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AFFINITY AND PHOSPHORYLATION CONSTANTS OF A SERIES OF *O,O*-DIALKYL MALAOXONS AND PARAOXONS WITH ACETYLCHOLINESTERASE*

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Abstract—The affinity constants (K_a) and the phosphorylation constants (k_p) were determined for a series of *O,O*-dialkyl malaoxons and paraoxons in which the phosphorylalkoxy groups were varied from methyl to butyl. The k_p values of the di-*n*-alkyl malaoxons and paraoxons ranged from 43 to 67 min^{-1} except for di-*n*-butyl malaoxon which was only 25 min^{-1}. Neither chain length nor leaving group appeared to affect k_p greatly, although there were minor variations.

Members in the paraoxon series bound better in each case than the comparable member of the malaoxon series. The K_a values were from two to ten times better for the paraoxon series than the comparable member of the malaoxon series. These values imparted the larger bimolecular rate constants to the paraoxon series. With di-*i*-propyl malaoxon and paraoxon poor binding occurred as well as a dramatic decrease in k_p (3 min^{-1}).

MAIN[1] demonstrated kinetically that complex formation prior to phosphorylation occurred with cholinesterase and organophosphorus inhibitors. A study by Main and Hastings[2] with a series of carbalkoxy homologs of malaoxon and serum cholinesterase showed that the affinity as well as the phosphorylation rates varied with the modifications in the "leaving group". Studies on the geometric isomers of Phosdrin demonstrated that the phosphorylation rate rather than affinity accounted for the higher inhibitory power of the *cis*-isomer when compared to the *trans*-isomer of Phosdrin.[3] Chiu and Dauterman,[4] studying the effect that structural differences in the "leaving group" of the various homologs of malaoxon and acetoxon had on the affinity and the phosphorylation rates, found that both values varied with structural changes. They also found that an α-carbethoxy group was necessary for good phosphorylation rates.

The terminology of Equation 1 will be employed, where P is the dialkyl phosphoryl group, X is the leaving group, E the enzyme, EPX the reversible complex and EP the phosphorylated enzyme.

$$E + PX \overset{K_a}{\rightleftharpoons} EPX \xrightarrow{k_p} EP \qquad (1)$$

The rate constants are K_a and k_p and $k_i = k_p/K_a$.

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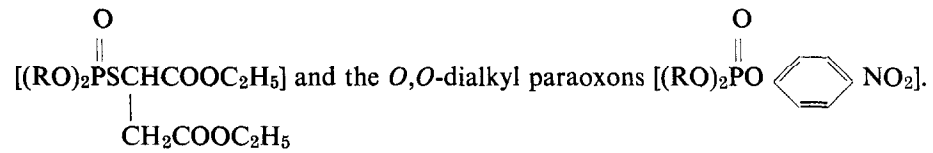
The present study was undertaken to determine the effect which comparable modification of the phosphorylating or "remaining" group (P) would have on affinity (K_a) and on rates of phosphorylation (k_p) and the overall inhibitory power (k_i). Two comparably substituted series of phosphoro di-alkoxy inhibitors were used: the *O,O*-dialkyl malaoxons



and the *O,O*-dialkyl paraoxons

In the malaoxon series, the leaving group (X) is aliphatic while in the paraoxon series, it is aromatic. However, the pK_a of the leaving groups were very similar; 6·96 for diethylthiomalate[2] and 7·15 for *p*-nitrophenol.[5] Consequently, the two series might be expected to offer an indication of the degree of interaction between the leaving and phosphorylating groups in determining affinity and rates of phosphorylation. Similarly, the results would also permit comparison of an aromatic with an aliphatic leaving group.

METHODS

Enzyme. Bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) was obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. The enzyme solution was prepared by dissolving 500 μM units in 50 ml of 10 mM sodium phosphate buffer, adjusted to pH 7·6, and kept in the cold under a drop of toluene. One μM unit of the enzyme hydrolyzed 1 μmole of acetylcholine per min at pH 8·0 at 37°. The solution was stable for weeks and was further diluted before use.

Organophosphorus compounds. The *O,O*-dialkyl S-(1,2-dicarbethoxy)ethyl phosphorothiolates referred to as the dialkyl malaoxons were prepared by heating on the steam bath 2 molar equivalents of trialkyl phosphite with 1 molar equivalent of tetraethyl dithiodisuccinate.[6, 7]

The time of heating of tetraethyl dithiodisuccinate and the various trialkyl phosphites was 22 hr with trimethyl and triethyl phosphites, 48 hr with the tripropyl phosphites, and 72 hr with tributyl phosphite. The excess phosphites were removed under vacuum and the compounds were run through a molecular still to remove the remaining traces of phosphites and diethyl 2-(alkylthio) succinates. The yields were as follows: Dimethyl malaoxon, 80%, 0·01 mm 83°; diethyl malaoxon, 77%, 0·15 mm, 135°; di-*n*-propyl malaoxon, 66%, 0·18 mm, 150°; di-*i*-propyl malaoxon, 47%, 0·15 mm, 140°; di-*n*-butyl malaoxon, 57%, 0·08 mm, 145°. Distillation temperatures were the minimum necessary to distill the product on a falling film molecular still with 1-cm path. All compounds were further purified by multimolecular adsorption chromatography.[8] The physical properties and trivial names are given in Table 1.

The *O,O*-dialkyl *p*-nitrophenyl phosphates referred to as dialkyl paraoxons were prepared and purified according to the method of Dauterman and O'Brien.[11]

Determination of K_a, k_p and k_i. The values of K_a, k_p and k_i were determined by using the procedure of Main and Iverson.[12] The rate of the inhibition reaction of the enzyme with various concentrations of inhibitors was expressed by plotting log v against t. The slopes were calculated by regression analysis and gave $2{\cdot}3\Delta\log v/\Delta t$

TABLE 1. PHYSICAL CONSTANTS OF PHOSPHORYLALKOXY HOMOLOGS OF MALAOXON

Compound	Boiling point		Phosphorus		Refractive index (n_D^{20})		
	C°	mm	Theor. (%)	Found[9] (%)	Calculated[10]	Found	Dev. (%)
Dimethyl malaoxon *O,O*-dimethyl S-(1,2-dicarbethoxy) ethyl phosphorothiolate	83	0·01	9·9	9·3	1·4673	1·4660	+0·09
Diethyl malaoxon *O,O*-diethyl S-(1,2-dicarbethoxy) ethyl phosphorothiolate	135	0·15	9·05	8·45	1·4674	1·4631	+0·29
Di-*n*-propyl malaoxon *O,O*-di-*n*-propyl S-(1,2-dicarbethoxy) ethyl phosphorothiolate	150	0·18	8·36	7·94	1·4675	1·4631	+0·30
Di-*i*-propyl malaoxon *O,O*-di-*i*-propyl S-(1,2-dicarbethoxy) ethyl phosphorothiolate	140	0·15	8·36	8·33	1·4675	1·4617	+0·40
Di-*n*-butyl malaoxon *O,O*-di-*n*-butyl S-(1,2-dicarbethoxy) ethyl phosphorothiolate	145	0·08	7·77	7·49	1·4676	1·4604	+0·49

values and their standard errors. These values were further used to plot the graphs of $i\Delta t/2{\cdot}3\Delta \log v$ against (i) according to the equation $i\Delta t/2{\cdot}3\Delta \log v = i/k_p + 1/k_i$ where $k_i = k_p/K_a$. All K_a, k_p and k_i values were obtained by regression analysis with the IBM 360 computer according to the method of Wilkinson.[13]

The rates of inhibition was measured at 5°, pH 7·6 in 10_mM sodium phosphate buffer. The residual enzyme activities were determined with 50 ml 3·0 mM acetylcholine at 25° and pH 7·6 on a Radiometer pH-stat.

TABLE 2. AFFINITY, PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE WITH *O,O*-DIALKYL MALAOXONS AT 5°, pH 7·6. THE HOMOLOG CONCENTRATION (i) AND THE CORRESPONDING INHIBITION VELOCITY ($i\Delta t/2{\cdot}3\Delta \log v$) FROM WHICH K_a, k_p AND k_i WERE CALCULATED ARE ALSO GIVEN

Dimethyl		Diethyl		Di-*n*-propyl		Di-*i*-propyl		Di-*n*-butyl	
	iΔt		iΔt		iΔt		iΔt		iΔt
i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)
2·00	66·5	3·125	131	2·00	113	4·50	4005	0·3125	39·1
1·50	58·0	2·500	117	1·50	105	3·00	3572	0·2000	34·2
1·00	52·0	2·000	108	1·00	96	2·50	3449	0·1500	32·4
0·50	43·4	1·000	87	0·50	92	2·00	3233	0·1250	31·5
0·02	37·3	0·050	70	0·10	79	1·00	2949	0·0800	30·6
0·001	38·6	0·010	73	0·002	77	0·50	2795	0·0600	28·9
						0·10	2573	0·0005	26·7

Constants	Dimethyl	Diethyl	Di-*n*-propyl	Di-*i*-propyl	Di-*n*-butyl
K_a (mM)	2·4 ± 0·15	3·6 ± 0·23	4·5 ± 0·10	8·9 ± 0·10	0·67 ± 0·02
k_p (min^{-1})	67·0 ± 2·6	52·0 ± 2·1	58·0 ± 1·2	3·30 ± 0·03	25·0 ± 0·70
k_i ($M^{-1}\ min^{-1}$)	$2{\cdot}8 \times 10^4$	$1{\cdot}4 \times 10^4$	$1{\cdot}3 \times 10^4$	$0{\cdot}04 \times 10^4$	$3{\cdot}8 \times 10^4$

RESULTS

The values of the inhibition velocity and kinetic constants of the dialkyl malaoxons are presented in Table 2, whereas the plot of the inhibition velocity $i\Delta t/2{\cdot}3\Delta \log v$ vs. the concentration of inhibitor i is presented in Fig. 1. With the malaoxon homologs, the best affinity ($1/K_a$) was found with di-*n*-butyl malaoxon and the poorest affinity with di-*i*-propyl malaoxon. The phosphorylation rate (k_p) of the dimethyl compound was the fastest and the di-*i*-propyl malaoxon was the slowest. However, the k_p values

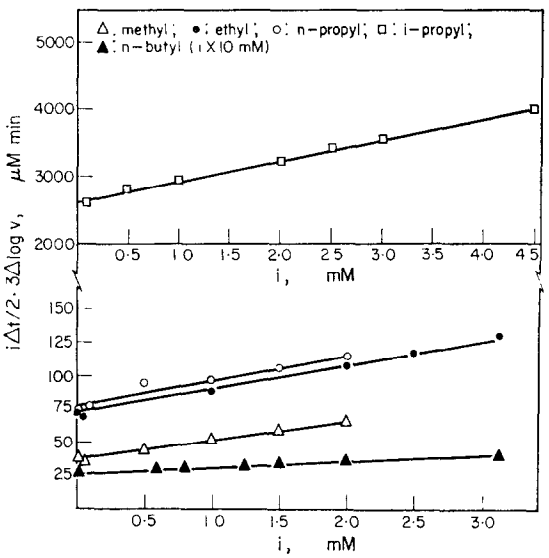


FIG. 1. Inhibition velocity ($i\Delta t/2{\cdot}3\Delta \log v$) vs. concentration (i) for the inhibition of acetylcholinesterase by *O,O*-dialkyl homologs of malaoxon.

of the methyl, ethyl and *n*-propyl homologs were of the same order. Overall, the best inhibitor of acetylcholinesterase in the malaoxon series based on the bimolecular rate constant (k_i) was di-*n*-butyl malaoxon. The average standard errors obtained with the log v vs. t plots at various concentrations of the malaoxon homologs were: Dimethyl $\pm$ 2·8%; diethyl $\pm$ 1·6%; di-*n*-propyl $\pm$ 3·0%; di-*i*-propyl $\pm$ 2·3%; di-*n*-butyl $\pm$ 2·2%.

The values of the inhibition velocity and kinetic constants of the dialkyl paraoxons are presented in Table 3 and the plot of the inhibition velocity $i\Delta t/2{\cdot}3\Delta \log v$ vs. the concentration of the inhibitor i is presented in Fig. 2. Here again the poorest affinity was found with the di-*i*-propyl paraoxon and the best affinity with the di-*n*-butyl compound. In the paraoxon series the di-*n*-butyl compound was the best inhibitor of acetylcholinesterase based on the k_i value and the di-*i*-propyl compound the poorest. The average standard errors associated with the paraoxon homologs were: Dimethyl $\pm$ 4·9% diethyl $\pm$ 1·7%; di-*n*-propyl $\pm$ 4·9%; di-*i*-propyl $\pm$ 4·2%; di-*n*-butyl $\pm$ 3·5%.

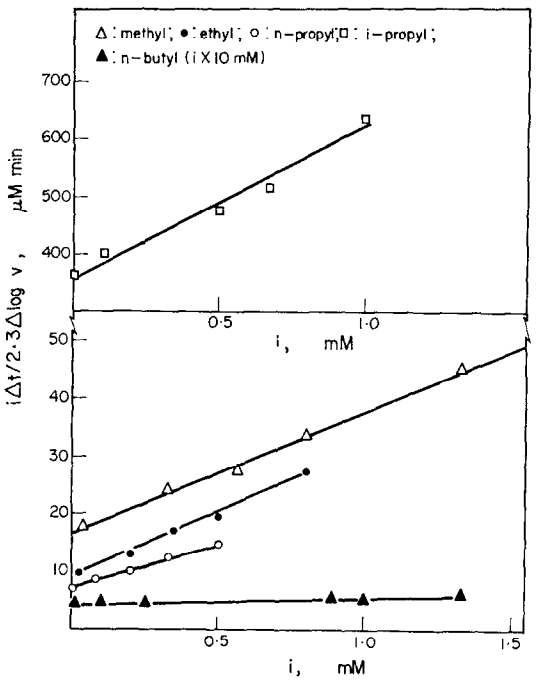


FIG. 2. Inhibition velocity (iΔt/2·3Δlog v) vs. concentration (i) for the inhibition of acetylcholinesterase by *O,O*-dialkyl homologs of paraoxon.

TABLE 3. AFFINITY, PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE WITH *O,O*-DIALKYL PARAOXONS AT 5°, pH 7·6. THE HOMOLOG CONCENTRATION (i) AND THE CORRESPONDING INHIBITION VELOCITY (iΔt/2·3Δlog v) FROM WHICH K_a, k_p AND k_i WERE CALCULATED ARE ALSO GIVEN

Dimethyl		Diethyl		Di-*n*-propyl		Di-*i*-propyl		Di-*n*-butyl	
	iΔt		iΔt		iΔt		iΔt		iΔt
i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)	i (mM)	2·3Δlog v (μM min)
2·66	69·8	0·8	27·4	0·500	14·8	1·000	637	0·133	6·2
1·33	45·4	0·5	19·2	0·333	12·5	0·667	515	0·100	5·4
0·80	34·2	0·35	17·2	0·200	9·6	0·500	474	0·089	5·5
0·567	27·5	0·20	12·9	0·0833	8·5	0·100	400	0·0267	4·7
0·333	24·5	0·01	9·7	0·0033	6·9	0·0067	364	0·0100	4·8
0·033	17·6							0·0065	4·7

Constants	Dimethyl	Diethyl	Di-*n*-propyl	Di-*i*-propyl	Di-*n*-butyl
K_a (mM)	0·88 ± 0·07	0·36 ± 0·05	0·43 ± 0·03	1·38 ± 0·14	0·27 ± 0·06
k_p (min^{-1})	50·2 ± 1·8	42·7 ± 2·8	63·1 ± 3·6	3·85 ± 0·27	65·0 ± 11·0
k_i (M^{-1} min^{-1})	0·57 × 10^5	1·20 × 10^5	1·45 × 10^5	0·028 × 10^5	2·41 × 10^5

DISCUSSION

A comparison of the K_a values within each of the inhibitor series showed only small variations. In the paraoxon series, the variation between the diethyl, di-*n*-propyl and di-*n*-butyl homologs was insignificant suggesting that the acyl-binding site was fully occupied by one or both of the methyl groups of the phosphorylalkoxy portion of the inhibitor. Since K_a is an equilibrium constant (i.e. k_{-1}/k_1) the calculation of the free energy of binding of the inhibitors is justified ($\Delta F = -RT \ln 1/K_a$). In the paraoxon series, going from the dimethyl to the diethyl analog, an increase of about 500 cal/mole in binding energy occurred while in the malaoxon series this change resulted in a decrease of 230 cal/mole. A greater dissimilarity occurred between the di-*n*-propyl and di-*n*-butyl malaoxons which was accompanied by an increase in binding energy of 1057 cal/mole. Despite these anomalies, which are minor compared to the potential increase in binding energy expected with the addition of two methylene groups (1000 cal/mole/methylene group),[14] it seems evident that the *n*-alkyl substituents at least beyond methyl are not involved in any significant way with initial binding based on the calculated free energy.

In both series, the di-*i*-propyl substituents resulted in a slightly less binding—about 700 cal/mole in each series. This is probably due to the steric effects of the di-*i*-propyl group.

Members in the paraoxon series bound better than the comparable member of the malaoxon series. On the average, this difference was of the order of a 1000 cal/mole and accounted almost entirely for the great inhibitory power (k_i) of the paraoxon series. Since the phosphorylalkoxy portion in the two series is the same, the greater binding capacity must be associated with the *p*-nitrophenoxy group when compared to the diethyl thiomalate moiety.

The phosphorylation rate constants (k_p) of the di-*n*-alkyl members were, with one exception, remarkably similar, both between members and between series. The exception was the di-*n*-butyl member of the malaoxon series which was 25 min^{-1} compared with the more typical values which ranged from 42·7 to 67 min^{-1}. Neither leaving group nor chain length appeared to affect k_p greatly, although there were minor variations. It is perhaps worth noting that the k_p values were quite high compared with others which have been reported.[15, 16]

Substitution of the branched-chain di-*i*-propyl group resulted in a dramatic decrease in k_p in both series, to about 3 min^{-1}, suggesting that the phosphorylalkoxy substituent does play a major role in determining k_p. The similar dissociation (or ionizing) constants of the leaving groups suggest a possible correlation with the similar k_p values,[17] but the similarity could be coincidental, particularly in view of the *i*-propyl substituent effect.

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