A Comparison of Acylation, Phosphorylation and Binding in Related Substrates and Inhibitors of Serum Cholinesterase

BY A. R. MAIN AND F. L. HASTINGS

*Departments of Biochemistry and Entomology,
North Carolina State University, Raleigh, N.C. 27607, U.S.A.*

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1. The K_m and catalytic-centre activities for human serum cholinesterase and methyl, ethyl, *n*-propyl and *n*-butyl butyrate substrates were determined and compared with the related inhibition constants of a similarly substituted organophosphate inhibitor series based on malaoxon. The results indicated that the catalytic-centre activities approximated to $k_{+2(a)}$, the acylation rate constant, and that K_m approximated to the equilibrium binding constant. The inhibition constants measured were K_a , the equilibrium binding constant, and $k_{+2(p)}$, the phosphorylation rate constant. 2. The effects of the alkyl substituents on $k_{+2(p)}$ and $k_{+2(a)}$ were closely parallel, and the decreasing order in each case was: *n*-butyl; methyl; *n*-propyl; ethyl. The Taft constants did not follow this order, suggesting that alkyl substituents did not primarily effect acylation or phosphorylation by electron induction. 3. For comparable homologues, the $k_{+2(a)}$ values were on average 435 times the $k_{+2(p)}$ values. The $k_{+2(p)}$ values at 25° and pH 7.6 ranged from 6.6 min.⁻¹ for the diethyl member to 22.6 min.⁻¹ for the di-*n*-butyl member. 4. The effect of the alkyl substituents on K_a and K_m were closely paralleled. The increasing order in each case was: *n*-butyl; *n*-propyl; ethyl; methyl. The K_a values were about 100 times less than the comparable K_m values. 5. Consideration of the binding energies suggested that only one of the two alkyl groups on the malaoxon homologues bound to the active site. 6. The possibility that malaoxon acted as a substrate as well as an inhibitor for cholinesterase was also investigated, but no evidence of a substrate reaction was found.

The substrate reaction of cholinesterases and their inhibition by organophosphates are considered to occur by analogous mechanisms (Aldridge, 1954; Wilson, 1960). The following equation can therefore be used to describe both reactions:

AB is either a carboxylic ester substrate or an organophosphate inhibitor, EAB is a reversible complex and EA is either the acylated or phosphorylated active site. The individual rate constants are designated by k_{+1} , k_{-1} etc. The inhibitor is then a poor substrate for which k_{+3} is relatively small, and in this case the esterase is converted largely into the inactive phosphorylated product, EA.

Equations have been derived for irreversible inhibition on the assumption that $k_{+3} = 0$ (Wilson, 1960; Main, 1964). An experimental procedure

based on these equations has been described by which the affinity constant K_a , i.e. k_{-1}/k_{+1} , and the phosphorylation rate constant $k_{+2(p)}$ were determined for the reaction of DFP* with acetylcholinesterase and serum cholinesterase (Main & Iverson, 1966). Final evaluation of $k_{+2(p)}$ and K_a depended on the linear form of the inhibition equation:

$$i\Delta t/2.3\Delta \log v = i/k_{+2(p)} + 1/k_i \quad (2)$$

where i is the inhibitor concentration, $2.3\Delta \log v/\Delta t$ is the first-order rate constant for a particular value of i and k_i , i.e. $k_{+2(p)}/K_a$, is the bimolecular reaction constant.

The results of the DFP-cholinesterase study

* Trivial names and abbreviations: DFP, di-isopropyl phosphorofluoridate; malaoxon, *OO*-dimethyl *S*-(1,2-diethoxycarbonyl ethyl) phosphorothiolate; malathion, *OO*-dimethyl *S*-(1,2-diethoxycarbonyl ethyl) phosphorodithioate.

(Main & Iverson, 1966) provided kinetic evidence for the existence of a reversible complex of significance in the inhibition reaction. Hitherto the reversible-complex hypothesis had depended on more indirect evidence since rate studies had not supported it.

Though the elements in the analogy between the substrate and inhibition reactions appear now to be complete, it remains to be seen how closely the comparison can be made. In addition, it would seem desirable to obtain further evidence to test the interpretation given K_a and $k_{+2(p)}$ in the DFP-cholinesterase study.

Two aspects of the substrate and inhibition reaction were compared. One was binding as reflected by K_m , the Michaelis constant of the substrate reaction, and by K_a , the affinity constant of inhibition. The other was a comparison of the acylation rates as measured by the catalytic-centre activities (Dixon & Webb, 1964) of the substrates and by the $k_{+2(p)}$ of the inhibitors.

Adams & Whittaker (1949) examined the specificity of human serum cholinesterase towards a series of alkyl butyrates. Different substituents significantly affected substrate velocity. It therefore seemed relevant to the purposes of the present study to see whether comparable substitution had a similar effect on the inhibition reaction. A series based on the inhibitor malaoxon appeared to be suitable for such a comparison since substitution could be made into the alkyl positions of the dicarboxylic esters on the leaving group of this compound. The relationship between the substrate and the inhibitor series is illustrated below:

R was methyl, ethyl, *n*-propyl or *n*-butyl in both series. The inhibitor series also included an isopropyl member.

If K_m and K_a were similarly affected by the alkyl substituents, this would support the validity of K_a as a measure of affinity and would strengthen the analogy between the substrate and inhibition reactions that Aldridge (1954) made on the basis of similar structural requirements.

The effect of the alkyl substituents on phosphorylation was examined from two points of view. The first, by comparison with the catalytic-centre activities, has been mentioned. The second involved determination of the pK values of the acid thiomalate esters that are a product of the phosphorylation reaction. The Taft (1956) constants of the alkyl substituents were also considered. In this way

the inductive effect of alkyl substitution on the phosphorylation rate constant could be considered.

In their specificity study, Adams & Whittaker (1949) did not determine the K_m and $V_{\max}$ values of the alkyl butyrates in a single-phase system. The K_m and $V_{\max}$ values of the alkyl butyrates for a single-phase system (the aqueous phase) were accordingly determined in the present work. The K_a and $k_{+2(p)}$ values for the inhibition reaction were determined by the procedure of Main & Iverson (1966).

Cholinesterases do not appear to use malathion, the non-inhibiting P = S analogue of malaoxon, as a substrate (Main & Braid, 1962). The possibility that serum cholinesterase could hydrolyse the carboxylic esters of malaoxon was investigated in the present work, but no evidence for such a reaction was found.

EXPERIMENTAL

Materials

Human serum cholinesterase. A preparation of this enzyme (acetylcholine acyl-hydrolase, EC3.1.1.8) purified about 700-fold and salt-free was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

For the determination of inhibition rates, 10000 activity units (1 unit hydrolysed 0.098 μ mole of acetylcholine/min. at 25° and pH 7.6 with 15 mm-acetylcholine as substrate) were dissolved and made up to 50 ml. with 20 mm-sodium phosphate buffer, pH 7.6, and stored in the cold under a drop of toluene. A further fivefold dilution in 20 mm-sodium phosphate buffer, pH 7.6, was made before use, giving a final concentration of 40 units/ml.

For substrate studies 10000 activity were dissolved in water, adjusted to pH 7.6 on a pH-stat and finally diluted to 50 ml. The number of units used with each substrate was: methyl *n*-butyrate, 100; ethyl *n*-butyrate, 200; *n*-propyl *n*-butyrate, 100; *n*-butyl *n*-butyrate, 100.

Alkoxy carbonyl homologues of malaoxon. The methyl, ethyl, *n*-propyl, isopropyl and *n*-butyl dicarboxylic ester homologues of malaoxon were prepared and purified by W. C. Dauterman of this Laboratory by the procedure of Ailman (1965). The purity and physical constants of each homologue are given by Dauterman & Main (1966). Each homologue was given a convenient name. Malaoxon, for example, was called ethoxycarbonyl malaoxon, and the *n*-butyl homologue was called *n*-butoxycarbonyl malaoxon.

Stock solutions were prepared by dissolving the appropriate weight of each homologue in 10 ml. of methanol. The concentrations were: methoxycarbonyl malaoxon, 1.00 M; ethoxycarbonyl malaoxon, 0.500 M; *n*-propoxycarbonyl malaoxon, 0.100 M; isopropoxycarbonyl malaoxon, 0.200 M; *n*-butoxycarbonyl malaoxon, 0.100 M. Aqueous solutions were prepared by blowing the appropriate volume of stock solution into the calculated volume of water and shaking to dissolve. The final concentration of methanol did not exceed 0.5% and in most instances was less than 0.2%.

Alkyl butyrates. The methyl, ethyl, *n*-propyl and *n*-butyl butyrate substrates were obtained from Eastman Organic Chemical Co., Rochester, N.Y., U.S.A. Each compound was distilled before use, and the boiling points and refractive

indices of the appropriate fractions were determined and were in agreement with published values.

Aqueous substrate solutions were prepared by weighing out sufficient ester to almost saturate a predetermined volume of water. The ester was dissolved in this volume by shaking vigorously for several minutes. Lower concentrations were prepared by dilution of such solutions.

Methods

Determination of K_m and $k_{+2(p)}$. The procedure of Main & Iverson (1966) was followed. Inhibition rates were measured at pH 7.6 and 25° in 10 mM-sodium phosphate buffer. An inhibition reaction vessel having a side-arm entry port in the main barrel was used. Esterase (0.5 ml. of a solution containing 40 activity units/ml.) was pipetted into the side arm and inhibitor solution (0.5 ml.) into the centre well. A vapour block was not required because of the low malaoxon vapour pressure. The shortest incubation time measured was 1 sec. and the range of times varied from 8 sec. to 30 min., depending on the inhibitor concentrations, which are given in Table 2. The plots of $\log v$ against t of the *n*-propoxycarbonyl, isopropoxycarbonyl and *n*-butoxycarbonyl homologues at the lowest concentrations are illustrated in Fig. 2. Residual substrate activities were measured with 30 ml. of 15 mM-acetylcholine at 25° and pH 7.6 on a Radiometer pH-stat.

Determination of K_m and V_{max} values for the alkyl substrate reaction. Activities were measured with a Radiometer pH-stat equipped with the 75 ml. glass cell jacketed for temperature control described by Main & Iverson (1966). The highest alkyl butyrate concentrations were dictated by the solubility of the ester and the lowest concentrations depended on the lowest velocity that could be accurately measured. Each determination was made with 30 ml. of substrate solution. The acid liberated by hydrolysis was neutralized with 20 mM-NaOH delivered from a 0.5 ml. syringe. The amount of esterase used with each substrate was sufficient to give a velocity of at least 1.5 μ moles/min. at the highest substrate concentration. From nine to 12 different concentrations of each ester were used in the determination of the K_m and V_{max} values. The concentration ranges were as follows: methyl *n*-butyrate, 10–100 mM; ethyl *n*-butyrate, 10–50 mM; propyl *n*-butyrate, 2–12 mM; *n*-butyl *n*-butyrate, 1–5 mM. In each case the K_m values (cf. Table 3) were close to the solubility limit so that valid estimations were possible. Each set of K_m and V_{max} values was determined twice in separate experiments and the values were in reasonable agreement. The values in Table 3 were obtained from the second set of experiments, which were executed with significantly improved precision. K_m and V_{max} and their standard errors were calculated by the method of Wilkinson (1961).

Determination of cholinesterase concentration. The cholinesterase concentration was determined by inhibiting two esterase solutions, one containing 20 and the other 100 units/ml., with various concentrations of DFP. Equal volumes of esterase and DFP solutions were incubated to constant inhibition at 25° and pH 7.6. The incubation times varied from 5 to 30 min. and 30 min. periods were used to obtain the I_{50} plots. When the final esterase concentration was 10 units/ml., the DFP concentrations were 0.5, 5, 10, 12.5, 15, 20 and 50 μ M. When the final esterase concentration was 50 units/ml., the DFP concentrations

were 5, 10, 20, 33, 50, 75, 100 and 150 μ M. At least four different DFP concentrations were in the region of 20–80% inhibition. The cholinesterase concentration/unit of activity calculated from the results with the lower esterase concentration was 2.29 μ M, whereas the higher esterase concentration gave a value of 2.40 μ M. The catalytic-centre activities were calculated by using the average concentration, i.e. 1 unit/ml. = 2.35 μ M.

RESULTS AND DISCUSSION

The inhibitory power of organophosphates is believed to depend on two factors. One is the affinity of the compound for the active site of the esterase. The other is the phosphorylation potential (Aldridge, 1954, 1957). It seems evident that phosphorylation potential would be reflected by $k_{+2(p)}$ whereas affinity would be measured by K_m .

The effect of substituents on the relative phosphorylation potentials of organophosphates has been related to the stability of the organophosphates to alkaline hydrolysis (Aldridge & Davison, 1952), to the Hammett or Taft constants of the substituents (Fukuto & Metcalf, 1956, 1959) and to the pK of the leaving groups (Ketelaar, 1951; Ahmed, Casida & Nichols, 1958).

Good correlations have been observed with series based on ring-substituted diethyl phenyl phosphates (e.g. Aldridge & Davison, 1952), and less precise, but suggestive, correlations have been observed with other series (Ooms, 1961). This has given rise to the hypothesis that phosphorylation potential and therefore inhibitory power depend, at least in part, on the electron density in the region of the phosphorus atom (O'Brien, 1960). An estimate of the electronic effects of alkyl substituents was therefore obtained and compared with the measured phosphorylation rate constants.

The effect of alkyl substituents on electron density is inductive, but with malaoxon the inductive effect may be quenched by the three and four atoms that are interposed between the substituents and the phosphorus atom (Royal, 1954). The quenching effect may, however, be decreased by the double substitution into each homologue. The Taft (1956) substituent constants give a measure of the relative inductive effects exerted by alkyl substituents; they are: methyl, 0; ethyl, -0.10; *n*-propyl, -0.115; *n*-butyl, -0.13; isopropyl, -0.19. They are given in order of decreasing phosphorylation potential as predicted by the 'electronic' hypothesis.

To obtain some estimate of the quenching effect on the one hand and of double substitution on the other, the pK values of the dialkyl thiomalate leaving groups actually involved were determined and the results are given in Table 1. They suggest that the substituents would affect the electron density to a small, but significant, degree. When

the pK value of dimethyl thiomalate was used as the zero reference, the ΔpK values indicated the same relationship with respect to methyl, ethyl and isopropyl as the Taft constants, but the order was not parallel with respect to ethyl, n -propyl and n -butyl, nor did the order of ΔpK values determined in water correspond precisely to that observed in 50% (v/v) ethanol. These disagreements are probably due to the relatively low accuracy of the pK determinations. The differences between the pK values of the ethyl, n -propyl and n -butyl compounds are in any case small. The order suggested by the Taft constants is probably correct.

Both the Taft constants and pK values suggest that the methoxycarbonyl homologue would have the greatest phosphorylation potential and the

isopropoxycarbonyl homologue the least. Only small differences would be expected in the phosphorylation potentials of the ethoxycarbonyl, n -propoxycarbonyl and n -butoxycarbonyl homologues.

The $k_{+2(p)}$ values of the alkoxy carbonyl homologues together with the $i\Delta t/2.3\Delta \log v$ values and the corresponding inhibitor concentrations are given in Table 2.

The $k_{+2(p)}$ values varied from 2.6 min.^{-1} for the isopropoxycarbonyl homologue to 22.6 min.^{-1} for the n -butoxycarbonyl homologue. The decreasing order was: n -butyl; methyl; n -propyl; ethyl; isopropyl. This order did not correspond to the order of the Taft substituent constants or of the measured pK values. The relationship between the Taft constants and the $k_{+2(p)}$ values therefore was not the one expected by the electronic hypothesis.

The catalytic-centre activities of the alkyl butyrate substrates are given in Table 3. If k_{+3} were rate-controlling, then the catalytic-centre activities of all the alkyl butyrates should be the same since the rate-controlling reaction would be de-butyrylation in each case. However, the catalytic-centre activities varied almost ten-fold overall. This suggested that $k_{+2(a)}$, the acylation rate constant, was rate-controlling. The effect of the substituents on the $k_{+2(a)}$ values, where $k_{+2(a)}$ is assumed to equal the catalytic-centre activity, in decreasing order was: n -butyl; methyl; n -propyl; ethyl. This order was identical with that of the relevant $k_{+2(p)}$ values.

Table 1. pK values of the acid thiomalate dialkyl esters at 25°

The values in water were determined with a Radiometer pH-stat; those in 50% (v/v) ethanol were obtained by manual titration with a pH-meter.

Alkyl substituent	pK	
	In water	In 50% ethanol
Methyl	6.68 ± 0.10	7.81 ± 0.03
Ethyl	6.96 ± 0.01	8.0 ± 0.02
n -Propyl	6.75 ± 0.06	7.96 ± 0.0
Isopropyl	6.97 ± 0.05	8.31 ± 0.09
n -Butyl	6.87 ± 0.04	8.0 ± 0.0

Table 2. Affinity constants K_a and phosphorylation constants $k_{(p)}$ for the inhibition of serum cholinesterase by alkoxy carbonyl homologues of malaoxon

Measurements were made at 25° and pH 7.6 in 10 mM-sodium phosphate buffer. The homologue concentration (i) and the appropriate function of the inhibition velocity from eqn. (2) ($i\Delta t/2.3\Delta \log v$) from which K_a and $k_{(p)}$ were calculated are also given. The determinations were made according to the procedure of Main & Iverson (1966). K_a and $k_{(p)}$ were calculated by the method of Wilkinson (1961). The bimolecular constant k_i was obtained from the relation $k_i = k_{(p)}/K_a$.

Alkyl substituent...	Methyl		Ethyl		n -Propyl		Isopropyl		n -Butyl	
	$i\Delta t$		$i\Delta t$		$i\Delta t$		$i\Delta t$		$i\Delta t$	
	i (mm)	$2.3\Delta \log v$ ($\mu\text{M min.}$)	i (mm)	$2.3\Delta \log v$ ($\mu\text{M min.}$)	i (mm)	$2.3\Delta \log v$ ($\mu\text{M min.}$)	i (mm)	$2.3\Delta \log v$ ($\mu\text{M min.}$)	i (mm)	$2.3\Delta \log v$ ($\mu\text{M min.}$)
	5.00	524	2.00	390	0.25	37.2	0.50	212	0.10	7.49
	4.00	378	1.50	331	0.20	28.6	0.40	189	0.08	6.70
	3.00	362	1.00	244	0.20	28.4	0.30	144	0.06	5.66
	2.00	246	0.50	160	0.15	25.0	0.20	100	0.04	5.18
	1.00	201	0.20	137	0.10	21.4	0.10	65	0.02	3.72
	0.50	228	0.01	92	0.05	13.2	0.001	20	0.0004	4.26
	0.01	189			0.001	9.4				
Constant	Units	Methyl	Ethyl	n -Propyl	Isopropyl	n -Butyl				
K_a	mm	2.04 ± 0.89	0.62 ± 0.08	0.085 ± 0.021	0.068 ± 0.013	0.070 ± 0.009				
$k_{(p)}$	min. ⁻¹	15.0 ± 2.7	6.60 ± 0.30	9.50 ± 0.82	2.60 ± 0.11	22.6 ± 1.6				
k_i	mm ⁻¹ min. ⁻¹	7.5	10.7	112	38	323				

The $k_{+2(a)}$ values were on average 435 times the $k_{+2(p)}$ values of the corresponding homologues. The relationship between the $k_{+2(p)}$ and $k_{+2(a)}$ values is illustrated in Fig. 1(b), where the effect of the substituents is seen to be closely parallel.

The inductive effect of the substituents evidently was not the dominant influence on the variations in $k_{+2(a)}$ as well as $k_{+2(p)}$. On the other hand, the correlation between the $k_{+2(a)}$ and $k_{+2(p)}$ values was remarkably close even though quantitatively they differed widely. This suggested that factors determining acylation rates would also determine phosphorylation rates.

Table 3. *Michaelis constants (K_m) and catalytic-centre activities of a homologous alkyl butyrate substrate series with serum cholinesterase*

Measurements were made at 25° and pH 7.6. Values are given as means $\pm$ S.D.

Substrate	K_m (mM)	$10^{-3} \times$ Catalytic-centre activity (min. ⁻¹)
Methyl butyrate	209 $\pm$ 41	9.33 $\pm$ 1.11
Ethyl butyrate	36 $\pm$ 4	1.17 $\pm$ 0.36
<i>n</i> -Propyl butyrate	11.7 $\pm$ 2.2	4.82 $\pm$ 0.62
<i>n</i> -Butyl butyrate	6.7 $\pm$ 1.7	13.6 $\pm$ 2.2

The velocities of cholinesterase substrate reactions typically do not bear a consistent relationship to the stabilities of the substrates hydrolysed, although such relationships have been observed (Ormerod, 1953). If acylation and phosphorylation are as closely related as the present results suggest, it would follow that relative phosphorylation potentials would not depend exclusively on the nucleophilic or electrophilic properties of the substituents, although with certain series such effects evidently predominated (e.g. Aldridge & Davison, 1952).

With the alkoxy-carbonyl malaoxon series, other as yet undetermined factors appeared to dominate the effect of alkyl substituents on $k_{+2(p)}$. It seems reasonable to suppose that properties peculiar to the enzyme were among these.

The K_m values of the alkyl butyrate series are given in Table 3 and they were in the increasing order: *n*-butyl; *n*-propyl; ethyl; methyl.

The corresponding K_a values for the alkoxy-carbonyl malaoxon series are given in Table 2 and they were in the increasing order: *n*-butyl; *n*-propyl; ethyl; methyl. The K_a values of the isopropyl and *n*-butyl homologues were about equal.

The order of K_a and K_m values was the same, indicating that the substituents had the same

Fig. 1. Effect of alkyl substituents on the substrate (○) and inhibitor (●) reaction constants of human serum cholinesterase. Substrate binding (pK_m) is compared with inhibitor binding (pK_a) in (a). Substrate acylation rate constants, $k_{+2(a)}$, assuming that the catalytic-centre activities equal $k_{+2(a)}$, are compared with phosphorylation rate constants, $k_{+2(p)}$ in (b). The substrates were methyl, ethyl, *n*-propyl and *n*-butyl butyrate. The inhibitors were the comparably substituted dialkoxy-carbonyl homologues of malaoxon. Inhibition rates were measured at 25° and pH 7.6 in 10 mM-sodium phosphate buffer according to the procedure of Main & Iverson (1966). Substrate velocities were measured at 25° and pH 7.6 without adding salts.

relative effects on binding in both the substrate and inhibitor series. The parallel was even closer than the order alone would indicate, as shown in Fig. 1(a). The effect of each additional methylene group on binding seemed to decrease in both series as the chain length increased.

K_a is essentially an equilibrium constant (i.e. k_{-1}/k_{+1}). If K_m is also assumed to approximate to k_{-1}/k_{+1} , then calculation of the free energy associated with each is justified. When this was done, the average change in ΔF per homologous member was 663 cal. for the inhibitor series and 685 cal. for the substrate series. The substrate series is mono-substituted whereas the inhibitor series is di-substituted. The close agreement between the average ΔF values suggested that only one of the two substituents in the di-substituted inhibitor series was actually bound to the active site.

The general expression for K_m derived from eqn. (1) is:

$$K_m = k_{+3}(k_{-1} + k_{+2(a)})/k_{+1}(k_{+2(a)} + k_{+3})$$

When $k_{+3} \gg k_{+2(a)}$, this reduces to:

$$K_m = (k_{-1} + k_{+2(a)})/k_{+1}$$

The closely parallel effects of the alkyl substituents on K_a and K_m together with the similar calculated ΔF values, suggested that $k_{-1} > k_{+2(a)}$, so that $K_m = k_{-1}/k_{+1}$ at least as an approximation.

The bimolecular velocity constant k_i is equal to $k_{+2(p)}/K_a$. If K_m approximates to k_{-1}/k_{+1} and the catalytic-centre activity approximates to $k_{+2(a)}$, then the ratios of these two constants for the substrate series should closely parallel the relative k_i values of the inhibitor series. When the *n*-butyl homologue of each series was given an arbitrary value of 100, the relative values for the $k_{+2(a)}/K_m$ ratio were: *n*-butyl, 100; *n*-propyl, 20; ethyl, 3.2; methyl, 2.2. The relative k_i values are: *n*-butyl, 100; *n*-propyl, 35; ethyl, 3.3; methyl, 2.3. The correspondence is close throughout and further supports the suggestion that the catalytic-centre activity = $k_{+2(a)}$ and that $K_m = k_{-1}/k_{+1}$.

Although affinity has been recognized as a potentially important factor contributing to inhibitory power, until recently there has been no direct way of estimating its significance relative to phosphorylation potential. Affinity, as measured by K_a , was primarily responsible for the wide variations (43-fold overall) between the inhibitory powers of the various malaoxon homologues. Variation in $k_{+2(p)}$ (three-fold overall in the *n*-alkyl series) was also of significance. With the isopropoxycarbonyl member the effect on $k_{+2(p)}$ was predominant.

There have been a number of studies in which poor correlations have been observed between the nucleophilic or electrophilic properties of substituents and inhibitory power (cf. O'Brien, 1960). Failure to

account for the affinity of the inhibitors may have been one contributing factor. Another may have been that substituents exert their effects on phosphorylation potential in more than one way and that electronic effects represent only one of these.

The possibility that malaoxon homologues may act as substrates of cholinesterase was suggested by the presence of the two carboxylic esters in these compounds. The substantial activity of cholinesterase towards the alkyl butyrates seemed to lend weight to this possibility.

However, when relatively concentrated solutions of cholinesterase and ethoxycarbonyl malaoxon were mixed in the reaction vessel of a pH-stat, no evolution of acid was observed, indicating that no significant substrate reaction occurred. In contrast, when carboxylesterase (EC type 3.1.1.1 hydrolase) and ethoxycarbonyl malaoxon were mixed under similar conditions, a vigorous reaction was observed (A. R. Main, unpublished work).

The procedures used to determine inhibition rates in the present work permitted this question to be examined further. The plots of $\log v$ against t (time) determined in the inhibition studies are linear when the inhibition reaction is first-order with respect to the esterase concentration. First-order kinetics hold when the inhibitor concentration remains essentially constant over the measured course of the reaction. For this reason i must be substantially greater than e_0 , the initial concentration of the enzyme, since a certain amount of inhibitor is lost through reaction with the esterase. If malaoxon were also a substrate, even a relatively slow reaction could significantly decrease i , particularly at the lower concentrations.

The lowest concentrations used of the *n*-butoxycarbonyl, *n*-propoxycarbonyl and isopropoxycarbonyl homologues were substantially greater than the esterase concentration, but were, nevertheless, of the same order. The initial concentrations of the *n*-propoxycarbonyl and isopropoxycarbonyl homologues were $21e_0$, whereas that of the *n*-butoxycarbonyl homologue was $8.5e_0$.

The $\log v$ against t at these lowest concentrations are illustrated in Fig. 2. Because of the low i/e_0 ratio, even a very slow substrate reaction should significantly alter these initial inhibition concentrations, and this would be reflected by non-linear plots of $\log v$ against t . However, the plots of the $\log v$ against t were linear, indicating that no significant substrate reaction occurred. The standard errors associated with the lines were: isopropoxycarbonyl malaoxon, $\pm 2.2\%$; *n*-propoxycarbonyl malaoxon, $\pm 2.46\%$; *n*-butoxycarbonyl malaoxon, $\pm 5.6\%$. The last point on the *n*-butoxycarbonyl malaoxon plot represented inhibition at a concentration that was 91.5% the initial value through loss of inhibitor to the inhibition reaction.

Fig. 2. Plots of $\log v$ against time at the lowest concentrations of the *n*-butoxycarbonyl (○), isopropoxycarbonyl (△) and *n*-propoxycarbonyl (□) malaoxon inhibitors of human serum cholinesterase. The linear relationships indicate that cholinesterase did not hydrolyse the carboxylic esters of the malaoxon homologues, i.e. malaoxon was not a substrate for cholinesterase. $\log v$ refers to the logarithm of the residual cholinesterase activity after incubation with inhibitor for the times indicated. The concentration of cholinesterase was $47 \mu\text{M}$. The concentrations of inhibitor were: *n*-butoxycarbonyl homologue, $0.4 \mu\text{M}$; *n*-propoxycarbonyl and isopropoxycarbonyl homologues, $1 \mu\text{M}$. Ratios of initial malaoxon inhibitor to esterase concentrations were: *n*-butoxycarbonyl homologue, 8.5:1; *n*-propoxycarbonyl and isopropoxycarbonyl homologues, 21:1. Inhibition rates were measured at 25° and pH 7.6 with 15 mM -acetylcholine.

The parallel effects of the alkyl substituents on the substrate and inhibitor reaction constants suggested that the alkyl group was bound to precisely the same position on the active site during both the initial binding step and the following acylation or phosphorylation reaction. However, the reactive phosphorus and carbonyl groups do not occupy the same positions relative to the alkyl substituents in substrate and inhibitor. This raises the question whether the phosphorylation and acylation reactions also occur at precisely the same position on the active site or whether separate acylation and phosphorylation sites exist.

The explanation for the similar effects and yet dissimilar orientation of the alkyl substituents with respect to the reactive bonds may be that with the

inhibitor molecules the esterases undergo different conformational changes during the reaction. The conformational change in each case would juxtapose the reactive bonds of either the substrate or inhibitor to the same group on the enzyme. The different degrees of conformation necessary to do this might explain in part the different magnitudes of the $k_{+2(p)}$ and $k_{+2(s)}$ values.

The failure of the alkoxy carbonyl malaoxons to act as substrates may reflect the inability of the enzyme to bind and then to juxtapose the relevant groups through conformational changes. It seems that malaoxon can bind to serum cholinesterase in only one way, and this results in phosphorylation rather than acylation.

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