XCII. THE FORMALDEHYDE-AZO-TEST FOR VITAMIN B₁.

BY HENRY WULFF KINNERSLEY AND
RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(Received March 5th, 1934.)

THERE is some vagueness as to the exact scope of the so-called Pauly reaction, such as is given by histidine with diazotised sulphanilic acid. In the broad sense, it might be considered to characterise any substance reacting in a weak alkaline solution to give the characteristic pink or red colour. In the particular sense however it is often now taken to indicate a substance reacting with the reagents studied in detail by Koessler and Hanke [1919; see also Hunter, 1922; 1925].

Jansen and Donath [1926] stated that their crystalline preparations of vitamin B₁ from rice polishings gave a strong Pauly reaction, *i.e.* gave a red colour when treated with diazotised sulphanilic acid in presence of sodium carbonate. On the other hand, Kinnersley and Peters [1927; 1928] found (*a*) that most of the substances in yeast concentrates of vitamin B₁ giving the Pauly reaction (Koessler and Hanke technique) did not fractionate with the activity and (*b*) that fractions of activity 10 γ per unit of B₁ did not contain more than 3 % of substances giving a Pauly reaction similar to that of histidine. Guha and Drummond [1929] also obtained active vitamin B₁ fractions from yeast giving feeble Pauly reactions. In ignorance of the true potency of vitamin B₁ itself, it was impossible to decide whether the final entity was responsible for the trace of pink found. In 1931, at the British Association, it was stated [Peters, 1931] that the trace of pink colour given in the Pauly reaction by active yeast concentrates and by Jansen's crystals could be enhanced by suitable choice of alkalinity, and that it was believed that the active entity gave a Pauly reaction¹. Shortly afterwards this opinion was confirmed in further work, the outcome of which is given in this paper. Meanwhile other researches have given contradictory results, *e.g.* the vitamin B₁ crystals of Ohdake [1932] are stated to give a slow pink diazo-reaction, whereas the crystalline preparations of Tschesche *et al.* [1931] and of Van Veen [1932] gave no Pauly reaction, though the latter author was of the opinion that interaction with diazotised sulphanilic acid took place. Our own crystalline preparations have been stated by us to give an entirely yellow reaction with Pauly's test as performed by Koessler and Hanke, *i.e.* in 2.5 % sodium carbonate. Hence we were correct in attributing our previous 3 % to impurities.

Nevertheless, in essence the opinion as expressed at the British Association Meeting is correct, namely that the vitamin would interact with the diazo-reagent to give a pink colour at a more alkaline reaction than the one employed in the Koessler and Hanke method. We have delayed publication on this question for over two years in the effort to collect conclusive evidence, and we are now presenting shortly some of our data (as a preliminary step). Two circumstances have assisted our progress: (1) the realisation that rather fine shades of alkalinity

¹ It should have been added "in this sense."

would influence the coupling of some substances in the yeast extracts and the degree of yellow rather than pink formed (it is well known that pronounced changes in the alkalinity influence these colours): (2) the discovery of the remarkable effect of formaldehyde.

TEST IN PRESENCE OF FORMALDEHYDE.

All vitamin B₁ crystalline preparations in our possession interact to produce a pink colour with the following reagent:

N NaOH	100 ml.
NaHCO ₃	5.76 g.
H ₂ O	100 ml.

Method of performing test. 0.5 ml. of the diazotised sulphanilic acid¹ (Koessler and Hanke) is added to 1.25 ml. of the reagent in a small test-tube and after 1 minute 1 drop (0.03 ml.) 40 % formaldehyde and then immediately the vitamin in 0.1–0.3 ml. solution of acidity greater than p_H 4.0. A pink colour develops slowly and increases in intensity for 30–60 minutes. After this time it remains approximately constant, though the pink becomes more prominent up to 24 hours owing to fading of the yellow. Standards can be made up with different concentrations of the vitamin from 2 to 6 or 7 pigeon doses and kept for about 14 days. The limit of sensitivity is about 1 vitamin B₁ unit, approximately 1.5–2.0 γ crystals. If made up in steps of 1 unit (pigeon dose) in small test-tubes (7×1.3 cm.) and corked, reasonably accurate estimates of colour can be made with 5–10 γ of vitamin crystals. Trials with a colorimeter have not given as satisfactory results with these dilute solutions as the simple comparative method. The latter can be combined with dilution if desired. With amounts of 1 unit, the colour is ill-defined. Even in the purest specimens of vitamin yet reached, the red is always tinged with yellow. 10 γ (5 pigeon doses in our sense) approximately match a mixture of Sørensen's acid and alkaline phosphates (2+8 volumes) of p_H 5 at 15°, containing in 103 ml., 100 ml. of phosphate mixture and 3.0 ml. 0.02 % phenol red.

Influence of formaldehyde.

The N/10 HCl extracts from charcoal [Kinnnersley *et al.* 1933]. In the purer preparations of 5 γ and under per vitamin B₁ unit, the formaldehyde stabilises the colour. In its absence the colour increases in intensity for a short time and then fades. This perhaps explains Ohdake's [1932] observations. The pink-coloured substance is extracted by butyl alcohol from alkaline solution, and so can often be separated from the yellow substances in impure solutions.

In the less pure preparations up to 100 γ per unit the formaldehyde has the effect of suppressing or inducing the gradual fading of the yellow colour caused by impurities (such as histamine), which react with the reagent at the particular alkalinity chosen to give a yellow colour. (At a less alkaline p_H such substances would of course give the usual pink.)

The 50 % alcohol extracts from charcoal and baryta extract of Jansen's acid clay standard. In these cases increase of the formaldehyde to a point at which the vitamin red colour is suppressed is not sufficient to inhibit the yellow colour. Orange colorations are therefore always produced. No attempt has yet been made to explore this further. By use of the Rosenheim-Schuster colorimeter, the red component can be selected and could probably be estimated, but this has not been properly explored.

¹ This reagent will keep for a week, if surrounded by ice in a thermos flask.

In all the impure extracts there is apt to be present in addition a substance giving a colour reaction at a more alkaline p_H . The alkalinity is chosen to reduce to a minimum the influence (a) of substances of the histamine type showing pink at more acid reaction and (b) of substances showing colours at a more alkaline reaction.

Vitamin B₁ couples at an acid as well as an alkaline reaction. The azo-colour is only produced at an alkaline p_H . The test cannot well be applied at all to extracts less pure than 500 γ /unit, as it is obscured by other reactions. In yeast extracts (and other sources investigated), there may be present at the earlier stages (in the extracts of acid charcoal for instance) a substance behaving similarly to the vitamin. It may be distinguished by adding to the pink solution concentrated HCl drop by drop to approximately p_H 8. At this p_H , the colour in question rapidly diminishes, whereas that of the vitamin persists for some time, even at an acid p_H . Beyond this, the test appears to have a fairly high degree of specificity; out of a large number of substances tried, one of the few giving the reaction is acetone, and the colour in this case is not permanent. The question however has not been thoroughly explored. The formaldehyde should be added to an acid solution of the vitamin immediately before the diazotised reagent. An explanation of the action of formaldehyde is not to hand; possibly it is in this case an oxidising action. It has been noticed that methylene blue reduced by sodium hydrosulphite at an acid p_H is not affected by addition of formaldehyde; making alkaline, however, induces much more rapid oxidation of the methylene white in presence than in absence of formaldehyde.

A preliminary exploration with this test has been made of a large number of extracts of seeds, roots, sugars, *etc.*, after treatment with lead acetate and charcoal for the extraction of the vitamin. In many cases there was a substance producing the vitamin type of colour unstable in acid; in no case was there reason to suspect a more active source of the vitamin than yeast. It should be emphasised that in first extracts the concentration of the vitamin is never sufficient to show the reaction.

Table I shows a comparison between vitamin activities by bird and formaldehyde-azo-test; it represents a few only of the numerous estimations made.

Table I.

Amounts (γ) having similar activities (= 4 pigeon day doses).

(1) Older preparations			(2) More recent tests		
No.	Bird γ	Formaldehyde-azo-test γ	No.	Bird γ	Formaldehyde-azo-test γ
53	10	10	92 A 50	8	8
Windaus <i>et al.</i>	17.8	18	19	10 (5)*	8.9
64.19	10	9.6	Van Veen	8 (10)†	6.8
56	11.2	13.8	93 A	10 (7)	12.8

(3) Tests during an early fractionation of a phosphotungstate fraction, p_H 5.5–4.8.

p_H refers to the p_H at which fractions were separated.

p_H	Bird (γ)	Formaldehyde-azo-test (γ)
5.3 +	51.5 (5)	51.5
5.2 +	125 (4)	120
5.0 –	157 (4)	230

* Figures in brackets refer to numbers of birds in tests. For probable errors see paper by Kinnerley *et al.* [1933], and for comparison with catatorulin tests Passmore *et al.* [1933].

Note. 1 pigeon day dose = approximately 1 vitamin B₁ unit.

† This is not the preparation recently mentioned in *Nature*.

Tests upon the preparations of Tschesche *et al.*, and Van Veen¹ are also included. The latter is from rice polishings, so that the reaction is given by oryzanin as well as by torulin.

There is no question from an examination of Table I of the close parallelism between the fractionation of vitamin B₁ and the substance in the extract giving the formaldehyde-azo-test. We are clear, however, that we have had in our hands occasionally forms of the vitamin which do not give the test at the same intensity as the bird test. Hence, though it can be said that with the above exception all substances giving this test in these extracts contain vitamin B₁, it is apparently not true that all forms of vitamin B₁ give the test.

Use of the test in fractionation.

The formaldehyde-azo-test is of the greatest value in the fractionation and has enabled us almost to dispense with the animal tests in fractionation of the phosphotungstate extract from the *N*/10 HCl extracts of the charcoal [Kinnersley *et al.* 1933]. By carrying out simultaneous tests with and without formaldehyde during the fractionation at *p*_H 4, it is possible to separate almost exactly all the vitamin from the substance which gives the yellow reaction, mostly destroyed by formaldehyde under our conditions². Until it is quite certain that pure vitamin B₁ has been obtained, it cannot be proved that the formaldehyde-azo-test is one of its properties. But it can be definitely asserted that the test is of the greatest value in following vitamin B₁ up to a highly potent crystalline stage. This assertion is based upon a large number of experiments over a period of two years. It is possible that the discrepancies still shown in Table I are not due to experimental error (*e.g.* 93 A); this should be decided by work in progress in collaboration with J. R. O'Brien.

SUMMARY.

An azo-test is described of high specificity for vitamin B₁. The specificity is due to the suitable choice of alkalinity for coupling and the use of formaldehyde as a stabilising agent.

We are indebted to the Medical Research Council for a personal grant to one of us. We are also grateful to Mr J. R. O'Brien for advice and help.

REFERENCES.

- Guha and Drummond (1929). *Biochem. J.* **23**, 880.
Hunter (1922). *Biochem. J.* **16**, 640.
— (1925). *Biochem. J.* **19**, 25.
Jansen and Donath (1926). *Proc. K. Akad. Wetensch. Amsterdam*, **29**, 1390.
Kinnersley and Peters (1927). *Biochem. J.* **21**, 789.
— (1928). *Biochem. J.* **22**, 422.
— O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 505.
Ohdake (1932). *Bull. Agric. Chem. Soc. Japan*, **8**, 111.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 542.
Peters (1931). *Chem. Brit. Assoc.* 141. (Heffer.)
Tschesche, Ruhkoff, Laqueur and Schultz (1931). *Nachricht. Ges. Wissenschaften Gött.* 207.
Van Veen (1932). *Rec. Trav. Chim. Pays-Bas*, **51**, 279.

¹ We again thank Dr Tschesche and Dr Van Veen for the gift of these preparations.

² Without formaldehyde, the pink colour gradually fades.