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Hydrophobic Regions on the Active Surface of Choline-esterases

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The methods of synthesis of *S*-alkyl(aryl) derivatives of methylphsophonothioic acid and methods for the investigation of their anticholine-esterase activity have been briefly described. Data confirming the anticholine-esterase activity of the compounds synthesised are presented.
It has been shown that, in the inhibition of choline-esterases by organophosphorus compounds, the hydrophobic sorption of these substances on the active surface of the enzymes plays an important and sometimes a decisive role.
It is suggested that the presence of hydrophobic regions on the active surface of choline-esterases tends to increase the probability of the sorption of acetylcholine (a choline esterase substrate) on this surface.
The bibliography includes 68 references.

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I. INTRODUCTION

Choline-esterases (CE) are important enzymes for vital processes whose purpose is to hydrolyse acetylcholine (AC), one of the mediators in nerve excitation. According to modern concepts[1-5], the catalytic centres in these enzymes consist of two distinct regions which are separated in space: anionic and esterase regions. The anionic region is probably formed by the carboxylate anion of a dibasic aminoacid included in the polypeptide chain of the enzyme. This may be an aspartic or glutamic acid residue. The role of the anionic region consists in the specific sorption of the trimethylammonium group of acetylcholine, which helps to ensure a favourable orientation of the substrate molecule on the active surface of the enzyme.

The esterase component, where the hydrolysis of acetylcholine takes place, is located at a distance of 4–5 Å from the anionic component. It has a complex structure in which the hydroxyl of serine, an aminoacid also included in the polypeptide chain of the enzyme, plays a central role. Proton-donating and proton-accepting groups are present in the immediate vicinity of the serine hydroxyl. These may be respectively the tyrosine hydroxyl and the imidazole group of histidine.

It is still unknown how the functional groups comprising each of these active components are located in the overall structure of the enzyme, i.e. whether they belong to the same polypeptide chain or to different chains, but it may be supposed that the mutual approach of these groups, leading to the formation of the active centre, takes place at the instant of interaction with the substrate and involves its direct participation[5,6].

The information available on the structure of the active surface of choline-esterase is largely due to large-scale and intense studies on the properties and activity of organophosphorus inhibitors (OPI), carried out at an increasing rate for more than 25 years[7-10].

The following mechanism of the hydrolysis of acetylcholine by choline-esterases has been generally accepted. Initially the substrate is sorbed on the active surface of the enzyme with formation of a Michaelis complex and subsequent acetylation of the serine hydroxyl. The last stage involves the deacetylation of the enzyme by water and the restoration of its initial structure. There is every reason to suppose that this is not spontaneous water hydrolysis but a more complex process.

It is known that the inhibition of choline-esterase by OPI consists in the phosphorylation of the serine hydroxyl in the esterase component of the enzyme. In contrast to the deacetylation of choline-esterases, which occurs in the enzymic hydrolysis of acetylcholine and other substrates, dephosphorylation takes place extremely slowly and therefore the inhibition is almost irreversible. It is significant that the hydrolysis of the phosphorylated enzyme requires the dissociation not of an *O*-acyl but of an *O*-alkyl bond, which is not characteristic of choline-esterases.

The activity of the inhibitor depends primarily on its phosphorylating capacity, i.e. on the effective positive charge of the phosphorus atom. However, the anticholine-esterase activity of OPI is not determined solely by their phosphorylating capacity[7,10-13]. Ionic and ion-dipole interactions also plays an important role. They are shown most clearly on introduction of a cationic group into the eliminated component† of the OPI molecule. OPI is then fixed on the anionic region of the enzyme as a result of which the probability of the reaction between the inhibitor and the esterase component is enhanced. First examples of such cationic OPI were investigated in 1955–1958—they contained ammonium[14-17] or sulphonium[18-26] groups.

† We refer to the group of atoms linked directly to the phosphorus atom and split off on phosphorylation of choline-esterase as the eliminated component of the OPI molecule.

In the study of the first ammonium OPI it was impossible to demonstrate sufficiently clearly the role of the 'onium group, since the tertiary analogues of thiophosphonylcholine are so highly protonated owing to the high basicity of the amino-nitrogen at biological pH values that conversion to a quaternary base enhances the anticholine-esterase activity only by a factor of 3–4.

The role of the positive charge was revealed much more clearly in studies on certain OPI containing sulphides and sulphonium groups[20–26]. In sulphide OPI the sulphur atom is incapable of being protonated under the usual conditions and therefore the appearance of a charge on conversion into sulphonium compounds increases the anticholine-esterase activity by a factor of 2000–3000.[26] Later a similar striking increase of activity was achieved also with nitrogen-containing OPI derived from aromatic amines of low basicity. Such amines are protonated to only a slight extent at pH 7–8 and therefore the appearance of a charge on their conversion into quaternary bases also increases the activity of such OPI by a factor of several thousand[27].

The position of the 'onium atom in the OPI molecule is very important. The greatest effect is achieved when the atom carrying the positive charge is located in the eliminated component of the OPI molecule at the same distance from the phosphorus atom as that between the quaternary nitrogen atom and the carbonyl carbon in acetylcholine.[8,24,25]

It should be noted that, on introduction of a cationic group into the OPI molecule, there is not only a possibility of ionic interaction but, by virtue of the inductive effect, there is a significant increase also in electrophilic phosphorylating capacity. These two effects are difficult to separate experimentally[24,26,28].

The electrophilic reactivity of OPI and their capacity to interact with the anionic component of the enzyme are the two properties of the inhibitor which have been widely discussed in numerous publications dealing with the relation between the structure of OPI and their activity.

However, as will be shown below, these two factors do not exhaust the possibilities of the interaction between OPI and the active surface of choline-esterase. It may be supposed that Coulombic attraction is not the only important factor in the interaction between the cationic "head" of acetylcholine and the anionic group of choline-esterases. The three methyl groups linked to the nitrogen atom also play a significant role in this interaction[29,30]. It has been found that uncharged 3,3-dimethylbutyl acetate, the t-butyl group of which imitates sterically the trimethylammonium group of acetylcholine, is hydrolysed by choline-esterase only a little more slowly than acetylcholine itself[31,32]. Hence it may be supposed that the introduction of the t-butyl group in the required position into the molecule of the choline-esterase inhibitor can also intensify its interaction with the enzyme. There is also some other evidence in support of this hypothesis, for example the high anticholine-esterase activity of the pinacolyl esters of alkylphosphonic acids[8,12].

The first results of studies on this problem[33,34] led to the hypothesis that, around the active centre of choline-esterases, there are several hydrophobic regions which can interact with the alkyl groups of both substrates and inhibitors[5].

The hypothesis of the "hydrophobic interaction" is nowadays widely used in enzymology. It has been shown for various enzymes, such as pepsin, chymotrypsin, trypsin, carboxypeptidase, and others, that the rate of the enzymic hydrolysis of substrates depends significantly on the size of their constituent hydrocarbon groups capable of being sorbed on the hydrophobic regions of the catalytic centre of the enzyme[35–39].

The mechanism of the inhibition of choline-esterases was not examined previously from this standpoint. This review presents the results of systematic studies on the "topography" of the hydrophobic regions located on the active surface of horse serum choline-esterase and the acetylcholine-esterase from bull erythrocytes.

II. MATERIALS FOR INVESTIGATION

Enzymes. We employed a preparation of choline-esterase from horse blood serum (BuCE, EC, 3.1.1.8), manufactured at the Mechnikov Institute, which had a purification factor of 60 and a specific activity of 1.2–1.6 μmole of AC per minute per milligramme of protein at 25°C, pH 7.5, and a substrate concentration of 0.02 M.

The stroma of bull erythrocytes carefully washed free of haemoglobin and then lyophilised, serves as a source of acetylcholine-esterase (ACE, EC, 3.1.1.7).[40,41]

OPI preparations. These consisted of *S*-alkyl(aryl) derivatives of methylphosphonothioic acid some of the physicochemical constants of which are listed in Table 1.

Table 1. Designation and constants of the organophosphorus inhibitor (OPI)

Series I

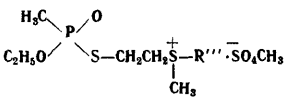
No.	R	*n*	B.p., °C/mmHg	n_D^{20}	d_4^{20}
1	2	3	4	5	6
1	CH_3	1	62—64/1	1.4760	1.0969
2	CH_3	2	85—86/2.5	1.4820	1.0712
3	CH_3	3	100—102/2	1.4810	1.0530
4	CH_3	4	108—110/3	1.4678	1.0306
5	CH_3	5	105—106/1	1.4712	1.0178
6	CH_3	6	124—126/2.5	1.4650	1.0040
7	CH_3	7	155—156/5	1.4690	0.9957
8	CH_3	8	142—144/2	1.4702	0.9841
9	CH_3	9	152—153/2	1.4705	0.9725
10	$(CH_3)_3C$	1	78—80/2	1.4640	1.0262
11	$(CH_3)_3C$	2	76—77/1	1.4746	1.0120
12	$(CH_3)_3C$	3	84—85/1	1.4710	1.0015
13	$(CH_3)_3C$	4	124—126/2	1.4700	0.9896
14	$(CH_3)_3C$	5	126—128/1.5	1.4670	0.9811
15	$(CH_3)_3C$	6	134—136/2	1.4700	0.9723
16	$(CH_3)_2CH$	1	120—121/2.5	1.4770	1.0610
17	$(CH_3)_2CH$	2	73—74/1	1.4710	1.0394
18	$(CH_3)_2CH$	3	84—85/1	1.4768	1.0270
19	$(CH_3)_2CH$	4	126—127/1	1.4698	1.0010
20	$(CH_3)_2CH$	6	132—134/1	1.4705	0.9862
21	C_6H_5	1	135—136/1.5	1.5462	1.1521
22	C_6H_5	2	136/2	1.5398	1.1369
23	C_6H_5	3	150—151/2	1.5346	1.0808
24	C_6H_5	4	137—139/1	1.5300	1.1068

Series II

1	2	3	4	5
25	CH_3	110—112/1.5	1.5211	1.1665
26	C_2H_5	98—99/1	1.5181	1.1429
27	n-C_4H_9	132—133/1	1.5082	1.0963
28	n-C_6H_{13}	137—138/1	1.5037	1.0650
29	n-C_8H_{17}	170—171/1	1.4998	1.0341
30	n-$C_{10}H_{21}$	192—195/1	1.4880	1.0097

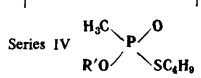
Table 1 Cont'd.

Series III



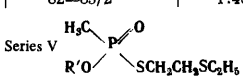
1	2	3	4	5
31	CH_3	—	1.5255	—
32	C_2H_5	—	1.5210	—
33	n-C_4H_9	—	1.5212	—
34	n-C_6H_{13}	—	1.4944	—
35	n-C_8H_{17}	—	1.4898	—
36	n-$C_{10}H_{21}$	—	1.5002	—

Series IV



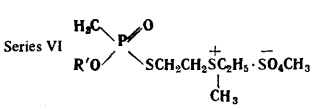
1	2	3	4	5
37	CH_3	64—65/1	1.4772	1.0825
38	n-C_3H_7	85—86/1	1.4692	1.0280
39	n-C_4H_9	92—93/1.5	1.4675	1.0125
40	n-C_5H_{11}	87/1.5	1.4700	0.9983
41	n-C_6H_{13}	116/2	1.4706	0.9930
42	n-C_7H_{15}	$70/2\cdot10^{-2}$	1.4693	0.9783
43	n-C_8H_{17}	$95/3\cdot10^{-2}$	1.4697	0.9656
44	n-C_9H_{19}	$110/2\cdot10^{-2}$	1.4690	0.9680
45	n-$C_{10}H_{21}$	$120/2\cdot10^{-2}$	1.4710	0.9643
46	$(CH_3)_2CH$	79/1.5	1.4703	1.0166
47	$(CH_3)_2CHCH_2$	80/1.5	1.4693	1.0053
48	$(CH_3)_2CH(CH_2)_2$	108/2	1.4693	0.9965
49	$(CH_3)_2CH(CH_2)_3$	$73/3\cdot10^{-2}$	1.4725	0.9929
50	$(CH_3)_2CH(CH_2)_4$	$76/3\cdot10^{-2}$	1.4693	0.9835
51	$(CH_3)_2CH(CH_2)_5$	$92—94/3\cdot10^{-2}$	1.4730	0.9818
52	$(CH_3)_3CCH_2$	82—83/2	1.4636	0.9891

Series V



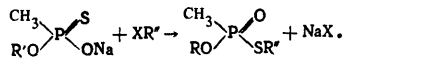
1	2	3	4	5
53	CH_3	100/1.5	1.5252	1.1728
54	n-C_3H_7	105/2	1.5101	1.1089
55	n-C_4H_9	$95—96/3\cdot10^{-2}$	1.5082	1.0960
56	n-C_5H_{11}	$104—105/2\cdot10^{-2}$	1.5040	1.0706
57	n-C_6H_{13}	$103—104/10^{-2}$	1.4984	1.0493
58	n-C_7H_{15}	$120—121/2\cdot10^{-2}$	1.4994	1.0430
59	n-C_8H_{17}	$117/10^{-3}$	1.4974	1.0365

Series VI

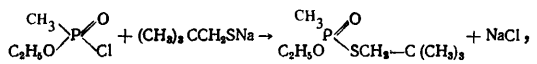


1	2	3	4	5
60	CH_3	—	1.5201	—
61	n-C_3H_7	—	1.5010	—
62	n-C_4H_9	—	1.5092	—
63	n-C_5H_{11}	—	1.5045	—
64	n-C_6H_{13}	—	1.5044	—
65	n-C_7H_{15}	—	1.4982	—
66	n-C_8H_{17}	—	1.5000	—

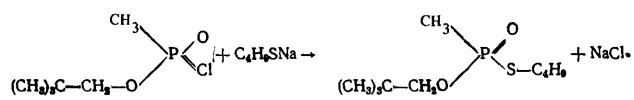
The general method for the preparation of S-alkyl O-ethyl methylphosphonothiolates and O-ethyl S-(ω-phenylalkyl)-methylphosphonothiolates (series I) and also O-alkyl S-n-butyl methylphosphonothiolates (series IV) consisted in the alkylation of sodium O-alkyl methylphosphonothioate with the corresponding alkyl halides or ω-halogenophenylalkanes [33,34,42-44]:



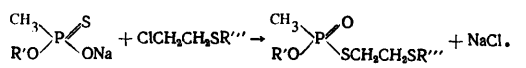
Exceptions were O-ethyl S-(2,2-dimethylpropyl) methylphosphonothiolate (Table 1, compound 1.10), which was synthesised by the reaction of O-ethyl methylphosphonochloridate with sodium 2,2-dimethylpropanethiolate [34]:



and S-n-butyl O-2,2-dimethylpropyl methylphosphonothiolate (Table 1, compound IV, 5.2), which was obtained by the interaction of O-2,2-dimethylpropyl methylphosphonochloridate with sodium butanethiolate:



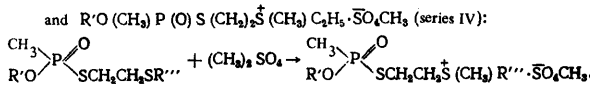
All the S-(β-alkylthioethyl) O-ethyl methylphosphonothiolates (series II) and O-alkyl S-(β-ethylthioethyl) methylphosphonothiolates (series V) were obtained by the reaction of sodium O-alkyl methylphosphonothioate with β-chloroethylalkyl sulphide [45,46]:



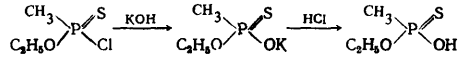
Next compounds of series II and V were converted by dimethyl sulphate into the corresponding S-methyl methosulphates:

$C_2H_5O(CH_3)P(O)S(CH_2)_2\overset{+}{S}(CH_3)R'''\cdot\overline{S}O_4CH_3$ (series III)

and $R'O(CH_3)P(O)S(CH_2)_2\overset{+}{S}(CH_3)C_2H_5\cdot\overline{S}O_4CH_3$ (series IV):



O-Alkyl methylphosphonothioates were obtained by the hydrolysis of O-alkyl methylthiophosphonochloridates [47]:



Some of the physical constants and the pK_a values of these substances are listed in Table 2.

III. RESEARCH METHODS

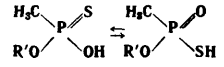
1. Estimation of Anticholine-esterase Activity

The anticholine-esterase activity of the OPI was estimated from the rate constant k_{II} for their interaction with the enzyme [4,48,49].

The enzyme (E) and the inhibitor (I) were incubated in the presence of 0.007 M phosphate buffer for a definite period (t, minutes) and then acetylcholine was added to the reaction mixture. The reaction between the OPI and the enzyme tended to cease completely in a dilute reaction mixture containing acetylcholine at a concentration corresponding to complete saturation of the enzyme by the substrate. The acetic acid formed in the hydrolysis of acetylcholine under the action of the residual concentration of the enzyme was determined by potentiometric titration with 0.1–0.12 N sodium hydroxide solution. The rate of reaction A_t corresponding to t minutes of incubation of E with

I was determined from the slope of the experimental linear plots of the volume of the solution of alkali against time. In some of the experiments A_t was found from the change in the concentration of acetylcholine in the reaction mixture, which was determined by Hestrin's method [50].

Table 2. Constants for *O*-alkyl methylphosphonothiolates:



R′	B.p., °C/mmHg	n_D^{20}	d_4^{20}	pK_a 60% alcohol	pK_a 80% alcohol thionic form	pK_a thiolic form
CH_3	98—99/3	1.5062	1.2527	3.27	3.78	3.48
C_2H_5	71—72/1	1.4927	1.1762	3.35	3.92	3.66
n-C_3H_7	92—93/1	1.4874	1.1285	3.42	3.96	3.59
n-C_4H_9	102—103/1	1.4830	1.0991	3.41	3.99	3.43
n-C_5H_{11}	106/2	1.4823	1.0686	3.46	3.99	—
n-C_6H_{13}	110/2	1.4814	1.0498	3.47	4.00	—
n-C_7H_{15}	$74/2\cdot10^{-2}$	1.4794	1.0315	3.46	3.97	—
n-C_8H_{17}	$84/2\cdot10^{-2}$	1.4790	1.0196	3.46	4.01	—
n-C_9H_{19}	$97/2\cdot40^{-2}$	1.4786	1.0087	3.49	4.00	—
n-$C_{10}H_{21}$	$115/2\cdot10^{-2}$	1.4780	0.9983	3.44	4.01	—

Since the reaction was carried out under standard conditions, the measured rates A_t were directly proportional to the corresponding concentrations of the active centres of the enzyme $[E]_t$. Accordingly the values of $[E]_t$ were calculated from the formula

$$[E_t] = [E]_0 A_t / A_0, \quad (1)$$

where A_0 is the rate of the enzyme reaction in the absence of the inhibitor with all other conditions kept constant and $[E]_0$ the initial concentration of the active centres of the enzyme in the reaction mixture. $[E]_0$ for the given initial enzyme solution was determined in a preliminary experiment by Berry's method [15,52] involving partial phosphorylation of the active centres of the enzyme (between 20 and 80%) with a highly active organophosphorus inhibitor—Gd-42 (Tables 1 and 6, compound III. 32)—at a known concentration, the inhibitor being completely consumed in the reaction. The end of the reaction was inferred from the constancy of A_t as a function of the duration of incubation of E with I. The data obtained were used to calculate k_{II} by the equation

$$k_{II} = \frac{2.303}{t([I]_0 - [E]_0)} \lg \frac{[E]_0([I]_0 - x)}{[I]_0([E]_0 - x)}, \quad (2)$$

where $[I]_0$ is the initial concentration of the OPI in the reaction mixture and x the decrease of the concentration of the enzyme and the OPI calculated from the difference $[E]_0 - [E]_t$. This formula is readily transformed into another more convenient for practical use [53]:

$$k_{II} = \frac{2.303}{t([I]_0 - [E]_0)} \lg \left[\frac{A_0}{A_t}\left(1 - \frac{[E]_0}{[I]_0}\right) + \frac{[E]_0}{[I]_0}\right]. \quad (3)$$

The concentration in the reaction mixture of the inhibitor of low activity was always many times higher than that of the enzyme, i.e. $[I]_0 \gg [E]_0$. In this case Eqns. (2) and (3) become

$$k_{II} = \frac{2.303}{t[I]} \lg \frac{A_0}{A_t}. \quad (4)$$

However, for the majority of the OPI, the constant k_{II} falls significantly with increase in time t. [54,55]

Kinetic studies have shown [55–57] that this type of relation between k_{II} and t follows from the generally accepted two-phase mechanism of the interaction between E and I:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_2} EI' + P_t, \quad (5)$$

where EI is the enzyme–inhibitor complex, analogous to the Michaelis complex, EI′ the phosphorylated enzyme, and Pt the corresponding mercaptan split off from the OPI on phosphorylation of the enzyme. Therefore we found the value of k_{II} corresponding to the beginning of the reaction between E and I.

For certain OPI, the constant k_{II} was determined at different temperatures, which made it possible to find the activation energy E_{act} for the inhibition of choline-esterases and to calculate the pre-exponential factors PZ in the Arrhenius equation.

2. Determination of the Rate Constants for Alkaline Hydrolysis

To estimate the electrophilic reactivity of the OPI, we determined the rate constants for their alkaline hydrolysis. An aqueous or aqueous alcoholic solution of the OPI was incubated at 25°C with a 2.5-fold excess of sodium hydroxide. The concentration of the OPI in the reaction mixture $[I]_0$ was usually 0.01 M and that of alkali $[B]_0$ was 0.025 M. The pH at the beginning of the reaction was 10.5–10.8. Samples were removed at definite time intervals and their sodium hydroxide concentration was determined by potentiometric titration. The pH at the beginning of the experiment fell by not more than 0.3. The hydrolysis rate constant k_{OH} was calculated from the formula

$$k_{OH} = \frac{2.303}{t([B]_0 - [I]_0)} \lg \frac{[I]_0([B]_0 - x)}{[B]_0([I]_0 - x)},$$

where x is the decrease of the concentration of NaOH and the OPI during the experiment, determined from the difference between the amounts of alkali consumed in the titration of a control reaction mixture ($t = 0$) and in the experiment after a period of incubation t.

The OPI containing a cationic group in the molecule are hydrolysed rapidly and their k_{OH} were found by direct titration of the reaction mixture at pH 10.5. [58] Since the concentration of OH^- ions in these experiments was constant, k_{OH} was calculated from the equation

$$k_{OH} = \frac{2.303}{t[OH]} \lg \frac{[I]_0}{([I]_0 - x)},$$

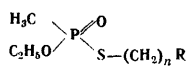
where x is the decrease of the inhibitor concentration determined from the amount of alkali consumed in the titration.

Determination of pK_a. To estimate the electrophilic reactivity of certain OPI, we also employed the ionisation constants (pK_a) of the corresponding *O*-alkyl methylphosphonothioates.

All the pK_a determinations were made by measuring the pH (using a silver, silver chloride electrode at 20°C) of partly neutralised (degree of neutralisation 30, 50 and 80%) 3.5×10^{-3}–50.1×10^{-3} M acid solutions. Since the ionic strength of the solution did not exceed 0.01, a correction for activity coefficients was not introduced. The calculation was based on the formula

$$K_a = [H^+] \frac{[Na^+] + [H^+] - \frac{K_W}{[H^+]}}{M - \left\{[Na^+] + [H^+] - \frac{K_W}{[H^+]}\right\}}.$$

Table 3. Inhibiting activity of series I compounds. *S*-Alkyl *O*-ethyl methylphosphonothiolates and *O*-ethyl *S*-(ω-phenylalkyl) methylphosphonothiolates with the general formula

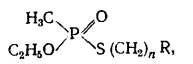


Series & no.	R	n	BuCE			ACE			k_{OH}, litre mole^{-1} min^{-1}
			k_{II}, litre mole^{-1} min^{-1}	E_{act}, kcal mole^{-1}	$10^{-10}pz$, litre mole^{-1} min^{-1}	k_{II}, litre mole^{-1} min^{-1}	E_{act}, kcal mole^{-1}	$10^{-10}pz$, litre mole^{-1} min^{-1}	
I.1	CH_3	1	$6.4 \cdot 10^1$	12.0	4	$2.2 \cdot 10^2$	11.0	3	0.15
I.2	CH_3	2	$1.4 \cdot 10^2$	12.0	12	$5.2 \cdot 10^2$	—	—	0.13
I.3	CH_3	3	$8.2 \cdot 10^2$	12.5	90	$1.2 \cdot 10^3$	—	—	0.15
I.4	CH_3	4	$2.6 \cdot 10^3$	12.0	170	$2.6 \cdot 10^3$	—	—	0.13
I.5	CH_3	5	$3.8 \cdot 10^4$	12.5	4100	$1.6 \cdot 10^4$	10.8	130	0.12
I.6	CH_3	6	$3.3 \cdot 10^4$	—	—	$2.1 \cdot 10^4$	—	—	0.13
I.7	CH_3	7	$4.1 \cdot 10^4$	—	—	$3.6 \cdot 10^4$	10.7	260	0.13
I.8	CH_3	8	$3.4 \cdot 10^4$	—	—	$3.1 \cdot 10^4$	—	—	0.13
I.9	CH_3	9	$3.8 \cdot 10^4$	—	—	$1.6 \cdot 10^4$	—	—	0.13
I.10	$C(CH_3)_3$	1	$2.1 \cdot 10^4$	12.5	3170	$2.0 \cdot 10^4$	11.0	120	0.10
I.11	$C(CH_3)_3$	2	$4.7 \cdot 10^3$	12.5	510	$1.6 \cdot 10^3$	11.0	16	0.16
I.12	$C(CH_3)_3$	3	$1.8 \cdot 10^4$	12.2	1660	$2.2 \cdot 10^3$	—	—	0.13
I.13	$C(CH_3)_3$	4	$9.1 \cdot 10^4$	12.0	5870	$7.2 \cdot 10^3$	—	—	0.15
I.14	$C(CH_3)_3$	5	$9.0 \cdot 10^4$	—	—	$1.1 \cdot 10^4$	—	—	0.12
I.15	$C(CH_3)_3$	6	$9.9 \cdot 10^4$	—	—	$1.9 \cdot 10^4$	—	—	0.15
I.16	$CH(CH_3)_2$	1	$6.7 \cdot 10^2$	—	—	$1.6 \cdot 10^3$	—	—	0.14
I.17	$CH(CH_3)_2$	2	$2.3 \cdot 10^3$	12.0	150	$1.3 \cdot 10^3$	—	—	0.16
I.18	$CH(CH_3)_2$	3	$2.5 \cdot 10^3$	—	—	$8.3 \cdot 10^2$	—	—	0.13
I.19	$CH(CH_3)_2$	4	$5.9 \cdot 10^4$	—	—	$9.0 \cdot 10^3$	—	—	0.16
I.20	$CH(CH_3)_2$	6	$9.3 \cdot 10^4$	—	—	$2.2 \cdot 10^4$	—	—	0.13
I.21	C_6H_5	1	$1.2 \cdot 10^5$	—	—	—	—	—	0.11
I.22	C_6H_5	2	$1.4 \cdot 10^4$	—	—	$1.2 \cdot 10^4$	—	—	0.14
I.23	C_6H_5	3	$8.5 \cdot 10^4$	—	—	$1.8 \cdot 10^4$	—	—	0.11
I.24	C_6H_5	4	$1.1 \cdot 10^5$	—	—	$3.4 \cdot 10^4$	—	—	0.12

Since *O*-alkyl methylphosphonothioates can exist in two tautomeric forms (Table 2), the pK_a values for both forms were calculated by the method described previously [59]. These values characterise the effective positive charge at the phosphorus atom in the acids and consequently to some extent also the phosphorylating capacity of the corresponding esters. Since the pK_a values of the acids vary relatively little when the size of R increases from CH_3 to $C_{10}H_{21}$ (Table 2), one may assume that the phosphorylating capacities of the corresponding OPI are also virtually identical. For other series of OPI, a similar conclusion follows from the constancy of the alkaline hydrolysis rate constant within each series (Tables 3, 5, and 6). Thus the OPI in each series react with the esterase component of choline-esterases as phosphorylating agents of virtually equal effectiveness.

A. Effect of the Structure of the Hydrocarbon Group in the Eliminated Components of OPI on their Anticholine-esterase Activity

The first series of OPI used to investigate the effect of hydrophobic sorption on the rate of phosphorylation of the enzyme have the general formula



where R = H, $C(CH_3)_3$, $CH(CH_3)_2$, or C_6H_5.

The results of these investigations are presented in Table 3 and show that the rate constants for alkaline hydrolysis (k_{OH}) and also the activation energies E_{act} for the compounds investigated in reactions with cholinesterases are almost constant, while the inhibition rate constants k_{II} differ sharply.

In the subseries of straight-chain alkane derivatives (compounds I.1–I.9) the constants k_{II} increase with the length of the chain of the alkyl group up to the hexyl derivative (I.5) for BuCE and up to the octyl derivative (I.7) for ACE. Further increase in the length of the alkyl chain in the case of BuCE does not cause significant changes in k_{II} and in the case of ACE actually leads to an appreciable decrease of the constant [33,34] (Fig. 1). Thus a simple increase in the length of the hydrocarbon group leads to a sharp increase of the rate of inhibition—by a factor of 170 for ACE and 600 for BuCE.

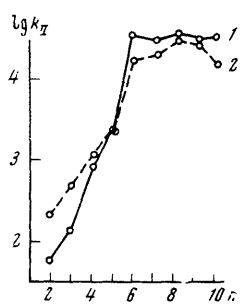


Figure 1. Variation of the rate constants k_{II} for the inhibition of BuCE (curve *1*) and ACE (curve *2*) by compounds of the type $C_2H_5O(CH_3)P(O)SC_nH_{2n+1}$ with the length *n* of the straight alkyl chain.

The second subseries of compounds, in which the alkylthio-substituent contained a t-butyl group at different distances from the phosphorus atom (compounds I.10–I.15), proved to be extremely interesting. The $C(CH_3)_3$ group is a complete steric analogue of the $N(CH_3)_3$ group in the acetylcholine molecule. The first member of this subseries (I.10) exhibits a fairly high anticholine-esterase activity. Then [compound (I.11)] k_{II} decreases. In experiments with BuCE subsequent members of the subseries (I.12–I.13) showed an increase of k_{II} with increase in the number of methylene groups *n*, but the change in k_{II} on going to compounds I.14 ($n = 5$) and I.15 ($n = 6$) is already small (Fig. 2). In experiments with ACE there was a continuous increase of k_{II} with *n*.

Next it was found that in this subseries of OPI all three methyl residues at the quaternary carbon atoms are important. The replacement of one methyl residue by a hydrogen atom, i.e. the replacement of the t-butyl group by an isopropyl group, results in a significant decrease of k_{II}, particularly for OPI with a small n (compounds I.16–I.18). A feature of the compounds of the third subseries which distinguishes them from those of the second is the absence of a decrease of k_{II} on going from the first member (I.16, $n = 1$) to the second (I.17, $n = 2$). The variation of k_{II} with n in this subseries has much in common with that for compounds of the first subseries.

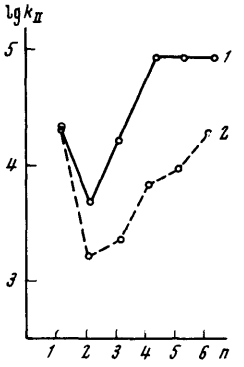


Figure 2. Variation of the rate constants k_{II} for the inhibition of BuCE (curve *1*) and ACE (curve *2*) by compounds of the type $C_2H_5O(CH_3)P(O)S(CH_2)C(CH_3)_3$ with the number of methylene groups n.

In the fourth subseries of the first series of OPI (I.21–I.24) under consideration an ω-phenylalkyl group is attached to sulphur. The high anticholine-esterase activity of the first member of the subseries (I.21, $n = 1$) is striking here. On going to the second member (I.22, $n = 2$), k_{II} decreases sharply. Further increase of n leads to an increase of k_{II}, up to $n = 4$.[44]

The very high anticholine-esterase activity of individual members of this series of OPI is particularly noteworthy. Thus the rate of inhibition of BuCE by compounds I.13, I.14, I.15, I.20, I.21, I.23, and I.24 is not inferior to that by an inhibitor as powerful as armin ($k_{II} = 2 \times 10^{-5}$).[49]

The data obtained will make it possible to elucidate the effect on k_{II} of the gradual conversion of the terminal methyl group into a t-butyl group. This effect is seen from an examination of Table 4, which lists the relative values of k_{II} for the OPI. k_{II} for the first compound in each of the five groups ($n = 1$, $n = 2$, $n = 3$, $n = 4$, and $n = 6$) was assumed to be unity.

Table 4 shows clearly that the gradual "conversion" of the methyl into a t-butyl group is accompanied in all cases, except for the inhibition of ACE by compounds with $n = 4$ and 6, by an increase of k_{II}. To what extent this increase is specific, i.e. whether it is associated with the presence of the tetrahedral t-butyl group as such or whether it is due to a simple increase in the "bulk" of the alkyl substituent, can be seen by comparing the constant k_{II} for compounds containing a t-butyl group with those for the corresponding compounds with an n-butyl group. The greatest effect of the t-butyl group for BuCE and ACE was observed with the compound in which $n = 1$. Here the distance between the quaternary carbon atom and the phosphorus atom is almost equal to the distance between the quaternary nitrogen atom and the carbonyl carbon atom in the AC molecule:

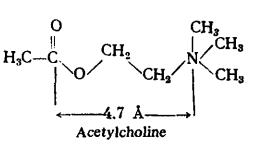


Table 4. Variation of the inhibiting activity of compounds of the type $C_2H_5O(CH_3)P(O)S(CH_2)_nR$ as the group R is varied from methyl to t-butyl for different n (based on the results in Table 1).

Compound	n	R	k_{II} (relative values)	
			BuCE	ACE
I.1	1	CH_3	1	1
I.2	1	CH_2CH_3	2	2
I.16	1	$CH(CH_3)_2$	10	7
I.10	1	$C(CH_3)_3$	330	90
I.4	1	C_4H_9	40	12
I.2	2	CH_3	1	1
I.3	2	CH_2CH_3	6	2
I.17	2	$CH(CH_3)_2$	17	2
I.11	2	$C(CH_3)_3$	34	3
I.5	2	C_4H_9	275	30
I.3	3	CH_3	1	1
I.4	3	CH_2CH_3	3	2
I.18	3	$CH(CH_3)_2$	3	1
I.12	3	$C(CH_3)_3$	22	2
I.6	3	C_4H_9	40	18
I.4	4	CH_3	1	1
I.5	4	CH_2CH_3	15	6
I.19	4	$CH(CH_3)_2$	23	3
I.13	4	$C(CH_3)_3$	35	3
I.7	4	C_4H_9	16	14
I.6	6	CH_3	1	1
I.7	6	CH_2CH_3	1	1.5
I.20	6	$CH(CH_3)_2$	3	2
I.15	6	$C(CH_3)_3$	3	1
I.9	6	C_4H_9	1	0.7

It is striking that here k_{II} for compound I.10 is approximately eight times higher than k_{II} for its isomer I.4. A similar effect of the complete conversion of the methyl into a t-butyl group is observed for compound I.3 ($n = 4$) and I.6 ($n = 4$) in relation to BuCE. In all other cases the isomers with a straight alkyl chain have a higher value of k_{II} than the compounds with a t-butyl group.

Table 3 shows that the observed increase of k_{II} as the hydrocarbon group of the OPI becomes longer and more branched is caused solely by a change in the probability factor—the pre-exponential factor PZ in the Arrhenius equation. The activation energy E_{act} is almost constant: it amounts to 12.0–12.5 kcal mole^{-1} for the interaction of the OPI investigated with BuCE and between 10.7 and 11.0 kcal mole^{-1} for the interaction with ACE. The rate constants for the non-enzymatic alkaline hydrolysis of these compounds proved to be invariant to an equal extent: they vary from 0.13 to 0.15 litre mole^{-1} min^{-1} at pH 10.5.[33,34] As stated above, these findings indicate that the electrophilic phosphorylating capacity of this series of OPI is identical. The differences between them are determined

by the different rates of their sorption on the membrane and consequently the different concentrations at any instant of the sorbed and appropriately oriented OPI molecules on the surface of the enzyme. This is equivalent to differences in the rate of formation of an enzyme–inhibitor EI complex, which has in fact been confirmed by careful kinetic analysis [56,57].

Since the reaction takes place in water, the increase in the extent of sorption of the OPI molecules with increase in the size of the *S*-alkyl substituent may be associated solely with the presence on the surface of the enzyme of hydrophobic regions: the hydrophilic medium (water) displaces the hydrocarbon groups of the OPI to these hydrophobic regions of the enzyme and thereby ensures their more complete sorption. Regions of this kind in the polypeptide chain of the enzyme may be formed by leucine, isoleucine, valine, and phenylalanine residues.

Evidently one of such hydrophobic regions, adapted for the sorption of the trimethylammonium group of acetylcholine, surrounds the anionic component of the active centre of the enzyme. One cannot exclude the possibility that the t-butyl group of compound I.10, in which the distance between the group and the phosphorus atom is close to that between the esterase and anionic sites of the enzyme, is sorbed precisely on this region. In this connection we must note yet again the higher value of k_{II} for compound I.10 compared with that for its isomer I.4.

Table 5. Inhibiting activity of series III compounds: *S*-methyl methosulphates of *S*-(β-alkylthioethyl) *O*-ethyl methylphosphonothiolates with the formula

Series & no.	R'''	k_{II}, litre mole^{-1} min^{-1}		k_{II}(ACE) / k_{II}(BuCE)	k_{OH} litre mole^{-1} min^{-1}
		BuCE	ACE		
III.31	CH_3	$1.0\cdot10^6$	$5.5\cdot10^7$	55.0	17.0
III.31	C_2H_5	$3.7\cdot10^6$	$2.5\cdot10^8$	67.5	17.0
III.33	n-C_4H_9	$1.1\cdot10^7$	$2.2\cdot10^8$	20.0	20.0
III.34	n-C_6H_{13}	$8.9\cdot10^7$	$1.1\cdot10^9$	12.4	21.4
III.35	n-C_8H_{17}	$1.0\cdot10^8$	$4.6\cdot10^8$	4.6	25.0
III.36	n-$C_{10}H_{21}$	$1.8\cdot10^8$	$4.0\cdot10^8$	2.2	19.0

The nearest homologue of substance I.10, i.e. substance I.11 (R = t-C_4H_9, n = 2), has a constant k_{II} smaller by a factor of 3.5. The distance between the phosphorus atom and the tertiary carbon atom in this compound (5.3 Å) is somewhat greater than the optimum value (4.7 Å) and the methyl groups sorbed on the hydrophobic surround of the anionic component prevent the exact orientation of the electrophilic phosphorus atom in relation to the hydroxy-group of the esterase site of the enzyme. Hence follows a sharp decrease of the probability factor PZ (by a factor of 6 for BuCE and 8 for ACE).

However, on further increase of n in this subseries an increase of k_{II} is again observed (up to n = 4 for BuCE and up to n = 6 for ACE). The rate of formation of the enzyme–inhibitor complex increases. Apparently on the surface of the enzyme there is yet another hydrophobic region of a similar type separated from the first by hydrophilic units. In BuCE it is undoubtedly complementary to the t-butyl group, as can be seen from the data in Table 4 (groups with n = 4 and n = 6). In ACE this is not so and, with the exception of the first compound (n = 1), the isomeric compounds with straight-chain alkyl substituents are in all cases more active than the compounds with the t-butyl group and the activity of the OPI with n = 6 altogether depends little on the structure of the terminal group. The isopropyl derivatives (Table 3) behave similarly to the straight-chain subseries but with the difference that these compounds reach the second hydrophobic region of the enzyme starting with n = 4 (compound I.19). This corresponds to the chain length with n = 6 in the subseries of the straight-chain alkyl derivatives.

The phenylalkyl derivatives containing the bulky phenyl group at the end of the chain show the same type of variation of k_{II} as in the subseries with the t-butyl group.

Although in relation to ACE this behaviour is shown less markedly, nevertheless the increase of k_{II} with increasing length of the hydrocarbon group in the compounds of the first and second subseries (Table 3) suggests that in ACE in the vicinity of the anionic site there is also a hydrophobic region, although its role is less pronounced than that of the hydrophobic region surrounding the anionic site.

It is noteworthy that a similar relation between the rate of inhibition of ACE and the structure of the hydrocarbon group in derivatives of di-O-ethyl phosphorothioate was found by O'Brien and Bracha [60]. Their investigations confirmed the above conclusions concerning the existence of hydrophobic regions in the vicinity of the anionic site of the enzyme.

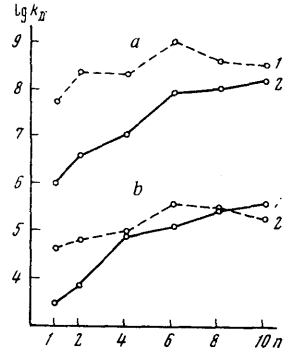
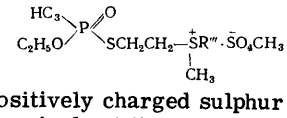


Figure 3. Variation of the rate constants k_{II} for the inhibition of BuCE (curves *1*) and ACE (curves *2*) by compounds of the type $C_2H_5O(CH_3)P(O)SC_2H_4\overset{+}{S}(CH_3)C_nH_{2n+1}$ (*a*) and $C_2H_5O(CH_3)P(O)SC_2H_4SC_nH_{2n+1}$ (*b*) with the length n of the straight alkyl chain.

In order to show that the second hydrophobic region in both BuCE and ACE is in fact located beyond the anionic site, compounds of series of II and III were investigated. Compounds of series III are of particular interest:



They contain a positively charged sulphur atom which should be fixed precisely at the anionic site of the enzyme. If the hypothesis of the existence and particular location of the above hydrophobic region is correct, an increase in the size of the hydrocarbon group R''' should entail an increase in the inhibiting activity of these compounds.

The data in Table 5 and Fig. 3 show that k_{II} does indeed increase with increase in the size of R''', particularly in reactions with BuCE. Compound III.36 is more active than III.31 by a factor of 180 in relation to BuCE and by a factor of 7.3 in relation to ACE. The increase of R''' is accompanied by an appreciable equalisation of the k_{II} for the two enzymes. While the first two compounds (III.31 and III.32) inhibit ACE 60–70 times faster than BuCE, the inhibition by the last compound III.36 is only 2.2 times faster [46,61] (Table 6).

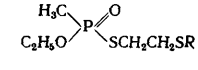
Table 6. Inhibiting activity of series II compounds. *S*-(β-alkylthioethyl) *O*-ethyl methylphosphonothiolates:

$C_2H_5O(CH_3)P(O)SC_2H_4SR'''$

Series and no.	R'''	k_{II}, litre mole^{-1} min^{-1}		$\frac{k_{II}(ACE)}{k_{II}(BuCE)}$	k_{OH} litre mole^{-1} min^{-1}
		BuCE	ACE		
II.25	CH_3	$3.2\cdot10^3$	$4.0\cdot10^4$	12.5	0.29
II.26	C_2H_5	$6.8\cdot10^3$	$6.0\cdot10^4$	8.8	0.28
II.27	n-C_4H_9	$7.8\cdot10^4$	$9.0\cdot10^4$	1.2	0.31
II.28	n-C_6H_{13}	$1.3\cdot10^5$	$4.0\cdot10^5$	3.1	0.28
II.29	n-C_8H_{17}	$3.0\cdot10^5$	$3.3\cdot10^5$	1.1	0.29
II.30	n-$C_{10}H_{21}$	$4.0\cdot10^5$	$2.0\cdot10^5$	0.5	0.27

It is significant that, apart from these findings, in this series of compounds the rate constants for non-enzymatic alkaline hydrolysis also vary within very narrow limits (16.0–22.0 litre mole^{-1} min^{-1}) and therefore here too one may speak of the constancy of the electrophilic reactivity of the OPI.

A similar relation between k_{II} and R obtains also for compounds with an uncharged sulphur atom (series II, Table 6 and Fig. 3):



Compound II.30 inhibits BuCE approximately 130 times faster and ACE only five times faster than compound II.25. In this series an increase in the size of R leads not only to an equalisation but actually to a reversal of the relative values of k_{II} for the enzymes investigated: substance II.25 inhibits ACE 12.5 times faster than BuCE, while the inhibition of ACE by compound II.30 is slower by a factor of 2 than that of BuCE (Fig. 3). Here too the rate of alkaline hydrolysis is virtually the same for all the substances.

Thus the data obtained leave no doubt about the existence of a hydrophobic region beyond the anionic site. This region is fairly extensive: in BuCE at a distance equivalent to 3–5 methylene groups from the anionic site there is a region which greatly increases the sorption of the t-butyl group.

In ACE this region is less distinctive and evidently this is why the hydrophobic properties of OPI do not play such an important role in the inhibition of ACE as in the inhibition of BuCE.

In the determination of the inhibiting effect of compounds of series II and III additional data were obtained on the different roles of the anionic sites in BuCE and ACE in the sorption of charged OPI. The ratio of the constants k_{II} for corresponding pairs of charged and uncharged OPI varies from 140 to 680 for BuCE and from 1400 to 4200 for ACE (Table 7).

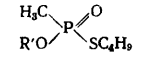
Thus in the sorption of OPI by enzymes the anionic region plays a more important role in ACE than in BuCE, while the hydrophobic environment of the anionic site is more important in BuCE.

Table 7. Comparative estimates of the inhibiting activities of *S*-(β-alkylthioethyl) *O*-ethyl methylphosphonothiolates $C_2H_5O(CH_3)P(O)SC_2H_4SR'''$ and their *S*-methyl methosulphates $C_2H_5O(CH_3)P(O)SC_2H_4S(CH_3)R'''.SO_4CH_3$ (according to data in Tables 5 and 6)

R		CH_3	C_2H_5	C_4H_9	C_6H_{13}	C_8H_{17}	$C_{10}H_{21}$
Ratio of k_{II} values for charged and uncharged OPI	BuCE	320	550	140	680	330	450
	ACE	1370	4200	2440	2750	1400	2000

B. Effect of the Structure of the Hydrocarbon Group in the Non-eliminated Components of the OPI on their Anticholinesterase Activity

The next three series of compounds (IV, V, and VI) were synthesised in order to elucidate the hydrophobic region surrounding the esterase component of the enzyme. The reactivity of a series of *O*-n-alkyl *S*-n-butyl methylphosphonothiolates was investigated first:



(series IV, Table 8), where R′ represents n-alkyl groups with a chain length comprising between 1 and 10 carbon atoms [47,62,63]. As mentioned above, the phosphorylating capacity of these compounds is virtually the same.

Table 8. Inhibiting activity of series IV compounds. *O*-n-alkyl *S*-n-butyl methylphosphonothiolates:

$R'O(CH_3)P(O)SC_4H_9$

Series and no.	R′	k_{II}, litre mole^{-1} min^{-1}		Series and no.	R′	k_{II}, litre mole^{-1} min^{-1}	
		BuCE	ACE			BuCE	ACE
IV.37	CH_3	$5.8\cdot10^1$	$2.6\cdot10^3$	IV.41	C_6H_{13}	$5.0\cdot10^4$	$4.2\cdot10^3$
I.3	C_2H_5	$5.2\cdot10^3$	$1.2\cdot10^3$	IV.42	C_7H_{15}	$1.1\cdot10^5$	$4.5\cdot0^3$
IV.38	C_3H_7	$4.5\cdot10^3$	$5.6\cdot10^3$	IV.43	C_8H_{17}	$3.6\cdot10^5$	$4.1\cdot10^3$
IV.39	C_4H_9	$1.2\cdot10^4$	$1.4\cdot10^4$	IV.44	C_9H_{19}	$7.0\cdot10^5$	$6.2\cdot10^3$
IV.40	C_5H_{11}	$1.6\cdot10^4$	$6.8\cdot10^3$	IV.45	$C_{10}H_{21}$	$6.2\cdot10^4$	$2.9\cdot10^3$

Table 8 shows that the anticholine-esterase activity of the compounds investigated depends significantly on the length of the *O*-alkyl group. In experiments with BuCE a gradual increase in the length of the group up to heptyl leads to an increase of the inhibition rate constant. The heptyl derivative proved to be a very powerful inhibitor of BuCE, its anticholine-esterase activity exceeding that of the methyl derivative by a factor of 18 000. A further increase in the length of the substituent entails some decrease of k_{II}.

A different type of relation with the structure is observed in the interaction of the OPI of this series with ACE. The anticholine-esterase activity increases with

hydrocarbon chain length only as far as butyl (the butyl derivative is 50 times more effective than the methyl derivative). The anticholine-esterase activity of the OPI with longer alkyl groups (between pentyl and decyl) is somewhat lower and varies within narrow limits. The variation of the anticholine-esterase activity in this series is determined, as in the previous series, mainly by the variation of the capacity of the OPI for the formation of an enzyme–inhibitor complex as a result of the sorption of the hydrocarbon components of the alkoxy-groups on the hydrophobic regions of the active surface of the enzyme.

The relations found in these experiments resemble those described above for studies on the series of *S*-n-alkyl *O*-ethyl methylphosphonothiolates (Table 3), but differ from them in important details.

Comparison of the effects of both series of OPI on BuCE showed that, with increase in the length of the alkoxy-group, the maximum effect is observed for the heptyl derivative, whereas variation of the length of the alkylthio-group shows the maximum effect with the hexyl derivative. Bearing in mind that in the first case the hydrocarbon group is linked to the phosphorus atom via an oxygen atom and in the second via a sulphur atom, one may assume that the maximum anticholine-esterase activity of these two series of compounds corresponds to the same length of the alkoxy- or alkylthio-substituents.

Table 9. Inhibiting activity of series V and VI compounds in relation to BuCE. *O*-n-alkyl *S*-(β-ethylthioethyl) methylphosphonothiolates and their *S*-methyl methosulphates:

$R'O(CH_3)P(O)SC_2H_4SC_2H_5$ (V)

$R'O(CH_3)P(O)SC_2H_4\overset{+}{S}(CH_3)C_2H_5\cdot\overset{-}{S}O_4CH_3$ (VI)

Series and no.	R′	k_{II}, litre mole^{-1} min^{-1}	Series and no.	R′	k_{II}, litre mole^{-1} min^{-1}
V.53	CH_3	$9.6\cdot10^3$	VI.60	CH_3	$7.7\cdot10^5$
II.26	C_2H_5	$4.4\cdot10^3$	III.32	C_2H_5	$3.8\cdot10^6$
V.54	C_3H_7	$5.8\cdot10^4$	VI.61	C_3H_7	$3.8\cdot10^7$
V.55	C_4H_9	$1.6\cdot10^5$	VI.62	C_4H_9	$4.8\cdot10^7$
V.56	C_5H_{11}	$1.7\cdot10^5$	VI.63	C_5H_{11}	$7.5\cdot10^7$
V.57	C_6H_{13}	$3.7\cdot10^6$	VI.64	C_6H_{13}	$1.8\cdot10^8$
V.58	C_7H_{15}	$2.8\cdot10^6$	VI.65	C_7H_{15}	$2.5\cdot10^8$
V.59	C_8H_{17}	$1.1\cdot10^6$	VI.66	C_8H_{17}	$2.8\cdot10^8$

This finding can have two explanations. The first involves the assumption that the hydrophobic regions located beyond the anionic and esterase components of the active centre of the enzyme are equidistant from these sites. The second involves the recognition that the sorption of the alkoxy- and alkylthio-groups takes place on the same region of the enzyme surface.

The second assumption must be immediately rejected in relation to ACE. In the series of *S*-n-alkyl *O*-ethyl methylphosphonothiolates the maximum activity on ACE (as well as on BuCE) is attained with the hexyl derivative (Table 3), whereas in the *O*-alkyl *S*-n-butyl methylphosphonothiolate series the butyl derivative is the most active (Table 8).

In order to obtain additional information about the localisation of the hydrophobic regions of choline-esterase with which alkoxy-groups may interact and in particular in order to find whether these groups are sorbed in the region of the anionic component of the enzyme, special experiments were carried out, consisting in the investigation of the anticholine-esterase activity of two series of OPI[62]—*O*-n-alkyl *S*-(β-ethylthioethyl) methylphosphonothiolates (series V) and the corresponding *S*-methyl methosulphates (series VI). The choice of these OPI was based on the fact that the reaction of choline-esterase with OPI of type VI, containing the sulphonium group, should necessarily involve the interaction of the 'onium group with the anionic site. Under these conditions, the alkoxy-group is incapable of being sorbed on the hydrophobic regions in the vicinity of the anionic centre. The results of the investigation of these two series of OPI in relation to BuCE are presented in Table 9.

Table 9 shows that, with increase in the length of the hydrocarbon component in the alkoxy-group in the OPI series of type V, there is an appreciable increase of k_{II}. The maximum value of k_{II} was noted for the heptyl derivative (higher by a factor of 2900 than for the methyl derivative). The constant k_{II} for the octyl derivative is somewhat lower. In the series of OPI of type VI the anticholine-esterase effect also increases with the length of the hydrocarbon component of the alkoxy-group. Comparison of these two series of inhibitors shows that all the *S*-methyl methosulphates have k_{II} values higher by 2–3 powers of 10 than their uncharged analogues.

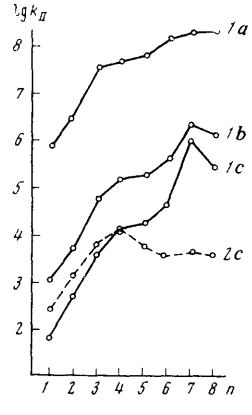


Figure 4. Variation of the rate constants k_{II} for the inhibition of BuCE (curves *1*) and ACE (curve *2*) by compounds of the type $C_nH_{2n+1}O(CH_3)P(O)SR$ with the length *n* of the straight alkyl chain: *a*) R = $C_2H_4S(CH_3)C_2H_5$; *b*) R = $C_2H_4SC_2H_5$; *c*) R = n-C_4H_9.

One may assume with certainty that the higher anticholine-esterase activity of the *S*-methyl methosulphates observed in all cases depends on the interaction between the charged sulphonium group and the anionic site of BuCE and in particular on the associated orienting effect. Therefore one may assume that the increase of the anticholine-esterase activity with increasing length of the hydrocarbon component of the alkoxy-group is also a consequence of the orienting effect, due to its sorption on a suitable hydrophobic region of BuCE.

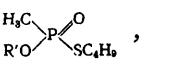
Since the anionic centre of BuCE in the interaction with series VI OPI is occupied by a group containing sulphonium sulphur, one may conclude that the hydrophobic region of the active surface of BuCE on which the alkoxy-group is sorbed is located outside the anionic site of the enzyme.

In all probability the alkoxy-group of uncharged OPI is also sorbed on the same hydrophobic region of choline-esterase. This conclusion follows from an examination of Fig. 4, where the variation of lg k_{II} with the number of carbon atoms in the alkoxy-group is plotted for the OPI of series IV, V, and VI.

Fig. 4 shows that the curves representing the effect on BuCE by the inhibitors of all three series vary in exactly the same sense and actually coincide in detail (the sharp rise on increasing n from 1 to 3, the inflection in the region with $n = 3-5$, and another sharper rise as n is increased to 7). This agreement can only be explained by the fact that the hydrocarbon components of the alkoxy-groups of the OPI of all three series interact with the same hydrophobic region of BuCE. The overall extent and location of this region probably correspond sterically to the heptyl group. However, the stepped form of the curve may be an indication that in fact we are dealing here with two hydrophobic regions separated by a hydrophilic group.

Fig. 4 also shows that the effects of the same series of OPI on BuCE and ACE (curves *1* and *2*) differ significantly. Whereas up to $n = 4$ the inhibition of both enzymes is almost the same, further increase in the length of the hydrocarbon group, which results in an appreciable increase of effectiveness in relation to BuCE, has completely no effect on the capacity of the OPI to inhibit ACE. We are inclined to explain this difference by the fact that in the region of the esterase centre ACE has not two but only one hydrophobic region, corresponding sterically to the butyl group.

To characterise more fully the steric configuration of the hydrophobic regions located in the vicinity of the esterase centre of the choline-esterases, a study was made of the effect on the choline-esterases of compounds containing a branched substituent in the alkoxy-group[64] (compounds IV.46–IV.51):



where R′ = $(CH_3)_2CH(CH_2)_n$.

Table 10. Inhibiting activity of series IV compounds. *S*-Butyl *O*-isoalkyl methylphosphonothiolates:

$R'O(CH_3)P(O)SC_4H_9$

Series & no.	$R'=(CH_3)_2CH(CH_2)_n$	k_{II}, litre mole^{-1} min^{-1}	
	n	BuCE	ACE
IV.46	0	$4.5\cdot10^2$	$3.2\cdot10^3$
IV.47	1	$4.4\cdot10^3$	$1.5\cdot10^4$
IV.48	2	$3.4\cdot10^4$	$6.1\cdot10^4$
IV.49	3	$3.9\cdot10^4$	$4.3\cdot10^5$
IV.50	4	$6.5\cdot10^4$	$9.3\cdot10^3$
IV.51	5	$3.1\cdot10^4$	$8.6\cdot10$

Table 10 shows that, with increase in the length of the hydrocarbon group, the anticholine-esterase activity of the compounds in relation to BuCE increases up to the isoheptyl derivative, while that in relation to ACE increases up to the isohexyl derivative. Further increase in the length of the group results in a decrease of k_{II}, the decrease for BuCE being slight (approximately by a factor of 2). On the other hand, in the case of ACE k_{II} for the isoheptyl derivative is smaller by a factor of 46 than for the isohexyl derivative. Moreover, it is seen that the first members of this series of compounds, up to the isohexyl derivative, are more active in relation to ACE than BuCE.

Above it was shown that an increase in the length of the alkoxy-group in the series of *O*-n-alkyl *S*-butyl methylphosphonothiolates does not result in a significant change of the electrophilic activity of the phosphorus atom in these compounds. It may be supposed that also in the series of *O*-isoalkyl derivatives the length of the hydrocarbon substituent will not have a significant influence on the magnitude of the effective positive charge of the phosphorus atom. This means that in this series too the factor determining the anticholine-esterase activity should be the hydrophobic interaction between the isoalkyl group and the hydrophobic regions on the surface of choline-esterase.

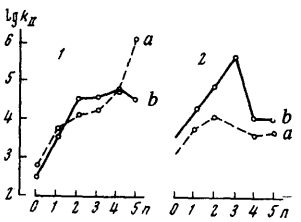


Figure 5. Variation of the rate constants k_{II} for the inhibition of BuCE (diagram *1*) ACE (diagram *2*) by compounds of the type $C_2H_5(CH_2)_nO(CH_3)P(O)SC_4H_9$ (*a*) and $(CH_3)_2CH(CH_2)_nO(CH_3)P(O)SC_4H_9$ (*b*) with the number of methylene groups n.

Since there is every reason to suppose that the hydrocarbon components of the isoalkyl and n-alkyl derivatives interact with the same parts of the enzyme, it is of interest to compare the effects of both series of compounds. Such a comparison is made in Fig. 5, where the isoalkyl derivatives are represented by compounds with an isopropyl substituent located at different distances from the phosphorus atom (curve *b*) while the series of OPI with straight-chain alkoxy-groups consists of compounds containing the ethyl group at the same distances (curve *a*). Thus compounds with the same length of a straight hydrocarbon chain are compared at each point. In the case of BuCE (Fig. 5) it is seen that initially (for $n = 0$ and 1) the branching of the substituent has no effect. For $n = 2-3$, i.e. in the region of the inflection in the curve, the branched-chain compounds are somewhat more effective, but on further increase in the length of the substituent the branched-chain group not only does not increase the anticholine-esterase activity but becomes a serious obstacle in the interaction between the OPI and the enzyme: k_{II} for the branched-chain compound with $n = 5$ is lower by a factor of 34 than for the corresponding straight-chain alkyl derivative.

In the interaction with ACE (Fig. 5), the branching of the substituent initially enhances appreciably the anticholine-esterase effect. The contribution of the branched-chain group is particularly pronounced for $n = 3$: k_{II} for this compound is higher by a factor of 63 than the constant for the corresponding OPI with a straight-chain substituent. Further separation of the isopropyl group from the phosphorus atom prevents the interaction of the OPI with ACE:

k_{II} diminishes by a factor of 46 and approaches the values of the rate constants for the straight-chain OPI. A still greater increase in the length of the substituent does not alter the anticholine-esterase activity of either series of OPI.

The above data confirm and complement the above considerations concerning the characteristics of the hydrophobic regions in the vicinity of the esterase component of the active centre of the choline-esterases. Probably in BuCE this region actually consists of two hydrophobic components. In general they are both complementary to hydrocarbon substituents with a straight-chain structure but the first, located closer to the esterase site, is not quite so rigorously specific and therefore the branching of the hydrocarbon substituent of the OPI does not prevent its sorption. The structure of the second (more remote) hydrophobic region is adapted solely for the sorption of a straight-chain substituent.

In ACE there is only one hydrophobic region. Its structure differs significantly from that of the first hydrophobic region of BuCE and is rigorously complementary to the isohexyl group (the maximum in curve *2b* in Fig. 4).

Yet another comparison is of interest for the characterisation of the configurational features of the hydrophobic region in the vicinity of the esterase component of the choline-esterases. Above, in the discussion of the anticholine-esterase properties of the OPI containing a branched-chain component in the alkylthio-group, it was noted that the introduction of a t-butyl substituent into this group significantly increases the capacity of the OPI to inhibit choline-esterase (Table 3).

An organophosphorus inhibitor containing the t-butyl group in the alkoxy-part of the molecule was synthesised [64]. Comparison of its activity with a series of other OPI described above shows how the gradual introduction of such a group affects the anticholine-esterase properties (Table 11).

Table 11. Anticholine-esterase effectiveness and the rate of alkaline hydrolysis (40°C) of OPI of the type $RCH_2O(CH_3)P(O)SC_4H_9$.

Series and no.	R	BuCE		ACE		k_{OH}, litre mole^{-1} min^{-1}
		k_{II}, litre mole^{-1} min^{-1}	relative value	k_{II}, litre mole^{-1} min^{-1}	relative value	
I.3	CH_3	$5.2\cdot10^2$	1	$1.1\cdot10^3$	1	0.145
IV.38	CH_3CH_2	$4.5\cdot10^3$	9	$5.8\cdot10^3$	5	0.160
IV.47	$(CH_3)_2CH$	$4.4\cdot10^3$	8	$1.5\cdot10^4$	13	0.146
IV.52	$(CH_3)_3C$	$9.5\cdot10^2$	2	$5.9\cdot10^3$	5	0.200
IV.39	n-C_4H_9	$1.6\cdot10^4$	30	$6.8\cdot10^3$	6	—

For comparison, the table also lists the anticholine-esterase activities of substances with R = n-C_4H_9, which is isomeric with the t-butyl group.

Table 11 shows that the degree of branching of the group R has little effect on the mobility of the P–S bond, which we estimated from the rate constant for alkaline hydrolysis. Consequently, the anticholine-esterase activities found must also be attributed to the different capacities of the hydrocarbon groups for sorption on the active surface of the enzyme.

Examination of the data for BuCE shows clearly yet again that the hydrophobic part of this enzyme has a structure complementing an unbranched hydrocarbon group. Indeed the replacement of one H atom by a CH_3 group on going from I.3 to IV.38 (increase in the length of the straight chain) increases the activity by a factor of 9, while the replacement of a second hydrogen atom by a methyl group (compound IV.47) has no effect despite the increase in the size of the group, and the introduction of a third methyl group (compound IV.52) causes a distinct decrease of activity. At the same time compound IV.39 is 15 times more active than the isomeric t-butyl derivative.

Compound IV.47 is the most effective in relation to ACE, the introduction of a third methyl group reducing the activity by a factor of 2.5. The straight-chain compound with R = n-C_4H_9 has no advantages compared with the t-butyl derivative.

Thus the hydrophobic region in ACE, in contrast to BuCE, complements best the isobutyl group. The above comparison leads to the conclusion that the fit of the hydrophobic regions in the vicinity of the esterase centre in both choline-esterases to the tetrahedral structure of the t-butyl group is poor. Therefore in the series of compounds investigated the introduction of such a group into the alkoxy-substituent results in a distinct hindrance of the sorption of the inhibitor on the active surface.

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Analysis of the results of the investigations leads to a general hypothesis concerning the location of the hydrophobic regions in the vicinity of the active centres of BuCE and ACE and certain characteristics of these regions [65].

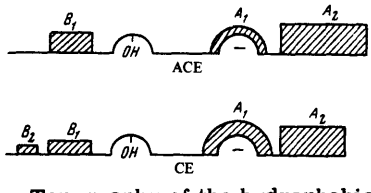


Figure 6. Topography of the hydrophobic regions on the active surface of ACE and BuCE.

The structure of the hydrophobic regions on the active surface of choline-esterase is shown schematically in Fig. 6. In the vicinity of the anionic centre there are two hydrophobic regions: A_1 surrounding directly the anionic centre and A_2 located at some distance beyond it. There is reason to believe that in BuCE region A_1 plays a relatively greater role than in ACE. Thus, according to Augustinsson [66], van der Waals forces play a major role together with Coulombic forces in the interaction of substrates and inhibitors with the anionic site in BuCE, while in ACE purely Coulombic forces predominate. The region A_2 is also different in the two choline-esterases: in BuCE its extent corresponds to a chain of six carbon atoms and in ACE to a chain of eight carbon atoms. The steric configuration of this region in both enzymes is such that it complements best groups containing the highly branched t-butyl substituent.

Near the esterase site of BuCE there are two hydrophobic regions separated by a hydrophilic group. The overall

extent of these hydrophobic regions corresponds to a chain of seven carbon atoms. They are both in general complementary to straight-chain groups, but the first (B_1) is not quite so rigorously specific and branched-chain groups can also be sorbed on it.

In ACE there is only one hydrophobic region, the structure of which differs significantly from that of the B_1 region in BuCE: it is rigorously complementary to the isohexyl group.

An important characteristic of the steric configuration of the B_1 regions in both enzymes is that they are completely unadapted for the sorption of groups containing the t-butyl substituent. Thus differences in the structures and extents of the hydrophobic regions located on the active surface of BuCE and ACE have been established. It is quite likely that these differences are to a large extent responsible for the different properties of the two cholinesterases.

The problem naturally arises of the biological value of this type of structure of the active surface of the cholinesterases. What is the purpose of the extensive hydrophobic regions surrounding the catalytic centre of the enzyme? It is hardly likely that the hydrophobic regions were formed in the evolutionary process solely as essential constructional details. The following hypothesis may be put forward: acetylcholine consists of highly hydrophilic molecules capable of being sorbed not only at the active site of choline-esterases but also at any other site on the polypeptide chains of the enzyme. Such sorption would lead to retardation of the hydrolysis of acetylcholine. The hydrophobic regions prevent the "parasitic" sorption of acetylcholine and thereby increase the likelihood of its sorption at the active centre of choline-esterase. They play the role of a kind of energy "hill" from which the acetylcholine molecule "rolls down" on to the active site of the enzyme. In this way the effective cross-section of the active site of the enzyme in the hydrolysis of acetylcholine is increased.

In this connection it is essential to consider also the problem of the structure of the active site of the choline receptor (CR). There is very significant evidence suggesting the hypothesis that there are hydrophobic regions also around the CR active site [5] and that there must be at least three of them: one fits the methyl groups of acetylcholine, another serves as the anionic site of the choline receptor, and the third is a site with affinity for the ester group. This hypothesis is supported by the enormous amount of factual data on the relation between the structure and the effect of substances blocking the choline receptor which have now accumulated. The empirical rule according to which acetylcholine must be made more "bulky" in order to prepare blocking substances was formulated long ago. Indeed, the introduction into the acetyl component of the acetylcholine molecule of phenyl, cyclohexyl, and large alkyl groups converts acetylcholine from an exciting to a blocking agent. An increase in the "bulk" of the cationic head has the same effect. It is striking that, as acetylcholine becomes more "bulky" at either end, its cholinomimetic, exciting effect is gradually weakened, then the blocking effect appears, and its intensity increases. This is easily understood. Not only rapid sorption but also easy desorption of the substance from the receptor is important for the exciting effect [67,68]. Retardation of desorption leads to the blocking effect. Naturally, the introduction of bulky hydrophobic groups into the acetylcholine molecule increases the interaction with the hydrophobic regions in the vicinity of the active site of the choline receptor and hinders desorption.

It is reasonable to suppose that, for the interaction of acetylcholine itself with choline-esterases, the biological purpose of the hydrophobic regions consists in a reduction of the "parasitic" sorption and an increase in the probability of the collision of the acetylcholine molecule with the active site of the choline receptor.

Returning now to the problem of choline-esterases we must finally conclude that the inhibiting capacity of the organophosphorus inhibitors depends, at any rate for the series of compounds which we investigated and possibly others as well, not only on the intrinsic phosphorylating capacity of the compound (electrophilic reactivity) but mainly on the rate of sorption, i.e. the rate of formation of the enzyme–inhibitor complex.

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