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Kinetic Constants Determined from Membrane Transport Measurements: Carbonic Anhydrase Activity at High Concentrations

(enzyme/diffusion-reaction coupling/CO_2 hydration)

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ABSTRACT **Facilitated diffusion rates can be used to determine kinetic constants for rapid reactions occurring within membranes and thin fluid layers. We have applied this technique to the study of the reversible CO_2 hydration reactions catalyzed by carbonic anhydrase (EC 4.2.1.1; carbonate hydro-lyase). The experimental method entails the diffusion of tracer $^{14}CO_2$ through Millipore filter membranes impregnated with aqueous bicarbonate solutions containing various concentrations of dissolved enzyme. A mathematical model of the simultaneous diffusion/reaction transport process is analyzed to predict the effective diffusion rate in terms of the relevant kinetic parameters. The solution to the mathematical model can be transformed to yield straight-line relations analogous to Lineweaver–Burk plots. The pseudo-first-order enzymatic rate constant for the hydration reaction can be determined from the slope or intercept of a plot of this straight-line relationship. Rate constants were accurately measured at high enzyme concentrations for reactions having half-times under a millisecond. The rate constants agree well with other reported kinetic constants for carbonic anhydrase, and the known pH-activity dependence and bicarbonate inhibition are quantitatively demonstrated. The specific activity is constant up to 4.0 mg/ml, which is believed to be the highest concentration at which the activity has been measured. The membrane transport technique has general applicability for other rapid reaction systems.**

There is much interest in the measurement of kinetic constants for enzymes contained within natural and artificial membranes (1–4). If the rate of transport of a reactive permeant through such a membrane is facilitated by reversible chemical reactions catalyzed by the entrapped enzyme, then measurement of the facilitated permeant flux can provide an attractive method for determining kinetic properties of the enzyme (5, 6). As opposed to conventional kinetic methods, the experimental measurement is a steady-state flux rather than a transient change in concentration, and the coupling between reaction and diffusion provides a great deal of flexibility in the design of the experiment. The differential equations governing the transport process can be solved analytically for first-order reaction kinetics, and the kinetic parameters can be determined from straight-line plots of the steady-state transport data. In the present study of CO_2 transport and carbonic anhydrase kinetics, the flux was measured by a radioactive tracer method that produces first-order reaction kinetics for the tagged permeant. In principle, the method is capable of measuring rate constants for reactions with half-times smaller than the dead times of conventional stopped-flow methods, which are typically of the order of a millisecond (7). We have determined rate constants for enzyme-catalyzed CO_2 hydration reactions having half-times well under a millisecond from pseudo-steady-state membrane transport data. This technique should be particularly useful in situations where traditional optical methods are impractical, e.g., in opaque hemoglobin solutions and in studies of the kinetics of membrane-bound enzymes.

The enzyme carbonic anhydrase (EC 4.2.1.1; carbonate hydro-lyase) catalyzes the reversible hydration of CO_2 to form bicarbonate ion, a process of considerable physiological importance (8). The turnover rate of carbonic anhydrase is among the highest known enzymatic rates, and the reaction velocity at physiological concentrations (2–3 mg/ml) is too rapid to be accurately measured by conventional stopped-flow techniques. We have obtained kinetic data for bovine carbonic anhydrase which indicate that the specific activity is constant up to concentrations of 4.0 mg/ml. The activity agrees well with the kinetic parameters obtained in dilute enzyme solutions by stopped-flow and thermal methods (9–12). These results provide justification for the extrapolation of dilute enzyme kinetics to the physiological conditions in the red cell, although there are undoubtedly other factors in the blood that influence the kinetic activity of the enzyme *in vivo* (13–15).

FACILITATED TRANSPORT OF CO_2

Facilitated transport through a membrane is a simultaneous reaction and diffusion process in which the normal Fickian diffusional transport of a permeant is augmented by the parallel diffusion of products of a reaction of the permeant with membrane components. For the case of CO_2-facilitated transport in an aqueous membrane, the chemical reactions are (16)

$$\begin{aligned} CO_2 + H_2O &\rightleftharpoons HCO_3^- + H^+ \\ CO_2 + OH^- &\rightleftharpoons HCO_3^- \end{aligned} \qquad [1]$$

The bicarbonate ion diffuses in parallel with the dissolved CO_2 and can augment the total CO_2 flux considerably under proper conditions. The relatively slow conversion of CO_2 to bicarbonate and the reverse reaction are catalyzed by carbonic anhydrase. The maximum flux augmentation is limited, however, by chemical reaction equilibrium, and is thus related to the equilibrium constant

$$K_{EQ} = \frac{[HCO_3^-]\,[H^+]}{[CO_2]} \qquad [2]$$

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The facilitated transport of CO_2 has been discussed in detail in the reports by Longmuir *et al.* (17), Ward and Robb (18), Enns (19), Suchdeo and Schultz (20), Meldon (21), Otto and Quinn (6), and Donaldson and Quinn (22).

EXPERIMENTAL METHOD

The facilitated transport of tracer $^{14}CO_2$ through Millipore filter membranes was measured in a diffusion cell having a gas flow proportional counter and a Geiger–Müller tube mounted in the upper and lower chambers, respectively. The diffusion cell was similar to the cell of Enns (19), and the complete apparatus is described more fully elsewhere (22, 23).

Millipore filter membranes (type AA, 0.8-μm pore size) were soaked in the solution to be studied for at least 4 hr prior to the transport experiments. The membranes were stored in the bicarbonate or bicarbonate/enzyme solutions and refrigerated at 6° between experiments. For a flux measurement, a membrane was mounted between two identical Plexiglas plates in which there were matching holes to permit gas transport. The sandwich was placed in the diffusion cell and both chambers were purged with a water-saturated gas mixture of 1% or 5% CO_2 in N_2 for 30 min. The bottom chamber was then closed off, and a small amount of $^{14}CO_2$ (about 25 μCi) was generated by releasing a drop of HCl into a small cup containing $Ba^{14}CO_3$. The purge in the top chamber was continued for another half-hour until the $^{14}CO_2$ count rate in the bottom chamber became constant. The top chamber was next sealed off and the rate of accumulation of $^{14}CO_2$ in the top chamber was recorded.

The concentration of $^{14}CO_2$ in the bottom chamber was less than 1% of the CO_2 in the purge gas and, consequently, did not significantly change the total CO_2 concentration. There was no pH gradient across the membrane, since both sides of the membrane were exposed to the same partial pressure of CO_2. No supporting buffers or other ions were used; the enzyme substrate-product system, CO_2-HCO_3^-, served as a buffer. Bovine carbonic anhydrase was obtained from Worthington Biochemical Corp. and used without further purification. The activity was determined by standard techniques (24), and the activity specified by Worthington was verified. The enzyme purity was estimated to be 75% by ultraviolet absorption and atomic absorption for Zn (23).

MATHEMATICAL MODEL

At steady state the transport of each species in the membrane is governed by an equation of the form

$$D_i \frac{d^2[i]}{dx^2} = R_i \qquad [3]$$

where x is the coordinate in the direction of transport, D_i is the diffusivity of species i, the brackets refer to molar concentrations, and R_i is the net rate of depletion of species i by chemical reaction. This system of equations is solved subject to the boundary conditions of constant CO_2 concentrations at the membrane boundaries and no flux of bicarbonate ion at the membrane boundaries (6, 22). The facilitation effect is expressed as an increase in the effective diffusivity of CO_2 in the membrane (D_{eff}) normalized by the ordinary diffusivity of molecular CO_2 (D_{CO_2}). The analytical expression for this ratio of diffusivities is

$$\frac{D_{eff}}{D_{CO_2}} = \frac{1 + F}{1 + (F/\phi)\tanh \phi} \qquad [4]$$

where

$$F = \frac{D_{HCO_3^-}}{D_{CO_2}} \frac{K_{EQ}}{[H^+]} \quad \text{and} \quad \phi = \frac{1}{2}\sqrt{\frac{kL^2}{D_{CO_2}}}\left(\frac{1 + F}{F}\right) \qquad [5]$$

in which $D_{HCO_3^-}$ is the bicarbonate diffusivity, L is the diffusional path length (or membrane thickness), and k is the combined pseudo-first-order rate constant for the CO_2 hydration reactions in Eq. [1]. As noted earlier, the pH is constant throughout the membrane under our experimental conditions and, hence, the reaction rates of the tagged $^{14}CO_2$ species are first-order; and since $[CO_2] \gg [^{14}CO_2]$, the total CO_2 concentration is uniform throughout the membrane and the equilibrium expression in Eq. [2] is applicable. An alternate, more useful form of $K_{EQ}/[H^+]$ is $[HCO_3^-]/[CO_2]$ from the equilibrium expression. A result similar to Eq. [4] has been presented by Danckwerts (25) and others for homogeneous first-order reactions in thin films. The normalized effective diffusivity in Eq. [4] can be reduced to simplified limiting forms for the cases of no reaction ($D_{eff}/D_{CO_2} = 1$), equilibrium reaction ($D_{eff}/D_{CO_2} = 1 + F$), and reaction-limited saturation ($D_{eff}/D_{CO_2} = \phi/\tanh \phi$). These limits are derived elsewhere (22).

Enzymatic Rate Constant. Bovine carbonic anhydrase has been shown to obey Michaelis–Menten kinetics and the Haldane relationship (under certain conditions; see refs. 9 and 26), and so the net reaction rate for the tracer $^{14}CO_2$ is

$$R_{^{14}CO_2} = \frac{k_3E_0}{K_m + [CO_2]} \times ([^{14}CO_2] - [H^{14}CO_3^-][H^+]/K_{EQ}) \qquad [6]$$

where $[CO_2]$ is the total molecular dissolved CO_2, including both tagged and untagged, K_m is the Michaelis constant, and k_3E_0 is the usual maximum velocity. It is assumed that there is negligible bicarbonate binding, and the enzyme activity dependence on pH is ignored for the moment. Both of these factors are discussed below.

Since the dissolved CO_2 concentration and the pH are constant throughout the membrane, the reaction kinetics are pseudo-first-order in both directions. A pseudo-first-order enzymatic rate constant k_{enz} can be defined for the hydration reaction as

$$k_{enz} = \frac{k_3E_0}{K_m + [CO_2]} \qquad [7]$$

Data Analysis. Eq. [4] is nonlinear in both F and ϕ, but it can be cast into a more nearly linear form by taking the reciprocal of both sides:

$$\frac{1}{D_{eff}/D_{CO_2}} = \frac{1}{1 + F} + \left(\frac{F}{1 + F}\right)\frac{\tanh \phi}{\phi} \qquad [8]$$

When $F \gg 1$, this double reciprocal form is essentially linear in the parameter $1/F$. Transport data for various values of F (i.e., various CO_2 or bicarbonate concentrations) can be plotted in this linear form, and the kinetic parameter ϕ can be obtained from the intercept. In addition, it is possible to experimentally vary the modulus ϕ by changing the rate constant (or perhaps the membrane thickness) while holding F constant. Data obtained in this manner can also be plotted in a linear fashion according to Eq. [8]. These double reciprocal plots are the membrane transport analog of the Lineweaver–Burk plot for homogeneous enzyme kinetics.

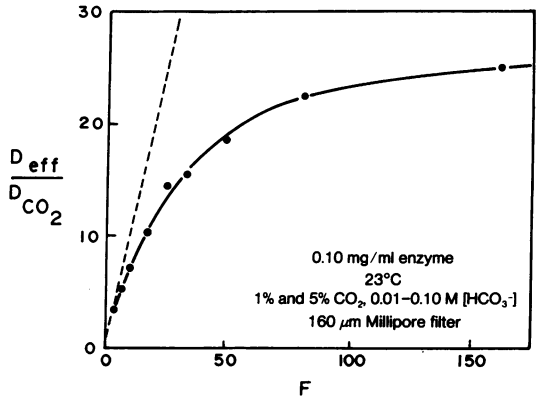

FIG. 1. Effective diffusivity ratio of CO_2 measured in Millipore filter membranes at constant enzyme concentration showing reaction-rate-limited transport. The broken line is the reaction equilibrium limit.

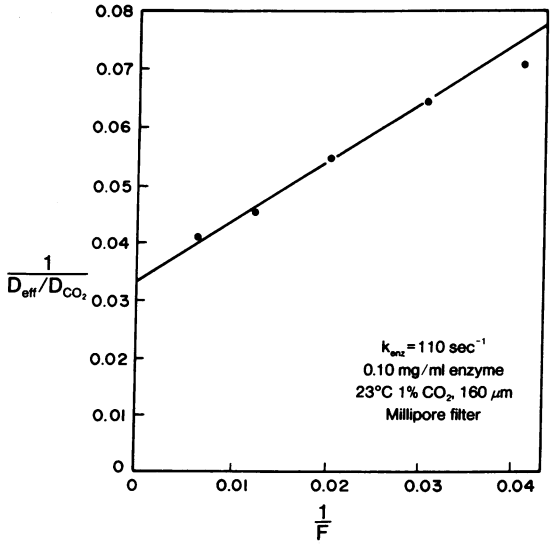

FIG. 2. Double reciprocal plot of large F data from Fig. 1. The line is the limiting linear form of Eq [8].

RESULTS

The $^{14}CO_2$ transport through Millipore filter membranes soaked in acidified water of pH < 4 was first measured in order to determine the transport resistance in the absence of facilitation and to characterize the diffusional path length in the Millipore filters. The facilitation arising from the uncatalyzed reactions was also examined. These aspects of the investigation are discussed elsewhere (22, 23).

Enzyme Adsorption. There was some irreversible adsorption of carbonic anhydrase onto the filter material. The activity of the adsorbed enzyme was negligible compared to the total enzymatic activity in the membranes at the enzyme concentrations reported here; however, the adsorbed enzyme did contribute significantly to the total activity at lower bulk enzyme concentrations. This behavior was established by repeatedly soaking the enzyme-impregnated filters in 50 mM $KHCO_3$ containing no carbonic anhydrase, and then measuring the CO_2 transport to determine the residual enzymatic activity. The total enzyme content of the filters was also determined by assaying for zinc. The filters were ashed, the residues dissolved in 5% nitric acid, and the samples assayed for Zn by atomic absorption spectroscopy. Appropriate standards and blank samples were also analyzed. The combination of the kinetic assays and the total enzyme assays (via Zn) indicated that the observed kinetic activity was due to enzyme in solution in the porous filter and not to adsorbed enzyme.

Constant Enzyme Concentration. The effective diffusivity ratio in Millipore filters soaked in solutions containing 0.10 mg/ml of carbonic anhydrase is shown in Fig. 1 as a function of the parameter F. The solid line illustrates the trend of the data, and the broken line represents the reaction equilibrium upper limit ($D_{eff}/D_{CO_2} = 1 + F$ in the limit of large ϕ). This limit has been attained experimentally under different membrane conditions at higher enzyme concentrations (22). The data of Fig. 1 fall well below the thermodynamic equilibrium limit, and the transport is, therefore, reaction-rate-limited. The effective diffusivity ratio is approaching the saturation plateau predicted by Eq. [4] at large F.

The parameter F is 25 or greater for a portion of the data in Fig. 1 and, thus, the criterion $F \gg 1$ is satisfied. The pH over this range is such that the enzyme activity is nearly maximal and constant. These data are plotted in the double reciprocal form of Eq. [8] in Fig. 2. The remainder of the data at lower F values cannot be used for this plot because they do not satisfy the criterion $F \gg 1$ and because of the variation of the enzyme activity with pH. The value of the modulus ϕ determined from the intercept in Fig. 2 is 29.4 and corresponds to a rate constant $k_{enz} = 110\ sec^{-1}$. The half-time of the enzymatic reaction is thus about 7 msec, and the uncatalyzed reaction with a half-time of about 12 sec is clearly negligible. The turnover number k_3 can now be calculated from Eq. [7], using a K_m of 12 mM (10) and an enzyme purity of 75%. The calculated turnover number is $5.3 \times 10^5\ sec^{-1}$, which may be compared with Kernohan's value of $10 \times 10^5\ sec^{-1}$ for the bovine enzyme measured by stopped-flow methods (10). The factor-of-two difference is most likely a result of bicarbonate inhibition (see subsequent discussion).

Values of K_m cannot be obtained independently from the present transport data. The usual method for determining the Michaelis constant is to measure the reaction rate as a function of substrate concentration [CO_2]. With the tracer system, K_m could be determined in principle from a plot of $1/k_{enz}$ against [CO_2] (Eq. [7]). The Michaelis constant for the bovine enzyme has been thoroughly investigated by Kernohan (9, 10) and found to be 12 mM at 25° and independent of pH.

pH Dependence of Enzyme Activity. The pH-dependent activity of carbonic anhydrase is well known, and corresponds to the addition of a proton to the basic form of an ionizing group at low pH, which inactivates the enzyme for the hydration reaction (9, 26, 27). This pH-dependent activity can be deduced from the data in Figs. 1 and 2. The pH for each point was calculated from the known bicarbonate and CO_2 concentration and the equilibrium relationship in Eq. [2], and the equilibrium constant K_{EQ} was corrected for ionic strength effects (28). The effective diffusivity ratios were converted to k_{enz} values, which were plotted as a function of pH. The sigmoidal result is shown in Fig. 3. The maximum k_{enz}, of course, is approximately 100 sec^{-1}, which was also determined from the intercept in Fig. 2.

The apparent pK for the pH-dependent activity is about 7.6 under these experimental conditions. This value is some-

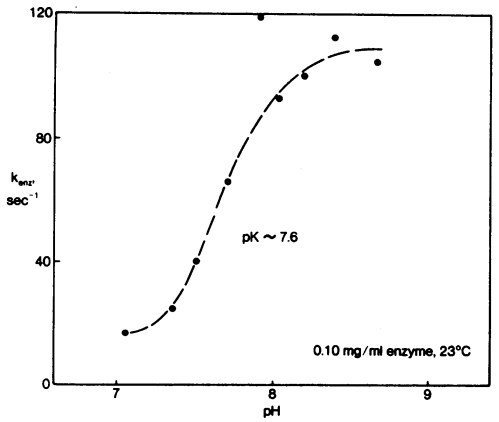

FIG. 3. Dependence of enzyme activity on pH.

what higher than the apparent pK of 6.9 reported by Kernohan (10). However, Kernohan's value was measured in the presence of chloride and no bicarbonate. The difference in apparent pK is probably due to the absence of chloride and the presence of bicarbonate in our membrane reaction system. Both of these ions are noncompetitive inhibitors, and are more effective at low pH than at high pH (10, 27). Unfortunately, a quantitative measure of the bicarbonate inhibition cannot be deduced from the data in Figs. 1 and 2 since these data represent a range of bicarbonate concentrations from 0.01 to 0.10 M in addition to the variation in pH. These transport experiments were not designed specifically to investigate the pH-activity relationship of the enzyme, but we wish to point out that the tracer transport technique is capable of revealing the pH-dependent activity and that the results are consistent with what is known about the enzyme. It is interesting to note that Lindskog (29) measured an apparent pK of 7.7 for the absorption spectrum change of Co(II)-carbonic anhydrase with pH in a solution containing only CO_2 and 0.10 M bicarbonate. In the presence of Cl^- (concentration unspecified), the pK was 7.1 (30).

High Enzyme Concentration. The transport results over an 80-fold range in bulk solution enzyme concentration are shown in Fig. 4. These data were obtained at constant 1% CO_2 and 50 mM bicarbonate, and a pH of 8.4. The enzyme solutions were prepared by dilution from a common stock solution. The solid line is simply a curve through the data points. The data are well below the equilibrium limit of 80, and the enzyme kinetic parameters can be deduced by replotting the data in the double reciprocal form in Fig. 5. The

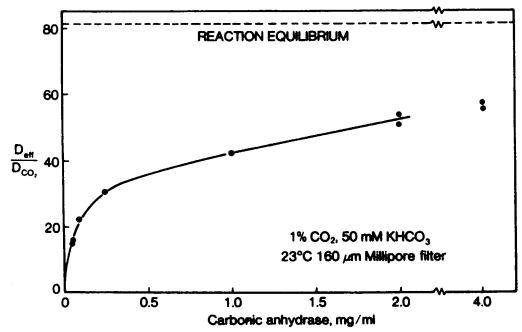

FIG. 4. Effective diffusivity ratio of CO_2 measured in Millipore filter membranes as a function of enzyme concentration.

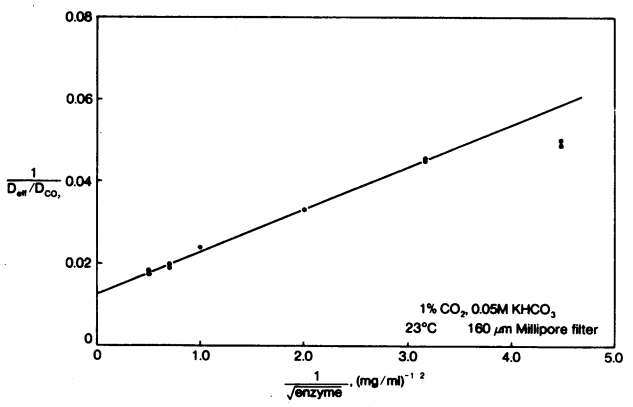

FIG. 5. Double reciprocal plot of data from Fig. 4. The points at 4.5 on the abscissa fall below the line as a result of enzyme adsorption on the Millipore filters.

parameter ϕ is large such that $\tanh \phi = 1$, and since the rate constant k_{enz} is proportional to enzyme concentration, the proper parameter for the abscissa is $[\text{enzyme}]^{-1/2}$. The linearity of the data at higher enzyme concentrations indicates that the enzyme specific activity is constant over the concentration range 0.1–4.0 mg/ml. The data at 0.05 mg/ml of carbonic anhydrase (at 4.5 on the abscissa) are below the line due to adsorption of carbonic anhydrase on the Millipore filters, as discussed previously. The turnover number k_3 can be determined from the slope of the straight line and Eqs. [5], [7], and [8]. Assuming $K_m = 12$ mM and 75% enzyme purity, k_3 is 5.5×10^5 sec^{-1}, in excellent agreement with 5.3×10^5 sec^{-1} determined from the 0.1 mg/ml data of Fig. 2.

Bicarbonate Inhibition. It is generally believed that there is little bicarbonate binding as a substrate for the dehydration reaction, but that bicarbonate is a noncompetitive inhibitor that combines with the enzyme at an anion-binding site (9). The extent of bicarbonate inhibition is reduced in the presence of Cl^- and other anions (which also inhibit), and it is believed that the bicarbonate is displaced from the anion-binding site by chloride and other anions.

The transport data of Figs. 4 and 5 over a range of enzyme concentrations were obtained at constant CO_2 and bicarbonate concentrations, and the bicarbonate inhibition effect can be estimated. The activity of the inhibited enzyme is expressed by

$$\frac{k_{enz}}{k_{0_{enz}}} = \frac{1}{1 + [HCO_3^-]/K_I} \qquad [9]$$

for a noncompetitive inhibitor, where $k_{0_{enz}}$ is the uninhibited activity and K_I is the bicarbonate binding (inhibition) constant. Based on Kernohan's turnover number of 10×10^5 sec^{-1} with no inhibition and our turnover number of 5.5×10^5 sec^{-1} in 50 mM bicarbonate at pH 8.4, the bicarbonate inhibition constant is about 60 mM. Kernohan (10) observed a reduction of 40% in the enzyme activity in the presence of 80 mM bicarbonate in pyrophosphate buffers at pH values from 8.2 to 8.6, and Maren (8) reported a 50% inhibition with 50 mM bicarbonate in a barbital buffer system in which the enzymatic hydration rate was independent of pH. These inhibition effects suggest that the binding constant is about 50–100 mM, in good agreement with our estimate of 60 mM. Extensive comparison and modeling of the anion inhibition is difficult since the inhibition effects are generally pH-dependent

for the bovine enzyme, but again our results are consistent with other bicarbonate inhibition observations.

CONCLUSIONS

We have demonstrated an equilibrium tracer membrane transport technique for the determination of kinetic constants for rapid reactions. To our knowledge, the data reported here are the first measurements of the kinetic activity of carbonic anhydrase at concentrations equivalent to those found in the red cell. The activity is in agreement with the results of several other studies at enzyme concentrations approaching the physiological concentration range (11, 12), and the kinetic behavior is consistent with observations at lower enzyme concentrations under similar conditions.

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