MICROESTIMATION OF CITRIC ACID; A NEW COLORIMETRIC REACTION FOR PENTABROMOACETONE*

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In an earlier publication\(^1\) a method was described for the determination of citric acid in blood serum for amounts as low as 5 \( \gamma \). While this method permitted determinations on from 0.5 to 1.0 ml. of serum, a need was felt for a method which would be suitable for smaller amounts for children. This need was felt most acutely when samples were drawn at frequent intervals from the same child.

The extinction coefficient for the sodium sulfide color with pentabromoacetone is relatively low, \( E_{\text{cm}}^{1 \%} = 92 \). The development of a new colorimetric reaction with a higher extinction coefficient was of critical importance in our studies.

Investigation of various substances available containing sulfur indicated that thiourea and substituted thiourea compounds form colored complexes with pentabromoacetone. For example, if a petroleum ether solution of pentabromoacetone is added to an alcoholic KOH solution containing thiourea, acetylthiourea, ethylthiourea, allylthiourea, sym-diethylthiourea, or sym-dimethylthiourea, yellow to greenish yellow colors are observed in the alcohol layer. In this strongly alkaline solution the colors slowly fade at room temperature.

Thiourea was chosen for study because of its solubility in water, ease of purification, and availability.

A 4 per cent thiourea solution dissolved in water exhibits a pH of 6.9. When this solution is shaken with a petroleum ether solution of pentabromoacetone for 15 to 30 minutes, a pinkish color, resembling a dilute permanganate solution, develops in the aqueous phase. This color has maximum absorption at 510 \( \mu \). Addition of acid such as dilute acetic acid destroys the color. If the pink solution is now made alkaline to pH 11, a yellow color develops which reaches its maximum intensity after 7 to 15 minutes, with a maximum absorption at 460 \( \mu \). Both colors are stable for at least an hour at room temperature. If the pentabromoacetone solution is too concentrated, a precipitate of the pink complex will form.

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Although the pink-colored complex is converted to the yellow compound on addition of alkali, these compounds are not in dynamic equilibrium with each other. Addition of alkali causes the disappearance of the 510 mμ peak, but the 460 mμ peak does not form until several minutes later.

For the purpose of analytical determination it is apparent that the pH had to be controlled in order to obtain a color with consistent extinction coefficient at one of the peaks. A procedure was developed, therefore, for the determination of citric acid in serum with the 460 mμ peak and pH 11.

In one procedure developed, citric acid is converted to pentabromoacetone as previously described, and the pentabromoacetone is extracted from the petroleum ether layer with a 4 per cent thiourea solution in a buffer of pH 7. Before reading the color, the pH is brought to 11 with NaOH solution. For micro quantities (1 to 10 μl) of citric acid the colors are read in the Coleman spectrophotometer with the 3 ml. capacity cuvettes and a 5 cm. light absorption path. For the Beckman spectrophotometer the color is developed in 1 ml. of solution and is read in the micro cups.

A modification which has recently shown itself to be suitable and simpler is to extract the pentabromoacetone into a solution containing 4 per cent thiourea and 2 per cent borax or sodium pyrophosphate, the borax or sodium pyrophosphate maintaining the pH at 9.2. In this manner the extraction time is less and the color may be read directly without changing the pH. The peak with a borax buffer is at 445 mμ and not at 460 mμ as with the phosphate buffer at pH 11. The extinction coefficient, however, is approximately the same.

The extinction coefficient plotted against wave-length for the thiourea-pentabromoacetone colors is illustrated in Fig. 1 for pH 11, 9.2, and 7.

For micro quantities of citric acid (0.2 ml. of serum) commercially available 2 ml. Pyrex volumetric flasks, test-tube shape, with ground glass stoppers, were utilized for the extractions. A mark was scratched on these tubes at 0.4 ml. with a diamond pencil. The stoppers were ground in by hand, a Palo-Myers grinding paste being used to prevent leakage. Silicone grease was also used for this same purpose.

A standard curve indicating that Beer's law is followed is shown in Fig. 2.

To prepare the standard curve, dilutions of the citric acid stock solution are made. The same procedure is followed as is described for blood, except that the precipitation with trichloroacetic acid and the boiling down are omitted. In determining the total amount of citric acid in the sample of unknown the results obtained from Fig. 2 must be multiplied by 6/5 to correct for the 5/6 aliquot taken after protein precipitation.

The curve as plotted from determinations on known amounts of citric acid actually represents 10/13 of the total amount of citric acid present, because a 10/13 aliquot of the petroleum ether, which contains the pentabromoacetone, is taken.
FIG. 1. Absorption spectra of the pentabromoacetone-thiourea complex at pH 7.0, 9.2, and 11.0 with 10 \( \gamma \) of pentabromoacetone made up in 3.5 ml. of the buffered 4 per cent thiourea solution.

FIG. 2. Standard curve obtained by plotting optical density against concentration of citric acid at 445 m\( \mu \) in the Coleman spectrophotometer with 3 ml. cuvettes with a 5 cm. light path; pH 9.2.
The procedures described have been used routinely in this laboratory for determination of citric acid for several months. When larger amounts of blood are available, the method may be adapted for determination in 1 ml. of serum. In this case the ordinary colorimeter (Klett) may be used with a No. 44 or No. 47 filter.

The specificity of the method is similar to that found for the sodium sulfide color. No measurable interference was found when aconitic acid, glucose, acetoacetic acid, hydroxybutyric acid, acetone, or pyruvic acid was added in amounts up to 50 mg. in determinations on 0.2 ml. of serum.

This procedure has advantages over the sodium sulfide method in that the color is stable at room temperature and smaller quantities may be determined, for the extinction coefficient is approximately twice that of the sodium sulfide color.

Typical results as obtained on four consecutive determinations are given in Table I.

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Determination of Citric Acid in 0.2 Ml. of Blood Serum</td>
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</table>

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Citric acid</th>
<th>Citric acid added</th>
<th>Citric acid recovered</th>
<th>Per cent recovered</th>
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</thead>
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<td>3.25</td>
<td>1.50</td>
<td>1.44</td>
<td>96.0</td>
</tr>
</tbody>
</table>

Method

Reagents—

Citric acid. Stock solution, 1 ml. = 1 mg. of anhydrous citric acid (analytical reagent), made up in 1 N H₂SO₄. This solution is diluted 1:100 with water daily to prepare the dilute standard; 1 ml. = 10 γ.

Sulfuric acid. 18 N (analytical reagent).

Potassium bromide-bromine reagent. Distilled water is saturated with bromine. The saturated bromine water is decanted from the bromine. In this solution, KBr (reagent grade) is dissolved to make it 1 N with respect to KBr.

Hydrogen peroxide. 6 per cent, made by diluting 10 ml. of 30 per cent H₂O₂ (analytical reagent) to 50 ml. This solution is stored in a refrigerator.

Petroleum ether, b.p. 90–100°. The commercial product is allowed to stand over 0.1 of its volume of concentrated H₂SO₄ for several days. The
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sulfuric acid is separated and the petroleum ether is washed several times with fresh sulfuric acid until the washings are colorless. The petroleum ether is shaken with saturated KMnO₄ solution made up in 0.5 N H₂SO₄. The mixture is shaken and allowed to stand for 30 minutes and the KMnO₄ is washed out with water. The petroleum ether is allowed to dry over K₂CO₃ (anhydrous), filtered, and distilled, the 90-100° fraction being collected.

KMnO₄ solution. 5 per cent (reagent grade).

Thiourea solution, pH 9.2. 2 gm. of Borax (analytical grade) are dissolved in 100 ml. of 4 per cent thiourea.

Procedure—0.2 ml. of serum is placed in a 3 ml. centrifuge tube and 1 ml. of 10 per cent trichloroacetic acid is rapidly blown in to precipitate the proteins in fine particles. Towards the end, the pipette is allowed to drain in order to obtain an accurate measurement. The mixture is shaken and allowed to stand for 10 minutes. The tubes are stoppered and then centrifuged at 2500 r.p.m. The supernatant liquid is poured off as completely as possible into a clean test-tube, without disturbing the precipitate. A 1 ml. aliquot is taken and placed in a 3 ml. test-tube with a ground glass stopper (2 ml. Pyrex volumetric flasks, test-tube shape) with a mark at 0.4 ml. 0.04 ml. of 18 N H₂SO₄ is added and the solution is evaporated to the 0.4 ml. mark by placing the tube in an oil bath maintained at 100-120°. 0.04 ml. of the KBr-bromine solution is added to the cooled solution and the mixture is allowed to stand for 10 minutes. 0.1 ml. of the 5 per cent KMnO₄ solution is added. The tube is shaken and allowed to stand for 10 minutes. It is then cooled to approximately 10° by placing the tubes in the ice box or in an ice bath. The excess permanganate is decolorized with approximately 2 drops (0.03 to 0.06 ml.) of cold 6 per cent hydrogen peroxide. 1.3 ml. of the purified petroleum ether is added to the tube from a burette, and the tube is stoppered with a minimum of silicone grease. The pentabromoacetone is then extracted by shaking in a machine for 10 minutes. The tube is centrifuged for 5 minutes at 2000 r.p.m. A 1.0 ml. aliquot of the petroleum ether layer is taken and placed in a 7 to 12 ml. glass-stoppered centrifuge tube.

3.5 ml. of the thiourea solution, pH 9.2, are added, and the tube is stoppered and shaken in a shaking machine for 5 minutes. The tube is then centrifuged at 2000 r.p.m. for 5 minutes. The petroleum ether layer is aspirated off and enough of the aqueous phase to be read is pipetted into the 3.0 ml. cuvettes with a 5 cm. light absorption path. The density is read in the Coleman spectrophotometer at 445 mμ. The color is read against a thiourea buffer solution which has been treated in a manner similar to the unknown (i.e., extracted with petroleum ether and centrifuged). A reading is taken at 650 mμ so as to correct for the difference in cloudiness be-
between the unknown and blank. Most often the cloudiness correction is negligible.

Two standards, 4 and 6 \( \gamma \) content, are run to check the standard curve with each set of determinations. The slope of the standard curve may vary slightly from day to day if marked changes in room temperature occur or if the pH of the buffer should change. This is noted and corrected for by the use of the standards.

**SUMMARY**

1. A colorimetric method is described for determining citric acid in biological fluids. Citric acid is converted to pentabromoacetone which is allowed to react with thiourea.

2. Thiourea and substituted thiourea compounds produce colored complexes when allowed to react with pentabromoacetone. The nature of the color produced varies with the pH of the solution.

3. The method is accurate within 5 per cent for amounts ranging from 1 to 20 \( \gamma \) of citric acid as determined by recoveries added to serum. The intensity of the colors obtained with change in concentration follows Beer's law.
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