The activity of carbonic anhydrase has been estimated by three procedures: manometric (1–3), colorimetric (2, 4–8), and electrometric (9). For investigations of the enzyme concentration of tissues the colorimetric method has often been used in preference to the manometric technique because of its relative simplicity. A disadvantage of the colorimetric method has been the inhibitory effects of the indicator (10) and of the carbonate-bicarbonate buffer, an effect also true of phosphate buffer in the manometric method, though to a lesser degree (8). Roughton and Booth (8) have found that veronal buffer is without noticeable effect on carbonic anhydrase and have suggested a colorimetric method which employs this buffer with brom-thymol blue. This method permits the measurement of enzyme activity in terms of moles of CO₂ hydrated, allowance being made for inhibition by the indicator. However, the influence of the indicator on the enzyme activity in this system has not been studied.

This paper describes a new electrometric method which is here employed for evaluation of the inhibitory effect of indicators on carbonic anhydrase preparations from various sources. A modification of the veronal colorimetric method suitable for routine determinations of carbonic anhydrase in tissues is also presented together with data on its reliability.

**Electrometric Method**

In the electrometric method suitable amounts of veronal buffer and saturated CO₂ solution are mixed at 0° to give a displacement of the hydrogen ion concentration from approximately pH 8 to 6.3 in a conveniently measurable period of 100 to 120 seconds for the uncatalyzed reaction. The apparatus employed (Fig. 1) utilizes two automatic measuring syringes (Becton, Dickinson improved Cornwall syringe No. 1250) to force buffer and CO₂ solutions simultaneously into a reaction vessel containing a glass electrode and the enzyme or experimental solutions or both. The pH changes may be followed (Fig. 2) or the time necessary for the solution to reach the end-point (pH 6.3) determined. After the reaction is completed, the reaction vessel is flushed and drained through a distilled water-evacuation system.
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Veronal Buffer—4.536 gm. of sodium barbiturate were dissolved in 950 ml. of double distilled water, and barbituric acid was added to bring the pH to 8.15. The amount required varied slightly with the source. The solution was then made up to 1 liter in a glass-stoppered flask. Precautions were taken to prevent carbon dioxide absorption.

Saturated CO₂ Solution—CO₂ from a Dewar flask containing solid CO₂ was bubbled through a gas bubbler into double distilled water at 0° for at least 1 hour before use.

Indicators—To study the effect of indicators on the catalyzed and uncatalyzed reactions, powdered brom-thymol blue (Harleco) and powdered phenol red (Merck) were made up in double distilled water and adjusted to pH 6.3 with NaOH.

Apparatus—Those portions of the apparatus shown in Fig. 1 are mounted on a metal support and placed in a glass container, 12 × 7½ × 8½ inches. Connected with the apparatus are (a) a 250 ml. Mariotte bottle of CO₂-saturated distilled water with a saturating device and a long tube to remove excess CO₂ from the vicinity of the apparatus, (b) a 250 ml. bottle of veronal buffer with an air vent protected by an NaOH solution, (c) a glass cooling coil connected with an elevated distilled water supply, and (d) a vacuum line. The total assembly was packed in a water-ice mixture in the glass container and placed in a wooden box with glass wool insulation between the two containers. It was found convenient to carry out the measure-
ments in a refrigerated room at 4°. All experimental solutions were kept at 0°.

Standard Beckman electrodes were mounted on a rack and pinion over the reaction vessel and connected through a shielded cable to a Leeds and Northrup pH meter. The pH meter and electrodes were standardized in position at 0° with phosphate buffers. All solutions used were made with double distilled water.

Operation—To place the system in operation, the plunger of the buffer syringe is removed and positive pressure induced through the CO₂ absorption system of the buffer bottle to fill the syringe through Stop-cock 4. (The construction of the stop-cock extensions indicates the orientation of the stop-cock.) The syringe is then reassembled and the contents forced into the reaction vessel (R) through Stop-cock 3 after Stop-cock 4 is closed. With the syringe plunger still down, Stop-cock 3 is turned to neutral position and Stop-cock 4 is opened. The slow release of the plunger allows the syringe to fill with fresh buffer. Stop-cock 4 is then closed. A similar procedure is used to fill the other side of the system with saturated CO₂ solution. With care all bubbles can be eliminated from the system.

The reaction vessel and electrodes are flushed in the following manner. After both sides of the system are filled, Stop-cocks 1 and 4 are closed and Stop-cocks 2 and 3 turned to the positions shown. Cold distilled water is allowed to flow into the reaction vessel through Stop-cock 2 until it is full. The stop-cock in the vacuum line (not shown) is then opened and the water withdrawn through Stop-cock 3 which is low enough for complete drainage. This process is repeated until the vessel and electrodes are clean (see below).

In making a determination all stop-cocks are turned to neutral position. 1.0 to 2.0 ml. of experimental solution or distilled water are carefully pipetted to the bottom of the reaction vessel. Both openings into the reaction vessel are covered by this quantity of solution, so that both the buffer and CO₂ solutions flow in under liquid. After temperature equilibrium is reached in this solution Stop-cocks 2 and 3 are turned to connect the reaction vessel with both syringes. Both plungers are depressed simultaneously, giving rapid mixing. Stop-cocks 2 and 3 are turned at once to neutral position and Stop-cocks 1 and 4 opened. Both plungers are then slowly released, refilling the syringes, and Stop-cocks 1 and 4 are then closed.

EXPERIMENTAL

Methods—Carbonic anhydrase preparations were made from human blood, gastric mucosa of Rana pipiens, and from whole rat brain. For blood carbonic anhydrase, hemolyzed erythrocytes and the modified crude chloroform extract of Roughton and Booth (11) were used. Neither prep-
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Aration showed appreciable change in activity when kept in a refrigerator several weeks in dilute solution. Mucosal and brain extracts were made by grinding fresh tissue thoroughly and diluting with about 15 volumes of distilled water. The suspension was then centrifuged 8 minutes at $850 \times g$ and the supernatant removed and diluted to give a reaction time of 45 to 70 seconds.

0.2 ml. of enzyme solution was added to 1.0 ml. of experimental solution or distilled water in the reaction vessel 30 seconds prior to the addition of 2.0 ml. each of $\mathrm{CO}_2$ solution and veronal buffer. Contact of enzyme and indicator was limited to this period plus the time of reaction.

![Graph](http://www.jbc.org/)

Fig. 2. Hydration of carbon dioxide in veronal buffer. Displacement of curve of enzyme plus brom-thymol blue indicates slight inhibition.

Results—The curves of a catalyzed and an uncatalyzed reaction are shown in Fig. 2; duplicate determinations were performed for the uncatalyzed reaction. As illustrated by the curves, the rate of change resulting from the low buffering power at pH 6.3 makes this a suitable end-point for both the electrometric method and the colorimetric method of Roughton and Booth (8) with brom-thymol blue.

The possibility of enzyme adsorption on the walls of the reaction vessel and the glass electrodes was examined by running a very potent crude blood preparation and then measuring the rate of the uncatalyzed reaction which followed after three and again after six flushings of the reaction vessel. Six flushings appear adequate.
Brom-thymol blue has an inhibitory action on carbonic anhydrase, the extent depending upon the indicator concentration and the particular enzyme preparation (Table I). With 1.92 mg. per cent of indicator, a concentration suitable for colorimetric determinations, the inhibition on rat brain and frog gastric mucosa preparations was about 11 per cent and 8 per cent respectively, with a negligible effect on blood preparations. In a similar series with phenol red (Table II) in a concentration of 2.88 mg. per cent, as ordinarily used in the Brinkman technique, the indicator was without significant effect except in the case of hemolysed blood in which there was an 8 per cent inhibition.

**Table I**

*Inhibition of Carbonic Anhydrase by Brom-thymol Blue*

All measurements were made with veronal buffer and electrometric equipment at 0°. The pH of the added indicator solution was adjusted at 6.3. The figures for the indicator represent final concentrations. Runs with enzyme and enzyme plus indicator were alternated.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Indicator</th>
<th>Enzyme + water</th>
<th>Enzyme + indicator</th>
<th>Per cent inhibition</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>1.92</td>
<td>53.3</td>
<td>59.5</td>
<td>11</td>
<td>0.001 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>3.84</td>
<td>48.4</td>
<td>62.9</td>
<td>30</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Frog gastric mucosa</td>
<td>1.92</td>
<td>52.3</td>
<td>56.5</td>
<td>8</td>
<td>0.02 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>3.84</td>
<td>51.1</td>
<td>62.6</td>
<td>23</td>
<td>0.001 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td>Human blood, hemolyzed</td>
<td>1.92</td>
<td>51.2</td>
<td>52.0</td>
<td></td>
<td>0.5 &lt; P &lt; 0.6</td>
</tr>
<tr>
<td></td>
<td>3.84</td>
<td>54.3</td>
<td>60.3</td>
<td>11</td>
<td>0.001 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td>Human blood, chloroform extract</td>
<td>1.92</td>
<td>51.7</td>
<td>53.1</td>
<td>3</td>
<td>0.4 &lt; P &lt; 0.5</td>
</tr>
<tr>
<td></td>
<td>3.84</td>
<td>53.3</td>
<td>56.0</td>
<td>4</td>
<td>0.1 &lt; P &lt; 0.2</td>
</tr>
<tr>
<td>No enzyme</td>
<td>3.84</td>
<td>113.2</td>
<td>113.7</td>
<td></td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

**Table II**

*Inhibition of Carbonic Anhydrase by Phenol Red*

All measurements were made with veronal buffer and electrometric equipment at 0°. The added indicator solution was adjusted to pH 6.3. Final concentration of phenol red was 2.88 mg. per cent. Runs with enzyme and enzyme plus indicator were alternated.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Enzyme + water</th>
<th>Enzyme + indicator</th>
<th>Per cent inhibition</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>sec.</td>
<td>sec.</td>
<td>sec.</td>
<td>56.7</td>
</tr>
<tr>
<td>Human blood, hemolyzed</td>
<td>sec.</td>
<td>56.5</td>
<td>61.2</td>
<td>8</td>
</tr>
<tr>
<td>&quot; &quot; chloroform extract</td>
<td>sec.</td>
<td>69.1</td>
<td>71.3</td>
<td>3</td>
</tr>
<tr>
<td>No enzyme</td>
<td>sec.</td>
<td>sec.</td>
<td>sec.</td>
<td>115.2</td>
</tr>
</tbody>
</table>
DETERMINATION OF CARBONIC ANHYDRASE

Colorimetric Method

Apparatus and Procedure—The colorimetric method is an adaptation of that described by Roughton and Booth (8) and utilizes a Cornwall automatic syringe to introduce cold saturated CO₂ solution into a veronal buffer containing brom-thymol blue in a test-tube. The time required for the pH to drop from approximately pH 8 to 6.3 is measured.

The CO₂ saturation system was similar to that used for the electrometric method. In making a determination, 2.0 ml. of saturated CO₂ solution are drawn into a cold syringe, and a small test-tube is placed over a 3 inch No. 20 gage needle and held by a small rubber stopper on the shaft of the needle. The syringe is then immersed in a water-ice mixture until needed. 2.0 ml. of the cold veronal buffer used in the electrometric method containing 5 mg. per cent of brom-thymol blue are then pipetted into a Kimball test-tube, 6 × ½ inches, with 1.0 ml. of the experimental solution or distilled water, stoppered, and placed in an ice-water mixture. After allowing time for temperature equilibration in the test-tube and the syringe, the syringe is quickly removed from the ice mixture, grasped with a cloth to remove excess water, and the small tube protecting the needle is pulled off. The CO₂ solution is rapidly ejected. With the size of test-tube indicated for the reaction vessel the syringe will come to rest with the tip of the needle immersed near the middle of the buffer solution and centered by the rubber stopper on the needle. After removing the syringe from the test-tube the needle is washed to remove any enzyme before the plunger is released. The needle may be coated with paraffin, though this was not done in the present study. The end-point is determined by matching with a comparator tube containing brom-thymol blue in veronal buffer at pH 6.3. Fogging of the ice bath can be prevented by Anti-Fog, No. 111, supplied by the American Optical Company.

Results—Eleven consecutive determinations of the uncatalyzed reaction gave an average of 103.5 seconds with a probable error of 1.0 second for a single determination. Two series of five runs each with enzyme gave averages of 86.4 and 80.4 seconds, with a probable error of 1.2 and 2.3 seconds for a single determination. Measurements were made with ice-packed equipment in a room at normal temperature.

DISCUSSION

The electrometric method with its provision for the automatic measurement of solutions and washing of the reaction chamber permits the measurement of the carbonic anhydrase activity of a considerable number of samples in a relatively short period of time. While the method has the
disadvantage that the enzyme is subjected to a range of hydrogen ion concentrations during the course of a single measurement, this range may be limited to any desired portion of the curve.

The electrometric apparatus may be used without the electrodes in colorimetric determinations by adding indicator to the stock buffer solution, though its chief application is with turbid or colored solutions in which brom-thymol blue cannot be used or in which details of the course of the reaction are required. It has been used here to test the effects of indicators on enzymes under conditions which duplicate the colorimetric technique in pH range, indicator concentration, and volume of solutions.

The effects of brom-thymol blue and phenol red on different carbonic anhydrase extracts call attention to certain considerations in the use of colorimetric methods. Since the extent of inhibition varies with the tissue extract, this value will have to be determined for the individual tissue before corrections can be made in calculations of enzyme activity by the procedure of Roughton and Booth (8). In any case, it is apparent that in the colorimetric method the indicator concentration should be kept as low as is practicable. The effects here described relate to the experimental situation in which indicator is mixed with enzyme immediately prior to the addition of the substrate, and may or may not obtain with prolonged contact. We are unable to say whether the marked inhibition found by Kiese and Hastings (10) may involve this factor or whether it is due to differences in extract and experimental method.

SUMMARY

1. An electrometric technique for the measurement of carbonic anhydrase activity is described. In the apparatus employed saturated carbon dioxide and veronal buffer are mixed with automatic measuring syringes and the pH change measured with the glass electrode.

2. The electrometric method was employed to study the inhibitory effect of brom-thymol blue and phenol red on the carbonic anhydrase activity of rat brain, frog gastric mucosa, and human erythrocyte preparations; the extent of inhibition was found to vary with the enzyme source and indicator concentration. An indicator concentration suitable for use in a colorimetric technique gave an inhibition of 0 to 11 per cent.

3. A modification of the Roughton and Booth veronal-brom-thymol blue colorimetric system is described and data on its reliability presented.

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ELECTROMETRIC AND COLORIMETRIC DETERMINATION OF CARBONIC ANHYDRASE
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