A NEW FLUOROMETRIC DETERMINATION OF THIAMINE*

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Several different types of assay procedure have been proposed for the quantitative determination of thiamine. In a discussion (1) of the difficulties encountered in some of the methods, it has been pointed out that colorimetric (2, 3) and fluorometric (4) methods often suffer from a lack of sufficient sensitivity or specificity.

The procedure described in the present communication, while not so sensitive as the microbiological assay (1), is sensitive enough for materials of relatively low potency. Furthermore, since the determination, unlike all others, depends upon the cyanogen bromide reaction, and since the more commonly encountered pyridine derivatives which might react with the reagent have been found to cause little or no interference, the method possesses a rather high degree of specificity.

Investigation of the cyanogen bromide reaction has shown (5) that thiamine, if present in relatively high concentration, produces a colored compound with this reagent. It was concluded that utilization of this reaction for the colorimetric determination of thiamine is not practicable because of the high concentration of the vitamin required. Further investigation has revealed, however, that under different and simpler experimental conditions thiamine produces with cyanogen bromide a highly fluorescent compound. This reaction has been made the basis for the following simple and rapid fluorometric determination of the vitamin. In addition to its simplicity and relatively high degree of sensitivity, the method has the added advantage of requiring the same reagents as are used in the determinations of nicotinic acid (6) and nicotinamide (7).

EXPERIMENTAL

The reaction between thiamine and cyanogen bromide to produce a highly fluorescent compound requires only the following two reagents: (1) a 4 per cent aqueous solution of cyanogen bromide, and (2) a buffer solution, adjusted to pH 6.6 (8), which consists of 988 ml. of water, 15 ml. of 15 per cent sodium hydroxide, 5 ml. of 85 per cent phosphoric acid, and 175 ml. of 95 per cent alcohol.

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The sample to be assayed, or the standard solution, is adjusted to contain between 0 and 0.5 γ of thiamine per ml. To 10 ml. of this solution are added 5 ml. of the buffer solution and 5 ml. of the cyanogen bromide reagent. After the mixture is allowed to remain at room temperature for 30 minutes, fluorescence readings are made in a suitable instrument. In this study the instrument used was a Klett photoelectric fluorometer. The reference fluorescing standard was a dilute solution of sodium fluorescein prepared by diluting 10 ml. of a 0.01 N NaOH solution, containing 1 γ of sodium fluorescein per ml., to 100 ml. with distilled water. Both lamp filters were Corning No. 597. The photocell filter for the sodium fluo-

<table>
<thead>
<tr>
<th>Table I</th>
<th>Determinations with Pure Thiamine Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine in sample</td>
<td>Fluorometer scale readings at varying times after adding CNBr</td>
</tr>
<tr>
<td>γ per ml.</td>
<td>10 min.</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>0.2</td>
<td>32</td>
</tr>
<tr>
<td>0.3</td>
<td>44</td>
</tr>
<tr>
<td>0.4</td>
<td>60</td>
</tr>
<tr>
<td>0.5</td>
<td>75</td>
</tr>
</tbody>
</table>

* Readings beyond the range of the fluorometer.

rescein solution was a Corning No. 038, while that for the unknown (thiamine) solution was a Corning No. 306 filter.

In preparing a standard curve, the solution containing no thiamine is used to adjust the fluorometer to its zero point. Thus, the scale readings are directly proportional to the concentration of thiamine, as shown by the 30 minute readings in Table I. Since this relationship is a simple direct proportion, a factor may be calculated for converting scale readings to concentration of thiamine. This should be done, however, each time analyses are carried out.

Effect of Time—Table I also indicates that the amount of fluorescence depends upon the length of time the solution is allowed to stand after addition of cyanogen bromide. It is important, therefore, that the time of making readings be carefully controlled. This can be done by allowing 30 seconds, or some other convenient interval, between additions of CNBr to successive samples. 30 minutes is the most satisfactory time interval to allow before measuring fluorescence. At that time the readings are
greatest, while still being proportional to concentration of thiamine. On longer standing the intensity of fluorescence continues to increase, but its relationship to concentration is not maintained.

**Specificity**—Since this determination depends upon reaction with cyanogen bromide, the greatest possibilities of interference might be expected from compounds containing the pyridine, and possibly thiazole, ring structures. Of such compounds, the ones most likely to be encountered in vitamin analyses would be nicotinic acid, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine. Of these, all except pyridoxal have been found to cause no interference when present in amounts over 100 times greater than the concentration of thiamine. In low concentration, pyridoxal likewise causes no interference, but when present in greater amounts (100 times the thiamine concentration) this compound does fluoresce. The fluorescence is slight, however, if the readings are made immediately after exposure of the solution to the light beam. Longer exposure causes a steady increase in fluorescence due to pyridoxal. On the other hand, the fluorescence due to thiamine steadily decreases from the moment of first exposure to the light. Hence, readings should be taken without delay. This aids not only in getting a more accurate reading for the fluorescence due to thiamine, but also in minimizing the error in the event of the presence of an unusually high concentration of pyridoxal.

While this reaction, when carried out as described, has a rather high degree of specificity for thiamine, it is interesting to note that minor changes in the procedure make it applicable to the determination of other vitamins. For example, additions of an aromatic amine and HCl (6) are the only changes needed to have a colorimetric assay procedure for nicotinic acid. The use of alkali, after addition of the CNBr, is the basis of a fluorometric procedure for nicotinamide (7, 9, 10). The addition of m-phenylenediamine to a pyridoxine-cyanogen bromide mixture produces a fluorescent compound in amount proportional to the concentration of pyridoxine. Lack of specificity and low sensitivity, however, cause the reaction to have a rather limited value as an assay method for vitamin B₆.

**Preparation of Sample**—As with all chemical methods, the thiamine must first be extracted from samples such as foods and biological tissues by boiling with a dilute acid and, in some cases, be freed from natural complexes by the action of enzymes. Such procedures are well established and have been described in other methods (2, 4, 11, 12).

For uncolored, non-turbid water or dilute acid extracts containing no highly fluorescent compounds, the assay as described above may be carried out directly on the extract. In such cases the blank consists of a sample to which 5 ml. of water, instead of cyanogen bromide, are added. The reading of this blank must be subtracted from all sample readings. When
the blank reading is relatively great, however, further purification of the sample is recommended, as with other methods (2, 4, 11, 12), by passage through a column of Zeolite.

**Results**

The results of recovery assays and of comparisons with the microbiological procedure (1) are shown in Table II. The good agreement between the two methods indicates the value of the cyanogen bromide determination.

**Table II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thiamine added</th>
<th>Thiamine found</th>
<th>Recovery of added thiamine</th>
<th>Microbiological method, Sarett and Cheldelin (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.00</td>
<td>2.10</td>
<td>108.0</td>
<td>2.00</td>
</tr>
<tr>
<td>B</td>
<td>2.50</td>
<td>4.80</td>
<td>96.0</td>
<td>5.08</td>
</tr>
<tr>
<td>C</td>
<td>2.50</td>
<td>7.60</td>
<td>97.6</td>
<td>3.08</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.50</td>
<td>8.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**SUMMARY**

The reaction between thiamine and cyanogen bromide to produce a fluorescent compound is the basis for a simple and rapid fluorometric determination of this vitamin. The method has a relatively high degree of sensitivity and specificity, and the results compare well with the microbiological assay.

**BIBLIOGRAPHY**

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