BA, which strongly points to the existence of a transitory ternary complex, inhibitor–substrate–carrier, in contrast with the proposal of Klingenberg & Appel (1980), who advocated a single site capable of binding substrates and inhibitors.

In the model of Figure 7, the α and β substructures of the carrier protein are associated. An alternative hypothesis would be that the α and β substructures represent two species of a heterogeneous mixture of carrier protein molecules, a fraction of which only is able to undergo the α to β transition upon FTP binding. This explanation, however, is unlikely since the CATR α site to CATR β site ratio has been reproducibly found to be 1 in more than 20 carrier protein preparations.

The model only summarizes the interactions between the substrate and inhibitor sites, as they are revealed by binding studies with the carrier protein in detergent; it, of course, does not intend to describe the situation of the carrier protein in the mitochondrial membrane with respect to its topology and functioning. However, from the present conclusion, it would be tempting to speculate that the membrane-bound ADP/ATP carrier is a dimer, with four potential interconvertible nucleotide binding sites, so that each subunit possesses a pair of sites. At a given time, the high-affinity site located on one side of one of the two subunits would take in charge ADP or ATP for transport; one may imagine that another site located on the second subunit, opposite to the first one, also interacts with ADP or ATP, but in that case with low affinity. The transport would then be sequential, in accordance with the kinetic data of Duyckaerts et al. (1980) and Barbour & Chan (1981), i.e., the nucleotide firmly bound by the first subunit would be transported and delivered to the other side of the membrane; concomitantly, the loose binding of the other nucleotide to the second subunit would become tighter, and transport of the second nucleotide would proceed in the opposite direction.

Acknowledgments

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Solvent Deuterium Isotope Effects in the Catalysis of Oxygen-18 Exchange by Human Carbonic Anhydrase II†

C. K. Tu and D. N. Silverman*

ABSTRACT: By measuring the rate of exchange at chemical equilibrium of ^{18}O between HCO_3^- and H_2O catalyzed by human carbonic anhydrase II in the absence of buffers, we have determined the rate of release from the enzyme of water bearing substrate oxygen. The ratio of this rate measured in H_2O to the rate measured in D_2O, the solvent deuterium isotope effect, is between 4 and 9 in the range of pH(D) from 5.8 to 8.0, with a value of 8.0 ± 0.7 at pH(D) 6.6 (uncorrected pH meter reading). The magnitude of this isotope effect at pH(D) 6.6 has an exponential dependence on the atom fraction of deuterium in solvent water. We conclude that an intramolecular proton transfer between a proton shuttle group on the enzyme and the active site is rate limiting for the release from the enzyme of water bearing substrate oxygen and involves a change in bonding of more than one proton. In contrast, the solvent deuterium isotope effect on the intermolecular proton transfer between the external buffer imidazole and the active site (or proton shuttle group) of the enzyme is small, 2.3 at pH(D) 7.0, as determined from initial velocity experiments. With a rate constant near 9×10^8 M^{-1} s^{-1}, this intermolecular transfer is limited to a significant extent by diffusion processes.

Carbonic anhydrase is a zinc-containing metalloenzyme that catalyzes the hydration of CO_2, as given in eq 1, in a process

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

that involves at least one proton transfer, that between the enzyme and its solution environment. Measurement of the catalysis in H_2O and D_2O by Steiner et al. (1975) using human carbonic anhydrase II (the high-activity isozyme) and by Pocker & Bjorkquist (1977) using the kinetically equivalent bovine carbonic anhydrase from red cells gave a solvent deuterium isotope effect between 3 and 4 for V_{max} and an isotope effect very close to unity for V_{max}/K_m in both the hydration and dehydration directions. A solvent deuterium isotope effect on V_{max}, for example, is the ratio of V_{max} measured in H_2O

† From the Department of Pharmacology, University of Florida, Gainesville, Florida 32610. *Received June 8, 1982.* This work was supported by a grant from the National Institutes of Health (GM25154).

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to V_{max} measured in D_2O. It was the interpretation of Steiner et al. (1975) that the proton transfer influencing V_{max} but not V_{max}/K_m occurred in a step that is separate and distinct from the catalytic interconversion of CO_2 and HCO_3^-. Support for this idea came from two equilibrium methods that showed no solvent deuterium isotope effect on the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium [Simonsson et al. (1979) measuring the exchange broadening of the ^{13}C NMR[1] resonance in $H^{13}CO_3^-$ and Silverman et al. (1979) measuring the rate of exchange of ^{18}O between CO_2 and water]. Steiner et al. (1975) proposed that the rate-limiting proton transfer occurs in an intramolecular step, shown in eq 3, that is necessary to move protons between the active site and solution:

$$E + CO_2 + H_2O \overset{k_1}{\rightleftharpoons} E^{CO_2}_{H_2O} \overset{k_2}{\rightleftharpoons} E^{H^+}_{HCO_3^-} \overset{k_3}{\rightleftharpoons} EH^+ + HCO_3^- \quad (2)$$

$$EH^+ \overset{k_4}{\rightleftharpoons} HE^+ \quad (3)$$

Here EH^+ denotes the form of enzyme with a protonated activity controlling group that has a pK_a near 7 at physiologic ionic strength; there is a wide body of indirect evidence to indicate that this pK_a represents the loss of a proton from zinc-bound water to form zinc-bound hydroxide (Lindskog, 1982). Equation 3 describes the transfer of a proton between the activity controlling group and a proton acceptor in the enzyme, designated HE^+, which acts as a proton shuttle group. In this proposal both E and HE^+ are considered active in the hydration of CO_2. Later studies showed that the transfer of protons from the enzyme to solution occurs mainly through a buffer-mediated process, as in eq 4 in which B denotes buffer

$$HE^+ + B \overset{k_5}{\rightleftharpoons} E + BH^+ \quad (4)$$

in solution (Silverman & Tu, 1975; Jonsson, et al. 1976). This proton transfer has been found to limit the rate of catalysis of the initial velocity of hydration of CO_2 only at buffer concentrations less than 10 mM (Jonsson et al., 1976). It should be noted that although eq 4 is consistent with the proposal of Steiner et al. (1975), which explains solvent deuterium isotope effects, there is no direct evidence to preclude direct proton transfer between B and EH^+.

If there is no solvent deuterium isotope effect associated with the catalytic interconversion of CO_2 and HCO_3^-, it could be associated with the proposed internal proton transfer of eq 3 and the intermolecular proton transfer of eq 4. This is the topic addressed here. The ^{18}O-exchange method was used since it provides information not only on the catalytic interconversion of CO_2 and HCO_3^- but also on a subsequent step in the catalysis, the rate of dissociation from the enzyme of water bearing substrate oxygen. It is our conclusion that a significant, primary solvent deuterium isotope effect of magnitude between 4 and 9 accompanies the intramolecular proton transfer, eq 3, near neutral pH, supporting the proposal of Steiner et al. (1975). Observation of this isotope effect as a function of deuterium content of solvent suggests proton transfer through water bridges. The magnitude of the solvent deuterium isotope effect on the intermolecular proton transfer between the external buffer imidazole and the enzyme (eq 4) was measured from the enhancement by imidazole of the initial velocity of hydration. This isotope effect was near 2, indicating that steps other than proton transfer, such as encounter or separation of buffer and enzyme, play significant roles in determining the rate of this intermolecular proton transfer.

Experimental Procedures

Materials and Enzyme. Oxygen-18-labeled bicarbonate was prepared by equilibrating $KHCO_3$ with ^{18}O-enriched water (up to 90 atom % ^{18}O), and carbon-13-labeled bicarbonate was prepared by acidifying ^{13}C-enriched barium carbonate (90 atom % ^{13}C) as described by Silverman et al. (1979). Imidazole was purified by recrystallization from benzene. Human carbonic anhydrase II was purified from outdated blood by the affinity chromatography procedure of Khalifah et al. (1977). The resulting enzyme was determined to be greater than 95% pure as determined by polyacrylamide gel electrophoresis, which showed one band. The concentration of human carbonic anhydrase II was obtained from the molar extinction coefficient of 5.4×10^4 M^{-1} cm^{-1} at 280 nm. Distilled, deionized water was passed through an ion-exchange column (Cole-Parmer 1506-35) prior to use. D_2O (99.8%) was placed overnight in activated charcoal; then the charcoal was filtered out, and the remaining D_2O was distilled. Solutions of CO_2 were prepared by dilution of saturated solutions of CO_2 made by bubbling CO_2 into water at 25 °C. This procedure is described by Pocker & Bjorkquist (1977).

pH Measurements. All measurements are presented as uncorrected pH meter readings. This was done to allow a partial cancellation of two factors: (1) the correction required of a pH meter reading to obtain pD (pD = meter reading + 0.4; Glasoe & Long, 1960) and (2) the change in pK_a for almost all acids with pK_a between 3 and 10 [$pK_a(D_2O) - pK_a(H_2O) \approx 0.5$; Bell, 1973]. This procedure shows more clearly than presentation of data at values of pH and pD the superposition of rates (Figure 1). It also shows the approximate agreement between the ionizations controlling catalytic rate and the difference $pK_a(D_2O) - pK_a(H_2O)$ found for small molecules.

Oxygen-18 Exchange. The exchange of ^{18}O between species of CO_2 and water occurs because of the hydration–dehydration cycle in these kinetic experiments carried out at chemical equilibrium (Mills & Urey, 1940):

$$CO^{18}O + H_2O \rightleftharpoons HCOO^{18}O^- + H^+ \rightleftharpoons CO_2 + H_2{}^{18}O \quad (5)$$

Carbonic anhydrase catalyzes this exchange. A second exchange process is the transfer of ^{18}O from ^{12}C-containing species of CO_2 to ^{13}C-containing species of CO_2. The uncatalyzed exchange is shown in eq 6 (Gerster et al., 1973). This

$$^{12}COO^{18}O^{2-} + {}^{13}CO_2 \rightleftharpoons {}^{13}COO^{18}O^{2-} + {}^{12}CO_2 \quad (6)$$

exchange is catalyzed by carbonic anhydrase through labeling of the enzyme with ^{18}O, not by catalysis of the reaction of eq 6 (Silverman et al., 1979). This exchange was measured by placing into solution ^{18}O-labeled bicarbonate not enriched in ^{13}C and ^{13}C-labeled bicarbonate not enriched in ^{18}O and observing the appearance of doubly labeled CO_2. The measurement of the atom fraction of ^{18}O in ^{12}C-containing CO_2, $^{(12)}\alpha$, and in ^{13}C-containing CO_2, $^{(13)}\alpha$, was made with a mass spectrometer by procedures described previously (Silverman et al., 1979):

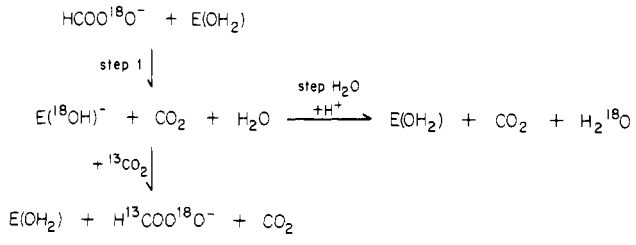
$$^{(12)}\alpha = \frac{(46) + 2(48)}{2[(44) + (46) + (48)]}$$

$$^{(13)}\alpha = \frac{(47) + 2(49)}{2[(45) + (47) + (49)]}$$

where (44), (45), (46), ... represent the heights of the mass

[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Scheme I



peaks of CO_2, $^{13}CO_2$, $CO^{18}O$, ...

The isotope exchange experiments were performed by a procedure described by Silverman et al. (1979). Each experiment was begun by placing into a CO_2 inlet vessel attached to a mass spectrometer 8.0 mL of a solution containing ^{18}O- and ^{13}C-labeled bicarbonate. The total ionic strength was maintained constant at 0.2 by adding Na_2SO_4 in all experiments carried out in the absence of buffer. Experiments using buffers were performed without Na_2SO_4 or other ions to maintain ionic strength. Sulfate is a weak inhibitor of carbonic anhydrase at low pH. Simonsson & Lindskog (1982) have found that 50 mM Na_2SO_4, a concentration comparable with those used in this work, inhibits by about 56% at pH 6 the activity of the hydrolysis of 4-nitrophenyl acetate catalyzed by human carbonic anhydrase II; the extent of inhibition decreases as pH is increased, and it is about 15% at pH 7. We have not corrected for the inhibitory effects of sulfate in the data reported here. All solutions in which ^{18}O exchange was measured contained 3 μM EDTA to sequester metal contaminants. This procedure does not affect the activity of carbonic anhydrase but does prevent inhibition of enzyme by cupric and mercuric ions (Tu et al., 1981). As a further precaution, all vessels were rinsed with EDTA solutions prior to use. Adjustment of pH(D) was made with NaOH (NaOD) and H_2SO_4 (D_2SO_4); the values of pH(D) reported are uncorrected pH meter readings. After adding bicarbonate, we waited for as long as several minutes to allow approach to chemical equilibrium; then measurements of isotopic content of CO_2 were made to determine uncatalyzed exchange rates. Enzyme was added in a volume less than 0.1 mL, and at least 20 s was allowed for mixing before measurements were taken. During an experiment the pH(D) remained constant to within 0.02 pH unit. All data presented here were obtained at 25 °C.

The interpretation of ^{18}O exchange catalyzed by carbonic anhydrase has been discussed thoroughly by Silverman et al. (1979) and Koenig & Brown (1981). The rates at which $^{(12)}\alpha$ and $^{(13)}\alpha$ decrease are biphasic as the exchanges in eq 5 and 6 proceed and can be described by eq 7 and 8. The rate

$$^{(12)}\alpha = b_1e^{-\theta t} + b_2e^{-(\theta+\phi)t} \quad (7)$$

$$^{(13)}\alpha = c_1e^{-\theta t} + c_2e^{-(\theta+\phi)t} \quad (8)$$

constant θ describes the exchange of ^{18}O between CO_2 and H_2O, a first-order process. This rate constant is expressed as the sum of its catalyzed and uncatalyzed components: $\theta = \theta_{cat} + \theta_{uncat}$. The rate constant ϕ describes 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 carbonic anhydrase (Gerster et al., 1973): $\phi = \phi_{cat} + \phi_{uncat}$. Silverman & Tu (1976) have described how θ_{cat} and ϕ_{cat} are obtained from experiments.

The rate constants for ^{18}O exchange θ_{cat} and ϕ_{cat} have been used by Silverman et al. (1979) to obtain the rates of two steps in the catalytic pathway, when the pathway is based on a zinc–hydroxide mechanism. In this mechanism a proton transfer step converts zinc-bound hydroxide to zinc-bound water, which allows water to exchange rapidly with solvent (see Scheme I). Koenig & Brown (1981) have presented the interpretation on the basis of a zinc-bound water mechanism in which zinc-bound water is the only catalytically active form of the enzyme. In Scheme I, step 1 is the dehydration of ^{18}O-labeled bicarbonate giving CO_2 and leaving ^{18}O at the active site. The step in which $^{13}CO_2$ reacts with labeled active site to yield $H^{13}COO^{18}O^-$ is a hydration step having the same rate as step 1 at equilibrium. The rate of step 1 at chemical equilibrium is denoted R_1. Step H_2O is the release to solvent of water bearing substrate oxygen, a step that is assumed to occur independently of step 1. The rate of step H_2O is denoted R_{H_2O}.

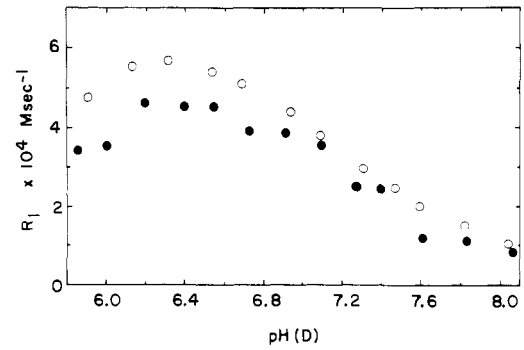


FIGURE 1: Catalyzed rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium R_1 as a function of uncorrected pH meter reading pH(D). The solvent was H_2O (○) or 98% D_2O (●) at 25 °C with a total concentration of all CO_2 species at 15 mM. Human carbonic anhydrase II was present at 2.5×10^{-9} M and EDTA at 3×10^{-6} M. The ionic strength was maintained at 0.2 with Na_2SO_4; no buffers were used.

Solution of the kinetic equations describing ^{18}O-exchange on the basis of Scheme I yields the following expressions for R_1 and R_{H_2O} (Silverman et al., 1979):

$$R_1 = 3[S_{tot}](\theta_{cat} + \phi_{cat}) \quad (9)$$

$$R_{H_2O} = \frac{R_1\theta_{cat}}{\phi_{cat}}\left(1 - \frac{[CO_2]}{3[S_{tot}]}\right) \quad (10)$$

$$[S_{tot}] = [CO_2] + [HCO_3^-]$$

Stopped-Flow Methods. Initial velocity measurements of the hydration of CO_2 were carried out on a Durrum-Gibson stopped-flow spectrophotometer by procedures described by Venkatasubban & Silverman (1980). The buffer–indicator pair of imidazole and *p*-nitrophenol was used, and the buffer factor relating changes in absorption to changes in $[H^+]$ was estimated by the method of Khalifah (1971). All experiments were performed at 25 °C and the ionic strength was maintained at 0.2 with Na_2SO_4.

Results

We have measured the two rates R_1 and R_{H_2O} using solutions containing human carbonic anhydrase II. These two rates are obtained from ^{18}O exchange processes: R_1 is the rate at chemical equilibrium of the interconversion of CO_2 and HCO_3^- and R_{H_2O} is the rate of release from the enzyme of water bearing substrate oxygen. The variation of R_1 with pH(D) (uncorrected pH meter reading) in H_2O and D_2O in the absence of buffer is given in Figure 1; the analogous plot for R_{H_2O} is given in Figure 2. These plots were calculated from θ_{cat} and ϕ_{cat} with eq 9 and 10. Values of θ_{cat} and ϕ_{cat} for selected values of pH(D) are given in Table I. These data were obtained with different enzyme solutions on different days and, hence, do not have sufficient controls to obtain isotope effects

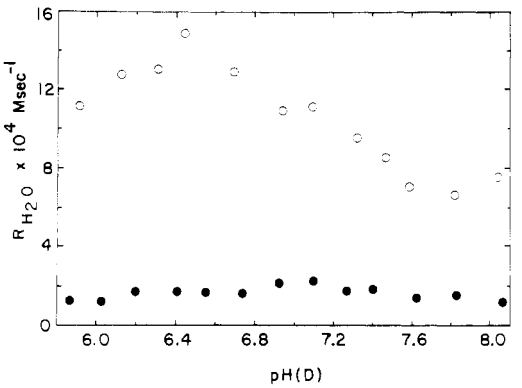


FIGURE 2: Rate of release from carbonic anhydrase of water bearing substrate oxygen R_{H_2O} measured in H_2O (○) or 98% D_2O (●). Experimental conditions are identical with those in Figure 1.

Table I: Variation with pH of Rate Constants for ^{18}O Exchange θ_{cat} and ϕ_{cat} Obtained in H_2O and D_2O in the Absence of Buffers[a]

	solvent H_2O[c]		solvent 98% D_2O	
pH(D)[b]	θ_{cat} (×10^3 s^{-1})	ϕ_{cat} (×10^3 s^{-1})	θ_{cat} (×10^3 s^{-1})	ϕ_{cat} (×10^3 s^{-1})
6.2	8.8	3.4	3.5	7.0
6.5	9.3	3.5	3.5	6.9
6.9	7.2	2.8	3.1	5.7
7.3	4.9	1.5	2.0	2.8
7.6	3.4	1.0	1.4	1.7
8.0	2.0	0.28	1.0	0.86

[a] Human carbonic anhydrase II was present at 2.5 × 10^{-9} M with $[CO_2]$ + $[HCO_3^-]$ at 15 mM. Solutions were maintained at 0.2 ionic strength by addition of Na_2SO_4 and contained no added buffers. The temperature was 25 °C. [b] Uncorrected pH meter reading. [c] The uncatalyzed values of these rate constants in H_2O at pH 7.4 are θ_{uncat} = 7.4 × 10^{-4} s^{-1} and ϕ_{uncat} = 1.1 × 10^{-4} s^{-1}.

with an accuracy greater than 15%. However, the data show a plateau in a region of pH(D) near 6.1–6.8 in which accurate measurements were attempted.

An accurate measurement of the solvent deuterium isotope effect on R_1 was 1.05 ± 0.03 (mean and standard deviation of four experiments) at pH(D) 6.6 under the conditions listed in the legend to Figure 3. The solvent deuterium isotope effect on R_{H_2O} was 8.0 ± 0.7 under the same conditions. Data obtained with an atom fraction of deuterium in solvent n = 0.49 suggest that there is no variation of R_1 with n but that R_{H_2O} plotted as a function of n is nonlinear and bulging down (Figure 3). The values of θ_{cat} and ϕ_{cat} from which this plot was calculated are given in the legend to Figure 3.

Values of θ_{cat} and ϕ_{cat} and R_1 and R_{H_2O} obtained in the presence of 50 mM imidazole at pH(D) 6.6 and 25 mM Tris–sulfate at pH(D) 8.0 are given in Table II.

A Hanes plot of the buffer dependence of the initial velocity of the hydration of CO_2 catalyzed by carbonic anhydrase II is presented in Figure 4. The data for both H_2O and D_2O

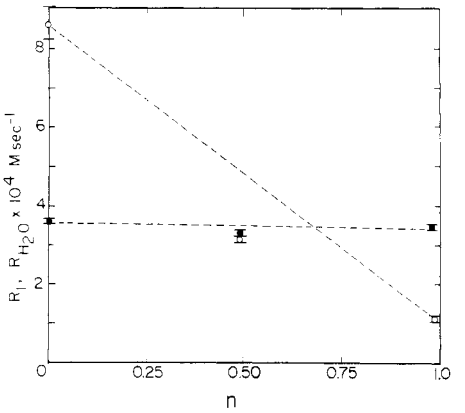


FIGURE 3: R_{H_2O} (○) and R_1 (●) at three values of n, the atom fraction of deuterium in solvent water. Data are the mean and standard deviation of four experiments. Human carbonic anhydrase II was present at 2.0 × 10^{-9} M and the total concentration of CO_2 species was 15 mM. The uncorrected pH meter reading was 6.6 at 25 °C. No buffers were used. Rate constants for ^{18}O exchange were as follows: at n = 0, θ_{cat} = (5.8 ± 0.07) × 10^{-3} s^{-1} and ϕ_{cat} = (2.26 ± 0.09) × 10^{-3} s^{-1}; at n = 0.49, θ_{cat} = (3.70 ± 0.12) × 10^{-3} s^{-1} and ϕ_{cat} = (3.58 ± 0.08) × 10^{-3} s^{-1}; at n = 0.98, θ_{cat} = (1.93 ± 0.12) × 10^{-3} s^{-1} and ϕ_{cat} = (5.77 ± 0.12) × 10^{-3} s^{-1}.

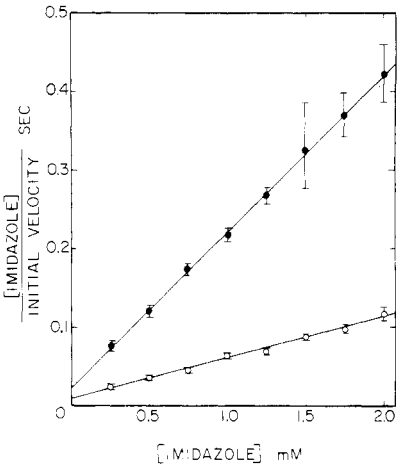


FIGURE 4: Hanes plot of buffer dependence of hydration of CO_2 in H_2O (○) and 99% D_2O (●) at an uncorrected pH meter reading of 7.0 and 25 °C. [Imidazole] is the concentration of the basic form of this buffer. The concentration of human carbonic anhydrase II was 1.1 × 10^{-7} M with indicator p-nitrophenol at 2.5 × 10^{-5} M and the total ionic strength maintained constant at 0.2 by the addition of Na_2SO_4. The initial concentration of CO_2 was 8.5 mM. Data are the mean and standard deviation of four experiments.

were obtained by using imidazole as buffer at a concentration of 8.5 mM CO_2 and an uncorrected pH meter reading of 7.0. The rate constant k_5 of eq 4 was obtained from the intercept in Figure 4 according to the following expression:

$$\frac{[B]}{v_{cat}} = \frac{1}{k_5[E]} + \frac{[B]}{k_{cat}[E]}\left[1 + \frac{K_m}{[CO_2]}\right] \qquad (11)$$

Table II: Rate Constants for ^{18}O Exchange θ_{cat} and ϕ_{cat} and Rates R_1 and R_{H_2O} Obtained in H_2O and D_2O in the Presence of Buffer[a]

	solvent H_2O				solvent 98% D_2O				
pH(D)[b]	θ_{cat} (×10^3 s^{-1})	ϕ_{cat} (×10^3 s^{-1})	R_1 (×10^3 M s^{-1})	R_{H_2O} (×10^3 M s^{-1})	θ_{cat} (×10^3 s^{-1})	ϕ_{cat} (×10^3 s^{-1})	R_1 (×10^3 M s^{-1})	R_{D_2O} (×10^3 M s^{-1})	R_{H_2O}/R_{D_2O}
6.6	6.5	1.9	0.38	1.2	2.3	5.2	0.34	0.14	8.6
8.0	2.5	0.67	0.29	1.1	1.6	1.6	0.29	0.29	3.8

[a] Solutions at pH(D) 6.6 contained human carbonic anhydrase II at 2.0 × 10^{-9} M with $[CO_2]$ + $[HCO_3^-]$ at 15 mM and 50 mM imidazole. Solutions at pH(D) 8.0 contained human carbonic anhydrase II at 4.7 × 10^{-9} M with $[CO_2]$ + $[HCO_3^-]$ at 30 mM and 25 mM Tris–sulfate. All solutions were maintained at 0.2 ionic strength with Na_2SO_4. The temperature was 25 °C.

This is a form of the initial velocity equation that is consistent with the observed hydration of CO_2 and protonation of external buffer catalyzed by human carbonic anhydrase II (Rowlett & Silverman, 1982). Here, [B] is the concentration of the basic form of external buffer, k_{cat} is the turnover number, and K_m is the Michaelis constant for CO_2 when buffer is present at large concentrations. Analysis of the intercept in Figure 4 gives $k_5 = (8.8 \pm 0.5) \times 10^8$ M^{-1} s^{-1} in H_2O and $k_5 = (3.8 \pm 0.3) \times 10^8$ M^{-1} s^{-1} in 98% D_2O (mean and standard deviation). The solvent deuterium isotope effect is $k_5^{H_2O}/k_5^{D_2O} = 2.3 \pm 0.3$. It should be pointed out that k_5 for imidazole is anomalously large among many buffers, including imidazole derivatives, which have been studied; other buffers of pK_a near 7 have values of k_5 closer to 2×10^8 M^{-1} s^{-1} (Jonsson et al., 1976; Rowlett & Silverman, 1982).

Discussion

The ^{18}O exchange method allows us to determine two rates in the catalysis of the hydration of CO_2 by carbonic anhydrase: R_1, the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, and R_{H_2O}, the rate of release from the enzyme of water bearing substrate oxygen. The values of R_1 obtained with the ^{18}O method have been shown (Silverman et al., 1979) to be the same as the values of R_1 determined by exchange broadening of the ^{13}C resonance line widths of $H^{13}CO_3^-$ in the NMR work of Simonsson et al. (1979). These studies are also in agreement that the change of solvent from H_2O to D_2O has no appreciable effect on the magnitude of R_1 at values of pH(D) (uncorrected pH meter reading) 6.9 and 7.4, results that have been confirmed in this study in the range of pH(D) from 5.8 to 8.0 (see Figure 1). The magnitude of the solvent deuterium isotope effect on R_1 found from four measurements at pH(D) 6.6 was 1.05 ± 0.03 (mean and standard deviation). The implications of this result on the catalytic pathway have been discussed by Simonsson et al. (1979): the data favor a mechanism in which the rate-limiting step in the interconversion of CO_2 and HCO_3^- does not involve proton transfer. This does not preclude a proton transfer in the catalytic interconversion of CO_2 and HCO_3^- but suggests that a proton transfer does not occur in the rate-limiting step. For example, release of product HCO_3^- could be rate limiting.

The main emphasis here concerns the large solvent deuterium isotope effect on R_{H_2O}, which is as great at 8 at pH(D) <7 and 3–5 at pH(D) near 8 (Figure 2), consistent with the result of four measurements at pH(D) 6.6 of 8.0 ± 0.7. The very different isotope effects on R_1 and R_{H_2O} certainly confirm that these rates are controlled by different steps in the catalytic pathway. We address here the possibility that the isotope effect on R_{H_2O} is caused by a conformational change upon change of solvents. We consider this possibility unlikely on the basis of the observation that several kinetic properties of the enzyme do not vary when the solvent is changed from H_2O to D_2O: R_1 as measured both by ^{18}O exchange and by ^{13}C NMR as described above and k_{cat}/K_m for initial velocity of hydration of CO_2 and dehydration of HCO_3^- (Steiner et al., 1975; Pocker & Bjorkquist, 1977). Moreover, the lack of a significant isotope effect on R_1 in the range of pH(D) 5.8–8.0 suggests that we have adequately compensated for the change in pK_a of ionizable groups controlling activity upon changing solvent from H_2O to D_2O. This was done by performing experiments in H_2O and D_2O at the same uncorrected pH meter readings, allowing the change in pK_a [$pK_a(D_2O) - pK_a(H_2O)$] of about 0.5 for almost all acids with a pK_a between 3 and 10 (Bell, 1973) to be cancelled by the correction required for a pH meter reading in 100% D_2O (pD = meter reading + 0.4; Glasoe & Long, 1960). Hence, although we report the isotope effect on R_{H_2O}, which is a rate, we believe that this is an isotope effect on rate constants and not an isotope effect on the concentration of that form of enzyme from which water dissociates.

The main conclusion of this study is that the large solvent deuterium isotope effect observed for R_{H_2O} indicates the presence of proton transfer in a step or steps that are rate limiting for R_{H_2O}. Several pertinent facts help us narrow the possibilities for which step this is. First, Silverman (1981) measured $1/T_1$ for H_2O has shown that the fractionation factor is indistinguishable from unity for exchangeable hydrogen in the inner coordination sphere of cobalt in Co(II)-substituted, bovine carbonic anhydrase. Consequently, it is unlikely that a deuterium isotope effect will be observed for the exchange of water between bulk solvent and its position as a ligand of the metal in carbonic anhydrase. Second, the large deuterium isotope effect on R_{H_2O} was observed in the absence of buffers. Consequently, the proton transfer involved in R_{H_2O} is not between external buffer and the enzyme. Substrate itself does not act as a proton transfer agent to affect R_{H_2O}, at least not at pH near 7 (Silverman et al., 1979), and the magnitude of R_{H_2O} precludes the possibility of a significant contribution of proton transfer between species of water and enzyme.[2] Hence, the proton transfer or transfers influencing the rate-limiting step must be intramolecular and must occur not in the interconversion of CO_2–HCO_3^- but in protonation steps that lead to the release to solvent of water bearing substrate oxygen. This proton transfer could very well be that described in eq 3, or it could be outside the catalytic pathway in experiments that measure the net production or consumption of protons at initial velocity.

To obtain an estimate of the number of hydrogens that change their fractionation factor in the pathway, we have plotted in Figure 3 R_{H_2O} as a function of three values of n, the atom fraction of deuterium in solvent. The larger standard deviation in R_{H_2O} for $n = 0$ results from the experimental error in the small value of ϕ_{cat}, an error that is then propagated into the value of R_{H_2O}. The values of ϕ_{cat} at $n = 0.49$ and 0.98 are larger, and the experimental error is a smaller fraction of the observed ϕ_{cat}. Despite the uncertainty of the true value of R_{H_2O} at $n = 0$, the data of Figure 3 are significantly nonlinear and bulging down. There is sufficient precision in Figure 3 to rule out a mechanism in which one proton is transferred in the transition state. This suggests the general form of the Gross–Butler equation for the proton transfer and indicates a transition state in which two or more hydrogens have fractionation factors different than in the reactant state (Schowen, 1978).

Venkatasubban & Silverman (1980) showed that for hydration of CO_2 catalyzed by bovine carbonic anhydrase at pH(D) 8.2, plots of both ln V_{max} and ln K_m vs. n are linear. Figure 5 demonstrates that ln R_{H_2O} vs. n is also linear. This behavior is consistent with a multiproton model in which many equivalent protons all contribute a small but approximately equal normal isotope effect. Venkatasubban & Silverman commented on the application of this model to the catalysis and concluded that the proton transfer mechanism involves

[2] A lower limit for the rate constant of the dissociation of water bearing substrate oxygen is $R_{H_2O}/[E]_{tot} \simeq (10^{-3}\ M\ s^{-1})/(3 \times 10^{-9}\ M) = 3.3 \times 10^5\ s^{-1}$ at pH 7.4 under conditions given in the legend to Figure 1. Proton transfer between solvent water and a proton acceptor group of $pK_a \sim 7$ is 10^2–10^3 s^{-1} (Eigen, 1964), insufficient to account for a turnover of 3.3×10^5 s^{-1}. The diffusion-controlled limit for proton transfer between H_3O^+ and a proton acceptor is $k_2[H_3O^+][E]_{tot} = (10^{10}\ M^{-1}\ s^{-1})(10^{-7}\ M)(3 \times 10^{-9}\ M) = 3 \times 10^{-6}\ M\ s^{-1}$, which is less than the observed value of R_{H_2O}.

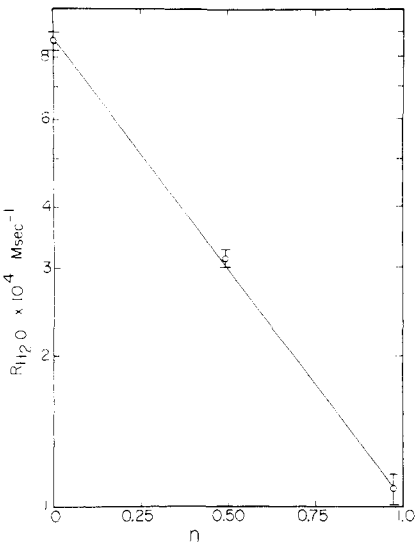
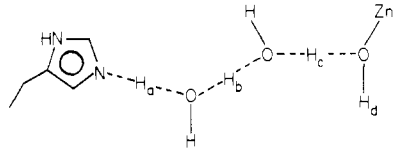


FIGURE 5: Data of Figure 3 plotted with a logarithmic ordinate.

solvating molecules in the active site to a significant extent. This identical observation in both a steady state and an equilibrium experiment supports further the comment that the same rate-limiting proton transfer is manifested in the isotope effects on V_{max} and R_{H_2O}. In their analysis, Venkatasubban and Silverman used the interpretation of Steiner et al. (1975) that the observed isotope effect was involved in an intramolecular proton transfer not part of the catalytic steps that actually convert CO_2 to HCO_3^-. The results of the work reported here have given a more direct confirmation of that interpretation since an isotope effect was observed on R_{H_2O} and not on R_1. A likely mechanism for a proton transfer is shown in eq 12, the same as presented by Venkatasubban &



(12)

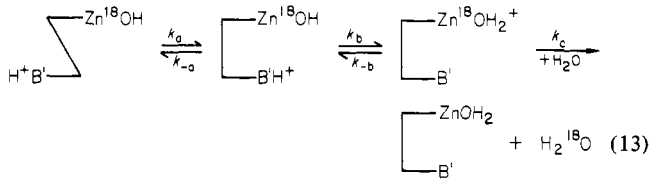
Silverman (1980), and involves water bridges in the transfer of a proton between the imidazole side chain of His-64 and the aqueous ligand of zinc. Three hydrogens (H_a, H_b, and H_c) change bonding in the transition state. The fourth (H_d) probably would not be seen since the fractionation factor for zinc-bound hydroxide is expected to be close to 1.0, the same value expected for zinc-bound water (Chiang et al., 1980; Silverman, 1981). The remaining hydrogens in the diagram do not change bonding in the proton transfer.

The most probable proton transfer group at pH values near 7 is the imidazole side chain of His-64, which was originally suggested and supported by Steiner et al. (1975). The imidazole ring of His-64 is about 6 Å from the zinc (Norstrand et al., 1975) in human carbonic anhydrase II, and observation of its magnetic resonance spectrum indicates a pK_a of 7.1 (Campbell et al., 1975). The proton transfer is to the active site; a large amount of data supports the hypothesis that the active site is zinc-bound water that dissociates to form zinc-bound hydroxide with a pK_a near 7 under the conditions of ionic strength used in this study (Lindskog, 1982).

The magnitude of the solvent deuterium isotope effect on R_{H_2O} is also significant. Having a value between 6 and 9 in the range of pH 6–7, this isotope effect is much greater than the maximum isotope effect of 3–4 observed for intermolecular proton transfer between nitrogen and oxygen acids and bases (Bergman et al., 1978; Cox & Jencks, 1978). These isotope effects are believed to reach their maximal value when the transition state for proton transfer is symmetrical, a condition that is met when the difference between the pK_a of proton donor and acceptor is close to zero. At $\Delta pK_a \sim 0$, the significant steps are encounter of reactants, proton transfer, and separation of products; only the proton transfer is normally expected to have an appreciable isotope effect. One interpretation of the large isotope effect observed for R_{H_2O} is that ΔpK_a is close to zero for the intramolecular proton transfer and that encounter of reactants and separation of products has less of a kinetically significant role in this process than in intermolecular proton transfers.

The participation of water bridges in the mechanism indicates proton transfer along hydrogen bonds connecting the proton donor and acceptor. For oxygen and nitrogen acids and bases, proton transfer through intervening water bridges is common (Grunwald & Eustace, 1975). As an example, the proton transfer between 2,4-lutidine and the 2,4-lutidinium ion has been found largely quatermolecular in nature, that is, involving two intervening water molecules. Rosenthal & Grunwald (1972) have found the rate constant for that proton transfer to be about 1×10^8 M^{-1} s^{-1} with a solvent deuterium isotope effect near 4. Proton transfer between the imidazolium ion and imidazole proceeds with one or more intervening water molecules and a rate constant of 1×10^8 M^{-1} s^{-1} (Ralph & Grunwald, 1968). NMR results confirm that rate constants for proton transfer processes with water participation can be of magnitude similar to this. A cyclic, intramolecular proton transfer in acetic acid involving two water molecules proceeds with a rate constant near 5×10^7 s^{-1} (Luz & Meiboom, 1963). The isotope effect for such transfers is characterized by a fractionation factor near 0.5 for the hydrogen in flight in the transition state. This has been the case for solvent participation in the hydrolysis of *o*-(dichloroacetyl)salicylate anion, which proceeds with changes in the fractionation factor of one hydrogen ($\phi^T = 0.46$, Minor & Schowen, 1973); the deacylation of acetylchymotrypsin, which also occurs with changes in the fractionation factor of one proton ($\phi^T = 0.42$, Pollock et al., 1973); and isomerization of ribonuclease A in which a rate-limiting ionization of the imidazole ring of a histidine precedes a conformational change ($\phi^T = 0.69$, Wang et al., 1975). From the model of Venkatasubban & Silverman (1980) and on the assumption of reactant-state fractionation factors of unity, as discussed previously, we calculate an isotope effect of 8.0 from the Gross–Butler equation involving transfer of three equivalent protons. [$k_0/k_n = (1 - n + 0.5n)^{-3} = 8$ for $n = 1$. This is obtained directly from the Gross–Butler equation in which n is the atom fraction of deuterium in solvent, the fractionation factor for the hydrogen in flight is 0.5 and reactant-state fractionation factors are unity.] This is to be compared with the experimental value $R_{H_2O}/R_{D_2O} = 8.0 \pm 0.7$ at pH(D) 6.6.

The rate R_{H_2O} must then include at least one intramolecular proton transfer in a rate-determining step and a second step that is the release of water from the enzyme to the solvent. It is necessary to ask at this point how an intramolecular proton transfer between oxygen and nitrogen acids and bases, which is usually a very fast reaction [see above and Eigen (1964)], can exert an isotope effect on R_{H_2O}, which appears to be comparatively slow ($R_{H_2O}/[E]_{tot} = 10^5$–$10^6$ s^{-1}). It is possible that an isotope effect is observed on R_{H_2O} because the rate-limiting proton transfer is preceded by an unfavorable equilibrium step in a complex reaction scheme. These steps in R_{H_2O} are shown in eq 13, in terms of a zinc–hydroxide mechanism (Lindskog, 1982). Step a is a conformational change including



(13)

perhaps a change in orientation of solvent within the active-site cleft that brings a proton-bearing side chain ($B'H^+$) of the enzyme into a position from which proton transfer can occur to the zinc-bound hydroxide. The deuterium isotope effect on such solvent reorganization steps is expected to be modest, of a magnitude near 1.3 (Schowen, 1978). Step b is the intramolecular proton transfer itself (probably involving water bridges), and step c is the exchange of zinc-bound water with water from bulk solvent. Step c is written as an irreversible step since $H_2{}^{18}O$ is essentially infinitely diluted by solvent $H_2{}^{16}O$.

A value of k_a/k_{-a} that is both small and independent of the deuterium content of solvent is consistent with the observation of an isotope effect for the intramolecular proton transfer in the relatively slow process described by R_{H_2O}. In this simplified mechanism, the observed isotope effect on R_{H_2O} consists of the isotope effect on k_b modified by commitment factors.[3] The slow overall rate is a consequence of conformational fluctuations in the enzyme, the proton-bearing residue and its solvent shell being in an orientation favorable for proton transfer only a small fraction of the time. Equation 13 is a simplified model meant to explain qualitatively the experimentally observed isotope effect. Another possibility not involving a conformational change is that the geometry for the intramolecular proton transfer in the active site may not be ideal. That is, the angle or more likely the distance required for the proton transfer may be constrained and unfavorable.

In the presence of buffer the observed isotope effect on R_{H_2O} at pH(D) 6.6 and 8.0 was nearly identical with that observed in the absence of buffer (Table II). This suggests that in the presence and absence of buffer the same step is rate limiting.

The isotope effect on the intermolecular proton transfer step described by k_5 of eq 4 is small, $k_5{}^{H_2O}/k_5{}^{D_2O} = 2.3$ when the buffer is imidazole under the steady-state conditions described in Figure 4. The rate constant $k_5 = 8.8 \times 10^8$ M^{-1} s^{-1} in H_2O describes the transfer of a proton between the buffer imidazole ($pK_a = 7.1$) and either the proton shuttle group (believed to be the imidazole side chain of His-64 with $pK \sim 7$; Campbell et al., 1975) or the active site itself ($pK_a \sim 7$). Hence, the proton transfer described by k_5 most likely occurs between groups of similar pK_a, is close to the diffusion-controlled limit, and has an isotope effect much lower than that observed for R_{H_2O}. We conclude that the intermolecular proton transfer described by k_5 for imidazole under the conditions described here is limited in rate more significantly by diffusion of reactants than is R_{H_2O}.

[3] The overall rate constant for R_{H_2O} as written in eq 13 is the composite of the following rate constants:

$$\frac{R_{H_2O}}{[E']} = k_t = \frac{k_a k_b / k_{-a}}{k_{-b}/k_c + k_b/k_{-a} + 1} \qquad (14)$$

In this equation [E'] is the concentration of the enzyme form on the left in eq 13. If one assumes that our procedure of comparing experiments at identical values of pH meter readings is correct and [E'] in H_2O equals [E'] in D_2O, then the isotope effect on R_{H_2O} is

$$\frac{R_{H_2O}}{R_{D_2O}} = \frac{{}^Dk_b + k_b/k_{-a} + k_{-b}{}^DK_{eq}/k_c}{1 + k_b/k_{-a} + k_{-b}/k_c}$$

The expressions k_b/k_{-a} and k_{-b}/k_c are commitments which can make R_{H_2O}/R_{D_2O} less than the intrinsic isotope effect ${}^Dk_b$ (Northrop, 1975). ${}^DK_{eq}$ is the isotope effect on k_b/k_{-b}. ${}^Dk_b$ is the solvent deuterium isotope effect on k_b.

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Kinetics of Oxygen Exchange at the Anomeric Carbon Atom of D-Glucose and D-Erythrose Using the Oxygen-18 Isotope Effect in Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

John M. Risley and Robert L. Van Etten*

ABSTRACT: The ^{18}O isotope induced shift in ^{13}C nuclear magnetic resonance (NMR) spectroscopy affords a new and convenient method for the study of oxygen exchange at the anomeric carbon atom of simple sugars. The efficacy of the technique was confirmed by a study of the oxygen exchange reaction of D-[1-^{13}C]glucose. At pH 7.0 and 61 °C, the incorporation of ^{18}O from solvent $H_2{}^{18}O$ onto the C-1 carbon atom of the diastereomeric α- and β-pyranose sugars was followed by ^{13}C NMR spectroscopy in a continuous assay mode. The pseudo-first-order rate constant for exchange of both the α and the β anomers was 9.5×10^{-5} s^{-1}, which is in agreement with a rate constant obtained in a previous study by a chemical conversion–mass spectrometry technique. The new technique was applied to a study of the oxygen exchange at the anomeric carbon atom of D-[1-^{13}C]erythrose, a furanose sugar for which no experimental data were available. In unbuffered, aqueous solutions the incorporation of the ^{18}O label from the medium ($H_2{}^{18}O$) onto the C-1 carbon atom of the α- and β-D-[1-^{13}C]erythrose and the D-[1-^{13}C]erythrose hydrate forms was followed by ^{13}C NMR at 10, 23, and 36 °C. From analysis of the data for the α and β diastereomers, the pseudo-first-order rate constants for exchange were 1.4×10^{-4} s^{-1} at 10 °C, 4.8×10^{-4} s^{-1} at 23 °C, and 8×10^{-4} s^{-1} at 36 °C, and the apparent energy of activation for the exchange reaction was 12.1 kcal/mol. Particularly in conjunction with the use of specifically ^{13}C-enriched sugars, the new technique for studying oxygen exchange reactions of carbohydrates has many distinct advantages over earlier approaches, including the ability to follow simultaneously the exchange reactions of all of the sugar species for which a ^{13}C NMR signal can be detected, the continuity of the assay, the avoidance of possible artifacts due to incomplete or selective derivatization reactions, and the simplicity of the data analysis.

Mutarotation (tautomerization) of simple sugar molecules in aqueous solution is a fundamental interconversion process and one upon which many of the chemical and biological properties of the carbohydrates depend (Schray & Benkovic, 1978). The nonenzymatic conversion of simple sugars between their acyclic and diastereomeric α and β forms has been studied extensively. A sugar molecule may undergo a "simple" or a "complex" mutarotation, and the macroscopic rate constants for the mutarotation reaction have been measured by a variety of techniques (Pigman & Isbell, 1968; Isbell & Pigman, 1969). The experimental evidence supports a mechanism for anomerization in aqueous solution that involves an aldehydo or keto sugar intermediate. Moreover, as with other carbonyl derivatives in aqueous solution, the postulated aldehydo or keto sugar intermediate is expected to be in equilibrium with the hydrate (*gem*-diol), formed by nucleophilic addition of water to the carbonyl carbon. Thus, in the most simple system, three simultaneous equilibria involving four species exist: (1) the α sugar with the aldehydo or keto sugar, (2) the β sugar with the aldehydo or keto sugar, and (3) the aldehydo or keto sugar with its hydrate.

In recent years, more sophisticated experimental techniques have allowed a greater understanding of the mutarotation reaction, including estimates and measurements of the microscopic rate constants that govern the equilibria (Wertz et al., 1981; Serianni et al., 1982). The mutarotation reaction is typically studied by dissolving a diastereomerically homogeneous sugar in water and then following the resultant changes in optical rotation or other property until equilibrium is reached. Support for the presence of an aldehydo or keto sugar intermediate comes from the studies of oxygen exchange reactions at the anomeric carbon atom. The technique for studying the latter reaction involves incubating either an ^{18}O-labeled[1] sugar in normal water or an unlabeled sugar in [^{18}O]water and analyzing the ^{18}O content of the sugar as a function of time. Despite the importance of such experiments in providing data on the rate of formation and reactivity of

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[1] Abbreviations: ^{18}O, oxygen-18; ^{13}C, carbon-13; NMR, nuclear magnetic resonance; E_a, Arrhenius energy of activation; FID, free induction decay; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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