The pH optimum at 23° for the insoluble urease was found at pH 6.0 as compared to the published values of pH 6.4 to pH 7.6 (ref. 10).

We have shown that urease, covalently coupled to an inorganic carrier, retains enzymatic activity. This enzyme–glass derivative has been employed continuously in a column over long periods without detectable losses in enzymatic activity.

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Stereospecific aging of phosphonylated cholinesterases

The conversion, called aging, of phosphonylated cholinesterases into a form which cannot be reactivated implies the release of an alkyl group from the phosphorus moiety of the inhibited enzyme[1,2]. The rate determining step in this process is probably a unimolecular fission of the C–O bond in the alkoxy group RO (Eqn 1)[3].

$$RO(CH_3)P(O)O\text{–}E + H_2O \rightarrow HO(CH_3)P(O)O\text{–}E + ROH \quad (1)$$

In 1964 BERENDS[4] observed that the aging of butyrylcholinesterase inhibited with racemic alkyl methylphosphonofluoridates did not obey first-order kinetics and suggested that this effect was connected with both possible configurations around the phosphorus atom.

This paper describes the influence of the configuration around the phosphorus atom and around the α-carbon atom of the previously mentioned alkoxy group on the aging rates of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) and butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8), inhibited with the stereoisomeric forms of cyclopentyl *S*-2-dimethylaminoethyl methylphosphonothioate, 1-methylheptyl methylphosphonofluoridate and 1,2,2-trimethylpropyl methylphosphonofluoridate (Compounds A, B and C of Table I, respectively).

TABLE I

BIMOLECULAR REACTION CONSTANTS (l mole^{-1} min^{-1}) FOR THE INHIBITION OF ACETYL- AND BUTYRYLCHOLINESTERASE WITH THE STEREOISOMERIC FORMS OF ASYMMETRIC ORGANOPHOSPHORUS COMPOUNDS AND FIRST-ORDER RATE CONSTANTS (min^{-1}) FOR AGING OF THE RESULTING PHOSPHONYLATED ENZYMES AT 25° AND pH 7 5

Complete phosphonylation of the enzymes was achieved by incubation with an excess of the inhibitor at pH 9 0 and 0° High pH and low temperature were chosen to prevent aging at this stage Excess of inhibitor was removed Maximally attainable reactivation was achieved after 24 h of incubation at pH 7 5 and 25° with 10 mM isonitrosoacetone after inhibition by Compound A and with 1 mM *N*-methylpyridinium-2-aldoxime methanesulphonate after inhibition by Compounds B or C Figures represent mean values of at least three determinations S D of mean was 7% or less

Compound	*Configuration*		*Acetylcholinesterase*		*Butyrylcholinesterase*	
	α-C	*P*	*Inhibition*	*Aging*	*Inhibition*	*Aging*
A Cyclo-$C_5H_9O(CH_3)P(O)S(CH_2)_2N(CH_3)_2$		(*R*)	7 4 10^8	3 7 10^{-2}	2 4 10^7	3 7 10^{-2}
		(*S*)	2 2 10^5	< 10^{-5}*	2 3 10^6	< 10^{-5}*
B *n*-$C_6H_{13}CH(CH_3)O(CH_3)P(O)F$	(*R*)	(*R*)	3 9 10^7	7 1 10^{-3}	4 2 10^8	2 1 10^{-3}
	(*R*)	(*S*)	≤ 10^5	—	1 4 10^7	—
	(*S*)	(*R*)	2 9 10^7	3 3 10^{-3}	2 1 10^8	2 2 10^{-4}
	(*S*)	(*S*)	≤ 10^5	—	1 5 10^7	—
C $(CH_3)_3CCH(CH_3)O(CH_3)P(O)F$	(*R*)	(*R*)	2 8 10^7	8 2 10^{-2}	6 4 10^6	1 1 10^{-2}
	(*R*)	(*S*)	≤ 10^4	—	6 4 10^6	< 10^{-5}*
	(*S*)	(*R*)	1 2 10^8	7 4 10^{-2}	8 5 10^7	7 5 10^{-2}
	(*S*)	(*S*)	≤ 10^4	—	2 9 10^5	-

* No decrease in the ability to reactivate during at least 24 h

The enantiomers of Compound A were prepared according to BOTER AND PLATENBURG[5] and were isolated as hydrogen oxalates (*R*)-isomer $[\alpha]^{25}_{578\,m\mu}$ — 25 6 ± 0 3° (*c* 1 04 in methanol), (*S*)-isomer $[\alpha]^{25}_{578\,m\mu}$ + 26 1 ± 0 3° (*c* 1 13 in methanol) The $(R)_C$- and $(S)_C$-isomers of Compounds B and C were prepared by the method of BRYANT *et al* [6], starting from the (*R*)-(—)- and (*S*)-(+)-enantiomers of octanol-2 and pinacolyl alcohol, respectively Compound B $(R)_C$-isomer $[\alpha]^{25}_{578\,m\mu}$ — 14 60 ± 0 03°, $(S)_C$-isomer $[\alpha]^{25}_{578\,m\mu}$ + 14 70 ± 0 03° (neat, 10 cm) Compound C $(R)_C$-isomer $[\alpha]^{25}_{578\,m\mu}$ — 12 78 ± 0 03° and $(S)_C$-isomer $[\alpha]^{25}_{578\,m\mu}$ + 14 42 ± 0 03° (neat, 10 cm)

The $(R)_C$- and $(S)_C$-isomers are mixtures of two diastereoisomers with a different configuration around the phosphorus atom In all but one case, the differences in the rate constants for inhibition are so large that only one isomer phosphonylates the enzyme Considering the results obtained on inhibition of acetylcholinesterase with closely related organophosphorus compounds of known absolute configuration[7], the $(R)_P$-configuration was assigned to the more reactive isomers of Compounds A, B and C

Acetylcholinesterase was a partially purified bovine erythrocyte enzyme (Winthrop Lab Inc) Butyrylcholinesterase was a horse serum enzyme purified according to a modified STRELITZ[8] procedure

Bimolecular reaction constants[9] for inhibition were determined by a ΔpH method according to OOMS AND BOTER[10] Rate constants of aging were calculated

from the decrease in maximally attainable reactivations with time as described previously[3]

The influence of the configuration around the phosphorus atom was demonstrated by the results obtained for Compound A (Table I) In experiments with the (*R*)-isomer, rapid aging obeying first-order kinetics was observed with both enzymes Phosphonylated enzymes obtained with the (*S*)-isomer, however, did not show any aging during 48 h

The influence of the configuration around the *α*-carbon atom was demonstrated using the stereoisomeric forms of Compound B (Table I)

Rate constants of aging of acetylcholinesterase and butylrylcholinesterase inhibited with the $(R)_C(R)_P$-isomer exceeded those obtained with the $(S)_C(R)_P$-isomer This influence is much smaller than that of the configuration around the phosphorus atom This finding was verified by results obtained with the isomers of Compound C (Soman) and acetylcholinesterase (Table I)

Due to lack of preference of butyrylcholinesterase for the $(R)_C(R)_P$- or $(R)_C(S)_P$-isomer of Compound C, a mixture of two phosphonylated enzymes was formed From the initial fast decrease in the attainable reactivation to 50% and after no further decrease for at least 24 h, it is concluded that only one of the phosphonylated enzymes is susceptible to aging (Fig 1a) It is assumed that, analogous to the results obtained with Compound A, in this case the enzyme was phosphonylated by the $(R)_C(R)_P$-isomer

By subtracting the mean value of the percentages of reactivation measured after 25 min of aging from the values determined between 0 and 25 min, an excellent first-order plot was obtained for the aging of butyrylcholinesterase inhibited with the $(R)_C(R)_P$-isomer (Fig 1b) Experiments performed with $(S)_C$-isomers gave the first-order rate constant for the aging of the enzyme phosphonylated by the $(S)_C(R)_P$-isomer The curve obtained for the aging of butyrylcholinesterase inhibited with

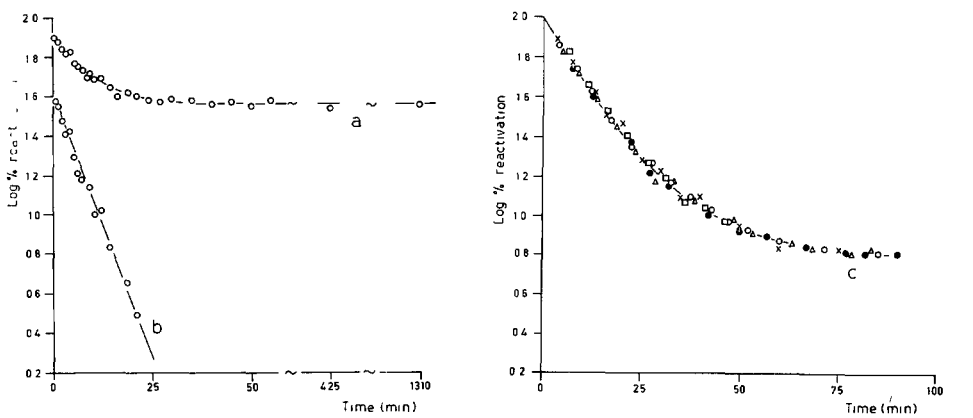

Fig 1 The decrease in the ability to reactivate (pH 7 5 and 25°) butyrylcholinesterase inhibited by stereoisomeric forms of Soman (a) Mixture of $(R)_C(R)_P$- and $(R)_C(S)_P$-isomer (b) $(R)_C(R)_P$-isomer (c) Racemic Soman ○, ×, △, ● and □, results obtained in five experiments On inhibition of the enzyme with an excess of inhibitor, the amounts of the four phosphonylated enzymes are proportional to the rate constants of inhibition of the isomers, assuming that the racemic compound consists of equal amounts of the isomers The curve is calculated by adding the reactivations (in %) of the simultaneously occurring aging reactions

racemic C is in excellent agreement with the curve calculated using the rate constants of inhibition and aging for the different isomers (Fig 1c)

From the influence of the pH on the aging rate, as found by MICHEL *et al* [2] and confirmed by us[11], it was concluded that a protonated group in the enzyme is involved in the aging reaction This group probably forms a hydrogen bond with a phosphorus bound oxygen atom which favors the unimolecular fission of the C–O bond in the alkoxy group RO (Eqn 1) The observed stereospecificity of aging due to the difference in configuration around the phosphorus atom suggests that hydrogen bond formation is not possible in the enzymes phosphonylated by the $(S)_P$-isomers This may be due to a different stereochemical orientation of the phosphonyl moieties of the $(R)_P$- and $(S)_P$-isomers on the enzyme induced by interactions with the active site which prevent free rotation around the P–O serine bond

The small influence of the configuration around the α-carbon atom in the alkoxy group is not unexpected in view of the proposed mechanism Small differences in hydrophobic interactions of the isomeric alkoxy groups with the enzyme might be responsible for the slightly different rates of aging

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