CV. INVESTIGATIONS ON VITAMIN B_2.

I. THE SOURCES OF VITAMIN B_2.
II. THE STABILITY OF VITAMIN B_2.
III. THE CHEMISTRY OF VITAMIN B_2.

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Since the recognition of the presence of at least two factors, B_1 and B_2, in the dietary complex known as "vitamin B," little work has been published on the nature and behaviour of vitamin B_2. The present investigation was prompted by the need for fuller knowledge of this subject. Preliminary reports of some of the results have appeared elsewhere [Guha, 1931, 2, 3].

Biological technique.

Young rats of about 50 g. in weight were kept in separate cages with screened bottoms and were fed on the basal diet described in a preceding paper [Guha, 1931, 1]. Each animal received separately in a dish a daily dose of 2 drops of cod-liver oil and of a preparation of vitamin B_1 from the beginning of the experimental feeding period. The B_1-preparation was obtained from brewer's top yeast by extracting with aqueous alcohol and then fractionating with neutral lead acetate [Guha, 1931, 1]. In some experiments the filtrate from the lead precipitate was further purified by adsorption on fuller's earth by the method described before. In others, however, the lead filtrate itself was used as the source of vitamin B_1 and was fed in a daily dose of 0·5 c.c., equivalent to 1–1·5 g. of the fresh yeast. This preparation contained a small amount of vitamin B_2, but as both positive and negative controls were kept, the results were checked throughout. Rats on the above dietary began to decline in weight in 2–4 weeks from the commencement of the experiment, according to the reserve of vitamin B_2 in their bodies. Test fractions were fed at this stage and if growth occurred at a rate of 10–12 g. per week for 2–3 weeks, the fractions were considered to be active. Fig. 1 shows that the B_1-preparation (lead filtrate) cannot by itself produce growth in 0·5 cc., but can produce slow growth in 3–4 times that dose. If, however, 0·5 cc. of the B_1-preparation is supplemented by 1 cc. of a 50 % solution of alkalised marmite [Guha, 1931, 1] as a source of vitamin B_2, good growth is obtained, whereas the alkalised marmite by itself cannot sustain growth even in a daily dose of 4 cc.

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The B_1-preparation made by means of fuller's earth is almost entirely devoid of vitamin B_2 and is probably to be preferred in all tests for vitamin B_2. The lead filtrate, however, is likely to contain other factors [Reader, 1930], which might be an advantage.

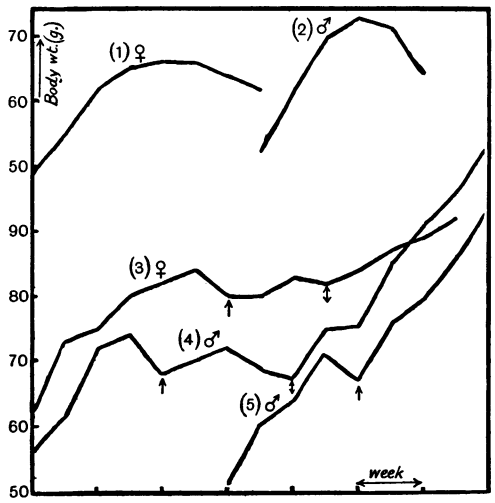

Fig. 1.

Curve (1). 0·5 cc. of vitamin B_1 preparation (lead filtrate) from the beginning of experiment.
Curve (2). 1 cc. of vitamin B_2 preparation (alkalised marmite) from the beginning of experiment.
Curve (3). 0·5 cc. of lead filtrate from the beginning. At ↑ given 1 cc. of lead filtrate. At ↕ given 3·0 cc. of lead filtrate.
Curve (4). 1 cc. of alkalised marmite from the beginning. At ↑ 4 cc. of the same. At ↕ 1 cc. of alkalised marmite *plus* 0·5 cc. of lead filtrate.
Curve (5). 1 cc. of alkalised marmite from the beginning. At ↑ 0·5 c.c. of lead filtrate added.

No case of refection was noticed in these experiments though uncooked rice starch was used in the basal diet. It is our custom, however, to check the results by observing whether the withdrawal of an active supplement is followed by a decline in weight.

I. The sources of vitamin B_2.

It was early realised that the recognition of the dual nature of "vitamin B" required a revision of the "vitamin B" values of foodstuffs, especially where these values were obtained by growth tests on rats. Aykroyd and Roscoe [1929] and Roscoe [1930] have recently assayed vitamin B_2 in certain materials.

The present estimation of vitamin B_2 in certain raw materials was carried out with the purpose of finding out which of these would be most useful for the extraction of the vitamin. Aqueous extracts of brewer's top yeast, baker's yeast, ox-liver, beef-muscle, commercial liver extract (Eli Lilly, No. 343)[1], and milk powder were examined.

[1] I am indebted to Dr C. Elvehjem for the commercial liver extract used in this research.

Brewer's yeast, baker's yeast, ox-liver and beef-muscle were extracted under identical conditions, the liver and muscle being finely minced before extraction. 300 g. of each of them were stirred gradually into 480 cc. of boiling distilled water and the boiling continued for 3 minutes.

These were filtered under suction and washed each with 100 cc. water. The muscle gave a practically colourless solution, while the liver yielded an opalescent brownish liquid with a faint green fluorescence.

Eli Lilly's commercial liver extract was fed both in the solid state and also as an aqueous extract which was prepared by stirring 50 g. of the liver extract with 200 cc. of cold water. This was filtered under suction from a small amount of insoluble residue which was washed with a little water. The deep brown-red filtrate was made up to 250 cc. to make a 20 % solution. The residue was apparently a remnant of the liver protein. It dissolved in $N/10$ NaOH and was precipitated by the careful addition of $N/10$ HCl at p_H about 4·6, which is close to the isoelectric point of the liver protein. Both the residue and the aqueous extract were tested for activity.

"Glaxo" milk powder, which was prepared from English winter milk, was fed both in the solid state and in an aqueous medium.

The results of these tests are shown in Figs. 2 and 3. It will be observed

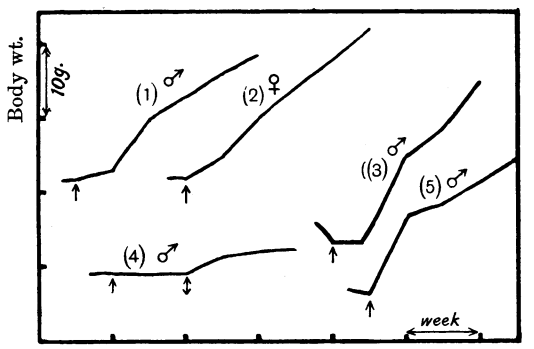

Fig. 2.

Curve (1). ↑ dose≡1·4 g. fresh brewer's yeast.
Curve (2). ↑ dose≡0·7 g. fresh baker's yeast.
Curve (3). ↑ dose≡0·94 g. fresh baker's yeast.
Curve (4). ↑ dose≡0·9 g. fresh beef muscle; ↕ dose≡1·8 g. fresh beef muscle.
Curve (5). ↑ dose=0·4 g. milk powder.

that the extract of baker's yeast is markedly richer in vitamin B_2 than that of the brewer's yeast, the former giving as good growth in a dose corresponding to 0·7–0·9 g. of fresh yeast as the latter in a dose equivalent to 1·4 g. Some hope was entertained about milk powder as a raw material for the fractionation of vitamin B_2, as the major constituents of this material are known. But it proved to be active only in a dose as large as 0·4 g. The muscle extract was comparatively inactive, which is rather surprising, considering that, according to Goldberger, lean meat is a rich source of the antipellagra factor. Vitamin B_2 may, however, be present in muscle in a combination from which

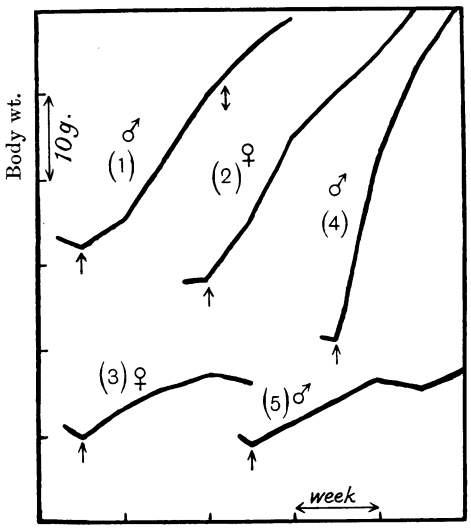

Fig. 3.

Curve (1). Aqueous extract of fresh ox-liver; ↑ dose≡0·3 g. of fresh liver (containing 6·0 mg. organic material); ↕ dose≡0·15 g. of fresh liver.
Curve (2). A cold aqueous extract of Eli Lilly's commercial liver concentrate; ↑ dose≡50 mg. of the liver concentrate.
Curve (3). The insoluble residue obtained after a cold aqueous extraction of the commercial liver concentrate; ↑ dose≡0·25 g. of the concentrate.
Curve (4). Commercial liver concentrate; ↑ dose=0·15 g.
Curve (5). Shows the vitamin B_1 potency of the cold aqueous extract of commercial liver concentrate; ↑ dose≡0·12 g. of the concentrate.

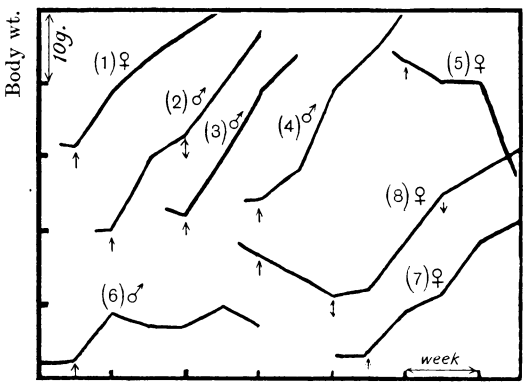

Fig. 4.

Curve (1). Aqueous extract of commercial liver concentrate, autoclaved at p_H 9·0 for ½ hour; ↑ dose≡60 mg. of the concentrate.
Curve (2). Same extract, autoclaved at p_H 9·0 for 3 hours; ↑ dose≡90 mg. of the concentrate; ↕ dose≡60 mg. of the concentrate.
Curve (3). Same extract, autoclaved at p_H 7·4 for 3 hours; ↑ dose≡60 mg. of the concentrate.
Curve (4). Same extract, autoclaved at p_H 6·2 for 3 hours; ↑ dose≡60 mg. of the concentrate.
Curve (5). Vitamin B_1 potency of the same extract, autoclaved at p_H 9·0 for ½ hour; ↑ dose≡180 mg. of the concentrate.
Curve (6). Brewer's yeast extract, autoclaved at p_H 9·0 for 3 hours; ↑ dose≡4·6 g. of fresh yeast.
Curve (7). Same extract, autoclaved at p_H 6·25 for 3 hours; ↑ dose≡1·7 g. of fresh yeast.
Curve (8). Aqueous extract of fresh ox-liver, autoclaved at p_H 9 for 3 hours; ↑ dose≡0·3 g. of fresh liver; ↕ dose≡0·9 g. of liver; ↓ dose≡0·6 g. of liver.

it is difficult to separate it by boiling water. The muscle residue was not tested for its activity. The aqueous extract of fresh ox-liver was extremely potent, being active in a daily dose containing less than 6 mg. total solids, which were equivalent to about 0·3 g. of the fresh liver. This extract should serve as a promising source of vitamin B_2 for fractionation and other purposes.

The cold aqueous extract of the commercial liver concentrate was highly active in a daily dose of 40–60 mg. The residue was practically inactive. This 20 % aqueous extract was used for the chemical study described in this paper. This material has some advantages over yeast, which has been so far generally used as a source of vitamin B_2. It is more potent, and it can be readily prepared from a stock of the solid liver concentrate whenever required. This ensures a greater uniformity of results. It is, moreover, comparatively poor in vitamin B_1, which is an advantage for certain purposes. The 20 % aqueous extract keeps well in the refrigerator.

II. The stability of vitamin B_2.

There has lately been a serious disagreement among different workers about the stability of vitamin B_2, especially to heat in an alkaline medium. Goldberger's work had shown that the factor concerned in the prevention of pellagra was relatively thermostable [Goldberger *et al.*, 1926]. In fact, the relative thermostability of vitamin B_2 has so far been the main factor in its differentiation from the more heat labile vitamin B_1. Other workers have also found that the vitamin B_2 potency of the commercial yeast extract, marmite, is not very seriously reduced by autoclaving for 3 hours at 120° at an alkaline reaction [Hassan and Drummond, 1927; Guha and Drummond, 1929; Narayanan and Drummond, 1930]. That a preparation of alkaline-autoclaved marmite can supplement a concentrated preparation of vitamin B_1 for the growth of vitamin B-deficient rats has been confirmed by us in hundreds of cases. On the other hand, other workers have stated that autoclaving in an alkaline medium destroys vitamin B_2 in brewer's yeast and its extracts, made by boiling with 0·01 % acetic acid, almost entirely [Williams, Waterman and Gurin, 1929; Chick and Roscoe, 1930].

It appeared to the writer at the outset that these discrepancies about the stability of vitamin B_2 might be due to the different behaviour of vitamin B_2 when present in association with different substances. That such is the case with vitamin B_1 is now well established [Kinnersley and Peters, 1928; Guha and Drummond, 1929]. An investigation was, therefore, instituted to find out the vitamin B_2 potency of some of the extracts, described in Part I, after autoclaving under identical conditions.

Aqueous extracts of brewer's top yeast, fresh ox-liver and the commercial liver concentrate were made as described and their p_H adjusted with $N/2$ NaOH. The treatment undergone by the extracts and the results on testing are summarised in the following table (see also Fig. 4).

Nature of material	Period of autoclaving (hours)	Temp. of autoclaving	Initial p_H	Final p_H	Percentage of inactivation (roughly)
Extract of fresh ox-liver	3	124–125°	9·0	6·2	75
Extract of commercial liver concentrate	3	124–125°	9·0	6·1	0
,, ,,	½	124–125°	9·0	6·5	0
,, ,,	3	124–125°	7·4	4·87	0
,, ,,	3	124–125°	6·2	4·65	0
Brewer's yeast extract	3	124–125°	9·0	7·5	90
,, ...	3	124–125°	6·25	5·62	0

Thus the curious fact is revealed that while vitamin B_2 in commercial liver extract and also in marmite [Hassan and Drummond, 1927] possesses a very high degree of stability, vitamin B_2 in the extracts made by boiling liver and fresh yeast with water is very markedly unstable to alkaline autoclaving. As the 20 % solution of the commercial liver extract contained about 5 times more solids than the brewer's yeast extract, the possibility could not be excluded that the greater dilution of the latter was helping its inactivation. The solution of the liver concentrate was therefore diluted 5 times and autoclaved at p_H 9 for 3 hours at 124–125°, when the p_H changed to 7·6. There was very slight inactivation, if any at all. It is probable that the stability of vitamin B_2 in a given preparation is due to the presence of some kind of protective material in it. Two possibilities present themselves: (1) that the commercial liver extract and marmite contain something which is added to them by the manufacturers and which has a protective action on vitamin B_2, (2) that there is a natural protective agent present in fresh liver and yeast, which is removed by the method of boiling with water, but which is not removed entirely in the manufacturing methods employed. The writer is inclined to think that the latter is the more probable explanation. The protective action of certain colloids, for instance of gelatin and saponin on gold sol, is well known.

Half an hour's autoclaving of the 20 % aqueous solution of the commercial liver extract at p_H 9 at 124–125°, while it did not affect vitamin B_2, destroyed vitamin B_1 almost entirely. Such a preparation would, therefore, appear very suitable for purposes where vitamin B_2, free from vitamin B_1, is needed. As this preparation contains much less solid matter and is apparently more palatable to the rats, it is to be preferred to autoclaved marmite.

III. The chemistry of vitamin B_2.

Few facts have been recorded in the literature on the concentration and chemistry of vitamin B_2. Chick and Roscoe [1929] observed that it was precipitated to a large extent from an aqueous yeast extract by neutral lead acetate. Narayanan and Drummond [1930] obtained a concentrate from yeast by successive fractionations with lead acetate and alcohol. Levene [1930] reports that he has obtained a preparation of vitamin B_2, which is potent for the growth of young rats in a daily dose of 0·7 mg.

The 20 % aqueous solution of Eli Lilly's liver extract, described in Part I, or the solid extract itself was used as the material for the present study. The potency of a fraction was, as usual, established by testing on at least two animals, frequently more. In no case was a serious discrepancy observed between the responses of two rats to a given fraction.

1. *Treatment with alcohol.* On treatment of 5 g. of the solid liver extract with 50 cc. of 58 % alcohol, most of the material dissolved giving a red solution. The residue (0·37 g.) was dissolved in water with a few drops of $N/10$ NaOH. About 65 % of the activity passed into the alcoholic extract, the rest being left in the residue (Fig. 5). There was no marked loss of activity

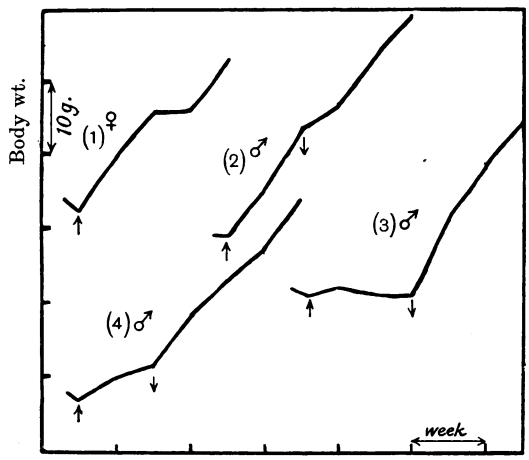

Fig. 5.

Curve (1). Fraction insoluble in 58 % alcohol; ↑ dose≡0·125 g. of the liver concentrate.
Curve (2). Fraction soluble in 58 % alcohol; ↑ dose≡0·125 g. of the liver concentrate; ↓ dose≡ 0·100 g. of the concentrate.
Curve (3). ↑ Picric acid precipitate, dose≡0·2 g. of the liver concentrate; ↓ picric acid filtrate, dose≡50 mg. of the liver concentrate.
Curve (4). Acetone-precipitated fraction; ↑ dose≡75 mg. of the liver concentrate; ↓ dose≡ 0·125 g. of concentrate (containing about 8 mg. organic material).

[*cf.* Chick and Copping, 1930]. But the treatment with 58 % alcohol does not apparently cause a clear-cut separation of vitamin B_2 from inactive material.

2. *Treatment with picric acid.* 100 cc. of the 20 % aqueous solution of the liver concentrate were treated with a moderate excess of a hot saturated aqueous solution of picric acid. After cooling, the clear red supernatant fluid was removed from the somewhat gummy precipitate, both were decomposed by hydrochloric acid and the picric acid was repeatedly extracted with ether and then with benzene. The material obtained from the picric acid precipitate was entirely inactive, while that from the picric acid filtrate retained practically all the activity (Fig. 5). This experiment shows that the vitamin is almost insoluble in ether and benzene. Most of the vitamin B_1 potency of the original liver extract was also obtained in the picric acid filtrate.

Fractionation with acetone. The picric acid filtrate which had a p_H of about 1, was treated with 6 times its volume of acetone, when an oily precipitate came down. After leaving the mixture in the refrigerator for 2 days in a corked flask, the clear reddish-orange supernatant liquid was poured off from the gummy brown residue. The residue, after being dried with a stream of air, was found to contain a little more than 50 % of the activity (Fig. 5). The mother-liquor was brought to p_H 6 and again treated with an excess of acetone, when a second oily precipitate separated, but both this precipitate and the filtrate were inactive.

The active fraction obtained by the first precipitation with acetone gave a negative purine test, a doubtful Millon's test and positive biuret and Adamkiewicz-Hopkins reactions. It also gave a very marked Molisch reaction, a fairly positive reaction with Tollen's reagent and a negative one with Bial's. It reduced Benedict's reagent and gave a positive sulphur reaction on prolonged boiling with lead acetate and concentrated sodium hydroxide.

The active picric acid filtrate fraction does not give a precipitate with flavianic acid even after standing for 3–4 hours.

3. *Action of benzoyl chloride.* 30 cc. of the 20 % solution of the liver concentrate were shaken up with 4 cc. of benzoyl chloride and excess of 20 % NaOH in the usual way. A considerable quantity of a yellow precipitate

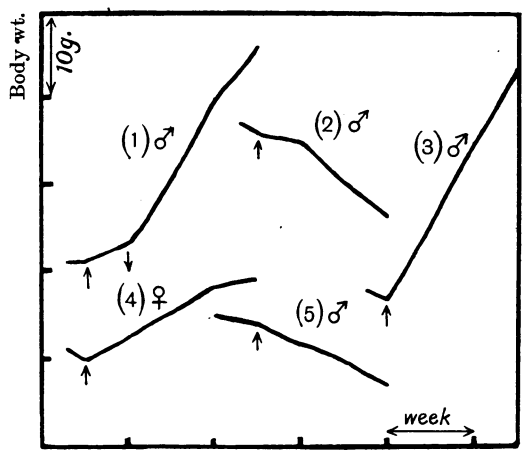

Fig. 6.

Curve (1). Filtrate from the precipitate obtained with benzoyl chloride; ↑ dose≡90 mg. of the liver concentrate; ↓ dose≡180 mg. of the concentrate.
Curve (2). The precipitate formed with benzoyl chloride; ↑ dose≡0·6 g. of the concentrate.
Curve (3). Filtrate from the precipitate obtained with nitrous acid; ↑ dose≡60 mg. of concentrate.
Curve (4). Filtrate from the precipitate obtained with phosphotungstic acid; ↑ dose≡0·3 g. of the concentrate.
Curve (5). Phosphotungstic acid precipitate fraction; ↑ dose≡0·3 g. of the concentrate.

came down, which was removed and dried in a desiccator (weight = 3·5 g.). The filtrate was treated with hydrochloric acid and the benzoic acid removed with ether. This filtrate retained about one-third of the activity, while the precipitate was inactive (Fig. 6).

4. *Action of nitrous acid.* Contrary to Levene [1928], Chick [1929] and Narayanan and Drummond [1930] reported that vitamin B_2 of yeast extracts was stable to nitrous acid. Vitamin B_2 of the 20 % solution of the liver concentrate was also found to be neither precipitated nor inactivated by nitrous acid.

30 cc. of the 20 % solution, acidified with hydrochloric acid, were treated in the usual way with a sufficient volume of a concentrated solution of sodium nitrite, as indicated by starch iodide paper. This was warmed at 60° for half an hour and the nitrous fumes expelled by boiling for 2 minutes in an open beaker. The precipitate, which had come down by treatment with nitrous acid, was inactive, while the filtrate retained the entire activity (Fig. 6).

5. *Reaction with phosphotungstic acid.* 25 cc. of the solution of liver extract were made 5 % acid with concentrated H_2SO_4 and treated with 45 cc. of saturated phosphotungstic acid in 5 % H_2SO_4. After standing overnight, the precipitate, which had removed most of the pigment from the solution, was filtered off. Both the precipitate and filtrate were freed from phosphotungstic acid by means of baryta, barium being finally removed with sulphuric acid. The precipitate fraction was entirely inactive, while the material obtained from the phosphotungstic filtrate was only very slightly active (Fig. 6). A combination of the precipitate and filtrate fractions did not give any more satisfactory results. It is highly probable that the vitamin was removed by adsorption on the surface of barium phosphotungstate and barium sulphate.

6. *Treatment with baryta.* 50 cc. of the 20 % solution were treated with 60 cc. of saturated baryta and left overnight. The resulting precipitate was removed and freed from barium by sulphuric acid (fraction *a*). The filtrate and washings from the barium precipitate were treated with an equal volume of alcohol and allowed to stand for 3 hours. The precipitate which had separated was filtered off and freed similarly from barium (fraction *b*). The filtrate from the second barium precipitate, which was still highly pigmented, was acidified with sulphuric acid and concentrated under reduced pressure (fraction *c*). Fractions (*a*) and (*b*) were inactive, while fraction (*c*) retained a little more than 30 % of the original activity (Fig. 7).

7. *Reaction with lead acetate.* 50 cc. of the aqueous liver extract, which was normally at p_H 4·6, on treatment with 20 cc. saturated neutral lead acetate (a slight excess) deposited a small quantity of precipitate. After standing overnight, this was removed and both precipitate and filtrate were decomposed by means of sulphuric acid. Vitamin B_2 was found to be partially precipitated by this procedure (Fig. 7).

Since treatment with lead acetate at p_H 4·6 did not cause a complete separation of vitamin B_2, the experiment was repeated at p_H 7 with no better results. The lead precipitates in either case were practically free from vitamin B_1. The lead precipitate fraction can thus be used as a source of vitamin B_2, free from vitamin B_1, but the method does not appear to have any great advantage over that described in Part II.

The lead precipitate fraction obtained at p_H 4·6 may be further fractionated by bringing it to p_H 6·8 and then treating it with 6 volumes of acetone. The active material is again precipitated as in the case of the picric acid precipitate.

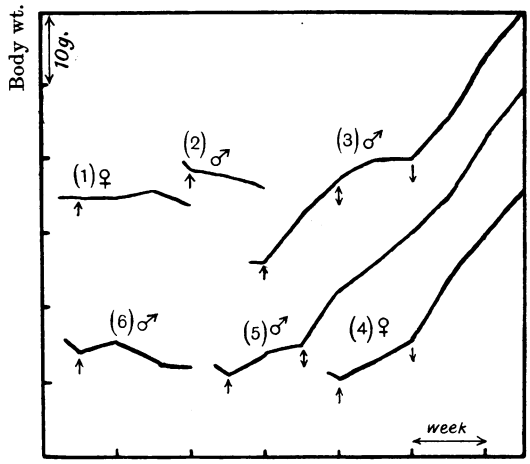

Fig. 7.

Curve (1). Fractionation with baryta; fraction *a*; ↑ dose≡0·2 g. of liver concentrate.
Curve (2). Fractionation with baryta; fraction *b*; ↑ dose≡0·2 g. of liver concentrate.
Curve (3). Fractionation with baryta; fraction *c*; ↑ dose≡0·2 g. of concentrate; ↕ dose≡0·1 g. of concentrate; ↓ dose≡0·15 g. of concentrate.
Curve (4). Lead filtrate; ↑ dose≡0·1 g. of concentrate; ↓ dose≡0·2 g. of concentrate.
Curve (5). Lead precipitate; ↑ dose≡0·1 g. of concentrate; ↕ dose≡0·15 g. of concentrate.
Curve (6). Shows the vitamin B_1 potency of the lead precipitate fraction; ↑ dose≡0·2 g. of concentrate.

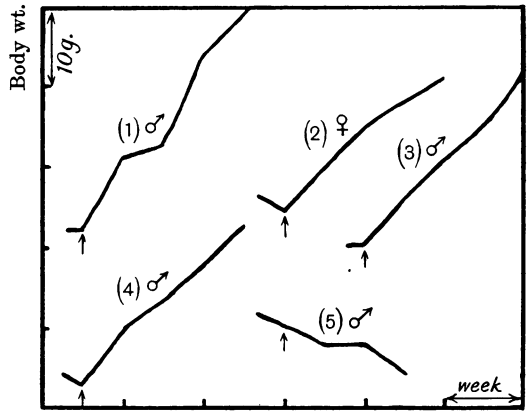

Fig. 8.

Curve (1). Silver nitrate precipitate fraction; ↑ dose≡0·1 g. of liver concentrate.
Curve (2). Silver nitrate filtrate fraction; ↑ dose≡0·3 g. of liver concentrate.
Curve (3). Filtrate from precipitate with litharge; ↑ dose≡75 mg. of concentrate.
Curve (4). Non-esterified fraction; ↑ dose≡0·15 g. of concentrate.
Curve (5). Esterified fraction; ↑ dose≡0·4 g. of concentrate.

8. *Reaction with silver nitrate.* 20 cc. of the aqueous liver extract were treated with 13 cc. of a 25 % solution of silver nitrate, the mixture left over-

night in the dark and the precipitate filtered off. Silver was removed from both the precipitate and filtrate by treatment with HCl and subsequent centrifugation (Fig. 8). The precipitate fraction was much more potent than the filtrate fraction. It appears that this method causes a more effective separation of vitamin B_2 from inactive material than does the reaction with lead acetate.

A portion of the silver precipitate fraction was brought to p_H 9 and autoclaved for 3 hours at 124–125°. The p_H changed to 8·0 and the preparation was found to be at least half inactivated. This shows that purification by certain methods may make the vitamin less stable to alkaline autoclaving, probably by removing some protective material (*cf.* Part II).

9. *Treatment with litharge.* 25 cc. of the aqueous liver extract were shaken up with 3 g. (excess) of litharge. After filtration the residue and the filtrate were freed from lead by sulphuric acid. Practically all the activity was present in the filtrate (Fig. 8).

10. *Esterification with ethyl alcohol.* 4 g. of the solid liver extract were covered with 120 cc. absolute alcohol and treated with dry hydrogen chloride in the usual way for 5 hours. In the last hour the temperature of the reaction vessel was maintained at 50–60°. Nearly all the material dissolved giving a dark brown-red solution. The contents of the flask were poured into a beaker and chilled to 0°. Two volumes of water were slowly added and then silver carbonate and silver oxide. When the p_H of the solution was still below 1, the precipitate, which appeared to consist wholly of silver chloride, was filtered off under suction and well washed and the washings added to the filtrate. The dark red solution was again brought to 0° and then very slowly treated with 20 % sodium hydroxide with constant stirring until the p_H was about 9·5 (the temperature should not rise above 5°). The solution which now measured 525 cc., was immediately extracted 4 times with a total volume of about 750 cc. ether. The ethereal layer was pale yellow, most of the pigment being in the aqueous layer. The ethereal layer was washed with very dilute hydrochloric acid and then with water, which removed some more of the colouring matter. After evaporating the ether by means of an electric fan at 30° the residue was taken up in water (fraction *a*). The aqueous layer was acidified with HCl, concentrated under reduced pressure, and filtered from the precipitated sodium chloride. As it was, however, still extremely saline and could not be fed to the animals, it was saturated with hydrogen chloride at 0°. After removing the large amount of precipitated sodium chloride the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, brought to p_H about 6 and fed to the animals (fraction *b*). Fraction *a* was inactive, while about 30–40 % of the original activity was present in fraction *b* (Fig. 8). The recovery appears to be good enough to warrant the conclusion that the vitamin is not esterifiable under the aforesaid conditions of experiment.

11. *Adsorption on charcoal.* It has been the experience of other workers

[Salmon, Guerrant and Hays, 1928; Narayanan and Drummond, 1930] that vitamin B_2 of yeast extracts is tenaciously adsorbed by certain substances, from which it is difficult to remove by elution. Such has also been the experience of the writer in using charcoal for the adsorption of vitamin B_2 from the liver concentrate.

100 cc. of the aqueous liver extract (p_H *circa* 4·6), were shaken up with 6·7 g. of norite charcoal for 5–7 minutes. After filtration, the charcoal weighed 12–15 g., and was potent in doses corresponding to 80–100 mg. of the original liver extract. The filtrate was practically inactive (Fig. 9).

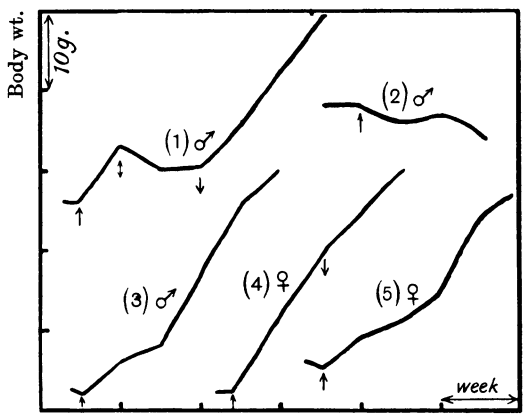

Fig. 9.

Curve (1). Activated charcoal; ↑ dose≡0·1 g. of liver concentrate; ↕ dose≡75 mg. of concentrate; ↓ dose≡90 mg. of concentrate.
Curve (2). Charcoal filtrate; ↑ dose≡0·18 g. of concentrate.
Curve (3). SO_2-treated preparation; ↑ dose≡60 mg. of the concentrate.
Curve (4). O_3-treated preparation; ↑ dose≡80 mg. of concentrate; ↓ dose≡60 mg. of concentrate.
Curve (5). H_2O_2-treated preparation; ↑ dose≡80 mg. of concentrate.

5 g. portions of this activated charcoal were extracted with 50 cc. each of 0·1 *N* HCl, 0·1 *N* NaOH, 30 % ethyl alcohol, containing 0·5 cc. concentrated hydrochloric acid and 45 % alcohol, containing 0·5 cc. of 20 % NaOH. The extractions were carried out by heating under reflux on a water-bath for 1 hour. The extract in every case was practically inactive. The charcoal residues were in some cases active, but the activity had been materially reduced. 5 g. of the activated charcoal were also extracted successively with 200 cc., 200 cc. and 100 cc. of 30 % propyl alcohol, the flask being heated each time at 75 ° for 10 minutes. The extract was slightly active, and the residue practically inactive. Another portion of 5 g. of the activated charcoal was extracted twice with 125 cc. of 0·2 % saponin ("pure," B.D.H.) in the cold, and the mixture centrifuged, but the centrifugate was inactive.

12. *Stability to sulphur dioxide.* Sulphur dioxide was passed into 20 cc. of the aqueous liver extract for 4 hours. The excess of SO_2 was boiled off in an open beaker. The vitamin is completely stable to this treatment (Fig. 9).

13. *Stability to hydrogen peroxide.* 20 cc. of the aqueous extract were treated with 5 cc. of Merck's perhydrol, heated for 1½ hours at 70° and then evaporated to dryness in an open basin in a water-bath. The residue was dissolved in water. No very marked inactivation occurred (Fig. 9).

14. *Stability to ozone.* 20 cc. of the aqueous extract were treated with a rapid stream of ozonised oxygen for 70 minutes. The colour of the solution gradually faded to a lemon-yellow. This was concentrated under reduced pressure. The vitamin was fairly stable to such treatment (Fig. 9).

15. *Action of trypsin.* 80 cc. of the aqueous liver extract were brought to p_H 8. 1 g. powdered trypsin (B.D.H.), which had been found in this laboratory to be active, was added. The mixture was incubated after the addition of a little toluene for 7 days at 37°. Another 80 cc. of the aqueous liver extract was similarly incubated without trypsin as a control. The p_H of the digested solution had changed to 7·2 in 7 days. Both the solutions were acidified with hydrochloric acid, concentrated *in vacuo* to remove toluene and tested. Both of them were active. The trypsin powder itself exhibited no activity even in relatively large doses.

16. *The behaviour of vitamin B_2 on electrodialysis.* Preliminary experiments carried out with Mr T. W. Birch on the electrodialysis of vitamin B_2 of the aqueous liver extract, indicate that the vitamin is probably a neutral substance. It remains concentrated in the middle compartment under the conditions tried, while small quantities appear to be present in both the anode and cathode compartments, this being apparently due to diffusion through the parchment membrane.

Discussion.

The behaviour of vitamin B_2 of the liver extract to basic precipitants like phosphotungstic acid, benzoyl chloride, picric acid, *etc.* indicates that the vitamin is probably not a basic substance. On the other hand, the fact that esterification of the extract leaves a fair amount of the vitamin in the non-esterified fraction, while the esterified fraction is inactive, argues against the acid nature of the vitamin. The occurrence of both vitamin B_2 and the factor specific for pernicious anaemia in large quantities in liver raises the question of a relation between the two substances. The fact that a lowering of the erythrocyte count has been observed in vitamin B_2 deficiency [Sure, Kik and Smith, 1931] also lends point to this question. The general chemical behaviour of the vitamin, as shown in this paper, is however in contrast to that of the factor for pernicious anaemia, which has recently been stated by Dakin, West and Howe [1930] to be a compound of β-hydroxyglutamic acid and l-γ-hydroxyproline.

The idea gained from general chemical evidence that vitamin B_2 is probably a neutral substance is apparently corroborated by the experiments on the electrodialysis of the vitamin. The precipitation of a large portion of the

activity with lead acetate and silver nitrate is probably due to the adsorption of the vitamin by the precipitates formed.

Certain other observations.

Among the rats, which were kept for periods of 10 weeks or so on a vitamin B_2-deficient diet in course of the experiments described in this paper, with only occasional administrations of small doses of vitamin B_2, a considerable proportion developed a curious form of depilation. The fur fell off not in patches but uniformly in such a manner as to give the rats a "close-cropped" appearance [Guha, 1931, 2]. Only in a very small proportion of cases did lesions appear near the mouth and on the paws. The hair began to grow within 2 or 3 days of the administration of the liver concentrate as a source of vitamin B_2 and within 7 or 8 days assumed the normal "lying-down" appearance. This condition of depilation has occurred fairly regularly when the deprivation of vitamin B_2 is not complete but sufficiently severe to cause the weight of the animals to remain between 60 and 80 g. over a period of 10–12 weeks [*cf.* Leader, 1930; Sure and Smith, 1931].

The above symptoms did not improve nor did growth resume on feeding lactalbumin, haemoglobin or haemin[1] in daily doses of 1 g., 0·5 g. and 25 mg. respectively [*cf.* Kollath, 1929; Bliss, 1930; Bliss and Thomason, 1931].

Summary.

1. The values of the following as sources of vitamin B_2 have been investigated—milk powder and aqueous extracts of brewer's yeast, baker's yeast, fresh ox-liver, beef-muscle and Eli Lilly's liver concentrate No. 343. The fresh ox-liver extract is apparently the most potent of these. An aqueous extract of the liver concentrate is also very potent, being effective in a daily dose of 40–60 mg. for the growth of young rats. The advantages of this liver concentrate over yeast as a source of vitamin B_2 are pointed out.

2. The stability of vitamin B_2 preparations obtained from different sources towards heat and alkali shows curious discrepancies. Thus the vitamin B_2 in aqueous extracts of yeast and fresh ox-liver is much less stable than that in marmite and in the commercial liver concentrate. It is probable that the stability of certain preparations is connected with the presence of some kind of protective material in them. An aqueous extract of the liver concentrate autoclaved at p_H 9 for half an hour at 124–125° provides an excellent source of vitamin B_2 free from vitamin B_1.

3. A chemical study of vitamin B_2 in a cold aqueous extract of the liver concentrate has been made. Picric acid, benzoyl chloride, phosphotungstic acid and flavianic acid do not precipitate the vitamin. Nitrous acid neither precipitates nor inactivates it. Lead acetate and silver nitrate precipitate it partially. Esterification leaves about 40 % of the vitamin in the non-esterified

[1] I am indebted to Mr Meldrum for this preparation of haemin.

fraction, the esterified fraction being inactive. It is not attacked by trypsin. Norite charcoal adsorbs the vitamin at the normal p_H (4·6) of the aqueous extract of the liver concentrate. It was not possible to elute it effectively by aqueous alcohol, 30 % propyl alcohol or by dilute saponin. On the basis of the present evidence it may be provisionally concluded that the vitamin is probably not a base, acid or peptide, but a neutral substance. Preliminary experiments on the electrodialysis of the vitamin support this tentative conclusion. The partial precipitation by lead acetate and silver nitrate is probably due to the adsorption of the vitamin on the precipitates formed. The vitamin is stable to sulphur dioxide, hydrogen peroxide and ozone.

4. Some of the rats which were maintained at a weight between 60 and 80 g. over periods of 12 weeks or so, and were not undergoing a drastic deprivation of vitamin B_2, developed a curious form of depilation, which was cured by administration of the liver extract.

5. Haemin, haemoglobin and lactalbumin could not ameliorate the above symptoms or produce growth in absence of vitamin B_2.

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