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Nuclear Magnetic Resonance Studies of Bovine Carbonic Anhydrase. Binding of Sulfonamides to the Zinc Enzyme*

A. Lanir and G. Navon

ABSTRACT: Nuclear magnetic resonance line broadening of sulfonamide inhibitors upon their binding to the zinc bovine carbonic anhydrase has been studied at various temperatures and two frequencies. Competition of different inhibitors for the same site was demonstrated. Comparison of the binding constants ratio determined by nuclear magnetic resonance technique with that determined by enzymatic activity confirmed that the broadening effect was due to specific binding to the active site. The dissociation rate constant of the enzyme–inhibitor complexes was found to be greater than 200 sec^{-1}. Frequency dependence of the broadening indicated a dipolar relaxation mechanism rather than a chemical shift dependent mechanism.

The rotational motion of the aromatic rings of several sulfonamide inhibitors was found to be close to that of the whole protein molecule, while that of methyl groups is much faster. Furthermore, it was concluded that, for the methyl protons, the main contribution to the dipolar relaxation is the intramolecular proton–proton interaction, and for the aromatic protons, the interaction with closely adjacent protein protons. The significance of these results to our understanding of the mode of binding is discussed.

Sulfonamides are known to be potent and specific inhibitors of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1.) (Mann and Keilin, 1940; Maren, 1967). All the strongest known sulfonamide inhibitors have an unsubstituted SO_2NH_2 group attached to aromatic or heterocyclic residue. Some N^1-substituted and aliphatic sulfonamides also inhibit the

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enzyme, though with a smaller binding constant (Krebs, 1948; Whitney *et al.*, 1967; Maren and Wiley, 1968). Recent 5.5-Å resolution X-rays data on crystalline human carbonic anhydrase C (Fridborg *et al.*, 1967) indicated a binding of the inhibitors in a narrow crevice leading to the zinc atom of the enzyme. Other indirect indications of the inhibitor direct linkage to the metal ion are spectral changes of the metal ions bands in cobalt and copper carbonic anhydrase upon sulfonamides binding (Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1965, 1968a,b).

Binding of a chloride ion to the zinc atom in carbonic anhydrase and its replacement by acetazolamide has been demonstrated by Ward (1969) using ^{35}Cl nuclear magnetic resonance. The binding mechanism of sulfonamide inhibitors has been thought to involve deprotonation of the SO_2NH_2 moiety. This was inferred from the pH dependence of the inhibition as measured by kinetics (Kernohan, 1966a; Lindskog and Thorslund, 1968), equilibrium dialysis methods (Coleman, 1967a,b), and optical absorption blue shift (Chen and Kernohan, 1967). It has been suggested that the interaction of the aromatic group with a hydrophobic binding site assists the binding (Chen and Kernohan, 1967). However, the mode of binding of sulfonamides to carbonic anhydrase is not very well understood and there are various reports about their mechanism of inhibition, *i.e.*, whether it is competitive or not (Kernohan, 1966b; Lindskog and Thorslund 1968; Maren and Wiley, 1968).

Nuclear magnetic resonance spectroscopy is being used currently as a method of studying the binding of small molecules to macromolecules (for reviews, see Jardetzky, 1964, and Sheard and Bradbury, 1971). In spite of the relative insensitivity of the method, it is possible to get information on minute concentrations of bound molecules by observing spectral changes of the excess unbound molecule. This is due to the averaging effect, brought about by chemical exchange. The present paper reports some results concerning the nature of sulfonamides binding to bovine carbonic anhydrase as studied by nuclear magnetic resonance technique.

Experimental Section

Bovine erythrocytes carbonic anhydrase prepared and purified by the method of Lindskog (1960) was obtained from Seravac. Preliminary nuclear magnetic resonance studies of inhibitors with this preparation have shown large broadening effects which could be interpreted as due to paramagnetic impurities. Measurements by atomic absorption spectroscopy have indeed demonstrated that this preparation contained about 0.25 copper atom/each zinc atom. Lindskog (1960) reported a similar value (0.24 Cu to Zn ratio) for his preparation. To eliminate any effect due to paramagnetic impurities, we have treated the enzyme in the following manner. Aqueous solution of the enzyme was dialyzed for 10 days against 0.01 M 1,10-phenanthroline solution in 0.1 M acetate buffer (pH 5.2) (Lindskog and Malmström, 1962). The dialysis solutions were changed every 48 hr. The enzyme was further dialyzed for 48 hr against three changes of deionized water, then for 24 hr against 10^{-2} M $ZnCl_2$, and for 48 hr against three changes of dionized water in order to remove any excess of Zn^{2+} ions. No detectable amount of copper could be found in the enzyme solution after the treatment. Its specific enzymatic activity was about 20% higher than that of the starting solution. The enzyme was then lyophilized and kept in the cold. In a later part of the work when carbonic anhydrase B was exclusively used, the commercial enzyme was first separated on DEAE-cellulose (Whatman DE52) column (Lindskog, 1960), and then the above procedure for removal of paramagnetic impurities was followed. Replacement of the carbonic anhydrase B and C mixture by the purified carbonic anhydrase B did not affect any of our results.

Inhibitors. Sulfanilamide was obtained from BDH, N^1-acetylsulfanilamide obtained from Fluka and *p*-toluenesulfonamide was of Koch and Light. The last two chemicals were recrystallized twice from water.

In order to eliminate any affect of small contaminations of unsubstituted sulfanilamide on our reported inhibition constant of N^1-acetylsulfanilamide, the inhibition constant was measured after each recrystallization step. No further change in its value was found after two recrystallization at 40°.

Enzymatic activity was determined by measuring the esterase activity toward the hydrolysis of *p*-nitrophenyl acetate (Pocker and Stone, 1967) by following the absorbance at 400 mμ and using the value of ϵ_{400} 1.82×10^4 for *p*-nitrophenolate ion (Armstrong *et al.*, 1967). The reaction cuvet contained 5×10^{-4} M substrate in 0.025 M Tris buffer–5% acetonitrile (pH 7.5) in 25°. The reaction was started by the addition of the substrate to the enzyme solution in the appropriate buffer. The reaction mixture was read against blank, containing all components except the enzyme.

Protein concentration was determined from measurements of the optical densities at 280 mμ using molar absorptivity of 57,000 M^{-1} cm^{-1} (Nyman and Lindskog, 1964).

Nuclear magnetic resonance spectra were obtained with a Varian HA 100 nuclear magnetic resonance spectrometer equipped with a Varian C-1024 time-averaging computer. Hexamethyldisiloxane was used as an external lock. For experiments other than those in which the temperature dependence was studied, the solution temperature was 27°. Nuclear magnetic resonance measurements at 60 MHz were conducted with a Jeol C-60 spectrometer.[1] Values of $1/T_2$ were obtained from the spectral line width using the expression $1/T_2 = \pi\Delta\nu$, where $\Delta\nu$ is the full line width at half-maximum peak height.

Results and Discussion

The three inhibitors used in our work were chosen according to their solubility in water and their relatively simple nuclear magnetic resonance spectra. The spectra of N^1-acetylsulfanilamide and *p*-toluenesulfonamide, together with the spectra broadened by the enzyme are given in Figures 1 and 2. No chemical shift was observed upon addition of the enzyme. The spectra of the phenyl protons which form a AA'BB' group of interacting protons break effectively to two overlapping AB spectra, and were treated as such. The two lines of the AB quartet which appear at the lower field were assigned to the protons ortho to the sulfamoyl group. The broadening of the four phenyl lines upon addition of the enzyme was the same within experimental error.

The average of these four broadening values was used in treating the data. The line of the methyl group of *p*-toluenesulfonamide was broad due to unresolved spin interaction with the adjacent phenyl protons. The broadening effects upon addition of the enzyme were large enough to make a detailed analysis of the spectra unnecessary. An approximate analysis of the spectra of the phenyl protons of N^1-acetylsulfanilamide is given by Jardetzky and Wade-Jardetzky (1965).

[1] We thank Mr. E. Sali for running these measurements.

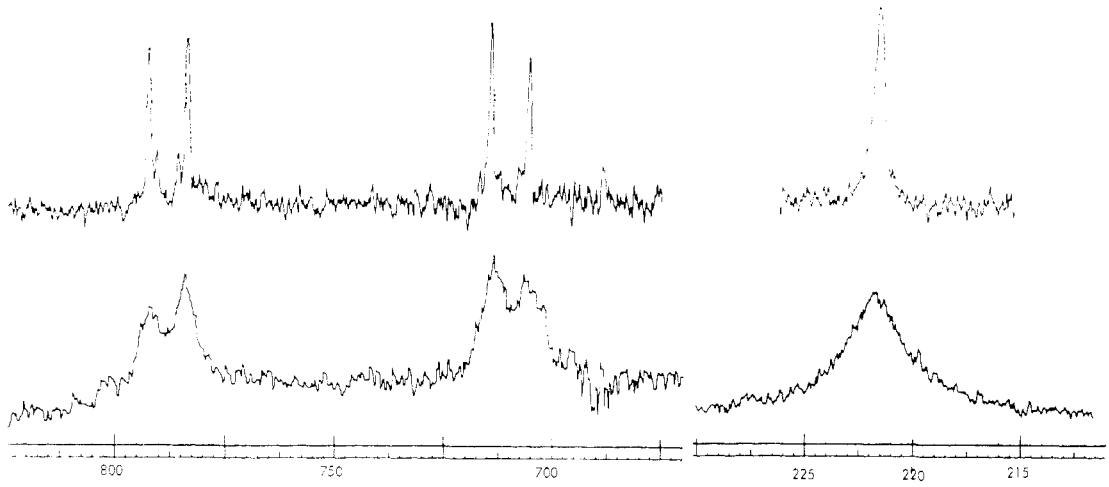


FIGURE 1: 100-MHz spectra of N^1-acetylsulfanilamide (0.02 M) in D_2O, pH 6.9, 27°. The numbers shown below the spectra are the shifts in cycles per second from external hexamethyldisiloxane. The upper trace is without enzyme. The lower one with 1.47×10^{-3} M bovine carbonic anhydrase.

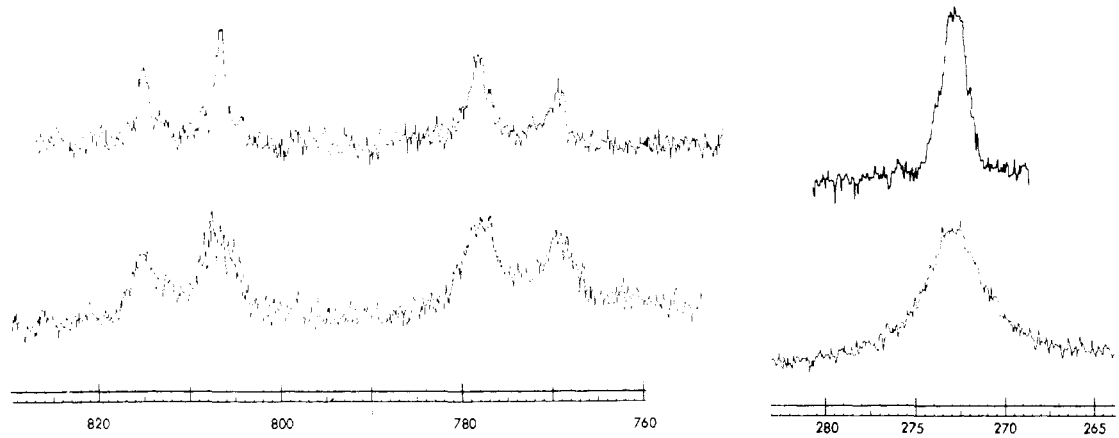


FIGURE 2: 100-MHz nuclear magnetic resonance spectra of *p*-toluenesulfonamide, 0.015 M in D_2O, pH 7.5, 27°. The numbers shown below the spectra are the shifts in cycles per second from external hexamethyldisiloxane. The upper trace is without enzyme. The lower one with 5.7×10^{-4} M bovine carbonic anhydrase.

Nuclear magnetic resonance spectral line broadening of small molecules due to partial binding to a macromolecule meets the requirements for Swift and Connick (1962) treatment which is general for nuclei exchange between two sites, one being in large excess of the other. In this case, one can see in the nuclear magnetic resonance spectrum only the major component and its broadening due to exchange with the minor one. In our case, the observed broadening of the inhibitors spectra is due to exchange with a small fraction of inhibitor bound to the enzyme. According to the equilibrium reaction

$$E + I \rightleftharpoons EI \tag{1}$$

If $1/T_{2(0)}$ is the relaxation rate of the free inhibitor and $1/T_2$ is the observed relaxation rate in the presence of the enzyme, then $1/T_{2p}$ defined by

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_{2(0)}} \tag{2}$$

is the net relaxation due to the interaction with the enzyme. According to Swift and Connick (1962)

$$\frac{1}{T_{2p}} = \frac{f\left(\frac{1}{T_{2M}}\right)^2 + \frac{1}{T_{2M}\tau_M} + \Delta\omega_M{}^2}{\tau_M\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right)^2 + \Delta\omega_M{}^2} \tag{3}$$

where T_{2M} is the transversal relaxation time of the bound inhibitors, τ_M is the exchange lifetime of bound inhibitor, $\Delta\omega_M$ is the chemical shift between bound and free inhibitor, and f is the fraction of inhibitor bound to the enzyme. In our measurements we have not observed any chemical shift of the inhibitor lines upon addition of the enzyme to the solution. Therefore $\Delta\omega_M{}^2$ can be neglected compared with the other terms in eq 3 and the expression for $1/T_{2p}$ is reduced to

$$\frac{1}{T_{2p}} = \frac{f}{T_{2M} + \tau_M} \tag{4}$$

For fast exchange $\tau_M \ll T_{2M}$, one observes the weighted

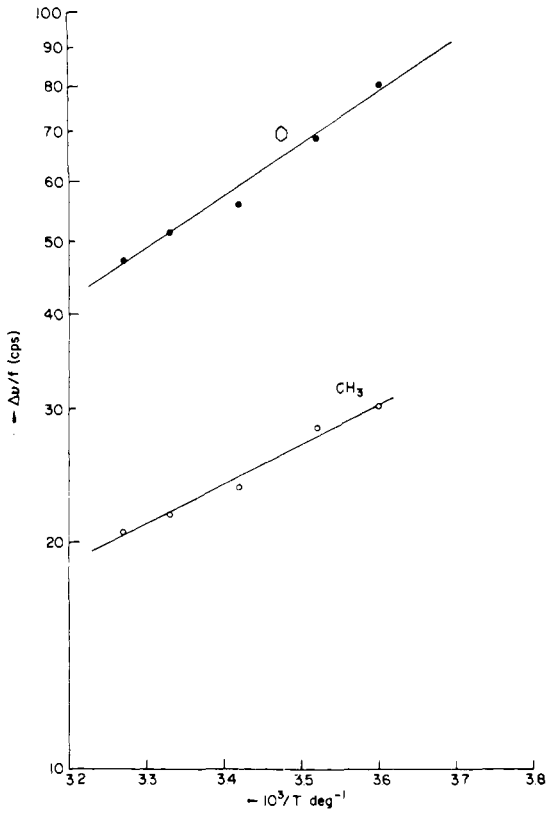


FIGURE 3: Temperature dependence of the specific broadening of N^1-acetylsulfanilamide, 0.02 M, pH 6.9. Enzyme concentration, 1.47×10^{-3} M. (○) Methyl protons; (●) phenyl protons.

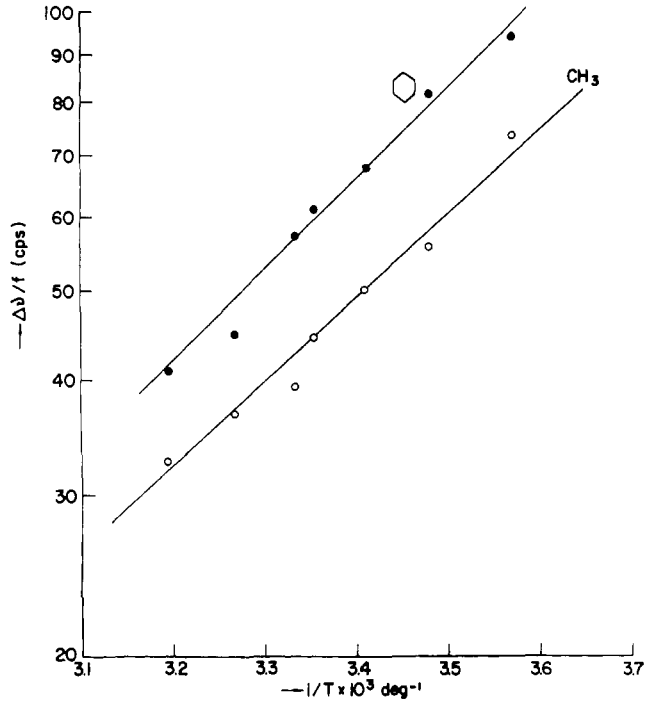


FIGURE 4: Temperature dependence of the specific broadening of *p*-toluenesulfonamide, 0.015 M, pH 7.5. Enzyme concentration, 5.74×10^{-4} M. (○) Methyl protons; (●) phenyl protons.

average width between the bulk and bound inhibitor

$$\frac{1}{T_{2p}} = \frac{f}{T_{2M}} \tag{5}$$

while for the slow exchange limit where $\tau_M \gg T_{2M}$

$$\frac{1}{T_{2p}} = \frac{f}{\tau_M} \tag{6}$$

i.e., every bound inhibitor molecule residing on the enzyme for a time long enough to enable it to relax. In this case, the broadening is directly proportional to the exchange reaction rate. It is possible to distinguish between these two cases using the temperature dependence of the broadening. Usually, for a variety of relaxation mechanisms (see below), $1/T_{2M}$ is a monotonically increasing function of the tumbling time of the enzyme–inhibitor complex and the tumbling time decreases upon temperature increase. Thus, T_{2M} should increase with temperature. On the other hand, τ_M which is inversely proportional to the dissociation reaction rate constant of the EI complex, is invariably decreasing upon temperature rise. The temperature dependence of the nuclear magnetic resonance line broadening of N^1-acetylsulfanilamide by carbonic anhydrase is illustrated by Arrhenius plot in Figure 3.

A similar plot for the *p*-toluenesulfonamide is given in Figure 4. The broadenings of the methyl and phenyl groups of these inhibitors are given in the plots separately. The broadening of the four lines due to the phenyl group were the same within our experimental error and the average values were recorded in the plot. As can be seen from Figures 3 and 4, the line widths decrease upon raising the temperature, clearly indicating that the fast-exchange limit prevails in the present system. $1/T_{2M}$ values and their apparent Arrhenius activation energies for the three inhibitors are given in Table I.

The following experiments were performed in order to exclude any possibility of broadenings due to viscosity effects or nonspecific binding. According to eq 5, the net broadening is proportional to the fraction of the bound inhibitor, *f*. Inhibitor concentrations, [I], used in our experiments were in a large excess over the total enzyme concentration, [E], and much greater than the dissociation constant of the various enzyme–inhibitor complexes (see Table I).

TABLE I: Dissociation Constants, Nuclear Magnetic Resonance Relaxation Rates, and Their Apparent Arrhenius Activation Energies for Inhibitors Bound to Bovine Carbonic Anhydrase.

		$1/T_{2M}$ (sec^{-1})[b]		E_a (kcal/mole)	
Inhibitor	K_{diss} (M)[a]	Methyl	Phenyl	Methyl	Phenyl
N^1-Acetylsulfanilamide	6.7×10^{-3}	72.5	201	2.5	3.3
p-Toluenesulfonamide	4.75×10^{-7}	140	192	4.2	4.6
Sulfanilamide	4.4×10^{-6}		225		

[a] Measured by the inhibition of the esterase activity of bovine carbonic anhydrase toward *p*-nitrophenyl acetate (Pocker and Stone, 1967). Solutions contained Tris-chloride buffer, 0.025 M, pH 7.5, 22°. [b] Relaxation rates at 27°. Estimated error: ±15%.

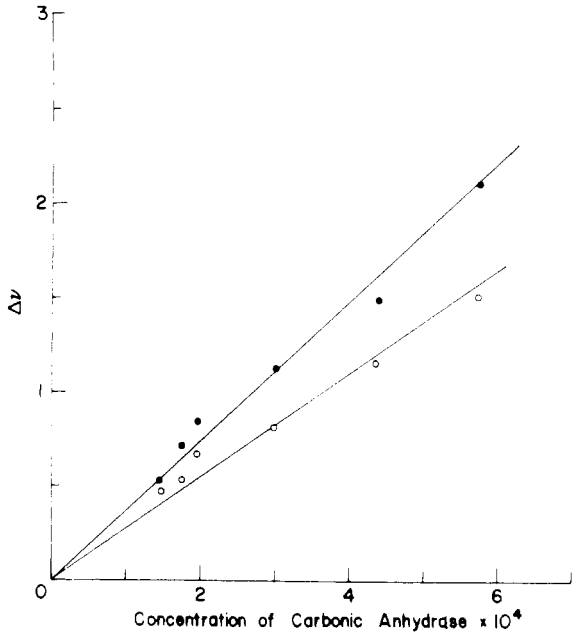


FIGURE 5: The dependence of the *p*-toluenesulfonamide inhibitor line broadening on enzyme concentration. Inhibitor concentration was 0.015 M, pH 7.5, 27°. (○) Methyl protons; (●) phenyl protons.

Consequently, the enzyme was totally bound to the inhibitor and the fraction, f, of bound inhibitor is given by

$$f = \frac{[EI]}{[I]} = \frac{[E]}{[I]} \tag{7}$$

The validity of this relation in our system was checked by the following three experiments. In the first experiment, the broadenings of the phenyl and methyl lines of *p*-toluenesulfonamide were found to be proportional to the enzyme concentration (see Figure 5). In Figure 6, $1/\Delta\nu$ is plotted *vs.*

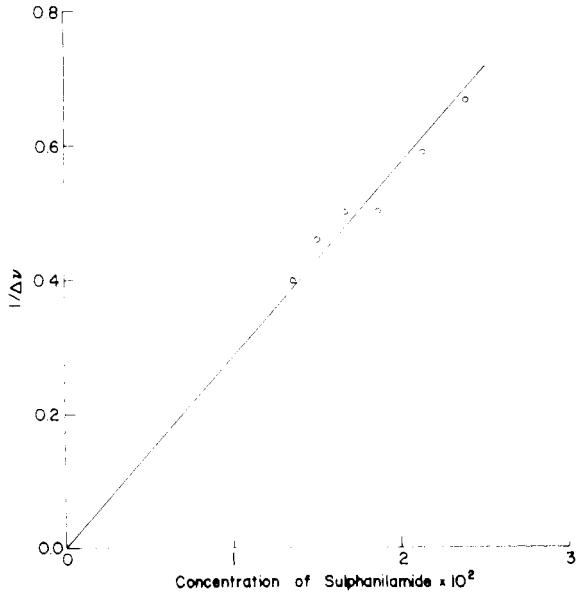


FIGURE 6: Concentration dependence of the reciprocal broadening of sulfanilamide. Enzyme concentration, 5.0×10^{-4} M, pH 7.5, 27°.

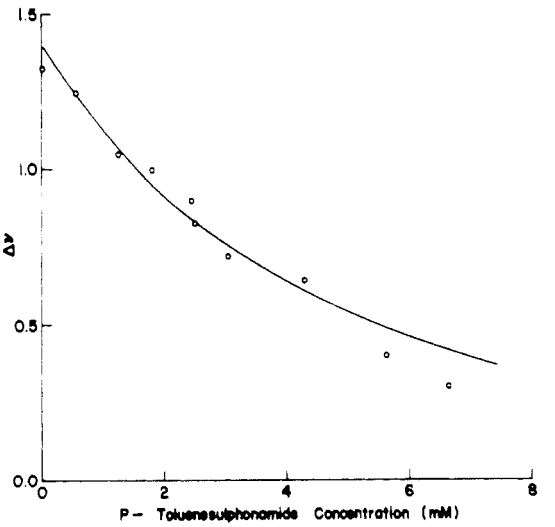


FIGURE 7: Sulfanilamide line broadening upon successive additions of *p*-toluenesulfonamide. The points are experimental. The line is a theoretical one, based on the ratio $K_p/K_s = 0.12$. Enzyme concentration is varied between 6.9×10^{-4} and 3.8×10^{-4} M. pH 7.5, 27°.

inhibitor concentration, keeping the enzyme concentration constant. A straight line is again obtained in accordance with eq 7. In another series of measurements in which solution of N^1-acetylsulfanilamide and carbonic anhydrase with a constant ratio of [I]/[E] = 13.6, was successively diluted by D_2O, so that the enzyme concentration was reduced from 1.47×10^{-3} to 1.47×10^{-4}, no significant change of line width was observed. The last two experiments clearly indicate that the line broadening is caused by the binding of the inhibitors to the protein according to eq 1 and not by viscosity increase.

The line broadening can be caused also by a nonspecific binding of inhibitor to an enzyme. Coleman (1967a,b) followed the binding of the sulfonamide inhibitor, acetazolamide, to carbonic anhydrase by studies of equilibrium dialysis. Up to his highest sulfonamide concentration which was about 10^{-4} M, he found a limiting 1:1 ratio of bound inhibitor to enzyme. In our experiments, however, inhibitor concentrations of the order of 10^{-2} were used, and we would like to eliminate additional nonspecific inhibitor binding. Comparison of the enzyme–inhibitor binding constant which prevail in our experiments to those obtained by activity inhibition measurements can indicate if the same binding site plays a role in both experiments.

For an excess of sulfanilamide concentration $[S] \gg [E_0]$, the concentration of the enzyme–inhibitor complex, [ES], is given by

$$[ES] = \frac{[E_0][S]}{K_s + [S]} \tag{8}$$

where K_s is the dissociation constant of the complex. Substituting $f = [ES]/[S]$ into eq 5 gives for the line broadening, $\Delta\nu$

$$\frac{1}{\Delta\nu} = \pi T_{2p} = \frac{\pi T_{2M}}{[E_0]}(K_s + [S]) \tag{9}$$

The dissociation constant, K_s, can be evaluated from a plot

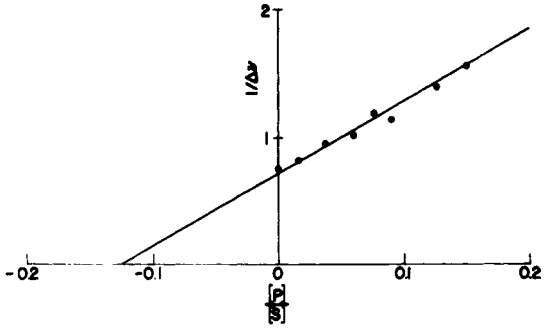


FIGURE 8: Reciprocal sulfanilamide line broadening as a function of concentration ratio [P]/[S] (see text).

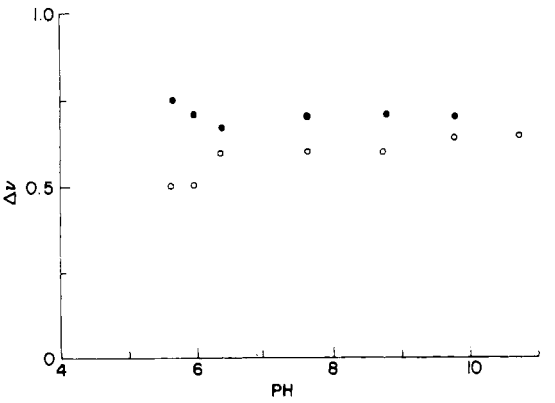


FIGURE 9: pH dependence of *p*-toluenesulfonamide (0.015 M) line broadening. Enzyme concentration, 2.1×10^{-4} M, 27°. (○) Methyl protons, (●) phenyl protons.

of $1/\Delta\nu$ *vs.* [S], provided that the inhibitor concentrations used are not too far from the value of K_s. Unfortunately, in our case, this requires the use of inhibitor concentration far below the limit of detection by the nuclear magnetic resonance method. Figure 6 shows indeed that the value of the intercept in the plot of $1/\Delta\nu$ *vs.* [S] is below our experimental error. Nevertheless, the ratio of binding constants of two inhibitors can be determined. When *p*-toluenesulfonamide is added gradually to a solution containing sulfanilamide and carbonic anhydrase, the broadening of the sulfanilamide nuclear magnetic resonance lines is diminished (see Figure 7). This is certainly due to a competition of these two inhibitors on the same binding site, and a gradual replacement of the sulfanilamide by *p*-toluenesulfonamide (designated by P). More quantitatively, considering an additional equilibrium reaction for the formation of the complex EP with a dissociation constant, K_p, we get

$$\frac{[S]}{[ES]} = \frac{1}{[E_0]}\left(K_s + [S] + \frac{K_s}{K_p}[P]\right) \qquad (10)$$

The term K_s is much smaller than [S], as was just concluded from Figure 6, and it can be neglected in eq 10. Then, similar to eq 9

$$\frac{1}{\Delta\nu} = \frac{\pi[S]T_{2M}}{[E_0]}\left(1 + \frac{K_s[P]}{K_p[S]}\right) \qquad (11)$$

In our experiment the ratio $[S]/[E_0]$ was kept constant upon the addition of *p*-toluenesulfonamide. A straight line is obtained upon plotting $1/\Delta\nu$ *vs.* [P]/[S] (Figure 8). The *x* axis negative intercept gives the value

$$-\left(\frac{[P]}{[S]}\right)_0 = \frac{K_p}{K_s} = 0.12$$

which matches well the dissociation constant ratio K_p/K_s which we found for these two inhibitors by activity inhibition measurements (see Table I). Similar K_p/K_s ratios of 0.11 and 0.12 were found by Krebs (1948) and Kakeya *et al.* (1969), respectively, for the inhibition of the hydration of CO_2 by bovine carbonic anhydrase. Displacement of N^1-acetylsulfanilamide by *p*-toluenesulfonamide was also observed, but as their binding constants are very different their ratio could not be measured accurately. The last results strongly indicate that the observed nuclear magnetic resonance line broadenings are due to inhibitor molecules, specifically bound to the active site.

The pH dependence of the line broadening of *p*-toluenesulfonamide is given in Figure 9. Within the experimental error the broadenings of both phenyl and methyl groups can be considered as pH independent. This is also true at pH values above 10.1 where deprotonation of the sulfamoyl group occurs. The dissociation constant of *p*-toluenesulfonamide–carbonic anhydrase complex at pH 7.5 is 4.75×10^{-7} M, while the inhibitor concentration used in this experiment was 0.015 M. Following eq 9 it is obvious that the broadening should be constant, even for a change in the binding constant, over several orders of magnitude.

Interpretation of T_{2M}. The temperature dependence studies described earlier showed us that the broadening is not limited by the exchange rate. Using eq 5 and the experimental values of *f* one can calculate T_{2M} which is the relaxation time of the bound inhibitor. In general, several mechanisms can cause inhibitor relaxation enhancement upon binding to the enzyme: (a) proton–proton dipolar interaction, (b) distribution of chemical shifts, and (c) chemical shift anisotropy.

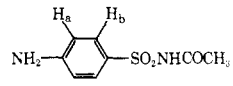
The last two relaxation mechanisms which are either distribution of chemical shift due to different binding modes of the inhibitor, all exchanging with the excess of free inhibitor, or relaxation caused by fluctuating magnetic field arising from chemical shift anisotropy, are proportional to the square of the magnetic field strength, H_0^2. The experimental magnetic field dependence for N^1-acetylsulfanilamide line broadenings, presented in Table III, shows narrowing of these lines upon increasing the magnetic field. Thus we can rule out any chemical shift effect on our results.

The contribution of the dipolar interaction to the transverse relaxation $1/T_{2M}$ for one pair of protons is given by the expression (Solomon, 1959; Abragam, 1961)

$$\frac{1}{T_{2M}} = \frac{3}{20}\frac{\gamma_p^4\hbar^2}{r^6}\left(3\tau_c + \frac{5\tau_c}{1+\omega_0^2\tau_c^2} + \frac{2\tau_c}{1+4\omega_0^2\tau_c^2}\right) \qquad (12)$$

where *r* is the proton–proton distance, τ_c is the tumbling correlation time, ω_0 is the Larmor angular frequency (3.77×10^8 and 6.28×10^8 rads per sec for proton resonances at 60 and 100 MHz, respectively), and γ_p is the gyromagnetic ratio for protons. If the proton under consideration interacts with more than one proton, it is a very good approximation to take the sum over all the dipolar interactions (Hubbard, 1958). Thus, in the case of the methyl group, considering only intramolecular interactions, the right-handed side of eq 12

TABLE II: Effect of Sulfamoyl Group Deprotonation on the Nuclear Magnetic Resonance Chemical Shifts.

Inhibitor	Formula	pK_a[a]	$\Delta\delta$ (Hz)[d] H_a	H_b	CH_3
N^1-Acetylsulfanilamide		5.38[b]	2.0	10.6	10.5
p-Toluenesulfonamide	$CH_3C_6H_5SO_2NH_2$	10.11[c]	8.4	7.3	4.0
Sulfanilamide	$NH_2C_6H_4SO_2NH_2$	10.48[c]	3.0	4.7	

[a] pK values for sulfamoyl protons dissociation reaction. [b] Bell and Roblin (1942). [c] Kakeya *et al.* (1969). [d] The changes of proton chemical shifts at 100 MHz upon deprotonation of the sulfamoyl groups. H_a and H_b refer to phenyl protons meta and ortho to the sulfamoyl group, respectively. All shifts are toward high field. No change in the spin-spin coupling constant, J, was observed. Solutions were in D_2O and contained 1% dioxane as an internal reference. Temperature 27°.

will be multiplied by a factor of 2. τ_c could be evaluated using eq 12 if all the distances of the interacting protons were known. However, this is impossible since neighboring protein protons, whose number and distances from the inhibitor protons are unknown, can also contribute to the dipolar interaction. Still, τ_c can be estimated by the frequency dependence of the line broadening. The theoretical ratio of the broadening at a Larmor frequency at 60 MHz to that of 100 MHz calculated from eq 12 is given in Figure 10. Using the experimental ratios listed in Table III correlation times of $(1.0 \pm 0.5) \times 10^{-8}$ and $(2.5 \pm 0.5) \times 10^{-9}$ sec were calculated for the phenyl and methyl groups of N^1-acetylsulfanilamide, respectively. Introducing these values of τ_c to eq 12 together with proton–proton distances of 2.48 and 1.80 Å for the phenyl and methyl groups, respectively, we can now estimate the relative contributions of the intramolecular and intermolecular dipolar interactions to the $1/T_{2M}$ found for these groups. For the phenyl protons the calculated intramolecular contribution amounted to only about 10% of the observed $1/T_{2M}$ whereas, for the methyl group, the corresponding value was found to be 86%. Although these values are only approximate, due to accumulating errors, they indicate clearly that the phenyl protons are broadened mainly by adjacent protein protons while the interaction of methyl protons with the enzyme protons is smaller and the main broadening is due to intramolecular interaction.

Chemical Shifts. It has been mentioned above that no proton chemical shifts were observed in our experiments. Deprotonation of the sulfamoyl group upon binding should, in general, cause a variation in the chemical shift. The sulfamoyl proton of N^1-acetylsulfanilamide has a pK value of 5.38 (Bell and Roblin, 1942). Therefore, it is ionized at the neutral pH where our experiments were carried out and we do not expect any protonation change upon binding. *p*-Toluenesulfonamide and sulfanilamide exist in our solutions in their un-ionized forms as is evident from the pK_a values listed in Table II. The chemical shifts of these inhibitors upon deprotonation are given also in that table. These values when multiplied by f are beyond our experimental error of chemical shift detection. Therefore the absence of observed shifts cannot serve as an evidence for a lack of deprotonation of bound inhibitors.

Chemical shifts can also be caused by aromatic rings located near the binding site. Such chemical shifts which are originated from ring currents are in the order of magnitude which should enable one to observe a chemical shift even for a small fraction of the bound inhibitors. Raftery *et al.* (1968, 1969) and Sykes (1969) observed chemical shifts of up to 0.9 ppm for *N*-acetyl groups of lysozyme inhibitors. This is in accordance with the known participation of tryptophan residues in the binding site of lysozyme (Blake *et al.*, 1967). Galley and Stryer (1968) concluded from the efficiency of triplet-triplet energy transfer from *m*-acetylbenzenesulfonamide to tryptophan residues in carbonic anhydrase, that tryptophan participates in the active site of the enzyme. The absence of chemical shift in our experiments does not support this conclusion, but on the other hand does not prove the opposite. A tryptophan residue might still be close to the

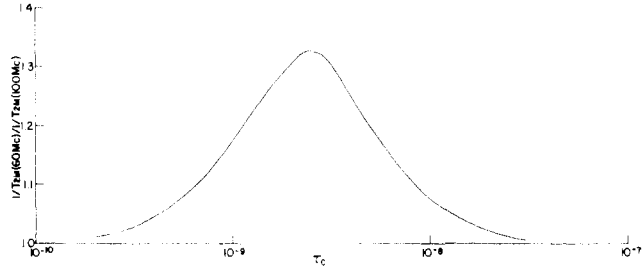


FIGURE 10: A theoretical plot of the ratio of dipolar spectral line broadening at 60 and 100 MHz, as a function of the correlation time, τ_c, according to eq 12.

TABLE III: Frequency Dependence of N^1-Acetylsulfanilamide Nuclear Magnetic Resonance Line Broadening.[a]

[E] (M)	Residue	$\Delta\nu$ (Hz) 60 MHz	100 MHz	Ratio
1.1×10^{-3}	Methyl	1.9	1.3	1.46
	Phenyl	4.5	4.3	1.05
6.2×10^{-4}	Methyl	0.9	0.65	1.38
	Phenyl	3.0	2.8	1.07

[a] Inhibitor concentration was 0.02 M, pH 6.9. Temperature 27°.

inhibitor but in such orientation, that the ring current effect is cancelled.

Kinetic Considerations. In many cases the inhibition of CO_2 hydration by sulfonamide inhibitors was found to follow a noncompetitive kinetics (Davis, 1959; Leibman *et al.*, 1961; Maren and Wiley, 1968). Recently, Kernohan (1966b) suggested a very slow rate of dissociation of the enzyme inhibitor complex, as the reason for the noncompetitive kinetic behavior. This was extended by Lindskog and Thorslund (1968) for the inhibition kinetics of bovine cobalt carbonic anhydrase. Typical values of the dissociation rate constant are in the range of 0.1–0.2 sec^{-1} which corresponds to enzyme–inhibitor complex lifetime of several seconds. Chen *et al.* (1969) using fluorescent stopped-flow spectrometry measured a second-order rate constant for the binding of a fluorescent dye to carbonic anhydrase and using the measured equilibrium constant for the dissociation reaction also derived a very low dissociation rate constant of the EI complex.

In our experiments, the temperature dependence of the nuclear magnetic resonance broadening is consistent, as was discussed above, with a fast-exchange region which means that exchange lifetime of bound inhibitor, τ_M, is shorter than its relaxation time T_{2M}. This is true for the loosely bound N^1-acetylsulfanilamide as well as for the tightly bound *p*-toluenesulfonamide. The values of T_{2M} for both inhibitors, at room temperature were 5.6×10^{-3} sec. Since $\tau_M = k_{off}^{-1}$, where k_{off} is the dissociation rate constant of the enzyme–inhibitor complex, we can derive a lower limit of $k_{off} > 2 \times 10^2$ sec^{-1}. This value is of several orders of magnitude higher than those of Lindskog and Thorslund (1968) and Chen and Kernohan (1967). Although the results have been obtained for different inhibitors, it is obvious that this cannot account for the large discrepancy in the values of dissociation rate constants. It might be mentioned at this point that experiments with manganese-substituted carbonic anhydrase enabled us to determine absolute values of 5×10^4 and 5×10^2 sec^{-1} for the first-order dissociation rate constants of the enzyme complexes with N^1-acetylsulfanilamide and *p*-toluenesulfonamide, respectively (A. Lanir and G. Navon, in preparation). It is possible that the nuclear magnetic resonance technique is sensitive to one step in a complex mechanism which is different from steps that are measured by the other methods. As an example we might be dealing with a primary fast enzyme–inhibitor association reaction that is followed by a slower conformational change of the enzyme protein. Still, it is important to point out that our results cannot be accounted for by assuming a minor fraction of a fast-exchanging enzyme–inhibitor complex. Such an assumption would lead to an unreasonable T_{2M} of this minor species. Note that in the calculation of T_{2M} from the observed broadening a value for the fraction of the bound inhibitor which exchanges with the free inhibitor in the bulk should be used. Assuming that only a small part of the bound inhibitor exchanges with the solution will lead to smaller values of f and consequently very short values of T_{2M}. In any case, we can conclude that at least one species of enzyme–inhibitor complex rapidly exchanges with its free constituents.

Correlation Time and Mode of Binding. Since the specific broadening of the ring protons upon binding was about the same for the three sulfonamide inhibitors used in our work (see Table I), it can be concluded that the mode of binding of the phenyl group of the three inhibitors is the same, and the same correlation time $\tau_c = (1.0 \pm 0.5) \times 10^{-8}$ sec determines their tumbling. This value is comparable to the τ_c calculated for the whole enzyme molecule using the Stokes–Einstein formula

$$\tau_c = \frac{4\pi\eta a^3}{3kT} \tag{13}$$

In this formula the enzyme is assumed to be a rigid sphere of radius, a, randomly rotating in a medium of viscosity, η (0.85 P for D_2O at 27°, Lewis and MacDonald, 1933). It was found from X-ray measurements (Fridborg *et al.*, 1967) that the overall dimensions of the molecules are $40 \times 45 \times 55$ Å. If a mean radius of 23.5 Å is taken as the effective radius of the enzyme in solution, the rotational correlation time for the enzyme as a whole is estimated to be $\tau_c = 1.5 \times 10^{-8}$ sec. There are also experimental measurements of the correlation time, τ_c, using fluorescent depolarization. Both Chen and Kernohan (1967) using 5-dimethylaminonaphthalene-1-sulfonamide bound to carbonic anhydrase and Brewer *et al.* (1968) using a fluorescein-labeled enzyme, obtained the same values for the relaxation time in the Perrin–Weber equation (Weber, 1952) $\tau_D = 3.0 \times 10^{-8}$ sec, and since $\tau_D = 3\tau_c$ then $\tau_c = 1.0 \times 10^{-8}$ sec which is in agreement with our results. The lack of independent rotational motion of the phenyl moiety of the inhibitors in relation to the enzyme indicates that this part of the inhibitor is tightly bound to the enzyme. The fact that about 90% of the phenyl proton line broadenings is due to intermolecular interaction with the enzyme's protons, leaving only 10% to intramolecular interactions, supports the above conclusion. Since the dipolar interaction is a very short-range interaction, inversely proportional to the sixth power of the distance between the interacting nuclei, there should be a close contact between the phenyl and the enzyme. On the other hand, the methyl group of the bound N^1-acetylsulfanilamide is less restricted, its τ_c is shorter than that of the protein and, furthermore, the major contribution to the broadening comes from intramolecular interactions. An inference might be made that the methyl group does not contribute to the strength of the enzyme–inhibitor complex. Dipolar interactions with the protein protons were not considered in earlier works (Gerig, 1968; Schmidt *et al.*, 1969; Sykes *et al.*, 1970).

Our results conform to the X-ray data (Fridborg *et al.*, 1967) in which the benzene ring was shown to lie in a narrow slot of the protein. The close interaction of the ring protons with those of the protein, found in the present work, is another expression of the hydrophobic binding which apparently also causes the fluorescent blue shift of bound inhibitor found by Chen and Kernohan (1967). The role of the aromatic ring in the binding process has also been demonstrated by binding of sulfonamide inhibitors to apocarbonic anhydrase. This binding could be observed in equilibrium dialysis experiments where high enough inhibitor concentration was used (Coleman, 1967a) and by our nuclear magnetic resonance line-broadening experiments (A. Lanir and G. Navon, unpublished results). In the later experiments the line broadenings obtained were similar to the present results with the zinc-containing enzyme, thus indicating a similar mode of binding.

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