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THE REACTION OF TRIS-(CHOLINE CHLORIDE) PHOSPHATE WITH EEL CHOLINESTERASE

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SUMMARY

Tris-(choline chloride) phosphate (TCCP) has been observed to slowly and progressively inhibit eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). The kinetics of the inhibition reaction are consistent with a mechanism involving binding of the inhibitor to the enzyme followed by a rate-limiting irreversible reaction producing inactive enzyme. The dissociation constant of the enzyme–inhibitor complex and the rate constant of the inactivation reaction have been determined to be 0.036 M and 0.2 min^{-1}, respectively. The quaternary reversible inhibitor tetraethylammonium bromide competes with TCCP for the reaction site. TCCP does not noticeably reduce the inhibition of enzyme by decamethonium bromide as does the structurally analogous compound flaxedil. The results obtained with TCCP are compared with available data on the relative enzymic reactivity of alkoxy and thiolo esters of phosphoric acid.

INTRODUCTION

Triesters of phosphoric acid were first observed to be inhibitors of cholinesterases (acetylcholine hydrolase, EC 3.1.1.7 and acylcholine acyl-hydrolase, E.C. 3.1.1.8) by Fukuto[1], who observed inhibition by *O,O*-diethyl *O*-(3,3-dimethyl-1-butyl) phosphate. They were also noted by Tammelin[2] while repeating earlier work by Koelle and Steiner[3] who investigated inhibition of cholinesterases by dialkoxyphosphorylthiocholines and analogues. Tammelin[2,4] obtained pI_{50} values for several diethylphosphorothiolates and their oxygen analogues. In each case the sulfur-containing compound was a considerably more potent inhibitor. More recently, O'Brien and co-workers[5–8] extended these studies to a large number of paired phosphates and phosphorothiolates which they compared in terms of I_{50} and second-order reaction rate constants, k_i (Eqn. 8). They found that with neutral compounds, the thiolates react more rapidly but that in most cases the differences are quite small (one order

Abbreviation: TCCP, tris-(choline chloride) phosphate; MES, morpholinoethane sulfonic acid.

of magnitude or less). Bracha and O'Brien[6] attributed these small differences to the general superiority of thiols as leaving groups*.

However, the enhancement in rate due to the presence of an S instead of the O in the corresponding phosphate esters[6], for which they coined the term "thiolo effect", is not always small. Two categories of deviations from the general pattern of thiolo effects can be noted, although the small number of examples in each category makes this generalization limited. We will refer to these deviations as abnormal thiolo effects.

With paired families of neutral phosphorus esters, $(EtO)_2P(O)X(CH_2)_nCH(C_2H_5)_2$ and $(EtO)_2P(O)X(CH_2)_nCH_3$, differences in inhibition rate between X = S and X = O where n = 2 or more are small and can be attributed to leaving group effects. The first category of deviants consists of these neutral esters when n = 1 or 0. Here the rate ratios are greater by several orders of magnitude[5,6]. In the second category are the basic compounds, including $(EtO)_2P(O)XCH_2CH_2N(C_2H_5)_2$ and its *N*-(2-fluoroethyl)-*N*-ethyl and *N*,*N*-di-(2-fluoroethyl) analogues[5–8]. Here, the rate ratios are even higher, reaching a value of 10^6. Such differences are entirely too large to attribute to leaving group effects and hence must be enzymic in origin.

Whether the abnormal thiolo effect is due to an unfavorable binding interaction between alkoxy inhibitor and enzyme or to a reduced rate of phosphorylation can only be determined by separating these two components of the second-order rate constant. Little data of this nature is available. O'Brien and his co-workers[5–7] examined several phosphorothiolates and reported their dissociation constants from bovine erythrocyte cholinesterase and also the rate constants for the irreversible inhibition step. Unfortunately, comparable data was not obtained for the oxygen analogues.

While examining the compound tris-(choline chloride) phosphate (TCCP) $P(O)(OCH_2CH_2N^+(CH_3)_3)_3 \cdot 3\,Cl^-$, slow irreversible inhibition of eel acetylcholinesterase was noted. Since it was convenient to determine binding and phosphorylation rate constants, this information was obtained. It was our hope that this information would contribute to our understanding of why some phosphates are unexpectedly poor inhibitors of acetylcholinesterase since K_p and k_p (see Eqn. 1) had not previously been separated for a non-sulfur containing phosphate.

KINETIC SCHEME

The irreversible inactivation of enzyme by inhibitor can be described by Eqns 1 and 2 adapted from Main[9] where E represents enzyme;

$$E + PX \overset{K_P}{\rightleftharpoons} E \cdot PX \overset{k_p}{\rightarrow} E' + X \qquad (1)$$

$$E + A \overset{K_A}{\rightleftharpoons} E \cdot A \qquad (2)$$

PX, an irreversible inhibitor with leaving group X; E', the phosphorylated enzyme; A, a competitive inhibitor; and K_A and K_P are dissociation constants for the enzyme complexes, which are assumed to be in equilibrium with their precursors. In the

* Tammelin[4] reported a relative rate factor of 5 times for reaction of OH^- with a phosphate and its isosteric phosphorothiolate. The latter is the more rapid reactant.

equations developed below, the concentrations of the species PX, $E \cdot PX$ and $E \cdot A$ are given respectively as $[P]$, $[EP]$ and $[EA]$. Since enzyme is conserved we have

$$[E]_0 - [E'] = [E] + [EA] + [EP] \tag{3}$$

which can be converted by substitution of the equilibrium expressions to

$$[E]_0 - [E'] = [EP]\left\{1 + \frac{K_P}{[P]}\left[1 + \frac{[A]}{K_A}\right]\right\} \tag{4}$$

The reaction can be followed by observing the activity of enzyme which is proportional to the remaining active enzyme concentration at time t, hence

$$\frac{d([E]_0 - [E'])}{dt} = -k_p[EP] \tag{5}$$

Substitution of Eqn 4 into Eqn 5 and integration gives the expression

$$R = \frac{\Delta \ln v}{\Delta t} = \frac{\ln ([E]_0 - [E'])_2 - \ln ([E]_0 - [E'])_1}{t_2 - t_1} = \frac{-k_p}{1 + \frac{K_P}{[P]}\left(1 + \frac{[A]}{K_A}\right)} \tag{6}$$

Inversion of Eqn 6 predicts that a linear double-reciprocal plot (Eqn 7) should be obtained for $1/R$ *vs* $1/[P]$, with its slope

$$\frac{1}{R} = \frac{K_P}{k_p}\left(1 + \frac{[A]}{K_A}\right) \cdot \frac{1}{[P]} + \frac{1}{k_p} \tag{7}$$

equal to the quantity $(1 + [A]/K_A)K_P/k_p$. The intercept on the ordinate is equal to $1/k_p$. When $[A] = 0$, the slope will be K_P/k_p allowing the separation of rate and equilibrium constants.

In the absence of A, from Eqn 6, the second-order reaction rate constant, k_i, is defined in Eqn 8.

$$k_i = \frac{R}{[P]} = \frac{k_p}{[P] + K_P} \tag{8}$$

With rapid reactants, $K_P \gg [P]$ so that $k_i = k_p/K_P$.

EXPERIMENTAL

TCCP was prepared by Ash-Stevens, Inc. under contract No. DAAA15-69-C-0584 according to the procedure described by Jackson[10]. Analysis observed: 38.90% C, 8.60% H, 9.06% N; analysis calculated: 38.92% C, 8.49% H, 9.08% N. Tetraethylammonium bromide was a recrystallized Eastman product obtained from Dr J. C. Kellet, Jr. The hygroscopic salt was dried at 110 °C then stored over P_2O_5. Phenyl acetate was an Eastman product. Decamethonium bromide $[(CH_3)_3N^+(CH_2)_{10}N^+(CH_3)_3 \cdot 2\ Br^-]$ was provided by Dr E. Bay. Eel acetylcholinesterase was purchased from Worthington and stored in stock solutions containing 0.225 M KCl, 0.1% gelatin and 0.02% NaN_3. Acetylcholine chloride was obtained in 100 mg preweighed ampoules from Nutritional Biochemicals Company. Stock solutions of 0.1101 M in acetylcholine were prepared by quantitatively dissolving the contents of one ampoule so as to make 5 ml of solution. Gallamine triethiodide [flaxedil; 1,2,3-tris-(2-triethylammonioethoxy)

benzene triiodide] was obtained from Davis and Peck and used without further purification.

Inhibition of the enzyme acetylcholinesterase by TCCP was measured in 0.1 M morpholinoethane sulfonic acid (MES) buffer[11], pH 6.61. Stock solutions of TCCP were prepared in MES buffer just prior to use. Inhibition reactions were initiated by adding a small (25 μl) volume of enzyme stock solution at a convenient concentration to an appropriate dilution of TCCP stock with MES buffer. The enzyme concentration range was estimated to be $1.2 \cdot 10^{-9}$ to $5 \cdot 10^{-9}$ M (ref. 12). The ionic strength was held constant at that of 0.066 M TCCP by the addition of solid KCl as needed, and 100 μl of tetraethylammonium bromide stock solution at 33.8 mM was added to reactions requiring a competitive inhibitor. The final concentration of tetraethylammonium bromide in inhibited reactions was 1.05 mM. The disappearance of active enzyme was followed by withdrawing 100-μl aliquots at intervals and assaying for enzyme activity in 3 ml of 0.1 M MES buffer, pH 6.61, containing 8 mM phenyl acetate. The hydrolysis of phenyl acetate could be conveniently followed at 270 nm on a Cary Model 14 recording spectrophotometer.

The observed phenyl acetate hydrolysis rates, v, were plotted as ln v *versus* incubation time with TCCP to obtain pseudo-first-order reaction rate constants R. First-order plots were linear for more than two half-times. These rate constants were plotted in the double-reciprocal form of Eqn 7 as shown in Fig. 1 to obtain the dissociation constant K_P and the first-order rate constant k_p.

The activity of Cl^- in TCCP solutions was measured with a Beckman Cl^- electrode with a calomel reference electrode on a Beckman research pH meter. A standard curve of electrode potential at 25 °C for known Cl^- concentrations was obtained by diluting stock KCl solutions in glass-distilled deionized water with the ionic strength made up with KNO_3.

Competitive inhibition constants K_i were calculated from observed K_m ($K_{m(obs)}$) values obtained with a Wilkinson[13] weighted regression analysis of reciprocal relationships. Strictly competitive inhibition was observed so that K_i values could be calculated using Eqn 9.

$$K_{m(obs)} = K_m \left(1 + \frac{[I]}{K_i}\right) \tag{9}$$

Partial reversal of decamethonium inhibition of acetylcholinesterase by flaxedil was obtained in 0.003 M MES buffer (pH 6.61) containing 8 mM phenyl acetate. Sufficient enzyme was added to a cuvette containing buffer, substrate and $2 \cdot 10^{-6}$ M decamethonium bromide to give a slow but measurable hydrolysis of the phenyl acetate as observed at 270 nm on the Cary Model 14 spectrophotometer. The comparative effects of TCCP and flaxedil were observed by adding them to this system at a concentration of $4 \cdot 10^{-5}$ M.

RESULTS AND DISCUSSION

Fig. 1 is a double-reciprocal plot according to Eqn 7. It indicates that the data are consistent with the mechanism assumed in Eqn 1, and that the inhibitor tetraethylammonium bromide is competitive with TCCP. K_A calculated for tetraethylammonium bromide from the data in Fig. 1 is $4.8 \cdot 10^{-4}$ M, in good agreement with a

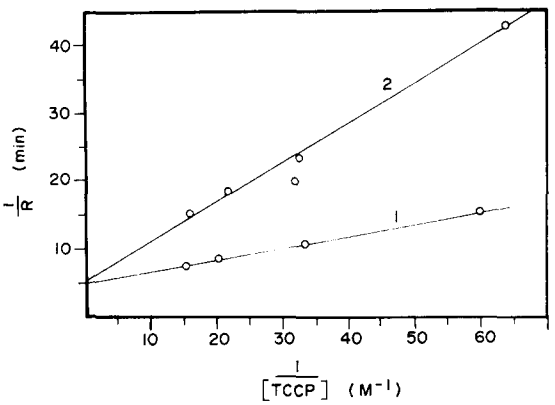

Fig. 1. Inhibition of acetylcholinesterase by TCCP; 0.1 M MES buffer, pH 6.61, 25 °C. Inhibitor: 1, none; 2, tetraethylammonium bromide ($1.05 \cdot 10^{-3}$ M).

competitive K_i for inhibition of phenyl acetate hydrolysis by tetraethylammonium bromide of $4.9 \cdot 10^{-4}$ M. The latter K_i was determined in 0.1 M MES buffer, pH 6.61, containing 0.357 M KCl to adjust the ionic strength to that present in the TCCP phosphorylation reactions. This result suggests that TCCP is binding to the enzyme active site and presumably this is also the site of its inactivation reaction. From the double-reciprocal plot in the absence of tetraethylammonium bromide the value of k_p and K_P can be calculated to be 0.2 min^{-1} and 0.036 M, respectively. Further, the competitive K_i value for TCCP determined from its inhibition of phenyl acetate hydrolysis is 0.0246 M. Its reasonably close agreement with the value of K_P provides additional support for TCCP reaction at the enzyme active site.

It had been suggested by Overberger *et al.*[14] that TCCP might exist in an ion-pair form so that the actual ionic strength of a solution would be much less than that calculated assuming complete dissociation of the Cl^-. To test this, the concentration of free Cl^- in a series of TCCP solutions was measured with a Cl^--sensitive electrode. A plot of observed Cl^- activity *versus* molar concentration of TCCP from 1 to 5 mM has a slope of 3.1, indicating complete dissociation of all chloride from the three quaternary nitrogens as the electrode is insensitive to ion pairs.

Due to the structural similarity between TCCP and gallamine triethiodide (flaxedil), TCCP was examined for the ability to reverse the inhibition of eel acetylcholinesterase by decamethonium which can be seen with flaxedil at low ionic strengths[15]. At concentrations of TCCP comparable to those at which we observed that flaxedil decreased the extent of inhibition produced by decamethonium by 3.94-fold the effect of TCCP was only 1.07-fold, an amount which easily can be attributed to the ionic strength contribution of the salt.

In Table I are listed K_P, k_p and k_i values for a series of dialkylphosphorothiolates, k_i values for related phosphates together with the values obtained in this work for TCCP. It is striking that TCCP, in spite of its three positive charges and choline leaving group, binds so very poorly to acetylcholinesterase. Like the other phosphates, V and VIII, its k_i value is also quite low, being perhaps 3–6 orders of magnitude smaller than those of the rapid phosphorylators. In Table II, comparisons are given of the values of K_P, k_p and k_i for TCCP and 4 of the phosphorothiolates listed in

TABLE I

KINETIC CONSTANTS FOR REACTION WITH ACETYLCHOLINESTERASE

Compound		K_p *(M)*	k_p *(min^{-1})*	k_i *(M^{-1}·min^{-1})*	*Ref.*
I	$(C_2H_5O)_2P(O)SCH_2CH_2CH(C_2H_5)_2$	$4.48 \cdot 10^{-5}$	4.49	$2.3 \cdot 10^5$	a
		$6.16 \cdot 10^{-5}$	2.58	$4.2 \cdot 10^4$	b
II	$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$	$7.16 \cdot 10^{-6}$	6.66	$1.4 \cdot 10^6$	a
		$2.8 \cdot 10^{-4}$	157	$5.6 \cdot 10^5$	c
III	$(CH_3O)_2P(O)SCH(CO_2C_2H_5)CO_2C_2H_5$	$7.7 \cdot 10^{-4}$	11	$1.4 \cdot 10^4$	b
IV	$(C_2H_5O)_2P(O)SCH_2CH_2N^+(C_2H_5)_3$	$2.48 \cdot 10^{-5}$	115[d]	$6.9 \cdot 10^6$	a
V	$(C_2H_5O)_2P(O)OCH_2CH_2N(C_2H_5)_2$	—	—	1.98	a
VI	$(C_2H_5O)_2P(O)SCH(CH_2N(CH_3)_2)_2$	$1.2 \cdot 10^{-4}$	55	$4.1 \cdot 10^5$	a
VII	$(C_2H_5O)_2P(O)OCH_2CH_2CH(C_2H_5)_2$	—	—	$7.3 \cdot 10^4$	e
VIII	$(C_2H_5O)_2P(O)OCH_2CH_3$	—	—	0.42	f
TCCP	$P(O)(OCH_2CH_2N^+(CH_3)_3)_3$	$3.6 \cdot 10^{-2}$	0.2	5.56	g

[a] Ref. 5; pH 7.4, phosphate, erythrocyte acetylcholinesterase; K_p and k_p at 25 °C; k_i at 38 °C.

[b] Ref. 6; pH 7.4, phosphate, erythrocyte acetylcholinesterase, 25 °C.

[c] Ref. 9; pH 7.0, erythrocyte acetylcholinesterase, 5 °C.

[d] These values (K_p and k_p) as reported are open to some question because of the considerable error observed in their measurement.

[e] Ref. 20; calculated from data in ref. 6.

[f] Ref. 1; rat brain acetylcholinesterase, 25 °C.

[g] This work, pH 6.61, eel acetylcholinesterase, 25 °C. The value of k_i here has been set equal to k_p/K_p; see Eqn 8 and accompanying discussion.

Table I. The marked reduction in k_i for TCCP cannot be assigned exclusively either to K_P or k_p. Diminished reaction is a result of decrements in both parameters.

The cause of the abnormal thiolo effect (at least in the nitrogen-containing phosphates) would appear to be due neither to strong but misoriented binding nor to rapid reaction but very poor binding. It is not due to appreciable differences in extent of protonation of the amino groups at neutral pH in paired compounds such as II and V. Both are extensively protonated since their pK_a values are close to 8 (ref. 5). Neither is the difference exclusively a result of the positive charge on the nitrogen atom. Fluorinated derivatives of II and V, the *N*-(2-fluoroethyl)-*N*-ethyl and *N*,*N*-di-(2-fluoroethyl) analogues, are unprotonated at the reaction pH; yet, the abnormal thiolo effect appears[8] (in spite of their isosteric relationships to I and VII; both of which react very rapidly with acetylcholinesterase). Finally, abnormal thiolo effects are not due to appreciable differences in their nucleophilic reactivity[4]. Thus, we find no simple unifying principle to include all of the observed results with the phosphates.

TABLE II

RATIOS OF KINETIC CONSTANTS OF TCCP TO THOSE OF SEVERAL DIALKYLPHOSPHOROTHIOLATES

Structures of compounds are given in Table I, k_i calculated from values in Table I.

Compound	K_p *(M) (TCCP/compound)*	k_p *(min^{-1}) (compound/TCCP)*	k_i *(M^{-1}·min^{-1}) (compound/TCCP)*
I*	580	13	$7.6 \cdot 10^3$
II**	5000	33	$2.5 \cdot 10^5$
III	47	55	$2.5 \cdot 10^3$
VI	300	275	$7.4 \cdot 10^4$

* Calculated from data in ref. 6.

** Calculated from data in ref. 5.

With acetyl esters, significant differences exist between some oxygen and sulfur (thiolo) esters. While Hillman and Mautner[16] have observed that acetylcholine and acetylthiocholine are very similar in turnover rate, Augustinsson[17] reported that there was a marked difference between the corresponding β-methylcholine and β-methylthiocholine esters. For the oxygen ester (mecholyl) the D isomer hydrolyzes much faster than the L isomer so that hydrolysis "stops" when $^1/_2$ of the ester has been consumed. With the analogous thiol ester, all of the ester (both isomers) is hydrolyzed by acetylcholinesterase in one rapid continuous step. Combined, these observations suggest the generalization that with thiol esters there is greater accommodation of the enzyme to adverse structural features. When the structures of the reacting paired (O and S) molecules do not contain adverse structural features, reaction rates with acetylcholinesterase are closely similar. When fit becomes strained, the rate of reaction with the oxygen analogue falls off, while the rate of reaction of the sulfur analogue remains at the normal elevated level.

The reason for the apparent adaptability of the enzyme with sulfur esters is not known. It could be related to sulfur's larger covalent radius, somewhat different bond angles, lower electronegativity (and reduced ability to form hydrogen bonds) or its greater polarizability. Based upon the well known evidence for substrate induced conformational changes in enzymes as a result of their interaction in the Michaelis complex[18], we are inclined to view abnormal thiolo effects in these terms. If one were to suppose greater interaction (hydrogen bonding) between the substrate oxygen atom and a proton at or very close to the esteratic site, it could well result in greater constriction in the enzyme conformation. Such restricted conformation, with its reduced flexibility, would be compatible with increased specificity, the reduced ability to accommodate to diverse structures.

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