torsion angles about the N(17)–C(16), C(16)–C(15), C(15)–C(13), C(13)–C(14), and C(14)–N(17) bonds are −3, −20, 35, −37, and 25°, respectively. The conformation of the other five-membered ring O–C(10)–C(9)–C(14)–C(8) is closer to a half-chair form than an envelope form, the torsion angles about the O–C(10), C(10)–C(9), C(9)–C(14), C(14)–C(8), and C(8)–O bonds being −17, 39, −45, 39, and −13°, respectively. The six-membered ring C(5)–C(6)–C(7)–C(8)–C(14)–C(13) is substantially flattened in comparison with an ideal cyclohexane ring, the deviations of atoms C(5) and C(8) from the plane of the other four atoms being 0.64 and −0.46 Å, and the mean of the valency angles in the ring 113.1°; the torsion angles about the bonds C(5)–C(6), C(6)–C(7), C(7)–C(8), C(8)–C(14), C(14)–C(13), and C(13)–C(5) are −56, 53, −48, 46, −44, and 51°, respectively. Because of the O–C(8) bridge between C(10) and C(14) the six-membered ring C(9)–C(10)–C(11)–C(12)–C(13)–C(14) does not adopt the normal half-chair form of a cyclohexene, and it is best described as an envelope form in which atom C(9) is the out-of-plane atom; in this ring the torsion angles about the bonds are C(9)–C(10) −76°, C(10)–C(11) 41°, C(11)–C(12) 1°, C(12)–C(13) −2°, C(13)–C(14) −41°, and C(14)–C(9) 79°.

The bromide ions in the crystal are associated with the alkaloid cations by hydrogen bonds of the N^+–H···Br (3.22 and 3.24 Å) and O–H···Br (3.27 and 3.30 Å) types.

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The Binding of 4-Hydroxy-3-nitrobenzenesulfonamide, a Reporter Group Labeled Inhibitor, to Carbonic Anhydrases

Sir:

The investigation of inhibitor and substrate binding to metalloenzymes provides valuable insight into the roles of metal ions in biological systems. Carbonic anhydrase is particularly suitable for this kind of investigation since it is a relatively well-studied metalloenzyme for which numerous primary sulfonamides are tightly bound specific inhibitors.[1] Strong evidence exists that the sulfonamide group binds directly to the Zn(II) at the active site replacing the water ligand present in the free enzyme.[2] We report here the results of a kinetic study of the binding to carbonic anhydrase of 4-hydroxy-3-nitrobenzenesulfonamide, a new inhibitor which has recently been prepared in this laboratory.[3] This sulfonamide possesses a chromophoric reporter[4] group whose absorption spectrum in the visible region undergoes a significant shift to longer wavelength on complex formation with carbonic anhydrase at pH 7.6.[3] Thus, the kinetics of binding could be followed directly at 420 mμ using a Durrum–Gibson stopped-flow spectrophotometer. Some previous studies on the kinetics of sulfonamide binding to carbonic anhydrase have been reported,[5,6] but these investigations employed an indirect more complicated method of measurement involving the rates of inhibition by the sulfonamides of the enzymatic hydration of carbon dioxide and the hydrolysis of *p*-nitrophenyl acetate.

In the presence of excess inhibitor a rapid first-order reaction is observed when 4-hydroxy-3-nitrobenzenesulfonamide is mixed with either bovine carbonic anhydrase or human carbonic anhydrase B.[7] The first-order rate constants, k_{obsd}, measured are related to the rate constants of eq 1,[6] where E is the free

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \quad (1)$$

$$k_{obsd} = k_{-1} + k_1[I]_0 \quad (2)$$

enzyme, I is the unbound inhibitor, and EI is the enzyme–inhibitor complex, and by eq 2 where $[I]_0$ is the initial sulfonamide concentration. Kinetic data obtained for the bovine enzyme plotted according to eq 2 are shown in Figure 1. The k_1 values found for this species and for human carbonic anhydrase B are given in Table I.

The dissociation constant, K_I, of the 4-hydroxy-3-nitrobenzenesulfonamide–bovine carbonic anhydrase complex was determined by equilibrium dialysis studies and from inhibition experiments. Our equilibrium dialysis data were plotted according to eq 3 where r is the ratio of moles of inhibitor bound/total moles of enzyme.[8] K_I was found to be (4.3 ± 1.7) ×

$$\frac{1}{r} = 1 + \frac{K_I}{[I]_{unbound}} \quad (3)$$

10^{-6} M at 25.0°, pH 7.6, and μ = 0.25 in the case of the bovine enzyme.

Stopped-flow kinetic experiments were performed at 410 mμ using the reporter group labeled sulfonamide as an inhibitor and employing 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as the substrate.[3] Both of these compounds were present in excess over the enzyme. The rate data were analyzed by eq 4 where

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Table I. Summary of Measurements at 25° of Inhibition Constants and Rate Constants for the Binding of Sulfonamides to Carbonic Anhydrases

Inhibitor	k_1, M^{-1} sec^{-1}	k_{calcd}, M^{-1} sec^{-1}	K_I, M^{-1}	pH of reaction solution	pK_a for sulfonamide group	Enzyme species
4-Hydroxy-3-nitrobenzenesulfonamide[a]	3.7×10^4	2.2×10^8	3.8×10^{-6}	7.6	10.9[d]	Bovine A + B
	7.5×10^4	4.5×10^8		7.6	10.9	Human B
Benzenesulfonamide	7×10^4 [b]	2.8×10^8	3.2×10^{-6} [b]	6.7	10.2	Bovine B
	1.6×10^5	1.8×10^8		7.7	10.2	Bovine B
Sulfanilamide	7×10^4 [c]	2.2×10^8	1.2×10^{-6} [c]	7.9	10.7	Co(II)-bovine B
Acetazolamide	5×10^6 [b]	2.6×10^7	2.2×10^{-8} [b]	6.7	7.2	Bovine B
	4×10^6 [c]	2.4×10^7	1.2×10^{-8} [c]	7.9	7.2	Co(II)- and Zn(II)-bovine B

[a] Results of this work. [b] Reference 5. [c] Reference 6. [d] Spectrophotometric titration gave a pK_a value of 4.89 ± 0.04 for the phenolic group and 10.9 ± 0.2 for the sulfonamide group in this inhibitor.

k_{meas} is the pseudo-first-order rate constant measured for the enzyme-catalyzed hydrolysis of the sultone,

$$\frac{[E]_0}{(k_{meas} - k_{spont})} = \frac{K_M}{k_{cat}} + \frac{K_M}{k_{cat}}\frac{[I]}{K_I} \quad (4)$$

and k_{spont} is the first-order rate constant observed for the spontaneous hydrolysis reaction at the same pH in the same buffer system. A value of K_I of (3.2 ± 0.3) × 10^{-6} M at 25.0°, pH 7.45, and μ = 0.125 was obtained in good agreement with the equilibrium dialysis results.[9]

Since $K_I = k_{-1}/k_1$ (see eq 1), if we take our average K_I value of 3.8 × 10^{-6} M, we calculate that k_{-1} for the bovine enzyme complex is 0.14 sec^{-1}. A really reliable intercept cannot be calculated from the plot shown in Figure 1 since a considerable extrapolation of the data points is required. Nevertheless, we have estimated from this plot that $k_{-1} \approx 0.17$ sec^{-1} which corresponds well with the value calculated above.

From Table I it can be seen that the K_I and k_1 values we obtained for the reported group labeled inhibitor 4-hydroxy-3-nitrobenzenesulfonamide are similar to those found for two other inhibitors whose sulfonamide groups have comparable ionization constants.[5,6] The pH dependencies reported for the quantities K_I[5,6] and k_1[5] can be accounted for by postulating that the enzyme–sulfonamide complexes are formed by the reaction of the ionized sulfonamides with the form of the enzyme in which the water ligand bound to the active site is un-ionized.[10] The k_{calcd} values listed in Table I are based on this hypothesis, and they have been calculated from the k_1 results with the assumption that the pK_a for the ionization of the water ligand in the enzyme is approximately 7.3.[3] The variation in k_{calcd} values is tenfold at most although the pK_a values for the ionization of the sulfonamide groups in acetazolamide and 4-hydroxy-3-nitrobenzenesulfonamide differ by 3.7 pH units. This kind of invariance of k_{calcd} and the k_{calcd} values themselves are reminiscent of the rate data measured for the reactions of various anionic ligands with Zn(II) to form inorganic complexes.[11]

(9) In ref 3 a K_I value of 7.38 × 10^{-7} M was reported for the sulfonamide inhibitor. However, for a variety of reasons we do not consider the results of these earlier inhibition experiments to be satisfactory.

(10) The alternative hypothesis that the un-ionized sulfonamides react with the ionized form of the enzyme cannot be discounted, however.

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Therefore, we suggest that the carbonic anhydrase–sulfonamide complex generation may be described in terms of rapid preequilibrium formation of an outer sphere complex between the sulfonamide anion and the Zn(II) ion at the active site which then loses water in the rate-determining step of inner sphere enzyme–inhibitor complex formation.

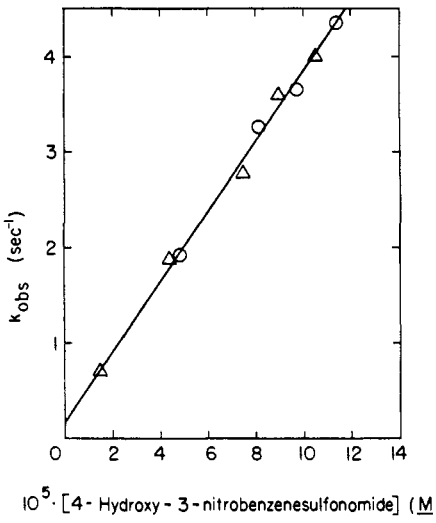

Figure 1. Rate data observed on mixing 4-hydroxy-3-nitrobenzenesulfonamide with bovine carbonic anhydrase: circled points, μ = 0.25; triangles, μ = 0.11; acetone concentration, 0.12–0.28%; temperature 25.0°; pH 7.6; Tris–sulfate buffer.

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Intermolecular Exchange in Phosphorus(V) Fluorides

Sir:

Facile ligand exchange is a well-recognized feature of pentacoordinate phosphorus compounds.[1] Gen-

(1) For pertinent reviews, see (a) E. L. Muetterties and R. A.