CXCIX. IMPROVEMENTS IN THE USE OF THE FORMALDEHYDE AZO REACTION FOR VITAMIN B₁

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THIS paper embodies further observations made upon and in relation to the formaldehyde azo reaction for vitamin B₁ [Kinnersley & Peters, 1934] and its possible application to semi-quantitative estimation of vitamin B₁. The development of the more sensitive thiochrome method of estimation [Jansen, 1936; Karrer & Kubli, 1937; Pyke, 1938] during the progress of this work, may ultimately make the method here described obsolete. Nevertheless, we think that some independent check may prove useful, and further, that the method may prove of value, when enough vitamin B₁ is available, owing to the stability of the standards, and to the fact that a dark room and a source of ultra-violet light are not required. Unfortunately, neither method is successful with phosphoric esters of vitamin B₁; these do not give the formaldehyde azo test (as stated by Lohmann & Schuster [1937]); they form a blue fluorescent compound with alkaline ferricyanide, but this does not pass into a butyl alcohol layer, which is a basis of separation from interfering fluorescent substances [Kinnersley & Peters, 1937]. We have found that phosphatase can be used in the pre-treatment of the extracts to liberate the vitamin B₁. In our previous communication we stated that the condition of alkalinity chosen and the presence of formaldehyde largely reduced interference by other substances; a particularly troublesome impurity giving a pseudo-vitamin B₁ colour reaction could be distinguished by its relative instability in acid solution. We have realized for some time that butyl alcohol would extract the bulk of the pink substance formed in the reaction. By an error the colour was compared in our previous paper [1934] with phenol red in phosphate buffer pH 5; it should have been "approximately pH 7.3". A considerable improvement is again made by shaking the butyl alcohol with dilute acid, when the pink substance passes back into the acid aqueous phase; the whole test now compares favourably as regards specificity with other methods and can be readily applied to vitamin B₁ concentrates from the International Standard Clay (1936) and also the cruder "charcoal" concentrates. We have found certain conditions which interfere with this and other azo tests. The work falls into two parts:

- I. The method and some conditions modifying it;
- II. Application to crude vitamin B₁ extracts.

I. The method and some conditions modifying it

(a) The original formaldehyde azo test has been modified as follows.

To 0.1–0.3 ml. of a solution containing 10–20 γ vitamin B₁ (acidity best about pH 6.0) and 30 % ethyl alcohol, is added after one minute a mixture of 1.25 ml. of the special reagent and 0.5 ml. diazotized sulphanilic acid. The alcohol now included has proved essential for full development of the pink colour [cf. Koessler & Hanke, 1919]. After standing for 2 hr. or more, the pink-coloured

solution is extracted twice successively with 2 ml. of butyl alcohol. The combined butyl alcohol extracts are then extracted with 2 ml. *N*/200 HCl and with 1 ml. twice (this should make the *pH* of the aqueous phase about 6.5). If not acid after shaking, more acid (trace of *N*/10) must be added; the pink HCl phase is now removed, and an equal volume of ethyl alcohol is added. The resulting coloured solution is compared with standards of vitamin B₁ similarly treated. They should be kept out of strong light except during tests. We have tried the colorimeter (visual and photoelectric), but are convinced that equal and even better accuracy in these dilute solutions is obtained by a naked eye comparison of similar small bore tubes. Our estimate of the accuracy of the method is $\pm 5\%$ for amounts of aneurin HCl of approximately 20 γ . Standards of 40 γ and over can be prepared.

(b) *Conditions modifying the test*

During our experience with this test some facts have been discovered which are briefly recorded. Reducing agents such as cysteine or traces of sodium hydro-sulphite, markedly delay the appearance of colour. Traces of some metals interfere both with azo reactions of the Pauly type for histamine and histidine, and also with the reaction for vitamin B₁.¹ Other systems have not been investigated. We do not know of any previous publication showing this effect upon azo reactions.

Histidine. Estimating 20 μ g. by the usual Pauly reaction according to the technique of Koessler & Hanke [1919], no interference was produced by U, Mn, Nb, Rb, Ta, Be, Pb, La, Th, Thallium, V, Cs, Cr, Fe in amounts of 20 γ , or by Al 10 γ , or In 25 γ ; interference was produced by Ni 5 γ , Co 10 γ , Hg 20 γ , Mn slightly, Cd 100 γ , Sn 9 γ , Ce 20 γ slight, Zn 10 γ , Cu 3 γ . The metals interfere mostly by reducing pinkness in the solution.

Vitamin B₁ formaldehyde azo reaction. Remarkably little interference was produced by most metals tried. Exceptions were found in Cu, Hg, Ag. With 10 γ vitamin B₁ Cl, HCl, 4 γ Hg were inhibitory and 10 γ Ag slightly so. The effect of Cu was of the greatest interest. A delayed reaction developed with minute traces of this, an amount equivalent to one atomic weight per mol. producing practically maximal effect. The action depends upon the presence of formaldehyde.

The question arises whether the presence of a metal could explain the delayed azo reaction previously reported by Kinnersley *et al.* [1935] during the formation of quinoxalines by oxidation with manganese oxides. If Mn compounds could produce the effect this would be reasonable; but they do not do so, nor is it possible to modify the reaction for vitamin B₁ by addition of the solution in which the vitamin is oxidising. Hence the original interpretation that the delayed azo reaction indicates a change in the vitamin molecule is still tenable.

II. *Application of reaction to crude vitamin B₁ extracts*

We have made a preliminary attempt to apply this to certain food materials, as well as to vitamin B concentrates, using for biological tests the birds required in the course of another research. After considerable trial, 50% acid ethyl alcohol has been employed to extract the crude, ground foodstuff. Both vitamin B₁ and its pyrophosphoric ester are very soluble in 50% ethyl alcohol and stable in faintly acid solution; 50% acid alcohol also removes vitamin B₁ from adsorbents such as charcoal. From what is now known of the chemistry of these substances it seems to us to be inconceivable that this extraction is not adequate; tests with 75% alcohol have not shown any larger recovery of the vitamin. In our experience it is essential to concentrate the vitamin and to remove it from certain interfering substances. For this purpose we cannot improve upon sodium phosphotungstate, which has been long used in this laboratory for the preparation of

¹ Some of these reactions were shown to the Biochemical Society in May 1936.

vitamin B₁ and which is known to precipitate it in very low concentration. The complete method is as follows.

A convenient amount (100 g. in the case of oatmeal) of the finely ground foodstuff is treated with 1000 ml. of boiling water; 2 ml. conc. HCl are added to bring it to pH 3.5, and the whole is heated for a few minutes to boiling. The rather thick mixture is placed on the boiling water bath for an hour or more and enough ethyl alcohol is then added gradually to make it up to 50 % by volume. The whole is filtered after cooling. Some materials require more solvents than others. The extract, after cooling overnight, and being freed from precipitate by Büchner filtration, is evaporated to 150 ml.; the water bath suffices for this as this vitamin is relatively stable in dilute acid solution. After further cooling and removal of insoluble matter by the centrifuge, Na phosphotungstate, 10 % at pH 6, is added to slight excess and the mixture is acidified to pH 1.0 with 20 % H₂SO₄. The phosphotungstate precipitate is allowed to stand 12 hr., collected in the centrifuge tube, and ground with baryta three times, according to our usual technique [Kinnersley *et al.* 1935]. After removal of baryta by H₂SO₄, the extract is concentrated if necessary at pH 3, care being taken to see that greater acidity does not develop during concentration; any free H₂SO₄ should be removed by BaCl₂ during this process. A convenient volume not exceeding 0.3 ml. is taken for the test.

Table I. *Comparison of vitamin B₁ in food materials by colour and biological tests. (γ per g. or ml. test material undried; no. of birds used in parentheses)*

Food material	Moisture content %	γ vitamin estimated by		Bird test		±2ε (γ)
		Colour test	Bird test	g. food given	γ vitamin found	
1. Yeast concentrate 41	—	120.0	145.00 (153)			
2. Wheat germ	7.0	20.0	13.50 (11)			
3. Pea	11.65	3.5	2.94 (81)	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">6</div> <div style="display: inline-block; vertical-align: middle;">3</div> <div style="display: inline-block; vertical-align: middle;">5</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">1.95 (11)</div> <div style="display: inline-block; vertical-align: middle;">3.27 (45)</div> <div style="display: inline-block; vertical-align: middle;">2.80 (25)</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">2.72-3.97</div> <div style="display: inline-block; vertical-align: middle;">2.22-3.55</div> </div>
4. Breakfast oats	10.0	3.0	2.54 (48)	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">6</div> <div style="display: inline-block; vertical-align: middle;">5</div> <div style="display: inline-block; vertical-align: middle;">3</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">2.23 (16)</div> <div style="display: inline-block; vertical-align: middle;">2.75 (12)</div> <div style="display: inline-block; vertical-align: middle;">2.67 (20)</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">2.39-2.98</div> </div>
5. Haricot bean	12.7	2.2	2.15 (5)			
6. Oatmeal	10.3	2.0	1.87 (41)	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">6</div> <div style="display: inline-block; vertical-align: middle;">5</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">2.00 (20)</div> <div style="display: inline-block; vertical-align: middle;">1.75 (21)</div> </div>	
7. Maize	9.7	2.0	1.54 (55)	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">6</div> <div style="display: inline-block; vertical-align: middle;">10</div> <div style="display: inline-block; vertical-align: middle;">5</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">1.41 (26)</div> <div style="display: inline-block; vertical-align: middle;">1.12 (6)</div> <div style="display: inline-block; vertical-align: middle;">1.80 (23)</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">1.09-1.82</div> </div>
8. Lentil	12.7	1.75	1.60 (12)			
9. Barley	12.0	1.2	0.71 (11)			

NOTES:

1. Yeast concentrate 41 was a 50% alcohol concentrate from charcoal used in routine work in the Laboratory.

2. The average day dose for the birds is calculated by the logarithmic method [Kinnersley & Peters, 1936].

3. The day dose is taken as equivalent to 2.5γ.

4. The individual tests are not here included, in order to economize space. They can be made available by writing to this laboratory.

5. In a few cases, the values of ±2ε are quoted as a guide. For barley there was wide variation in the 11 tests of which the average is given.

6. Values for assays of vitamin B₁ quoted in the review by Fixsen & Roscoe [1938] are wheat germ 10-55γ, pea 1.0-3.0γ, haricot bean 1.3-2.5γ, oatmeal 8γ, breakfast oats 3.5γ, whole maize 0.75-1.5γ, lentil 1.0-5.2γ, all per g.

Using the pigeons available in the laboratory in the course of another research, we have obtained the following preliminary results. So far as we know the curative and protective test is valid for these cruder concentrates, though Kinnersley & Peters [1936] showed that it would not apply to the pure vitamin. The figures given in columns 5 and 6 of Table I give reasonably satisfactory agreement between the larger and smaller doses except in the case of the 6 g. dose for the pea, but here it will be noted that the number of tests is fewer. Inspection of Table I shows that parallel results are obtained with the azo method and biological tests; the order of arrangement in the table is based on the colour test and it agrees with that obtained by the bird test. The results of the bird tests have been calculated upon the assumption that one day dose equals 2.5 γ vitamin B₁; in 1936, we concluded that one day dose must be equivalent to 2.0 γ *per diem* as the result of tests of the catatorulin type and also of colour reactions. Since then, however, we have found that our extract of the acid clay may have deteriorated and that a better value is 2.5 γ (cf. Sampson & Keresztesy [1937], also Pyke [1938]). Though there are some marked deviations from previous estimates of similar foodstuffs in the literature (see note 6 to Table I), our results as a whole are of the same order.

Phosphoric esters of vitamin B₁ (cocarboxylase etc.). We have attempted to decide how far the presence of phosphoric esters of vitamin B₁ interfere in the case of (a) yeast extracts and (b) foodstuffs. The most suitable method for breaking up the phospho-compounds is enzymic. After considerable trial, and with the final advice of Dr Kay to whom we are grateful, we adopted takaphosphatase (as present in takadiastase 0.5 %), acting at approximately pH 4. The result of one experiment is given in Table II.

Table II. *Effect of takadiastase on cocarboxylase (crude)*

Exp. 1. Solutions A, B, C were made up as stated below and incubated at 38° for one hour, after which the vitamin B₁ and phospho-esters were determined by a thiochrome method upon an aliquot. The results in the Table are expressed as equivalents of vitamin B₁.

Solution	A	B	C
Takadiastase	100 mg. in 10 ml.	Nil	100 mg. in 5 ml.
Cocarboxylase	20 ml.	10 ml.	Nil
Phosphate buffer pH 4	50 ml.	25 ml.	12.5 ml.
Water	Nil	5 ml.	Nil
Content in esterified or free vitamin B ₁			
Before incubation:			
Vitamin B ₁ : Esterified	600	600	Nil
Free	100	100	Nil
After incubation:			
Vitamin B ₁ : Esterified	50*	500	Nil
Free	500*	100	Nil
% "free" vitamin liberated by phosphatase	91	15	—

* These values were checked by azo colour tests which were in agreement. The crude cocarboxylase solution contained preformed vitamin B₁.

Both by azo test and fluorescence it was found that approximately 90 % of the combined vitamin B₁ had been converted into the free form by this treatment.

Application of this treatment to yeast extracts showed that in the initial extracts combined as well as free vitamin B₁ might be present, but there was no reason to think that this applied to the foods. Hence this possible objection to the use of both the azo test and the modification of Jansen's thiochrome methods in these foodstuffs appears in practice to be without foundation.

Though vitamin B₁ added can be recovered quantitatively, it is conceivable that some of the vitamin B₁ present in various forms in the foodstuffs may escape extraction with dilute acid or precipitation by phosphotungstate. But any such amounts are likely to be small in view of the known properties of the vitamin and its compounds. In any case, a method of this nature is valuable chiefly to establish a minimum. In view of the fair parallelism seen in Table I, we think that the method merits further exploration.

SUMMARY

1. The formaldehyde azo test for vitamin B₁ has been improved and extended to include a preliminary investigation of the vitamin content of foodstuffs, in which colour tests have been compared with biological tests. Parallel results have been obtained.

2. Certain metals in small amounts interfere with this test, and also with reactions of the Pauly type.

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