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INHIBITION OF ACETYLCHOLINESTERASE BY THE ENANTIOMERS OF ISOPROPYL *S*-2-TRIMETHYLAMMONIOETHYL METHYLPHOSPHONOTHIOATE IODIDE. AFFINITY AND PHOSPHORYLATION CONSTANTS

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SUMMARY

The rate of inhibition of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) by the enantiomers of isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide was measured in the presence of the substrate phenyl acetate.

It was shown from the aging reaction of the phosphorylated acetylcholinesterase formed on incubation of the enzyme with a preparation of the less reactive (+)-enantiomer, that 60% of the enzyme was phosphorylated by the (+)-enantiomer and 40% by the (−)-enantiomer.

The dissociation constant of the enzyme–inhibitor complex K_d, the phosphorylation rate constant k_p and the bimolecular rate constant k_i for the (+)-enantiomer were estimated from the combined results of inhibition and aging experiments and are $1.9 \cdot 10^{-4}$ M, 2.6 min^{-1} and $1.4 \cdot 10^{4}$ $l \cdot mole^{-1} \cdot min^{-1}$, respectively.

The amount of the (−)-enantiomer in the preparation of the (+)-enantiomer turned out to be only 0.02%.

The values of K_d, k_p and k_i for the (−)-enantiomer were determined directly from the inhibition reaction with the (−)-enantiomer and are $3.5 \cdot 10^{-7}$ M, 15 min^{-1} and $4.3 \cdot 10^{7}$ $l \cdot mole^{-1} \cdot min^{-1}$, respectively.

INTRODUCTION

In the course of investigations concerning the properties of the active sites of esterases carried out in this laboratory, Boter and Ooms[1-4] studied the stereospecificity exhibited by various esterases with regard to their reaction with optically active organophosphorus compounds. In these studies the ratio (r_a) of the bimolecular rate constants (k_i) of the inhibition reaction obtained for the stereoisomers of an organophosphorus compound was taken as a measure for stereospecificity. In the case of inhibition of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7)

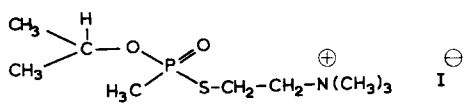
by enantiomers of these compounds large values of r_a were observed; *e.g.* an r_a value of 4200 was reported for isopropyl methylphosphonofluoridate (Sarin).

The inhibition reaction of cholinesterase with methanesulphonates[5] and with organophosphorus compounds[6] was shown to proceed *via* two steps: the formation of an enzyme–inhibitor complex EI controlled by the dissociation constant $K_d (= k_{-1}/k_1)$, followed by sulphonylation or phosphorylation, respectively, leading to an irreversible inhibited enzyme EP.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_p} EP \qquad (1)$$

When the inhibitor concentration is far below the value of K_d, the overall inhibition rate is determined by the bimolecular rate constant k_i which equals k_d/K_d. This condition was fulfilled in the experiments of Boter and Ooms.

The present study was undertaken to determine the influence of asymmetry around the central P atom in an organophosphorus inhibitor on the affinity ($1/K_d$) of acetylcholinesterase for these compounds as well as on the rate of phosphorylation (k_p). Isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide (I) was chosen as the inhibitor for this study, since a high r_a value has already been observed for this compound[4] and the resolved enantiomers can be synthesized in a high degree of optical purity. The rate of inhibition of acetylcholinesterase by the (+)-



I

and (−)-enantiomers of I was followed spectrophotometrically in the presence of the substrate phenyl acetate by means of a method reported previously[7].

Owing to the high r_a value of I, incubation of acetylcholinesterase with a large excess of a preparation of the less reactive enantiomer, as necessary for the determination of K_d and k_p, could result in a partial or complete inhibition of the enzyme with the more reactive enantiomer, depending on the optical purity of the preparation. The high stereospecificity of the aging reaction of phosphorylated enzymes[8] was used to obtain more information about the optical purity of the preparation of the less reactive enantiomer. The optical purity of this preparation and the values of K_d and k_p of the less reactive enantiomer were estimated from the combined results of aging and inhibition experiments with the preparation of the less reactive enantiomer.

MATERIALS AND METHODS

Materials

Bovine erythrocyte acetylcholinesterase was obtained from Winthrop Laboratories. The synthesis of the (+)- and (−)-enantiomers of isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide [$(\alpha)_D^{25}$ (+22.8 ± 0.3)° and (−22.6 ± 0.3)° (c = 1, methanol), respectively] was described previously[4].

All chemicals employed were of analytical grade. The phosphate buffer was prepared by titrating 0.05 M Na_2HPO_4 with 0.05 M KH_2PO_4 to pH 7.0.

Inhibition experiments

All experiments were carried out at 5 °C in 0.05 M phosphate buffer (pH 7.0) containing 2.5% methanol. A general description of the method was given previously[7]. Phenyl acetate concentrations of $4 \cdot 10^{-3}$ M and $3 \cdot 10^{-3}$ M were used in the case of the (−)- and (+)-enantiomer, respectively. The final enzyme concentration was 2.2 units/ml corresponding with approx. $4.8 \cdot 10^{-9}$ M of active centers. Amounts of 20 to 100 μl of the inhibitor solution made in water–methanol mixtures were added to the substrate solution (1.9–2.0 ml). The cuvette was placed in a thermostated holder equipped with a magnetic stirring motor. After addition of the enzyme solution (0.1 ml) rapid mixing was obtained by using a magnetic stirrer as described by Conrad[9]. Mixing times of approx. 1.5 s were obtained. A 0.1-s impulse-sender (Sodeco, Genève, KN 561) was used in the programmer as described previously[7], in order to be able to take readings of the absorbance as fast as every 0.5 s.

The rate constants of the inactivation (k_{obs}) were calculated according to Eqn 4: $\ln(P_\infty - P_t) = \ln P_\infty - k_{obs}t$ (see Derivation of kinetic equations). Values of k_{obs} were calculated from plots of $\ln(P_\infty - P_t)$ against t by means of the method of least squares. If P_∞, the concentration of hydrolyzed substrate formed after complete reaction, could not be determined, k_{obs} was calculated by using the method of Swinbourne[10].

The K_m value of phenyl acetate determined in the absence of inhibitor was $5.2(\pm 0.8) \cdot 10^{-4}$ M.

Aging experiments

The aging experiments were carried out according to the method given by Benschop and Keijer[11]. To form a phosphorylated enzyme with the preparation of the (+)-enantiomer, acetylcholinesterase was inhibited under conditions identical with those of the inhibition experiments. No detectable enzyme activity was left after incubation for 5 min with the preparation of the (+)-enantiomer ($9 \cdot 10^{-4}$ M) in the presence of phenyl acetate. The excess of inhibitor and the substrate were removed on a Sephadex G-25 column at 5 °C, pH 9.0. The aging reaction was performed at 35 °C, pH 6.0. More than 90% of reactivation was achieved by incubation of the inhibited enzyme for 24 h at 25 °C, pH 7.6, in the presence of $5 \cdot 10^{-2}$ M monoisonitrosoacetone.

DERIVATION OF KINETIC EQUATIONS

Kinetic equations were derived assuming the substrate and inhibitor concentration to be constant during the reactions.

Inhibition by one inhibitor in the presence of substrate

The inhibition of acetylcholinesterase in the presence of a substrate can be represented by the following reaction scheme:

$$\text{Products} \leftarrow ES \leftrightarrows S + E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_p} EP \qquad (2)$$

in which E, I, S, EI, EP and ES stand for free enzyme, inhibitor, substrate, enzyme–inhibitor complex, phosphorylated enzyme and enzyme–substrate complex, respec-

tively. The rate of inactivation of the enzyme can be described by Eqn 3*, derived by Smissaert[12] on the basis of Scheme 2.

$$\ln (P_\infty - P_t) = \ln P_\infty - \frac{k_p I}{K_d(1 + S/K_m) + I} t \tag{3}$$

The dissociation constant K_d is defined as $K_d = k_{-1}/k_1$, K_m is the Michaelis constant for the substrate used, and P_t and P_∞ are the concentrations of hydrolyzed substrate formed at time t and after complete reaction, respectively.

Eqn 3 can be written in the form

$$\ln(P_\infty - P_t) = \ln P_\infty - k_{obs} t \tag{4}$$

by introduction of k_{obs} as the rate constant of inactivation of the enzyme

$$k_{obs} = \frac{k_p I}{K_d(1 + S/K_m) + I} \tag{5}$$

A more general expression for Eqn 5 is given by Eqn 6

$$k_{obs} = \frac{A I}{B + I} \tag{6}$$

where $A = k_p$ (7)

$$B = K_d (1 + S/K_m) \tag{8}$$

When $I \ll K_d(1 + S/K_\infty)$, k_{obs} becomes proportional to the inhibitor concentration and the bimolecular rate constant $k_i = k_p/K_d$ governs the reaction.

Inhibition by a mixture of two inhibitors in the presence of substrate

The procedure used by Smissaert to obtain the kinetic equations for Scheme (2) was extended for the derivation of the kinetics for the following scheme:

$$\begin{array}{l} \text{Products} \leftarrow ES \rightleftarrows S + E \\ \qquad\qquad\qquad\qquad\quad + I_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI_1 \xrightarrow{k_{p1}} EP_1 \\ \qquad\qquad\qquad\qquad\quad + I_2 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI_2 \xrightarrow{k_{p2}} EP_2 \end{array} \tag{9}$$

The concentrations of the enzyme–inhibitor complexes and of the enzyme–substrate complex as fraction of the total enzyme concentration are given by

$$\frac{EI_1}{E_0} = \frac{E \cdot I_1}{E_0 K_{d1}} \tag{10}$$

$$\frac{EI_2}{E_0} = \frac{E \cdot I_2}{E_0 K_{d2}} \tag{11}$$

$$\frac{ES}{E_0} = \frac{E \cdot S}{E_0 K_m} \tag{12}$$

where $E_0 = E + EI_1 + EI_2 + EP_1 + EP_2 + ES$, and $K_{d1} = k_{-1}/k_1$ and $K_{d2} = k_{-2}/k_2$.

* Brackets indicating concentrations have been omitted in this and the following equations.

The total concentration of the enzyme, which has not been irreversibly inhibited, will be represented by ε

$$\varepsilon = \frac{E + EI_1 + EI_2 + ES}{E_0} = 1 - \frac{EP_1}{E_0} - \frac{EP_2}{E_0} \tag{13}$$

By substitution of the Eqns 10, 11 and 12 in Eqn 13 it follows that

$$\varepsilon = \frac{K_{d1}\, EI_1\, D}{I_1 \cdot E_0} = \frac{K_{d2}\, EI_2\, D}{I_2 \cdot E_0} \tag{14}$$

where D stands for $1 + I_1/K_{d1} + I_2/K_{d2} + S/K_m$.
The phosphorylation rates are

$$\frac{d\, EP_1}{dt} = k_{p1}\, EI_1 \quad \text{and}$$

$$\frac{d\, EP_2}{dt} = k_{p2}\, EI_2, \quad \text{or}$$

$$\frac{d\, EP_1/E_0}{dt} = k_{p1}\frac{EI_1}{E_0} \quad \text{and} \tag{15}$$

$$\frac{d\, EP_2/E_0}{dt} = k_{p2}\frac{EI_2}{E_0} \tag{16}$$

Dividing of Eqn 15 by Eqn 16 followed by integration, leads to Eqn 17 with the aid of Eqn 14. For k_{p1}/K_{d1} and k_{p2}/K_{d2} the bimolecular rate constants k_{i1} and k_{i2} are introduced.

$$\frac{EP_1}{EP_2} = \frac{k_{i1}\, I_1}{k_{i2}\, I_2} \tag{17}$$

Summation of the Eqns 15 and 16 and substitutions for

$\frac{EI_1}{E_0}$ and $\frac{EI_2}{E_0}$ according to Eqn 14 give

$$\frac{d\, EP_1/E_0}{dt} + \frac{d\, EP_2/E_0}{dt} = \frac{d(EP_1 + EP_2)/E_0}{dt} = -\frac{d\varepsilon}{dt} = \frac{k_{i1}\, I_1 + k_{i2}\, I_2}{D}\,\varepsilon \tag{18}$$

Integration leads to

$$\ln \varepsilon = -\frac{k_{i1}\, I_1 + k_{i2}\, I_2}{D}\, t \tag{19}$$

From the Eqns 13, 14 and 19 it follows that

$$1 - \frac{EP_1}{E_0} - \frac{EP_2}{E_0} - \frac{EI_1}{E_0} - \frac{EI_2}{E_0} = \frac{E + ES}{E_0} = (1 - I_1/K_{d1} - I_2/K_{d2})\, \exp.\left(-\frac{k_{i1}\, I_1 + k_{i2}\, I_2}{D}\, t\right) \tag{20}$$

Since the rate of the substrate splitting is proportional to $E + ES$ according to the Michaelis–Menten equation, the quotient of the rate of the substrate splitting in the presence of inhibitor (v_i) divided by the rate of the substrate splitting in the absence of inhibitor (v_0) can be substituted for $(E + ES)/E_0$. Consequently,

$$v_i = \frac{dP}{dt} = v_0(1 - I_1/K_{d1}D - I_2/K_{d2}D)\, \exp.\left(-\frac{k_{i1}\, I_1 + k_{i2}\, I_2}{D}\, t\right) \tag{21}$$

In this equation P represents the concentration of one of the products formed after substrate hydrolysis (alcohol or acid). Integration of Eqn 21 leads to

$$P_t = \frac{v_0(D - I_1/K_{d1} - I_2/K_{d2})}{k_{i1} I_1 + k_{i2} I_2} \left[1 - \exp.\left(-\frac{k_{i1} I_1 + k_{i2} I_2}{D} t\right)\right] \tag{22}$$

When $t = \infty$,

$$P_\infty = \frac{v_0(D - I_1/K_{d2} - I_2/K_{d2})}{k_{i1} I_1 + k_{i2} I_2}$$

Hence,

$$\ln (P_\infty - P_t) = \ln P_\infty - \frac{k_{i1} I_1 + k_{i2} I_2}{D} t \tag{23}$$

From Eqn 23 it follows that the inactivation of the enzyme according to Scheme 2 and to Scheme 9 can be described by the same general formula

$$\ln(P_\infty - P_t) = \ln P_\infty - k_{obs} t \tag{4}$$

The rate constant of enzyme inactivation according to Scheme 9 is

$$k_{obs} = \frac{k_{i1} I_1 + k_{i2} I_2}{D} \tag{24}$$

In the present study the ratio of the inhibitor concentration in the preparation of the less reactive enantiomer is not exactly known. However, since the less reactive inhibitor (I_2) is present in a large excess to the more reactive inhibitor (I_1), the concentration of the less reactive inhibitor can be set equal to the known total concentration of the inhibitors (I). Hence, Eqn 24 will be rewritten as a relation between k_{obs} and the concentration of the less reactive inhibitor only, in order to be able to carry out a mathematical analysis of inhibition experiments with the preparation of the less reactive inhibitor. By introduction of the constant q which is equal to $k_{i1} I_1 / k_{i2} I_2$, it follows from Eqn 24 that

$$k_{obs} = (q + 1) \frac{k_{i2} I_2}{D} = (q + 1) \frac{k_{i2} I}{D} \tag{25}$$

According to Eqn 17 q is equal to the value of EP_1/EP_2. In the present study its value was determined from the results of aging experiments. Eqn 25 can be rewritten to a more general formulation as given by

$$k_{obs} = \frac{A I}{B + I}$$

where I is the total concentration of the inhibitor in the mixture and approximately equal to I_2, and

$$A = \frac{(q + 1) k_{p1}}{q k_{p2} + k_{p1}} k_{p2} \tag{26}$$

$$\text{and } B = \frac{k_{p1}}{q k_{p2} + k_{p1}} (1 + S/K_m) K_{d2} \tag{27}$$

RESULTS

Aging experiments

Acetylcholinesterase phosphorylated with the more reactive (−)-enantiomer of isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide showed rather slow aging obeying first-order kinetics. The half-life time of the reaction performed at 35 °C and pH 6.0, was 144 min. A biphasic course, as given in Fig. 1, was found for the aging reaction of acetylcholinesterase inhibited by the preparation of the less reactive (+)-enantiomer. No further decrease in the attainable reactivation of the inhibited enzyme was observed after 21 h of aging. A first-order plot was obtained for the first part of the curve when the mean value of the enzyme activity measured after 21 h of aging was subtracted from the values obtained between 0 and 6 h (Fig. 1). The half-life time calculated from this plot, 148 min, is consistent with that found for the aging of acetylcholinesterase inhibited by the (−)-enantiomer.

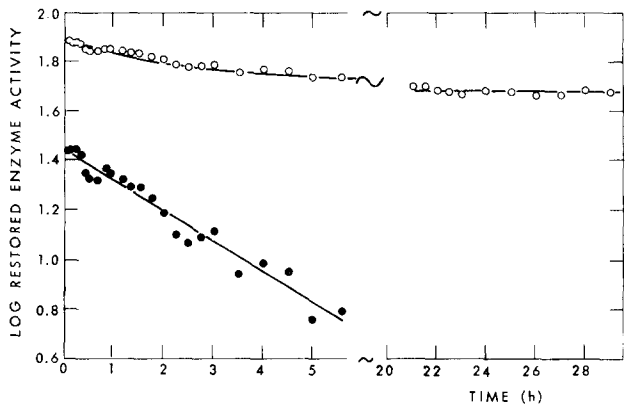


Fig. 1. Dependence of restored enzyme activity in arbitrary units on time of incubation at pH 6.0 and 35 °C of acetylcholinesterase inhibited with the preparation of the (+)-enantiomer of isopropyl *S*-2-trimethylammonioethyl methylphosphonothioate iodide (○—○). ●—●, restored enzyme activities diminished with the mean value of the restored enzyme activities measured after 21 h of incubation.

Keijer and Wolring[8] observed almost absolute stereospecificity of the aging reaction of phosphorylated acetylcholinesterase. From their study it appears that acetylcholinesterase inhibited by the less reactive stereoisomer of organophosphorus compounds having the phosphorus atom as a centre of chirality does not show any aging during at least 48 h. Hence, we concluded that two phosphorylated enzymes are formed after inhibition of acetylcholinesterase with the preparation of the less reactive enantiomer. 64% of the enzyme was inhibited by the less reactive (+)-enantiomer (no aging) and 36% by the more reactive (−)-enantiomer. Hence, $EP_1/EP_2 = 0.56$.

Inhibition experiments

Values of k_{obs} obtained in inhibition experiments with the (−)-enantiomer and with the preparation of the (+)-enantiomer are listed in Table I. It has already been

TABLE I

RATES OF INACTIVATION (k_{obs}) OF ACETYLCHOLINESTERASE DETERMINED FROM INHIBITION EXPERIMENTS WITH THE (−)-ENANTIOMER AND WITH THE PREPARATION OF THE (+)-ENANTIOMER OF ISOPROPYL *S*-2-TRIMETHYLAMMONIOETHYL METHYLPHOSPHONOTHIOATE IODIDE

The experiments were carried out in 0.05 M phosphate buffer, pH 7.0, containing 2.5% methanol, in the presence of the substrate phenyl acetate at 5 °C. Each value is the mean of three measurements. By fitting these data to the formula $k_{obs} = A\,I/(B + I)$ (Eqn 6) the values of A and B with their standard errors were calculated.

(−)-Enantiomer		*Preparation of the (+)-enantiomer*	
Inhibitor concn (μM)	k_{obs} *(min⁻¹)*	*(+)-Enantiomer (mM)*	k_{obs} *(min⁻¹)*
1.20	4.05	1.50	2.06
1.05	3.85	1.35	2.12
0.90	3.66	1.20	1.80
0.75	2.71	1.05	1.82
0.60	2.49	0.90	1.49
0.45	1.79	0.75	1.62
0.30	1.36	0.60	1.21
A (min^{-1})	15 ± 5	3.7 ± 0.7	
B (M)	$(31 \pm 13)\cdot 10^{-7}$	$(12 \pm 4)\cdot 10^{-4}$	

demonstrated[7] that the inhibition of acetylcholinesterase by the (−)-enantiomer carried out in the presence of the substrate phenyl acetate can be described by a reaction scheme in which the inhibitor interacts with the free enzyme only. By analogy with these results we can also apply Scheme 9 on the reactions of the preparation of the (+)-enantiomer.

TABLE II

DISSOCIATION CONSTANTS (K_d), RATE CONSTANTS OF PHOSPHORYLATION (k_p) AND BIMOLECULAR RATE CONSTANTS (k_i) FOR THE INHIBITION OF ACETYLCHOLINESTERASE BY THE ENANTIOMERS OF ISOPROPYL *S*-2-TRIMETHYLAMMONIOETHYL METHYLPHOSPHONOTHIOATE IODIDE IN 0.05 M PHOSPHATE BUFFER, pH 7.0, CONTAINING 2.5% METHANOL, AND AT 5 °C

	k_p *(min⁻¹)*	K_d *(M)*	k_i *(l·mole⁻¹·min⁻¹)*
(−)-Enantiomer	15	$3.5\cdot 10^{-7}$	$4.3\cdot 10^{7}$
(+)-Enantiomer	2.6	$1.9\cdot 10^{-4}$	$1.4\cdot 10^{4}$

Hence, the values of k_{obs} were fitted to Eqn 6: $k_{obs} = A\,I/(B + I)$ obtained on the basis of Scheme 2 and 9, according to the non-linear regression method of Wilkinson[13]. The values of k_p, K_d and $k_i (= k_p/K_d)$ for both enantiomers were found by inserting the values obtained for A and B in Eqns 7 and 8, and 26 and 27 (Table II). The values of K_m ($5.2\cdot 10^{-4}$ M), $q (= k_{i1}\,I_1/k_{i2}\,I_2 = EP_1/EP_2 = 0.46)$ and k_{p1} (15 min^{-1}) used in these calculations were obtained from experiments in the absence of inhibitor, aging experiments, and inhibition experiments with the (−)-enantiomer, respectively.

By substituting the values obtained for k_{i1} and k_{i2} in Eqn 17 it follows that

$I_1/I_2 = 2 \cdot 10^{-4}$. In this paper the kinetic equations were derived on the condition that the concentrations of substrate and inhibitors are constant during the reactions. Although the amount of the (−)-enantiomer in the preparation of the (+)-enantiomer is only 0.02%, the (−)-enantiomer was present in all inhibition experiments with the preparation of the (+)-enantiomer in a sufficient large excess with regard to the enzyme to fulfill this condition.

DISCUSSION

The pronounced stereospecific behaviour of acetylcholinesterase was already shown by Boter[4] by comparison of the bimolecular rate constants for the inhibition by enantiomers of a number of organophosphorus compounds. The question arises, if stereochemical factors are critical in the binding of the inhibitor to the enzyme or in the subsequent phosphorylation reaction.

The large ratio of the K_d values in the present study suggests that in the binding step the (−)-enantiomer presents a more favourable complementary conformation to the active site of the enzyme than the (+)-enantiomer. Thus, the free energy of the enzyme-inhibitor complex of the (−)-enantiomer is lower than that of the (+)-enantiomer. The slight difference between the k_p values indicates that starting from the more stable enzyme–inhibitor complex the phosphorylation reaction proceeds at least as fast as by starting from the less stable complex with the (+)-enantiomer. Consequently, the free energy of the transition state of the phosphorylation reaction with the (−)-enantiomer is decreased with regard to that with the (+)-enantiomer. Hence, the stereospecific behaviour of the enzyme with respect to this inhibitor is also based on stereochemical factors stabilizing the transition state of the phosphorylation step.

The reaction of α-cyclodextrin (cyclohexa-amylose) with organophosphorus compounds shows striking correspondence to the inhibition reaction of acetylcholinesterase. Complex formation and subsequent phosphorylation as well as stereospecific behaviour of α-cyclodextrin were observed by Van Hooidonk en Breebaart-Hansen[14] in their investigations with isopropyl methylphosphonofluoridate (Sarin). In that case, however, the enantiomer having a higher affinity phosphorylates α-cyclodextrin at a slower rate. The difference with the present data suggests that the more flexible and complex enzyme structure contributes more efficiently to the acceleration of the reaction with organophosphorus compounds than the rigid and rather small α-cyclodextrin molecule.

The determination of optical purity of the preparation of the less reactive enantiomer by a combination of inhibition and aging experiments holds also for inhibition reactions carried out at low inhibitor concentrations. With the aid of Eqn 17 and 25, where D becomes equal to $1 + S/K_m$ in that case (Eqn 14), the ratio of I_1/I_2 could be calculated from the data of aging and inhibition experiments.

The degree of the optical purity of resolved inhibitors is not limiting in the determination of bimolecular rate constants. Boter and Van Dijk[3] determined the high ratio of k_i values for the reaction of acetylcholinesterase with the enantiomers of Sarin from the inhibition by the racemic mixture only. If, however, K_d and k_p values are to be measured, a very high degree of optical purity is required for the less reactive enantiomer, which is the more important as stereospecificity increases. The desired purity will probably be obtainable only for a few selected inhibitors. Hence,

it will be very difficult to investigate the more general validity of the present results for acetylcholinesterase inhibition.

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