pounds appeared at identical chemical shift although those of **8** were more of a structured doublet than a multiplet. Butadiene was used as the trap because other dienes (e.g., cyclopentadiene) freeze at −110°.
(12) No adduct was observed at −60°.
(13) Prepared by T. T. Coburn to whom the authors express grateful appreciation.
(14) This surprising product finds ample precedent in the reaction of other carbenes with methyl ethers although the fate of the displaced CH_2 is not known.[15]
(15) Cf. W. Kirmse, "Carbene Chemistry", 2nd ed, Academic Press, New York, N.Y., 1971.
(16) For examples of ring openings of 3*H*-pyrazoles to diazoalkanes, see G. L. Closs and W. A. Boll, *J. Am. Chem. Soc.*, **95**, 3904 (1963); *Angew. Chem., Int. Ed. Engl.*, **2**, 399 (1963); M. Franck-Newmann and C. Buchecker, *Ibid.*, **9**, 526 (1970).
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The Mechanism of Carbonic Anhydrase Studied by ^{13}C and ^{18}O Labeling of Carbon Dioxide

Sir:

The very rapid rate of hydration of CO_2 catalyzed by carbonic anhydrase (EC 4.2.1.1) has been explained by the hypothesis that there is a proton transfer step in the mechanism involving the enzyme and buffers in solution.[1-3] Supporting this explanation, a buffer dependence of the carbonic anhydrase-catalyzed exchange of oxygen-18 between bicarbonate and water at equilibrium and alkaline pH has been reported.[4] We present here results which further support this hypothesis by measuring the buffer dependence of the catalysis by bovine carbonic anhydrase of two types of ^{18}O exchange involving species of CO_2 and water. Type I: the ^{18}O exchange between bicarbonate and water[5,6] has been measured near neutral pH where the enzyme is most active. Type II: the exchange of ^{18}O between species of CO_2 in solution, measured as the exchange of ^{18}O between ^{12}C- and ^{13}C-containing molecules,[7,8] has been measured in the same pH region.

The atom fraction of ^{18}O in carbon dioxide, α, includes all ^{12}C- and ^{13}C-containing molecules. However, we designate as $^{(12)}\alpha$ the atom fraction of ^{18}O in ^{12}C-containing CO_2 only.[8] In type I exchange, the decay of $(\alpha - \alpha_\infty)$ is a first-order process with the following rate constant:[4]

$$\theta_1 = \theta_{cat} + \theta_{uncat} = (f_{HCO_3^-})(k_c + k_{uncat})/3$$

where k_{uncat} is the rate constant for the uncatalyzed dehydration of HCO_3^- and k_c is the first-order rate constant describing the catalyzed dehydration of HCO_3^- at equilibrium. The fraction of all CO_2 species existing as bicarbonate is $f_{HCO_3^-}$. The kinetic equations describing type II exchange have been derived.[8] The first-order rate constant ϕ ($\phi = \phi_{cat} + \phi_{uncat}$) describes the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2 in solution. The sum $(\theta_1 + \phi)$ is obtained as the slope of a plot of $-\ln(^{(12)}\alpha - \alpha)$ vs. time.[8]

The preparation of ^{18}O- and ^{13}C-enriched bicarbonate, as well as bovine carbonic anhydrase, is identical with that described earlier.[4,8] The isotopic enrichments were measured on a Finnigan 3000 mass spectrometer and monitored continuously using a CO_2 inlet system which allows CO_2 to pass across a membrane in contact with the reaction solution.[8] All solutions were 1.6×10^{-9} *M* bovine carbonic anhydrase (BCA), 10 m*M* total CO_2 species, and were maintained at an ionic strength of 0.2 with Na_2SO_4.

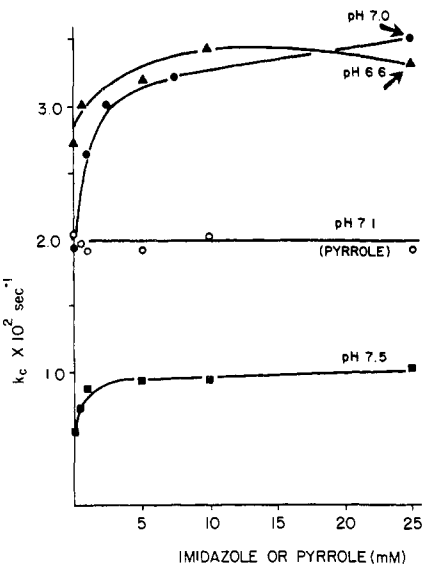


Figure 1. The first-order rate constant k_c at 25° for catalyzed dehydration of bicarbonate at equilibrium as a function of concentration of imidazole at pH 7.5 (■), pH 7.0 (●), and pH 6.6 (▲) or pyrrole (○) at pH 7.1. The concentration of bovine carbonic anhydrase was 1.6×10^{-9} *M* and the total concentration of CO_2 species was 10 m*M*. Ionic strength was maintained at 0.2 with the noninhibitory Na_2SO_4.

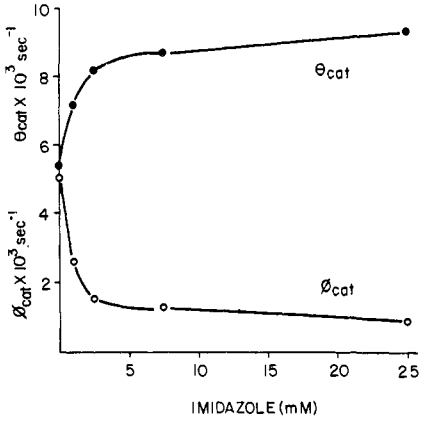
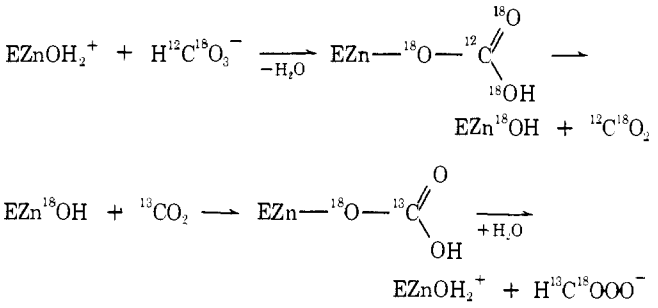


Figure 2. The first-order rate constants at 25° and pH 7.0 for the catalyzed exchange of ^{18}O between bicarbonate and water, θ_{cat} (●), and for the catalyzed exchange of ^{18}O between ^{12}C- and ^{13}C-containing CO_2 species, ϕ_{cat} (○), as a function of imidazole concentration. The concentration of bovine carbonic anhydrase was 1.6×10^{-9} *M* and the total concentration of CO_2 species was 10 m*M* with ionic strength held constant at 0.2 using Na_2SO_4. The ^{13}C enrichment was 44-46% in a final volume of 8 ml.

As in previous studies at alkaline pH,[4] k_c near neutral pH is dependent on low concentrations of buffer (imidazole, as shown in Figure 1, *N*-methylmorpholine, and 2,4-lutidine), but becomes essentially invariable at higher buffer concentrations. This buffer dependence is not obtained using compounds similar to imidazole in structure but lacking a proton transfer capability, such as 1,3-dimethylimidazolium sulfate and pyrrole (shown in Figure 1). That k_c is relatively large even in the absence of added buffer may be attributed to the buffering capabilities of the substrate and enzyme themselves, and to the fact that alternating hydration–dehydration steps at equilibrium do not require proton transfer.

Figure 2 compares the effect of imidazole buffer at pH 7.0 on θ_{cat} and ϕ_{cat}. The rate constant for the uncatalyzed exchange, ϕ_{uncat}, is small ($\sim 6 \times 10^{-5}$ sec^{-1}) and independent of imidazole concentration under these conditions. A

Scheme I



pattern very similar to Figure 2 is obtained using the buffers *N*-methylmorpholine at pH 7.5-7.8 and 2,4-lutidine at pH 6.9-7.3. Both θ_{cat} and ϕ_{cat} can be abolished by the carbonic anhydrase inhibitor ethoxzolamide at 10^{-7} *M*. Furthermore, no catalyzed type I or type II exchange can be observed using the apoenzyme of BCA at 1.6×10^{-9} *M*.

Figure 2 indicates that as the buffer increases θ_{cat} increases and ϕ_{cat} decreases proportionately. From the symmetry of these two curves and from the fact that they both measure a property of labeled oxygen, we conclude that the two exchange processes described by θ_{cat} and ϕ_{cat} are related. These characteristics are consistent with a scheme in which ^{18}O labels the active site, as postulated earlier.[8] Furthermore, this behavior combined with the results in Figure 1 suggests general features of the steps in the catalytic mechanism which involve proton transfer. Such a mechanism is presented in Scheme I, which shows ^{18}O bound to the zinc of the active site. Although there is no evidence from these experiments that bicarbonate forms an inner sphere complex with this metal, other experiments indicate that bicarbonate coordinates directly to zinc.[9,10] In the absence of added buffers there is a slow rate of protonation of $EZn^{18}OH$. Magnetic resonance relaxivity data[11,12] establish that the residence time of the proton on water or hydroxide bound to the metal in Co(II) or Mn(II) BCA is relatively long in a neutral or low pH region even in the presence of buffers. Consequently, this basic form of the labeled enzyme has a relatively long lifetime, increasing the likelihood that it reacts with CO_2 to form $HCOO^{18}O^-$. This step retains ^{18}O in the CO_2 system; that is, this is a step which does not exchange ^{18}O with water; it is a step which, if prevalent, would cause a low value of θ_{cat} and a high value of ϕ_{cat}. As buffer is added, the rate of proton transfer to the enzyme increases, and the rate of formation of $EZn^{18}OH_2^+$ increases. As shown in Scheme I, $^{18}OH_2$ is displaced from the active site by bicarbonate (also by hydroxide ion or certain other anions, or possibly by another water molecule), a step which results in the exchange of ^{18}O with water increasing θ_{cat} and, since ^{18}O is displaced from the active site, decreasing ϕ_{cat}. At higher buffer concentrations, the data show a change in rate-determining step; the maximum enzyme activity is reached and further buffer does not affect type I or II exchange. Consequently, a mechanism such as shown in Scheme I in which ^{18}O labels the active site and can exchange a proton with buffer is compatible with the data of Figure 2.

It is also pertinent to note in Figure 2 that the rate constant ϕ_{cat} is not abolished in solutions with larger buffer concentrations. Even with 50 m*M* imidazole at pH 7 the catalyzed type II exchange occurs. Apparently, under these conditions, the ^{18}O-labeled active site can react with CO_2 to give labeled bicarbonate at a rate which is still significant compared to the rate of equilibration of ^{18}O label with the solvent.

The importance of the data in Figure 2 then is to confirm the interpretation of θ_{cat} as indicative of a proton transfer step involving buffer and is to establish as a likely site of proton transfer the oxygen in the active site which is involved in catalytic hydration of CO_2. This need not be a direct proton transfer but may occur through intervening amino acid side chains and water bridges. Just as the proton transfer step can be rate determining in equilibrium oxygen exchange at low buffer concentration, we anticipate that this step will be rate determining in the *nonequilibrium* reaction at low buffer concentration. For example, in the catalytic dehydration, the catalysis will be limited by how rapidly the proton transfer can convert EZnOH into $EZnOH_2^+$. We consider these ^{18}O exchange experiments to be consistent with the hypothesis that the maximal activity of carbonic anhydrase-catalyzed hydration and dehydration of CO_2 is dependent on the presence of buffers capable of providing protons to or accepting protons from the carbonic anhydrase active site.

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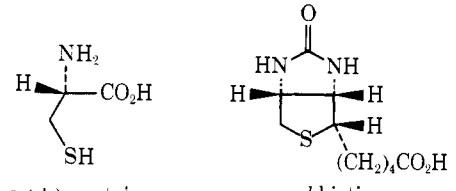
A Stereospecific Total Synthesis of *d*-Biotin from L-(+)-Cysteine

Sir:

We wish to record the total synthesis of d-biotin from its biogenetic precursor[1] L-(+)-cysteine[2] via a pathway which avoids a chemical resolution sequence characteristic of all previous syntheses.[3]



L-(+)-cysteine *d*-biotin

To this end, L-(+)-cysteine was converted into (4*R*)-carboxy-(2*S*)-phenylthiazolidine (**1**, R = H),[4] mp 159-160°, $[\alpha]^{25}D$ −135.1 (*c* 1.02, DMSO), by condensation with benzaldehyde. The nitrogen atom was further protected by reaction with methyl chloroformate in aqueous base to yield the urethane **1** (R = CO_2CH_3), mp 129-130°, $[\alpha]^{25}D$