CHEMICAL DETERMINATION OF VITAMIN B₁

I. REACTION BETWEEN THIAMINE IN PURE AQUEOUS SOLUTION AND DIAZOTIZED p-AMINOACETOPHENONE

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In the recent literature several chemical methods for the determination of thiamine are described. Some of these admittedly lack specificity (1–3). The determination of the blue fluorescence in ultraviolet light of thiochrome, one of the oxidation products of thiamine, has been most widely investigated (4–12). Its application to the quantitative determination of thiamine is complicated by similar blue fluorescence given by other substances (4–7, 9), the difficulty in correcting for such non-specific fluorescence (6, 13), the presence in biological materials of substances which may combine with thiochrome to form non-fluorescent compounds (7) or which may affect the intensity of the fluorescence (14, 15), the instability of the fluorescence during measurement (16), and the fact that thiochrome is an intermediate in the oxidation of thiamine (4–8), the yield of which must be influenced by other oxidizing and reducing substances (4, 6–8) as well as the amount of thiamine and reagent present (4–7).

In 1934, Kinnersley and Peters (17) reported that the reaction between thiamine and diazotized sulfanilic acid in alkaline solution was made more sensitive by the presence of formaldehyde. This reaction has been repeatedly criticized because of lack of specificity. Their procedures were subsequently improved (18) so that semiquantitative data were obtained when the reaction was applied to the determination of thiamine in biological materials. Prebluda and McCollum (19) modified the reagent using diazotized p-aminoacetophenone; the formaldehyde addition was

not included in the test. The diazonium salt of 2,4-dichloroaniline has also been reported (20) as a reagent for the chemical determination of thiamine in biological materials. The specificity of this reagent for the vitamin and the adequacy of the procedures used in its application require confirmation.

In the present studies we have investigated the use of diazotized p-aminoacetophenone as a reagent for the chemical determination of thiamine. The method has been found specific for the vitamin, made quantitative, more sensitive, and successfully applied to analyses of a number of natural sources of thiamine, including yeast, rice polish, wheat germ, and liver.

EXPERIMENTAL

Thiamine reacts with diazotized p-aminoacetophenone in alkaline solution to produce an insoluble red pigment. In our investigations we have used this reagent exactly as described by Prebluda and McCollum¹ (19). For the estimation of small amounts of the vitamin, a solvent was sought which would extract quantitatively the thiamine derivative to yield a solution suitable for colorimetric evaluation. The solvent should not only extract quantitatively the pigment from the aqueous phase, but should also be completely immiscible with water, differ appreciably in specific gravity so that the separation into the two phases may take place with satisfactory rapidity, possess a low vapor pressure in order that no change in volume of the solution may occur during the colorimetric evaluation, and finally be a stable, non-reactive compound so that the character of the reaction product may not be altered by its use. Xylene possesses all of these qualifications and has been used throughout for this purpose.

Specificity of Method for Detecting Thiamine—Inasmuch as we always use the technique of adsorption on synthetic zeolite (permutit) and elution in preparing concentrates for testing, only those compounds similarly adsorbed were studied. A series of tests was conducted with 5 mg. quantities of a number of such

¹ We are very much indebted to Dr. E. V. McCollum for supplying us with detailed directions for the preparation of the reagent, prior to publication of the complete report.
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The compounds investigated were ammonium nitrate, hydroxylamine, hydrazine, ethylamine, trimethylamine, benzylamine, choline, guanidine, atropine, pilocarpine, nicotine, quinine, histamine, adrenalin, tyrosine, histidine, arginine, and lysine. Of these compounds some did react with the reagent to yield colored derivatives. Histamine and histidine both gave orange colors; tyrosine, pink; and adrenalin, red, which changed to violet and finally faded to yellow. However, none of these colored derivatives was extracted by the xylene but all remained in the aqueous phase.

Degradation products of thiamine which are not biologically active were found to produce no color with the diazonium salt. This was true after sulfite cleavage which yields intact pyrimidine and thiazole derivatives (22), after the action of alkali and heat which results in the opening of the thiazole ring (23), after simple deamination, and after oxidation to the thiochrome stage (13). Because in both of these last two reactions the free amino group on the pyrimidine ring no longer exists, Barger and associates (13) believe that it is this group which couples with diazonium salts to yield colored derivatives.

Probably the best proof of specificity of the method is derived from tests conducted on a number of biological materials (24). In all cases the substance responsible for the red color in the xylene layer was found to be as completely adsorbed and eluted as thiamine and also as unstable to alkali and heat.

**Time Required for Completion of Reaction**—100 micrograms of thiamine chloride in 10 cc. of water at pH 7 were allowed to react at room temperature with 20 cc. of the Prebluda-McCollum reagent for variable periods of time. The reaction product was then extracted in each case with 2 cc. of xylene and the color evaluated in a microcolorimeter with the 24 hour sample as the standard for comparison. The results of this study are presented in Fig. 1. The reaction was observed to have gone 75 per cent to completion within 5 to 30 minutes and by the 13th hour maximal and constant values were obtained. In all our subsequent

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1 Dr. Howard B. Lewis of the Department of Biological Chemistry, University of Michigan, kindly supplied us with many of these compounds.

2 Cork stoppers are used.
tests the xylene extraction was carried out after the reaction had been allowed to proceed for at least 15 hours (overnight).

**Influence of Variations in Volume of Thiamine Solutions upon Reaction**—A series of tests was performed in which 20 cc. of the reagent were added to vitamin solutions containing in all cases 100 micrograms of thiamine chloride but varying in volume from 1 to 20 cc. The results of these tests are given in Fig. 2. When comparison is made with the standard vitamin solution, 10 cc. in volume, an increase in the recovery of the vitamin is noted in the more dilute solutions and *vice versa*.

![Fig. 1. Time required for completion of the reaction between thiamine and diazotized p-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test.](http://www.jbc.org/)

In similar fashion, tests conducted with varying quantities of the reagent but with all other factors maintained constant indicate that greater recoveries of the vitamin derivative are possible when decreasing amounts of the reagent are used. This is true to within certain limits, after which the quantity of the reagent may be insufficient to couple with all of the vitamin. These variable recoveries were found to be due to the fact that the thiamine concentrations are determined by an alkaline reagent which
tends to destroy the vitamin and as the ratio of the volume of the reagent to that of the vitamin solution is decreased this destruction is reduced. These findings are presented in Fig. 3.

In order to minimize the destructive action of the alkaline reagent upon the vitamin, tests were conducted at 0-5° with all solutions initially chilled to this temperature range. Only 70 per cent recoveries were obtained after a 24 hour reaction period, probably owing to a decrease in the velocity of the chemical reaction. Attempts to make a reagent containing the diazonium salt of p-aminoacetophenone in a less alkaline solution were unsatisfactory.

We have preferred to use the proportion of 2 parts of the reagent to 1 part of the vitamin solution because tests on biological materials have indicated that other substances are always present in the final test solutions, which also react with the reagent, so that an excess of reagent is desirable.

Influence of pH upon Reaction—In the reaction between diazotized p-aminoacetophenone in alkaline solution and thiamine the

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Fig. 2. Influence of variations in volume of the thiamine solution upon the reaction between thiamine and diazotized p-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test.
initial hydrogen ion concentration of the latter solution was found to exert a profound effect upon the percentage recovery of the vitamin derivative. In the investigation of this problem 142 tests were conducted. The most significant series of these determinations is presented in Fig. 4. Pure aqueous solutions of the vitamin, containing in all cases 100 micrograms of thiamine chloride but varying amounts of acid or alkali, were tested. All other factors influencing the reaction were maintained constant.

Examination of Fig. 4 indicates that, as the acidity of the vitamin solution is decreased, greater recoveries of the vitamin derivative are obtained. For maximal and constant recoveries it is necessary to neutralize to a point just alkaline to litmus and to follow this immediately with the addition of the reagent. If the solution is made too alkaline, losses as large as 10 per cent may occur despite the immediate addition of the reagent. The variable results obtained on the alkaline side are probably due not to any
effect of pH upon the reaction but to a destruction of the thiamine; such an interpretation is indicated in Fig. 4 by the broken line.

Reproducibility of Method—In all cases the volume of the thiamine solutions was 10 cc., the reaction was neutral to litmus, 20 cc. of the reagent were added, and after a 24 hour reaction period at room temperature the vitamin derivative was extracted by xylene. The results of this study are summarized in Table I.

![Figure 4](http://www.jbc.org/) Influence of pH upon the reaction between thiamine and diazotized p-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test. The variable results on the alkaline side (broken line) are probably not due to any effect of pH upon the reaction, but to a destruction of the thiamine.

In this series the maximal deviation from the average is ±4 per cent, with the average deviation ±2 per cent. The greatest differences are found in the tests conducted with the solutions containing 5 micrograms of thiamine chloride per cc. This concentration of the vitamin represents the lower limits of the sensitivity of the method for accurate analyses without the modifications to be described (24).

Intensity of Color Obtained As an Index of Thiamine Concentration...
Vitamin B<sub>1</sub>. I

**Reproducibility of Chemical Method for Determination of Thiamine**

<table>
<thead>
<tr>
<th>Thiamine concentration micrograms per cc.</th>
<th>No. of determinations</th>
<th>Deviation from average</th>
<th>Maximal per cent</th>
<th>Average per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12</td>
<td>±6</td>
<td>±3.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>±3</td>
<td>±1.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>±3</td>
<td>±2</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>12</td>
<td>±4</td>
<td>±1.5</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>12</td>
<td>±3</td>
<td>±2</td>
<td></td>
</tr>
</tbody>
</table>

Average ........................................... ±4 ±2

**Chemical Determination of Thiamine Concentration in Solutions Ranging from 0.4 to 2.0 Times the Standard**

<table>
<thead>
<tr>
<th>Thiamine concentration in standard</th>
<th>Ratio of thiamine concentration in sample to standard solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>0.60</td>
</tr>
<tr>
<td>Colorimetric readings*</td>
<td>mm.</td>
</tr>
<tr>
<td>Thiamine concentration micrograms per cc.</td>
<td>mm.</td>
</tr>
<tr>
<td>10</td>
<td>59.0</td>
</tr>
<tr>
<td>20</td>
<td>49.0</td>
</tr>
<tr>
<td>40</td>
<td>54.0</td>
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<tr>
<td>80</td>
<td>65.2</td>
</tr>
<tr>
<td>Theoretical</td>
<td>50.0</td>
</tr>
</tbody>
</table>

* Standard solutions of the vitamin derivative in xylene set at 20 mm.

10 and 20 micrograms per cc. solutions as the standards, show good agreement with the theoretical values. With the 40 and 80 micrograms per cc. solutions as standards, discrepancies occur which become greater as the differences between sample and standard increase. However, in all of these tests such disagree-
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ment does not indicate errors in the observed values, because of the excellent reproducibility of the individual determinations. It indicates that, for estimating the thiamine concentration by this method without the modifications to be described (24), it is essential to have available a previously determined reference curve bracketing the value obtained.

SUMMARY

The characteristics of the reaction between thiamine in pure aqueous solution and diazotized p-aminoacetophenone with respect to specificity, time for completion of the reaction, influence of variations in volume and pH of the solutions, reproducibility, and applicability to the quantitative determination of the vitamin concentration were studied. Xylene was found to be a selective solvent for the quantitative extraction of the reaction product. With the use of xylene, the reaction is specific for thiamine and with controlled conditions may be used for the quantitative determination of it in pure aqueous solution.

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