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Combined Inhibition of Cholinesterases by Organophosphorus Compounds

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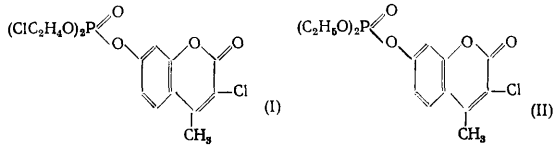
The review is devoted to studies on the combined inhibition of the catalytic activity of cholinesterases by organophosphorus compounds. This type of inhibition depends significantly on the presence of hydrophobic substituents in the organophosphorus molecule. It is suggested that there is a possibility of "concerted" or "non-concerted" sorption of the hydrophobic groups of the inhibitor on the hydrophobic regions in the vicinity of the anionic and esterase sites on the active surfaces of cholinesterases. These effects apparently determine the nature of the inhibition of cholinesterases.
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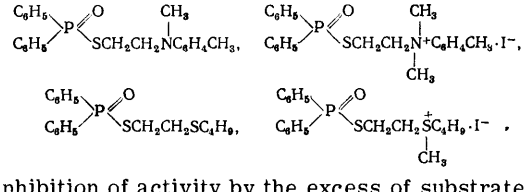
I. INTRODUCTION

The esters of phosphorus(V) acids, which exhibit a phosphorylating capacity, are known to be capable of inhibiting cholinesterases—vitally important enzymes which regulate the transmission of nerve excitation from the nerve to the innervated cell. Until recently it was believed that organophosphorus compounds are capable of only irreversible inhibition of cholinesterases by phosphorylating the serine hydroxyl, which forms part of the esterase centre in these enzymes. However, in 1969, Aldridge and Reiner[1] observed in experiments with coumarinyl phosphates (I) and (II) two types of inhibition of the catalytic activity of acetylcholinesterase (ACE) of bovine erythrocytes: (a) irreversible inhibition progressing in the course of time, and caused by the phosphorylation of the active centre of the enzyme; (b) reversible inhibition established instantaneously and non-progressive. According to the authors[1], the latter type of inhibition is associated with the interaction of the organophosphorus inhibitor (OPI) with the section of the active surface of the enzyme responsible for the inhibition of the activity of ACE by the excess of substrate.



(I) (II)

In 1971, a similar complex type of inhibition of catalytic activity was observed by Kabachnik and coworkers for another enzyme—butyrylcholinesterase (BuCE) of horse blood serum, in experiments with certain diphenylphosphinothiate esters:



Since inhibition of activity by the excess of substrate is not characteristic of BuCE, the authors[2] explained the presence of the reversible component in the enzyme inhibition by the possibility of the "non-productive" sorption of the inhibitor on the active surface of the enzyme (E), which does not entail the orientation of the inhibitor on the active surface of BuCE favourable for the phosphyrylation of the serine hydroxyl. The authors suggested that the enzyme–inhibitor complex (EI_r) formed is incapable of subsequent conversion into the phosphorylated enzyme (EIn'), in contrast to the usual Michaelis type complex (EIn): it is analogous to the enzyme–inhibitor complex obtained when cholinesterases are acted upon by reversible inhibitors, for example tetralkylammonium ions†. In other words, instead of the usual phosphorylation mechanism

$$E + In \rightleftarrows EIn \xrightarrow{k_2} EIn',$$

the following mechanism has been proposed for this complex and so-called combined type of inhibition[2]:

$$E + In \left\{ \begin{array}{l} \overset{k_1}{\rightleftarrows} EIn \xrightarrow{k_2} EIn' \\ \overset{K_{i,r}}{\rightleftarrows} EI_r \end{array} \right. ,$$

where k_1, k_{-1}, and k_2 are the rate constants for the corresponding reaction stages and $K_{i,r}$ is the equilibrium dissociation constant of the "non-productive" complex EI_r.

Combined inhibition is manifested primarily by the fact that, with increase of the inhibitor concentration [In], there is a significant decrease of the experimental bimolecular rate constant for the phosphorylation of the enzyme k_{II}, which is calculated from the equation

$$k_{II} = \frac{1}{[In]\, t} \ln (v_0/v_t),$$

where v_0 is the rate of the enzymatic hydrolysis of the substrate in the absence of the inhibitor and v_t the rate (after t min) of incubation of the enzyme with the inhibitor S, the concentration of which is many times greater than

† The extensive experimental data available at the present time permit the hypothesis that the complex EI_r does not differ significantly from the complex EIn, and may consist of a section of the enzyme–inhibitor complex which does not enter into the enzyme phosphorylation reaction. We shall therefore henceforth regard the complex EI_r as "non-productive" and the complex EIn as "productive".

the enzyme concentration. Reversible inhibition by these compounds is manifested both in the phosphorylation of the enzyme, where it is estimated in terms of the equilibrium inhibitor constant $K_{i,r}$, and in the enzymatic hydrolysis of the substrate (acetylcholine), where it is estimated in terms of the constant $K_{i,S}$.

The most reliable parameter of the combined inhibition is the decrease of the catalytic activity of the enzyme when $t \to 0$.

In order to calculate the rate constants for the irreversible inhibition of BuCE and ACE and of the equilibrium constants $K_{i,S}$ and $K_{i,r}$, the following equations were used[2]:

$$k = \frac{1}{[In]\,t}\left(\ln\frac{v_0}{v_t} - \ln\frac{v_0}{v_{0,i}}\right),$$

where k is the experimental bimolecular rate constant for the given value of t, and $v_{0,i}$ the rate of the enzymatic hydrolysis of acetylcholine in the presence of the inhibitor at zero time of the incubation with the enzyme:

$$K_{i,S} = \frac{K_m}{(K_m + [S])[v_0/(v_{0,i} - 1)]},$$

where K_m is the Michaelis constant and [S] the substrate concentration. The true bimolecular rate constant k_a, which is independent of the inhibitor concentration, and the inhibitor constant $K_{i,r}$ were determined graphically from the values of k for different [In] using the relation

$$k = \frac{k_a}{1 + K_{i,r}}.$$

A study of the kinetics of the interaction of BuCE with diphenylphosphinothioate esters[2] yielded data in good agreement with mechanism (2) and the equations which followed from it. This first study initiated a detailed investigation of the relation between the chemical nature of OPI and their ability to inhibit cholinesterases via the combined mechanism.

Preparations of butyrylcholinesterase (BuCE, CE 3.1.1.3) from horse blood serum and of acetylcholinesterase (ACE, CE 3.1.1.7) from human blood erythrocytes were used. The enzyme activities were determined from the rate of hydrolysis of acetylcholine chloride or acetylcholine iodide (AX).

Table 1. Constants for the inhibition of BuCE by the compounds $(C_6H_5)_2P(O)SCH_2CH_2R$ (A) and $(C_6H_5)_2P(O)OCH_2.CH_2R$ (B).[2]

R	$10^{-5}\,k_a$, litre mole^{-1} min^{-1}	$10^7\,K_{i,r}$, mole litre^{-1}	$10^7\,K_{i,S}$, mole litre^{-1}	$10^6\,K_i$, mole litre^{-1}
		A		B
SC_4H_9	0.25	21	33	12
$^+S(CH_3)C_6H_5 \cdot I^-$	15	4.8	2.4	1.8
$N(CH_3)C_6H_4$ (*m*-CH_3)	0.05	0.41	13	3.9
$^+N(CH_3)_2C_6H_4$ (*m*-CH_3)$\cdot I^-$	10	0.55	0.8	0.58

The rate of hydrolysis of AC under the influence of BuCE and ACE were determined by potentiometric titration of the acetic acid formed in the reaction at a constant pH (7.5 or 7.8) using pH-meters with glass electrodes[3,4]. In the study of the inhibition of the catalytic activity of cholinesterases by purely reversible inhibitors, the inhibitor constants K_i were determined by a graphical method[5]. The results of these studies are described in the present review.

II. INTERACTION OF CHOLINESTERASES WITH DIPHENYLPHOSPHINOTHIOATE ESTERS

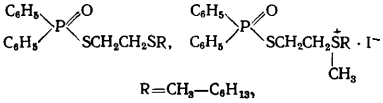
As stated above, Kabachnik and coworkers[2] investigated the inhibition of BuCE by certain diphenylphosphinothioate esters (Table 1).

The phosphinothioates investigated proved to be strong inhibitors of BuCE with a combined activity. The bimolecular irreversible inhibition rate constants obtained for them are close to the values for the analogous *OO*-diethyl *S*-alkylphosphorothioates and *O*-ethyl *S*-alkyl methylphosphonothioates[6,7]. In terms of their reversible activity, they are greatly superior to typical reversible inhibitors such as tetra-alkylammonium ions[8]. The inhibitor constants of the phosphinothioates are smaller approximately by an order of magnitude than the values of K_i for their oxygen analogues, which are also powerful but only reversible inhibitors (Table 1). Thus both phosphinothioates and their oxygen analogues are capable of forming very stable enzyme–inhibitor complexes, probably because of the presence in their composition of two or three phenyl groups, which greatly intensify the hydrophobic interaction of OPI with BuCE, the latter having comparatively large hydrophobic regions in the vicinity of both the anionic and the esterase sites[9-11].

The reversible inhibition by the phosphinothioates investigated is of a competitive type, as shown by the constancy of $K_{i,S}$ calculated by Eqn.(14) for different [In] and by the direct proportionality between $1/k$ and [In]. When [S] is reduced by a factor of 20 for a given [In], the slope remains the same (Fig. 1), but the intercept b diminishes, i.e. the substrate exhibits a protective function in relation to the reversible inhibition of the enzyme, which is again characteristic of competitive inhibition.

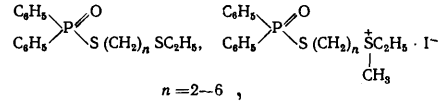
There is a definite relation between the phosphorylation rate constants k_a and the equilibrium dissociation constants of reversible enzyme–inhibitor complexes ($K_{i,S}$ and $K_{i,r}$): the higher the value of k_a, the lower the constants $K_{i,S}$ and $K_{i,r}$, i.e. the more stable are the complexes. Inhibitors with cationic groups have higher values of k_a and lower values of $K_{i,S}$ and $K_{i,r}$ than "uncharged" compounds.

The data obtained in the study of the inhibiting activity of *S*-(β-alkylthioethyl) diphenylphosphinothioates and their methiodides[12,13] (series Ia and IIa, Tables 2 and 3), i.e.



R=CH_3–C_6H_{13},

as well as *S*-(ω-ethylthioalkyl) diphenylphosphinothioates[14,15] (series IIIa and IVa, Tables 4 and 5), i.e.



$n = 2-6$,

in relation to BuCE demonstrated a considerable influence of the hydrophobic interactions on the combined inhibition. It follows from Tables 2 and 3 that, with increase of the length of the alkyl group attached to sulphide or sulphonium sulphur (series Ia and IIa), a continuous increase of both

irreversible (increase of k_a) and reversible (decrease of $K_{i,S}$ and $K_{i,r}$) inhibition of the catalytic activity of BuCE is observed[13]. An analogous increase of inhibition with increase of the distance between the phosphorus and sulphide or sulphonium sulphur atoms in compounds of series IIIa and VIa (Tables 4 and 5) has also been noted[15]. A characteristic feature is that, with increase of the P–S distance, the irreversible inhibition hardly changes (series IIIa, Table 5) or diminishes, particularly at the beginning as n increases from 2 to 3 (Table 5).

Table 2. Constants for the inhibition of BuCe by the compounds $(C_6H_5)_2P(O)SCH_2CH_2SR$ (series Ia) and $(C_6H_5)_2P(O)OCH_2CH_2SR$ (series Ib).[13]

R	$10^{-4}k_a$, litre mole^{-1}	$10^6K_{i,r}$, mole litre^{-1}	$10^6K_{i,S}$, mole litre^{-1}	10^6K_i, mole litre^{-1}
	series Ia			series Ib
CH_3	0.33	9.3	11	28
C_2H_5	0.42	7.2	7.3	23
C_3H_7	0.77	3.6	4.1	16
C_4H_9	2.5	2.1	3.4	8.4
C_5H_{11}	7.2	0.90	0.95	4.0
C_6H_{13}	13	0.55	0.48	1.2

Table 3. Constants for the inhibition of BuCE by the compounds $(C_6H_5)_2P(O)SCH_2CH_2\overset{+}{S}(CH_3)R.I^-$ (series IIa) and $(C_6H_5)_2P(O)OCH_2CH_2\overset{+}{S}(CH_3)R.I^-$ (series IIb).[13]

R	$10^{-5}k_a$, litre mole^{-1} min^{-1}	$10^7K_{i,r}$, mole litre^{-1}	$10^7K_{i,S}$, mole litre^{-1}	10^6K_i, mole litre^{-1}
	series IIa			series IIb
CH_3	3.0	14	6.6	16
C_2H_5	4.9	10	4.4	13
C_3H_7	6.7	6.1	2.7	6.7
C_4H_9	15	4.8	1.2	1.8
C_5H_{11}	22	3.1	0.89	0.94
C_6H_{13}	27	1.2	0.56	0.51

Table 4. Constants for the inhibition of BuCE by the compounds $(C_6H_5)_2P(O)S(CH_2)_nSC_2H_5$ (series IIIa) and $(C_6H_5)_2P(O)O(CH_2)_nSC_2H_5$ (series IIIb).[15]

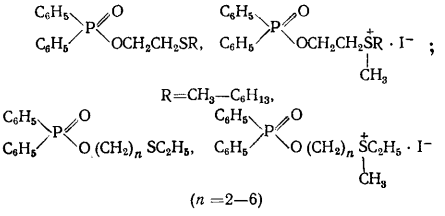
n	$10^{-3}k_a$, litre mole^{-1} min^{-1}	$10^6K_{i,r}$, mole litre^{-1}	$10^6K_{i,S}$, mole litre^{-1}	10^6K_i, mole litre^{-1}
	series IIIa			series IIIb
2	4.2	7.2	7.3	23
3	5.5	3.7	3.2	20
4	5.0	1.5	2.1	14
5	5.2	1.3	1.6	7.4
6	4.8	1.2	0.9	4.8

It follows from the results that the equilibrium constants $K_{i,S}$ and $K_{i,r}$ do indeed characterise the formation of a "non-productive" enzyme–inhibitor complex EIr, different from the Michaelis complex EIn which is rapidly converted into the phosphorylated enzyme EIn'. This has been shown most convincingly by the results of experiments on compounds in series IVa: with increase of n, there is an appreciable increase of the sorbability of the inhibitor, but at the same time the conditions for the formation of the "productive" complex are impaired owing to the increasing incompatibility of the distance between the phosphorus and sulphonium sulphur atoms in the inhibitor molecule and the distance between the esterase and anionic sites of the catalytic centre of the enzyme[15]. The influence of this incompatibility on the irreversible inhibition has been thoroughly investigated for the compounds $CH_3(C_2H_5C)P(C)S(CH_2)nC_2H_5$ and their methosulphates[16].

Table 5. Constants for the inhibition of BuCE by the compounds $(C_6H_5)_2P(O)S(CH_2)n\overset{+}{S}(CH_3)C_2H_5.I^-$ (series IVa) and $(C_6H_5)_2P(O)O(CH_2)n\overset{+}{S}(CH_3)C_2H_5.I^-$ (series IVb).[15]

n	$10^{-4}k_a$ litre mole^{-1} min^{-1}	$10^7K_{i,r}$, mole litre^{-1}	$10^7K_{i,S}$, mole litre^{-1}	10^6K_i, mole litre^{-1}
	series IVa			series IVb
2	49	10	4 4	13
3	6.0	9.2	3.1	3.0
4	5.3	6.4	2.9	2.0
5	3.0	4.8	2.5	1.6
6	2.5	4.5	1.9	1.2

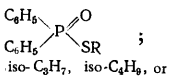
The observed enhancement of the sorbability of the inhibitors on the active surface of BuCE with increase of the size of the alkyl group attached to the sulphide or sulphonium sulphur in compounds of series Ia and IIa and with increase in the length of the polymethylene chain in compounds of series IIIa and IVa takes place owing to the improvement of the binding of the diphenylphosphinothioates to the esterase site of BuCE. Evidently it is caused by the enhanced interaction of the inhibitor with the enzyme near the anionic site. A characteristic feature is that the changes in $K_{i,S}$ and $K_{i,r}$ are in all cases correlated with the change in the K_i for the corresponding oxygen analogues[13,15]:



(n = 2–6)

(series Ib, IIb, IIIb, and IVb, Tables 2–5), which indicates the reliability of the results.

The study of the anticholinesterase activity of diphenylphosphinothioate thioesters[17,18] (series V, Table 6), i.e.



R=$CH_3C_6H_{13}$; iso-C_3H_7, iso-C_4H_9, or iso-C_6H_{11}

showed that these compounds inhibit cholinesterases via the combined mechanism. Both the reversible and irreversible inhibiting activities depend on the length and degree of branching of the alkylthio-group. Thus, when straight-chain alkyl substituents in the alkylthio-groups are displaced by branched substituents, a sharp decrease

of both the irreversible and reversible anticholinesterase activities is observed in relation to ACE.[17] In experiments with BuCE [18] such replacement actually increases the inhibiting activity of the compounds (Table 6). Therefore compounds with straight-chain substituents inhibit ACE more strongly [k_a(ACE)/k_a(BuCE) > 1], while compounds with branched substituents inhibit BuCE more strongly [k_a(ACE)/k_a(BuCE) < 1].

Table 6. Constants for the inhibition of cholinesterases by the compounds $(C_6H_5)_2P(O)SR$ (series V).[18]

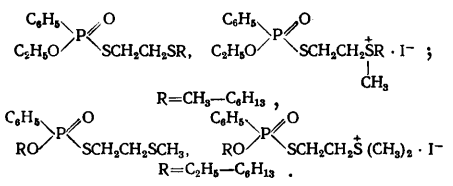
R	$10^{-2}k_a$, litre mole^{-1} min^{-1}	$10^5K_{i,r}$, mole litre^{-1}	$10^5K_{i,S}$, mole litre^{-1}	$10^{-2}k_a$, litre mole min^{-1}	$10^5K_{i,r}$, mole litre^{-1}	$10^6K_{i,S}$, mole litre^{-1}	k_a(ACE)/ k_a(BuCE)
	ACE			BuCE			
CH_3	2.2	7.7	36	1.2	4,3	26	1.8
C_2H_5	4.9	4.6	19	1.5	3.5	14	3.3
C_3H_7	12	1.9	3.2	1.9	3.2	7.6	6.3
C_4H_9	25	0.62	1.3	2.2	1.8	5.0	11.4
C_5H_{11}	46	0.44	0.72	2.4	1.7	4.3	19.2
C_6H_{13}	58	0.29	0.64	2.5	1.4	2.8	23.2
iso-C_3H_7	0.3	50	50	2.9	4.5	16	0.10
iso-C_4H_9	0.5	14	14	8.5	3.7	6.1	0.06
iso-C_5H_{11}	0.7	82	8.2	27	2.2	3.2	0.026

It follows from these results that the enhancement of the hydrophobic sorption of inhibitors on the active surfaces of cholinesterases via the alkylthio-groups increases the anticholinesterase activity of OPI of the types investigated only when the given effect is consistent with the hydrophobic sorption of phenyl groups on the hydrophobic regions located near the esterase site of the enzyme, i.e. when the increase of the activity of the OPI does not impair the "productive" sorption of the inhibitor on the active surface of the enzyme. If the impairment occurs, then, instead of an increase, a decrease of the anticholinesterase activity should take place owing to the "non-productive" sorption, which occurs when ACE interacts with OPI having branched alkylthio-groups[17].

On the whole the result of experiments with diphenylphosphenothioate esters confirm the existing ideas concerning the "productive" and "non-productive" sorption developed to explain the causes of the selective catalytic activity of chymotrypsin[19,20] and cholinesterases[21,22].

III. REACTIONS OF CHOLINESTERASES WITH PHENYLPHOSPHONOTHIOATE, CYCLOHEXYLPHOSPHONOTHIOATE, AND BENZYLPHOSPHONOTHIOATE THIOESTERS

In order to investigate the influence of hydrophobic sorption on the manifestation of combined inhibition by OPI, various phenylphosphonothioate esters were synthesised and their anticholinesterase activity was investigated[23,24]:



These compounds differ from those discussed above by the fact that they contain a straight-chain alkoxy-group at the phosphorus atom instead of the bulky phenyl group (Tables 7 and 8).

Table 7. Constants ($10^{-4}k_{II}$, litre mole^{-1} min^{-1}) for the inhibition of cholinesterases by the compounds $C_6H_5(C_2H_5O)P(O)SCH_2CH_2SR$ (series VI).[25]

R	ACE	BuCE	R	ACE	BuCE
CH_3	6.1	1.3	C_4H_9	23	66
C_2H_5	3.1	3.7	C_5H_{11}	7.1	19
C_3H_7	2.6	1.1	C_6H_{13}	1.7	15

Table 8. Constants ($10^{-7}k_{II}$, litre mole^{-1} min^{-1}) for the inhibition of cholinesterases by the compounds $C_6H_5(C_2H_5O)P(O)SCH_2CH_2\overset{+}{S}(CH_3)R.I^-$ (series VII).[25]

R	ACE	BuCE	R	ACE	BuCE
CH_3	15	0.63	C_4H_9	5.7	7.6
C_2H_5	7.6	1.8	C_5H_{11}	12	6.5
C_3H_7	11	5.4	C_6H_{13}	14	3.7

The replacement of one phenyl group in compounds of series Ia and IIa by an alkoxy-group led to a sharp alteration of the nature of the inhibition of ACE and BuCE. In contrast to the analogous diphenylphosphinothioate esters, *O*-ethyl phenylphosphonothioates of both sulphide (series VI, Table 7) and sulphonium (series VII, Table 8) types cause only the irreversible inhibition of cholinesterases[25]. The $\lg(v_0/v_t)-t$ linear plots for these substances pass through the origin of coordinates. Furthermore, the values of k found are almost independent of the concentration of the inhibitors. The absence of reversible inhibition indicates a complementarity between the compounds and the active centre of the enzyme such that "non-productive" sorption is either completely absent or is so weak that it cannot be recorded with the aid of the methods described.

Thus, when the alkylthio-group, which may be sorbed on the hydrophobic region near the anionic site, became sufficiently elongated, the combined type of inhibition could not be detected either for ACE or for BuCE.

Next an attempt was made to find inhibitors of the combined type among compounds analogous to those discussed above but having alkoxy-groups of different lengths, capable of being sorbed on the hydrophobic regions near the esterase centre. *O*-Alkyl *S*-(β-methylthioethyl) phenylphosphonothioates (series VIII, Table 9) and their methiodides (series IX, Table 10) were selected as compounds of this kind[26]. In experiments with BuCE combined inhibition was observed for compounds with long alkoxy-groups (C_5H_{11}, C_6H_{13}). For compounds with short alkoxy-groups, only irreversible inhibition was observed[26]. In the experiments with ACE the reversible component was shown only for the last two compounds of the sulphide type (series VIII, Table 9). Substances of the sulphonium type (series (IX) showed only irreversible inhibition (Table 10), which

permitted the conclusion that in the case of ACE an increase of the tendency of the inhibitor towards hydrophobic sorption on the active surface of the enzyme, both near the anionic and esterase sites, is not accompanied by the formation of a "non-productive" enzyme–inhibitor complex, as in the case of BuCE.[26]

Table 9. Constants for the inhibition of cholinesterases by the compounds $C_6H_5(RO)P(O)SCH_2CH_2SCH_3$ (series VIII).[26]

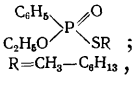
R	$10^{-4}\,k_{II}$, litre mole^{-1} min^{-1}	$10^{6}\,K_{i,r}$, mole litre^{-1}	$10^{6}\,K_{i,S}$, mole litre^{-1}	$10^{-4}\,k_{II}$, litre mole^{-1} min^{-1}	$10^{6}\,K_{i,r}$, mole litre^{-1}	$10^{7}\,K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
C_2H_5	6.1	—	—	1.3	—	—
C_3H_7	5.5	—	—	7.4	—	—
C_4H_9	15	—	—	26	—	—
C_5H_{11}	17*	4.1	1.3	38	2.1	6.0
C_6H_{13}	19*	2.9	1.5	74	3.9	2.3

*The values of k_a (litre mole^{-1} min^{-1}) are quoted.

Table 10. Constants for the inhibition of cholinesterases by the compounds $C_6H_5(RO)P(O)SCH_2CH_2\overset{+}{S}(CH_3)_2.I^-$ (series IX).[26]

R	$10^{-7}\,k_{II}$, litre mole^{-1} min^{-1}	$10^{-7}\,k_{II}$, litre mole^{-1} min^{-1}	$10^{6}\,K_{i,r}$, mole litre^{-1}	$10^{6}\,K_{i,S}$, mole litre^{-1}
	ACE		BuCE	
C_2H_5	15	0.63	—	—
C_3H_7	5.5	3.4	—	—
C_4H_9	24	11	—	—
C_5H_{11}	20	2.2	1.9	4.8
C_6H_{13}	12	3.1	4.4	5.9

In the study of the anticholinesterase activity of phenylphosphonothioate thioesters without any polar substituents in the alkylthio-group,



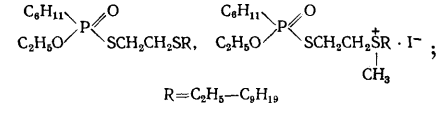
(series X, Table 11) the eliminated part of the OPI molecule was found to influence the nature of the anticholinesterase activity[27]. In contrast to *O*-ethyl *S*-(β-alkylthioethyl) phenylphosphonothioates and their methiodides (series VI–IX), all the compounds of this series inhibited ACE and BuCE via the combined mechanism. Evidently the absence from compounds of series X of dipolar and particularly cationic groups in the eliminated part of the molecule impairs the conditions for the correct orientation of the molecule on the active surface of the enzyme, on the one hand, and decreases the electrophilic phosphorylating capacity, on the other, which leads to a distinct combined inhibition of the catalytic activity of cholinesterases.

However, the dipolar and cationic groups in the eliminated part of the OPI molecule are by no means always able to ensure "productive" sorption. This was manifested not only for diphenylphosphinothioate esters, but also for two phenylphosphonothioate esters (Tables 9 and 10). In the first instance the orienting influence of the mercapto-group was interferred with by the two phenyl substituents, and in the second by the phenyl and alkoxy-substituents next to the C_5H_{11} and C_6H_{13} groups. In the presence of smaller alkyl groups concerted sorption of the phosphoryl component and of the mercapto-group was observed and there was no reversible component in the inhibiting activity of the OPI.

Table 11. Constants for the inhibition of cholinesterases by the compounds $C_6H_5(C_2H_5O)P(O)SR$ (series X).[27]

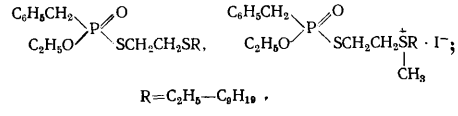
R	$10^{-3}\,k_a$, litre mole^{-1} min^{-1}	$10^{5}\,K_{i,r}$, mole litre^{-1}	$10^{5}\,K_{i,S}$, mole litre^{-1}	$10^{-3}\,k_a$, litre mole^{-1} min^{-1}	$10^{5}\,K_{i,r}$, mole litre^{-1}	$10^{5}\,K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
CH_3	0.29	76	6.3	1.0	20	5.0
C_2H_5	0.38	11	11	0.14	150	6.1
C_3H_7	0.26	51	15	0.28	29	5.7
C_4H_9	0.58	29	8.0	0.77	12	2.6
C_5H_{11}	1.15	5.7	1.8	4.5	4.5	0.64
C_6H_{13}	2.10	2.2	3.0	51	0.62	0.62

The influence of hydrophobic substituents at the phosphorus atom on the combined inhibition of cholinesterases by OPI was also investigated for a number of cyclohexylphosphonothioate thioesters[28] (series XI and XII, Tables 12 and 13);



R=C_2H_5–C_9H_{19}

and for benzylphosphonothioate thioesters[29] (series XIII and XIV, Tables 14 and 15):



R=C_2H_5–C_9H_{19}.

Table 12. Constants for the inhibition of cholinesterases by the compounds $C_6H_{11}(C_2H_5O)P(O)SCH_2CH_2SR$ (series XI).[30]

R	$10^{-3}\,k_a$, litre mole^{-1} min^{-1}	$10^{5}\,K_{i,r}$, mole litre^{-1}	$10^{5}\,K_{i,S}$, mole litre^{-1}	$10^{-3}\,k_a$, litre mole^{-1} min^{-1}	$10^{5}\,K_{i,r}$, mole litre^{-1}	$10^{6}\,K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
C_2H_5	1.5	29	9.7	0.65	13	28
C_3H_7	2.8	8.0	4.8	6.3	2.0	17
C_4H_9	4.0	6.0	6.6	25	0.9	1.4
C_5H_{11}	2.9	7.2	3.7	23	1.0	3.1
C_6H_{13}	15	1.0	2.9	48	0.53	2.5
C_7H_{15}	8.3	2.0	6.3	5.7	2.2	7.9
C_8H_{17}	9.5	1.3	1.5	16	0.45	0.73
C_9H_{19}	15	0.3	1.3	5.0	0.29	0.89

It was found that the presence of the cyclohexyl group instead of the phenyl group at the phosphorus atom in the case of compounds of the sulphide type leads to a pronounced combined inhibition of BuCE (Table 12).[30] An

analogous effect for BuCE has been observed in the presence of the corresponding benzylphosphonothioate thioesters (Table 14),[31] the latter compounds being more active than derivatives of cyclohexylphosphonothioic acid[30].

Table 13. Constants for the inhibition of cholinesterases by the compounds $C_6H_{11}(C_2H_5O)P(O)SCH_2CH_2\overset{+}{S}(CH_3)R.I^-$ (series XII).[30]

R	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^6 K_{i,r}$, mole litre^{-1}	$10^6 K_{i,S}$, mole litre^{-1}
	ACE		BuCE	
C_6H_5	1.0	0.57	14	70
C_7H_7	0.7	0.84	5.8	63
C_8H_9	1.1	9.5	0.42	8.4
C_9H_{11}	3.3	16	1.5	12
$C_{10}H_{13}$	9.4	12	5.2	25
C_7H_{15}	10	7.8	5.0	20
C_8H_{17}	12	3.9	1.4	11
C_9H_{19}	2.8	2.5	0.85	9

Table 14. Constants for the inhibition of cholinesterases by the compounds $C_6H_5CH_2(C_2H_5O)P(O)SCH_2CH_2SR$ (series XIII).[31]

R	$10^{-4}k_a$, litre mole^{-1} min^{-1}	$10^6 K_{i,r}$, mole litre^{-1}	$10^6 K_{i,S}$, mole litre^{-1}	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^7 K_{i,r}$, mole litre^{-1}	$10^8 K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
C_2H_5	1.5	25	19	0.74	85	95
C_3H_7	2.6	14	16	7.5	59	69
C_4H_9	2.9	19	12	27	2.5	3.2
C_5H_{11}	1.3	23	21	19	8.9	4.1
C_6H_{13}	3.3	9.8	8.3	18	7.2	5.6
C_7H_{15}	21	1.0	1.9	6.5	9.5	7.0
C_8H_{17}	22	3.6	2.2	22	16	6.5
C_9H_{19}	24	3.2	1.9	13	12	4.9

The interaction of cyclohexylphosphonothioate and benzylphosphonothioate thioesters of the onium type (series XII and XIV, Tables 13 and 15) with BuCE takes place differently. In this case the only inhibitors with a combined type of activity are derivatives of cyclohexylphosphonothioic acid (Table 13).[30] Benzylphosphonothioate thioesters of the onium type cause only the irreversible inhibition of BuCE (Table 14).[31]

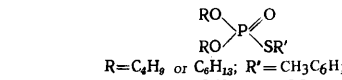
Table 15. Constants ($10^{-7}k_{II}$, litre mole^{-1} min^{-1}) for the inhibition of cholinesterases by the compounds $C_6H_5CH_2(C_2H_5O)P(O)SCH_2CH_2\overset{+}{S}(CH_3)R.I^-$ (series (XIV).[31]

R	ACE	BuCE	R	ACE	BuCE
C_2H_5	1.0	6.5	C_6H_{13}	0.94	8.2
C_3H_7	0.82	21	C_7H_{15}	1.4	13
C_4H_9	1.2	12	C_8H_{17}	1.6	88
C_5H_{11}	1.3	34	C_9H_{19}	2.8	12

In experiments with ACE only irreversible inhibition was observed in all cases (Tables 13 and 15). These data and also those obtained in the study of the analogous phenylthiophosphonothioate thioesters (series VI–IX) show that the combined inhibition of the catalytic activity of cholinesterases is in most cases promoted by the presence of hydrophobic groups in the inhibitor molecule, particularly those in the phosphorylating component, and is hindered by the presence of polar groups. This is manifested most clearly in the case of ACE.

IV. INTERACTION OF CHOLINESTERASES WITH *OO*-DIALKYL *S*-ALKYL PHOSPHOROTHIOATES

Combined inhibition of cholinesterases does not occur solely when bulky hydrophobic substituents are present at the phosphorus atom. This effect is shown distinctly in those instances where there are two alkoxy-groups at the phosphorus atom. Thus *OO*-dialkyl *S*-alkyl phosphorothioates



R=C_4H_9 or C_6H_{13}; R'=$CH_3C_6H_{13}$ iso-C_4H_9, or iso-C_5H_{11},

with two butoxy-[32] and two hexyloxy-groups[33] (series XV and XVI, Tables 16 and 17) cause the combined inhibition of cholinesterase, the reversible component being in all cases more pronounced in the interaction with BuCE than with ACE.

Table 16. Constant for the inhibition of cholinesterases by the compounds $(C_4H_9O)_2P(O)SR$ (series XVI).[32]

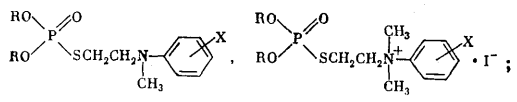
R	$10^{-4}k_a$, litre mole^{-1} min^{-1}	$10^6 K_{i,r}$, mole litre^{-1}	$10^6 K_{i,S}$, mole litre^{-1}	$10^{-5}k_a$, litre mole^{-1} min^{-1}	$10^7 K_{i,r}$, mole litre^{-1}	$10^7 K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
CH_3	0.75	34	11	0.12	27	11
C_2H_5	1.2	19	31	0.13	16	31
C_3H_7	0.85	31	24	0.86	33	14
C_4H_9	1.2	4.5	9.9	3.2	7.1	5.5
C_5H_{11}	2.1	1.7	4.4	7.7	1.9	1.9
C_6H_{13}	2.2	7.5	3.4	61	0.1	0.26
iso-C_4H_9	2.4	31	4.7	6.2	1.6	1.4
iso-C_5H_{11}	9.4	18	3.7	2.0	16	1.0

Table 17. Constants for the inhibition of cholinesterases by the compounds $(C_6H_{13}O)_2P(O)SR$ (series XVI).[33]

R	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^8 K_{i,r}$, mole litre^{-1}	$10^8 K_{i,S}$, mole litre^{-1}	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^7 K_{i,r}$, mole litre^{-1}	$10^7 K_{i,S}$, mole litre^{-1}
	ACE			BuCE		
CH_3	4.9	43	2.4	4.5	3.9	2.2
C_2H_5	3.3	79	30	1.2	31	0.64
C_3H_7	13	3.7	4.9	3.3	1.7	1.2
C_4H_9	21	1.9	2.6	2.8	6.0	1.1
C_5H_{11}	14	1.9	1.9	1.3	42	2.7
C_6H_{13}	8.1	2.3	2.4	2.1	7.8	4.6
iso-C_4H_9	5.3	5.0	4.2	1.0	40	4.8
iso-C_5H_{11}	15	2.1	1.5	5.9	2.9	1.6

The combined inhibition of the catalytic activity of cholinesterases is apparently fairly characteristic of phosphorothioate thioesters even when there are small alkoxy-groups at the phosphorus atom. This has been

shown for a series of *OO*-dialkyl *S*-[β-(*NN*-arylmethylamino)ethyl] phosphorothioates and their methiodides [34,35]:



R=C_3H_7, C_4H_9, iso-C_3H_7; X=H, *n*-, *m*-CH_3; *p*-, *m*-CH_3O, *p*-, *m*-Cl

It was found that OPI of the above type, having straight-chain alkoxy-groups at the phosphorus atom, give rise to combined inhibition [36,37]. This applies both to "uncharged" compounds (series XVII–XIII, Tables 18 and 19) and to compounds of the onium type (series XIX and XX, Tables 20 and 21). In all cases combined inhibition is shown more in relation to BuCE than to ACE.

Table 18. Constants for the inhibition of cholinesterases by the compounds $(C_3H_7O)_2P(O)SCH_2CH_2N(CH_3)C_6H_4R$ (series XVII). [36]

R	ACE		BuCE	
	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{7}K_i$, S, mole litre^{-1}	$10^{-7}k_{II}$, litre mole^{-1} min^{-1}	$10^{8}K_i$, S, mole litre^{-1}
p-OCH_3	0.9	10.2	0.2	6.8
p-CH_3	1.6	7.6	0.7	2.2
m-CH_3	1.8	5.5	0.8	2.8
H	1.7	8.5	1.6	1.0
m-OCH_3	1.5	7.5	0.9	2.2
p-Cl	4.7	1.7	1.3	1.0
m-Cl	3.1	3.4	2.1	1.2

Table 19. Constants for the inhibition of cholinesterases by the compounds $(C_4H_9O)_2P(O)SCH_2CH_2N(CH_3)C_6H_4R$ (series XVIII). [37]

R	ACE		BuCE	
	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{7}K_i$, S, mole litre^{-1}	$10^{-7}k_{II}$, litre mole^{-1} min^{-1}	$10^{8}K_i$, S, mole litre^{-1}
p-OCH_3	2.7	2.3	0.6	1.6
p-CH_3	13	1.5	2.5	6.9
m-CH_3	4.7	3.2	2.7	5.7
H	6.8	2.6	2.0	9.0
m-OCH_3	3.2	3.9	4.3	2.1
p-Cl	12	0.9	3.0	3.5
m-Cl	5.4	2.6	2.6	3.7

When there are two branched isopropoxy-groups at the phosphorus atom, combined inhibition is not observed in all cases [38]. Thus the reversible component is observed in the inhibition of ACE and BuCE only by "uncharged" compounds (series XXI, Table 22). The only exception is the *p*-methoxy-derivative, which causes only the irreversible inhibition of BuCE. Only some OPI of the onium type exhibit the combined type of inhibition in relation to ACE, while BuCE is only irreversibly inhibited by these compounds [38] (series XXII, Table 23). Thus BuCE does not always tend to be more readily inhibited via the combined mechanism by *OO*-dialkyl *S*-alkyl phosphorothioate esters, in contrast to the phosphonothioate thioesters.

Table 20. Constants for the inhibition of cholinesterases by the compounds $(C_3H_7O)_2P(O)SCH_2CH_2\overset{+}{N}(CH_3)_2C_6H_4R.I^-$ (series XIX). [36]

R	ACE		BuCE	
	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{8}K_i$, S, mole litre^{-1}	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{10}K_i$, S, mole litre^{-1}
p-OCH_3	1.5	9.8	2.1	3.3
p-CH_3	3.6	3.3	2.8	1.8
m-CH_3	2.6	5.5	3.1	2.1
H	2.8	3.2	6.4	2.8
m-OCH_3	2.2	18	3.6	1.9

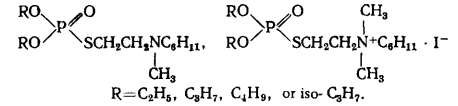
Table 21. Constants for the inhibition of cholinesterases by the compounds $(C_4H_9O)_2P(O)SCH_2CH_2\overset{+}{N}(CH_3)_2C_6H_4R.I^-$ (series XX). [37]

R	ACE		BuCE	
	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{8}K_i$, S, mole litre^{-1}	$10^{-8}k_{II}$, litre mole^{-1} min^{-1}	$10^{10}K_i$, S, mole litre^{-1}
p-OCH_3	2.6	4.1	2.6	2.5
p-CH_3	6.1	1.7	3.6	3.6
m-CH_3	3.7	3.2	4.9	2.6
H	6.3	1.9	6.2	7.4
m-OCH_3	4.2	2.6	3.2	2.1

Table 22. Constants for the inhibition of cholinesterases by the compounds (iso-$C_3H_7O)_2P(O)SCH_2CH_2N(CH_3)C_6H_4R$ (series XXI). [38]

R	ACE			BuCE		
	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^{8}K_i$, r, mole litre^{-1}	$10^{8}K_i$, S, mole litre^{-1}	$10^{-8}k_a$, litre mole^{-1} min^{-1}	$10^{7}K_i$, r, mole litre^{-1}	$10^{7}K_i$, S, mole litre^{-1}
p-OCH_3	2.5	77	32	1.0	—	—
p-CH_3	4.3	30	16	4.2	25	4.8
m-CH_3	3.7	32	9.4	2.4	29	10
H	0.9	15	12	2.9	35	7.0
m-OCH_3	3.4	65	28	3.7	34	7.4
p-Cl	43	13	2.6	64	0.18	0.31
m-Cl	45	0.46	2.3	40	5.0	1.0

Analogous results were also obtained in experiments with *OO*-dialkyl *S*-alkyl phosphorothioate esters containing the cyclohexyl group at the nitrogen atom in the β-position relative to the thioester group [39]:



R=C_2H_5, C_3H_7, C_4H_9, or iso-C_3H_7.

Compounds with isopropoxy- or n-butoxy-groups at the phosphorus atom, exhibit the combined type of inhibition only in relation to ACE, the effect being observed with

both "uncharged" compounds (series XXIII, Table 24) and with methiodides (series XXIV, Table 25). Organophosphorus inhibitors of the above types cause only the irreversible inhibition of BuCE.

Table 23. Constants for the inhibition of cholinesterases by the compounds (iso-$C_3H_4O)_2P(O)SCH_2CH_2\overset{+}{N}(CH_3)_2C_6H_4R.I^-$ (series XXII).[38]

R	$10^{-3}\ k_a$, litre mole^{-1} min^{-1}	$10^6\ K_i$, r, mole litre^{-1}	$10^7\ K_i$, S, mole litre^{-1}	$10^{-7}\ k_{II}$, litre mole^{-1} min^{-1}
	ACE			BuCE
p-OCH_3	12	—	—	2.3
p-CH_3	16	—	—	12
m-CH_3	30	1.4	0.5	8.1
H	9.3	5.5	1.0	5.8
m-OCH_3	29	2.4	62.	13

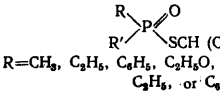
Table 24. Constants for the inhibition of cholinesterases by the compounds $(RO)_2P(O)SCH_2CH_2N(CH_3)C_6H_{11}$ (series XXIII).[39]

R	$10^{-3}\ k_a$, litre mole^{-1} min^{-1}	$10^6\ K_i$, r, mole litre^{-1}	$10^7\ K_i$, S, mole litre^{-1}	$10^{-7}\ k_{II}$, litre mole^{-1} min^{-1}
	ACE			BuCE
C_2H_5	4.6	—	—	3.2
C_3H_7	24	—	—	42
iso-C_3H_7	0.18	30	51	1.3
C_4H_9	35	0.34	0.19	22

Table 25. Constants for the inhibition of cholinesterases by the compounds $(RO)_2P(C)SCH_2CH_2\overset{+}{N}(CH_3)_2C_6H_{11}.I^-$ (series XXIV).[39]

R	$10^{-6}\ k_a$, litre mole^{-1} min^{-1}	$10^6\ K_i$, r, mole litre^{-1}	$10^6\ K_i$, S, mole litre^{-1}	$10^{-7}\ k_{II}$, litre mole^{-1} min^{-1}
	ACE			BuCE
C_2H_5	2.0	—	—	6.3
C_3H_7	0.1	—	—	67
iso-C_3H_7	13	1.4	1.1	5.0
C_4H_9	0.13	$3.0\cdot10^{-3}$	$6.3\cdot10^{-3}$	43

The above experimental data permit the conclusion that the combined inhibition of the catalytic activity of cholinesterases is most effectively promoted by hydrophobic substituents in the phosphorylating component of the CPI molecule. However, there are also instances where substituents in the eliminated component of the CPI molecule exert a decisive influence. This has been demonstrated for the diphenylmethyl esters of various phosphorus(V) thio-acids[40]:



R=CH_3, C_2H_5, C_6H_5, C_2H_5O, or C_6H_5O; R′=C_2H_5O, C_2H_5, or C_6H_5O.

The tendency of these compounds to exhibit the combined type of inhibition in relation to ACE and BuCE is very pronounced even in those cases where there are groups with short hydrophobic substituents at the phosphorus atom. The presence of two phenoxy-groups at the phosphorus atom leads to the loss of ability by OPI to cause the irreversible inhibition of ACE, but BuCE undergoes combined inhibition under these conditions (series XXV, Table 26).

Table 26. Constants for the inhibition of cholinesterases by the compounds $RR'P(O)SCH(C_6H_5)_2$ (series XXV).[40]

R	R′	$10^{-4}\ k_a$, litre mole^{-1} min^{-1}	$10^6\ K_i$, r, mole litre^{-1}	$10^7\ K_i$, S, mole litre^{-1}	$10^{-4}\ k_a$, litre mole^{-1} min^{-1}	$10^6\ K_i$, r, mole litre^{-1}	$10^7\ K_i$, S, mole litre^{-1}
		ACE			BuCE		
CH_3	C_2H_5O	49.5	0.99	9.2	1.62	6.5	8.3
C_2H_5O	C_2H_5O	18.4	0.74	6.75	9.43	17.7	8.95
C_2H_5	C_2H_5	0.143	100	34	0.052	2.15	78.7
C_6H_5	C_2H_5O	2.18	10.5	18	23	9.78	1.26
C_6H_5O	C_6H_5O	—	—	12	3.96	22.6	773

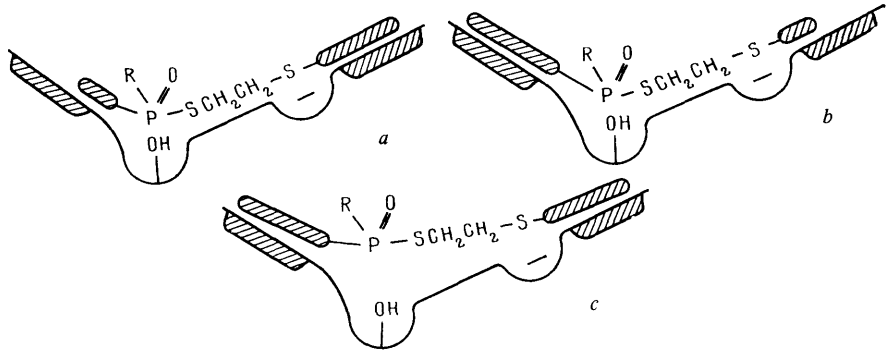
V. CONCLUSION

The results obtained show that BuCE is much more readily inhibited by substances capable of hydrophobic sorption on the active surface of the enzyme than is ACE. This conclusion had been reached earlier[9-11]. In subsequent studies it was clearly confirmed and developed further. In particular, relations were discovered between the structure of OPI with different hydrophobic substituents and their inhibiting activity in relation to ACE and BuCE.[41]

Comparison of the anticholinesterase activities of the diphenylphosphinothioates, phenylphosphonothioates, benzylphosphonothioates, and cyclohexylphosphonothioates presented in this review, as well as the methylphosphonothioates investigated earlier[7] clearly demonstrates differences between the sensitivities of ACE and BuCE to these compounds as a function of the structure of the phosphoryl component in their molecules.

S-Alkylthioethyl compounds with a methyl group at the phosphorus atom exert the strongest irreversible inhibiting activity in relation to ACE. This highly specific enzyme has apparently become adapted to acetylcholine and for this reason interacts faster with substances containing the $CH_3-P=O$ group, which simulates the $CH_3-C=O$ group of acetylcholine. Substances with a phenyl group at the phosphorus atom resemble methylphosphonothioate esters. *S*-Alkylthioethyl esters of phosphonothioic acids with benzyl and particularly cyclohexyl groups at the phosphorus atom inhibit ACE much less.

Thus substances of the type $A(C_2H_5O)P(O)SCH_2CH_2SR$ and $A(C_2H_5O)P(O)SCH_2CH_2\overset{+}{S}(CH_3).I^-$ can be arranged in the following sequence in terms of the irreversible inhibition of ACE as a function of the structure of A: $CH_3 > C_6H_5 > C_6H_5CH_2 > C_6H_{11}$.



Schematic illustration of the "compatible" (*a* and *b*) and "incompatible" (*c*) sorption of OPI on the active surfaces of cholinesterases.

A different type of behaviour is observed when the above compounds act on BuCE—the enzyme which hydrolyses butyrylcholine at the maximum rate and not acetylcholine. Sulphide derivatives of *O*-ethyl alkylphosphonothioates and of diphenylphosphinothioic acid can be arranged in the following sequence in terms of irreversible inhibition: $C_6H_5CH_2(C_2H_5O) > C_6H_5(C_2H_5O) > CH_3(C_2H_5O) > (C_6H_5)_2 > C_6H_{11}(C_2H_5O)$.

Sulphonium derivatives can be arranged in the same sequence in terms of their inhibiting activity subject to an important stipulation with regard to methylphosphonothioate esters: their positions in the series depend on the size of the alkyl group R.

The above series is valid only for $R < C_6H_{13}$. For $R > C_6H_{13}$, the inhibiting activity of methyl derivatives increases and is not inferior to that of benzyl derivatives: $(C_2H_5O)C_6H_5CH_2 \geq (C_2H_5O)CH_3 > (C_2H_5O)C_6H_6 > (C_6H_5)_2 > (C_2H_5O)C_6H_{11}$.

Thus in the presence of large substituents, which firmly attach the inhibitor in the vicinity of the anionic site of BuCE as a result of hydrophobic interaction, the methyl or benzyl group A at the phosphorus atom is "better" for productive sorption and subsequent phosphorylation of the enzyme than the phenyl or cyclohexyl group, while in the presence of small substituents the phenyl group A is "better" than the methyl group. The complementarity of the inhibitor molecule to the active surface of the enzyme, which ultimately determines the occurrence of "productive" sorption, in general depends on the compatibility of the ion–ion, ion–dipole, and hydrophobic interactions, as mentioned above. When these interactions are compatible, the sorbed molecule is oriented on the active centre in such a way that the phosphorus atom and the serine hydroxyl of the enzyme are in close proximity and phosphorylation may occur; this is illustrated schematically in the Figure. When the effects are "incompatible", the inhibitor molecule is arranged in such a way that the phosphorus atom and the serine hydroxyl are separated in space, phosphorylation is difficult, and the combined type of inhibition is shown clearly.

For certain structures of the inhibitors, hydrophobic sorption may promote the correct orientation of the inhibitor on the active surface of the enzyme, as a result of which the rate of irreversible inhibition increases. However, for other structures the sorption may interfere with the "correct orientation": for example, in those cases where the inhibitor molecule, fixed at two hydrophobic sites, is arranged in such a way that the phosphorus atom is separated from the serine hydroxyl of the enzyme active centre. Analogous steric hindrance may arise also when certain sulphide and sulphonium derivatives act on the enzyme, whereupon, as a result of the "incompatibility" of the sorption at the anionic site and in the corresponding hydrophobic region, the ester group of the inhibitor is incorrectly oriented relative to the esterase site of the enzyme.

Effects of this kind, which lower the rate constant for irreversible inhibition and cause the appearance of the reversible component, probably exert the most marked influence when the inhibitor molecule contains bulky hydrophobic groups, particularly those linked directly to the phosphorus atom (two phenyl groups, a cyclohexyl group, and a benzyl group).

Effects of this kind were explained previously by the absence of complementarity between the hydrophobic group of the inhibitor and the hydrophobic region located in the immediate vicinity of the esterase site of the enzyme. In the light of new experimental data, a revised treatment of the above effects is now necessary. The "hydrophobic region–esterase site" complementarity rather than with the complementarity to the hydrophobic region itself is relevant.

Thus it has been shown[9-11] that the introduction of hydrophobic groups into the eliminated or non-eliminated component of the inhibitor molecule enhances its irreversible inhibiting activity, particularly in relation to butyrylcholinesterase. On the basis of the data described, one may now claim that the introduction of bulky hydrophobic groups, particularly several such groups, may lead to the opposite effect, owing to the incompatibility of the orientation of the inhibitor molecule with the active surface of the enzyme: instead of an enhancement, a weakening of the anticholinesterase activity and the appearance of the reversible component are observed.

Thus the data described above show that the anticholinesterase activity of compounds and the nature of their inhibiting effect depend on three factors: on the electrophilic phosphorylating capacity of the inhibitor, on its tendency to undergo hydrophobic sorption on the active surface of the enzyme, and on the complementarity of the

inhibitor molecule to the "hydrophobic region–esterase centre" system.

One must recall that the difference between productive and non-productive sorption apparently consists solely in the localisation of the inhibitor molecule on the active surface of the enzyme; the vigorous interactions ensuring both types of sorption are virtually identical. Accordingly, the stabilities of the "productive" (EIn) and "non-productive" (EI_r) complexes should be similar. From this point of view, the quantities $K_{i,S}$ and $K_{i,r}$ are of great interest, because they can yield information about the equilibrium constant for the formation of the Michaelis enzyme–inhibitor complex. When the above quantities are analysed, the most striking fact is that in the vast majority of instances the quantities $K_{i,S}$ and $K_{i,r}$ for BuCE are significantly smaller than for ACE. This was shown particularly clearly in experiments with *S*-alkyl diphenylphosphinothioates [18], *O*-ethyl *S*-alkyl phenylphosphonothioates [27], and *OO*-dibutyl *S*-alkyl phosphorothioates [32], in the sorption of which the hydrophobic interaction plays an extremely significant role. The hydrophobic interaction is known to be more characteristic of BuCE than of ACE. Hence it becomes understandable why BuCE forms more stable complexes than ACE on interaction with the compounds investigated. This applies to both "non-productive" and "productive" complexes.

Attention must be drawn to the fact that frequently there are differences between the quantities $K_{i,S}$ and $K_{i,r}$, characterising the reversible component of the inhibition of cholinesterases. In most instances $K_{i,S}$ is smaller almost by an order of magnitude than $K_{i,r}$, i.e. the inhibiting effect of certain compounds in the hydrolysis of acetylcholine is manifested to a greater extent than in the phosphorylation of the enzyme.

One of the possible causes of this divergence is that the reversible inhibition of the enzymatic hydrolysis of acetylcholine may be associated with the interaction of the OPI, for example via the anionic site, not only with the free enzyme but also with the acetylated enzyme. This hinders deacetylation and thereby reduces the rate of the enzymatic hydrolysis of acetylcholine and therefore affects $K_{i,S}$.[42] Such reversible interaction of the OPI with the phosphorylated enzyme cannot influence $K_{i,r}$, because the phosphorylated enzyme is the final product of the process investigated.

The second cause may be associated with the presence of the "productive" enzyme–inhibitor complex EIn in the reaction medium when the rate of the enzymatic hydrolysis of AX is determined, because there are insufficient grounds for the belief that, following the addition of AX to the reaction mixture of the enzyme and the OPI, the complex EIn is fully dissociated into the inhibitor and the enzyme, which then catalyses the hydrolysis of AX. Even under conditions where the concentration of AX is many times greater that of the OPI, part of the enzyme may be present in the form of the complex EIn if the latter is very stable, Within the narrow inhibitor concentration ranges employed in experiments, the dependence of the rate constant k for the irreversible inhibition on [In] can therefore be either comparatively weak or may not exist at all. In the latter case it is altogether impossible to determine $K_{i,r}$.

It is noteworthy that, in view of the presence of two stages in the phosphorylation process, k should depend on the incubation time t of the enzyme with the inhibitor and on the inhibitor concentration [In] even in the absence of "non-productive" sorption, provided that these parameters vary within wide limits. Methods for the calculation of the rate constants of the individual stages of the process via mechanism (1) are based on the determination of the dependence of k on t and [In].[43,44]

Thus the combined inhibition of cholinesterases by certain organophosphorus inhibitors, which is manifested clearly in experiments and about the occurrence of which there is no doubt, may be caused not only by the "non-productive" sorption of the inhibitor on the enzyme but also by the presence of an enzyme–inhibitor complex in the enzymatic hydrolysis of the substrate.

Evidently, at the level of the modern experimental technique for the investigation of the interaction of cholinesterases with OPI, where there is as yet no possibility of distinguishing experimentally the "productive" and "non-productive" enzyme–inhibitor complexes, the reversible component must be regarded as a result of the binding of the enzyme both in the complex EI_r and in the Michaelis complex EIn.

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