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STEREOSPECIFICITY OF HYDROLYTIC ENZYMES IN THEIR REACTION WITH OPTICALLY ACTIVE ORGANOPHOSPHORUS COMPOUNDS—I.

THE REACTION OF CHOLINESTERASES AND PARAOXONASE WITH S-ALKYL P-NITROPHENYL METHYLPHOSPHONOTHIOLATES

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Abstract—Reaction velocities of the irreversible inhibition of acetylcholinesterase and butyrylcholinesterase by a series of optically active S-alkyl *p*-nitrophenyl methylphosphonothiolates have been determined, as well as the rates of hydrolysis of these phosphorus compounds by sheep serum paraoxonase. Acetylcholinesterase proved to be much more stereospecific than butyrylcholinesterase. A rough correlation between the stereospecificity of acetylcholinesterase and inhibitor activity exists.

Sheep serum paraoxonase likewise shows a definite stereospecificity for this particular series of compounds. It hydrolyses preferentially the enantiomeric series with the lowest anticholinesterase activity.

INTRODUCTION

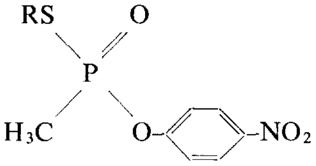
THE stereospecificity exhibited by hydrolytic enzymes in their reaction with tetracovalent asymmetric organophosphorus compounds is a phenomenon, recognized in the last few years.

In 1955 Michel[1] reported that sarin (isopropyl methylphosphonofluoridate) inhibited acetylcholinesterase as if two components were present in equal amounts; one of which reacted rapidly, the other slowly. This effect was explained in terms of optical isomerism. In the same year Hoskin and Trick[2] showed that an enzyme in rat serum hydrolyzed the toxic dextrorotatory isomer of tabun (ethyl N,N-dimethylphosphoramidocyanidate) much more rapidly than the almost non-toxic levorotatory isomer. In a subsequent study Augustinsson[3] showed that tabun was hydrolyzed by purified preparations of hog liver and kidney as if it contained two components of different effectiveness as substrates, one component being hydrolyzed over ten times faster than the other. This result was explained by assuming that the components were the optical isomers: however experiments to show any change in rotation, as had been carried out by Hoskin and Trick, failed. Purified human plasma enzyme did not distinguish between the isomers whereas rabbit plasma showed a slight stereospecificity. Later investigations by Adie *et al.*[4,5] demonstrated that the same enzyme in rat serum which is responsible for the hydrolysis of tabun, also hydrolyzed sarin; in this case no evidence of different reaction rates for the two possible optical isomers of sarin was found.

Aaron *et al.*[6] isolated the two enantiomers of ethyl S-2-ethylmercaptoethyl ethylphosphonothiolate and found that the levorotatory isomer reacted from 10 to 20 times faster with cholinesterase than the dextro isomer depending on the enzyme source. Similar results were obtained by Fukuto and Metcalf,[7] using fly-brain cholinesterase; the levorotatory isomer proved to be about 10 times more toxic to several insects than the dextro isomer.

Hilgetag and Lehmann[8] resolved O,S-dimethyl *p*-nitrophenyl phosphorothiolate. They found that the levo isomer was a better inhibitor for rat-brain cholinesterase and also about 5 times more toxic than the dextro isomer.

Finally Krzyżanowski[9] could demonstrate that the dextrorotatory isomer of ethyl S-2-chloroethyl ethylphosphonothiolate was twice as active as the corresponding levo isomer against acetylcholinesterase. From these literature data a more or less marked stereospecificity of the inhibition of cholinesterases by asymmetric organophosphorus compounds and of the hydrolysis of these compounds by so called "phosphorylphosphatases" can be concluded. In connection with our studies on structure-activity relationships in enzyme inhibition we have investigated in a more systematic way the influence of asymmetry in tetracovalent organophosphorus compounds on the rate of inhibition with a number of hydrolytic enzymes. This paper deals with the influence of the size of the alkyl group in a series of S-alkyl *p*-nitrophenyl methylphosphonothiolates



on the stereospecificity of the inhibition reaction of acetylcholinesterase (acetylcholine acetyl-hydrolase E.C.3.1.1.7) and butyrylcholinesterase (acylcholine acyl-hydrolase E.C.3.1.1.8) and on the stereospecific hydrolysis of the compounds by paraoxonase.

The stereospecificity exhibited by chymotrypsin, trypsin, aliesterase and acetylesterase will be made the subject of another publication in this series.

EXPERIMENTAL

Enzyme preparations

Acetylcholinesterase (AChE) from bovine erythrocytes was obtained commercially (Winthrop Laboratories) and had a specific activity of 8·8 units/mg N using $4{\cdot}1 \times 10^{-3}$ M acetylcholine chloride as a substrate.*

Butyrylcholinesterase (BuChE) was purified from horse serum according to a somewhat modified version of the method of Strelitz.[10] The specific activity of the enzyme was 46 units/mg N, using $1{\cdot}1 \times 10^{-2}$ M acetylcholine chloride as a substrate.

Paraoxonase was obtained from sheep serum. The purification method of Main[11] was followed up to and including stage 4. The precipitate was dissolved in 0·1 M sodium bicarbonate and the pH was adjusted to 7·6 with 2 N ammonia. The solution was dialyzed for two days against sodium bicarbonate in the cold. Starting with 800

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*In accordance to the recommendation of the International Union of Biochemistry enzymatic units will be defined as μ moles substrate hydrolyzed per minute at 25·0°.

ml. of sheep serum, 44 ml. of solution was obtained with a protein content of 40 mg/ml. and an activity of 0·048 units/mg protein using 8 × 10^{-3} M paraoxon as a substrate under conditions as described by Main. The enzyme solution was stable for at least 6 months if kept frozen at —15°.

Assay methods for the cholinesterases

For standardization purposes and for the calculation of molarities, the titrimetric assay method was used (c.f. Augustinsson[12]). The conditions were: 4·1 × 10^{-3} M acetylcholine chloride for AChE and 1·1 × 10^{-2} M for BuChE as the substrate, pH 7·5, t=25·0° using a Radiometer Titrigraph SBR_2/SBU_1. However this method does not lend itself for series of successive determinations as are required in kinetic experiments. For that reason we used a slightly modified electrometric assay method described by Michel[13] in our inhibition experiments.

The modification consisted of the use of twice diluted buffers; the reaction medium contained 10^{-3} M acetylcholine chloride for AChE and 1·5 × 10^{-3} M acetylcholine chloride for BuChE. The procedure was carried out as follows: 1 ml. of enzyme dilution was added to 11 ml. of the reaction medium thermostated at 25·0°. The pH was determined at 0 min and at 30 min for AChE and 21 min for BuChE using an Electronic Instruments Ltd. pH meter, model 39 A. The dilution of the enzymes was chosen so that the maximum pH drop was 0·70 pH units. Under these conditions the $\triangle$pH was proportional to the concentration of the enzymes.

Cholinesterase inhibition rates

Dilutions of the cholinesterases in 0·0066 M veronal buffer (pH 7·7) were made. The acetylcholinesterase solution contained 1·8 units/ml. ($\sim$ 8 × 10^{-9} M), the butyrylcholinesterase solution contained 2·2 units/ml. ($\sim$ 6 × 10^{-8} M). (Molarities are used here for moles "active sites" per litre.) In a thermostatic bath at 25·0° equal volumes of enzyme solution and inhibitor dilution (also in veronal buffer) were mixed at time zero. After 1, 2, 3, 4, 6, 8, 10 and 12 min, 1 ml. portions of this reaction mixture were assayed as described before. The dilution in the reaction medium and the presence of the substrate blocked the inhibition reaction completely.

Percentage residual enzyme could be calculated and the rate constants were determined graphically using either pseudo-first order or second-order kinetic formulae (c.f. Ooms[14]). At least three different inhibitor concentrations were used for each rate determination: the accuracy of the rate constants proved to be ± 5 per cent.

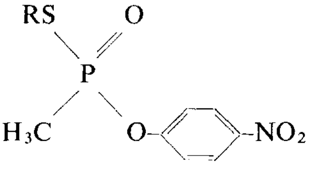
Paraoxonase experiments

A 0·08 M solution of the S-alkyl *p*-nitrophenyl methylphosphonothiolate in methanol was diluted 100 times with 0·067 M phosphate buffer (pH 6·0) containing 0·1% (w/v) gum acacia.

To 20 ml. of this solution was added 0·05 ml. enzyme solution or 0·05 ml. bicarbonate (0·01 M). The appearance of *p*-nitrophenolate was measured by determination of the extinction of these solutions in a Zeiss PMQ II spectrophotometer at 400 mμ every 5 min during 35 min. The cell compartment of the spectrophotometer was thermostated at 25·0°. Reaction velocities were determined graphically after correction for the spontaneous hydrolysis. In all cases a linear relation between reaction time and extinction was observed indicating zero-order or pseudo-zero order kinetics.

S-alkyl p-nitrophenyl methylphosphonothiolates

The optically active S-alkyl *p*-nitrophenyl methylphosphonothiolates (I) have been synthesized by the resolution of *p*-nitrophenyl hydrogen methylphosphonothioate (II) and subsequent reaction of the enantiomers with alkyl halides.*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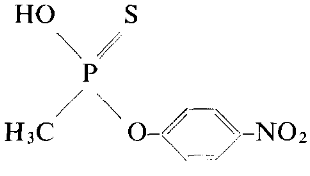
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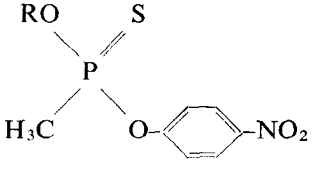
The resolution has been accomplished using strychnine as the resolving base. Separation of the diastereoisomeric strychnine salts was carried out by repeated crystallization from methanol. The less soluble alkaloid salt (III) had m.p. 215·5° (decomp.), $[\alpha]_D^{23}$ − 16·4 ± 1·0° (c = 1·0 in chloroform); the more soluble diastereoisomer (IV) had m.p. 195° (decomp.), $[\alpha]_D^{21}$ − 12·6 ± 1·0° (c = 1·0 in chloroform).

The enantiomers of acid II were liberated from their alkaloid salts and were converted into dicyclohexylamine salts as the acids themselves turned out to be very unstable. This procedure also enabled us to prepare these salts in nearly optically pure form by repeated crystallization, as the racemic compound (m.p. 155 – 157°) appeared to be much less soluble in the crystallizing solvent (ethyl acetate/ether, v/v, 1/5). The isolated dicyclohexylamine salts had m.p. 131 – 132° and specific rotations −34·6 ± 0·1° (from III) and + 35·2 ± 0·1° (from IV) (c = 2·0 in methanol, 25°, $\lambda = 589 m\mu$).

Reaction of these salts with a tenfold excess of the alkyl bromide (for the first member of the series methyl iodide was used as the alkylating agent) in benzene solution, filtration of the quantitatively precipitated dicyclohexylammonium halide and concentration of the filtrate delivered the desired S-alkyl *p*-nitrophenyl methylphosphonothiolates as slightly yellow coloured, indistillable oils (though in some cases a crystalline product was obtained, see Table 1). Yields varied from 83 up to 99 per cent. The products turned out to be optically stable for several weeks, stored in undiluted form at room temperature. The racemic compounds were prepared in essentially the same way starting with the dicyclohexylamine salt of the racemic acid II.

Paperchromatographic analyses showed that all products contained small amounts of *p*-nitrophenol (1–3 per cent.). No detectable amounts of the corresponding thionate isomers (V)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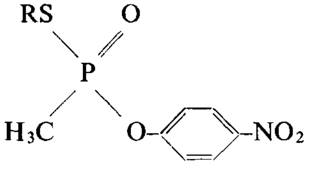
were present, indicating that S-alkylation had taken place exclusively.[15]

*A detailed description of the preparations will be published elsewhere.

Starting with the dicyclohexylamine salt of (—) II the first member of the series was isolated in the levo form; all others were dextrorotatory. In order to indicate that these five products possess the same configuration we gave them the prefix Dp. Consequently the members of the enantiomeric series were given the prefix Lp.

TABLE 1. PHYSICAL CONSTANTS AND ANALYSES OF S-ALKYL *p*-NITROPHENYL METHYLPHOSPHONOTHIOLATES



R	isomer	m.p. or n_D^{25}	$(\alpha)_D$*	%C		%P		%S	
				calc.	found	calc.	found	calc.	found
CH_3	(±)	91–92°	—	38·86	39·1	12·53	12·5	12·97	12·9
	Dp(−)	101–102·5°	−8·2±0·2°		39·2		12·5		12·9
	Lp(+)	101–102·5°	+8·0±0·2°		39·1		12·5		13·0
C_2H_5	(±)	1·5718	—	41·37	41·4	11·85	11·6	12·27	12·3
	Dp(+)	1·5718	+40·0±0·2°		41·5		11·9		12·3
	Lp(−)	1·5721	−40·6±0·3°		41·4		11·7		12·2
$n\text{-}C_3H_7$	(±)	1·5637	—	43·63	43·7	11·25	11·3	11·65	11·8
	Dp(+)	1·5639	+45·9±0·3°		43·7		11·2		11·6
	Lp(−)	1·5639	−45·0±0·3°		43·8		11·1		11·6
$n\text{-}C_4H_9$	(±)	1·5555	—	45·67	45·7	10·71	10·8	11·08	11·1
	Dp(+)	1·5555†	+44·0±0·3°		45·8		10·8		11·1
	Lp(−)	1·5562†	−43·4±0·2°		46·4		10·6		10·9
$n\text{-}C_5H_{11}$	(±)	1·5498	—	47·51	47·6	10·21	10·3	10·57	10·5
	Dp(+)	1·5498	+42·8±0·3°		47·5		10·2		10·6
	Lp(−)	1·5502	−42·7±0·5°		47·5		10·1		10·5

* measured in benzene p.a. at room temperature; c=3·6 – 3·9 ml.
† crystallization had taken place after a storage of several months at room temperature; m.p. 33 - 36°.

RESULTS AND DISCUSSION

In order to visualise the phenomenon of the stereospecificity we have introduced the "ratio of activity" (r_a) which will be defined as the "activity" (rate constant or rate of hydrolysis) of the Lp compounds divided by the "activity" of the corresponding Dp compounds of this particular series. In Table 2 the reaction rates found for the different isomers, together with their r_a values, are collected.

The results of Table 2 indicate that all the enzymes investigated do show more or less a stereospecificity. That this specificity is not due to traces of a more active impurity in the Lp-series may be concluded from the fact that, except in the cases where r_a is very close to unity, the "rate constants" for the racemic mixtures are in between the values for the two optically active forms. (One should keep in mind that there does not exist a rate constant for the racemic mixture: the "constant" will always be composed of the individual rate constants of the enantiomers; in the case of a very reactive inhibitor, when enzyme and inhibitor are present in nearly equimolecular amounts, no constant value for the racemic modification will even be found.)

Of the cholinesterases, AChE does show by far the greatest stereospecificity,

TABLE 2. RATE CONSTANTS OF THE REACTION OF THE STEREOISOMERS OF S-ALKYL *p*-NITROPHENYL METHYLPHOSPHONOTHIOLATES WITH CHOLINESTERASES AND THE RATE OF HYDROLYSIS OF THE COMPOUNDS BY SHEEP SERUM PARAOXONASE WITH THEIR RESPECTIVE "RATIOS OF ACTIVITY".

Alkyl group	Isomer	Acetyl-cholinesterase rate constant ($1/M^{-1}/min^{-1}$)	r_a	Butyryl-cholinesterase rate constant ($1/M^{-1}/min^{-1}$)	r_a	Paraoxonase rate in μ moles substrate hydrolyzed/min/mg protein × 10^3	r_a
CH_3	(±)	$1{\cdot}3 \times 10^4$	1·2	$3{\cdot}7 \times 10^4$	0·66	16·8	0·81
	Dp(−)	$1{\cdot}2 \times 10^4$		$4{\cdot}0 \times 10^4$		20·8	
	Lp(+)	$1{\cdot}5 \times 10^4$		$2{\cdot}6 \times 10^4$		16·8	
C_2H_5	(±)	$1{\cdot}2 \times 10^5$	13·1	$9{\cdot}4 \times 10^4$	0·86	37·9	0·26
	Dp(+)	$1{\cdot}4 \times 10^4$		$8{\cdot}8 \times 10^4$		50·1	
	Lp(−)	$1{\cdot}8 \times 10^5$		$7{\cdot}6 \times 10^4$		12·9	
n-C_3H_7	(±)	$6{\cdot}6 \times 10^5$	36·4	$3{\cdot}6 \times 10^5$	2·0	37·4	0·19
	Dp(+)	$4{\cdot}3 \times 10^4$		$2{\cdot}5 \times 10^5$		40·0	
	Lp(−)	$1{\cdot}6 \times 10^6$		$5{\cdot}0 \times 10^5$		7·7	
n-C_4H_9	(±)	$1{\cdot}2 \times 10^6$	50·5	$8{\cdot}5 \times 10^5$	2·9	36·7	0·27
	Dp(+)	$5{\cdot}5 \times 10^4$		$3{\cdot}5 \times 10^5$		39·5	
	Lp(−)	$2{\cdot}8 \times 10^6$		$1{\cdot}0 \times 10^6$		10·6	
n-C_5H_{11}	(±)	$8{\cdot}7 \times 10^5$	29·1	$6{\cdot}9 \times 10^5$	1·0	32·4	0·06
	Dp(+)	$6{\cdot}2 \times 10^4$		$7{\cdot}4 \times 10^5$		34·3	
	Lp(−)	$1{\cdot}8 \times 10^6$		$7{\cdot}6 \times 10^5$		2·0	

indeed the value $r_a = 50$ for the *n*-butyl homologue is the highest reported up to now for an organophosphorus compound. In the series r_a rises with increasing size of the alkyl group with a maximum for the *n*-butyl compound. There appears to be a correlation between the degree of stereospecificity and the rate constant of the faster reacting isomer, or in other words, between the stereospecificity and the specificity. If one assumes a multipoint interaction in the formation of the transition state (c.f. Dixon and Webb[16]) then a maximum difference between the rate of a compound with an optimum fit and that of its antipode is understandable. Butyrylcholinesterase shows much less stereospecificity, the highest ratio of activity found is 3, again for the *n*-butyl compound. Here the antimers of the ethyl- and the *n*-pentyl homologue react with virtually the same rate.

The hydrolysis of the compounds under the influence of paraoxonase does show more stereospecificity again, here the maximum ratio is observed with the *n*-pentyl compound. In contrast with the inhibition of acetylcholinesterase the change in the rate of the slowest reacting isomer seems to be the cause of the maximum ratio. It is assumed that in the hydrolysis reaction the phosphorylphosphatases are phosphorylated intermedially as in the case of the cholinesterases, the difference being the high rate of dephosphorylation in the case of the first mentioned enzymes.[17] Our experiments do not show which step is rate determining or even if the same step is rate determining for all the members of the series.

A comparison between the stereospecificity of the cholinesterases and of paraoxonase has to be handled carefully for these reasons. In all cases the members of the Dp-series are hydrolyzed faster than the corresponding members of the Lp-series. The last mentioned series however contains the compounds which react faster with acetylcholinesterase, being therefore the most toxic.* Thus our results contradict those of Hoskin and Trick[2] who found that the toxic isomer of tabun was hydrolyzed faster than its enantiomeric form.

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*In the Medical-Biological Laboratory of the National Defence Research Organization T.N.O., LD_{50}'s (rat s.c. after 24 hr) were determined according to Litchfield and Wilcoxon[18] for the different forms of S-n.butyl *p*-nitrophenyl methylphosphonothiolate: (±) 1210 μg/kg (1061-1379), Lp(-)699 μg/kg (666-734). No symptoms developed when a dose of 3720 μg/kg of the Dp(+) -isomer was administered.