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HYDROPHOBIC AREAS ON THE ACTIVE SURFACE OF CHOLINESTERASES

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I. INTRODUCTION

Two separate active sites can be distinguished on the active surface of cholinesterases: an anionic site and an esteratic one (10, 12, 39, 43, 58, 67, 90). The anionic site is capable of coulombic interaction with the trimethylammonium group of acetylcholine and this interaction facilitates a favorable orientation of the substrate molecule on the enzyme surface so that the hydrolysis of acetylcholine is carried out by the esteratic site.

We do not know anything about the position of the functional groups that form these sites in the general structure of the enzyme. It is not known whether they belong to the same polypeptide chain or to different chains. One may suppose that the final formation of the active center occurs during its interaction with the substrate, which induces some conformational changes in the enzyme protein molecule (35, 60).

The hydroxyl of serine is the main functional group of the esteratic site; it is widely held that the high reactivity of this serine hydroxyl is the result of its interaction with the imidazole group of histidine. At the anionic site, the carboxylic groups of glutamic and aspartic acid seem to be the only possible functional groups with the requisite negative charge at physiological pH 7.3 to 7.4.

The functional groups of the esteratic and anionic sites that react directly with the substrate are probably very similar, if not identical, in the two main

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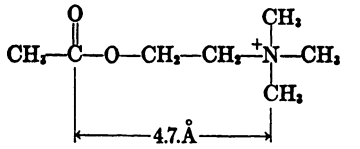
types of cholinesterase: specific or acetylcholinesterase (E.C. 3.1.1.7) and non-specific or butyrylcholinesterase (E.C. 3.1.1.8). It is therefore logical to suppose that the type of cholinesterases is determined not by the differences in the structure of their active centers, but by some differences in the structure of various areas around the active centers.

Our knowledge of the structure of the active surface of cholinesterase is due to a great extent to the fruitful studies of the interaction of the enzyme with its organophosphorus inhibitors carried out with increasing intensity over more than a quarter of a century (see reviews: 13, 44, 49, 51–53, 69, 87, 90). The inhibition of cholinesterases by organophosphorus inhibitors is known to be due to the phosphorylation of the serine hydroxyl group at the esteratic site of the enzyme. In contrast to the deacetylation of cholinesterases, which takes place during the enzymatic hydrolysis of acetylcholine, their dephosphorylation proceeds very slowly, and therefore the inhibition is of an irreversible type. Possibly this is due to the fact that for the hydrolysis of a phosphorylated enzyme the O-alkyl bond (a) must be broken whereas the enzyme is adapted to break the O-acyl bond (b) (53):



The anticholinesterase potency of an organophosphorus inhibitor is determined not only by its phosphorylating ability (51–53, 69, 90), which in turn is determined by the effective positive charge of the phosphorus atom, but also by ionic and ion-dipole interactions. The role of the ionic interaction can be clearly demonstrated when a cationic group is introduced into the "leaving part" of the organophosphorus inhibitor molecule. (We call the "leaving part" of the organophosphorus inhibitor molecule the atomic group directly bound to the phosphorus atom which is split off from the organophosphorus inhibitor molecule in the course of phosphorylation of cholinesterase.) In this case the organophosphorus inhibitor is fixed on the anionic site of the enzyme, and this reaction increases the probability of the interaction between the inhibitor and the esteratic site.

The first cationic organophosphorus inhibitors containing an ammonium or a sulfonium group were studied in 1955 to 1958. The study of ammonium organophosphorus inhibitors did not reveal any great difference between the potencies of phosphorylthiocholine and its tertiary analogue. The high basicity of tertiary compounds makes them highly protonated at neutral pH, and therefore the quaternization increases their potency only 3- to 4-fold (46, 59, 82, 83). The role of positive charge was shown more clearly by comparing the organophosphorus inhibitors containing a sulfide sulfur with their sulfonium analogues (38, 40, 41, 65, 79, 80, 81, 86, 92, 93). The sulfide sulfur cannot protonize, and therefore the charged sulfonium compounds are 2000 to 3000 times more potent than their sulfide analogues (92). A similar dramatic increase of inhibitory potency was

obtained subsequently by quaternization of certain organophosphorus inhibitors containing aromatic amine groups of low basicity. Such amines are very poorly protonized at pH 7 to 8 and their quaternization, by insuring the presence of a permanent positive charge, increases their potency more than a thousandfold (30).

The position of the onium atom in the organophosphorus inhibitor molecule is also very important. The maximal effect is reached when the charged atom is in the leaving part of the molecule and is separated from the phosphorus atom by the same distance that separates the nitrogen atom from the carbonyl carbon in the acetylcholine molecule (44, 65, 86).

It is important to add that the introduction of a cationic group in the organophosphorus inhibitor molecule not only permits coulombic interaction with the anionic group of the cholinesterase molecule, but also produces an inductive effect within the organophosphorus inhibitor molecule itself, increasing the phosphorylating ability of the inhibitor (55, 57, 61, 86, 92).

The two main properties of organophosphorus inhibitors, their phosphorylating activity and their ability to interact with the anionic site of the enzyme, have been widely studied. But, as shown below, the ability of organophosphorus inhibitors to interact with the active surface of cholinesterase is not limited by these two properties.

It has been known for a long time that the interaction of the cationic head of acetylcholine with the anionic site of cholinesterase or the cholinoreceptor involves not only coulombic forces. The three methyl groups attached to the nitrogen atom also play an important role (19, 67, 89). Moreover, Whittaker and coworkers (8, 88) showed that 3,3-dimethylbutyl acetate, whose tertiary butyl group sterically imitates the trimethylammonium group of acetylcholine, is hydrolyzed by the cholinesterase only slightly less rapidly than acetylcholine itself. This finding can be regarded as the result of hydrophobic sorption of the methyl groups of acetylcholine and of its carbon isostere on the hydrophobic areas surrounding the anionic site of the enzyme.

The idea of "hydrophobic sorption" or "hydrophobic interaction" is well known in modern enzymology and pharmacology (17, 18, 48, 56, 68, 84, 85). Strictly speaking there are no special "hydrophobic interactions." The van der Waals forces are universal and what are called "hydrophobic interactions" are the result of a pure Langmuir effect in which the hydrophilic medium pushes out the hydrophobic parts of the dissolved substance towards the hydrophobic regions of the enzyme surface. It is generally held that these interactions play an important role in the formation of complexes between enzymes and their substrates, coenzymes, and inhibitors and in the reactions of receptors with the transmitter substances, their mimetics, and antagonists.

The attentive study of early (78) and more recent (15, 16, 21, 47, 63, 70, 73, 74) publications clearly shows that changes in the structure and in the degree of branching of the hydrocarbon radicals of an organophosphorus inhibitor can considerably influence its potency. This effect may be striking when a branched alkyl radical containing a tertiary butyl group is introduced into the molecule

(*e.g.*, pinacoline esters of alkylfluorophosphonic acids) (44). In view of these results it seemed logical to expect that on the active surface of cholinesterase there must be one or more hydrophobic areas at which the sorption of the hydrophobic alkyl radicals of substrates or inhibitors can occur. The first results of investigation of this problem confirmed this supposition (31, 42, 51). The need then arose for a systematic study of these hydrophobic regions: their localization, their length and width, their steric structure, and the details of their structure and localization in different types of cholinesterases, *etc.*

II. METHODS OF EXPLORING HYDROPHOBIC AREAS

The chief method of exploring hydrophobic areas in the region of the active center of cholinesterase is to compare the anticholinesterase potencies of a series of organophosphorus inhibitors differing in the structure of their hydrocarbon radicals. Certain conditions seem essential for this approach.

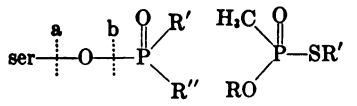
1) Within each series of organophosphorus inhibitors only the structure of the hydrocarbon radical may be changed and these changes must be gradual and logical.

2) Each series must be large enough; for instance, a homologous series must contain at least 8 to 10 compounds.

3) It must be appreciated that the changes in the structure of the alkyl radicals will influence the positive charge on the phosphorus atom by means of inductive and other effects. Therefore the phosphorylating activity of each member of the series must be determined and corresponding corrections introduced. Meaningful results can be obtained only if the phosphorylating activity is constant throughout the series.

4) It is very important to determine the anticholinesterase activity by kinetic methods and to characterize it by the reaction rate constants. With any other method it is difficult to compare the results of different authors.

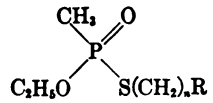
Unfortunately, for the most part, these conditions were not met in early investigations of anticholinesterase potency, and it is sometimes very difficult to use such results to get information about the hydrophobic areas on the active surface of cholinesterase. In the investigations of the reviewers' research group the above mentioned conditions have been strictly met. Purified cholinesterase of horse serum (butyrylcholinesterase, E.C. 3.1.1.8) made in the Mechnikov Institute of Vaccines and Sera (Moscow) and the purified acetylcholinesterase (E.C. 3.1.1.7) from bovine erythrocytes (34, 94) were used. All the organophosphorus inhibitors investigated were thiol derivatives of methylphosphonic acid with the general structure



The rate constants of their reactions with butyrylcholinesterase and acetylcholinesterase (9, 90) and of their aqueous-alkaline hydrolysis, as well as some physical constants are given in table 1. These organophosphorus inhibitors were synthesized (1, 3, 4, 31, 32, 42, 76) by the following methods.

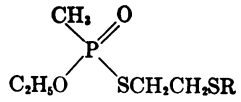
TABLE 1

Structure, physical constants, anticholinesterase potency, and the rate of alkaline hydrolysis of the derivatives of methylphosphonic acid (1, 2, 3, 31, 32, 42, 76)



Series I

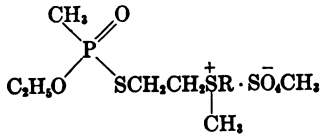
Series and Number	R	n	Bp/mm	n_D^{20}	d_4^{20}	k_a, $M^{-1} \times min^{-1}$		
						BuChE*	AChE	OH
I.1	CH_3	1	62–64/1	1.4760	1.0969	6.4×10^1	2.2×10^2	0.15
I.2	CH_3	2	85–86/2.5	1.4820	1.0712	1.4×10^3	5.2×10^2	0.15
I.3	CH_3	3	100–102/2	1.4810	1.0530	8.2×10^3	1.2×10^3	0.15
I.4	CH_3	4	108–110/3	1.4678	1.0306	2.6×10^3	2.6×10^3	0.13
I.5	CH_3	5	105–106/1	1.4712	1.0178	3.8×10^4	1.6×10^4	0.12
I.6	CH_3	6	124–126/2.5	1.4650	1.0040	3.3×10^4	2.1×10^4	0.13
I.7	CH_3	7	155–156/5	1.4690	0.9957	4.1×10^4	3.6×10^4	0.13
I.8	CH_3	8	142–144/2	1.4702	0.9841	3.4×10^4	3.1×10^4	0.13
I.9	CH_3	9	152–153/2	1.4705	0.9725	3.8×10^4	1.6×10^4	0.13
I.10	$(CH_3)_3C$	1	78–80/2	1.4640	1.0262	2.1×10^4	2.0×10^4	0.10
I.11	$(CH_3)_3C$	2	76–77/1	1.4746	1.0120	4.7×10^3	1.6×10^3	0.16
I.12	$(CH_3)_3C$	3	84–85/1	1.4710	1.0015	1.8×10^4	2.2×10^3	0.13
I.13	$(CH_3)_3C$	4	124–126/2	1.4700	0.9896	9.1×10^4	7.2×10^3	0.15
I.14	$(CH_3)_3C$	5	126–129/1.5	1.4670	0.9811	9.0×10^4	1.1×10^4	0.12
I.15	$(CH_3)_3C$	6	134–136/2	1.4700	0.9723	9.9×10^4	1.9×10^4	0.15
I.16	$(CH_3)_2CH$	1	120–121/2.5	1.4770	1.0610	6.7×10^2	1.6×10^3	0.14
I.17	$(CH_3)_2CH$	2	73–74/1	1.4710	1.0394	2.3×10^3	1.3×10^3	0.16
I.18	$(CH_3)_2CH$	3	84–85/1	1.4768	1.0270	2.5×10^3	8.3×10^2	0.13
I.19	$(CH_3)_2CH$	4	126–127/1	1.4698	1.0010	5.9×10^4	9.0×10^3	0.16
I.20	$(CH_3)_2CH$	6	132–134/1	1.4705	0.9862	9.3×10^4	2.2×10^4	0.13
I.21	C_6H_5	1	135–136/1.5	1.5462	1.1531	1.2×10^5		0.11
I.22	C_6H_5	2	136/2	1.5398	1.1369	1.4×10^4	1.2×10^4	0.14
I.23	C_6H_5	3	150–151/2	1.5346	1.0808	8.4×10^4	1.8×10^4	0.11
I.24	C_6H_5	4	136–139/1	1.5300	1.1068	1.1×10^5	3.4×10^4	0.12



Series II

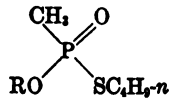
Series and Number	R	Bp/mm	n_D^{20}	d_4^{20}	k_a, $M^{-1} \times min^{-1}$		
					BuChE	AChE	OH
II.25	CH_3	110–112/1.5	1.5211	1.1665	3.2×10^3	4.0×10^1	0.29
II.26	C_2H_5	98–99/1	1.5181	1.1429	6.8×10^3	6.0×10^4	0.28
II.27	n-C_4H_9	132–133/1	1.5082	1.0963	7.6×10^4	9.0×10^4	0.31
II.28	n-C_6H_{13}	137–138/1	1.5037	1.0650	1.3×10^5	4.0×10^5	0.28
II.29	n-C_8H_{17}	170–171/1	1.4998	1.0341	3.0×10^5	3.3×10^5	0.29
II.30	n-$C_{10}H_{21}$	192–195/1	1.4880	1.0097	4.0×10^5	2.0×10^5	0.27

TABLE 1—*Continued*



Series III

Series and Number	R	n_D^{20}	k_a, $M^{-1} \times min^{-1}$		
			BuChE	AChE	OH
III.31	CH_3	1.5255	1.0×10^6	5.5×10^7	17.0
III.32	C_2H_5	1.5210	3.7×10^6	2.5×10^8	17.0
III.33	n-C_4H_9	1.5212	1.1×10^7	2.2×10^8	20.0
III.34	n-C_6H_{13}	1.4944	8.9×10^7	1.1×10^9	21.4
III.35	n-C_8H_{17}	1.4898	1.0×10^8	4.6×10^8	25.0
III.36	n-$C_{10}H_{21}$	1.5002	1.8×10^8	4.6×10^8	19.0



Series IV

Series and Number	R	Bp/mm	n_D^{20}	d_4^{20}	k_a, $M^{-1} \times min^{-1}$		
					BuChE	AChE	OH
IV.37	CH_3	64–65/1	1.4772	1.0825	5.8×10^1	2.6×10^2	0.26
IV.38	n-C_3H_7	85–86/1	1.4692	1.0280	4.5×10^2	5.6×10^2	0.14
IV.39	n-C_4H_9	92–93/1	1.4675	1.0125	1.2×10^4	1.4×10^4	0.16
IV.40	n-C_5H_{11}	87/1.5	1.4700	0.9988	1.6×10^4	6.8×10^2	
IV.41	n-C_6H_{13}	116/2	1.4706	0.9783	5.0×10^4	4.2×10^2	0.16
IV.42	n-C_7H_{15}	$70/2 \times 10^{-2}$	1.4693	0.9783	1.1×10^6	4.5×10^2	
IV.43	n-C_8H_{17}	$95/2 \times 10^{-2}$	1.4697	0.9656	3.6×10^5	4.1×10^2	
IV.44	n-C_9H_{19}	$110/2 \times 10^{-2}$	1.4690	0.9080	7.0×10^5	6.2×10^2	
IV.45	n-$C_{10}H_{21}$	$120/2 \times 10^{-2}$	1.4710	0.9643	6.2×10^4	2.9×10^2	
IV.46	$(CH_3)_2CH$	79/1.5	1.4703	1.0166	4.5×10^2	3.2×10^2	0.08
IV.47	$(CH_3)_2CHCH_2$	80/1.5	1.4693	1.0053	4.4×10^2	1.4×10^4	0.15
IV.48	$(CH_3)_2CH(CH_2)_2$	108/2	1.4698	0.9965	3.4×10^4	6.1×10^4	0.15
IV.49	$(CH_3)_2CH(CH_2)_3$	$43/3 \times 10^{-2}$	1.4725	0.9929	3.9×10^4	4.3×10^5	0.15
IV.50	$(CH_3)_2CH(CH_2)_4$	$76/3 \times 10^{-2}$	1.4693	0.9835	6.5×10^4	9.3×10^2	0.14
IV.51	$(CH_3)_2CH(CH_2)_5$	$92–94/3 \times 10^{-2}$	1.4730	0.9818	3.0×10^4	8.6×10^2	
IV.52	$(CH_3)_3CCH_2$	82–83/2	1.4636	0.9891	9.5×10^2	5.9×10^2	0.12

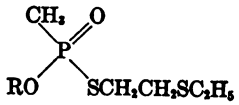
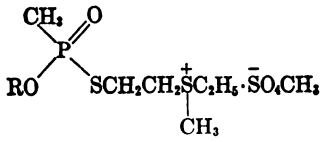


TABLE 1—*Continued*

Series V

Series and Number	R	Bp/mm	n_D^{20}	d_4^{20}	k_a, $M^{-1} \times min^{-1}$ BuChE*
V.53	CH_3	100/1.5	1.5251	1.1728	9.6×10^3
V.54	n-C_3H_7	105/2	1.5101	1.1089	5.8×10^4
V.55	n-C_4H_9	95–96/3 $\times 10^{-3}$	1.5082	1.0960	1.6×10^5
V.56	n-C_5H_{11}	104–105/2 $\times 10^{-3}$	1.5040	1.0706	1.7×10^5
V.57	n-C_6H_{13}	103–104/1 $\times 10^{-3}$	1.4984	1.0493	3.7×10^5
V.58	n-C_7H_{15}	120–121/2 $\times 10^{-3}$	1.4994	1.0430	2.8×10^6
V.59	n-C_8H_{17}	117/1 $\times 10^{-4}$	1.4974	1.0365	1.1×10^6

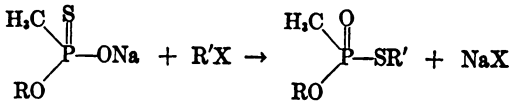


Series VI

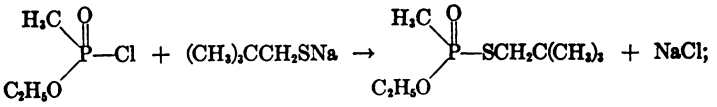
Series and Number	R	n_D^{20}	k_a, $M^{-1} \times min^{-1}$ BuChE*
VI.60	CH_3	1.5201	7.7×10^5
VI.61	n-C_3H_7	1.5010	3.8×10^7
VI.62	n-C_4H_9	1.5092	4.8×10^7
VI.63	n-C_5H_{11}	1.5045	7.5×10^7
VI.64	n-C_6H_{13}	1.5044	1.8×10^8
VI.65	n-C_7H_{15}	1.4982	2.5×10^8
VI.66	n-C_8H_{17}	1.5000	2.8×10^8

The abbreviations used are: BuChe, butyrylcholinesterase; AChE, acetylcholinesterase.

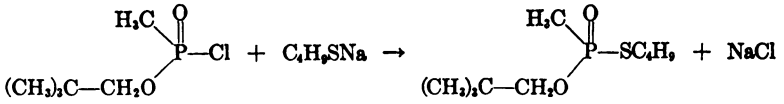
Thiol esters of methylphosphonic acid were usually prepared by the alkylation of the sodium alkyl methylthiophosphonates with the corresponding alkyl halides (4, 42, 76).



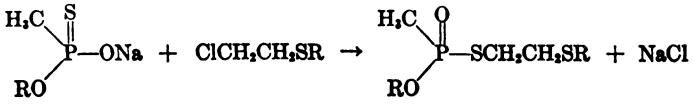
An exception was made in two cases: in the case of O-ethyl-S-neopentyl methylthiophosphonate (table 1, compound I.10), which was obtained from ethyl methylphosphonyl chloride by the action of sodium neopentyl mercaptide (31)



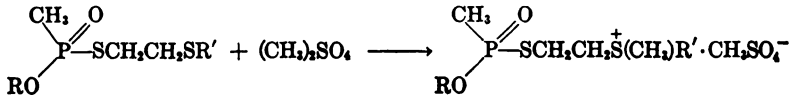
and in the synthesis of O-neopentyl-S-butyl methylthiophosphonate (table 1, compound IV.52), which was obtained by reaction of neopentyl methylphosphonyl chloride and sodium butyl mercaptide (1)



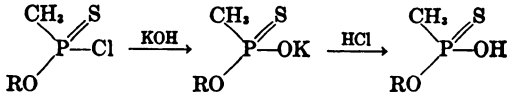
All the O-ethyl S-β-alkylmercaptoethyl methylthiophosphonates (series II) and O-alkyl-S-ethylmercaptoethyl methylthiophosphonates (series V) were synthesized by the action of β-chlorethyl alkyl sulfide on the sodium alkyl methylthiophosphonates (32, 54):



The onium compounds (series III and IV) were obtained from the alkylmercaptoalkyl phosphonates by treatment with dimethyl sulfate



Hydrolysis of the alkyl methylphosphonyl chlorides gave O-alkyl methylthiophosphonates



The individuality and purity of all synthesized compounds were characterized by elementary analysis and also by thin-layer chromotography (1, 3, 4, 31, 32, 42, 76).

For estimation of the anticholinesterase potency of organophosphorus inhibitors the bimolecular rate constant of their interaction with the enzyme (k_a)[4] (9, 20, 90, 91) was determined. These constants were calculated from Aldridge's equation:

$$k_a = \frac{2.3}{[I] \cdot t} \lg \frac{V_o}{V_t}$$

where $[I]$ is the concentration of inhibitor which surpasses by far the concentration of the active centers of the enzyme; V_o and V_t are the rates of acetylcholine hydrolysis in the absence of the inhibitor and after the incubation of the enzyme with organophosphorus inhibitor during t minutes, respectively. Each organophosphorus inhibitor was tested in 2 to 4 different concentrations

[4] In the Russian literature k_{II} is used instead of k_a.

and with each concentration the time t varied (from 2 to 5 or from 5 to 15 minutes). The rate of acetylcholine hydrolysis was determined by the estimation of the increase in the concentration of the acetic acid by potentiometric titration or of the decrease in the concentration of acetylcholine (in the last case Hestrin's method was used) (45). The reaction of the enzyme with organophosphorus inhibitor and with acetylcholine occurred in a phosphate buffer (0.07 M) at pH 7.5 at 25°. The nonenzymatic alkaline hydrolysis was determined at pH 10.5 at 25°.

III. HYDROPHOBIC AREAS IN THE REGION OF THE ANIONIC SITE

The role of the hydrocarbon radicals in the leaving part of the organophosphorus inhibitor molecule was studied (31, 32, 42, 76) with the compounds of series I (I.1 to I.24; see table 1). All these compounds possess the same phosphorylating activity, as can be seen from the identity of rate constants of nonenzymatic alkaline hydrolysis of these compounds. The values of these constants at pH 10.5 varied from 0.13 to 0.15 M^{-1} min^{-1}. With the compounds containing normal alkyl radicals (I.1 to I.9) the rate constants of inhibition (k_a) of both butyrylcholinesterase and acetylcholinesterase rose sharply with increasing length of the carbon chains (fig. 1). In the case of butyrylcholinesterase the increase in the k_a values was observed only up to the hexyl radical, but in the case of acetylcholinesterase up to the octyl. Once this cut-off point was reached, further lengthening of the alkyl chain did not change the k_a values (butyrylcholinesterase) or even decreased them slightly (acetylcholinesterase). Since all these compounds possess equal phosphorylating activity and their radicals are not capable of any specific reactions, the increase in the inhibitory potency with the lengthening of the alkyl chain can be attributed only to the increase in the sorption of the organophosphorus inhibitor onto the surface of cholinesterase due to hydrophobic interaction with the hydrophobic areas close to the anionic site. The abrupt cut-off point that this increase has in the case of both enzymes (n = 5 with butyrylcholinesterase, and n = 7 with acetylcholinesterase) indicates that the corresponding hydrophobic areas on the enzyme surface probably are of limited size and can accommodate only six or eight methylenes.

The compounds in which thioalkyl radicals contain a tertiary butyl group at different distances from the phosphorus atom (I.10 to I.15) proved to be very interesting. The first number of this series possesses a comparatively high anticholinesterase potency (fig. 2). Its k_a value for butyrylcholinesterase is 2.1×10^4 M^{-1} min^{-1}. For the second member of this set there is a decrease in the value of k_a and for the next members, there is a gradual increase of k_a as the number of methylene groups increases. For butyrylcholinesterase the cut-off point is reached at n = 4 and k_a becomes constant. For acetylcholinesterase a continuous increase in k_a values (with n = 2 to 6) is observed. A similar relationship has been observed with the less extensive series of phenyl derivatives (I.21 to I.24).

The zigzag form of the curve can be explained, not withstanding the fact that the phosphorylating activity of all the compounds is the same and the tertiary butyl group is incapable of any polar interactions, if we assume the existence of

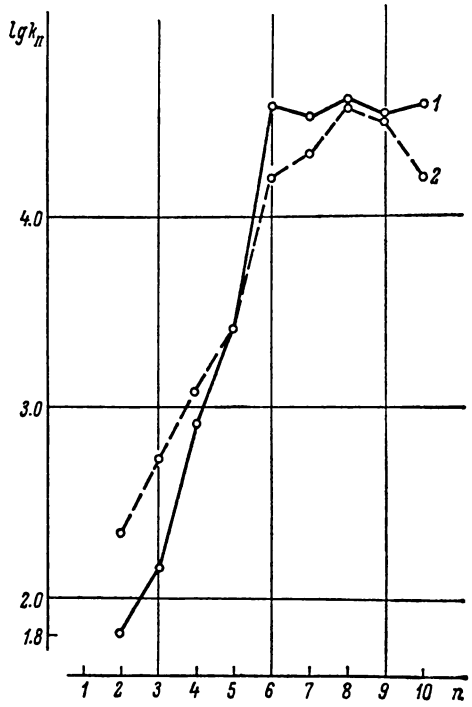
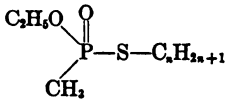
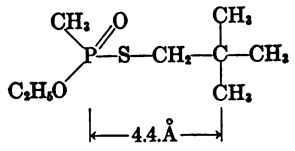


FIG. 1. The anticholinesterase potency of organophosphorus inhibitors with the general formula



in reactions with butyrylcholinesterase (1) and with acetylcholinesterase (2) (31, 53, 76).

some peculiar hydrophobic fold or deepening on the enzyme surface on which the bulky tertiary butyl group can be sorbed in a complementary manner. If the distance between the tertiary butyl group and the phosphorus atom corresponds to the interval between the hydrophobic fold and the esteratic site of the enzyme the value of k_a must be high, as in the case of compound I.10 (see fig. 2). In this compound the distance between the tertiary butyl group and the phosphorus atom is practically equal to the distance between the trimethylammonium group and the carbonyl carbon atom in the acetylcholine molecule, that is, it corresponds to the interval between the anionic and the esteratic sites in the enzyme.



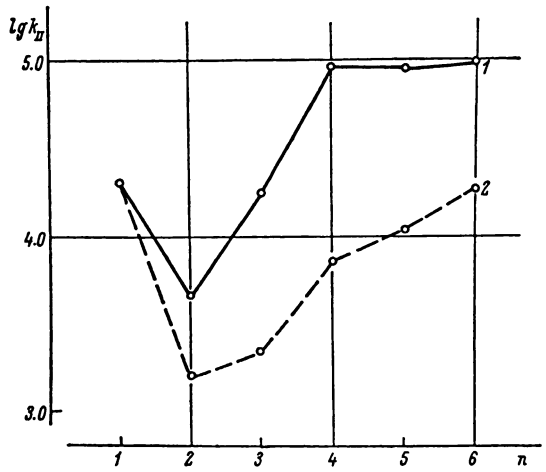
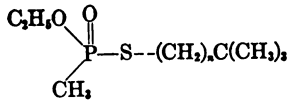


FIG. 2. The anticholinesterase potency of organophosphorus inhibitor with the general formula



in reactions with butyrylcholinesterase (1) and with acetylcholinesterase (2) (31).

The high anticholinesterase potency of this neopentyl compound could be explained by assuming that the anionic group is surrounded by a hydrophobic region on which the tertiary butyl group of this organophosphorus inhibitor can be sorbed, much as the methyl groups of the trimethylammonium "head" of acetylcholine are sorbed.

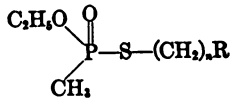
All three methyl radicals attached to the tertiary carbon atom are important for the interaction with the enzyme, as is clearly shown in table 2, which demonstrates the effect on the k_a values of gradual transformation of the end methyl group into a tertiary butyl group. This effect depends on the distance between the tertiary butyl group and the phosphorus atom.

The most striking effect is observed when $n = 1$. If one hydrogen atom of the terminal methyl group is replaced by a methyl group the k_a value for butyrylcholinesterase is only doubled; if two hydrogen atoms are replaced the increase is 10-fold; and if all the three hydrogens are replaced by methyl groups, so that a neopentyl compound is formed, the increase is 330-fold. The isomer of the neopentyl compound containing the terminal straight chain butyl group is much weaker. It is clear that the high activity of the neopentyl compound results from the presence of the tetrahedral tertiary butyl group and cannot be attributed simply to the increase in size of the alkyl radical.

A quite different picture is observed when $n = 2$. The transformation of the terminal methyl radical into a tertiary butyl group increases the k_a value for

TABLE 2

Changes in inhibitory potency in the series with gradual changes in the group R from methyl to tertiary butyl and with different numbers of methylenes (n)



The values of k_a for the compounds with a methyl group are taken as 1 (31).

R	k_a									
	$n = 1$		$n = 2$		$n = 3$		$n = 4$		$n = 6$	
	BuChE*	AChE	BuChE	AChE	BuChE	AChE	BuChE	AChE	BuChE	AChE
CH_3	1	1	1	1	1	1	1	1	1	1
CH_2CH_3	2	2	6	2	3	2	15	6	1	1.5
$CH(CH_3)_2$	10	7	17	2	3	1	23	3	3	2
$C(CH_3)_3$	330	90	34	3	22	2	35	3	3	1
C_4H_9	40	12	275	30	40	18	16	14	1	0.7

*The abbreviations used are: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase.

butyrylcholinesterase only 34-fold and, most important, the isomer of normal structure proved to be much more potent. This implies that butyrylcholinesterase possesses no hydrophobic area complementary to the tertiary butyl group at a corresponding distance from the phosphorus atom.

With further increase in the distance between the tertiary butyl group and the phosphorus atom the effect described above becomes weak and with $n = 6$ it almost disappears.

Similar relationships are observed with acetylcholinesterase but the hydrophobic regions seem to be less specific for the tertiary butyl group than with butyrylcholinesterase.

Kinetic studies have shown (31, 42) that the observed steep increase in the rate constants of inhibition with the lengthening or branching of the hydrocarbon radical of organophosphorus inhibitor is due to the changes in the steric factor. The pre-exponential factor (pZ) of the Arrhenius equation increased by several orders of magnitude in this series of compounds. At the same time the values of activation energy remained practically constant: for the interaction with butyrylcholinesterase, 12.0 to 12.5 kcal/mole, and with acetylcholinesterase, 10.7 to 11 kcal/mol. This is additional evidence that the true phosphorylating ability is the same in all the compounds studied, and the difference in the inhibition rate constants are only due to the different rates or equilibria of sorption onto the enzyme surface.

This point of view is supported by the determination of the rate constants

for the separate reaction stages of an organophosphorus inhibitor (I) with the enzyme (E):

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI' + \text{thioalcohol}$$

where EI is the complex formed by the sorption of the inhibitor on the enzyme and EI' is the phosphorylated enzyme. It turns out that the difference between different organophosphorus inhibitors is only in the k_1 values, and that the values of k_{-1} and k_2 are practically the same (29, 36).

Thus it seems clear that at least two separate hydrophobic areas are present in the region of the anionic site of the enzyme: one immediately surrounding the anionic group, and the other located outside the anionic site, beyond it.

Additional proof of the existence of the second hydrophobic patch beyond the anionic site was obtained by studying series III (III.31 to III.36, table 1). These compounds contain a positively charged sulfur atom which must be precisely fixed at the anionic site of the enzyme (37). If there really is a hydrophobic area near the anionic site, the inhibitory power should increase with the lengthening of the hydrocarbon radical in this series of organophosphorus inhibitors. It can be seen from figure 3 (as well as from table 1) that k_a values in fact increase with the lengthening of the hydrocarbon chain, especially in the reactions with butyrylcholinesterase. The decyl derivative (III.36) inhibits butyrylcholinesterase 180 times more strongly than the methyl derivative (III.31). In the case of acetylcholinesterase the increase was only 8-fold. It is evident that the comparison of the influence of the alkyl groups in series III, on the one hand, and in series I and II, on the other is valid only within certain limits. When the onium organophorphorus inhibitors are sorbed the ionic interaction may produce conformational changes in the enzyme that would cause the dislocation of the hydrophobic areas.

A similar relationship has been observed with the compounds containing an uncharged sulfur atom (series II, II.25 to II.30). The comparison of these two series (II and III) shows that the introduction of a cationic group increases the activity in reactions with acetylcholinesterase much more than the activity of the same compounds in reactions with butyrylcholinesterase. For the ethyl derivatives II.26 and III.32 this increase was 500-fold in the case of butyrylcholinesterase but more than 4000-fold with acetylcholinesterase.

These data are compatible with the view that the ionic interactions are more important in the reactions of organophosphorus inhibitors with acetylcholinesterase and hydrophobic interactions are more significant in their reactions with butyrylcholinesterase. This point of view was put forward by Augustinsson (11) in connection with the study of the reactions of some substrates and inhibitors (pyridine derivatives) with cholinesterases.

The hydrophobic area located beyond the anionic site is comparatively extensive. In butyrylcholinesterase a part of this hydrophobic patch is complementary to the tertiary butyl group, whereas in acetylcholinesterase this part is

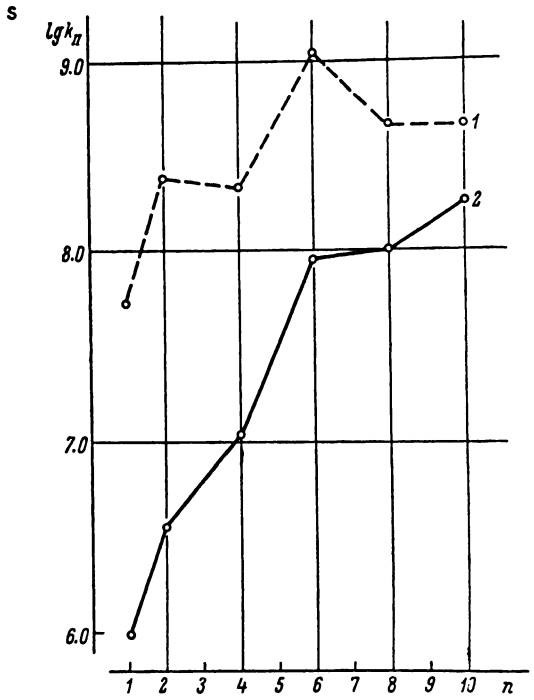
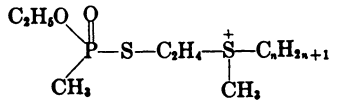


FIG. 3. The anticholinesterase potency of organophosphorus inhibitors with the general formula



in reactions with acetylcholinesterase (1) and butyrylcholinesterase (2) (37).

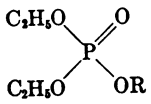
not so pronounced. Perhaps this is the reason why the hydrophobic properties of organophosphorus inhibitor are less important for the inhibition of acetylcholinesterase than for the inhibition of butyrylcholinesterase.

The conclusions cited above were confirmed by Bracha and O'Brien in their interesting studies of the anticholinesterase potency of several series of trialkylphosphates and phosphothiolates containing alkyl groups of different length and different degree of branching (23–26). They used the acetylcholinesterase of bovine erythrocytes and expressed the anticholinesterase potency as I50 values, that is, the molar concentration of the inhibitor producing 50% inhibition of the enzyme. It is therefore difficult to compare the absolute potency of their thiolphosphates with the potency of thiolphosphonates examined in our investigations. But, as the duration of the incubation of the enzyme with the inhibitor was the same (10 minutes) in all trials, we have calculated from the I50 values the bimolecular constants, k_a, with Aldridge's (9) expression

$$k_a = \frac{0.695}{I50 \cdot t}$$

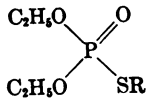
TABLE 3

Structure, physical constants, and anticholinesterase potency of the derivatives of phosphoric and thiophosphoric acid calculated according to references 25 and 26



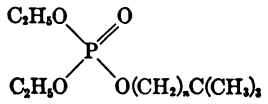
Series VII

Series and Number	R	Bp/mm	n_D^{20}	I50*	k_a†, $M^{-1} \times min^{-1}$
VII.67	$CH(C_2H_5)_2$	112–113/10	1.4172	4.7×10^{-4}	1.5×10^{2}
VII.68	$CH_2CH(C_2H_5)_2$	96–97/0.3	1.4194	$>10^{-3}$	$<7.0 \times 10^{1}$
VII.69	$CH_2CH_2CH(C_2H_5)_2$	93–94/0.08	1.4183	9.5×10^{-7}	7.3×10^{4}
VII.70	$(CH_2)_3CH(C_2H_5)_2$	108–109/0.1	1.4223	8.5×10^{-7}	8.2×10^{4}
VII.71	$(CH_2)_4CH(C_2H_5)_2$	111–112/0.08	1.4294	8.5×10^{-7}	8.2×10^{4}
VII.72	$(CH_2)_5CH(C_2H_5)_2$	118–119/0.05	1.4276	4.8×10^{-7}	1.4×10^{5}



Series VIII

Series and Number	R	Bp/mm	n_D^{20}	I50*	k_a†, $M^{-1} \times min^{-1}$
VIII.73	$CH(C_2H_5)_2$	130–132/10	1.4605	6.0×10^{-6}	1.2×10^{4}
VIII.74	$CH_2CH(C_2H_5)_2$	93–94/0.1	1.4629	5.6×10^{-7}	1.2×10^{5}
VIII.75	$CH_2CH_2CH(C_2H_5)_2$	110–111/0.3	1.4573	3.0×10^{-7}	2.3×10^{5}
VIII.76	$(CH_2)_3CH(C_2H_5)_2$	115–116/0.9	1.4553	1.9×10^{-7}	3.6×10^{5}
VIII.77	$(CH_2)_4CH(C_2H_5)_2$	120–121/0.05	1.4550	1.6×10^{-7}	4.3×10^{5}
VIII.78	$(CH_2)_5CH(C_2H_5)_2$	126–127/0.05	1.4610	1.6×10^{-7}	4.3×10^{5}



Series IX

Series and Number	n	Bp/mm	n_D^{20}	I50*	k_a† $M^{-1} \times min^{-1}$
IX.79	0	87/8	1.4095	5.2×10^{-5}	1.3×10^{3}
IX.80	1	72–73/0.2	1.4120	4.2×10^{-5}	1.7×10^{3}
IX.81	2	83–84/0.3	1.4188	4.6×10^{-6}	1.5×10^{4}
IX.82	3	91–92/0.1	1.4219	2.4×10^{-6}	2.9×10^{4}
IX.83	4	93–94/0.08	1.4240	8.4×10^{-7}	8.3×10^{4}

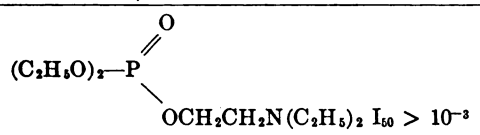
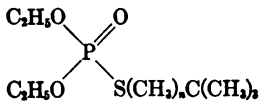
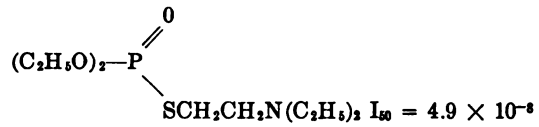


TABLE 3 *Continued*

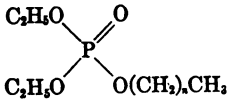


Series X

Series and Number	*n*	Bp/mm	n_D^{20}	I50*	k_a†, $M^{-1} \times min^{-1}$
X.84	2	92–93/0.08	1.4585	1.6×10^{-6}	4.3×10^{4}
X.85	3	105–106/0.1	1.4618	6.4×10^{-7}	1.1×10^{5}
X.86	4	112–113/0.05	1.4561	2.3×10^{-7}	3.0×10^{5}

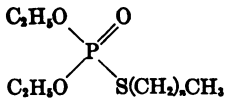


$I_{50} = 4.9 \times 10^{-8}$



Series XI

Series and Number	*n*	Bp/mm	n_D^{20}	I50*	k_a†, $M^{-1} \times min^{-1}$
XI.87	1	100/18	1.4060	$>10^{-2}$	<7.0
XI.88	2	96–97/6	1.4090	4.5×10^{-5}	1.5×10^{3}
XI.89	3	81–82/1.0	1.4124	3.3×10^{-4}	2.1×10^{2}
XI.90	4	89–90/1.0	1.4168	8.5×10^{-6}	8.2×10^{3}
XI.91	5	97–98/1.0	1.4210	2.1×10^{-6}	3.3×10^{4}
XI.92	7	156–157/1.0	1.4256	1.65×10^{-6}	4.2×10^{4}
XI.93	9	125–126/0.05	1.4282	7.7×10^{-7}	9.0×10^{4}



Series XII

Series and Number	*n*	Bp/mm	n_D^{20}	150*	k_a†, $M^{-1} \times min^{-1}$
XII.94	1	127–129/12	1.4604	1.4×10^{-5}	6.2×10^{3}
XII.95	2	135–137/12	1.4602	7.0×10^{-6}	9.9×10^{3}
XII.96	3	85–86/0.1	1.4596	1.0×10^{-5}	7.0×10^{3}
XII.97	4	93–94/0.1	1.4600	2.4×10^{-6}	2.9×10^{4}
XII.98	5	152–153/10	1.4583	7.0×10^{-7}	9.9×10^{4}
XII.99	6	116–117/0.1	1.4577	8.5×10^{-7}	8.2×10^{4}
XII.100	7	134–136/0.1	1.4577	7.3×10^{-7}	9.5×10^{4}
XII.101	8	139–140/0.1	1.4602	7.3×10^{-7}	9.9×10^{4}
XII.102	9	143–145/0.05	1.4617	5.9×10^{-7}	1.1×10^{4}

* In series VII, VIII, IX, and X the values I_{50} were obtained after 10 minutes incubation at 37° and pH 7.4; in series XI and XII, at 25°.

† Here and in the following series (VII to XII) k_a is calculated by Aldridge's equation: $k_a = 0.695/I_{50} \cdot t$.

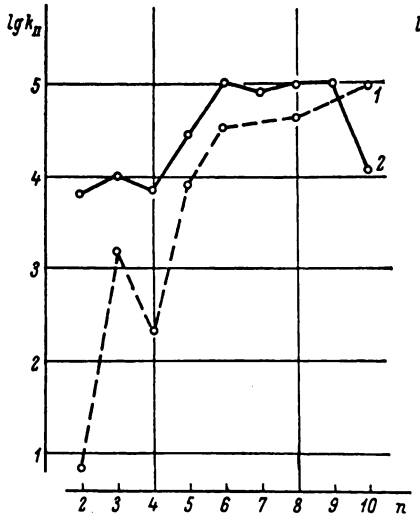


FIG. 4

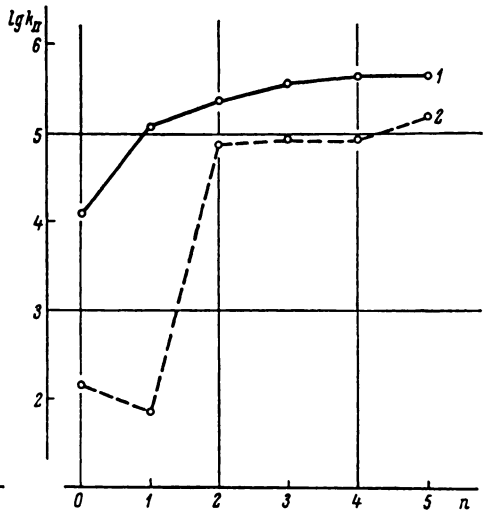
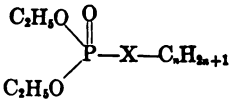


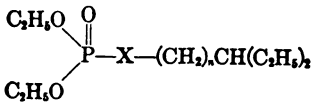
FIG. 5

FIG. 4. The anticholinesterase potency of organophosphorus inhibitors with the general formula



in reactions with acetylcholinesterase. 1. X = O; 2. X = A (26).

FIG. 5. The anticholinesterase potency of organosphosphorus inhibitors with the general formula



in reactions with acetylcholinesterase. 1. X = S; 2. X = O (25).

and so compared the potency of different phosphates and thiolphosphates. The results obtained with the derivatives of the thiolphosphoric acid as well as some of their physical constants are given in table 3, which is compiled from the data of Bracha and O'Brien (25, 26). It is very interesting to compare these results with the data given in table 1. We can see in table 3 and in figure 4 that in the series of thiolphosphates with normal alkyl groups (compounds XII.94 to XII.102) the lengthening of the chain increases the anticholinesterase potency only up to the compound with a 6-carbon chain (fig. 4). Further lengthening of the chain to C_9 does not influence the inhibitory power, and with the 10-carbon derivative the inhibitory potency is considerably decreased. Also with thiolphosphates having a branched chain (VIII.73 to VIII.78), the lengthening of the unbranched main chain increases the activity only up to the total length of C_6 (fig. 5).

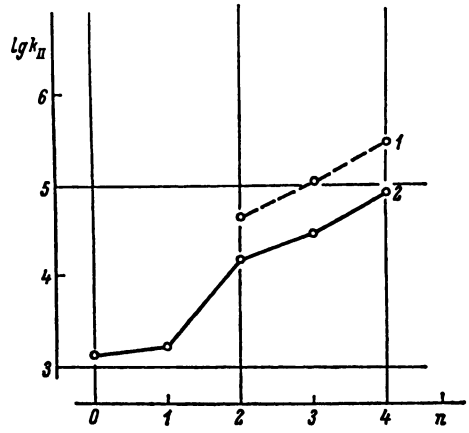
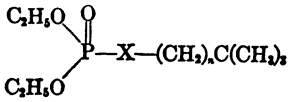


FIG. 6. The anticholinesterase potency of organophosphorus inhibitor with the general formula



n reactions with acetylcholinesterase. 1. X = S; 2. X = O (26).

The short series (X) of thiolphosphates with a tertiary butyl group shows that the anticholinesterase potency increases with the removal of the tertiary butyl group from the phosphorus atom (fig. 6). Unfortunately this series did not extend beyond the compound with 6-carbon total chain.

Very interesting results were obtained by Bracha and O'Brien with the series VII, IX and XI, that is with trialkylphosphates. In the three series the phosphates with shorter alkyl radicals are considerably less potent than the corresponding thiolphosphates (table 3; figs. 4 to 6). With the increase in the length of the alkyl chain this difference becomes much less pronounced, with the phosphates becoming only a little less potent than the corresponding thiolphosphates and the shape of both curves becoming very similar.

Bracha and O'Brien explained this phenomenon in the following way. With small alkyl radicals hydrophobic sorption is not great and the phosphates are far less potent than the corresponding thiolphosphates because the phosphorylating ability of phosphates is much lower. With longer alkyl radicals the equilibrium constant ($k_1 : k_{-1}$) makes a significant contribution to the total potency. With longer radicals this equilibrium constant increases and the phosphates nearly equal the thiolphosphates.

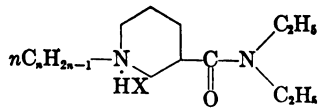
All these results led Bracha and O'Brien (23–26) to the conclusion that on the active surface of acetylcholinesterase there are hydrophobic areas of limited size which can accommodate only a certain number of methylenes, and that the hydrocarbon radicals of inhibitors are sorbed on these areas.

Bracha and O'Brien concluded that the hydrophobic area responsible for the reaction of the enzyme with the phosphates and thiolphosphates is located near

the esteratic site of the enzyme (26), whereas we believe that it is in the vicinity of the anionic site of the enzyme. In both series the maximal potency is reached with the main chain containing 6 carbon atoms. It indicates that both the phosphates and the thiolphosphates are sorbed on the same hydrophobic areas. A quite different relationship between the potency and the length and degree of branching of the alkyl chain is observed when these alkyl radicals are sorbed near the esteratic site of the enzyme. Therefore it seems more likely that the hydrocarbon groups of Bracha and O'Brien's compounds were sorbed on the hydrophobic areas in the vicinity of the anionic site.

It must be noted also that with the thiolphosphates investigated by Bracha and O'Brien (25, 26) the maximal activity in reactions with acetylcholinesterase was observed with C_6 whereas with the thiolphosphonates investigated by the reviewers the potency increased up to C_8 (76). This difference may be connected with different structures of the phosphorylating part of the organophosphorus inhibitors.

Interesting results indicating the presence of a hydrophobic area beyond the anionic site of butyrylcholinesterase were recently published by Purcell and Beasley (72). They investigated the inhibition of hydrolysis of acetylcholine by butyrylcholinesterase under the influence of a series of N'-alkylnipecotamide homologues with general formula:



The constant K_i, that is, the dissociation constant of the enzyme-inhibitor complex, was used to evaluate the potency of these reversible inhibitors. It was established that the inhibitory power continuously increased (the K_i values decreased) with the increase of the length of the alkyl radical from C_4 to C_{12}. From C_4 to C_9 this increase was steeper, and with further lengthening (from C_{10} to C_{12}) less steep (fig. 7). The authors calculated the free energy of binding of one methylene group to be 0.39 kcal/mol for the series C_4 to C_9, and 0.13 kcal/mole for the series C_{10} to C_{12}. The value 0.39 kcal/mole is characteristic for the interaction of one methylene group with hydrocarbon chains in aqueous medium.

There is a striking similarity in the relationship between the length of the alkyl radical and the values of pK_i of the derivatives of N'-alkylnipecotomide (fig. 7), on the one hand, and the value of log k_a for the series of methylsulfomethylates of O-ethyl-S-(β-alkylmercaptoethyl)-methylthiophosphonates (organophosphorus inhibitor series III, fig. 3, butyrylcholinesterase), on the other hand. In contrast to the organophosphorus inhibitor of the series I, II, VII, VIII, IX, X, XI, and XII, in the case of series III no definite maximum is observed when the potency is plotted against the length of the alkyl chain. Probably the interaction of the onium (ammonium or sulfonium) group of the organophosphorus inhibitor with the anionic site of the enzyme can induce some conformational change that modifies the length of the hydrophobic area.

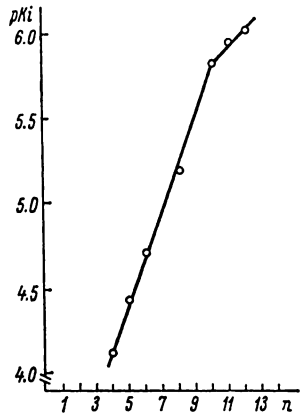
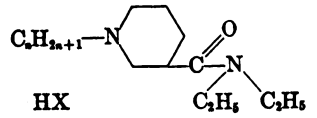


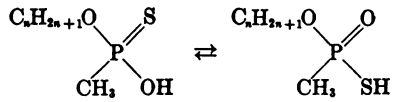
FIG. 7. The anticholinesterase potency of the series of N'-alkylnipecotamide in reactions with butyrylcholinesterase (72).



IV. HYDROPHOBIC AREAS IN THE REGION OF THE ESTERATIC SITE

To explore the hydrophobic areas in the region of the esteratic site three series of organophosphorus inhibitors have been used (IV, V and VI, see table 1) in which the influence of the structure of the hydrocarbon groups in the phosphorylating part of the molecule on the inhibitory power was followed.

All the members of series IV (IV.37 to IV.45) have the same phosphorylating activity (2, 5, 77), as borne out by the equal pK_a values of the corresponding acids that can exist in thion and thiol forms



The values of the pK_a's for these acids (in 60% alcohol) proved to be constant from the propyl derivative to the higher members of the series (for the thion form, 3.96 to 4.01; for the thiol form, 3.43 to 3.59) (5). The rate constants of the aqueous-alkaline hydrolysis of substances I.3, IV.38, IV.39, and IV.41 are also equal.

The anticholinesterase potencies of these compounds are given in figure 8 (curves 3 and 4). It is seen that the inhibitory power depends on the length of the O-alkyl carbon chain. In the case of butyrylcholinesterase (curve 3), gradual lengthening of the chain up to the heptyl group leads to a marked increase of the rate constants of inhibition. The heptyl derivative turns out to be a very potent inhibitor of butyrylcholinesterase, 18,000 times more potent than the methyl derivative. Further lengthening of the chain leads to some lowering of the k_a value.

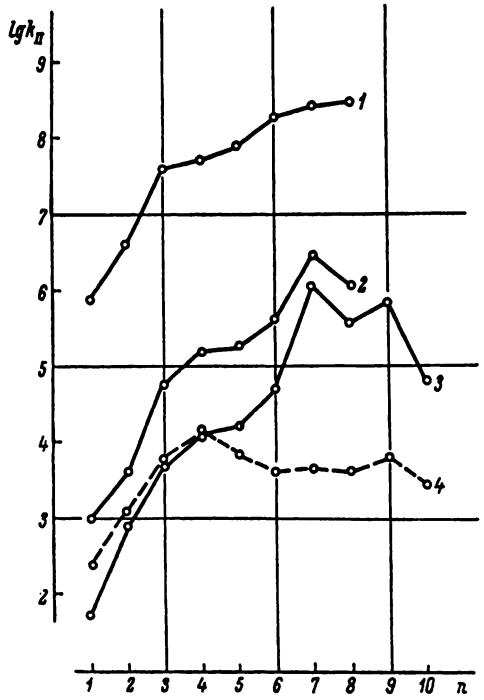
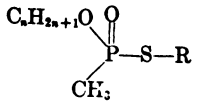


FIG. 8. The anticholinesterase potency of organophosphorus inhibitor with the general formula



1. R = C_2H_4—$\overset{+}{S}$(CH_3)—C_2H_5 (butyrylcholinesterase) 2. R = C_2H_4—S—C_4H_9 (butyrylcholinesterase) 3. R = nC_4H_9 (butyrylcholinesterase) 4. R = nC_4H_9 (acetylcholinesterase) (2, 6).

The interaction of this series of organophosphorus inhibitors with acetylcholinesterase (curve 4) has a quite different character. The lengthening of the chain of the O-alkyl group increases the anticholinesterase potency only up to the butyl derivative (which is 50 times more potent than the methyl one). The potency of organophosphorus inhibitors containing longer carbon chains (from pentyl to decyl) is somewhat lower and varies within narrow limits.

The changes in the anticholinesterase activity is essentially due (as in the previous series) to the changes in the ability of the organophosphorus inhibitors to form an enzyme-inhibitor complex by means of the sorption of the hydrocarbon radicals of the alkoxyl groups on the hydrophobic areas of the enzyme surface.

It was necessary to check whether the alkoxyl group is actually sorbed in the region of the esteratic site and not near the anionic site. Therefore two series of organophosphorus inhibitor were studied: series V and series VI (6). The reaction

of cholinesterase with organophosphorus inhibitors of series VI containing a sulfonium sulfur atom must involve the interaction of the onium group with the anionic site. In this case the alkoxyl group cannot be sorbed onto the hydrophobic areas in the region of the anionic site.

The results obtained with series V and VI are given in figure 8 (curves 1 and 2). It can be seen that with the lengthening of the carbon chain of the alkoxyl group in series V (curve 2) a considerable increase in the k_a values is observed. The maximal k_a value is reached with the heptyl derivative (2900 times higher than with the methyl derivative). With the octyl derivative some lowering of the k_a value is observed. In series VI (curve 1) an increase of the anticholinesterase effect with the lengthening of the alkoxyl-chain is also observed. A comparison of these two series (V and VI) shows that the k_a values for all the sulfonium derivatives are 2 to 3 orders of magnitude higher than for their uncharged analogues. In both series the increase of the anticholinesterase activity with the lengthening of the hydrocarbon chain indicates that this chain is sorbed onto a suitable hydrophobic area.

With the compounds of series VI the anionic site of the enzyme must be occupied by the group containing the sulfonium sulfur atom. One may conclude that the hydrophobic area on which the alkoxyl group is sorbed cannot be in close proximity to the anionic site; this area must be located near the esteratic site of the enzyme. The alkoxyl group of the uncharged organophosphorus inhibitor (series V) is probably sorbed onto the same hydrophobic area of the enzyme. This follows from the comparison of all the curves given in figure 8. It can be seen that the curves showing the reactions with butyrylcholinesterase are very similar in detail in the three series: a steep rise with the increase of n from 1 to 3, a bend in the region when $n = 3$ to 5, and a steep rise again when n is increasing up to 7. This coincidence can be understood only if the alkoxy groups of all the three series interact with the same hydrophobic region of butyrylcholinesterase. The total length of this area probably corresponds to the heptyl group. But the bend in the course of the curves may indicate that in reality there are two hydrophobic areas separated by some hydrophilic group.

A considerable difference in the mode of action of the same organophosphorus inhibitor on butyrylcholinesterase and on acetylcholinesterase (curves 3 and 4) can be seen in figure 8. Up to $n = 4$ the inhibition of both enzymes is practically the same. But with further lengthening of the chain the activity increases only with butyrylcholinesterase. This difference can be explained if we assume that in the vicinity of the esteratic site of acetylcholinesterase there is only one hydrophobic area that can accommodate only a butyl radical.

The study of the compounds containing a branched radical in the alkoxyl group (IV.46 to IV.51) gives some information about the special configuration of hydrophobic areas located in the vicinity of the esteratic site of cholinesterases (1).

There is every reason to suppose that the hydrocarbon radicals of the isoalkyl and of the normal alkyl derivatives interact with the same parts of the enzyme surface. Therefore it is of interest to compare the action of both series,

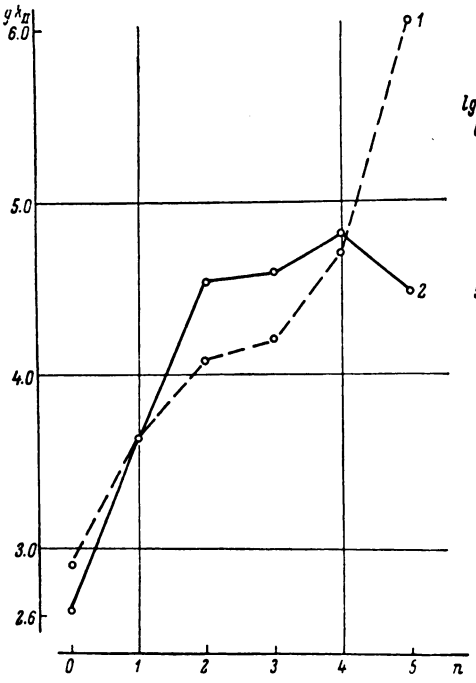


FIG. 9

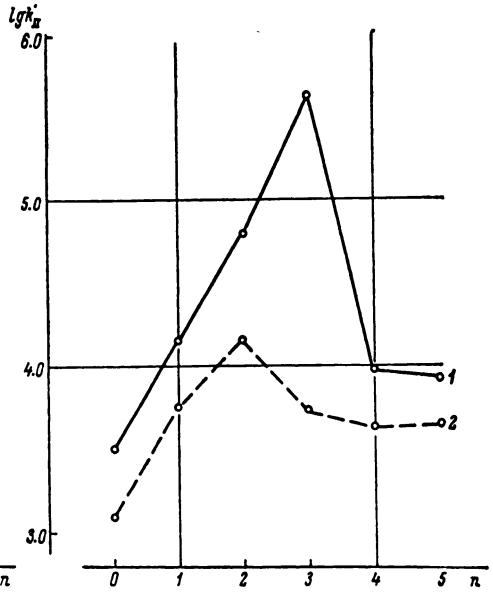
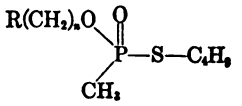


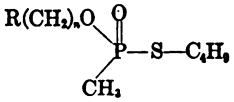
FIG. 10

FIG. 9. The anticholinesterase potency of organophosphorus inhibitor with the general formula



in reactions with butyrylcholinesterase. 1. R = C_2H_5; 2. R = $(CH_3)_2CH$ (1).

FIG. 10. The anticholinesterase potency of organophosphorus inhibitor with the general formula

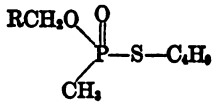


in reactions with acetylcholinesterase. 1. R = $(CH_3)_2CH$; 2. R = C_2H_5 (1).

as in figures 9 and 10. In these, the isoalkyl derivatives are represented by compounds having an isopropyl group at different distances from the phosphorus atom, and the organophosphorus inhibitors with a normal radical are represented as compounds having an ethyl group at the same distance. Thus at each point the compounds that are compared have the same length of the straight-chain part of the hydrocarbon chain. With butyrylcholinesterase (fig. 9) it can be noticed that with $n = 0$ and 1 the branching of the radical is without effect. With $n = 2$ and 3, *i.e.*, in the region when the curve is bending, the iso-

TABLE 4

Anticholinesterase potency of organophosphorus inhibitors of the series



The k_a value for the compound with R—CH_3 is taken as 1 (1).

Series and Number	R	k_a	
		BuChE*	AChE
I.3	CH_3	1	1
IV.38	CH_3CH_2	9	5
IV.47	$(CH_3)_2CH$	8	13
IV.52	$(CH_3)_3C$	2	5
IV.39	C_4H_9	30	6

* The abbreviations used are: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase.

compounds are somewhat more effective. With further lengthening of the chain the iso-group not only does not enhance the interaction with the enzyme but greatly interferes with this interaction. For instance with $n = 5$ the k_a value for the iso-compound is 35 times lower than that of the normal alkyl derivative.

With acetylcholinesterase (fig. 10) the branching of the radical causes a marked effect when $n = 3$. The k_a value for this iso-compound is 60 times greater than that of the corresponding organophosphorus inhibitor with a normal radical. Further separation of the isopropyl group from the phosphorus atom hinders the interaction with acetylcholinesterase: the k_a value diminishes by a factor of 45 and approaches the k_a values for the normal series.

In both series the anticholinesterase potency does not change with further lengthening of the chain.

The above data confirm and amplify the suppositions regarding the hydrophobic areas in the vicinity of the esteratic site of cholinesterases. It seems true that in butyrylcholinesterase this area consists of two hydrophobic parts. The two are complementary to the normal radicals, but the first part, nearer to the esteratic site, is not so strictly limited, and therefore branching of the hydrocarbon chain does not interfere with its sorption. The second, more distant, part of the hydrophobic area is suitable for the sorption only of straight-chain radicals.

In acetylcholinesterase there is probably only one hydrophobic area. Its structure differs considerably from the structure of the first part of the similar area in butyrylcholinesterase and is strictly complementary to the isohexyl radical.

It has been shown above that the introduction of a tertiary butyl group in the thioalkyl radical of an organophosphorus inhibitor increases its anticholines-

terase potency. An organophosphorus inhibitor containing a tertiary butyl group in the alkoxyl part of the molecule has also been studied (table 1, IV.52; table 2) and the effect of gradual formation of the group was followed (1). Table 4 shows that the structure of the hydrophobic area near the esteratic site of butyrylcholinesterase is complementary to an unbranched chain. Indeed the replacement of one hydrogen atom by a methyl group (*i.e.*, the lengthening of the normal chain) increases the activity 9-fold (compounds I.3 and IV.38). The replacement of the second hydrogen by a methyl group (compound IV.47) has no effect. The introduction of the third methyl group produces a net decrease in potency (compound IV.52). At the same time its isomer of normal structure (IV.39) turned out to be 15 times more active than the compound with a tertiary butyl group.

In the case of acetylcholinesterase, compound IV.47 proved to be the most effective and the introduction of the third methyl group lowered the activity 2.5-fold. The isomeric compound with a normal butyl radical has no advantage compared with the tertiary butyl derivative. Thus the hydrophobic area of acetylcholinesterase, in contrast to that of butyrylcholinesterase, is most complementary to the isobutyl radical in this series of organophosphorus inhibitors.

We can conclude from this comparison that the hydrophobic areas in the vicinity of the esteratic site of both acetylcholinesterase and butrylcholinesterase do not correspond to the tetrahedral structure of the tertiary butyl group. Therefore the introduction of such a group in the alkoxyl radical of this series of organophosphorus inhibitors interferes with their sorption on the active surface of the enzyme. This is one of the main features of the hydrophobic areas in the vicinity of the esteratic site. As shown above, the hydrophobic areas in the vicinity of the anionic site of cholinesterases are, by contrast, adapted to the accommodation of the tertiary butyl group. The organophosphorus inhibitors containing such a group in the leaving part of the molecule possess a high anticholinesterase potency.

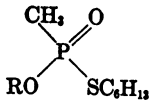
V. THE POSSIBILITY OF SUMMATION OF THE EFFECTS OF HYDROPHOBIC SORPTION IN THE REGIONS OF THE ANIONIC AND ESTERATIC SITES

It has been shown above that in the series of O-ethyl-S-alkyl-methylthiophosphonates (series I, table 1), among the derivatives containing an unbranched thioalkyl radical the compound with an —S-*n*-hexyl group (I.5) proved to be the most potent; in the series of O-alkyl-S-*n*-butylmethylthiophosphonates (series IV, table 1), the most potent compound was the O-heptyl derivative (IV.42). These data and others led to the conclusion that the hydrophobic patch near the anionic site of butyrylcholinesterase can accommodate only an *n*-hexyl radical, and the hydrophobic area located in the vicinity of the esteratic site is complementary to the *n*-heptyl group. It seemed natural to expect that a compound containing groups complementary to both hydrophobic areas must be the most potent; that is, a summation of the effects of the hydrophobic sorption would be observed.

Unexpectedly, such a compound, that is, O-heptyl-S-hexyl-methylthiophos-

TABLE 5

Structure, physical constants, and anticholinesterase potency of the XIII series of organosphophorus inhibitors (7)



Series and Number	R	Bp/mm	n_D^{20}	d_4^{20}	k_a, $M^{-1} \times min^{-1}$	
					BuChE*	AChE
XIII.103	CH_3	82/2	1.4750	1.0339	2.1×10^3	6.1×10^3
I.5†	C_2H_5	(105–106)/1	1.4712	1.0178	3.8×10^4	1.6×10^4
XIII.104	*n*-C_3H_7	102/1.5	1.4700	0.9986	1.7×10^5	3.1×10^4
XIII.105	*n*-C_4H_9	120/2	1.4699	0.9872	1.4×10^5	5.8×10^4
XIII.106	*n*-C_5H_{11}	$97/3 \times 10^{-2}$	1.4701	0.9781	6.3×10^4	3.8×10^3
XIII.107	*n*-C_6H_{13}	$(84–85)/1 \times 10^{-2}$	1.4694	0.9702	6.0×10^4	7.6×10^3
XIII.108	*n*-C_7H_{15}	$(105–106)/1 \times 10^{-2}$	1.4700	0.9621	2.7×10^5	5.6×10^3

* The abbreviations used are: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase.

† See table 1.

phonate, proved to be less potent than its S-butyl homologue. This paradox was elucidated by the study of the whole series of O-*n*-alkyl-S-*n*-hexylmethylphosphonates (7). The results are given in table 5. In figure 11 this series is compared with organophosphorus inhibitors containing a thiobutyl group in the leaving part. Figure 11a shows that with butyrylcholinesterase the S-hexyl derivatives are more potent than the S-butyl compounds only in the case of comparatively short O-alkyl chains (up to C_3H_7). With further lengthening of the O-alkyl chain this difference diminishes: two O-hexyl compounds already have equal k_a values, and the O-heptyl-S-hexylthiophosphonate proved to be even less potent than its S-butyl homologue.

The following explanation is suggested. The thioalkyl group is sorbed at the hydrophobic area located near the anionic site of the enzyme (area A_2 in fig. 13), which can accommodate an *n*-hexyl group. In the region of the esteratic site there are probably two hydrophobic patches separated by some hydrophilic group (areas E_1 and E_2 in fig. 13). One may suggest that the —S-hexyl derivatives are more firmly fixed at the hydrophobic area near the anionic site (A_2) and that in such "strict" conditions the O-alkyl group can be accommodated only on the first, more proximate hydrophobic patch near the esteratic site (E_1) and cannot reach the more distant patch (E_2). Therefore the S-hexyl derivatives containing a long O-alkyl group have no advantage over the corresponding S-butyl derivatives. This supposition does not contradict the results obtained with acetylcholinesterase (fig. 11b). Acetylcholinesterase probably possesses only one hydrophobic patch, near its esteratic site (E), corresponding to the first hydrophobic patch in the region of the esteratic site of butyrylcholinesterase (E_1). The firm

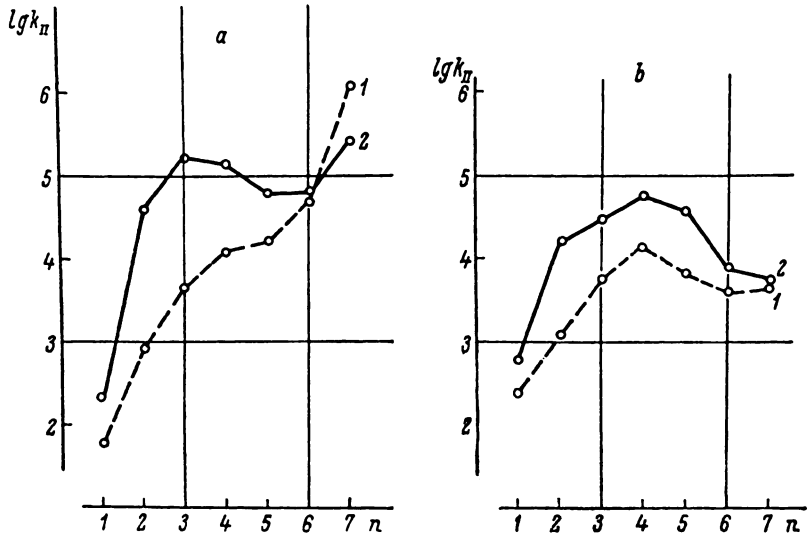
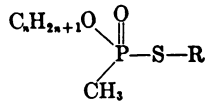


FIG. 11. The anticholinesterase potency of organophosphorus inhibitors with the general formula



in reactions with butyrylcholinesterase (a) and with acetylcholinesterase (b). 1. R = nC_4H_9; 2. R = nC_6H_{13} (7).

fixation of the S-hexyl group near the anionic site does not interfere with the sorption of the O-alkyl radical near the esteratic site. Therefore all the S-hexyl derivatives turn out to be more potent than the S-butyl ones. In both these series the O-butyl compound is the most potent.

VI. THE INFLUENCE OF IONIC STRENGTH AND THE EFFECT OF ORGANIC SOLVENTS ON THE HYDROPHOBIC INTERACTION BETWEEN ORGANOPHOSPHORUS INHIBITORS AND CHOLINESTERASES

Such factors as change in the ionic strength of the medium or the addition of organic solvents are known to influence hydrophobic interactions strongly.

The study of the effect of ionic strength (27, 28, 64) has shown that the rate constants of the reaction of charged organophosphorus inhibitors with cholinesterase decrease with increasing concentration of salts (NaCl, KCl). Sometimes this decrease is very pronounced. For example, in the case of butyrylcholinesterase the k_a value for compound VI.60 (table 1) in the presence of 0.2 M KCl was 15 times lower than in the solution that contained no salts. With the uncharged organophosphorus inhibitor a gradual increase in the salt concentration produced first a slight decrease in the k_a values and then increased them. This

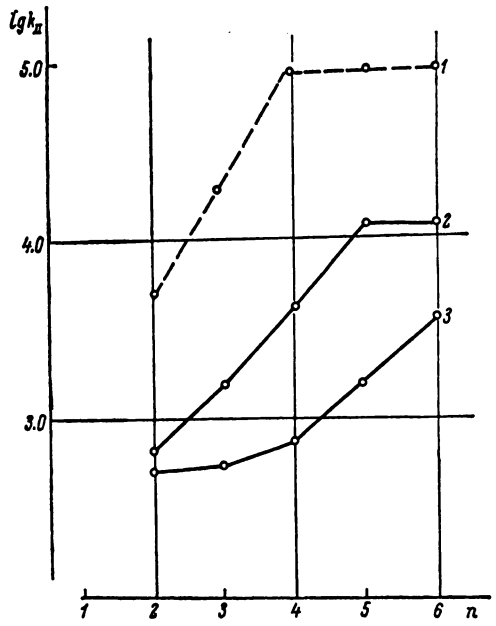
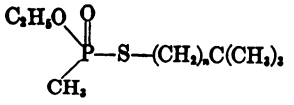


FIG. 12. The anticholinesterase potency of organophosphorus inhibitor with the general formula



n reactions with butyrylcholinesterase in the absence of isobutyl alcohol (1) and in the presence of 0.216 M (2) and 0.54 M (3) of isobutyl alcohol (33).

increase was more pronounced with compounds containing large hydrocarbon radicals, for example, with compound V.58 (table 1), containing a heptyl group.

The influence of pH on the inhibitory power of organophosphorus inhibitor in solutions of high ionic strength proves to be very interesting. With charged organophosphorus inhibitor an increase in ionic strength diminishes the anticholinesterase activity in the interval of pH from 6 to 10. However, at pH 5.5, when the dissociation of the anionic group is suppressed and the sorption of charged organophosphorus inhibitor is due mainly to the hydrophobic interactions, the increase in the ionic strength of the solution increases the k_a values. With uncharged organophosphorus inhibitors the increase in the ionic force led to an increase of the anticholinesterase potency at all the pH values, including pH 5.5. The results obtained are fully consistent with the theory of the effect of ionic strength on coulombic and hydrophobic interations (87).

The study of the action of alcohols (33, 62, 75) is of great interest because the sorption of alcohol molecules on the hydrophobic areas should interfere with the hydrophobic interaction of the enzyme with organophosphorus inhibitors con-

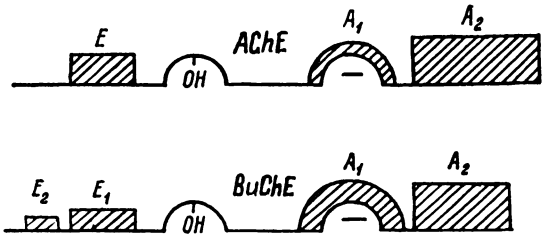


FIG. 13. The scheme of the disposition of the hydrophobic areas on the active surface of acetylcholinesterase and butyrylcholinesterase.

taining large alkyl groups and reduce the rate constants of inhibition. Indeed in reactions with butyrylcholinesterases the k_a value for the organophosphorus inhibitor I.13, containing a 5,5-dimethyl-hexyl radical, was reduced 100 times by the presence of 0.54 M isobutyl alcohol. At the same time with a poorly hydrophobic compound containing an ethyl radical (I.I) the lowering of k_a was only to one third.

These investigations suggested that the alcohols not only hinder the hydrophobic interaction but may also alter the structure of the hydrophobic areas themselves. This assumption is based on the results obtained with butyrylcholinesterase and organophosphorus inhibitors of the first series (I.11 to I.15) (see fig. 12). In the absence of alcohol the k_a values increase with increasing length of the hydrocarbon radical up to $n = 4$; in the presence of 0.2 M isobutanol, up to $n = 5$; and in the presence of 0.54 M isobutanol, up to $n = 6$. Thus alcohol produces the same effect as an increase in the length of the hydrophobic area. The influence of alcohols on the reaction of butyrylcholinesterase with series IV organophosphorus inhibitors (I.38 to IV.45), in which hydrocarbon chains are sorbed onto the hydrophobic areas near the esteratic site, did not reveal any changes in the structure of these hydrophobic regions under the influence of alcohols, though there was a distinct lowering of the k_a values.

VII. CONCLUSIONS

A. The "topography" of the hydrophobic areas

The analysis of all the results described above allows us to form a general idea about the localization of the hydrophobic areas on the active surface of butyrylcholinesterase and acetylcholinesterase and about some of the peculiarities of these areas (6, 31, 52). A diagram of the localization of the hydrophobic areas on the active surface of cholinesterases is given in figure 13. In the region of the anionic site there are two hydrophobic areas: A_1 directly surrounding the anionic group and A_2 located at some distance from the anionic group, "behind" it, outside of the true anionic site. Probably area A_1 plays a more important role in butyrylcholinesterase than in acetylcholinesterase. Area A_2 is not equal in the different enzymes: in butyrylcholinesterase its length corresponds to 6-carbon chain and in acetylcholinesterase it can accommodate an 8-carbon chain. The spatial configuration of this area makes it complementary to the radicals con-

taining the highly branched tertiary butyl group separated by 4 to 6 methylene groups from the thiol sulfur atom.

The scheme supposes that in the vicinity of the esteratic site of butyrylcholinesterase there are two hydrophobic areas (E_1 and E_2) separated by a hydrophilic group. The total length of this hydrophobic area corresponds to a 7-carbon chain. Both parts of this hydrophobic area (E_1 and E_2) seem to be complementary to the radicals of straight-chain structure, but the first part (A_1) is not so strictly limited and branched hydrocarbon radicals can also be sorbed onto it.

In the vicinity of the esteratic site of acetylcholinesterase there is probably a single hydrophobic area whose structure differs considerably from that of the area E_1 in butyrylcholinesterase: the area in acetylcholinesterase is strictly complementary to the isohexyl radical. Neither the area E_1 in butyrylcholinesterase nor the area E in acetylcholinesterase is suitable for the sorption of radicals containing a tertiary butyl group. Thus marked peculiarities in the structure and length of different hydrophobic patches on the active surface of butyrylcholinesterase and acetylcholinesterase have been established. It seems very probable that these peculiarities are responsible for the different properties of the two cholinesterases.

B. The possible biological significance of the hydrophobic areas on the active surface of cholinesterases and cholinoreceptors

It is natural to ask what is the biological purpose of the structure described above on the active surface of cholinesterase. It is relatively easy to explain the purpose of the hydrophobic area E_1 immediately surrounding the anionic group: it is adapted to interaction with the methyl groups attached to the nitrogen atom of acetylcholine. But what is the purpose of the spacious hydrophobic regions A_2 and E (E_1 and especially E_2), which are situated beyond the catalytic center of the enzyme? It does not seem likely that these hydrophobic areas were formed in the course of evolution only as accidental details of the enzyme structure. The following assumption seems more probable (52). The acetylcholine molecule, containing a charged nitrogen group and a carbonyl group, is very hydrophilic. It can be sorbed not only at the active center but at many other polar groups of the polypeptide chains of the enzyme. Such a "parasitic" sorption would decelerate the interaction of the acetylcholine molecule with the active center. The extensive hydrophobic areas (A_2, E_1, and E_2) interfere with such a parasitic sorption. The hydrophilic acetylcholine molecules are "thrown out" of these hydrophobic areas and the probability of their collision with the active center of the enzyme increases. One could say that these hydrophobic regions constitute energetic "hills" from which the acetylcholine molecule is rolled down to the active center. Thus the "effective section" of the enzyme is actually enlarged, and this increases the rate of hydrolysis of acetylcholine.

In this connection another problem, that of the structure of the active center of the cholinoreceptor, must be touched upon. There are serious reasons to suppose that hydrophobic areas are present around the active center of the cholinoreceptor (53, 66). The existence of a hydrophobic area adapted to the interaction

with the methyl groups of the cationic head of the acetylcholine molecule was recently postulated by Barlow (14). This area directly surrounding the anionic group of the receptor is probably similar to area E_1 in cholinesterase. In the region of the so called "esterophilic site" (53) of the receptor a small hydrophobic area adapted to the interaction with the methyl group attached to the carbonyl carbon of acetylcholine also has been postulated (14). Belleau assigned an important role to hydrophobic interactions in the reactions of acetylcholine with the active centers of both cholinesterases and cholinoreceptors (17, 18).

But, in addition, some other, more spacious hydrophobic areas must also exist close to the active center of the receptor. This idea is supported by the enormous amount of experimental data that has accumulated in the field of structure-activity relationships for the cholinolytic agents. Long ago Bovet and Bovet-Nitti (22) formulated the empirical rule that increasing the molecular weight of the acetylcholine molecule by attaching hydrocarbon groups to its acidic and alcoholic ends converts it into a cholinolytic agent. (They called this process "allourdissement" of the acetylcholine molecule.) Actually it is well known that the introduction of hydrophobic radicals, such as phenyl, cyclohexyl, or large aliphatic radicals, in the acidic part of the acetylcholine molecule gives potent blocking drugs. With gradual "allourdissement" of the acetylcholine molecule its cholinomimetic activity gradually diminishes and the cholinolytic properties become more and more pronounced until a certain limit is reached. The replacement of methyl groups attached to the nitrogen atom by ethyl groups has a similar effect (14, 50, 66). This effect seems quite comprehensible. For the cholinomimetic action not only rapid sorption, but also rapid desorption of the drug from the receptor is needed. The deceleration of the desorption leads to a blocking effect (71). The introduction of heavy hydrophobic groups in the acetylcholine molecule enhances the interaction with the hydrophobic areas situated outside the active center of the receptor and hinders the desorption.

It is natural to suppose that in the case of the action of acetylcholine itself the biological function of such hydrophobic areas in the vicinity of the active center of the receptor is the same as with cholinesterase: it enlarges the "effective area" of the active center of the receptor and increases the probability of an effective collision of the acetylcholine molecule with the receptor surface.

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