Runge, M. S., Schlaepfer, W. W., & Williams, R. C., Jr. (1981) *Biochemistry 20*, 170-175.
Sandoval, I. V., & Weber, K. (1978) *Eur. J. Biochem. 92*, 463-470.
Schlaepfer, W. W. (1977) *Brain Res. 136*, 1-9.
Schlaepfer, W. W., & Micko, S. (1978) *J. Cell Biol. 78*, 369-378.
Schlaepfer, W. W., & Freeman, L. A. (1980) *Neuroscience (Oxford) 5*, 2305-2314.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765-768.
Siman, R., Baudry, M., & Lynch, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3572-3576.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866-4871.
Suzuki, K., Ishiura, S., Tsuji, S., Katamoto, T., Sugita, H., & Imahori, K. (1979) *FEBS Lett. 104*, 355-358.
Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., & Imahori, K. (1981) *J. Biochem. (Tokyo) 90*, 275-278.
Tashiro, T., & Ishizaki, Y. (1982) *FEBS Lett. 141*, 41-44.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Truglia, J. A., & Stracher, A. (1981) *Biochem. Biophys. Res. Commun. 100*, 814-822.
Tsuji, S., & Imahori, K. (1981) *J. Biochem. (Tokyo) 90*, 233-240.
Wheelock, M. J. (1982) *J. Biol. Chem. 257*, 12471-12474.
Yoshida, N., Weksler, B., & Nachman, R. (1983) *J. Biol. Chem. 258*, 7168-7174.
Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., & Murachi, T. (1983) *J. Biol. Chem. 258*, 8883-8889.
Zimmerman, U.-J. P., & Schlaepfer, W. W. (1982) *Biochemistry 21*, 3977-3983.

Molecular Basis of the Oxygen Exchange from CO_2 Catalyzed by Carbonic Anhydrase III from Bovine Skeletal Muscle†

D. N. Silverman* and C. K. Tu
Departments of Pharmacology and Biochemistry, University of Florida, Gainesville, Florida 32610
Received March 31, 1986; Revised Manuscript Received July 28, 1986

ABSTRACT: The exchange of ^{18}O from CO_2 to H_2O in aqueous solution is caused by the hydration–dehydration cycle and is catalyzed by the carbonic anhydrases. In our previous studies of ^{18}O exchange at chemical equilibrium catalyzed by isozymes I and II of carbonic anhydrase, we observed simple first-order depletion of ^{18}O from CO_2 with the ^{18}O distribution among the species $C^{18}O^{18}O$, $C^{16}O^{18}O$, and $C^{16}O^{16}O$ described by the binomial expansion (i.e., a random distribution of ^{18}O). Using membrane-inlet mass spectrometry, we have measured ^{18}O exchange between CO_2 and H_2O catalyzed by native zinc-containing and cobalt(II)-substituted carbonic anhydrase III from bovine skeletal muscle near pH 7.5. The distributions of ^{18}O in CO_2 deviate from the binomial expansion and are accompanied by biphasic ^{18}O-exchange patterns; moreover, we observed regions in which ^{18}O loss from CO_2 was faster than ^{18}O loss from HCO_3^-. These data are interpreted in terms of a model that includes ^{18}O loss from an enzyme–substrate or intermediate complex. We conclude that more than one ^{18}O can be lost from CO_2 per encounter with the active site of isozyme III, a process that requires scrambling of oxygens in a bicarbonate–enzyme complex and cycling between intermediate complexes. This suggests that the rate of dissociation of $H_2{}^{18}O$ (or $^{18}OH^-$) from isozyme III is comparable to or faster than substrate and product dissociation.

The loss of ^{18}O from CO_2 in aqueous solution is caused by the hydration–dehydration cycle and is catalyzed by carbonic anhydrase (Mills & Urey, 1940):

$$C^{18}O^{18}O + H_2{}^{16}O \rightleftharpoons HC^{18}O^{18}O^{16}O^- + H^+ \rightarrow C^{18}O^{16}O + H_2{}^{18}O \quad (1)$$

Here, $H_2{}^{18}O$ is very greatly diluted by solvent $H_2{}^{16}O$, and the loss of ^{18}O from CO_2 is considered irreversible. In our previous studies of ^{18}O exchange at chemical equilibrium catalyzed by isozymes I and II of carbonic anhydrase (Silverman et al., 1979; Tu & Silverman, 1977), we observed rates of depletion of ^{18}O from CO_2 that were monophasic and first order with the ^{18}O distribution in CO_2 described by the binomial expansion at all times. We describe here two new observations for ^{18}O exchange catalyzed by isozyme III from bovine skeletal muscle. First, the ^{18}O exchange is biphasic, described by the sum of two first-order rates; and second, the ^{18}O distribution among labeled CO_2 is not described by the binomial expansion at times before isotopic equilibrium. We concluded that there is scrambling of oxygens in an intermediate, enzyme–substrate complex and that more than one ^{18}O can be lost to solvent per encounter of CO_2 with the active site of carbonic anhydrase III. These conclusions are based on observations of ^{18}O contents of HCO_3^- as well as CO_2 and three criteria for nonrandom ^{18}O distribution, which we describe. The atom fractions of ^{18}O in CO_2 were measured with a mass spectrometer using a membrane inlet.

Although this is the first report of the emergence of nonrandom ^{18}O distributions in catalysis by an isozyme of carbonic anhydrase, such an effect has been thoroughly studied for various phosphatases. Boyer et al. (1977) have described this

† This work was supported by a grant from the National Institutes of Health (GM25154).
* Address correspondence to this author at the Health Center, University of Florida, Gainesville, FL 32610.

0006-2960/86/0425-8402$01.50/0

effect for adenosinetriphosphatase from sarcoplasmic reticulum of skeletal muscle measuring the distribution of ^{18}O in inorganic phosphate using a mass spectrometer. These studies were extended to the phosphate–water exchange catalyzed by yeast inorganic pyrophosphatase by Hackney and Boyer (1978). Using the ^{18}O shift on the ^{31}P NMR signals of inorganic phosphate, Bock and Cohn (1978) have observed the distribution of ^{18}O in inorganic phosphate in the presence of native zinc-containing and cobalt(II)-substituted alkaline phosphatase. These data are analyzed in terms of the partitioning of phosphate between a noncovalent enzyme–phosphate complex and the covalent phosphorylated enzyme.

Experimental Procedures

^{18}O-Exchange Method. Measurements of ^{18}O content of CO_2 were made with a mass spectrometer using a CO_2-permeable inlet that allowed very rapid, for our purposes continuous, measurement of the isotopic content of CO_2 in solution. The ^{18}O content of CO_2 was expressed as τ, the fraction of all oxygens in CO_2 that are ^{18}O:

$$\tau = \frac{[C^{18}O^{16}O] + 2[C^{18}O^{18}O]}{2([C^{16}O^{16}O] + [C^{18}O^{16}O] + [C^{18}O^{18}O])} = \frac{46 + 2(48)}{2(44 + 46 + 48)}$$

where 44, 46, and 48 are the heights of the corresponding mass peaks. The ^{18}O content of CO_2 was also expressed as c, the fraction of all oxygens that are ^{18}O and appear in doubly labeled CO_2:

$$c = \frac{[C^{18}O^{18}O]}{[C^{16}O^{16}O] + [C^{18}O^{16}O] + [C^{18}O^{18}O]} = \frac{48}{44 + 46 + 48}$$

The ^{18}O content of bicarbonate τ' was determined by measuring τ for the CO_2 generated by rapid acidification of aliquots of the reaction solution. The ^{18}O content of CO_2 τ in the reaction solution at the time the sample was taken was also known from the membrane inlet, and the pH was known, allowing calculation of ^{18}O content in HCO_3^- (Mills & Urey, 1940):

$$\tau' = \frac{[HC^{16}O^{16}O^{18}O] + 2[HC^{16}O^{18}O^{18}O] + 3[HC^{18}O^{18}O^{18}O]}{3[HCO_3^-]_{total}}$$

In our previous studies of isozymes I and II of carbonic anhydrase in homogeneous solution, we found $\tau - \tau_\infty$ and $c - c_\infty$ each to follow a first-order decrease as described by a single exponential (Silverman et al., 1979; Tu & Silverman, 1977). In contrast, with certain homogeneous solutions containing bovine isozyme III, the depletion of ^{18}O from CO_2 is described by the sum of two exponentials:

$$\tau - \tau_\infty = Ae^{-\theta_1 t} + Be^{-\theta_2 t} \quad (2)$$

$$c - c_\infty = Ce^{-\gamma_1 t} + De^{-\gamma_2 t} \quad (3)$$

Each of these rate constants can be expressed as the sum of its catalyzed and uncatalyzed components:

$$\theta = \theta_{uncat} + \theta_{cat} \qquad \gamma = \gamma_{uncat} + \gamma_{cat}$$

These are the characteristics of random ^{18}O distributions maintained throughout ^{18}O depletion from CO_2 when uncatalyzed or catalyzed by isozyme II (Gerster, 1971): (1) The distribution of CO_2 among the masses 44, 46, and 48 is adequately fit by the binomial expansion at any time during the depletion. For an ^{18}O atom fraction in CO_2 given by τ, the fractions of total CO_2 with masses 44, 46, and 48 are $(1 - \tau)^2$, $2\tau(1 - \tau)$, and τ^2, respectively (here and throughout the text we write τ and c instead of $\tau - \tau_\infty$ and $c - c_\infty$ for brevity). (2) $c/\tau^2 = 1.0$ at any time during the depletion, a direct result of the binomial expansion. (3) The ratio $\gamma/\theta = 2.0$ for the rates of ^{18}O exchange during the depletion. These three characteristics are equivalent criteria for random distributions. Deviations from these criteria define the extent to which the ^{18}O distribution is nonrandom. The cascade of ^{18}O loss $C^{18}O^{18}O \rightarrow CO^{18}O \rightarrow COO$ maintains the binomial distribution (here we write O instead of ^{16}O). However, if this cascade becomes shortened to $C^{18}O^{18}O \rightarrow COO$, then the binomial distribution no longer holds and $c \neq \tau^2$ and $\gamma \neq 2\theta$. This would occur, as proposed here, when more than one ^{18}O is lost to solvent water per encounter of $C^{18}O_2$ with enzyme.

We report here a third rate constant for ^{18}O exchange: $\phi = \phi_{uncat} + \phi_{cat}$. This rate constant pertains to an experiment in which ^{18}O-containing species of CO_2 not enriched in ^{13}C are present in solution with ^{13}C-containing species of CO_2 not enriched in ^{18}O. The rate constant ϕ measures the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2, an exchange that occurs slowly in the absence of enzyme and is catalyzed by carbonic anhydrase. The catalysis occurs because dehydration of $H^{12}COO^{18}O^-$ can label the active site with ^{18}O, which then reacts with $^{13}CO_2$ to achieve the ^{18}O transfer measured by ϕ_{cat}. This is described in more detail by Silverman et al. (1979), who also describe how ϕ is obtained from experiments.

Materials and Enzyme. Oxygen-18 labeled bicarbonate was prepared by dissolving $KHCO_3$ in enriched water (up to 90 atom % ^{18}O, Mound Laboratory, Miamisburg, OH), allowing approach to isotopic equilibrium, and then removing water by vacuum distillation. Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was obtained from Sigma Chemical Co. Carbonic anhydrase III was obtained from fresh bovine and equine flank steak by gel filtration (Ultrogel AcA 44, LKB) followed by ion-exchange chromatography (DEAE-Sephacel, Pharmacia). The apoenzyme of bovine carbonic anhydrase III was made by dialysis in solutions containing the chelator 2-carboxy-1,10-phenanthroline, as described by Engberg and Lindskog (1984). Their procedure was also used to prepare Co(II)-substituted carbonic anhydrase III by dialyzing the apoenzyme against 10 mM $CoCl_2$, followed by dialysis of the enzyme sample against six changes of a large volume of water. The resulting enzyme as well as the native enzyme was determined to be greater than 95% pure as determined by polyacrylamide gel electrophoresis, which showed one band corresponding to a molecular weight near 30000. A more rigorous method to test for contamination by the red cell isozymes is to measure inhibition by ethoxzolamide of catalysis of CO_2 hydration. The value of K_i is about 10^4 times smaller in the inhibition of the red cell isozymes I and II than of muscle isozyme III (Sanyal et al., 1982). By this criterion, we found no contamination by red cell isozyme in our purified samples of isozyme III. We have no evidence of dimerization during the period we used isozyme III; purifications with and without dithiothreitol gave the same results. Bovine carbonic anhydrase II was obtained from erythrocytes and purified by the affinity chromatography procedure described by Khalifah et al. (1977).

Procedure. Isotope-exchange experiments were performed in the range of pH 7.3–7.8 with pH adjustments made with NaOH or H_2SO_4. In all experiments, the total ionic strength

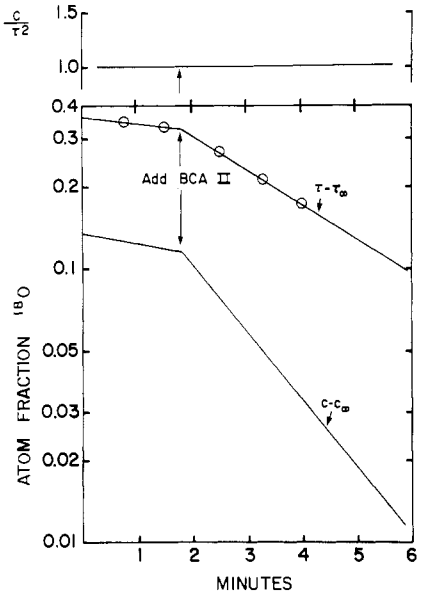


FIGURE 1: Atom fractions of ^{18}O in CO_2, $\tau - \tau_\infty$ and $c - c_\infty$, and (upper figure) ratio c/τ^2 as a function of time. These are two sets of data with the solid line representing continuous, experimentally determined CO_2 data and (O) representing discontinuous HCO_3^- data. Native bovine carbonic anhydrase II was added at the time indicated by the arrows to achieve a total enzyme concentration of 2.4×10^{-9} M. The pH was 7.5, and total inorganic carbon ($CO_2 + HCO_3^-$) was present at 15 mM. Total ionic strength of solution was adjusted to 0.2 with Na_2SO_4; 20 mM Hepes was present as buffer.

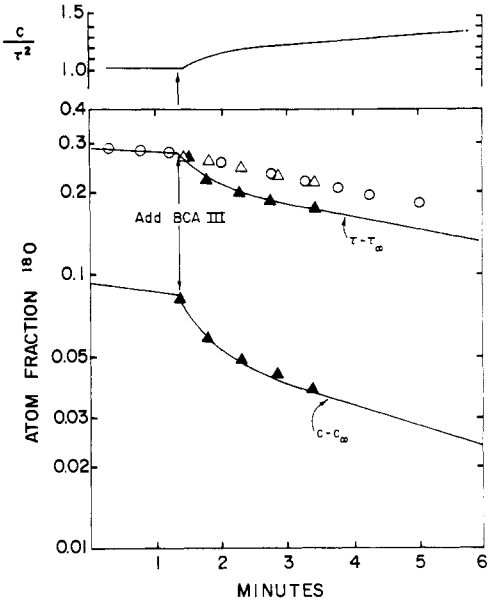


FIGURE 2: Lines are ^{18}O atom fractions in CO_2 measured by membrane-inlet mass spectrometry, which gives a continuous measurement of ^{18}O content. (O) ^{18}O atom fractions in HCO_3^- obtained by rapid acidification of samples and measurement of the ^{18}O content of the resulting CO_2. Native bovine carbonic anhydrase III was added at the time indicated by the arrows to achieve an enzyme concentration of 1.2×10^{-7} M. The buffer was Hepes at 20 mM with pH 7.5 and ionic strength adjusted to 0.2 with Na_2SO_4. (▲) Calculated by numerical integration of eq 10–13 and the analogous equations for c to fit the experimental data for CO_2 represented by the lines. (△) Calculated to fit the data for HCO_3^- represented by open circles. The set of rate constants used to obtain these calculated values are $k_1 = 1.0 \times 10^6$ M^{-1} s^{-1}, $k_{-1} = 4.2 \times 10^3$ s^{-1}, $k_2 = 7.5 \times 10^3$ s^{-1}, $k_{-2} = 2.5 \times 10^3$ s^{-1}, $k_3 = 2.0 \times 10^3$ s^{-1}, $k_{-3} = 1.3 \times 10^7$ M^{-1} s^{-1}, $k_4 = 1.5 \times 10^5$ s^{-1}, and $k_{-4} = 500$ s^{-1}.

of the solution was 0.2, maintained by adding the appropriate amount of Na_2SO_4. Before each experiment, the concentration of bovine carbonic anhydrase II was determined from the molar absorptivity at 280 nm, $\epsilon_{280} = 5.6 \times 10^4$ M^{-1} cm^{-1} (Lindskog, 1960), and that of bovine carbonic anhydrase III from $\epsilon_{280} = 6.4 \times 10^4$ M^{-1} cm^{-1} (Engberg & Lindskog, 1984). The concentration of bovine Co(II) carbonic anhydrase III was confirmed by its absorption at 640 nm and pH 7.0, $\epsilon_{640} = 270$ M^{-1} cm^{-1} (Engberg & Lindskog, 1984).

Each experiment was begun by placing in the membrane-inlet vessel 8.0 mL of a solution in which ^{18}O-labeled bicarbonate had been dissolved. A period as long as several minutes elapsed before measurements were made to allow approach to chemical equilibrium. At this time, the uncatalyzed ^{18}O-exchange rate was measured. Then enzyme was added in a volume less than 0.1 mL. During each experiment, pH was constant to within 0.02 pH unit. All data were obtained at 25 °C.

Stopped-flow experiments of catalyzed CO_2 hydration were carried out at 25 °C with the procedure of Khalifah (1971), and the data were analyzed by methods described by Rowlett and Silverman (1982).

RESULTS

We report two basic observations for ^{18}O exchange catalyzed by carbonic anhydrase III at chemical equilibrium but before isotopic equilibrium: first, the loss of ^{18}O from CO_2 catalyzed by isozyme III was biphasic with the ^{18}O content of CO_2 less than that of HCO_3^-; second, the ^{18}O distribution in CO_2 during catalysis cannot be described by the binomial distribution. We describe now the first observation. The ^{18}O depletion from CO_2 and from HCO_3^- after the addition of bovine carbonic anhydrase II is described by a single, first-order decrease in ^{18}O content as shown in Figure 1 (for which $\theta_{cat} = 3.9 \times 10^{-3}$ s^{-1} and $\gamma_{cat} = 7.8 \times 10^{-3}$ s^{-1}). This is consistent with previous measurements of ^{18}O exchange catalyzed by isozyme II and I (Silverman et al., 1979; Tu & Silverman, 1977). The uncatalyzed ^{18}O exchange is also described by a single, first-order depletion, shown also in Figure 1 as the rate before addition of enzyme ($\theta_{uncat} = 6.0 \times 10^{-4}$ s^{-1}, $\gamma_{uncat} = 1.2 \times 10^{-3}$ s^{-1}). The ^{18}O depletion from CO_2 in the presence of bovine carbonic anhydrase III is biphasic (Figure 2). The sum of two exponentials, eq 2 and 3, provided an excellent description of the data as determined by computer fit with the Simplex method (Nelder and Mead, 1965): $\theta_1 = 5.2 \times 10^{-2}$ s^{-1}, $\theta_2 = 1.7 \times 10^{-3}$ s^{-1}, $\gamma_1 = 4.8 \times 10^{-2}$ s^{-1}, and $\gamma_2 = 2.9 \times 10^{-3}$ s^{-1}. The ^{18}O depletions from CO_2 catalyzed by bovine cobalt(II) carbonic anhydrase III (Figure 3) and equine carbonic anhydrase III (data not shown) were also biphasic and also excellently fit by the sum of two exponentials.

The ^{18}O depletion from HCO_3^- in the presence of isozymes II and III and cobalt(II)-substituted isozyme III was simple first order and monophasic (the circles in Figures 1–3). At pH 7.5, the ^{18}O content of HCO_3^-, τ', is the same as that of CO_2, τ, in the uncatalyzed region of the exchange (Mills & Urey, 1940). In the presence of bovine isozyme III and Co(II) isozyme III, the ^{18}O content of CO_2 was less than that of HCO_3^- (Figures 2 and 3). The ^{18}O content of HCO_3^- in the presence of equine isozyme III was not measured. In experiments such as shown in Figure 2, use of a higher concentration of isozyme III resulted in a greater difference between ^{18}O content of CO_2 and HCO_3^- (i.e., between τ and τ') at times before isotopic equilibrium.

The appearance of CO_2 with ^{18}O content lower than that of HCO_3^- when in the pressence of Co(II)-substituted bovine isozyme III was confirmed in a nonequilibrium experiment. Helium gas was passed at rapid flow rate through a fritted disk to produce fine bubbles in a solution containing 200 mM Hepes buffer and 1×10^{-6} M Co(II) carbonic anhydrase III.

Table I: Percent of Total CO_2 Present as $^{12}C^{16}O^{16}O$ (Mass 44), $^{12}C^{18}O^{16}O$ (Mass 46), and $^{12}C^{18}O^{18}O$ (Mass 48) at Specified Times in the Experiments Shown in Figures 1–3[a]

	isozyme II at 6.00 min		isozyme III at 5.39 min		Co(II) isozyme III at 5.47 min	
	obsd	calcd random	obsd	calcd random	obsd	calcd random
$^{12}C^{16}O^{16}O$ (%)	81.6	81.6	74.0	73.4	80.0	79.6
$^{12}C^{16}O^{18}O$ (%)	17.4	17.5	23.3	24.6	18.7	19.2
$^{12}C^{18}O^{18}O$ (%)	1.0	0.9	2.7	2.0	1.3	1.2
τ[b]	0.097	0.097	0.143	0.143	0.107	0.107
(c/τ^2)[c]	1.0	1.0	1.3	1.0	1.2	1.0

[a] These distributions are compared with those predicted for random occurrence as given by the binomial distribution. For a random distribution with an ^{18}O atom fraction in CO_2 given by τ, the fractions of total CO_2 appearing with masses 44, 46, and 48 are $(1-\tau)^2$, $2\tau(1-\tau)$, and τ^2. [b] Atom fraction of ^{18}O in CO_2, see Experimental Procedures. [c] Here c is the atom fraction of ^{18}O in doubly labeled CO_2.

Table II: Steady-State Kinetic Parameters for Hydration of CO_2 Catalyzed by Bovine Carbonic Anhydrases II and III and Cobalt(II)-Substituted Bovine Carbonic Anhydrase III at pH 7.5

	isozyme II	isozyme III	isozyme III calcd	Co(II) isozyme III
$k_{cat}^{CO_2}$ (s^{-1})	6.0×10^5[b]	2.5×10^3[a]	1.2×10^3[d]	1.3×10^3[a]
$k_{cat}^{CO_2}/K_m^{CO_2}$ (M^{-1} s^{-1})	6.3×10^7[b]	2.5×10^5[a]	4.5×10^5[d]	1.4×10^5[a]
$(k_{cat}^{exch}/K_{eff}^{CO_2})_{app}$ (M^{-1} s^{-1})[c]	6.0×10^7	5.1×10^5		7.4×10^5

[a] Steady-state data measured by stopped-flow spectrophotometry as described in text, using Hepes buffer at 28 mM and phenol red as indicator. Temperature was 25 °C, and ionic strength was adjusted to 0.2 with Na_2SO_4. [b] Pocker and Bjorkquist (1977). [c] Measured by ^{18}O exchange according to Silverman et al. (1979). For isozyme III, $(k_{cat}^{exch}/K_{eff}^{CO_2})_{app}$ is the rate of interconversion of CO_2 and HCO_3^- (at $[CO_2] \ll K_{eff}$) at chemical equilibrium R_1 divided by $[CO_2][E_{tot}]$ and measured for the slower phase of ^{18}O exchange. [d] Calculations were performed as described in the Appendix with the rate constants given in the legend to Figure 2.

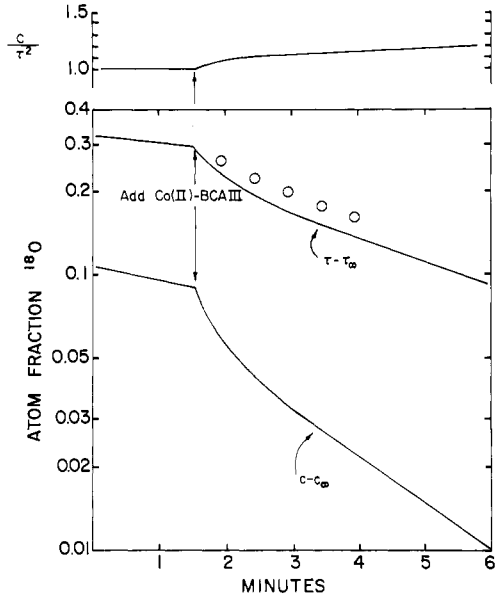


FIGURE 3: Experiment for which conditions were identical with those described in the legend to Figure 2 except that cobalt(II)-substituted bovine carbonic anhydrase III was added to achieve a total enzyme concentration of 2.0×10^{-7} M and the pH was 7.4.

Bicarbonate (initial ^{18}O content 36%) was added quickly to give an initial concentration of 60 mM and initial pH of 8.0. The ^{18}O content of CO_2 was measured in the CO_2 as it escaped with the helium (20% after 9.0 min) and in the HCO_3^- remaining in solution (31% after 9.0 min).

We describe now our second observation, that concerning the distribution of ^{18}O in CO_2 during catalysis. Oxygen-18 exchange from CO_2 to water catalyzed by bovine carbonic anhydrase II fit the criteria for maintaining distribution of oxygens in CO_2 as described by the binomial expansion ($c/\tau^2 = 1.0$, $\gamma_{cat}/\theta_{cat} = 2.0$ shown in Figure 1 and Table III, the distribution of ^{18}O shown in Table I). This ^{18}O exchange when catalyzed by native and cobalt(II)-substituted bovine carbonic anhydrase III did not maintain a distribution of oxygens in CO_2 that can be described by the binomial distribution (Figures 2 and 3 and Tables I and III). The ratio $\gamma_{cat}/\theta_{cat}$ measures the extent to which the ^{18}O distribution varies from random and is given in Table III for two isozymes. The steady-state rate constants for CO_2 hydration at pH 7.5 catalyzed by the bovine isozymes studied here appear in Table II. The ^{18}O distribution in CO_2 in the presence of equine carbonic anhydrase III was also not described by the binomial distribution; under conditions identical with those described

Table III: Ratios of Rate Constants for ^{18}O Exchange $\gamma_{cat}/\theta_{cat}$ Observed during Catalysis by Bovine Carbonic Anhydrases II and III and Cobalt(II)-Substituted Bovine Carbonic Anhydrase III[a]

	$(\gamma_{cat}/\theta_{cat})_1$	$(\gamma_{cat}/\theta_{cat})_2$
isozyme II	2.0[b]	
isozyme III	1.0	1.4
isozyme III calcd	1.0[c]	1.4[c]
Co(II) isozyme III	1.0	1.5

[a] A ratio $\gamma_{cat}/\theta_{cat} = 2.0$ indicates a distribution of ^{18}O that is random and is described by the binomial expansion; $\gamma_{cat}/\theta_{cat} \neq 2.0$ cannot be fit by the binomial distribution. Oxygen-18 measurements were made at 25 °C and pH 7.3 with total inorganic carbon ($CO_2 + HCO_3^-$) at 15 mM and enzyme at the concentrations given in Figures 1–3. Total ionic strength of solution was 0.2 adjusted with Na_2SO_4. No buffers were used. Subscript 1 indicates the more rapid phase of ^{18}O depletion. A standard deviation no greater than ±10% was found for three to six repetitions of these ratios. [b] ^{18}O exchange catalyzed by isozyme II exhibited a monophasic depletion. [c] Calculations were performed as described in the Appendix with the rate constants given in the legend of Figure 2.

Table IV: Rate Constants for ^{18}O Exchange Catalyzed by Co(II)-Substituted Bovine Carbonic Anhydrase III as a Function of Total Substrate Concentration at pH 7.25[a]

$[CO_2] + [HCO_3^-]$ (mM)	$(\theta_{cat})_2$ ($\times 10^3$ s^{-1})	$(\gamma_{cat}/\theta_{cat})_2$	$(\phi_{cat})_2$ ($\times 10^3$ s^{-1})
10	3.7	1.5	0.14
20	3.3	1.5	0.22
33	3.0	1.5	0.36
40	3.0	1.5	0.41
67	2.4	1.7	0.46
100	2.2	2.0	0.56

[a] Enzyme concentration was 2.0×10^{-7} M with total ionic strength of solution held constant at 0.2 by adding the appropriate amount of Na_2SO_4. Subscript 2 refers to the slower phase of ^{18}O depletion.

in Figure 2, except that equine isozyme III was used at an undetermined concentration, θ_2 was $(2.9 \pm 0.3) \times 10^{-3}$ s^{-1} and $(\gamma_{cat}/\theta_{cat})_2$ was 1.7 ± 0.2 (mean and standard deviation from four measurements). Table IV shows the variation with total substrate concentration of three rate constants for ^{18}O exchange catalyzed by bovine Co(II) carbonic anhydrase III.

Discussion

There are two qualitative features of ^{18}O exchange catalyzed by carbonic anhydrase III (Figure 2) that are different than ^{18}O exchange catalyzed by carbonic anhydrase II (Figure 1) and that we interpret here. First, in catalysis by isozyme III the ^{18}O content observed in CO_2 was less than that observed in HCO_3^- at times before isotopic equilibrium. And second, in catalysis by isozyme III the ^{18}O distribution in CO_2 was observed to be different than the binomial distribution (i.e., not random). We have interpreted these data using a model that in computer simulations is quantitatively consistent with the data using a reasonable set of kinetic constants. In the model, ^{18}O is lost from an enzyme–substrate complex or some analogous intermediate of isozyme III. In contrast, our previous results with carbonic anhydrase II have been explained by ^{18}O loss from enzyme after product release. In the discussion that follows, we draw two conclusions from these observations: ^{18}O can be exchanged from an enzyme–substrate complex (as $H_2{}^{18}O$, most likely) to bulk solvent, and there is a significant probability that more than one ^{18}O can be exchanged per encounter of CO_2 with the active site. This requires that the dissociation rates of CO_2 and HCO_3^- from isozyme III are comparable to or smaller than the ^{18}O dissociation rate.

The following scheme is consistent with a wide body of data for catalysis by carbonic anhydrase II (Lindskog, 1983) as well as carbonic anhydrase III (Kararli & Silverman, 1985):

$$CO_2 + \gtrless ZnOH^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \gtrless Zn^{OH^-}_{CO_2} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \gtrless ZnHCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \gtrless ZnOH_2 + HCO_3^- \quad (4)$$

$$\gtrless ZnOH_2 + B \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \gtrless ZnOH^- + BH^+ \quad (5)$$

The proton transfer to buffer B in eq 5 can be rate limiting for isozyme II, but there is no evidence that this step is rate-limiting for isozyme III. The loss of ^{18}O from an enzyme–substrate complex could occur as shown in eq 6 or 7.

$$\gtrless Zn^{CO_2}_{^{18}OH^-} \overset{H_2O}{\rightarrow} \gtrless Zn^{CO_2}_{OH_2} + {}^{18}OH^- \quad (6)$$

$$\gtrless Zn^{CO_2}_{^{18}OH^-} \overset{H^+}{\rightleftharpoons} \gtrless Zn^{CO_2}_{H_2{}^{18}O} \overset{+H_2O}{\rightarrow} \gtrless Zn^{CO_2}_{H_2O} + H_2{}^{18}O \quad (7)$$

The model proposed here includes the mechanism of eq 4 and 5 and loss of ^{18}O from an enzyme–substrate complex, such as in eq 6 or 7. The model is specifically defined by eq 10–13 of the Appendix. We presume that eq 7 is a likely pathway because of the observed solvent hydrogen isotope effect on ^{18}O loss from isozyme III (Tu et al., 1983), in analogy with isozyme II. There is no direct evidence for eq 6, but with a catalytic turnover of 10^3 s^{-1} for isozyme III, the concentration of OH^- at pH 7 is great enough so that the reverse of this reaction could contribute in the catalytic hydration of CO_2. There are other possibilities, such as transfer of a proton from H_2O to the active site.

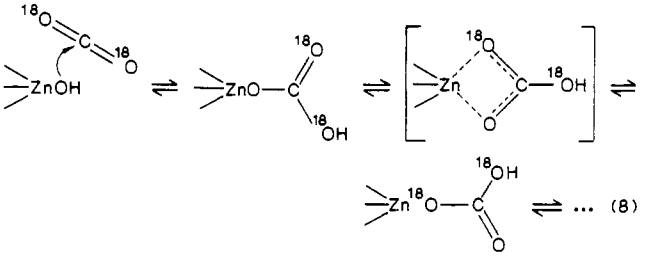
The Appendix shows the kinetic equations that describe ^{18}O exchange both uncatalyzed and catalyzed according to eq 4 and 5 with ^{18}O loss from intermediate complexes, as for example in eq 6 or 7. This set of equations was solved numerically, as described in the Appendix, to give calculated ^{18}O contents of CO_2 and HCO_3^-. The differences between these results and the experimental data were minimized by a numerical, least-squares procedure. The results of the minimization are shown as the triangles in Figure 2 with the rate constants obtained by the minimization given in the legend. We have no evidence that this is a unique solution, but it yields reasonable rate constants, as described in the Appendix, and also reproduces the ^{18}O depletions shown in Figure 2.

Why in the presence of isozyme III (and before isotopic equilibrium) is the ^{18}O content of CO_2 less than the ^{18}O content of HCO_3^-? We cannot account for the observation by considering only the enhancement of the rate of interconversion of CO_2 and HCO_3^-, because this does not explain different ^{18}O contents of CO_2 and HCO_3^-. The answer is that ^{18}O must be lost from an intermediate complex. The data are consistent with these steps: isozyme III binds CO_2, undergoes conversion to bound intermediate (such as enzyme–bicarbonate complex), and returns to dissociate CO_2 with loss of ^{18}O to solvent. This process is nonproductive binding and would not be observed in an experiment that measures a decrease of substrate concentration. This ^{18}O-exchange property of isozyme III is very likely a reflection of the tight binding of HCO_3^- to the active site [the tight binding of anions to the site is reported by Sanyal et al. (1982)]. Our interpretation requires that the dissociation of HCO_3^- has a rate slower than or comparable to the release of CO_2 and the processes involved in the release of ^{18}O to solvent. This is apparent in the rate constants, which in calculations fit the data of Figure 2. About 30 s after addition of isozyme III under the conditions of these experiments, a second region of ^{18}O depletion was observed in which the loss of ^{18}O from CO_2 was slower but was comparable to the rate of depletion of ^{18}O from HCO_3^-. Here the loss of ^{18}O from CO_2 in the catalytic process is balanced by the production of ^{18}O-containing CO_2 caused by the dehydration of HCO_3^-.

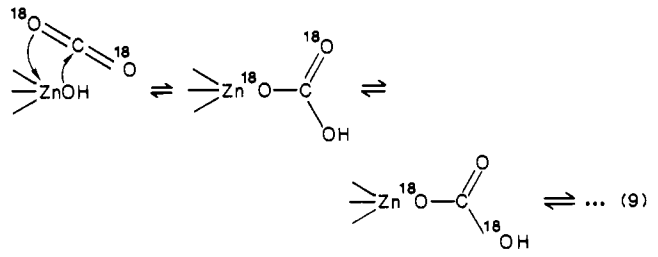
Another conclusion required by our observations is that there be scrambling of oxygens on the enzyme; this permits an oxygen to be removed from the intermediate complex other than the oxygen added to CO_2. The scrambling of oxygens must occur in the bicarbonate–enzyme complex:

$$\gtrless ZnOH^- + C^{18}O_2 \rightleftharpoons \gtrless Zn(HCO^{18}O^{18}O^-) \overset{\text{scramble}}{\rightleftharpoons} \gtrless Zn(HC^{18}O^{18}OO^-) \rightleftharpoons \gtrless Zn^{18}OH^- + CO^{18}O$$

It is known that bicarbonate binds to the metal in carbonic anhydrase I (Led et al., 1982; Williams & Henkens, 1985). This scrambling with isozyme III is perhaps a result of an intermediate bidentate complex of HCO_3^- with zinc at the active site. There is evidence for bidentate ligands and pentacoordinated metal in isozymes I and II (Bertini et al., 1982), and Van Niekerk et al. (1953) reported a bidentate chelation for Zn(II)(acetate)$_2$:



Another possibility is internal rotation with accompanying proton transfer:



... (9)

We also observed that ^{18}O distribution among the various isotopic species of CO_2 was not described by the binomial expansion (i.e., was not random) in the presence of native or cobalt(II)-substituted bovine isozyme III. In the presence of isozymes I or II, this distribution is random. This shows another characteristic of the ^{18}O exchange pathway: more than one ^{18}O can be lost from the CO_2–HCO_3^- system in a single encounter of substrate with enzyme. This conclusion is supported by comparable studies on the ^{18}O distribution in inorganic phosphate in the presence of phosphatases (Boyer et al., 1977; Hackney & Boyer, 1978; Bock & Cohn, 1978) and on the ^{18}O distribution in CO_2 in the presence of red cells (Silverman et al., 1981; Tu et al., 1978). These references show that the binomial distribution of ^{18}O in CO_2 is consistent with the cascade of ^{18}O loss $C^{18}O^{18}O \rightarrow C^{16}O^{18}O \rightarrow C^{16}O^{16}O$. For isozyme III our observations require that the cascade is shortened to $C^{18}O^{18}O \rightarrow C^{16}O^{16}O$, which suggests cycling between intermediates accompanied by more than one loss of ^{18}O per encounter of $C^{18}O_2$ and isozyme III resulting in nonrandom ^{18}O distributions. This conclusion is supported by the numerical calculations based on our model of eq 4–7. The calculations reproduce the nonrandom ^{18}O distributions as shown by the filled triangles in Figure 2, which fit experimental data for both c and τ, and the calculated values of $\gamma_{cat}/\theta_{cat}$, which fit experimental values in Table III ($\gamma_{cat}/\theta_{cat} < 2.0$ indicates nonrandom ^{18}O distributions in CO_2, as described under Experimental Procedures). This result shows that the two basic observations presented here (^{18}O content of CO_2 less than that of HCO_3^- and nonrandom ^{18}O distributions) can be explained by a single mechanism; that is, they need not reflect independent phenomena. From the values $\gamma_{cat}/\theta_{cat} = 1.0$ in the rapid phase of catalyzed ^{18}O exchange (Table III), we estimate that the probability is high that $C^{18}O_2$ exchanges both ^{18}O labels for ^{16}O per single encounter with the active site of isozyme III.

To be consistent with our data, such a cycle must have ^{18}O loss to solvent comparable to or faster than dissociation of CO_2 or HCO_3^-. Further evidence for this conclusion comes from the rates of ^{18}O exchange between ^{12}C- and ^{13}C-labeled species of CO_2. The rate constant θ_{cat} measures the catalyzed loss of ^{18}O from all CO_2. The rate constant ϕ_{cat} measures the catalyzed exchange of ^{18}O from ^{12}C-containing to ^{13}C-containing species of CO_2 and requires that $H^{12}CO_2{}^{18}O^-$ react to form an ^{18}O-labeled active site and in a subsequent step $^{13}CO_2$ bind to that site to form product containing both ^{13}C and ^{18}O (Silverman et al., 1979). Table IV shows that ϕ_{cat} was small relative to θ_{cat} (less than 5% of θ_{cat} for low substrate concentration). This means that the rate of release of ^{18}O from the active site is large compared with the rate of reaction of ^{18}O at the active site with $^{13}CO_2$. As the substrate concentration increases and the rate of encounter of CO_2 with isozyme III increases, so does the value of ϕ_{cat} increase relative to θ_{cat} (Table IV). It is interesting that at the highest substrate concentration shown in Table IV the distribution of ^{18}O was fit by the binomial expansion ($\gamma/\theta = 2.0$). A possible explanation is that the catalytic rate of scrambling of ^{18}O was too small to detect in the presence of the large reservoir of ^{18}O.

Because the data of Table IV at total substrate concentration of 100 mM obey the binomial expansion, we can calculate the rate of dissociation from isozyme III of ^{18}O using the method of Silverman et al. (1979): 2×10^4 s^{-1}. This dissociation rate is faster than the rate of release from the enzyme of CO_2 or HCO_3^-, which according to the fit of the data (see Appendix) are 4×10^3 s^{-1} and 2×10^3 s^{-1}, respectively. For HCO_3^-, this result can be reconciled with the known tight binding of anions to the active site of isozyme III (Sanyal et al., 1982). To require that CO_2 dissociation be slow with respect to ^{18}O exchange steps in eq 7 is a difficult restriction to satisfy because CO_2 binding to carbonic anhydrase has always been thought to be weak and consequently the binding site has not been determined. It is possible that the site from which CO_2 dissociates slowly is not the active site but another site nearby. Bovine isozyme III is known to have a lysine residue at position 64, unlike isozymes II and I which have histidine at this position (Tashian et al., 1980). However, we believe that carbamoylation of lysine-64 in bovine isozyme III does not provide a binding site of CO_2 that affects ^{18}O exchange because the ^{18}O-exchange properties of bovine isozyme III and equine isozyme III were qualitatively similar and equine isozyme III has an arginine at position 64 (Wendorff et al., 1985).

Comparison of Figures 2 and 3 and the data of Table III show that there was a similarity of the biphasic ^{18}O-depletion patterns as well as a similarity in the measure of ^{18}O distribution $\gamma_{cat}/\theta_{cat}$ for catalysis at pH 7.5 for the native and cobalt(II)-substituted isozymes III. This is in contrast to the case for alkaline phosphatase for which ^{18}O-exchange patterns in phosphate are very different for native zinc-containing and cobalt(II)-substituted enzymes (Bock & Cohn, 1978). In fact, the steady-state rate constants for CO_2 hydration at pH 7.5 are similar for the native and cobalt(II)-substituted bovine isozyme III (Table II). The substitution of cobalt for zinc in isozyme III has resulted in no qualitative differences in ^{18}O-exchange properties. Work is in progress to assess quantitative differences, a task that will require that the ^{18}O-scrambling properties found for isozyme III be incorporated into our previous evaluation of ^{18}O exchange (Silverman et al., 1979). Moreover, our previous interpretation of ^{18}O exchange catalyzed by isozyme III from cat muscle (Tu et al., 1983) needs to be reevaluated in this regard.

This work has shown that for bovine and equine isozyme III there may be ^{18}O exchange to solvent water without catalytic interconversion of HCO_3^- to CO_2. These considerations explain why $k_{cat}^{exch}/K_{eff}^{CO_2}$ for isozyme III determined by ^{18}O exchange (Table II) is greater than $k_{cat}^{CO_2}/K_m^{CO_2}$ determined by rate of appearance of product in stopped-flow experiments. In principle and in practice for isozymes I and II, these two measures of enzymic activity are equal (Koenig et al., 1974; Simonsson et al., 1979). One more difficulty arises because of this work. This paper suggests that product dissociation is rate-contributing for overall catalysis of CO_2 hydration by bovine isozyme III. Kararli and Silverman (1985) have presented evidence that a proton-transfer step is rate-contributing in maximal turnover of CO_2 hydration catalyzed by feline isozyme III. These are not mutually exclusive conclusions but do warrant further work.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of George Wynns. We thank Drs. Sven Lindskog and Paul Engberg for a sample of the chelator 2-carboxy-1,10-phenanthroline and Dr. Andrzej Wierzbicki for his assistance with the computations.

APPENDIX

The goal of this Appendix is to describe the simulation of the ^{18}O-exchange data of Figure 2 with the simple kinetic model of eq 4 and 5 of the text. In this model it is assumed that ^{18}O is lost to solvent water from an enzyme–substrate complex, such as shown in eq 6 and 7. Four rate equations, eq 10–13 describe the rate of change of the atom fractions of

$$\frac{-d\tau_1}{dt} = k(\tau_1 - \tau_1') + k_1[E](\tau_1 - \tau_2) \quad (10)$$

$$\frac{-d\tau_1'}{dt} = k'(\tau_1' - 2\tau_1/3) + k_{-3}[E](\tau_1' - \tau_2') \quad (11)$$

$$\frac{-d\tau_2}{dt} = k_2(\tau_2 - \tau_2') + k_{-1}(\tau_2 - \tau_1) \quad (12)$$

$$\frac{-d\tau_2'}{dt} = k_{-2}(\tau_2' - 2\tau_2/3) + k_3(\tau_2' - \tau_1') \quad (13)$$

^{18}O in CO_2 and bicarbonate both free in solution and bound to enzyme. Each equation is the sum of two parts: one describes the on- and off-rate of substrate and product, and a second describes the ^{18}O loss to solvent due to the hydration–dehydration reaction [as originally derived by Mills and Urey (1940)] either from the uncatalyzed reaction or from the enzyme–substrate or intermediate complex. The rate constants are those shown in eq 4 with k and k' representing the rate constants for the uncatalyzed hydration of CO_2 (0.035 s^{-1}) and dehydration of HCO_3^- [5.5×10^4 M^{-1} s^{-1} (H^+)]. The atom fractions of ^{18}O in free CO_2 and HCO_3^- are τ_1 and τ_1', respectively. The fraction of all oxygens that are ^{18}O in bound CO_2 and HCO_3^- are τ_2 and τ_2'. The term $k_1[E]\tau_2$ in eq 10 and similar terms in eq 11–13 were derived from terms such as $k_1[C^{18}O^{18}O]_{free}/[CO_2]_{bound}$ by using the relationship $K_{eq}^{CO_2} = [E][CO_2]_{free}/[CO_2]_{bound} = k_{-1}/k_1$. The validity of the equations is based on the assumptions that chemical equilibrium has been attained and that kinetic isotope effects are negligible. The rate equations for the analogous ^{18}O atom fractions involving double labeling with ^{18}O, c_1, c_2, c_1', and c_2' are identical with eq 10–13 with the exception that each coefficient 2/3 is changed to 1/3.

Reasonable initial values were chosen for the rate constants, and the set of differential equations was solved by Gear's method (Gear, 1971) using program DGEAR from the International Mathematical and Statistical Library (IMSL, Inc.). This results in calculated values of the atom fractions τ at various times, which were compared with the experimental data by a least-squares criterion. By use of an optimization procedure (program ZXMIN also from IMSL), the initial parameters were refined, and the set of equations was solved again. This process was repeated until the calculated values of τ and c converged to values close to the experimental values (see Figure 2). The final rate constants were as listed in the legend to Figure 2. By use of the relations between these constants and the steady-state parameters for the mechanism of eq 4 and 5 (Segel, 1975), the rate constants given in the legend to Figure 2 yield these values: $k_{cat}^{CO_2} = 1.2 \times 10^3$ s^{-1} and $k_{cat}^{CO_2}/K_m^{CO_2} = 4.5 \times 10^5$ M^{-1} s^{-1}. These constants can be compared with experimental values given in Table II for isozyme III. These calculations are also consistent with the requirement that $k_4/k_{-4} = K_a/[H^+] \geq 300$, where K_a is the acid dissociation constant of the catalytic group. Moreover, these calculations are consistent with $k_1k_2k_3k_4/k_{-1}k_{-2}k_{-3}k_{-4} = K_{eq}/[H^+] \simeq 25$ (our calculations give 32), where K_{eq} is the equilibrium constant for the catalyzed reaction, $10^{-6.1}$.

Registry No. CO_2, 124-38-9; H_2O, 7732-18-5; O_2, 7782-44-7; HCO_3^-, 71-52-3; carbonic anhydrase, 9001-03-0.

REFERENCES

Bertini, I., Luchinat, C., & Scozzafava, A. (1982) *Struct. Bonding (Berlin) 48*, 45–92.

Bock, J. L., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4082–4085.

Boyer, P. D., deMeis, L., Carvalho, M., & Hackney, D. D. (1977) *Biochemistry 16*, 136–140.

Engberg, P., & Lindskog, S. (1984) *FEBS Lett. 170*, 326–330.

Gear, C. W. (1971) *Numerical Initial Value Problems in Ordinary Differential Equations*, Prentice-Hall, Englewood Cliffs, NJ.

Gerster, R. (1971) *Int. J. Appl. Radiat. Isot. 22*, 339–348.

Hackney, D. D., & Boyer, P. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3133–3137.

Kararli, T., & Silverman, D. N. (1985) *J. Biol. Chem. 260*, 3484–3489.

Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.

Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.

Koenig, S. H., Brown, R. D., London, R. E., Needham, T. E., & Matwiyoff, N. A. (1974) *Pure Appl. Chem. 40*, 103–113.

Led, J. J., Neesgaard, E., & Johansen, J. T. (1982) *FEBS Lett. 147*, 74–80.

Lindskog, S. (1960) *Biochim. Biophys. Acta 39*, 218–226.

Lindskog, S. (1983) *Zinc Enzymes* (Spiro, T. G., Ed.) pp 78–121, Wiley, New York.

Mills, G. A., & Urey, H. C. (1940) *J. Am. Chem. Soc. 62*, 1019–1026.

Nelder, J. A., & Mead, R. (1965) *Comput. J. 8*, 308–313.

Pocker, Y., & Bjorkquist, D. W. (1977) *Biochemistry 16*, 5698–5707.

Rowlett, R. S., & Silverman, D. N. (1982) *J. Am. Chem. Soc. 104*, 6737–6741.

Sanyal, G., Swenson, E. R., Pessah, N. I., & Maren, T. H. (1982) *Mol. Pharmacol. 22*, 211–220.

Segel, I. (1975) *Enzyme Kinetics*, pp 534–543, Wiley, New York.

Silverman, D. N., Tu, C. K., Lindskog, S., & Wynns, G. C. (1979) *J. Am. Chem. Soc. 101*, 6734–6740.

Silverman, D. N., Tu, C. K., & Roessler, N. (1981) *Respir. Physiol. 44*, 285–298.

Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1979) *Eur. J. Biochem. 93*, 409–417.

Tashian, R. E., Hewett-Emmett, D., Stroup, S. K., Goodman, M., & Yu, Y. L. (1980) *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 165–176, Springer-Verlag, Berlin.

Tu, C. K., & Silverman, D. N. (1977) *J. Biol. Chem. 252*, 3332–3337.

Tu, C. K., Wynns, G. C., McMurray, R. E., & Silverman, D. N. (1978) *J. Biol. Chem. 253*, 8178–8184.

Tu, C. K., Sanyal, G., Wynns, G. C., & Silverman, D. N. (1983) *J. Biol. Chem. 258*, 8867–8871.

Van Niekirk, J. N., Schoening, F. R. L., & Talbot, J. H. (1953) *Acta Crystallogr. 6*, 720.

Wendorff, K. M., Nishita, T., Jabusch, J. R., & Deutsch, H. F. (1985) *J. Biol. Chem. 260*, 6129–6132.

Williams, T. J., & Henkens, R. W. (1985) *Biochemistry 24*, 2459–2462.