III. THE CONCENTRATION OF VITAMIN B_2.

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WHILE a considerable amount of work has been done to elucidate the chemical nature of vitamin B_1 as yet few observations of a similar character have been recorded regarding the more recently discovered vitamin B_2. Of the few facts known concerning the latter substance, the most important are those that indicate its comparative stability to heat and to somewhat drastic treatment with aqueous alkalis. Several investigators have stated that it is sparingly soluble in alcohol of greater concentration than 80 %, while it has also been recorded that it is absorbed by fuller's earth [Salmon *et al.*, 1928] and silica gel [Levene, 1928]. The attempts to separate the two vitamins B_1 and B_2 have been based on these properties, but so far little progress has been made in the isolation of vitamin B_2. The most potent concentrate recorded in the literature appears to be that obtained by Levene [1928], by means of adsorption on silica gel. This preparation supplied the requirements of the young rat in doses of only 2 mg. of organic matter daily. He observed that the activity was destroyed by treatment with nitrous acid.

The present investigation was undertaken as part of the general study of vitamin-like substances present in yeast extracts.

EXPERIMENTAL.

In the foregoing paper a description is given of an investigation on the chemical nature of "bios." During the earlier part of this investigation it was uncertain whether the yeast stimulant was identical with the factor known as B_2 or not, but it was soon ascertained that precipitation of the baryta-hydrolysates of yeast extracts with lead acetate effected a separation of the two substances. The vitamin B_2 is precipitated by lead acetate whilst "bios" remains in the filtrate. The precipitation of vitamin B_2 by this reagent has been recorded by Rosedale [1927], and by Chick and Roscoe [1929]. The present communication describes the efforts that were made to separate the factor B_2 from the materials precipitated by lead acetate (see Table II [Narayanan, 1930]).

A. *Technique.* In these experiments young rats (usually black and white, bred in these laboratories) of approximately 50 g. in weight were placed on a

vitamin-free diet consisting of caseinogen 20 %, starch 74 %, and a special salt mixture 4 %. They were also given 40 mg. daily of cod-liver oil of tested vitamin A and D strength and each rat was also given a supply of a concentrate containing vitamin B_1 sufficient to permit satisfactory growth if the diet was otherwise adequate. The concentrate was made by the method of Kinnersley and Peters [1927].

Each rat was kept in a separate cage and when the growth had quite, or very nearly, ceased, a daily dose of the extract to be tested was given. An extract under test was assumed to be active when the rats grew at the rate of at least 10 g. a week.

B. *Tests on known substances.* A number of substances, which for one reason or other might possibly have been suspected of being related to vitamin B_2, were tested. All these tests proved negative. The list of substances tested is given below with the doses employed.

Table I.

Substance	Dosage (mg.)	Average growth in a week (g.)
Nucleic acid (yeast)	1–10	– 2
Guanine hydrochloride	1–5	Nil
Adenine sulphate	1–5	,,
Funk's compound, M.P. 234°	1–5	1
Nicotinic acid hydrochloride	1–5	Nil
Betaine hydrochloride	1–5	– 1
Inositol	1–10	– 1 to + 1
Potassium pyrophosphate	1–10	– 3
Non-saponifiable matter from yeast fat	1–5	Nil

C. *Preparation of lead acetate fraction.* The precipitate produced by the addition of lead acetate to the extract hydrolysed by alkali, or to the aqueous solution of the material extracted from yeast by alcohol [see Narayanan, 1930] was decomposed by suspending in warm water and slowly adding 10 % sulphuric acid while the mixture was stirred mechanically. When acid to Congo red, the lead sulphate was removed by filtration, and the filtrate neutralised with sodium hydroxide. The filtrate fraction was found to be active and promoted growth in daily doses representing 10–15 mg. of organic matter. (N content 6·7 %.) (Curve no. I, Fig. 1.)

The filtrate containing material not precipitated by lead acetate produced practically no growth, even in daily doses as high as 400 mg. of organic matter.

D. *Adsorption on fuller's earth.* Preliminary experiments were made to discover if the factor is readily adsorbed by fuller's earth, and, if so, the best conditions for effecting concentration by this means. A preliminary series of trials with the liquid resulting from decomposition of the lead acetate precipitate demonstrated that over a range of p_H extending from 6·8 to 0·1 the adsorption increased as the acidity was raised. At p_H 0·1 the removal of the active factor by fuller's earth was almost complete, and the main bulk of a large preparation was consequently treated in the following manner.

The active liquid from the lead acetate precipitate was rendered approximately 0·9 *N* with sulphuric acid and treated with 3 g. of fuller's earth for every 100 cc. of the liquid.

The mixture was well stirred mechanically for half an hour, filtered and the earth well washed with a small quantity of 0·9 *N* sulphuric acid. The filtrate and washings were similarly treated a second time with 1·5 g. of fuller's earth for each 100 cc. Approximately 40 % of the total solids and organic matter of the original extract was adsorbed. The resulting earth contained practically all the active factor, and promoted growth in doses of 40 mg. of earth; corresponding to 6 mg. of adsorbed organic matter. (Curve no. II, Fig. 1.)

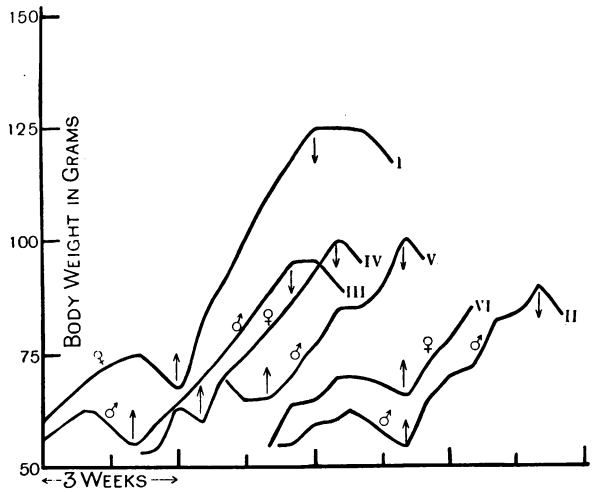


Fig. 1.

↑ = Supplement given. ↓ = Supplement withdrawn.

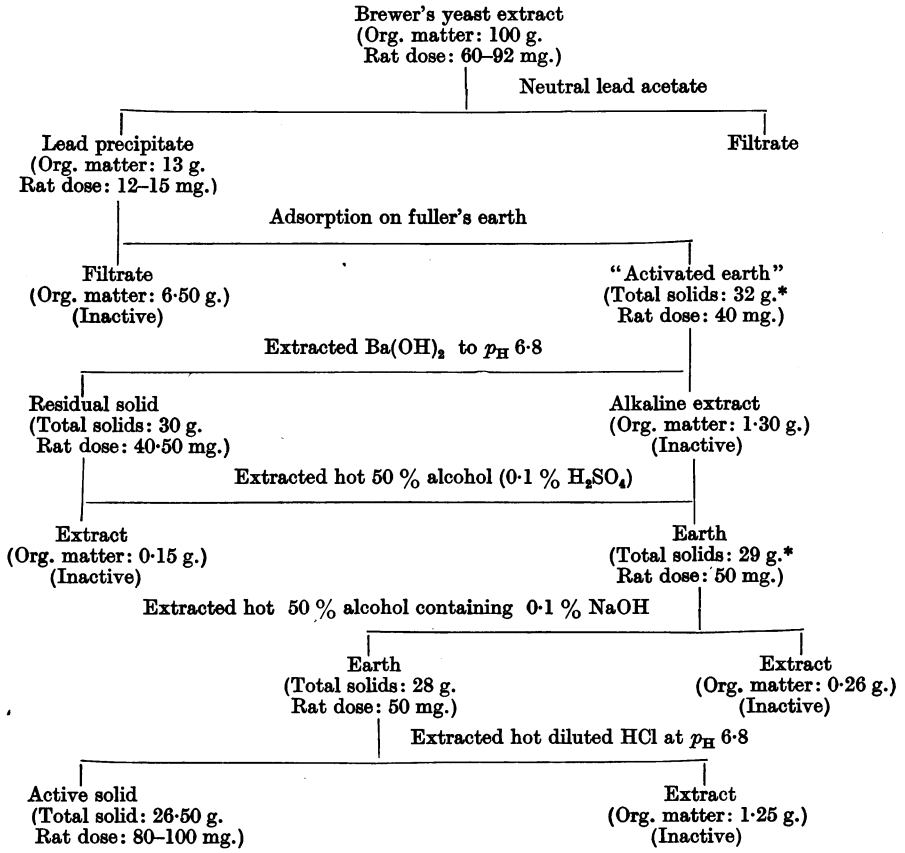
The "activated" fuller's earth was now triturated with cold saturated baryta until alkaline to bromocresol purple, filtered, and the precipitate well washed with distilled water until the washings failed to give a test for barium. The filtrate and washings contained 26 % of the solids adsorbed on the earth, but did not promote growth when tested. On the other hand, the earth after extraction with baryta produced growth in doses of 40 to 50 mg. of earth, corresponding to 5 mg. of residual adsorbed organic matter. (Curve no. III, Fig. 1.)

The earth was now extracted with 50 % alcohol containing 0·1 % of sulphuric acid. Twenty-six g. of earth, containing approximately 2·7 g. of organic matter, were twice extracted at 60 to 70° for 1 hour with 50 cc. of the acid alcohol. The extracts and washings were evaporated at reduced pressure, and the residual fluid carefully neutralised. On testing the extract it was found to be inactive. It contained 0·16 g. of organic matter, representing 6 % of the total adsorbed substances. The residual fuller's earth was found to be still active in doses of 50 mg. of earth. (Curve no. IV, Fig. 1.)

The residual earth was now extracted in a similar manner with 50 % alcohol containing 0·1 % of sodium hydroxide. The extract after removal of the alcohol and neutralisation was found to contain 0·2 g. of organic matter, but to be quite inactive. The residual solid weighed 20·5 g. and still retained its original activity, *i.e.* 50 mg. of earth. (Curve no. V, Fig. 1.)

A quantity of this residual "activated" earth, representing a total of approximately 400 daily doses, was again extracted, this time with dilute HCl at a p_H of 6·8, under the same conditions as before. The extract was concentrated and neutralised. It contained 0·75 g. of organic matter, but was inactive. The activity of the earth was found to have been somewhat reduced by this treatment as it required doses of 80–100 mg. to produce an average growth of 10–12 g. a week. (Curve no. VI, Fig. 1.)

The following scheme illustrates the experiments described.



* Total solids = total weight of "activated" fuller's earth.

E. *Adsorption on "norite" charcoal.* In view of the failure that attended the efforts to concentrate vitamin B_2 by the use of fuller's earth, an attempt was made to adsorb the active factor on "norite" charcoal. After a series of trials the following procedure was adopted.

The extract from the decomposition of the lead acetate precipitate from 7 lb. of yeast was rendered approximately 0·9 *N* with sulphuric acid, and treated with 5 g. of norite for every 100 cc. of the liquid. The mixture was well stirred for half an hour and filtered. The charcoal was then washed well with 0·9 *N* H_2SO_4, and the filtrate and the washings were again treated with 2 g. of charcoal per 100 cc. The combined charcoals weighed 42 g. when dry. The filtrate and washings were reserved for further experiment.

The charcoal fraction was suspended in 50 cc. of a solution of 1 % sodium hydroxide in 50 % alcohol, added until the reaction of the mixture was p_H 6·8, and the mixture heated on the water-bath for 1 hour, care being taken to keep the reaction at p_H 6·8. The alcoholic solution was filtered and concentrated under reduced pressure. The concentrate contained 40 % of the organic matter of the original extract, but was practically inactive. The residual charcoal, however, showed slight activity in 60 mg. doses. (Curve no. VII, Fig. 2.)

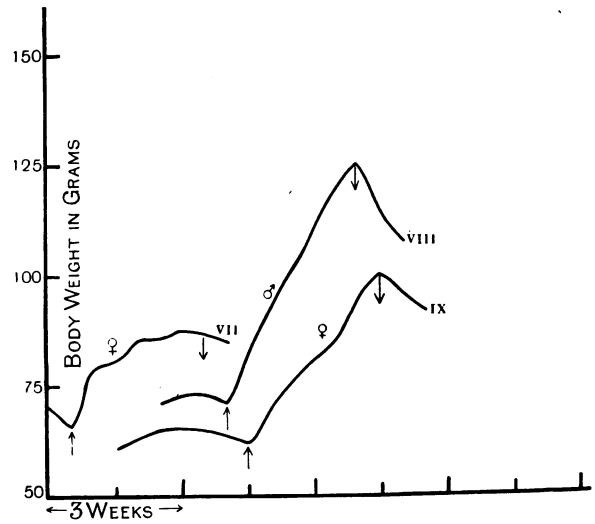


Fig. 2.

↑ = Supplement given. ↓ = Supplement withdrawn.

The reaction of the filtrate and washings from this charcoal treatment was adjusted to p_H 2·3 by the addition of sodium hydroxide and a similar treatment with norite carried through. The combined charcoals were similarly extracted at 60–70° with 50 % alcohol at p_H 6·8. The alcoholic extract contained 50 % of the residual organic matter, but did not produce any appreciable growth in 5–6 mg. doses. The extracted charcoal was quite inactive. It was concluded that norite does not appreciably adsorb vitamin B_2 at this p_H.

The filtrate from the charcoal treatments was concentrated under reduced pressure to a thick syrup and extracted with hot 50 % alcohol. A granular precipitate consisting mostly of inorganic matter separated, and was discarded on being found to be quite inactive. The alcoholic extract contained practically

all the residual organic matter but was also inactive. The loss of activity in the experiment has not been satisfactorily accounted for.

F. *Attempts to concentrate vitamin B_2 by means of alcohol.* In view of the failure of the efforts to concentrate vitamin B_2 by adsorption methods, it was decided to attempt fractionation by alcohol. The fraction resulting from decomposition of the lead acetate precipitate was treated with absolute alcohol until the solution contained approximately 50 %. A considerable amount of a slimy precipitate was obtained which was filtered off and washed with small quantities of 50 % alcohol. The alcoholic extracts and washings were concentrated under reduced pressure. This extract was found to be active in daily doses of 9 mg. of organic matter (curve no. VIII, Fig. 2), and contained about 88 % of the activity of the original fraction.

The 50 % alcoholic extract was further treated by raising the percentage of alcohol to approximately 70 %, when another slimy dark-brown precipitate was deposited. This was removed by filtration and well washed with small quantities of 70 % alcohol. The filtrate was concentrated, but on being tested on rats was found to be inactive. The precipitate contained most of the activity, giving good growth in daily doses of 6 mg. of organic matter [cf. Sherman and Sandels, 1929]. Further investigation of this fraction is in progress. (Curve no. IX, Fig. 2.)

Some properties of vitamin B_2.

(*a*) *Treatment with acids and alkalis.* In the course of these experiments, further confirmation was obtained of the observation that the active factor, vitamin B_2, withstands rather drastic treatment with acids and alkalis.

No appreciable destruction appears to occur when either a yeast extract or brewer's yeast is hydrolysed by 10–15 % sulphuric or hydrochloric acid at boiling point for 24 hours, or when they are treated with 10–15 % barium hydroxide solution in an autoclave at 110–120° for 1 to 3 hours. (Curves nos. X, XI, XII, Fig. 3.)

(*b*) *Treatment with hydrogen peroxide.* The active factor is apparently stable to hydrogen peroxide. 100 cc. of the lead precipitate fraction, after removal of the lead, was treated with an excess of hydrogen peroxide, the mixture being warmed on the water-bath at 60–70° for 30 minutes. The resulting liquid was equal in activity to the original untreated material. (Curve no. XIII, Fig. 4.)

(*c*) *Treatment with nitrous acid.* In contradiction of Levene's statement [1928] and in confirmation of the more recent observation of Chick [1929] no appreciable destruction of the active factor was observed when treated with nitrous acid. 100 cc. of the lead precipitate fraction (containing 1·5 g. of organic matter) were treated with 2·5 g. of sodium nitrite and the mixture was cooled on ice. 10 % hydrochloric acid was then slowly added and the mixture allowed to stand for 10 minutes. The solution was then warmed to 60–70°, and

finally neutralised with sodium hydroxide solution. The resulting liquid showed no appreciable deterioration of its original activity. (Curve no. XIV, Fig. 4.)

(*d*) *Solubility.* Apart from its extreme solubility in water, vitamin B_2 is very soluble in 50–60 % alcohol, but only sparingly so in alcohol of greater

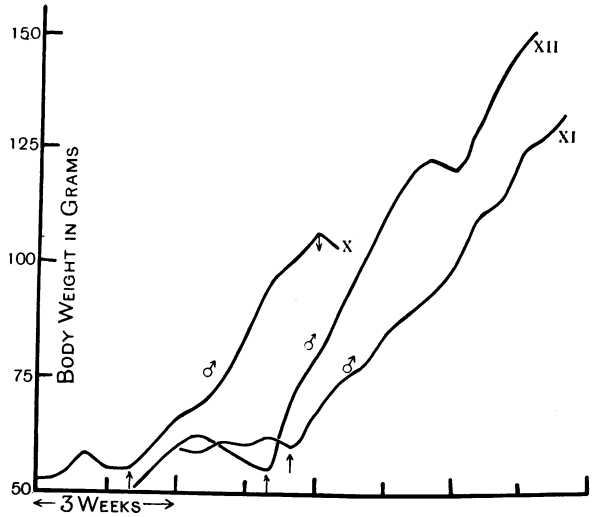


Fig. 3.

↑ =Supplement given. ↓ =Supplement withdrawn.

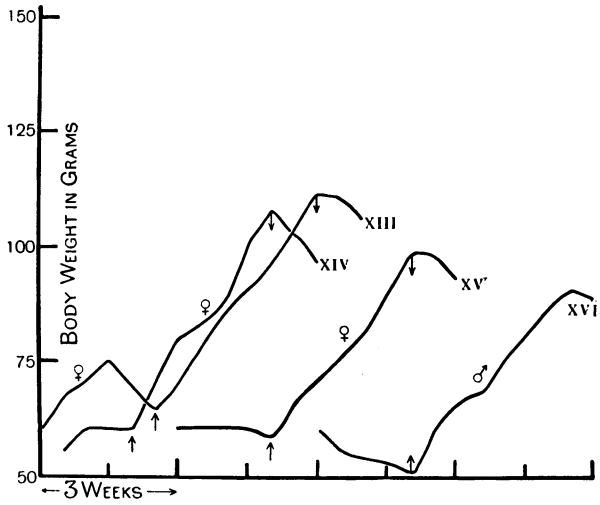


Fig. 4.

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concentration, and practically insoluble in higher alcohols, of the type of butyl alcohol, as the following experiment shows.

The extraction was made in a continuous extraction apparatus essentially of the form described by Dudley [1919].

The extract from 7 lb. of yeast was concentrated to a thick brown syrup, and extracted in two lots with *n*-butyl alcohol at 60–70° for 48 hours. The butyl alcohol extract was separated from the aqueous mixture, and the latter solution was again subjected to extraction for a further period of 48 hours. The extracts were combined and concentrated under reduced pressure to remove all the solvent. The concentrate contained 5 % of the total solids of the original extract, but was found useless as a source of vitamin B_2. The residual aqueous liquor retained practically all the activity. (Curves nos. XV and XVI, Fig. 4.)

Summary.

(1) An attempt to concentrate vitamin B_2 by means of adsorption on fuller's earth is described. While adsorption was complete at the extreme acid range of p_H (0·05–0·10), great difficulty was encountered in removing the adsorbed active factor.

(2) "Norite" charcoal is not an efficient adsorbent of the active factor.

(3) The active factor is relatively insoluble in alcohol of greater concentration than 70 % by volume. By this process a concentrate has been obtained, of which the daily dose which supplies the requirements of the young rat contains 6 mg. of organic matter. Further fractionation of this concentrate is in progress.

(4) The general finding that the active factor is stable to heat, acids and alkalis is confirmed. It is also found that vitamin B_2 is not destroyed by either hydrogen peroxide or nitrous acid. It is not appreciably soluble in butyl alcohol.

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