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INDOPHENYL ACETATE AND ACETYLCHOLINESTERASE: BINDING OF A NON-SPECIFIC SUBSTRATE ON THE MARGIN OF THE ACTIVE CENTER

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Summary

1. Indophenyl acetate is a very poor substrate of eel or bovine acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), with a V less than 5% of that of phenyl acetate, but it is a labile ester and in imidazole buffer is hydrolyzed, non-enzymically, even faster than phenyl acetate. 2. Indophenyl acetate completely protects the enzymes against inhibition by diisopropylphosphorofluoridate but promotes inhibition by methanesulfonyl fluoride. 3. With either of these inhibitors the measured rate of inactivation of eel acetylcholinesterase is the same whether activity is determined with this poor substrate or with a good substrate, acetylthiocholine. With bovine enzyme the inactivation rate is 25% lower when assayed with the former substrate. However this preparation contains a minor enzyme component which is involved in hydrolysis of indophenyl acetate but not good substrates, and which is not readily inhibited. When this is taken into account the inactivation rates for bovine acetylcholinesterase, too, are found to be the same in either assay.

These and other observations in the literature can be explained if indophenyl acetate, because of its size, cannot fully penetrate into the active center and is bound in adjoining non-polar regions of the protein. From this external position it makes only intermittent contact with the esteratic site. Hence it is slowly hydrolyzed and fails to protect the enzyme against methanesulfonyl fluoride, though it does protect, possibly sterically, against the larger inhibitor diisopropylphosphorofluoridate.

Introduction

Indophenyl acetate is a unique substrate of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), since it exhibits the following remarkable prop-

erties: (1) Enzyme treated with irreversible inhibitors that react in or near the anionic site (those of the aziridinium class [1—3] and *p*-(trimethylammonium)-benzenediazonium fluoroborate [4]) is more active than the native enzyme with this substrate, but less efficient with other neutral substrates, and totally or almost totally inactive towards cationic substrates such as acetylcholine. (2) Enzyme treated with tetranitromethane, which probably reacts with a tyrosine residue, suffers a complete loss of activity with acetylthiocholine but no loss with indophenyl acetate [5]. (3) Indophenyl acetate hydrolysis is reported to be non-competitively inhibited by acetylcholine, phenyl acetate, naphthyl acetate and tetraethylammonium ion, which is very odd if the same active center hydrolyzes all substrates [6]. (4) The apparent rate constant for inactivation of bovine acetylcholinesterase by the organophosphorus inhibitor Amiton was reported to be 3—4 times lower when enzyme activity was assayed with indophenyl acetate rather than acetylcholine [3], and the rate became 1000 times lower in the indophenyl acetate assay if similar experiments were carried out with enzyme which had first been alkylated with an aziridinium compound (see point (1), above).

The first three classes of observations mentioned above (1—3) could be explained in terms of a single species of active center for all substrates, including indophenyl acetate, because the inhibitors may be considered to act upon the binding subsite and not the catalytic subsite in the enzyme (in the absence of knowledge to the contrary this generalization includes tetranitromethane). The last mentioned observations, under point 4, present a far more serious difficulty. They cannot be explained in this way, because organophosphorus inhibitors destroy the catalytic apparatus itself. If corroborated, the observations would prove that the enzyme contains more than one type of active center, with different degrees of catalytic efficiency towards substrates (and organophosphorus inhibitors). There is, however, a trivial explanation for this, and rather than labor the point it seems preferable to anticipate the following description of some of the experiments, which show that commercial preparations of bovine erythrocyte (but not eel) acetylcholinesterase contain a small but significant amount of contaminating enzyme activity; this enzyme hydrolyzes indophenyl acetate but not good substrates of acetylcholinesterase, and is not readily inactivated by organophosphorus inhibitors. All the work described above on indophenyl acetate was carried out with enzyme from this source; the observations are probably basically sound (because in the present study hydrolysis of this substrate was mainly (83%) due to acetylcholinesterase and only in small part (17%) to the contaminant activity), but they must nevertheless be treated with some reservation.

The mechanism of indophenyl acetate hydrolysis has here been investigated by making use of the irreversible inhibitor, methanesulfonyl fluoride, which is believed to react with an essential serine residue in the catalytic (esteratic) site [7,8]. Methanesulfonyl fluoride is a convenient probe of substrate binding because molecules adsorbed at the active center normally protect the enzyme only if they interact strongly with the esteratic subsite and physically obstruct its approach. This is in part due to the small size of the inhibitor, but also to its insensitivity to deformation of the active center; for it has been shown that denaturing agents which severely reduce enzyme reactivity with

p-nitrophenyl acetate or diisopropylphosphorofluoridate have relatively little effect on methanesulfonyl fluoride reaction [9]. Furthermore, cations activate this reaction even when they are bound unspecifically in nonpolar regions adjacent to the active center [10].

This property of methanesulfonyl fluoride was exploited in an earlier study. While some cationic substrates such as acetylcholine protected the enzyme, others which are poor substrates did not, indicating that they can become bound at the anionic site without involving the esteratic site [10]. Phenyl esters, which protected the enzyme and which compete with acetylcholine, invariably occupy the esteratic site. Judging by the observations noted above, indophenyl acetate, though an analog of phenyl acetate, may not be adsorbed in the active center at all.

Materials and Methods

Acetylcholinesterase from the electric eel and from bovine erythrocytes was obtained from Sigma Chemical Co. (Types V and I respectively). Chemicals were of reagent grade, and indophenyl acetate, from Pfaltz and Bauer, N.Y., was recrystallized from butanol before use.

All enzymic reactions were studied at 26° C in solutions containing 20 mM potassium phosphate buffer and 4% methanol, pH 8.0. Rates of hydrolysis of indophenyl acetate, phenyl acetate, or *o*-nitrophenyl acetate were followed in a Beckman DB-G spectrophotometer equipped with a thermostatted cell compartment, by measurement of the absorption of indophenol at 625 nm, of phenol at 270 nm, or of *o*-nitrophenol at 412 nm. Acetylthiocholine hydrolysis was determined in a modified Ellman assay [11,12].

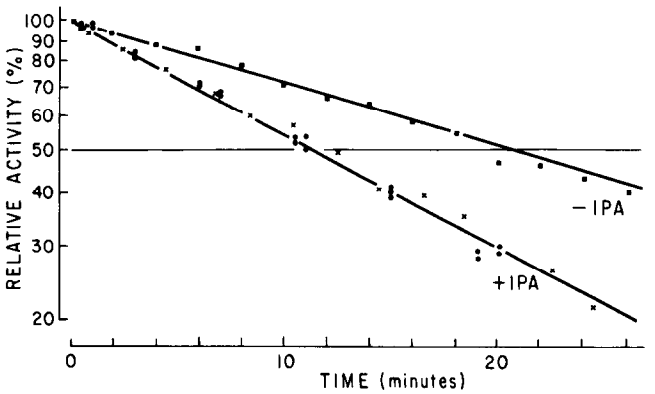

Fig. 1. Inactivation of eel acetylcholinesterase by 0.145 mM methanesulfonyl fluoride, either in the presence or absence of 0.66 mM indophenyl acetate (IPA). Enzyme activity was determined with acetylthiocholine in both cases. In the presence of indophenyl acetate, activity in the acetylthiocholine assay is denoted • and that in the indophenyl acetate assay X. The calculated second order rate constants are $2.3 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ in the absence of indophenyl acetate and $4.2 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ in its presence. Temp. 26° C, pH 8.0, in 20 mM potassium phosphate buffer, with 4% methanol.

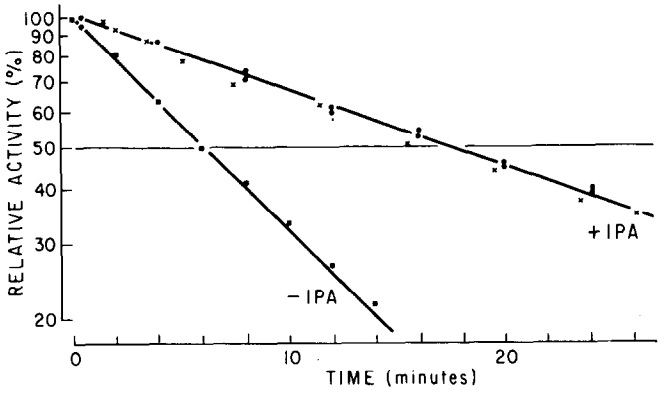

Fig. 2. Inactivation of eel acetylcholinesterase by 0.0058 mM diisopropylphosphorofluoridate in the presence or absence of 0.66 mM indophenyl acetate. Conditions and assays, symbols and abbreviations as in Fig. 1. The calculated second order rate constants are $1.9 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ in the absence of indophenyl acetate and $7.0 \cdot 10^3$ $M^{-1} \cdot min^{-1}$ in its presence.

Experimental Results

Rates of inactivation of eel and bovine acetylcholinesterase by methanesulfonyl fluoride and diisopropylphosphorofluoridate were determined either in the presence or absence of indophenyl acetate, at pH 8.0 and 26°C. Enzyme activity in aliquots withdrawn at intervals from the reaction mixture was determined with acetylthiocholine. In reaction solutions containing indophenyl acetate, the rate of hydrolysis of the latter was monitored continuously by recording the optical density at 625 nm. In the course of the inhibition reaction no more than 10% of this substrate was hydrolyzed. Rates of indophenyl acetate

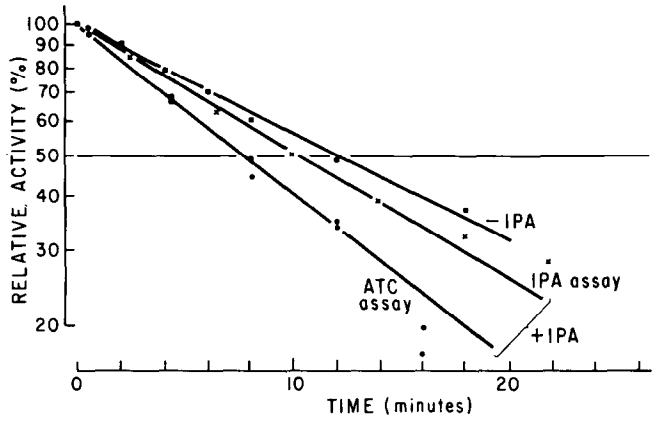

Fig. 3. Inactivation of bovine acetylcholinesterase by 0.145 mM methanesulfonyl fluoride, with or without indophenyl acetate (IPA) (0.66 mM). Conditions and assays, symbols and abbreviations as in Fig. 1. Second order rate constants calculated from acetylthiocholine (ATC) assays: $4.0 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ (−IPA); $6.1 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ (+IPA). In the presence of indophenyl acetate the half time for inactivation is 7.8 min in the acetylthiocholine assay and 10.0 min in the indophenyl acetate assay. In the latter assay 58.6% of the initial activity remained after 7.8 min (on the basis of the straight line in the figure drawn through the experimental points).

hydrolysis were determined at intervals from tangents drawn on the curved recorder tracing. In these experiments, therefore, enzyme activity was known from the hydrolysis rates of both indophenyl acetate and acetylthiocholine. Plots of relative activity (on a log scale) against time are shown in Figs. 1—4, where the concentrations of inhibitors are given. The concentration of indophenyl acetate was 0.66 mM in all cases. It is seen that this substrate protects the enzyme against diisopropylphosphorofluoridate, but increases the rate of inactivation by methanesulfonyl fluoride.

In order to decide whether this protection was partial or complete, K_M values for the substrate were determined, and at the same time relative maximum velocities. The reaction conditions were the same as in the previous experiments, and initial rates were measured at substrate concentrations from 0.08 to 0.83 mM with eel enzyme, and from 0.17 to 0.66 mM with bovine enzyme. The calculated K_M values were $4.54 \pm 0.04 \cdot 10^{-4}$ M and $4.74 \pm 0.05 \cdot 10^{-4}$ M for the eel and bovine enzymes, respectively. The corresponding maximum velocities, relative to a value of 1.00 for acetylthiocholine in the above assays, were 0.029 and 0.077, respectively, assuming that rates were proportional to enzyme concentrations.*

From the concentration of indophenyl acetate and its K_M value, the expected reduction in the measured inactivation rate constant may be calculated, assuming that the enzyme · substrate complex does not react. The rate constant should be reduced by a factor of $1/(1+[S]/K_M)$, which comes out to be 0.39 and 0.42 for the eel and bovine enzymes. The observed ratios were

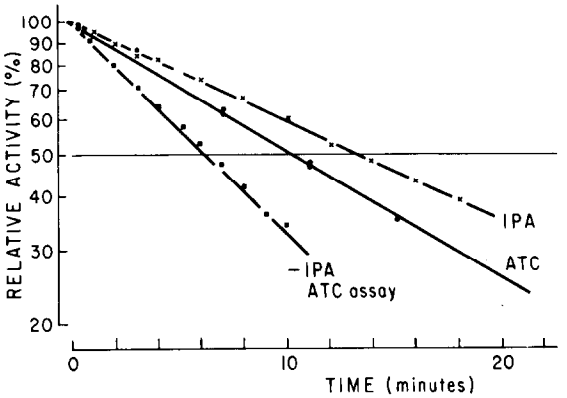

Fig. 4. Inactivation of bovine acetylcholinesterase by diisopropylphosphorofluoridate with or without indophenyl acetate (IPA). In the presence of indophenyl acetate the inhibitor concentration was 0.0035 mM and in its absence 0.0023 mM. Conditions and assays, symbols and abbreviations as in Fig. 1. Second order rate constants calculated from acetylthiocholine (ATC) assays: $4.7 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ (—IPA); $2.0 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ (+IPA). In the presence of indophenyl acetate the half time for inactivation is 10.1 min in the acetylthiocholine assay and 13.2 min in the indophenyl acetate assay, and in the latter the activity remaining after 10.1 min was 59.0% of the original.

* As is shown below, the bovine preparation contains a small quantity of a contaminant enzyme activity acting upon indophenyl acetate but not acetylthiocholine. The measured V for this enzyme is therefore high; the corrected value is 0.064, rather than 0.077.

0.37 and 0.43 respectively. Hence indophenyl acetate must fully protect the enzymes against diisopropylphosphorofluoridate.

The inactivation rates as measured in the two assays are identical for eel acetylcholinesterase with either inhibitor. This suggests that the same active center is involved in hydrolysis of both substrates. However with bovine enzyme the rate of inactivation (Fig. 3 and 4) is slightly less when measured by means of indophenyl acetate. (The half times are increased by 30%). One possible explanation is that the bovine preparation contains an enzyme which catalyzes hydrolysis of indophenyl acetate but not acetylthiocholine, and which is less readily inactivated by the inhibitors. Such a mechanism could give the observed results, with the appearance of linearity in the experimental plots, provided that the second enzyme accounts for only a small fraction of indophenyl acetate hydrolysis, possibly one quarter or less (a requirement that may be demonstrated by adding a quantity that declines logarithmically to another that is constant, and plotting the logarithm of the sum). To decide if such activity is present, the enzyme preparations were subjected to a reversible inhibitor, 3-hydroxyphenyltrimethylammonium iodide. This cation should be specific for acetylcholinesterase because its relatively high affinity for the enzyme (K_i is of the order of 10^{-7} M) depends on the hydroxyl group at the meta-position in the phenyl ring [13]. Moreover, in the case of an insect acetylcholinesterase, only 3-hydroxyphenyltrimethylammonium, and not the unsubstituted or the 2- or 4- substituted analogs, protected the enzyme against methanesulfonyl fluoride, implying that the hydroxyl group bonds firmly with a part of the active center closely involved in catalysis [10].

Rates of indophenyl acetate hydrolysis were determined, under the same conditions as before, but in the presence of several different concentrations of the reversible inhibitor. With eel enzyme and a substrate concentration of 0.69 mM ($[S]/K_M = 1.5$), the activity in the presence of 0.01 mM 3-hydroxyphenyltrimethylammonium ion was approximately 3.5% of the uninhibited value, and with 0.1 mM inhibitor was 2.5%. As expected, virtually all the enzyme acting upon indophenyl acetate in the eel preparation appears to be acetylcholinesterase. Not so with the bovine preparation, where activity in the presence of increasing concentrations of inhibitor dropped to a minimum value of about 17%. (Measured activities with 0.01, 0.02, 0.1 and 0.2 mM inhibitor were 25%, 20%, 19.0% and 17.1%, respectively).

In the case of a good substrate, on the other hand, activity in the bovine preparation was completely inhibited. The substrate chosen was *o*-nitrophenyl acetate, which has a V value of 90% of that of acetylcholine [14]. Its K_M is 0.26 mM. At 0.37 mM ($[S]/K_M = 1.4$) its rate of hydrolysis was reduced to less than 1% by 0.02 mM inhibitor and to an undetectable level by 0.1 mM inhibitor. The conclusion from these assays is that hydrolysis of good substrates by the bovine enzyme preparation is entirely due to acetylcholinesterase. However, the rate at which indophenyl acetate is hydrolyzed, though mainly attributable to the action of this enzyme (83%) is partly due to the action of another enzyme that does not act upon good substrates to any significant extent.

To determine whether this contaminant can be inhibited by methanesulfonyl fluoride and diisopropylphosphorofluoridate, the bovine enzyme was treated with these inhibitors for 15 or 30 min at 10 times the concentrations

TABLE I

Second order rate constants ($M^{-1} \cdot min^{-1}$) for inactivation of acetylcholinesterase by methanesulfonyl fluoride and diisopropylphosphorofluoridate, in the presence or absence of 0.66 mM indophenyl acetate (IPA)

Source of enzyme	Methanesulfonyl fluoride		Diisopropylphosphorofluoridate	
	−IPA	+IPA	−IPA	+IPA
Eel	$2.3 \cdot 10^2$	$4.2 \cdot 10^2$	$1.9 \cdot 10^4$	$7.0 \cdot 10^3$
Bovine	$4.0 \cdot 10^2$	$6.1 \cdot 10^2$	$4.7 \cdot 10^4$	$2.0 \cdot 10^4$

used in the first experiments (the final concentrations were now 1.45 mM and 0.035 mM respectively). All activity was found to be lost in both assays (indophenyl acetate and acetylthiocholine). The second enzyme must therefore be vulnerable to these inhibitors, though it reacts much less rapidly than does acetylcholinesterase.

The question remains whether the observed rates of inactivation as measured in the two assays can be accounted for quantitatively (Fig. 3 and 4). Assuming that the contaminant 17% of the activity towards indophenyl acetate has not yet been appreciably inhibited when half the acetylcholinesterase activity has been destroyed, it is a simple matter, as is now shown, to predict the fractional activity towards indophenyl acetate that should subsist at that time. In the beginning, before any inactivation has occurred, hydrolysis of indophenyl acetate is 17% due to the insensitive enzyme and 83% to cholinesterase. At the half time for inactivation, as signalled by the acetylthiocholine assay, the original 17% of insensitive enzyme is intact, but the activity of acetylcholinesterase should have been reduced to a relative value of 41.5%. Hence the total activity should now be (17 + 41.5)% = 58.5% of the initial value. In the experiments the observed values were remarkably close to this, being 58.6% in the experiment with methanesulfonyl fluoride (Fig. 3) and 59.0% in that with diisopropylphosphorofluoridate (Fig. 4).

It therefore seems safe to conclude that with both eel and bovine acetylcholinesterase, rates of inactivation are identical whether activity is determined with acetylthiocholine or indophenyl acetate, and that the same active center in these enzymes may act upon both substrates. Inactivation rate constants for the two irreversible inhibitors are summarized in Table I.

One last question regarding indophenyl acetate hydrolysis is explored here. This ester is an incomparably poorer substrate than the closely related compound phenyl acetate; for the latter is more rapidly hydrolyzed than even acetylcholine [15]*. We may ask whether this sluggishness is due to an especially stable ester bond in the poor substrate, or to an aberrant interaction with the active center. The rates of non-enzymic hydrolysis of indophenyl acetate and phenyl acetate were therefore compared; in the presence of 20 mM imidazole buffer at pH 7.9, the first order rate constants were found to be

* The relative maximum velocities of indophenyl acetate and phenyl acetate, assuming that the V for phenyl acetate is 36% higher than that of acetylthiocholine [15], are 0.021 and 0.047 for eel and bovine acetylcholinesterase respectively.

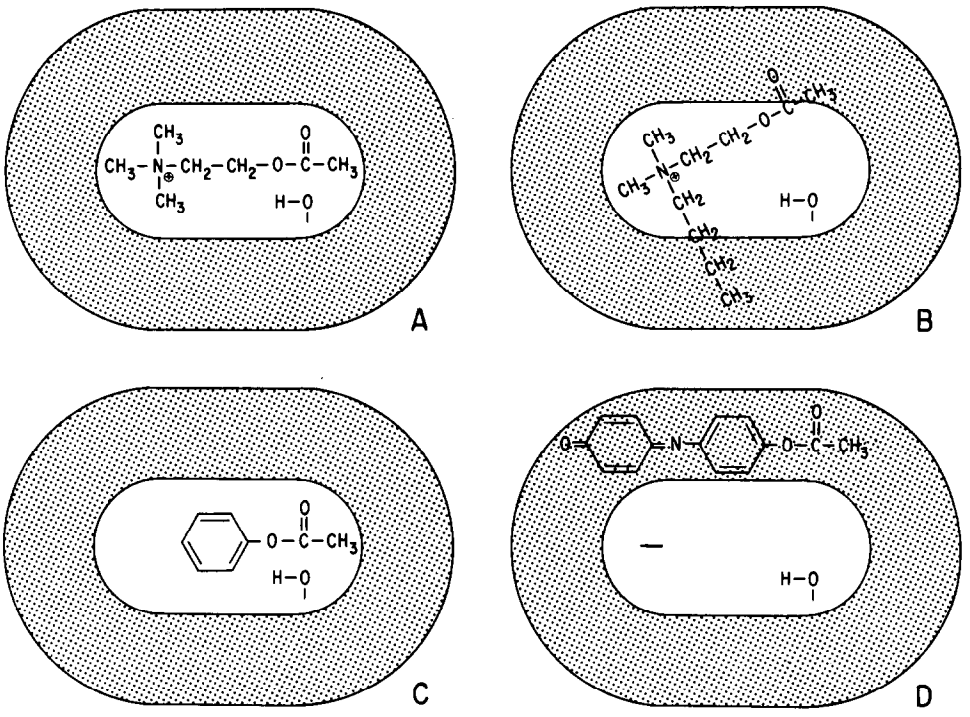

Fig. 5. Proposed interactions of four substrates with the active center of acetylcholinesterase, shown diagrammatically. The inner light area represents the active center crevice; the anionic site is on the left and the esteratic site, which contains a serine hydroxyl group (OH), on the right. The surrounding shaded area represents a region of the protein that unspecifically adsorbs nonpolar molecules. (A) Acetylcholine completely fills the active center. (B) *N,N,N*-dimethyl-*n*-butylaminoethyl acetate is too large to fit into the crevice, and this prevents its acetyl group from interacting closely with the esteratic site. Hence it is slowly hydrolyzed and fails to protect against methanesulfonyl fluoride. (C) Phenyl acetate fits into the active center, is rapidly hydrolyzed and protects against methanesulfonyl fluoride. (D) Indophenyl acetate is bound on the margin of the active center. Consequently it is slowly hydrolyzed and it fails to protect against methanesulfonyl fluoride.

0.023 min^{-1} and 0.012 min^{-1}, respectively. Indophenyl acetate is obviously a very labile ester, and its slow enzymic hydrolysis must be a consequence of biological specificity.

Discussion

In all probability indophenyl acetate fails to become adsorbed in the active center of acetylcholinesterase (though this is not to say that it cannot lodge nearby). The reasons for believing so are as follows: (1) As was just noted, this ester is more unstable in non-enzymic hydrolysis than phenyl acetate, and therefore if the acetyl groups of both were bound in the same position in the esteratic site, we would expect comparable hydrolysis rates. (2) Previous work [10] indicated that substrate analogs slightly larger than acetylcholine do not fully penetrate into the active center, which may therefore be a crevice just large enough to hold the natural substrate. Phenyl acetate should fit into this region, but the larger indophenyl acetate molecule would be excluded.

TABLE II

Affinities and hydrolysis rats for acetylcholinesterase substrates (bovine erythrocyte acetylcholinesterase*).

Substrate	K_M(M)	Relative V	Relative V/K_M	Reference
Acetylcholine	$2.4 \cdot 10^{-4}$	1.00	1.00	15
Acetylthiocholine	$1.3 \cdot 10^{-4}$	0.83	1.53	15
N,N,N-dimethyl-*n*-butyl-aminoethyl acetate	$6.2 \cdot 10^{-4}$	0.54	0.21	10
Phenyl acetate	$1.3 \cdot 10^{-3}$	1.13	0.21	15
o-Nitrophenyl acetate	$2.6 \cdot 10^{-4}$	0.89	0.81	14
Indophenyl acetate**	$4.7 \cdot 10^{-4}$	0.053	0.027	—

* With eel acetylcholinesterase the parameters for indophenyl acetate relative to acetylcholine are $V = 0.024$, and $V/K_M = 0.01$. The K_M value for indophenyl acetate is $4.5 \cdot 10^{-4}$ M.

** Measurements of indophenyl acetate hydrolysis were carried out in 20 mM potassium phosphate buffer, pH 8.0, with 4% methanol. In the determination of V and K_M for other substrates no buffer was present, but instead 0.1 M NaCl + 0.04 M $MgCl_2$, pH 7.5. With the neutral substrates, phenyl acetate and *o*-nitrophenyl acetate, 2% methanol was included in the reaction mixture, but no methanol was present with the cationic substrates. However 2% methanol had an entirely negligible effect on acetylcholine hydrolysis.

(3) Experiments with anionic site-directed inhibitors, as noted at the beginning, and the present experiments with methanesulfonyl fluoride, indicate that neither the anionic nor esteratic subsite is important for indophenyl acetate attachment. However, identical rates of enzyme inactivation as measured with acetylthiocholine and indophenyl acetate argue that the same active center hydrolyzes both. Complete protection against diisopropylphosphorofluoridate together with acceleration of methanesulfonyl fluoride reaction, in the presence of indophenyl acetate, then indicates that this substrate is probably bound very near the active center but that it makes infrequent contact with the esteratic site.

There is ample evidence to show that lipophilic protein regions are commonly associated with enzyme active centers [16], and many observations support their existence in acetylcholinesterase [17]. To explain the observations on indophenyl acetate it appears to be sufficient to postulate that this substrate is adsorbed in such a region, which is in close proximity to the active center crevice. It is adsorbed here because, as indicated above, it is too large to fit into the active center, and because its planar nonpolar structure favors hydrophobic bonding, which is by nature rather unspecific. This is an unfavorable position from the standpoint of catalysis; the consequence is infrequent contact with the esteratic site, a low rate of hydrolysis, and failure to protect against methanesulfonyl fluoride. There is nevertheless interference with the working of the catalytic center, as shown by complete protection against diisopropylphosphorofluoridate. Chemical modification in the region of the anionic site necessarily disorients a specifically bound substrate molecule like acetylcholine, blocking its hydrolysis, but with indophenyl acetate the possibility must exist that the orientation will be slightly improved, accelerating its hydrolysis, as observed [1—3]. By way of summary, the proposed binding of various substrates is illustrated in a highly schematic way in Fig. 5, and sub-

strate parameters for compounds of interest are listed in Table II.

How indophenyl acetate promotes methanesulfonyl fluoride reaction is not explained by the present experiments. The mechanism could involve an increase either in the affinity of the inhibitor for the esteratic site, or in its reactivity in the complex with the enzyme, assuming that such a complex is formed. It may be noted that *n*-butanol and guanidine also accelerate the reaction [9], suggesting that a substrate structure, as in indophenyl acetate, may not be a requirement.

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