CXCV. THE ALCOHOL-SOLUBILITY OF THE ANTI-DERMATITIS, MORE HEAT-STABLE VITAMIN B₂ CONSTITUENT OF THE VITAMIN B COMPLEX.

BY HARRIETTE CHICK AND ALICE MARY COPPING.

From the Department of Experimental Pathology, Lister Institute.

(Received October 28th, 1930.)

THE relative insolubility in alcoholic solutions of 70 % strength upwards of vitamin B₂ as compared with the antineuritic vitamin B₁, has been shown by several independent workers using materials derived from different sources [Goldberger *et al.*, 1926; Sherman and Sandels, 1929; Chick and Roscoe, 1929; Narayanan and Drummond, 1930]. Osborne and Wakeman [1919] used alcohol fractionation in the preparation of their "vitamin B" concentrate. Alcohol was added to a watery yeast extract, obtained by boiling brewer's yeast in dilute acetic acid, until the concentration in the mixture was 52 % (by weight), and the precipitate was removed. The additional precipitate formed on raising the concentration of alcohol in the filtrate to 79 % was found to contain "most of the water-soluble vitamin originally present in the yeast." It is probable that in the vitamin tests of these authors the growth of the experimental rats was limited by the amount of vitamin B₁ present, this factor being soluble in 70 % alcohol, but there can be little doubt that vitamin B₂ was abundantly present in the precipitate.

Chick and Roscoe [1929] added alcohol to a concentrated watery extract of brewer's yeast until the mixture contained 83–93 % alcohol by weight, and although vitamin B₂ could not be detected in the alcoholic solutions which were rich in vitamin B₁, the vitamin B₂ potency of the insoluble material was nevertheless found to be less than that of the original yeast extract. This result suggested that vitamin B₂ had suffered some destruction from the strong alcoholic environment.

Sherman and Sandels [1929] noticed a similar diminution in the total vitamin B₂ value when air-dried baker's yeast was extracted with 60–80 % alcohol. No loss, however, was detected when 95 % alcohol was used, the insoluble material in this case possessing potency equal to that of the original yeast. Chick and Roscoe also detected no loss when their yeast extract was evaporated to complete dryness and then treated with 96 % alcohol. Fixsen (unpublished experiments) using dried egg-white obtained a similar result with 98 % or absolute alcohol. From these experiments it would appear that

vitamin B₂ was sensitive to alcohols of strength ranging from about 60 to 95 %, but could resist stronger solutions.

Narayanan and Drummond [1930] have recently published results differing somewhat from the above. They employed alcoholic fractionation to prepare a strong vitamin B₂ concentrate from brewer's yeast. Starting from the vitamin B₂-containing fraction, obtained by decomposition of the precipitate formed in the yeast extract with lead acetate, alcohol was added to 50 % strength and the precipitate removed. To the filtrate "which contained about 88 % of the activity of the original fraction" alcohol was further added to approximately 70 %. The "slimy, dark brown, precipitate" was found to contain most of the original vitamin B₂ activity, and the organic matter contained in the daily dose required to maintain satisfactory growth in young rats was as low as 6 mg.

The value of such a vitamin B₂ concentrate for the further study of the vitamin B complex is obvious, and we have attempted to repeat the work of Narayanan and Drummond. Our efforts, described in the following paragraphs, have, however, met with little success.

We used the solution (Fraction C) obtained by decomposition of the precipitate formed (at p_H 7.5) when basic lead acetate is added to the extract obtained by boiling washed brewer's yeast in 0.01 % acetic acid solution [Chick and Roscoe, 1929]. The precipitate was decomposed and lead removed by H₂S, after acidification with sulphuric acid, and the filtrate concentrated on a water-bath until the equivalent of 745 g. yeast (dry weight) was contained in 3050 cc. (Fraction C), the p_H being 1.5.

The vitamin B₂ content of this and the succeeding fractions was estimated by the method previously described [Chick and Roscoe, 1928]. Before testing, the solution was brought to *ca.* p_H 3.0 and most of the sulphate present removed by addition of baryta and barium chloride. The final product was evaporated until 1 cc. contained the equivalent of 0.35 g. yeast (Control solution I). A daily dose of 1 cc. of this product maintained an average weekly increase in the body weight of young rats of 15.5 g., *i.e.* contained adequate vitamin B₂ for optimal growth ("normal" standard = average weekly increase of 10–12 g.).

Exp. 1. *Fractionation with alcohol at p_H 1.5.* To 500 cc. of Fraction C ($\equiv$ 122 g. yeast) 96 % alcohol (by weight) was added until the concentration in the mixture was 56 %. A small precipitate (Precipitate II, dry weight 1.3 g.) separated, which was found to be inactive in doses equivalent to 6 g. yeast.

One-half of the filtrate ($\equiv$ 61 g. yeast) was reserved for testing. The alcohol was driven off by distillation under reduced pressure and the solution concentrated further on a water-bath. Baryta was added to remove sulphate and to bring the solution to *ca.* p_H 3.0. This fraction (Filtrate II) possessed about half the vitamin B₂ potency of the Fraction C to which it corresponded. The daily dose required for normal growth was equivalent to 0.35–0.7 g. yeast (average weekly increase in weight 10–12 g.).

To the other half of Filtrate II ($\equiv$ 61 g. yeast) 96 % alcohol was added till the concentration of the mixture was 70 %. A very small precipitate was formed on neutralising the solution; this proved inactive in doses equivalent to 1.4 g. yeast (Precipitate III). The filtrate (Filtrate III), tested after preliminary treatment similar to that of Filtrate II, was also found to be inactive in doses equivalent to 1.4 g. yeast. A combined dose of Precipitate III and Filtrate III, each equivalent to 1.4 g. yeast, was equally ineffective.

Exp. 2. *Fractionation with alcohol at p_H 3.2.* An experiment similar to Exp. 1 was carried out with 500 cc. of Fraction C ($\equiv$ 122 g. yeast) after neutralisation to p_H 3.2. The precipitate formed with 56 % alcohol (Precipitate IV) contained more sulphate and was larger than Precipitate II in Exp. 1. Traces only of activity, however, were detected in doses equivalent to 1.5 g. yeast. The filtrate (Filtrate IV) was equally inactive.

When Filtrate IV was subsequently fractionated with 70 % alcohol the small precipitate thrown down on neutralisation (Precipitate V), the filtrate (Filtrate V) and a combination of both, were all found to be inactive. The doses given were the equivalents of 1.5–2.2 g. yeast.

The results of these two experiments may be summarised thus. Precipitation with 56 % alcohol at p_H 1.5 (Exp. 1) caused the disappearance of about one-half of the vitamin B_2 originally present, while subsequent treatment with 70 % alcohol caused destruction or inactivation of the remainder. With less acid material, p_H 3.2 (Exp. 2), inactivation appeared to take place with 56 % alcohol.

Experiments were also made with a vitamin B_2 preparation prepared from egg-white [Chick, Copping and Roscoe, 1930]. When fractionation was attempted with 50 % alcohol at p_H 4.0, destruction of at least half of the vitamin occurred; with 70 % alcohol destruction appeared to be complete. An attempt to make a vitamin B_2 preparation by boiling acidified fresh egg-white with 50 % alcohol instead of water also proved unsuccessful.

The discrepancy between these results and those of Narayanan and Drummond (and possibly those of Osborne and Wakeman also) may be due to differences in the reaction of the solutions employed. The vitamin B_2 preparation fractionated by them was previously neutralised while those here described were acid, p_H 1.5 and 3.2 respectively. In the present study, however, vitamin B_2 appeared to be more stable in the more acid solution.

Such discrepancies are by no means uncommon in the scanty literature which exists concerning the properties of vitamin B_2 . For example, this vitamin was found by Levene [1928] to be sensitive to the action of nitrous acid; but completely resistant by Chick and Roscoe [1929] and Narayanan and Drummond [1930]. Chick and Roscoe [1930] and Chick and Copping [1930] have found vitamin B_2 to be destroyed by action of alkalis (p_H 9–10) at high temperatures 120–125°; Reader [1929] and Narayanan and Drummond [1930], on the other hand, have found it to be resistant.

Vitamin B_2 (in common with the additional constituents of the B group

of vitamins recently described by Williams and Waterman [1927, 1928] and Reader [1929, 1930]) is probably not a well-defined or stable chemical entity. Its solubility and precipitation limits are irregular and uncertain and are greatly influenced by small variations in the conditions under which they are studied. These conditions include the dilution and hydrogen ion concentration of the solution and the presence and concentration of salts and other substances. There must also be other disturbing substances which are at present unrecognised. In this laboratory it has often proved impossible to repeat a given observation under apparently similar conditions. Much more investigation is needed to eliminate these difficulties and, in the meantime, the necessity for including exact details of procedure in all published work is obvious.

A further complication arises from the fact that the usual method for estimation of vitamin B₂ consists in a growth test with young rats and therefore is very liable to erroneous interpretation unless strictly controlled. A more satisfactory criterion would be the cure of the dermatitis which occurs in rats on diets deprived of this vitamin, but the skin lesions are irregular and slow of development (8–30 weeks) so that such tests are impracticable on a large scale.

SUMMARY.

When alcohol to 56 % was added to a solution of vitamin B₂ at p_H 1.5, prepared from brewer's yeast, the precipitate was inactive and the filtrate possessed only one-half the potency of the original material. After further addition of alcohol to 70 % the solution was completely inactive. When the original solution was less acid (p_H 3.2) total inactivation occurred with 56 % alcohol.

REFERENCES.

- Chick (1929). *Biochem. J.* **23**, 514.
— and Copping (1930). *Biochem. J.* **24**, 932.
— — and Roscoe (1930). *Biochem. J.* **24**, 1748.
— and Roscoe (1928). *Biochem. J.* **22**, 790.
— — (1929). *Biochem. J.* **23**, 504.
— — (1930). *Biochem. J.* **24**, 105.
Goldberger, Wheeler, Lillie and Rogers (1926). *U.S. Public Health Reports*, **41**, 297.
Levene (1928). *J. Biol. Chem.* **79**, 465.
Narayanan and Drummond (1930). *Biochem. J.* **24**, 19.
Osborne and Wakeman (1919). *J. Biol. Chem.* **40**, 383.
Reader (1929). *Biochem. J.* **23**, 689.
— (1930). *Biochem. J.* **24**, 77.
Sherman and Sandels (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 536.
Williams and Waterman (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 1.
— — (1928). *J. Biol. Chem.* **78**, 311.