LVII. AN ATTEMPT TO SEPARATE VITAMIN B_2 FROM VITAMIN B_1 IN YEAST AND A COMPARISON OF ITS PROPERTIES WITH THOSE OF THE ANTINEURITIC VITAMIN B_1.

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(*Received May 7th, 1929.*)

YEAST contains both constituents of the "water-soluble B" vitamin, vitamins B_1 and B_2, in abundance, and seeing that the antineuritic (vitamin B_1) concentrate prepared by the method of Peters [1924] is free from vitamin B_2 [Chick and Roscoe, 1927] our first endeavour towards the separation of this vitamin was to scrutinise every stage of the process to find where the disappearance occurred.

Our method has been that of Kinnersley and Peters [1925, 1927] with a few modifications. 10–12 kg. of pressed, brewery yeast were washed three or four times with cold tap water. The pressed yeast was puddled with water to the consistency of cream, filtered on a Gooch filter, using slight suction, and finally sucked dry or pressed in a hand press, this process being repeated three or four times until the filtrate was of a pale straw colour. After removal of a small sample for determination of the dry weight, the washed, pressed yeast (in the proportion of 1 kg. pressed, or 200 g. dry, yeast to 2–3 litres water) was thrown into boiling tap water containing 0·02 % acetic acid; this was raised again to the boiling point and allowed to boil for 5 minutes and filtered. This extract ("Fraction 5") about 20–30 litres in volume, was then treated with 25 % lead acetate solution till no more precipitate was formed (*ca.* 55–80 cc. per litre of extract), the whole allowed to stand overnight and then filtered. To the filtrate ("Fraction 4") baryta, at first solid and then as saturated solution, was added, till no more precipitate formed. As the resulting solution is alkaline, filtration was carried out at once and the filtrate acidified with strong H_2SO_4 until a p_H of about 4·0 was reached (using the indicator bromocresol green or Congo red). The solution, heated to assist agglutination of the precipitated $BaSO_4$, was filtered, and to the filtrate Hopkins's reagent (10 % solution of $HgSO_4$ in water containing 70 cc. H_2SO_4 per litre) was added to the point of most complete precipitation (25–50 cc. per litre of filtrate). In order to remove any traces of lead and mercury that might be present, H_2S was passed through the filtrate to saturation and the whole allowed to stand several hours in corked bottles; on heating the solution,

the metallic sulphides settled well and could readily be filtered. If the previous precipitation with baryta is omitted, these sulphides tend to remain in the colloidal form and are separated with difficulty. The reaction of the filtrate was then carefully adjusted to the neutral point (using litmus or phenol red) and treated twice with norite charcoal which had been purified before use by boiling with dilute hydrochloric acid and thorough washing with distilled water to remove all traces of acid. The charcoal was filtered off, washed and sucked dry on a Gooch filter and the filtrate treated with a second quantity. 60–70 g. norite per kg. dry yeast were used in each adsorption. Both portions of charcoal were boiled two or three times with fresh quantities of dilute acid alcohol (50 cc. alcohol, 50 cc. water, 1 cc. concentrated hydrochloric acid) and the combined extracts reduced to a convenient bulk (the equivalent of 6 g. yeast contained in 1 cc.) by distilling under reduced pressure at a temperature of 40 to 50°.

After each precipitation a portion of the filtrate was set aside and examined by the method already published [Chick and Roscoe, 1928] for its content of vitamin B_2. It is necessary to work quantitatively, determining in each case the minimum dose required to maintain a standard degree of growth (a weekly increase in body weight of 11–14 g.) and referring the dose given to the equivalent amount of the original dried yeast.

Table I. *Details of preparation from Yeast V of Peters's antineuritic vitamin B_1 concentrate, and results of tests of filtrates at each stage for content of vitamin B_2*.*

Process	Material tested	Dose given, expressed as equivalent of dry yeast g.	No. of rats observed	Average growth g. per week
	Dried yeast (from Table III, Chick and Roscoe [1927])	0·2	—	11
		0·4	—	23
1. Extraction of pressed, washed Yeast V with boiling water containing 0·01 % acetic acid	*Fraction* 5. Filtrate from 1 after evaporation to small bulk	0·25	3	12
		0·5	3	21
		1·0	3	29
2. Precipitation with Pb acetate at p_H 4·7 (800 cc. 25 % Pb acetate soln. per kg. dry yeast)	*Fraction* 4. Filtrate from 2 after evaporation to small bulk and removal of Pb with H_2SO_4	1·0	2	14
3. Precipitation with baryta and acidification with H_2SO_4 to p_H *ca.* 4·0 to precipitate $BaSO_4$	*Fraction* 3. Filtrate from 3 after evaporation and partial neutralisation with removal of any further traces of $BaSO_4$	2·0	2	6
		3·4	2	15†
4. Precipitation with acid mercuric sulphate, Hopkins's reagent (260 cc. per kg. dry yeast), and removal of traces of Hg by H_2S	*Fraction* 2. Filtrate from 4 after evaporation to small bulk	1·0	1	0
		2·0	1	0
		3·0	1	2
5. Adsorption of vitamin B_1 by treatment with norite (100 g. norite per kg. dry yeast, in two portions) after neutralisation	*Fraction* 1. Filtrate from norite after evaporation to small bulk	4·0	2	2·5

* Method of Chick and Roscoe [1928]. Growth of young rats observed for 2–4 weeks on a diet deprived of B_2 vitamins, but receiving vitamin B_1 as 0·1 cc. (=0·6 g. yeast) Peters's antineuritic concentrate.

† A similar fraction prepared from yeast VII was used for part of this test. These tests followed on those of the 2·0 g. doses.

In Table I are set out the details of one such preparation, viz. from Yeast V. There was little loss of vitamin B_2 in Fraction 5, the first extract

from the yeast with acidified boiling water. The average weekly growth-increments of young rats receiving daily doses equivalent to 0·25 g. dried yeast were 12 g., about the same as that of rats receiving 0·2 g. daily of dried yeast; daily doses equivalent to 0·5 g. dried yeast had about the same effect as 0·4 g. dried yeast.

During precipitation with lead acetate there was a loss. Daily doses of the filtrate, Fraction 4, equivalent to 1·0 g. dry yeast produced an average weekly increase in weight of only 14 g. in two male rats, whereas an equivalent amount of Fraction 5 induced about twice as much growth (29 g.).

The precipitation with baryta caused a further loss in vitamin B_2 and, after precipitation with mercuric sulphate and subsequent removal of traces of mercury from the filtrate with SH_2, the filtrate contained no significant amount of this vitamin. Doses equivalent to 2 or 3 g. of the original dried yeast did not produce more growth than occurred in some control animals on the basal diet.

One may, therefore, conclude that about one half to three-fourths of the vitamin B_2 contained in the original yeast is carried down with the lead acetate precipitate; of the remainder, the greater part (about two-thirds) disappears with the precipitate formed by baryta, and the rest with that given with acid mercuric sulphate. Attention was, therefore, directed to the precipitation with lead acetate.

Influence of hydrogen ion concentration on the amount of vitamin B_2 carried down by lead acetate.

In the Peters process as described above, the precipitation takes place at a p_H of about 4·5–4·7 (bromocresol green). We tried precipitation at p_H 2·6 (bromophenol blue), this being the degree of acidity attained during the precipitation with Hopkins's reagent in the above process, when the last remaining traces of vitamin B_2 were found to be adsorbed. Precipitation with lead acetate at p_H 6·3–8·9 (using bromocresol purple and phenol red as indicators) was also tried.

At p_H 2·6 less than one-half the vitamin B_2 present was removed by lead acetate, at p_H 4·7 about three-fourths. In neutral or slightly alkaline solution the experiments indicated that all was carried down. Whereas of the original extracts the equivalent of 0·25 g. to 0·5 g. dry yeast, respectively, supported normal growth, little or no growth was induced by doses of the filtrates equivalent to 1·0 or 2·0 g. dry yeast (for details see Table II, Fractions 4, Yeast VIII and Yeast X). Unfortunately, as will emerge later, none of these precipitates with lead acetate was consistently free from vitamin B_1.

Recovery of vitamin B_2 from the lead acetate precipitate.

Vitamin B_2 can be recovered by decomposing the lead acetate precipitate with sulphuretted hydrogen. If the reaction is acid, however, the lead sulphide remains in colloidal solution, owing to the action of the yeast gum which

possesses an alkaline isoelectric point. If the solution is brought to p_H 8–9 (thymol blue) the lead sulphide separates readily, but in that case vitamin B_2 is adsorbed on the precipitate and the solution correspondingly impoverished. To prevent loss in this way the reaction should not be less acid than p_H 3·0 (bromophenol blue). The technical trouble caused by the yeast gum at a higher p_H can be overcome by hydrolysing it in the original extract.

Table II. *Influence of hydrogen ion concentration upon the removal of vitamin B_2 (and B_1) from an extract of brewers' yeast by precipitation with lead acetate.*

Estimation of vitamins B_2 and B_1 in the filtrates and in the solutions obtained by decomposition of the precipitate by methods of Chick and Roscoe [1928] and [1929] respectively.

Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Av. growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Av. growth g. per week	Dose (expressed as equivalent in g. of dry yeast) required to give normal (11–14 g. weekly) increase in weight as source of: Vitamin B_2	Vitamin B_1
Yeast VIII:							
Fraction 5. Extract with dilute acetic acid	0·12	2	7	—	—	0·25	—
	0·25	4	11	—	—		—
	0·5	1	18	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 2·6	0·5	3	13	—	—	0·5	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 4·7	1·0	1	13	—	—	1·0	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 6·3	0·5	1	0	—	—	No growth with 1·0	—
	1·0	1	0	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 8·9	1·0	3	0	—	—	„	—
Yeast X:							
Fraction 5	0·25	4	9	—	—	0·25 to 0·5	—
Fraction 5, after hydrolysis 1 hour 100° at p_H 1·5	0·5	3	13	—	—		—
	0·25	1	13	—	—		—
Fraction 4. Filtrate after precipitation with Pb acetate at p_H 7·2	1·0	1	7	—	—	>2·0	—
	2·0	3	4	—	—		—
Fraction C_1 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 4·7	0·5	1	10	1	1	0·5 to 1·0	>2·0
	1·0	1	14	1	0·2		>2·0
	2·0	—	—	1	0·3		>2·0
Fraction C_2 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 7·5	0·5	3	11	1	7·5	0·5 to 1·0	0·5 to 1·0
	1·0	1	14	1	14		0·5 to 1·0
Yeast XI:							
Fraction 5	0·25	2	11	—	—	0·25	—
Fraction C_1, from Pb acetate precipitate formed at p_H 4·7	0·25	2	11	2	12	0·25	0·25
	0·5	2	9	1	18		0·25
	1·0	1	19	—	—		0·25
Fraction C_2, from Pb acetate precipitate formed at p_H 7·5	0·5	2	9	—	—	0·5 to 1·0	<1·0
	1·0	1	15	1	25		<1·0
Yeast XII:							
Fraction 5	0·12	—	—	2	15	0·25 to 0·4	0·12
	0·25	2	8·5	—	—		0·12
	0·4	2	14	—	—		0·12
Fraction C_2, from Pb acetate precipitate formed at p_H 7·3	0·25	—	—	4	15	0·5	0·25
	0·5	6	14·5	1	20		0·25

The following procedure was adopted. To 1 litre of the original dilute acetic acid yeast extract (Fraction 5) were added 10 cc. concentrated hydrochloric acid, making the p_H about 1·5 (thymol blue). The mixture was heated in steam at 100° for 1 hour, neutralised, the p_H adjusted to about

7·5 (phenol red) and 10 % solution of basic lead acetate added till precipitation was complete. The liquor was decanted, the precipitate drained on a Gooch filter and washed with a small amount of distilled water. If not wanted immediately the precipitate can be dried and worked up later, but if kept in the moist condition it is liable to become infected with moulds. The precipitate was suspended in water, the reaction adjusted to p_H 3·0 (bromophenol blue), sulphuretted hydrogen passed to saturation and the whole shaken for about 2 hours. After standing, a clear yellow fluid should separate from the lead sulphide. This was filtered, the residue washed, again suspended in water and treated with sulphuretted hydrogen, the operation being repeated two or three times until the top fluid was colourless. The combined filtrates and washings, which were strongly acid, were neutralised with sodium hydroxide or baryta (preferably the latter) to *ca.* p_H 3·0 and evaporated on a water-bath to a convenient bulk (1–2 cc. containing the equivalent of 1 g. yeast), during which a small additional amount of lead sulphide was often deposited. This degree of heating does not seem to impair the activity of the preparation. The sodium sulphate present in this final product, referred to as Fraction C for brevity, sometimes caused diarrhoea in the doses administered to the rats; it can, however, be replaced by sodium chloride if precipitated by barium chloride, but care must be taken to avoid any excess.

Table II gives the details of such preparations made from Yeasts X, XI and XII respectively. In addition to the tests for vitamin B_2, the products, Fractions C, were also tested for content of vitamin B_2, using the method described in the accompanying paper [Chick and Roscoe, 1929].

The Fractions C_2, obtained from Yeasts X and XI by decomposition of the lead acetate precipitates formed at p_H 7·3 to 7·5, contained about one-half of the vitamin B_2 present in the respective original yeast extracts (Fractions 5). Fraction C_2, obtained in the same manner from Yeast XII, contained nearly the whole of the vitamin B_2 of the corresponding Fraction 5; normal growth was obtained with the equivalent of 0·5 g. yeast, the dry weight of the dose (less ash) being 0·03 g. It was, however, admixed with vitamin B_1 and although it contained only about one-half of that present in the original Fraction 5, it was richer in this vitamin than in vitamin B_2.

By precipitation with lead acetate in neutral or alkaline solution more vitamin B_1 is carried down than in the weakly acid solution (p_H 4·7) of Peters's original process. It was hoped that by precipitation at p_H 4·7 a method of separation would be obtained. From Yeast X a Fraction C_1, almost free from vitamin B_1 was indeed obtained from the lead acetate precipitate formed at this p_H. However, this was not found to occur uniformly (compare Fraction C_1 prepared from Yeast X with Fraction C_1 from Yeast XI, Table II). The reason for the discrepancy is not obvious but may be due to differences in the relative proportions of the two vitamins contained in the original yeast extract.

It is possible that by working throughout in more acid solutions, at p_H 2·0

to 3·0, a preparation of vitamin B_2 free from B_1 might be obtained, but it would be very weak. In the experiments made with Yeast VIII (Table II), when the lead acetate precipitation occurred at a low p_H, the amount of vitamin B_2 adsorbed was much reduced.

Yeast extract seems to be an unfavourable medium for separation of these two vitamins by the type of process here employed, and it is possible that success might be attained by working with some other material. Rosedale [1927] used a method resembling Peters's for preparing an antineuritic concentrate from rice polishings. On decomposing the lead acetate precipitate with sulphuretted hydrogen, he obtained a substance which maintained health and weight in pigeons but had no curative effect on polyneuritis, although affording temporary relief by causing evacuation of the bowel. At the present time little is known, except by inference, of the relation of vitamin B_2 to the nutrition of the pigeon, but it is probable that Rosedale's preparation contained vitamin B_2 without admixture of vitamin B_1.

Other methods tried for separating vitamin B_2 from B_1 in yeast extracts.

The vitamin B_2 preparations described above could doubtless be freed from vitamin B_1 by heating at a high temperature, but the vitamin B_2 content would be reduced at the same time. The vitamin B_1 in yeast was destroyed and the vitamin B_2 reduced to about one-half the original amount by heating to 120° for 5 hours [Chick and Roscoe, 1927, Table III].

Our endeavour was to effect a separation by other means than heat, and to this end we tried the following: (1) dialysis, (2) solubility in strong alcohol and (3) ultra-violet light. None was successful, but as the work afforded some information as to the properties of these two vitamins it seems worth while to place it on record.

Dialysis.

Fraction 5 (acetic acid extract) from Yeast X, which contained both vitamins B_1 and B_2, was placed in a bag of cellophane (a cellulose membrane prepared from viscose) and dialysed against distilled water. The solutions were acidified to p_H about 3·0) and dialysis was carried on in a refrigerator to prevent putrefaction. After 4 days, the inside and outside liquors were tested for content of vitamins B_1 and B_2 respectively. No difference could be detected in the concentration of vitamin B_2 in the fluid inside and outside the membrane. Vitamin B_1 also passed freely through the membrane (see Table III, exp. 1).

Solubility in alcohol.

There are many facts in the literature indicating that the antineuritic (vitamin B_1) component of the complex "water-soluble B" is more readily soluble in strong alcohol than the more heat-stable (vitamin B_2) component.

Table III. *Influence of dialysis, ultra-violet irradiation, and alcohol upon vitamins B_1 and B_2 respectively contained in a dilute acetic extract (Fraction 5) from yeast.*

Methods of assay as in Tables I and II.

Exp.	Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Average growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Average growth g. per week
1	*Dialysis:*					
	Yeast X, Fraction 5 (extract in dilute acetic acid)	0·25	4	9	—	—
		0·5	3	13	—	—
	Yeast X, Fraction 5. Dialysed: outside liquor	0·25	1	8	1	10
		0·5	1	12	1	14
	inside liquor	0·25	1	8	—	—
		0·5	1	15	—	—
2	*Effect of ultra-violet light:*					
	Yeast XII, Fraction 5	0·12	—	—	2	15
		0·25	2	9	—	—
		0·4	4	14	—	—
	Yeast XII, Fraction 5. Irradiated with ultra-violet light for 6 hours	0·12	—	—	2	11·5
		0·25	1	4	—	—
		0·5	1	8	—	—
	for 12 hours	0·12	—	—	2	7
		0·25	—	—	1	8
		0·5	1	4	—	—
		0·75	1	9	—	—
3	*Solubility in alcohol:*					
	Yeast XII, Fraction 5. See above exp. 2.					
	Yeast XII, Fraction 5, after drying and exposure to air for 19 days	0·4	2	13·5	—	—
	Yeast XII, Fraction 5, after drying and exposure to 94 % alcohol for 19 days	0·4	2	11	—	—
	Yeast XII, Fraction 5. Fraction soluble in 92 % alcohol	0·25	—	—	2	12
		0·5	2	2	—	—
		1·0	2	0·5	—	—
	Yeast XII, Fraction 5. Fraction insoluble in 92 % alcohol	0·12	—	—	1	10
		0·25	—	—	1	16
		0·5	3	9	—	—

The antiberiberi, antineuritic vitamin, has long been known to be soluble in alcohol in strengths ranging from 88 % to absolute [Fraser and Stanton, 1910; Eijkman, 1911; Chamberlain and Vedder, 1911; Kinnersley and Peters, 1925, and others]. It was further observed by Schaumann [1911] that extracts made with 96 % alcohol from yeast or rice bran, while potent in the cure and prevention of polyneuritis, could not maintain the body weight of the experimental animals, whereas equivalent amounts of the original materials could do both. Drummond [1917] found it impossible to obtain a satisfactory extract of the complex formerly known as "water-soluble B" with absolute alcohol, but succeeded with 70 %. This discrepancy in alcohol-solubility between the antineuritic vitamin and the complex water-soluble B was one of the facts throwing doubt on the supposed identity of these two dietary factors [Mitchell, 1919].

After demonstrating the existence of the two vitamins in water-soluble B, Goldberger and his colleagues [1926] found an 85 % (by volume) alcoholic extract of white maize to be rich in the antineuritic vitamin B_1, but deficient

in the heat-stable P-P (vitamin B_2) component. They write, "we have gained the impression that 'P-P' is relatively much more soluble in acidulated water than in 85 % alcohol, whereas the antineuritic factor is soluble in both." In a recent paper Sherman and Sandels [1929] find vitamin B_2 ('vitamin G') to be insoluble in 95 % alcohol.

We have fully confirmed the conclusions of Goldberger and his colleagues and consistently find vitamin B_1 soluble, and vitamin B_2 insoluble, in alcohol of concentrations from 83 to 93 % by weight. But we have not been able to effect a satisfactory separation of the two vitamins in this manner. After the final recovery from the insoluble residue the vitamin B_2 was reduced in amount, suggesting that this vitamin was destroyed by contact with alcohol or by some other conditions experienced during the operation. Sherman and Sandels [1929], whose work appeared after these experiments were complete, came to a like conclusion.

The following illustrates the method used. Fraction 5, the dilute acetic acid extract from Yeast XII, was concentrated on a water-bath. Of the concentrate, 50 cc., equivalent to 82 g. yeast, were dropped slowly into 2½ litres of 94 % (by weight) alcohol with vigorous stirring. A precipitate was formed immediately. The mixture was shaken for 2 hours and allowed to stand several days after which the clear yellow top liquor was siphoned off. The insoluble matter was filtered and, after washing, shaken with a fresh amount of 94 % alcohol and the operation repeated until the alcoholic extract was colourless.

The combined alcoholic extracts and washings, of which the specific gravity corresponded to strengths of alcohol varying from 91·5 to 93 % alcohol, were concentrated to a small bulk at a low temperature under reduced pressure and finally taken to dryness at 37° with a fan. This dry residue, which was very acid, and the dried insoluble precipitate (= 7·7 g.) were dissolved in water and separately tested for content of vitamins B_1 and B_2 respectively.

The results (Table III, exp. 3) showed that about one-half the vitamin B_1 present in the original material was in the alcohol-soluble fraction. Vitamin B_2 was not detected in this fraction, but the amount present in the alcohol-insoluble portion was only equal to about one-half of that originally present.

In order to see whether this destruction of vitamin B_2 were due to the contact with alcohol or to oxidation when in the dry condition, a sample of the concentrated yeast extract used for the above experiment was evaporated to complete dryness and portions were allowed to remain at room temperature exposed to the air, and covered with 94 % alcohol respectively. After 19 days the alcohol was blown off the latter at 37° with a fan and the dry residue taken up in water and tested for vitamin B_2 content. This was compared with that of both the original solution and the solution made from the air-dried material. The results, set out in Table III, exp. 3, showed that a slight diminution in potency had taken place in the material which had been in contact with alcohol, but the difference was not significant and seemed too small to account for the loss experienced in the experiment described above.

Further investigation is needed to elucidate this matter. It may be that the destructive effect of alcohol on vitamin B_2 depends on the reaction of the solution.

Action of ultra-violet light.

Hogan and Hunter [1928] reported experiments on growing rats in which "vitamin B" was supplied in the diet respectively by autoclaved yeast or yeast irradiated by ultra-violet light. From the results they concluded that the heat-labile antineuritic (vitamin B_1) component was resistant to the action of ultra-violet light, whereas the heat-stable component (vitamin B_2) was destroyed by irradiation. If this were so, an easy method would be available for preparing an antineuritic material free from vitamin B_2, which could replace Peters's concentrate in the study of vitamin B_2.

The action of ultra-violet light was tested upon the dilute acetic acid extract, Fraction 5, from Yeast XII, which is rich in both vitamins (Table III). The material was concentrated to a small volume (2 cc. containing the equivalent of 1 g. yeast) and poured over the surface of a flat white dish, to an average depth of about 2 mm. and irradiated at a distance of 40 cm. from a mercury vapour arc lamp. From time to time a little distilled water was added to prevent drying and the whole well mixed. After 6 hours' irradiation the content of vitamin B_2 was found to be reduced to about one-half, while that of vitamin B_1 had also suffered, though to a less extent. After 12 hours' irradiation, more than half the vitamin B_1 originally present had been destroyed. Further destruction of vitamin B_2 had also occurred, but this was by no means complete (see Table III, exp. 2).

Summary.

1. The Peters process, by which an antineuritic, vitamin B_1, concentrate free from vitamin B_2 is prepared from yeast, has been scrutinised to find out at which stage the removal of vitamin B_2 occurs.

2. About one-half to three-quarters of the vitamin B_2 present in the original yeast was carried down in the precipitation with lead acetate at p_H 4·7. The removal was more complete if the precipitation were carried out in neutral or slightly alkaline solution.

3. If the lead acetate precipitate is decomposed with sulphuretted hydrogen, a clear solution containing vitamin B_2 can be separated from the lead sulphide if certain precautions be taken.

The dose of this preparation required to maintain normal growth in young rats on diets deprived only of vitamin B_2, contained 0·03 g. dry weight (less ash) and was the equivalent of 0·5 g. of the original yeast.

4. This preparation contained more or less vitamin B_1, depending on the reaction at which precipitation with lead acetate had been carried out and probably on the amount (relative to vitamin B_2) contained in the original yeast.

5. Vitamin B_2 is insoluble, and vitamin B_1 is soluble, in alcohol of 92 % by weight. The method employed to effect a separation with strong alcohol destroyed vitamin B_2. The attempt to prepare a vitamin B_2 concentrate free from vitamin B_1, by the use of alcohol, was unsuccessful.

6. Both vitamins dialyse freely through cellophane (a viscose preparation of cellulose).

7. Ultra-violet light was found to exert a destructive action on both vitamins, vitamin B_2 being destroyed at a quicker rate than vitamin B_1. The result obtained with vitamin B_2 confirms the previous observation of Hogan and Hunter [1928]. Their conclusion that vitamin B_1 is resistant to ultra-violet light is not, however, confirmed.

In conclusion we desire to express our thanks to Sir Charles Martin for helping us by his continued advice and criticism.

REFERENCES.

Chamberlain and Vedder (1911). *Philippine J. Sci.* **6**, 251.
Chick and Roscoe (1927). *Biochem. J.* **21**, 698.
—— —— (1928). *Biochem. J.* **22**, 790.
—— —— (1929). *Biochem. J.* **23**, 498.
Drummond (1917). *Biochem. J.* **11**, 255.
Eijkman (1911). *Arch. Schiff. Trop. Hyg.* **15**, 698.
Fraser and Stanton (1910). *Philippine J. Sci.* **5**, 55.
Goldberger and Lillie (1926). *U.S. Public Health Reports*, **41**, 1025.
—— Wheeler, Lillie and Rogers (1926). *U.S. Public Health Reports*, **41**, 297.
Hogan and Hunter (1928). *J. Biol. Chem.* **78**, 433.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
—— —— (1927). *Biochem. J.* **21**, 777.
Mitchell (1919). *J. Biol. Chem.* **40**, 399.
Peters (1924). *Biochem. J.* **18**, 858.
Rosedale (1927). *Biochem. J.* **21**, 1266.
Schaumann (1911). *Trans. Soc. Trop. Med. Hyg.* **5**, 59.
Sherman and Sandels (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 536.