Transition-State Properties and Rate-Limiting Processes in the Acetylation of Acetylcholinesterase by Natural and Unnatural Substrates[1]

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Abstract: The acetylation of acetylcholinesterase at pH 7.5 and 25.00 °C proceeds at the same rate to within a few percent for acetylcholine, $CD_3CO_2CH_2CH_2N(CH_3)_3^+$, $CH_3CO_2CD_2CH_2N(CH_3)_3^+$, and $CH_3CO_2CH_2CD_2N(CH_3)_3^+$ and possibly slightly more slowly for $CH_3CO_2CH_2CH_2N(CD_3)_3^+$. Acetylation by $CH_3CO_2C_6H_4NO_2$-*p* and $CD_3CO_2C_6H_4NO_2$-*p* occurs with identical rates, while acetylation by $CD_3CO_2CH_3$ seems about 10% slower than by $CH_3CO_2CH_3$ under somewhat different conditions. Acetylation by *p*-nitrophenyl acetate is retarded upon addition of deuterium oxide (atom fraction of deuterium *n*) according to $(k_E^n/k_E^0) = (1 - n + n[0.45])/(1 - n + n[0.70])$. The results are consistent with a model for acylation of acetylcholinesterase in which some event in the binding of acetylcholine is rate limiting for the natural substrate. Other stages of the acylation process (which are rapid with the natural substrate) become sufficiently slow to be observed with other, unnatural substrates.

Acetylcholine (ACh, **1**) is a substance responsible for the transmission of information across nervous synapses and neuromuscular junctions.[2] Its hydrolytic destruction (eq 1), effected by the enzyme acetylcholinesterase (AChE, EC 3.1.1.7),[3,4] is apparently of such great biological advantage[2] that AChE has evolved as a powerful catalyst. The AChE-catalyzed hydrolysis of ACh is about 10^8 times faster[5] than the nonenzymic (hydroxide-promoted) reaction at pH 7 and 25 °C.

$$\underset{\mathbf{1}}{CH_3CO_2CH_2CH_2N(CH_3)_3^+} + H_2O \rightarrow CH_3CO_2^- + H^+ + HOCH_2CH_2N(CH_3)_3^+ \quad (1)$$

AChE shows a number of mechanistic affinities with the simple digestive enzymes known as serine proteases, including the double-displacement (acylation-deacylation) pathway of eq 2:

$$E\text{-}OH + CH_3CO_2R \rightarrow E\text{-}OCOCH_3 + ROH \quad (2a)$$

$$E\text{-}OCOCH_3 + H_2O \rightarrow E\text{-}OH + CH_3CO_2^- + H^+ \quad (2b)$$

in which a serine hydroxyl serves as a nucleophilic function and a histidine-containing unit as a general acid-base catalytic entity.[3,4] AChE has sometimes been thought of as an advanced form of the serine proteases, its assignment having been elevated from a routine digestive function to a critical role in neurotransmitter inactivation with a corresponding increase in structural complexity.[4] While the serine proteases are commonly single-subunit globular enzymes of molecular weight ~25 000, AChE when examined electron microscopically exhibits a tetrameric structure with an appended "tail", with aggregates of two or three of these units also being present. Under the conditions used for mechanistic study, AChE appears to be a noncovalent dimer of disulfide-linked dimers [thus $(\alpha_2)_2$] with each monomeric subunit (α) possessing a molecular weight of 76 000 and a single active site.

The kinetic parameter k_E ($\equiv k_{cat}/K_m$) is the second-order rate constant for the acylation step (eq 2a) with hydrolytic enzymes[6,7] of this type, and mechanistic probes based on k_E will illuminate the transition states for the conversion of free reactants to acetyl-enzyme. Rosenberry[8] has taken note of the following properties of the acetylation process: (1) The magnitude of k_E with ACh is around 10^8 M^{-1} s^{-1}, only about tenfold below the diffusional limit. (2) There is practically no depression of k_E for ACh in deuterium oxide, although for k_{ES} ($\equiv k_{cat}$, for which deacetylation is largely rate limiting[3]) there is a substantial solvent isotope effect: $k_{ES}^{H_2O}/k_{ES}^{D_2O} \simeq 2.3$. (3) In the dependence of k_E on pH, different substrates give different values of the inflection point (apparent pK_a), which is consistent with more than a single rate-limiting step for acetylation, with the identity of the dominant step varying with substrate structure. Rosenberry proposes in a hypothesis unifying these observations that a conformation change in the enzyme-substrate encounter complex determines the rate with ACh, so that acid-base catalysis has not yet begun. Thus, a large rate constant k_E with no solvent isotope effect is observed. With less well-adapted substrates, other steps begin to influence the rate.[8]

Here we present a further exploration of this question, making use of β-deuterium secondary isotope effects[9-12] and solvent isotope effects.[13,14]

Results

AChE with ACh. Isotopic modifications **2**–**5** of ACh and the unlabeled substrate were subjected to hydrolysis with catalysis by AChE under first-order ([ACh] < K_m) conditions. The rate constants and isotope effects are shown in Table I.

$$\underset{\mathbf{2}}{CD_3CO_2CH_2CH_2N(CH_3)_3^+}$$

$$\underset{\mathbf{3}}{CH_3CO_2CH_2CH_2N(CD_3)_3^+}$$

$$\underset{\mathbf{4}}{CH_3CO_2CD_2CH_2N(CH_3)_3^+}$$

$$\underset{\mathbf{5}}{CH_3CO_2CH_2CD_2N(CH_3)_3^+}$$

AChE with *p*-Nitrophenyl Acetate. Similar experiments employed *p*-nitrophenyl acetate and its d_3 analogue, in both protium oxide (pH 7.50) and deuterium oxide (pD 8.05) at 25.00 ± 0.03 °C, 0.10 M phosphate or Tris buffers. Different enzyme preparations were employed in the two solvents, so then an accurate solvent isotope effect cannot be computed from the data; the isotopic substrates can be properly compared with each other in H_2O and in D_2O, however. The pD value of 8.05 "corresponds" to pH 7.50 in lying at the same relative point on the pL/rate profile.[14] The first-order rate constants were 10^5k (s^{-1}) = 1299 ± 17 (CH_3 ester, H_2O), 1310 ± 13 (CD_3 ester, H_2O), and 612 ± 1 (CH_3 ester, D_2O), 615 ± 4 (CD_3 ester, D_2O). The isotope effects are thus k_{3H}/k_{3D} = 0.992 ± 0.016 (H_2O), 0.995 ± 0.008 (D_2O).

AChE with Methyl Acetates. Initial rates were obtained

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Table I. First-Order Rate Constants for Acetylcholinesterase[a]-Catalyzed Hydrolysis of Isotopically Substituted Acetylcholine Perchlorates[b] at pH 7.50 ± 0.05[c] and 25.00 ± 0.01 °C

substrate	10^5k,[d] s^{-1}	k_E^H/k_E^D, observed	$100[(k_E^{NH}/k_E^{ND})^{1/N} - 1]$,[e] % per D
$CH_3CO_2CH_2CH_2N(CH_3)_3^+$	480 ± 8	0.99 ± 0.04	−0.3 ± 1.3
$CD_3CO_2CH_2CH_2N(CH_3)_3^+$	483 ± 14		
$CH_3CO_2CH_2CH_2N(CH_3)_3^+$	523 ± 25	1.06 ± 0.05	0.7 ± 0.6
$CH_3CO_2CH_2CH_2N(CD_3)_3^+$	495 ± 11		
$CH_3CO_2CH_2CH_2N(CH_3)_3^+$	525 ± 28	1.00 ± 0.07	0.0 ± 3.5
$CH_3CO_2CD_2CH_2N(CH_3)_3^+$	526 ±22		
$CH_3CO_2CH_2CH_2N(CH_3)_3^+$	420 ± 8	1.00 ± 0.03	0.0 ± 1.5
$CH_3CO_2CH_2CD_2N(CH_3)_3^+$	418 ± 11		

[a] Enzyme was Sigma Type III electric eel acetylcholinesterase, used at a concentration of about 64 ng/mL. [b] Initial substrate concentrations were about 3.2×10^{-5} M. [c] The pH was maintained at 7.50 by Radiometer pH Stat ([NaCl] = 0.16 M). [d] Mean of three-five determinations. [e] N = number of isotopically substituted hydrogens.

under conditions used by Krupka[15] for these much less reactive substrates. Although the data should not be considered as so reliable as those for other substrates, duplicate experiments conducted at different times yielded identical results. At pH 6.45 ± 0.05, 25.00 ± 0.01 °C, 0.10 M sodium chloride, 0.04 M magnesium chloride, the mean velocities (10^6 fraction of buret s^{-1}) were 387 ± 14 ($CH_3CO_2CH_3$) and 352 ± 13 ($CD_3CO_2CH_3$). This yields $k_{3H}/k_{3D} = 1.10 \pm 0.06$.

Nonenzymic Basic Hydrolysis. (a) ACh. The nonenzymic, basic hydrolysis reactions of the isotopic ACh modifications **1**–**5** were studied at pH 11.00 and 25.00 ± 0.01 °C by monitoring the change in absorbance at 400 or 522 nm in solutions of alizarin yellow GG, diethylamine/diethylammonium buffer, 0.009–0.018 M initial substrate concentration, with the use of automatic spectrophotometric data acquisition techniques, or by automatic titration (pH Stat) in 0.1 M sodium chloride solutions with 6.67×10^{-4} M initial concentrations of substrate. The isotope effects were 0.981 ± 0.005 [$(CD_3CO_2CH_2CH_2N(CH_3)_3^+$, −0.6 ± 0.2% per D], 0.997 ± 0.027 [$CH_3CO_2CH_2CH_2N(CD_3)_3^+$, −0.03 ± 0.3% per D], 1.004 ± 0.012 ([$CH_3CO_2CD_2CH_2N(CH_3)_3^+$, +0.2 ± 0.6% per D], 0.995 ± 0.012 [$CH_3CO_2CH_2CD_2N(CH_3)_3^+$, −0.3 ± 0.7% per D].

(b) *p*-Nitrophenyl Acetate. The spectrophotometric method was employed to measure first-order rate constants k_0^{31} for CH_3CO_2Ar and CD_3CO_2Ar at constant, excess sodium hydroxide concentrations, 25.00 ± 0.03 °C, 1 M sodium chloride. The linear least-squares fit of k_0^{31} to [NaOH] gave:

$$10^3k_0^{3H}(s^{-1}) = (8496 \pm 63\ M^{-1}\ s^{-1})[NaOH] - (0.5 \pm 0.2)$$

$$10^3k_0^{3D}(s^{-1}) = (8762 \pm 54\ M^{-1}\ s^{-1})[NaOH] - (0.4 \pm 0.2)$$

The isotope effect is thus $k_{3H}/k_{3D} = 0.970 \pm 0.009$.

Proton Inventory for *p*-Nitrophenyl Acetate with AChE. Values of k_{Ee_0} were determined in mixtures of H_2O and D_2O and are given in Table II. Extrapolation to 100% D_2O (see Discussion) gives $k_E^{H_2O}/k_E^{D_2O} = 1.56$.

Model Vibrational Analysis Calculation for Binding of ACh to AChE. To estimate the magnitude of the isotope effect for ACh derivatives of the type **4** to **5**, which would originate solely from torsional restriction upon binding of these compounds at the AChE active site, a model vibrational analysis was performed with the BEBOVIB-IV program of Sims and Burton, in a manner we have otherwise described.[11] A molecule XCH_2CH_2X (where X is an atom of mass 59, the same as CH_3CO_2 and $-N(CH_3)_3^+$) was taken as the reactant. It was given a cis, eclipsed geometry with standard bond lengths[16] [CH 1.073 Å; CX 1.453 Å (average of CN and CO); CC 1.537 Å], angles[16] (109.5°), and force constants[16,17] [stretches (mdyn/Å): CH 4.8, CX 5.0, CC 4.5; bends (mdyn-Å/rad^2): HCC 0.55, HCX 0.50, HCH 0.32, CCX 0.98; torsions:[17] 0.027 mdyn-Å/rad^2; all off-diagonal force constants are 0]. The enzyme-bound state was assumed to have exactly the same geometry and, except for the torsional force constant, the same force field including 0 off-diagonal elements. In one calculation the torsional force constant was increased to 0.36 mdyn-Å/rad^2 and in a second calculation to 1.00 mdyn-Å/rad^2. For each of these bound states, two masses were used for X. In one case, X was kept at a mass of 59, simulating a situation in which the enzyme restricts rotation about the ethano bridge of ACh, while leaving the acetyl and trimethylammonio (X) groups dynamically independent of enzyme structure. In the second case, the mass of X was increased to 1000 to simulate strong attachment to extensive regions of enzyme structure. The isotope effects differed only slightly for these two assumptions. The calculated isotope effects are given in Table III.

Table II. First-Order Rate Constants for Acylation of Acetylcholinesterase[a] by *p*-Nitrophenyl Acetate[b] at pH 7.50 and Equivalent[c] in Binary Mixtures of Protium Oxide and Deuterium Oxide at 25.00 ± 0.03 °C

atom fraction of deuterium, n	$10^5k_E^nC_0$, s^{-1}	atom fraction of deuterium, n	$10^5k_E^nC_0$, s^{-1}
0.000	389 387	0.593	323 324
0.099	379 378	0.692	307 306
0.198	369 371	0.792	293 291
0.296	357 354	0.891	274 274
0.395	344 347	0.988	254 254
0.494	338 335		

[a] Enzyme was Worthington ECHP 53N525 from electric eel at C_0 = 13 μg/mL. [b] Substrate was injected in acetonitrile solution to produce 1.66% (v/v) acetonitrile and an initial substrate concentration of 6.33×10^{-5} M. [c] 0.03 M Tris buffer (0.006 M Tris, 0.024 M Tris-HCl) in all solvents.

Discussion

Acetylation of AChE by ACh. To within several percent (usually around 1% per deuterium or better), isotopic substitution at all positions in ACh, excepting only the *N*-methyl groups, produces no change in the value of k_E (Table I). The deuteration of the *N*-methyl groups appears to decrease the rate but the effect—if present at all—is only just detectable at the limit of one standard deviation (67% confidence level). Deuteration at all other positions has no effect at this confidence level. From this it may be concluded that the force field of ACh in the rate-determining transition state leading to acetyl-enzyme very strongly resembles the force field for free ACh in aqueous solution, with the possible exception of force constants associated with the *N*-methyl group. It may in turn, then, be deduced that the structure of ACh (again possibly excepting the *N*-methyl region) is substantially *unaltered* in the rate-determining transition state from the structure in free aqueous solution.

Isotopic substitution on the ethano bridge of ACh (compounds **4** and **5**) would have been expected to increase the value of k_E to produce an inverse isotope effect if the rate-determining transition state had involved substantial torsional restriction about this bridge (Table III). Such restriction would

Table III. Calculated Isotope Effects for Binding of Eclipsed *cis*-XCL_2CH_2X (L = H,D) to a Hypothetical AChE Active Site at 25 °C

mass of X in bound state[a]	torsional force constant in bound state,[b] mdyn-Å/rad^2	MM1[c]	EXC[c]	ZPE[c]	$(K_{2H}/K_{2D})^c$
59	0.027	1.0000	1.0000	1.0000	1.0000
59	0.36	1.0000	1.0502	0.8494	0.8921
59	1.00	1.0000	1.0825	0.6776	0.7337
1000	0.027	1.0386	0.9799	0.9814	0.9987
1000	0.36	1.0386	1.0302	0.8327	0.8909
1000	1.00	1.0386	1.0632	0.6635	0.7326

[a] Reactant-state value is 59. [b] Reactant-state value 0.027. [c] Contributions for XCD_2CH_2X vs. XCH_2CH_2X.

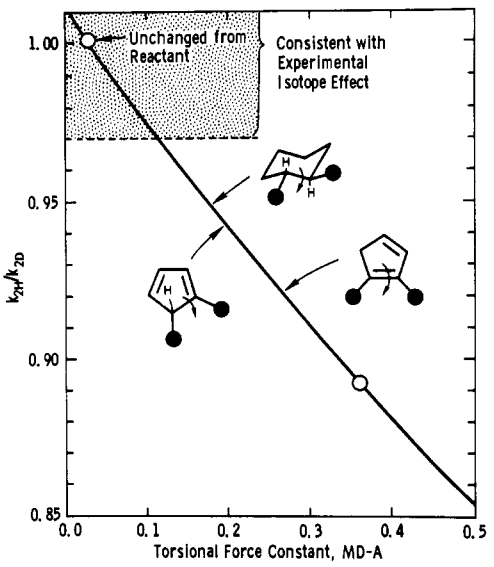
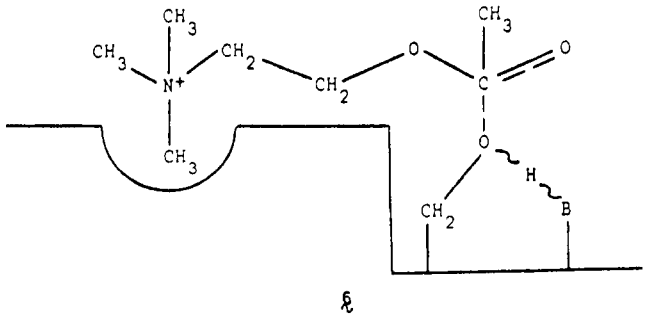


Figure 1. Plot of estimated k_{2H}/k_{2D} vs. torsional force constant assumed for ACh in the bound state for reaction with AChE of $CH_3CO_2CL_2CH_2N(CH_3)_3^+$ (**4**, L = H, D) or $CH_3CO_2CH_2CL_2N(CH_3)_3^+$ (**5**, L = H, D), assuming the isotope effect to arise solely from changes in torsional force constant and/or mass upon binding to the enzyme. The circles show two calculated points and the solid line is a quadratic fit to these and a third for a unit torsional force constant. The shaded area at the top encompasses the mean and one standard deviation of the experimental isotope effect for **5**. Also shown are values of torsional force constants that have been used in vibrational analyses of cyclohexane[19] and cyclopentadiene.[20] Because of the complexity of the force fields used in these analyses, the force constants shown can be considered to give only the roughest kind of approximation to the torsional restriction.

be expected if, in the transition state, strong enzyme–substrate interactions were present as in the commonly conceived structure **6**. An appreciation for the degree of restriction which



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can be accommodated by the experimental data can be developed by a study of Figure 1. Here a plot is given of the anticipated isotope effect for double deuteration (k_{2H}/k_{2D}) as a function of the transition-state torsional force constant (the line was obtained by fitting the three calculated values of Table III to a quadratic function). Indicated in the figure are values of torsional force constants characteristic of some common structures in organic compounds, and also the range of force constants consistent with the more precise of the two relevant experimental measurements (that for compound **5**). Clearly only a minimal torsional restriction in the transition state can be reconciled with the measurements.

Deuteration in the acetyl group (compound **2**, giving rise to a β-deuterium secondary isotope effect) is also expected to increase the rate if nucleophilic interaction at the carbonyl group (as, for example, in **6**) is a dominant feature in the transition state.[12] Electrophilic interaction at carbonyl should produce a normal effect.[12] A value of k_{3H}/k_{3D} of around 0.87 is an approximate limit for complete adduct formation.[12] The mean isotope effect for acetylation of AChE by ACh **1** and **2** (Table I) is slightly inverse but well within experimental error of unity. This is consistent with either (a) a transition state with no strong interaction of any kind at carbonyl, or (b) a transition state in which both electrophilic and nucleophilic interactions exist but balance each other nearly exactly. With respect to (b), it may be noted that in acid-catalyzed alkyl ester hydrolysis (where electrophilic and nucleophilic interactions might have cancelled), the balance strongly favors the nucleophilic interaction ($k_{3H}/k_{3D} = 0.93 \pm 0.02$).[12] On the other hand, the apparent nucleophilic interaction at the ACh carbonyl group in *nonenzymic*, base-promoted hydrolysis is not very strong ($k_{3H}/k_{3D} = 0.98$). We believe in this and similar cases[12] that this is not because the structure of the actual transition state for nucleophilic bond information is "early", with a long bond to nucleophile and near-trigonal carbonyl, but instead that such a transition state is only partially rate determining in basic hydrolysis. Its intrinsic isotope effect, which could be quite inverse, may be diluted by unit or normal isotope effects for solvent-reorganization transition states which participate in determining the rate, to generate a resultant effect of 0.98. Thus, we favor the hypothesis that nucleophilic bond-formation processes play a minimal role, and perhaps no role at all, in the rate-determining step for acetylation of AChE by ACh.

Deuteration of the *N*-methyl groups of ACh might produce an effect on k_E if the trimethylammonio group is removed from aqueous solution and finds itself, at the transition state, in another environment such as the "anionic binding site" postulated for AChE.[3] It is, however, difficult to estimate the magnitude and direction to be expected for this effect, both because the nature of force-constant changes for such environmental alterations is not well understood and because the physical character of the enzymic binding site for the trimethylammonio group is not well known. The observation of a small, normal, apparent isotope effect can, therefore, be taken as consistent with, but not compelling evidence for, some transition-state interaction of the positive role of ACh with AChE in the acetylation transition state.

The isotope effects thus indicate that some process prior to nucleophilic attack at carbonyl, most likely some event in the course of substrate binding, is the rate-determining step for acetylation of AChE by ACh.

Acetylation of AChE by Unnatural Substrates. The reaction of *p*-nitrophenyl acetate with AChE occurs about 1000 times more slowly than the reaction with the natural substrate. It is similar in that the β-deuterium secondary isotope effect (no more than a few tenths of a percent per deuterium) indicates no net interaction at substrate carbonyl in the transition state. Here the nonenzymic reaction with hydroxide ion reveals considerably more nucleophilic interaction at this center than in the enzymic reaction, although we consider it likely[12] this effect is, even so, strongly diluted by incursion of solvent reorganization as a kinetic determinant. The reaction with AChE of *p*-nitrophenyl acetate *differs* from that of ACh (for which[8] $k_{H_2O}/k_{D_2O} \simeq 1$) in exhibiting a substantial solvent isotope effect $k_{H_2O}/k_{D_2O} = 1.56$. This indicates reorganization at exchangeable hydrogenic centers of AChE. The rate-determining process thus appears not to be the same as that for ACh, although as in the latter case, there seems still to be little or no net nucleophilic or electrophilic interaction at carbonyl. A considerable increase in leaving-group electron density in the rate-determining transition state for reaction of activated alkyl and aryl acetates is indicated by the ρ^* value of 2.80 ± 0.33 seen by Järv, Kevatera, and Aaviksaar.[18]

The interpretation of the solvent isotope effect of 1.56 for acetylation of AChE by *p*-nitrophenyl acetate can be aided by consideration of the "proton-inventory" data in partially deuterated solvents. A plot of k_E^n vs. n (≡ atom fraction of deuterium in the binary mixture of protium and deuterium oxides), shown in Figure 2, reveals that the rate falls off as deuterium is introduced, not in a linear fashion, but in the way described by K. B. Schowen[14] as "bowed upward". Such curves have also been observed for the acylation of α-chymotrypsin and α-lytic protease.[21] They were explained in those cases in terms of eq 3:

$$k_E^n = k_E^0(1 - n + n\phi_T)/(1 - n + n\phi_R) \qquad (3)$$

which corresponds to the conversion of a reactant-state protonic site with fractionation factor[14] ϕ_R to a more loosely bound transition-state site with ϕ_T. The data of Table II yield, as shown in Figure 2, $\phi_R = 0.70$ and $\phi_T = 0.45$. These are similar to the values found with the two serine proteases.[21]

One possibility of many which could generate the results observed is if a conformational change of the enzyme, involving relaxation of the catalytic acid–base machinery to its "resting" state, after formation of the acetyl-enzyme but before or concomitant with alcohol product release should determine the rate. Since the carbonyl group is trigonal in such a transition state, no β-D effect would be seen, but a substantial ρ^* and solvent isotope effect would be found.

The data for acetylation of AChE by methyl acetate are not closely comparable with the others, but it does seem clear that $k_H > k_D$ for this very slowly reacting substrate. This signals (a) that a transition state has been exposed in which force-constant changes in the substrate acetyl group are apparent, and (b) that these changes are indicative of some electrophilic character in the interaction at carbonyl. The latter might arise either from removal of nucleophilic solvation at trigonal carbonyl,[12] carbonyl attack or leaving-group expulsion with a strong predominance of electrophilic (acid catalytic) function by the enzyme, or a transition state for product release from acyl-enzyme in which a trigonal carbonyl remains unsolvated. The solvent isotope effect[8] of about 2 for methyl acetate could be taken to suggest the second of these.

These data seem most readily consistent with a model in which some event in substrate binding is rate determining for acetylation of AChE by ACh, but that other processes become rate determining with the other acetate esters studied.

Thus, ACh seems so well fitted by evolutionary selection to the AChE structure and dynamics that all events subsequent to its arrival at the active site are very rapid. Only by altering the substrate to an unnatural form can the characteristics of these subsequent stages be exposed for study.

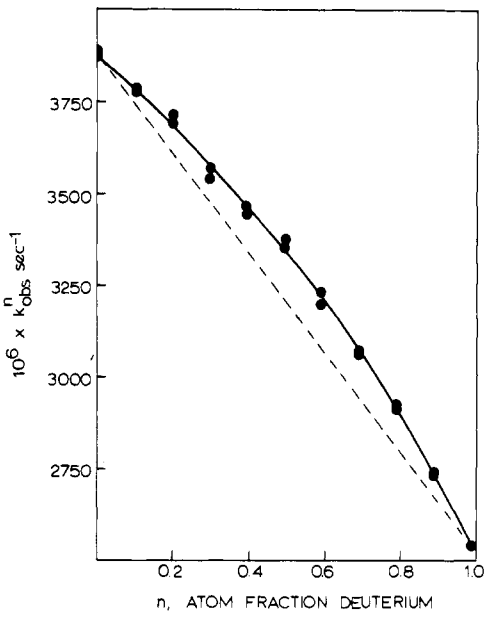

Figure 2. Proton inventory plot for acetylation of AChE by *p*-nitrophenyl acetate ($k_{obs}^n \equiv k_E^n C_0$). The solid line is a plot of eq 3 with $\phi_T = 0.45$, $\phi_R = 0.70$. The dashed, straight line connects the points in pure light and heavy water.

Experimental Section

Materials. Inorganic salts, buffer components, and preparative reagents were reagent-grade chemicals, which were used as purchased, or dried, recrystallized, or distilled as necessary. Protium oxide was distilled, deionized, and degassed with nitrogen. Deuterium oxide (Stohler, 99.8 atom % deuterium) was distilled (100–102 °C fraction) and stored under nitrogen.

Substrates. Acetylcholine perchlorates containing deuterium were prepared and characterized as previously described.[22] The levels of deuteration attained were: **2,** 93.7%; **3,** 96.0%; **4,** 99.6%; **5,** 99.6%. *p*-Nitrophenyl acetates were prepared by reaction of *p*-nitrophenol with acetyl chloride (in the labeled case, from Diaprep CD_3CO_2D, 99.5% deuterated) as otherwise described.[12] Methyl acetates[12] were prepared from methanol and acetic acid by acidic esterification, with no protium detectable at the acetyl position in the NMR spectrum of the labeled compound.

Enzyme. Acetylcholinesterase from *Electrophorus electricus* was obtained either as a solution of 10.7 mg of protein mL^{-1} with an activity of 10 000 micromolar units/mL (Sigma Type III) or as a lyophilized powder with an activity of 2000 micromolar units per mg of solid (Worthington).

AChE Kinetics. (a) ACh. Enzyme solutions in 0.16 M aqueous sodium chloride were prepared at a concenration of 64 ng/mL by dilution of the Sigma enzyme preparation. Substrate stock solutions were prepared at millimolar levels in the same solvent, with solid substrates (ACh perchlorates) weighed by a Cahn Electrobalance. Reaction rates were determined by automatic titration (2.2×10^{-3} M sodium hydroxide, 0.25-mL buret) at pH 7.50 ± 0.05 and 25.000 ± 0.005 with a Radiometer pH Stat (G 202C glass electrode, K401 calomel electrode). Runs were initiated by injection of 100 ± 1 μL of substrate stock solution with an Eppendorf pipet into 10.00 ± 0.02 mL of thermostated enzyme solution in the sample chamber. Runs with protiated and deuterated substrates were conducted in alternation. First-order rate constants were calculated by a nonlinear least-squares method.

(b) PNPA. With *p*-nitrophenyl acetate, the rates were determined spectrophotometrically with use of automated data acquisition.[23] Reactions were conducted at enzyme concentrations of 17–20 μg mL^{-1} at a pH of 7.50 in protium oxide, attained with phosphate buffer (1.6560 g L^{-1} $NaH_2PO_4 \cdot H_2O$, 12.4950 g L^{-1} Na_2HPO_4; used for the β-D measurement in H_2O), or with Tris buffer (0.2524 g L^{-1} Tris, 1.2440 g L^{-1} Tris-HCl; used for solvent isotope effects and β-D measurement in D_2O). Corresponding pL in H_2O/D_2O mixtures was

achieved by use of the same buffer composition throughout.[14] Substrate concentrations were initially 47.5–73.1 μM. H_2O/D_2O mixtures were made up by weight from buffer stock solutions in the pure isotopic solvents.

Runs were conducted by, first, thermal equilibration of 3.00 mL of buffer in a quartz cuvette in the Cary 16 cell compartment, as monitored by a thermistor probe. Then 100 μL of substrate stock solution was added, the solution was manually mixed, and data acquisition (400 nm) begun. Rate constants were calculated from the computer-stored data by a nonlinear least-squares method.

(c) **Methyl Acetate.** Rates were determined by the same pH Stat technique as above. Enzyme concentration was 5 μg mL^{-1} (0.10 M NaCl, 0.04 M $MgCl_2$). Substrates were introduced neat by Eppendorf pipet (50 μL into 10.00 ± 0.02 mL) with the final concentrations calculated using an appropriate correction for the densities of $CH_3CO_2CH_3$ (0.965 ± 0.005 g mL^{-1}) and $CD_3CO_2CH_3$ (1.014 ± 0.007 g mL^{-1}). The resulting substrate concentrations were 0.0651 M ($CH_3CO_2CH_3$) and 0.0658 M ($CD_3CO_2CH_3$). A 2.5-mL buret with 0.01 M sodium hydroxide titrant was employed, the reactions being followed to less than 2% completion. A linear least-squares fit to the initial-rate data was made.

Nonenzymic Kinetics. The rates of basic hydrolysis of ACh perchlorates were measured by pH Stat methods and of *p*-nitrophenyl acetate by automated spectrophotometry in ways very similar to those used for the enzymic reactions. The only unusual procedures were those denoted methods A and B in the Results section. These differed only in the wavelength of spectrophotometric observation. In both, a quartz cuvette in the Cary 16 cell compartment was charged with 3.00 mL of a solution 5×10^{-2} M in diethylamine and in diethylamine hydrobromide and 10^{-3} M in alizarine yellow GG. After thermal equilibration of this solution was complete, either 50, 100, or 200 μL of a 0.3 M solution of ACh was added by Eppendorf pipet, and data collection was initiated. Thereafter, the procedures were the same as for the *p*-nitrophenyl acetate experiments.

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Communications to the Editor

Lithium and Sodium 2,6-Di-*tert*-butylphenoxides and the Crystal and Molecular Structure of $[Li(OC_6H_2Me\text{-}4\text{-}Bu^t_2\text{-}2,6)(OEt_2)]_2$

Sir:

A family of alkali metal di-*tert*-butylphenoxides, $(MOAr\cdot L)_n$, has been prepared (selected examples and data are in Table I; M = Li or Na, L = OEt_2 or THF). These compounds are versatile phenoxide-transfer reagents for preparing corresponding complexes of other metals, which in turn are already showing promise of structural and chemical interest.[1-5] The present paper is our first (but see also notes 1–5) dealing with the chemistry and structure of the 2,6-di-*tert*-butylphenoxides of the elements and the studies are complementary to researches on bulky amides[6] and alkyls[7] (see also note 8), e.g., on metal complexes having $Me_3SiCH_2^-$, $(Me_3Si)_2CH^-$, $(Me_3Si)_2N^-$, $Me_2C(CH_2)_3CMe_2N^-$, or ArO^- as ligands.[9]

The crystal and molecular structure of $(LiOAr'\cdot OEt_2)_2$ (Ar' = 2,6-di-*tert*-butyl-4-methylphenyl) (**1**), illustrated in Figure 1, shows the compound to be the di-μ-phenoxo-dilithium dietherate having three-coordinate lithium atoms and an overall pseudospherical shape with pendant alkyl groups at the periphery, which accounts for its unexpected solubility in hydrocarbon solvents.

Compound **1** is a rare binuclear lithium compound to be stable under ambient conditions in the crystalline state. The previously reported small lithium clusters include the dimer of bicyclo[1.1.0]butan-1-yllithium tetramethylethylenediamine,[10] trimers,[11] or tetramers;[12] hexamers (as in cyclohexyllithium[13] or trimethylsilyllithium[14]) or chain polymers are also a feature with organic ligands.[8] Mass spectra led to the suggestion that $LiCH_2SiMe_3$ is the tetramer and $LiOBu^t$ the hexamer;[15] the corresponding sodium alkoxide has a more complicated crystal and molecular structure, $(NaOBu^t)_9(NaOBu^t)_6$.[16] In general, alkyllithiums are covalent rather than ionic;[17] however, when stabilization of the carbanion is possible and the ligand ceases to be monohapto, solvated ion pairs are found, e.g., in $LiCPh_3$ (TMEDA).[18]

The lithium and sodium phenoxides are obtained according to the equations

$$2LiBu^n + 2ArOH \xrightarrow{OEt_2} (LiOAr\cdot OEt_2)_2 + 2C_4H_{10} \quad (1)$$

$$Na + ArOH \rightarrow {}^1/_n(NaOAr\cdot OEt_2)_n + {}^1/_2H_2 \quad (2)$$

In one preparative experiment, 15.3 g of predried 2,6-di-*tert*-butyl-4-methylphenol was dissolved in 50 mL of dry, degassed OEt_2 and cooled to 0 °C; 43.25 mL (49.6 mmol in *n*-C_6H_{14}) of *n*-butyllithium was slowly added with stirring and cooling during 0.5 h. A white precipitate was separated by filtration, and solvent was removed from the filtrate at 20 °C (10^{-2} mmHg). Recrystallization of the residue from *n*-

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