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THE AFFINITY AND PHOSPHORYLATION CONSTANTS OF THE OPTICAL ISOMERS OF *O,O*-DIETHYL MALAOXON AND THE GEOMETRIC ISOMERS OF PHOSDRIN WITH ACETYLCHOLINESTERASE*

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Abstract—The affinity constant (K_a) of the *d*-isomer of *O,O*-diethyl malaoxon with acetylcholinesterase at 5° was $2{\cdot}3(\pm 0{\cdot}23) \times 10^{-3}$M and for the *l*-isomer $4{\cdot}9(\pm 0{\cdot}51) \times 10^{-3}$M. The phosphorylation rate (k_p) of the *d*-isomer ($63{\cdot}0 \pm 3{\cdot}2$ min^{-1}) was twice that of the *l*-isomer. Overall, the inhibitory power (k_i) was four times greater for the *d*-isomer than for the *l*-isomer. With the geometric isomers of Phosdrin, the *cis*-isomer had a K_a of $1{\cdot}7(\pm 0{\cdot}06) \times 10^{-3}$M and the *trans*-isomer had a K_a of $3{\cdot}2(\pm 0{\cdot}65) \times 10^{-3}$M. The k_p of the *cis*-Phosdrin ($59{\cdot}0 \pm 1{\cdot}20$ min^{-1}) was ten times that of the *trans*-isomer. The 20-fold difference in the inhibitory power of the *cis*-isomer was largely attributed to the difference in the phosphorylation rate.

IT IS GENERALLY recognized that stereospecific reactions of an enzyme with either substrate or inhibitor are due to the asymmetric nature of the enzyme surface. The stereospecificity exhibited by various esterases in regard to their reactions with organophosphorus compounds has been recognized for about one decade. Previous studies have been almost exclusively made with compounds having an asymmetric phosphorus atom (Michel,[1] Hoskin and Trick,[2] Adie *et al.*,[3] Aaron *et al.*,[4] Fukuto and Metcalf,[5] Ooms and Boter[6]). Recently Ooms and van Dijk[7] found that chymotrypsin showed marked stereospecificity for organophosphorus inhibitors and was extremely sensitive to the asymmetric phosphorus atom but less sensitive to the asymmetric alkoxy group of three alkylphosphonofluoridates. Little attention has been paid to the "leaving group" of organophosphorus inhibitors, which have carbon isomers, and their effect on the inhibition of cholinesterase.

Recently the role of complex formation prior to phosphorylation has been indicated and evaluated by Main *et al.*[8–10] By using the kinetics of Main,[8] the present investigation was undertaken to determine the effect that the optical isomers of diethyl malaoxon and the geometric isomers of Phosdrin had on the affinity (K_a) of acetylcholinesterase for these compounds as well as on the rate of phosphorylation (k_p) and the overall inhibitory power (k_i).

EXPERIMENTAL

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

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Organophosphorus compounds. The *d*- and *l*-isomers of *O,O*-diethyl malaoxon* were prepared and purified according to the method of Hassan and Dauterman[11]. A Phosdrin† mixture containing 60·2±1·4% *cis*-Phosdrin by weight and the *cis*- and *trans*-isomers of Phosdrin, each with purities greater than 98 per cent, were kindly supplied by Shell Development Company (Modesto, Calif.). *Cis*-Phosdrin is the form in which the allylic methyl and carbmethoxy groups are in a *cis* relation[12] (Fig. 1).

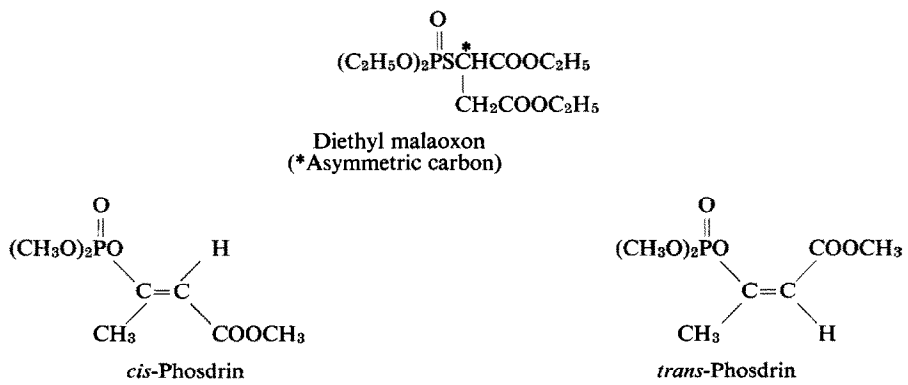

FIG. 1. Structures of diethyl malaoxon and Phosdrin.

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 organophosphate isomers as well as for their mixture according to the method of Main and Iverson.[10] From these findings k_i was calculated, since $k_i = k_p/K_a$. All of the inhibitions were performed at 5°, pH 7·6. The residual enzyme activity was measured in 50 ml of 3·0 mM acetylcholine at 25°, pH 7·6, on a Radiometer pH-stat.

RESULTS

The values of the inhibition velocity and kinetic constants of the optical isomers of diethyl malaoxon are presented in Table 1, whereas the plot of the inhibition velocity $\Delta t/2{\cdot}3\,\Delta \log v$ vs. concentration of inhibitor i is presented in Fig. 2. The K_a value of the *d*-isomer was about half that of the *l*-isomer, whereas the k_p value of the former was twice as fast as that of the latter. Consequently, the *d*-isomer had a k_i value about four times higher than the *l*-isomer with acetylcholinesterase.

For the determination of the kinetic constants of the optical isomers of diethyl malaoxon, at least five lines were fitted by linear regression analysis for each isomer. Each line represented the rate of inhibition at a certain concentration. The average standard errors associated with the lines were: *dl*-mixture, ±1·6%; *d*-form; ±2·1%; *l*-form, ±1·3%. However, only the lines at the highest concentration of 3·125 mM for each isomer were illustrated in Fig. 3. The *d*-isomer of diethyl malaoxon reacted much faster than the corresponding *l*-isomer, whereas the racemic mixture fell in between these two isomers in the inhibition of acetylcholinesterase. The rates of inhibition at lower concentrations were also determined but are not illustrated. The linearity of the log v vs. t relationship indicated that the rate of inhibition was a first-order reaction. First-order conditions were satisfied as the result of a substantial

* *O,O*-diethyl-S-(1,2-dicarbethoxy)ethyl phosphorothiolate.
† *O,O*-dimethyl-1-carbomethoxy-1-propen-2-yl phosphate.

excess of the inhibitor concentration compared to the enzyme concentration in order to obtain a desirable degree of inhibition within a relatively short incubation time under the experimental conditions.

TABLE 1. AFFINITY, PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE WITH OPTICALLY ACTIVE *O,O*-DIETHYL MALAOXON*

Racemic (*dl*) mixture		*d*-Form		*l*-Form	
i (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)	*i* (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)	*i* (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)
3·125	131	3·125	86	3·125	260
2·50	117	2·50	74	2·50	236
2·00	108	1·50	60	2·00	221
1·00	87	0·10	39	0·50	181
0·05	70	0·01	38	0·01	164
0·01	73				

Constants	Racemic	*d*-Form	*l*-Form
K_a (mM)	3·6±0·23	2·3±0·23	4·9±0·51
k_p (min^{-1})	52·0±2·1	63·0±3·2	31·0±2·2
k_i (M^{-1} min^{-1})	$1{\cdot}4\times10^4$	$2{\cdot}8\times10^4$	$0{\cdot}63\times10^4$

* 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.

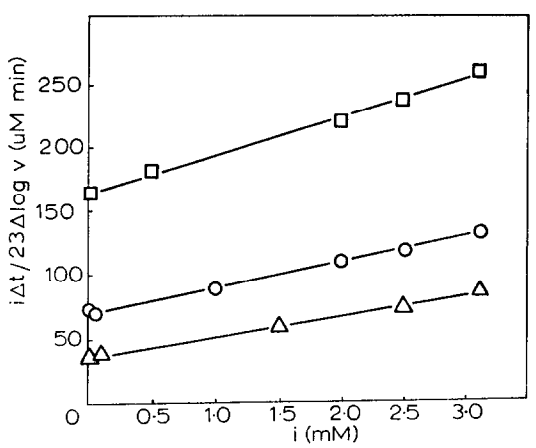

FIG. 2. Inhibition velocity ($i\Delta t/2{\cdot}3\ \Delta\log v$) vs. concentration (*i*) for the inhibition of acetylcholinesterase by various optical isomers of diethyl malaoxon. △—*d*-isomer; ○—racemic form; □—*l*-isomer.

Phosdrin, which is composed of a *cis-trans* mixture, also displayed first-order kinetics in the inhibition reaction with this enzyme. Although the plots representing the rates of inhibition at various concentrations of each isomer are not illustrated, the values of inhibition velocity calculated by linear regression analysis and their corresponding inhibitor concentrations are presented in Table 2 and plotted in Fig. 4. The average standard errors associated with the log *v* vs. *t* plot were: *cis-trans*-Phosdrin, ±1·6%; *cis*-Phosdrin, ±2·2%; *trans*-Phosdrin ±2·7%.

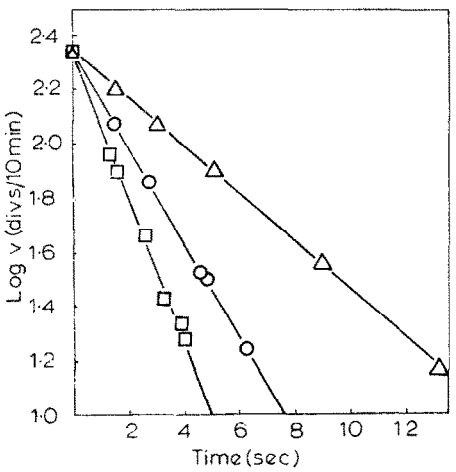

FIG. 3. Illustration of the linearity of the log v vs. t relationship for enzyme inhibition with diethyl malaoxon and its optical isomers. The velocity v refers to the hydrolysis rate of acetylcholine at 25° by the residual enzyme after inhibition for time t with various concentrations at 5°, pH 7·6. The lines were fitted by linear regression analysis. The slope of each line represents the rate of inhibition at 3·125 mM of the various isomers. △—*l*-form; ○—*dl*-mixture; □—*d*-form.

As shown in Table 2, the K_a values of Phosdrin and its geometric isomers, carboxyester-containing vinyl phosphates, were in the same order as those of methyl *i*-propoxon (1·6 mM) and its close homolog, methyl acetoxon (2·4 mM).[13] This implied that the linkages of P–S–C and P–O–C = C to the carboxyester group had more or less the

TABLE 2. AFFINITY, PHOSPHORYLATION AND BIMOLECULAR CONSTANTS (K_a, k_p AND k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE WITH THE GEOMETRIC ISOMERS OF PHOSDRIN*

Cis-trans-Phosdrin		*Cis*-Phosdrin		*Trans*-Phosdrin	
i (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)	i (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)	i (mM)	$i\Delta t/2{\cdot}3\ \Delta \log v$ (μM min)
2·00	96·4	2·00	61·4	2·00	917
1·50	88·7	1·50	53·7	1·50	881
1·00	73·8	1·00	45·0	1·25	787
0·50	60·0	0·50	36·0	1·00	753
0·01	49·0	0·10	28·7	0·50	646
		0·01	28·3	0·01	599

Constants	*Cis-trans*-Phosdrin	*Cis*-Phosdrin	*Trans*-Phosdrin
K_a (mM)	2·1 ± 0·30	1·7 ± 0·06	3·2 ± 0·65
k_p (min^{-1})	42·0 ± 3·60	59·0 ± 1·20	5·6 ± 0·77
k_i ($M^{-1}\ min^{-1}$)	$2{\cdot}00 \times 10^4$	$3{\cdot}5 \times 10^4$	$0{\cdot}18 \times 10^4$

* 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.

same magnitude of binding, although *cis*-Phosdrin showed a better binding than *trans*-Phosdrin. However, the phosphorylation rates varied with the isomers considerably. The k_p value of *cis*-Phosdrin was approximately ten times faster than *trans*-Phosdrin.

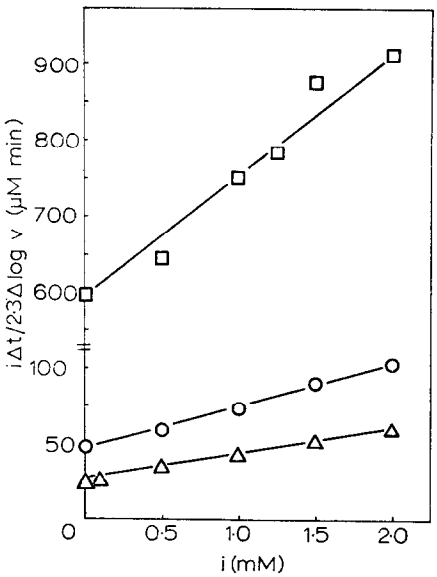

FIG. 4. Inhibition velocity ($i\Delta t/2{\cdot}3\ \Delta \log v$) vs. concentration ($i$) for the inhibition of acetylcholinesterase by Phosdrin and its geometrical isomers. △—*cis* phosdrin; ○—mixture of 60% *cis* and 40% *trans* phosdrin; □—*trans* phosdrin.

DISCUSSION

The findings indicated a marked stereospecificity of this enzyme for the *d*-isomer of diethyl malaoxon. As expected, the kinetic constants of the racemic mixture approxmated the mean of the values for the two isomers. The constants of the racemic mixture are always composed of the individual constants of the two isomers provided no interaction such as competition occurs. The slight difference in the optical purity of the two isomers was not enough to affect the kinetic results.

Recently Hassan and Dauterman[11] examined the inhibition of the optical isomers of diethyl malaoxon with erythrocyte acetylcholinesterase and rat liver carboxylesterase in relation to the toxicity of these compounds to mice and houseflies. As expected, the *d*-isomer was more toxic than the *l*-isomer, whereas the racemic mixture was intermediate in toxicity to both organisms. The acute toxicity of these compounds in general paralleled the inhibition reactions *in vitro* of acetylcholinesterase. The present kinetic results were in good agreement with the earlier findings.

Apparently, geometric isomerism of Phosdrin affected the phosphorylation rate far greater than the affinity for this enzyme. The overall inhibitory power of the *cis*-isomer was nearly 20 times that of the *trans*-isomer. This further revealed that the phosphorylation potential rather than affinity was mainly responsible for contributing to a wide difference in inhibitory power between *cis*- and *trans*-Phosdrins. Like the racemic mixture of diethyl malaoxon, the kinetic constants of the *cis-trans*-Phosdrin mixture were, in each case, essentially the average of the values for the two isomers.

A pronounced difference in the phosphorylation potential between *cis*- and *trans*-Phosdrin as a consequence of a different spatial configuration in the vinyl group of the compound suggested that the surface of this enzyme is stereospecific with respect to geometric asymmetry. The measurement of Stuart–Briegleb atomic models showed

that the distance between the phosphorus atom and the carbonyl carbon atom for *cis*-Phosdrin was in the range of 2·7–4·3 Å and approximated the distance between the esteratic site and the anionic site of acetylcholinesterase (2·5–4·5 Å).[14] In the case of *trans*-Phosdrin, this distance was much smaller (1·6–4·0 Å) due to a *trans* arrangement of the carboxyester group. This probably results in a less complementary fit of the phosphoryl group to the esteratic site for phosphorylation when binding occurred at the anionic site. Consequently, the *trans*-isomer was not as reactive as the corresponding *cis*-isomer with this enzyme.

The toxicological data of the Phosdrin isomers show an interesting example of how the biological properties of a compound can be the consequence of a different spatial arrangement of certain groups. *Cis*-Phosdrin is 20 times more toxic to the mouse than *trans*-Phosdrin.[15, 16] A comparison of the bimolecular rate constants (k_i), as a measure of the overall inhibitory power, shows the *cis*-isomer to be 20 times more reactive than the *trans*-isomer. The parallelism between toxicity and inhibition *in vitro* expressed by the inhibitory power is clearly demonstrated.

REFERENCES

1. H. O. MICHEL, *Fedn. Proc.* **14**, 255 (1955).
2. F. C. G. HOSKIN and G. S. TRICK, *Can. J. Biochem. Physiol.* **33**, 963 (1955).
3. P. A. ADIE, F. C. G. HOSKIN and G. S. TRICK, *Can. J. Biochem. Physiol.* **34**, 80 (1956).
4. H. S. AARON, H. O. MICHEL, B. WITTEN and J. I. MILLER, *J. Am. chem. Soc.* **80**, 456 (1958).
5. T. R. FUKUTO and R. L. METCALF, *J. econ. Ent.* **52**, 739 (1959).
6. A. J. J. OOMS and H. L. BOTER, *Biochem. Pharmac.* **14**, 1839 (1965).
7. A. J. J. OOMS and C. VAN DIJK, *Biochem. Pharmac.* **15**, 1361 (1966).
8. A. R. MAIN, *Science, N.Y.* **144**, 992 (1964).
9. A. R. MAIN and F. L. HASTINGS, *Biochem. J.* **101**, 584 (1966).
10. A. R. MAIN and F. IVERSON, *Biochem. J.* **100**, 525 (1966).
11. A. HASSAN and W. C. DAUTERMAN, *Biochem. Pharmac.* **17**, 1431 (1968).
12. J. B. STOTHERS and E. Y. SPENCER, *Can. J. Chem.* **39**, 1389 (1961).
13. Y. C. CHIU and W. C. DAUTERMAN, unpublished results.
14. W. B. NEELY, *Molec. Pharmac.* **1**, 137 (1965).
15. E. Y. SPENCER, *Can. J. Biochem. Physiol.* **39**, 1790 (1961).
16. A. MORELLO, E. Y. SPENCER and A. VARDANIS, *Biochem. Pharmac.* **16**, 1703 (1966).