A Study of the Thymol Turbidity Test with Special Reference to Buffer Behavior

Formulation of a Modified Reagent Using Tris-hydroxymethylaminomethane

John G. Reinhold

With the technical assistance of Judith Heitkemper Young and Edythe Daitz

Replacement of barbiturate buffer by a tris-hydroxymethylaminomethane buffer in the thymol test reagent of Maclagan greatly improves the stability of the reagent. Thymol turbidity measurements made by means of the modified reagent agreed closely with those made with barbiturate-containing reagents. However in hyperlipemic sera, the latter reagent gave higher turbidities. A reaction of barbiturate with serum chylomicrons and triglycerides explains the difference.

It is believed that the modified reagent is less likely to be affected by casual changes in serum lipid concentration and that therefore it should prove to be more dependable as an aid in the study of liver disease.

Heating is not an essential operation in preparation of thymol test reagents.

The pH is of critical importance in measurement of thymol turbidity. The large change in pH of barbiturate and Tris buffers with change in temperature makes careful control of temperature essential. Tables showing the appropriate reagent pH at various laboratory temperatures are presented.

Although thymol turbidity measurement was originally proposed by Maclagan (1) as a semiquantitative test for the diagnosis of liver diseases, its potentialities as an aid to diagnosis may be substantially increased by careful control of certain factors that affect the reaction of thymol with serum proteins and lipids (2). Among the more impor-
tant of such factors are pH, the temperature at which pH is measured, and the stability of the thymol reagent.

Barbiturate, which was incorporated into the reagent as a buffer, deteriorates in the presence of thymol and oxygen (3). Deterioration is accelerated by heat applied in preparing the reagent, but it occurs also during storage at room temperatures, especially if these are elevated. Evidence of decomposition and an accompanying change in reactivity may occur within a day after a preparation is completed, and it is sometimes necessary to discard reagents within a week. Modification of the original procedure of Maclagan, with curtailed heating, delays, but does not prevent, deterioration (3). Storage of the reagent in the cold is effective but introduces new possibilities of error due to losses of thymol by crystallization. The use of alcohol as a solvent and stabilizer introduces a new component which may itself react unpredictably with abnormal serum proteins.

Since decomposition of barbiturate appeared to be responsible for the deterioration, studies of other buffers have been made in order to learn whether its replacement would result in a thymol reagent with improved stability. The present paper describes thymol reagents in which tris-hydroxymethylaminomethane and either hydrochloric or maleic acids replace sodium barbital and barbital. Methods for preparing these reagents have been simplified. Moreover, it has been found that in large part the erratic performance often associated with the thymol test has resulted from a failure to recognize the considerable influence of temperature on the pH of barbital buffers. Indeed, there is reason to believe that the disagreement concerning the optimal pH of thymol turbidity test reagents has occurred in part because of failure to take into account the temperature effect.

Preparation of Thymol Test Reagents

**Thymol-Tris-HCl Reagent**

Two methods are available. Method A requires heating the thymol solution; Method B, substitutes the use of mechanical mixing.

**Method A**

Heat 1000 ml. of deionized (or distilled) water to boiling in a flask of 2-L capacity. Allow it to cool to 80°–90°. Place 6 gm. of thymol crystals (colorless) and 1.21 gm. of tris-hydroxymethylaminomethane in a flask, and add the hot water. When it has cooled to 25° add some
thymol crystals and shake vigorously. Filter, and add exactly 7.30 ml. of 1.00 N HCl to the filtrate. Determine the pH, preferably at 25°. If the pH is not 7.55 ± 0.03, add more HCl or Tris, mix, and determine pH again. If the temperature differs from 25°, make the appropriate correction from Table 1.

Table 1. Change of pH with Temperature (Thymol-Tris Reagent)

<table>
<thead>
<tr>
<th>Temperature (°)</th>
<th>pH</th>
<th>Temperature (°)</th>
<th>pH</th>
<th>Temperature (°)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>7.83</td>
<td>21</td>
<td>7.67</td>
<td>26</td>
<td>7.52</td>
</tr>
<tr>
<td>17</td>
<td>7.80</td>
<td>22</td>
<td>7.64</td>
<td>27</td>
<td>7.49</td>
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<tr>
<td>18</td>
<td>7.77</td>
<td>23</td>
<td>7.61</td>
<td>28</td>
<td>7.46</td>
</tr>
<tr>
<td>19</td>
<td>7.74</td>
<td>24</td>
<td>7.58</td>
<td>29</td>
<td>7.42</td>
</tr>
<tr>
<td>20</td>
<td>7.71</td>
<td>25</td>
<td>7.55</td>
<td>30</td>
<td>7.39</td>
</tr>
</tbody>
</table>

Method B

Place approximately 12 gm. of thymol and 1.21 gm. of tris-hydroxymethylaminomethane in a flask of 1- to 2-L capacity. Agitate the solution by means of a mechanical stirring device or shaking machine for at least 90 min. Filter, and add 7.30 ml. of 1 N HCl. Determine the pH as described above.

Thymol-Tris-maleate Reagent

In this case, 0.425 gm. per liter of maleic acid is substituted for the HCl used in Methods A or B.

Experimental

Thymol-barbital reagent was prepared by Method A, with 3.09 gm. barbital and 1.69 gm. of sodium barbital substituted for Tris and HCl. The pH was measured by means of Beckman Model G or Radiometer glass electrode assemblies and potentiometers. The pH of these reagents was corrected for temperature by increasing or decreasing it by 0.02 for each degree of deviation from 25°, as described elsewhere (7).

The thymol test was performed as described previously (3) except that temperature control of the test solutions themselves was abandoned. Results were recorded as Shank-Hoagland units (6). All readings were made with the aid of Evelyn photocolorimeters calibrated by means of colloidal glass suspensions (4).
Results

Figure 1 shows that the thymol turbidities measured in reagents buffered with Tris agree closely with those buffered with barbital over a wide range of values. The four reagents used in studies presented in Fig. 1 were typical of the 22 preparations that were used for examination of 864 sera. Although results agreed sufficiently well to permit the Tris-buffered reagent to replace those in which barbital buffer was
used, a small but significant tendency for the barbital-buffered reagent to give higher results may be seen by inspection of the means in Table 2. As will be shown, this is mainly due to the peculiar behavior of lactescent sera. Tris-buffered reagents measure elevated thymol turbidities as accurately as those within normal limits (Table 3).

Examination of the sera yielding the most marked differences when tested by the two reagents disclosed that many were lactescent. Others, although clear, had high triglyceride concentrations. In Fig. 2 it may be seen that lactescent sera did not exhibit the same relationship between the two reagents as did clear sera. Calculation of the ratio of thymol turbidity measured in barbiturate-containing reagents to that measured in Tris-buffered reagents showed that this differed significantly in lactescent sera (Table 3). Lactescent specimens were mainly from blood donors who had not fasted for more than a few hours. That barbital was responsible for the higher turbidities in barbital-buffered thymol reagents is proved by experiments in which serum was added to barbital and Tris buffer solutions from which thymol was omitted. In the presence of barbital, turbidity approximated the incre-

Table 2. Comparison of Thymol Test Reagent Performance

<table>
<thead>
<tr>
<th>Number of preparations tested</th>
<th>No. of sera</th>
<th>Tri-HCl</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heated</td>
<td>Untreated†</td>
<td>Tris-malate</td>
<td>Barbiturate</td>
</tr>
<tr>
<td>14 (1959-60)</td>
<td>643</td>
<td>3.97</td>
<td>—</td>
<td>—</td>
<td>4.31</td>
</tr>
<tr>
<td>8 (1961)</td>
<td>221</td>
<td>4.63</td>
<td>—</td>
<td>—</td>
<td>4.97</td>
</tr>
<tr>
<td>6</td>
<td>125</td>
<td>4.61</td>
<td>4.61</td>
<td>—</td>
<td>4.46</td>
</tr>
<tr>
<td>6</td>
<td>213</td>
<td>4.62</td>
<td>5.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>4.61</td>
<td>—</td>
<td>4.50</td>
<td>—</td>
</tr>
</tbody>
</table>

*Mean thymol turbidity, in Shank-Hoagland units.
†Prepared by mechanically stirring or shaking; not heated.

Table 3. Results* with Use of Thymol Reagents in Examination of Sera Showing Lactescence or Elevated Thymol Turbidity

<table>
<thead>
<tr>
<th>Type of serum</th>
<th>No.</th>
<th>Tri-HCl</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heated</td>
<td>Untreated</td>
<td>Barbiturate</td>
<td>Ratio</td>
</tr>
<tr>
<td>Clear</td>
<td>221</td>
<td>4.63</td>
<td>—</td>
<td>4.76</td>
<td>0.97</td>
</tr>
<tr>
<td>Lactescent</td>
<td>41</td>
<td>5.53</td>
<td>—</td>
<td>6.14</td>
<td>0.90</td>
</tr>
<tr>
<td>Thymol turbidity &gt; 4 U.</td>
<td>64</td>
<td>6.69</td>
<td>—</td>
<td>6.92</td>
<td>0.97</td>
</tr>
<tr>
<td>Clear</td>
<td>61</td>
<td>7.08</td>
<td>6.87</td>
<td>1.03</td>
<td></td>
</tr>
</tbody>
</table>

*Shank-Hoagland units.
ment of thymol turbidity found when the barbital-buffered thymol reagent was used. No turbidity, other than that of the lactescent serum, developed in the Tris-buffered reagent.

The Use of Maleate Buffer

Tris in combination with maleate is a more effective buffer at pH 7.55 than is Tris with HCl. However, the results were identical when maleic acid was substituted for HCl in the thymol test reagents (Table 2). Maleic acid has the advantage of not requiring the accurate standardization that normal HCl does. The latter, however, is

Fig. 2. Lactescent sera (shown by crosses) fall mainly below the diagonal, which represents exact agreement between the two reagents. The higher readings of such sera in thymol-barbital reagent are caused by a reaction of barbiturate with lipid.
more generally available and has been used in nearly all of the present studies.

Stability of Tris-buffered Thymol Reagents

Deterioration of thymol turbidity reagents prepared with barbital buffer may be manifested by appearance of opalescence or by fall in pH. In the writer's experience, deterioration has usually necessitated discarding barbiturate-buffered reagents within 2 weeks of their preparation. Reagents prepared by Maclagan's original method had a useful life of about one week. Figure 3 shows that the Tris-buffered reagent remains clear and usable for many months. A turbidity reading of more than 0.5 units has been adopted as evidence of significant deterioration in these studies.

Unheated Thymol Reagents

The purpose of heating presumably is to insure saturation of the solution with thymol. There is no evidence of a change in absorption spectrum that would suggest interaction of thymol and barbiturate (3). Indeed the ability of Tris to replace barbiturate indicates that the latter has no essential function other than that of buffer. Because heating is troublesome, especially if large volumes of thymol reagent are made, a number of preparations have been tested in which mechanical agitation either by a shaker or motor-driven stirrer replaced heating. The thymol concentration was doubled to assure more rapid and complete saturation. Thymol turbidities measured with such a reagent tended to be somewhat higher than those obtained when heat was used to assure saturation (Tables 2 and 3). Compared with barbiturate-buffered reagent, unheated Tris-buffered reagent also gave slightly higher results.

Fig. 3. The improved stability of thymol reagent prepared with tris instead of barbital buffer is shown. Most thymol reagents buffered with barbital were no longer suitable for use after 2 weeks, as shown by turbidity readings exceeding 0.5 Shank-Hoagland units.
Effect of Temperature Upon pH of the Reagents

It is well known that pH changes with temperature, but this fact has not received adequate consideration in formulation of thymol reagents. It is especially important that temperature be taken into account when Tris buffers are used, because a change of about 0.03 pH. This is about the maximum pH deviation that can be tolerated in thymol turbidity measurements. The preferred method for preventing error due to the temperature effect is to adjust the solutions and glass electrode to 25°. If this is not feasible, temperature corrections for Tris-buffered reagents shown in Table 1 may be applied. Temperature corrections for barbital-buffered thymol reagents are published elsewhere (7).

In 1953, a report from this laboratory (5) claimed that thymol turbidity was markedly affected by the ambient temperature. These studies have been repeated and have not been confirmed. When reagents are used that have been correctly adjusted to the required pH at any given temperature, ambient temperature is no longer important (Table 4). The effect attributed to ambient temperature most probably was a result of the influence of temperature upon the pH of the thymol reagents at the time of preparation, since these were saturated and adjusted at widely differing temperatures in the earlier study.

Thymol flocculation occurs in the same degree and to the same extent in the thymol-Tris as in thymol-barbital reagents.

### Discussion

The substitution of tris-hydroxymethylaminomethane for barbital as buffer in preparation of the reagent for the thymol test results in a much more stable reagent. Deterioration, if it occurs, is delayed many months. The pH also remains more stable despite the longer life and resultant increase in expectancy of change. The elimination of barbiturate and of the hazards created by the availability of a potent restricted drug is also advantageous.
The pH of Tris-HCl buffers is more easily adjustable than is that of barbital-sodium barbital because of the much higher solubility of Tris. Tris-hydroxymethylaminomethane is available from several sources in high purity, and in our experience has not shown the deviations in buffering capacity encountered with occasional lots of sodium barbiturate.

Discrepancies between the thymol turbidities yielded by Tris- and barbiturate-buffered thymol reagents are limited, with rare exceptions, to sera with elevated triglyceride concentrations. This represents an important advantage of Tris over barbital. The susceptibility of the latter to the effects of the casual hyperlipemia occurring after meals has been one of the principal shortcomings when the thymol test has been applied to study of nonfasting persons, as, for example, in the screening of blood donors for detection of carriers of viral hepatitis. Evidence has been obtained which suggests that it is a reaction of barbiturate with lipid that is responsible for the difference. The change in serum colloid structure believed to be mainly responsible for production of abnormal thymol turbidity in the presence of liver disease is the rise in concentration and altered character of gamma globulin. A complex of thymol, beta lipoprotein, and gamma globulin forms as a result, which rapidly reaches dimensions that causes separation of a phase, with resultant turbidity. The reaction with chylomicrons and triglyceride is quite different (2). Although disturbances in serum lipid composition occur in liver disease and may contribute to the occurrence of abnormal thymol turbidity, the tendency of barbiturate-buffered thymol reagents to react with lipid is a disadvantage because of the occurrence of elevated thymol turbidities in some healthy persons after ingestion of fatty foods. This type of artifactual elevation decreases the specificity of the thymol test as a diagnostic aid in detection of liver involvement. Tris-buffered thymol reagents, therefore, should yield results of improved diagnostic usefulness.

The upper limit of thymol turbidity observed when specimens from fasting healthy persons are examined by means of Tris-buffered thymol reagents remains unchanged. In this laboratory 4.0 Shank-Hoagland (c) units represents the limit below which thymol turbidities of 95 per cent of all fasting healthy persons will fall. On the other hand, it is anticipated that limits will be lower than those now in use when thymol-Tris reagents are used for examination of sera from postprandial blood specimens.
Importance of pH

The most critical and troublesome factor in performance of the thymol turbidity measurement is the adjustment of the pH of the reagent. It has been possible in the present study to detect by casual inspection of results, reagents that deviated from the norm, by as little as 0.02-0.05 pH, because of the consistently different thymol turbidities that such aberrant reagents yielded. Hence, reagents with relatively slight deviations in pH may cause significant inaccuracies.

The difficulties of pH adjustment are enhanced by the substantial changes occurring in pH with change in temperature. Careful measurement of the temperature of the solution in the pH meter and appropriate correction if this differs from 25° is essential. Elsewhere (7) the writer has pointed out that the differences between Maclagan's reagent buffered at pH 7.80 and that of Mateer et al. (8) buffered at pH 7.55 are much less than would be expected, probably because of the differences in the temperatures prevailing in their laboratories when their respective reagents were developed. The universal use of phosphate buffers for standardization of pH contributes to such errors since phosphate buffers, in contrast to barbiturate or Tris buffers, undergo little change in pH with temperature.

Previous attempts have been made to substitute other buffers for barbiturate in the thymol test. Phosphate decreases the yield of turbidity markedly and cannot be used. Glycyglycine was proposed by Christensen and Christensen (9). It is more expensive than Tris and not as easily purified. Moreover, Tris is already available in many laboratories because of its extensive use in enzyme measurements.

One pooled serum preparation used as a laboratory control consistently gave lower results with the thymol-barbital than with thymol-Tris reagents, contrary to the usual finding. Much of the phospholipid in this specimen had been destroyed by bacterial action in the course of preparation of the pool, and the lack of phospholipid or presence of lysolecithin may have been responsible. Phospholipid has been associated with thymol-barbital reactivity (10). A few serum specimens showed a similar reversed relationship, as may be seen in Fig. 1. All other pooled serum preparations behaved as did fresh sera.

Addendum

Thymol purchased during the past 6 months from a source that for many years has proven satisfactory has yielded opalescent thymol test
solutions regardless of the buffer used. However, the opalescence was so marked in barbital-buffered solutions as to make the solutions virtually unusable. The crystals of the faulty thymol have a distinctly pink cast instead of the yellow that usually characterizes unsuitable thymol. The importance of using pure thymol must again be emphasized.

References