Kinetic Studies of Human Carbonic Anhydrases B and C*

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Carbonic anhydrase preparations from human erythrocytes contain at least three distinct molecular forms of the enzyme (1-4), two of which—denoted here as carbonic anhydrases B and C—have been studied in detail. They differ widely in specific activity, and also in their isoelectric points (1, 4), their amino acid composition (4-6), and their chromatographic behavior (3, 4). Earlier studies on the kinetics of human carbonic anhydrase (7-9) were made on preparations that presumably were mixtures of these isoenzymes. DeVoe and Kistiakowsky (9) found that the kinetics of their preparation did not fit the Michaelis-Menten equation. It seemed of interest, therefore, to study the kinetic behavior of the two major forms of the enzyme, now available in quite pure form, and to compare these results with those obtained on earlier preparations of the human and the bovine enzymes. The studies reported here are particularly concerned with the effect of pH on the kinetic parameters of carbonic anhydrases B and C. We have already given a preliminary report of these findings (10).

Carbonic anhydrase (EC 4.2.1.1) catalyzes the reaction which we may write adequately for present purposes as

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad (1)$$

This reaction proceeds at an appreciable rate even in the absence of enzyme, although apparently too slowly to permit normal functioning of many organisms and tissues. The catalysis of this reaction can conveniently be studied in either direction. Since the over-all equilibrium constant of Equation 1 is near $10^{-4.4}$, the ratio of the concentrations of HCO$_3^-$ and CO$_2$ at neutral pH is therefore not far from unity.

The results to be reported here show great differences in the kinetic constants of carbonic anhydrases B and C. These two enzymes probably differ more in this respect than any pair of isoenzymes for which data have yet been reported.

**EXPERIMENTAL PROCEDURE**

We employed a stopped flow photometric apparatus to follow the rates of Reaction 1 at 25.0 ± 0.1°C, in the absence and in the presence of enzyme, as a function of the concentration of substrate (CO$_2$ or HCO$_3^-$). We made most of the measurements in phosphate buffers over the pH range 6.3 to 7.5, and used p-nitrophenol as indicator, at concentrations between 25 and 100 μM. We followed the reaction by measurement of absorbance at 400 μm, as previously described (11); the length of the optical path was 1 cm.

To determine the catalytic effect of the enzyme it is necessary to subtract the rate in the absence of enzyme from the total rate. We have already reported values for the rate constants for hydration and dehydration in the absence of enzyme, and have described the apparatus and the method of measurement (11) (see also Ho and Sturtevant (12)). Except for the few differences noted below, the techniques for making rate measurements in the presence of enzyme were identical with those already described.

*Substrate and Buffer Solutions—We have already described in detail the preparation, composition, and concentration of these solutions (11). We made measurements on the enzyme solutions at 25° in three phosphate buffers, at a total phosphate concentration of 0.025 M, and at pH 6.30, 7.05, and 7.55. The CO$_2$ solutions ranged in concentration from 1.66 to 14.4 mM, and the bicarbonate solutions, from 12.5 to 100 mM.

We made up all solutions in deionized water drawn from a mixed bed ion exchange agent, and rinsed all glassware thoroughly in deionized water before use.

*Method of Calculation of Results—Our earlier paper (11) gives details of the method of calculation. We assumed that although the runs with the enzyme solutions might approach zero order kinetics at high substrate concentrations, the calculation of the data by a first order equation would still be valid. In fact, the results demonstrated that under the conditions used the enzymatic runs were much more nearly first than zero order.

**Enzyme Preparations—**Enzymes B and C were prepared from human erythrocytes by methods previously described (4). In one case the starting material was a hemolysate made from the red cells of about 20 individuals. Hemoglobin was removed by the chloroform-ethanol treatment. The crude fraction so obtained was stored as a lyophilized powder at 4° for about 2 years prior to use in this work. Some of it was then chromatographed on hydroxylapatite (4) to prepare carbonic anhydrases B and C (Table I).

The other preparation of the two carbonic anhydrases was made from the erythrocytes of a single individual. In this case we separated the carbonic anhydrases from the hemoglobins in the original hemolysate by chromatography on Sephadex G-75 (4), and then separated the component forms of the enzyme by chromatography on hydroxylapatite (Table I). After fractionation, we lyophilized the enzyme solutions and stored the dried powders in a dessicator at 4°.

Stock solutions of the enzymes (0.8 to 4 mg in 10 ml of deionized H$_2$O) were made from the dried preparations whenever they were needed. These solutions were centrifuged at 12,000 × g for 10 minutes to remove a small amount of insoluble material.

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that rendered them turbid, and then stored in the cold. From the readings of the absorbance at 280, 290, 320, 360, 400, and 410 m\(\mu\), corrections were made for scattering (1 to 6\%) and for the contribution of hemoglobin to the reading at 280 m\(\mu\). We assumed a value of 59 at 410 m\(\mu\) and of 20.8 at 280 m\(\mu\) for the contribution of hemoglobin to the reading at 280 m\(\mu\). We determined the rates in 0.025 m phosphate buffer, pH 7.05; the CO\(_2\) concentration was 4.90 mm.

**Table I**

Separation of carbonic anhydrases B and C from crude fraction on hydroxylapatite columns

<table>
<thead>
<tr>
<th>Source of crude fraction</th>
<th>Ass*</th>
<th>Specific activity†</th>
<th>Yield of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Chloroform-ethanol procedure</td>
<td>67</td>
<td>12</td>
<td>12.5-14</td>
</tr>
<tr>
<td>Sephadex procedure</td>
<td>43</td>
<td>8</td>
<td>12-17</td>
</tr>
</tbody>
</table>

* Expressed as the percentage, appearing in that fraction, of the total absorbance (at 280 m\(\mu\)) of the material applied to the column. B and C denote enzymes B and C.

† Activity is expressed as Wilbur-Anderson kilounits of enzyme per mg, measured by a modification of the original method of Wilbur and Anderson (13, 4). These units should not be confused with the official IUB units discussed in the text.

**Table II**

Hydration velocity as function of enzyme concentration

The enzyme used was carbonic anhydrase C, Sephadex preparation, in 0.025 m phosphate buffer, pH 7.05; the CO\(_2\) concentration was 4.90 mm.

<table>
<thead>
<tr>
<th>Enzyme concentration (E(_0))</th>
<th>Velocity ((V_i), Equation 2)</th>
<th>(\frac{V_i}{E_o})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M \times 10^4)</td>
<td>(\mu \text{sec}^{-1} \times 10^4)</td>
<td>(\text{sec}^{-1} \times 10^{-4})</td>
</tr>
<tr>
<td>3.1</td>
<td>4.15</td>
<td>3.35</td>
</tr>
<tr>
<td>6.4</td>
<td>8.7</td>
<td>3.35</td>
</tr>
<tr>
<td>9.3</td>
<td>13.0</td>
<td>3.40</td>
</tr>
<tr>
<td>12.6</td>
<td>17.1</td>
<td>3.35</td>
</tr>
</tbody>
</table>

The velocity of the enzyme-catalyzed reaction is defined by the equation

\[ V_{\text{total}} - V_{\text{th}} = V_i \text{ (hydration) or } V'_i \text{ (dehydration)} \]

where \(V_{\text{total}}\) and \(V_{\text{th}}\) denote the observed total velocity and the velocity in the absence of enzyme, respectively. We usually used sufficient enzyme to make \(V_{\text{total}}\) at least twice as great as \(V_{\text{th}}\). The velocity of the enzyme-catalyzed reaction was a linear function of the concentration of carbonic anhydrase C (Sephadex preparation) over a 4-fold range (Table II).

The velocity of the enzyme-catalyzed hydration reaction, \(v_i\) in Equation 2, was measured at six CO\(_2\) concentrations for each pH value. The results were plotted according to Lineweaver and Burk (19), and values of the kinetic parameters \(V\) and \(K_m\) were determined from the graphs. Fig. 1 shows the results at one pH (7.55). Each value of \(v_i\) shown in the figure was the average of four runs; the average deviation from the mean in any set was 4\%. This statement applies also to the results at other pH values. We determined the rates in 0.025 m phosphate buffer at three pH values, 6.30, 7.05, and 7.55, for both enzymes B and C. Table III summarizes the results.

1 The following symbols are used: \(K_m\) denotes the Michaelis constant and \(V\) the maximum velocity in the hydration reaction, while \(V'_m\) and \(V'_i\) denote the corresponding parameters for the dehydration reaction. \(E_o\) is the total molar enzyme concentration, and \(v_i\) (or \(v'_i\)) is the enzyme-catalyzed velocity of hydration (or dehydration) in moles per liter per second.
We also made hydration measurements on carbonic anhydrase C (Sephadex preparation) in pH 7.05 buffer containing one-half the usual p-nitrophenol concentration, i.e. 25 instead of 50 µM. We found no significant change in the values of $K_m$ and $V/E_o$ as a result of this variation of indicator concentration.

In another experiment the phosphate buffer concentration was reduced to 0.0125 M, which was half its usual value. The pH at this dilution was 7.10, as compared with 7.05 for the buffer at 0.025 M with the same ratio of primary to secondary phosphate. In this case, $K_m$ decreased about 10% to 12.5 mM and $V/E_o$ remained unchanged. The variation in $K_m$ is close to the limit of experimental error.

In an attempt to extend the range of measurements to higher pH values, we made some hydration measurements in 0.025 M sodium Veronal buffer, pH 7.85, containing m-cresol purple as indicator. However, this buffer-indicator system inhibited the enzyme, and the results were discarded.

**Dehydration Measurements**—All dehydration runs were made in the 0.025 M phosphate buffer, pH 7.05. Again each recorded velocity was the average of four runs. Because of the presence of significant amounts of CO2 in the bicarbonate solutions, it was necessary to correct for the back-reaction (hydration) in the dehydration runs. We applied the method described by DeVoe and Kistiakowsky (9) for making this correction. Fig. 2 shows the uncorrected and corrected Lineweaver-Burk plots of the data, and Table IV presents the resulting dehydration parameters for the two curves.

We estimate the maximum probable error in the measured velocities as ±5%. Since $v_{total}$ was at least twice as great as $v_i$ in all runs, the maximum error in $v_i$ or $v_i'$ should be less than ±10%. Empirically $K_m$ was found reproducible within ±10%, and the maximum velocity, $V$, was reproducible to ±6%. The uncertainty in the relative values of $E_o$ was probably ±3%. The absolute value of $E_o$ is less certain, however, for it depends on the assumed values of the molecular weight and of the absorbance at 280 µm for a 1% solution.

**DISCUSSION**

The question arises whether the enzymes have the same kinetic properties if the hemoglobin is initially removed by the chloroform-ethanol or by the Sephadex method. The only significant difference that we have found between the preparations obtained by the two methods was in the value of $V/E_o$ for carbonic anhyd-
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activity, the values of substrate concentrations approximately 2.5 times as great as ours. Clearly a mixture of two isoenzymes possessing much the same kinetic properties, whichever method of preparation is used to remove hemoglobin from the hemolysate.

The value of \( V/E_0 \) for enzyme C (Sephadex preparation) is 25 to 50 times that of enzyme B. This large difference is in qualitative agreement with previous estimates of relative specific activity (1-4). Laurent et al. (3) give a figure of 20, whereas Nyman (1) and Rickli et al. (4) give much lower values, of the order of 3 to 5. These differences in the reported relative specific activities are explainable in terms of the different assay methods used. The \( K_m \) values for enzyme B are considerably smaller than those for enzyme C; hence, at low substrate concentrations, the relative velocities of the reactions catalyzed by the two enzymes differ much less than the relative \( V/E_0 \) values.

The Commission on Enzymes of the International Union of Biochemistry has recommended that an enzyme unit be defined as "that amount which will catalyze the transformation of 1 micromole of substrate per minute" under optimal conditions, and preferably at 25°C (20). We have calculated such values for enzymes B and C from the values of \( V/E_0 \) at pH 7.55 (Table III), although the rates at this pH are probably not quite optimal. A kilounit of enzyme B, then, is \( 2.3 \times 10^{-4} \) mg, and of enzyme C, \( 7.7 \times 10^{-4} \) mg. Their specific activities are accordingly 44,000 and 1,300,000 units, respectively, per mg of protein.

Previous kinetic measurements on the human enzyme have generally been made with material which presumably contained both enzymes B and C. DeVoe and Kistiakowsky (9) found that their data, obtained at 0°C in dilute phosphate buffers, did not fit a straight line in a Lineweaver-Burk plot. They suggested that substrate activation of the enzyme might explain the fact that \( n \) did not show an asymptotic approach to a maximum value at high CO\(_2\) concentrations. Because the dehydration kinetics fitted the Michaelis-Menten equation, they rejected the alternative hypothesis that there might be two kinds of independent active sites.

The presence of two enzymes with very different kinetic behavior may well provide the explanation for the failure to obey Michaelis-Menten kinetics that DeVoe and Kistiakowsky observed. However, it must be pointed out that the solubility of CO\(_2\) and the Michaelis constants are such that at 25°C, the temperature of our experiments, it is impossible to approach saturation of the enzyme and to answer this question conclusively. Kernohan, Forrest, and Roughton (21), who worked both at 20°C and at 35°C, have commented on this also. For technical reasons it proved impossible to make measurements at 0°C with our apparatus. DeVoe and Kistiakowsky, at 0°C, were able to achieve substrate concentrations approximately 2.5 times as great as ours.

Human carbonic anhydrases B and C differ widely in specific activity, the values of \( V/E_0 \) being about 30 times as great for enzyme C as for enzyme B. We know of no other case in which two isoenzymes differ so much in this respect, but similar instances may well be found in future. These findings emphasize the need for a searching inquiry into the homogeneity of enzyme preparations prior to kinetic studies. Clearly a mixture of two isoenzymes possessing very different kinetic characteristics may exhibit anomalous behavior. Although enzyme C is so much more active than enzyme B, the latter is present in much larger amount, and should make a significant contribution to the catalysis of the hydration and dehydration reactions in vivo.

In contrast to their results with the human enzyme, DeVoe and Kistiakowsky (9) found the kinetics of the bovine enzyme to give a good fit to the Michaelis-Menten equation. It is now known that there are at least two different forms of the bovine enzyme, but these resemble one another rather closely in specific activity (22). Probably this is also true of their kinetic constants.

All previously reported values of \( V/E_0 \) for hydration have been much higher for the bovine than for the human enzyme. Such values for bovine carbonic anhydrase have ranged from \( 10^4 \) to \( 10^5 \) sec\(^{-1} \) at 0°C and pH 7 (9,23), while reported values for the human enzyme have not exceeded 1800 at 0°C (7). The values recorded in Table III are clearly of a higher order than this, especially for enzyme C, even when allowance is made for the difference in the temperature of measurement.

Kernohan (24), in studies on bovine carbonic anhydrase, found that \( K_m \) was independent of pH between 6.7 and 7.7, and that \( V \) increased with pH as if it depended on the ionization of one group with a pK of 6.9. In the dehydration reaction he found \( K_m \) to be very large, and \( V \) decreased with increasing pH, as if it depended on the concentration of the acid form of the same group in the enzyme, with a pK of 6.9. Kernohan worked with imidazole buffers, in the presence of chloride ions at defined concentrations, and his enzyme preparation presumably contained both enzymes A and B of Lindskog (22). He suggested that in the dehydration reaction, bicarbonate ion might act not only as a substrate but also as an inhibitor. On this hypothesis it would resemble other anions such as chloride, which is well known to be inhibitory, as Kernohan points out. It is obvious that Kernohan's findings differ strikingly from ours. Without further studies we cannot decide how far this represents a true difference between the human and bovine enzymes, and how far it may be due to the difference in the buffer systems employed, as well as to the presence of chloride ions in Kernohan's experiments.

We fail to confirm the report (9) that both phosphate and p-nitrophenol act formally as activators and competitive inhibitors of the enzyme. These effects, if they exist, must be small.

Calculation of Haldane Relation for Carbonic Anhydrase—The equilibrium constant for the reaction of Equation 1 may be defined by

\[
K' = \frac{[H^+][HCO_3^-]}{[CO_2]} \tag{3}
\]

where the prime superscript on \( K \) denotes the value at a particular ionic strength.

The Haldane relation (25, 26) for carbonic anhydrase is, from Equation 2,

\[
\frac{(V)K_m}{(K_m')V} = \frac{K_1}{a_H} \tag{4}
\]

where \( \gamma_H \) and \( a_H \) are the activity coefficient and activity of \( H^+ \), respectively. Since the right-hand side of the equation is a function of pH and ionic strength, values of the four parameters on the left must be taken from measurements at the same pH and ionic strength. Table V gives the results of the calculation.

We have used the kinetic data at pH 7.05 (corrected for the back-reaction) taken from Tables III and IV.

The discrepancy between the two sides of Equation 4 is about
TABLE V
Haldane relation for carbonic anhydrases B and C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(\frac{(V') (K'_m)}{(K_m) (V)})</th>
<th>(K'_m (\text{vmax})^2 (\text{pH}))</th>
<th>(K'_m) (vmax) (\text{pH})</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>8.5</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>C</td>
<td>8.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

28%. This seems rather larger than one might expect from the estimated accuracy of the data, particularly in view of the fact that some possible sources of inaccuracy (for instance, the buffer factor discussed previously (11)) will cancel out in the product on the left-hand side of Equation 4. The close agreement between the experimental results for enzyme B and enzyme C suggests that some systematic error is involved. We have already pointed out (11) the uncertainty in the value to be assigned to the hydrogen ion concentration calculated from \(a_n\) in the dehydration runs without enzyme. The same uncertainties obviously exist in the enzymatic work as well. In addition we note that if the carbonic anhydrase catalysis is affected by the changing ionic strength as the substrate concentration is varied in different runs, then the values of \(K'_m\) and \(V'\) will be erroneous. DeVoe (29) has also discussed this subject. We believe that the uncertainties in the enzymatic dehydration results are greater than those in the hydration studies, and that they are chiefly responsible for the discrepancy.

DeVoe and Kistakowski (9) found that most of their data for the bovine enzyme and the “nonactivated” human enzyme satisfied the Haldane relation within 10%. Kernohan (24) also obtained good agreement with his data on the bovine enzyme.

The present data for \(K_m\) and \(V/E_0\) cover too narrow a pH range to permit determination of the dissociation constants of any H⁺ ion equilibria influencing the catalysis. It is, unfortunately, difficult to extend the pH range of such measurements. Below pH 6 the equilibrium lies in favor of dehydration of H₂CO₃, and the back-reaction becomes considerable in hydration measurements. Above pH 8 the velocity of the reaction of CO₂ with hydroxyl ion (30) becomes large. In addition the problem of specific ion effects on the enzyme arises when more than one buffer is used.

Possible further studies to extend these observations are so numerous that we shall not attempt to discuss them here. However, we have made a preliminary kinetic study of the cobalt analogues of the native zinc enzymes B and C. The cobalt derivatives of the bovine enzyme have been intensively studied by Lindskog and Malmstrom (31) and Lindskog (32). Dr. Egon E. Rickli in this laboratory prepared the cobalt derivative of human carbonic anhydrase B and found that it possessed about 40% of the activity of the native zinc enzyme by the Wilbur-Anderson (13) assay. We have found, in preliminary studies of the cobalt enzymes at pH 7.05, the following values for the hydration reaction: carbonic anhydrase B, \(K_m = 1.8 \times 10^{-3}\) mM, \(V/E_0 = 9,000 \text{ sec}^{-1}\); carbonic anhydrase C, \(K_m = 5.9 \times 10^{-3}\) mM, \(V/E_0 = 260,000 \text{ sec}^{-1}\). We have not characterized our preparations of the cobalt enzymes spectroscopically, so that their freedom from zinc is not completely established. Therefore these values must be regarded as tentative, but we believe them to be approximately correct.

SUMMARY

We have studied the kinetic behavior of the two major isoenzymes of human carbonic anhydrase in the pH range 6.3 to 7.5, in phosphate buffers at 25°C, by spectrophotometric measurements in a stopped flow apparatus. Under the conditions employed, both the hydration and the dehydration kinetics fitted the Michaelis-Menten equation. There are striking differences between the kinetic parameters of carbonic anhydrases B and C. At pH 7.0 the Michaelis constants for hydration are 2.6 mM for enzyme B and 14 mM for enzyme C, and the maximum rate values per enzyme molecule \(V/E_0\) are 15,000 sec⁻¹ (B) and 620,000 sec⁻¹ (C). For both enzymes, \(K_m\) decreases and \(V/E_0\) increases with increasing pH in this range. The activity of our preparation of enzyme C is the highest yet reported for a human carbonic anhydrase preparation. Some of the anomalous kinetic behavior reported by others may be explained by the presence of a mixture of components.

Application of the Haldane equation shows a moderate discrepancy between the known equilibrium constant for the ionization of carbonic acid and the value calculated from the kinetic data. This discrepancy may be due to changes in the activity coefficients of the hydrogen and bicarbonate ions as the concentration of substrate is varied in studies of the dehydration reaction.

Preliminary data for the kinetic constants of the cobalt derivatives of enzymes B and C are reported.

Acknowledgments—We thank Professor J. M. Sturtevant for arranging for the fabrication of the stopped flow unit according to his design, and Dr. R. B. Pennell of the Protein Foundation, Jamaica Plain, for the supplies of human erythrocytes. We have had valuable discussions with Dr. I. R. Gibbons and with Dr. J. C. Kernohan, who also kindly permitted us to read his paper (24) before publication.

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