The Relationship of Enzyme Inhibitory Activity to the Structure of *n*-Alkylphosphonate and Phenylalkylphosphonate Esters*

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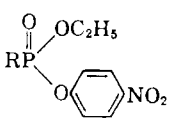
This study is concerned with a comparison of the inhibitory activities of *O*-*p*-nitrophenyl *O*-ethyl alkyl and phenylalkylphosphonates toward human red cell acetyl cholinesterase, chymotrypsin, and trypsin. In the series of alkylphosphonates, the ability to inactivate cholinesterase decreases markedly from the propyl to the butylphosphonate. A further increase in the length of the alkyl chain causes little change in anticholinesterase activity until the decylphosphonate is reached, when a further decrease occurs. The presence of the phenyl group close to the phosphorus depresses anticholinesterase activity, and this decrease in activity is not completely overcome until there are three carbon atoms between the aromatic ring and the phosphorus. The antichymotrypsin activity attains an optimum with the heptyl and phenylpropylphosphonates respectively; the peak antitryptic activity is obtained with the hexylphosphonate in the alkylphosphonate series and the phenylpropylphosphonate in the phenylalkylphosphonate series. These results support the idea that the elements of structure responsible for facilitating the inactivation of a given enzyme by organophosphorus inhibitors are the same as those responsible for facilitating the activity of substrates for the same enzymes.

The interest in organophosphorus compounds as both pesticides and potential chemical warfare agents has led to numerous studies attempting to relate their chemical structure to their activity as inhibitors of acetyl cholinesterase (Fukuto, 1957; O'Brien, 1960). In addition to cholinesterase, a number of other enzymes of presumed physiologic and pathologic importance can also be inactivated by these same substances. Organophosphorus compounds capable of more or less specifically inhibiting such enzymes *in vivo* should be extremely valuable in many ways. Such inhibitors do not exist at present, and before they can be made more must be known about the relationship of the structure of organophosphorus inhibitors to their ability to inhibit enzymes other than acetyl cholinesterase. Relatively few investigators have interested themselves in this aspect of the problem (Aldridge, 1953; Mounter *et al.*, 1957; Ooms, 1961). It was therefore the purpose of this study to investigate the ability of a series of alkyl and phenylalkylphosphonate esters of the general structure:



to inhibit particularly chymotrypsin, but also trypsin and human red cell cholinesterase.

The viewpoint guiding the choice of compounds for investigation was the generally accepted one that the organophosphorus compounds inhibit by phosphorylating the active site of the enzyme in a manner comparable to the way in which the substrate acylates the same site. Thus, as Aldridge (1953) pointed out and others have confirmed (Fukuto, 1957; Heath, 1960; Ooms, 1961), one would expect that elements of structure which contribute to a substrate's being a "good" substrate would also contribute to the organophosphorus compound's being a "good" inhibitor.

On this basis, two series of phosphonate esters were chosen for study; in the first R was a straight alkyl chain whose length was increased regularly from 3 carbons to 10 except that the 9 carbon phosphonate was not synthesized. These compounds were chosen in order to correlate their inhibitory activity for chymotrypsin and trypsin with the activity of the analogous straight-chain fatty acid esters of hydroxybenzoic acid as substrates for the same enzymes (Hofstee, 1957, 1959).

In the second series, R was the phenylalkyl group in which the number of carbon atoms in the straight chain between the phosphorus and the benzene ring varied between zero and four. The reason for study of these compounds is the well-known ability of chymotrypsin to hydrolyze particularly easily substrates containing aromatic amino acids in the proper position.

EXPERIMENTAL

Material.—The *O*-*p*-nitrophenyl *O*-ethyl phosphonates were synthesized as previously described (Fukuto and Metcalf, 1959), except that, in the case of the pentyl, heptyl, octyl, decyl, phenylethyl, phenylpropyl, and phenylbutylphosphonates, oxalyl chloride (Pelchowicz, 1961) instead of PCl_5 was used to form the corresponding phosphonochloridates, and that the styrylphosphonate was prepared from styrene by the method of Anisimov (1954). The physical constants and elemental analyses of those phosphonates not previously reported (Fukuto and Metcalf, 1959) are seen in Table I.

Single lots of crystalline salt-free chymotrypsin (Worthington, Lot No. 66-65) and of crystalline trypsin (Worthington, Lot No. 717) were used throughout. Hemolyzed human red cells were the source of acetyl cholinesterase. Heparinized blood was collected into 0.15 M NaCl and centrifuged, and the cells were washed two times with saline. The cells were brought to the original volume of the blood with 0.15 M NaCl and then lysed with six volumes of 0.01% saponin in distilled water. If the hemolysate was not used immediately it was stored in the frozen state at −20°.

Procedures.—The assay of acetyl cholinesterase was that described by Michel (1949). Chymotrypsin and trypsin were assayed essentially the same way except that a calcium-Tris-imidazole buffer was substituted for the veronal phosphate buffer used by Michel, and the

* This material has been reviewed by the Office of the Surgeon General, Department of the Army, and there is no objection to its presentation and/or publication. This review does not imply any indorsement of the opinions advanced or any recommendation of such products as may be named.

TABLE I
PHYSICAL CONSTANTS OF O-ETHYL-O-p-NITROPHENOL ALKYLPHOSPHONATES

Alkyl Group	Distillation[a] Temp.	Distillation[a] Pressure (mm Hg)	$N_D^{25°}$	Empirical Formula		Analysis Calcd.	Analysis Found
Phenylethyl	139°	0.02	1.5577	$C_{16}H_{18}NO_5P$	C	57.31	57.46
					H	4.37	5.60
					N	4.18	3.90
Styryl	(m.p. 68–69° from benzene-heptane)			$C_{16}H_{16}NO_5P$	C	57.66	57.92
					H	4.84	4.79
					N	4.20	4.13
Phenylpropyl	139°	0.02	1.5402	$C_{17}H_{20}NO_5P$	C	58.34	58.44
					H	5.73	5.84
					N	4.02	4.16
Phenylbutyl	118°	0.005	1.5249	$C_{18}H_{22}NO_5P$	C	59.45	59.46
					H	6.06	5.80
					N	3.85	3.62
n-Heptyl	139°	0.025	1.5055	$C_{16}H_{24}NO_5P$	C	54.71	54.58
					H	7.29	7.59
					N	4.26	4.40
n-Octyl	139°	0.020	1.5046	$C_{16}H_{26}NO_5P$	C	56.00	55.80
					H	7.58	7.81
					N	4.08	4.34
n-Decyl	145°	0.01	1.4945	$C_{18}H_{30}NO_5P$	C	58.22	57.72
					H	8.08	8.40
					N	3.77	3.99
					P	8.36	7.76

[a] Distillation temperatures listed are the minimum necessary to distill product on a falling film molecular still with a 1.0 cm path.

final volume was 3.0 ml. For both trypsin and chymotrypsin, the *p*H of the assay mixture was 8.0, and the concentration of calcium was 0.02 M, of Tris 0.01 M, and of NaCl 0.08 M. For the assay of trypsin, the imidazole concentration was 0.001 M and 0.02 M tosyl-L-arginine methyl ester (TAMe) was the substrate, whereas for chymotrypsin the imidazole concentration was 0.004 M, and 0.02 M acetyl-L-tyrosine ethyl ester was the substrate. For both trypsin and chymotrypsin, a linear relationship was demonstrated between the enzyme concentration and the fall in *p*H (ΔpH) when the decrease in *p*H was not over 2 *p*H units. The drop in *p*H was linear from 0.25 μg to 3 μg of trypsin per 3 ml of final reaction mixture (Fig. 1) and from 0.25 μg to 1.6 μg of chymotrypsin per 3 ml of final reaction mixture (Fig. 2).

Varying concentrations of inhibitor were dissolved in acetone and 0.1 ml of the desired concentration was added to 1.9 ml of the enzyme buffer mixture at 25°. In each case, the buffer was the same as that used in the assay of the respective enzyme. The concentration of chymotrypsin to which the inhibitor was added was 50 μg/ml. At the end of 15 minutes the inhibitor-chymotrypsin mixture was diluted 1:25 with distilled water and 1 ml of this was transferred to 2 ml of the substrate-buffer mixture. The final concentrations of the buffer and substrate in the assay were as given previously. The 1:25 dilution was made in order to ensure that the action of the inhibitor on the enzyme was completely stopped.

The concentration of trypsin to which the inhibitor was added was 1 μg/ml in the same buffer as used for assay. At the end of 15 minutes the reaction was stopped by the addition of the substrate. The reaction of the inhibitor on cholinesterase was stopped at the end of 15 minutes by the addition of 0.1 ml of 4% acetylcholine chloride.

In each case the logarithm of the per cent residual enzyme activity was plotted against the concentration of inhibitor (Aldridge and Davison, 1952). The resulting straight line was used to obtain I_{50}, the molar concentration of inhibitor required for 50% inactivation when the inhibitor and enzyme were in contact for 15 minutes at 25°. For convenience, the I_{50} was transformed into its negative logarithm, the pI_{50}. When the inhibitor is in great excess over the enzyme, I_{50} is inversely proportional to the second order reaction constant (O'Brien, 1960, p. 78). In the case of the inhibition of chymotrypsin by the hexyl, heptyl, phenylethyl, styryl, and phenylpropylphosphonates, the inhibitor and enzyme concentrations were of the same order of magnitude. In these instances, second order rate constants, k_2, were determined by assaying for residual chymotrypsin activity, x, at varying times in a mixture of initial enzyme concentration, b, and initial inhibitor concentration, a. The slope of the straight line obtained when plotting t against log $\frac{a-x}{b-x}$ was employed to calculate k_2. With the hexyl and heptyl phosphonates, the residual enzyme activity was determined by means of the assay technique just described; with the styryl, phenylethyl, and phenylpropylphosphonates, residual enzyme activity was assayed by means of the *p*H stat. The second order rate constants were transformed into pI_{50}, for purposes of comparison, by means of the relation $k_2 = 0.0464/I_{50}$ (O'Brien, 1960, p. 78).

The first order hydrolysis constants were determined at 37° in 0.066 M phosphate buffer, *p*H 8.3, as previously described (Fukuto and Metcalf, 1959). Only the hydrolysis rates of those compounds which had not been determined before (Fukuto and Metcalf, 1959) were measured here. However, in the case of the *O*-*p*-nitrophenyl *O*-ethyl *n*-pentylphosphonate, the hydrolysis constant was measured anew and found to be 3.75×10^{-4} min.$^{-1}$, compared to the value of 3.62×10^{-4} min.$^{-1}$ found by Fukuto and Metcalf (1959).

RESULTS

The results obtained with the alkylphosphonates are summarized in Figure 3. As is seen in the uppermost curve, there is a very small decrease in the rate of hydrolysis from the 3 carbon to the 7 carbon phos-

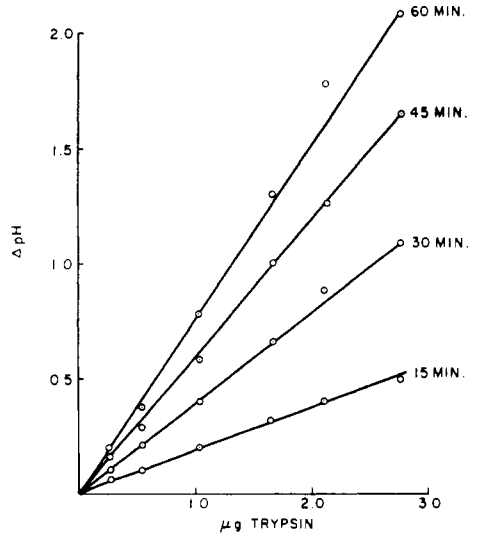


FIG. 1.—The linear relationship between the fall in *p*H (Δ*p*H) and the amount of trypsin per 3 ml of reaction mixture at various times of incubation at 25°.

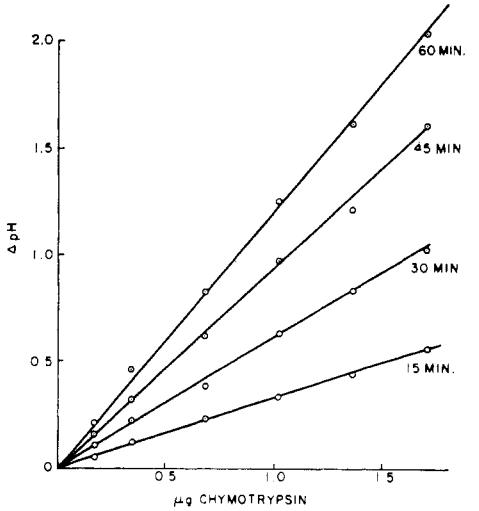


FIG. 2.—The linear relationship between the fall in *p*H (Δ*p*H) and the amount of chymotrypsin per 3 ml of reaction mixture at various times of incubation at 25°.

phonate, but a much more distinct fall in hydrolysis rate with those inhibitors containing 8 and 10 carbon atoms. The inhibitory activity against acetyl cholinesterase decreases decidedly from the propylphosphonate to the butylphosphonate with relatively little change after that until the decylphosphonate is reached. On the other hand, the shape of the third curve, portraying the behavior of these same compounds with chymotrypsin, is quite different from the upper two. With chymotrypsin, the inhibitory activity increases over a 1000 fold from the 3 carbon to the 7 carbon phosphonate, and then as the alkyl chain is further lengthened decreases almost as sharply.

Although these compounds were much less active against trypsin, their behavior toward this enzyme paralleled that toward chymotrypsin, except that with trypsin there was a maximum in inhibitory activity with the 6 carbon phosphonate instead of the 7 carbon compound. Ooms (1961) studied many compounds, including the *O*-*p*-nitrophenyl *O*-isopropyl alkylphosphonates, and investigated the way in which their inhibitory activity against bovine red cell cholinesterase, chymotrypsin, and trypsin, as well as other en-

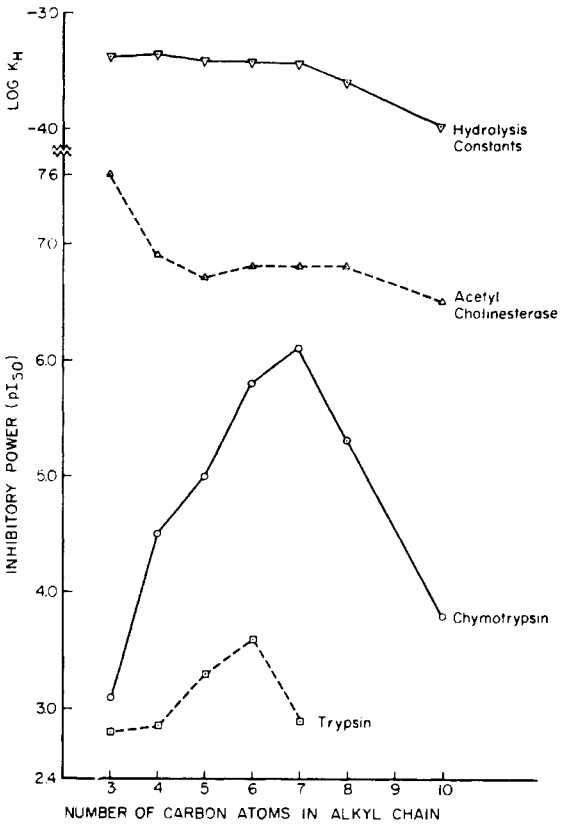


FIG. 3.—Hydrolysis constants and inhibitory activities (pI_{50}) toward acetyl cholinesterase, chymotrypsin, and trypsin, plotted against the number of carbon atoms in the alkyl chain of a series of *O*-*p*-nitrophenyl-*O*-ethyl *n*-alkylphosphonates. The hydrolysis constants of the propyl and butylphosphonates were taken from Fukuto and Metcalf (1959).

zymes, varied when the length of the alkyl chain was increased from 2 to 5 carbon atoms. The results just described agree with those of Ooms in terms of the effect of variation of chain length on the relative inhibitory activities against the several enzymes, but the inhibitory activities of the *O*-*p*-nitrophenyl *O*-ethyl alkylphosphonates studied here were distinctly greater than those of the corresponding *O*-*p*-nitrophenyl *O*-isopropyl alkylphosphonates investigated by Ooms.

Figure 4 summarizes the behavior of the phenylalkylphosphonates. The uppermost curve demonstrates a decided decrease in the rate of hydrolysis from the phenyl to the benzylphosphonate; with further lengthening of the distance between the phenyl group and the phosphorus, this fall in rate is very small. The anticholinesterase activity of the phenylphosphonate is relatively low, and that of the benzyl phosphonate is still lower, but once there is more than one carbon between the phenyl group and the phosphorus the apparent steric interference of the phenyl group is progressively overcome.

There is a steady increase in antichymotrypsin activity from the phenyl to the phenylpropylphosphonate, but the addition of one more carbon atom makes the phenylbutylphosphonate 2200 times less active than the phenylpropylphosphonate in this regard.

The same compounds show a quite different pattern of inhibition against trypsin. There is a 30-fold fall in activity from the phenyl to the phenylethylphosphonate, whereas the addition of one carbon atom increases the inhibitory activity of the phenylpropylphosphonate 20 times, and the addition of one more carbon makes the

antitrypsin activity of the phenylbutylphosphonate 120 times less than that of the phenylpropylphosphonate.

A further effect of small changes in structure can be seen when we compare the activities of the phenylethylphosphonate with the corresponding styrylphosphonate, the unsaturated analog of phenylethylphosphonate. The introduction of the double bond between the α and β carbon atoms increased the hydrolysis rate 4 times, the anticholinesterase activity 1.5 times, and the antichymotrypsin activity 1.2 times, whereas the antitrypsin activity was increased almost 90-fold compared to the phenylethylphosphonate. Considering the marked effect of the styryl compound on the inhibition of trypsin, it would be of interest to isolate the *cis* and *trans* isomers and compare their inhibitory activities; this has not been done.

Discussion

The results described here, as well as those described by others (Aldridge, 1953; Ooms, 1961), show in unequivocal fashion that quite small changes in the structure of the compound can cause quite large changes in the inhibitory activity toward a given enzyme. Furthermore, the results also make it clear that the manner in which the inhibitory activity varies with changes in the structure of the inhibitor differs quite markedly with the different enzymes. Thus, it would appear that, at least with certain enzymes and certain organophosphorus compounds, the requirements for optimum inhibitory activity are approximately as rigid as the requirements for optimum substrate activity.

A number of investigators have shown for acetyl cholinesterase (Aldridge and Davison, 1952; Fukuto, 1957; Heath, 1961; Ooms, 1961) as well as other enzymes (Aldridge, 1953; Ooms, 1961) that a distinct similarity in structure exists between the substrate and certain of the organophosphorus inhibitors. However, Ooms (1961) has pointed out that when one compares the reaction of the esterase with the substrate on the one hand, and with the inhibitor on the other, one is comparing two processes. In the action of the enzyme on the substrate, one has to consider not only the binding of the substrate to the enzyme and its subsequent acylation but also the deacylation step. For the action of the enzyme on the organophosphorus inhibitor, one has to consider solely the combination of inhibitor and enzyme and the subsequent phosphorylation. Only where the acylation reaction is slow compared to the deacylation reaction, and thus is rate determining, will there exist a basis for the comparison of the two processes (Ooms, 1961). That this caution is warranted is seen from an examination of the present data.

Hofstee (1959) concluded from his studies on the fatty acid ester substrates of chymotrypsin that the acylation reaction was rate limiting. Thus, at least in this instance, this basic requirement for a valid comparison between substrate and inhibitor activities is met. Hofstee (1957, 1959) found that, when the maximum enzyme velocity (V_m) was plotted against the number of carbon atoms in the fatty acid of the ester, a sharp optimum was obtained at the 7 carbon fatty acid with chymotrypsin and at the six carbon fatty acid with trypsin. In directly comparing substrate with inhibitory activity, if one considers the carboxyl group of the ester to correspond to the phosphoryl group of the inhibitor, one would expect that the *n*-hexylphosphonate would give the optimum inhibitory activity against chymotrypsin, whereas the fact is that the *n*-heptylphosphonate is the best inhibitor. On the same basis, one would expect the pentylphosphonate to be the best inhibitor of trypsin, whereas the *n*-hexylphosphonate inhibits trypsin the best. These

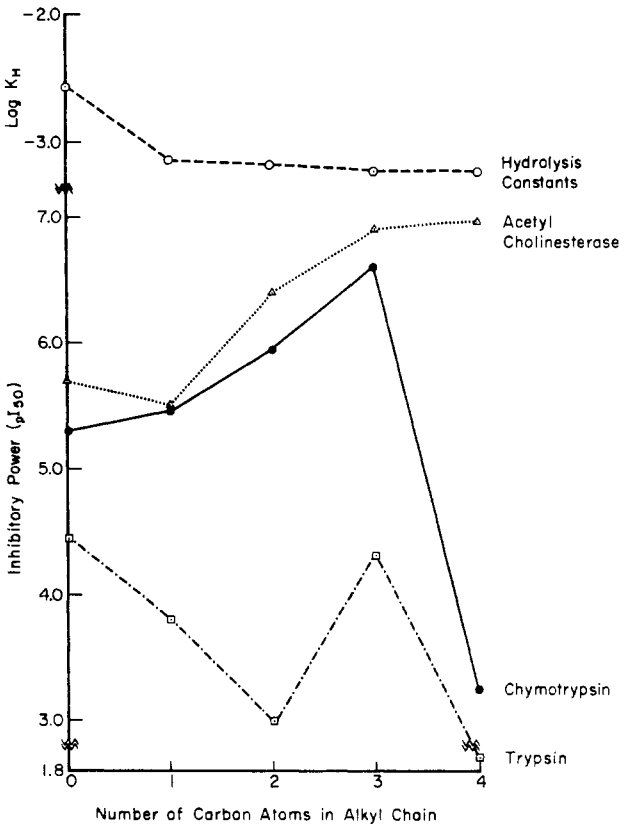


FIG. 4.—Hydrolysis constants and inhibitory activities (pI_{50}) toward acetylcholinesterase, chymotrypsin, and trypsin, plotted against the number of carbon atoms in the alkyl chain of the *O*-*p*-nitrophenyl-*O*-ethyl phenylalkylphosphonates. The hydrolysis constants of the phenyl and benzylphosphonates were taken from Fukuto and Metcalf (1959).

discrepancies between expectation and fact may, however, reflect an incorrect criterion for the "goodness" of substrate action, rather than anything real. Hofstee (1959) showed for chymotrypsin, that if one plots V_m/K_m[1] against the number of carbons in the fatty acid, the optimum substrate activity is shifted to the ester of the 8 carbon fatty acid, corresponding quite precisely to the optimum inhibitory activity found with the *n*-heptylphosphonate. V_m is the maximum velocity of substrate splitting when the enzyme is saturated with substrate; for the system Hofstee studied, V_m/K_m is the reaction constant of the first order reaction between enzyme and substrate that is approximated at low substrate concentrations (Hofstee, 1952) when the enzyme is not saturated. With respect to chymotrypsin, at the low concentrations of inhibitor used in the cases of the *n*-heptyl and *n*-octyl phosphonates it is impossible that the enzyme was saturated. It would therefore seem that in this situation V_m/K_m is the better measure to compare substrate activity to inhibitor activity.

Whether this discrepancy between substrate and inhibitory activities with trypsin would also disappear if V_m/K_m were used in place of V_m as the measure of enzyme activity is not known. In the phenylalkyl series, the peak inhibition was given by the phenylpropylphosphonate; on the basis that the aromatic amino acids are the best substrates, we expected the phenylethylphosphonate to be the best inhibitor. However, in the hydrolysis of acetyl-L-tyrosine ethyl ester by chymotrypsin, there is evidence that the break-

[1] K_m is the Michaelis constant.

down of the chymotrypsin acetyl-L-tyrosine intermediate is rate limiting (Dixon and Neurath, 1957; Hofstee, 1959, p. 187). If true, this would prohibit a direct comparison between the compound giving the optimum in substrate activity and the inhibitor giving optimum activity for these series of compounds.

The present results do show, however, that although a direct and detailed comparison of the effect of structure on substrate and on inhibitor activity must await further information, one can successfully use the expectation of a relation between them as a general guide in the search for more specific organophosphorus inhibitors. The results also make clear that in this search it will not be sufficient to find a structure which increases activity toward a given enzyme, but, in addition, the molecule will have to contain structural features which decrease reactivity toward other enzymes. This is given point by the demonstration that although the changes in length of the carbon chain of the alkylphosphonates had a distinct effect on the inhibitory activity toward chymotrypsin and trypsin, the inhibitory activity against acetyl cholinesterase was high with all of these compounds and was relatively little affected by these changes in structure. As a corollary of this, all of the compounds studied here have a high acute mammalian toxicity.

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The Mechanism of Action of Acetylcholinesterase: Substrate Inhibition and the Binding of Inhibitors

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The effects of the cationic inhibitors, tetramethylammonium and choline, on the inhibition of acetylcholinesterase by its substrate have been investigated. All the experimental results can be explained by a mechanism involving the formation of (*a*) an unreactive complex between the acetyl enzyme and the substrate and (*b*) a complex, at least partially reactive, between the acetyl enzyme and the inhibitor. This mechanism predicts a small noncompetitive component in inhibition by such cationic substances. It also predicts that plots of the reaction velocity against the substrate concentration at various concentrations of an inhibitor should form a family of curves having certain characteristic features with regard to (1) the optimum substrate concentration, (2) the reaction velocity at the optimum substrate concentration, and (3) the ability or inability of the inhibitor to increase the reaction rate at substrate concentrations above the optimum. The family of curves can be considered to fall into one of several broad classes, the behavior observed depending on the particular enzyme, substrate, inhibitor, and experimental conditions. It is shown that the experimental observations of this study and others conform to the predictions. In contrast to this behavior, the inhibition by *cis*-2-dimethylaminocyclohexanol, which contains a large noncompetitive component, can only be explained if the acetyl enzyme-inhibitor complex is unreactive.

It has long been known that the rate of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine declines at high concentrations of the substrate. Recently it was shown that this substrate inhibition is probably due to the formation of a binary complex between a molecule of acetylcholine and the acetyl enzyme (Krupka and Laidler, 1961). The latter is a reaction intermediate derived from the Michaelis complex; it is formed when the acetyl group of the substrate is transferred from choline to a group at the active center of the enzyme (Wilson, 1960). Accordingly, the hydrolysis of the substrate may be represented as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + A$$
$$\qquad\qquad\qquad\qquad\quad \underset{C}{+}$$
$$EA + S \rightleftharpoons EAS$$

where E is the enzyme, S acetylcholine, ES the Michaelis complex, and EA the acetyl enzyme. C