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STEREOSPECIFICITY OF HYDROLYTIC ENZYMES ON REACTION WITH ASYMMETRIC ORGANOPHOSPHORUS COMPOUNDS—III

THE INHIBITION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY ENANTIOMERIC FORMS OF SARIN

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Abstract—Inhibition experiments carried out with acetylcholinesterase from bovine erythrocytes and horse serum butyrylcholinesterase indicate that optically pure (−)-sarin reacts at least 4200 times faster than optically pure (+)-sarin with the first mentioned enzyme, whereas the enantiomers inhibit butyrylcholinesterase with virtually equal rates. The high stereospecificity exhibited by acetylcholinesterase was applied to determine the optical purities of several sarin preparations.

IN CONTINUATION of our studies on the stereospecific inhibition of hydrolytic enzymes[1,2] the inhibitor sarin (isopropyl methylphosphonofluoridate) was used. Michel[3] reported that sarin inhibits electric eel acetylcholinesterase as if two components, being present in equal amounts, react at different rates. The biphasic course of the reaction was interpreted in terms of optical isomerism. Later on Ooms[4] estimated that the ratio of activities (r_a) of the enantiomers of sarin when inhibiting acetylcholinesterase from bovine erythrocytes exceeds a factor of one hundred. In both investigations the inhibitor was used in its racemic form.

Some time ago (+)- and (−)-sarin were synthesized in this laboratory. Stereospecificities of bovine erythrocytes acetylcholinesterase (AChE, EC 3.1.1.7) and horse serum butyrylcholinesterase (BuChE, EC 3.1.1.8) on inhibition with the sarin isomers were mentioned briefly.[5]

This paper describes the inhibition experiments, performed to establish the stereospecificity of both esterases, in detail.

Probably as the result of racemization appearing during isolation the optical purities of the various sarin preparations were less than one hundred per cent. The high stereospecificity shown by AChE was used to determine the respective optical purities.

MATERIALS AND METHODS

Sarin

Three dextrorotatory and two levorotatory samples of sarin were used; further on these will be designated as (+)- and (−)-sarin respectively. The absolute values of

the specific rotations varied from 11·1–14·6° (c 1·5–2·0 in acetone, 25°, λ–578 mμ). Chemical purities were checked by gas–liquid chromatography, using experimental conditions reported before.[6]

For the inhibition experiments stock solutions in dry isopropanol, 0·11–0·14 M in sarin, were prepared. These proved to be optically stable as no changes in optical rotations were observed over periods of several weeks.

Enzymes and inhibition experiments

A detailed description of the enzyme preparations and the procedure used for the determination of the rate constants of inhibition have been reported before.[1]

In the different experiments the initial concentration of AChE in the enzyme–inhibitor mixture (pH 7·7, 25°) varied from $4{\cdot}5 \times 10^{-9}$ M to $5{\cdot}3 \times 10^{-9}$ M, that of (+)-sarin varied from $1{\cdot}5 \times 10^{-8}$ M to $5{\cdot}4 \times 10^{-8}$ M whereas (−)-sarin was used in concentrations ranging from $1{\cdot}3 \times 10^{-9}$ M to $5{\cdot}4 \times 10^{-9}$ M. Rate constants of inhibition were calculated according to second-order kinetics using equation (1).

Studying inhibition of BuChE the initial enzyme concentration ranged from $1{\cdot}1 \times 10^{-8}$ M to $1{\cdot}2 \times 10^{-8}$ M; (+)-sarin and (−)-sarin concentrations varying from $1{\cdot}2 \times 10^{-8}$ M to $1{\cdot}5 \times 10^{-8}$ M were applied. Because of the almost identical concentrations of enzyme and inhibitor the kinetic equation (2) was used.[7]

RESULTS AND DISCUSSION

Acetylcholinesterase. When the enzyme was inhibited with nearly equimolar concentrations of racemic sarin the second-order equation

$$kt = \frac{2{\cdot}303}{a - b} \log \frac{b(a - x)}{a(b - x)} \qquad (1)$$

was used to describe the course of the inhibition process. In equation (1) a and b represent the initial enzyme and inhibitor concentration respectively and x stands for the concentration of enzyme (or inhibitor) which has reacted at time t. Curves were obtained when $\log [(a\text{-}x)/(b\text{-}x)]$ was plotted as a function of time, indicating that the inhibition process did not obey the kinetics of equation (1). According to previous work[3,4] it must be assumed that AChE shows a strong preference for one of the enantiomers of sarin. Thus, half of the actual initial inhibitor concentration (b) may be inserted into equation (1). In this way the forementioned curves turned into straight lines, the slope of which gave the rate constant of inhibition. From a dozen experiments a rate constant $(1{\cdot}4 \pm 0{\cdot}1) \times 10^7$ (l mol^{-1} min^{-1}) was derived, which pertains to the more potent enantiomer. This value was substituted into equation (1) and percentage activity–time curves were constructed. These coincided completely with the experimental curves thus providing additional evidence that under these circumstances the slower reacting isomer of sarin plays a role of minor importance in the inhibition process.

Using the above-mentioned rate constant it was possible to establish which of the enantiomers of sarin is the more active towards AChE. In Fig. 1 the decrease in enzyme activity is plotted as a function of time for representative (+)- and (−)-sarin preparations. In both cases the course of the inhibition experiment is compared with the curve calculated for racemic sarin of the same concentration. It is seen that the (−)-isomer constitutes the more active component in sarin.

From the curves presented in Fig. 1 and similar percentage activity–time relationships obtained for other sarin preparations it is possible to estimate the optical purities of the inhibitor preparations. The percentage of inhibited enzyme has become almost constant after about 2·5 hr; the corresponding concentration equals that of the (−)-isomer present in the inhibitor. This concentration is used as a first approximation for b in equation (1). A more precise value is calculated by numerical variation of b until the slope of the line obtained when $\log[(a-x)/(b-x)]$ is plotted as a function of time corresponds with a rate constant $(1{\cdot}4 \pm 0{\cdot}1) \times 10^7$ (l mol^{-1} min^{-1}). Optical purities thus determined for five sarin preparations are collected in Table 1. A combination of these values with the specific rotations derived from polarimetric experiments gives reasonably consistent values for the calculated rotation of optically pure sarin.

In an attempt to determine the rate constant of inhibition connected with the (+)-isomer the enzyme concentration was raised to $1{\cdot}0 \times 10^{-7}$ M. The enzyme was incubated with $2{\cdot}8 \times 10^{-7}$ M of a sarin sample containing only a small amount (18 per cent) of the (−)-isomer (prep. 3, Table 1). It may be calculated that under

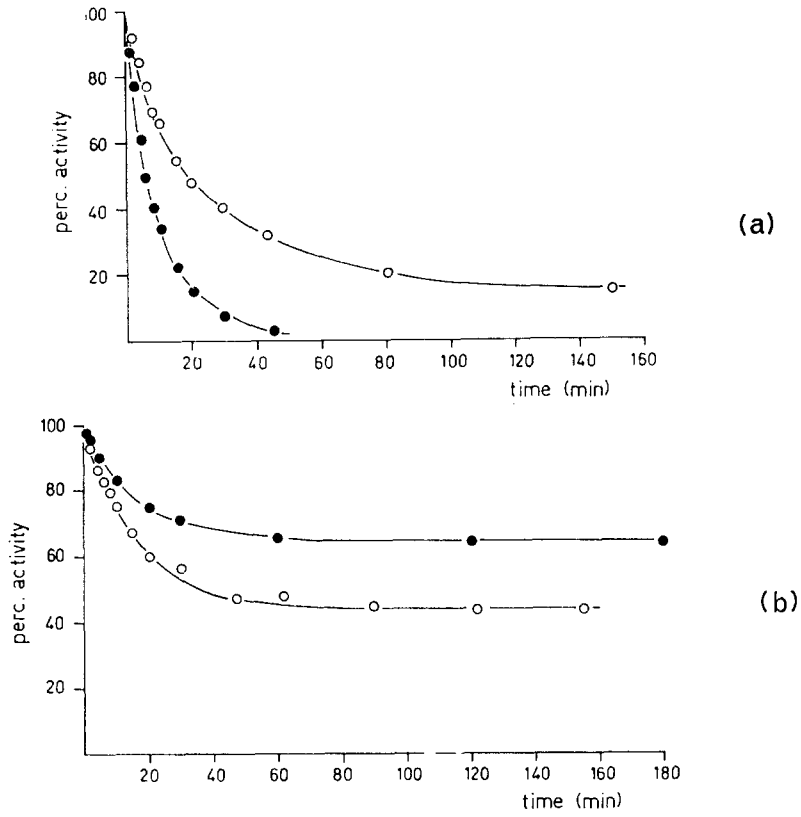

FIG. 1 Experimental curve (○——○) representing (a) the inhibition of AChE ($5{\cdot}3 \times 10^{-9}$M) with (+)—sarin ($2{\cdot}0 \times 10^{-8}$M, prep. 1, Table 1) and (b) the inhibition of this enzyme ($5{\cdot}0 \times 10^{-9}$M) with (—)—sarin ($3{\cdot}5 \times 10^{-9}$M, prep. 5, Table 1). Calculated curves (●——●) for racemic sarin [(a): $2{\cdot}0 \times 10^{-8}$M, (b):$3{\cdot}5 \times 10^{-9}$M] are included.

TABLE 1. COMPARISON OF THE SPECIFIC ROTATIONS OF VARIOUS SARIN PREPARATIONS WITH THEIR OPTICAL PURITIES* OBTAINED FROM INHIBITION EXPERIMENTS, AFFORDING PREDICTED VALUES FOR THE SPECIFIC ROTATION OF OPTICALLY PURE SARIN

prep.	$[\alpha]_{578}^{25}$†	opt. purity (%)	$[\alpha]_{578}^{25}$ opt. pure sarin
1	+ 11·1 ± 0·6	54 ± 0·5	+ 20·6 ± 1·1
2	+ 14·6 ± 0·7	76 ± 0·5	+ 19·2 ± 0·9
3	− 11·7 ± 0·5	64 ± 0·5	− 18·3 ± 0·9
4	− 11·0 ± 0·8	56 ± 1·5	− 19·6 ± 1·6
5	− 12·0 ± 0·6	58 ± 2·0	− 20·7 ± 1·3
		mean abs. value	19·7 ± 0·5

* The optical purity or enantiomeric purity is defined as the excess of one enantiomer over the other expressed as a percentage of the total.
† Conc. 1·5–2·0 g/100 ml in acetone.

these circumstances 50 per cent of the enzyme will be inhibited by the (−)-isomer within a few minutes, leaving the same percentage of enzyme for inhibition by the (+)-isomer. The latter inactivation process was followed for 6 hr from which a rate constant $3{\cdot}3 \times 10^3$ (l mole^{-1} min^{-1}) was calculated as a mean value of two experiments. This rate constant is due to the (+)-isomer itself or reflects the reactivity of the (−)-isomer formed by racemization of the (+)-enantiomer during the experiment. Bearing in mind the susceptibility of sarin to racemization[5] this possibility cannot be excluded. In that case the rate constant for the (+)-isomer will be even lower. It may be concluded that the ratio of activity (r_a) of the sarin isomers towards AChE equals or exceeds a factor of 4200.

Butyrylcholinesterase. The enzyme was incubated with the optically active sarin preparations summarized in Table 1 and racemic sarin using concentrations mentioned in the preceding section. According to the kinetic equation[7]

$$kt = \frac{1}{d - x} - \frac{1}{d} \tag{2}$$

where d represents the mean value of the almost equal concentrations of enzyme and inhibitor, it was found that in all cases straight lines were obtained when $1/d - x$ was plotted as a function of time until most of the enzyme activity had disappeared. From nine inhibition experiments a mean rate constant $(6{\cdot}0 \pm 0{\cdot}6) \times 10^6$ (l mol^{-1} min^{-1}) was derived. It follows that BuChE shows no measurable preference for one of the stereoisomeric forms of sarin. This result is in agreement with the observation that on inhibition of the enzyme with an excess of racemic sarin roughly equal amounts of the two possible stereoisomeric phosphonylated enzymes were formed.[9]

Summarizing, it may be concluded that AChE is substantially more stereospecific than BuChE on inhibition with sarin.† This phenomenon indicates that the steric requirements of the active site of the enzyme are much more stringent in the case of AChE. Results obtained using S-alkyl *p*-nitrophenyl methylphosphonothioates[1] and isopropyl S-2-subst.ethyl methylphosphonothioates[10] as inhibitors substantiate this idea.

† The toxicities of the enantiomers of sarin parallel the AChE inactivation rates. In the Medical-Biological Laboratory of the National Defence Research Organization T.N.O., LD_{50}'s (s.c. rat, ♀, after 24 hr) were determined according to Litchfield and Wilcoxon[8] for three sarin samples : prep. 2 [12 per cent (−)-isomer] 359 μg/kg (326–395), rac. mixture [50 per cent (−)-isomer] 185 μg/kg (173–198), prep. 5 [79 per cent (−)-isomer] 97 μg/kg (89–106). Figures in parentheses refer to 95 per cent confidence-limits.

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