Preparation of Amine Salts of Thymolphthalein Monophosphate and Their Use for Measuring Phosphatase Activity

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An easy and quick preparation of amine salts of thymolphthalein monophosphate is described. All of the salts prepared are stable, nonhygroscopic, soluble powders. The 2-amino-2-methyl-1,3-propanediol salt is preferred for use as a substrate for measuring phosphatase activities because of its high solubility and easy preparation.

Thymolphthalein monophosphate has been demonstrated to be a useful substrate for determining phosphatase activities in reports on the use of the magnesium (1, 2) and the sodium salts (3–6). Described here is the preparation of amine salts of thymolphthalein monophosphate and the use of these materials as substrates for the measurement of phosphatase activities.

Preparation of 2-Amino-2-Methyl-1,3-Propanediol–Thymolphthalein Monophosphate (AMPD–TMP)

Place 5 g (4.27 mmol) of magnesium thymolphthalein monophosphate (Eastman Kodak, Rochester, N. Y. 14650) in a 500-ml Erlenmeyer flask and add 100 ml of 2 mol/liter HCl. Stir vigorously until the magnesium salt is well dispersed, add 100 ml of diethyl ether and continue stirring until a uniform suspension is obtained. Transfer the mixture to a separatory funnel and separate the phases. Extract the aqueous phase with an additional 75 ml of diethyl ether and equilibrate the combined ether extracts with 20 ml of saturated aqueous sodium chloride solution.

Transfer the ethereal solution of thymolphthalein monophosphoric acid to a flask containing 5 g of anhydrous sodium sulfate. After 10 min, filter the ethereal solution and wash the sodium sulfate with two 15-ml portions of ether. Prepare a solution of 1.15 g (10.95 mmol) of 2-amino-2-methyl-1,3-propanediol in 10 ml of methanol, and add 25 ml of diethyl ether. Add the filtrate to this solution, mix well, and refrigerate 10 min. The precipitate is collected with vacuum filtration, washed three times with 15-ml portions of ether, and air dried. A sintered-glass funnel allows the filter cake to be moved about, to expedite filtration and drying.

Results and Discussion

Other amines were investigated and with slight modifications of the procedure presented above, salts were obtained from 2-amino-2-methyl-1-propanol, tris(hydroxymethyl)aminomethane, diethanolamine, and dicyclohexylamine. All the salts are white to off-white, free flowing, stable, nonhygroscopic powders. While most of the salts are acceptable for preparation of phosphatase substrates, AMPD–TMP is clearly superior in its properties and its ease of preparation and is shown to be an excellent source of thymolphthalein monophosphate.

AMPD–TMP obtained by this procedure is largely the monoamine salt, whereas with excess amine the diamine salt is the product. The composition of the salts was determined by nuclear magnetic resonance spectroscopy, comparing the intensities of the aromatic envelope and the hydroxymethylene signals of the AMPD.\(^2\)

For comparison, substrate solutions were prepared according to Roy (3) with AMPD–TMP and sodium thymolphthalein monophosphate (5). Both were prepared at a concentration of 300 mg/dl and the absorbances at 440 nm were...
indicated the concentrations were equivalent. Table 1 shows that the amine salt is an acceptable material for use in alkaline phosphatase determinations.

Use of AMPD-TMP has several advantages. Preparation from the magnesium salt is considerably easier and faster than the preparation of the sodium salt (5). Including the time for the preparation, AMPD-TMP costs about 20% as much as commercially available sodium thymolphthalein monophosphate. In addition, one cannot be confident of what one is buying; two lots of sodium TMP obtained from one supplier were essentially devoid of TMP. A distinct advantage of AMPD-TMP over magnesium thymolphthalein monophosphate is its solubility. The magnesium salt has low solubility and substrate solutions prepared from it have a limited range.

References

Table 1. Results Obtained from Sodium TMP and AMPD-TMP as Substrates for Alkaline Phosphatase

<table>
<thead>
<tr>
<th></th>
<th>Sodium TMP</th>
<th>AMPD-TMP</th>
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<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>x</td>
<td>43.2</td>
<td>42.9</td>
</tr>
<tr>
<td>SD</td>
<td>32.6</td>
<td>33.0</td>
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<tr>
<td>CV, %</td>
<td>75.6</td>
<td>77.0</td>
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<tr>
<td>Range</td>
<td>17–154</td>
<td>16–153</td>
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A paired t-test on the 24 pairs of observations showed no significant difference at the 95% level.

*Measurements were carried out according to Roy (5), and are reported in International Units.

Magnesium gluconate is shown to be a potential Standard Reference Material for magnesium determination.

The criteria for an ideal standard for serum magnesium determinations include excellent stoichiometry and a favorable weighing factor, good solubility in water, little change in weight on contact with moist air, good storage stability, and in addition the ion involved should not interfere in the analytical method(s) selected for magnesium.

The status of standards for the atomic absorption determination of serum magnesium has been reviewed (1). Magnesium metal, although available in high-purity, must be acid-washed to remove surface oxide, then dried, weighed, dissolved in acids, and diluted to volume. These operations are somewhat tedious and care must be exercised to avoid loss of material. Magnesium oxide adsorbs moisture and carbon dioxide rapidly; special precautions are demanded, therefore, in the weighing and transfer of this standard after firing to high-assay material. Magnesium acetate tetrahydrate has been used as a standard by some workers; however, basic acetate may be present and a product of exact stoichiometry appears difficult to attain. Anhydrous magnesium acetate has been specially synthesized since simple dehydration of the tetrahydrate leads to basic acetate formation (2). This anhydrous salt, during the few minutes of weighing, picks up moisture somewhat less rapidly than does high-assay magnesium oxide (l) (Figure 1).

A study of the literature suggested that magnesium salts of sugar acids might have more desirable properties for use as a magnesium standard. Magnesium gluconate dihydrate, Mg(C₆H₁₇O₇)₂·2H₂O, has a solubility of 16 g/dl of water at 25 °C, and in the presence of acids or bases up to pH 11. Aqueous solutions are neutral in pH and can be sterilized by autoclaving at 120 °C. The weighing factor for this salt is most favorable (see Table 1). Magnesium gluconate has been available commercially for pharma-