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STUDIES ON THE OPTICALLY ACTIVE ISOMERS OF *O, O*-DIETHYL MALATHION AND *O, O*-DIETHYL MALAOXON*

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Abstract—The optically active isomers of *O,O*-diethyl malathion and *O,O*-diethyl malaoxon have been prepared by two different synthetic routes. Partially purified carboxylesterase (99-fold) from rat liver has been prepared for kinetic studies involving inhibition rate constants (k_i) and Michaelis constants (K_m) for malaoxons and malathions respectively. The *d*-isomers of malathion and malaoxon always proved to be more toxic toward mice and houseflies than the corresponding *l*-isomers. The higher toxicity of the *d*-isomers is believed to be related to higher bimolecular rate constants of *d*-malaoxon (k_i) with acetylcholinesterase and carboxylesterase.

Although *d*-malaoxon is a better inhibitor for carboxylesterase, *d*-malathion proved to be the better substrate for this enzyme. *In vivo*, however, the inhibition reaction dominated the substrate reaction, resulting in the *d*-isomers being more toxic.

IT IS WELL recognized that the stereospecificity exhibited by enzymes in their reactions with certain asymmetric compounds is the consequence of the asymmetric nature of the enzyme surface. With the phosphonates, four different substituents arranged around the phosphorus atom result in two optically active forms. Studies with Tabun, ethyl *N*-dimethylphosphoramidocyanidate[1] and with Sarin, isopropyl methylphosphonofluoridate,[2] showed a high degree of specificity with regard to the hydrolysis of the isomers. Aaron *et al.*[3] reported that the *l*-isomer of *O*-ethyl S-(2-ethyl mercaptoethyl) ethylphosphonothiolate reacted 10–20 times faster than the *d*-isomer in the inhibition of cholinesterases from four different sources. Hilgetag and Lehmann[4] reported that the antipodes of *O*,S-dimethyl *O*-(*p*-nitrophenyl) thiophosphates did not differ in their toxicity to Drosophila, but the *l*-isomer was 5 times better in the inhibition of rat brain cholinesterase and also 5 times more toxic to rats than the *d*-isomer. Recently Ooms and Boter[5] reported that the *d*-isomers of a series of S-alkyl *p*-nitrophenyl methylphosphonothioates were more toxic and reacted faster with acetylcholinesterase than the *l*-isomers.

Our study is concerned with malathion, *O,O*-dimethyl S-(1,2 dicarbethoxy) ethyl phosphorodithioate, which does not have an asymmetric phosphorus but has an asymmetrical carbon in the "leaving group," the succinate portion of the molecule.

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This work was undertaken to examine the reactions of the optical isomers of the *O,O*-diethyl homologs of malathion* and malaoxon† with acetylcholinesterase and liver carboxylesterase, and to relate these findings to the acute toxicity of the compounds to mice and houseflies. Studies were conducted with liver carboxylesterase because this enzyme is involved in the detoxication of malathion and appears to be responsible for the low mammalian toxicity.[6]

EXPERIMENTAL

Chemical synthesis

Dextrorotatory O,O-*diethyl malathion.* *l*-Aspartic acid was treated with nitrous acid and sodium bromide[7] to give *l*-bromosuccinic acid melting with decomposition at 177–180°. The specific rotation of the product $[\alpha]_D^{20}$ was —75·5° in ethyl acetate (C = 6). The *l*-bromosuccinic acid was converted to the diethyl ester by passing dry HCl in a solution of the acid in absolute ethanol (yield = 92 per cent.)[8] The $[\alpha]_D^{20}$ of the ester was —44·8° in ethyl acetate (C = 6), and its refractive index n_D^{20} was 1·4507. The levorotatory ester was then refluxed for 8 hr with an equimolecular amount of *O,O*-diethyl potassium phosphorodithioate in acetone. The solution was filtered, evaporated and the residue was extracted with chloroform. The extract was washed with 5 per cent bicarbonate solution and then with water. After evaporation of chloroform, the oily residue was purified on a Florisil column according to Patchett and Batchelder.[9] The specific rotation of *d*-malathion was +70·8° with a yield of 57 per cent, n_D^{20} = 1·4914.

Dextrorotatory O,O-*diethyl malaoxon.* The *l*-diethylbromosuccinate was allowed to react with an equimolecular amount of *O,O*-diethyl sodium phosphorothiolate in acetone. The reaction mixture was refluxed for 8 hr and the isolation of the compound was the same as described for diethyl malathion. The compound was obtained with $[\alpha]_D^{20}$ = + 26·0, n_D^{20} = 1·4625, and a yield of 42 per cent.

Levorotatory O,O-*diethyl malathion.* The *l*-malic acid was converted to the corresponding diethyl ester by passing dry HCl gas for 2 hr in a solution of the acid in absolute ethanol.[10] The reaction mixture was kept at 2° for 3 days. The alcohol was removed and the ester distilled at (109° at 2 mm) n_D^{20} = 1·4349, $[\alpha]_D^{20}$ = — 16·0° in acetone (C = 6). The *l*-diethylmalate was then treated with PBr_5 in an ether solution to give *d*-diethylbromosuccinate,[8] n_D^{20} = 1·4502, $[\alpha]_D^{20}$ = + 42·8°. This ester was refluxed for 8 hr with an equivalent amount of *O,O*-diethyl potassium phosphorodithioate in acetone to give the levorotatory *O,O*-diethyl malathion. The purification of the latter followed the same procedure as with the *d*-isomer. The specific rotation of *l*-malathion was — 70·0°, n_D^{20} = 1·4914 and a yield of 55 per cent.

Levorotatory O,O-*diethyl malaoxon.* This compound was prepared by condensing *d*-diethyl bromosuccinate with *O,O*-diethyl sodium phosphorothiolate as described for the *d*-isomer. The *l*-malaoxon was obtained with $[\alpha]_D^{20}$ = — 27·5°, n_D^{20} = 1·4632, and a 44 per cent yield.

O,O-diethyl malathion and *O,O*-diethyl malaoxon will be referred to as malathion and malaoxon respectively. Although the absolute configuration of the optically active isomers could not be determined, it may be assumed that the *dextro*-isomers have the *d*-configuration and the *levo*-isomers the *l*-configuration. This assumption

* *O,O*-diethyl-S-(1,2 dicarbethoxy) ethyl phosphorodithioate.
† *O,O*-diethyl-S-(1,2 dicarbethoxy) ethyl phosphorothiolate.

is based on the configuration of the starting materials (*l*-aspartic and *l*-malic acids), considering that a reverse of rotation is associated with a reverse of configuration. Consequently, the *dextro*-isomers will be described by *d*-configuration and the *levo*-isomers by *l*-configuration. The racemic mixtures are the *dl*-compounds.

Rat liver carboxylesterase (*carboxylic-ester hydrolase*) *EC* 3.1.1.1. The procedure adopted for the purification of the enzyme from rat liver is a modification of the method of Connors *et al.*[11] The reagents for the isolation procedure were reagent grade and were used without further purification. *p*-Nitrophenyl butyrate was prepared in this laboratory and served as a substrate.

Enzymatic activity

The catalytic activity of the enzyme was measured colorimetrically at 347 mμ, in much the same manner as described by Main *et al.*[12]

The protein concentration was determined by the biuret method.[13]

Purification procedure for liver carboxylesterase

Frozen livers* from rats of both sexes were thawed overnight at 2°, then rinsed with distilled water. About 150 g liver (fresh weight) was homogenized with 700 ml ice-cold acetone in a Servall Omnimixer. The homogenate was filtered and the residue washed with 700 ml ice-cold acetone. The material was then spread in a thin layer and left to dry overnight at room temperature. After carefully grinding the material to a powder (about 40 g), it was extracted with 400 ml distilled water by stirring in the cold for 15 hr. After centrifugation, the residue was further extracted with 200 ml distilled water for 10 hr. After removing the residue, the two supernatants were combined to constitute Supernatant I.

For every 100 ml of Supernatant I, 62 ml cold saturated ammonium sulphate solution (adjusted with ammonia to pH 7·5) was added. The mixture was kept at 2° for 15 hr, and the precipitate was removed by centrifugation. Solid ammonium sulphate was added with stirring to Supernatant II to increase the saturation from 0·38 to 0·65. The mixture was allowed to stand in the cold for 15 hr. Precipitate II, isolated by centrifugation, was dissolved in about 200 ml of 0·15 M phosphate buffer, pH 7·5. The buffered solution was brought quickly to 53° and held at this temperature for 5 min. The solution was then chilled in ice for 2 hr, centrifuged and Supernatant IV was saved.

For every 90 ml of Supernatant IV, 30 ml of ice-cold acetone was added with stirring; the solution was allowed to stand in the cold for 12 hr and then centrifuged. To every 100 ml of Supernatant V, 30 ml ice-cold acetone was added with stirring and the solution was allowed to stand in the cold for 12 hr. After centrifugation, Precipitate V was extracted with distilled water (about 50 ml) and treated with calcium phosphate gel (150 mg for every 100 mg protein) with stirring for 10 min. After removing the gel by centrifugation, the solution was run into a calcium phosphate column, 1·0 cm dia. and 30 cm high. Equilibration buffer (0·1 M phosphate buffer, pH 7·5) was then run through until no measurable activity was detectable in the eluate.

Solid copper acetate (400 mg for every 100 mg protein) was added with stirring to the latter, and the mixture allowed to stand in the cold for 6 hr, centrifuged, and Supernatant VII was exhaustively dialyzed against distilled water. The precipitated

* Pel-Freez Biologicals, Inc., Rogers, Ark.

proteins were removed by centrifugation. The enzyme solution (Supernatant VIII) could be stored in the refrigerator for at least 6 weeks without loss of activity. The results of the enzyme purification are summarized in Table 1. The yield of a series of different preparations ranged from 21–31 per cent. The purification ranged from 70- to 99-fold.

TABLE 1. PURIFICATION OF CARBOXYLESTERASE FROM RAT LIVER

Purification step	Sp. act. (units/mg protein)	Purfication (fold)	Enzyme* activity	Yield (% of initial activity)
Acetone powder	1·26	1	22,700	100
Supernatant I	3·01	2·4	18,680	82
Supernatant II	4·2	3·3	18,170	80
Precipitate II	5·7	4·5	15,120	67
Supernatant IV	7·0	5·6	15,100	67
Supernatant V	19·4	15·4	15,000	66
Precipitate V	43·5	34·5	14,150	62
Eluate	56·2	44·6	11,750	52
Supernatant VII	111·9	88·8	7,050	31
Supernatant VIII	124·5	98·8	6,600	29

* One unit of enzyme is that amount which causes an initial rate of hydrolysis of 1 μmole substrate/min under the conditions described.

Toxicity studies

The toxicity of the parent compounds was determined by oral administration in corn oil (0·2 ml/25 g mouse) to 7- to 8-week-old, 20–25 g female Duplin mice, ICR strain, starved for 24 hr prior to administration.

Tri-o-cresyl phosphate* (TOCP) was administered diluted in corn oil at 0·5 g/kg to fed mice. After an interval of 24 hr without food, the mice were treated again with the compounds under investigation. Mortality was recorded 24 hr after final treatment. The statistical confidence limits were determined by using angular transformation.

Determinations of LD_{50} were also made with the female adult housefly *Musca domestica* (L.) NAIDM strain. Contact insecticidal toxicity was based on 24-hr mortality counts after topical application of the insecticidal solutions in 1·23 μl acetone to individual flies. The LD_{50} values were computed by using the method of Finney[14] for probit analysis, which was adapted to a program for the IBM 1620 computer.

Determination of K_m and V_{max} for malathions with rat liver carboxylesterase

The reaction rates of malathions with carboxylesterase were determined with a Radiometer pH stat. The acid liberated as a result of the enzyme action was continuously titrated at a constant pH with a standard alkali. The procedure used in this paper is essentially the same as that described by Main and Braid.[15] K_m, V_{max} and the S.E's were calculated according to Wilkinson's method.[16]

Determination of bimolecular rate constants of malaoxons with bovine erythrocyte acetylcholineesterase† and rat liver carboxylesterase

* Eastman Organic Chemicals Department, Distillations Products Industries, Rochester, N.Y.
† Sigma Chemical Co., St. Louis, Mo.

Reactions rates for both enzymes were determined in a Radiometer pH stat.[17, 18] Acetylcholine served as a substrate for cholinesterase, and dibutyl malate for carboxylesterase.

RESULTS

Toxicity studies

The acute LD_{50} values of malathions and malaoxons for mice are given in Table 2. The *d*-malathion was more toxic than the *l*-isomer. Of the malaoxons tested, the *d*-isomer was again the most toxic.

TABLE 2. ACUTE ORAL TOXICITY OF MALATHIONS AND MALAOXONS TO MICE

Compound	Pretreatment	LD_{50} (mg/kg)	95% Confidence limits (mg/kg)
d-Malathion	None	1014	468–2164*
d-Malathion	TOCP	132	115– 146
	Difference	882	
l-Malathion	None	2357	1362–2357
l-Malathion	TOCP	189	138– 242
	Difference	2168	
dl-Malathion	None	1942	1324–2720
dl-Malathion	TOCP	159	95– 238
	Difference	1783	
d-Malaoxon	None	163	147– 177
d-Malaoxon	TOCP	40	29– 50
	Difference	123	
l-Malaoxon	None	249	191– 325
l-Malaoxon	TOCP	100	85– 125
	Difference	149	
dl-Malaoxon	None	243	218– 280
dl-Malaoxon	TOCP	66	57– 75
	Difference	177	

* Lack of fit.

TOCP, an inhibitor of carboxylesterase,[15] was administered to partially inhibit the enzyme *in vivo*. As a result, the toxicity was greatly increased, i.e. the LD_{50} was reduced to 8–13 per cent in the malathion series and to 25–40 per cent in the malaoxon series. If it is assumed that carboxylesterase was inhibited *in vivo* by TOCP to the same extent in all studies, then the difference between the LD_{50} values with and without pretreatment with TOCP should more or less represent the contribution of carboxylesterase toward detoxication of the compound (Table 2). Since malaoxon is the actual inhibitor of the target enzyme "acetylcholinesterase," its toxicity, as expected, was found to be greater than that of the parent substance.

Table 3 shows the LD_{50} values of malathions and malaoxons to houseflies. The *d*-isomer in both series was almost twice as toxic as the corresponding *l*-isomer. The optically inactive substances showed values intermediate between those of the active isomers.

K_m and V_{max} for malathions

The Michaelis constant and the maximum reaction velocity of the malathions with carboxylesterase are shown in Table 4. The *d*-isomer showed the highest affinity

TABLE 3. ACUTE TOXICITY OF MALATHIONS AND MALAOXONS TO HOUSEFLIES

Compound	LD_{50} (μg/fly)	95% confidence limits (μg/fly)
d-Malathion	0·13	0·05–0·33
l-Malathion	0·27	0·12–0·42
dl-Malathion	0·19	0·10–0·27
d-Malaoxon	0·18	0·07–0·49
l-Malaoxon	0·36	0·16–0·56
dl-Malaoxon	0·27	0·03–0·51

TABLE 4. ACTIVITY OF CARBOXYLESTERASE TOWARD MALATHIONS

Compound	$K_m \pm$ S.E. (mM)	$V_{max} \pm$ S.E. (μmoles/mg/min)	Relative enzymatic half-life ($0{\cdot}695\ (K_m/V_{max})$)
d-Malathion	0·084 ± 0·007	0·761 ± 0·011	0·077
dl-Malathion	0·205 ± 0·032	0·937 ± 0·043	0·152
l-Malathion	0·159 ± 0·008	0·559 ± 0·008	0·218

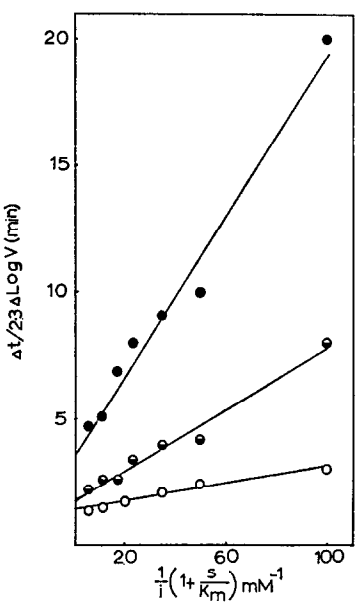

FIG. 1. The plot of $\Delta t/2{\cdot}3\ \Delta \log v$ against $(1 + s/K_m)/i$ for determination of k_i of malaoxons with carboxylesterase. The slope is $1/k_i$; ○ = *d*-malaoxon, ● = *l*-malaoxon, ◒ = *dl*-malaoxon.

toward the enzyme, as indicated by the low K_m value. Also, its maximum reaction velocity was higher than that of the *l*-isomer. These properties classify the *d*-isomer as a better substrate than the *l*-isomer. This is also indicated by the relative enzymatic half-life "or toxicological half-life." According to Main and Braid,[15] this is the time necessary to reduce a given substrate concentration, at a constant enzyme concentration, to half of its initial value.

Bimolecular rate constants (k_i) of malaoxons

Carboxylesterase. Since malaoxon, like the parent substance, serves as a substrate for this enzyme, it was necessary to determine the inhibition rate constant without interference of the malaoxon substrate reaction. Main and Dauterman[18] described a procedure for this situation by determining k_i in the presence of a substrate (dibutyl malate). Fig. 1 shows the plot of $\Delta\, t/2{\cdot}3\, \Delta \log v$ against $(1 + s/K_m)/i$ where the slope is the reciprocal of k_i. The Michaelis constant (K_m) of dibutyl malate with the enzyme preparation was determined to be 0·044 mM. The concentration of dibutyl malate used was 0·30 mM. The rate constants of the malaoxons are listed in Table 5. The *d*-isomer showed a faster rate of inhibition than the *l*-isomer (more than 8-fold).

TABLE 5. BIMOLECULAR REACTION CONSTANTS OF MALAOXONS

Compound	$k_i \times 10^4$ (M^{-1} min^{-1})	
	Bovine erythrocyte acetylcholinesterase*	Rat liver carboxylesterase†
d-Malaoxon	2·80	5·40
l-Malaoxon	0·63	0·65
dl-Malaoxon	1·49	1·70

* Measurements were made at 5°, pH 7·6, with 20 mM sodium hydroxide solution for titration.

† Measurements were made at 27°, pH 7·0, with 2 mM sodium hydroxide solution for titration

Acetylcholinesterase. The inhibition of this enzyme by the malaoxons showed the same trend as the toxicity data *in vivo*; the *d*-isomer was the better inhibitor (Table 5).

DISCUSSION

The synthesis of the optically active isomers of diethyl malathion and diethyl malaoxon followed two different routes. The formation of (+) diethylbromosuccinate from (−) diethyl malate follows the typical Walden inversion example.[19, 20] The inversion which took place after the reaction of diethyl bromosuccinate with the alkali salts of dialkoxy phosphorothiolate (or dithioate) is not unexpected. The reaction involves nucleophilic displacement and severing a bond to the asymmetric carbon, which may lead either to inversion or retention of configuration.[21] However, the bimolecular nature of the reaction suggests that inversion of configuration should take place.[22]

The toxicity data presented in this paper clearly demonstrate that the *d*-isomer is more toxic than the corresponding *l*-isomer for both the malathion and malaoxon series. The difference in LD_{50} (with and without TOCP) is greater in the malathion series, and would certainly indicate that both malathion and malaoxon are substrates for carboxylesterase.[6, 23] This has also been confirmed in studies with the purified carboxylesterase. The drastic modification of the LD_{50} in the presence of TOCP clearly indicates the importance of carboxylesterase in detoxifying malathion and malaoxon. A striking feature in Table 2 is that, with the TOCP-pretreated series, the LD_{50} values of the racemic (*dl*) malathion and malaoxon occur almost exactly halfway

between the values of the optically active isomers. This may be explained on the basis that, with carboxylesterase inhibited, the toxicity parallels more or less the rate constants (k_i) of the malaoxons with the targeten zyme acetylcholinesterase (Table 5). This receives support from the pattern of the LD_{50} values for the houseflies (Table 3), where the contribution of the carboxylesterase to the detoxication of malathion is negligible.[24]

The inhibition of acetylcholinesterase and carboxylesterase by malaoxon is more or less of the same order. For example, the rate of inhibition of cholinesterase by the *d*-isomer is about twice as fast as the rate of inhibition of carboxylesterase. This conclusion is based on comparing k_i values at the same temperature; (k_i is doubled for every 10-degree rise in temperature). The better inhibition of acetylcholinesterase and carboxylesterase by *d*-malaoxon indicates a better fit on the enzyme surface. This in turn suggests that the binding site for both enzymes is similar, in-so-far as configurtion is required.

Like optical isomers involving phosphorus as the asymmetric atom,[3, 4] the isomers of malathion and malaoxon displayed a difference in rates of reaction with enzymes. For example, *d*-malaoxon was 4 and 8 times better than *l*-malaoxon in the inhibition of acetylcholinesterase and carboxylesterase respectively; however, the difference in toxicity was of a lower order.

The kinetic data presented in this investigation indicate that *d*-malathion is a better substrate for carboxylesterase than the *l*-isomer (Table 4). However, the corresponding malaoxons showed a different pattern (Table 5), as indicated by the bimolecular rate constants (k_i). The *d*-isomer is a more potent inhibitor for both carboxylesterase and acetylcholinesterase than the *l*-isomer. It is probable that *in vivo* the inhibition of acetylcholinesterase and carboxylesterase by *d*-malaoxon dominates the detoxication (or hydrolysis of the *d*-isomers of malathion and malaoxon), thus accounting for the greater toxicity of the *d*-isomers. Accordingly, it may be concluded that the most important feature governing the acute toxicity of malathion in mammals is the rate of inhibition of acetylcholinesterase and carboxylesterase by malaoxon. These rates are in turn dependent on the rate of oxidation of malathion to its P=O analogue.

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