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The inhibition of cholinesterase by organophosphorus compounds in the presence of substrate

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SUMMARY

The inhibition reactions of acetyl- and butyrylcholinesterase with organophosphorus compounds, carried out in the presence of the substrates acetylthiocholine and phenyl acetate, were checked to observe whether they obey the reaction scheme in which the inhibitor interacts only with the free enzyme. Based on the results obtained, a method is given to determine the bimolecular rate constants of the inhibition of the cholinesterases with organophosphorus compounds. This method is especially suitable in the case of strong and/or unstable inhibitors.

The bimolecular rate constant of the reaction of cholinesterase with an organophosphorus compound is usually estimated by a discontinuous method as described by Aldridge[1]. Essentially the method consists of withdrawing samples for enzyme activity assay at some (6 to 10) intervals after mixing of enzyme and inhibitor.

Main and Dauterman[2] suggested to measure the inhibition of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) titrimetrically in the presence of substrate. By assuming Scheme 1 to describe the reactions taking place

$$EI \leftarrow I + E + S \rightleftharpoons ES \rightarrow E + \text{products} \qquad (1)$$

where *E, I, S, EI, ES* stand for free enzyme, inhibitor, substrate, inhibited enzyme and Michaelis complex, respectively, the bimolecular rate constant of the inhibition reaction can be calculated.

Attempts to check Scheme 1 for the reaction of cholinesterases with organophosphorus compounds were reported by Brestkin *et al.*[3] and Volkova[4,5] who estimated titrimetrically the inhibition of butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) in the presence of various concentrations of acetylcholine and phenyl acetate. Only in the case of phenyl acetate the reaction could be described by Scheme 1. However, the bimolecular rate constant is strongly dependent on the ionic strength of the reaction mixture. Hence, it is reasonable to assume the bimolecular rate constant to depend on the acetylcholine

concentration used since this concentration was varied over a wide range in a solution of low ionic strength.

In order to develop a spectrophotometric determination of the bimolecular rate constant of the inhibition of cholinesterase by organophosphorus compounds, we tested the validity of the proposed mechanism in the following experiments. The reactions of acetyl- and butyrylcholinesterase with isopropyl methylphosphonofluoridate (Compound A) and (*R*)-(−)-isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide (Compound B), were estimated spectrophotometrically in the presence of five or six different concentrations of the substrates acetylthiocholine and phenyl acetate.

Compound A was prepared according to Boter and Van den Berg[6]. The synthesis of Compound B was described previously[7].

Bovine erythrocyte acetylcholinesterase was obtained from Winthrop Laboratories. Butyrylcholinesterase was purified from horse serum according to a somewhat modified version of the method of Strelitz[8].

The rate constants of the inactivation (k_{obs}) were calculated according to Eqn.2.

$$\ln(P_\infty - P_t) = \ln P_\infty - k_{obs}\, t \tag{2}$$

where P_t and P_∞ are the concentrations of hydrolyzed substrate formed at time t and after complete reaction, respectively. Absorbance values belonging to the zero-order part of the enzymic hydrolysis were determined in the absence of inhibitor. Based on these data, P_t values were taken in such a way that the substrate concentration can be considered to be constant. If P_∞ could not be determined, k_{obs} was calculated by using the method of Guggenheim[9].

An equation for the bimolecular rate constant (k_i) of the irreversible inhibition proceeding in the presence of substrate was derived by Smissaert[10,11] according to Scheme 1

$$k_i = \frac{k_{obs}(1 + [S]/K_m)}{[I]} \tag{3}$$

where K_m is the Michaelis constant for the substrate used. From Eqn.3 it follows that

$$\frac{1}{k_{obs}} = \frac{1}{k_i\,[I]} + \frac{[S]}{k_i\,[I]\,K_m} \tag{4}$$

If the values of k_{obs} obtained in the inhibition experiments of acetylcholinesterase in the presence of phenyl acetate and of butyrylcholinesterase in the presence of acetylthiocholine were plotted against the concentration of the substrate according to Eqn.4, straight lines were obtained. The intercept at the $[S]$-axis was found to be equal to the value of K_m estimated for the substrate used in separate experiments in the absence of inhibitor. The bimolecular rate constant of the inhibition reaction can be calculated from the intercept at the $1/k_{obs}$-axis or, if K_m of the substrate used is known, from the individual k_{obs} values according to Eqn.3. For these two systems Scheme 1 describes the reactions taking place.

The mechanism of the inhibition of acetylcholinesterase in the presence of acetylthiocholine and of butyrylcholinesterase in the presence of phenyl acetate turned out to be more complicated (Table I). The data obtained might be explained by assuming extra interactions of the inhibitor with enzyme or enzyme–substrate complexes.

TABLE I

BIMOLECULAR RATE CONSTANT (k_i) OF THE INHIBITION OF ACETYL- AND BUTYRYLCHOLINESTERASE BY ISOPROPYL METHYLPHOSPHONOFLUORIDATE(A) AND BY (*R*)-(-)-ISOPROPYL *S*-2-TRIMETHYLAMMONIOETHYL METHYLPHOSPHONOTHIOATE IODIDE(B) ESTIMATED SPECTROPHOTOMETRICALLY IN THE PRESENCE OF THE SUBSTRATES ACETYLTHIOCHOLINE AND PHENYL ACETATE

All experiments were carried out in 0.05 M phosphate buffer (pH 7.0) at 25°. Measurements were made with a Zeiss PMQ II spectrophotometer equipped with a Wagner digital voltmeter W44 log, a Kienzle digital printer D4 and a programmer. After periods of 1 sec an impulse sender (Sodeco, Genève, KN 551) decreased a preselected number adjusted in a counting mechanism (Kübler F3 EW 15–2) with 1 unit. After counting down to zero the programmer closed the contact of the printer. The apparatus was zeroed on a blank containing all the reagents except the cholinesterase. The substrate solution was pipetted into the cuvette. Using acetylthiocholine as a substrate, 1.67 moles 5,5'-dithiobis-2-nitrobenzoic acid was added to 1 mole acetylthiocholine in the substrate solution. In the case of inhibition experiments, 50 µl inhibitor solution in methanol was injected into the substrate solution. At zero time the enzyme solution (final concentration: acetylcholinesterase 0.02 and 0.15 unit/ml, and butyrylcholinesterase 1.04 and 0.24 units/ml using acetylthiocholine and phenyl acetate, respectively) was added and after rapid mixing readings of the absorbance were taken every 15 sec at 412 nm and 272.5 nm using acetylthiocholine and phenyl acetate, respectively. The spontaneous hydrolysis of both substrates could be neglected. The value of k_i was calculated from the intercept at the $1/k_{obs}$-axis obtained by plotting $1/k_{obs}$ values against the substrate concentration (Eqn.4). The values mentioned in the table are means of three measurements carried out at five or six substrate concentrations. S.D. of mean was 5% or less. The bimolecular rate constants of inhibition in the absence of substrate were determined according to the procedure of Ooms and Boter[12]. K_m values of acetylthiocholine and phenyl acetate estimated in the absence of inhibitor were found to be $5.7 \cdot 10^{-5}$ M and $1.3 \cdot 10^{-3}$ M, respectively, for acetylcholinesterase, and $0.91 \cdot 10^{-4}$ M and $5.0 \cdot 10^{-3}$ M, respectively, for butyrylcholinesterase.

Substrate and inhibitor	*Acetylcholinesterase*			*Butyrylcholinesterase*		
	Substrate concn. (mM)	*Inhibitor concn. (µM)*	k_i *($l \cdot mole^{-1} \cdot min^{-1}$)*	*Substrate concn. (mM)*	*Inhibitor concn. (µM)*	k_i *($l \cdot mole^{-1} \cdot min^{-1}$)*
Acetylthiocholine and A	0.1–0.3	0.10	*	0.1–0.4	0.15	$3.5 \cdot 10^6$
		0.15	*		0.18	$3.7 \cdot 10^6$
Acetylthiocholine and B	0.1–0.3	0.049	*	0.1–0.4	9.8	$1.2 \cdot 10^5$
		0.074	*		7.5	$1.0 \cdot 10^5$
Phenyl acetate and A	1–4	0.10	$0.73 \cdot 10^7$	2–5	0.19	**
		0.15	$0.74 \cdot 10^7$		0.15	**
Phenyl acetate and B	1–4	0.050	$1.8 \cdot 10^7$	2–5	4.9	**
		0.074	$1.6 \cdot 10^7$		3.0	**
In the absence of substrate						
A			$1.1 \cdot 10^7$			$3.7 \cdot 10^6$
B			$1.7 \cdot 10^7$			$1.0 \cdot 10^5$

*Plotting $1/k_{obs}$ against [*S*] no straight line was obtained.
**Plotting $1/k_{obs}$ against [*S*] a straight line was obtained. However, the intercept on the [*S*]-axis is not equal to $-K_m$ of the substrate used.

From the present results it follows that the determination of the bimolecular rate constant of the inhibition reaction of acetylcholinesterase and of butyrylcholinesterase can be carried out spectrophotometrically in the presence of a fixed concentration of phenyl acetate and of acetylthiocholine, respectively. In the method as described by Aldridge[1] the

inhibition reaction is followed discontinuously by adding the substrate to the enzyme–inhibitor mixture after different times of preincubation. The substrate added is assumed to block the inhibition reaction instantaneously. The present continuous method for the determination of k_i values lacks these disadvantages leading to a more accurate and less time-consuming technique. Hence, the present assay is especially suitable for the determination of the bimolecular rate constants of the inhibition by instable and/or potent inhibitors and for the separate estimation of the equilibrium constant of the complex-formation step and the rate constant of the phosphorylation step of the inhibition reaction[13].

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