# The Reaction of Acetylcholinesterase with Diethylphosphoryl Esters of Quaternary and Tertiary Aminophenols

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SUMMARY

The second order rate constant values were measured for the inhibition of acetylcholinesterase (electric eel) by a number of diethylphosphoryl esters (anhydrides) containing different leaving groups. These groups include the tertiary, quaternary, and some diquaternary derivatives of hydroxyquinoline, hydroxyisoquinoline, hydroxystilbazole, and hydroxyphenylazopyridine. The values of the rate constants for the uncharged inhibitors formed a monotonic sequence when plotted against the value of the $pK_a$ of the leaving group. As the $pK_a$ value of the leaving group increased, the rate fell slowly at first but then very rapidly as the $pK_a$ value exceeded 7–8. The cationic inhibitors, except for two compounds, were far more active than would be expected for the $pK_a$ values of the leaving groups, and it was concluded therefore that these inhibitors were more active because they were attracted to the enzyme by the anionic site.

## INTRODUCTION

Acetylcholinesterase often reacts much more rapidly with compounds containing a quaternary ammonium function than with uncharged compounds of similar structure. However, only a few organophosphates containing quaternary ammonium functions in the leaving group have been studied as inhibitors of this enzyme. Of these, the diethylphosphoryl esters of 3-hydroxy-1-methylquinolinium (1, 2), 3-hydroxy-*N*-trimethylanilinium (1, 2), and thiocholine (3) are very active inhibitors. Yet the last two are not more potent than tetraethyl pyrophosphate, which of course, does not contain a cationic structure. On the other hand, since diethylphosphoric acid is more acidic than the conjugate acids of the other leaving groups, diethyl phosphate might a priori be expected to be a better leaving group. (It is not known whether the acid or the anion is the leaving group.)

The question then is whether enzyme specificity is involved in the phosphorylation of the enzyme; that is, whether a group containing a cationic ammonium function is better able to serve as a leaving group in the inhibition reaction than its chemical properties would demand. The problem, of course, is to know what to expect for the reactivity of a compound containing a given leaving group. We find that the reactivity of neutral phosphorylating inhibitors varies smoothly and monotonically with the $pK_a$ value of the leaving group, within a factor of three. If we then assume that a cationic inhibitor would in the absence of molecular complementarity react at the same rate as a neutral compound with a leaving group having the same $pK_a$ value, we should have a basis for judging whether the cationic charge is contributing per se to the inhibitory reaction.

The reaction of an inhibitor with the enzyme can be represented as

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$$\underset{E}{HE} + \underset{I}{(EtO)_2POX} \overset{K}{\rightleftharpoons} \underset{E\cdot I}{HE\cdot(EtO)_2POX} \overset{k}{\rightarrow} \underset{E'}{(EtO)_2POE} + HX$$

The scheme indicates the formation of a reversible initial complex between the enzyme and the inhibitor. A kinetic study can yield the dissociation constant $K$ and the zero order rate constant $k$. Samples of a solution containing enzyme and inhibitor are withdrawn at various times and added to a solution of acetylcholine to measure the concentration of uninhibited enzyme.

The equations to be solved for the rate of inhibition are

$$E^0 = (E) + (E \cdot I) + (E') = (E) + (E')$$

$$\frac{(I)(E)}{(E \cdot I)} = K$$

$$-\frac{d(E)}{dt} = k(E \cdot I)$$

where $E^0$ is the total enxyme concentration. If the enzyme solution is diluted during assay so that (I) < K, the measured activity corresponds to the sum (E) + (E·I) = (E). Under these circumstances, with the acetylcholine concentration 20 times greater than the value of $K_m$, very little enzyme in the complex form, E·I, will be present, i.e., any E·I present during the inhibition reaction will dissociate during the assay to yield free enzyme, E. The solution of the equations is

$$\ln \frac{E}{E^0} = \frac{-kt}{1 + \frac{K}{(I)}}$$

for $(I) \gg E^0$.

The pseudo first order rate constant is given by

$$k_1^0 = \frac{k}{1 + \frac{K}{(I)}}$$

and if $(I) \ll K$

$$k_1^0 = \frac{k(I)}{K}$$

therefore,

$$k_2^0 = \frac{k_1^0}{(I)} = \frac{k}{K}$$

where $k/K$ is now the second order rate constant, $k_2^0$. By measuring the values of the pseudo first order rate constant at several values of (I), $K$ and $k$ can be evaluated unless $(I) \ll K$. In the latter case only the ratio $k/K$ is obtained, and it is apparent that this ratio is equal to the second order rate constant because under these circumstances (E·I) ≪ (E). In this study it was found that the measured values for the pseudo first order rate constants were proportional to (I). This means that $(I) \ll K$, and therefore only the second order rate constants were evaluated.

A number of new organophosphates, the tertiary, monoquaternary, and diquaternary esters of *O*-diethylphosphorylaminophenols (4), were studied.

## MATERIALS AND METHODS

*Enzyme.* Acetylcholinesterase (EC 3.1.-1.7) was prepared from the electric organ of *Electrophorus electricus* (5). At 25°, pH 7 and in a medium consisting of 0.1 M NaCl, 0.02 M $MgCl_2$, 0.005% gelatin, $10^{-5}$ EDTA, $10^{-3}$ M acetylcholine (bromide), the enzyme had a specific activity of 100 mmoles of acetylcholine hydrolyzed per minute per milligram of protein, measured by automatic titration with a pH stat. The $K_m$ value was $9.1 \times 10^{-5}$ M.

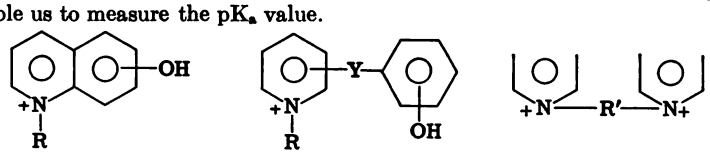
*Assay method.* The enzyme activity was measured by the decrease in acetylcholine concentration in 2 min. The acetylcholine concentration was determined by the hydroxamic acid method (6). The medium was the same as above except that the initial concentration of acetylcholine was $2.7 \times 10^{-3}$ M and the solution contained 0.02 M sodium phosphate buffer.

The inhibition reaction was started by the addition of inhibitor to a solution of enzyme. The medium was the same as in the assay above except that acetylcholine was not persent. The rate of inhibition was followed by withdrawing 0.1-ml samples at usually 8, but sometimes at as many as 18, time intervals and assaying the remaining activity with 1.0 ml of acetylcholine solution. The logarithm of the enzyme activity was plotted as a function of time in accord with pseudo first order kinetics;

TABLE 1

*Second order rate constant values for the inhibition of eel acetylcholinesterase*

All compounds tested were the diethylphosphoryl esters of the agents listed under "Leaving group." The conditions were 25° and pH 7 in a medium of 0.1 M NaCl, 0.02 M $MgCl_2$, 0.005% gelatin, and $10^{-5}$ M EDTA. The dimensions of the rate constant values are liters · $mole^{-1}$ $min^{-1}$. The quaternary and tertiary amines were the picrate derivatives. Some picrate compounds were not sufficiently soluble in 10% methanol to enable us to measure the $pK_a$ value.




| Compound No. | Leaving group | | | | | Second order rate constant values, $k_2°$ | $pK_a$ of leaving group |
|---|---|---|---|---|---|---|---|
| | $N^a$ | $N^b$ | R | R′ | Y | | |
| 1 | 5[c] | | $CH_3$ | | | $6.5 \times 10^4$ | 6.8 |
| 2 | 5[c] | | H | | | $8.9 \times 10^3$ | |
| 3 | 5[c] | | | $-CH_2OCH_2-$ | | $8.1 \times 10^5$ | |
| 4 | 3 | | $CH_3$ | | | $1.2 \times 10^8$ | 5.3 |
| 5 | 3 | | H | | | $5.5 \times 10^4$ | 7.7[d] |
| 6 | 5 | | $CH_3$ | | | $2.4 \times 10^6$ | 6.1 |
| 7 | 5 | | H | | | $3.0 \times 10^3$ | 8.6[d] |
| 8 | 6 | | $CH_3$ | | | $9.3 \times 10^6$ | 7.0 |
| 9 | 6 | | H | | | $4.1 \times 10^3$ | 8.8[d] |
| 10 | 6 | | | $-CH_2OCH_2-$ | | $2.7 \times 10^8$ | |
| 11 | 7 | | $CH_3$ | | | $1.2 \times 10^8$ | 5.7 |
| 12 | 7 | | H | | | $1.0 \times 10^5$ | 8.6[d] |
| 13 | 8 | | $CH_3$ | | | $5.3 \times 10^2$ | 6.3 |
| 14 | 8 | | H | | | $1.4 \times 10$ | 9.7[d] |
| 15 | 3 | 4 | $CH_3$ | | —N=N— | $2.8 \times 10^6$ | 6.9 |
| 16 | 3 | 4 | H | | —N=N— | $4.7 \pm 10^4$ | |
| 17 | 4 | 4 | $CH_3$ | | —CH=CH— | $1.8 \times 10^4$ | 8.3 |
| 18 | 4 | 4 | H | | —CH=CH— | $4.9 \times 10^3$ | |
| 19 | 4 | 4 | | $-CH_2OCH_2-$ | —CH=CH— | $1.1 \times 10^5$ | |
| 20 | 4 | 3 | $CH_3$ | | —CH=CH— | —[e] | 8.8 |
| 21 | 4 | 3 | | $-CH_2OCH_2$ | —CH=CH— | $5.0 \times 10^3$ | |
| 22 | 4 | 2 | $CH_3$ | | —CH=CH— | —[e] | 8.3 |
| 23 | 4 | 2 | | $-CH_2OCH_2-$ | —CH=CH— | $1.2 \times 10^3$ | |
| 24 | $(CH_3)_3N^+CH_2CH_2SH$ | | | | | $1.6 \times 10^6$ | 7.9 |
| 25 | $(CH_3)_2$ <br> $+N-CH_2-CH_2-SH$ <br> $(CH_2)_5$ <br> $+N-CH_2-CH_2-SH$ <br> $(CH_3)_2$ | | | | | $3.6 \times 10^7$ | |
| 26 | $(CH_3)_3N^+-CH_2CH_2CH_2SH$ | | | | | $5.5 \times 10^4$ | 9.1[f] |
| 27 | HF | | | | | $2.7 \times 10^5$ | 3.5 |
| 28 | $(C_2H_5O)_2P(=O)-OH$ | | | | | $2.1 \times 10^6$ | 1.4 |

[a] Number refers to the position of substitution on the ring of compounds 1 through 14; and the position of the bridge (Y) attachment on the pyridine ring of compounds 15 through 23.

[b] Position of substitution on the benzene ring of compounds 15 through 23.

[c] Hydroxy-1-methyl quinolinium replaced by 5-hydroxy-2-methylisoquinolinium.

[d] Measured in 10% methanol.

[e] Very slow inhibition at $10^{-5}$ M inhibitor concentration.

[f] Estimated value.

straight lines were obtained. In most cases a 10-fold range of inhibitor concentration was used.

In some cases the phosphorylation was too rapid for accurate measurement. (The concentration of inhibitor must be at the very least twice the concentration of enzyme.) In these cases, a reversible inhibitor, tetraethylammonium ion was added to the enzyme solution just before the phosphate inhibitor was added. The reaction rate of the phosphorylating agent and the enzyme was thus decreased by a known factor, and the time intervals became large enough for convenient manipulations.

*Determination of the value of $pK_a$.* The values of $pK_a$ for the leaving group compounds were taken as the pH of a half-neutralized solution (1.5 equivalents of NaOH for tertiary picrates), measured with a glass electrode. Some of the phenols were available only as the picrates and were not suitably soluble in water. Measurements were therefore made in 10% methanol, but some of the compounds were still not sufficiently soluble. The low solubility of the picrates of diethylphosphoryl esters was not a problem for inhibition measurements because most of the compounds were quite active even in very low concentrations.

## RESULTS AND DISCUSSION

In this work the phosphorylating group was kept constant as a diethylphosphoryl moiety and the leaving group was varied. The new compounds studied were diethoxyphosphinyloxy derivatives of isoquinoline, quinoline, phenylazopyridine, stilbazole, and the corresponding *N*-methylquaternary compounds (4). These compounds were picrates. A number of bisquaternary derivatives of these compounds containing a dimethylene ether bridge were studied, and also a bisquaternary derivative of diethoxyphosphinyl dimethylaminoethanethiol containing a pentamethylene bridge. Older, more familiar compounds were also studied.

The second order rate constant and the $pK_a$ values of the leaving groups are given in Table 1, and the measurements of a typical experiment are shown in Fig. 1. In each of eight pairs, the quaternary compound is a more effective inhibitor than the tertiary analog. The second order rate constant value for inhibition by the quaternary compound is one to four orders of magnitude larger than for the tertiary inhibitor. The tertiary amines are aromatic and therefore largely uncharged in an aqueous solution at pH 7.0.

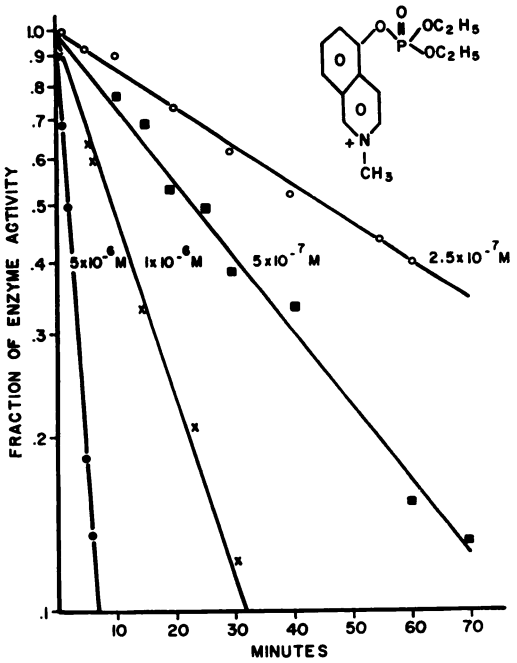



FIG. 1. *A semilogarithmic plot of percent inhibition of eel acetylcholinesterase versus time*

The conditions of measurement were pH 7, 25° in a medium of 0.1 M NaCl, 0.02 *M* $MgCl_2$, 0.005% gelatin, $10^{-5}$ M EDTA, 0.02 M sodium phosphate buffer, and $2.7 \times 10^{-3}$ M acetylcholine bromide. The half-times were inversely proportional to the concentration of the inhibitor in accord with second order kinetics.

The diquaternary compounds are about ten times more active on a mole basis than the corresponding monoquaternary compounds. This is distinctly larger than the 2-fold greater value expected for the rate constant, if the only consideration in the comparison were the fact that the diquaternary compound contains two reacting functional groups.

A plot of the logarithms of the rate con-

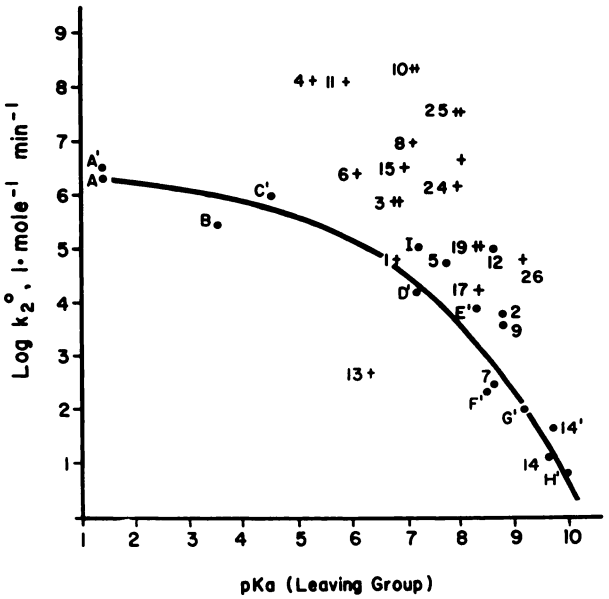



FIG. 2. *Relationship between the values of the second order rate constants for inhibition of eel acetylcholinesterase and the $pK_a$ values of the leaving groups*

The lettered points are for diethylphosphoryl derivatives containing a leaving group without an amine function; the leaving groups are A: $OPO(OEt)_2$; B: F; C: $SC_6H_4$-4-$NO_2$; D: $OC_6H_4$-2-$NO_2$; E: $OC_6H_4$-3-$NO_2$; F: $OC_6H_4$-2-Cl; G: $OC_6H_4$-4-Cl; H: $OC_6H_5$; I: $OC_6H_4$-4-$NO_2$. The prime marks indicate data from Aldridge (7). The lettered points indicate that the reactivity of the neutral phosphorylating compounds varies monotonically with the $pK_a$ value of the leaving group. The numbered points are for tertiary amines, the crosses for quaternary amines, and the double crosses for bis compounds. The numbers refer to the compounds listed in Table 1. The unnumbered cross is the *O*-diethylphosphoryl ester of 3-hydroxyphenyltrimethyl ammonium ion (1, 2). The bis compounds were plotted at the $pK_a$ value of the monoquarternary leaving group.

stant values versus the $pK_a$ values of the leaving groups is given in Fig. 2. Data for a number of other compounds are recorded in this plot including data for the reaction of uncharged inhibitors with acetylcholinesterase from red blood cells of sheep and human beings. The justification for using the data obtained with other enzymes is given in Table 2, from which we note that the values of second order rate constants for inhibition by three uncharged inhibitors and two charged inhibitors are about the same with these three enzymes. In this way we can use the data of Aldridge (7), who has shown a linear log-log relationship between the rate of inhibition and the rate of hydrolysis of the inhibitor, to help establish whether a log inhibition versus $pK_a$ (leaving group) relationship exists for *uncharged* inhibitors. In Fig. 2 we note that a reasonable monotonic sequence exists for the uncharged inhibitors with a deviation of about a factor of three.

We use this curve as the basis for estimating the rate of inhibition expected for a compound as a function of the $pK_a$ value of the leaving group. If, as in the case of compound 4+, the rate of inhibition should be much larger than given by the curve for $pK_a = 5.25$, we would conclude that some special feature of the compound is enhancing its rate of reaction with acetylcholinesterase. The use of this curve as a reference line is supported by the studies of Aldridge (9) which show a linear relationship between the log rate of inhibition of the enzyme and the log rate of hydrolysis of the compound. There are other examples, in nonenzyme chemistry, of linear log rate *vs.* log rate relationships that curve in a

TABLE 2

*Comparison of rate constant values with enzyme source*

The rate constant values for the inhibition of acetylcholinesterase from red blood cells (RBC) and electric tissue indicates the similarity of these enzymes when compared in this manner. The letter "a" indicates that the data are from our laboratory; the numbered references appear in the bibliography.

| Compound | Reference | Enzyme source | Temp. (°C) | pH | $k_2°$ |
|---|---|---|---|---|---|
| Tetraethylpyrophosphate (TEPP) | *a* | Eel | 25 | 7.0 | $2.1 \times 10^6$ |
| | 8 | Eel | 25 | 7.4 | $2.1 \times 10^6$ |
| | 8 | Human RBC | 25 | 7.4 | $2.1 \times 10^6$ |
| | 9 | Sheep RBC | 37 | 7.6 | $3.3 \times 10^6$ |
| Diethoxyphosphorylthiocholine (phospholine) | *a* | Eel | 25 | 7.0 | $1.5 \times 10^6$ |
| | 3 | Ray (Electric) | 25 | 7.5 | $1.2 \times 10^5$ |
| | *a* | Human RBC | 25 | 7.4 | $1.6 \times 10^6$ |
| Diethoxyphosphoryl-*p*-nitrophenol (Paraoxon) | *a* | Eel | 25 | 7.0 | $2.4 \times 10^6$ |
| | 10 | Sheep RBC | 37 | 7.6 | $1.1 \times 10^6$ |
| | 11 | Rabbit RBC | 37 | 7.6 | $2.6 \times 10^6$ |
| Diethylphosphoryl-3-hydroxy-1-methylquinolinium | *a* | Eel | 25 | 7.0 | $1.22 \times 10^8$ |
| | 1 | Human RBC | 37 | 7.4 | $1.05 \times 10^8$ |
| | 1 | Bovine RBC | 37 | 7.4 | $1.15 \times 10^8$ |
| Diethylphosphoryl-8-hydroxyquinoline | *a* | Eel | 25 | 7.0 | 13.9 |
| | 10 | Sheep RBC | 37 | 7.6 | 46.0 |

manner very similar to that depicted in Fig. 2, where log rate values are plotted *vs.* the $pK_a$ values of the leaving groups (12). The familiar linear relationship between values of log rate and $pK_a$ in the Hammett equation corresponds to $\Delta pK_a$ values of less than 2.

The rate of inhibition is highly dependent upon the acidity of the leaving group at high $pK_a$ values but tends toward a more moderate dependence as the acidity is increased. Diethylphosphoryl-4-hydroxynitrobenzene (paraoxon, log $k_2° = 6$; $pK_a$ nitrophenol = 7.0) is an exception to the curve. This compound is also more active than would be expected from its rate of hydrolysis (7). The curve suggests that the $pK_a$ of a leaving group must be no larger (with a diethylphosphoryl transferring group) than 10, if some reasonably significant inhibitory activity is to be expected. An extrapolation of the curve to $pK_a = 16$, a procedure which is necessarily very crude, suggests that the second order rate constant for an inhibitor containing an aliphatic alcohol as a leaving group is of the order of magnitude of $10^{-9}$ liter·mole$^{-1}$min$^{-1}$. The extreme importance of the acidity of the leaving group at low acidities offers an explanation for the failure to obtain phosphorylation with *O*-diethoxyphosphorylcholine ($pK_a$ choline = 14) even though the thiocholine derivative is very active. The estimated rate constant for *O*-diethylphosphorylcholine is $10^{-3}$ liter·mole$^{-1}$min$^{-1}$ based on the dependence of activity on $pK_a$ and the observed value for the thiocholine inhibitor.

We have already noted that quaternary inhibitors are orders of magnitude more active than the corresponding tertiary uncharged analogs. However, the leaving groups are also very much more acidic. Are the quaternary inhibitors more active because the leaving groups are more acidic or because the enzyme shows specifiicty toward cationic inhibitors? Figure 2 shows that seven monoquaternary inhibitors are very much more active than we would expect from the $pK_a$ values of the leaving groups, one is only one order of magnitude more active, one is about as active, and one is much less active than we might have anticipated. It is reasonable therefore to conclude that the enhanced activity of the quaternary compounds derives from interaction of the ammonium function with the anionic site of the enzyme, i.e., some

degree of molecular complementarity is involved.

One of the tertiary compounds, *O*-diethylphosphoryl-7-hydroxyquinoline, compound 12, is also much more active than should be expected. There is a possible explanation for this within the ideas we have been discussing. The $pK_a$ value of this compound (not the leaving group) is 4.0. Therefore at pH 7.0 there is one part per thousand in the cationic form. Now the tertiary analog of S-diethylphosphorylthiocholine, number 24, which is cationic at pH 7.0 is about one-third as active as the quarternary compound (13), and therefore we may assume that cationic tertiary inhibitors are within one order of magnitude as active as the related quarternary inhibitors. With this assumption the high activity of compound number 12 is accounted for. Compounds 5 and 9 might be a little too high for the same reasons, but for the purposes of this discussion their position relative to the curve for uncharged inhibitors is satisfactory.

This explanation is supported qualitatively by the observation that the inhibition rate for compound number 12 decreases from pH 7 to pH 8 by a factor of about 3 whereas the rate for compound number 1 increases about 70%. Tetrathylphyrophosphate inhibition is also more rapid at pH 8.0 than at pH 7.0. Therefore if the experiments shown in Fig. 2 had been carried out at pH 8.0 rather than pH 7.0, the points 5, 9, and 12 would have fallen closer to the curve.

The very low activity of compound numbers 13 and 14 can easily be explained by the very marked steric hindrance of the diethylphosphinyloxy function in the 8 position on the quinoline ring.

Among the quaternary compounds, it also appears that the acidity of the leaving group is a decidedly important parameter determining activity. Aside from quaternary function, no special feature of molecular complementarity is revealed in these data. *S*-Diethylphosphorylthiocholine (phospholine), which has the same leaving group as acetylthiocholine and might therefore be expected to be especially active if other features of molecular complementarity were involved, is very active in relation to the curve for uncharged inhibitors, but not more so than several other compounds.

These data suggest that the dominant features in determining the inhibitory activity of diethylphosphoryl esters are the $pK_a$ values of the leaving groups and the presence of a quaternary ammonium function.

Since the second order rate constant for the acetylation of acetylcholinesterase by reaction with acetylcholine is about $9 \times 10^{10}$ liters·mole$^{-1}$min$^{-1}$, it is apparent that some of the quaternary inhibitors are remarkably active. Compounds number 10 and 11 combine good stability in solution with high activity and might therefore be of some practical use.

It is known that methanesulfanylfluoride and dimethylcarbamylfluoride react very much more rapidly with acetylcholinesterase when quaternary ammonium ions are added to the medium (14, 15). The question arises whether this accelerating effect of cationic ammonium ions is closely related to the enhanced rate of reaction we have found in this study when the inhibitor contains a quaternary ammonium function. There are a number of differences that would seem to make a close relationship doubtful. The enhancements observed here are considerably larger, in several cases by a factor of 1000, whereas a factor of 50 is representative of the higher accelerations previously observed (14, 15). Acceleration was not observed with pyridinemethiodide nor with phenyltrimethyl ammonium ion. And finally acceleration does not occur with diethylphosphorylfluoride. On the contrary, cationic ammonium ions inhibit this phosphorylating reaction.

The enhancement in reaction rate reported in these studies cannot be accounted for merely on the basis of increased binding of the inhibitor, for this could produce only a factor of thirty in increased rate. In this respect these results resemble the acceleration results, for in the latter case the accelerator is not even part of the inhibiting molecule. It would appear that

something else has to occur. Possibly an appropriate change in the conformation of the enzyme is induced.

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