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THE AFFINITY AND PHOSPHORYLATION CONSTANTS OF A SERIES OF BRANCHED-CHAIN HOMOLOGS OF DIETHYL MALAOXON AND ACETOXON WITH ACETYLCHOLINESTERASE*

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Abstract—The affinity constants (K_a) and the phosphorylation constants (k_p) were determined for a series of homologs of diethyl malaoxon where the "leaving group" consisted of either mercaptomalonate, mercaptosuccinate, α-mercaptoglutarate or β-mercaptoglutarate esters. The K_a(mM) values with respect to acetylcholinesterase had the following order: succinate > β-glutarate > α-glutarate > malonate, and ranged from 3·6 for succinate to 0·15 for malonate. The order of k_p(min^{-1}) values was α-glutarate ⩾ malonate ⩾ succinate ⩾ β-glutarate and varied from 77 for α-glutarate to 0·5 for β-glutarate. The K_a values of the relevant acetoxon homologs were of the same order as those of the comparable malaoxon homologs, suggesting that only one carbethoxy group was necessary for initial binding. Compounds in this study which lacked an α-carbethoxy group were poor cholinesterase inhibitors because they were unable to phosphorylate the enzyme.

ALTHOUGH affinity has been suggested as an important factor in contributing to the inhibition of acetylcholinesterase by organophosphates,[1] only recently has there been a method available for measuring its quantitative significance.[2] Main and Iverson[3] quantitatively demonstrated that the differences in the inhibitory properties of di-isopropyl phosphorofluoridate (DFP) with serum cholinesterase and erythrocyte actetylcholinesterase were attributed to the different affinities that DFP had for the two enzymes. A subsequent study on serum cholinesterase with a series of carbalkoxy homologs of malaoxon also provided evidence to reach a similar conclusion.[4] Studies on the optical isomers of *O,O*-diethyl malaoxon and the geometric isomers of Phosdrin demonstrated that the phosphorylation rate rather than the affinity accounted for the higher inhibitory power of the *d*-isomer of malaoxon and the *cis*-isomer of Phosdrin when compared to the corresponding *l*- and *trans*-isomers respectively.[5]

The present study was undertaken to determine the effect that structural differences in the "leaving group" of the various homologs of malaoxon and acetoxon had on the affinity (K_a), the rate of phosphorylation (k_p) and the overall inhibitory power (k_i) with acetylcholinesterase. The branched-chain homologs of malaoxon used in this study are: malonate, with an α, α′-carbethoxy group; succinate, with an α, β-carbethoxy group; α-glutarate with an α, γ-carbethoxy group; and β-glutarate, with a β,β′-

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carbethoxy group. The acetoxon homologs, which are monocarboxyesters, also consist of either α- or β-carbethoxy groups in the leaving moiety (Fig. 1).

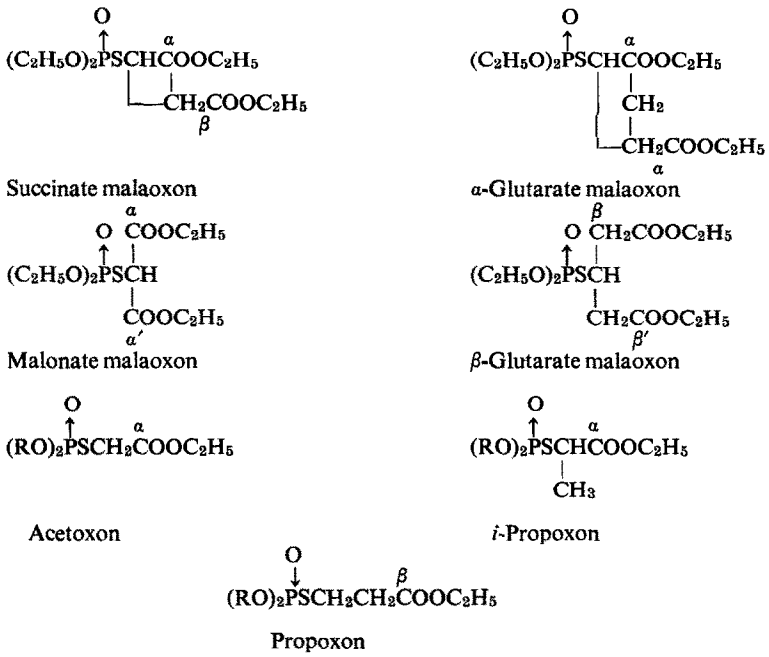

FIG. 1. Structure of branched-chain homologs of diethyl malaoxon and acetoxon. R = methyl or ethyl.

EXPERIMENTAL

Enzyme. Bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7) was obtained from the Sigma Chemical Company (St. Louis, Mo.).

Organophosphorus compounds. The branched-chain homologs of *O,O*-diethyl malaoxon [*O,O*-diethyl S-(dicarbethoxy)alkyl phosphorothiolates] were prepared according to the method of Chiu *et al.*[6] The methyl acetoxon homologs [*O,O*-dimethyl S-(carbethoxy) alkyl phosphorothiolates] were prepared by heating on a steam bath for 24 hr two molar equivalents of trimethyl phosphite with one molar equivalent of either diethyl dithiodiacetate, diethyl-3,3′-dithiodipropionate or diethyl 2,2′-dithiodipropionate.[7] The excess trimethyl phosphite was removed under vacuum and the compounds were further purified by molecular distillation.

The acetoxon homologs were prepared according to the method of O'Brien *et al.*[8] by refluxing for 10 hr in acetone equimolar amounts of sodium *O,O*-diethyl phosphorothiolate[9] with each of the following intermediates: ethyl chloroacetate, ethyl 3-bromopropionate and ethyl-2-bromopropionate. On cooling, the salt was filtered off and acetone was removed under vacuum. The compound was extracted with chloroform and washed with water. The chloroform layer was dried over anhydrous sodium sulfate and the chloroform was removed under vacuum. The compounds were molecular-distilled and further purified by multimolecular chromatography.[10] The physical constants and trivial names are given in Table 1.

TABLE 1. PHYSICAL CONSTANTS OF THE VARIOUS HOMOLOGS OF ACETOXON

Compound	b.p. C°	b.p. mm	Phosphorus Theor. (%)	Phosphorus Found* (%)	Refractive index (n_D^{20}) Calculated†	Found	Dev. (%)
Methyl acetoxon *O,O*-dimethyl S-(carbethoxy)methyl phosphorothiolate	100	0·10	13·57	12·98	1·4660	1·4680	−0·13
Methyl *i*-propoxon *O,O*-dimethyl S-1-(carbethoxy)-ethyl phosphorothiolate	110	0·10	12·79	12·48	1·4662	1·4675	−0·09
Methyl propoxon *O,O*-dimethyl S-2-(carbethoxy)ethyl phosphorothiolate	85	0·15	12·79	12·57	1·4662	1·4645	+0·19
Acetoxon *O,O*-diethyl S-(carbethoxy)methyl phosphorothiolate	125	0·05	12·09	11·78	1·4663	1·4631	+0·22
Isopropoxon *O,O*-diethyl S-1-(carbethoxy)ethyl phosphorothiolate	130	0·04	11·46	10·70	1·4684	1·4600	+0·57
Propoxon *O,O*-diethyl S-2-(carbethoxy)ethyl phosphorothiolate	120	0·04	11·46	10·95	1·4684	1·4628	+0·38

* Reference 11.
† Reference 12.

TABLE 2. AFFINITY PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE WITH BRANCHED-CHAIN HOMOLOGS OF DIETHYL MALAOXON*

Malonate		α-Glutarate		β-Glutarate		Succinate	
	$i\Delta t$		$i\Delta t$		$i\Delta t$		$i\Delta t$
i (mM)	$2{\cdot}3\Delta\log v$ (μM min)	i mM)	$2{\cdot}3\Delta\log v$ (μM min)	i (mM)	$2{\cdot}3\Delta\log v$ (μM min)	i (mM)	$2{\cdot}3\Delta\log v$ (μM min)
0·100	3·93	0·250	11·56	2·00	8139	3·125	131
0·075	3·52	0·200	10·95	1·50	7190	2·500	117
0·067	3·67	0·150	10·34	1·00	6141	2·000	108
0·040	2·95	0·100	9·56	0·50	5142	1·000	87
0·030	2·76	0·050	8·98	0·01	5082	0·050	70
0·0005	2·41	0·001	8·57	—	—	0·010	73

Constants	Malonate	α-Glutarate	β-Glutarate	Succinate
K_a (mM)	0·15 ± 0·03	0·64 ± 0·03	2·1 ± 0·06	3·6 ± 0·23
k_p (min^{-1})	63·0 ± 9·7	77·0 ± 3·0	0·50 ± 0·01	52·0 ± 2·1
k_i ($M^{-1}min^{-1}$)	42·0 × 10^4	12·0 × 10^4	0·02 × 10^4	1·4 × 10^4

* The 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.

Measurement of inhibition rates. The affinity constant K_a (i.e. k_{-1}/k_{+1}) and the phosphorylation rate (k_p) were determined for the various malaoxon and acetoxon homologs according to the method of Main and Iverson.[3] From these findings k_i was calculated, since $k_i = k_p/K_a$. All of the inhibition reactions were performed at 5°, pH 7·6. The residual enzyme activity was measured in 50 ml of 30 mM acetylcholine plus 2% *n*-butanol by volume at 25°, pH 7·6 on a Radiometer pH-Stat.

RESULTS

The values of the inhibition velocity and kinetic constants of the branched-chain homologs of diethyl malaoxon are presented in Table 2. The plot of the inhibition velocity $i\Delta t/2{\cdot}3\Delta\log v$ vs. concentration of inhibitor i is presented in Fig. 2.

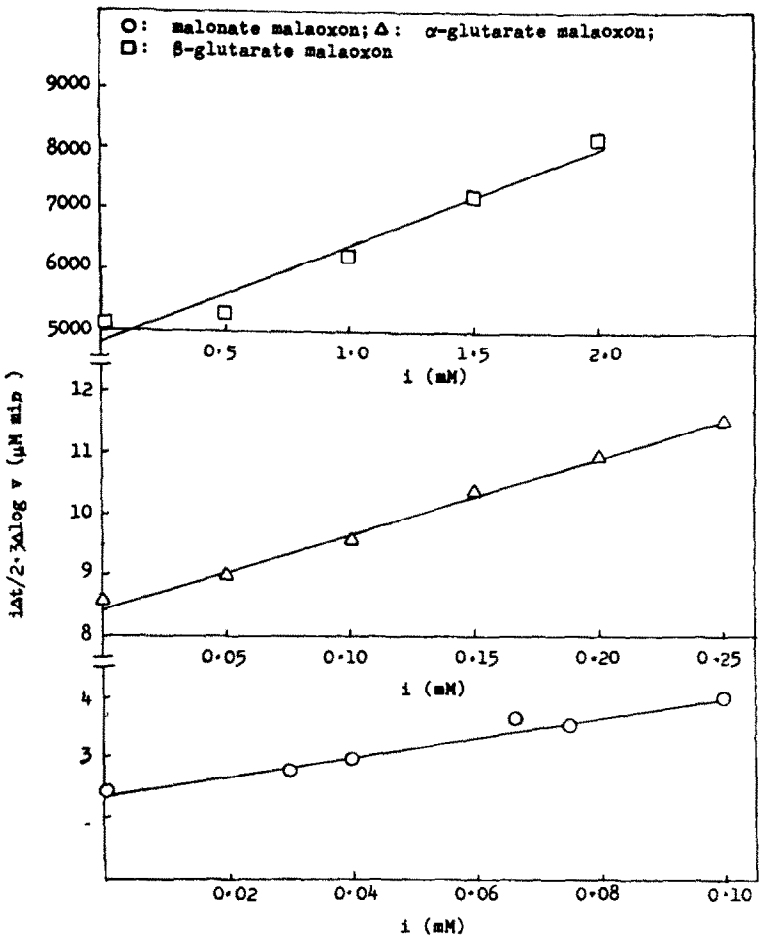

FIG. 2. Inhibition velocity $i\Delta t/2{\cdot}3\Delta\log v$ vs. concentration i for the inhibition of acetylcholinesterase with branched-chain homologs of diethyl malaoxon.

The K_a values varied from 0·15 to 3·6 mM for malonate and succinate homologs, respectively, and had a decreasing order: succinate > β-glutarate > α-glutarate > malonate. The results indicated that the affinity of the malonate homolog was 4·3, 14 and 24 times better than the α-glutarate, β-glutarate and succinate homologs respectively.

The k_p values had the following order: α-glutarate ⩾ malonate ⩾ succinate ⩾ β-glutarate, ranging from 0·5 min^{-1} for β-glutarate to 77 min^{-1} for α-glutarate. The k_p values of the α-glutarate, malonate and succinate homologs were all of the same order. There was approximately a 100-fold difference in k_p between succinate and β-glutarate homologs. The average standard errors associated with the first-order

rate constants were: malonate, ±2·6 per cent; succinate, ±1·6 per cent; α-glutarate, ±1·8 per cent; β-glutarate, ±2·6 per cent.

The overall inhibitory power as expressed by k_i (M^{-1} min^{-1} × 10^4) for this series of compounds followed the order of malonate > α-glutarate > succinate ≫ β-glutarate, varying from 42 to 0·02 for the malonate and β-glutarate homologs respectively. The data indicated that the malonate homolog had the highest k_i value and the β-glutarate homolog had the lowest k_i value, with approximately a 2000-fold difference in anticholinesterase activity *in vitro*.

In order to examine whether the α-carbethoxy group played an important role in both binding and phosphorylation, three each of the methyl and the ethyl series of acetoxon homologs, which have only one carboxyester group, were studied. The results are given in Table 3 and Fig. 3. Because of the low inhibitory potency of both propoxon and methyl propoxon, only approximate k_i values could be determined.

TABLE 3. AFFINITY, PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION REACTION OF ACETYLCHOLINESTERASE WITH VARIOUS HOMOLOGS OF ACETOXON*

Methyl acetoxon		Methyl *i*-propoxon		Acetoxon		*i*-Propoxon	
	$i\Delta t$		$i\Delta t$		$i\Delta t$		$i\Delta t$
i (mM)	$2{\cdot}3\Delta\log v$ (μM min)	i (mM)	$2{\cdot}3\Delta\log v$ (μM min)	i (mM)	$2{\cdot}3\Delta\log v$ (μM min)	i (mM)	$2{\cdot}3\Delta\log v$ (μM min)
2·67	516	3·33	253	2·00	138	2·50	250
2·00	429	2·50	203	1·50	121	2·00	217
1·33	400	2·00	180	1·00	109	1·00	159
1·00	329	1·50	160	0·50	87	0·50	137
0·01	232	0·01	124	0·01	70	0·01	112

Constants	Methyl acetoxon	Methyl *i*-propoxon	Acetoxon	*i*-propoxon	Methyl propoxon	Propoxon
K_a (mM)	2·4 ± 0·54	1·6 ± 0·24	2·3 ± 0·27	1·8 ± 0·13	—	—
k_p (min^{-1})	9·9 ± 1·3	20·0 ± 1·3	32·0 ± 2·3	17·0 ± 0·65	—	—
k_i ($M^{-1}min^{-1}$)	4·1 × 10^3	12·3 × 10^3	13·4 × 10^3	9·7 × 10^3	0·015 × 10^3	0·045 × 10^3

* 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.

With the methoxy series, the k_p value of methyl *i*-propoxon was about twice that of methyl acetoxon, whereas the K_a value of these two compounds was of the same magnitude. The results further indicated that the side-chain methyl group in methyl *i*-propoxon did not increase the nucleophilic effect upon the P—S—C bond so as to decrease the phosphorylation potential as anticipated by the electronic induction. Methyl propoxon had little inhibitory potency toward erythrocyte acetylcholinesterase. This drastic decrease in inhibition was reflected in an approximate k_i value which was about 275 and 820 times smaller than those of methyl acetoxon and methyl *i*-propoxon respectively.

With the ethyl series of acetoxon, the kinetic constants did not follow the same pattern as those of the foregoing series. The k_p value of acetoxon was nearly twice that of *i*-propoxon. However, the affinity of these two compounds was in the same order. Again, the addition of a methylene group as in propoxon also resulted in a very

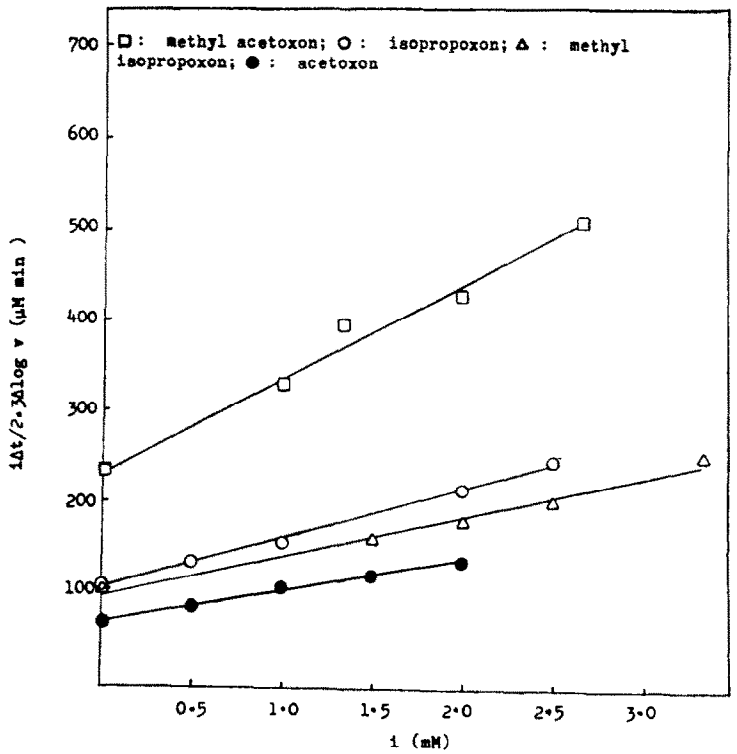

FIG. 3. Inhibition velocity $i\Delta t/2{\cdot}3\Delta\log v$ vs. concentration i for the inhibition of acetylcholinesterase with branched-chain homologs of acetoxon.

low potency in the inhibition reaction with this enzyme. The approximate k_i value of propoxon was nearly 300 and 200 times less than that of acetoxon and *i*-propoxon respectively.

DISCUSSION

A comparison of the K_a values of the branched-chain homologs of malaoxon demonstrated that the affinity of the malonate homolog was the best in the series. It has been demonstrated that molecules with relatively high symmetry and with a compact form generally have enhanced affinity.[13] Therefore, the symmetrical α- and α'-carbethoxy group in the leaving moiety probably is responsible for binding at the active site of the enzyme. However, the K_a values of the α-glutarate and β-glutarate malaoxons as well as the succinate homologs were still quite good, indicating that the β- or the γ-carboxyester group could also bind at the active site of the enzyme.

The K_a values of the relevant acetoxon homologs were in the same order as those of the comparable malaoxon homologs. For example, the K_a values of both methyl acetoxon and methyl malaoxon were the same with 2·4 mM,* while the K_a of acetoxon was 2·3 mM when compared to 3·6 mM for diethyl malaoxon. The data suggest that only one carboxyester group was essential for the initial binding to an active site. Since both β-glutarate malaoxon and the malonate malaoxon, which are dicarbethoxy

* Y. C. Chiu, A. R. Main and W. C. Dauterman, unpublished results.

compounds, are able to bind to the enzyme, it is obvious that either the α- or the β-carbethoxy group can bind in this manner and probably only one carbethoxy group can bind in this manner and probably only one carbethoxy group does bind.

A comparison of the phosphorylation rates of the branched-chain homologs showed the K_p of α-glutarate, malonate and succinate malaoxons to be of the same order. However, the k_p of the β-glutarate homologs was at least 100 times slower than that of the malaoxon homologs containing an α-carbonyl carbon, suggesting that the direct linkage of the P—S—C bond to the α-carbonyl carbon was essential for phosphorylation.

With the acetoxon series, the k_p values were lower than in the malaoxon series. However, methyl propoxon and propoxon were the only compounds which did not have an α-carboxyester group and their inhibitory power was so low that K_a and k_p could not be determined.

A comparison of the k_i values showed that the inhibitory power of the organophosphates lacking an α-carbonyl carbon was extremely low. With this in mind, the distance between the functional groups in the malaoxon and acetoxon homologs was determined by using Stuart–Briegleb models. The distance between the phosphorus atom and carbonyl atom ranged from 2·7 to 4·7 Å, 3·3 to 6·0 Å and 3·3 to 6·7 Å for α-, β- and γ-carbonyl carbons respectively. Compounds containing an α-carbonyl carbon approximated the distance between the esteratic and anionic site of acetylcholinesterase (2·5 to 4·5 Å).[14] Here the relative size probably allowed the α-carbonyl carbon to bind at the anionic site and the phosphate portion of the inhibitor to phosphorylate the esteratic site. With compounds such as β-glutarate malaoxon, the initial reaction allowed the β-carbethoxy group to bind at the anionic site, but the distance range of 3·3 to 6·0 Å was too large to allow the phosphate portion of the molecule to phosphorylate the esteratic site at the same time. This is a possible explanation for the low potency of the compounds lacking an α-carbethoxy group in the malaoxon and acetoxon series.

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