The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. VIII. Deuterium Solvent Isotope Effects and Apparent Activation Parameters for the Enzyme-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate*

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ABSTRACT: The kinetics of the carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate have been studied in both H_2O and D_2O at 25.0°. Plots of activity as a function of pD have the sigmoid shape analogous to that obtained in H_2O, but with the inflection at pH 7.5 being displaced to a pD of 8.1. In addition, a second rise in activity occurs at pD > 10 which appears to correspond to the initial part of a second titration curve of a deuterio acid of $pK_a > 10$. The value of $k_{enzyme}^{H_2O}/k_{enzyme}^{D_2O}$ corresponding to the ratios of catalytic coefficients k_{enzmye} in H_2O and D_2O, respectively, was found to be around unity. The kinetic effects of D_2O solvent on K_m and on the turnover number, k_2, were separated and found to be: $K_m^{H_2O}/K_m^{D_2O} \simeq 0.5$ and $k_2^{H_2O}/k_2^{D_2O} \simeq 0.75$. The kinetic deuterium isotope effect for the turnover number is shown to be almost identical with that obtained for OH^- *vs.* OD^- attack on *p*-nitrophenyl acetate.

The enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate was also investigated at four temperatures ranging from 15.0 to 45.0°. The pH–rate profile was determined at each temperature and the sigmoid curve obtained was found to correspond to the titration of an acid whose pK_a is shifted to lower values with increase in temperature, $\Delta pK_a/\Delta T \simeq -0.02$. Likewise the Michaelis–Menten parameters were determined at several pH values at each temperature. The activation energy for the dissociation of the enzyme–substrate complex, K_{diss}, was found to be 11 kcal/mole. At constant pH, an apparent energy of activation of around 12 kcal/mole was deduced for the breakdown of the enzyme–substrate complex in the region of the first inflection; this value is similar to that found for the hydroxide ion catalyzed hydrolysis of *p*-nitrophenyl acetate. We have independently shown that the turnover number, k_2, depends upon a group whose heat of ionization is *ca.* 7 kcal/mole and that the proper energy of activation associated with the breakdown of enzyme–substrate complex is 7.7 kcal/mole. Consistency of the data with the previously proposed reaction pathway is noted.

The mechanism of catalysis of esters has received widespread attention (Bender, 1960). In particular, *p*-nitrophenyl acetate has often been employed in such studies (Bender and Turnquest, 1957; Bruice and Lapinski, 1958; Jencks and Carriuolo, 1960; Bruice and Schmir, 1957; Bruice and Benkovic, 1963; Sacher and Laidler, 1964). Basic hydrolysis of *p*-NPA[1] has been established as following the $B_{AC}2$ scheme.[2] Furthermore, there has been a direct correlation between basicity and nucleophilic reactivity toward esters (Jencks and Carriuolo, 1960), although basicity alone does not determine reactivity. For this reason *p*-NPA is an excellent substrate for an investigation of the effects of deuterium oxide (Bender *et al.*, 1962) and temperature (Bruice and Schmir 1957).

Deuterium oxide solvent isotope effects have been extensively studied for acid-catalyzed reactions (Wiberg, 1955; Long and Bigeleisen, 1959) and for nucleophilic reactions (Bender *et al.*, 1962). The isotope effect of the former is significantly larger than that normally associated with the latter. Therefore, from the effect of deuterium oxide on the rate of the bovine carbonic anhydrase catalyzed hydrolysis of *p*-NPA, one should be able to distinguish between general base and nucleophilic catalysis by the enzyme. The reaction was formally analyzed in terms of the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

In all probability the actual sequence of events is more complex (Pocker and Dickerson, 1968); however, from the effect of D_2O on each step in this over-simplified kinetic scheme, it should at least be possible to determine whether a proton is transferred in the rate-determining part of any of these three elementary steps. Consequently, the effects of D_2O on the various steps were studied and are reported in this paper.

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: *p*-NPA, *p*-nitrophenyl acetate; BCA, bovine carbonic anhydrase.

[2] The notation $B_{AC}2$ symbolizes a base-catalyzed ester hydrolysis which is bimolecular and proceeds with acyl–oxygen fission.

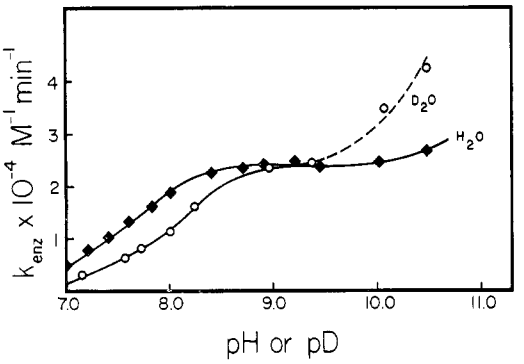

FIGURE 1: The BCA-catalyzed hydrolysis of *p*-NPA as a function of pH or pD in Tris and ephedrine buffers at 25.0°. BCA $\simeq 3 \times 10^{-6}$ M, 10% (v/v) acetonitrile, $\mu = 0.09$; ◆, H_2O; ○, D_2O.

In a like manner, the effects of temperature have been determined for *p*-NPA hydrolysis catalyzed by water, hydroxide ion, hydronium ion, and imidazole (Bruice and Schmir, 1957). There have also been many investigations of temperature effects on the various kinetic parameters involved in enzymatic reactions (Laidler, 1958). We have obtained the activation parameters of the Michaelis–Menten coefficients and comparison is made with those obtained in chemical hydrolysis as well as in the other enzyme systems.

Experimental Section

The purification and properties of *p*-nitrophenol and *p*-nitrophenyl acetate as well as bovine carbonic anhydrase have been described (Pocker and Stone, 1967). Likewise the kinetic technique has been previously described in great detail (Pocker and Stone, 1967). The D_2O was obtained from General Dynamics Corp. and was greater than 99.7% D_2O.

A Beckman Model DU-2 equipped with an insulated cell compartment thermostated to the desired temperature was employed in all spectrophotometric determinations. All pH measurements were recorded with a glass electrode on a Beckman research pH meter thermostated at the desired temperature.

D_2O Studies. Tris and ephedrine buffers in D_2O were prepared by acidifying 0.05 M stock Tris or ephedrine solutions in 99.7% D_2O with concentrated HCl. Such a procedure produced only minor fluctuations of the per cent D_2O present and these were neglected. The pD was determined by adding 0.40 unit onto the pH meter reading (Glasoe and Long, 1960; Li *et al.*, 1961), pD = pHmr + 0.40. Prior to determination of the pH meter reading the electrodes were soaked in D_2O for 2–3 hr. The meter was standardized against Beckman standard buffers. All work was done at 25.0°.

A pD–rate profile was determined as was done in water. The *p*-NPA concentration was held at 0.3 mM and the reaction was monitored at 348 mμ. The buffer rates as well as the enzymatic coefficients were obtained at ten pD values in the range 7–10.5. Tris buffers were employed at pD 7.16, 7.57, 7.72, 8.01, 8.23, 8.45, and 8.95 with respective BCA concentrations of 8.83×10^{-6}, 7.30×10^{-6}, 11.09×10^{-6}, 6.26×10^{-6}, 10.43×10^{-6}, 7.17×10^{-6}, and 9.76×10^{-6} M. Ephedrine buffers at pD 10.06 and 10.49 were employed with BCA concentrations of 11.43×10^{-6} and 9.17×10^{-6} M, respectively. Values of k_{enzyme} were calculated as before. The profile was then plotted and compared with that in H_2O.

TABLE I: Dependence of Esterase Activity upon pD.[a]

pD	Buffer	k_{buffer} ($\times 10^2$ min^{-1})	k_{enzyme} ($\times 10^{-4}$ M^{-1} min^{-1})
7.16	Tris	0.007	0.37
7.57		0.030	0.66
7.72		0.033	0.92
8.01		0.048	1.00
8.23		0.078	1.63
8.45		0.074	1.60
8.95		0.249	2.27
9.37		0.484	2.46
10.06	Ephedrine	2.22	3.71
10.49		5.71	4.19

[a] Solutions were 10% (v/v) acetonitrile, $\mu = 0.09$, at 25.0°.

Using a K_{D_2O} of 1.35×10^{-15} at 25.0° (Covington *et al.*, 1966) and the assumption that the OD^- is the major constituent of the buffer rate, a coefficient for this component was determined. This was then compared with that for hydroxide ion.

Michaelis–Menten parameters were determined at four pD values. Values of pD of 7.57, 7.72, 8.01, and 8.23 were employed with BCA concentrations of 5.11×10^{-6}, 5.11×10^{-6}, 4.96×10^{-6}, and 5.22×10^{-6} M, respectively. The hydrolysis was followed at 465 mμ. From 1.5 to 5.0 mM *p*-NPA solutions were used to deduce Lineweaver–Burk plots. All work was done in 10% (v/v) acetonitrile with μ at 0.09. From this data K_m and k_2 values were determined and a plot of K_m against k_2 was made. This data allowed the determination of all the Michaelis–Menten parameters in D_2O which were then compared with H_2O results.

Temperature Studies. The effect of temperature was studied over the range 15.0–45.0° in H_2O. Eight Tris or ephedrine buffers were employed at each temperature. The hydrolysis was monitored at 348 mμ and 0.2 mM *p*-NPA was used. Typical reaction procedure and conditions were employed. The pH values recorded were those experimentally obtained for the buffer at the given temperature. The pH–rate profiles were then determined at each temperature and compared.

Michaelis–Menten studies at each temperature were performed. At 15.0° pH values of 7.44, 7.79, 7.99, and 8.22 were used to deduce K_m and k_2; at 35.0° pH 7.32, 7.49, 7.77, and 8.11 were employed; and at 45° pH values of 7.24, 7.54, and 7.87 were employed. For all cases typical procedure for determination of Lineweaver–Burk plots was followed.

TABLE II: Comparison of Coefficients in H_2O and D_2O.[a,b]

Coefficient[c]	H_2O	D_2O	k_H/k_D
$k_{enzyme} \times 10^{-4}$	1.15 (pH 7.5)	1.15 (pD 8.0)	1
k_1	2×10^4	2×10^4	1
k_{-1}	40	84	0.5
k_2	18 (pH 7.07)	27 (pD 7.57)	0.67
	24 (pH 7.22)	32 (pD 7.72)	0.73
	54 (pH 7.51)	63 (pD 8.01)	0.86
	73 (pH 7.73)	101 (pD 8.23)	0.72
$K_m \times 10^4$	28 (pH 7.07)	55 (pD 7.57)	0.51
	32 (pH 7.22)	65 (pD 7.72)	0.50
	46 (pH 7.51)	70 (pD 8.01)	0.66
	62 (pH 7.73)	94 (pD 8.23)	0.66
$k_{OB^-} \times 10^{-3}$	OH^-	OD^-	
	0.89	1.19	0.75

[a] Comparisons made at the point where $BH^+/B = BD^+/B$, *i.e.*, at pD = pH + 0.5. [b] Studies done at 25.0° in 10% acetonitrile. [c] The units are: M^{-1} min^{-1} for k_{enzyme}, k_1, and k_{OB^-}; min^{-1} for k_{-1} and k_2; and M for K_m.

From the Michaelis–Menten data plots of K_m against k_2 were made at each temperature. Thus, all of the Michaelis parameters were determined as a function of temperature. Arrhenius plots were then made for k_1, k_{-1}, and k_2. These plots were used to derive E_a and A which could then be employed in the calculation of $\Delta H^{\pm}$, $\Delta S^{\pm}$, and $\Delta F^{\pm}$ for each of the parameters.

Results

D_2O. The enzymatic coefficient as a function of pD was studied for the BCA-catalyzed hydrolysis of *p*-NPA over the pD range 7.1–10.5. Values for the buffer component and the enzymatic coefficient are listed in Table I. The pD–rate profile is plotted in Figure 1 along with the pH–rate profile. The inflection point at pH 7.5 in H_2O is shifted to pD 8.0 in D_2O. Both curves are sigmoidal but at high pD a much more noticeable rise in k_{enzyme} is observed in D_2O, reflecting a second inflection of pK > 10. In H_2O this second inflection is only barely detectable due primarily to the potent catalysis by hydroxide ion. While deutroxide ion is a more powerful catalyst, $k_{OD^-} = 1190$ M^{-1} min^{-1}, the pK of D_2O is 14.87 at 25.0° and much less [OD^-] is present (Figure 2). The entire lower portion of the pD profile is shifted approximately 0.5 pH unit which is consistent with observed shifts of 0.55 to higher pH in D_2O (Li *et al.*, 1961). It is best to compare values of k_{enzyme} in D_2O and H_2O at points where the ratio of the basic form of the enzyme to its conjugate acid is equal in H_2O and D_2O, *i.e.*, at points where pD = pH + 0.55. Inspection of the profiles shows a larger displacement at the second inflection. If this latter inflection corresponds to the titration of a zinc–aquo complex one might well expect it to be shifted by 0.87 unit as the pK of D_2O is 14.87 as compared with 14 for H_2O. Thus, the corresponding values

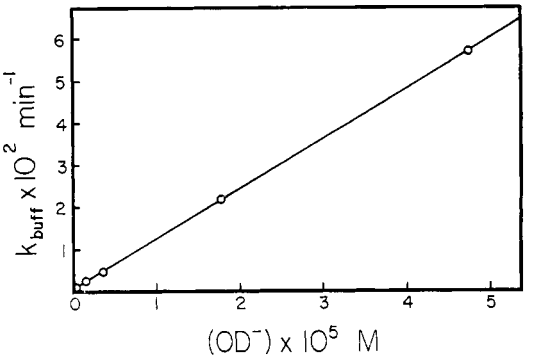

FIGURE 2: Plot of k_{buffer} *vs.* (OD^-).

of pH to pD should be obtained by pD = pH + 0.87. Unfortunately, very little of the second inflections are obtainable with *p*-NPA but were confirmed with slower reacting substrates (Pocker and Storm, 1968).

As in all enzyme reactions one must consider the possibility that D_2O may change the hydrogen-bonded structure of the enzyme and therefore produce partial or total inactivation. The enzyme was not irreversibly denaturated as the concentrations weighed out were in agreement with those determined spectrally. Furthermore, prolonged standing of enzyme in D_2O solutions did not alter the kinetic results. Since the kinetic parameters were found to be greater in D_2O, there is an inherent reasonableness to a lack of denaturation by D_2O.

The Michaelis–Menten parameters were studied as a function of pD (Figure 3) and results are listed in Table II. As in water a formal analysis of the Michaelis–Menten scheme may be obtained. It was found that K_m was a linear function of k_2 in D_2O (Figure 4). The best straight line has a slope $1/k_1 \sim 9.5 \times 10^{-4}$ M min and an inter-

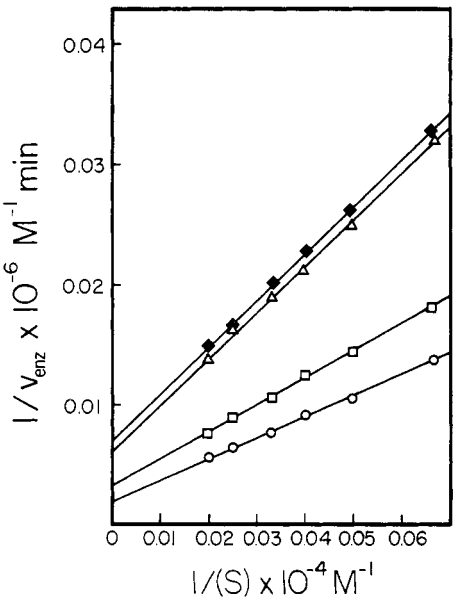

FIGURE 3: Lineweaver–Burk plots for the BCA-catalyzed hydrolysis of *p*-NPA in D_2O. Tris buffer, 10% (v/v) acetonitrile, μ = 0.09; ◆, pD 7.57, BCA = 5.11×10^{-6} M; △, pD 7.72, BCA = 5.11×10^{-6} M; □, pD 8.01, BCA = 4.96×10^{-6} M; ○, pD 8.23, BCA = 5.22×10^{-6} M.

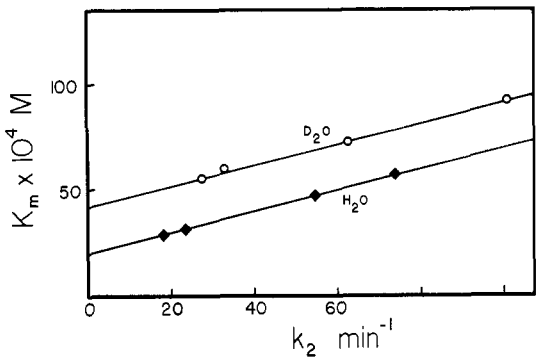

FIGURE 4: The variation of K_m with k_2 for the BCA-catalyzed hydrolysis of *p*-NPA in D_2O at 25.0°.

cept k_{-1}/k_1 of 42×10^{-4} M. Thus the formal binding constant k_1/k_{-1} of 238 M^{-1} arises from a k_1 with an apparent minimum value of *ca.* 2×10^4 M^{-1} min^{-1} and a k_{-1} with an apparent minimum value of *ca.* 84 min^{-1}.

The results in D_2O are summarized in Table II along with the corresponding values in H_2O. The value for k_{enzyme} in H_2O at pH 7.5 when compared with the value in D_2O at pD 8.1 gave a k_H/k_D ratio of approximately 1.0 ± 0.1. The k_H/k_D ratio for k_1 was also 1.0, while for k_{-1} the value was 0.5. The values for the k_2 ratio were 0.75 ± 0.1 at various pH and pD values, while K_m gave a k_H/k_D ratio of about 0.6. The chemical catalysis by hydroxide and deuterioxide gave a k_H/k_D ratio of 0.75.

It has long been known that if an enzyme-catalyzed reaction is studied over a range of temperatures the over-all rate passes through a maximum. Two independent processes were found to produce this effect, the catalyzed reaction itself and the thermal inactivation of the enzyme. The thermal inactivation of carbonic anhydrase was found to occur at around 80°. In the lower temperature range, up to 45°, inactivation is slow and has no appreciable effect on the rate of the catalyzed reaction; the over-all rate therefore increases with a rise in temperature, in the manner of ordinary chemical reactions. The BCA-catalyzed hydrolysis of *p*-NPA was examined at temperatures of 15, 25, 35, and 45°. Enzymatic pH

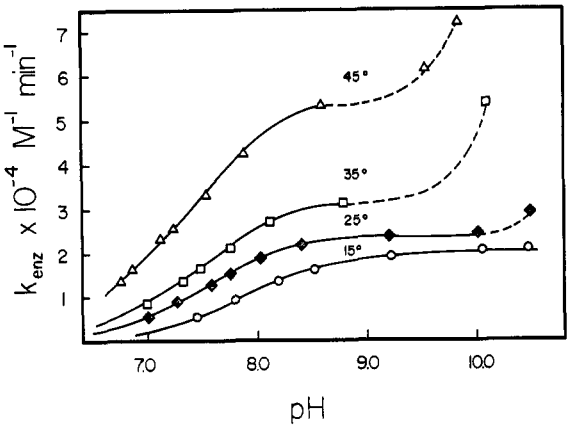

FIGURE 5: The BCA-catalyzed hydrolysis of *p*-NPA as a function of pH at various temperatures; 10% (v/v) acetonitrile, μ = 0.09, BCA 3×10^{-6} M, *p*-NPA 2×10^{-4} M; ○, 15°; ◆, 25°; □, 35°; △, 45°.

TABLE III: K_m and k_2 as a Function of pH at 15, 25, 35, and 45°.

Temp (°C)	pH	$K_m \times 10^4$ (M)	k_2 (min^{-1})
45	7.24	54	90
	7.54	61	150
	7.87	78	334
35	7.32	49	45
	7.49	69	91
	7.77	78	124
	8.11	110	219
25	7.07	28	18
	7.22	32	24
	7.51	46	54
	7.73	62	73
15	7.44	14	8
	7.79	24	18
	7.99	56	46
	8.22	86	66

profiles were obtained at each temperature, and found to be sigmoidal with the basic form of the enzyme being the active form (Figure 5).[3] The pK_a values are shifted to lower pH by about 0.2 unit/10°. Such a value agrees well with the temperature coefficient, $\Delta pK_a/\Delta T$, for imidazole which is also −0.02 unit/deg (Perrin, 1964).

The variation of rate with temperature is a composite of effects. The kinetics were formally analyzed in terms of the temperature effect on the binding process and on the subsequent hydrolysis step. Lineweaver–Burk plots were used to obtain values of K_m and k_2 as a function of pH at each temperature (Table III). Since K_m was found to be a linear function of k_2 (Figure 6), formal values of k_1, k_{-1}, and k_2 were determined for each temperature studied.

The Arrhenius law was followed and the parameters were obtained from the equation $k = Ae^{-E_a/RT}$. Plots of ln k *vs.* $1/T$ were made for k_1, k_{-1}, and k_2 (Figures 7a–c), respectively. From such plots values for E_a, A, $\Delta F^{\pm}$, $\Delta S^{\pm}$, and $\Delta H^{\pm}$ may be evaluated for the various coefficients (Table IV). The Arrhenius procedure has been applied to K_m and k_2 for a large number of enzyme systems with very satisfactory success. Improper application of the law to rates rather than individual rate constants has often incorrectly led to the conclusion that it does not apply to biological processes.

Discussion

p-NPA has proved to be a convenient substrate for

[3] In Figure 5 experimental curves are given. Values of pK_a were deduced from the best fit to the theoretical equation: $k_{enzyme} = k^{max}_{enzyme}/(1 + [H^+]/K_a)$. It should be noted that pK_a values determined in such a fashion from data reported in both Figures 1 and 5 are based on the assumption that $k_{enzyme} \rightarrow 0$ as pH or pD decreases below 6.5. We have actually substantiated the correctness of this assumption for the more detailed pH–rate profile of *p*-nitrophenyl acetate at 25°.

TABLE IV: Activation Parameters.[a]

Coefficient	E_a[b] (kcal/mole)	$\Delta H^{\ddagger}$[c] (kcal/mole)	ln A	$\Delta S^{\ddagger}$[d] (eu)	$\Delta F^{\ddagger}$[e] (kcal/mole)
k_1	11.5	10.9	29.2	−10.3	14.0
k_{-1}	22.5	21.9	41.1	13.5	17.8
K_{diss}		11.0[f]		24.0[f]	3.7[f]
k_2[g]					
At pH 7.5	12.0	11.4	24.0	−20	17.4
At half-maximum	7.7	7.1	16.6	−35	17.7
At maximum	7.7	7.1	17.4	−34	17.3
K_a[h]		6.9	27.9	−13.3	10.9

Catalyst	*p*-NPA Hydrolysis[i] E_a	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
HOH	20.7	20.1	−6.0	21.9
OH^-	11.1	10.5	−19.2	16.2
Imidazole	8.4	7.8	−27.2	15.9

[a] In 10% (v/v) acetonitrile, μ = 0.09, at 25.0°. [b] From plots of ln k *vs.* $1/T$. [c] $\Delta H^{\ddagger} = E_a - 0.6$ kcal at 25.0°. [d] $\Delta S^{\ddagger} = R \ln A - 68.5$ cal/deg at 25.0° for coefficients reported in terms of min^{-1}. [e] $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ at 25.0°. [f] ΔH from ln K_{diss} *vs.* $1/T$; ΔF from $-RT \ln K_{diss}$; and ΔS from $(\Delta H - \Delta F)/T$. [g] Parameters reported at a constant pH of 7.5 and at points where the fraction of active enzyme is constant, *i.e.*, where the same degree of ionization of the enzyme exists at each temperature. The values at half-maximum and maximum are therefore not at constant pH. [h] From the variation of the pK_a of the first inflection with temperature. [i] Bruice and Schmir (1957).

detailed kinetic study because of the ease of estimation of nitrophenol by spectrophotometry. Since no burst of *p*-nitrophenol was observed in the BCA-catalyzed hydrolysis of *p*-NPA, the reaction is provisionally analyzed in terms of the three rate coefficients, k_1, k_{-1}, and k_2. It is fully realized that each of the above steps may be

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

much more complex and can be further subdivided. Formally, however, k_2 may be taken to represent the hydrolysis process whereas the ratio k_1/k_{-1} represents the formal equilibrium between E and S with ES. While the proposed reaction scheme unquestionably is oversimplified, it allows a detailed analysis of the effect of deuterium oxide solvent and temperature on the above kinetic parameters.

Inspection of the pD–rate profile (Figure 1) reveals a sigmoidal curve analogous to the pH–rate profile. The plateau region of both profiles is found to be at almost identical k_{enzyme} values. Furthermore, comparison of k_{enzyme} values in the region of the first inflection, *i.e.*, where BH^+/B equals BD^+/B, also indicates identical values. The solvent isotope effect on k_{enzyme} may then be said to be around unity. Further inspection reveals that activity at 25.0° in H_2O depends upon a group of p$K_a \simeq$ 7.5 which is shifted to 8.1 in D_2O. We have earlier suggested that this pK_a could be associated with the ionization of an imidazole group of the histidine residue in the enzyme (Pocker and Stone, 1967). This suggestion receives some support from the observation that in D_2O the pK_a of this group is shifted by 0.5–0.6 pK_a unit (Li *et al.*, 1961). Similar results have been obtained for the BCA-catalyzed hydration of acetaldehyde (Pocker and Meany, 1965) as well as for the hydrolysis of *p*-nitrophenyl propionate (Pocker and Storm, 1968). Also a second rise in activity, as described by Pocker and Storm (1968) for certain esters, is noted for the hydrolysis of *p*-NPA in D_2O.

The results of the D_2O solvent isotope studies have been summarized in Table II. Each of the kinetic parameters is decreased in H_2O relative to that in D_2O by a factor of 0.5–1.0. These ratios may be rationalized in terms of what is known about the nonenzymatic reaction in H_2O and D_2O. The value of $k_2^{H_2O}/k_2^{D_2O}$ was found

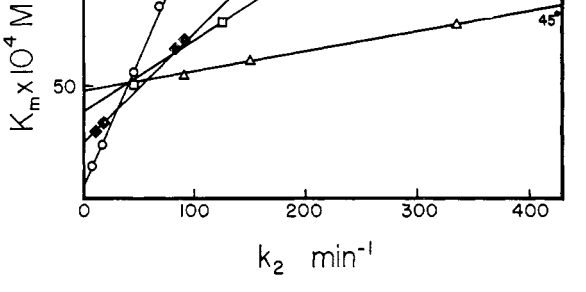

FIGURE 6: The variation of K_m with k_2 for the BCA-catalyzed hydrolysis of *p*-NPA at various temperatures. ○, 15°; ◆, 25°; □, 35°; △, 45°.

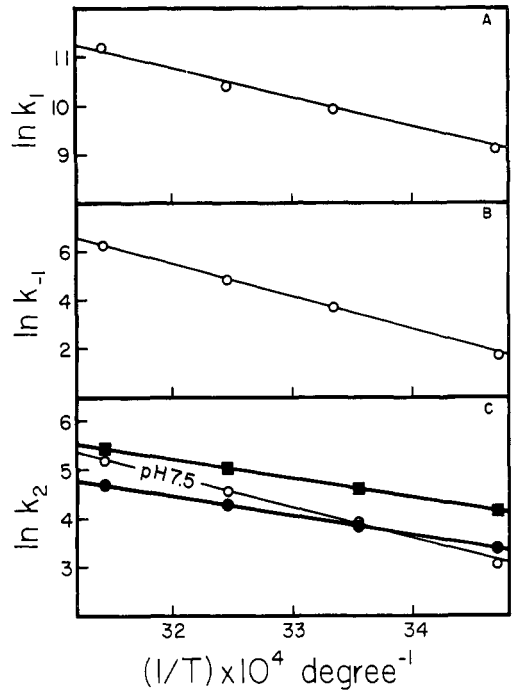

FIGURE 7: Arrhenius plot of ln k_1 *vs.* $1/T$ (a), of ln k_{-1} *vs.* $1/T$ (b), and of ln k_2 *vs.* $1/T$ (c). (○) At constant pH (7.5); (●) from points corresponding to half-ionization; (■) from points corresponding to complete conversion into conjugate base (plateau region). Data derived from Table III and Figure 6.

to be about 0.75 in the region of the first inflection at pH 7.5. This factor is much too small to be ascribed entirely to a proton transfer in the rate-controlling step (Wiberg, 1955; Long and Bigeleisen, 1959). Likewise, these values are too small to be attributed to solvation of ions which possess ratios of 1 to 1.5 (Swain *et al.*, 1960). It must be emphasized that extreme care should be taken in such evaluations since counterbalancing effects in the complex enzymatic process may be of importance. There is, however, a striking similarity between the value of $k_2^{H_2O}/k_2^{D_2O}$ in the pH interval 7–8 and the value of k_{OH^-}/k_{OD^-}. This result is very much in accord with the proposed enzymatic reaction pathway which formally involves a transfer of OH^- from enzyme to substrate. Other less detailed investigations of carbonic anhydrase catalyzed reactions have revealed similar results. For the acetaldehyde hydration reaction a $k_2^{H_2O}/k_2^{D_2O}$ ratio of 0.5 was observed at pH 7.3, *vs.* pD 7.8, (Pocker and Meany, 1965); while *p*-nitrophenyl propionate hydrolysis also was found to be 0.5 at pH 7.8 *vs.* pD 8.4 (Pocker and Storm, 1968). Thus it appears that a hydroxyl transfer (nucleophilic attack by ^-OH) is in general accord with the D_2O solvent isotope studies on BCA esterase and hydrase activity.

The formal binding constant in the formation of the enzyme-substrate complex was found to be greater in H_2O by a factor of 2 over that in D_2O. In other words the substrate would be more tightly bound to the enzyme in H_2O than in D_2O. Since most compounds are less soluble in D_2O than in H_2O because D_2O forms stronger hydrogen bonds with itself, it is somewhat surprising that a more stable ES complex is formed in H_2O. One does find, *e.g.*, that the $K_m^{H_2O}/K_m^{D_2O}$ ratio for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate is about 1.3 (Bender and Hamilton, 1962). This result is consistent with the ester, which has large hydrocarbon-like parts, being "squeezed out" of solution and onto the hydrophobic enzyme more in D_2O than in H_2O. We, on the other hand, find a $K_m^{H_2O}/K_m^{D_2O}$ ratio of around 0.5–0.7. Likewise for the BCA-catalyzed hydrolysis of *p*-nitrophenyl propionate a value of 0.6 was determined (Pocker and Storm, 1968). The corresponding value for acetaldehyde hydration was found to be 0.3 (Pocker and Meany, 1965). Thus, in each of the cases studied the formal binding constant in the formation of the BCA–substrate complex was found to be greater in H_2O by a factor of 2–3 over that in D_2O. Using BCA, the effect seems to arise from a different behavior of the enzyme in the solvent. Although the dielectric constants for both H_2O and D_2O are essentially the same, the effect of hydrogen bonding may be of great importance for the increased stability in water. Scheraga has claimed that enzymes may maintain their native structure in D_2O better than in H_2O (Kresheck *et al.*, 1965). This observation is consistent with the results obtained in this investigation, if we are indeed dealing with an enzyme conformation change upon substrate binding. Likewise such an isotope effect could be explained through the participation of both hydrogen bonding and hydrophobic forces (Pocker and Storm, 1968) in the formation of an ES complex.

By working at temperatures below which the enzyme is not thermally inactivated it is possible to study the effect of temperature on the enzyme-catalyzed reaction itself. The effect of temperature on the pH–rate profile can be seen in Figure 5. We find that the sigmoidal curve in the pH region 6–9 is displaced toward lower pH as temperature is increased. We also note that k_{enzyme} values in the "plateau" region of the curve increase with temperature. Furthermore, at high pH, a second rise in activity appears at elevated temperature and is also shifted to lower pH with increased temperature. Unfortunately, it is difficult to quantitatively evaluate the latter shift in the rate profile.

Activation parameters associated with the esterase activity of chymotrypsin and trypsin are available (Snoke and Neurath, 1950; Kaufman *et al.*, 1949; Gutfreund, 1955). The effect of temperature on k_2 for chymotrypsin, trypsin, and other enzymes has been compiled (Laidler, 1958). It is interesting to note that the entropies of activation associated with the breakdown process are all negative. Activation parameters for the hybrid Michaelis constant, K_m, showed no general trends. Energies of activation varied from 0 to 20 kcal per mole and entropies varied from −40 to +40 eu. Both solvent and structural effects may be of importance in considering the magnitude of entropies of activation. The former effect refers to the interaction of solvent with the reaction system, and the latter to the possibility that the enzyme itself may actually undergo some reversible change in conformation during the process of reaction.

Although no temperature studies of carbonic anhydrase esterase activity have been reported, an energy of

activation for the breadkown of the carbonic anhydrase–CO_2 complex is reported as 9 kcal/mole (Kiese, 1941). The breakdown of the BCA–*p*-NPA complex was studied at constant pH in the region of the first inflection. An apparent activation energy of *ca.* 12 kcal/mole and an entropy of −20 eu was found. Since the per cent active enzyme varies with temperature at constant pH, the above parameters are apparent and include a term of a group of $pK_a = 7.5$ at 25° for the ionization process. The true k_2 activation parameters were determined at the point where the group associated with the enzymatic activity was half-ionized at each temperature. This procedure leads to an energy of activation of 7.7 kcal/mole and an entropy of −35 eu. The apparent activation energy in the region of the first inflection is 8–9 kcal/mole lower than that for the "spontaneous" (uncatalyzed) hydrolysis of the ester. This sizeable reduction in the energy of activation reflects the capacity of the enzyme to efficiently promote ester hydrolysis. Indeed the scheme we envision involves the binding of the substrate to the enzyme which serves to bring into the right juxtaposition the various groups, followed by the actual hydrolysis step which involves the base-assisted transfer of a water molecule (formally a hydroxyl group) to the substrate. The apparent activation energy is very similar to that found for hydroxide ion catalyzed hydrolysis of *p*-NPA (Table IV). It is interesting to note that the activation parameters for CO_2 hydration likewise follow this pattern, *i.e.*, the enzyme-catalyzed hydration is *ca.* 9 kcal/mole less than the "spontaneous" hydration but is roughly equivalent to that found for hydroxide attack.

We have previously suggested that the inflection around physiological pH arises from the neutralization of an imidazolium group, the basic form of which is essential to BCA esterase activity (Pocker and Stone, 1967). The change of this pK_a with temperature leads to an enthalpy of ionization of *ca.* 7 kcal/mole for the group associated with enzymatic activity. A heat of ionization of *ca.* 6–7 kcal/mole is very characteristic for imidazole (Cohn and Edsall, 1942). This result, coupled with the fact that the pK_a of this group is ~7.5 in H_2O and 8.1 in D_2O at 25°, is in excellent accord with our previous implication of an imidazole group at the active site of carbonic anhydrase.

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