A New, Specific and Reversible Bifunctional Alkylborinic Acid Inhibitor of Acetylcholinesterase†

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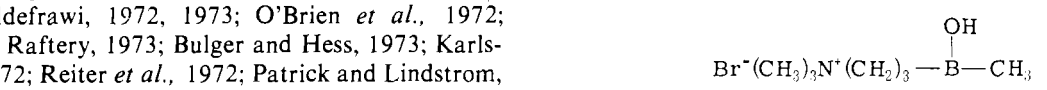
ABSTRACT: A powerful reversible bifunctional inhibitor of acetylcholinesterase has been synthesized. The inhibitor is an alkylborinic acid analog of acetylcholine, *N,N*-trimethylpropylammonium bromide methaneborinic acid. The steady-state kinetic parameters of acetylcholinesterase-catalyzed hydrolysis of acetylcholine in presence of this inhibitor were measured between pH 5.4 and pH 8.5 at 25°. The inhibition is competitive. The extent of inhibition depends on the state of ionization of a group of the enzyme with an apparent pK of ~6.7. Ionization of the borinic acid of the inhibitor interferes with the inhibition reaction and indicates that the acid pH form of the inhibitor binds preferentially to the enzyme. Inhibition of acetylcholinesterase by a number of related inhibitors was also measured for comparison. Preliminary electrophysiological experiments indicate that the inhibitor has an additional advantage over inhibitors used previously in that it interacts very poorly with membrane components which initiate changes in electrical potential of electroplax.

It is well established that acetylcholine initiates changes in electrical potential at nerve synapses and vertebrate neuromuscular junctions, and in the electric organ of certain fish. The structure and function of the membrane component to which acetylcholine binds, the acetylcholine receptor, are being investigated in a number of laboratories (Nachmansohn, 1968; Fulpius *et al.*, 1972; Olsen *et al.*, 1972; Eldefrawi and Eldefrawi, 1972, 1973; O'Brien *et al.*, 1972; Schmidt and Raftery, 1973; Bulger and Hess, 1973; Karlsson *et al.*, 1972; Reiter *et al.*, 1972; Patrick and Lindstrom, 1973; Biesecker, 1973; Klett *et al.*, 1973; Fu *et al.*, 1974). The use of acetylcholine itself in investigations of the membrane component is hampered by the intimate association of the receptor with acetylcholinesterase which catalyzes the hydrolysis of acetylcholine very efficiently (Froede and Wilson, 1971). The use of many irreversible or reversible acetylcholinesterase inhibitors is limited because, at the high concentrations of reagents required, they may react with the membrane component of interest (Bartels and Nachmansohn, 1969).

For these reasons we have searched for, and synthesized, a highly specific, reversible inhibitor which binds considerably better to acetylcholinesterase than to the acetylcholine receptor.

The design of a number of specific enzyme inhibitors has been based on the hypothesis that reactive intermediates, or transition states, of compounds whose reactions are being catalyzed, bind considerably better to enzymes than do the compounds themselves (Pauling, 1948; Lienhard, 1973; Wolfenden, 1972). One such compound is phenylethylboronic acid, a powerful inhibitor of chymotrypsin, prepared by Koehler and Lienhard (1971). They suggested that the γ oxygen of the reactive serine residue of this enzyme forms an adduct with the trigonal boron of the inhibitor in the enzyme complex to form a stable tetrahedral intermediate. Because acetylcholinesterase is a member of the same class of serine proteases as chymotrypsin, and is believed to operate by a similar mechanism (Froede and Wilson, 1971), we synthesized an alkylborinic acid analog (ABA) of acetylcholine, *N,N,N*-trimethylpropylammonium bromide methaneborinic acid



In this paper we report also steady-state kinetic measurements of the acetylcholine-catalyzed hydrolysis of acetylcholine in the presence of the new inhibitor in the range pH 5.4–8.5 at 25°. The steady-state kinetic parameters of some related inhibitors were also measured for comparison. A few preliminary electrophysiological experiments using ABA have also been carried out.[1]

Experimental Section

Materials

Enzyme. Acetylcholinesterase was prepared from electroplax of *Electrophorus electricus* as described by Leuzinger and Baker (1967), and the 35% (v/v) ammonium sulfate precipitate was used in the experiments. Stock solutions of the enzyme were prepared by dialyzing the precipitate against 0.01 M phosphate buffer (pH 7.0) and stored at 4°. Prior to use in the experiments, 0.08 ml of this solution was added to 1 ml of cold 0.1 M sodium chloride solution.

N,N,N-Trimethylammonium pentan-4-one iodide (I) and 4-(*N,N,N*-trimethylammonium)butyramide chloride (II) were gifts of Dr J. Fastrez. *N,N,N*-Trimethylaminopropanesulfonic acid, a gift of Dr J. L. Fu, was recrystallized from ethanol–water and dried in air. 2-Phenylethaneboronic acid was a gift of Dr G. E. Lienhard. Choline-

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[1] We are grateful to Dr. Eva Bartels and Dr. David Nachmansohn, Department of Biochemistry and Neurology, Columbia University, for electrophysiological experiments with the inhibitor and electroplax. At concentrations greater than 2×10^{-4} M, the inhibitor initiates electrical potential changes in electroplax.

methylanesulfonyl chloride was purchased from Nutritional Biochemicals and used without further purification. Acetylcholine bromide was purchased from Eastman Organic Chemicals and was recrystallized from ethanol-water before use.

N,N,N-Trimethylbutylammonium iodide was prepared by reacting methyl iodide and *N,N*-dimethylbutylamine in benzene, collecting the white precipitate, and recrystallizing it from ethyl acetate. Its mass spectrum was consistent with the expected fragmentation of *N,N,N*-trimethylbutylammonium iodide. The uncorrected melting point was 236–238° (lit. 232°). Methaneboronic acid was synthesized using the reaction of methylmagnesium bromide with trimethyl borate by the method of McCusker *et al.* (1957) as described by Nesmeyanov and Sokolik (1967). The resulting boronic acid was recrystallized twice from benzene and ligroin, mp 99° uncorrected (lit. 96–100°), and allowed to dry in the air. *Anal.* Calcd for CH_5BO_2: C, 20.1; H, 8.03; B, 18.1. Found: C, 20.24, 19.95; H, 7.98, 8.08; B, 18.07, 18.06.

The proton magnetic resonance spectrum on an A-60 Varian instrument in D_2O at approximately 40° showed the following signals (expressed in ppm downfield relative to an external tetramethylsilane standard): a singlet at 0.2 (CH_3-B); a singlet at 4.75 (H-O-D).

Potentiometric titration of 0.05 M methaneboronic acid with 1.0 M sodium hydroxide under nitrogen at 23°, using degassed, nitrogen-saturated solutions in the presence of 0.5 M mannitol to lower the apparent pK_a to approximately 7.0 (Steinberg, 1964), yielded an equivalent weight of 59.5 (molecular weight 59.8). Similarly, titration in the absence of mannitol at 25.2°, ionic strength 1.0 maintained with sodium chloride, yielded a pK_a value of 10.5. The base was added with a Hamilton microliter syringe.

N,N,N-Trimethylpropylammonium bromide methaneborinic acid was synthesized as follows. Di-*n*-butyl methaneborate ($CH_3B(O\text{-}n\text{-butyl})_2$) was prepared by the method of Torssel (1954) and Brindley *et al.* (1955); 9.8 g (0.17 mol) of methaneboronic acid and 5 ml of 1-butanol/g of methaneboronic acid were heated at atmospheric pressure and the water–butanol azeotrope was collected. When water production stopped, the product was distilled under reduced pressure (approximately 13 mm). Its boiling point and nuclear magnetic resonance (nmr) spectrum were fully consistent with the di-*n*-butyl ester of methaneboronic acid.

N,N-Dimethylaminopropyl Chloride. *N,N*-Dimethylaminopropyl chloride hydrochloride was treated with slightly less than 1 equiv of sodium hydroxide and extracted with diethyl ether. Subsequent slow distillation of ether, followed by distillation at reduced pressure, yielded *N,N*-dimethylaminopropyl chloride which was stored in the cold and used within 1 day.

The magnesium Grignard of the amine chloride was prepared under nitrogen in reagent grade tetrahydrofuran which had been dried by distillation from preformed methylmagnesium chloride directly into the reaction vessel; 0.11 mol of Grignard was prepared in about 150 ml of tetrahydrofuran from 13.4 g of *N,N*-dimethylaminopropyl chloride; 16.4 g (0.1 mol) of di-*n*-butyl methaneborate was added to approximately 100 ml of freshly distilled tetrahydrofuran, and the 150 ml of *N,N*-dimethylaminopropylmagnesium chloride was added slowly under a stream of nitrogen, with vigorous stirring, to the borate. During this addition the reaction vessel was cooled in a Dry Ice–acetone bath. The vessel was then allowed to reach room temperature overnight. Stirring and a slow stream of nitrogen were maintained. The reaction mixture was heated briefly to drive any alkylboron compounds which may have formed into a Dry Ice trap, and the Grignard mixture was hydrolyzed by addition of saturated ammonium chloride solution (Fieser and Fieser, 1967), extracted with diethyl ether, dried over magnesium sulfate, and its volume reduced by distillation. Excess methyl bromide was distilled into the cooled distillation vessel, and the precipitate was filtered and dried in the air. Two recrystallizations from benzene–acetonitrile yielded white plate-like crystals, mp 155°. *Anal.* Calcd for $C_7H_{19}NOBr$: C, 37.6; H, 8.52; N, 6.30; B, 4.83; Br, 35.7. Found: C, 37.56; H, 8.59; N, 6.39; B, 4.97; Br, 35.6.

The pmr (D_2O) spectrum and its integration lead to the following assignments (expressed in ppm downfield from an external tetramethylsilane standard): singlet 0.2 (CH_3B), multiplet 0.7 (CH_2B); multiplet 1.8 ($-CH_2-$); singlet 3.0 ($-^+N(CH_3)_3$); multiplet 3.2 (CH_2N^+); singlet 4.6 (H–O–D).

Titration of 0.0272 g of *N,N,N*-trimethylpropylammonium bromide methaneborinic acid with 1 N sodium hydroxide, ionic strength 0.5 maintained with added 2 M sodium chloride, at 25.2° in a 10 ml volume under nitrogen, yielded a pK_a value of 8.4, and an equivalent weight of 243 (molecular weight 224), as is shown in Figure 1b.

Sodium hydroxide solutions and standard buffers were either made up from BD ampoules or were Fisher Certified solutions. Reagent grade salts were used without further purification. Distilled water was used throughout. Microanalyses were performed by Schwartzkopf Microanalytical Laboratory Inc.

Methods

Instruments. A Cary Model 14 recording spectrophotometer was used to determine the formation of complexes. A Radiometer pH meter, Type TTT1, was used for the pH-Stat steady-state kinetic experiments. It was standardized with an appropriate standard buffer whose pH was within 2 pH units of that of the samples. Between each run the electrode and the reaction vessel were rinsed with 0.01 M hydrochloric acid in order to inactivate any enzyme remaining, and were then rinsed several times with distilled water.

A Corning Model 12 research pH meter equipped with a scale expander was used to determine the titration curves. The meter was standardized at pH 7 and pH 10 at 25° with appropriate buffers.

Procedures. The initial rate of acetylcholinesterase-catalyzed hydrolysis of acetylcholine bromide was measured by the consumption of sodium hydroxide in the pH-Stat. All rates were determined at 25.0–25.2° under nitrogen with 15-ml reaction mixtures containing 0.2 M sodium chloride and 10^{-3} or 5×10^{-3} M sodium phosphate. Each kinetic run was organized as follows. A solution of buffer, substrate, and inhibitor, when present, was incubated in the thermostated reaction vessel at 25°. The reaction was initiated by the addition of 20 μl of diluted enzyme stock solution, and the uptake of 5×10^{-3} M sodium hydroxide, with time, was recorded. Over the pH range investigated, there was no significant background "uncatalyzed" hydrolysis of acetylcholine bromide.

At each pH value and with each inhibitor, sets of initial rate measurements were made at four to six substrate concentrations, in the absence of an inhibitor, and in the pres-

ence of one or more constant concentrations of inhibitor. The substrate concentrations varied from a value of about one-third that of the K_m at the ionic strength employed to a value of about three times that of the K_m (1.4×10^{-4} M). Acetylcholine bromide stock solutions (10^{-2} M) were prepared fresh daily immediately prior to use.

The activity of the diluted enzyme stock did not change significantly during the time these experiments were carried out. The following arbitrary standard assay was carried out periodically; 20 μl of diluted enzyme stock solution were added, at pH 7.8, to 10 ml of a solution containing 2.5×10^{-4} M sodium phosphate, 0.2 M sodium chloride, and 10^{-3} M acetylcholine bromide. The latter concentration is about five times the K_m of the catalytic reaction (Table I). Typically, 0.123 ml of 5×10^{-3} M sodium hydroxide consumed per minute was the rate observed when less than 10% of the substrate was consumed. In order to compare steady-state kinetic data obtained with different enzyme preparations, all velocity measurements were normalized using this reaction velocity. This normalization procedure assumes that v is directly proportional to the enzyme concentration. An approximation of the concentration of enzyme in such a preparation can be made from the data of Leuzinger and Baker (1967).

Values of the dissociation constant of the enzyme–inhibitor complex, K_I, were derived from $1/v$ *vs.* $1/[S]$ plots using the equation

$$K_I = [I]\text{slope}_0/(\text{slope}_I - \text{slope}_0) \qquad (1)$$

in which slope_0 is the slope of the plot when no inhibitor is present, and slope_I is the slope of the plot for each set with inhibitor present.

Values of K_{app} for the formation of a complex between chromotropic acid and $CH_3B(OH)_2$ were determined spectrophotometrically. Varying concentrations of methaneboronic acid were added to a series of solutions containing 0.4 M sodium acetate (pH 7.0) and 8×10^{-5} M freshly prepared chromotropic acid disodium salt, ionic strength 0.5 maintained with added sodium chloride.

The range of methylboronic acid concentrations used was 4×10^{-5} to 5×10^{-3} M. The uv spectra were determined on the spectrophotometer at room temperature (approximately 23°). Three distinct isosbestic points at 3530, 3490, and 3380 Å were observed. A plot of $1/\Delta A$ at 3620 Å *vs.* $1/[CH_3B(OH)_2]_{total}$, where $1/\Delta A$ is the reciprocal of the difference between the absorbance of chromotropic acid alone and the absorbance of chromotropic acid in the presence of methaneboronic acid at 3620 Å, is linear with a slope of 8.66×10^{-4} and a $1/\Delta A$ intercept at 1.14 OD units^{-1}.

Results

The steady-state kinetic measurements of acetylcholinesterase-catalyzed hydrolysis of acetylcholine bromide in the presence and absence of inhibitors at pH 7.5 and 25° are tabulated in Table I, in which are given the coordinates of lines calculated from the rate data by means of a digital computer program written for the Lineweaver–Burk (1934) form of the Michaelis–Menten rate equation. Data weighting was performed as discussed by Wilkinson (1961). The table gives the slopes of the lines, which are proportional to the steady-state kinetic parameter, K_m/V_{max}. The table also gives the ordinate intercept which is proportional to $1/V_{max}$ and the abscissa intercept which is proportional to $1/K_m$ when no inhibitor is present, and to $1/K_m[1 + (I_0/K_I)]^{-1}$ in presence of a competitive inhibitor.

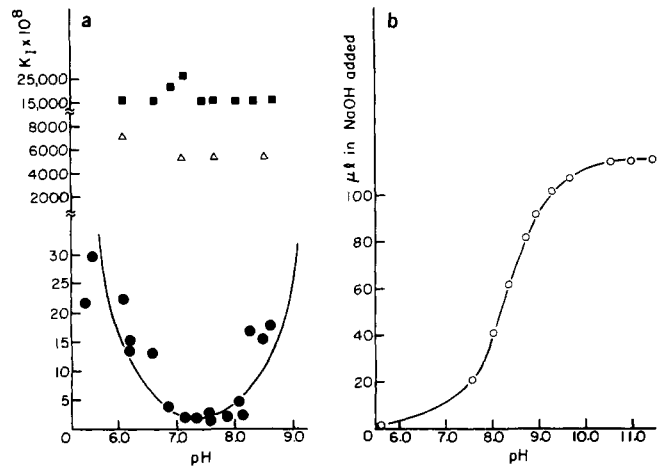


FIGURE 1: (a) Semilogarithmic plot of $K_{I(obsd)}$ *vs.* pH for the inhibition of acetylcholinesterase-catalyzed hydrolysis of acetylcholine bromide by *N,N,N*-trimethylpropylammonium bromide methaneborinic acid (ABA). The solid lines have been calculated from the derived parameters (Table I). For comparison, the K_I values of the carbon analog of ABA, compound I, in the pH region investigated, are also shown, Δ. The K_m value for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine is indicated by ■. All experiments were performed at 25°, 0.2 M NaCl. (b) Titration curve for *N,N,N*-trimethylpropylammonium bromide methaneborinic acid (1.21×10^{-2} M) at 25° with 1 N sodium hydroxide, the ionic strength of 0.5 being maintained with 2 M sodium chloride solution under nitrogen. The curve is corrected for solvent titration by subtracting values obtained for an equivalent titration but without substrate. Error due to ignoring correction for the volume of titrant added is <1% at the highest volume of titrant added.

It can be seen from the table that the ordinate intercepts are the same, within experimental error, in the presence and absence of inhibitor (ABA). The data in Table I indicate that in the presence of ABA the slope of the line increases. Since the V_{max} values are constant, the $K_{m(obsd)}$ values of the catalytic reaction are increased. Identical V_{max} values, but different $K_{m(obsd)}$ values in the presence of ABA indicate competitive inhibition between substrate and inhibitor. Similar results were obtained throughout the pH range investigated, pH 5.4–pH 8.5. The dissociation constant, K_I, of the inhibitor, at various pH values is also listed in Table I. It should be noticed from the data in Table I and Figure 1a that K_I is pH dependent, and the affinity of the inhibitor for the enzyme increases from pH 5.5 to 7.5 and then decreases. The data in Table I and Figure 1a indicate that the apparent K_m of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine is essentially independent of pH in the range pH 7.5–8.5.

A minimum mechanism which accounts for these data is shown in Scheme I. According to this mechanism only the

Scheme I

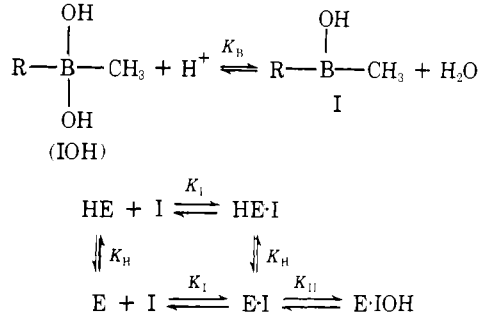


TABLE I: Constants for the Inhibition of Acetylcholinesterase-Catalyzed Hydrolysis of Acetylcholine Bromide by Quaternary Ammonium and Boron-Containing Inhibitors, 25°, 0.2 M NaCl.

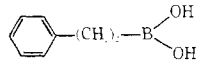
Inhibitor	[I] (M)	pH	K_I (M)	Slope ($\times 10^{-5}$)	Parameters of a 1/[S] *vs.* 1/*v* Plot in the Absence of Inhibitor: Ordinate (*y*) Intercept	Parameters of a 1/[S] *vs.* 1/*v* Plot in the Absence of Inhibitor: Abscissa (*x*) Intercept ($\times 10^4$)
$(H_3C)_3{}^+N—(CH_2)_3\ SO_3^-$	0.1	5.5	9.3×10^{-2}	1.0	0.06	−0.63
Cholinesulfonate	0.1	7.4	7.4×10^{-2}	1.1	0.07	−0.72
	0.1	8.5	9.5×10^{-2}	1.0	0.06	−0.65
$(H_3C)_3{}^+N—(CH_2)_2OSO_2CH_3\ Cl^-$	10^{-3}	7.5	2.1×10^{-5}	1.0×10^{-5}	0.07	−0.72
Methanesulfonylcholine chloride						
O	10^{-3}	6.0	6.9×10^{-5}	3.1×10^{-5}	0.2	−0.7
‖	10^{-3}	7.0	4.9×10^{-5}	1.2×10^{-5}	0.09	−0.7
$(H_3C)_3{}^+N—(CH_2)_3—C—CH_3\ I^-$	10^{-3}	7.5	5.1×10^{-5}	1.0×10^{-5}	0.07	−0.7
N,N,N-Trimethylammonium-pentan-4-one iodide (I)	10^{-3}	8.4	5.1×10^{-5}	1.0×10^{-5}	0.07	−0.07
CH_3	10^{-6}	5.40	2.1×10^{-7}	4.8×10^{-6}	0.03	−0.68
|	10^{-6}	5.5	2.9×10^{-7}	5.8×10^{-5}	0.42	−0.72
$H_3C—{}^+N—(CH_2)_3B(OH)CH_3\ Br^-$	10^{-8}	6.0	2.2×10^{-7}	2.9×10^{-5}	0.20	−0.70
|	$10^{-6} \times 5$	6.1	1.4×10^{-7}			
CH_3	10^{-7}	6.5	1.6×10^{-7}	1.8×10^{-5}	0.13	−0.70
N,N,N-Trimethylpropylammonium bromide methaneborinic acid	10^{-8}	6.5	1.3×10^{-7}	1.70×10^{-5}	0.10	−0.60
	$10^{-7} \times 5$	6.8	1.1×10^{-7}	1.5×10^{-5}	0.09	−0.61
	10^{-7}	6.80	4.1×10^{-8}	1.70×10^{-5}	0.08	−0.52
	$10^{-7} \times 5$	7.10	1.6×10^{-8}	1.3×10^{-5}	0.08	−0.66
	$10^{-6} \times 2$					
	10^{-7}	7.30	1.5×10^{-8}	1.1×10^{-5}	0.08	−0.70
	$10^{-7} \times 2$	7.50	2.4×10^{-8}	1.0×10^{-5}	0.07	−0.68
	0	7.5		1.6	0.07	−0.4
	10^{-8}	7.5	2.7×10^{-8}	2.8		
	$10^{-8} \times 2.3$	7.5	2.6×10^{-8}	4.7		
	$10^{-7} \times 5.0$	7.5	3.0×10^{-8}	7.7		
	$10^{-7} \times 1$	7.5	3.2×10^{-8}	14.0		
	$10^{-7} \times 2$	7.80	1.8×10^{-8}	1.0×10^{-5}	0.07	−0.70
	$10^{-7} \times 2.5$	8.00	4.3×10^{-8}	1.1×10^{-5}	0.06	
	10^{-7}	8.1	2.3×10^{-8}	9×10^{-6}	0.07	−0.73
	$10^{-7} \times 5$	8.20	1.7×10^{-7}	1.0×10^{-5}	0.07	−0.68
	$10^{-7} \times 1.5$	8.4	1.6×10^{-7}	0.9×10^{-5}	0.07	−0.7
	10^{-6}	8.50	1.8×10^{-7}	1.2×10^{-5}	0.07	−0.68
$(H_3C)_3{}^+N—(CH_2)_2—OH\ Cl^-$	$10^{-3} \times 5$	7.5	4×10^{-4}	2.1	0.14	−0.69
Choline chloride						
$(CH_3)_3{}^+N—(CH_2)_3—CH_3\ I^-$	$10^{-3} \times 5$	7.5	1.1×10^{-4}	1.1	0.07	−0.69
N,N,N-Trimethylbutylammonium iodide						
$(CH_3)_3{}^+N—CH_3\ I^-$	10^{-3}	7.5	1.1×10^{-3}	1.4	0.07	−0.54
Tetramethylammonium iodide	$10^{-3} \times 2$	7.5	1.0×10^{-3}	1.4	0.07	−0.54
$CH_3—B(OH)_2$	$10^{-3} \times 5$	7.5	3.2×10^{-3}	2.1	0.12	−0.58
Methaneboronic acid						
	10^{-4}	7.5	3.7×10^{-5}	1.6	0.11	−0.65
	10^{-3}	7.5	2.5×10^{-5}	1.6	0.11	−0.65
	10^{-4}	5.5	1×10^{-4}	4.6	0.32	−0.7
2-Phenylethaneboronic acid	$10^{-4} \times 5$	8.3	1.9×10^{-5}	1.1	0.07	−0.7
O						
‖	10^{-3}	7.5	3.1×10^{-3}	1.6	0.11	−0.7
$(H_3C)_3{}^+N—(CH_2)_3—C—NH_2\ Cl^-$	10^{-2}	7.5	1.9×10^{-3}	1.6	0.11	−0.7
(*N,N,N*-Trimethylammonium)-butyramide chloride (II)						

TABLE II: Values for the Competitive Inhibition of the Acetylcholinesterase-Catalyzed Hydrolysis of Acetylcholine Bromide by Mixtures of Inhibitors, at pH 7.50, 25°.[a]

Inhibitor	[Inhibitor] (M)	$Slope_0$ ($\times 10^{-5}$)	$Slope_I$ ($\times 10^{-5}$)	$Slope_{both}$ ($\times 10^{-5}$)	$Slope_{calcd}$ ($\times 10^{-5}$)	$Slope_{obsd}/Slope_{calcd}$
$(CH_3)_4N^+ I^-$	2×10^{-3}	1.4	4.0	7.1	5.6	1.3
$CH_3B(OH)_2$	5×10^{-3}	1.4	2.9			
$(CH_3)_3N^+(CH_2)_3CH_3 I^-$	5×10^{-4}	1.1		7.9	7.5	1.1
$CH_3B(OH)_2$	5×10^{-3}	1.1	3.0			
$(CH_3)_3N^+(CH_2)_3 I^-$	5×10^{-3}	1.1	0.5	0.8	0.5	1.6
$CH_3B(OH)_2$	5×10^{-3}	1.1	3.1			
$(CH_3)_3N^+(CH_2)_3CONH_2 I^-$	5×10^{-3}	1.7	6.7	0.35	0.3	1.2
$(CH_3)_3N^+(CH_2)_2OH Cl^-$	5×10^{-3}	1.7				
$C_6H_5(CH_2)_2B(OH)_2$	5×10^{-4}	1.8		0.5	0.3	1.9
$(CH_3)_3N^+(CH_2)_2OH Cl^-$	10^{-3}	1.8				
$(CH_3)_3N^+(CH_2)OH Cl^-$	5×10^{-3}	2.1	0.3	0.46	0.3	1.4
$CH_3B(OH)_2$	5×10^{-3}	2.1	0.02			
$(CH_3)_3N^+(CH_2)_2OH$	5×10^{-3}	1.9	0.2	1.0	0.5	2.2
$CH_3B(OH)_2$	2.4×10^{-3}	1.9	0.15			

[a] Any given set of experiments was run on an identical enzyme preparation. $Slope_0$ = slope of line in 1/[S] *vs.* 1/*v* plot in absence of inhibitor; $slope_I$ = slope of line in the presence of each separate inhibitor; $slope_{both}$ = slope of line in the presence of both inhibitors; $slope_{calcd}$ = slope of line calculated on assumption that the competitive inhibitors do not interact.

un-ionized form, I, of the inhibitor binds to the enzyme. K_B represents the apparent acid dissociation constant of the inhibitor. Furthermore, binding to acetylcholinesterase depends on the ionization of an amino acid residue of the enzyme. The form of the enzyme in which this particular residue is unprotonated, E, forms a final, stable inhibitor complex, E · IOH, which represents a stable complex between the boron and an amino acid residue in the active site of the enzyme involving an intramolecular proton transfer. The enzyme configuration in which the residue is protonated, HE, does not form such a complex. K_H is the acid dissociation constant of this ionizing group in the free enzyme and of the enzyme-inhibitor complex. K_I is the dissociation constant of the enzyme-inhibitor complexes, E · I and HE · I. K_{II} is the equilibrium constant for the conversion of EI to E · IOH.

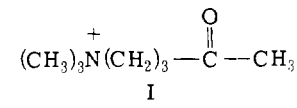
When the initial inhibitor concentration is much greater than the initial enzyme concentration I_0, the pH dependence of $K_{I(obsd)}$ is given by

$$K_{I(obsd)} = \left(K_I K_{II} \frac{[H^+] + K_B}{[H^+]}\right)\left(K_{II} + \frac{K_H}{K_H + [H^+]}\right)^{-1} \quad (2)$$

At low pH when $[H^+] \gg K_B$ and K_H, and $K_{II} > K_H/[H^+]$, $K_{I(obsd)} = K_I$.

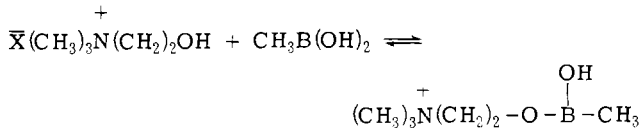
The observed pH dependence of $K_{I(obsd)}$ is adequately accounted for by the following parameters: $K_I K_{II} = 2.8 \times 10^{-8}$, $pK_H = 6.7$, and $pK_B = 8.2$. The solid line in Figure 1a was computed using these parameters. The titration curve of the inhibitor at 25.2°, 0.5 ionic strength, is shown in Figure 1b. The apparent dissociation constant, $pK_B(app)$, is 8.4 under these conditions.

A priori the pH dependence of $K_{I(obsd)}$ below pH 7.0 can be explained by a simpler model in which the inhibitor binds only to E and not to HE. The data with the carbon analog of ABA, compound I, do not favor this model. The data in Figure 1a and Table I indicate that compound I inhibits the enzyme strongly, but by a factor of 10^3 less than ABA. The dissociation constant of this inhibitor is independent of pH between pH 5.5 and pH 8.5. The values of K_I for a number of other inhibitors are also listed in Table I for comparison.



I

Attempts were made to generate the ester between choline and methaneboronic acid. The formation of this ester



can be observed by measuring the decrease in absorbance of the chromotropic-boronic acid complex (see Experimental Section). At pH 7.0, using up to 0.1 M choline chloride and 4×10^{-3} M methaneboronic acid in the presence of 8×10^{-4} M chromotropic acid, at an ionic strength of 1.0 M (maintained with added sodium chloride), no significant change in absorbance was observed. Controls were run for blank absorbance by 0.1 M choline and 4×10^{-4} M methaneboronic acid over the range 4000–32000 Å. Assuming that we could see only a 20% decrease in free methaneboronic acid the experiments of Koehler *et al.* (1972) suggest that the association constant for ester formation from choline and methaneboronic acid is less than 2.

The possibility of using the choline–methaneboronic acid ester as an inhibitor of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine was explored. The steady-state kinetic measurements of the enzyme-catalyzed hydrolysis of acetylcholine at pH 7.5 and 25° in the presence of methaneboronic acid, choline chloride, and equimolar mixtures of methaneboronic acid and choline chloride are presented in Table II. The combination of compounds inhibits the enzyme about twice as well as calculated, indicating that the ester is a better inhibitor than choline chloride or methaneboronic acid alone. Table II includes values for competitive inhibition of the acetylcholinesterase-catalyzed hydrolysis

of acetylcholine by mixtures of alcohols and boronic acids to serve as comparison.

Discussion

The extent of the inhibition of ABA depends on the state of ionization of a group on the enzyme with an apparent pK of 6.7, and on the ionization of the inhibitor itself with apparent pK of 8.2 (Figure 1a). The effect of hydrogen ions on the inhibition of acetylcholinesterase by ABA at low pH is similar to the effect on the inhibition of chymotrypsin by phenylethylboronic acid, and on the rate of reactions catalyzed by acetylcholinesterase and other serine proteases. In many serine proteases this ionizing group has been identified as a histidine residue in the active site of the enzyme. Its unprotonated state yields both maximum catalytic efficiency and, in our experiments, optimum binding of ABA.

Above neutral pH the effect of hydrogen ions on the binding of ABA to acetylcholinesterase is different from that observed with phenylethylboronic acid and α-chymotrypsin.

Unlike α-chymotrypsin (Hess, 1971), acetylcholinesterase does not undergo pH-dependent conformational change in the pH range investigated. Unlike 2-phenylethaneboronic acid of pK_a 10.0 (Koehler and Lienhard, 1971), ABA has a pK_a of 8.4 (Figure 1b). We conclude that the second ionizing group which affects the binding of ABA is the borinic acid itself. This leads to the interesting conclusion that the tetrahedral adduct of the inhibitor with hydroxide ion does not bind to the catalytic site of the enzyme. This may be due to the geometry of the binding site of acetylcholinesterase. The known crystal structure of the active site region of another serine protease, α-chymotrypsin, indicates no obvious impediment to the binding of tetrahedral compounds (Matthews *et al.*, 1967; Henderson, 1970). Another explanation of this observation, consistent with suggestions made by Koehler and Lienhard (1972) for the inhibition of chymotrypsin by 2-phenylethaneboronic acid, is that the boron must be in trigonal form so that it can form a stable tetrahedral adduct with the γ oxygen of a serine residue in the active site of the enzyme. Direct evidence supporting this suggestion has recently been obtained using laser Raman spectroscopy (Hess *et al.*, 1975).

The derivative synthesized and used in the present studies is the most powerful bifunctional reversible inhibitor known. It has the added advantage over inhibitors used previously that it interacts only very poorly with membrane components which initiate changes in electrical potential allowing suppression of enzyme activity by a factor of 10^4. One of the powerful and frequently used reversible inhibitors (Table I), 3-hydroxyphenyltrimethylammonium bromide (Wilson and Quan, 1958), binds by a factor of 10 less well to the enzyme than ABA does, and perturbs the membrane potential of electroplax at 10 times lower concentrations (Podleski, 1967), allowing suppression of enzyme activity in membrane studies by a factor of only 10^2.

Combinations of various alcohols of quaternary alkylamines with boronic acids used to inhibit the enzyme did not give clear-cut results because of the unfavorable equilibrium in the presence of water. The data in Table II indicate that these compounds do not act as ideally noninteracting inhibitors. However, the results with a combination of choline bromide and methaneboronic acid suggest that ester formation may occur and that the ester is a better inhibitor than the alcohol or acid alone.

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