Diffusion of Carbon Dioxide through Lipid Bilayer Membranes

Effects of Carbonic Anhydrase, Bicarbonate, and Unstirred Layers

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ABSTRACT   Diffusion of ^{14}C-labeled CO_2 was measured through lipid bilayer membranes composed of egg lecithin and cholesterol (1:1 mol ratio) dissolved in n-decane. The results indicate that CO_2, but not HCO_3^-, crosses the membrane and that different steps in the transport process are rate limiting under different conditions. In one series of experiments we studied one-way fluxes between identical solutions at constant pCO_2 but differing $[HCO_3^-]$ and pH. In the absence of carbonic anhydrase (CA) the diffusion of CO_2 through the aqueous unstirred layers is rate limiting because the uncatalyzed hydration-dehydration of CO_2 is too slow to permit the high $[HCO_3^-]$ to facilitate tracer diffusion through the unstirred layers. Addition of CA (ca. 1 mg/ml) to both bathing solutions causes a 10–100-fold stimulation of the CO_2 flux, which is proportional to $[HCO_3^-]$ over the pH range 7–8. In the presence of CA the hydration-dehydration reaction is so fast that CO_2 transport across the entire system is rate limited by diffusion of HCO_3^- through the unstirred layers. However, in the presence of CA when the ratio $[HCO_3^- + CO_3^=]:[CO_2] > 1,000$ (pH 9–10), the CO_2 flux reaches a maximum value. Under these conditions the diffusion of CO_2 through the membrane becomes rate limiting, which allows us to estimate a permeability coefficient of the membrane to CO_2 of 0.35 cm s^{-1}. In a second series of experiments we studied the effects of CA and buffer concentration on the net flux of CO_2. CA stimulates the net CO_2 flux in well buffered, but not in unbuffered, solutions. The buffer provides a proton source on the upstream side of the membrane and proton sink on the downstream side, thus allowing HCO_3^- to facilitate the net transport of CO_2 through the unstirred layers.

INTRODUCTION

One of the most important compounds in nature is carbon dioxide, which plays a central role in photosynthesis, respiration, and acid-base balance. Although

CO_2 exists in several different chemical forms, at least two of these forms, i.e. HCO_3^- and $CO_3^=$, cannot easily diffuse across most cell membranes. However, HCO_3^- and $CO_3^=$ often dominate the total CO_2 movements through the aqueous solutions bathing the membrane. Thus, the transport of CO_2 across a cell membrane or epithelium involves not only diffusion of dissolved CO_2 gas across the membrane, but also reactions among the several chemical forms of CO_2 in the aqueous unstirred layers.

The hydration of CO_2 to H_2CO_3 is an intrinsically slow process ($t_{1/2} \simeq 14$ s). However, in many plant and animal cells the reaction is catalyzed by carbonic anhydrase, which has one of the highest turnover numbers of any known enzyme. In man CA occurs in at least eight different tissues where it plays a conspicuous, although apparently nonessential role in the transport of CO_2, HCO_3^-, and/or H^+ (see Maren, 1967). The physiological role of carbonic anhydrase in many plant and animal cells is not understood, and the function of the enzyme in some cases may not be that of a carbonic anhydrase (Maren et al., 1976).
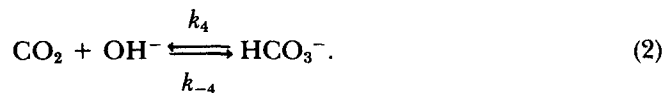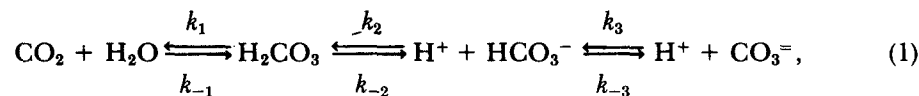
A number of previous studies have described the "facilitation" of CO_2 diffusion by HCO_3^- and $CO_3^=$, and the enhancement of this facilitated diffusion by carbonic anhydrase (Enns, 1967, 1974; Ward and Robb, 1967; Broun et al., 1970; Schultz et al., 1974; Suchdeo and Schultz, 1974; Donaldson and Quinn, 1974, 1975). Most of the experimental studies describe CO_2 diffusion between two gas phases separated by a hydrophilic membrane, such as a porous filter soaked with aqueous test solutions. A few studies have dealt also with facilitated diffusion of CO_2 across hydrophobic membranes (e.g. silicone rubber) separating aqueous solutions.

In the present study we measured the diffusion of CO_2 across lipid bilayer membranes and associated unstirred layers. The specific aims of this study are to: (a) identify the several different rate-limiting processes which occur during CO_2 diffusion across a lipid bilayer and unstirred layers under different conditions; (b) show how chemical reactions catalyzed by carbonic anhydrase in the unstirred layers facilitate the diffusion of CO_2 across the system; (c) estimate the permeability of the lipid bilayer to CO_2; (d) test the effects of carbonic anhydrase on halide transport across lipid bilayers.

THEORY

Chemical Reactions and Equilibria Involving CO_2

Carbon dioxide exists in four different chemical forms which are interconverted via the following four reactions (see Edsall and Wyman, 1958; Kern, 1960).

$$CO_2 + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_2CO_3 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H^+ + HCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} H^+ + CO_3^=, \tag{1}$$

$$CO_2 + OH^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} HCO_3^-. \tag{2}$$

At neutral pH the uncatalyzed hydration-dehydration of CO_2 by reactions (1) or (2) is a slow process having the following approximate rate constants at 25°C:

$k_1 = 3.7 \times 10^{-2}$ s^{-1}; $k_{-1} = 14$ s^{-1}; $k_4 = 8.5 \times 10^3$ liter mol^{-1} s^{-1}; and $k_{-4} = 2 \times 10^{-4}$ s^{-1}. The k_2's and k_3's, on the other hand, are essentially instantaneous, i.e. diffusion limited. Carbonic anhydrase at concentrations of about 2 mg/ml (60 μM) increases the hydration-dehydration of CO_2 by about 10^5. When present, CA usually occurs in such high concentrations that the hydration-dehydration of CO_2 is not the rate-limiting step in biological transport processes (Maren, 1967; Maren et al., 1976).

The equilibrium relations between CO_2, H_2CO_3, HCO_3^-, and $CO_3^=$ are as follows (25°C, ionic strength = 0.1):

$$K_{H_2CO_3} = \frac{[H_2CO_3]}{[CO_2]} = 2.6 \times 10^{-3};$$ (3)

$$pK'_{CO_2} = pH - \log\frac{[HCO_3^-]}{[CO_2]} = 6.1;$$ (4)

$$pK_{HCO_3^-} = pH - \log\frac{[CO_3^=]}{[HCO_3^-]} = 10.0.$$ (5)

Predicted Permeability of a Lipid Bilayer to CO_2

The permeability of a lipid bilayer membrane to CO_2 ($P_{CO_2}^M$) is given by the expression,

$$P_{CO_2}^M = \frac{K_p D_{CO_2}^M}{\Delta X^M},$$ (6)

where K_p is the partition coefficient for CO_2 (membrane interior/water), ΔX^M is the membrane thickness, and $D_{CO_2}^M$ is the diffusion coefficient for CO_2 in the membrane. Although accurate values for K_p, $D_{CO_2}^M$, and ΔX^M are not known, information from some other systems allows us to predict an approximate value for $P_{CO_2}^M$. The K_p for CO_2 in an olive oil-water system is about 1.7 at 25°C (Battino et al., 1968), and the thickness of a lipid bilayer membrane is about 5×10^{-7} cm (White, 1975). A reasonable estimate for $D_{CO_2}^M$ is 5×10^{-8} cm^2 s^{-1}, which is the apparent diffusion coefficient for methanol in the human red cell membrane at 21°C (Solomon, 1974). Inserting these values into Eq. (6) yields a value for $P_{CO_2}^M$ of 0.2 cm s^{-1}. For comparison, the permeability of the human red cell membrane to CO_2 has been estimated to be 0.15 cm s^{-1} (Roughton, 1959) and 0.58 cm s^{-1} (Forster, 1969), assuming that CO_2 goes through the lipid part of the membrane.

Both the diffusion coefficient and the partition coefficient of CO_2 will vary, of course, with the composition and structure of the membrane. For example, the addition of cholesterol to lecithin-decane bilayers decreases the permeability to small nonelectrolytes by decreasing the membrane fluidity or the partition coefficient, or both (Finkelstein and Cass, 1967; Finkelstein, 1976). In our experiments we used a 1:1 molar ratio of egg lecithin and cholesterol in decane. Although the 1:1 molar ratio produces a "highly ordered" membrane (Erdei et al., 1975), the presence of unknown amounts of decane may increase membrane fluidity. In planar lipid bilayers, molar ratios of egg lecithin:cholesterol:decane of about 1:1:1 were recently reported by Bunce and Hider (1974), who used membrane-forming solutions similar to those used in our experiments.

Model for Diffusion of a Permeant Weak Acid across a Lipid Bilayer Membrane and Unstirred Layers

The diffusion of a highly permeant (nonionic) weak acid (or weak base) and its relatively impermeant anion (or cation) across lipid bilayer membranes has been studied by LeBlanc (1969) and Neumcke (1971) using electrical techniques, and by Gutknecht and Tosteson (1973) using tracer techniques. If a substance exists in two or more chemical forms, one of which cannot cross the membrane, then chemical reaction(s) between the permeant and impermeant forms in the unstirred layers will affect rate of diffusion of the permeant form across the membrane. An equation which describes such a diffusion process contains at least three reciprocal terms, i.e. a term describing diffusion of both species through the unstirred layers, a term describing diffusion of the permeant species through the membrane, and a term describing the chemical reaction(s) between the diffusing species (see Gutknecht et al., 1972). If the reaction is fast compared to diffusion through the membrane and unstirred layers, then for a weak acid (HA) and its anion (A⁻) diffusing across a membrane under symmetrical conditions, the equation can be written

$$\frac{1}{J_A} = \frac{1}{P_{A^-}^{UL}[A^-] + P_{HA}^{UL}[HA]} + \frac{1}{P_{HA}^{M}[HA]}, \tag{7}$$

where J_A is the one-way flux of HA + A⁻ (mol cm⁻² s⁻¹), $P_{A^-}^{UE}$, P_{HA}^{UL}, and P_{HA}^{M} are the permeability coefficients of the unstirred layers and the membrane to A⁻ and HA (cm s⁻¹), and [A⁻] and [HA] are the concentrations of A⁻ and HA (mol cm⁻³).

Fig. 1. shows the theoretical relation between the total flux J_A and the anion concentration [A⁻] at constant [HA], i.e. as the pH increases. If the chemical reaction is fast compared to the rate of diffusion through the membrane and unstirred layers, then a plot of J_A vs. [A⁻] at constant [HA] will yield a sigmoid curve from which the three permeability coefficients, P_{HA}^{UL}, $P_{A^-}^{UL}$, and P_{HA}^{M}, can be estimated because each permeability coefficient controls a different part of the curve. The upper dashed line in Fig. 1 shows that if there were no unstirred layers, (i.e. perfect mixing in the two solutions), then the flux would be large and insensitive to [A⁻]. The lower dashed line shows that if there were unstirred layers but no chemical reactions (i.e. that the reaction was slow compared to diffusion), then the flux would be much lower but still insensitive to [A⁻].

MATERIALS AND METHODS

Lipid bilayer (optically black) membranes were made by the brush technique of Mueller and Rudin (1969). Unless otherwise specified, the membranes were formed from a mixture of egg lecithin (30 mg/ml) and cholesterol (15 mg/ml) in n-decane, to give a lecithin:cholesterol mole ratio of 1:1. Membranes were formed on a 1.6-mm diam hole in a polyethylene partition which separated two magnetically stirred solutions of 1.2 ml each. The solutions were equilibrated with CO_2 and N_2 in various proportions, depending upon the experimental conditions. The solutions also contained $NaHCO_3$, Na_2CO_3, carbonic anhydrase, and NaCl as specified with each experiment. Sodium phosphate, Tris-Cl, and Na-HEPES buffers were added as specified. The temperature was 22-24°C. In order to vary the ([HCO₃⁻] + [CO₃⁼]) at constant [CO₂] (i.e. at constant pCO₂), we

varied the pH as shown in Eq. (4) and (5). Since egg lecithin is isoelectric over a pH range of 3–11 (Papahadjopoulos, 1968), we assumed that altering the pH of our solutions over the experimental range of 7.1–9.6 did not alter the properties, e.g. surface charge, of the membranes.

After a stable membrane had been formed, 1–3 μCi of $NaH^{14}CO_3$ were injected into the rear compartment, which was then covered with either Teflon tape or a Teflon plug. The rate of appearance of radioactivity in the front compartment was measured by continuous perfusion (1–2 ml/min) and collection of samples at 3-min intervals. The samples were collected by aspiration into a vacuum trap containing dilute NaOH. During
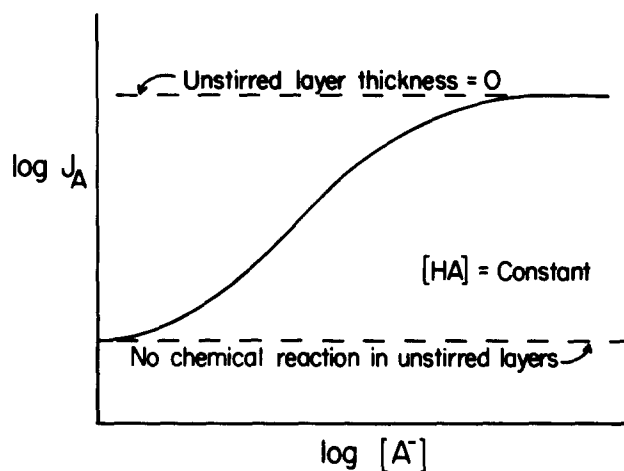

FIGURE 1. Theoretical flux of a permeant weak acid as a function of its anion (A^-) concentration at a constant concentration of HA. The aqueous solutions are assumed to be symmetrical, except for the addition of tracer to one side, and the chemical reaction, $HA \rightleftarrows A^- + H^+$, is assumed to be fast compared to the diffusion of HA and A^- across the membrane and unstirred layers. On the lower flat part of the solid curve, pH $<$ pK and [HA] $>$ [A^-]. On the upward part of the curve, pH $>$ pK and [A^-] $>$ [HA]. On the upper flat part of the curve, pH $\gg$ pK and [A^-] $\gg$ [HA]. The upper dashed line shows the flux expected if the bulk solutions were perfectly mixed (no unstirred layer), and the lower dashed line shows the flux expected if no reaction between HA and A^- occurred in the unstirred layers (i.e., if diffusion were much faster than the chemical reaction).

the flux experiments the rear compartment was sampled periodically with a microsyringe. All the samples were dried in 2-inch planchets and counted in a low background planchet counter. The one-way flux of CO_2 was calculated by the equation:

$$J_{CO_2} = \frac{^{14}C^F}{t\,A\,SA^R}, \tag{8}$$

where J_{CO_2} is the flux (mol cm^{-2} s^{-1}), $^{14}C^F$ is the total amount of tracer (cpm) entering the front compartment during the time interval, t (s), A is the surface area of the membrane (cm²), and SA^R is the specific activity of the tracer in the rear compartment (cpm/mol).

We calculated the membrane resistance, using Ohm's law, from the membrane potential produced by applying a known voltage pulse across the membrane in series with a known resistance. The membrane potential was recorded as the potential difference

between two calomel-KCl electrodes which made contact with the front and rear solutions.

Carbonic anhydrase (carbamate hydrolase, E.C. 4.2.1.1.) from bovine erythrocytes, cholesterol, acetazolamide, n-decane, Tris, and HEPES buffers, were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Egg lecithin and phosphatidylserine (bovine) were obtained from either Supelco, Inc. (Bellefonte, Pa.) or from Lipid Products (Surrey, England). Sodium ^{14}C-bicarbonate was obtained from the Amersham/Searle Corp. (Arlington Heights, Ill.), and ^{82}Br$^-$ (as KBr) was obtained from International Chemical and Nuclear Corp. (Irvine, Calif.).

RESULTS

Relation betweem Membrane Conductance and CO_2 Flux

In the absence of carbonic anhydrase (CA) the membrane conductance (G_m) ranged from 1×10^{-8} to 2×10^{-7} mho cm^{-2}. In the presence of CA (0.1-2.0 mg/ml), G_m was more variable, ranging from 10^{-8} to 10^{-5} mho cm^{-2}. However, there was no correlation between the CA concentration and G_m or between the CO_2 flux and G_m, i.e. membranes which had the lowest conductances sometimes had the highest fluxes, and vice versa. In addition to increasing the variability in G_m, CA also increased the fragility of the membranes. This made the tracer experiments difficult but not impossible, since we were able to complete most experiments within 25 min.

In the results which follow we will not present electrical data with each flux value because the observed flux under all conditions was almost entirely electrically silent. In order to compare the observed one-way flux with the ionic flux of HCO$_3^-$ if G_m were due entirely to HCO$_3^-$ diffusion we used the following equation (Hodgkin, 1951):

$$J_{HCO_3^-} = \frac{R}{z^2} \frac{T}{F^2} g_{HCO_3^-}, \tag{9}$$

where $J_{HCO_3^-}$ is the predicted one-way flux of HCO$_3^-$, R is the gas constant, T is the absolute temperature, z is the ionic valence, F is the faraday, and $g_{HCO_3^-}$ is the HCO$_3^-$ conductance. Taking $g_{HCO_3^-}$ as 10^{-5} mho cm^{-2} (the highest observed G_m), this equation gives an upper limit for $J_{HCO_3^-}$ of 2.5×10^{-12} mol cm^{-2} s^{-1}, which is less than 10% of the lowest observed flux, i.e. 2.7×10^{-11} mol cm^{-2} s^{-1}. Furthermore, since $P_{HCO_3^-} = J_{HCO_3^-} /[HCO_3^-]$ when $V_m = 0$, we can also estimate an upper limit of $P_{HCO_3^-}$ to be about 2×10^{-7} cm s^{-1}. Thus, it appears that tracer crosses the membrane as CO_2, not as HCO$_3^-$ or CO$_3^=$. (We will consider later the possibility that part of the flux is due to H_2CO_3 or to exchange diffusion of HCO$_3^-$.)

Effect of CA and $[HCO_3^-]$ on the CO_2 Flux under Symmetrical Conditions

Fig. 2 shows the one-way flux of CO_2 (J_{CO_2}) as a function of $([HCO_3^-] + [CO_3^=])$ at constant $[CO_2]$ in the presence and absence of CA. In this experiment the two bathing solutions were identical except for the addition of tracer to the rear compartment. In the absence of CA and at pH 7-8 (lower curve in Fig. 2), J_{CO_2} is about 2.5×10^{-11} mol cm^{-2} s^{-1}. This flux is about equal to the rate at which 10^{-5}

M CO_2 can diffuse across a layer of water 10^{-2} cm thick, which is the approximate thickness of the unstirred layer in our system, as well as in other lipid bilayer systems (see Gutknecht and Tosteson, 1973; Finkelstein, 1976). This suggests that the membrane permeability to CO_2 ($P^M_{CO_2}$) is higher than the combined permeability of both unstirred layers to CO_2 ($P^{UL}_{CO_2}$). Furthermore, as expected, the uncatalyzed hydration-dehydration of CO_2 at neutral pH is too slow to permit the high HCO_3^- to enhance the tracer flux by more than about 15%. At pH > 8, however, the reaction between CO_2, OH^-, and HCO_3^-, as
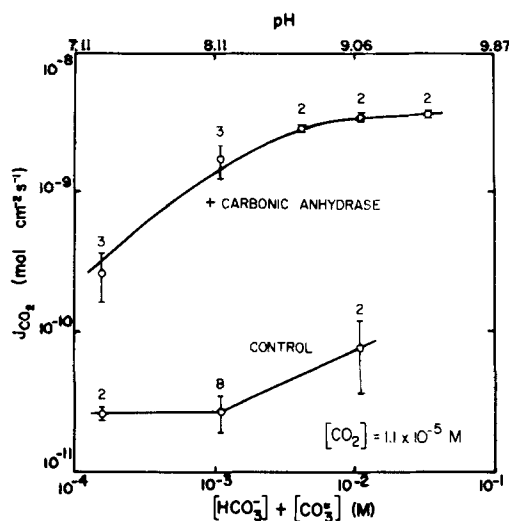

FIGURE 2.   One-way flux of CO_2 as a function of ($[HCO_3^-]$ + $[CO_3^=]$) at constant $[CO_2]$ (0.25 mm Hg; 1.1 × 10^{-5} M) in the presence and absence of carbonic anhydrase (CA). The front and rear solutions are identical except for the addition of tracer ($^{14}CHO_3^-$) to the rear compartment. The solutions contain $NaHCO_3$, Na_2CO_3, and sufficient NaCl to make the ionic strength ca. 0.1. The pH 7 solutions are buffered with sodium phosphate (5 mM), and the pH 8-9 solutions are buffered with Tris-Cl (5 mM). The CA concentration is 1.0–1.6 mg/ml. The vertical bars are standard errors, and the number above each bar is the number of membranes. The solid curve connecting the upper five points is calculated from Eq. (10), which is discussed in the text.

shown in Eq. (2), should facilitate appreciably the flux of CO_2, as suggested by the lower curve in Fig. 2.

The upper curve in Fig. 2 shows the effect of CA on J_{CO_2}. At pH 7–8, CA (1.0–1.6 mg/ml) causes a 10–80-fold stimulation of J_{CO_2}, which now becomes proportional to ($[HCO_3^-]$ + $[CO_3^=]$). Over the pH range of 7–8, J_{CO_2} is about equal to the rate at which HCO_3^- can diffuse across the unstirred layer. This suggests that in the presence of CA the hydration-dehydration of CO_2 is so fast that chemical equilibrium between CO_2 and HCO_3^- exists throughout the unstirred layer and, furthermore, that $P^M_{CO_2}$ is so high that 10^{-5} M CO_2 can diffuse across the membrane faster than 10^{-4}–10^{-3} M HCO_3^- can diffuse across the unstirred

layer. Consequently, the rate-limiting step in CO_2 transport over the pH range of 7-8 appears to be the diffusion of HCO_3^- across the unstirred layer, even though HCO_3^- cannot readily cross the membrane.

A "saturation" of the flux is observed at pH >9, where the ratio $([HCO_3^-] + [CO_3^=]):[CO_2] > 10^3$. One explanation for this saturation is that the $([HCO_3^-] + [CO_3^=])$ is now sufficiently high that the anions can diffuse across the unstirred layer faster than CO_2 can cross the membrane, in which case the transmembrane diffusion of CO_2 becomes the rate-limiting step in the transport process and further increases in $[HCO_3^-] + [CO_3^=]$ at constant $[CO_2]$ cannot increase the flux. An alternative explanation is that the catalyzed reaction between CO_2 and HCO_3^- is rate limiting. If this were true, then the saturation value of the flux should be increased by increasing the CA concentration. However, a graph of J_{CO_2} vs. [CA] at high pH (Fig. 3) also shows that J_{CO_2} saturates at about 4×10^{-9}
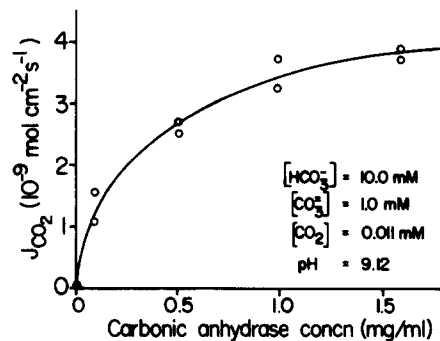

FIGURE 3. Flux of CO_2 as a function of CA concentration at constant $[HCO_3^-]$ and pCO_2. The front and rear solutions are identical except for the addition of tracer to the rear compartment. The solutions contain NaCl (90 mM), $NaHCO_3$ (10 mM), Na_2CO_3 (1.0 mM), and CO_2 (0.011 mM), pH 9.12. Each point represents a single membrane.

mol cm^{-2} s^{-1}, which is similar to the maximum flux in Fig. 2. Thus, the membrane, rather than the reaction rate, appears to be rate limiting under the conditions of our experiments.

In Fig. 2 the solid curve connecting the upper five points is calculated from a simplified form of Eq. (7), i.e.,

$$\frac{1}{J_{CO_2}} = \frac{1}{P_{A^=}^{UL}[A^-]} + \frac{1}{P_{CO_2}^{M}[CO_2]}, \tag{10}$$

where $A^- = [HCO_3^-] + [CO_3^=]$. In using this equation over the pH range 7.1-9.5 we are assuming that: (a) HCO_3^- and $CO_3^=$ are the only species carrying significant amounts of tracer through the unstirred layers; (b) CO_2 is the only species which crosses the membrane in significant amounts; and (c) chemical equilibrium between CO_2, HCO_3^-, and $CO_3^=$ exists throughout the unstirred layer, i.e. that the reaction is fast compared to diffusion across the membrane and unstirred layer. In other words, the upper curve in Fig. 2 is analogous to the

right-hand side of Fig. 1. By fitting this equation to the data, we can estimate both $P_{A^-}^{UL}$ and $P_{CO_2}^M$, because these two permeability coefficients control different parts of the curve. The values used to generate the upper curve in Fig. 2 are $P_{A^-}^{UL}$ = 1.8 × 10⁻³ cm s⁻¹ and $P_{CO_2}^M$ = 0.36 cm s⁻¹.

A more accurate way of estimating $P_{CO_2}^M$ is obtained by converting Eq. (10) into a linear form, as shown in Fig. 4. In this graph $P_{CO_2}^M$ is the reciprocal of the slope and $P_{A^-}^{UL}$ is the reciprocal of the intercept. A linear regression analysis of the upper five points in Fig. 2 yields a value for $P_{CO_2}^M$ of 0.35 ± 0.04 cm s⁻¹ (SE). Alternatively, Eq. (10) can be rearranged so that $1/P_{A^-}^{UL}$ is the slope of a linear regression of $[CO_2]/J_{CO_2}$ on $[CO_2]/[A^-]$.
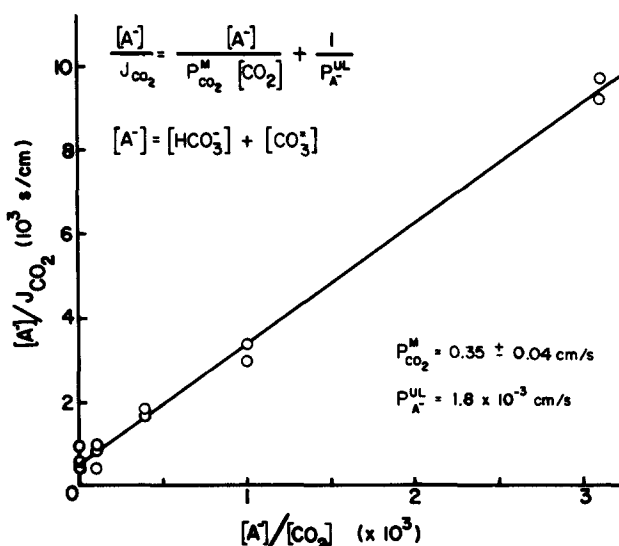

FIGURE 4. A linear regression analysis of the CO_2 flux as a function of ($[HCO_3^-]$ + $[CO_3^=]$) at constant $[CO_2]$. Eq. (10) has been rearranged so that $P_{CO_2}^M$ is the reciprocal of the slope and $P_{A^-}^{UL}$ is the reciprocal of the intercept. Each point represents a single membrane.

This method can be used to estimate the separate permeabilities of a membrane and unstirred layer to any permeant weak acid or weak base, provided that: (a) the permeability of the nonionic form greatly exceeds the permeability of the ionic form; and (b) the reaction is fast compared to diffusion through the membrane and unstirred layers.

Inhibition and Reversibility of the Carbonic Anhydrase Effect

Two implicit assumptions in the experiments described so far are that: (a) CA is acting in the unstirred layers rather than in or on the membrane; and (b) CA is enhancing the flux by catalyzing the hydration-dehydration of CO_2 rather than by some unspecified "protein effect." Although we cannot prove the validity of these two assumptions, we have done two additional experiments which support the assumptions.

In the first experiment we tested the "reversibility" of the CA effect by forming a membrane in the presence of CA (1 mg/ml) and then washing the membrane on both sides for 10 min with CA-free solutions before injecting tracer and measuring the CO_2 flux. The composition of the solutions was identical to that described for Fig. 2 at pH 8.1. The CO_2 flux in these membranes was $(1.6 \pm 0.7) \times 10^{-11}$ (3) (SE) mol cm^{-2} s^{-1}, similar to the control membranes which had never "seen" CA, i.e. $(2.7 \pm 0.8) \times 10^{-11}$ (8). This rapid reversibility of the CA effect is consistent with our assumption that CA is acting primarily in the unstirred layers rather than in or on the membrane.

In the second experiment we tested the ability of acetazolamide (Diamox) to inhibit the CA-stimulated flux. The first two lines in Table I show that acetazolamide alone (1 mM) has no effect on J_{CO_2}. The last two lines of Table I show that acetazolamide abolishes 90% $\pm$ 5% of the CA-stimulated flux, which supports our assumption that CA is acting predominantly by catalyzing the interconver-

TABLE I

EFFECTS OF ACETAZOLAMIDE AND CARBONIC ANHYDRASE ON THE ONE-WAY FLUX OF CO_2 THROUGH LIPID BILAYER MEMBRANES

Acetazolamide (1 mM)	Carbonic anhydrase (1 mg/ml)	CO_2 flux
		$10^{-11} \ mol \ cm^{-2} \ s^{-1}$
None	None	2.7±0.8 (8)
Front and rear	None	2.8±1.9 (4)
None	Front and rear	140±45 (3)
Front and rear	Front and rear	14.0±4.7 (5)

Front and rear solutions contained NaCl (95 mM), NaHCO$_3$ (1 mM), CO$_2$ (0.011 mM) (pCO$_2$ = 0.25 mm Hg), and Tris-Cl$^-$ buffer (5 mM), pH 8.1. Results are quoted in the form: mean ± SE (number of membranes).

sion of CO_2 and HCO_3^-. However, the residual CO_2 flux in the presence of CA plus acetazolamide is higher than expected, i.e. three to seven times greater than the control flux. From the known binding constant for acetazolamide and CA, we expected that the inhibition of the CA-stimulated flux would be >99.9% (see Maren, 1967). We have no explanation for the residual fivefold stimulation of J_{CO_2} in the presence of both CA and acetazolamide, except the possibility that a small fraction of the CA-stimulated flux involves an electrically silent transport of HCO_3^- which is not affected by acetazolamide.

Effects of CA and Buffers on the Net Flux of CO_2

In all of the above experiments we measured one-way fluxes under symmetrical conditions. In the following experiments we tested the effects of CA and buffer concentration on the net flux of CO_2. In these experiments the rear solution contained 1.7 mM CO_2 (pCO$_2$ = 40 mm Hg) and 40 mM HCO_3^- (pH 7.5), and the front solution contained a CO_2-free solution. Under these conditions, the one-way flux of CO_2 is equal to the net flux. Table II shows that, as expected, CA stimulates the net flux of CO_2, but only when the solutions are well buffered (Na-HEPES, 50 mM).

To explain the stimulating effect of buffer on the net flux of CO_2 we must consider the role of H^+ in the interconversion of CO_2 and HCO_3^-, as shown in Eq. (1) (see also Enns, 1974). As CO_2 diffuses from rear to front, leaving behind the impermeant HCO_3^-, the rear unstirred layer becomes more alkaline, and the front unstirred layer becomes more acidic. The low $[H^+]$ in the rear unstirred layer limits the net rate of production of CO_2 from HCO_3^-, and the high $[H^+]$ in the front unstirred layer retards the net conversion of CO_2 to HCO_3^-. If the bulk solutions are poorly buffered, steep $[H^+]$ and $[OH^-]$ gradients develop in the unstirred layers. Because H^+ and OH^- are in low concentrations and cannot readily cross the membrane, the diffusion of H^+ or OH^- through the unstirred layers becomes the rate-limiting step in the transport of CO_2. The buffer supplies H^+ on the upstream side and absorbs H^+ on the downstream side, which allows the CA and high $[HCO_3^-]$ to facilitate the net transport of CO_2.

TABLE II

EFFECTS OF CARBONIC ANHYDRASE AND BUFFER
CONCENTRATION ON THE NET FLUX OF CO_2 THROUGH
LIPID BILAYER MEMBRANES

Carbonic anhydrase (1 mg/ml)	Buffer (Na-HEPES, 50 mM, pH 7.5)	CO_2 net flux
		10^{-9} mol cm^{-2} s^{-1}
None	Front and rear	1.1 ± 0.9 (3)
Front and rear	None	0.7 ± 0.5 (4)
Front and rear	Front and rear	12.7 ± 4.3 (5)

The rear solution also contained $NaHCO_3$ (40 mM), CO_2 (1.7 mM) (pCO_2 = 40 mm Hg), pH 7.5. The front solution contained either Na-HEPES buffer, as indicated above, or 50 mM NaCl. Results are quoted in the form: mean $\pm$ SE (number of membranes).

Effect of Carbonic Anhydrase on Bromide Fluxes

Several recent reports suggest that in certain plant and animal cells CA may be directly involved in carrier-mediated anion transport (e.g. Findenegg, 1974; Emanovic et al., 1976). Therefore, we tested the effects of two types of CA on Br^- transport across bilayers made from two different phospholipids. The experimental technique was similar to that used for $^{14}CO_2$, but small amounts of sodium thiosulfate were added to the aqueous solutions to eliminate the trace amounts of Br_2 which normally exist in NaBr solutions (see Gutknecht et al., 1972).

As shown in Table III, CA has no significant effect on Br^- fluxes across lecithin-decane or phosphatidylserine-decane membranes. We tested both a "high activity" enzyme (CA from bovine erythrocytes) and a "low activity" enzyme (human CA "B" from human erythrocytes) (see Maren et al., 1976), which was a gift from Dr. Robert Henkens (Duke University). The Br^- fluxes in both the presence and absence of CA were low, although still substantially higher than the maximum flux predicted from the membrane conductance (see

Gutknecht et al., 1972; Toyoshima and Thompson, 1975). These results suggest that the small residual CO_2 flux in the presence of CA and acetazolamide (Table II) is not due to an electrically silent anion (i.e. HCO_3^-) transport, and they provide no support for (although they do not disprove) the idea that CA acts as an anion "permease" in some biological membranes.

DISCUSSION

Theoretical specific activity profiles for CO_2, H_2CO_3, HCO_3^-, and $CO_3^=$ under three different conditions are shown in Fig. 5. Fig. 5 A shows schematically the

TABLE III

EFFECTS OF CARBONIC ANHYDRASE ON THE ONE-WAY FLUX OF BROMIDE THROUGH LIPID BILAYER MEMBRANES

Membrane composition	Carbonic anhydrase	Membrane conductance	Br^- flux
		10^{-8} mho cm^{-2}	10^{-12} mol cm^{-2} s^{-1}
Phosphatidylserine (bovine) and n-decane	None	<1.0-1.1	0.7-5.4 (3)
Phosphatidylserine (bovine) and n-decane	Front and rear (bovine, 1 mg/ml)	<1.0-3.0	2.4-8.8 (3)
Egg lecithin and n-decane	None	<1.0-3.0	1.5-1.7 (2)
Egg lecithin and n-decane	Front and rear (bovine, 1 mg/ml)	<1.0	0.7 (1)
Egg lecithin and n-decane	Front and rear (human CA "B", 1 mg/ml)	3-5	<1.0-1.1 (2)

Both front and rear solutions contained NaBr (100 mM), $Na_2S_2O_3$ (1 mM), and Na^+ phosphate buffer (2 mM), pH 7.3. The phospholipid concentrations in decane were 25-30 mg/ml. Data are presented as the range and, in parentheses, the number of membranes.
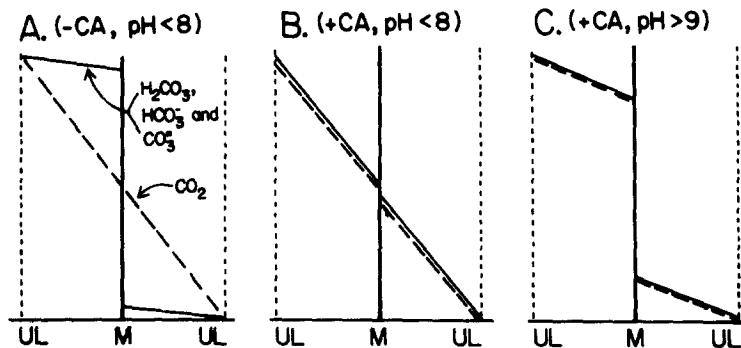

FIGURE 5.   Theoretical specific activity profiles for CO_2 (dashed lines) and H_2CO_3, HCO_3^-, and $CO_3^=$ (solid lines) across a lipid bilayer membrane (M) and unstirred layers (UL) under three different conditions. See text for details.

specific activity gradients expected if the diffusion of CO_2 is faster than the hydration-dehydration of CO_2 (e.g., CA absent, pH 7-8). Note the steep tracer gradients for both HCO_3^- and H_2CO_3 across the membrane, whereas the CO_2 gradient occurs almost entirely across the unstirred layer. Although we know from conductance measurements that the rate of ionic diffusion of HCO_3^- through the membrane is negligible compared to the total flux, we have not excluded the possibility that H_2CO_3 contributes significantly to the flux. However, we think this is unlikely because: (a) J_{CO_2} is close to that expected for CO_2 alone; (b) the concentration of H_2CO_3 is only about 0.002 times the concentration of dissolved CO_2 (Eq. [3]); and (c) the lipid solubility of H_2CO_3, which is much

more polar than CO_2, is probably much lower than the lipid solubility of CO_2. An experimental measurement of $P^M_{H_2CO_3}$ is impossible in the presence of CO_2, but we have measured a membrane permeability coefficient for acetic acid of about 10^{-3} cm s^{-1} (unpublished data), which supports indirectly our assumption that $P^M_{H_2CO_3} \ll P^M_{CO_2}$.

Fig. 5 B and C shows theoretical specific activity profiles for CO_2, H_2CO_3, HCO_3^-, and $CO_3^=$ in the presence of high concentrations of CA (e.g., upper curve in Fig. 2). In Fig. 5 B at pH 7–8 ($[HCO_3^-]/[CO_2] = 9$–80), the specific activity gradients for both CO_2 and HCO_3^- are approximately linear across the entire system. These linear gradients are a consequence of the very high $P^M_{CO_2}$ compared to $P^{UL}_{HCO_3^-}$ and the rapid catalyzed interconversion of CO_2 and HCO_3^- in the unstirred layers. Fig. 5 C shows theoretical specific activity profiles at pH >9, i.e. $([HCO_3^-] + [CO_3^-])/[CO_2] > 10^3$. CO_2, H_2CO_3, HCO_3^-, and $CO_3^=$ are in equilibrium throughout virtually all of the unstirred layer and there is a steep specific activity gradient for all four species across the membrane, which reflects the fact that transmembrane diffusion of CO_2 is rate limiting.

Similar profiles could be drawn for the net transport of CO_2, except that concentrations would be substituted for specific activities. For example, the results shown in Table II, lines 1 and 2, could be represented by a concentration profile such as that in Fig. 5 A, whereas the results shown in Table II, line 3, would be characterized by a profile such as that shown in Fig. 5 B. Complete profiles showing all the diffusing and reacting species would be much more complicated because gradients in buffer concentrations, as well as H^+ and OH^- concentrations, would have to be included (see, e.g., Stehle and Higuchi, 1972; Gutknecht and Tosteson, 1973).

The permeability coefficient for CO_2 of 0.35 cm s^{-1} which we estimate for an egg lecithin-cholesterol-decane bilayer is similar to that estimated for the red cell membrane by Roughton (1959) and Forster (1969), but lower than the estimate of Gros and Moll (1971). Our value is about 10 times lower than the CO_2 permeability of "soluble" monolayers of hexadecyltrimethylammonium bromide (Princen et al., 1967) and about 100 times higher than the CO_2 permeability of "insoluble" condensed monolayers of long-chain aliphatic alcohols and fatty acids, in which the permeability coefficients approach those expected for CO_2 diffusion in solids (Blank and Roughton, 1960). Since our membranes contained a high mole fraction of cholesterol, which decreases lipid fluidity, we expect some biological membranes to have CO_2 permeabilities higher than 0.35 cm s^{-1}. On the other hand, values of $P^M_{CO_2}$ lower than 0.35 cm s^{-1} might occur in membranes which contain, in addition to cholesterol, high mole fractions of highly saturated lipids such as cerebroside or sphingomyelin (see, e.g., Hicks et al., 1974; Finkelstein, 1976).

In conclusion, we have measured the diffusion of CO_2 across lipid bilayers composed of egg lecithin, cholesterol, and decane, and we have identified several processes which may be rate limiting under different conditions. In the absence of CA and at pH <8, the diffusion of CO_2 through the unstirred layer and the uncatalyzed hydration-dehydration are rate limiting in the transport process. In the presence of CA and at pH 7–8, the main rate-limiting step is the diffusion of HCO_3^- through the unstirred layers. In the presence of CA and at

pH >9, diffusion of CO_2 across the membrane becomes rate limiting. Diffusion of H^+ (or H^+ equivalents) through the unstirred layer may become rate limiting when CA is present and the solutions are poorly buffered. This latter process can be rate limiting only when net CO_2 transport is occurring, whereas the other processes can be rate limiting either for one-way (self exchange) fluxes or for net fluxes. The catalyzed hydration-dehydration of CO_2 does not appear to be rate limiting under our experimental conditions due to the high turnover number of CA, the high concentrations of CA, the low concentrations of CO_2 and HCO_3^-, and the fairly low permeability of the lecithin-cholesterol-decane membrane to CO_2.

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