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Fractional Diffusion-Limited Component of Reactions Catalyzed by Acetylcholinesterase†

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ABSTRACT: The values of k_{cat}/K_m for the reactions of four substrates, *p*-nitrophenyl acetate (PNPA), propionyl-β-methylthiocholine (PrMSCh), 3,3-dimethylbutyl thioacetate (DBTA), and acetylthiocholine (AcSCh), with acetylcholinesterase were determined as a function of increasing viscosity (η_{rel}) in sucrose-containing and in glycerol-containing buffers. Glycerol, or possibly some contaminant of it, was found to be a nonspecific inhibitor and sucrose a nonspecific activator of the enzyme as reflected in the dependence of k_{cat}/K_m values measured for PNPA and PrMSCh upon the concentration of these reagents. The rates of reactions of these two substrates, the first neutral and the second cationic, are chemically limited rather than diffusion limited, and they thus serve as quantitative controls or internal standards to monitor the effects of the viscosogens on the enzyme, which are not related to diffusion. The additional effect on k_{cat}/K_m over the controls observed for the rapidly reacting substrates AcSCh (cationic) and DBTA (neutral) serves as a measure of the extent to which these values of k_{cat}/K_m measure diffusion-controlled processes. The reaction rate of DBTA with the enzyme is 24% diffusion controlled as measured in glycerol-containing buffers and 16–20% as determined in sucrose-containing buffers, while that for AcSCh is 100% (in glycerol) and 24–40% (in sucrose) diffusion controlled.

The reactions of acetylcholinesterase, the enzyme that catalyzes the hydrolysis of acetylcholine (AcCh)[1] at neuromuscular junctions, with good substrates exhibit some of the highest values of k_{cat}/K_m known, e.g., 1.6×10^9 M^{-1} s^{-1} for AcSCh at ionic strength = 0.0032, decreasing to 2×10^8 M^{-1} s^{-1} at ionic strength = 0.13 (Nolte et al., 1980). The magnitude of this figure has suggested that the rate of the reaction may be diffusion controlled (Rosenberry, 1975; Nolte et al., 1980; Hasinoff, 1982). Further evidence in support of this conjecture includes the lack of a solvent kinetic effect on k_{cat}/K_m for AcCh (Rosenberry, 1975) and the sensitivity of this parameter to increases in viscosity (Hasinoff, 1982). On the other hand, the correlation demonstrated by Hasan et al. (1980) of k_{cat}^n/K_m, where k_{cat}^n is the value of k_{cat} normalized

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); AcSCh, acetylthiocholine; PrMSCh, propionyl-β-methylthiocholine; PNPA, *p*-nitrophenyl acetate; DBTA, 3,3-dimethylbutyl thioacetate; AcCh, acetylcholine.

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by the values of the alkaline hydrolysis rate constants, with apparent molal volumes for a series of substituted acetate esters may be interpreted in terms of the rates of reaction of even the best substrates being chemically controlled. Smissart (1981) has summarized other evidence supporting the later conclusion. Thus, the question of the extent to which the reaction rates of acetylcholinesterase with good substrates are diffusion controlled remains unanswered.

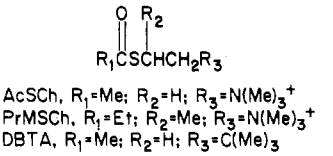
Scheme I

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

The viscosity variation method has been used to determine values for the rate constant for formation of the enzyme–substrate complex, k_1, and for the partition ratio of this complex, k_{-1}/k_2 (Scheme I), for partially diffusion controlled reactions catalyzed by chymotrypsin (Brouwer & Kirsch, 1982) and by β-lactamase (Hardy & Kirsch, 1984). The rate constant, k_2, in Scheme I will be replaced by the *net rate constant* for this step in mechanisms in which more than one enzyme–substrate complex preceeds the first irreversible step in the reaction (Cleland, 1975). The method is thus independent of the number of preequilibrium complexes. These earlier studies emphasized the importance of employing a control substrate, i.e., one whose rate of reaction is chemically limited rather than diffusion controlled, to monitor the nonspecific effect of the viscosogenic reagent on the protein. Varying the viscosity with a second cosolute served as a further control in these studies. No significant perturbations of enzyme activity induced by the viscosogen were observed with either chymotrypsin or β-lactamase, and thus, the results and conclusions would not have been affected had the less reactive substrates not been tested. These controls proved, however, to be essential for the present work, where glycerol is found to be an inhibitor of acetylcholinesterase and sucrose a nonspecific activator in reactions with substrates whose rates of reaction are not completely diffusion limited. The knowledge of the viscosogen concentration dependence of the values of k_{cat}/K_m for the control substrates proved indispensible for determining the quantitative extent of diffusion control for the rates of reaction of acetylcholinesterase with the rapidly reacting cationic substrate AcSCh and with its uncharged homologue DBTA.

Materials and Methods

Materials. Acetylcholinesterase from electric eel was purchased from Sigma as a lyophilized powder containing ca. 5% NaCl. It was dissolved in 0.1 M NaCl–0.05 M sodium phosphate buffer, pH 7.5, and stored in the freezer at −10 °C. The values of k_{cat}/K_m typically obtained with commercial enzyme preparations (Steinberg et al., 1970; Hasan et al., 1980; Naveh et al., 1981) are ca. one-fifth that measured with fresh preparations (Rosenberry, 1975). Sucrose and glycerol (analytical reagent grade) were obtained from Mallinckrodt and used without further purification. Similar results were observed with glycerol assayed at 95 or 99% purity. AcSCh, PrMSCh, and DTNB were from Baker, Sigma, and Aldrich, respectively. PNPA was prepared by the method of Chattaway (1931). 3,3-Dimethyl-1-bromobutane and 3,3-dimethyl-1-thiobutane were prepared by the general procedures outlined by Vogel (1962). A total of 8 g (78 mmol) of 3,3-dimethyl-1-butanol was mixed with 16.4 g of 48% HBr and 5.5 g of sulfuric acid and refluxed for 2 h. The lower layer was then separated and dried with sodium sulfate to give 3,3-dimethyl-1-bromobutane [8 g (49 mmol) in a 64% yield]. 3,3-Dimethyl-1-thiobutane was prepared by adding 5.2 g (68 mmol) of thiourea in 5 mL of H_2O to 8 g of 3,3-dimethyl-1-bromobutane. The mixture was refluxed for 3 h. The liquid layer was distilled off and discarded, and 6 g of NaOH in 65 mL of water was added to the solid and the mixture refluxed for 1.5 h. The upper layer, containing the bulk of the mercaptan, was separated, dried with sodium sulfate, and combined with the lower layer after it had been acidified with 5.4 mL of sulfuric acid in 11 mL of H_2O. The neutral thiol was extracted with ether and the ether evaporated off. A total of 2.5 g (21 mmol) of thiol was obtained. To this was added 2.7 g (34 mmol) of acetyl chloride and the mixture refluxed for 3 h. The fraction distilling between 165 and 186 °C was collected to give 2.5 g (16 mmol) of DBTA. Anal. Calcd: C, 59.95; H, 10.06; S, 20.00. Found: C, 60.04; H, 9.89; S, 20.13. The structures of AcSCh, PrMSCh and DBTA are



Methods. Experiments at pH 7.5 were performed with buffer solutions of 0.05 M sodium phosphate containing 0.1 M NaCl, which were prepared by mixing a solution containing 0.1 M NaCl and 0.05 M $Na_2HPO_4 \cdot H_2O$ with one containing 0.1 M NaCl and 0.05 M NaH_2PO_4 to pH 7.5 ($I_c \approx 0.22$). The pH-variation experiments were done in buffers of $I_c \approx 0.15$ prepared by mixing solutions of 0.05 M NaH_2PO_4 and 0.017 Na_2HPO_4, each containing 0.1 M NaCl, to the desired pH. Sodium ion concentration was held constant at 150 mM to avoid variations in the extent of inhibition due to specific binding of this ion (Smissaert, 1981). Sucrose and glycerol solutions were prepared in the same manner except that each buffer component contained the specific amount (percent weight by volume) of viscosogen. All the solutions were filtered through a Millipore membrane (0.22 μm) to remove suspended material.

The relative viscosity ($\eta_{rel} = \eta/\eta^0$) was measured with an Ostwald viscometer at 25 °C in the same buffer with no added viscosogen as a reference. Approximate relative viscosities for the sucrose solutions are as follows: 14% sucrose, $\eta_{rel} = 1.5$; 24% sucrose, $\eta_{rel} = 2.2$; 32% sucrose, $\eta_{rel} = 2.9$. These agree well with the values reported earlier by Brouwer & Kirsch (1982) except for 32% sucrose for which these workers reported $\eta_{rel} = 3.2$. The values of η_{rel} determined for glycerol solutions were 1.3, 1.8, and 2.3 for 10, 20, and 30% (w/v), respectively. The pH values of the solutions were measured with a Radiometer Model pHM 64 meter with a type B electrode.

The enzymatic hydrolysis reactions were followed continuously with a Cary Model 118C spectrophotometer equipped with a thermostated cuvette holder maintained at 25 °C. Reactions of acetylcholinesterase with PNPA were monitored at 400 nm where $\Delta\epsilon = 16\,000$ (Rosenberry, 1975). Reactions of the enzyme with the thio esters (AcSCh, PrMSCh, and DBTA) were followed with DTNB, as described by Ellman et al. (1961). The concentration of DTNB was 0.25–0.33 mM, about 10-fold greater than that of the substrates.

Reactions were initiated by the addition of 50–100 μL of an ice-cooled solution of acetylcholinesterase in pH 7.5 buffer to a reaction mixture equilibrated at 25 °C. The latter solution was prepared by mixing 5–20 μL of substrate solution at room temperature into the appropriate buffer–viscosogen solution in a quartz cuvette to the final volume of 2.0 mL less the amount of enzyme mixture to be added. Solutions of cationic

Table I: Dependence of k_{cat}/K_m on Viscosity for Acetylcholinesterase-Catalyzed Hydrolysis of Acetylthiocholine (AcSCh) and 3,3-Dimethylbutyl Thioacetate (DBTA)[a]

viscosogen [% (w/v)]	η_{rel}	DBTA[c] k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1}) (SE)[d]	DBTA[c] $(k_{cat}/K_m)_{rel}$ (SE)	DBTA[c] cor $(k_{cat}/K_m)_{rel}$ (SE)[e]	AcSCh[b] k_{cat}/K_m ($\times 10^{-6}$ M^{-1} s^{-1}) (SE)	AcSCh[b] $(k_{cat}/K_m)_{rel}$ (SE)	AcSCh[b] cor $(k_{cat}/K_m)_{rel}$ (SE)[f]
				Glycerol			
0	1	12.18 (0.34)	1.00 (0.04)	1.00 (0.04)	11.0 (0.2)	1.00 (0.03)	1.00 (0.03)
10	1.3	8.16 (0.22)	0.67 (0.03)	0.91 (0.05)	6.58 (0.58)	0.60 (0.05)	0.69 (0.06)
20	1.8	6.22 (0.22)	0.51 (0.02)	0.85 (0.04)	4.28 (0.10)	0.39 (0.01)	0.56 (0.03)
30	2.3	4.68 (0.25)	0.38 (0.02)	0.75 (0.04)	2.90 (0.10)	0.26 (0.01)	0.45 (0.02)
				Sucrose			
0	1	7.35 (0.36)	1.00 (0.05)	1.00 (0.05)	17.0 (0.5)	1.00 (0.05)	1.00 (0.06)
14	1.5	7.39 (0.36)	1.01 (0.07)	0.86 (0.07)	15.4 (0.9)	0.91 (0.06)	0.75 (0.06)
24	2.2	7.37 (0.39)	1.00 (0.07)	0.73 (0.05)	14.3 (0.4)	0.84 (0.04)	0.62 (0.04)
32	2.9	7.08 (0.46)	0.96 (0.8)	0.65 (0.06)	14.2 (0.1)	0.83 (0.3)	0.57 (0.03)

[a] In 0.05 M sodium phosphate buffers, 0.1 M NaCl, pH 7.5, 25 °C; $I_c \approx 0.22$. [b] [AcSCh], 6–14 μM; [acetylcholinesterase], 0.20–0.53 nM. [c] [DBTA], 39 μM; [acetylcholinesterase], 1.2 nM; [CH_3CN], 1% (v/v). [d] Average and standard error from three to six determinations. [e] $(k_{cat}/K_m)_{rel}^{DBTA}/(k_{cat}/K_m)_{rel}^{PNPA}$. [f] $(k_{cat}/K_m)_{rel}^{AcSCh}/(k_{cat}/K_m)_{rel}^{PrMSCh}$.

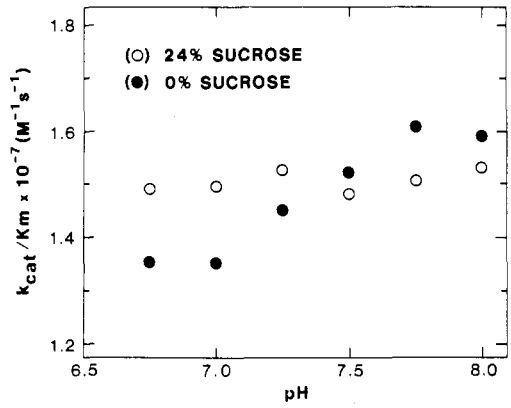


FIGURE 1: pH dependence of k_{cat}/K_m for the acetylcholinesterase-catalyzed hydrolysis of acetylthiocholine (AcSCh) in the presence (O) and absence (●) of 24% (w/v) sucrose. Conditions: 0.05 M phosphate buffers with NaCl added to $I_c \approx 0.2$, 6.25 μM AcSCh, 0.3–0.6 nM enzyme, 25 °C.

substrates were prepared in phosphate–NaCl buffer (pH 7.5), and neutral substrates were dissolved in acetonitrile. These solutions were kept in the refrigerator for not more than 20 days. Initial substrate concentrations were at least 20-fold lower than the K_m to ensure pseudo-first-order reaction conditions. The rates of the reactions were analyzed by nonlinear least-squares regression to the pseudo-first-order rate equation.

Results and Discussion

Effect of Sucrose on the pH Dependence of k_{cat}/K_m for AcSCh. The initial experiments on viscosity variation were done with sucrose as added cosolute. Since it is convenient to work at a single pH at the maximum of the pH vs. k_{cat}/K_m profile, it is important to determine if the viscosogen shifts the pH profile. The effect of sucrose on the k_{cat}/K_m vs. pH profile for the reaction of AcSCh with acetylcholinesterase is shown in Figure 1. Little variation is found in the value of this parameter with pH, either in 24% or in 0% sucrose-containing solutions over the pH range 6.5–8.0. The latter results agree well with those reported by Rosenberry (1975) for AcCh. All further experiments were therefore carried out at a pH(app) of 7.5.

Sucrose as Viscosogenic Cosolute. In earlier work reported from this laboratory for the reactions of chymotrypsin (Brouwer & Kirsch, 1982) and β-lactamase (Hardy & Kirsch, 1984), increasing sucrose concentration caused regular decreases in values of k_{cat}/K_m for rapidly reacting substrates, i.e., for those substrates whose rates of reaction are partially

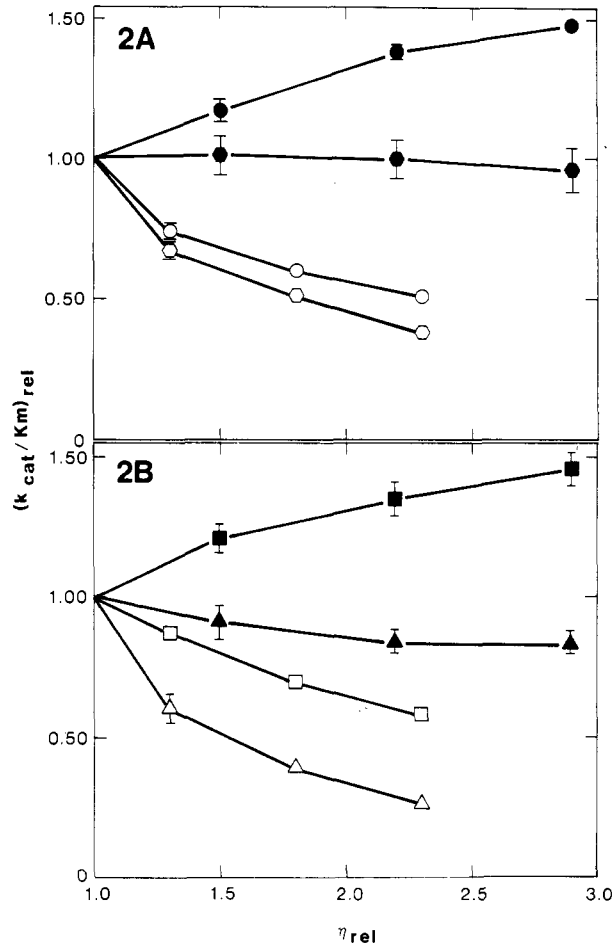


FIGURE 2: Plots of $(k_{cat}/K_m)_{rel}$ vs. η_{rel} for the reactions of acetylcholinesterase with neutral (A) and cationic (B) substrates in glycerol- (open symbols) or sucrose- (closed symbols) containing buffers. (A) 3,3-Dimethylbutyl thioacetate (DBTA), (●, O); *p*-nitrophenyl acetate (PNPA) (●, O). (B) Acetylthiocholine (AcSCh) (▲, △), propionyl-β-methylthiocholine (PrMSCh) (■, □). Conditions: 0.05 M sodium phosphate buffers, 0.1 M NaCl, pH(app) 7.5, $I_c \approx 0.22$, 25 °C. Error flags smaller than the plotting symbols are not shown. The lines are drawn for clarity only and have no theoretical significance.

diffusion controlled. The addition of sucrose to buffer solutions contrastingly affords only a slight decrease in the values of k_{cat}/K_m for AcSCh (Figure 2B and Table I). This was an unexpected observation, because there is good reason to believe that this substrate, whose value of k_{cat}/K_m is on the order of 10^8 M^{-1} s^{-1} (Rosenberry, 1975; Nolte et al., 1980), should be

Table II: Dependence of k_{cat}/K_m on Glycerol and Sucrose Concentrations for Acetylcholinesterase-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate (PNPA) and Propionyl-β-methylthiocholine (PrMSCh)[a]

viscosogen [% (w/v)]	η_{rel}	PNPA[b] k_{cat}/K_m ($\times 10^{-4}$ M^{-1} s^{-1})[d]	PNPA[b] $(k_{cat}/K_m)_{rel}$ (SE)[e]	PrMSCh[c] k_{cat}/K_m ($\times 10^{-6}$ M^{-1} s^{-1})[d]	PrMSCh[c] $(k_{cat}/K_m)_{rel}$ (SE)
		Glycerol			
0	1	4.47 (0.07)	1.00 (0.02)	3.58 (0.05)	1.00 (0.02)
10	1.3	3.32 (0.12)	0.74 (0.03)	3.13 (0.03)	0.87 (0.02)
20	1.8	2.67 (0.04)	0.60 (0.01)	2.51 (0.08)	0.70 (0.02)
30	2.3	2.28 (0.01)	0.51 (0.01)	2.07 (0.07)	0.58 (0.02)
		Sucrose			
0	1	3.66 (0.04)	1.00 (0.02)	1.28 (0.04)	1.00 (0.04)
14	1.5	4.28 (0.12)	1.17 (0.04)	1.55 (0.05)	1.21 (0.05)
24	2.2	5.05 (0.08)	1.38 (0.03)	1.73 (0.06)	1.35 (0.06)
32	2.9	5.42 (0.05)	1.48 (0.02)	1.87 (0.06)	1.46 (0.06)

[a] In 0.05 M sodium phosphate buffers, 0.1 M NaCl, pH 7.5, 25 °C; $I_c \approx 0.22$. [b] [PNPA], 30–100 μM; [acetylcholinesterase], 13–29 nM; [CH_3CN], 1–2% (v/v). [c] [PrMSCh], 20–35 μM; [acetylcholinesterase], 0.98–1.46 nM. [d] Averages from four to six determination. The errors reported are the average standard errors for the individual runs, because the specific activities of the two enzyme lots differed. This has no effect on the relative values reported in the last column. [e] Propagated average of SE's of the independent sets of experiments.

reacting at or near the diffusion-controlled limit. To investigate further, a much more slowly reacting substrate for the same enzyme was examined, since the rate constants for the latter should be largely or completely chemically limiting and therefore independent of viscosity. *p*-Nitrophenyl acetate (PNPA) was chosen for this purpose because of the observation of Rosenberry (1975) that k_{cat}/K_m for this substrate exhibits a solvent isotope effect, $(k_{cat}/K_m)_{H_2O}/(k_{cat}/K_m)_{D_2O} = 1.9$, implying chemical participation of the solvent in the first irreversible step of the reaction, whereas k_{cat}/K_m for AcCh exhibits a solvent isotope effect of only 1.07 (Rosenberry, 1975) consistent with that expected for a diffusion-controlled reaction. That sucrose *nonspecifically* activates the enzyme is shown by the observation that the values of k_{cat}/K_m for PNPA are sharply increased in high concentrations of sucrose (Figure 2A); e.g., k_{cat}/K_m in 24% sucrose ($\eta_{rel} = 2.2$) is increased by 38% over the figure recorded in buffers containing no sucrose (Table II). Increases in the values of k_{cat}/K_m effected by sucrose with substrates whose rates of reaction are chemically limited were not noted in earlier work with chymotrypsin (Brouwer & Kirsch, 1982) or β-lactamase (Hardy & Kirsch, 1984). The present observation of this phenomenon illustrates the importance of the use of a control substrate (i.e., one whose rate of reaction with the enzyme is chemically limited rather than diffusion limited) before evaluating the additional effect of sucrose on diffusion for the more rapidly reacting substrates.

Charged vs. Uncharged Substrates. The active site of acetylcholinesterase is known to have substantial net negative charge as evidenced by the sharp dependence of association rate constants on ionic strength (Nolte et al., 1980), the specific inhibition by sodium ion (Smissaert, 1981), and the extra affinity of charged inhibitors compared to analogous neutral species (Hasan et al., 1981). Diffusion rate constants for collisions of molecules of opposite charge tend to be ca. 1 order of magnitude greater than those for comparable uncharged species (Eigen, 1964); therefore, a more slowly reacting positively charged substrate for the enzyme was sought as a control for the cationic AcSCh. The low value of k_{cat}/K_m reported by Steinberg et al. (1970) of 6×10^6 M^{-1} s^{-1} for propionyl-β-methylcholine, which is a factor of ca. 10 less than that reported by them for AcCh, suggested the former as a likely candidate for the desired chemically controlled substrate. The thio ester analogue was used in the present work in order to facilitate the DTNB-coupled spectrophotometric assay. Comparative assays of the two substrates with the same enzyme sample gave $(k_{cat}/K_m)_{AcSCh}/(k_{cat}/K_m)_{PrMSCh} = 7$. Thus, the thio and oxy esters react with approximately the same rate constant ratios.

The effect of increasing viscosity in sucrose-containing buffers on the values of k_{cat}/K_m for PrMSCh and for AcSCh (filled symbols) is shown in Figure 2B. High concentrations of sucrose significantly increase the value of this kinetic parameter for PrMSCh; e.g., at $\eta_{rel} = 2.9$, k_{cat}/K_m is equal to 1.46 times that observed in the absence of sucrose. Thus, the rates of reaction of both the neutral and cationic control substrates are increased in a similar fashion by sucrose. These results for the two slowly reacting substrates PNPA and PrMSCh, whose values of k_{cat}/K_m indicate that they are largely, if not completely, chemically limited, show that sucrose functions as a nonspecific activator of the enzyme and suggests that the insensitivity to sucrose concentration recorded for AcSCh (Figure 2B) represents an offset of a decrease effected by the excess viscosity in the sucrose-containing buffers vs. an increase in rate of reaction caused by the nonspecific activation due to sucrose.

The sucrose effect was probed further with a rapidly reacting neutral substrate DBTA, which has a reported value of k_{cat}/K_m of ca. 1×10^6 M^{-1} s^{-1} (Hasan et al., 1980), which is 25-fold greater than that of PNPA and about one-tenth that of AcSCh measured under similar conditions (Tables I and II). The latter comparison, in particular, suggested that the reaction rate of DBTA might also be partly diffusion controlled. The effect of sucrose on k_{cat}/K_m for this substrate is also shown in Figure 2A and in Table II. The value of this rate constant for DBTA is virtually independent of added sucrose and behaves in this sense very similarly to what has been described above for AcSCh.

Glycerol as Viscosogen. Given the additional complexity in analysis introduced by sucrose-induced activation of the enzyme, it was decided to investigate the dependence of the values of k_{cat}/K_m in the presence of glycerol as added viscosogen for all four substrates. The data are shown in Figure 2 and Tables I and II. Glycerol strongly *decreases* all of the values of k_{cat}/K_m, providing in this respect a sharp contrast to the apparent nonspecific *activation* effected by sucrose. The decrease in k_{cat}/K_m for the most reactive substrates DBTA and AcSCh is dependent on greater than the first power of the reciprocal of viscosity; e.g., at $\eta_{rel} = 2.3$, k_{cat}/K_m for AcSCh is reduced to nearly one-fourth the value obtained in the absence of glycerol (Table I) vs. the theoretical limit of 1/2.3.

A plausible hypothesis to account for the glycerol effect is that this compound, or possibly some contaminant of it,[2] is

Table III: Analysis of the Viscosity Dependence of the Kinetics of the Reactions of Acetylthiocholine (AcSCh) and 3,3-Dimethylbutyl Thioacetate (DBTA) according to Equations 2 and 3

substrate	viscosogenic reagent	k_1^0(calcd) ($\times 10^6$ M^{-1} s^{-1}) (SE)[d]	k_{-1}^0/k_2 (SE)	% diffusion controlled, $(1 + k_{-1}/k_2)^{-1} \times 100$ (SE)	RSS[b]
		Competitive Model[c]			
AcSCh	glycerol	10.2 (1.6)	0.0 (0.2)	100 (17)	0.082
	sucrose	4.0 (0.5)	1.5 (0.4)	40 (6)	0.281
DBTA	glycerol	0.51 (0.09)	3.2 (0.8)	24 (5)	0.033
	sucrose	0.26 (0.04)	4.1 (0.7)	20 (3)	7.5
		Noncompetitive Model[d]			
AcSCh	glycerol	9	<0	>100	0.26
	sucrose	6.8 (0.8)	3.2 (0.3)	24 (2)	0.288
DBTA	glycerol	1.1	<0	>100	0.07
	sucrose	0.45 (0.07)	5.3 (1.2)	16 (3)	7.9

[a] The absolute values of k_1^0 are approximately 5-fold greater than those given here (see text). [b] Residual sums of squares from the regression. [c] From the fits to eq 2, where the viscosogen affects k_1 values. [d] From the fits to eq 3, where the viscosogen affects k_{-1}/k_2 values.

a competitive or noncompetitive inhibitor of the enzyme. The high K_m values for PNPA (Rosenberry, 1975) and PrMSCh (M. Bazelyansky and J. F. Kirsch, unpublished observations) prevent an accurate exploration of the dependence of k_{cat} and K_m individually on glycerol concentration, but the fact that k_{cat}/K_m for both of the slower substrates is roughly equally decreased by 3.0 M glycerol (30% w/v) is consistent with glycerol acting as a competitive inhibitor. Within the framework of this hypothesis, the competitive aspect of glycerol inhibition would be equally manifest for all four substrates, but the excess inhibition seen for DBTA and AcSCh results from the additional effect of increased viscosity, which becomes apparent only for those substrates whose rates of reaction are partially diffusion controlled.

Determination of the Effect of Increasing Viscosity on the Rates of Reaction of AcSCh and DBTA with Acetylcholinesterase. The values of the association rate constant k_1^0 and the ES partition ratio $k_{-1}^0/k_2 = P$ for Scheme I are related to the observed values of k_{cat}/K_m ($=k_a$) and the relative viscosity ($\eta_{rel} = \eta/\eta^0$) (Nakatani & Dunford, 1979):

$$k_a = \frac{k_1^0(\eta^0/\eta)}{1 + (\eta^0/\eta)P^0} \quad (1)$$

where the superscript zero refers to reactions at $\eta_{rel} = 1$. Glycerol and sucrose can influence the values of k_a for all substrates by affecting either k_1 or P. Glycerol, in the former instance, acts as a competitive inhibitor by decreasing the fraction of available enzyme and thus the apparent value of k_1, while sucrose would act as a "competitive activator". Sucrose could increase the apparent value of k_1 if available and blocked binding sites exist in an equilibrium subject to dynamic modification by sucrose. Analysis of the data according to the competitive model is achieved by regression analysis on eq 2 for k_a vs. η_{rel} and C_2. In eq 2 $C_2 = (k_{cat}/K_m)_{rel}$

$$k_a = \frac{C_2 k_1^0(\eta^0/\eta)}{1 + (\eta^0/\eta)P^0} \quad (2)$$

for the control substrate (i.e., PrMSCh for AcSCh and PNPA for DBTA; cf. Table II).

A competitive model requires that the fractional inhibition observed with both control substrates, PrMSCh and PNPA, be the same at a given concentration of sucrose or glycerol, but adherence of the data to this condition does not prove that such a model obtains. The data shown in Table II, columns 4 and 6, show quite a good correlation for the effect of sucrose on PNPA and PrMSCh and an approximaae one for that of glycerol.

An alternative model is one in which sucrose or glycerol combines with the enzyme–substrate complex and changes k_{cat}/K_m by decreasing or increasing the value of P, i.e., by affecting k_{-1} or k_2. This noncompetitive activation/inhibition model is described by

$$k_a = \frac{k_1^0(\eta^0/\eta)}{1 + (\eta^0/\eta)C_1P^0} \quad (3)$$

where $C_1 = (K_m/k_{cat})_{rel}$ for the control substrates.

All four sets of data (AcSCh and DBTA in sucrose and in glycerol solutions) were fit to both eq 2 and 3 with the results compiled in Table III. The glycerol data are not well accommodated by the noncompetitive inhibition model of eq 3 for which the least-squares procedure minimized to give physically impossible negative values of the partition ratios for both substrates with RSS values of 0.26 and 0.07 for AcSCh and DBTA, respectively. These data are much better described by the competitive inhibition model of eq 2, which gave the much lower RSS values of 0.082 and 0.033, respectively. Thus, it is reasonable to conclude that glycerol acts primarily as a simple competitive inhibitor. The data obtained in viscosogenic sucrose solutions give about equally good fits to both models (RSS = 0.281 vs. 0.288 for AcSCh and 7.5 vs. 7.9 for DBTA) and thus do not allow a decision as to whether sucrose affects principally k_1 or P. The values of k_1 and P for both models are, however, within a factor of 2 of each other. The data summarized in Table III show that the DBTA reaction rate is 24% diffusion controlled as determined from the kinetics in glycerol-containing buffers and 16–20% with sucrose as viscosogenic reagent. We find in agreement with earlier studies, that the reaction of AcSCh with the enzyme has a considerable diffusion-controlled component, although the quantitative analysis obtained with sucrose and with glycerol shows fair agreement at best (Table III).

It is of interest that the value of k_{cat}/K_m for DBTA, a substrate whose rate of reaction is partially diffusion controlled, is somewhat lower than that for PrMSCh, a substrate with a chemically controlled rate of reaction (Tables I and II). The likely explanation can be derived from an examination of the effects of charge and steric hindrance on the microscopic rate

[2] The molar concentration ratios of glycerol to substrates exceeded 10^9 in some experiments; thus, the inhibition observed and pro forma attributed to glycerol could be due to some very minor contaminant of this reagent. The control substrates, whose rates of reaction are chemically limited rather than diffusion limited, serve to monitor and to allow the evaluation of the extent of inhibition or activation effected by any and all components of the viscosogenic cosolutes.

constants of eq 1. The value of k_1^0 will be larger for a cationic than for a neutral substrate reacting with an anionic active site. Similarly, k_{-1}^0 for the ES complex formed with neutral substrate will be larger than the corresponding rate constant for the ion-paired complex (Nolte et al., 1980). The value of k_2 for PrMSCh must be much less than k_{-1}, probably for steric reasons. Thus, $k_{cat}/K_m = (k_1^0/k_{-1}^0)k_2$ for PrMSCh while $k_{cat}/K_m = k_1^0/(1 + \sim 4)$ for DBTA (cf. Table III), with a calculated k_1^0 value (for these data; cf. below) of $5k_{cat}/K_m = 5 \times 10^6$ M^{-1} s^{-1}. Microscopic rate constants for PrMSCh cannot be calculated because neither k_1^0 nor k_2/k_{-1}^0 can be determined separately, but the observed value of k_{cat}/K_m (Table III) can be accommodated for example with $k_1^0 = 5 \times 10^7$ M^{-1} s^{-1} and $k_2/k_{-1} = 0.07$, i.e., with a k_1^0 value 10-fold greater than that for the neutral substrate and a k_2/k_{-1} value consistent with a chemically controlled reaction rate.

Estimates of Absolute Values of the Association Rate Constants k_1^0 for DBTA and AcSCh. The fact that the commercial enzyme used in the present work is ca. one-fifth as active, as estimated from the values of k_{cat}/K_m, as the best fresh preparations employed by previous investigators (see Materials and Methods) precludes the calculation of k_1^0 from the data obtained herein. The values of the partition ratios k_{-1}^0/k_2 are, however, independent of enzyme activity. These latter values taken together with the present observed values of k_{cat}/K_m and the factor of 5 for reduced enzyme activity give the relationship

$$k_1^0 = 5(k_{cat}/K_m)_{obsd}^0(1 + P) \quad (4)$$

from which $k_1^0 \approx (2\text{–}3) \times 10^7$ M^{-1} s^{-1} is found for DBTA and a range of $(1\text{–}3) \times 10^8$ M^{-1} s^{-1} is calculated for AcSCh at $I_c = 0.22$. The latter value might be as much as a factor of ca. 3 higher on the basis of the value of 1.5×10^8 M^{-1} s^{-1} for k_{cat}/K_m reported by Nolte et al. (1980). The cationic substrate does associate with the enzyme with a rate constant that is about 1 order of magnitude greater than that obtaining for the isosteric neutral species. This observation provides further evidence for an anionic binding locus at the active site.

Registry No. AcSCh, 4468-05-7; DBTA, 21770-87-6; PNPA, 830-03-5; PrMSCh, 81352-88-7; acetylcholinesterase, 9000-81-1; glycerol, 56-81-5; sucrose, 57-50-1.

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