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Nuclear Magnetic Resonance Studies of Carbonic Anhydrase. Binding of Sulfacetamide to the Manganese Enzyme†

Amos Lanir and Gil Navon*

ABSTRACT: The effect of manganese(II)–bovine carbonic anhydrase on the proton magnetic relaxation times T_1 and T_2 of sulfacetamide was studied. The activity and stability of the manganese–enzyme complex were determined. The specificity of sulfacetamide binding to the active site was demonstrated by its replacement by *p*-toluenesulfonamide and azide ion. The equilibrium constant for the inhibitor–enzyme complex determined by the nuclear magnetic resonance method was found to agree with that obtained by kinetic measurements, providing further evidence for the specificity of the binding. The enhancement of the relaxation rate, $1/T_2$, appeared to be controlled by both the exchange lifetime and the dipolar relaxation mechanisms. Their relative contributions were separated using the measured T_1/T_2 ratios and the correlation time for the dipolar interaction could be determined. It was found that the electronic spin relaxation is the dominating correlation time at lower temperatures, while the rotational correlation time, which has a different temperature dependence, dominates at higher temperatures. Substituting the correlation times in the Solomon–Bloembergen equations for relaxation rates of the bound inhibitor, the distances between the manganese ion and protons of the methyl group, and phenyl protons ortho and meta to the sulfonamide group in the bound inhibitor molecule were calculated as 4.6 ± 0.2, 5.6 ± 0.3, and 6.6 ± 0.4 Å, respectively. These distances fit with a model in which the sulfonamide nitrogen is directly bound to the metal ion. However, this model is not unique. From the exchange lifetime and the equilibrium binding constants, assuming one-step inhibition mechanism, the rate constants for the association and dissociation reactions and their temperature dependences were derived.

Sulfonamides are known to be potent inhibitors of the zinc metalloenzyme, carbonic anhydrase (EC 4.2.1.1) (Maren, 1967). X-Ray crystallography has shown that sulfonamide inhibitors are bound in a cavity in the enzyme close to the zinc atom (Fridborg *et al.*, 1967; Liljas *et al.*, 1969). Still the detailed structure of the enzyme–inhibitor complex is not yet available. There are several other spectroscopic indications that sulfonamides are bound near the metal and probably occupy a position within the coordination sphere (Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1967b, 1968). In the complex the sulfonamides are in the anionic state, although there are still different arguments on whether the deprotonation step occurs before or after the binding process (Kernohan, 1966; Chen and Kernohan, 1967; Lindskog and Thorslund, 1968; Lindskog, 1969; King and Burgen, 1970; Taylor *et al.*, 1970b). Fluorescence shifts observed with dansylamide (Chen and Kernohan, 1967) and nuclear magnetic resonance (nmr) studies of sulfonamide binding (Lanir and Navon, 1971; Navon and Lanir, 1972) confirmed the stabilization of the complex through hydrophobic forces involving the aromatic ring which is tightly bound to the enzyme. In order to be highly effective inhibitors, the sulfonamides must possess an unsubstituted sulfonamide group (Maren, 1967), although certain modifications of this group can be made without destroying the binding affinity completely (Krebs, 1948; Whitney *et al.*, 1967; Pocker and Stone, 1968).

The zinc in the native carbonic anhydrase can be reversibly replaced by various bivalent metal ions of the first transition series (Lindskog and Malmstrom, 1962; Lindskog, 1963).

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Optical and magnetic properties of the cobalt enzyme were used as a probe for changes at the immediate environment of the active site upon sulfonamide binding. (For a review, see Lindskog, 1970.) The present paper takes advantage of the magnetic properties of manganese ion in carbonic anhydrase, and we report a quantitative study of its effect on the proton relaxation times of the inhibitor sulfacetamide.

The only physical method available at the moment for direct measurements of geometrical parameters of enzyme–substrate complexes is X-ray diffraction, with the obvious imitation of the need of single crystals and the assumption of the equivalence of the structure of the enzyme–substrate complexes in the crystalline and the dissolved states. Nuclear magnetic relaxation time measurements have the potential of obtaining these parameters in solutions. One very promising way of estimating distances of various parts of substrate or inhibitor from a certain point in an enzyme is the use of paramagnetic ions containing enzymes. This method is summarized in excellent reviews by Mildvan and Cohn (1970) and Cohn (1970) and was used by several authors: Navon *et al.* (1968, 1970) in carboxypeptidase inhibitors and Mildvan and Scrutton (1967) and Mildvan *et al.* (1967) in pyruvate carboxylase and pyruvate kinase systems. However, in most of these works, the high accuracy of this method could not be achieved due to either slow exchange rates, where only upper limits for the distances could be obtained, or uncertainties in the values of the correlation times. We report in the present paper the evaluation of interatomic distances between various protons of the inhibitor sulfacetamide (N^1-acetylsulfanilamide) and the metal ion in manganese–bovine carbonic anhydrase B, as well as the kinetic parameters of the enzyme–inhibitor dissociation and association reactions.

Experimental Section

Preparation of Mn–Enzyme. B ovine carbonic anhydrase B was separated from crude carbonic anhydrase (Seravac) by DEAE-cellulose chromatography (Lindskog, 1960). The apoenzyme was prepared (Lindskog and Malmstrom, 1962) by dialysis against 5×10^{-3} M 1,10-phenanthroline (Merck) in 0.1 M acetate buffer (pH 5.2) at 5° for at least 14 days. The chelator was removed by dialysis against frequent changes of deionized water until there was no absorption at 270 nm. Mn(II)–carbonic anhydrase was obtained by dialyzing the apoenzyme against a 250-fold excess of 5×10^{-4} M $MnCl_2$. A precipitate of denatured material was centrifuged off. Blank solutions of Mn(II) ions were prepared from the last dialysis solutions.

Protein concentrations were determined from measurements of the optical densities at 280 nm using a molar absorptivity of 5.7×10^4 cm^{-1} M^{-1} and a molecular weight of 30,000 (Nyman and Lindskog, 1964).

Sulfonamide. Sulfacetamide (N^1-acetylsulfanilamide) was purchased from Fluka and was recrystallized three times from water. The compound showed a pK value of 5.38, and it is ionized in the neutral range of pH. The inhibitor was found to be free from metal ions, as was determined by the dithizone method (Sandell, 1959). In order to eliminate the possibility of contaminations by other sulfonamides with unsubstituted $-SO_2NH_2$ groups, which are much stronger inhibitors than sulfacetamide, the inhibition constant of the sulfacetamide was determined after each recrystallization. No change of the inhibition constant was found after more than three recrystallizations.

Concentrations of Mn(II) were determined by atomic absorption (Perkin-Elmer) and from the amplitude of the electron paramagnetic resonance (epr) spectrum of Mn(II) obtained on a Varian V-4500 epr spectrometer. The sample was contained in a quartz capillary tube maintained at 25° for the epr measurements.

Enzymatic activity was determined using *p*-nitrophenyl acetate as a substrate following the procedure of Armstrong *et al.* (1966).

Magnetic Resonance Techniques. Nuclear magnetic resonance spectra (100 MHz) were obtained with a Varian HA-100 spectrometer, equipped with a Varian C-1024 time-averaging computer. The probe temperature was measured by the peak separation of dry methanol and was 30 ± 1° unless otherwise stated. Hexamethyldisiloxane was used as an external lock. Values of $1/T_2$ were obtained from the spectral line width using the expression $1/T_2 = \pi\Delta\nu_{1/2}$ where $\Delta\nu_{1/2}$ is the full line width at half-maximum peak height. T_1 values were obtained using the progressive saturation method. H_1 was calibrated by progressive saturation of a standard manganese solution. T_1 and T_2 of the standard solution were measured independently by pulse method using a pulse nmr attachment to a Varian HA-100 high-resolution spectrometer (Ginsburg *et al.*, 1970).

The nuclear magnetic resonance spectrum of the phenyl protons of sulfacetamide break effectively into two overlapping AB spectra. The broadening of every two lines of the AB quartet, upon additions of the Mn–enzyme, was the same within our experimental error, and their average has been used in treating the data.

Results

Binding of Mn(II) Ions to Apocarbonic Anhydrase B. The equilibrium constant K_{Mn} of the manganese–apoenzyme complex was estimated by using both epr and equilibrium dialysis. In the first method, concentrations of free Mn(II) ions were determined from the epr signal height in solutions of apoenzyme to which a known amount of Mn(II) was added (Cohn and Townsend, 1954). The control samples contained the same buffer without the enzyme. The method was calibrated with a standard $MnCl_2$ solution. The enzyme concentration range was 1×10^{-4} to 1.5×10^{-4} M. In the equilibrium dialysis method a correction due to the Donnan effect is applied. The correction is dependent on the ionic concentration and on the average charge of the macromolecule in the prevailing pH (see, *e.g.*, Tanford, 1961). The isoelectric point of bovine carbonic anhydrase B was reported to be 5.9, and the average charge at pH 7.0 to be 3.6 (Nilsson and Lindskog, 1967). Considering the buffer concentration (Tris sulfate, 0.1 M) this correction was found to be negligible in our case.

The interaction of Mn(II) with n identical and noninteracting sites on the enzyme molecule is given by eq 1 (Hughes and

$$\frac{[E]_T}{[Mn^{2+}]_b} = \frac{K_{Mn}}{n[Mn^{2+}]_f} + \frac{1}{n} \quad (1)$$

Klotz, 1956). The results of the two methods are plotted in Figure 1. The value of n, determined from the reciprocal of the ordinate intercept, is 1.0 ± 0.4, indicating one molecule of bound manganese ion per molecule of enzyme. The value of K_{Mn} at pH 7.0 was obtained from the reciprocal of the abcissa intercept of Figure 1 and was found to be $(3.9 \pm 0.5) \times 10^{-4}$ M at 25°. This value is in a reasonable agreement to that given by Lindskog and Nyman (1964) for Mn(II)–human carbonic anhydrase B complex, $K_{Mn} = 1.58 \times 10^{-4}$, which was measured by equilibrium dialysis at pH 5.5. The

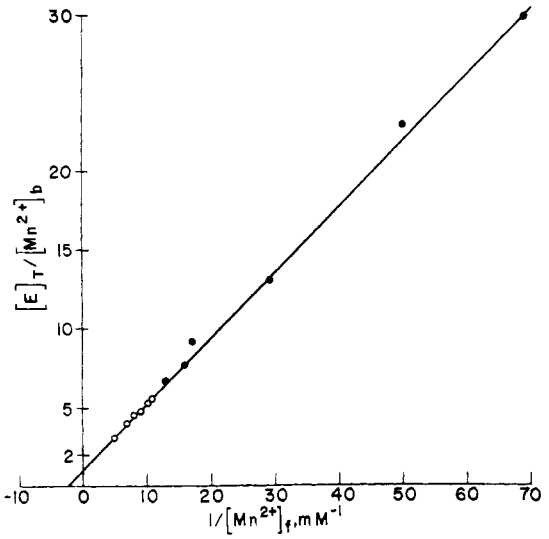

FIGURE 1: Hughes and Klotz type of plot of the ratio of bovine carbonic anhydrase to bound manganese *vs.* the reciprocal of the free manganese concentration. Samples contained (1–1.5) $\times$ 10^{-4} M apocarbonic anhydrase in 0.1 M Tris-sulfate buffer (pH 7.0). (○) Free manganese ion concentrations were determined from the epr signal height; (●) equilibrium dialysis experiment. Total manganese(II) concentrations were estimated by atomic absorption.

binding constants of Mn(II) ions to the apoenzyme were determined also at 4° (K_{Mn} = 4.8 $\times$ 10^{-4} M) and 31°(K_{Mn} = 3.0 $\times$ 10^{-4} M). The enthalpy of the binding reaction obtained from these results is 3.0 $\pm$ 0.5 kcal/mole. This value is close to an enthalpy increase of about 3–4 kcal/mole found for the formation of the relatively inactive Ni(II)-, Cu(II)-, and Cd(II)-enzyme complexes (Henkens *et al.*, 1968).

Activity of the Manganese Enzyme. The apoenzyme is very sensitive to small contaminations of Zn(II) ions in solution. Since the small fraction of the zinc enzyme present in our solution exhibits high esterase activity as compared with the manganese enzyme, it was important to determine accurately

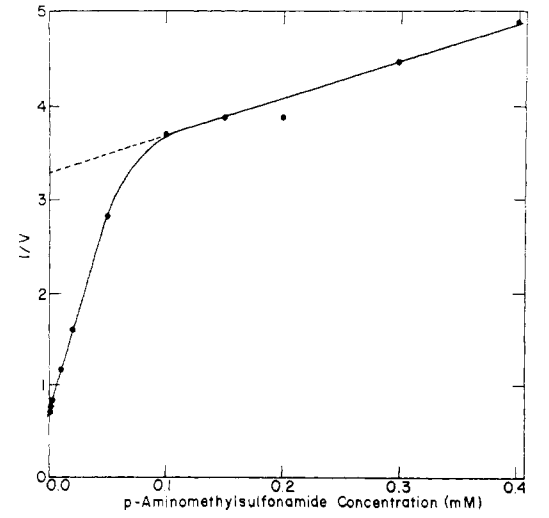

FIGURE 2: Selective inhibition of manganese and zinc carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate by *p*-aminomethylsulfonamide. *V* is given in arbitrary units. The assay medium contained Tris-sulfate buffer (pH 7.5, ionic strength 0.1 M), 3% acetone (v/v), 1.0 mM *p*-nitrophenyl acetate. Free Mn(II) concentration was 3 $\times$ 10^{-4} M. Temperature, 25°.

the relative concentrations of the zinc and manganese enzymes. These concentrations were estimated by the selective inhibition of the two metalloenzymes by the strong inhibitor *p*-methylaminosulfonamide. For a mixture of zinc and manganese enzymes (denoted as ZnE and MnE, respectively), providing that the substrate concentration is smaller than the Michaelis constants and that we have an excess of inhibitor, [I], on the metalloenzymes concentrations, the substrate hydrolysis rate is given by eq 2, where k_{Zn} and k_{Mn} are the

$$V = \frac{k_{Zn}[ZnE]}{1 + K_{IZn}[I]} + \frac{k_{Mn}[MnE]}{1 + K_{IMn}[I]} \quad (2)$$

activities ($k = k_{cat}[S]/K_m$), and K_{IZn} and K_{IMn} are the binding constants of the inhibitor to the corresponding metalloenzymes. The plot of $1/V$ *vs.* the concentration of the inhibitor can be separated into two straight lines (Figure 2). The slope of the first line to a good approximation is

$$\frac{K_{IZn}}{k_{Zn}[ZnE] + k_{Mn}[MnE]} \quad (3)$$

The second slope which prevails after full titration of the zinc enzyme is

$$\frac{K_{IMn}}{k_{Mn}[MnE]} \quad (4)$$

The reciprocal intercept on the ordinate of the first line is the total V_0.

$$V_0 = k_{Zn}[ZnE] + k_{Mn}[MnE] \quad (5)$$

The reciprocal intercept of the extrapolated second line on the ordinate gives the value of $V_{Mn} = k_{Mn}[MnE]$. From these two intercepts we could obtain the value of k_{Zn}·[ZnE]. By comparing this value with the specific activity of the native zinc enzyme we could determine the residual zinc enzyme concentration. Using the manganese-apoenzyme binding constant, the MnE concentration was then calculated, and, consequently, the activity of the manganese enzyme, which was found to be 4.5 $\pm$ 0.5% of the activity of the native zinc enzyme. Lindskog and Nyman (1964) quoted 8 $\pm$ 2% residual hydration of CO_2 activity of the manganese-substituted bovine enzyme. However, a value of 3.9%, where *p*-nitrophenyl acetate was the substrate, and which is in agreement with our results, was reported by Thorslund and Lindskog (1967) in a later work. Using the intercept to slope ratios for the two straight lines in Figure 2, the inhibition constants of both manganese and zinc enzymes by *p*-aminomethylsulfonamide were obtained. These were found to be (1.3 $\pm$ 0.3) $\times$ 10^{-3} and (9.6 $\pm$ 0.3) $\times$ 10^{-6} M, respectively.

Effects of Mn-Enzyme on Sulfacetamide Line Broadening. Manganese-carbonic anhydrase B has a very pronounced broadening effect on the nmr spectral lines of sulfacetamide. The nmr spectrum of sulfacetamide consists of three groups of lines which can easily be assigned to the acetyl protons, protons ortho to the sulfonamide group, and the protons in the meta position. For the same concentration range the broadenings were more than 100-fold larger than that of the diamagnetic zinc enzyme (Lanir and Navon, 1971). The different proton groups of sulfacetamide were broadened by different amounts. The broadening effect was largest for the acetyl protons, sec-

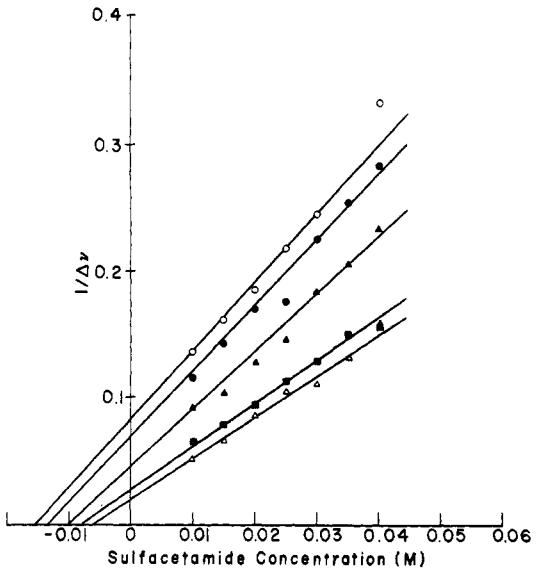

FIGURE 3: Concentration dependence of the reciprocal net line broadening of the methyl group of sulfacetamide bound to manganese carbonic anhydrase, at various temperatures: (○) 1°, (●) 7°, (▲) 12°, (■) 22.5°, (△) 30°. The manganese enzyme concentration was 1.06×10^{-5} M.

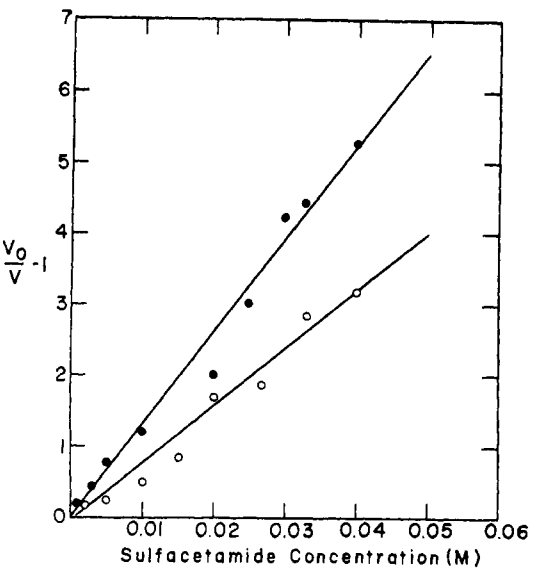

FIGURE 4: Inhibition of manganese carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate by sulfacetamide: (●) 14°, (○) 21°. The assay medium contained Tris-sulfate buffer (0.1 M, pH 7.5), 3% acetone (v/v), 1.0 mM *p*-nitrophenyl acetate.

ond in order for the ortho phenyl protons, and the smallest broadening was observed for the protons in the meta position to the sulfonamide group. Smaller broadening effects of the Mn–enzyme were found for the nitrogen-unsubstituted sulfonamides, which possess better inhibition activity. However, they exhibit more complicated behavior and will be considered separately.

The interpretation of the broadening was done using eq 6 which is valid in the present situation where a small fraction (f) of bound inhibitor is exchanging with an excess of free inhibitor in the bulk and in the absence of chemical shift

$$\frac{1}{T_{ip}} = \frac{f}{\tau_M + T_{iM}} \qquad i = 1, 2 \tag{6}$$

where $1/T_{ip}$ is the net relaxation rate caused by the Mn-enzyme.

$$\frac{1}{T_{ip}} = \frac{1}{T_i(\text{obsd})} - \frac{1}{T_i(0)} \tag{7}$$

τ_M is the exchange lifetime of the bound inhibitor, which equals k_{off}^{-1} for a one-step inhibition mechanism.

$$E + I \rightleftharpoons EI \tag{8}$$

T_{1M} and T_{2M} are the longitudinal and transverse relaxation times of the bound inhibitor, respectively. Since in our case $[I]_0 > [EI]$, f is given by eq 9, where K_I is the dissociation equilibrium constant of reaction 8.

$$f = \frac{[EI]}{[I]_0} = \frac{[E]_0}{K_I + [I]_0} \tag{9}$$

Equilibrium Constant for the Sulfacetamide–Mn(II)–Bovine Carbonic Anhydrase Complex. Sulfacetamide is not a very strong inhibitor and was only partially bound in the concentration used. In order to calculate f, it was necessary to measure K_I at each temperature. This was done by measuring the dependence of the line-broadening values on the inhibitor concentration. Since the nmr line due to the methyl group is a singlet and has the highest amplitude, it was chosen for the equilibrium constant determination. Using eq 6 and 9 and the expression $1/T_{2p} = \pi\Delta\nu_p$ one obtains (Navon *et al.*, 1970)

$$\frac{1}{\Delta\nu_p} = \frac{\pi(T_{2M} + \tau_M)}{[E]_0}(K_I + [I]) \tag{10}$$

A plot of $1/\Delta\nu_p$ *vs.* [I], keeping the enzyme concentration constant, gave us a straight line. Its negative x-axis intercept is equal to the equilibrium constant, K_I. Such a plot for 5 different temperatures is given in Figure 3.

To eliminate any nonspecific binding of the inhibitor to the enzyme, we compared the dissociation constant obtained from the nmr method to that controlling the inhibition of the Mn–bovine carbonic anhydrase esterase activity.

$$\frac{V_0}{V} = 1 + \frac{[I]}{K_I} \tag{11}$$

Figure 4 presents a plot of $(V_0/V) - 1$ *vs.* the concentration of the inhibitor for two temperatures. An Arrhenius plot of the equilibrium constants determined by the two methods is given in Figure 5A. The fact that the binding constants obtained by the two methods fall in the same line strongly indicates that even if an additional nonspecific binding of sulfacetamide to the enzyme occurs, such binding has no effect on our results. Moreover, this agreement between the binding constant values excludes any possible small contaminations of N^4-acetylsulfanilamide or sulfanilamide, since these inhibitors can cause a pronounced effect on the measured inhibition constant of sulfacetamide, but do not contribute to its line broadenings. The apparent ΔH of sulfacetamide binding to the manganese enzyme was found to be 5.8 kcal/mole.

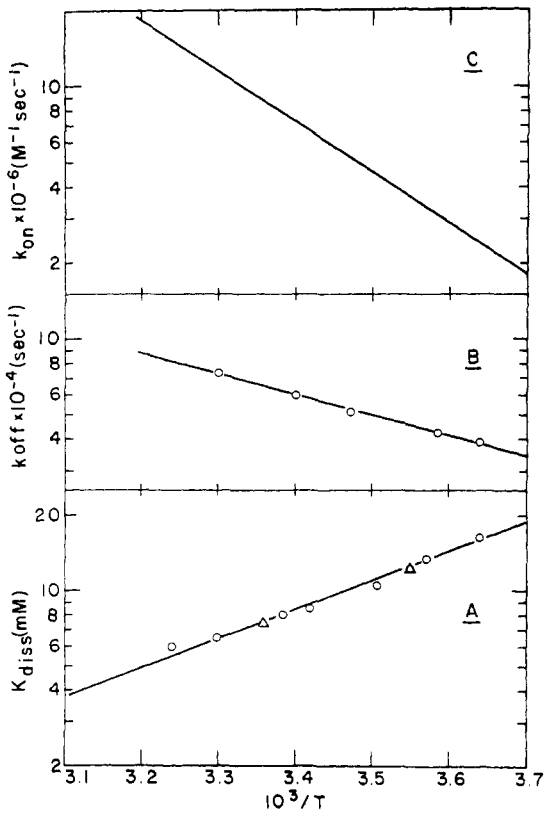

FIGURE 5: Arrhenius plots for the binding parameters of sulfacetamide to manganese–bovine carbonic anhydrase. (A) The dissociation equilibrium constant of the enzyme–inhibitor complex K_I, (○) found from nmr method, (△) from inhibition measurements. (B) The dissociation rate constant of the complex, k_{off}. (C) The complex formation rate constant, k_{on}, calculated from the measured values of K_I and k_{off}.

Nmr Titrations. In order to determine whether the observed nmr line broadening of sulfacetamide is due to a specific binding to the active site of manganese–bovine carbonic anhydrase, its displacement was followed using two specific inhibitors: *p*-toluenesulfonamide, which has an unsubstituted sulfonamide group, and the anionic inhibitor, N_3^- ion. The narrowing of the line width of the sulfacetamide methyl group upon gradual addition of the inhibitors is given in Figures

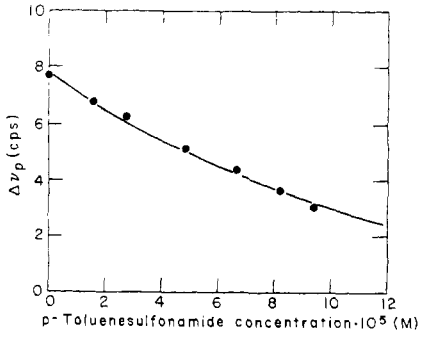

FIGURE 6: The net line broadening of the methyl group of sulfacetamide upon titration with *p*-toluenesulfonamide. The points are experimental. The line is a theoretical one. pH 7.5, 30°. Initial concentrations of the manganese(II)–bovine carbonic anhydrase and sulfacetamide were 1.41×10^{-5} M and 0.04 M, respectively. At the last point they are diluted by a factor of 1.60.

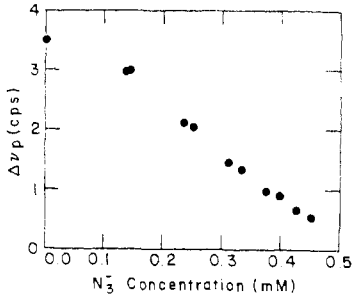

FIGURE 7: The net line broadening of the methyl group of sulfacetamide, upon successive addition of azide ion. pH 7.5, 30°. Initial concentrations of the manganese(II)–bovine carbonic anhydrase and sulfacetamide were 0.79×10^{-5} M and 0.04 M, respectively. At the last point they are diluted by a factor of 1.83.

6 and 7. For a simple competition between two inhibitors, I_1 and I_2, where $[I_1] \gg [EI_1]$ and $[I_2] \gg [EI_2]$ (Lanir and Navon, 1971)

$$\frac{1}{\Delta\nu_p} = \frac{\pi(\tau_M + T_{2M})}{[E]_0}\left(K_{I_1} + [I_1] + \frac{K_{I_1}}{K_{I_2}}[I_2]\right) \qquad (12)$$

TITRATION WITH *p*-TOLUENESULFONAMIDE. Since the solution was diluted during the titration, and, consequently, the concentrations of E_0 and I_1 were not constant, the dissociation constant of the *p*-toluenesulfonamide–Mn(II)–bovine carbonic anhydrase complex of 3.5×10^{-5} M was estimated from the *x*-axis negative intercept in the plot of $[E]_0/[\pi\Delta\nu_p \cdot (T_{2M} + \tau_M)] - [I_1]$ *vs.* $[I_2]$. The calculated broadening, assuming this value of K_I, is represented by the solid line in Figure 6.

TITRATION WITH AZIDE. Here the titration curve (Figure 7) could not be fitted using a simple competition (eq 12), and there is an apparent lag in the sulfacetamide displacement, up to N_3^- concentration of about 1.3×10^{-4} M. It is possible that this lag is partially due to an additional and stronger binding of azide ions not to the active site. A further discussion on this point will be given in a subsequent publication. An order of magnitude estimation of the equilibrium constant for the binding of azide to the sulfacetamide binding site gives $K_I \cong 3 \times 10^{-4}$ M and can be compared with the value of 6×10^{-4} M for the azide–zinc(II)–bovine carbonic anhydrase complex (Riepe and Wang, 1968; Pocker and Stone, 1968).

Separation of T_{2M} and τ_M and Calculation of Distances. Using the values of K_I and the stability constant of Mn(II)–bovine carbonic anhydrase complex, we calculated the fraction of bound inhibitor, *f*, at each temperature. Substituting in eq 6, $T_{2M} + \tau_M$ values for the three proton groups were derived and are given in Figure 8. In order to separate the contributions of the exchange lifetime, τ_M, and the relaxation time, T_{2M}, let us consider their temperature dependencies: τ_M, being the reciprocal of the dissociation constant, k_{off}, of eq 8, is invariably decreasing with temperature. Furthermore, in a case where τ_M dominates the relaxation mechanism (slow exchange limit) one expects a single broadening value for all proton groups of the molecule, which exchanges as one unit. In our case, however, different broadening values were obtained for the various groups. Also the temperature dependency of the broadening was not characteristic of an exchange-limited mechanism. It is obvious, therefore, that the contribution of T_{2M} is very important. The relaxation rates of bound inhibitor protons, due to dipolar interaction with a paramagnetic metal ion located at a distance *r*, has been

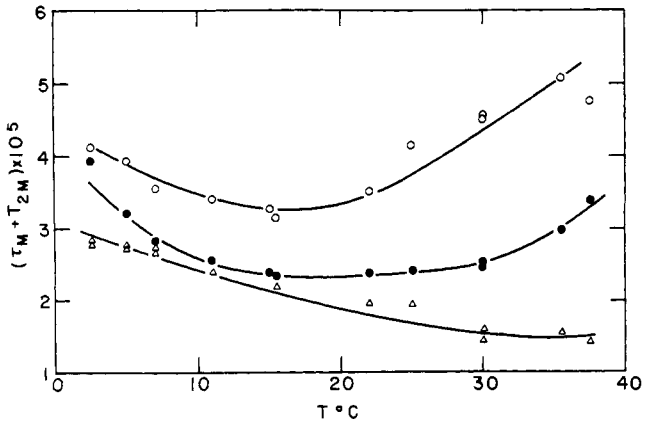

FIGURE 8: Temperature dependence of $(T_{2M} + \tau_M)$ of sulfacetamide bound to Mn(II)-bovine carbonic anhydrase. (○) Phenyl protons meta to the sulfonamide group. (●) Phenyl protons ortho to the sulfonamide group. (△) Methyl protons of the acetyl group.

given by Solomon (1955). For a manganese-containing macromolecule, where $\omega_s\tau_c \gg 1$ and where the contact hyperfine interaction (Bloembergen, 1957) can be neglected (Peacocke *et al.*, 1969)

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{\mu^2\gamma_I^2}{r^6}\left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (13)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{\mu^2\gamma_I^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (14)$$

In order to calculate distances between the various protons and the manganese ion, using eq 13 and 14, one needs to separate the contributions of τ_M and T_{2M} from their combined experimental value (see Figure 8), and also to know τ_c. The minimum obtained in the plot of $T_{2M} + \tau_M$ as a function of temperature does not necessarily originate from the different temperature dependencies of T_{2M} and τ_M. In fact, as τ_M is the same for all the three proton groups, it can be seen that by subtracting the $T_{2M} + \tau_M$ values due to the methyl protons from those due to the phenyl protons one still obtains a plot containing a minimum even though the difference is not dependent on τ_M. We concluded, therefore, that the temperature dependency of T_{2M} has a minimum. This behavior is similar to that obtained by Peacocke *et al.* (1969) for proton relaxation in solutions of *Escherichia coli* ribosomal RNA containing Mn(II) ions, which they interpreted as due to different temperature dependencies of τ_s and τ_r in the expression

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_M^{-1} \quad (15)$$

In this relation τ_r is the rotational correlation time which decreases with temperature. On the other hand, τ_s, the electronic spin relaxation time, increases with temperature (Bernheim *et al.*, 1959) and dominates τ_c, and thus T_{2M}, at lower temperatures. In our case, τ_M is very long compared with the two other correlation times and can be omitted.

The evaluation of τ_M, T_{2M}, and τ_c is accomplished as follows. The ratio

$$\frac{T_{1p}}{T_{2p}} = \frac{T_{1M} + \tau_M}{T_{2M} + \tau_M} \quad (16)$$

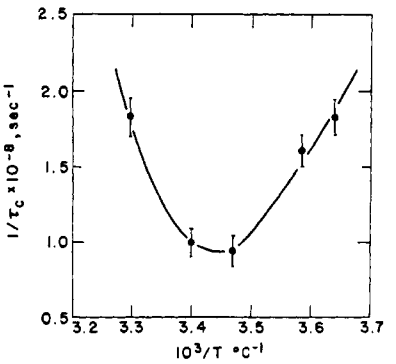

FIGURE 9: Temperature dependence of τ_c^{-1}

and the quantity $T_{2M} + \tau_M$ are measured for each of the three proton groups. Thus, we obtain three equations of the form

$$\left(\frac{T_{1M}}{T_{2M}}\right)[(T_{2M} + \tau_M) - \tau_M] + \tau_M = \frac{T_{1p}}{T_{2p}}(T_{2M} + \tau_M) \quad (17)$$

In (17) the ratio

$$\frac{T_{1M}}{T_{2M}} = \frac{7}{6} + \frac{2}{3}\omega_I^2\tau_c^2 \quad (18)$$

is obtained from eq 13 and 14 and is independent of the proton group. The three equations of the form 17 overdetermine the two constants $(T_{1M})/T_{2M}$ and τ_M. A consistent set of solutions which satisfies all three equations is obtained for each temperature. The correlation time for the dipolar interaction, τ_c, was derived from eq 18 and the temperature dependency of τ_c^{-1} is shown in Figure 9.

Substituting τ_c in eq 14 for $1/T_{2M}$, values of the distances from the metal ion to the various proton groups could be derived. All the parameters are summarized in Table I. The average distances, with their estimated errors, are 4.6 ± 0.2, 5.6 ± 0.3, and 6.6 ± 0.4 Å for the methyl protons, the ortho phenyl protons, and those which are meta to the sulfonamide group, respectively. The distances fit very well with a model in which the sulfonamide nitrogen is directly bound to the metal ion. Nevertheless, other models cannot be excluded.

The values of $k_{off} = \tau_M^{-1}$ of the sulfacetamide–Mn(II)–bovine carbonic anhydrase complex at various temperatures are plotted in Figure 5B. Combining the experimental values of τ_M and the equilibrium constants, the kinetic rate constants k_{on} for the enzyme–inhibitor binding reaction could be derived using the expression $K_I = k_{off}/k_{on}$ (see Figure 5C).

Discussion

From the relaxation times induced by paramagnetic ions, both kinetic and structural information can be obtained. This was the reason for substituting the naturally occurring zinc atom with the paramagnetic manganese ion. The manganese enzyme retains 4.5% of the activity of the zinc enzyme. Sulfacetamide, though N^1-substituted sulfonamide, still has a considerable binding constant to Mn(II)–bovine carbonic anhydrase. This was demonstrated both by enzymatic inhibition and by the appreciable relaxation rate enhancement of the various proton groups of the bound inhibitor. Moreover, this enhancement indicates the proximity of the sulfacetamide binding site to the paramagnetic metal ion. The

TABLE I: Experimental and Calculated Parameters for Sulfacetamide Bound to Mn(II)–Carbonic Anhydrase B.

Temp (°C)	T_{1p}/T_{2p}			$\tau_M \times 10^5$ (sec)	T_{1M}/T_{2M}	$\tau_e \times 10^9$ (sec)	(Å)		
	CH_3	H_{ortho}[a]	H_{meta}[a]				CH_3	H_{ortho}[a]	H_{meta}[a]
30	1.95	5.1	6.1	1.35	9	5.45 ± 0.5	4.5	5.8	6.8
21	3.4	9.9	14.5	1.68	28	10.1 ± 1.0	4.6	5.8	6.8
15	3.9	8.1	13.3	1.94	32	10.9 ± 1.1	4.6	5.3	6.4
6	1.9	3.1	4.7	2.35	11	6.2 ± 0.6	4.6	5.5	6.3
2	1.9	3.9	3.5	2.53	9	5.45 ± 0.5	4.8	5.75	6.3

[a] H_{ortho} and H_{meta} refer to the phenyl protons position in relation to the sulfonamide group in the inhibitor.

specificity of the binding was confirmed by the competition of *p*-toluenesulfonamide and the azide anion for the same binding site. Also the fact that the inhibition constant of Mn(II)–bovine carbonic anhydrase esterase activity by sulfacetamide agrees with the equilibrium constant obtained from the nmr method indicates the specific binding of the inhibitor to the active site of the manganese enzyme.

It was mentioned by several authors that the mechanism of sulfonamides binding to carbonic anhydrase is a multistep process, and the stabilization of the complex cannot be established through a single type interaction. In contrast to previous suggestions (Kernohan, 1966; Thorslund and Lindskog, 1967; Lindskog and Thorslund, 1968; Taylor *et al.* 1970a,b) proposed as the first step, a rapid preequilibrium association of the protonated sulfonamide with the anionic form of the enzyme, it seems that the initial complex involves only a hydrophobic interaction of the aromatic ring with the enzyme. Such a close contact between the aromatic moiety of sulfonamides and the hydrophobic cleft of the enzyme was shown in previous papers (Lanir and Navon, 1971; Navon and Lanir, 1972). Consequently, this initial step must be independent of the metal ion and, to a good approximation, also of the substitution on the sulfonamide group. Accordingly, the binding rate constant $k_{on} = 9 \times 10^6$ M^{-1} sec^{-1} at 25° obtained in the present work (Figure 5c) for the sulfacetamide–bovine Mn(II)–carbonic anhydrase complex is similar to the binding rate constants, ranging from 1×10^5 to 1×10^7 M^{-1} sec^{-1} for various unsubstituted sulfonamides to the zinc enzyme (Chen *et al.*, 1969; Taylor *et al.*, 1970a,b; Taylor and Burgen, 1971; Olander and Kaiser, 1970).

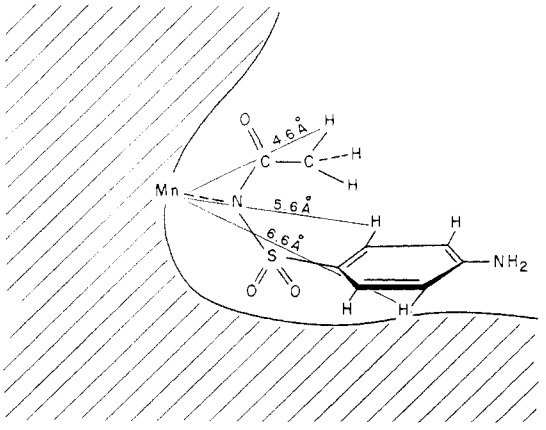

FIGURE 10: Possible structure of the carbonic anhydrase–sulfacetamide complex.

Since it is evident (see above) that sulfonamides, when bound to the enzyme, are in their anionic form, the assumption of a primary binding of the protonated form leads to a dissociation of a proton from the amido group of the sulfonamide as a second step. It was found that the replacement of the zinc atom by manganese caused reduction by a factor of about 25 in the esterase activity of bovine carbonic anhydrase and probably more in the CO_2 hydration activity (Coleman, 1967a). This fact can be correlated with the reduction in the binding affinity of sulfonamide inhibitors to Mn(II)–bovine carbonic anhydrase relative to the zinc enzyme. The binding constants of *p*-toluenesulfonamide to bovine carbonic anhydrase are 4.75×10^{-7} M (Lanir and Navon, 1971) and 3.5×10^{-5} M for the zinc and manganese metalloenzymes, respectively, *i.e.*, a reduction factor of 70 in the binding affinity. Similar reductions by factors of 20–150 were found for several other sulfonamides (unpublished results). On the other hand, no difference was found for sulfacetamide binding to the manganese and zinc enzymes. The resemblance of the metal ion dependencies of the activity and the binding of sulfonamides which are protonated at neutral pH indicates an analogy between the hydration of CO_2, or the hydrolysis mechanisms, and the binding of these sulfonamide inhibitors. A common basic group may play a role in both catalytic activity and the deprotonation step in the inhibitor binding. This analogy was also pointed out, from different considerations, by Taylor *et al.* (1970b). However, the binding of sulfacetamide which is deprotonated at neutral pH is metal ion independent. This suggests that the step in the sulfonamide binding process that is controlled by the metal ion is the proton-release step.

Structural information about the conformation of bound inhibitor can be obtained by X-ray diffraction measurements on the crystalline enzyme. Such measurements for the human carbonic anhydrase C at low resolution (Fridborg *et al.*, 1967) have indicated binding of the sulfonamide close to the metal ion, but no detailed geometry of the enzyme–inhibitor complex was obtained yet. Nevertheless, it should be pointed out that the conformation of the enzyme–inhibitor complex in the crystalline state can differ from the conformation in solution (Johansen and Vallee, 1971). Nmr has the potential for direct measurements of geometrical parameters in solution, and we applied the method for estimating the manganese–proton distances in sulfacetamide–Mn(II)–bovine carbonic anhydrase system. The distances reported in the present work can fit to a model in which the metal ion is directly bound to the nitrogen atom in the $SO_2\bar{N}R$ group of the sulfacetamide. Such a model is drawn in Figure 10. In that case, the conformation of the bound inhibitor is quite

uniquely determined. However, other structures in which the metal ion is not directly bound to the sulfonamide nitrogen atom are also consistent with the observed distances and cannot be ruled out.

It can be seen from Figure 9 that the dependency of τ_c^{-1} in temperature has a minimum. A simple interpretation to this effect is that while at high temperatures τ_c is controlled by τ_r and becomes longer as the temperature decrease, τ_s, which has an opposite temperature dependency, dominates at lower temperatures. Assuming equal contributions of τ_r and τ_s at the minimal τ_c, one gets a value of about 2×10^{-8} sec for both τ_r and τ_s. This value is in the right order of magnitude for both τ_r and τ_s. τ_r can be compared with the approximate value of $\tau_r = (1.5 \pm 0.5) \times 10^{-8}$ sec estimated for the whole enzyme (Chen and Kernohan, 1967;[1] Brewer *et al.*, 1968; Lanir and Navon, 1971; Hower *et al.*, 1971). Such a value for τ_s of the manganese ion can be compared to the value of 2×10^{-8} sec obtained for manganese–carboxypeptidase A at 100 MHz (Navon, 1970). The preliminary frequency dependence of sulfacetamide line broadening by Mn(II)–bovine carbonic anhydrase at 100 and 60 MHz revealed that at 60 MHz the broadenings were smaller by 10–20% for the various lines. This cannot be explained unless a frequency dependence of τ_c is assumed. For frequency-independent τ_c, an opposite frequency dependence is expected. Therefore the observations agree with a considerable contribution of τ_s to τ_c. τ_s would then have to increase with frequency as is expected (Bloembergen and Morgan, 1961) and as was found experimentally (Navon, 1970; Reuben and Cohn, 1970).

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Conformation of Oligoinosinates: Chain-Length Dependence and Comparison to Other Oligonucleotides†

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ABSTRACT: The oligomer series of ribosyl inosinates [$(Ip)_{2-14}I$] have been prepared by alkaline hydrolysis of poly(I) and were carefully identified. The optical properties, ultraviolet absorption and circular dichroism (CD), of this series have been studied. These studies were complemented by pmr studies of IpI and $(Ip)_2I$. In addition, using pig liver nuclei ribonuclease, pIpI and $p(Ip)_2I$ were also prepared, carefully characterized, and studied by the above techniques. The CD spectra of oligoinosinates were found to be *strongly* dependent on temperature and salt concentration. These results indicate the sensitivity of CD to electrostatic perturbations exerted on the conformation by the phosphate groups. The proton magnetic resonance (pmr) results indicate that these oligomers have an all anti conformation and the screw axis of the stack is most likely right handed. In contrast to CD, the pmr results show essentially no salt dependence, indicating that all the oligomers have similar conformational properties (*i.e.*, all anti). The uv and CD properties of the oligo(I) series were analyzed together with the published data of rA, dA, dT, rU, rC, and dC oligomers. ϵ *vs.* $1/n$ plots produce two groups: those yielding a straight line (dA, dT, rU) and those having a nonlinear curve (rI, rA, rC, dC). The extinction coefficient of the interior bases (ϵ_{int}) can be deduced for oligomers longer than dimer. Oligomers of dA, dT, and rU have ϵ_{int} which are independent of chain length (n). This observation implies that the nearest-neighbor interaction is the only significant factor in hypochromicity. The chain-length effect on CD spectral parameters is different for each polynucleotide: (1) rA, $[\theta]_{peak}$ and $[\theta]_{trough}$ both increase with n (conservative type); (2) rC, $[\theta]_{trough}$ remains essentially constant with n but $[\theta]_{peak}$ increases with n; (3) dA, $[\theta]_{trough}$ remains essentially constant with n but $[\theta]_{peak}$ decreases with n; (4) rI, $[\theta]_{trough}$ remains essentially constant with n, but $[\theta]_{peak}$ decreases at first and then increases with increasing n; (5) rU, the optical activity is not dependent on n as expected for a nonstacking coil. The conservative CD spectrum derived from base–base interactions is found to be the exception rather than the rule. Also, the chain length necessary for attaining the same physical parameter as the polymer is shorter for uv hypochromicity than for CD spectral parameters.

Much effort, including that from our laboratory, has been devoted to the research on monomeric units of nucleic acids—bases, nucleosides, and nucleotides (see review, Ts'o, 1970). Similarly, dinucleoside monophosphates and dinucleotides have been extensively studied (Brahms *et al.*, 1966, 1967; Davis and Tinoco, 1968; Ts'o *et al.*, 1969a; Chan and Nelson, 1969; Warshaw and Cantor, 1970; Kondo *et al.*, 1970; Tazawa *et al.*, 1970; Fang *et al.*, 1971; Miller *et al.*, 1971; and references cited therein). With this valuable information as backbround, our laboratory has launched a systematic investigation on the properties of oligonucleotides. The first paper in this series concerns the thermodynamic and optical properties of the 1:1 oligoinosinate–polycytidylate complexes (Tazawa *et al.*, 1972).

In this study, the oligomer series of ribosyl inosinate (r-$(I)_{2-14}$) have been carefully prepared and identified. The optical properties—both ultraviolet (uv) absorbance and circular dichroic (CD) spectra—of the oligo(rI) were investigated, and the results complemented by comprehensive proton magnetic resonance (pmr) studies on the dimer and trimer of inosinate. Reports in the literature about the optical properties of oligo(rA), oligo(dA), oligo(rC), oligo(dC), oligo(rU), and oligo(dT) have been analyzed in comparison to those observed in the present studies on oligo(rI).

Experimental Section

Materials. The sources of the chemicals and enzymes used are: poly(I), IDP, and UDP, Miles Laboratory, Elkhart, Ind.; [^{14}C]IDP and [^{3}H]UDP, Schwarz BioResearch, Orangeburg, N. Y.; *Micrococcus luteus* polynucleotide phosphorylase (EC 2.7.7.8), P-L Biochemicals, Inc., Milwaukee, Wis.; pancreatic RNase A (EC 2.7.7.16), Sigma Chemical Co., St. Louis, Mo.; *Escherichia coli* alkaline phosphatase (EC 3.1.3.1), spleen phosphodiesterase (EC 3.1.4.1), and venom phosphodiesterase (EC 3.1.4.1), Worthington Biochemical Corp., Freehold, N. J. The maximum molar extinction coefficient

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