Effects of Quaternary Ligands on the Inhibition of Acetylcholinesterase by Arsenite†

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ABSTRACT: Arsenite inhibits acetylcholinesterase in a second-order reaction. The rate and equilibrium constants depend upon pH and have values on the order of 10^2 M^{-1} min^{-1} and 10^{-5} M (dissociation), respectively. Some quaternary ammonium ligands completely block the arsenite inhibition of the enzyme, others decrease the rate of the reaction and some, notably pyridine-2-aldoxime methiodide, greatly accelerate the rate of the reaction, up to 220-fold. Accelerators may bind at a separate enzyme site distinct from the anionic site involved in substrate binding. Although the kinetic data are consistent with a covalent reaction between arsenite and acetylcholinesterase, chemical evidence excludes the involvement of sulfhydryl groups which are usually implicated in arsenite inhibition.

A sizeable number of enzymes are inhibited by arsenite[1] (Boyer et al., 1963), and evidence has been presented that the mechanism of inhibition involves reaction of the arsenite with neighboring thiol groups to form cyclic thioarsenite diesters (Peters, 1949). Acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7; AcChE)[2] does not display the characteristic sensitivity of sulfhydryl enzymes to organic mercury compounds and alkylating agents (Mounter and Whittaker, 1953; Karlin, 1967), nor does the purified enzyme from electric eel contain free sulfhydryl groups (Rosenberry, 1975). AcChE is, nevertheless, readily inhibited by arsenite (Thompson, 1947; Mounter and Whittaker, 1953). In the following, we present evidence that arsenite reversibly inhibits AcChE with second-order kinetics and that the reverse reaction is very slow. Quaternary ligands are known to modulate the effect of acylating agents and other chemical agents on AcChE, and in some cases markedly accelerate the reaction (Kitz and Wilson, 1963; Metzger and Wilson, 1963; Roufogalis and Thomas, 1969; Kitz et al., 1970; Fuchs et al., 1974). We demonstrate that, while some quaternary ligands block the inhibition of AcChE by arsenite, others, notably pyridine-2-aldoxime methiodide (2-PAM), strongly accelerate both the onset of inhibition and its reversal. We also present data indicating that disulfide and sulfhydryl functions are most probably not involved in inhibition of AcChE by arsenite.

Experimental Section

Materials. Dithiothreitol (DTT), *N*-ethylmaleimide, acetylthiocholine iodide (AcThCh), acetylcholine chloride (AcCh), 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), pyridine-2-aldoxime methiodide (2-PAM), *p*-chloromercurisulfonate (PCMS), and Tris-HCl (Trizma) were obtained from Sigma Chemical Co. (St. Louis, Mo.); toxogonin (bis(4-hydroxyiminomethylpyridinium-1-methyl) ether dichloride), iodine, and sodium arsenite were from E. Merck (Darmstadt, W. Germany), tetramethylammonium bromide (TMA) and EDTA were from Fluka, A.G. (Buchs, Switzerland), propidium diiodide (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridium diiodide) was from Calbiochem (Los Angeles, Calif.), and Tensilon (3-hydroxyphenyldiethylmethylammonium bromide) was from Roche Laboratories (Nutley, N.J.). Phenyltrimethylammonium iodide (PTMA), obtained from BDH Chemicals (Poole, England) was recrystallized from ethanol before use. *N*-Methyl-7-dimethylcarbamoxyquinolinium iodide (M7C) was prepared according to Rosenberry and Bernhard (1971).

Pyridine methiodide (PMI) was prepared by stirring pyridine with a slight excess of methyl iodide in anhydrous acetone overnight at room temperature. The pyridine methiodide which precipitated was recrystallized from 2-propanol. Yield 85%, mp 111–112 °C. Anal. Calcd: I, 57.5; N, 6.3. Found: I, 56.5; N, 6.3.

Acetylcholinesterase (AcChE) was the 11S species from the electric organ of the electric eel, *Electrophorus electricus*, purified to a specific activity above 6000 units/mg of protein by affinity chromatography as described by Dudai et al. (1972). One unit is the amount of AcChE hydrolyzing 1 μmol of AcCh per min under the assay conditions of the above authors.

Buffers. Phosphate buffers were prepared by the addition of appropriate quantities of 1 M HCl and 0.5 M NaCl to 0.05 M Na_2HPO_4. Tris buffers were prepared by adding 2 M NaOH to 0.125 M Tris-HCl. To achieve lower buffer concentrations, both buffers were diluted with 0.125 M NaCl. All buffers contained 0.1 mg of gelatin/mL and 10^{-5} M EDTA.

Acetylcholinesterase Determinations. Enzyme activity was assayed by the method of Ellman et al. (1961), in phosphate buffer, pH 7.4, $\mu = 0.125$, containing 1×10^{-4} M Nbs_2, at 22 °C, using 3×10^{-4} M acetylthiocholine as substrate. The reaction was followed at 412 nm in a Gilford 2400-S recording spectrophotometer.

Enzymatic activity on acetylcholine was measured titrimetrically, according to Dudai et al. (1972), and on indophenyl

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[1] Although arsenite exists mainly as undissociated arsenious acid ($pK_a = 9.2$) below pH 9.0, it is customary to refer to the inhibition as arsenite inhibition.

[2] Abbreviations used are: AcChE, acetylcholinesterase; 2-PAM, pyridine-2-aldoxime methiodide; DTT, dithiothreitol; AcThCh, acetylthiocholine; AcCh, acetylcholine; PCMS, *p*-chloromercurisulfonate; TMA, tetramethylammonium; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); PTMA, phenyltrimethylammonium; PMI, pyridine methiodide; M7C, *N*-methyl-7-dimethylcarbamoxyquinolinium iodide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

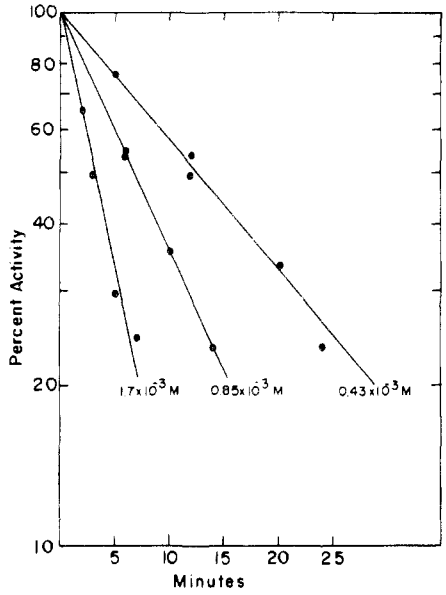

FIGURE 1: Rate of inhibition of AcChE by arsenite. Inhibition was performed on solutions of AcChE in 0.05 M phosphate, pH 7.4, $\mu = 0.125$, at 22 °C, by addition of arsenite to final concentrations of 1.7×10^{-3}, 0.85×10^{-3}, and 0.425×10^{-3} M. The percentage of enzymatic activity remaining is plotted on a logarithmic scale as a function of time after addition of the arsenite.

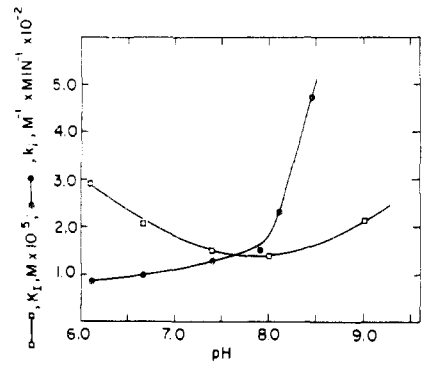

FIGURE 2: pH-dependence of inhibition of AcChE by arsenite. The second-order rate constant, k_i (M^{-1} min^{-1}), for inhibition of AcChE by arsenite is plotted as a function of pH in 0.05 M phosphate, $\mu = 0.125$, at 22 °C. The dissociation constant, K_i(M), plotted as a function of pH, was measured in the presence of 10^{-3} M 2-PAM in the same phosphate buffer, except at pH 9.0 where 0.125 M Tris, $\mu = 0.125$, was employed.

acetate spectrophotometrically, according to Kramer and Gamson (1958), by following the increase in absorbance at 625 nm.

Assay of Chymotrypsin. Enzymatic activity of chymotrypsin was monitored spectrophotometrically by following the increase in absorption at 400 nm on hydrolysis of the synthetic substrate succinyldiglycyl-L-phenylalanyl-*p*-nitroanilide, which was a gift from Dr. Shmaryahu Blumberg. The assay was performed in 0.5 M KCl-0.01 M Tris, pH 8.0, at 22 °C.

Arsenite Inhibition Experiments. Enzyme solutions containing 2–10 units/mL of AcChE in the desired buffer were incubated with the appropriate concentrations of arsenite, with and without quaternary ligands, for varying times at 22 °C. Small aliquots (5–20 μL containing 0.01–0.05 unit of AcChE activity) were added to 2–3 mL of the assay buffer, and the change in absorbance at 412 nm was recorded. Although the reaction with arsenite was terminated by the large dilution, no reversal of inhibition occurred during the assay because the reverse reaction is very slow (see Results). Interference with the assay by the added quaternary ligand was precluded by the large dilution, together with the presence of a relatively high substrate concentration in the assay mixture.

Because arsenite is a rather potent inhibitor, low concentrations must be employed for equilibrium measurements of enzyme activity. At such concentrations, the rate of inhibition is so slow that several days would be required to approach equilibrium. Equilibrium measurements were, therefore, performed in the presence of 2-PAM, thus taking advantage of our observation (see Results) that this ligand greatly accelerates the reaction of AcChE and arsenite without changing the position of equilibrium.

Fluorescence Measurements. The reaction of AcChE with M7C, as measured by appearance of the fluorescent product 7-hydroxy-*N*-methylquinolinium, was followed in a Perkin-Elmer Model MPF-3L recording fluorescence spectrophotometer, according to the procedure of Rosenberry and Bernhard (1971).

Results

On addition of arsenite to a solution of AcChE a decrease in enzyme activity with time is observed which follows pseudo-first-order kinetics at a fixed arsenite concentration (Figure 1). The reaction is seen to be second order, since the half-times for the reaction are inversely proportional to the arsenite concentration. Because the reaction does not go to completion, but approaches a reversible equilibrium (Figure 2), this effect must be taken into account in plotting the data obtained with lower concentrations of arsenite than those used in the experiments shown in Figure 1. The appropriate equation is:

$$\left(1 - \frac{E_e}{E^0}\right) \ln \left(\frac{E - E_e}{E^0 - E_e}\right) = -k_i I t \qquad (1)$$

where E is the enzyme activity at time t, E_e is the enzyme activity at equilibrium, E^0 is the initial enzyme activity, I is the concentration of inhibitor (arsenite), and k_i is the second-order rate constant.

Since the second-order rate constant increases only slowly on raising the pH from 6 to 8 (Figure 2), it appears that undissociated arsenious acid rather than arsenite is the kinetically active species in this pH range. Above pH 8, the rate rises rapidly. The reason for this rapid rise is not known, but may reflect an increasing role of arsenite anion as an actual reactant in addition to undissociated arsenious acid.

When measurements were made in Tris buffer at pH 8.0 and 8.45, instead of in phosphate buffer, the rate of inhibition was found to be greatly enhanced and to depend upon the concentration of Tris. The addition of phosphate to the Tris buffer did not affect the rate of arsenite inhibition, and in phosphate buffer alone, at pH 8.0, the rate was found to be unaffected by the concentration of phosphate. Thus, the increased rate of inhibition in Tris must be caused by a positive effect of Tris on the rate of arsenite inhibition.

The equilibrium constant (K_I) which we have evaluated for the inhibition of AcChE by arsenite is the analytical equilibrium constant which is calculated without regard to the state of ionization of arsenite or of the enzyme and is, therefore, expected to be pH dependent. However, the observed equilibrium constant does not vary greatly with pH and changes only by a factor of little more than two in the pH range 6–9 (Figure 2). A change in K_I above pH 8 might be expected to result from the ionization of arsenious acid to arsenite; the observed increase in K_I may indicate that arsenite has less

affinity for AcChE than arsenious acid. However, both the increase in K_I at alkaline pH, as well as the increase at acid pH, may reflect changes in the enzyme itself. The value of the equilibrium constant, 1.5×10^{-5} M at pH 7.4, is lower than the concentrations of arsenite needed to inhibit various aldehyde dehydrogenases by 50% (Jakoby, 1963) and is similar to the concentrations reported for the lipoic acid containing enzyme pyruvate oxidase (Gunsalus, 1953).

The relative activity of the inhibited enzyme was independent of the substrate concentration used in the assay. This is expected, because reversal of inhibition is so slow (even in the presence of substrate, as shown below) that dilution of the inhibited enzyme freezes the extent of the reaction with arsenite. Thus, under our assay conditions arsenite behaves as if it were an irreversible inhibitor. The rate constant for reversal of inhibition calculated from the equilibrium constant and the rate constant for inhibition is about 1.9×10^{-3} min^{-1}. In one trial, we observed 1.1×10^{-3} min^{-1}.

Enzymatic activity was rapidly restored by diluting AcChE, inhibited with 2×10^{-3} M arsenite, 100-fold into a 4×10^{-5} M iodine solution. Iodine slowly inhibits the enzyme at this concentration, but, by assaying samples at 1-min intervals or by addition of thiosulfate to destroy excess iodine, we obtained nearly complete recovery of the enzyme in 5–10 min (Figure 3). Iodine must oxidize not only the free arsenite but also the enzyme-bound arsenite to explain the rapid recovery of enzyme in these experiments. Similarly, by the addition of 10^{-3} M DTT, slower, but still rapid, recovery of enzyme activity was achieved.

Substances are known which react with AcChE in such a way that they inhibit its activity towards AcCh and AcThCh, yet enhance or do not affect its activity toward the poor substrate indophenyl acetate (Purdie and McIvor, 1966; Wofsy and Michaeli, 1967; Changeux et al., 1969; Fuchs et al., 1974). In one trial, we found that arsenite inhibited the action of AcChE on indophenyl acetate to the same extent as on AcThCh.

AcChE reacts readily with the carbamylating agent *N*-methyl-7-dimethylcarbamoxyquinolinium iodide (M7C) (Kitz et al., 1967) to form a carbamyl enzyme derivative that is analogous to the normal acetyl enzyme formed as an intermediate during the hydrolysis of substrates. This reaction can be followed either by monitoring inhibition of the enzyme (Kitz et al., 1967) or by observing the formation of the fluorescent *N*-methyl-7-hydroxyquinolinium ion (Rosenberry and Bernhard, 1971).

Using the fluorescence technique, with an enzyme concentration of about 1×10^{-8} and 1×10^{-5} M M7C, in 0.05 M phosphate, pH 7.4, $\mu = 0.125$; at 22 °C, we found that the enzyme reacted almost completely with M7C in about 1 min, as expected from previous work (Rosenberry and Bernhard, 1971). However, enzyme inhibited by 10^{-2} M arsenite for 10 min produced no measurable increase in fluorescence.

Similarly, we found that, if it reacts at all, enzyme which was first carbamylated reacted with arsenite 100 times less rapidly than free enzyme. In this experiment, enzyme was incubated with 1×10^{-5} M M7C for 10 min and one portion was diluted 5000-fold into substrate solution in a pH stat. The rate of return of enzyme activity, corresponding to decarbamylation of the enzyme (Wilson et al., 1961; Kitz et al., 1970), was measured during a period of 30 min. Another portion was incubated for 12 min with 10^{-2} M arsenite before dilution in the pH stat. The half-time for inhibiting the enzyme at this concentration of arsenite is only 0.5 min, so that if the carbamyl enzyme were able to react with arsenite, the reaction should have gone to completion and almost no recovery of enzyme activity in the pH stat should have been observed, since the reversal of arsenite inhibition is very slow. Still, enzyme activity did recover and at a rate only about 25% slower than in the control experiment. It therefore appears that the carbamyl enzyme was not inhibited by arsenite and that the relatively small loss of enzyme activity arose from inhibition of the free enzyme that exists in low concentration in a dynamic steady state in the presence of the carbamylating agent. Alternatively, arsenite might react with the carbamyl enzyme two orders of magnitude more slowly than with the free enzyme.

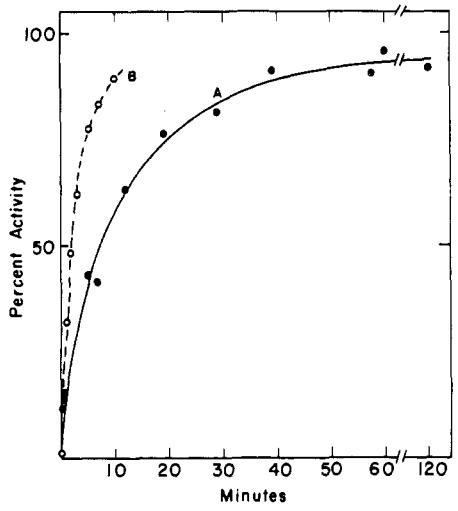

FIGURE 3: Reversal of arsenite inhibition of AcChE by dilution into solutions of 2-PAM or iodine. (A) AcChE was inhibited in 1×10^{-4} M arsenite containing 1×10^{-4} M 2-PAM in 0.05 M phosphate, pH 8.0, $\mu = 0.125$, at 22 °C. After equilibrium had been achieved, the enzyme was diluted 100-fold into 10^{-4} M 2-PAM in the same buffer. The calculated expected initial inhibition was 88% and the calculated expected final inhibition was 7%. The rate constant for recovery calculated from the plot is 0.081 min^{-1}. Under these conditions, the second-order rate constant for arsenite inhibition is 5.3×10^3 M^{-1} min^{-1}. The ratio of these two rate constants gives 1.53×10^{-5} M for the equilibrium constant, as compared to 1.37×10^{-5} M measured directly. (B) AcChE was incubated in 2×10^{-3} M arsenite in 0.05 M phosphate, pH 8.0, $\mu = 0.125$, at 22 °C for 15 min, and then diluted 100-fold into a solution containing 4×10^{-5} M I_2 and 0.02 M KI in the same buffer. Aliquots were treated with 10^{-3} M sodium thiosulfate at various times in order to remove the iodine.

Most reactions of AcChE are affected by quaternary ammonium ions (Froede and Wilson, 1971). Depending upon the reactant and the ligand, the effect may be negative (inhibitory) or positive (accelerating), or there may be little or no effect. We measured the rate of inhibition of AcChE by arsenite in the presence of a number of quaternary ammonium ions, all of which are competitive inhibitors of substrate hydrolysis (Table I). We found that phenyltrimethylammonium ion (PTMA) and Tensilon (3-hydroxyphenyldiethylmethylammonium ion) are negative effectors of arsenite inhibition. At high concentrations both ligands completely prevent reaction of AcChE with arsenite, and at lower concentrations they decrease the rate of arsenite inhibition, in accordance with a dissociation constant that is in agreement with the dissociation constants found for substrate inhibition, i.e., 5×10^{-5} M for PTMA and 2×10^{-8} M for Tensilon (Tables I and II). The fraction of enzyme that is free in the presence of ligand, L, is given by:

$$1/(1 + [L]/K_L)$$

and the expected rate constant is:

$$k_i/(1 + [L]/K_L)$$

TABLE I: Dissociation Constants for Binding of Quaternary Ligands to AcChE as Determined by Inhibition of Substrate Hydrolysis and by Modulation of Arsenite Inhibition.

Ligand	Dissociation constant[a] (M)				$k_i°$ (M^{-1} min^{-1})
	A	B	C	D	
2-PAM	3.3×10^{-5}	5×10^{-4}		3×10^{-5}	2.8×10^{4}
PMI	3.0×10^{-5}	3×10^{-4}			1.2×10^{3}
Toxogonin	4.1×10^{-5}	5×10^{-5}			7.5×10^{3}
PTMA	5.2×10^{-5}		5×10^{-5}		
TMA	6.5×10^{-4}		6×10^{-4}		
Propidium	3.2×10^{-6}		3×10^{-6}		
Tensilon	2.0×10^{-8}		2×10^{-8}	2×10^{-8}	
Tris (pH 8.0)		9×10^{-2}			9×10^{3}
No ligand					1.28×10^{2}

[a] Dissociation constants for the binding of the various ligands were obtained as described in the text: (A) From the inhibition of substrate hydrolysis; (B) from the acceleration of arsenite inhibition; (C) from the blocking of arsenite inhibition; (D) from the retardation of the acceleration of arsenite inhibition. $k_i°$ is the maximal second-order rate constant for arsenite inhibition in the presence of those ligands which served as accelerators.

TABLE II: Effects of Tensilon, PTMA, and 2-PAM on the Rate of Inhibition of AcChE by Arsenite.[a]

$HAsO_2$ (M)	$t_{1/2}$ (min)	2-PAM (M)	Tensilon (M)	PTMA (M)	k_i'[b] (M^{-1} min^{-1})	$k_{i,calcd}$[c] (M^{-1} min^{-1})	$k_{i,exp}$[d] (M^{-1} min^{-1})
6.7×10^{-3}	1.7	0	2.5×10^{-8}		1.28×10^{2}	58	61
6.7×10^{-3}	3.2	0	6.4×10^{-8}		1.28×10^{2}	31	32
6.7×10^{-4}	1.55	3.3×10^{-5}	6.4×10^{-8}		1.9×10^{3}	7.2×10^{2}	6.7×10^{2}
1.5×10^{-4}	4.0	3.8×10^{-5}	2.5×10^{-8}		2.2×10^{3}	1.4×10^{3}	1.2×10^{3}
6.7×10^{-3}	3.7	0		2×10^{-4}	1.28×10^{2}	26	28
1.3×10^{-2}	3.5	0		5×10^{-4}	1.28×10^{2}	12	15

[a] The reactions were performed in 0.05 M phosphate, pH 7.4, $\mu = 0.125$, at 22 °C. [b] k_i' is the rate constant observed for the inhibition of AcChE by arsenite in the absence of Tensilon or PTMA but in the presence of 2-PAM (when added). [c] $k_{i,calcd}$ is the rate constant calculated for arsenite inhibition from eq 3, using as K_I values for 2-PAM, tensilon, and PTMA the dissociation constants obtained from the inhibition of substrate hydrolysis by these ligands. [d] $k_{i,exp}$ is the corresponding rate constant observed experimentally.

where K_L is the ligand-binding dissociation constant and k_i is the second-order rate constant for arsenite inhibition in the absence of ligand.

The active site of AcChE consists of two subsites. One is the esteratic site, where the catalytic process occurs, and the other is the anionic site which binds quaternary ammonium (and other substituted amine) structures. Thus, our results indicate that the binding of PTMA and Tensilon at the anionic site completely prevents reaction of the enzyme with arsenite. This suggests that the site that reacts with arsenite is close to the anionic site. The effect of a ligand bound at the anionic site may be mediated by a change in conformation or the effect may be largely steric.

Tetramethylammonium ion (TMA) and propidium (Taylor and Lappi, 1975) also retard arsenite inhibition (Table I), but even at high concentrations (5 to 20 times the value of K_L) the negative effect is not complete and corresponds in each case to only about a 40–45% slowing of the rate of inhibition. Half this effect occurs at a concentration near the dissociation constant of the ligand, as determined by inhibition of substrate hydrolysis, i.e., 6×10^{-4} and 3×10^{-6} M for TMA and propidium, respectively.

The effect of pyridine-2-aldoxime methiodide (2-PAM) was tested because it had previously been found to accelerate the rate of inhibition of AcChE by tetranitromethane (Fuchs et al., 1974). We found that 2-PAM enormously accelerates the rate of inhibition by arsenite (Figure 4). Although there is considerable scatter of points, the acceleration data follow a binding curve at pH 7.4 with a dissociation constant of about 5×10^{-4} M and a maximum acceleration of 220 times the rate of inhibition in the absence of added ligand. The expected equation for the observed rate constant for arsenite inhibition in the presence of an accelerator, A, that binds at a single site with a dissociation constant, K_A, is

$$\frac{k_i}{1 + [A]/K_A} + \frac{k_i°}{1 + K_A/[A]} \quad (2)$$

where k_i is the rate constant in the absence of A and $k_i°$ is the maximum rate constant that is approached as [A] increases without limit and saturates the enzyme. Our data fit this expression, but the first term is negligible for most values of [A]. The dissociation constant thus obtained for acceleration by 2-PAM does not agree with the value obtained from the inhibition of substrate hydrolysis, 3.3×10^{-5} M, but is an order of magnitude larger. This result can be explained by assuming that the binding of 2-PAM that causes acceleration occurs not at the anionic site, but at a second binding site, which we shall call the accelerator site for the arsenite inhibition reaction or, in this paper, simply the accelerator site. We must emphasize that this site may not be the binding site involved in the acceleration of the reactions of other substances with AcChE. We must also note that the effect that is produced by a ligand that binds at the accelerator site will probably depend upon the nature of the ligand. Thus, we assume that there are two binding sites for 2-PAM, although further documentation for the existence of the second site is desirable. The simplest circumstance that might prevail is that the sites bind ligand more or less independently and that only the second site, the accel-

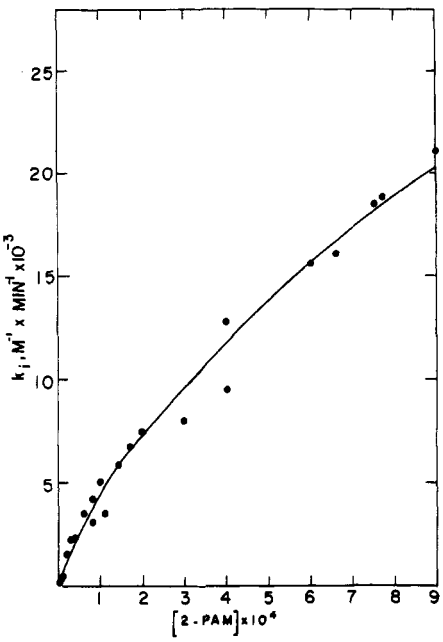

FIGURE 4: Acceleration by 2-PAM of inhibition of AcChE by arsenite. The second-order rate constant, k_i (M^{-1} min^{-1}), for the inhibition of AcChE by arsenite is plotted as a function of the concentration of 2-PAM. The reaction was performed in 0.05 M phosphate, pH 7.4, μ = 0.125, at 22 °C. At 2-PAM = 2×10^{-3} M, $k_i = 25 \times 10^3$ M^{-1} min^{-1} (not shown). The curve indicates a dissociation constant (K_I) of about 5×10^{-4} M for 2-PAM acting as an accelerator, with a maximum accelerated second-order rate constant of 28×10^3 M^{-1} min^{-1}.

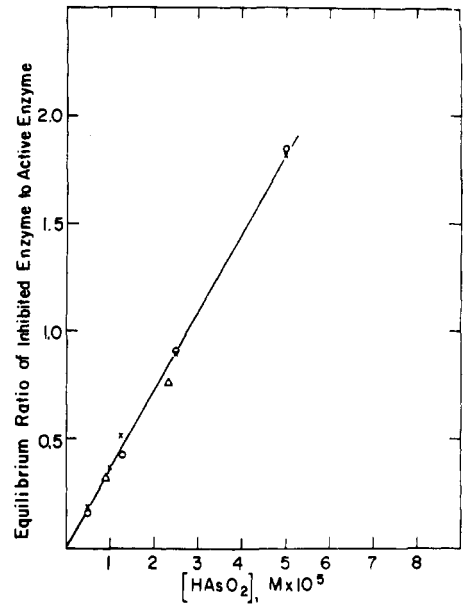

FIGURE 5: Dependence on arsenite concentration of the equilibrium ratio of active to inactive AcChE. Measurements were performed at pH 8.0 in the presence of 10^{-4} or 10^{-3} M 2-PAM using 0.05 M phosphate, μ = 0.125, or in the absence of 2-PAM, using 0.125 M Tris, μ = 0.125. Enzyme was incubated with the indicated concentrations of arsenite and assayed at various times (up to 3 h for the lowest concentrations) until no further decrease in enzyme activity was observed. The ratio of inhibited enzyme to active enzyme is equal to the concentration of arsenite divided by the equilibrium constant for dissociation. The value of the equilibrium constant in this experiment is 1.37×10^{-5} M. (O-O) 1×10^{-3} M 2-PAM-0.05 M phosphate; (x-x) 1×10^{-4} M 2-PAM-0.05 M phosphate; (Δ-Δ) 0.125 M Tris.

erator site, has to be occupied for acceleration to occur, regardless of whether the anionic site is also occupied. This situation also leads to eq 2. Even if there is considerable interaction between the two sites, they would appear to behave more or less independently because k_i° is so much larger than k_i. For the following reason, it appears that the sites are more or less independent. We know that acceleration occurs when both sites are occupied, for if one of the sites had to be vacant, the observed dissociation constant would be less than or equal to the smaller dissociation constant (3.3×10^{-5} M). We also know that one site can be vacant, because, if both sites had to be occupied for acceleration to occur, the curve would be sigmoid. Similarly, the curve would be sigmoid if there were marked positive cooperativity in binding, and if there were marked negative cooperativity in binding the observed dissociation constant for acceleration would be less than 3.3×10^{-5} M. Thus, our data are consistent with the existence of an accelerator site that, in the binding of 2-PAM and in its acceleration of arsenite inhibition, behaves more or less independently of the anionic site. 2-PAM also accelerates at pH 8.0 and to a slightly higher extent than at pH 7.4. We did only a few experiments at this pH because the rate constant changes rapidly with pH at pH 8.0.

N-Methylpyridinium ion is also an accelerator but a very much weaker one than 2-PAM. The acceleration follows a binding curve with a dissociation constant of 3×10^{-4} M, and a maximum acceleration of about ninefold is attained. Again, the dissociation constant for acceleration is an order of magnitude larger than the value obtained from the inhibition of substrate hydrolysis, so that the evidence for a distinct binding site that causes acceleration is reinforced.

Toxogonin, a bisquaternary derivative of 4-PAM (Engelhard and Erdmann, 1964), is also an accelerator, but in this case the dissociation constant obtained from the acceleration curve is about the same as the dissociation constant obtained from substrate inhibition, 4×10^{-5} M. An attractive explanation for the difference in behavior of this compound, as compared to the two previous accelerators, is that toxogonin, which is a diquaternary compound, can span both the anionic and accelerator sites; however, we have no corrobatory evidence on this point. The maximum acceleration of 50-fold is quite substantial.

We have already noted that Tris is an accelerator. The binding of this compound, as determined from acceleration studies, is quite weak, with a dissociation constant of about 0.09 M (pH 8.0). Tris is a potent accelerator with a maximum acceleration of about 70-fold.

We have noted that the reaction of arsenite with the enzyme is so slow that it is difficult to measure the equilibrium constant for the reaction. The reaction in the presence of 2-PAM or Tris is much more rapid, and we therefore measured the equilibrium in 10^{-4} and 10^{-3} M 2-PAM in phosphate buffer at pH 7.4 and 8.0 and in 0.125 M Tris, pH 8.0, in the absence of 2-PAM (Figure 5). We found that the equilibrium constant was independent of the concentration of 2-PAM and was the same as found in Tris buffer. These results indicate that the equilibrium constant is not changed by 2-PAM or Tris.

Since inhibition by arsenite is rapid in 2-PAM solution, recovery of inhibited enzyme should also be rapid. We found that enzyme inhibited with 1×10^{-4} M arsenite (in 10^{-4} M 2-PAM) recovered rapidly when diluted 100-fold into 10^{-4} M 2-PAM, with a rate constant of 0.081 min^{-1}; this is in agreement with the value of 0.073 min^{-1} calculated from the rate constant for inhibition and the equilibrium constant (Figure 5).

We have already noted that Tensilon prevents the inhibition of AcChE by arsenite. We also examined the combined effect of Tensilon and 2-PAM on the rate of arsenite inhibition. Al-

though Tensilon offsets the effect of 2-PAM, relatively high concentrations of Tensilon were needed, depending upon the concentration of 2-PAM. The results were consistent with competitive binding of Tensilon and 2-PAM. The appropriate constant for Tensilon was its known dissociation constant from substrate inhibition and the appropriate constant for 2-PAM was also the one obtained from substrate inhibition and not the dissociation constant obtained from acceleration (Table I). The equation for the observed second-order rate constant used in these calculations was

$$k_i = k_i'/\{1 + [T]/[K_T(1 + [P]/K_p)]\} \quad (3)$$

where k_i' is the rate constant observed in the absence of Tensilon, T, but in the presence of 2-PAM, P. The constant for Tensilon is K_T and for 2-PAM, K_p. The equation is simply the rate constant in the presence of 2-PAM multiplied by the fraction of enzyme that has not bound Tensilon. Our results suggest that 2-PAM interferes with the binding of Tensilon by competing with it for the anionic site and that Tensilon bound at the anionic site prevents arsenite inhibition even if 2-PAM is simultaneously bound at the accelerator site.

Taylor and Lappi (1975) have suggested that propidium can bind to AcChE at a site distinct from the substrate-binding site. We therefore measured the rate of arsenite inhibition in the presence of both propidium and 2-PAM in order to learn if this site is the same as the accelerator site. We used only one concentration of 2-PAM, 2.4×10^{-4} M. This concentration of 2-PAM is high for binding at the anionic site, but low for binding at the accelerator site. Thus, it should be difficult for propidium to occupy the anionic site, but relatively easy for it to displace 2-PAM from the accelerator site if propidium binding can occur at either of these sites (or is competitive with 2-PAM binding at either of these sites). We found that 8×10^{-6} M propidium, i.e., a concentration almost *three* times its dissociation constant for inhibition of substrate hydrolysis, decreased acceleration only very slightly and 8×10^{-5} M propidium decreased acceleration by only about 50%. The result at the lower propidium concentration indicates that propidium cannot displace 2-PAM from the accelerator site and, therefore, does not bind there, at least not with a binding constant of 3×10^{-6} M. The result at the higher propidium concentration, along with the result at the lower concentration, is consistent with the proposal that 2-PAM binding at the anionic site is competitive with propidium, whether or not propidium actually binds there. In this scheme, propidium at the higher concentration is bound at its site (and has displaced 2-PAM from the anionic site) but has left 2-PAM at the accelerator site. This enzyme molecule, even though it contains 2-PAM at the accelerator site, does not react with arsenite at its full acceleration potential because propidium bound at its site partially interferes with the arsenite reaction just as it does in the absence of 2-PAM.

Propidium at a concentration of 8×10^{-5} M in the presence of 2.4×10^{-4} M 2-PAM did not affect the equilibrium constant for arsenite inhibition.

We measured the effect of the substrate acetylcholine (AcCh) on the rates of arsenite inhibition and recovery from arsenite inhibition. The rate of recovery was measured in the presence of AcCh by diluting inhibited enzyme 1000-fold into 1×10^{-3} M AcCh in a pH stat; as time went on, the rate of hydrolysis increased. The rate constant for recovery was found to be 1.8×10^{-3} min^{-1} as compared to the "expected value" of 1.9×10^{-3} min^{-1} calculated from the rate constant for inhibition and the equilibrium constant, both in the absence of substrate. In determining the effect of AcThCh upon recovery, inhibited enzyme was diluted 125-fold into assay medium containing 4×10^{-4} M AcThCh in a cuvette, and again the rate of hydrolysis was found to increase with time, corresponding in this case to a rate constant for dissociation of the enzyme-arsenite complex of 1.5×10^{-3} min^{-1}. Thus, it appears that AcCh and AcThCh have little or no effect upon the rate of recovery of inhibited enzyme.

The effect of AcThCh on the rate of arsenite inhibition was also determined in a cuvette under continuous recording. In 3.2×10^{-4} M AcThCh, pH 7.4, using 8×10^{-4} M arsenite, the half-time for arsenite inhibition was 5 min, corresponding to a rate constant of 1.7×10^2 M^{-1} min^{-1}, compared to 1.3×10^2 M^{-1} min^{-1} in the absence of substrate. In both 4×10^{-3} and 1.2×10^{-2} M AcThCh, the half-time was the same, 3.1 min, corresponding to a rate constant of 2.8×10^2 M^{-1} min^{-1}. Thus, there is a small but distinct increase of about twofold in the rate of arsenite inhibition at high concentrations of AcThCh.

The effect of AcThCh on the acceleration of arsenite inhibition by 2-PAM was also investigated, but only one experiment was done. In a cuvette, in the presence of 4×10^{-5} M 2-PAM, 4×10^{-4} M AcThCh, and 8×10^{-4} M arsenite, the half-time for inhibition at pH 7.4 was 2.7 min, corresponding to a rate constant of 3.2×10^2 M^{-1} min^{-1}, whereas in the absence of AcThCh and in the presence of this concentration of 2-PAM the rate constant is 2.3×10^3 M^{-1} min^{-1}. Thus, AcThCh almost completely prevented acceleration of arsenite inhibition in this experiment. The acceleration of recovery of inhibited enzyme by 2-PAM in the range $0.4\text{–}2.4 \times 10^{-4}$ M was also decreased by 3.2×10^{-4} M AcThCh, but the decrease was only 40–50% under these conditions.

We made some attempts to elucidate the chemical nature of arsenite inhibition. In particular, we investigated the possibility that arsenite as a chemical reductant reduces a disulfide bond to form neighboring dithiols which then combine reversibly with a second molecule of arsenite to form a "cyclic" thioarsenite ester. If arsenite acts as a reductant, arsenate is formed. Therefore, we measured the equilibrium constant for arsenite inhibition in the presence of 1×10^{-4} M 2-PAM in 0.1 M arsenate at pH 7.4, 7.8, and 8.8. We found no difference from the value in 0.1 M phosphate buffer. We also brought enzyme to equilibrium in 10^{-4} M 2-PAM–0.05 M phosphate, pH 8.0, at 78% inhibition with 5×10^{-5} M arsenite, and bubbled oxygen through the solution for 30 min. No change in AcChE activity resulted. Thus, these two experiments indicate that if any reductive step occurs in the inhibition, it is not involved in the final equilibrium reaction. We tried to find evidence for the production of thiol groups in the arsenite inhibition of AcChE by adding other SH reagents to a solution at equilibrium with 78% inhibition by arsenite, obtained as above. The extent of inhibition remained unchanged by the addition of 10^{-3} M Nbs_2 or 10^{-2} M *N*-ethylmaleimide, but the addition of 1.2×10^{-3} M *p*-chloromercuribenzenesulfonate increased the enzyme activity from 22 to 60%. The results with the first two reagents suggest that no sulfhydryl groups had been formed. The interpretation of the result with the mercurial is uncertain but it probably does not involve an SH group, because the concentration of the organomercurial that is required to increase the activity is much higher than those usually used to titrate sulfhydryl groups in proteins (Riordan and Vallee, 1967). None of these reagents have any effect on AcChE activity in the absence of arsenite, even after incubation for 1 h, nor do 10^{-2} M thiosulfate or 10^{-2} M sulfite have any effect. After incubation with thiosulfate and sulfite, which were removed by dilution, the enzyme was still inhibited by

arsenite at the same rate as in control experiments. Although the reductive hypothesis is attractive, our results are against its validity.

In order to test whether other serine enzymes might be sensitive to arsenite, we incubated chymotrypsin in 10^{-2} M arsenite at pH 7 at room temperature for 30 h. No inhibition of activity was attained after this incubation when the enzyme was assayed spectrophotometrically using succinyldiglycyl-L-phenylalanyl-*p*-nitroanilide as substrate, either with or without 10^{-2} M arsenite in the assay medium.

Discussion

The chemical nature of the inhibition of AcChE by arsenite remains obscure. As mentioned in the Introduction, arsenite inhibition is usually believed to involve formation of a cyclic dithioarsenite ester with neighboring sulfhydryl groups in the enzyme molecule (Peters, 1949) and this view is strengthened, in the case of various dehydrogenases, by the observation that they are sensitive to arsenite only in their reduced state (see, for example, Jakoby, 1963). Mounter and Whittaker (1953) already stressed that it was unlikely that arsenite inhibited AcChE via sulfhydryl groups, since the enzyme was relatively insensitive both to other sulfhydryl reagents and to organic arsenicals, such as Lewisite (β-chlorovinyldichloroarsine), which are usually more potent than arsenite itself. We considered and tested the additional possibility that arsenite might act either by reducing a disulfide bond in AcChE or by such reduction followed by combination with the sulfhydryl groups so produced. Our experimental data do not support such a model.

Mounter and Whittaker (1953) suggested that arsenite might bind electrostatically to the active site of AcChE. However, the slowness of the inhibition which we observed in our kinetic studies suggests either a covalent reaction or a change in conformation of the protein. If a conformational transition were the rate-determining step, the reaction with arsenite would not be second order. Arsenite was shown by Crafts (1871) to form triesters with low-molecular-weight alcohols. Although such triesters are unstable in aqueous solution, if three suitable groups in the AcChE molecule were adventitiously placed in just the proper orientation, one could speculate that a fairly stable complex would ensue. Whatever the nature of the complex, we do know that its formation is not a fundamental characteristic of the catalytic site of serine esterases because we were unable to inhibit chymotrypsin with arsenite.

The existence in AcChE of at least one more site, in addition to the anionic site, that can bind quaternary ammonium ligands, has been known for many years, based mainly upon the greater potency of bisquaternary, as compared to monoquaternary, ligands as AcChE inhibitors and reactivators (Bergmann et al., 1950; Bergmann and Segal, 1954; Wilson and Ginsburg, 1958; Hobbiger et al., 1958; Poziomek et al., 1958; Changeux, 1966). More recent information has been obtained from studies on fluorescence changes attending the binding of suitable quaternary ligands (Mooser et al., 1972; Taylor and Jacobs, 1974). In the present study, a possible second binding site for 2-PAM was revealed by the observation of different binding constants for substrate inhibition and for acceleration of arsenite inhibition. Kitz et al. (1970) earlier suggested the participation of a second ligand-binding site in acceleration of decarbamylation of AcChE by gallamine.

2-PAM increases the rate of reaction of arsenite with AcChE, and also the rate of dissociation of the resulting complex, without changing the equilibrium constant. This result indicates that 2-PAM is a catalyst that binds equally well to the free enzyme and to the arsenite–enzyme complex. The increase in the rates of association and dissociation must mean that 2-PAM binds much better to the protein in the transition state of the reaction than it does to the free enzyme or the enzyme–arsenite complex.

The small acceleration of arsenite inhibition caused by substrate is not easily interpreted because there are several enzyme forms present in the steady state of substrate hydrolysis. One of these forms, the acetyl enzyme, which may account for 80% or more of the steady-state mixture (Wilson and Cabib, 1956), may not react at all with arsenite judging from our observation that the dimethylcarbamyl enzyme does not react with arsenite except perhaps very slowly. This is not a firm conclusion, because the dimethylcarbamyl group is larger than the acetyl group and may thus perhaps prevent reaction of arsenite with the enzyme by steric hindrance. If we nonetheless assume that the acetyl enzyme cannot react with arsenite, we should have to ascribe the small acceleration observed in arsenite inhibition to its reaction with nonacetylated AcChE. The small fraction of nonacetylated enzyme would thus have to be subject to sizeable acceleration by the substrate itself in order to overcome the nonreactivity of the acetyl enzyme and to yield a "net acceleration".

Tris is commonly used as a buffer in AcChE assays, and is not known to inhibit the enzyme. Nevertheless, it is a powerful accelerator of arsenite inhibition, albeit with a low affinity for the enzyme. It is interesting to note in this connection that the rate of recovery of activity of an organophosphoryl-AcChE derivative has been observed to be enhanced in Tris buffer (Y. Ashani, personal communication).

2-PAM is a "site-directed" reactivator of AcChE that has been phosphorylated by organophosphate inhibitors (Wilson and Ginsburg, 1955). At which site does 2-PAM bind when it serves as a reactivator? From previous work, it is known that the 2-PAM dissociation constants for binding to the free enzyme determined from substrate inhibition and to the phosphoryl enzyme determined from reactivation differ only by a factor of two (Maglothin et al., 1976). Thus, it is apparent that 2-PAM binds at the anionic site as a reactivator.

Taylor and Lappi (1975) have reported that propidium at very low ionic strength, and with AcChE from *Torpedo californica,* binds at a site different from the anionic site. The propidium-binding site is not the same as the accelerator site, at least in electric eel enzyme, since we found that propidium could only partially diminish the acceleration produced by 2-PAM. Thus, our results, taken together with those of Taylor and Lappi (1975), indicate that there are three sites, the substrate-binding site and two others, which can bind quaternary ammonium ligands, depending upon the structure of the ion. Taylor and Lappi (1975) have also suggested the existence of more than one peripheral site from different considerations. It is not known whether any of these additional sites play any role in the physiological function of AcChE.

Acknowledgments

I. B. Wilson was a Katzir-Katchalsky Fellow and is grateful to Professor Nathan Sharon of the Department of Biophysics for his hospitality.

The participation of Dr. Yadin Dudai in some of the initial experiments and of Ms. Lili Anglister in the carbamylation experiments is gratefully acknowledged, as is the assistance of Mr. Nathan Tal in the chymotrypsin assays.

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