The ^1H n.m.r. spectra of solutions of N-methyl-2,4,6-trinitroaniline (VIId) and sodium methoxide in dimethyl sulfoxide (Figure 2) result from a mixture of the anion obtained by methoxide addition at the 3 position, IVd (R = $NHCH_3$, R′ = OCH_3), and the anion obtained by abstraction of the NH proton, IId (X = NCH_3).[6] With less than 1 equiv. of methoxide proton exchange between VIId and IId is very rapid, and for these two species an averaged spectrum is observed (Figure 2A). With slightly more than 1 equiv. of methoxide a dramatic spectral change occurs (Figure 2B). This change is undoubtedly due to a sharp decrease in the rate of rotation around the nitrogen to ring carbon bond. This rotation occurs in the free amine, VIId, and when exchange between IId and VIId is rapid produces an averaged resonance for the two aromatic ring protons.[10] The ratio of IId to IVd is solvent dependent, decreasing from about 9:1 in 10% methanol in DMSO to about 1:1 in 50% methanol in DMSO.

N-Phenyl-2,4,6-trinitroaniline reacts with methoxide to give a single anion whose structure appears to be IIe. That NH proton loss is occurring is verified by the large increase in chemical shift difference between the *ortho* and *meta* protons of the N-phenyl group on reaction (see footnote *c* of Table I). 2,4,6-Trinitroaniline also gives an equilibrium mixture of two anions. The assignment of structure IVc (R = NH_2, R′ = OCH_3) to the major component follows from a comparison of its n.m.r. spectrum with that of the anions IVa, IVb, IVd, and IVf. Comparison of the chemical shift of the ring protons in the second anion with those of the corresponding protons in Ib, IId, and IIe argues strongly for structure IIc. The ratio of IIc to IVc is about 1:9 in 10% methanol in DMSO and decreases as the methanol concentration is increased.

Of the compounds so far examined, *only trinitroanisole gives a Meisenheimer-type complex.* If the present results may be assumed to be applicable to other solvents, the results of numerous previous studies may be readily understood without invoking a myriad of dubious hypotheses.[3a,c,e,4c–e] A detailed discussion of the factors influencing the relative rates of formation of the isomeric ions and their thermodynamic stabilities together with the results of further studies on these systems will be presented at a later date.

(10) Rapid proton exchange is also observed between IVd and the dianion obtained by proton abstraction from IVd (see Figure 2C).[11] These results will be presented in a forthcoming publication.

(11) K. L. Servis, unpublished results.

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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. The Enzyme-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate

Sir:

Carbonic anhydrase (CA) is not a specific catalyst for the reversible hydration of CO_2 but powerfully catalyzes the hydration of other carbonyl systems.[1]

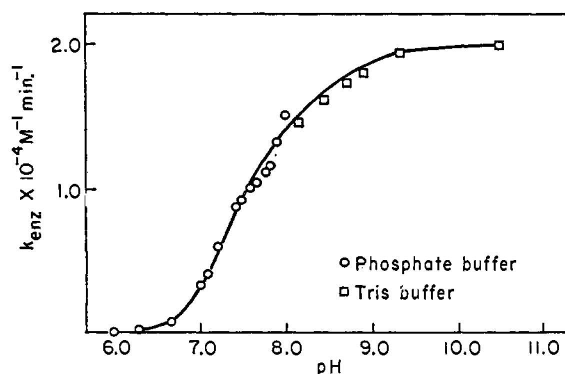

Figure 1. The hydrolysis of *p*-nitrophenyl acetate as catalyzed by bovine carbonic anhydrase.

We wish to report that *CA also acts as an esterase.*[2] Thus the enzyme efficiently catalyzes the hydrolysis of *p*-nitrophenyl acetate, a reaction which may prove to be of great value in the elucidation of the mode of action of CA because it can be studied over a very wide pH range by spectrophotometrically following the appearance of the nitrophenolate ion at 400 mμ (ϵ $\sim$2.1 × 10^4), the rate of *p*-nitrophenolate ion appearance being identical with that of substrate disappearance. Excellent pseudo-first-order kinetics are obtained; the reaction proceeds to completion (>99% hydrolysis) with stable infinities being observed.

Up to pH 9, the nonenzymic components were less than 15% of the total rate, while at higher pH they became progressively significant because of hydroxide ion catalysis. The buffers, either phosphate or Tris, were approximately 0.01 *M* and the ionic strength, μ, was held at 0.09 with added NaCl. The aqueous solutions were 10% by volume in acetonitrile. (The slope of k_{obsd} *vs.* [E] is defined as k_{enz}.) Under these conditions a pH–rate profile was obtained at 25.0° over the pH range 6.0 to 10.5[3] using bovine CA (Figure 1). Both phosphate and Tris buffers gave essentially identical k_{enz}. In acidic media the enzyme is very ineffective (k_{enz} = 82 min.$^{-1}$ M^{-1} at pH 6) while a plateau is reached in basic media where the limiting k_{enz} = 20,000 min.$^{-1}$ M^{-1} (no drop off in rate is observed up to pH 10.5); the inflection point occurs at pH 7.5. It is instructive to compare the enzyme effectiveness with that of other catalytic species present in the buffer systems: the catalytic constants, k_c, for the proton and hydroxide ion, under our conditions, are 58 and 890 min.$^{-1}$ M^{-1}, respectively, while the catalytic constants for HPO_4^{2-}, $H_2PO_4^-$, H_2O, and Tris are appreciably smaller.[4] The zinc ion associated with each molecule of CA is an obligatory component for its

(1) Y. Pocker and J. Meany, Abstracts of the Sixth International Congress of Biochemistry, 1964, Vol. IV-132, New York, N. Y., p. 327; *J. Am. Chem. Soc.*, **87**, 1809 (1965).

(2) We felt that CA would possess esterase activity because of the similarity between hydration and certain bimolecular hydrolytic mechanisms [M. L. Bender, *ibid.*, 73, 1626 (1951); **75**, 5986 (1953)]. Others have approached this activity somewhat differently [R. E. Tashian, D. P. Douglas, and Y. L. Yu, *Biochim. Biophys. Res. Commun.*, **14** (3), 256 (1964); F. Schneider and M. Liefländer, *Z. Physiol. Chem.*, **334**, 279 (1963); J. T. Edsall, private communication; S. Lindskog and P. O. Nyman, private communication.]

(3) In order to determine the catalytic efficiency of CA at high pH the inhibitory effects of basic anions had to be avoided. Pure 0.25 *M* Tris in 10% acetonitrile gives a solution whose pH as recorded by a glass electrode is 10.5 and whose "buffer" capacity is such that during initial hydrolysis the observed pH changes can be neglected.

(4) T. C. Bruice and R. Lapinski, *J. Am. Chem. Soc.*, **80**, 2265 (1958).

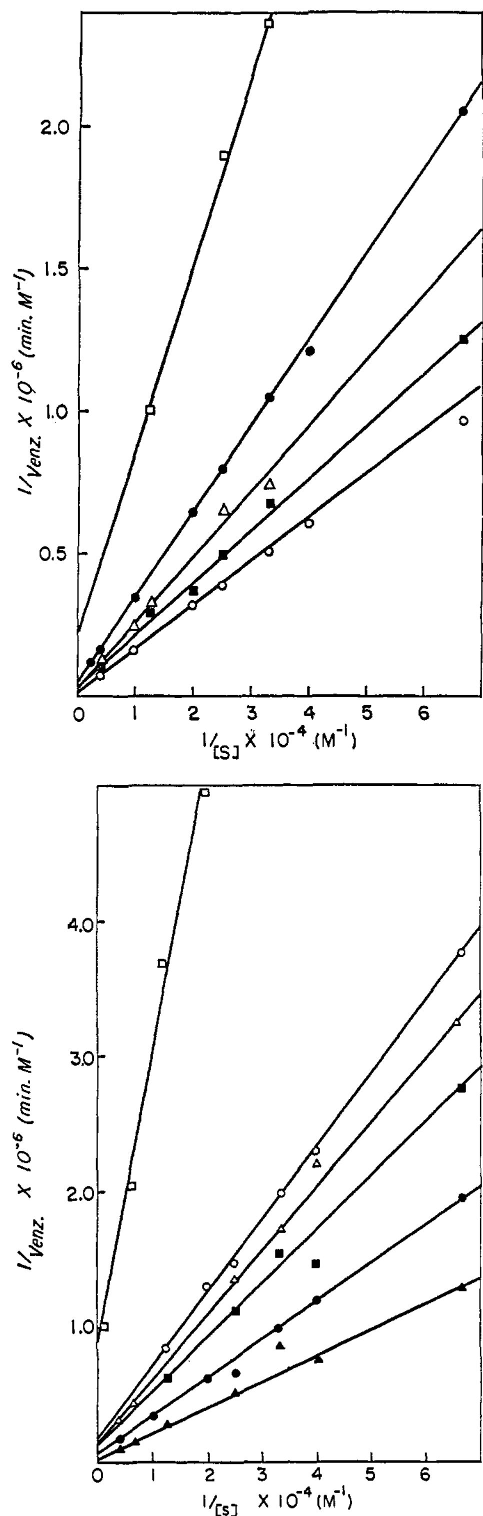

Figure 2. (a) Lineweaver–Burk plots ([CA] = 3.75×10^{-6} *M*) as a function of pH and inhibitor: □, pH 7.00; ●, pH 7.55; △, pH 7.62; ■, pH 8.45 with 5×10^{-7} *M* acetazolamide; ○, pH 8.45. (b) Lineweaver–Burk plots ([CA] = 3.75×10^{-6} *M*) at pH 7.55 in the presence of 0.09 *M* salt: □, NaI; ○, $NaNO_3$; △, NaBr; ■, NaOAc; ●, NaCl; ▲, NaF.

catalytic activity,[5] and we have shown that while catalysis by zinc ion in Tris or malonate buffers is very mild, marked enhancement is observed in imidazole buffers well in excess of that accorded by imidazole alone: k_{HIm^+}, very small; k_{Im} = 15 min.$^{-1}$ M^{-1} (similar k_{Im} is reported by others[6]); $k_{Zn^{2+}}$ (in imidazole buffers[7]; pH 6.9–7.2; $[Zn^{2+}]$ up to 5×10^{-3} *M*; ([Im] + $[HIm^+]$) = 0.036 *M*) $\simeq$ 40 min.$^{-1}$ M^{-1}.

(5) S. Lindskog and B. G. Malmström, *J. Biol. Chem.*, **237**, 1129 (1962).

We find that the dependence of the enzymatic rate on substrate concentration follows the classical Michaelis–Menten relationship. Lineweaver–Burk plots at constant enzyme concentration give values of K_M and V_M which increase with pH (Figure 2a). The observed variation of K_M is a linear function of V_M, indicating that the apparent binding constant, k_1/k_{-1}, is invariant in the pH range 6–10.5. This implies that the inflection point in the pH–rate profile refers to the titration of the hydrolyzing rather than the binding site. These observations accord with the hypothesis that the binding site must be composed of a group or groups whose pK_a lies outside the region 6–10.5, while the hydrating site has a pK_a of 7.5, as would accord with the suggestion that unprotonated imidazole and water coordinated to zinc participate in hydrolysis.

Salt (anionic) inhibition follows Michaelis–Menten kinetics and is noncompetitive, the order of inhibition being[8] $F^- < Cl^- < AcO^- < Br^- < NO_3^- < I^- < CNS^-$ (Figure 2b). Combining the pH and salt inhibition data with the equation[9] $K_M = a + bV_M$, it is found that $a = 2.5 \times 10^{-4}$ *M* and b = 13.4 min., leading to an apparent $k_1 = 2.0 \times 10^4$ M^{-1} min.$^{-1}$ and an apparent k_{-1} = 4.9 min.$^{-1}$. Acetazolamide,[10] one of the most powerful noncompetitive inhibitors of the hydrase activity of CA, is also found to inhibit noncompetitively its esterase activity with respect to *p*-nitrophenyl acetate hydrolysis, with an inhibition constant $K_i = 3.7 \times 10^{-6}$ *M* at pH 8.45. The totality of these observations, particularly the effect of pH, as well as the inhibitory effects of anions and of acetazolamide on k_2 for both hydrase and esterase activity suggest that the underlying mechanisms of CA-catalyzed hydration and hydrolysis are similar.

Acknowledgment. This investigation was supported by Public Health Service Research Grants from the National Institutes of Health.

(6) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **79**, 1656 (1957); T. C. Bruice and G. L. Schmir, *ibid.*, **79**, 1663 (1957).
(7) W. L. Koltun, R. N. Dexter, R. E. Clark, and F. R. N. Gurd, *ibid.*, **80**, 4188 (1958), report that zinc ions prevent catalysis by imidazole inasmuch as zinc–imidazole complexes have no detectable effect on the rate of hydrolysis of *p*-nitrophenyl acetate. Their observations might be due to the fact that they worked at a lower Hp (5.5–6.5).
(8) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 319 (1946), have obtained similar results for CO_2 hydration.
(9) R. P. Davis, *J. Am. Chem. Soc.*, **80**, 5209 (1958).
(10) T. H. Maren, E. Mayer, and B. C. Wadsworth, *Bull. Johns Hopkins Hosp.*, **95**, 199 (1954); Y. Pocker and J. E. Meany, *Biochemistry*, **4**, 2535 (1965); Y. Pocker and D. G. Dickerson, unpublished observations.

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Hydrogen Exchange in Benzyl Methyl Sulfoxide. Kinetic and Spectroscopic Nonequivalence of Methylene Protons

Sir:

Although measurement of Hammett substituent constants for the methylsulfonyl and methylsulfinyl groups[1]