

# Catalysis by acetylcholinesterase in two-hydronic-reactive states

## Integrity of deuterium oxide effects and hydron inventories

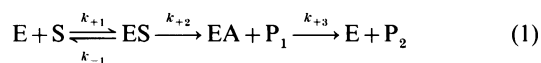
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Low  $^2\text{H}_2\text{O}$  effects (1.0–1.5) for the parameter  $k_{\text{cat.}}/K_m$  in the hydrolysis of various substrates by acetylcholinesterase (AcChE) is due to normal  $^2\text{H}_2\text{O}$  effects (1.8–2.8) for the parameter  $k_{\text{cat.}}$  and  $^2\text{H}_2\text{O}$  effects of 1.0–2.5 for the parameter  $K_m$ . The analyses and interpretations of  $^2\text{H}_2\text{O}$  effects in the literature utilizing the parameter  $k_{\text{cat.}}/K_m$ , which led to the proposal of ‘isotope insensitivity’ of the catalytic steps and the hypothesis of a rate-limiting substrate-induced-fit conformational change, are incorrect. Since  $k_{\text{cat.}}$  is the only parameter that can represent the hydron-transfer step solely, the  $^2\text{H}_2\text{O}$  effect can most appropriately be evaluated by using this parameter. Calculations and comparison of acylation ( $k_{+2}$ ) and deacylation ( $k_{+3}$ ) rate constants show that acylation is rate-determining for most substrates and the improved binding  $-0.84$  to  $-2.09$  kJ/mol ( $-0.2$  to  $-0.5$  kcal/mol) in  $^2\text{H}_2\text{O}$  obscures the normal  $^2\text{H}_2\text{O}$  effect on  $k_{\text{cat.}}$  when the ratio  $k_{\text{cat.}}/K_m$  is utilized. Consistent with this, measurements of the inhibition constant ( $K_{\text{I(com.)}}$ ) for a reversible inhibitor, phenyltrimethylammonium, lead to  $K_{\text{I(com.)}}^{\text{H}_2\text{O}} = 39 \pm 3 \mu\text{M}$  and  $K_{\text{I(com.)}}^{^2\text{H}_2\text{O}} = 24.5 \pm 3.5 \mu\text{M}$ , an  $^2\text{H}_2\text{O}$  effect of  $1.59 \pm 0.26$ . pH-dependence of  $k_{\text{cat.}}$  in  $^2\text{H}_2\text{O}$  is subject to variability of the  $\text{p}K_{\text{(app.)}}$  values, as evaluated in terms of the two-hydronic-reactive states (EH and  $\text{EH}_2$ ) of AcChE, and is due to an uneven decrease in  $^2\text{H}_2\text{O}$  of the kinetic parameters  $\tilde{k}_{\text{cat.}}$  for the  $\text{EH}_2$  state relative to  $\tilde{k}_{\text{cat.}}$  for the EH state, thus leading to variable shifts in  $\text{p}K_{\text{(app.)}}$  values of between 0.5 and 1.2 pH units for this parameter. The observed pH-independent limiting rate constants for  $k_{\text{cat.}}/K_{\text{m(app.)}}$  are made to vary between 0.5 and 1.0 in  $^2\text{H}_2\text{O}$  by effects on kinetic parameters for the  $\text{EH}_2$  state,  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m$  varying between 0.2 and 0.7 relative to the EH state, with  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m$  varying between 0.4 and 1.0. The effects observed on  $k_{\text{cat.}}/K_{\text{m(app.)}}$  are ultimately the result of variable effects of  $^2\text{H}_2\text{O}$  on  $\tilde{k}'_{\text{cat.}}$  and  $\tilde{K}'_m$  for the  $\text{EH}_2$  state relative to  $\tilde{k}_{\text{cat.}}$  and  $\tilde{K}_m$  for the EH state of AcChE. These effects are responsible for the variable shifts and more than 0.5 pH unit of the  $\text{p}K_{\text{app.}}$  values in  $^2\text{H}_2\text{O}$  for pH- $k_{\text{cat.}}/K_m$  profiles. The upward-bowing hydron inventories for  $k_{\text{cat.}}/K_m$  are the result of linear hydron inventories for  $k_{\text{cat.}}$  and downward-bowing on  $K_m$  and are not due to the rate-limiting substrate-induced fit process as claimed in the literature. Analysis of the hydron-inventory data for  $k_{\text{cat.}}$  in terms of the two-hydronic-reactive-state model provided fractionation factors  $\phi^T$  ranging between 0.5 and 0.6 for the transferred hydrons in the respective reactive states.

### INTRODUCTION

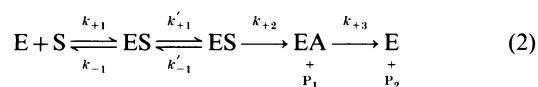
In an attempt to evaluate mechanistically relevant components of the reaction steps of hydrolysis of various substrates by acetylcholinesterase (AcChE, EC 3.1.1.7), extensive studies of these reactions in isotopically different solvent mixtures have been carried out. For a minimal enzyme kinetic model represented by eqn. (1), both acylation and deacylation (with rate constants  $k_{+2}$  and  $k_{+3}$  respectively) would involve rate-determining hydron-transfer processes through participation of His-440 (Gibney *et al.*, 1990; Sussman *et al.*, 1991) as a general acid–base catalyst.



where E = free enzyme, S = substrate, ES = enzyme–substrate complex, EA = acylenzyme, and  $\text{P}_1$  and  $\text{P}_2$  = products. Depending on whether acylation or deacylation is rate-determining with a given substrate,  $k_{+2}$  or  $k_{+3}$  represents  $k_{\text{cat.}}$ . Indeed, with some substrates, neither  $k_{+2}$  nor  $k_{+3}$  is clearly rate-determining, but both contribute to overall  $k_{\text{cat.}}$ , as is the case with the natural substrate, acetylcholine. It is expected that the experimentally observed rate constant  $k_{\text{cat.}}$ , whether acylation or deacylation

is rate-determining, will give an  $^2\text{H}_2\text{O}$  effect,  $k_{\text{cat.}}^{\text{H}_2\text{O}}/k_{\text{cat.}}^{^2\text{H}_2\text{O}} \approx 2.0$ –3.0.

The overall studies for a series of substrates revealed significant deviations from the expected behaviour both with respect to effects of  $^2\text{H}_2\text{O}$  as well as shapes of hydron inventories. The deviations from the expected (approx. 2.0–3.0)  $^2\text{H}_2\text{O}$  effect evaluated in the literature using the parameter  $k_{\text{cat.}}/K_m$  with a number of substrates, where data showed little or no effect (approx. 1.0–1.5) and upward-bowing curves for hydron inventories, led to analysis of the results by using complex equations derived from a proposed model described in eqn. (2). This model involves an additional reversible step, describing a rate-limiting induced-fit conformational change that precedes the acylation step.



It was originally introduced to provide an explanation for the variation in the  $\text{p}K_{\text{app.}}$  values as well as low  $^2\text{H}_2\text{O}$  effects (Rosenberry, 1975*a,b,c*) and later used by Quinn & Swanson (1984), Acheson *et al.* (1987*a,b*), Barlow *et al.* (1987), Quinn

Abbreviations used: AcChE, acetylcholinesterase; AcSCh, acetylthiocholine;  $K_{\text{I(com.)}}$ , competitive inhibition constant; ONA, *o*-nitroacetanilide; ONFA, *o*-nitroformanilide; ONCA, *o*-nitrochloroacetanilide; PMPCA, *p*-methoxyphenylchloroacetate; PCA, phenylchloroacetate; PNPA, *p*-nitrophenyl acetate; ONPA, *o*-nitrophenyl acetate; PMPF, *p*-methoxyphenyl formate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PTA, phenyltrimethylammonium  $k_{\text{cat.}}^{\text{H}_2\text{O}}$ ,  $K_{\text{m}}^{\text{H}_2\text{O}}$  and  $k_{\text{cat.}}/K_{\text{m}}^{\text{H}_2\text{O}}$  are observed in pure water and those with  $^2\text{H}_2\text{O}$  represent corresponding values observed in pure  $^2\text{H}_2\text{O}$ ;  $k_{\text{cat.}}^n$ ,  $K_{\text{m}}^n$  and  $k_{\text{cat.}}/K_{\text{m}}^n$  are values in progressively increasing ( $n = 0.0$ –1.0) atom fraction of deuterium; hydron, the International Union of Pure and Applied Chemistry (1986) (Chem. Int. 8, 21) recommends that the ion  $\text{H}^+$  be called the ‘hydron’ when an individual isotope is not being specified.

(1987) and Acheson & Quinn (1990) for the data obtained thereafter. Also the model has been utilized by these latter authors to explain the unusual upward-bowing hydron-inventory results for the parameter  $k_{\text{cat.}}/K_m$ .

The most recent analysis of the pH-dependence of AcChE-catalysed reactions has shown that the variation in the observed apparent  $pK_a$  values for substrates with varied structural features and ionic states was a result of two-hydronic-reactive states of AcChE (Salih, 1991). This kinetic model involving two overlapping kinetically influential ionizations, one of the general acid-base catalyst of  $pK_a$  6.5 and the other a modulatory group with  $pK_a$  5.5, provided a rational evaluation of the pH-dependence of both  $k_{\text{cat.}}/K_m$  and  $k_{\text{cat.}}$ . Further analysis showed that the variation in  $pK_{\text{app.}}$  values observed from pH- $k_{\text{cat.}}/K_m$  profiles was predominantly due to differences in binding of a given substrate to the two-hydronic-reactive,  $\text{EH}_2$  and  $\text{EH}$ , states of AcChE. Analysis in the present paper of the experimental data reported in the literature confirm the expected results that the  $^2\text{H}_2\text{O}$  effect,  $k_{\text{cat.}}^{\text{H}_2\text{O}}/k_{\text{cat.}}^{\text{H}_2\text{O}}$ , falls around 1.8–2.8 and hydron inventories for  $k_{\text{cat.}}$  give a linear relationship (see the Results and discussion section). Use of the parameter  $k_{\text{cat.}}/K_m$  in evaluation of the  $^2\text{H}_2\text{O}$  effect and hydron inventories is particularly misleading, since it involves the binding constant  $K_m$ .

The present paper deals with rational analysis and explanations of: (1) the observed  $^2\text{H}_2\text{O}$  effects on the parameter  $k_{\text{cat.}}/K_m$  by determining such effects on the separate parameters,  $k_{\text{cat.}}$  and  $K_m$ ; (2)  $^2\text{H}_2\text{O}$  effects on the shapes of the pH-dependence curve of  $k_{\text{cat.}}$ ,  $k_{\text{cat.}}/K_m$  and apparent shifts of more than 0.5 pH unit of the corresponding  $pK_{\text{app.}}$  values in connection with the two-hydronic-reactive states of AcChE (Salih, 1991); (3) interpretation of the unusual hydron inventories on  $k_{\text{cat.}}/K_m$  for hydrolysis of a wide range of substrates by AcChE with respect to the corresponding hydron inventories on the separate parameters,  $k_{\text{cat.}}$  and  $K_m$ .

## MATERIALS AND METHODS

### Chemicals

*Electrophorus electricus* AcChE, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and acetylthiocholine (AcSCh) iodide were from Sigma; phenyltrimethylammonium iodide (PTA), m.p. 226–227 °C (lit. 227 °C) and  $^2\text{H}_2\text{O}$  (99.9%) were from Aldrich.

### Kinetics

Enzyme assays were carried out by the method of Ellman *et al.* (1961) using an Hitachi Perkin-Elmer spectrophotometer, at  $\lambda = 410$  nm and  $\epsilon_{410} = 13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ . Initial velocities were recorded at pH 7.45, 1.02 mol/l and equivalent buffer with pD = 7.86 in a 1 ml cell containing 0.32 mM-DTNB and various concentrations of AcSCh (0–0.22 mM), in the presence of 0.63 nM-AcChE. The concentrations of AcSCh were determined spectroscopically from the complete hydrolysis of the substrate at  $\lambda = 410$  nm and using  $\epsilon_{410}$ . The enzyme concentration was calculated from the  $V_{\text{max}}$  in Fig. 1, using  $k_{\text{cat.}} = 1.5 \times 10^4 \text{ s}^{-1}$  (Cohen *et al.*, 1989). For reactions in  $^2\text{H}_2\text{O}$ , DTNB, AcSCh and AcChE stock solutions were made in  $^2\text{H}_2\text{O}$  equivalent buffer, pD = 7.86.

The buffer system used was  $\text{NaHPO}_4/\text{KH}_2\text{PO}_4$  and made as described by Long (1971). The equivalent buffer in pure  $^2\text{H}_2\text{O}$  was made by dissolving calculated solid buffer salts directly in  $^2\text{H}_2\text{O}$  to provide the same salt concentrations as in  $\text{H}_2\text{O}$ . The pD value was then calculated from the pH reading, using a PHM82 standard digital pH-meter (glass electrode) with an accuracy of  $\pm 0.01$ , and pD = pH-meter reading + 0.4 (Glascoe & Long, 1960).

### Data analysis

Initial velocities obtained in buffers of pure water and pure  $^2\text{H}_2\text{O}$  in the absence and presence of the reversible inhibitor, PTA (0–0.238 mM), were analysed by reciprocal plots of  $1/V_i$  against  $1/[S]$  by linear regression. The primary slopes obtained this way were then plotted against [PTA] and linear regression analysis provided the competitive inhibition constant  $K_{i(\text{com.})}$  as the negative  $x$ -axis intercept. The results are summarized in Fig. 1.

### Calculation of separate values for pH-independent rate constants, $K_{\text{cat.}}/K_m$ , $k_{\text{cat.}}$ and $K_m$ in $^2\text{H}_2\text{O}$

The separate values of the pH-independent rate constants  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}}$ ,  $k_{\text{cat.}}^{\text{H}_2\text{O}}$  and  $K_m^{\text{H}_2\text{O}}$  in  $^2\text{H}_2\text{O}$  were calculated from the reported values of the ratios  $(k_{\text{cat.}}/K_m^{\text{H}_2\text{O}})/(k_{\text{cat.}}/K_m^{\text{H}_2\text{O}})$ ,  $(k_{\text{cat.}}^{\text{H}_2\text{O}})/(k_{\text{cat.}}^{\text{H}_2\text{O}})$  in combination with the values of  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}}$ ,  $k_{\text{cat.}}^{\text{H}_2\text{O}}$  and  $K_m^{\text{H}_2\text{O}}$  reported in the literature (Table 1).

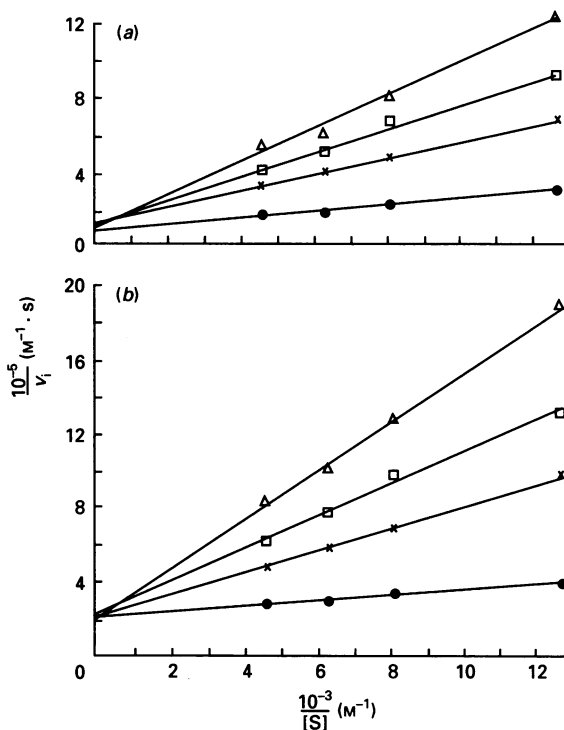


Fig. 1. Reciprocal plots,  $1/V_i$  against  $1/[S]$  for the hydrolysis of AcSCh by 0.63 nM-AcChE at pH 7.45, 1.02 mol/l and equivalent buffer in  $^2\text{H}_2\text{O}$ , pD = 7.86 at 22 °C in the absence and presence of the reversible inhibitor PTA

(a) In  $\text{H}_2\text{O}$ : ●, no inhibitor; ×, 0.095 mM; □, 0.158 mM; △, 0.238 mM. Slopes of the linear regression lines ●,  $13.3 \pm 1.8$  s; ×,  $40.8 \pm 0.7$  s; □,  $60.0 \pm 1.9$  s and △,  $86.9 \pm 8.0$  s; and corresponding intercepts  $(1.07 \pm 0.15) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ ;  $(1.43 \pm 0.06) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ ;  $(1.39 \pm 0.16) \times 10^5 \text{ M}^{-1} \cdot \text{s}$  and  $(1.20 \pm 0.68) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ . From the slope and intercept of the data with no inhibitor,  $K_m^{\text{H}_2\text{O}} = 0.125 \pm 0.024$  mM and  $V_{\text{max}}^{\text{H}_2\text{O}} = (9.4 \pm 1.3) \times 10^{-6} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ . (b) in  $^2\text{H}_2\text{O}$ : for the same inhibitor concentrations as in (a) slopes ●,  $11.8 \pm 1.8$  s; ×,  $57.5 \pm 1.6$  s; □,  $83.0 \pm 6.5$  s; and △,  $127.0 \pm 5.4$  s; and corresponding intercepts,  $(2.15 \pm 0.15) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ ;  $(2.08 \pm 0.14) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ ;  $(2.3 \pm 0.55) \times 10^5 \text{ M}^{-1} \cdot \text{s}$  and  $(2.01 \pm 0.45) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ . From the slope and intercept of the data with no inhibitor,  $K_m^{\text{H}_2\text{O}} = 0.056 \pm 0.009$  mM and  $V_{\text{max}}^{\text{H}_2\text{O}} = (4.7 \pm 0.3) \times 10^{-6} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ . Secondary plots, slope versus [PTA], gave slopes and intercepts for data in water: slope =  $(3.1 \pm 0.1) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ , intercept =  $12.2 \pm 1.4$  s (correlation coefficient 99.9%); and in  $^2\text{H}_2\text{O}$ : slope =  $(4.8 \pm 0.2) \times 10^5 \text{ M}^{-1} \cdot \text{s}$ , intercept =  $11.1 \pm 2.7$  s (correlation coefficient 99.9%) leading to  $K_{i(\text{com.})}^{\text{H}_2\text{O}} = 39.3 \mu\text{M}$  and  $K_{i(\text{com.})}^{\text{H}_2\text{O}} = 24.5 \pm 3.5 \mu\text{M}$  respectively.

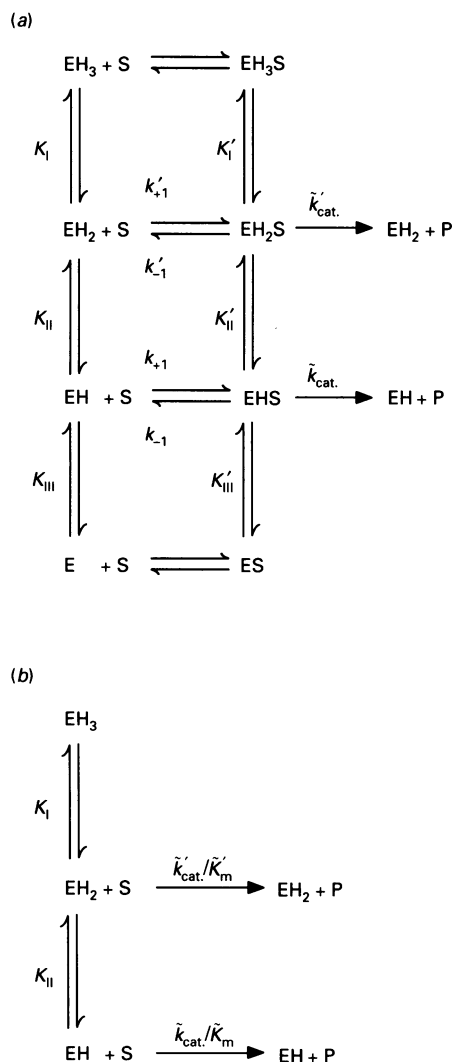
**Table 1. Kinetic parameters of substrate catalysis in water and  $^2\text{H}_2\text{O}$  by AcChE obtained from the literature and the calculated separate kinetic parameters in  $^2\text{H}_2\text{O}$  for  $k_{\text{cat}}(\text{app.})$ ,  $K_{\text{m}}(\text{app.})$ ,  $k_{\text{cat.}}/K_{\text{m}}(\text{app.})$  and the ratio  $K_{\text{H}_2\text{O}}^{k_{\text{cat.}}}/K_{\text{m}}^{k_{\text{cat.}}}$  revealing the  $^2\text{H}_2\text{O}$  effect on these parameters**

The data reported in the literature were obtained at pH 7.3 in the work by Acheson *et al.* (1987a,b 1990), at pH 7.5 in the work of Hogg *et al.* (1980) and at pH 8.5 in the work of Rosenberry (1975a,b), which are optimum pHs, and equivalent pDs were used; buffers or salt solutions of 0.1–0.2 mol/l, were used at 25 °C (except for PCA and *p*-methoxyphenylchloracetate (PMPCA) which were at 20 °C). Further details are available from the specific references in the Table. The propagation of the standard errors in ratios  $x/y$  and multiples  $xy$  were calculated according to the method of Ku (1966). For some of the values from the literature, standard errors were not provided.

Substrate	$k_{\text{cat.}}/K_{\text{m}}^{k_{\text{cat.}}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )	$K_{\text{m}}^{k_{\text{cat.}}}$ (mM)	$k_{\text{H}_2\text{O}}^{k_{\text{cat.}}(\text{app.})}$ ( $\text{s}^{-1}$ )	$K_{\text{m}}^{k_{\text{cat.}}(\text{app.})}$ (mM)	$k_{\text{H}_2\text{O}}^{k_{\text{cat.}}(\text{app.})}$ ( $\text{s}^{-1}$ )	$k_{\text{cat.}}/K_{\text{m}}^{k_{\text{cat.}}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )	$k_{\text{cat.}}/K_{\text{m}}^{k_{\text{cat.}}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )	Reference	Present work		
									$k_{\text{cat.}}/K_{\text{m}}^{k_{\text{cat.}}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )	$K_{\text{m}}^{k_{\text{cat.}}}$ (mM)	$k_{\text{H}_2\text{O}}^{k_{\text{cat.}}}$ ( $\text{s}^{-1}$ )
ONFA*	$1.94 \times 10^3$	2.11	4.1	1.85 ± 0.09	1.41 ± 0.03	$(1.38 \pm 0.03) \times 10^3$	$2.22 (\pm 0.1)$	Acheson <i>et al.</i> (1978b)	$1.61 \pm 0.09$	$2.22 (\pm 0.1)$	$1.31 \pm 0.07$
ONCA†	$3.60 \times 10^4$	1.70	60	$2.00 \pm 0.02$	$1.28 \pm 0.03$	$(2.81 \pm 0.06) \times 10^4$	$30 (\pm 0.3)$	Acheson <i>et al.</i> (1990)	$1.07 \pm 0.03$	$30 (\pm 0.3)$	$1.59 \pm 0.03$
ONCA*	$1.03 \times 10^5$	0.80	82	$1.75 \pm 0.03$	$1.31 \pm 0.02$	$(7.86 \pm 0.12) \times 10^4$	$47 (\pm 0.9)$	Acheson <i>et al.</i> (1990)	$0.60 \pm 0.02$	$47 (\pm 0.9)$	$1.33 \pm 0.03$
ONA*	$7.8 \times 10^3$	20	156	2.00	$1.55 \pm 0.03$	$(5.03 \pm 0.1) \times 10^3$	78	Acheson <i>et al.</i> (1987b)	$15.5 \pm 0.3$	78	$1.29 \pm 0.03$
Methyl acetate*	$1.3 \times 10^3$				2.00	$6.5 \times 10^2$		Rosenberry (1975a)			
PMPCA*	$2.5 \times 10^5$	0.80	200	$1.60 \pm 0.10$	$0.93 \pm 0.02$	$(2.69 \pm 0.06) \times 10^5$	$(1.25 \pm 0.07) \times 10^2$	Acheson <i>et al.</i> (1987a)	$0.47 \pm 0.03$	$(1.25 \pm 0.07) \times 10^2$	$1.70 \pm 0.1$
PCA*	$2.18 \times 10^6$	0.17	370	$1.81 \pm 0.04$	$1.0 \pm 0.10$	$(2.18 \pm 0.22) \times 10^6$	$(2.04 \pm 0.04) \times 10^2$	Acheson <i>et al.</i> (1987a)	$0.09 \pm 0.01$	$(2.04 \pm 0.04) \times 10^2$	$1.89 \pm 0.21$
PNPA*	$2.51 \times 10^5$	5.1	$12.8 \times 10^2$	2.00	$1.93 \pm 0.07$	$(1.30 \pm 0.05) \times 10^5$	$6.4 \times 10^2$	Rosenberry (1975a)	$4.92 \pm 0.19$	$6.4 \times 10^2$	$1.04 \pm 0.04$
	$2.24 \times 10^4$				1.56	$1.44 \times 10^4$		Hogg <i>et al.</i> (1980)			
ONPA†	$6.9 \times 10^6$	0.36	$2.50 \times 10^3$	$2.02 \pm 0.09$	$0.95 \pm 0.15$	$(7.26 \pm 1.2) \times 10^6$	$(1.24 \pm 0.06) \times 10^3$	Acheson <i>et al.</i> (1990)	$0.17 \pm 0.03$	$(1.24 \pm 0.06) \times 10^3$	$2.12 \pm 0.37$
ONPA*	$8.8 \times 10^6$	0.26	$2.00 \times 10^3$	$1.89 \pm 0.06$	$1.07 \pm 0.03$	$(8.22 \pm 0.24) \times 10^6$	$(1.06 \pm 0.03) \times 10^3$	Acheson <i>et al.</i> (1990)	$0.13 \pm 0.01$	$(1.06 \pm 0.03) \times 10^3$	$2.02 \pm 0.08$
Isoamyl acetate*	$6.31 \times 10^5$	3.3	$2.08 \times 10^3$	$2.84 \pm 0.70$	$1.26 \pm 0.28$	$(5.00 \pm 1.1) \times 10^5$	$(7.32 \pm 1.44) \times 10^2$	Rosenberry (1975a)	$1.46 \pm 0.05$	$(7.32 \pm 1.44) \times 10^2$	$2.26 \pm 0.08$
PMPP*	$3.1 \times 10^6$	2.02	$6.26 \times 10^3$	$2.65 \pm 0.10$	$1.09 \pm 0.01$	$(2.84 \pm 0.02) \times 10^6$	$(2.36 \pm 0.08) \times 10^3$	Acheson <i>et al.</i> (1987b)	$0.83 \pm 0.03$	$(2.36 \pm 0.08) \times 10^3$	$2.43 \pm 0.09$
Acetylcholine*	$1.6 \times 10^8$	0.10	$1.6 \times 10^4$	$2.35 \pm 0.30$	$1.15 \pm 0.15$	$(1.39 \pm 0.16) \times 10^8$	$(6.8 \pm 0.76) \times 10^3$	Rosenberry (1975a)	$0.05 \pm 0.01$	$(6.8 \pm 0.76) \times 10^3$	$2.00 \pm 0.24$
AcSCH*	$1.15 \times 10^8$	0.13	$1.5 \times 10^4$	$2.00 \pm 0.13$	$0.93 \pm 0.16$	$(1.24 \pm 0.22) \times 10^8$	$(7.46 \pm 0.48) \times 10^3$	Present work	$0.06 \pm 0.01$	$(7.46 \pm 0.48) \times 10^3$	$2.17 \pm 0.36$
Phenyl acetate*†	$7.94 \times 10^6$	2.16	$1.71 \times 10^4$					Rosenberry (1975b)			
		2.40		$2.36 \pm 0.2$	$1.50 \pm 0.10$			Kovach <i>et al.</i> (1986)			
		1.41	$2.72 \times 10^3$	$2.26 \pm 0.06$	$1.33 \pm 0.02$	$(1.44 \pm 0.02) \times 10^6$	$(1.2 \pm 0.03) \times 10^3$	Acheson <i>et al.</i> (1987a)	$0.83 \pm 0.02$	$(1.2 \pm 0.03) \times 10^3$	$1.70 \pm 0.05$

\* AcChE from *Electrophorus electricus* (lytic 11 S form).

† AcChE from human erythrocyte.



Scheme 1. Kinetic model of AcChE activity

EH<sub>3</sub>, EH<sub>2</sub>, EH and E are hydronic states of the free enzyme molecule;  $\tilde{K}'_m = k'_{-1}/k'_{+1}$  and  $\tilde{K}_m = k_{-1}/k_{+1}$  are the dissociation constants for the enzyme-substrate complexes, EH<sub>2</sub>S and EHS, which are productive;  $K_1$ ,  $K_{\text{II}}$  and  $K_{\text{III}}$  are free enzyme molecular acid dissociation constants;  $K'_1$ ,  $K'_{\text{II}}$  and  $K'_{\text{III}}$  are acid dissociation constants of the respective substrate complexes, EH<sub>2</sub>S, EH<sub>2</sub>S and EHS; and  $\tilde{k}'_{\text{cat.}}$ ,  $\tilde{k}_{\text{cat.}}$ ,  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m$  and  $\tilde{k}_{\text{cat.}}/\tilde{K}_m$  are the respective pH-independent limiting rate constants. When acylation is rate-determining, (a) may be written as in (b) for the overall catalysis in the acid region of the pH.

### Computer simulation of pH-rate profiles involving two-hydronic-reactive states of AcChE in pure water and pure <sup>2</sup>H<sub>2</sub>O

Computer-simulation work was carried out by using reported and/or calculated values normalized to 1.0 for ease of computation. (1) When reactions in water (H<sub>2</sub>O) were considered, the error-free values of the rate constant  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}}$  as a function of pH or pD were generated using eqn. (3) (applicable to the kinetic model in Scheme 1b),  $\text{p}K_1 = 5.5$ ,  $\text{p}K_{\text{II}} = 6.5$ , and values for the pH-independent rate constants  $\tilde{k}_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = 1.0$  and  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m^{\text{H}_2\text{O}}$  varied between 0.0 and 1.0 (Table 4) by the method described previously (Salih, 1991). (2) For reactions in <sup>2</sup>H<sub>2</sub>O, the general procedure of obtaining error-free data for pD-rate profiles was as in (1), except that  $\text{p}K_1 = 6.0$  and  $\text{p}K_{\text{II}} = 7.0$  were used because

ionization constants of weak acids in <sup>2</sup>H<sub>2</sub>O ideally increase by approx. 0.5 pH unit. Also, values of the pH-independent rate constants  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m^{\text{H}_2\text{O}}$  and  $\tilde{k}_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}}$  used here were normalized against 1.0, 1.0 being the value of a particular rate constant in water (Table 4). For pH- $k_{\text{cat.}}$  profiles, the computer simulations were carried out using  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m^{\text{H}_2\text{O}} = 0.5$  and  $\tilde{k}_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = \text{variable}$  (0.0–0.5). A fixed value of  $\tilde{k}'_{\text{cat.}}/\tilde{K}'_m^{\text{H}_2\text{O}} = 0.5$  was used for ease of computation instead of varying the constant between 0.35 and 0.63 (actual experimental data in Table 4). Eqn. (4), which is applicable to Scheme 1(a), was used to generate the data points for pH- $k_{\text{cat.}}$  profiles. It is of the same form as eqn. (3) except that the ionization constants  $K'_1$  and  $K'_{\text{II}}$  represent those of the enzyme-substrate complexes and are indeed numerically the same as  $K_1$  and  $K_{\text{II}}$  in the present context.

$$\frac{k_{\text{cat.}}(\text{app.})}{K_m} = \frac{\tilde{k}'_{\text{cat.}}/\tilde{K}'_m}{1 + \frac{[\text{H}^+]}{K_1} + \frac{[\text{H}^+]}{K_{\text{II}}}} + \frac{\tilde{k}_{\text{cat.}}/\tilde{K}_m}{1 + \frac{[\text{H}^+]^2}{K_1 K_{\text{II}}} + \frac{[\text{H}^+]}{K_{\text{II}}}} \quad (3)$$

$$k_{\text{cat.}}(\text{app.}) = \frac{\tilde{k}'_{\text{cat.}}}{1 + \frac{[\text{H}^+]}{K'_1} + \frac{[\text{H}^+]}{K'_{\text{II}}}} + \frac{\tilde{k}_{\text{cat.}}}{1 + \frac{[\text{H}^+]}{K_{\text{II}}} + \frac{[\text{H}^+]^2}{K'_1 K'_{\text{II}}}} \quad (4)$$

The pH-rate error-free data obtained in (3) and (4) were then fitted to a simple sigmoidal curve by using eqn. (5), providing  $\text{p}K_{\text{app.}}$  values and the corresponding limiting rate constants as described previously (Salih, 1991).

$$\frac{k_{\text{cat.}}(\text{app.})}{K_m} = \frac{\tilde{k}_{\text{cat.}}/\tilde{K}_m}{1 + \frac{[\text{H}^+]}{K_a}} \quad (5)$$

### Rate-determining steps for a series of substrates hydrolysed by AcChE

For a minimal enzyme mechanistic model represented by eqn. (1),  $k_{+2}$  and  $k_{+3}$  are the kinetic parameters for acylation and deacylation respectively. It has been estimated that  $k_{+2}/k_{+3} = 2.12$  (Froede & Wilson, 1984) for the 14–18 S form of *Electrophorous* AcChE and  $k_{+2}/k_{+3} = 6$  (Wilson & Cabib, 1956) for the lytic 11 S form from *Electrophorous*, both with the substrate acetylcholine, at pH 7.0 and 25 °C. Since most of the kinetic work reported in the literature was carried out using the lytic 11 S form of the enzyme and  $k_{\text{cat.}}$  values reported in the literature at pH 7.0–7.5, both of the above estimated values have been utilized in the present work for comparison. Using  $k_{\text{cat.}} = 1.5 \times 10^4 \text{ s}^{-1}$  for the 11 S form of AcChE with acetylcholine at pH 7.0 and  $k_{\text{cat.}} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$  provides  $k_{+3} = 2.2 \times 10^4 \text{ s}^{-1}$  (for  $k_{+2}/k_{+3} = 2.12$ ) and  $k_{+3} = 1.8 \times 10^4 \text{ s}^{-1}$  (for  $k_{+2}/k_{+3} = 6$ ). For all the substrates utilized by AcChE leading to 'acetyl-acetylcholinesterase', the deacylation rate constant,  $k_{+3}$ , would be expected to be  $2.2 \times 10^4 \text{ s}^{-1}$  or  $1.8 \times 10^4 \text{ s}^{-1}$ . Calculations for a series of substrates using the above approach are summarized in Table 2.

### Hydron inventories for $k_{\text{cat.}}/K_m$ , $k_{\text{cat.}}$ and $K_m$

Hydron inventories for  $k_{\text{cat.}}/K_m$  in the literature for AcChE-catalysed reactions are predominantly, and apparently complicated by, the upward-bowing shapes for substrates where acylation is rate-determining, whereas those for  $k_{\text{cat.}}$  are simple and linear. The interest in the present context is to evaluate the hydron inventories in terms of the separate kinetic parameters,  $k_{\text{cat.}}$  and  $K_m$ , that constitute the parameter  $k_{\text{cat.}}/K_m$  and compare the overall effect. For example, the hydron inventory on  $k_{\text{cat.}}/K_m$  is reported as  $(k_{\text{cat.}}/K_m^n)/(k_{\text{cat.}}/K_m^{\text{H}_2\text{O}})$  for  $n = 0.0$ – $1.0$ , the value of  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}}$  is a constant; so, using hydron-inventory curves, the experimentally observed values of  $k_{\text{cat.}}/K_m^n$  (but not reported in

**Table 2.** Calculated values of acylation rate constants  $k_{+2(\text{app.})}$  ( $\text{s}^{-1}$ ) for a series of substrates using reported values of  $k_{\text{cat.}(\text{app.})}$  from Table 1 and deacylation constants,  $k_{+3(\text{app.})} = 2.2 \times 10^4 \text{ s}^{-1}$  ( $1.8 \times 10^4 \text{ s}^{-1}$ )

Calculated values of  $k_{+2}$  are based on  $k_{+2}/k_{+3} = 2.12$  for the *Electrophorous* 14–18 S form of AcChE, and those in parentheses are based on  $k_{+2}/k_{+3} = 6$  for the *Electrophorous* lytic 11 S form of AcChE, both with acetylcholine and at pH 7.0. The values of  $k_{\text{cat.}}$  in the literature were obtained at pH 7.3, in 0.1 M-sodium phosphate containing 0.1 M-NaCl, except for PNPA, isoamyl acetate and phenyl acetate which were obtained at pH 8.5 and in 0.1 M-NaCl. The dashed line separates substrates where acylation is rate determining (above dashed line) from those where both acylation and deacylation contribute to the rate determination.

Substrate	$k_{+2(\text{app.})}$ ( $\text{s}^{-1}$ ) $\times 10^{-2}$	$\frac{k_{+3(\text{app.})}}{k_{+2(\text{app.})}}$
ONFA*	0.04 (0.04)	5500 (4500)
ONCA†	0.60 (0.60)	367 (300)
ONCA*	0.82 (0.82)	268 (220)
ONA*	1.57 (1.57)	140 (115)
PMPCA*	2.02 (2.02)	109 (89)
PCA*	3.76 (3.77)	59 (48)
PNPA*	13.59 (13.78)	16 (13)
ONPA*	22.00 (22.50)	10 (8)
Isoamyl acetate*	22.97 (23.52)	10 (8)
ONPA†	28.20 (29.03)	8 (6)
-----		
PMPF*	87.50 (95.98)	3 (2.0)
Acetylcholine*	587.67 (1440)	0.4 (0.1)
Phenyl acetate*†	771.80 (3501)	0.3 (0.1)

\* AcChE from *Electrophorous electricus* (lytic 11 S form).

† AcChE from human erythrocyte.

the literature as such) for a range  $n = 0.0$ – $1.0$ , can be calculated. The values of  $K_m^{\text{H}_2\text{O}}$  and  $K_m^{2\text{H}_2\text{O}}$  are reported and/or calculated (Table 1), as well as those for  $k_{\text{cat.}}^{\text{H}_2\text{O}}$  and  $k_{\text{cat.}}^{2\text{H}_2\text{O}}$ . The knowledge of numerical values of  $k_{\text{cat.}}^{\text{H}_2\text{O}}$  and  $k_{\text{cat.}}^{2\text{H}_2\text{O}}$  together with the linearity of hydron inventories for the parameter  $k_{\text{cat.}}$  (i.e.  $k_{\text{cat.}}^n/k_{\text{cat.}}^{2\text{H}_2\text{O}}$  = linear), allowed estimation of the individual  $k_{\text{cat.}}^n$  values for a range  $n = 0.0$ – $1.0$ . The calculated values of  $k_{\text{cat.}}^n$ , together with the corresponding values calculated for  $k_{\text{cat.}}/K_m^n$ , provided values of  $K_m^n$  at each  $n$  atom fraction of deuterium. The results of such analyses are graphically represented in Fig. 4, in the usual way as  $(k_{\text{cat.}}/K_m^n)/(k_{\text{cat.}}/K_m^{2\text{H}_2\text{O}})$ ,  $k_{\text{cat.}}^n/k_{\text{cat.}}^{2\text{H}_2\text{O}}$  and  $K_m^n/K_m^{2\text{H}_2\text{O}}$  for three substrates; these observations apply to other substrates as well, but are not plotted for clarity.

#### Evaluation of the kinetic parameters $k_{\text{cat.}}/K_m$ and $K_m$ in terms of two-hydronic-reactive states of AcChE in $^2\text{H}_2\text{O}$

The kinetic parameters in  $^2\text{H}_2\text{O}$  for two-hydronic-reactive states were determined by using the values of  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}}$ ,  $k_{\text{cat.}}/K_m^{2\text{H}_2\text{O}}$ ,  $k_{\text{cat.}}^{\text{H}_2\text{O}}$ ,  $k_{\text{cat.}}^{2\text{H}_2\text{O}}$ ,  $K_m^{\text{H}_2\text{O}}$ ,  $K_m^{2\text{H}_2\text{O}}$  in Table 3, leading to values of  $k_{\text{cat.}}/K_m$ ,  $k_{\text{cat.}}/K_m$ ,  $K_m$  and  $K_m$  (Table 4). For example, for substrate *o*-nitroformanilide (ONFA) in Table 3,  $k_{\text{cat.}}/K_m^{\text{H}_2\text{O}} = 1.0$ ,  $k_{\text{cat.}}^{\text{H}_2\text{O}} = 1.0$  and  $K_m^{\text{H}_2\text{O}} = 1.0$  and  $k_{\text{cat.}}/K_m^{2\text{H}_2\text{O}} = 0.71$ ,  $k_{\text{cat.}}^{2\text{H}_2\text{O}} = 0.54$  and  $K_m^{2\text{H}_2\text{O}} = 0.76$ . Since the values for a two-hydronic-reactive-state model (Table 4) in pure water are  $\tilde{k}'_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = 0.8$ ,  $\tilde{k}_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = 1.0$ ,  $\tilde{K}_m^{\text{H}_2\text{O}} = 1.25$ ,  $\tilde{K}_m^{\text{H}_2\text{O}} = 1.0$  and  $\tilde{k}'_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = 1.0$  [the method for obtaining these values from pH–rate profiles is outlined in Salih (1991)], then in  $^2\text{H}_2\text{O}$  when  $\tilde{k}'_{\text{cat.}}/\tilde{K}_m^{2\text{H}_2\text{O}} = 0.54$  and  $\tilde{k}_{\text{cat.}}/\tilde{K}_m^{2\text{H}_2\text{O}} = 0.71$ , it follows that  $\tilde{k}'_{\text{cat.}}/\tilde{K}_m^{2\text{H}_2\text{O}} = 0.57$  and using  $\tilde{k}'_{\text{cat.}}/\tilde{K}_m^{\text{H}_2\text{O}} = 1.0$  = 0.54,  $\tilde{K}_m^{2\text{H}_2\text{O}} = 0.54/0.71 = 0.76$  and  $\tilde{K}_m^{2\text{H}_2\text{O}} = 0.54/0.57 = 0.95$ .

#### Fractionation factors $\phi^T$ for the transferred hydrons in two-hydronic-reactive states

Since the parameter pertinent to the hydron-transfer process is  $k_{\text{cat.}}$ , assessment of the fractionation factors for the two-hydronic-reactive states was carried out for this parameter only. The observed apparent  $*k_{\text{cat.}}$  for this system can be represented as in eqn. (6):

$$*k_{\text{cat.}} = f_{\text{EH}_2} \tilde{k}'_{\text{cat.}} + f_{\text{EH}} \tilde{k}_{\text{cat.}} \quad (6)$$

where  $f_{\text{EH}_2}$  and  $f_{\text{EH}}$  are fractions of the enzyme in the respective  $\text{EH}_2$  and  $\text{EH}$  states at a given pH and can be calculated accurately from the knowledge of  $\text{p}K_1 = 5.5$  and  $\text{p}K_{11} = 6.5$ . In terms of hydron inventory, eqn. (6) becomes eqn. (7), where  $\phi^T$  and  $\phi^R$  are fractionation factors for the transferred hydrons in the respective reactive states. The fractionation factors  $\phi^R$  for the reactant states are assumed to be unity (Schowen & Schowen, 1982; Venkatasubban & Schowen, 1985).

$$*k_{\text{cat.}}^n = f_{\text{EH}_2} \tilde{k}'_{\text{cat.}}^{\text{H}_2\text{O}} (1 - n + n\phi^T) + f_{\text{EH}} \tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}} (1 - n + n\phi^T) \quad (7)$$

Dividing eqn. (7) by  $*k_{\text{cat.}}^{\text{H}_2\text{O}}$ , where when  $k_{\text{cat.}}^{\text{H}_2\text{O}} = \tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}$ , then at pH values around 7.0–8.0  $*k_{\text{cat.}}^{\text{H}_2\text{O}} = \tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}$  (Salih, 1991) and, since  $f_{\text{EH}_2} + f_{\text{EH}} = 1.0$ , leads to eqn. (8).

$$\frac{*k_{\text{cat.}}^n}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} = 1 - n + n\phi_{\text{app.}}^T \quad (8)$$

where  $\phi_{\text{app.}}^T = f_{\text{EH}_2} \phi^T + f_{\text{EH}} \phi^T$ .

For substrates where  $\tilde{k}'_{\text{cat.}} \neq \tilde{k}_{\text{cat.}}$ , the relevant equation would be eqn. (9):

$$\frac{*k_{\text{cat.}}^n}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} = \left( f_{\text{EH}_2} \frac{\tilde{k}'_{\text{cat.}}^{\text{H}_2\text{O}}}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} + f_{\text{EH}} \frac{\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} \right) (1 - n) + n \left( f_{\text{EH}_2} \frac{k_{\text{cat.}}^{\text{H}_2\text{O}}}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} \phi^T + f_{\text{EH}} \frac{\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}}{*k_{\text{cat.}}^{\text{H}_2\text{O}}} \phi^T \right) \quad (9)$$

Where  $\phi_{\text{app.}}^T = f_{\text{EH}_2} (\tilde{k}'_{\text{cat.}}^{\text{H}_2\text{O}}/*k_{\text{cat.}}^{\text{H}_2\text{O}}) \phi^T + f_{\text{EH}} (\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}/*k_{\text{cat.}}^{\text{H}_2\text{O}}) \phi^T$ . When  $\tilde{k}'_{\text{cat.}} < \tilde{k}_{\text{cat.}}$ , at neutral pH  $\phi_{\text{app.}}^T$  approaches  $f_{\text{EH}} (\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}/*k_{\text{cat.}}^{\text{H}_2\text{O}}) \phi^T$ , with the observed  $*k_{\text{cat.}}^{\text{H}_2\text{O}}$  being slightly less than  $\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}$  and  $f_{\text{EH}} (\tilde{k}_{\text{cat.}}^{\text{H}_2\text{O}}/*k_{\text{cat.}}^{\text{H}_2\text{O}})$  approaching 1.0.

Eqns. (8) and (9) were used to carry out linear-regression analysis on the data for  $k_{\text{cat.}}$  from Fig. 4 and those from the literature, providing values of  $\phi_{\text{app.}}^T$  and hence  $\phi^T$  and  $\phi^T$ . For substrates *p*-nitrophenyl acetate (PNPA), *o*-nitrochloroacetanilide (ONCA), ONFA and phenylchloroacetate (PCA), linear-regression analysis was carried out according to eqn. (8) with  $\tilde{k}'_{\text{cat.}} = \tilde{k}_{\text{cat.}}$  and  $*k_{\text{cat.}} = \tilde{k}_{\text{cat.}}$ . In determining the fractionation factors for *o*-nitrophenyl acetate (ONPA), eqn. (9) was utilized with  $\tilde{k}'_{\text{cat.}} = 0.2 \tilde{k}_{\text{cat.}}$  [determined from the reported  $\text{p}K_{\text{app.}} = 6.35$  for the pH– $k_{\text{cat.}}$  profile (see the Results and Discussion section for further details)] and  $*k_{\text{cat.}} = 0.88$ . The results are summarized in Table 6.

## RESULTS AND DISCUSSION

### Integrity of the $^2\text{H}_2\text{O}$ effect on $k_{\text{cat.}}$ , $K_m$ and $k_{\text{cat.}}/K_m$

Critical analysis of the results reported in the literature on the effects of  $^2\text{H}_2\text{O}$  on the parameter  $k_{\text{cat.}}/K_m$ , used by Rosenberry (1975a,b,c), Hogg *et al.* (1980), Quinn & Swanson (1984), Acheson *et al.* (1987a,b), Barlow *et al.* (1987), Quinn (1987) and Acheson & Quinn (1990) to interpret such investigations led to a more extensive and collective screening in the present study of the data by separating this 'quotient' into its individual components, namely  $k_{\text{cat.}}$  and  $K_m$  (Table 1). Evaluation in the present paper of the effect of  $^2\text{H}_2\text{O}$  on these latter parameters allowed direct comparison of the consequences on the corresponding

**Table 3.** Kinetic parameters for substrates in Table 1 normalized to 1.0 when reactions were carried out in water and the corresponding calculated relative values in  $^2\text{H}_2\text{O}$ 

Substrate	$k_{\text{cat.}}/K_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$k_{\text{cat.}}/K_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$k_{\text{cat.}}^{\text{H}_2\text{O}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat.}}^{\text{H}_2\text{O}}$ ( $\text{s}^{-1}$ )	$K_{\text{m}}^{\text{H}_2\text{O}}$ (mM)	$K_{\text{m}}^{\text{H}_2\text{O}}$ (mM)
PMPF*	1.0	0.92 ± 0.01	1.0	0.38 ± 0.02	1.0	0.41 ± 0.01
Isoamyl acetate*	1.0	0.79 ± 0.17	1.0	0.35 ± 0.07	1.0	0.44 ± 0.02
ONPA†	1.0	1.06 ± 0.18	1.0	0.50 ± 0.02	1.0	0.47 ± 0.08
Acetylcholine*	1.0	0.87 ± 0.10	1.0	0.43 ± 0.05	1.0	0.49 ± 0.06
ONPA*	1.0	0.93 ± 0.03	1.0	0.53 ± 0.02	1.0	0.50 ± 0.02
PCA*	1.0	1.00 ± 0.10	1.0	0.55 ± 0.01	1.0	0.55 ± 0.06
PMPCA*	1.0	1.08 ± 0.02	1.0	0.63 ± 0.04	1.0	0.58 ± 0.04
Phenyl acetate†	1.0	0.75 ± 0.01	1.0	0.44 ± 0.01	1.0	0.59 ± 0.02
ONCA†	1.0	0.78 ± 0.02	1.0	0.50 ± 0.01	1.0	0.63 ± 0.02
ONCA*	1.0	0.77 ± 0.01	1.0	0.57 ± 0.01	1.0	0.74 ± 0.02
ONFA*	1.0	0.71 ± 0.02	1.0	0.54 ± 0.03	1.0	0.76 ± 0.04
ONA*	1.0	0.65 ± 0.01	1.0	0.50	1.0	0.78 ± 0.02
p-Nitrophenyl acetate*	1.0	0.52 ± 0.02	1.0	0.50	1.0	0.96 ± 0.04
Methyl acetate*	1.0	0.50	1.0	0.50	1.0	1.0

\* AcChE from *Electrophorus electricus* (lytic 11 S form).

† AcChE from human erythrocyte.

**Table 4.** pH-independent limiting rate constants evaluated for two-hydronic-reactive states of AcChE [Scheme 1 and eqns. (3 and 4)] in water and in  $^2\text{H}_2\text{O}$  using the data in Table 3 and the method of Salih (1991).

Substrate	$\tilde{k}_{\text{cat.}}'/\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$\tilde{k}_{\text{cat.}}/\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ (mM)	$\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ (mM)	$\tilde{k}_{\text{cat.}}'/\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$\tilde{k}_{\text{cat.}}/\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	$\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ (mM)	$\tilde{K}_{\text{m}}^{\text{H}_2\text{O}}$ (mM)
PMPF*	0.80	1.0	1.25	1.0	0.73	0.92	0.51	0.41
Isoamyl acetate*	0.90	1.0	1.1	1.0	0.73	0.79	0.48	0.44
ONPA†		1.0		1.0		1.06		0.47
Acetylcholine*	0.20	1.0	1.0	1.0	0.17	0.87	0.49	0.49
ONPA*		1.0		1.0		0.93		0.50
PCA*		1.0		1.0		1.0		0.55
PMPCA*		1.0		1.0		1.08		0.58
Phenyl acetate†	0.9	1.0	1.10	1.0	0.68	0.75	0.65	0.59
ONCA†	0.6	1.0	1.70	1.0	0.47	0.78	1.07	0.63
ONCA*	0.6	1.0	1.7	1.0	0.46	0.77	1.24	0.74
ONFA*	0.8	1.0	1.25	1.0	0.57	0.71	0.70	0.76
ONA*	0.8	1.0	1.25	1.0	0.35	0.65	0.96	0.78
p-Nitrophenyl acetate*	0.3	1.0	3.30	1.0	0.16	0.52	3.18	0.96
Methyl acetate*	0.45	1.0	2.2	1.0	0.23	0.50	2.22	1.00

\* AcChE from *Electrophorus electricus* (lytic 11 S form).

† AcChE from human erythrocyte.

ratio,  $k_{\text{cat.}}/K_{\text{m}}$ . It is found that the low  $^2\text{H}_2\text{O}$  effect of approx. 1.0–1.5 (from one substrate to another) on  $k_{\text{cat.}}/K_{\text{m}}$  was in fact due to the normal  $^2\text{H}_2\text{O}$  effect of approx. 1.8–2.8 on parameter  $k_{\text{cat.}}$  and reduced values of  $K_{\text{m}}$  ( $^2\text{H}_2\text{O}$  effect of approx. 1.0–2.5). This effect can be visualized by eqn. (10):

$$(k_{\text{cat.}}/K_{\text{m}})^{\text{H}_2\text{O}}/(k_{\text{cat.}}/K_{\text{m}})^{\text{H}_2\text{O}} = \frac{k_{\text{cat.}}^{\text{H}_2\text{O}}}{k_{\text{cat.}}} \times \frac{K_{\text{m}}^{\text{H}_2\text{O}}}{K_{\text{m}}} = 2.0 \times \frac{K_{\text{m}}^{\text{H}_2\text{O}}}{K_{\text{m}}} \quad (10)$$

When the  $^2\text{H}_2\text{O}$  effect on  $k_{\text{cat.}}$ ,  $k_{\text{cat.}}^{\text{H}_2\text{O}}/k_{\text{cat.}}$  is predominantly around 2.0 (see Table 3), then the observed values for  $(k_{\text{cat.}}/K_{\text{m}})^{\text{H}_2\text{O}}/(k_{\text{cat.}}/K_{\text{m}})^{\text{H}_2\text{O}}$  ranging between 1.0 and 1.5 are a function of the values of  $K_{\text{m}}^{\text{H}_2\text{O}} < K_{\text{m}}^{\text{H}_2\text{O}}$  (Table 3).

This view and interpretation is in direct contrast with the hypotheses and interpretations offered by a number of investigators in the literature, where the kinetic model of a rate-determining induced-fit conformational change was utilized, and

the explanation of the low  $^2\text{H}_2\text{O}$  effect on  $k_{\text{cat.}}/K_{\text{m}}$  was inclined to stress the so-called isotope insensitivity of the catalytic steps. In actuality, the experimental data for all the substrates studied thus far in the literature show that the catalytic step  $k_{\text{cat.}}$ , whether acylation or deacylation is rate-determining (both of which involve hydron transfer), is isotope sensitive with a normal  $^2\text{H}_2\text{O}$  effect of 1.8–2.8. The parameter  $k_{\text{cat.}}/K_{\text{m}}$  does not reflect solely the catalytic step involving hydron transfer ( $k_{\text{cat.}}$ ) but also  $K_{\text{m}}$ . It is clear that when  $^2\text{H}_2\text{O}$  has a strong effect on  $K_{\text{m}}$  (see Tables 1 and 3), use of the parameter  $k_{\text{cat.}}/K_{\text{m}}$  is not appropriate and can be misleading in such studies. Consistent with the conclusions of the evaluation of the effect of  $^2\text{H}_2\text{O}$  on  $K_{\text{m}}$  is the  $^2\text{H}_2\text{O}$  effect of  $1.59 \pm 0.26$  found for the  $K_{\text{I}(\text{com.})}$  values of the reversible inhibitor, PTA (Fig. 1). For most of the substrates,  $K_{\text{m}}$  represents the dissociation constant for the enzyme–substrate complex and the decreased value of this constant indicates improved binding in  $^2\text{H}_2\text{O}$ . Such an analysis is made effective by ensuring that the  $K_{\text{m}}$

**Table 5.** Calculated binding energies in hydronium oxide  $\Delta G^{\text{H}_2\text{O}}$  and deuterium oxide  $\Delta G^{\text{D}_2\text{O}}$  and the improved binding energies  $\Delta\Delta G = \Delta G^{\text{D}_2\text{O}} - \Delta G^{\text{H}_2\text{O}}$  in deuterium oxide for substrates where acylation is rate determining

For calculations  $\Delta G = -2.3RT \log_{10} (1/K_m)$  was used where  $R = 8.31 \text{ kJ/mol}$  ( $1.987 \text{ cal/mol}$ )  $\cdot \text{K}^{-1}$ ,  $T = (273 + 25 \text{ }^\circ\text{C}) \text{ K}$  except for substrates PCA and PMPCA where reactions were carried out at  $20 \text{ }^\circ\text{C}$ . The values of  $K_m^{\text{H}_2\text{O}}$  and  $K_m^{\text{D}_2\text{O}}$  used were from Table 1. The propagation of the errors in  $1/K_m$  and in  $\log_{10} (1/K_m)$  were calculated according to the method of Ku (1966). Values in parentheses are given in kcal/mol.

Substrate	$\Delta G^{\text{H}_2\text{O}}$ (kJ/mol)	$\Delta\Delta G^{\text{D}_2\text{O}}$ (kJ/mol)	$\Delta G$ (kJ/mol)
ONA*	-9.67 (-2.31)	$-10.33 \pm 0.13$ ( $-2.47 \pm 0.03$ )	$-0.67 \pm 0.13$ ( $-0.16 \pm 0.03$ )
ONFA*	-15.23 (-3.64)	$-15.90 \pm 0.33$ ( $-3.80 \pm 0.08$ )	$-0.67 \pm 0.33$ ( $-0.16 \pm 0.08$ )
ONCA*	-17.66 (-4.22)	$-18.41 \pm 0.13$ ( $-4.40 \pm 0.03$ )	$-0.75 \pm 0.13$ ( $-0.18 \pm 0.03$ )
PTA†	$-24.89 \pm 0.42$ ( $-5.95 \pm 0.1$ )	$-26.02 \pm 0.79$ ( $-6.22 \pm 0.19$ )	$-1.13 \pm 0.88$ ( $-0.27 \pm 0.21$ )
ONCA‡	-15.77 (-3.77)	$-16.95 \pm 0.13$ ( $-4.05 \pm 0.03$ )	$-1.17 \pm 0.13$ ( $-0.28 \pm 0.03$ )
PMPCA*	-17.36 (-4.15)	$-18.66 \pm 0.33$ ( $-4.46 \pm 0.08$ )	$-1.20 \pm 0.33$ ( $-0.31 \pm 0.08$ )
PCA*	-21.13 (-5.05)	$-22.68 \pm 0.63$ ( $-5.42 \pm 0.15$ )	$-1.55 \pm 0.63$ ( $-0.37 \pm 0.15$ )
ONPA*	-20.42 (-4.88)	$-22.13 \pm 0.21$ ( $-5.29 \pm 0.05$ )	$-1.72 \pm 0.21$ ( $-0.41 \pm 0.05$ )
ONPA*	-19.62 (-4.69)	$-21.46 \pm 1.00$ ( $-5.13 \pm 0.24$ )	$-1.84 \pm 1.00$ ( $-0.44 \pm 0.24$ )
Isoamyl acetate*	-14.14 (-3.38)	$-16.15 \pm 0.21$ ( $-3.86 \pm 0.05$ )	$-2.01 \pm 0.21$ ( $-0.48 \pm 0.05$ )

\* AcChE from *Electrophorus electricus* (lytic 11 S form).

† From  $K_{i(\text{com.})}$  inhibition constants.

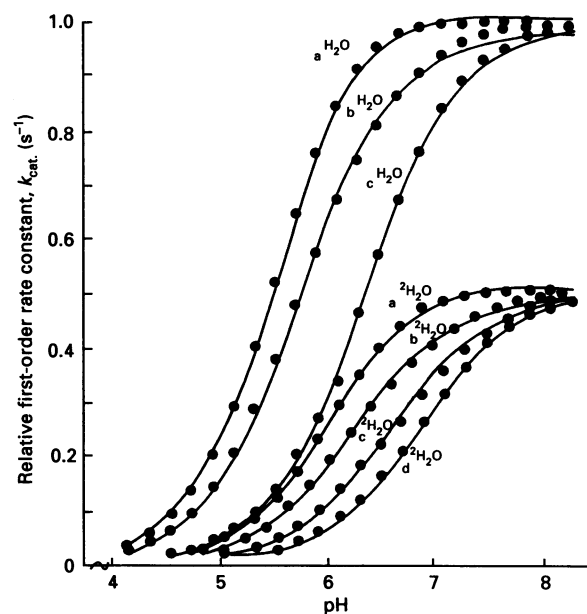
‡ AcChE from human erythrocyte.

values for the substrates under investigation are without complications from the kinetic constants  $k_{+2}$  and  $k_{+3}$ . The magnitude of the second-order rate constant  $k_{\text{cat.}}/K_{m(\text{app.})}$  ( $\text{M}^{-1} \cdot \text{s}^{-1}$ ) compared with the value of  $k_{+1}$  was used as the basis for deciding whether acylation was rate-determining (Salih, 1991). In the present analysis, calculation of the rate constant  $k_{+2}$  using the reported values of  $k_{+3}$  and  $k_{\text{cat.}}$  provided alternative and/or additional information for evaluation of the rate-determining step. The results summarized in Table 2 indicate that use of a value for the second-order rate constant  $< 5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$  is consistent with our knowledge of  $k_{+2}$  and  $k_{+3}$  in deciding the rate-determining step. The acylation would be rate-determining when  $k_{+2} \ll k_{+3}$ , a condition that can be defined by  $k_{+3}/k_{+2} \geq 10$  or  $k_{\text{cat.}} \leq 2 \times 10^3 \text{ s}^{-1}$ . Table 3 shows that, in  $^2\text{H}_2\text{O}$ , the  $K_m$  values are consistently lower to varying degrees, i.e.  $K_m^{\text{H}_2\text{O}}/K_m^{\text{D}_2\text{O}}$  ranging between approx. 1.0 and 2.5. When acylation is rate-determining, such data indicate that the binding of substrates to AcChE in  $^2\text{H}_2\text{O}$  is improved by  $\Delta\Delta G = \Delta G^{\text{D}_2\text{O}} - \Delta G^{\text{H}_2\text{O}} = -0.837$  to  $-2.092 \text{ kJ/mol}$  ( $-0.2$  to  $-0.5 \text{ kcal/mol}$ ), (Table 5). Effects of this kind suggest a significant role for hydrogen bonding during binding of substrates to AcChE. Support for this comes from the fact that non-covalent hydrogen bonds of water molecules are stronger by  $-1.004 \text{ kJ/mol}$  ( $-0.24 \text{ kcal/mol}$ ), with  $^2\text{H}_2\text{O}$  than with hydronium oxide (Nemethy & Scheraga, 1964). Assuming four hydrogen bonds per water molecule in bulk water provides  $-0.251 \text{ kJ/mol}$  ( $-0.06 \text{ kcal/mol}$ ) of ( $-\text{O} \cdots \text{D}-$ ) bond. The stronger binding of substrates to AcChE in  $^2\text{H}_2\text{O}$  can be accounted for by two types of force which can operate during binding. One is the general change in the overall electric field experienced by the substrates within the active centre due to exchange of hydrons with deuterons, and the other is the contribution towards the overall binding energy of the hydrogen bonds. Whether this latter case is a result of direct hydrogen bonding between substrate and active-centre components or is due to an enthalpic process or indeed a combination of the two is not easy to discern.

#### pH-dependence of $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$ in $^2\text{H}_2\text{O}$ for two-hydronic-reactive states of AcChE

Fig. 2 shows selected pH- $k_{\text{cat.}(\text{app.})}$  profiles revealing both changes in profile shape and  $\text{p}K_{\text{app.}}$  values in  $^2\text{H}_2\text{O}$  which would be observed when two reactive states of the enzyme contribute

towards overall catalysis (Scheme 1a). When  $\tilde{k}'_{\text{cat.}} = \tilde{k}_{\text{cat.}} = 1.0$  and  $\text{p}K_1 = 5.5$  and  $\text{p}K_{11} = 6.5$ , the value for  $\text{p}K_{\text{app.}}$  is 5.5 (Fig. 2, curve a $^{\text{H}_2\text{O}}$ ). This was shown to be the case for all the substrates for which pH- $k_{\text{cat.}}$  profiles were experimentally constructed (Salih, 1991). For this particular case, where the  $k_{\text{cat.}}$  values



**Fig. 2.** Apparent sigmoidal pH- $k_{\text{cat.}}$  rate profiles and variability of  $\text{p}K_{\text{app.}}$  in water and in  $^2\text{H}_2\text{O}$

The data points were computer-generated using eqn. (4) which is applicable to two-hydronic-reactive states of AcChE. For reactions in water  $\text{p}K_1 = 5.5$ ,  $\text{p}K_{11} = 6.5$  and pH-independent limiting rate constants  $\tilde{k}_{\text{cat.}} = 1.0$ , with  $\tilde{k}'_{\text{cat.}}$  for a $^{\text{H}_2\text{O}} = 1.0$ , for b $^{\text{H}_2\text{O}} = 0.7$  and for c $^{\text{H}_2\text{O}} = 0.2$ . For reactions in  $^2\text{H}_2\text{O}$   $\text{p}K_1 = 6.0$ ,  $\text{p}K_{11} = 7.0$  and pH-independent limiting rate constants  $\tilde{k}_{\text{cat.}} = 0.5$ , with  $\tilde{k}'_{\text{cat.}}$  for a $^{2\text{H}_2\text{O}} = 0.5$ , for b $^{2\text{H}_2\text{O}} = 0.35$ , for c $^{2\text{H}_2\text{O}} = 0.2$  and for d $^{2\text{H}_2\text{O}} = 0.1$ . Solid lines are theoretical for eqn. (5), providing best sigmoidal fit for data points with characteristic  $\text{p}K_{\text{app.}}$  values for a $^{\text{H}_2\text{O}} = 5.5$ , for b $^{\text{H}_2\text{O}} = 5.7$ , for c $^{\text{H}_2\text{O}} = 6.35$  and for a $^{2\text{H}_2\text{O}} = 6.0$ , for b $^{2\text{H}_2\text{O}} = 6.2$ , for c $^{2\text{H}_2\text{O}} = 6.57$  and for d $^{2\text{H}_2\text{O}} = 6.85$ .

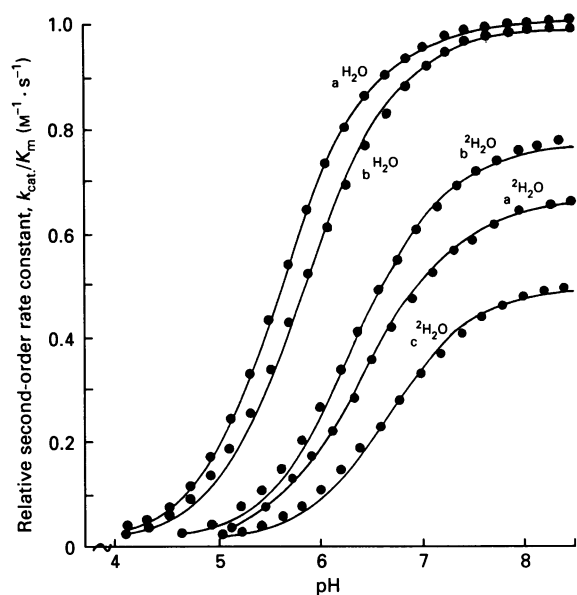


Fig. 3. Apparent sigmoidal  $\text{pH}-k_{\text{cat}}/K_m$  rate profiles and variability of  $\text{p}K_{\text{app}}$  values in water and in  $^2\text{H}_2\text{O}$

The data points were computer-generated using eqn. (3) which is applicable to two-hydronic-reactive states of AcChE. For reactions in water,  $\text{p}K_1 = 5.5$ ,  $\text{p}K_{11} = 6.5$  and pH-independent limiting rate constants  $\tilde{k}_{\text{cat}}/\tilde{K}_m = 1.0$  with  $\tilde{k}'_{\text{cat}}/\tilde{K}'_m$  for  $\text{a}^{\text{H}_2\text{O}}$  = 0.8 and for  $\text{b}^{\text{H}_2\text{O}}$  = 0.6. For reactions in  $^2\text{H}_2\text{O}$  the data points were generated using  $\text{p}K_1 = 6.0$ ,  $\text{p}K_{11} = 7.0$  and pH-independent limiting rate constants for  $\text{a}^{^2\text{H}_2\text{O}}$   $\tilde{k}'_{\text{cat}}/\tilde{K}'_m = 0.35$ ,  $\tilde{k}_{\text{cat}}/\tilde{K}_m = 0.65$ ; for  $\text{b}^{^2\text{H}_2\text{O}}$   $\tilde{k}'_{\text{cat}}/\tilde{K}'_m = 0.47$ ,  $\tilde{k}_{\text{cat}}/\tilde{K}_m = 0.78$ ; for  $\text{c}^{^2\text{H}_2\text{O}}$   $\tilde{k}'_{\text{cat}}/\tilde{K}'_m = 0.16$ ,  $\tilde{k}_{\text{cat}}/\tilde{K}_m = 0.52$ . The solid lines are theoretical for eqn. (5), providing best sigmoidal fit for data points with characteristic  $\text{p}K_{\text{app}}$  values for  $\text{a}^{\text{H}_2\text{O}}$  = 5.62, for  $\text{b}^{\text{H}_2\text{O}}$  = 5.83 and for  $\text{a}^{^2\text{H}_2\text{O}}$  = 6.43, for  $\text{b}^{^2\text{H}_2\text{O}}$  = 6.33 for  $\text{c}^{^2\text{H}_2\text{O}}$  = 6.67.

for the two reactive states are equivalent, two possibilities exist in  $^2\text{H}_2\text{O}$ : (i) both of the  $k_{\text{cat}}$  values for the two-reactive states,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}}$  and  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}}$  may evenly decrease and  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = \tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.5$ , with  $\text{p}K_{\text{app}}$  shifting from 5.5 to 6.0 due to a shift in  $\text{p}K_1$  and  $\text{p}K_{11}$  by 0.5 pH unit each in  $^2\text{H}_2\text{O}$  (Fig. 2, curve  $\text{a}^{^2\text{H}_2\text{O}}$ ) [e.g. for substrate *o*-nitroacetanilide (ONA)]; (ii) for a given substrate the two  $k_{\text{cat}}$  values in  $^2\text{H}_2\text{O}$  may decrease unevenly, hence  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.2$  and  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.5$  (Fig. 2, curve  $\text{c}^{^2\text{H}_2\text{O}}$ ). An  $^2\text{H}_2\text{O}$  effect on  $\text{p}K_{\text{app}}$  represents a shift of 1.07 pH units, which is more than the expected 0.5 pH unit for the pure change in the ionization constants,  $\text{p}K_1$  and  $\text{p}K_{11}$ .

A second case is when the  $k_{\text{cat}}$  values for the two reactive states are non-equivalent,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} \neq \tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}}$  (e.g.  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.7$ ,  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 1.0$ ) (e.g. Fig. 2, curve  $\text{b}^{\text{H}_2\text{O}}$ ). Similarly to the first case, in  $^2\text{H}_2\text{O}$  the  $k_{\text{cat}}$  values may decrease evenly ( $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.35$ ,  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.5$ ) leading to the  $\text{pD}-k_{\text{cat}}$  profile represented in Fig. 2 (curve  $\text{b}^{^2\text{H}_2\text{O}}$ ); with  $\text{p}K_{\text{app}}{}^{\text{H}_2\text{O}} = 5.7$  shifting to  $\text{p}K_{\text{app}}{}^{^2\text{H}_2\text{O}} = 6.2$ , a change of 0.5 pH unit occurs. Alternatively, the  $k_{\text{cat}}$  values for the two reactive states may decrease unevenly, for example,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.2$  and  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.5$ , then the  $\text{pD}-k_{\text{cat}}$  profile would be as in Fig. 2 (curve  $\text{c}^{^2\text{H}_2\text{O}}$ ) with  $\text{p}K_{\text{app}} = 6.57$ , a shift of 0.87 pH unit. It is expected that certain substrates would have different  $k_{\text{cat}}$  values in addition to different  $K_m$  values for the  $\text{EH}_2$  and  $\text{EH}$  states. For *o*-nitrophenyl acetate (ONPA), the experimentally constructed  $\text{pH}-k_{\text{cat}}$  profile gives  $\text{p}K_{\text{app}}{}^{\text{H}_2\text{O}} = 6.35$  (Acheson *et al.*, 1987b), and analysis in terms of two-hydronic-reactive states of AcChE using eqn. (4) revealed that, for this substrate,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.2$  and  $\tilde{k}_{\text{cat}}{}^{\text{H}_2\text{O}} = 1.0$  for the  $\text{EH}_2$  and  $\text{EH}$  states respectively (Fig. 2, curve  $\text{c}^{\text{H}_2\text{O}}$ ). In general, therefore, the effect of  $^2\text{H}_2\text{O}$  on the two rate constants

$\tilde{k}'_{\text{cat}}$  and  $\tilde{k}_{\text{cat}}$  can lead to a shift in the observed  $\text{p}K_{\text{app}}$  values greater than the 0.5 pH unit expected for a pure change in ionization constants in  $^2\text{H}_2\text{O}$ .

The pH-dependence of  $k_{\text{cat}}/K_m$  in water and  $^2\text{H}_2\text{O}$  are summarized in Fig. 3. Fig. 3 (curve  $\text{a}^{\text{H}_2\text{O}}$ ) gives a  $\text{p}K_{\text{app}} = 5.6$  with  $k_{\text{cat}}/K_m(\text{app.}) = 1.0$  and Fig. 3 (curve  $\text{a}^{^2\text{H}_2\text{O}}$ ) gives a  $\text{p}K_{\text{app}} = 6.43$  and  $k_{\text{cat}}/K_m(\text{app.}) = 0.65$ . These are corresponding profiles of the  $\text{pH}-k_{\text{cat}}$  profiles in Fig. 2 (curves  $\text{a}^{\text{H}_2\text{O}}$  and  $\text{a}^{^2\text{H}_2\text{O}}$ ), exemplified by substrate ONA. Fig. 3 (curve  $\text{a}^{^2\text{H}_2\text{O}}$ ) shows that the halving of  $k_{\text{cat}}$  in  $^2\text{H}_2\text{O}$ ,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}} = 0.5$ , is slightly compensated for in the ratio  $k_{\text{cat}}/K_m = 0.65$ , by a decrease in  $K_m(\text{app.})$  to 0.78. Fig. 3 (curve  $\text{b}^{\text{H}_2\text{O}}$ ) ( $\text{p}K_{\text{app}}{}^{\text{H}_2\text{O}} = 5.9$ ) shows the corresponding  $\text{pH}-k_{\text{cat}}/K_m$  profile for Fig. 2 (curve  $\text{a}^{\text{H}_2\text{O}}$ ) ( $\text{p}K_{\text{app}} = 5.5$ ). In  $^2\text{H}_2\text{O}$  the profile takes the shape of Fig. 3 (curve  $\text{b}^{^2\text{H}_2\text{O}}$ ) ( $\text{p}K_{\text{app}} = 6.33$ ) and the limiting rate constant  $k_{\text{cat}}/K_m(\text{app.}) = 0.78$ , i.e. for substrate ONCA, halving of  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}}$  to 0.5 is almost compensated for by a decrease in  $K_m(\text{app.})$  to 0.63. Fig. 3 (curve  $\text{c}^{^2\text{H}_2\text{O}}$ ) illustrates the  $\text{pH}-k_{\text{cat}}/K_m$  profile in  $^2\text{H}_2\text{O}$  ( $\text{p}K_{\text{app}} = 6.7$ ) and  $k_{\text{cat}}/K_m(\text{app.}) = 0.52$ , where halving of the  $k_{\text{cat}}$  value in  $^2\text{H}_2\text{O}$  is not compensated for by  $K_m(\text{app.})$ , i.e. the situation for PNPA.

In general, the observations in the literature of variable shifts and greater than 0.5 pH unit of  $\text{p}K_{\text{app}}$  values and pH-independent rate constants in  $^2\text{H}_2\text{O}$  can be accounted for by, and are consistent with, a two-hydronic-reactive state model.

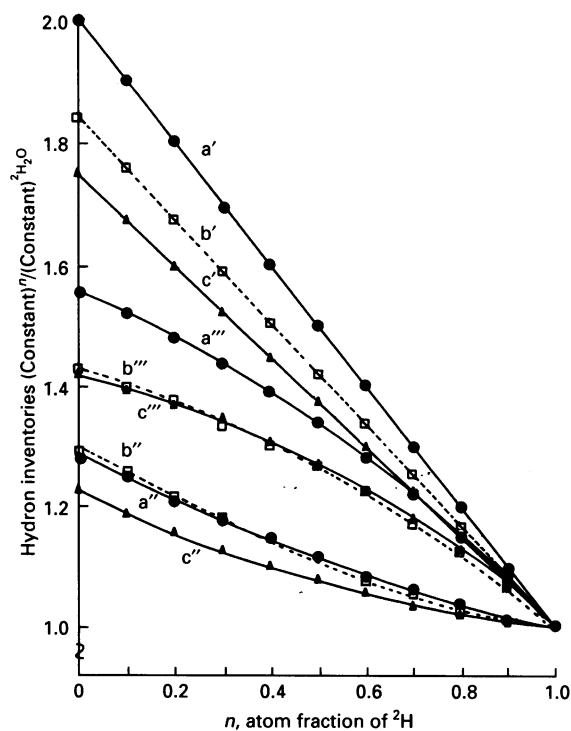


Fig. 4. Hydron inventories for  $k_{\text{cat}}$ ,  $K_m$  and  $k_{\text{cat}}/K_m$  for three substrates where acylation is rate-determining

The hydron inventories for  $k_{\text{cat}}/K_m$  ( $\text{a}'''-\text{c}'''$ ) are from the literature, obtained at pH 7.3 and equivalent pDs, in 0.1 M-sodium phosphate containing 0.1 M-NaCl. Those for  $k_{\text{cat}}$ , ( $\text{a}'-\text{c}'$ ) are determined from a knowledge of the linearity of hydron inventories for this parameter and the experimentally determined values in pure water,  $\tilde{k}'_{\text{cat}}{}^{\text{H}_2\text{O}}$ , and pure  $^2\text{H}_2\text{O}$ ,  $\tilde{k}'_{\text{cat}}{}^{^2\text{H}_2\text{O}}$ , by connecting these two points with a straight line. The hydron inventories for  $K_m$  ( $\text{a}''-\text{c}''$ ) were constructed by using the estimated  $k_{\text{cat}}$  values at each  $n$  atom fraction of  $^2\text{H}_2\text{O}$  and calculating the separate values of  $K_m^n$  from  $k_{\text{cat}}/K_m^n$  as outlined in the Materials and methods section, for substrates PNPA (●), ONFA (□) and ONCA (▲).



Table 6. Fractionation factors,  $\phi^T$ , for the transferred hydrons in two-hydronic-reactive states

Substrate	pH	Fraction of enzyme in EH <sub>2</sub> and EH states		Apparent fractionation factors, $\phi_{app}^T$	Fractionation factors for EH <sub>2</sub> and EH states		Contributions to $\phi_{app}^T$	
		$f_{EH_2}$	$f_{EH}$		$\phi^{T_{EH_2}}$	$\phi^{T_{EH}}$	EH <sub>2</sub>	EH
ONPA	7.3	0.15	0.85	0.55	0.55	0.55	0.02	0.53
PNPA	7.5	0.12	0.88	0.50	0.50	0.50	0.06	0.44
ONCA	7.3	0.15	0.85	0.58	0.58	0.58	0.09	0.49
PCA	6.12	0.75	0.25	0.55	0.55	0.55	0.41	0.14
ONFA	7.3	0.15	0.85	0.55	0.55	0.55	0.08	0.47

### Evaluation of hydron inventories for $k_{cat}$ , $K_m$ and $k_{cat}/K_m$

Hydron inventories carried out by using the parameter  $k_{cat}/K_m$  with AcChE gave predominantly upward-bowing curves (Hogg *et al.*, 1980); Quinn & Swanson, 1984; Acheson *et al.*, 1987b; Quinn, 1987). Just as use of  $k_{cat}/K_m$  is not appropriate for evaluation of <sup>2</sup>H<sub>2</sub>O effects (because of effects on  $K_m$ ), this parameter is not appropriate for evaluation of hydron inventories. Fig. 4 shows hydron-inventory plots for  $K_m$  and  $k_{cat}$ . (calculated in the present paper) and those reported in the literature for  $k_{cat}/K_m$ , exemplifying the effects of the progressive increase in <sup>2</sup>H concentration on  $K_m$  and  $k_{cat}$ , and the overall resultant effect on the ratio  $k_{cat}/K_m$ . The calculated data for substrates where acylation is rate-determining show that  $K_{m(app)}$  values decrease with increasing <sup>2</sup>H<sub>2</sub>O in a downward-curve fashion. The observations in the literature of upward-bowing hydron inventories for  $k_{cat}/K_m$  are due to linear hydron inventories for  $k_{cat}$  and downward bowing of those for  $K_m$  and are not the result of rate-limiting induced-fit conformational change. The analyses for Fig. 4 were carried out assuming linear hydron inventories for  $k_{cat}$ . This assumption was derived from the observations of linear inventories constructed experimentally for substrates phenylacetate, PCA and ONPA (Kovach *et al.*, 1986; Acheson *et al.*, 1987a; Acheson & Quinn, 1990). The possibility of the hydron inventories for  $k_{cat}$  with substrates in Fig. 4 being downward-bowing (i.e. more than one hydron transfer involved in the rate-determining step) would only mean the hydron-inventory plots for  $K_m$  calculated in the present paper being even more profoundly downward-bowing. The linear hydron inventories for  $k_{cat}$  are in accord with eqns. (8) and (9) for the two-hydronic-reactive states, indicating that AcChE utilizes a single hydron transfer in the catalytically significant transition state in both reactive states. This is further substantiated by the linearity of hydron inventories for  $k_{cat}$  at pH 7.0–7.5 where contribution of the EH state,  $\phi^T$ , dominates the observed  $\phi_{app}^T$  and at pH 6.0 where contribution of the EH<sub>2</sub> state,  $\phi^{T_{EH_2}}$ , dominates the observed  $\phi_{app}^T$  (Table 6). In addition to the substrates in this Table, the <sup>2</sup>H<sub>2</sub>O effects on  $k_{cat}$  (Table 1) indicate that these conclusions have general applicability. The fractionation factors evaluated by using eqns. (8) and (9) show that the  $\phi_{app}^T$  values range between 0.5 and 0.6 for a number of substrates. The transferred hydrons in the two reactive states require the two fractionation factors,  $\phi^{T_{EH_2}}$  and  $\phi^T$ , to be equivalent. This may have been expected since mechanistically the participant acid–base catalyst His-440 is common to both reactive states (Salih, 1991). However, the contribution of each state to the observed  $\phi_{app}^T$  can vary significantly depending on the pH of the hydron-inventory study. Therefore the overall isotope effects observed are the result of combined contributions from the two reactive states, unless the pH of the experiment is high enough or low enough that either the EH or EH<sub>2</sub> state would account for all of the isotope effect.

Although authors are frequently tempted to postulate the double-hydron transfer (charge-relay) mechanism (Taylor, 1991; Doctor *et al.*, 1990; Soreg & Proddy, 1988), at present there is no evidence for this in AcChE and such analogies derived from other serine proteinases do not have a favourable outcome. This is because, even for the most extensively studied serine proteinases, the catalytic triad involving the serine, histidine and aspartate residues proposed originally by Blow *et al.* (1969) from X-ray-crystallographic studies to act as a charge-relay has been shown to be not a charge-relay system (Steitz & Shulman, 1982; Polgar & Halansz, 1982). Also, <sup>13</sup>C n.m.r. and neutron-diffraction studies show that it is the imidazole of the triad that is hydronated and not the aspartate (Bachovchin *et al.*, 1981; Kossiakov & Spencer, 1981). Consistent with this, the analysis by Warshel *et al.* (1989) led to the conclusion that the double-hydron transfer mechanism does not apply in serine enzyme catalysis, and the transition state is stabilized by electrostatic interactions rather than charge-relay. In view of the above details, it is reasonable to propose only a catalytic triad in AcChE and not the charge-relay.

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