Oxonase and Esterase Activities of Erythrocyte Carbonic Anhydrase†

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ABSTRACT: The present investigation reveals that bovine carbonic anhydrase, in contrast with the serine esterases, effectively catalyzes the hydrolysis of dimethyl 2,4-dinitrophenyl phosphate. It is thus the first well-characterized enzyme to behave unambiguously as an oxonase. The oxonase and esterase activities of bovine carbonic anhydrase toward dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate, respectively, exhibit a number of clear similarities. Both are characterized below pH 9 by typical approximately sigmoidal pH–rate profiles, defined by pk_{enz} values of 7.37 and 7.53, for each of which maximal activity requires the basic form of the enzyme. In the pH region 6–8, the Michaelis constants describing the interactions of both substrates with bovine carbonic anhydrase are, to a first approximation, linear functions of the respective turnover numbers. The formal dissociation constants, namely, the values of K_M extrapolated to zero turnover, of the enzyme–substrate complexes for dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate are 5.8×10^{-3} and 1.4×10^{-3} M, respectively, while the relative efficiencies of the bovine carbonic anhydrase catalyzed hydrolyses of these two substrates are directly reflected in the values 6.85 and 118 assumed by the ratios k_{enz}/k_{OH^-} at the inflexions in the pH–rate profiles. The apparent dissociation constants in the presence of dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate, respectively, for the complex formed between the enzyme and the specific inhibitor acetazolamide, at pH 8.95, are 3.0×10^{-7} and 3.1×10^{-7} M, thereby implicating to a similar extent the participation of the active-site zinc ion of bovine carbonic anhydrase in both oxonatic and esteratic processes. Unlike the noncompetitive pattern consistently observed for carboxylate esters, however, acetazolamide inhibition of the enzyme catalyzed hydrolysis of dimethyl 2,4-dinitrophenyl phosphate is formally competitive in type. Furthermore, the appearance of the rise in activity commonly observed above pH 9 with bovine carbonic anhydrase rate profiles occurs at a point for 2,4-dinitrophenyl acetate over 1.5 pH units higher than that found in the case of dimethyl 2,4-dinitrophenyl phosphate; such a marked substrate dependence argues strongly in favor of the involvement both of a well-defined substrate binding site and of associated conformational changes in the enzyme.

The observation that diisopropyl phosphorofluoridate does not inactivate carbonic anhydrase (Malmström et al., 1964; Liefländer & Zech, 1968) suggested analogues of paraoxon, namely, diethyl *p*-nitrophenyl phosphate, as potentially fruitful substrates for the enzyme. Indeed, the purification and partial characterization of an A-type esterase from sheep serum (Main, 1960a,b), which has a second-order catalytic rate coefficient with respect to the hydrolysis of paraoxon nine times smaller in magnitude than that for the hydrolysis of *p*-nitrophenyl acetate, is of interest: the enzyme is reversibly inhibited completely by millimolar concentrations of EDTA. Furthermore, an alkaline phosphatase preparation from kidney has been found to exhibit paraoxonase activity (Malý & Janok, 1956). The presently available information, although scanty, gives the general impression that enzymes which possess appreciable oxonase[1] activity may in fact be metal activated enzymes or metalloenzymes, but, whatever the case, the demonstration of such behavior on the part of carbonic anhydrase confers upon it the distinction of being the first well-characterized enzyme to exhibit this particular property.

The oxonase activity of bovine carbonic anhydrase with respect to dimethyl 2,4-dinitrophenyl phosphate is compared in detail with the esterase activity toward 2,4-dinitrophenyl acetate. The similar values observed in the presence of both substrates for the kinetically determined dissociation constants of the complex formed between the enzyme and the specific inhibitor acetazolamide clearly implicate the involvement of the active-site zinc ion to the same extent in the two catalytic processes.

Experimental Section

Materials

Dimethyl 2,4-dinitrophenyl phosphate[2] was prepared (DeRoos & Toet, 1958) from a sample of dimethyl phosphorochloridate (Poshkus & Hereweh, 1957), and the product recrystallized from 50% (v/v) ether–petroleum ether to a constant melting point of 63.5–64.7 °C, a procedure which gave a 65% yield overall (with respect to starting trimethyl phosphite) of very pale yellow crystals: ^{1}H NMR δ^{CDCl_3} 4.00 (d, $J_{P\text{-}OMe}$ = 12.5 Hz, 6, P(*OMe*)$_2$), 7.87 (d of d, 1, Ar $H_{6,B}$), 8.50 (d of d, J_{AB} = 9.0 Hz, J_{AX} = 2.5 Hz, 1, Ar $H_{5,A}$), 8.85 (d of d, J_{BX} = 1.0 Hz, 1, Ar $H_{3,X}$).

† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received June 6, 1977; revised manuscript received August 25, 1977.* Support for this work by grants from the National Institute of Arthritis, Metabolism and Digestive Diseases of the U.S. Public Health Service and the National Science Foundation is gratefully acknowledged. Taken in part from the dissertation of S.S. submitted to the University of Washington on July 20, 1976, in partial fulfillment of the Ph.D. degree.

[1] The term "oxonase" is derived directly from the common names given to pesticides such as dialkyl *p*-nitrophenyl phosphates (alkyl paraoxons) and *O,O*-dialkyl *S*-(1,2-dicarbethoxy)ethyl phosphorothiolates (malaoxons) and should be distinguished from "phosphatase" which generally refers to enzyme-catalyzed hydrolysis of phosphate monoesters.

[2] Caution! Care should be exercised at all times in the handling of dimethyl 2,4-dinitrophenyl phosphate since it may be expected to exhibit powerful anticholinesterase activity. Indeed, Fukuto & Metcalf (1956) found diethyl 2,4-dinitrophenyl phosphate to be the most potent inhibitor of the Fly-brain cholinesterase among 24 diethyl substituted phenyl phosphates tested, although it actually possessed a relatively low contact toxicity presumably owing to its hydrolytic degradation before reaching the site of action.

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2,4-Dinitrophenyl acetate was prepared using the method suggested by Bender & Nakamura (1962).

Bovine carbonic anhydrase was a product of Schwarz/Mann Research Laboratories. The ratio of the active to the total enzyme concentration, determined by the titration of bovine carbonic anhydrase oxonase and esterase activities with the specific inhibitor acetazolamide (vide Pocker & Stone, 1967), was found to vary between 67% and 80% for the various batches of enzyme used. The catalytic activities of pure bovine carbonic anhydrase isozyme B, purchased from Miles-Seravac Ltd. (Lindskog, 1960), were found to be within 4% of those exhibited by the active enzyme component in the Schwarz/Mann preparations.

5-Acetamido-1,3,4-thiadiazole-2-sulfonamide (acetazolamide), obtained from the American Cyanamid Co. (Lederle Laboratory Division), was found to be analytically pure (Guilbert, 1971).

Buffer components employed were phosphate (pH 5.8–8.2), tris(hydroxymethyl)aminomethane (pH 7.4–9.4), *N,N*-dimethylglycine (pH 9.2–10.4), triethylamine sulfate (pH 10.0–11.2), and basic phosphate (pH 11.0–12.0), the ionic strength being maintained at 0.10 where appropriate with added sodium sulfate. Of these, only *N,N*-dimethylglycine and triethylamine took part in side reactions with substrate in that they produce some methyl 2,4-dinitrophenyl phosphate diester (monoanion) by nucleophilic attack at the methyl group of dimethyl 2,4-dinitrophenyl phosphate (see Results), but the rate of displacement at the methyl carbon atom was never more than 40% of the overall rate of attack in buffer alone.

Potassium dihydrogen phosphate (Mallinckrodt), disodium hydrogen phosphate (Mallinckrodt), sodium sulfate (Allied Chemical), potassium chloride (Baker), sulfuric acid, and hydrochloric acid were of the analytical or reagent grade commonly available commercially and were used without further purification. Tris(hydroxymethyl)aminomethane (Tris) from Aldrich was recrystallized three times from absolute ethanol and sublimed at 140 °C. *N,N*-Dimethylglycine was prepared (Pocker & Guilbert, 1972) from *N,N*-dimethylglycine hydrochloride (Nutritional Biochemicals Corp.). Triethylamine was heated to reflux with acetic anhydride and then fractionally distilled from the mixture; the distillate was shaken with solid potassium hydroxide, then heated to reflux with powdered barium oxide, and subsequently distilled from the same.

Sodium hydroxide solutions were prepared so as to be completely free of carbonate.

Miscellaneous. The molar extinction coefficient in aqueous 10% (v/v) acetone for 2,4-dinitrophenoxide ($\epsilon_{360} = 1.42 \times 10^4$ M^{-1} cm^{-1}) was determined using a highly purified sample.

Instrumentation

The rates of ester hydrolysis were determined spectrophotometrically on a Beckman Model DU-2 instrument which was equipped with a solid state log converter, designed and assembled by Mr. Sheldon Danielson of the departmental electronics shop, and an insulated cell compartment consisting of a water bath thermostated to 25.0 ± 0.05 °C.

Rapid rates of hydrolysis were monitored on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.). The drive-syringe assembly and stopped-flow cell were thermostated by means of an insulated water bath compartment maintained at 25.0 ± 0.05 °C.

Methods

Buffer Preparations and Hydroxide Solutions. The total concentration of buffer species used with the enzyme kinetic studies was 0.030 M throughout, except for the pH range between 10.0 and 12.0, wherein the ionic strength 0.10 of the triethylamine sulfate and basic phosphate buffers was maintained by the buffer species alone. The rate coefficients for the hydroxide ion catalyzed hydrolyses of the esters were determined using a set of six aqueous sodium hydroxide solutions, the ionic strengths of which were made up to 0.10 with potassium chloride.

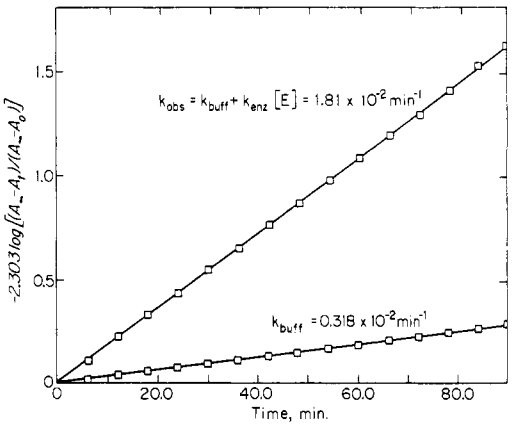

FIGURE 1: Typical first-order rate plots illustrated by the hydrolysis of dimethyl 2,4-dinitrophenyl phosphate in the presence and absence of bovine carbonic anhydrase at 25.0 °C. [Dimethyl 2,4-dinitrophenyl phosphate] = 5.30×10^{-5} M, and [bovine carbonic anhydrase] = 1.18×10^{-5} M in aqueous 10% (v/v) acetone–0.03 M Tris–sulfate buffer, ionic strength 0.10, pH 9.04, monitored at 360 nm. Such plots assign a value of 1.25×10^3 M^{-1} min^{-1} to k_{enz}.

Preparation of Bovine Carbonic Anhydrase Solutions. Solutions of the enzyme in a given buffer were prepared directly before use.

Kinetics. The hydrolyses of the 2,4-dinitrophenyl esters were followed spectrophotometrically primarily at 360 nm, but also at higher wavelengths when high substrate concentrations were employed.

Analysis of Rate Data

(*i*) *First-Order Rates.* At low substrate concentrations, $[S]_0 \ll K_M$, pseudo-first-order rate coefficients, k_{obsd}, were deduced from the data obtained with the Beckman DU-2 instrument by evaluating the best slope for first-order plots using the standard least-squares method. The plots were linear for at least 2 half-lives and the correlation coefficient of the slope for all runs was better than 0.9990. A typical first-order plot is exemplified in Figure 1. The spectrophotometric data displayed as an oscilloscope trace from the stopped-flow instrument was similarly treated; any plots for which the correlation coefficient was smaller than 0.9990 (found for less than 5% of the total number of rates run) were excluded.

For reactions involving 0.35 to 1.15×10^{-3} M dimethyl 2,4-dinitrophenyl phosphate or 2,4-dinitrophenyl acetate initial rates were followed at 459 nm for periods which ranged between 15% and 75% of the half-life of the reaction so that for a given set of rates in which only the substrate concentration was varied, the absolute concentrations of products were the same, respectively, at the beginning and end of the time-span during which each reaction was monitored.

(*ii*) *Initial Zero-Order Velocities.* In the case of reactions involving 1.06 to 3.06×10^{-3} M dimethyl 2,4-dinitrophenyl phosphate, initial velocities, v_{obsd}, were deduced from linear traces of the optical density at 360 nm between 0.80 and 1.20 units.

Analysis of k_{obsd} and v_{obsd}. In a given buffer, plots of k_{obsd} against enzyme concentration were linear over the range from

TABLE I: Pseudo-First-Order Rate Coefficients Characterizing the Hydroxide Ion Catalyzed Hydrolysis of the Ester Substrates.[a]

Ester	k_{obsd} (min^{-1}) at hydroxide ion concn of 0.0245	0.0392	0.0539	0.0686	0.0833	0.0979	k_{OH^-} (M^{-1} min^{-1})
Dimethyl 2,4-dinitrophenyl phosphate[b]	2.18	3.87	5.58	7.02	8.05	9.35	9.82×10
2,4-Dinitrophenyl acetate[b]	0.888×10^2	1.30×10^2	1.87×10^2	2.35×10^2	2.89×10^2	3.54×10^2	3.45×10^3

[a] In aqueous 10% (v/v) acetone, ionic strength 0.10 at 25.0 °C. [b] The pseudo-first-order rate coefficients given at each hydroxide ion concentration represent on the average the mean from eight individual runs monitored on the Durrum-Gibson stopped-flow apparatus.

3×10^{-7} to 5×10^{-5} M. The slopes of such plots, k_{enz}, were obtained below pH 9.0 from runs monitored in the presence of four different enzyme concentrations using initial substrate concentrations of about 5×10^{-5} M. At pH values above 9.0, k_{enz} values were determined using two different enzyme concentrations at each of which the rates were run in duplicate with the Beckman DU-2 instrument, and in duodecuplicate on the Durrum-Gibson stopped-flow apparatus. The dependence of the enzymatic rate upon the substrate concentration was formally analyzed below pH 8.0 in terms of the simple Michaelis and Menten scheme according to the formalism of Lineweaver and Burk. Between three and five duplicate runs each were followed at five different substrate concentrations in determining K_M and V_M.

The apparent value of the inhibition constant (cf. Webb, 1963) for the specific inhibitor acetazolamide was obtained, from the region in which the fractional inhibition was no longer directly proportional to the sulfonamide concentration, by using the relationship $K_i = (1 - i)([I]_0 - i[E]_0)/i_0$, where $[E]_0$ and $[I]_0$ are the total concentrations of bovine carbonic anhydrase and inhibitor, respectively, and i the fractional inhibition.

Results

Reactions of the Ester Substrates with Nucleophiles

Hydroxide Ion Catalysis. The respective data for the two esters studied and the second-order rate constants k_{OH^-} are listed in Table I. The results are within 7% of those reported by Kirsch & Jencks (1964) for 2,4-dinitrophenyl acetate.

The question arises as to whether a certain proportion of the hydroxide ion catalyzed hydrolysis of dimethyl 2,4-dinitrophenyl phosphate is accompanied by carbon–oxygen bond cleavage resulting from direct nucleophilic displacement at the aromatic ring. Khan & Kirby (1970) have shown, however, that the hydrolysis of 2-(2,4-dinitrophenoxy)-2-oxo-1,3,2-dioxaphosphorinan in 0.01 to 0.03 M NaOH solutions is accompanied solely by phosphorus–oxygen bond fission.

Nucleophilic Attack at the Methyl Carbon of Dimethyl 2,4-Dinitrophenyl Phosphate. Both *N,N*-dimethylglycinate and triethylamine were observed not only to produce 2,4-dinitrophenoxide anion but also to displace the 2,4-dinitrophenyl methyl phosphate monoanion (Kirby & Younas, 1970) by nucleophilic attack at the methyl group of dimethyl 2,4-dinitrophenyl phosphate without significant subsequent SN2 displacement at the methyl carbon atom of the phosphate diester monoanion thus formed under the experimental conditions employed (*cf.* Kirby & Varvoglis, 1967).

It is clear that the second-order rate coefficients for nucleophilic attack at the methyl carbon atom of dimethyl 2,4-dinitrophenyl phosphate are equal in magnitude to the respective slopes of plots of k_{obsd} vs. $[N](A_{\infty,calcd} - A_0)/(A_{\infty,calcd} - A_{\infty,obsd})$, where k_{obsd} is the observed pseudo-first-order constant for hydrolysis, [N] is the molar concentration of *N,N*-dimethylglycinate or triethylamine, $A_{\infty,obsd}$ is the absorbance value at 360 nm observed after roughly 10 half-lives, $A_{\infty,calcd}$ is that found after the complete hydrolysis of an identical concentration of the phosphate triester in aqueous 10% (v/v) acetone phosphate buffer or hydroxide, and A_0 is the absorbance extrapolated to the time of initiation of the rate. Thus, the rate coefficients for the displacement of 2,4-dinitrophenyl methyl phosphate by *N,N*-dimethylglycinate and triethylamine from dimethyl 2,4-dinitrophenyl phosphate were found to be 3.49×10^{-1} and 5.80×10^{-2} M^{-1} min^{-1}, respectively. These results are of the generally expected order of magnitude (cf. Gregory & Bruice, 1967).

Bovine Carbonic Anhydrase Catalyzed Hydrolysis of the Ester Substrates

The simplest scheme consistent with the enzymatic hydrolysis of the esters is illustrated by the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P_1 + ES_1 \xrightarrow{k_3} P_2 + E$$

The interaction of diethyl *p*-nitrophenyl phosphate ("paraoxon" or "E600") with chymotrypsin and the other serine esterases, in which one molecule of *p*-nitrophenol is liberated during the inhibition of one enzyme molecule ($k_3 = 0$ in the above scheme: Hartley & Kilby, 1950), contrasts with bovine carbonic anhydrase catalysis of dimethyl 2,4-dinitrophenyl phosphate hydrolysis. Furthermore, as distinct from the trypsin catalyzed hydrolysis of *p*-nitrophenyl acetate (Hartley & Kilby, 1954), plots of 2,4-dinitrophenoxide appearance vs. time for the bovine carbonic anhydrase catalyzed hydrolyses of both 2,4-dinitrophenyl acetate and dimethyl 2,4-dinitrophenyl phosphate pass directly through the origin. Under the most favorable circumstances, the ratio of the maximum readily attainable $[S]_0$ to K_M was 0.55 for dimethyl 2,4-dinitrophenyl phosphate and 0.85 in the case of 2,4-dinitrophenyl acetate.

Thus, the inability to detect the "burst" effect (Gutfreund & Sturtevant, 1956; Spencer & Sturtevant, 1959; Sturtevant, 1960; Kézdy & Bender, 1962; Bender et al., 1966, 1967) would seem to suggest that in the case of bovine carbonic anhydrase the dimethylphosphoryl-enzyme and acetyl-enzyme intermediates either lack discrete existence or are hydrolyzed very rapidly ($k_3 > k_2$ in the above scheme, a supposition that would not be unreasonable if the ES_1 intermediates were actually the zinc–dimethyl phosphate and zinc–acetate complexes, respectively).

The pH dependencies of k_{enz} for dimethyl 2,4-dinitrophenyl phosphate over the pH range from 6 to 11 and 2,4-dinitrophenyl acetate between pHs 6 and 12 are plotted in Figures 2 and 3, respectively, in both of which the smooth curves have been essentially empirically generated. These curves suggest that enzymatic activity at physiological pH is governed by a base the conjugate acid of which has an apparent pK_a of about 7, and that at higher pH there appears a second basic species of which the conjugate acid exhibits a strongly substrate de-

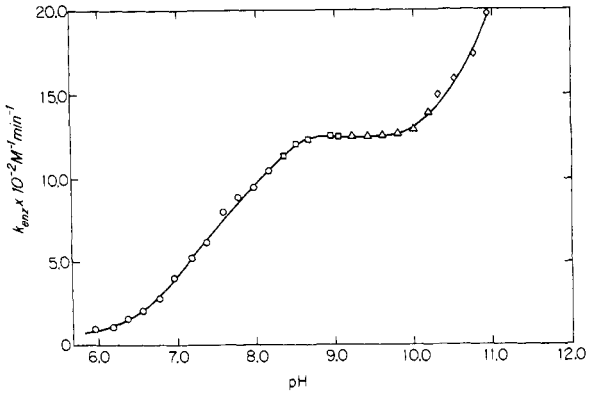

FIGURE 2: Bovine carbonic anhydrase catalyzed hydrolysis of dimethyl 2,4-dinitrophenyl phosphate as a function of pH in aqueous 10% (v/v) acetone, ionic strength 0.10, at 25.0 °C. (○) Phosphate; (□) Tris; (△) *N,N*-dimethylglycine; (◇) triethylamine. [Dimethyl 2,4-dinitrophenyl phosphate] = 5.1×10^{-5} M; [bovine carbonic anhydrase] = $0.5\text{–}4.7 \times 10^{-5}$ M.

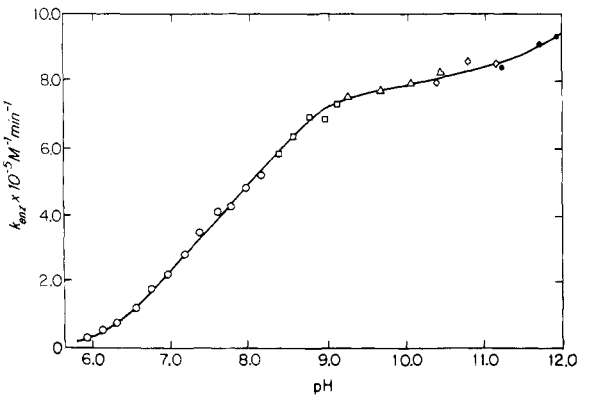

FIGURE 3: Bovine carbonic anhydrase catalyzed hydrolysis of 2,4-dinitrophenyl acetate as a function of pH in aqueous 10% (v/v) acetone, ionic strength 0.10, at 25.0 °C. (○) Phosphate; (□) Tris; (△) *N,N*-dimethylglycine; (◇) triethylamine; (●) basic phosphate. [2,4-Dinitrophenyl acetate] = 5.4×10^{-5} M; [bovine carbonic anhydrase] = $0.3\text{–}20.6 \times 10^{-6}$ M.

pendent formal pK_a value.

The biphasic pH dependence of k_{enz} can be described by one of two models, the first utilizing the ideal behavior of a single dibasic group, the second employing that of two independent monobasic groups (Pocker & Watamori, 1973). Since both give rise to expressions for k_{enz} of identical form (Wells et al., 1975), the data for dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate cannot distinguish between these two schemes. Furthermore, estimates of the apparent pK_a values from the variation of k_{enz} with pH are only indirectly related to the actual values for the activity-linked groups on the enzyme even under the most ideal conditions (cf. Khalifah & Edsall, 1972).

Plots of log k_{enz} vs. pH reveal that, while the esterase activity toward 2,4-dinitrophenyl acetate approximates closely to a sigmoidal pH function about the lower inflexion, the oxonase activity toward dimethyl 2,4-dinitrophenyl phosphate deviates appreciably from this form (Sarkanen, 1976). Indeed the influence of $[H^+]$ upon k_{enz} may be expected to be more complicated than that reflected purely by the factor $1/(1 + [H^+]/K_a)$ (cf. DeVoe & Kistiakowsky, 1961). Furthermore, the pH dependence of both the turnover number (Khalifah, 1971) and anionic inhibition (Whitney & Brandt, 1976) of human carbonic anhydrase B are not consistent with the titration of a single unperturbed activity-linked group. The

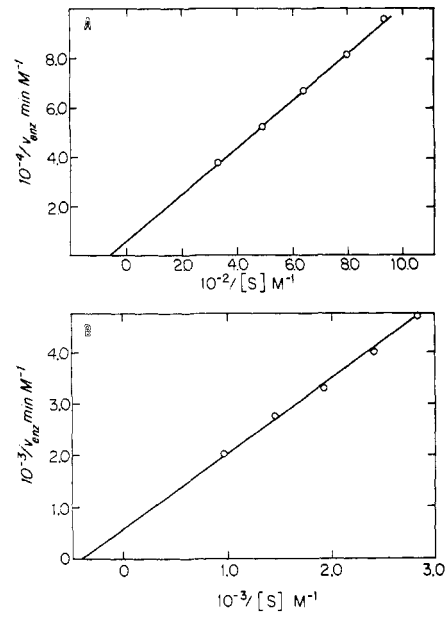

FIGURE 4: Typical Lineweaver-Burk plots reflecting bovine carbonic anhydrase catalyzed hydrolyses of ester substrates in aqueous 10% (v/v) acetone-0.03 M phosphate buffer, ionic strength 0.10, at 25.0 °C. (A) Dimethyl 2,4-dinitrophenyl phosphate at pH 7.38; [bovine carbonic anhydrase] = 1.57×10^{-5} M; $k_2 = 10.2$ min^{-1}; $K_M = 1.52 \times 10^{-2}$ M. (B) 2,4-Dinitrophenyl acetate at pH 7.18; [bovine carbonic anhydrase] = 2.46×10^{-6} M; $k_2 = 6.73 \times 10^2$ min^{-1}; $K_M = 2.38 \times 10^{-3}$ M.

formal pK_a value characteristic of enzymatic activity is thus most reasonably defined by the point where the observed magnitude of k_{enz} is half of that on the plateau, allowing the assignment of pK_a 7.37 to the oxonase and pK_a 7.53 to the esterase activity studied in the present work.

Calculation of the pseudo-first-order rate coefficients for nucleophilic attack at the methyl carbon atom of dimethyl 2,4-dinitrophenyl phosphate in the presence and absence of reasonable concentrations of the enzyme indicates that bovine carbonic anhydrase, up to a level of 2.5×10^{-5} M, is without observable effect upon the rate of displacement at the methyl group of the phosphate triester substrate (Sarkanen, 1976).

Michaelis-Menten Parameters. Under the most favorable conditions $v_{enz}/[S]_0$ could be reduced, upon increasing the initial substrate concentration to its upper limit, by 45% for 2,4-dinitrophenyl acetate and 30% in the case of dimethyl 2,4-dinitrophenyl phosphate. Typical Lineweaver-Burk plots for the enzyme catalyzed hydrolyses of dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate are given in Figure 4. The magnitudes of the values for the Michaelis-Menten parameters using the Lineweaver-Burk treatment and the Hoffstee (1952) method (Dowd & Riggs, 1965) have in the present work consistently been found to be within 5% of one another (Sarkanen, 1976). When the magnitude of K_M is greater than the highest attainable initial substrate concentrations, the Lineweaver-Burk plot acts as a better representative of physical reality since the value of k_2/K_M, namely, k_{enz}, can be accurately determined while the absolute magnitudes of the components K_M and k_2 are less certain.

A careful examination of the data indicates that in the pH interval from 6 to 8 K_M is, at least to a first approximation, a linear function of the turnover number k_2 for both substrates (Figure 5). It is thus possible to assign formal values to k_1 and k_{-1} from the plots of K_M vs. k_2 of Figure 5 in which the slopes of the lines are identified with $1/k_1$ and the intercepts at the ordinate with k_{-1}/k_1. These values along with the respective apparent dissociation constants k_{-1}/k_1 and the $\Delta G°$ values

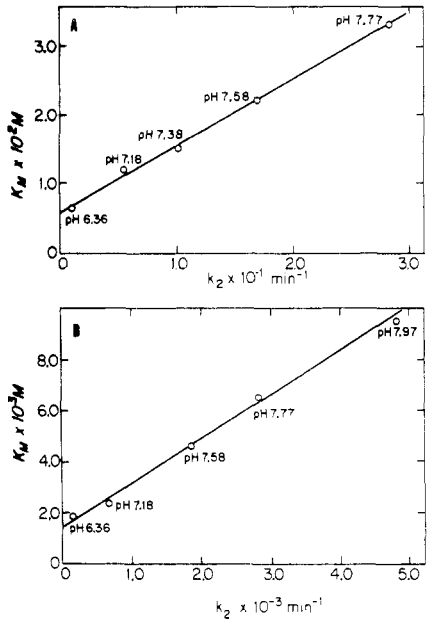

FIGURE 5: The dependencies of the Michaelis constant on turnover number for the bovine carbonic anhydrase catalyzed hydrolyses of the ester substrates as the pH is varied in aqueous 10% (v/v) acetone–0.03 M phosphate buffer, ionic strength 0.10, at 25.0 °C. (A) Dimethyl 2,4-dinitrophenyl phosphate; $k_1 = 1.03 \times 10^3$ M^{-1} min^{-1}; $k_{-1} = 6.01$ min^{-1}; $k_{-1}/k_1 = 5.84 \times 10^{-3}$ M; (B) 2,4-dinitrophenyl acetate; $k_1 = 5.80 \times 10^5$ M^{-1} min^{-1}; $k_{-1} = 8.12 \times 10^2$ min^{-1}; $k_{-1}/k_1 = 1.40 \times 10^{-3}$ M.

TABLE II: Comparison of the Formal Dissociation Constants of the Bovine Carbonic Anhydrase–Ester Complexes for Dimethyl 2,4-Dinitrophenyl Phosphate, 2,4-Dinitrophenyl Acetate, and *p*-Nitrophenyl Acetate.[a]

k_1 (M^{-1} min^{-1})	k_{-1} (min^{-1})	k_{-1}/k_1 (M)	$\Delta G°$ (cal)[b] for binding
Dimethyl 2,4-Dinitrophenyl Phosphate			
1.03×10^3	6.01	5.84×10^{-3}	3050
2,4-Dinitrophenyl acetate			
5.80×10^5	8.12×10^2	1.40×10^{-3}	3890
p-Nitrophenyl acetate[c]			
2.00×10^4	4.00×10	2.00×10^{-3}	3680

[a] Aqueous 10% (v/v) acetone–0.03 M phosphate buffer, ionic strength 0.10, at 25.0 °C. [b] For binding, $\Delta G° = RT \ln(k_1/k_{-1})$ in calories at a formal level of analysis. [c] Aqueous 10% (v/v) acetonitrile–Tris-Cl buffer, ionic strength, 0.09 (Pocker & Stone, 1967).

for the binding of each substrate to the enzyme are listed in Table II, as well as those previously reported by Pocker & Stone (1967) for *p*-nitrophenyl acetate.

Inhibition by Acetazolamide. Certain aromatic sulfonamides have been found to be very potent selective inhibitors of carbonic anhydrase activity toward both hydration (Mann & Keilin, 1940; Pocker & Meany, 1965; Kernohan, 1966; Leibman et al., 1967) and hydrolysis (Pocker & Stone, 1965; Armstrong et al., 1966; Pocker & Storm, 1968). All but 2.5% of bovine carbonic anhydrase oxonase activity toward dimethyl 2,4-dinitrophenyl phosphate at pH 7.18 could be eradicated by acetazolamide, although the necessary concentration of enzyme was high enough to render the apparent dissociation constant of the bovine carbonic anhydrase–acetazolamide complex, K_i, experimentally inaccessible under these conditions. A plot of enzymatic activity vs. the ratio of inhibitor

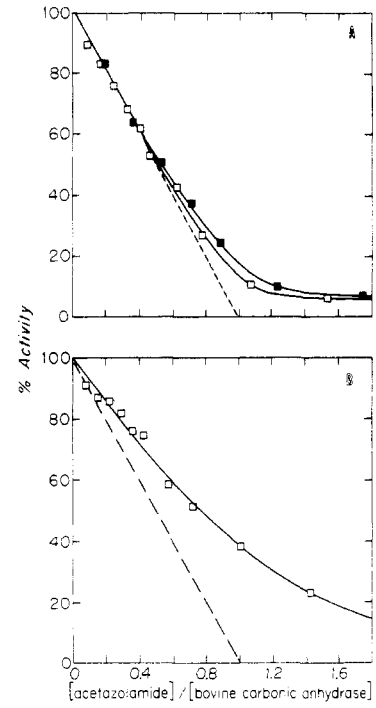

FIGURE 6: Percent enzymatic activity with respect to ester hydrolysis as a function of the ratio of acetazolamide concentration to that of bovine carbonic anhydrase at pH 8.95 in aqueous 10% (v/v) acetone–0.03 M Tris buffer, ionic strength 0.10, at 25.0 °C. (A) Dimethyl 2,4-dinitrophenyl phosphate, $K_i = 3.0 \times 10^{-7}$ M; (■) [enzyme] = 1.14×10^{-5} M; (□) [enzyme] = 1.30×10^{-5} M; (B) 2,4-dinitrophenyl acetate, $K_i = 3.1 \times 10^{-7}$ M; [enzyme] = 1.39×10^{-6} M.

TABLE III: Comparison of the Apparent K_i Values Describing Acetazolamide Inhibition of Bovine Carbonic Anhydrase Catalyzed Hydrolyses of Various Substrates.

Substrate	pH	$K_i \times 10^8$ (M)
Dimethyl 2,4-dinitrophenyl phosphate[a]	7.18	*f*
2,4-Dinitrophenyl acetate[a]	7.18	1.6[g]
Carbon dioxide[b]	7.47	0.4[h]
3-Acetoxy-2,6-dinitropyridine[c]	7.47	2.0[h]
p-Nitrophenyl acetate[d]	8.45	20[h]
3-Acetoxy-2-nitropyridine[e]	8.90	28[h]
Dimethyl 2,4-dinitrophenyl phosphate[a]	8.95	30[i]
2,4-Dinitrophenyl acetate[a]	8.95	31[j]

[a] Present work: aqueous 10% (v/v) acetone, ionic strength 0.10, 25.0 °C. [b] Pocker & Bjorkquist (1977): aqueous phosphate, ionic strength 0.10, 25.0 °C. [c] Pocker & Watamori (1971); aqueous 0.5% (v/v) acetonitrile, ionic strength 0.15, 25.0 °C. [d] Pocker & Stone (1968a): aqueous 10% (v/v) acetonitrile, ionic strength 0.09, 25.0 °C. [e] Pocker & Watamori (1973): aqueous 0.5% (v/v) acetonitrile, ionic strength 0.15, 25.0 °C. [f] Enzyme concentration required for accurately measurable catalytic activity is too high to allow the magnitude of the apparent K_i to be estimated from the data; inhibition is competitive in type. [g] ±25%. [h] Inhibition is noncompetitive in type. [i] ± 30%. [j] ± 9%.

concentration to that of enzyme, however, suggests that there is one prominent oxonatic site per enzyme molecule (cf. Figure 6A). The apparent K_i for the enzyme–acetazolamide complex in the presence of 2,4-dinitrophenyl acetate at pH 7.18 was found to be 1.6×10^{-8} M (Table III), the data indicating the presence of one esteratic site per molecule.

At pH 8.95 (Figure 6), where the apparent K_i for the enzyme–acetazolamide complex in the presence of normal ester substrates is about an order of magnitude higher than at pH

7.18 (Pocker & Watamori, 1973), all but 2.0% of bovine carbonic anhydrase oxonase activity toward dimethyl 2,4-dinitrophenyl phosphate could be eradicated by acetazolamide, while inhibition was virtually complete (>99.5%) in the case of the acetate ester. The values of the apparent K_i for the bovine carbonic anhydrase–acetazolamide complex in the presence of the two substrates are compared in Table III with a representative selection of those previously reported.

As far as the carbonic anhydrase catalyzed hydrolysis of esters is concerned, inhibition by aromatic sulfonamides has, with few exceptions, consistently been found to bring about a reduction in the value of V_M without significantly affecting the magnitude of K_M (Verpoorte et al., 1967; Pocker & Stone, 1968a; Pocker & Watamori, 1973). The enzyme-catalyzed hydrolysis of dimethyl 2,4-dinitrophenyl phosphate does not appear to be consistent with this pattern. At pH 7.18, 5.1×10^{-6} M acetazolamide acts in such a way as to increase by 50% the value of the K_M characteristic of dimethyl 2,4-dinitrophenyl phosphate without having a measurable effect upon V_M (Sarkanen, 1976). The value of the apparent K_i cannot be estimated, however, since the concentration of free inhibitor is very small and unknown.

Discussion

The present study has clearly demonstrated that bovine carbonic anhydrase effectively catalyzes the hydrolysis of dimethyl 2,4-dinitrophenyl phosphate, a process which involves the active site of the enzyme in an entirely conventional manner. The value of k_{enz} describing the oxonase activity of the enzyme toward this phosphate triester is 600-fold smaller (Figures 2 and 3) than that for the esterase activity with respect to 2,4-dinitrophenyl acetate at 25.0 °C. Bovine carbonic anhydrase in these respects resembles the partially characterized A-type esterase isolable from sheep serum (Main, 1960a,b), which was found to catalyze the hydrolysis of paraoxon nine times less rapidly than that of *p*-nitrophenyl acetate. It is thus the first well-characterized enzyme which has been observed to behave unambiguously as an oxonase. It would not cause much surprise if mammalian serum esterases exhibiting oxonase activity are subsequently all found to possess certain common features within their active centers (see introductory section). It is not an unreasonable expectation that a further more detailed chemical understanding of the oxonatic property among mammalian carbonic anhydrases and serum esterases might pave the way toward the design of efficient insecticides which display little or no contact toxicity in regard to mammals themselves.

As has consistently been found for the natural and all the normal nonnatural substrates of the enzyme (Pocker & Meany, 1965; Pocker & Stone, 1967; Pocker & Storm, 1968; Pocker & Watamori, 1971; Pocker & Guilbert, 1972; Pocker et al., 1977), the bovine carbonic anhydrase oxonase activity toward dimethyl 2,4-dinitrophenyl phosphate and esterase activity with respect to 2,4-dinitrophenyl acetate both exhibit below pH 9 approximately sigmoidal pH–rate profiles (Figures 2 and 3), characterized by pk_{enz} values (indirectly related to the pK_a of the activity controlling group: Khalifah & Edsall, 1972) of 7.37 and 7.53, respectively.

In conformity with previous findings concerning the esterase activity of bovine carbonic anhydrase (Pocker & Stone, 1967, 1968b; Pocker & Storm, 1968; Pocker & Watamori, 1971; Pocker et al., 1977), to a first approximation the Michaelis constants for the two substrates are empirically linear functions of the respective turnover numbers (Figure 5) between pH 6 and 8. While the apparent values of the dissociation constants characterizing the complexes formed between bovine carbonic anhydrase and dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate, respectively, lie well within an order of magnitude of one another, the ratio of the formal rates of association, k_1, for the two substrates (Table II) is within 10% of the corresponding k_{enz} ratio. These formal k_1 values are much smaller in magnitude than expected if they were purely to characterize a diffusion-controlled process of association of ester with bovine carbonic anhydrase. The appearance of new titratable group(s) upon the combination of certain aromatic sulfonamides (the binding of which within the primary coordination sphere of the metal ion differs significantly from that of the natural and nonnatural substrates) with human carbonic anhydrase B (King & Roberts, 1971; Pesando & Grollman, 1975) has been interpreted as indicative of a conformational change involving a small number of amino acid residues (although this is not supported by x-ray crystallographic results: Bergstén et al., 1972; Vaara, 1974) but it is not clear at present how this is related to the binding of ester substrates.

A slight shoulder-like perturbation is prominently displayed near the points of inflexion in the pH-rate profiles for dimethyl 2,4-dinitrophenyl phosphate, 2,4-dinitrophenyl acetate, and other non-natural substrates (cf. Figures 2 and 3 with Pocker & Meany (1965) and Pocker & Stone (1967)). The pH variation of the turnover number for human carbonic anhydrase B was found not to be consistent with the unperturbed titration of a single group either (Khalifah, 1971; Khalifah & Edsall, 1972), and indeed two ionizing groups have been found to influence the pH dependence of both the anionic inhibition of the human B enzyme (Whitney & Brandt, 1976) and the chemical shift of the ^{13}C resonance from its histidine 200 N^{τ}-carboxy[^{13}C]methyl derivative (Strader & Khalifah, 1976). The pK_a of the nonligand active site histidine in the bovine B isozyme could be as high as, but not greater than, 6.4 (Cohen et al., 1972), while the pK_a of the zinc-bound water molecule is presumably about 7.0, i.e., very similar to that in the human C enzyme (Campbell et al., 1975) with which the bovine B isozyme shares complete homology of the active site residues (Sciaky et al., 1974).

The Michaelis constants extrapolated to zero turnover for the bovine carbonic anhydrase catalyzed hydrolyses of *p*-nitrophenyl carboxylate esters suggested that the progressive increase per incremental methylene in the apparent free energy of binding (725 ± 50 cal per CH_2) is adequately accounted for by an increase in the free energy of hydrophobic interactions (Pocker & Storm, 1968). The present work reveals that a similar pattern is not always observed. Thus, the apparent free energy of binding for 2,4-dinitrophenyl acetate with the enzyme (Table II) is 450 cal more favorable than that for 2,4-dinitrophenyl propionate under the same conditions (Pocker et al., 1977).

A reasonable case may be made that the magnitude of the kinetically determined apparent dissociation constant, K_i, for the carbonic anhydrase–acetazolamide complex in the presence of a given substrate might reflect the degree of the molecular distribution of the compound between two related potentially productive binding loci on the enzyme. The binding of aromatic sulfonamides to apocarbonic anhydrase is 10^4 times less affine than that to the native enzyme (Coleman, 1967; King & Burgen, 1976), with the essential zinc ion of which the $-SO_2NH_2$ group becomes directly coordinated (Fridborg et al., 1967; Liljas et al., 1972; Vaara, 1974). Indeed, 4-nitrobenzenesulfonacetamide competes, but 10^4 times less effectively, with 4-nitrobenzenesulfonamide for the human C isozyme (King & Burgen, 1976). The apparent K_i values for the bovine carbonic anhydrase–acetazolamide complex in the

TABLE IV: Comparison of the Hydrolysis of Esters and Hydration of Aldehydes and Carbon Dioxide Catalyzed by Hydroxide Ion with That by Bovine Carbonic Anhydrase at the Inflexion in the pH-Rate Profile.

Substrate	pH	k_{enz} (M^{-1} min^{-1})	K_M (M)	$k_{enz,corr}$[a] (M^{-1} min^{-1})	k_{OH^-} (M^{-1} min^{-1})	k_{enz}/k_{OH^-}	$k_{enz,corr}/k_{OH^-}$
Dimethyl 2,4-dinitrophenyl phosphate[b]	7.38	6.73×10^2	1.52×10^{-2}	6.73×10^2	9.82×10	6.85	6.85
2,4-Dinitrophenyl acetate[b]	7.58	4.07×10^5	4.58×10^{-3}	1.23×10^5	3.45×10^3	118	35.6
3-Acetoxy-2-nitropyridine[c]	7.47	7.11×10^4	4.02×10^{-3}	1.88×10^4	2.10×10^3	33.9	8.96
3-Acetoxy-2,6-dinitropyridine[c]	7.47	9.31×10^5	2.19×10^{-3}	1.34×10^5	1.14×10^4	81.7	11.8
5-Nitro-3*H*-1,2-benzoxathiole 2,2-dioxide	7.32	1.52×10^7[h]	3.1×10^{-2}[i]	3.1×10^7[i]	7.86×10^4[j]	193	390
Acetaldehyde[d]	7.20	7.38×10^4	6.5×10^{-1}	3.16×10^6	4.75×10^5	0.155	6.65
Propionaldehyde[e]	7.04	3.96×10^4	2.0×10^{-1}	5.21×10^5	1.41×10^5	0.281	3.70
2-Pyridinecarboxaldehyde[f]	7.20	3.29×10^5	1.4×10^{-2}	3.03×10^5	1.8×10^6	0.18	0.168
Carbon dioxide[g]	6.75	1.61×10^9	1.55×10^{-2}	1.64×10^9	3.60×10^5	4470	4570

[a] $k_{enz,corr} = k_{enz}K_M/1.52 \times 10^{-2}$. [b] Present work: aqueous 10% (v/v) acetone, ionic strength 0.10, 25.0 °C. [c] Pocker & Watamori (1971), and Watamori (1971); aqueous 0.5% (v/v) acetonitrile, ionic strength 0.15, 25.0 °C. [d] Pocker & Meany (1965, 1967b) and Pocker & Dickerson (1969): aqueous solution, ionic strength 0.01–0.1, 0.0 °C; rate constants refer to forward process. [e] Pocker & Dickerson (1968, 1969): aqueous solution, ionic strength 0.01–0.1, 0.0 °C; rate constants refer to forward process. [f] Pocker & Meany (1967a, 1968): aqueous solution, ionic strength 0.01–0.1, 0.0 °C; rate constants refer to forward process. [g] Pocker & Bjorkquist (1977): aqueous solution, ionic strength 0.1, 25.0 °C. [h] Kaiser & Lo (1969): aqueous 0.5% (v/v) acetone–0.05 M Tris buffer, ionic strength 0.25, 25.0 °C. [i] K_M for 5-nitro-3*H*-1,2-benzoxathiole 2,2-dioxide was estimated on the basis of the assumption that its ratio to that for 2,4-dinitrophenylmethanesulfonate (1.2×10^{-2} M at pH 7.18, aqueous 10% (v/v) acetone, ionic strength 0.10, 25.0 °C) is approximately equal to the ratio of the K_M for *p*-nitrophenyl acetate (1.22×10^{-2} M at pH 7.58, aqueous 10% (v/v) acetone, ionic strength 0.10, 25.0 °C) to that for 2,4-dinitrophenyl acetate (4.58×10^{-3} M at pH 7.58: see this table): Sarkanen (1976). [j] Kaiser & Lo (1969): aqueous 3.3% (v/v) acetonitrile–0.01 M Tris buffers, ionic strength 0.09, 25.0 °C.

presence of various substrates cover the range between the two extremes (Pocker & Bjorkquist, 1977; Pocker & Watamori, 1971; Pocker & Storm, 1968; Pocker & Meany, 1965; Pocker & Dickerson, 1968). Those for the bovine carbonic anhydrase–acetazolamide complex in the presence of dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate, respectively, possess identical values (Table III). The participation of the zinc ion may thus be implicated to a similar extent in both the oxonatic and the esteratic processes.

In contrast to the acetazolamide inhibition of carbonic anhydrase esterase activity when characterized by an apparent K_i within an order of magnitude of that in the presence of CO_2 itself (Verpoorte et al., 1967; Pocker & Stone, 1968a; Pocker & Storm, 1968; Pocker & Watamori, 1973), that of bovine carbonic anhydrase oxonase activity toward dimethyl 2,4-dinitrophenyl phosphate is formally competitive. The kinetic implications stemming from the interaction of the sulfonamide with carbonic anhydrase can be understood completely only in terms of the relative rates of association and dissociation of the respective complexes formed by the combination of the enzyme with the inhibitor and the substrate at both the primary and the secondary sites within the active center (cf. Lindskog & Thorslund, 1968).

While the apparent K_i's for the bovine carbonic anhydrase–acetazolamide complex in the presence of dimethyl 2,4-dinitrophenyl phosphate and 2,4-dinitrophenyl acetate are identical in magnitude, the respective appearances of the presumed inflexions in the rate profiles for these two substrates at high pH (Figures 2 and 3) are over 1.5 pH units apart from one another. The precise origin of the enhanced catalytic activity above pH 9 has not been satisfactorily accounted for (cf. Pocker & Storm, 1968; Pocker & Watamori, 1973; Wells et al., 1975); that a well-defined substrate binding site(s) must be involved, nevertheless, is evident from the very prominent substrate dependence exhibited here by this property of carbonic anhydrase. Bovine carbonic anhydrase B N^{τ}-alkylated at the nonligand active site histidine by bromoacetazolamide, with the $-SO_2NH_2$ group of the inhibitor "frozen" into the primary coordination sphere of the metal ion, was found to display a high pH–rate profile very similar to that expected from the pH dependence observed for the native enzyme, assuming two independent activity controlling groups (Wells et al., 1975). However, at least two states of the bound sulfonamide are present (Grell, 1974; Taylor, 1975) and the native enzyme possesses *two equivalent* potential ligand binding sites at the metal ion (Cockle, 1974; Haffner & Coleman, 1975). There appears to be a change in ligand geometry surrounding the metal ion from four to five coordinate as the pH is increased from 6 to 9 (Lindskog, 1963; Lindskog & Nyman, 1964; Dennard & Williams, 1966; Taylor et al., 1970), suggesting that under acidic conditions carbonic anhydrase embodies a zinc–aquo complex, while at higher pH it is transformed to a zinc–aquo–hydroxo form. Providing that the zinc-bound water molecule does not exchange with solvent at an appreciable rate, such an hypothesis readily accounts for the apparent contradiction between the proton relaxation data for Co(II) carbonic anhydrase (Fabry et al., 1970) and the enhanced ^{35}Cl quadrupolar relaxation of chloride in the presence of the native enzyme (Ward, 1969, 1970). It is instructive that the hydrogen-bonded system encompassing the zinc-bound water molecule, threonine-197 (199), and glutamic acid-105 (106) is a particularly prominent feature of the active site of carbonic anhydrase (Kannan et al., 1971, 1975; Vaara, 1974). It is then reasonable to suppose that the high pH rise in activity may be governed by the formation of a zinc–dihydroxo species, and that only one of the metal bound hydroxide ions would be effectively displaced in the enzyme histidine N^{τ}-alkylated by bromoacetazolamide. The reactivity of the zinc–dihydroxo complex could be enhanced by the dissociation of a proton from the imidazole moiety of one of the three histidines ligated to the metal ion, a process which could depend at least in part upon a substrate induced conformational change in the structure of the enzyme (Pocker & Guilbert, 1974).

The relative efficacies of the bovine carbonic anhydrase catalyzed hydrolysis or hydration of its various substrates may be most readily compared by considering the ratio of k_{enz} to

k_{OH^-} (Table IV). It is apparent that the efficiency of bovine carbonic anhydrase toward the hydrolysis of 2,4-dinitrophenyl acetate is 17 times larger than that toward dimethyl 2,4-dinitrophenyl phosphate. Owing to the large value of k_{enz} for 5-nitro-3*H*-1,2-benzoxathiole 2,2-dioxide, "certain special interactions" with the active site cavity of the enzyme satisfying a presumed orientation requirement for particularly efficient turnover have been invoked (Coleman, 1971, 1973, 1975). However, the data in Table IV reveal that such an hypothesis is gratuitous: the value of k_{enz}/k_{OH^-} (193) for 5-nitro-3*H*-1,2-benzoxathiole 2,2-dioxide is very similar to that (118) for 2,4-dinitrophenyl acetate.

The turnover number may be compared directly with k_{OH^-} by scaling each k_{enz} to a fixed value of K_M. The resulting ratios $k_{enz,corr}/k_{OH^-}$ are also listed in Table IV; with the exception of the aldehydes, their values are within an order of magnitude of the respective k_{enz}/k_{OH^-}. The efficiency of bovine carbonic anhydrase with respect to catalysis of dimethyl 2,4-dinitrophenyl phosphate hydrolysis may now be put in perspective: while k_{enz} for 3-acetoxy-2,6-dinitropyridine is 1380 times the corresponding parameter for the phosphate triester, $k_{enz,corr}/k_{OH^-}$ for the dinitropyridyl acetate is only 41% larger. Perhaps the most important observation is that the respective ratios of $k_{enz,corr}/k_{OH^-}$ for the various substrates cannot be arranged in any self-consistent pattern. An attempt to account for the sensibly wide variation in the values of these ratios by viewing the association of the substrates with the enzyme on an ad hoc basis in terms of a nonuniform hydrophobic locus, which constrains the bound molecules to adopt significantly different orientations (both productive and nonproductive) with respect to the activity-linked group(s) on the enzyme, might therefore appear to be reasonable.

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