MEASUREMENT OF CARBONIC ANHYDRASE ACTIVITY OF BLOOD AT BODY TEMPERATURE*

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Most measurements of blood carbonic anhydrase activity recorded in the literature have been made by the method of Meldrum and Roughton (1) or some modification of it. These procedures are carried out in a cold water bath at 10° or below. This temperature is disadvantageous technically, and, moreover, affords no direct information as to the activity of the enzyme at mammalian body temperature; it is well known that low temperatures exaggerate the apparent activity of the enzyme markedly (1–4). In addition, the enzyme, under some circumstances, may be partially inactivated at low temperature (2, 5, 6). Methods based on that of Meldrum and Roughton (1) are unsatisfactory, also, in that violent shaking is required to counteract the retarding effect of cold on the diffusion of the evolved gas; variations in the rate and angle of rotation of the shaker used give rise to apparent variations in enzyme activity. Differences in size and shape of the vessels used also cause large differences in the values obtained (7). Mitchell, Pozzani, and Fessenden (8) have shown also that calculation of enzyme activity by means of the formula of Meldrum and Roughton (1) is not valid mathematically and yields misleadingly high values of enzyme activity. Mitchell et al. found that the catalyzed and the uncatalyzed reactions used in the estimation of carbonic anhydrase activity were unimolecular in character; accordingly they introduced the utilization of the principle of Guggenheim (9), based on velocity constants, in the measurement of activity of the enzyme. The present method is a modification of the procedure of Mitchell et al. (8), designed to estimate carbonic anhydrase activity in blood at 37°. It is to be noted that the velocity constant of the reaction is only one of several variables in the method; so that the values obtained are related to, but not the same as, the velocity constant.

Method

The basis for the determination of carbonic anhydrase activity is the usual one of its catalysis of the decomposition of carbonic acid at approximately pH 6.8.

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CARBONIC ANHYDRASE ACTIVITY OF BLOOD

Reagents—

Phosphate buffer. Solutions of 0.2 M monopotassium acid phosphate (KH₂PO₄) and 0.2 M disodium phosphate (Na₂HPO₄) are prepared and stored in separate containers. Just before use equal amounts of these solutions are mixed.

Bicarbonate solution. 0.2 M sodium bicarbonate is dissolved in 0.02 M sodium hydroxide. This solution is sealed in 20 ml. glass ampules, one of which is broken just before use, and the alkali mixed with an exactly equal quantity of distilled water.

Apparatus—The manometric method of Meldrum and Roughton (1) has been adapted to the use of standard Warburg vessels, manometers, constant temperature water bath, and shaking apparatus, as suggested by Stadie et al. (10). The water bath is maintained at 37°, and the vessels are shaken through an arc of about 30°, making 63 complete oscillations per minute. The rate of evolution of carbon dioxide is estimated from changes in pressure as read in the manometer; this rate is so rapid under the conditions of the method that it is directly proportional to the differences in manometric readings (11), and variations in the volumes of carefully made Warburg flasks and manometers are far too small to be of significance as sources of error in the present method.

Determination of Enzyme Activity—Heparinized venous blood is used; this is laked and diluted with distilled water in volumetric flasks and brought to the dilutions (in ml.) usually used, 1:20, 1:50, and 1:200. 0.5 ml. of diluted blood is placed in the Warburg flask; two glass beads and 1.0 ml. of phosphate buffer are added. 1 ml. of bicarbonate solution is added to the side arm. For each day's determinations a thermobarometer flask is prepared containing 2.5 ml. of distilled water.

The reaction vessel containing the reagents and enzyme is attached to its manometer and placed in the water bath for 10 minutes of equilibration. It is then removed, tilted gently three times to mix the solutions and to rinse the side arm, and returned to the water bath. The shaker is then started. The manometer stop-cock is left open for varying intervals of shaking, allowing the carbon dioxide rapidly evolved during the initial part of the reaction to escape, since the measurement of the rapid initial changes is inaccurate. Control reactions are allowed to proceed with the manometer stop-cock open for 90 seconds, catalyzed reactions with 1:200 blood for 50 seconds, catalyzed reactions with 1:50 blood for 40 seconds, and catalyzed reactions with 1:20 blood for 30 seconds. After the stated interval, the shaker is stopped, and the manometer is rapidly adjusted to the zero mark, the manometer stop-cock closed, and shaker and stop-cock are started again. Thereafter, the shaker is stopped to permit manometer readings every 30 seconds for up to 7 minutes.
eter readings are made at the same time and the appropriate corrections applied to the readings of the reaction manometer. The recently introduced circular Warburg apparatus makes stopping of the shaker unnecessary.

The control, or uncatalyzed, reaction is observed in a similar manner, except that in place of blood 0.5 ml. of a 1:200 dilution of plasma in distilled water is used. This control solution was found to give smoother and more consistent curves than when distilled water alone was used as diluent. Neither enhancing nor inhibitory effects on enzyme activity were found when this or even higher concentrations of human plasma were used.

All estimations were made in duplicate or triplicate, closely agreeing determinations being averaged.

Calculations—Calculation of activity of each sample is made from the data according to the method suggested by Mitchell, Pozzani, and Fessenden (8), and activity is expressed in terms of $K$, related to unimolecular velocity constants.

Pressure readings are taken at 30 second intervals as long as measurement of the rate of evolution of gas is accurate and these are corrected
for thermobarometer changes. These corrected readings are then paired, the differences taken, and the logarithm of these differences plotted against time (9) (Fig. 1). As the reaction proceeds, the back reaction of hydration becomes increasingly significant and some of the terminal points may have to be discarded in order to secure a straight line. The slope of the straight line thus obtained is multiplied by 2.303 to give the value \( K \) related to the velocity constant for the reaction.

Enzyme activity is expressed as the difference \( (K) \) between \( K_0 \), the value related to the velocity constant of the control reaction, and \( K \), that related to the velocity constant of the reaction in the presence of the enzyme as follows:

\[
K = K_0 - K
\]

Values of \( K \) for each dilution are then plotted against the logarithm of the amount of blood used in the experiment (Fig. 2) and a straight line drawn. By extending this line to zero dilution, an extrapolated value for 0.5 ml. of undiluted blood is obtained, this value being then multiplied by 2 to give the enzyme activity for 1.0 ml. of whole blood.

**Table I**

Effect of Freezing and Thawing on 1:200 Dilution of Human Blood

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>( K^* ) before</th>
<th>( K^* ) immediately after</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.20</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.21</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>5</td>
<td>0.16</td>
<td>0.11</td>
</tr>
</tbody>
</table>

\( K^* = \) activity.

**DISCUSSION**

It is evident that values for blood carbonic anhydrase activity obtained at body temperature are more significant for mammalian physiology than those obtained at 0–10°, particularly since working in the cold exaggerates the apparent activity of the enzyme (1–4). Moreover, extreme cold partially inactivates the enzyme, as was shown when attempts were made in the present study to resort to freezing and thawing in order to avoid the need of laking the blood in water. When the blood was frozen at -5° and thawed three times, the activity of the enzyme was markedly reduced (Table I).

The findings of Meldrum and Roughton (1) showed an absence of linear relation between concentration of the enzyme and its apparent activity.
when blood is tested. The data of van Goor (12), Lambie (13), Philpot and Philpot (14), and of the present study are in accord. It was found here that when blood was used in high dilution, i.e. 1:200, the calculated activity per ml. of whole blood found was greater than in lower dilution, i.e. 1:20 or 1:50. However, when the logarithm of the amount of blood used was plotted against the activity found, a straight line could be drawn. Extrapolating this line to zero dilution gave the activity of 0.5 ml. of undiluted blood. The advantage of using the activity of undiluted blood for comparison of one specimen with another is apparent; it eliminates errors which might arise through the use of variously diluted bloods in the case of anemia or polycythemia. It might be considered that extrapolation introduces significant errors, as indeed it does; such errors are smaller, however, than those caused by basing conclusions on observations made only at high dilutions, as is the rule in methods previously described.

**Table II**

Observations in Forty-Two Normal Subjects

<table>
<thead>
<tr>
<th></th>
<th>Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, % erythrocytes</td>
<td>38.0 - 56.0</td>
<td>45.2</td>
</tr>
<tr>
<td>Hemoglobin, gm. per ml. blood</td>
<td>0.133 - 0.199</td>
<td>0.138</td>
</tr>
<tr>
<td>Erythrocytes, billions per ml. blood</td>
<td>3.62 - 5.93</td>
<td>4.87</td>
</tr>
<tr>
<td>Carbonic anhydrase, units per ml. blood</td>
<td>1.2 - 2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; erythrocytes</td>
<td>2.6 - 5.8</td>
<td>4.05</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; gm. hemoglobin</td>
<td>8 - 17</td>
<td>13</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; billion erythrocytes</td>
<td>0.25 - 0.56</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The need for violent shaking to insure adequate diffusion of the gas evolved is obviated by the use of a high bath temperature.

By the present method and with the calculations used here the carbonic anhydrase activity is 1.2 to 2.6 (mean 1.8) units per ml. of normal blood (Table II; Fig. 3); i.e., the value related to the velocity constant of the reaction which occurs when 1 ml. of blood causes the release of carbon dioxide from the reaction mixture is 1.2 to 2.6 times greater than that of the uncatalyzed reaction. Since all of the enzyme is within the red blood cells, its concentration might be expressed as 2.6 to 5.8 units per ml. of erythrocytes (average 4.05) (Table II). In agreement with the findings of Booth (15) no evidence of an inactivator was found in human plasma. The activity of the enzyme found here is almost certainly somewhat lower than the activity of the enzyme as it exists in the erythrocytes of the body, for the reaction mixture has a final pH of 6.90, a level at which carbonic anhydrase activity is decreased to some extent.
Data on the influence of various ions upon carbonic anhydrase activity have been reviewed by Roughton and Booth (16), who made extensive additional observations; it is clear that the chemical environment in which the enzyme is placed in the method here described is so markedly different from that which obtains in the red blood cell as to make it impossible to estimate the quantitative effects of the action of carbonic anhydrase in intact blood from results obtained with methods of the type described here. The work of Mitchell et al. (8) removed some of the mathematical invalidities from the earlier methods. The present method removes invalidities due to low temperature and to the lack of linear relation between enzyme concentration and its activity. However, conclusions as to the amount of acceleration caused by carbonic anhydrase in the hydration of carbon dioxide in the blood in vivo cannot be made from data now available.

**Fig. 3.** Carbonic anhydrase activity of whole blood in forty-two normal subjects. The solid squares indicate men; the clear squares women.

**SUMMARY**

A method, in which a value related to unimolecular velocity constants is employed, has been devised for measuring carbonic anhydrase activity of blood at body temperature with standard Warburg apparatus. It has the advantages of eliminating some errors inherent in earlier methods, such as those consequent to low temperatures, lack of linear relation between enzyme concentration and activity, and mathematical invalidity of calculations used. On the other hand it affords no true indication of activity of the enzyme in vitro because of the differences in pH and in ionic constitution of the media used as against the interior of erythrocytes in blood.

**BIBLIOGRAPHY**

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