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An Azo Colour Reaction for the Determination of Vitamin B₁ in National Flour

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Jansen & Donath [1926 *a, b*; 1927] reported that vitamin B₁ concentrates gave a Pauly colour reaction with diazotized sulphanilic acid. Kinnersley & Peters [1927; 1928], Windaus, Tschesche, Ruhkopf, Laquer & Schultz [1931] and Van Veen [1932], however, considered that vitamin B₁ did not give a Pauly reaction as ordinarily carried out or as modified by Koessler & Hanke [1919]. By increasing the alkalinity of the reaction as carried out by Koessler & Hanke, Kinnersley & Peters [1927] found that vitamin B₁ gave a pink colour instead of a yellow one, but this was tinged with yellow and faded gradually. Later, Kinnersley & Peters [1934] found that the pink colour could be intensified and stabilized by the addition of formaldehyde but that the yellow colour still persisted. Barger, Bergel & Todd [1935] suggested that it is the free amino group which reacts with diazonium salts. Todd & Bergel [1936], however, consider the formaldehyde-azo colour reaction of Kinnersley & Peters depends on the presence of a β -hydroxy-ethyl group in position 5 and a hydrogen atom in position 2 of the thiazole nucleus.

Diazotized *p*-aminoacetophenone was used by Prebluda & McCollum [1936; 1937; 1939] as a qualitative test for vitamin B₁ and by Platt & Yang [1937] and Yang & Platt [1939] for the quantitative estimation of vitamin B₁ in biological material, the reaction being carried out in the presence of the clay on which they adsorbed the vitamin B₁. Willstaedt & Bárány [1938] and v. Euler [1940-1] employed diazotized 2:4-dichloroaniline.

Melnick & Field [1939] also used Prebluda and McCollum's reaction in the quantitative estimation of vitamin B₁. They carried out a preliminary adsorption of the vitamin on 'Decalso', eluted it with hot acid KCl solution and developed the colour in

the presence of phenol, which, they state, makes the reaction more sensitive, has a protective action on the oxidation of the vitamin B₁ in alkaline solution and prevents the inhibition of colour reaction caused by various substances. Emmett, Peacock & Brown [1940] adopted Melnick & Field's procedure except that they adsorbed the vitamin B₁ on 'Superfiltrol' instead of on 'Decalso', and carried out the colour reaction in the presence of the adsorbate, on which the dye was quantitatively adsorbed and subsequently eluted with ethanol. Auerbach [1940] has simplified Prebluda & McCollum's reagents and applied the reaction to the quantitative estimation of vitamin B₁ in certain pharmaceutical preparations, omitting the use of any adsorbent.

Prebluda & McCollum [1939] tested the specificity of the *p*-aminoacetophenone reaction, using several pyrimidine and thiazole derivatives. Some of the thiazole derivatives gave purple-red compounds with the alkaline diazo reagent. The free thiazole portion of the molecule reacted much more slowly and less completely than vitamin B₁ itself. They concluded that the presence of the methylene bridge between the thiazole and pyrimidine portions of the molecule was necessary for complete reaction. Melnick & Field found that degradation products of vitamin B₁ did not react. This was true after sulphite cleavage, which yields intact pyrimidine and thiazole derivatives; after the action of alkali and heat, which results in the opening of the thiazole ring; after simple deamination, and after oxidation to the thiochrome stage.

Kirch & Bergeim [1942] have recently described a colour reaction for vitamin B₁ with diazotized ethyl *p*-aminobenzoate, the dye being subsequently dissolved in *iso*-amyl alcohol. They claim that preliminary adsorption is unnecessary under their con-

ditions for the determination of vitamin B₁ in urine, but they have not applied the reaction to any other biological material. Using pure vitamin B₁ solutions, we have found this colour reaction to be much less sensitive than with diazotized *p*-aminoacetophenone using the procedure described below. We have attempted to apply their reaction to flour extracts and to 'Superfiltrol' adsorbates of flour extracts. In both cases, the colour reaction was completely inhibited, a bright yellow colour being extracted by the solvent.

We have recently examined the procedure of Melnick & Field and find that although their method of adsorption and elution is fairly satisfactory, it is a tedious and complicated procedure. A more simple method has been devised which was finally adopted for the estimation of vitamin B₁ in national flour.

EXPERIMENTAL

Extraction of vitamin B₁

The vitamin has been extracted from flour with 0.5*N* HCl and, in order to be sure of complete extraction, it has been found advisable to stir up the flour suspension at intervals over a period of several hours and finally to leave the suspension to settle overnight. Since flour contains a negligible amount of phosphorylated vitamin B₁, preliminary enzymic digestion has been omitted.

It has been found that during extraction the whole of the vitamin B₁ passes into the liquid phase. Thus, in calculating the vitamin B₁ content of flour, it is permissible to take the volume of the extracting fluid as the volume of the flour extract, so that if, as in the following procedure, 25 g. flour are extracted with 100 ml. 0.5*N* HCl, the vitamin B₁ contained in 40 ml. of the filtered extract is equivalent to 10 g. original flour.

Adsorption of vitamin B₁ from the flour extract

Quantity of adsorbent. This has been kept as small as possible so as to minimize the amount of dye precipitated on it at a later stage in the process. One adsorption of flour extract (=10 g. flour) on 50 mg. adsorbent removed approximately 90% of the vitamin B₁, a second adsorption on a further 50 mg. removing all the remaining vitamin B₁ from solution. Two successive adsorptions on 50 mg. 'Superfiltrol' have consequently been employed.

pH for adsorption. Quantitative adsorption of the vitamin B₁ from flour extracts was obtained without any adjustment of pH.

Time and conditions for adsorption. Shaking for a total period of 1 hr. on a tumbler rotating at approximately 60 r.p.m. has been found adequate for complete adsorption of the vitamin B₁ from flour extracts.

Adjustment of the adsorbate prior to the colour development

pH adjustment. Prebluda & McCollum [1939] advocated adjusting the pH to 5.0-6.0 immediately before carrying out the reaction. Melnick & Field [1939] give experimental evidence for adjusting the pH of vitamin B₁ in alcoholic

solution to 7.0, but in practice add alkali until their solution is blue to thymol blue. Emmett *et al.* [1940] adjusted the pH in the presence of alcohol to 7-8.

We found that solutions at pH from 2-9, adjusted in the presence of alcohol, gave erratic results. Consistent and reproducible results were, however, obtained from pH 4-7, if the pH adjustment was made immediately before the addition of the ethanol.

Concentration of ethanol. The presence of a high concentration of ethanol in the final stages of the reaction is advantageous as it helps to elute vitamin B₁ from the adsorbent, stabilizes it, intensifies the colour produced [Melnick & Field, 1939] and also keeps the dye in solution. The concentration of ethanol (40%) cannot be exceeded, otherwise salts from the alkaline diazo reagent are precipitated.

Factors affecting the colour reaction

Preparation of alkaline diazo reagent. An alkaline reagent similar to that used by Kinnerley & Peters [1927] was incorporated in the reagents of Prebluda & McCollum [1939] and was used unmodified by Melnick & Field [1939], except that the latter workers carried out the colour reaction in the presence of phenol and ethanol. Prebluda & McCollum allowed the alkali and diazo reagent to react for at least 15 min. before carrying out the colour reaction. This procedure, we have found, diminishes considerably the colour produced.

Like Koessler & Hanke, we have found it necessary to add the alkaline diazo reagent to the alcoholic suspension of vitamin B₁ exactly 1 min. after mixing the diazo reagent and alkali.

The relative concentrations of alkali and diazo reagent have been found to be very critical. Employing the same reagents as Prebluda & McCollum, we have ascertained the effect on the colour development of varying the relative concentrations of diazo reagent and alkaline reagent. The two curves in Fig. 1 show the effects of (a) varying the amount of alkaline reagent from 1.0 to 5.0 ml., keeping the volume of diazo reagent constant at 2.5 ml., and (b) varying the amount of diazo reagent from 1.0 to 5.0 ml., keeping the volume of alkaline reagent constant at 2.5 ml. In both cases the reaction was carried out in the presence of a flour adsorbate, treated as described below, the colorimeter readings/100 mg. wet adsorbent being plotted against the volume of reagent (Fig. 1).

From these results it was concluded that for estimating the vitamin B₁ contained in 10 g. national flour, 2.5 ml. diazo reagent to 2.25 ml. alkaline reagent are required.

In the following method, the concentrations of acid *p*-aminoacetophenone solution and the alkaline reagent have for convenience been adjusted so that 2 ml. of both diazo reagent and alkali contain the optimal amounts of the reactants. The final diazo mixture is considerably less alkaline than that used by Prebluda & McCollum.

Presence of phenol. According to Melnick & Field, phenol intensifies the colour produced. Using our reagents we have, however, obtained much better colour development than did Melnick & Field employing phenol in addition. We consider the presence of phenol unnecessary.

Reaction time. Melnick & Field state that it is necessary to leave the reaction mixture overnight for complete colour development. Using the procedure described below, the maximum is reached at the end of 1 hr.

Extraction of the dye. Toluene is a more efficient solvent for the dye than xylene (used by Melnick & Field [1939]) in the presence of the adsorbent. The colour in toluene is, moreover, much more stable in bright daylight. Toluene, however, volatilizes more rapidly than xylene and pre-

from the flour adsorbate reaction mixture alone with that from a flour extract to which vitamin B₁ had been added immediately before the colour reaction was carried out. The tints of the standard and unknown were identical by this procedure.

Fig. 1. The colour developed when flour adsorbate reacts with the diazo reagent in alkaline solution under different conditions: (a) with constant vol. (2.5 ml.) of diazo reagent, (b) with constant vol. (2.5 ml.) of alkaline reagent.

cautions have to be taken to prevent evaporation at any stage.

Emmett *et al.* [1940] adsorbed the dye quantitatively on 'Superfiltrol' and subsequently eluted it with ethanol. In order to simplify and shorten the procedure, we have attempted to keep most of the dye in solution by using a high concentration of ethanol, and have extracted the dye directly from solution by toluene. Extraction of the dye from the reaction mixture was found to be much more difficult if delayed until maximum colour development is reached, since, by that time, most of the dye is carried down on the adsorbent. Toluene was therefore added 20 min. after the beginning of the reaction. This procedure also accelerated the attainment of full colour development, which is never reached if extraction of the dye is begun during the initial 10 min.

Choice of standard

In early tests, the dye derived from flour extracts was compared with that from a standard vitamin B₁ solution, the colour reaction being carried out in the presence of 'Superfiltrol'. In the absence of 'Superfiltrol', the colour extracted from the vitamin B₁ reaction mixture was more purple than that extracted from a flour reaction mixture.

Values of more than 100% for the recovery of vitamin B₁ were obtained when the vitamin was added to flour extracts before adsorption. Similar values were obtained if vitamin B₁ in solution was added after adsorption and immediately before the colour reaction was carried out. In both cases, i.e. with 20 µg. vitamin B₁ added before and after adsorption, a value of 22.6 µg. vitamin B₁ was obtained. It thus appears that the presence of the flour adsorbate intensifies the colour produced, and it was therefore decided to compare the colour of the dye extracted

Method adopted

Reagents

- (1) 0.5N HCl (approx.).
- (2) 2% NaOH (w/v).
- (3) Alkaline reagent: 3% NaHCO₃ (w/v) in 0.6N NaOH.
- (4) *p*-Aminoacetophenone solution: 0.8% *p*-aminoacetophenone in 3.5 HCl (w/w). Keep in a dark bottle in the cold.
- (5) 4.4% NaNO₂ (w/v) (Analar). Keep in the cold, for not longer than 1 month.
- (6) Diazo reagent: Measure 1.5 ml. *p*-aminoacetophenone solution into a 50 ml. volumetric flask and cool to 0–5°. Add 1.5 ml. NaNO₂ solution (previously cooled to 0–5°), mix and leave in the cold for 10 min. Add a further 6 ml. NaNO₂ solution, mix and leave for 10 min. Make up to 50 ml. with ice-cold distilled water and allow to stand at least 15 min. before use. (This reagent should be prepared on the day of the test and must be kept at 0–5°.)
- (7) 'Superfiltrol' (activated clay).
- (8) Toluene (rectified).
- (9) 60% C₂H₅OH (v/v) in distilled water.
- (10) Standard vitamin B₁ solution: (a) Stock solution containing 150 µg. vitamin B₁ (hydrated—4.65% moisture)/ml. in 0.01N HCl containing 25% ethanol. (b) Solution for use: Dilute the stock solution with distilled water on the day of the test so that it contains 30 µg. vitamin B₁/ml.

Extraction of vitamin B₁ from flour and treatment of the adsorbate. (i) Stir 25 g. national flour into 100 ml. 0.5*N* HCl in a beaker, and leave at room temperature throughout the day preceding the test. Stir thoroughly at intervals and leave to stand overnight. Pour off the supernatant fluid and filter through a No. 1 Whatman filter paper (a satisfactory extract cannot be obtained by centrifuging).

(ii) Pipette 40 ml. of the filtrate into a bottle of 100–150 ml. capacity, provided with a glass stopper. Add 50 mg. activated clay, stopper and shake for 30 min. (a tumbler rotating at 60 r.p.m. is satisfactory). Transfer the contents of the bottle to a tapering 50 ml. centrifuge tube, wash out of the bottle as much of the adsorbate as possible with 5 ml. 0.5*N* HCl and add the washings to the contents of the centrifuge tube. Centrifuge until the adsorbate is well packed. Pour the supernatant fluid back into the bottle and shake for a further 30 min. with another 50 mg. activated clay. Transfer the contents to the centrifuge tube containing the first adsorbate and centrifuge. Discard the supernatant fluid. Wash out the bottle with five successive portions of 5 ml. 0.5*N* HCl, transfer the washings to the centrifuge tube containing the adsorbate and resuspend the adsorbate in the washings. Centrifuge again and discard the supernatant fluid.

(iii) Add to the washed adsorbate 1 ml. distilled water, 2 drops bromo-cresol-green and adjust to pH 5 (approx.) by the dropwise addition of 2% NaOH, keeping the adsorbate in suspension by stirring with a glass rod. Pour the suspension through a small glass funnel into a 25-ml. glass-stoppered measuring cylinder. Wash out the centrifuge tube with five successive portions of 2 ml. 60% ethanol, adding the washings to the suspension in the cylinder. Proceed immediately with the following step.

Development of colour. To 2 ml. NaOH-NaHCO₃ solution contained in a dry test-tube add 2 ml. diazo reagent. Invert to mix and, exactly 1 min. after mixing, add to the suspension of adsorbate in the measuring cylinder. Stopper and mix by inversion at intervals of a few minutes. After 20 min. introduce 5 ml. toluene into the cylinder. Restopper and continue to mix by inversion (done gently so as to avoid emulsion formation) at frequent intervals for a total period of 1 hr. or longer. Withdraw the toluene layer into another stoppered cylinder containing 10 ml. 0.5*N* HCl and wash by inversion. Transfer the washed toluene layer to a 5 ml. tapering centrifuge tube, shake in a small amount of anhydrous Na₂SO₄, mix and close the tube with a well-fitting cork. Centrifuge for a very short time, avoiding any risk of loss of solvent by evaporation.

Preparation of standard solution. To a second washed adsorbate, prepared as described in (i) and (ii), add 1 ml. of diluted standard vitamin B₁ solution and 2 drops of indicator and continue from this stage exactly as before with one difference, i.e. extract the dye with 10 ml. toluene instead of 5 ml.

Comparison of solutions. The two solutions are compared in a Duboscq type of colorimeter fitted with cups having a stem capacity of 5 ml.

Accuracy of method

(a) *Solutions of pure vitamin B₁.* The accuracy was determined on amounts of 20, 30 and 40 μ g. pure vitamin B₁ in solution. The procedure adopted was the same as that described above for flour extracts except that preliminary adsorption on 'Superfiltrol' was omitted and it was found necessary to extract the dye with toluene after 60 min. instead of after 20 min., since the colour production is retarded in the absence of 'Superfiltrol'. The accuracy of the estimations (in triplicate) carried out by three different observers is set out in Table 1.

Table 1. *Statistical analysis of the results of vitamin B₁ determinations*

Amount of pure vitamin B ₁ in test sample (μ g.)	20	30	40
Average of 9 observations (μ g.) ...	20.11	30.12	39.98
Standard deviation ...	0.4106	0.6099	1.0208
Value of <i>P</i> ...	0.95 0.99	0.95 0.99	0.95 0.99
Limits of error:			
For determinations in duplicate	2.8 3.7	2.8 3.7	3.5 4.7
For determinations in triplicate	2.3 3.0	2.3 3.0	2.9 3.8
For determinations in quadruplicate	2.0 2.6	2.0 2.6	2.5 3.3

(b) *Extracts of national flour.* A number of results for the vitamin B₁ contents of national flours, using our azo method as described in this paper, obtained by different observers, have been published in a Medical Research Council report [1943]. The accuracy of the results obtained compares favourably with that of the thiochrome method.

SUMMARY

A simple colorimetric method is described for the routine estimation of vitamin B₁ in national flour using diazotized *p*-amino-acetophenone. The method, with minor modifications, can be used for the estimation of pure vitamin B₁.

We are indebted to Dr Katharine Coward for her statistical analysis of the results set out in Table 1.

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A Quantitative Method for the Determination of Uroporphyrin in Pathological Urines

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The chief difficulty attending the isolation of porphyrins from urine and their separation from one another is the relatively sparing solubility of these pigments in organic solvents. Coproporphyrins I and III are extractable by the acetic acid-ether technique discovered by Saillet [1896] and adopted by Fischer [1926] and may thus be quantitatively determined. For preparative purposes, although large volumes of solvent are required, a high degree of purification may be achieved by repeated transferences between ether and dilute HCl. Uroporphyrin III is similarly extractable by ethyl acetate from solutions adjusted to a pH of 3.0-3.2 [Waldenström, Fink & Hoerbürger, 1935], but again large volumes of solvent are required for this laborious process, which is not suitable for quantitative purposes. Uroporphyrin I is not extracted by any known solvent immiscible with water.

Recourse must, therefore, be had to adsorptive procedures for the concentration and purification of the uroporphyrins prior to their spectrophotometric or fluorimetric determination. Many different materials have been proposed as suitable adsorbents for porphyrins. They include Ca and Pb phosphates, alumina, talc, BaSO₄, etc., and Waldenström [1935] has elaborated a chromatographic procedure for the adsorption of porphyrins from urine upon alumina. In this, as in all other

procedures, adsorption takes place at or near the isoelectric point of the porphyrin. At this reaction the greater part of the dark brown or red water-soluble pigment of unknown constitution, always present in porphyrinuric urines, is also adsorbed, so that from a quantitative point of view the operation is of little value, and further purification of the uroporphyrin fraction is difficult. Attempted purification of the porphyrins from urine by acidifying with acetic acid [Nebelthau, 1899] is not always successful, and in any precipitate which forms coproporphyrin is mixed with uroporphyrin.

While searching for a new adsorptive technique, it was discovered that kieselguhr has the very desirable and remarkable property of adsorbing both coproporphyrins and uroporphyrins from solution in dilute HCl whilst rejecting almost entirely the dark, brown-red, non-porphyrin pigment. Elution by suitable buffers brings about a fairly sharp separation of the coproporphyrins from the uroporphyrins, but it has not been found possible to separate by this means the I and III isomers of either pigment, no doubt owing to the fact that their isoelectric points are so similar. Based upon the kieselguhr technique, a good quantitative method has been worked out for the determination of total porphyrin, and hence of uroporphyrin, in porphyrinuric urines, thus for the first time enabling