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METHANESULFONYL FLUORIDE INACTIVATION OF ACETYLCHOLINESTERASE IN THE PRESENCE OF SUBSTRATES AND REVERSIBLE INHIBITORS

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SUMMARY

Reaction of acetylcholinesterase with methanesulfonyl fluoride is accelerated in the presence of those N-alkyl analogs of acetylcholine that are relatively poor substrates (i.e. slowly hydrolyzed compared to acetylcholine), but is greatly retarded by those that are good substrates, such as acetylcholine itself. The observations are not explained by acetyl enzyme formation. They may be understood if such substrates are first bound at the anionic site and then intermittently contact the esteratic site; attenuation of this second interaction accounts for slow hydrolysis as well as failure to protect the enzyme against methanesulfonyl fluoride, which attacks the esteratic site. Ligands which contain a phenyl ring, including both poor and good substrates, generally protect the enzyme. A phenyl-binding region may therefore be closely associated with the esteratic site.

Acceleration of methanesulfonyl fluoride reaction by cationic substrates apparently results from non-specific and normally inhibitory modes of substrate attachment. Like these substrates, reversible cationic inhibitors (which also accelerate the reaction) are now known to be bound in non-specific regions adjacent to the active center. It follows that the activation mechanism is unlikely to involve specific, and normally functional, conformational changes in acetylcholinesterase.

INTRODUCTION

Reaction of the active center of acetylcholinesterase with methanesulfonyl fluoride, which abolishes its catalytic activity, is accelerated by a variety of reversible cationic inhibitors [1–3], as is reaction of two other acylating agents, dimethylcarbamyl fluoride and acetyl fluoride [4, 5]. This has often been regarded as evidence that such cations induce a specific conformational change in the enzyme related to that which occurs during normal enzyme function [3, 6, 7]. Despite the reasonableness of this interpretation, and its appeal in connection with the mechanism of the cholinergic receptor [3, 8], there has been no proof that conformational changes are really involved. An alternative explanation involves environmental effects of a positive charge near the site of reaction [1]. The findings reported here show that non-specific forces due to normally inhibitory binding modes cause acceleration of the reaction.

It is therefore argued that acceleration is not likely to be mediated by normal rearrangements of enzyme structure.

MATERIALS AND METHODS

The partial purification of acetylcholinesterase from house fly heads followed a method described previously [9]. The enzyme moved as a single somewhat diffuse band in gel electrophoresis. In kinetic studies with substrates and reversible inhibitors the preparation acted as a single enzyme rather than a mixture, indicating that any extraneous protein did not affect rate measurements [10]. Bovine erythrocyte acetylcholinesterase was a partially purified preparation supplied by Sigma Chemical Company.

Rates of substrate hydrolysis were determined by titration of acid released with 0.01 M NaOH, and kinetic constants were measured, as described before [10]; though for routine assay of residual enzyme activity after partial inactivation, a modified Ellman procedure was employed [11].

In all cases reaction mixtures contained 0.1 M NaCl and 0.04 M $MgCl_2$ and were maintained at 26 °C. The pH was held constant in a pH-stat reaction vessel, with a N_2 stream directed over the liquid surface to prevent CO_2 absorption. Three different methods were employed in determining activity loss in the presence of methanesulfonyl fluoride. In one, aliquots of the reaction mixture were removed at intervals for assay with acetylthiocholine as substrate (Ellman method). Due to the dilution of methanesulfonyl fluoride involved, and protection of enzyme by substrate, further activity loss was negligible. With this procedure a series of samples were conveniently assayed over a period of time. In a second method the whole reaction mixture was diluted 2-fold with a solution of acetylcholine, the rate of whose hydrolysis was determined by titration with NaOH. In this case inactivation at only one time was determined for a given enzyme–inhibitor reaction mixture. The two methods gave comparable rate constants for methanesulfonyl fluoride reaction. In a third method the rate of acetylcholine hydrolysis was recorded in the presence of sufficient methanesulfonyl fluoride to inhibit the enzyme by 50% over a 5–15-min period. Hydrolysis rates at various times were then determined from tangents on the curved recording chart tracing. This continuous method was convenient when the effect of the substrate on methanesulfonyl fluoride reaction was to be determined. Rate constants were calculated from the equation $2.3 \log (v_0/v) = k$ [methanesulfonyl fluoride] t, where v_0 and v are rates of substrate hydrolysis before and after treatment of enzyme with methanesulfonyl fluoride for t min. Such plots were linear with time. The reproduceability of the determinations was good, as may be judged from the data in Figs 1 and 2, and from the standard errors for the constants based on the linear plots shown (see Results).

EXPERIMENTAL RESULTS

Inactivation rate constants for methanesulfonyl fluoride at pH 7.5 in the presence of various substrates, with either bovine erythrocyte or fly head acetylcholinesterase, are listed in Tables I and II. The effects are most simply interpreted in the case of fly acetylcholinesterase, because with this enzyme the rate-limiting step

TABLE I

METHANESULFONYL FLUORIDE INACTIVATION RATES FOR FLY ACETYLCHOLINESTERASE IN THE PRESENCE OF SUBSTRATES

The inactivation rate constant in the absence of substrate is $1.1 \cdot 10^3$ $M^{-1} \cdot min^{-1}$. K_m values and relative maximum velocities are also shown. pH 7.5, temp. 26 °C.

Substrate	Concentration ($M \times 10^3$)	K_m (M)	V	$k \times 10^{-2}$ ($M^{-1} \cdot min^{-1}$)
Acetylcholine	3.3	$4.9 \cdot 10^{-5}$	1.0	1.5
Acetylthiocholine	2.5	$4.3 \cdot 10^{-5}$	0.85	3.1
Dimethyl-*n*-propyl-aminoethyl acetate	3.0	$4.6 \cdot 10^{-5}$	1.0	5.0
Dimethyl-*n*-butyl-aminoethyl acetate	3.0	$9.9 \cdot 10^{-5}$	0.66	8.7
Diethyl-*n*-butylaminoethyl acetate	2.0	$2.5 \cdot 10^{-4}$	0.30	82.0
3-Mercaptophenyltrimethylammonium acetate	6.7	$<10^{-4}$	0.43	6.4
Phenyl acetate	7.9	$4.0 \cdot 10^{-4}$	0.92	0.40
o-Nitrophenyl acetate	3.3	—	0.6	3.4
p-Nitrophenyl acetate	3.4	—	0.5	4.0

in hydrolysis of all substrates appears to be reaction of the enzyme–substrate complex rather than the acetyl-enzyme intermediate formed from it, and therefore the concentration of the acetyl enzyme must be negligible [10]. Hence protection can be attributed to hindrance of methanesulfonyl fluoride attack at the esteratic site by the intact substrate molecule.

TABLE II

METHANESULFONYL FLUORIDE INACTIVATION RATES FOR BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE IN THE PRESENCE OF SUBSTRATES

The inactivation rate constant in the absence of substrate is $1.9 \cdot 10^2$ $M^{-1} \cdot min^{-1}$. pH 7.5, temp. 26 °C.

Substrate	Concentration ($M \times 10^3$)	K_m (M)	V	$k \times 10^{-2}$ ($M^{-1} \cdot min^{-1}$)
Acetylcholine	3.3	$2.4 \cdot 10^{-4}$	1.0	0.29
Acetylthiocholine	2.5	$1.3 \cdot 10^{-4}$	0.83	0.98
Dimethyl-*n*-propylaminoethyl acetate	3.0	$5.3 \cdot 10^{-4}$	0.63	5.0
Dimethyl-*n*-butylaminoethyl acetate	3.0	$6.2 \cdot 10^{-4}$	0.54	4.5
3-Mercaptophenyltrimethylammonium	5	$1.5 \cdot 10^{-4}$	0.3	0.30
Phenyl acetate	7.9	$1.3 \cdot 10^{-3}$	1.13	0.32
p-Nitrophenyl acetate	1.7	$4.0 \cdot 10^{-3}$	0.2	1.5
	3.4			1.3

While good substrates generally protect fly acetylcholinesterase against methanesulfonyl fluoride to varying degrees, one acetylcholine analog that is a poor substrate does not, and actually increases the rate of reaction 8-fold. These findings indicate that occupation of the esteratic site by a bound substrate molecule is not continuous. Uncharged esters protect, particularly phenyl acetate, which is more effective than even acetylcholine. Presumably such substrates necessarily overlap the esteratic site, whereas charged substrates, bound at the anionic site, do not.

The same trends are seen in erythrocyte acetylcholinesterase, though with the best substrates of this enzyme, acetylcholine, acetylthiocholine and phenyl acetate, the interpretation is less simple. Deacetylation is here a rate-limiting step in hydrolysis, and an acetyl enzyme intermediate is therefore the preponderant enzyme species at saturating substrate concentrations [12]. This intermediate cannot react with methanesulfonyl fluoride because both methanesulfonyl fluoride and substrate react with the same group in the active center [13–15]. Apart from acetyl enzyme formation, the intact substrate may sterically hinder approach of methanesulfonyl fluoride to the esteratic site as in fly acetylcholinesterase.

In the case of less rapidly hydrolyzed substrates, those with enlarged onium groups (the N-propyl and N-butyl analogs of acetylcholine), the rate of methanesulfonyl fluoride reaction is increased. Here V is relatively low and acetylation must be rate-limiting, i.e. most of the enzyme is in the form of a complex with substrate, rather than an acetyl-enzyme intermediate. By contrast those substrates containing a phenyl ring do protect against methanesulfonyl fluoride, even though V and the steady-state level of acetyl-enzyme are low. Thus, 3-mercaptophenyltrimethylammonium acetate gives protection, as does *p*-nitrophenyl acetate.

Methanesulfonyl fluoride inactivation rate constants in the presence of reversible inhibitors are listed in Tables III and IV. With erythrocyte acetylcholinesterase, a number of cations are found to give protection, including bulky symmetrical ions such as tetrabutylammonium. Hydroxy-substituted phenyltrimethylammonium ions (*ortho*, *meta* or *para*) protect the enzyme strongly, and weak protection is given by the unsubstituted parent compound. This is to be contrasted with the large accelerations brought about by choline (Table IV), alkyltrimethylammonium ions [2], or the *N*-propyl and *N*-butyl analogs of acetylcholine (Table II).

Comparison of a choline analog with its acetyl ester shows that dimethylbutylaminoethanol and dimethylbutylaminoethyl acetate produce similar rate acceler-

TABLE III

METHANE SULFONYL FLUORIDE INACTIVATION RATES FOR FLY ACTYLCHOLINESTERASE IN THE PRESENCE OF REVERSIBLE INHIBITORS

pH 7.5, 26 °C. Cation	Concentration (M)	K_i (M)	Observed $k \times 10^{-3}$ ($M^{-1} \cdot min^{-1}$)
—			1.1
Tetraethylammonium	$3.18 \cdot 10^{-3}$	$8.6 \cdot 10^{-4}$	6.7
	saturating		13.0
Tetrapropylammonium	$1.26 \cdot 10^{-4}$	—	14.0
Tetrabutylammonium	$2.70 \cdot 10^{-5}$	$1.22 \cdot 10^{-5}$	10.0
	saturating		16.0
Tetrapentylammonium	$3.07 \cdot 10^{-4}$	$9.5 \cdot 10^{-6}$	3.3
Tetraphenylarsonium	$9.83 \cdot 10^{-6}$	—	2.2
Decamethonium	$8.2 \cdot 10^{-5}$	—	8.4
N,N,N-Triethylgallamine	$3.14 \cdot 10^{-4}$	—	1.1
2-Hydroxyphenyltrimethylammonium	$1.8 \cdot 10^{-4}$	$1.4 \cdot 10^{-4}$	1.7
3-Hydroxyphenyltrimethylammonium	$1.9 \cdot 10^{-4}$	$7.2 \cdot 10^{-6}$	0.13
4-Hydroxyphenyltrimethylammonium	$1.5 \cdot 10^{-4}$	$1.7 \cdot 10^{-4}$	4.4
Proflavin	$1.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	1.2

TABLE IV

METHANESULFONYL FLUORIDE INACTIVATION RATES FOR BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE IN THE PRESENCE OF REVERSIBLE INHIBITORS

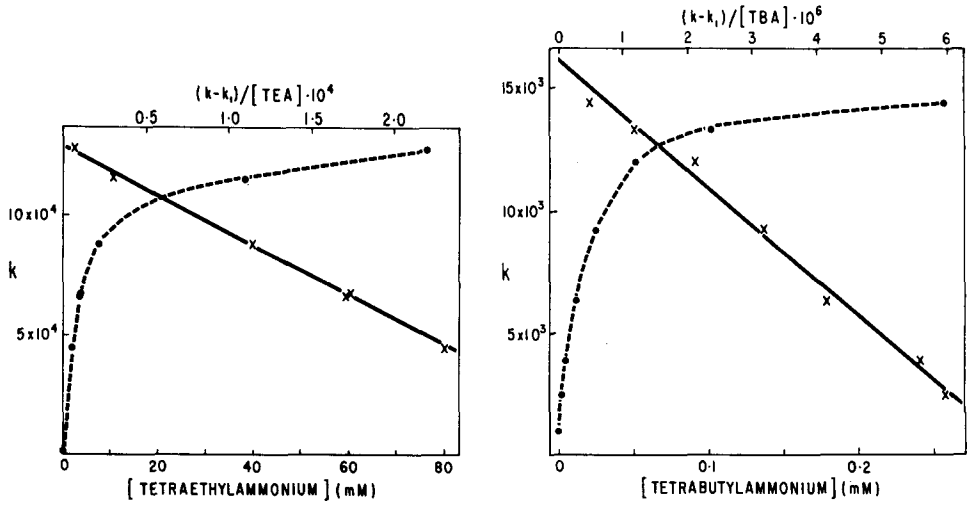
pH 7.5, 26 °C.

Cation	Concentration (M)	K_i (M)	Observed $k \times 10^{-2}$ ($M^{-1} \cdot min^{-1}$)
—			1.9
Tetraethylammonium	$6.34 \cdot 10^{-2}$	$9.9 \cdot 10^{-4}$	4.0
Tetrapropylammonium	$1.25 \cdot 10^{-4}$	$7.6 \cdot 10^{-5}$	1.3
Tetrabutylammonium	$2.83 \cdot 10^{-5}$	$5.7 \cdot 10^{-5}$	1.1
Choline	$1.8 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	10.1
N,N,N-dimethyl-*n*-butylaminoethanol	$2.5 \cdot 10^{-3}$	$3.2 \cdot 10^{-4}$	5.2
N,N,N-diethyl-*n*-butylaminoethanol	$1.04 \cdot 10^{-2}$	$6.0 \cdot 10^{-4}$	8.9
Phenyltrimethylammonium	$2.1 \cdot 10^{-3}$	$8.4 \cdot 10^{-5}$	1.4
2-Hydroxyphenyltrimethylammonium	$1.8 \cdot 10^{-4}$	$1.9 \cdot 10^{-4}$	0.14
3-Hydroxyphenyltrimethylammonium	$1.8 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$	0.075
4-Hydroxyphenyltrimethylammonium	$1.8 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$	0.21
Proflavin	$2.1 \cdot 10^{-4}$	—	3.0

ations (2.6-fold) in erythrocyte acetylcholinesterase, though neither acceleration is as large as that with choline, which in turn is only 1/4 of that given by tetraethylammonium. The latter was the most potent accelerator tested.

Protection of fly head acetylcholinesterase by reversible cationic inhibitors appears to be extremely rare, and only one example has been found, that of 3-hydroxyphenyltrimethylammonium (Table III).

The dependence of reaction rates with fly acetylcholinesterase on the concentration of tetraethyl or tetrabutylammonium ions (pH 7.5) is shown in Figs 1 and 2.



Figs 1 and 2. Effects of tetraethylammonium and tetrabutylammonium ions on the rates of methanesulfonyl fluoride reaction with fly head acetylcholinesterase. The dotted line shows the relationship between the rate constant and the cation concentration. The solid line shows its relation to $(k - k_1)$/[cation], according to Eqn 5 (Appendix). The units for this second plot are indicated above.

To determine the affinity constants for these ions, the data were replotted according to Eqn 5 in the Appendix (Inset in Figs 1 and 2). The approximate linearity in these plots indicates that a single ion activates. From a least-squares analysis, the dissociation constants are found to be $3.69 \cdot 10^{-3} \pm 0.15 \cdot 10^{-3}$ M for tetraethylammonium, and $2.21 \cdot 10^{-5} \pm 0.09 \cdot 10^{-5}$ M for tetrabutylammonium. The inactivation rate constants in the presence of a saturating concentration of the cations are $13.0 \cdot 10^{4} \pm 0.21 \cdot 10^{4}$ and $16.0 \cdot 10^{3} \pm 0.36 \cdot 10^{3}$ $M^{-1} \cdot min^{-1}$, respectively.

The effect of pH on reaction of methanesulfonyl fluoride with erythrocyte acetylcholinesterase is shown in Fig. 3. The relationship agrees closely with that of the acetylation reaction for substrate [16], both in the measured pK_a (which is 5.6 for methanesulfonyl fluoride, phenyl acetate or isoamyl acetate), and in the shape of the curve, which is abnormal in that rates do not fall as rapidly below pH 5.5 as expected for a single ionization.

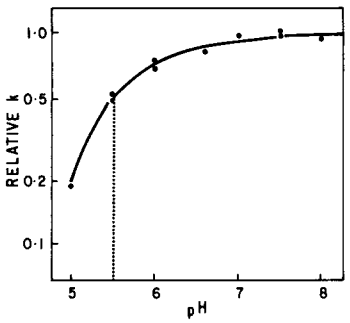


Fig. 3. Effect of pH on the rate of methanesulfonyl fluoride reaction with bovine erythrocyte acetylcholinesterase.

DISCUSSION

A 2-fold correlation comes to light among the observations on cationic substrates and substrate analogs. Pertinent data summarized in Table V show that activation of methanesulfonyl fluoride reaction is associated with weakened substrate binding (relative to that of the parent alcohol) and with reduced maximum velocities. This is explained if all cationic substrates initially bind in the anionic site and if the ester group of the substrate then makes intermittent contact with the esteratic site. Frequency of contact presumably depends on the overall goodness of fit between the substrate and the active center. Weakened interaction at the esteratic site would necessarily have three effects: it would reduce the catalytic rate, specifically the rate of acetylation, reduce substrate affinity, and weaken protection against methanesulfonyl fluoride, which attacks the esteratic site without overlapping the anionic site. Thus a good substrate such as acetylcholine tends to involve the entire active center in its attachment, while poor substrates involve only part.

These trends are most apparent in a comparison of the two enzymes. With bovine acetylcholinesterase enlargement of the onium group of acetylcholine, by substitution of a methyl group by propyl or butyl, greatly reduces the acetylation rate and results in promotion of methanesulfonyl fluoride reaction (Table II). It is safe to say that with this substrate acetylation is much slower than with acetylcholine,

TABLE V

SUMMARY OF FINDINGS ON ACETYLCHOLINE AND CHOLINE ANALOGS ($R_3N^+C_2H_4OCOCH_3$ AND $R_3N^+C_2H_4OH$): AFFINITIES, SUBSTRATE ACTIVITIES, AND EFFECTS ON METHANESULFONYL FLUORIDE REACTION

As noted in the text the rate limitation for acetylcholine hydrolysis by erythrocyte acetylcholinesterase (AChE) is in the deacetylation step. Hence the affinity of this substrate is derived from data on the related dimethylaminoethyl acetate (see ref. 21); maximum velocity measurements with other substrates give underestimates of the reduction in the rates of acetylation, as shown in the column second from the right. Affinity values are taken from ref. 21. Me, methyl; Et, ethyl; Bu, butyl.

Enzyme	R_3	Relative affinity (ester)	Relative affinity (alcohol)	Affinity of ester / Affinity of alcohol	Relative acetylation rate (ester)	k/k_0 (methanesulfonyl fluoride)
Fly AChE	Me_3	1.0	1.0	7.3	1.0	0.14
	Me_2Bu	0.5	1.5	2.4	0.66	0.79
	Et_2Bu	0.2	4.0	0.35	0.30	7.5
Bovine	Me_3	1.0	1.0	1.7	1.0	0.15
AChE	Me_2Bu	1.0	3.3	0.51	<0.25	2.4
	Et_2Bu	0.7	1.7	0.72	<0.08	—

because with the latter acetylation is faster than deacetylation, and a substantial reduction in V occurs only if the acetylation rate becomes rate-limiting, i.e. slower than deacetylation, which is a common step with all the substrates. With the same substrates and fly acetylcholinesterase there is little or no reduction in maximum velocity, which in this case is a measure of a rate-limiting acetylation step for all acetyl esters. Accordingly the substrates protect the enzyme against methanesulfonyl fluoride. With further substitution, however, in the N,N,N-diethylbutyl analog, a more substantial reduction in hydrolysis rate is attained, and at the same time methanesulfonyl fluoride attack is promoted. In all cases larger substrates produce greater accelerations; hence the behavior cannot depend on interference with the approach of methanesulfonyl fluoride to the active center.

That the effects are not determined by the chemical properties of the esters, but by interactions between them and the enzyme active center, is proven by the case of the dimethyl butyl analog of acetylcholine (Table V). With erythrocyte acetylcholinesterase, its acetylation rate is very much lower than for acetylcholine. In consequence the substrate is more weakly bound than its choline analog, and it increases the rate of methanesulfonyl fluoride reaction. The opposite result is found with fly acetylcholinesterase, where the deacetylation rate is not greatly reduced (it is 2/3 that for acetylcholine) and where, as a result, the substrate is more strongly bound than its choline analog, and reaction with methanesulfonyl fluoride is slightly retarded rather than promoted.

The rate-limiting step in acetylcholine hydrolysis by erythrocyte acetylcholinesterase is deacetylation, but switches to acetylation when the trimethylammonium group of the substrate is altered, either by enlargement as in the examples here, or by substitution of hydrogen for N-methyl groups, as in dimethylaminoethyl acetate, methylaminoethyl acetate, and aminoethyl acetate [12, 17]. A simple explanation may now be given this behavior, in that a misfit at the anionic site leads to misalignment of the rest of the substrate molecule and failure to adhere to the esteratic site. Observations on the entropy of substrate binding [17] may be accounted for in

the same way. While the entropy change for complex formation has a large negative value with acetylcholine, it is positive with the H-substituted substrates listed above, and increasingly so with each additional substitution of H for methyl. Both ends of the acetylcholine molecule become simultaneously attached to the enzyme, and this high degree of ordering is associated with a negative entropy. The other substrates are linked mainly at one end (the ammonium group) and with increasing substitution attraction for the esteratic site should gradually become less strong and the maximum velocity lower; at the same time increasing freedom of substrate movement in the complex produces a more positive entropy.

Perhaps the greatest interest of the present findings lies in their bearing on cation acceleration of methanesulfonyl fluoride reaction. Three different hypotheses have been advanced to explain this phenomenon. The first, and simplest, is that activation is due to environmental effects of a positive charge near the reaction site [1]. In a second, an induced-fit mechanism, a cation such as that in the substrate acetylcholine induces a specific conformational change in the protein, which mobilizes catalytic groups in the esteratic site [6, 7]. According to a third interpretation the phenomenon is related to the regulation of enzymes by small molecules [3, 18]. This suggestion was made in order to explain certain similarities in the binding of cations to the enzyme and the cholinergic receptor of nerve preparations. Cations were regarded as being bound in either of two ways: in one, characteristic of substrates, the molecule overlaps the esteratic site, and in another, characteristic of agonists acting upon the cholinergic receptor, the molecule interacts, not with the esteratic site, but with another region adjoining the anionic site; this interaction alters the protein structure so as to accelerate methanesulfonyl fluoride reaction [18]. This second type of interaction was thought to be related to the normal function of the enzyme molecule and was probably suggested by the possibility that acetylcholinesterase and the cholinergic receptor protein are identical. However it is now fairly certain that this is not so, and that the two activities are associated with completely distinct proteins (see ref. 19). The application of the hypothesis to acetylcholinesterase is therefore dubious, since cations compete with the substrate and protect the enzyme against inhibitors larger than methanesulfonyl fluoride, such as organophosphates, and since no clear evidence of normal regulatory activity exists, aside from inhibition.

The induced-fit hypothesis, in turn, appears to be ruled out by the following considerations, some of which apply also to the former hypothesis:

(1) Cation activation is not essential for efficient substrate hydrolysis, as shown by the case of neutral substrates, such as phenyl acetate which is hydrolyzed more rapidly than acetylcholine [12], and the carbon analog of acetylcholine, 3,3-dimethylbutyl acetate, hydrolyzed half as fast [20].

(2) Good substrates, which are most likely to induce specific conformational changes promoting catalysis, protect the enzyme against methanesulfonyl fluoride, while those poor substrates with enlarged onium groups, though less likely to induce such conformational changes, accelerate reaction with methanesulfonyl fluoride. It is understandable that poor substrates should offer less protection than good substrates, because their acyl groups may interact less strongly with the esteratic site; but activation is unexpected if it results from a precise, functional conformational change induced by the substrate.

(3) Two distinct modes of ligand attachment have been demonstrated in

acetylcholinesterase [21]. In one, which is characteristic of substrates and is therefore thought to involve "specific", or productive, binding forces, weaker binding results from enlargement of the onium group in acetylcholine analogs. In a second, characteristic of reversible inhibitors and therefore "non-specific" or non-productive, enlargement of the onium group in choline analogs produces stronger binding. The more firmly substrates are held by these non-specific forces the lower becomes their maximum hydrolysis rate, and this is the criterion of non-productive binding forces, as used here. A wide variety of ligands, almost all of which are bound in the non-specific mode, are now found to accelerate reaction with methanesulfonyl fluoride (Tables III and IV). Large symmetrically substituted cations such as tetrapentylammonium and tetraphenylarsonium ions, which accelerate sulfonylation of fly acetylcholinesterase, cannot possibly occupy the same locus as the ammonium group of acetylcholine, since they would then necessarily overlap the esteratic site and protect the enzyme. It cannot be said that they are bound at some distant allosteric site, since they protect the enzyme against larger irreversible inhibitors such as organophosphates. They must therefore be bound on the periphery of the anionic site where they obstruct entry of the larger inhibitors. In the case of erythrocyte acetylcholinesterase, rate enhancement is even seen with proflavin, a molecule bearing little resemblance to acetylcholine. Thus non-specific ligand interactions depress the rate of acylation by substrates but accelerate the rate of sulfonylation by methanesulfonyl fluoride.

(4) In fly acetylcholinesterase two anionic sites are easily demonstrated, and attachment of any cationic inhibitor to the second site, in the presence of a cationic substrate bound at the first, diminishes catalytic efficiency [10, 22]. In the case of methanesulfonyl fluoride reaction, binding of a second cation, which must occur at the highest concentrations of tetraethyl and tetrabutylammonium ions employed (Figs 1 and 2), does not depress the rate. These concentrations, $7.8 \cdot 10^{-2}$ and $2.5 \cdot 10^{-4}$ M, are much above K_i' values for addition of the cations to the enzyme–substrate complex, which are $5.7 \cdot 10^{-3}$ and $7.4 \cdot 10^{-5}$ M, respectively (Hellebrand, K., and Krupka, R. M., unpublished). In these cases the concentration dependence is not as simple as might have been expected. The dissociation constants for binding of tetraethylammonium to the free enzyme (K_i) and the complex with acetylcholine (K_i') are $8.60 \cdot 10^{-4} \pm 0.11 \cdot 10^{-4}$ and $5.68 \cdot 10^{-3} \pm 0.40 \cdot 10^{-3}$ M, respectively (ref. 21 and Hellebrand, K., and Krupka, R. M., unpublished) and that for binding in enhancement of methanesulfonyl fluoride reaction is $3.69 \cdot 10^{-3} \pm 0.15 \cdot 10^{-3}$ M. The discrepancy between the latter and K_i appears to be too large to be ascribed to experimental error. The same pattern is seen with tetrabutyl ammonium, where $K_i = 1.22 \cdot 10^{-5} \pm 0.19 \cdot 10^{-5}$ M, $K_i' = 7.41 \cdot 10^{-5} \pm 0.56 \cdot 10^{-5}$ M, and the constant for methanesulfonyl fluoride enhancement is $2.21 \cdot 10^{-5} \pm 0.09 \cdot 10^{-5}$ M. The explanation could be that acceleration actually results from addition of the cation to the second, i.e. inhibitory, anionic site.

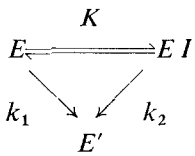
These considerations do not rule out the occurrence of functional conformational changes in the enzyme following substrate binding, but they do indicate that any such mechanism is probably not involved in the cation enhancement of sulfonylation. Serious consideration should therefore be given to the possibility that the true explanation is simply a field effect of a positive charge near the leaving fluoride anion.

As a matter of lesser interest, the observations also point to the existence of a phenyl-binding region closely associated with the esteratic site. This is shown by protection against methanesulfonyl fluoride afforded by phenyl acetate and its derivatives (Tables I and II). In the case of erythrocyte acetylcholinesterase and the substrate 3-mercaptophenyl-trimethylammonium acetate, protection is given even though the maximum velocity is lower than that of other cationic substrates that accelerate methanesulfonyl fluoride attack. In this case the quaternary nitrogen group and the phenyl ring are probably both bound, the latter close to the esteratic site, impeding methanesulfonyl fluoride entry, but not so as the present the acetyl group to the enzyme in an orientation leading to rapid hydrolysis.

With fly acetylcholinesterase acceleration by reversible cationic inhibitors is more widespread than with erythrocyte acetylcholinesterase, and occurs not only with bulky symmetrical ammonium ion derivatives, which protect the latter enzyme, but even with some phenylammonium ions. The single observed exception is 3-hydroxyphenyltrimethylammonium ion, which protects, even though its *ortho* and *para*-substituted analogs accelerate. This behavior is probably related to that in erythrocyte acetylcholinesterase, where the *meta* compound gives stronger protection than its isomers. Affinity studies showed that the hydroxyl group of the meta compound forms a strong attachment with some group in the enzyme, assumed to be a constituent of the active center essential in catalysis [23]. The present observations support this idea. They also suggest either that the phenyl-binding region in the insect enzyme is not as closely associated with the esteratic site as in erythrocyte acetylcholinesterase, or that a phenyl ring can occupy another area more remote from the esteratic site. Only bonding at the 3-hydroxy position enforces overlap with the esteratic site.

APPENDIX

Enzyme (E) inactivation by methanesulfonyl fluoride in the presence of a reversible cationic inhibitor (I) may be represented as follows:



(1)

where EI is the complex of E and I, and E' is enzyme that has reacted with methanesulfonyl fluoride (MSF). The rate of activity loss is given by

$$\frac{dE'}{dt} = k_1[\text{MSF}]\,[E] + k_2[\text{MSF}]\,[EI] \tag{2}$$

$$= \frac{k_1 + k_2[I]/K}{1 + [I]/K}\,[E]_0 \cdot [\text{MSF}] \tag{3}$$

$$= k[E]_0 \cdot [\text{MSF}] \tag{4}$$

where k is the experimentally measured reaction constant for methanesulfonyl fluoride. From Eqns 3 and 4 it can be shown that,

$$k = k_2 - K(k - k_1) / [I] \quad (5)$$

The values of k_2 and K may be determined from plots according to Eqn 5; k_1 is already known, being the rate constant in the absence of I.

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