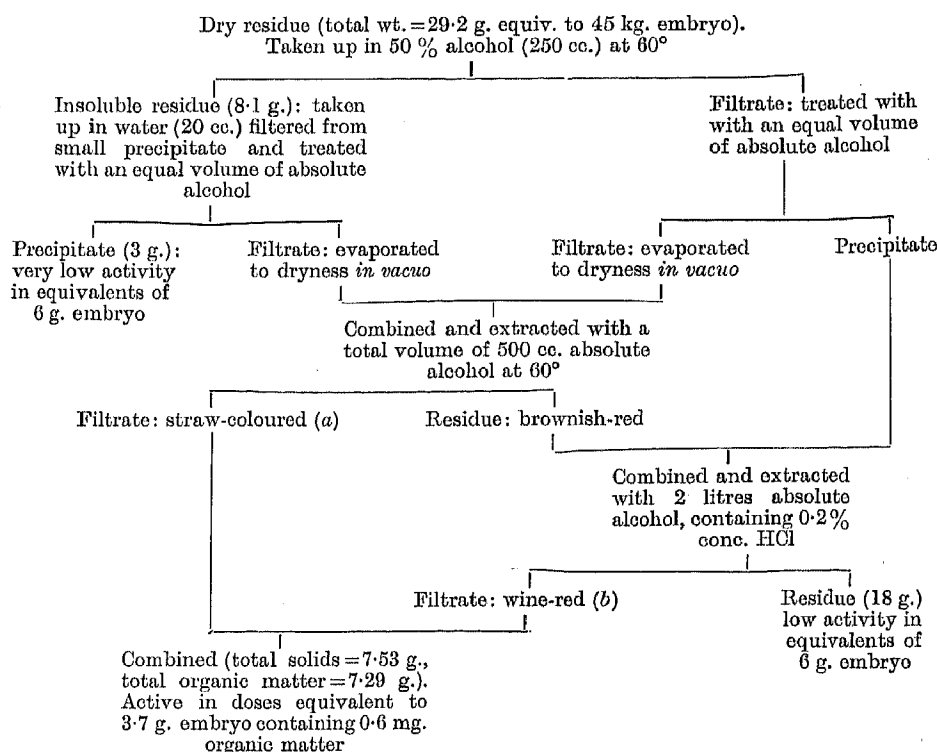
Stage 3. Fractionation with phosphotungstic acid. The extracts corresponding to p_H 4 and 5 were combined and the p_H raised to 5 by adding 2 *N* NaOH. The colour of the solution darkened and a light flocculent precipitate separated. This was removed as it was found to carry no activity. Sufficient sulphuric acid was added to the filtrate to make it 5 % acid, and the solution was then treated with 1.5 kg. phosphotungstic acid in saturated solution in 5 % sulphuric acid. The phosphotungstic acid added represented a considerable excess over that required for complete precipitation, but it was found that the precipitation of the active material was apparently

¹ We have been able to confirm the statement of Kinnersley and Peters [1928] that vitamin B₁ is selectively adsorbed from yeast extracts by norite at p_H 7. The preparation so obtained is curative for pigeons and also promotes good growth in rats, when supplemented by vitamin B₂.

dependent on such excess. Nearly all the active material was found in the precipitate removed after standing for 40 hours. The precipitate and filtrate were both rendered free from phosphotungstic acid by treatment with concentrated hydrochloric acid and amyl alcohol and ether.

Stage 4. Adsorption on silver oxide. The acid aqueous solution of the substances that had been precipitated by phosphotungstic acid was shaken up with an aqueous suspension of freshly precipitated silver oxide (free from alkali), sufficient to produce a reaction neutral or very faintly alkaline to litmus. The resulting precipitate, which was found to contain highly active material, was separated from the inactive filtrate, washed with a little water and extracted twice with 2 litres of 50 % alcohol containing 1 % concentrated HCl above the amount of HCl required to combine with any free silver oxide. After the removal of the alcohol *in vacuo* the red solution proved active in doses equivalent to 2 g. embryo, containing 1.3 mg. organic solids. It contained 17.8 % nitrogen.

Stage 5. Fractionation with alcohol. Further attempts to concentrate the active material usually resulted in a certain amount of loss. The solution resulting from the extraction of the silver oxide precipitate was evaporated to dryness *in vacuo* and fractionated in the following manner. It represents one of the methods of alcoholic fractionation followed and gave fairly good results.



Stage 6. Treatment with picrolonic acid. Solutions (a) and (b) were combined and evaporated to dryness *in vacuo* at 40–50°. The residue was taken up in water (150 cc.) and brought to p_H 6.5 with 2 *N* NaOH, when the small precipitate produced was filtered off and found to be inactive. The filtrate was treated with an absolute alcoholic solution of picrolonic acid in slight excess, whereby a semi-crystalline yellow precipitate was produced. Both precipitate and filtrate were rendered free from picrolonic acid by means of hydrochloric acid and ether. The precipitate was found to be inactive, even in doses equivalent to 12 g. embryo, while the filtrate was active in doses equivalent to 4.5–6 g. embryo and carrying 0.4–0.5 mg. organic matter. Nearly 11 % of the activity of the original extract of wheat germ was present in this product. The precipitate and the filtrate contained respectively 22.0 % and 16.5 % nitrogen (Micro-Kjeldahl).

Pigeon-curative tests with the picrolonic filtrate.

Pigeon No.	Dose injected (mg.)	Days of protection	Day-dose (mg.)
1	0.15	5	0.03
2	0.15	4	0.037
3	0.2	5	0.04
4	0.2	4	0.05
5	0.3	6	0.05
6	0.3	6	0.05

Average day-dose = 0.043 mg.

Some of the pigeons were cured again by means of the same extract on a second and even a third onset of convulsions. A few died without exhibiting symptoms a second time. One pigeon was protected for 15 days after the injection of 0.15 mg. but this was excluded from consideration, as probably an abnormal case.

Attempts at further concentration. Several methods of concentrating further the active material in this preparation were investigated without success. Amongst these was a second precipitation with phosphotungstic acid followed by fractional crystallisation from hot water and from mixtures of acetone and water. Attempts were made to adsorb the active principle selectively on silica gel at p_H 3, 4 or 5, followed by subsequent extraction at p_H 9.5 [Levene and van der Hoeven, 1925; Levene, 1928], but with no success. Fractionation with platonic chloride likewise yielded unsatisfactory products and resulted in marked loss of activity, which could be traced neither in the platinum precipitate nor in the filtrate. Representative curves illustrating the potency of the extract at different stages in this method of fractionation are shown in Fig. 1.

Second method of fractionation.

This method approximates to that followed by Jansen and Donath [1926] in their fractionation of rice-polishings and has yielded interesting results.

Stage 1. Adsorption by fuller's earth. 2 kg. of the original wheat embryo extract, equivalent to 10 kg. embryo, were dissolved in 20 litres of distilled

water. The p_H of the resulting solution was usually 4.5. If not, it was adjusted to this reaction and agitated vigorously for half an hour with 1 kg. of fuller's earth, which had been previously tested for its adsorbing capacity [Seidell, 1926]. After leaving overnight in the cold the supernatant fluid was syphoned off. It possessed little physiological activity. The bottom layer was filtered and the fuller's earth residue washed with a little water, acidulated with hydrochloric acid to p_H 4.5. The residue was extracted by grinding in small portions of 80–100 g. with a total volume of 1500–2000 cc. of saturated baryta, each extraction being carried out as quickly as possible and followed by rapid filtration. The duration of contact between fuller's earth and baryta should be as short as is consistent with good extraction. The filtrate was received in filter flasks containing an excess of sulphuric acid so as to remove the barium and restore an acid reaction quickly. The precipitated $BaSO_4$ was removed and the filtrate concentrated *in vacuo* to 750 cc. The resulting solution was found to be highly active in doses equivalent to 1 g. embryo and containing 2.9 mg. total solid, of which 2.6 mg. was organic with 13.3 % nitrogen.

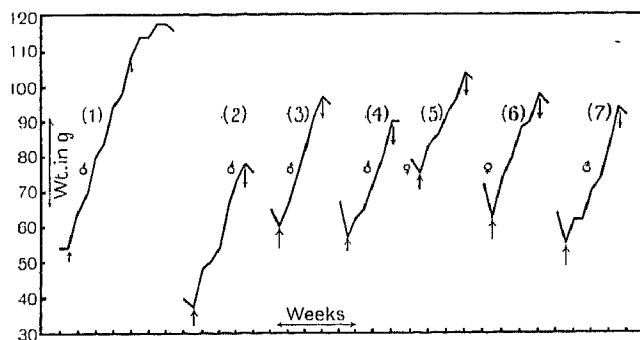


Fig. 1.

Curve (1). 50 % alcoholic extract of wheat embryo; ↑ dose, equivalent to 0.5 g. wheat embryo.

Curve (2). Filtrate from lead precipitate; ↑ dose, equivalent to 0.75 g. wheat embryo.
Curves (3) and (4). Charcoal fractions at p_H 4 and 5 respectively; ↑ dose, equivalent to 2 g. wheat embryo.

Curve (5). Silver fraction; ↑ dose, equivalent to 2 g. wheat embryo.

Curve (6). Combined (a) and (b) fractions; ↑ dose, equivalent to 3.7 g. wheat embryo.

Curve (7). Picrolonic filtrate fraction; ↑ dose, equivalent to 5 g. wheat embryo.

↓ Supplement withdrawn.

Stage 2. Fractionation with silver nitrate and baryta. The solution after the removal of barium had a p_H below 1.8. 45 cc. 50 % silver nitrate (excess) were added with stirring. A drop of the solution then gave a brown precipitate almost immediately with excess of baryta. The mixture was treated gradually with constant stirring with sufficient saturated baryta to raise the p_H to 4.5. The precipitate was filtered off and the filtrate brought to p_H 6.5 by adding more baryta with constant stirring. After standing overnight in the cold the dark brown precipitate, which carried almost all the activity, was filtered

repeatedly through the same filter under suction, until the filtrate was nearly clear. More baryta was now added to the filtrate to bring the p_H to 8 and the precipitate filtered off quickly. The silver precipitates obtained at the three reactions were separately decomposed by triturating with concentrated hydrochloric acid and the silver chloride was removed. The solutions corresponding to p_H 4.5 and 8 were entirely inactive even in doses equivalent to 6 g. embryo. The solution corresponding to p_H 6.5 was opalescent and greyish-brown even after repeated filtration. It was active in doses equivalent to 1.5 g. embryo, representing 0.21 mg. total solids, of which 0.18 mg. was organic matter. It contained 12.5 % nitrogen.

Stage 3. Fractionation by phosphotungstic acid. The acidity of the above extract having been measured by titration, enough sulphuric acid was added to make it 5 % acid, when it was treated with a small excess of a saturated solution of phosphotungstic acid in 5 % sulphuric acid. After standing in the refrigerator for 38 hours the phosphotungstic precipitate was filtered off under suction and washed with 5 % sulphuric acid (moist weight, 6 g.). It was fractionated by grinding in a mortar with 75 cc. of 50 % acetone. The solution after filtration gave a clear red filtrate, leaving a small precipitate, which was washed with a little 50 % acetone and the washings were added to the main filtrate. The clear solution was poured into 300 cc. of 5 % sulphuric acid, and the precipitate produced, after standing in the cold for 40 hours, was collected, washed with 5 % sulphuric acid, dried (1.7 g.) and then ground and dissolved in 60 cc. of 50 % acetone. Saturated baryta (60–70 cc.) was then gradually added until the solution reacted alkaline to phenolphthalein. On shaking violently the precipitate settled rapidly and was immediately filtered off. The filtrate was quickly acidified with *N* sulphuric acid and the excess acid removed by addition of the requisite amount of baryta. The product was active for rats in doses equivalent to 3 g. embryo, which contained 0.10 mg. total solid. On testing this fraction on pigeons the following results were obtained:

Pigeon No.	Dose injected (mg.)	Days of protection	Day-dose (mg.)
1	0.1	8	0.0125
2	0.1	7	0.0143
3	0.1	6	0.0167

Average day-dose = 0.0143 mg.

Stage 4. Fractionation with platonic chloride. The nearly colourless solution obtained at the preceding stage was first concentrated at 40° and finally evaporated to dryness in a vacuum desiccator over soda-lime. The residue was a semi-crystalline slightly reddish coloured product, weighing 0.2677 g., representing 8.3 kg. embryo. It was extracted with absolute alcohol (250 cc.) in small portions at 60°. The amount which remained undissolved was 0.0777 g. but it produced subnormal growth in rats even in doses equivalent to 15 g. embryo. The alcoholic filtrate on concentration in a vacuum desiccator over calcium chloride produced a precipitate, which was inactive even in doses

equivalent to 12 g. embryo, and was, therefore, discarded. The remaining solution on treatment with a slight excess of a 5 % solution of platinum chloride in absolute alcohol gave a yellowish-orange precipitate which was filtered off after standing for 24 hours at 0°. After decomposing the aqueous suspension of the precipitate with hydrogen sulphide a colourless solution was obtained, which was again evaporated to dryness over soda-lime. The residue weighed 24.9 mg. equivalent to 7754 g. embryo, and was found to be active in doses equivalent to 4.6 g. embryo and containing 0.015 mg. total solids. About 11 % of the activity of the original extract was thus retained in this material. The product was colourless and for the greater part micro-crystalline but obviously impure. The alcoholic filtrate from the platinum chloride precipitate gave a peculiar orange precipitate on concentration and treatment with a little water, but both this precipitate and the filtrate were entirely inactive. The potency of the active fraction as demonstrated by tests on pigeons is shown in the following table:

Pigeon No.	Dose injected (mg.)	Days of protection	Day-dose (mg.)
1	0.0148	4	0.0037
2	0.0296	9	0.0033
3*	0.0592	7	0.00846

Average day-dose = 0.00515 mg.

* This pigeon showed only slight emprosthotos on the seventh day after cure.

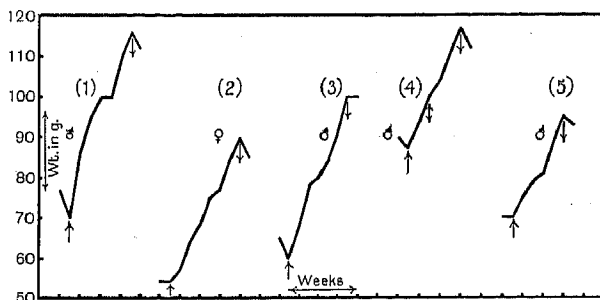


Fig. 2.

- Curve (1). Fraction adsorbed on fuller's earth; ↑ dose, equivalent to 1 g. wheat embryo.
 Curve (2). Silver nitrate-baryta fraction (p_H 6.5); ↑ dose, equivalent to 1.5 g. wheat embryo.
 Curve (3). Phosphotungstic fraction; ↑ dose, equivalent to 3 g. wheat embryo.
 Curve (4). Platinum chloride precipitate fraction; ↑ dose, equivalent to 9 g. wheat embryo.
 ↓ dose, equivalent to 4.6 g. wheat embryo.
 Curve (5). Platinum chloride precipitate fraction; ↑ dose, equivalent to 4.6 g. wheat embryo.

↓ Supplement withdrawn.

Fig. 2 illustrates the potency of the different fractions obtained by this method for the growth of rats.

Stage 5. Fractionation with gold chloride. The active preparation from the platinum compound was extracted with 20 cc. absolute alcohol at 60–70°. Nearly one-third of the total substance was removed in the form of an insoluble

inactive material. The soluble fraction (13.4 mg.) was concentrated slightly and treated with 5 cc. of a freshly prepared 5% absolute alcoholic solution of gold chloride. As only a very slight turbidity was produced the liquid was warmed until clear and allowed to evaporate slowly in a desiccator over calcium chloride. A very small amount of micro-crystalline deposit was formed, which did not, however, appear to be homogeneous and did not show the characteristic appearance of the gold double salt obtained by Jansen and Donath and described by Eijkman [1927]. The gold salt was separated, when the solution had been concentrated to about 1.5 cc., washed with a very small amount of absolute alcohol and decomposed in aqueous suspension with hydrogen sulphide. The gold filtrate was also similarly decomposed after removal of alcohol. The filtrates were evaporated to dryness in a vacuum desiccator over soda-lime. The amount of the material obtained from the gold precipitate was 4.9 mg. and that from the gold filtrate was 6.6 mg., both of which were equivalent to 6.3 kg. of embryo. The tests of these products on the growth of rats provided results that appear to us both curious and important. The insoluble gold salt gave a fraction that produced sub-normal growth in quite large amounts, *e.g.* 0.028 mg., whereas the soluble gold fraction was entirely inactive even in quantities of 0.038 mg., each of the doses being equivalent to 36 g. of embryo. When such sudden loss of activity apparently occurs, it is always advisable to investigate whether a resolution of the active principle into two or more constituents has been effected. In order to test this possibility a mixture of 0.014 mg. of the material obtained from the insoluble gold salt and 0.019 mg. of the soluble fraction was tried and found to produce good growth. This fact suggests, therefore, that the action of vitamin B₁ is probably to be ascribed to more than one factor. On this basis the gold precipitate would seem to contain most of one factor and a little of the other, whereas the gold filtrate would appear to be composed almost exclusively of the second factor. It is further significant that the substance from the insoluble gold salt gave a very strong Pauly reaction, whilst the soluble gold fraction gave a very faint response. Confirmatory evidence is being sought in further work in which, it is hoped, larger amounts of material will be available. These results obtained from experiments with rats (Fig. 3) appear to be borne out by curative tests on pigeons.

	Dose injected (mg.)	Pigeon No.	Days of protection	Day-dose (mg.)
Gold precipitate	0.0277	1	6	0.0046
Gold filtrate	0.072	2	11	0.0065
Gold precipitate + Gold filtrate	0.014 + 0.019	3	13	0.0025

It will be seen that the mixture of the gold precipitate and filtrate is more active than the precipitate alone, which itself is more active than the filtrate. Tests with the same solutions a month later showed that they had suffered a marked loss of activity, but not uniformly. The average day-doses of the gold precipitate, filtrate and the mixture were respectively 0.0092, 0.054, 0.0087 mg.

Certain properties and reactions of the vitamin B₁ concentrates.

(a) *Influence of hydrogen ion concentration and the period of heating on the stability of the vitamin.* In general a preparation of the "antineuritic" factor or "vitamin B" has been found to be fairly stable to acids even when subjected to prolonged heating. Thus Steenbock [1917] obtained a preparation from egg-yolk which retained its antineuritic potency after treatment with hot hydrochloric acid. Drummond [1917] reported that the activity of marmite as shown by rat-tests was not much reduced by boiling with 1 % hydrochloric acid for 12 hours, though boiling with 20 % sulphuric acid inactivated the material considerably. McCollum and Simmonds [1918] likewise found that an active extract of wheat germ retained its activity for rats after treatment with hydrochloric acid. It has, in fact, been now and again asserted that acid hydrolysis produces a more active extract. For this statement, however, there

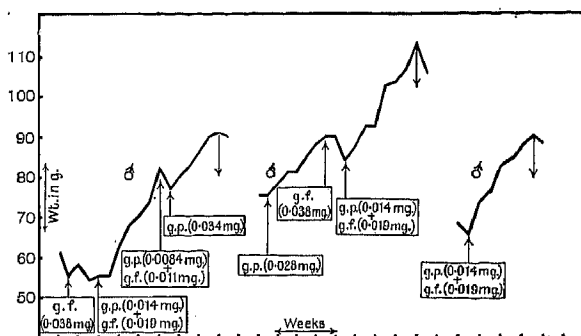


Fig. 3.

g.p. = Material obtained from the gold precipitate.
 g.f. = Material obtained from the gold filtrate.
 ↓ = Supplement withdrawn.

is as yet little convincing evidence. Towards alkali, on the other hand, the substance has been found to be markedly unstable, especially on the application of heat. Cooper [1913] demonstrated the destructive action of ammonia on a vitamin preparation from horse-flesh. Osborne and Leavenworth [1920-21], as also Kinnersley and Peters [1928], have observed the same effect of caustic alkalis on the vitamin potency of yeast concentrates. The latter workers found that the destructive action of alkali was enhanced by the presence of alcohol. There are, however, a few quantitative discrepancies in the literature on the subject, which we are inclined to attribute partly to the variable behaviour of the substance in the presence of accompanying matter. Thus we found a sample of marmite to be somewhat more stable to alkali than a relatively pure preparation of the wheat-embryo extract.

Sherman and Burton [1926] have recently investigated the vitamin B potency of tomato-juice under varying conditions using the constant weight of the test-rats during an experimental feeding period of 8 weeks' duration as the criterion. Our experiments on the stability of one of our comparatively

concentrated preparations (charcoal concentrate) boiled at different hydrogen ion concentrations for different periods of time have yielded results which are in general accord with those of Sherman and Burton. These results are summarised in the following table. The hydrogen ion concentrations were adjusted by the addition of hydrochloric acid or sodium hydroxide.

p_{H}	Time of boiling (hours)	Av. weekly growth on original dose* g.	Av. weekly growth on double original dose g.
1	1	+11.0	+12.0
1	8	+12.0	+14.0
1	24	+12.0	+14.0
5	1	+9.0	+10.0
5	8	+8.5	+10.0
5	24	+3.5	+8.0
9	$\frac{1}{2}$	+0.6	+9.5
9	$\frac{1}{3}$	0	+9.0
9	1	-9.0	+5.0

* "Original dose" stands for the dose in which the preparation produced good growth (weekly gain of 10-12 g.) prior to heating with acid or alkali. A supply of alkali-autoclaved marmite was of course always provided to supply the factor B_2 .

While quantitative deductions from the above experiments are not warranted because of the inadequacy of the number of tests it is clear that this study leads to certain general conclusions. Boiling for 24 hours at p_{H} 1 does not cause any appreciable inactivation. At p_{H} 5, however, the activity deteriorates slowly with the period of heating, the preparation requiring at least double the original dose to promote good growth after being boiled for 24 hours at this reaction. Boiling for 1 hour at p_{H} 9 inactivates it to the extent of more than 50%; half an hour's boiling results in relatively less destruction, whereas with 15 minutes' boiling the loss of potency is still less. It may be concluded that the inactivation is, as might be expected, roughly proportional to the concentration of hydroxyl ions (see Sherman and Burton [1926]) and to the period of heating.

(b) *Action of nitrous acid.* McCollum and Simmonds [1918] observed that wheat embryo extract treated with nitrous acid gas, when administered to rats as the sole source of vitamin B, was still able to promote good growth. It was pointed out that this experiment argued against the possibility of the vitamin being a primary or secondary amine. Peters [1924] found that treatment with nitrous acid did not cause any diminution of the antineuritic potency of his yeast-concentrates. Levene [1928] has made the same observation regarding his concentrated preparations from yeast. We can also confirm these findings, having observed that the picrolonic acid filtrate fraction was not appreciably inactivated after treatment with nitrous acid both as regards the pigeon and the rat. The fraction tested was treated in the usual way with hydrochloric acid and sodium nitrite and then warmed for half an hour and finally boiled for a few minutes to expel the nitrous fumes. The charcoal concentrate obtained by the method of Kinnersley and Peters [1928] from brewer's yeast was also found to retain its activity regarding the rat and the pigeon after similar treatment (Fig. 4).

(c) "Bios"-activity of concentrates. For some years several investigators [Williams, 1920; Eddy and Stevenson, 1919-20; Funk and Dubin, 1920] held the view that "bios" and vitamin B were identical, so much so that several methods were devised involving yeast growth as the criterion of the presence of vitamin B. It is now certain, however, that vitamin B₁ and "bios" are different. The former is peculiarly labile to alkali treatment, whereas the latter is extremely stable. In fact, several workers have shown that alkali-treatment of certain preparations destroys the vitamin B potency without reducing the activity for yeast growth [Souza and McCollum, 1920; Whipple, 1920]. Fleming [1921] found that an extract of rice even after prolonged boiling with sodium hydroxide, which was present to the extent of 10 %, retained its power of stimulating yeast growth unimpaired. It has been likewise found in this laboratory that the "bios"-activity of marmite is little affected by autoclaving with alkali at 12 lb. pressure for 3 hours.



ERRATUM

Vol. 23, p. 892 in the third column of the table

for 0.6 read 6.0

↓ dose, same as that of untreated concentrate.
↓ Supplement withdrawn.

We conducted several experiments with the fractions obtained at the first and last stages of fractionation of wheat embryo to observe their growth on *Saccharomyces cerevisiae* (National Collection of Type Cultures, Chapman 2160). All the fractions tested were highly potent as regards the rat and the pigeon. The results are summarised in the following table. The data for marmite are given for comparison. The respective doses for the growth of the rat are also stated.

Preparation tested	Rat-dose of preparation (B ₁ -potency) (mg.)	Addendum of preparation per cc. of Reader's medium (mg.)	Count of cells inoculated per cc.*	Count of cells per cc. after incubation for 48 hours*	Results after 7 days
Baryta-autoclaved marmite	—	0.25	0.02-0.04	32	Copious growth
Lead filtrate of embryo extract	51	0.25	0.02-0.04	25	Copious growth
Picrolonic filtrate	0.4-0.5	0.04	0.1	Practically nil	Very slight growth
Mixture of materials obtained from the gold precipitate and filtrate	0.033	0.018	0.1	Practically nil	Slight growth (6.8 counts per cc.)

* Expressed in units of 250,000 cells per cc.

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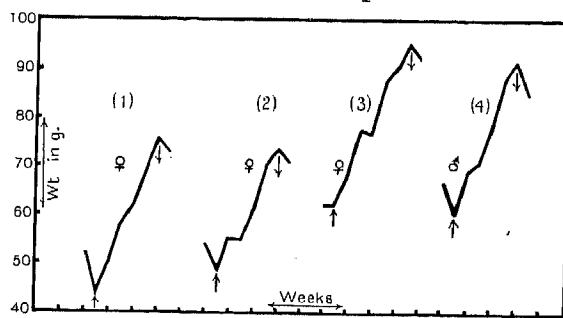


Fig. 4.

Curves (1) and (2). Picrolonic filtrate fraction treated with HNO₃; ↑ dose, equivalent to 6 g. wheat embryo.
 Curves (3) and (4). Peters's charcoal concentrate from brewer's yeast, treated with HNO₃. ↑ dose, same as that of untreated concentrate.
 ↓ Supplement withdrawn.

We conducted several experiments with the fractions obtained at the first and last stages of fractionation of wheat embryo to observe their growth on *Saccharomyces cerevisiae* (National Collection of Type Cultures, Chapman 2160). All the fractions tested were highly potent as regards the rat and the pigeon. The results are summarised in the following table. The data for marmite are given for comparison. The respective doses for the growth of the rat are also stated.

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It is apparent from the above figures that the "bios"-activity of the lead filtrate, which was obtained at the earliest stage of fractionation, was comparable with that of marmite heated with baryta. But the much purer concentrates obtained at the last stages had very little "bios" effect, though they were added in quantities which bore a much larger ratio to their respective rat-doses than the lead filtrate, and though the yeast inoculum was also considerably larger. Such slight growth as was noticed after incubation for 7 days is known to occur with various substances and is not specific for "bios."

(d) *Certain reactions.* The active picrolonic filtrate gave a very strong Pauly reaction, which was comparable with that given by histidine. The crystalline substance obtained by Jansen and Donath was also stated to give an intense red coloration with Pauly's reagent. This was part of the evidence which led them to suggest a glyoxaline formula for the substance they had prepared. All the active concentrates prepared from yeast by Kinnersley and Peters [1928] were also found to give a positive response to the Pauly reaction. They observed, however, that the substances responsible for the reddish reaction did not fractionate with the curative activity, but they were not led to any definite conclusions from this observation.

Heated with 40% sodium hydroxide, the picrolonic filtrate fraction emitted a strong smell suggestive of alkylamines but no smell of scatole or indole was noticed (cf. Levene [1928]). No sulphur reaction with lead acetate was obtained. Millon's, xanthoproteic and purine reactions were negative, which would appear to exclude the possibility of the vitamin being a hydroxyphenyl or purine derivative, which has been suggested from time to time. The platinum fraction and the preparation obtained from the gold precipitate gave a very strong Pauly reaction, whereas the gold filtrate gave a very faint one. The gold precipitate also produced a very faint blue coloration with Folin and Denis's reagent. Using the preparation obtained by Jansen and Donath, Eijkman also observed a blue coloration with this reagent, which, however, was stated to be much weaker than that produced by a solution of uric acid of the same concentration. This observation is important in view of statements in the literature claiming that Folin and Denis's reagent is a valuable guide in the detection of vitamin B.

DISCUSSION.

In the first place it will be noticed from our results that there is no fixed ratio between the rat-dose and the pigeon-dose of the various concentrates investigated. Thus the rat-doses of the picrolonic filtrate, of the phosphotungstic fraction and of the platinic chloride fraction were respectively 10 times, 7 times and 3 times the pigeon-dose. Whether this fact indicates a difference between the rat-factor and the pigeon-factor or whether it reflects on the accuracy of the present biological methods of assay is uncertain, but probably the latter is the more likely explanation.

As regards the properties of vitamin B₁, its behaviour appears to be determined to an extraordinary extent by the presence of other substances and by the previous treatment of a given preparation, a fact which has been pointed out by Kinnersley and Peters [1928]. These investigators observed that the solubility of their curative preparations from yeast in alcohol varied with their activity and with the hydrogen ion concentration of the solutions treated with alcohol. Similar variability has been observed with fractionation by silver nitrate. In our first method of fractionation it will be observed that the active material was not precipitated by platinic chloride, whilst in the second it was. In the first method the precipitation of the active substance appeared to be dependent on the presence of an excess of phosphotungstic acid, whereas in the second the amount of this reagent required was much less. Moreover, charcoal adsorbed most of the active material at p_{H} 4 and 5 in our first method, whereas the curative substance in yeast extracts has been found by Kinnersley and Peters to be adsorbed selectively by the same reagent in the zone of neutrality—an observation which we have been able to confirm. Jansen and Donath [1926] described a procedure in which the activity was found to be associated with the precipitate obtained by treatment of their concentrate from rice-polishings with picrolonic acid, whereas in the first procedure which we adopted for the concentration of wheat-embryo extract we have been able to recover most of the activity in the picrolonic acid filtrate. Similar apparently conflicting statements regarding the properties of vitamin B₁ have appeared repeatedly in the literature. It has been suggested that the factors which various workers were attempting to isolate from different sources were probably not identical. In this paper, however, it has been shown that even when the same biological techniques are adopted and the same substance chosen as the raw material, the properties of the active factor are profoundly modified according to the treatment undergone. It does not, therefore, appear strange that the substance should exhibit even greater differences in behaviour in the hands of investigators, starting from different raw materials and adopting different biological methods.

From the experiments in connection with fractionation by gold chloride it appears reasonable to infer that the activity of vitamin B₁ is probably to be ascribed to more than one factor. In this respect, the rat-experiments and pigeon-experiments appear to corroborate each other. The literature is replete with discussions on the identity or otherwise of the antineuritic factor of Eijkman and vitamin B of McCollum and Davis. But there are comparatively few references to the complex nature of vitamin B₁ and very little clear-cut experimentation has been made on this point. Random suggestions have been made that the curative property possibly does not belong to a single chemical individual but to a combination of different substances. Recently, Kinnersley and Peters [1928] have stated that about 12 % of the activity of their yeast extracts was always associated with the lead precipitate, even upon a reprecipitation. This along with other evidence has led them to suggest that they

were probably "dealing with two forms of vitamin B₁, either differently combined or perhaps oxidised and reduced." Evidence has also been given lately from different quarters about the existence of a third factor of the vitamin B complex besides B₁ and B₂*. From the experiments recorded in this paper it is not possible to conclude whether the two (or more) components in the gold precipitate and the filtrate were originally present in the raw material or whether a resolution of vitamin B₁ was effected by the different reagents employed in the fractionation. Jansen and Donath [1926] have stated, in adducing proof of the purity of their "vitamin hydrochloride," that on recrystallisation as well as on conversion of the gold salt back to the hydrochloride, the substance retained most of its activity, and that the mother-liquor was much less active than the crystallised hydrochloride. But the physiological activity of the gold precipitate and the filtrate together does not appear to have been investigated. It is not unlikely that they might have found the mixture more active than either component. Conclusive evidence about the multiple nature of vitamin B₁ can, however, be obtained only when a more complete separation of the two (or more) components is effected by fractional crystallisation of the gold salt or by other means.

SUMMARY.

- (1) The preparation of a concentrate of vitamin B₁ from wheat embryo is described, the pigeon-curative day-dose of which is 0.005 mg., and which promotes good growth in rats in daily doses of 0.015 mg., when supplemented by vitamin B₂.
- (2) Evidence is presented which points to the multiple nature of vitamin B₁.
- (3) Certain properties and reactions of the concentrates are described.

We wish to express our thanks to the Medical Research Council for a grant out of which the expenses of this research were defrayed. One of us (B. C. G.) also desires to thank the Trustees of the Tata Education Scheme for a scholarship, and the High Commissioner of India for a personal grant.

* We have also observed that ordinary marmite contains a factor or factors which can supplement to some extent vitamin B₁ and B₂ preparations, which, by themselves, are unable to produce normal growth in rats, even when administered in quite large doses [cf. Reader, 1928].

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