CIV. INVESTIGATIONS ON THE PREPARATION AND BEHAVIOUR OF VITAMIN B₁ CONCENTRATES FROM YEAST.

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INTRODUCTION.

IN a previous publication a method was described for the concentration of vitamin B₁ from wheat embryo, which had been attended with a considerable measure of success [Guha and Drummond, 1929]. A semi-crystalline preparation was obtained, of which the pigeon-curative day-dose was found to be of the order of 0.005 mg. It also promoted good growth in young rats, placed on a vitamin B₁-deficient diet, in a daily dose of 0.015 mg. The preparation, however, was obviously impure, and when subjected to fractionation by gold chloride behaved as if the vitamin had been split into two fractions, as a mixture of the substances obtained from the gold precipitate and filtrate appeared to be more active than either. As, however, a very small amount of material was at our disposal, the number of pigeon and rat tests was very inadequate, and, therefore, our results had to be taken with reserve. Recently, however, Reader [1930] has stated that the experimental "beriberi" condition in rats is a complex syndrome, resulting from a deficiency of two factors, B₁ and B₄. Furthermore, the crystals obtained by Jansen and Donath [1926] from rice polishings, which were originally claimed to be vitamin B₁ hydrochloride, have also been stated to contain vitamin B₄ [Jansen, Kinnersley, Peters and Reader, 1930]. Further information on this subject will be awaited with interest.

The present investigation was undertaken with the double object of finding out how far the method of fractionation, which had been previously applied to wheat embryo, could be successfully employed for the concentration of the vitamin from yeast, and whether any evidence about the multiple nature of vitamin B₁ could be obtained when yeast was used in place of wheat embryo as the raw material. Brewery bottom yeast was employed by Seidell [1926] for the concentration of vitamin B₁, but as his method of biological assay depended on the prevention of loss of weight of pigeons on a polished rice diet, it is clear from recent work on pigeons that the tests were complicated by other factors besides B₁ [Williams and Waterman, 1928; Carter, Kinnersley

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and Peters, 1930, 1, 2; Guha, 1929]. Baker's yeast has usually been employed by Peters and his co-workers and he has recently published improvements in his technique by fractional precipitation by means of phosphotungstic acid at different hydrogen ion concentrations [Kinnersley and Peters, 1930].

The fractionation recorded in the present paper was carried out with brewer's top yeast, as its aqueous extract was found by means of preliminary experiments, based on rat-growth tests, to be somewhat richer in vitamin B₁ than an aqueous extract of baker's yeast. The method has been standardised up to the platinum stage by several repetitions. The preparation obtained at this stage promotes good growth in young rats in a daily dose of 0.075–0.10 mg. It is expected that the material collected by this method of fractionation in several operations might be further fractionated with advantage. Incidentally the relation, if any, between vitamin B₁ on the one hand and secretin, co-enzyme and bios on the other has been investigated. The absorption spectra and certain other properties of B₁ concentrates are also described.

EXPERIMENTAL.

Biological technique.

The method of biological assay described before [Guha and Drummond, 1929] has been used in these experiments with a few modifications. Young rats, about 50 g. in weight, were fed on a basal diet of the following composition:

Rice starch	...	...	...	...	65 %
"Light white casein" (B.D.H.)	...	...	...	...	21 %
Palm kernel oil	...	...	...	...	10 %
Salt mixture (Osborne and Mendel)	...	...	...	...	4 %

This diet was supplemented daily with 2 drops of cod-liver oil and 1.5 cc. of a 50 % solution of autoclaved marmite in order to supply vitamins A, D and B₂. The latter preparation was obtained by mixing the commercial yeast extract, marmite, with baryta, bringing the mixture roughly to p_H 9 and autoclaving for 1½ hours at 124°, barium being finally removed by means of sulphuric acid. Numerous experiments have proved that such a preparation contains large quantities of vitamin B₂ and negligible quantities of B₁. When the weight of the young animals began to decline on this diet they were fed with the vitamin B₁ fraction to be tested, and the standard of growth was taken to be at least 10–12 g. per week. Each fraction was tested on at least 2 rats, usually more. Rat-growth tests were adopted as a matter of routine, while these were periodically checked by pigeon-curative tests. The pigeon-curative day-dose was computed by dividing the amount injected by the number of days for which protection was afforded.

The technique of assaying vitamin B₁ has recently been discussed by several investigators [Smith, 1930; Kinnersley, Peters and Reader, 1930].

Smith describes an apparently promising method, based on curative tests with rats, by which he seems to have obtained very regular results. In our experience, rat-growth tests have yielded fairly uniform results. Fig. 1 illustrates the fact that under strictly controlled conditions it is possible to get

Fig. 1. Experiments with an aqueous-alcoholic extract of brewer's yeast.

Curve (1). ↑ dose=0.15 cc. Curve (2). ↑ dose=0.225 cc.
 „ (3). ↑ dose=0.33 cc. „ (4). ↑ dose=0.45 cc.

approximately proportionate growth responses to graded doses of a given vitamin B₁ preparation. We have adhered to this method in order to have results comparable with those obtained from wheat germ. It is possible, however, that at the last stages of fractionation, Smith's curative method might give more trustworthy results. It would, doubtless, be more economical.

Tests with known substances.

In the earlier paper, it was stated that various substances like nucleic acid, betaine, nicotinic acid, which had at one time or other been credited with properties of B₁, were found to be inactive by the rat-growth tests. The following substances have now been fed in the doses stated, again with negative results.

Substance tested						Dose (mg.)	
Adenine picrate	...	...	...	...	...	2	and 4
Guanine hydrochloride	...	...	...	...	...	2	„ 4
Uracil	...	...	...	...	...	2	„ 4
Synthetic <i>dl</i> -thyroxine	...	...	...	...	...	0.5	„ 1
Histamine acid phosphate	...	...	...	...	...	0.1	„ 0.2
"Adrenalinum" (B.D.H.) (faintly acidified with HCl)						0.1	„ 0.2

The relative vitamin B₁ values of extracts of brewer's top yeast and baker's yeast.

Aqueous extracts of brewer's top yeast and baker's yeast, made by extraction with boiling water and boiling for 3 minutes were, compared for vitamin B₁ activity. The brewer's yeast gave somewhat more active extracts

in most cases. One sample of baker's yeast was, however, found particularly rich in vitamin B₁ (Fig. 2). It is probable that there is a considerable variation

Fig. 2. Tests with aqueous extracts of brewer's top yeast and baker's yeast.

Curve (1). ↑ dose $\equiv$ 0.5 g. baker's yeast.

„ (2). ↑ dose $\equiv$ 0.5 g. brewer's top yeast.

„ (3) illustrates the potency of a particularly active variety of baker's yeast;
↑ dose $\equiv$ 0.5 g. yeast.

in the vitamin B₁ potency of different samples of both varieties of yeast [see also Quinn, Whalen and Hartley, 1930]. Pirie's method of extraction of yeast for glutathione [Pirie, 1930] also extracts vitamin B₁ effectively from both brewer's and baker's yeast.

Procedure of fractionation.

Brewer's yeast was employed for the fractionation of vitamin B₁ recorded in this paper. This method has met with success up to the platinum stage every time it was tried, though, unfortunately, the same degree of concentration was not achieved at this stage as had been obtained with wheat germ [Guha and Drummond, 1929]. The following account represents a fairly standardised method.

Stage 1. Extraction with aqueous alcohol. 3178 g. of fresh brewer's top yeast were extracted with 2.5 l. of 95 % alcohol at 60–70° for 4–6 hours. The residue, after filtration, was re-extracted with 1.75 l. of 50 % alcohol at the same temperature for the same period. After concentration under reduced pressure, the first extract was active in a daily dose, representing 0.64 g. of the fresh undried yeast, which contained 40 mg. total solids, of which 33.5 mg. were organic. The active dose of the second extract was equivalent to 1.6 g. of the fresh yeast and carried 25 mg. of the total solids, of which 22.4 mg. were organic (Fig. 3).

Stage 2. Fractionation by lead acetate. The two extracts of the preceding stage were combined and precipitated with 120–150 g. of neutral lead acetate in aqueous solution. The filtrate from the lead precipitate was freed from lead

by hydrogen sulphide in a slightly acid medium, the hydrogen sulphide being later removed by concentration *in vacuo* to a volume of 1500 cc. This material was active in daily doses containing 40 mg. of total solids, of which 30 mg. were organic, equivalent to 0.75 g. of the fresh yeast (Fig. 3).

Fig. 3.

- Curve (1). The 95 % alcoholic extract of brewer's yeast; ↑ dose ≡ 0.64 g. of fresh yeast.
 „ (2). The second 50 % alcoholic extract of brewer's yeast; ↑ dose ≡ 1.6 g. of fresh yeast.
 „ (3). The lead filtrate; ↑ dose ≡ 0.75 g. yeast.
 „ (4). The fuller's earth extract; ↑ dose ≡ 1.38 g. yeast.
 „ (5). Silver nitrate-baryta fraction at p_H 6.8; ↑ dose ≡ 1.5 g. yeast.

Pigeon-curative tests with 4 birds showed that the average day-dose contained 12 mg. total solids of which 9.3 mg. were organic.

Stage 3. Adsorption by fuller's earth. The lead filtrate was brought, if necessary, to p_H 4.5 and agitated for 20 minutes with 50 g. fuller's earth¹ and left overnight. The supernatant fluid was syphoned off and the bottom layer sucked dry. The fuller's earth residue, which weighed 67 g., was extracted by grinding up with 200 cc. of saturated baryta and immediately filtering with strong suction. The total operation of grinding and filtering took 5–7 minutes. The filtrate was received in a slight excess of concentrated sulphuric acid. The fuller's earth residue was washed with a little baryta and then with water. After the removal of the precipitated barium sulphate from the filtrate, the fraction proved to be active in doses containing 1.4 mg. total solids, of which 1.3 mg. were organic. The dose was equivalent to 1.38 g. yeast (Fig. 3). Curative tests with 3 pigeons showed that the average day-dose contained 0.57 mg. total solids of which 0.53 mg. was organic.

Stage 4. Fractionation by silver nitrate and baryta. The above fuller's earth extract, which was acid to Congo red, was treated with 15 cc. of 50 % silver nitrate solution, which represented an excess, and then treated with the requisite quantity of saturated baryta to bring the p_H to 4.5 and allowed to settle for 2 hours. The precipitate which was very slightly active was discarded

¹ In our experience different samples of fuller's earth have been found to differ greatly in their adsorbing capacity. It is therefore desirable to stock a quantity of fuller's earth, after its suitability has been established. In these experiments Boots's fuller's earth was used. Experiments with the "acid clay" used by Jansen and Donath show that it is also a very efficient adsorbent for the vitamin.

and the filtrate brought to p_H 6.8 by adding the requisite amount of saturated baryta. After being allowed to stand overnight, the brown precipitate was filtered off (wet weight 5 g.) and decomposed by hydrochloric acid. On filtration, a straw-coloured filtrate was obtained, of which the rat day-dose contained 0.345 mg. total solids, of which 0.29 mg. was organic matter (Fig. 3). This was equivalent to 1.5 g. yeast. Further fractionation of the main filtrate at p_H 8 yielded negative results. Curative tests on 2 pigeons with the silver fraction precipitated at p_H 6.8 showed that the average day-dose contained 0.16 mg. total solids of which 0.13 mg. was organic.

Stage 5. Fractionation by phosphotungstic acid. The fraction obtained at p_H 6.8, which is thus seen to contain almost all the activity, had a volume of 200 cc., and this was treated with the required quantity of concentrated H_2SO_4 to make roughly 5 % acid, and then with 14 cc. of a saturated solution of phosphotungstic acid in 5 % H_2SO_4 , which represented a slight excess. The precipitate was removed after 24 hours, filtered under strong suction (dry weight = 3.5 g.), dissolved in 70 cc. of 50 % acetone and filtered from a very small amount of undissolved residue. The clear red-brown filtrate was treated gradually with 50 cc. of 5 % H_2SO_4 with constant stirring. At this stage a slight turbidity remained, which did not disappear on warming to 60° . A gummy material separated, which, on leaving the mixture for a week in the refrigerator, turned granular. This orange-yellow precipitate (*a*) was filtered, sucked dry (weight = 1 g.) and decomposed by dissolving in 50 cc. of 50 % acetone and shaking up with 30–40 cc. baryta, which were sufficient to make the solution alkaline to phenolphthalein. The precipitate of barium phosphotungstate was filtered off immediately and the filtrate acidified with sulphuric acid. After filtration from $BaSO_4$ and the removal of acetone *in vacuo* the clear pale straw-coloured filtrate proved to be active in a daily dose containing 0.29 mg. total solids, equivalent to 1.6 g. yeast (Fig. 4)¹. The filtrate obtained after removal of precipitate (*a*) on treatment with an excess (300 cc.) of 5 % H_2SO_4 yielded a further quantity of precipitate, which was found to be inactive.

The filtrate obtained after the removal of the original phosphotungstic precipitate, on standing in the refrigerator for several days, deposited a very small amount of colourless crystals which appeared as a homogeneous mass of rhombohedra under the microscope. After decomposition by baryta, it proved to be entirely inactive. On evaporation and examination it was found to be crystalline and homogeneous. In view of the small amount of material, however, its identity was not further investigated.

¹ It is not easy to understand why Kinnersley and Peters [1930] failed to obtain the vitamin in the acetone-soluble phosphotungstic fraction. The phosphotungstates of the vitamin fractions from wheat germ [Guha and Drummond, 1929], from rice polishings [Jansen and Donath, 1926], as also from brewer's yeast (as shown in the present communication) are apparently acetone-soluble. It is possible, however, that this solubility is not a property of the vitamin *per se* but is due to the presence of the acetone-soluble phosphotungstates of other materials, which may not be precipitated under the somewhat altered conditions of Kinnersley and Peters.

The average pigeon-curative day-dose of the active phosphotungstic acid fraction, tested with 3 birds, contained 0.052 mg. total solids.

Stage 6. Extraction with absolute alcohol. The above active phosphotungstic fraction was evaporated to dryness in a vacuum desiccator containing soda lime and was extracted four times with hot (70–80°) absolute alcohol. The pale straw-coloured extract, after filtration, measured 116 cc., and a portion of it, after removal of alcohol, was found to be active in doses containing 0.274 mg. total solids, equivalent to 1.76 g. yeast (Fig. 4).

Fig. 4.

- Curve (1). Phosphotungstic acid fraction; ↑ dose ≡ 1.6 g. yeast.
 „ (2). Alcoholic fraction; ↑ dose ≡ 1.76 g. yeast.
 „ (3). Platinum precipitate fraction; ↑ dose ≡ 2.37 g. yeast.
 „ (4). Platinum precipitate fraction; ↑ dose ≡ 3.16 g. yeast.

Stage 7. Fractionation by platinic chloride. The alcoholic extract was concentrated in a vacuum desiccator to a small bulk, and, after the removal of a light pale yellow precipitate which had separated, the filtrate (30 cc.) was treated with 6.5 cc. (excess) of a 5 % solution of platinic chloride in absolute alcohol. It rapidly deposited a micro-crystalline orange-yellow precipitate, which was left in the refrigerator overnight. As previous experiments showed that decomposition of the platinum precipitate by hydrogen sulphide frequently results in a considerable loss of activity¹, the platinum precipitate, after being filtered off and washed with a little absolute alcohol, was decomposed by means of potassium chloride by the method of Ewins [1914]. The platinum filtrate was also similarly decomposed. The platinum precipitate was active in doses containing 0.075–0.10 mg. total solids, equivalent to 2.37–3.16 g. yeast (Fig. 4). The platinum filtrate was entirely inactive in doses equivalent to 5 g. yeast. A mixture of half the effective dose of the platinum precipitate and a dose of the platinum filtrate, equivalent to 2.5 g. of original yeast, was almost entirely inactive.

Curative tests with 5 birds showed that the average day-dose of the platinum precipitate fraction contained 0.047 mg. total solids.

¹ In two instances about 80 % of the activity disappeared on treatment of the platinum precipitate with hydrogen sulphide. Such a large loss under the same treatment was not found to occur with wheat germ fractions [cf. Guha and Drummond, 1929], though the KCl method is undoubtedly a better one for such purposes.

Stage 8. Fractionation by gold chloride. The solution obtained by the decomposition of the platinum precipitate was evaporated to dryness in a vacuum desiccator. The residue was extracted with 13 cc. of hot absolute alcohol, filtered from a small amount of insoluble residue, and treated with 2 cc. of a 4 % solution of gold chloride in absolute alcohol. As no precipitate was formed, the solution was concentrated to a very small volume (0.5 cc.) in a desiccator, and then filtered from a very minute amount of an orange-yellow semi-crystalline precipitate. The gold precipitate, thus formed, was much more soluble in alcohol than in water.

The gold precipitate was dissolved in 10 cc. of 90 % alcohol and shaken up with precipitated metallic silver, according to the method of Dudley [1929], to precipitate the gold. The gold filtrate was also similarly freed from gold. The above operation, however, resulted in a great loss of activity. The fraction from the gold precipitate produced only slight growth in doses containing 0.054 mg. total solids, equivalent to 6 g. of the original yeast, while the gold filtrate was inactive in the same dose but produced slight growth in doses, containing 0.2 mg. total solids, equivalent to 11.2 g. of yeast. A mixture of 0.0357 mg. of the gold precipitate and 0.1 mg. of the gold filtrate resulted also in very slight growth (Fig. 5).

Fig. 5.

- Curve (1). Gold precipitate fraction; ↑ dose = 0.054 mg. total solids.
 „ (2). Gold filtrate fraction; ↑ dose = 0.2 mg. total solids.
 „ (3). ↑ dose = 0.0357 mg. of the gold precipitate fraction *plus* 0.1 mg. of the gold filtrate fraction.

Curiously, though the rat-growth tests gave such unsatisfactory results, the pigeon-curative tests indicated that there was quite considerable activity in both the gold precipitate and filtrate fractions, the former having twice

Gold precipitate fraction			Gold filtrate fraction			Gold precipitate fraction (g.p.) <i>plus</i> Gold filtrate fraction (g.f.)			
Pigeon no.	Dose injected (mg.)	Days of protection	Pigeon no.	Dose injected (mg.)	Days of protection	Pigeon no.	Dose injected (mg.)		Days of protection
							g.p.	g.f.	
1	0.05	3	1	0.1	8	1	0.05 + 0.05		8
2	0.05	2	2	0.15	2	2	0.05 + 0.05		5
3	0.05	6	3	0.25	8	3	0.05 + 0.05		9
4	0.05	7	4	0.3	16				
Average day-dose = 0.012 mg.			Average day-dose = 0.024 mg.			Average day-dose = 0.014 mg.			

the activity of the latter. A combination of the two fractions did not show any markedly enhanced effect. These results are shown in the above tables.

These curious results are discussed later.

Notes on the course of fractionation.

(1) The extraction of the brewer's yeast, if carried out simply by boiling with water, also gives satisfactory results and the fractionation can be followed to the platinum stage as described above.

(2) The precipitation by lead acetate at Stage 2 does not effect any considerable concentration because, while some inactive material is removed, a certain amount of loss occurs due, probably, to adsorption. But unless this preliminary purification is carried out, the subsequent fractionation by fuller's earth at Stage 3 does not appear to be so satisfactory. Adsorption by charcoal also ceases to be selective and occurs imperfectly at p_H 3, 4, 5 and 6, unless the lead stage is introduced. Kinnersley and Peters [1928] insert the lead stage before adsorption with charcoal.

(3) Levene and Van der Hoeven [1924] considered that barium ions precipitate a portion at least of the vitamin. Seidell [1926] was, therefore, led to the use of sodium hydroxide instead of saturated baryta for the elution of the vitamin from the "activated" fuller's earth. As it was not desired to introduce soluble inorganic material unnecessarily, the question was re-investigated. Under identical conditions elutions by equal volumes of $N/8$ NaOH and saturated baryta are equally effective, though one extraction by $N/8$ NaOH is more effective than one by $N/8$ Ba(OH)₂. There does not therefore appear to be any ground for assuming that the vitamin forms an insoluble complex with barium ions.

(4) It has been found that kieselguhr at p_H 4.5, can also adsorb the vitamin which can be eluted by saturated baryta. The adsorption by kieselguhr is, however, far less efficient than that by fuller's earth, as only about one-third of the vitamin is adsorbed by the former.

Vitamin B₁ and secretin.

Three different preparations of vitamin B₁, one obtained at Stage 1 by aqueous-alcoholic extraction, another at Stage 2 after lead fractionation and a third prepared by extracting with water, adsorbing with norite charcoal and fractionating with phosphotungstic acid, which were all highly active, were examined for their secretagogue activity. The tests were kindly carried out by Dr Brocklehurst and Mr Cunningham on amytal-anaesthetised cats. The third concentrated preparation did not show any appreciable effect at all, while the first two impure preparations had such a great depressor effect that in one case the animal was almost instantly killed. The nature of this depressor principle is not understood.

These observations confirm the earlier experiments of Anrep and Drummond [1921], as opposed to those of Uhlmann [1918] and of Voegtlin and Myers [1919].

Vitamin B₁ and "bios."

In confirmation of the earlier observations with wheat germ preparations [Guha and Drummond, 1929] it has been found that the "bios" activity of the yeast fraction obtained at Stage 5 is negligible, though this latter fraction was potent for rats in a daily dose of 0.3 mg. It is, however, of interest to point out in this connection that Williams and Roehm [1930] consider that while vitamin B₁ cannot stimulate the growth of yeast by itself, it can do so in conjunction with a material obtained from yeast extracts, which is not adsorbed by fuller's earth. Jansen and Donath's crystals are stated to exhibit this effect in minute amounts. In fact, from a parallel study of the growth of different varieties of yeast, Williams and Bradway [1931] have come to the conclusion that while "bios," as required by Wildier's strain of yeast and presumably by Narayanan's [1930], has not been fractionated, the strain used by the Toronto workers requires multiple factors, of which apparently inositol is one. Williams and Bradway consider that there are four "bios" factors and there might be some relation between vitamin B₁ and one of these. The writer's observations do not cover this point, as the tests were carried out with the same strain of yeast as was used by Narayanan.

Vitamin B₁ and co-enzyme.

Kinnersley and Peters [1928] and Euler and Myrbäck [1929] consider that vitamin B₁ and co-enzyme are not identical. As the most potent co-enzyme preparations have been found to give a strong Molisch reaction and to contain phosphorus [Euler and Myrbäck, 1930] these properties have been investigated in some of our preparations.

2 cc. of the fraction obtained from the gold precipitate, which contained at least 10 rat day-doses, gave a negative reaction. Of the fractions obtained by the electrodialysis of a purified vitamin B₁ concentrate at p_H 8.5 (Birch and Guha, unpublished results) the fraction obtained in the cathode compartment, which contained at least 40 % of the activity, gave a negative Molisch reaction, while the fraction of the middle compartment gave a positive one. 2 cc. of a preparation obtained from rice polishings, potent for rats in 0.02 cc., kindly supplied by Prof. J. C. Drummond, which had been concentrated up to the platinum stage, still gave a positive reaction. This indicates, incidentally, that certain neutral impurities may be got rid of more readily by electrodialysis under suitable conditions than by metallic fractionation. Unlike co-enzyme preparations, however, Drummond's concentrate was practically phosphorus-free. All these observations indicate that vitamin B₁ is probably not very closely related in structure to co-enzyme. Also co-enzyme is more readily destroyed by heat under certain conditions than vitamin B₁ and is susceptible to the action of nucleotidases. Vitamin B₁ does not appear to be very sensitive to the action of enzymes.

The absorption spectra of certain vitamin concentrates.

It was considered of interest to investigate whether vitamin B₁ concentrates show any selective absorption, which may be attributed to the vitamin. A search of the literature showed that Damianovich [1922] had observed an absorption band between 248 and 260 $\mu\mu$ given by an active brewer's yeast extract, which appeared to indicate pyrimidine compounds. Mukherji [1929] has recently observed that the crystals, which he obtained from rice polishings by Jansen and Donath's procedure, exhibited marked absorption at 390–330 $\mu\mu$.

The absorption spectra of (1) the cathode fraction obtained from yeast by fractionating up to Stage 5 and then electrolysing at p_H 8.5, and of (2) Drummond's rice-polishings concentrate, were examined with the ultra-violet spectrophotometer of the Hilger pattern (Fig. 6). The yeast concentrate

Fig. 6.

Curve (1). The absorption spectrum of the electrodialysed yeast fraction. 100 cc. of the solution contained 40 rat day-doses. Thickness of cell = 2 cm.

Curve (2). The absorption spectrum of Drummond's rice-polishings concentrate. 100 cc. of the solution contained 500 rat day-doses. Thickness of cell = 2 cm.

showed a very strong absorption in the region of 260 $\mu\mu$, which apparently accords with the observation of Damianovich. The same type of band, however, was observed by Dixon and Keilin to be given by a protein precipitate obtained by treatment with sulphur dioxide in course of fractionation for cytochrome from baker's yeast (private communication). This substance was kindly provided by Dr Keilin and, on being tested, it was found to be devoid of vitamin B₁ potency even in 10 mg. doses. This band is therefore not specific for B₁. The nature of the substance causing this absorption is unknown. Drummond's concentrate, which was much more potent than the electrolysated fraction, did not show this band (it was examined in three dilutions). The band observed by Mukherji was not shown markedly by either of the fractions. It is unlikely that a study of the absorption spectra of vitamin B concentrates will yield any significant results. It should be pointed out that

if vitamin B₁ has the glyoxaline structure as suggested by Jansen and Donath [1926], it may not have any selective absorption, as is indicated by the observations of Marchlewski and Nowotowna [1925-6] on histidine.

Certain other properties of the vitamin concentrates.

1. *Behaviour towards flavianic acid.*

The fuller's earth extract did not give any appreciable precipitate with flavianic acid even after standing for 5 hours.

2. *Optical activity.*

The phosphotungstic fraction was examined for optical activity with reference to the mercury green line and at 18°, but there was no perceptible rotation, while the fraction obtained at the lead stage showed only a slight activity.

3. *Ninhydrin and Adamkiewicz-Hopkins reactions.*

The preparation obtained after fractionation with phosphotungstic acid and alcohol gave a negative ninhydrin reaction. A fraction purified by electro-dialysis at p_H 8.5 gave a very feeble Adamkiewicz-Hopkins reaction, while Drummond's fraction obtained from rice polishings, did not give this reaction.

4. *Pauly reaction.*

The Pauly reaction given by Jansen and Donath's crystals was one of the grounds on which they ascribed a glyoxaline structure to the vitamin [Jansen and Donath, 1926]. This reaction is given rather more strongly by the potent vitamin B₁ concentrates from yeast obtained by Kinnersley and Peters, and both reactions are less intense than that given by histidine [Jansen *et al.*, 1930]. We had observed a strong Pauly reaction with our most potent concentrate from wheat germ [Guha and Drummond, 1929]. The preparations obtained at the platinum stage by the method described in this paper have been found to give a very poor Pauly reaction compared with histidine. This reaction as given by these different concentrates cannot, therefore, be considered as trustworthy evidence about the chemical nature of vitamin B₁.

DISCUSSION.

The fractionation described in this paper gives very regular results, if followed in detail. It is difficult to understand the phenomenon observed at the stage of gold fractionation. The rat-growth tests failed to agree well with the curative tests on pigeons. One explanation might be that there is a difference between the rat-growth factor (or factors) and the pigeon curative factor (or factors). On the other hand, it is necessary to point out that the particular pigeon tests with the gold fractions showed very large individual variations and it is probable that the results are untrustworthy. This error could only be partially eliminated if an unusually large number of pigeons

were used. The results obtained with the rats are much more regular and these indicate that there is an enormous loss of vitamin B₁ at the gold fractionation stage, and the supplementary effect of the fractions obtained from the gold precipitate and filtrate, if any, cannot be expected to be observed under the circumstances. The question therefore must be considered still unsettled.

About the actual chemical nature of vitamin B₁ nothing can yet be said with confidence. Jansen and Donath's crystals have now been stated to contain vitamin B₄ [Jansen *et al.*, 1930]. Williams, Waterman and Gurin [1930] in a recent investigation could not obtain the pure crystals, while Mukherji [1929] states that by following Jansen and Donath's procedure in the main and working with rice polishings he has obtained crystals identical with those described by Jansen and Donath. It is not clear, however, that the crystals were pure. The tests, which were carried out on rice-birds (*Munia maja*) showed that 30 mg. of the crystals per day were effective for protection from "polyneuritis." Lower doses do not appear to have been tried. The Pauly reaction given by different vitamin B₁ concentrates from rice polishings and yeast cannot yet safely be attributed to the vitamin *per se*.

SUMMARY.

1. Adenine, guanine, uracil, *dl*-thyroxine, histamine and adrenaline cannot replace vitamin B₁ in the diet of rats.
2. A fairly standardised method of concentration of vitamin B₁ is described by which, starting from brewer's yeast, a preparation can be obtained at the platinum stage, which promotes good growth in rats in a daily dose of 0.075–0.1 mg. when supplemented by vitamin B₂. Its pigeon-curative day-dose is of the order of 0.047 mg.
3. No clear-cut answer has been obtained to the question whether fractionation by gold chloride would provide any evidence for the splitting of vitamin B₁. The rat-growth tests and pigeon-curative tests of these fractions show serious discrepancies.
4. Vitamin B₁ does not appear to be closely chemically related to co-enzyme. Its relation to secretin and "bios" is discussed.
5. Some vitamin B₁ concentrates are optically inactive and give negative ninhydrin and Adamkiewicz-Hopkins reactions. The Pauly reaction given by the purer yeast concentrates is comparatively weak. Vitamin B₁ is not apparently precipitated by flavianic acid from a fairly concentrated preparation.
6. The absorption spectra of certain concentrates are described.

I wish to express my thanks to Sir F. G. Hopkins and Prof. J. C. Drummond for their stimulating interest, and also to Dr M. Dixon for his interest and generous help in the work on the absorption spectra of the concentrates.

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