Acetylcholinesterases of Organophosphate-Susceptible and -Resistant Spider Mites

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Cholinesterases of susceptible (S) and organophosphate resistant (R) spider mites were compared with respect to a series of thioester substrates and irreversible inhibitors. The pH dependence of the rate of inhibition was studied with some of them. Bovine erythrocyte AChE has been involved in part of the comparison. Both mite enzymes show the phenomenon of substrate inhibition. The results suggest that the specificity of acetylation for the leaving group of the substrate is rather low and that deacetylation is probably more or less rate limiting in acetic ester hydrolysis. Irreversible inhibition of the R-enzyme seems to be impeded most seriously by the presence of a bulky group in the acyl moiety. The essential alteration in the R-enzyme is thought to be a slightly different position of an imidazole residue relative to the serine hydroxyl involved in the reaction.

The acetylcholinesterase (AChE) of an organophosphate resistant strain (R) of spider mites (*Tetranychus urticae* Koch) has been shown to be less sensitive to *in vitro* inhibition by these compounds than that of a normal susceptible strain (S). Moreover, the rate of substrate hydrolysis per milligram of tissue has been shown to be relatively low in AChE-preparations of the R-strain. (Smissaert, 1964, 1965a,b; Helle, 1965; Voss and Matsumura, 1964, 1965; Zahavi and Tahori, 1968.)

The purpose of further work is twofold. First, a knowledge of reactivities in both enzymes will be helpful in a more rational search for better and more specific pesticides for the control of organophosphate-susceptible and -resistant spider mites. The second aim is to find out the cause of the lower reactivity of the R-enzyme with substrates and irreversible inhibitors. Since the extremely high reaction velocities provide a key problem in enzyme chemistry, the answer would certainly add to the insight into the importance of the factors which jointly are capable to effect the high catalytic rates. This paper provides an initial contribution to the first point and a working hypothesis for the second. A further paper, in preparation, will deal with pH effects on substrate hydrolysis and reversible inhibition by ammonium ions (Smissaert, 1969).

MATERIALS AND METHODS

Strains and Culture. The two strains employed, originate from "Bayer" Leverkusen, Germany, and have been described before (Smissaert, 1964). Mites were bred on beanplants at 25 °C. using a 16-hour photoperiod. Mobile stages of both sexes were collected by an air suction device and stored at −15 °C.

Enzyme Assay. The rate of substrate hydrolysis of the thioesters was measured by the Ellman technique (1961). On complete hydrolysis of $5.10^{-5}M$ solutions of the substrates used, the final optical densities were always found to be about 0.680 as expected. Thus, the thiols produced react completely with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction mixture (0.7 ml.) was made in a semimicrocell (10-mm. lightpath) and the rate of increase in O.D. at 412 mμ was measured by the aid of a Beckman-B or -DBG spectrophotometer connected with a recorder. Blanks for spontaneous hydrolysis and reaction of protein sulfhydryl groups were run and normally found to be negligible. Routinely two enzyme concentrations differing by a factor of two were applied. The final concentrations of a typical reaction mixture were: Acetylthiocholine iodide (ASCh) = $5 \times 10^{-4}M$; DTNB = $3 \times 10^{-4}M$; sodium pyrophosphate-HCl buffer pH 7.0 = $0.05M$; sodium chloride = $0.07M$; ionic strength $\mu \simeq 0.4$. The temperature was kept at 33 °C. In the experiments on the influence of substrate concentration, that of the iodine ion had been kept constant by addition of sodium iodide.

Irreversible Inhibition was studied by preincubation of the enzyme preparation with varying inhibitor concentrations in sodium pyrophosphate buffer pH 7.5 at 27 °C. Stock solutions of less water soluble inhibitors were made in acetone, while the final acetone concentration during inhibition was 0.5%.

Samples were taken after periods from 2 to 30 minutes, diluted at least sevenfold by the substrate reaction mixture (ASCh = $3 \times 10^{-4}M$) and assayed immediately as described. The linear OD-time records showed the inhibition to be constant during assay in all cases except that after physostigmine inhibition.

Standard Enzyme Preparation. Unless stated otherwise, the enzyme preparation was obtained as follows: Homogenates of 200 mg. of mites per milliliter of deionized water were made by the aid of an icecold Potter Elvehjem all glass homogenizer. After centrifugation (30 minutes, 170,000 × g) the precipitate, containing about 70% of total AChE-activity, was mixed with an excess of toluene and stored at 2 °C. for a few days with several replacements of the toluene. The dry material was then homogenized in sodium pyrophosphate buffer pH 7.5 ($0.08M$)-3% sodium chloride and shaken for about an hour at 31 °C. The supernatant (30 minutes, 170,000 × g) was dialyzed overnight in Sartorius collodion bags (Cat. No. 15100) under reduced pressure. The toluene treatment removes lipophilic material and most of the colored substances while the extraction procedure at 31 °C. causes some denaturation of labile nonspecific esterases. Specific activity was yet extremely low—*e.g.* 0.02 μmol ASCh hydrolyzed per minute per milligram protein for the S- and usually up to three times lower in R-preparations.

In some experiments, partly purified preparations were used as indicated. Since the procedure has not yet been standardized, only a brief description follows. Starting with the enzyme preparation described, the following steps were applied. Heat denaturation in the presence of metahydroxyphenyltrimethylammoniumbromide and other quaternary reversible inhibitors of AChE. Column chromatography over benzylated diethyl-amino-ethyl cellulose (Kremzner and Wilson, 1963), Sephadex G 200 and carboxymethyl cellulose

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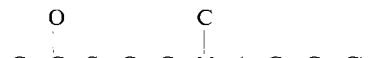
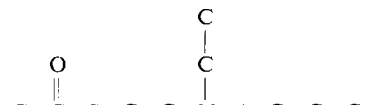
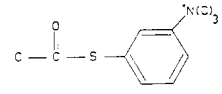
Table I. Approximate Values of V_m and K_m for Three Enzymes Indicated[a]

No.	Substrate Structure (H's omitted)	S-enzyme V_m	S-enzyme $K_m(M)$	R-enzyme V_m	R-enzyme $K_m(M)$	Erythrocyte-enzyme V_m	Erythrocyte-enzyme $K_m(M)$
1		1.0	3×10^{-5}	1.0	5×10^{-5}	1.0	7×10^{-5}
2		0.89	1×10^{-4}	0.88	2×10^{-4}	0.02	5×10^{-5}
3		0.90	4×10^{-4}	0.90	8×10^{-4}	0.03	6×10^{-5}
4		0.82	2×10^{-4}	0.74	3×10^{-4}	0.23	2×10^{-3}
5		0.78	2×10^{-4}	0.85	2×10^{-4}	0.05	1×10^{-3}
6		0.75	3×10^{-5}	0.27	2×10^{-4}	0.35	1.5×10^{-4}
7		1.47	3×10^{-5}	0.76	2×10^{-4}	0.62	2×10^{-4}
8		0.19	7×10^{-5}	0.36	3×10^{-3}	0.02	5.5×10^{-4}

[a] V_m values for ASCh had been taken as unity for each of the enzymes. Conditions: pH 7.0; temp. 33 °C; ionic strength $\mu \simeq 0.4$. A slightly purified and a standard preparation was used in the experiments with the R- and S-enzyme, respectively. Numbers of the substrates refer to the names in the section materials.

in the usual way. The purification factor was rather disappointing and varied up to about 100-fold. The main purpose was the removal of nonspecific esterases, but the result was, so far, not quite successful.

Substrates. The numbers in parentheses refer to those in Table I. Compounds 2, 3, 4, 5, 6, and 9 are not known from the literature.

(1) (2-Mercaptoethyl)-trimethylammonium iodide acetate and

(7) (2-mercaptoethyl)-trimethylammonium iodide propionate were made by published procedures (Alberton and Clinton, 1945; Clinton *et al.*, 1948; Hansen, 1957).

(2) (2-Mercaptoethyl)-methyl-di-*n*-propylammonium iodide acetate. Crude 2-di-*n*-propylaminoethyl chloride hydrochloride (Cadogan, 1955) was converted into the corresponding thiol by reaction with thiourea in water and treatment of the resulting solution with sodium hydroxide (Clinton *et al.*, 1948). Esterification of 2-di-*n*-propylaminoethanethiol-1, b.p. 87–88°/12 mm., n_D^{25} 1.4621 with acetic anhydride gave the thioacetate b.p. 122–124°/13 mm., n_D^{25} 1.4688. The 2-di-*n*-propylaminoethyl thioacetate reacted rather rapidly with excess of methyl iodide.

After standing for some days in the dark, the product was recrystallized from alcohol and freshly distilled ether. White solid m.p. 113.5°.

Anal. (Calcd for $C_{11}H_{24}INOS$ (M.W. 345.28): C, 38.3; H, 7.0; I, 36.8; N, 4.1; S, 9.3. Found: C, 38.2; H, 7.1; I, 37.1; N, 4.1; S, 9.2.

(3) (2-Mercaptoethyl)-ethyl-di-*n*-propylammonium iodide acetate. 2-Di-*n*-propylaminoethylthioacetate was refluxed with ethyl iodide in methyl ethyl ketone for two days. After standing a week, the solvent and the excess of ethyl iodide were removed by evaporation under reduced pressure and the product was recrystallized from alcohol and freshly distilled ether. White solid m.p. 101°.

Anal. Calcd for $C_{12}H_{26}INOS$ (M.W. 359.31): C, 40.1; H, 7.3; I, 35.3; N, 3.9; S, 8.9. Found: C, 40.5; H, 7.1; I, 35.1; N, 3.8; S, 8.9.

(4) (3-Mercaptopropyl)-trimethylammonium iodide acetate. From 3-dimethylaminopropyl chloride hydrochloride, 3-di-methylaminopropylthiol-1 was obtained by the method of Clinton *et al.* (1948). Colorless clear liquid b.p. 47°/9 mm., n_D^{25} 1.4647, n_D^{20} 1.4662. Andrews *et al.* (1953) reported b.p. 40–41°/12 mm., n_D^{20} 1.4666. Esterification with acetic anhydride gave a liquid b.p. 91–92°/11 mm., n_D^{25} 1.4686. Quaternization with methyl iodide in pure ether gave white crystals m.p. 148–149° after recrystallization from methanol–hexane.

Anal. Calcd for $C_8H_{18}INOS$ (M.W. 303.20): C, 31.7; H, 6.0; I, 41.9; N, 4.6; S, 10.6. Found: C, 32.0; H, 5.5; I, 42.1; N, 4.7; S, 10.3.

(5) (4-Mercaptobutyl)-trimethylammonium iodide acetate. 4-Dimethylaminobutanethiol-1 (Morrison and Andrews, 1954; Avison, 1951) reacted with acetic anhydride giving a liquid b.p. 96–108°/9 mm. This liquid treated with methyl iodide in pure ether gave a white compound m.p. 176° after recrystallization from alcohol and freshly distilled ether.

Anal. Calcd for $C_9H_{20}INOS$ (M.W. 317.23): C, 34.1; H, 6.4; I, 40.0; N, 4.4; S, 10.1. Found: C, 34.2; H, 6.1; I, 39.9; N, 4.6; S, 10.1.

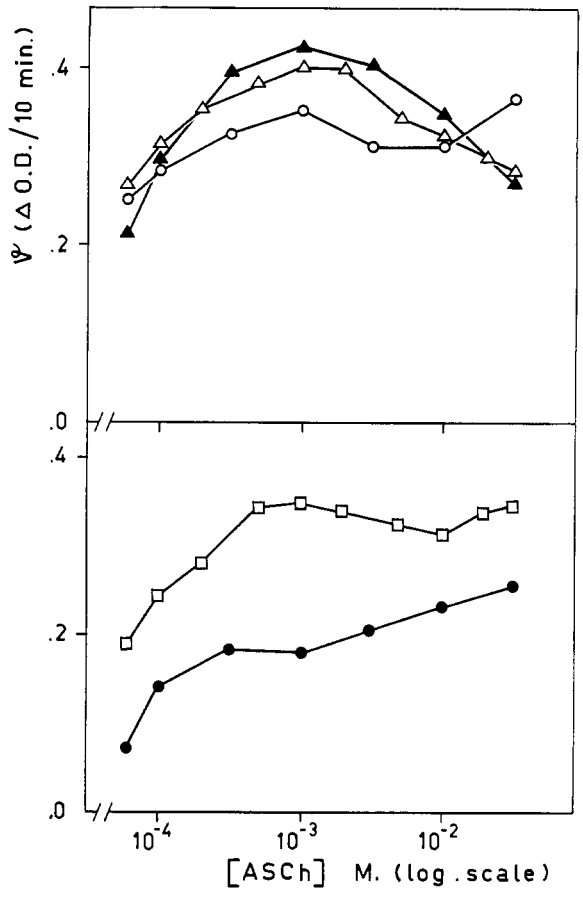


Figure 1. Effect of ASCh concentration on the rate of enzymic hydrolysis (v) expressed as O.D./10 minutes. pH = 7.0; temp. 33°C.

Upper part, S-enzyme; crude homogenate (μ = 0.7) ○, standard preparation (μ = 0.7) ▲, and (μ = 0.4) △
Lower part, R-enzyme; crude homogenate (μ = 0.7) ●; standard preparation (μ = 0.4) □

(6) (3-Mercaptophenyl)-trimethylammonium iodide acetate. Freshly distilled 3-dimethylaminophenol was converted into *O*-3-dimethylaminophenyl dimethyl thiocarbamate by treatment with dimethyl thiocarbamoyl chloride in dimethylformamide in the presence of sodium hydride under nitrogen. The yellow product was recrystallized from methanol and melted at 71–72°. Heating at 280° for about one hour yielded *S*-3-dimethylaminophenyl, dimethyl thiocarbamate, a yellow oil b.p. 132–136°/0.05 mm. (Newman and Karnes, 1966). Hydrolysis by sodium hydroxide in methanol gave 3-dimethylaminothiophenol b.p. 68–72°/0.1 mm., n_D^{25} 1.6178 (Bordwell and Boutan, 1956, report b.p. 82–83°/0.6 mm., n_D^{25} 1.6173). The thiophenol reacted slightly exothermic with acetic anhydride, giving a slightly colored oil b.p. 92–98°/0.1 mm., n_D^{25} 1.5968. This oil was treated with methyl iodide in ether and the product recrystallized from alcohol and freshly distilled ether, melted at 157°.

Anal. Calcd for $C_{11}H_{16}INOS$ (M.W. 337.22): C, 39.2; H, 4.8; I, 37.6; N, 4.2; S, 9.5. Found: C, 39.3; H, 4.9; I, 37.7; N, 4.0; S, 9.3.

(8) (2-Mercaptoethyl)-trimethylammonium iodide butyrate, was obtained from Fluka Schweiz.

(9, not in Table I) 3,3-Dimethylbutylthioacetate. 2,2-Dimethylbutanol-4 with phosphorus tribromide gave 3,3-dimethylbutyl bromide b.p. 58–62°/51 mm., n_D^{20} 1.4490 (Strating and Backer, 1936). To 74 mmol. of bromide in 40 ml. of alcohol was added 84 mmol. of freshly distilled thioacetic acid and 84 mmol. of potassium hydroxide. After refluxing for 6 hours and standing overnight the precipitate was filtered off and the filtrate mixed with ether. The ether solution was washed with water to which sodium chloride had been added, dried with sodium sulfate, and fractionated. This resulted in 3.5 g. (29%) of a clear yellow liquid b.p. 66 °C/6 mm, n_D^{25} 1.4574.

Anal. Calcd for $C_8H_{16}OS$ (M.W. 160.28): C, 60.0; H, 10.1; S, 20.0. Found: C, 60.2; H, 9.9; S, 19.9.

The thiol of this substrate does not react quantitatively with DTNB, under the conditions described and there was also a large contribution by nonspecific esterases in the hydrolysis (Aarseth *et al.*, 1968). It was therefore not used in this work.

Inhibitors. The numbers in parentheses refer to those in Table II. All the samples were 95% pure or better.

(1) *O*-Ethyl-*S*-2-triethylammonium ethylphosphonothiolate iodide, a mixture of the two stereoisomers was obtained from H. S. Hopf (I.C.I. Ltd.; Jealotts' Hill Res. St. Bracknell, Berks, G.B.).

(2− and 2+) *O*-Isopropyl-*S*-2-trimethylammoniumethyl methylphosphonothiolate iodides were obtained from H. L. Boter (Chemical Laboratory, National Defense Research Organization T.N.O. The Netherlands) (Boter and Platenburg, 1967).

(3) *O*-Isopropyl-*S*-2-isoamyl methylphosphonothiolate, a mixture of the two isomers, was obtained from the same source as compound 2.

(4) *O,O*-Diethyl-*O*-(2-isopropyl-4-methylpyrimidyl-6) phosphate, was obtained from Geigy A.G., Basel, Switzerland.

(5) *O,O*-Diethyl-*S*-(2-diethylamino-ethyl) phosphorothiolate hydrogen oxalate, was obtained from the same source as compound 1.

(6) *O,O*-Diethyl-*O*-(*p*-nitrophenyl) phosphate, was obtained from Bayer, Leverkusen, Germany.

(7) *O,O*-Diisopropyl-fluorophosphate, was obtained from BDH.

(8) *O,O*-Dimethyl-*S*-(*N*-methylcarbamoylmethyl)phosphorothioate, was obtained from Bayer.

(9) Physostigmine sulfate, was obtained from BDH.

(10) Methylphenylcarbamoyl chloride was synthesized by the method of Metzger and Wilson (1964). A 20% solution of phosgene in toluene was used instead of a saturated solution of phosgene in benzene. The product melted at 85–86° and boiled at 130°/11 mm.

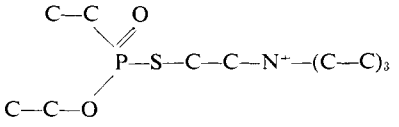
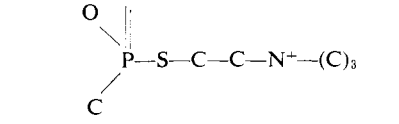
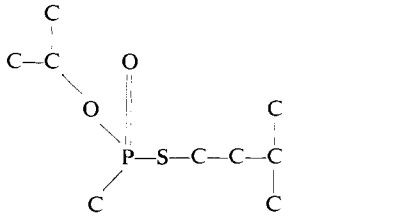
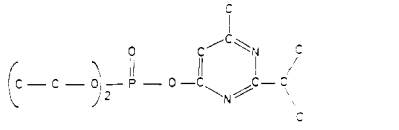
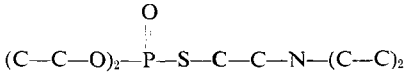
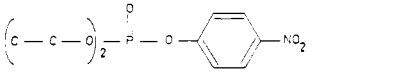
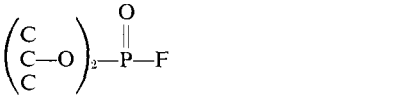
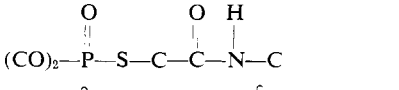
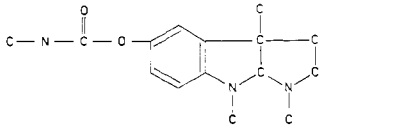
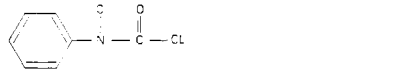
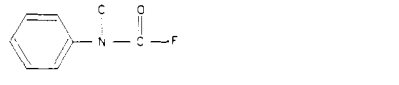
(11) Methylphenylcarbamoyl fluoride could be obtained from the corresponding chloride by treatment with SbF_3 (Metzger and Wilson, 1964). After a few careful distillations, the product had a constant boiling point of 107–108°/9 mm, n_D^{25} 1.5088.

Further Compounds. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was used as a buffer in a single experiment and made according to Good *et al.* (1966). Other compounds used were commercially available and reagent grade.

RESULTS AND DISCUSSION

Effect of Substrate Concentration and Substrate Specificity. Figure 1 shows the effect of ASCh concentration on the rate of enzymic hydrolysis for both enzymes with crude homogenates (Smissaert, 1964) and the standard preparation.

Table II. Approximate Bimolecular Rate Constants (k_i, M^{-1} min^{-1}) for the Mite-Enzyme, and Three Other AChE[a]

No.	Name	Inhibitor Structure (H's omitted)	k_i Values (M^{-1} Min.$^{-1}$) for Enzymes Indicated S	R	S/R	Other AChE
1	QAT		10^7	10^3	10000.	4×10^{7b} 5×10^{8c}
2−			3×10^6	10^3	3000.	5.3×10^{7d}
2+			3×10^3	4×10^2	8.	4.5×10^{4d}
3			3×10^4	20.	1500.	5.4×10^{2d}
4	Diazoxon		3×10^6	2×10^4	150.	4×10^{7c}
5	Tetram		6×10^5	1.5×10^3	400.	3.3×10^{6e} 4×10^{7c}
6	Paraoxon		1.5×10^5	2.5×10^2	600.	4.6×10^{5e} 5×10^{6c}
7	DFP		10^3	10^2	10.	4.6×10^{4e}
8	Omethoat		1.3×10^4	2.5×10^2	50.	
9	Physostigmine		3×10^6	3×10^5	10.	2×10^{6f}
10			3×10^4 (1.2×10^4)	65.(?) (70)	460.(?) (170)	8.7×10^{5b} 1.4×10^{5g}
11			2.5×10^5 (9.2×10^4)	2×10^2 (1.9×10^2)	1250. (490)	3×10^{6b} 7.4×10^{5g}

[a] Conditions: pH 7.5 (Nos. 8, 9, 10, 11 at pH 7.0); temp. 27 °C; ionic strength $\mu \simeq 0.5$; acetone 0.5%; in brackets, acetone 10%. Numbers refer to the names in the section materials. [b] Bovine erythrocytes AChE, this work. [c] Flyhead AChE, van Asperen and Oppenoorth (1960). [d] Bovine erythrocytes AChE, Boter, personal communication (1967). [e] Bovine erythrocytes AChE, Ooms (1961). [f] Electric eel, Wilson *et al.* (1961). [g] Electric eel, Metzger and Wilson (1964).

Inhibition by excess substrate is shown when the latter but not when the former preparation is used. It is clear that the crude preparations contain at least one nonspecific enzyme which hydrolyzes ASCh at high concentrations. Its K_m value is likely to be in the order of $3 \times 10^{-2}M$. The results also show that its contribution to total ASCh hydrolysis is relatively large in the crude R-preparation, presumably due to the decreased activity of the AChE of this strain. Based on experiments with crude preparations of normal mite AChE, it had been concluded that there is no inhibition by excess substrate in acetylcholine hydrolysis (Voss, 1960; Dauterman and Mehrotra, 1963; Voss and Matsumura, 1965; Sakai, 1967; Motoyama and Saito, 1968). It seems likely, however, that there is also inhibition by excess sub-

strate in acetylcholine (ACh) hydrolysis and the results were due to the contribution of more than one enzyme.

Similar experiments were carried out with other thioesters and values of V_m and K_m estimated by the aid of a so-called Eadie plot (Dixon and Webb, 1958, p. 21) as shown in Figure 2. A thirty-fold purified preparation of the R-enzyme was used in these experiments. Inhibition by physostigmine showed that enzymic hydrolysis of these substrates was solely due to AChE. Moreover it was shown that the bimolecular rate constant of organophosphate inhibition (compound I, Table II) was, up to nearly complete inhibition, identical whether ASCh or substrate 5 (Table I) was used to assay the enzyme activity. Experiments with Bovine Erythrocyte AChE (Sigma) were included for comparison and the results collected in Table I. V_m values for ASCh were arbitrarily taken as unity for each of the three enzymes. It should be noticed that the V_m values of the R-enzyme have to be divided by a factor of nearly three if comparison with the S-strain AChE is made on the basis of enzyme activity per milligram mite tissue.

There is some evidence (Smissaert, unpublished data) that an acetylated enzyme is an intermediate in acetylester hydrolysis by both mite AChE's, as shown before with Eel-electric-tissue (Wilson and Cabib, 1956) and bovine erythrocyte-AChE (Krupka, 1964). Therefore the formal scheme is:

$$E + S \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + A \qquad (1)$$

where E, S, ES, and EA refer to concentrations of the free enzyme, free substrate, reversible enzyme-substrate complex, and acylated enzyme, respectively. Variation of V_m in the hydrolysis of the acetates should be due to that in acetylation rate constant (k_2) since the deacetylation rate (k_3) is identical. As shown in Table I there is generally much less variation in V_m of the two mite AChE's then that of the erythrocyte enzyme. Dauterman and Mehrotra (1963), applying crude mite homogenates to the oxygen analogs of the first four substrates of Table I, reported similar relative activities as compared with ACh at the lowest concentrations ($10^{-3}M$–$3 \times 10^{-3}M$) used. However, at $3 \times 10^{-3}M$ the oxygen analog of substrate 5 was hydrolyzed at a rate of about a quarter of that for ACh. The effects of increasing substrate concentrations were, as mentioned before, quite different from those found in this work.

There are two possible interpretations for the lack of variation of V_m in the mite enzymes. First, deacetylation (k_3) could be the rate-limiting step with all these substrates ($k_2 \gg k_3$) and therefore V_m would hardly be affected by a decrease in acetylation rate. Second, the mite AChE's may, of course, be less specific in catalysis of acetylation than the erythrocyte enzyme. There is at present no conclusive evidence for either of these possibilities. They are, for reasons not discussed, both difficult to accept and therefore a combination of the two seems to be the most probable explanation. A pronounced increase in K_m for the ester of 3-mercapto phenyltrimethylammonium (substrate 6) for the R-enzyme, relative to that of S, is noticed and corresponds with the higher dissociation constant of the oxygen analog of the leaving group with this enzyme (Smissaert, 1969). This substrate shows the highest interstrain difference in V_m values found so far. With propionylthiocholine a higher V_m is obtained than with ASCh in case of the S-enzyme, while the reverse is true for the R-strain AChE. The former probably means that the rate of hydrolysis of the propionyl-enzyme (k_3) is faster than deacetylation. Yet ACh seems to be the

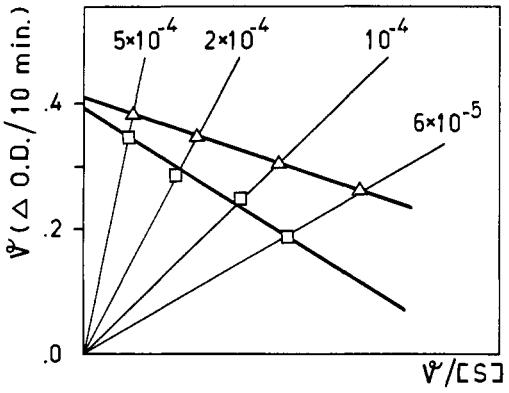


Figure 2. Eadie plot of effect of ASCh concentration on v. ($v = V_m - K_m \times v/[S]$)

Same data and legend as in Figure 1
Polar coordinates denote ASCh concentrations as indicated in M

normal substrate in spider mites (Mehrotra, 1961). Substitution of butyryl in ASCh causes a fivefold decrease in V_m in the S- and a threefold decrease in the R-enzyme. The mite enzyme shows a similar ratio of ASCh to butyryl-thiocholine V_m-values as the flyhead AChE (Kunkee and Zweig, 1963). Published data for insect AChE with other substrates are difficult to compare. The studies of Metcalf *et al.* (1955) and Dauterman and Mehrotra (1963) suggest that the specificity of the flyhead AChE also is generally less than that of bovine erythrocytes although more pronounced than in the mite enzymes.

Irreversible Inhibition. The purpose of the first experiment to be described, is to provide additional and conclusive evidence that the AChE's molecules of the two strains are indeed different. The high ratio of bimolecular rate constants of paraoxon inhibition should enable one to discriminate between the two enzymes in a mixture. The two enzyme preparations and mixtures of them, in which each contributes half of the total hydrolytic activity, were preincubated with paraoxon at $10^{-6}M$ and $10^{-4}M$. Enzyme activity remaining after varying periods was assayed as described. Figure 3 shows a first-order plot of the results. Mixing of the enzymes the day before the experiment did not affect the results. The inhibition rate of neither of the two enzymes is influenced by the presence of a preparation of the other one. Therefore the results fit the conclusion drawn before based on diazoxon inhibition of an enzyme preparation obtained from the female offspring of crosses between the two strains (Smissaert, 1964).

A second point is whether the inhibition of the R-enzyme by the relative slowest inhibitors is really due to phosphorylation of the active site. If paraoxon reacts with the site of substrate hydrolysis, the fraction $[(ES + EA)/E_0]$ of the enzyme saturated by a substrate such as ACh would be protected to inhibition. Therefore the rate of paraoxon inhibition was measured in the presence of varying concentrations of ACh. Because ACh is hydrolyzed during inhibition, a constant and short period of 2 minutes was applied. Blanks with ACh and without paraoxon were involved for the calculation of 100% activity and to check whether the ACh concentration was essentially constant. Because the solubility of paraoxon was a limiting factor with such a short inhibition period for this enzyme, the acetone concentration was raised to 10% to solubilize $2 \times 10^{-3}M$ paraoxon without causing inactiva-

tion by acetone. Figure 4 shows the results plotted according to the equation:

$$k_i/k_{i\,app} = 1 + S/K_m \qquad (2)$$

where $k_{i\,app}$ = the calculated bimolecular rate constant for paraoxon inhibition uncorrected for the effect of ACh and k_i = the bimolecular rate constant in the absence of ACh. The data should fit this equation in case protection by ACh is proportional to the saturation of the active site as measured by S/K_m. [K_m throughout this paper is the substrate concentration effecting a rate of hydrolysis half the theoretical maximum (V_m) and according to steady state kinetics from Scheme 1, $K_m = k_3(k_{-1} + k_2)/k_1(k_2 + k_3)$].

At the ACh concentration reducing $k_{i\,app}$ to half the value of k_i, half the enzyme is protected and therefore saturated with ACh. Thus at $k_i/k_{i\,app} = 2$, the ACh concentration should be equal to K_m. The value obtained from Figure 4 is $1.1 \times 10^{-3}M$. Next, the value of K_m for the R-enzyme with ACh under these conditions (10% acetone etc.) had to be determined. For technical reasons this was done by measuring reversible inhibition of the ASCh hydrolysis by ACh. K_m was estimated from competitive inhibition in the usual way (Dixon and Webb, 1958, p. 25). The value obtained was $0.86 \times 10^{-3}M$ which compares reasonably with that from protection to paraoxon inhibition. The value for K_m is about 5 times increased by the presence of 10% acetone because previous determinations in the absence of acetone provided a K_m value of $2 \times 10^{-4}M$ for this enzyme (Smissaert, unpublished). A similar experiment, at a lower acetone concentration, showed that the effect of acetone on K_m obtained from protection to paraoxon inhibition equals that from inhibition of ASCh-hydrolysis. Therefore, the results provide convincing evidence that paraoxon reacts with a group in the active site of the R-enzyme. It was noticed that V_m of ASCh hydrolysis was not significantly affected by the presence of 10% acetone.

Inhibition experiments were carried out with a series of inhibitors (Table II). The results, apart from those with physostigmine, fitted pseudo first-order conditions while the calculated second-order (or bimolecular) rate constants (k_i) were independent from the inhibitor concentration applied. In the case of physostigmine the inhibition approaches a well known, steady state level (Wilson *et al.*, 1960). In this case, rough estimates of the bimolecular rate constants (k_i) were calculated from the initial slopes in first order plots of log remaining activity *vs.* time.

Values of k_i for the S-strain AChE are generally at least 10-fold lower than those for electric eel, bovine erythrocyte, and flyhead. AChE's as shown by some data from the literature also provided in Table II. Those of the R-enzyme are lower in all cases studied, the ratios of k_i values for the S- and R-enzymes range from 10 to 10,000 (Table II).

There are two pairs of compounds which deserve some special attention. The two optical isomers of *S*-2-trimethylammoniumethyl *O*-isopropylmethylphosphonothiolate (Table II compounds 2+ and 2−); Boter and Platenburg (1967) reported on the tertiary analogs before. Although the k_i values for the S-enzyme are about tenfold lower than those of bovine erythrocyte AChE (Table II), the 1000-fold ratio of k_i values for the two stereoisomers is surprisingly similar for the two enzymes. The 3000-fold ratio of k_i values for the S- and R-enzymes with the − isomer is one of the highest found so far. Possibly as a consequence of the same interactions causing these extreme ratios, the interstrain ratio (k_i of S/k_i of R) for the + isomer (8×) and the effect of isomeric configuration in the R-enzyme (2.5×) is rather low. It is tempting to speculate that the *O*-isopropyl group is interfering with proper binding or (and) phosphorylation in the normal AChE's in case the + configuration is used, while the same may occur in the R-enzyme with both isomers. At the moment, however, we don't even know whether the stereospecificity is formally due to an effect on the dissociation constant of the initial reversible enzyme-inhibitor complex (K_i) or on the first-order rate constant of phosphorylation (k_2) ($k_i = k_2/K_i$) (Kitz and Wilson, 1962; Main, 1964).

Another pair of compounds to be considered in some detail are methylphenyl-carbamyl-fluoride and -chloride (compounds 10 + 11, Table II). The stimulus to study these compounds came from a paper by Metzger and Wilson (1964), who showed that the fluoride was a better inhibitor of electric eel AChE than the chloride. Although part of the explana-

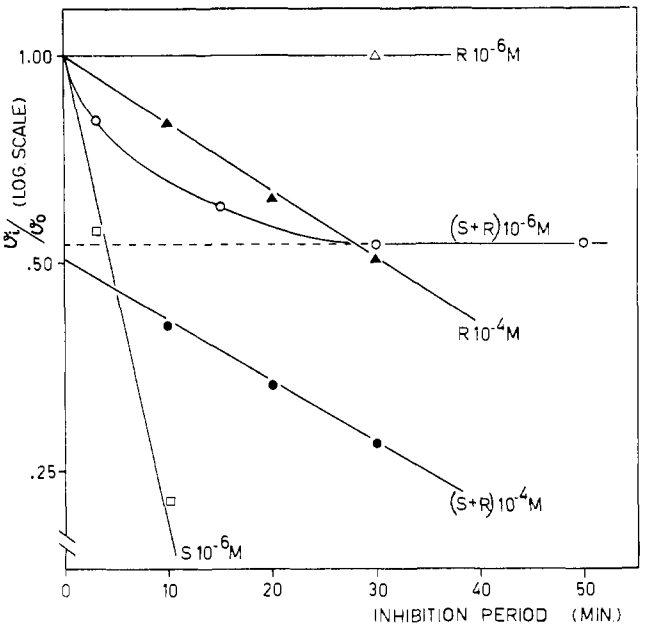


Figure 3. Paraoxon inhibition, first-order plot of the fraction remaining enzyme activity (v_i/v_o) *vs.* inhibition period for the S- and R-enzyme and a mixture (S + R) in which each contributes half of the total hydrolytic activity. Paraoxon concentrations 10^{-6} and 10^{-4} *M* as indicated

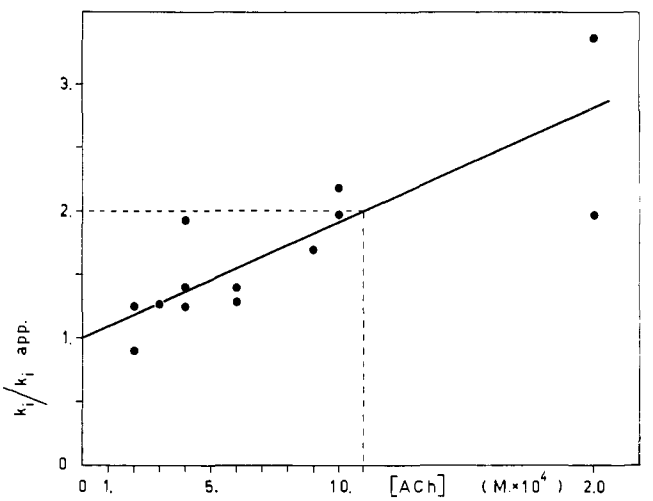


Figure 4. Protection by ACh of the R-enzyme to paraoxon inhibition. ($k_i/k_{i\,app} = 1 + [ACh]/K_m$)

See text for explanation

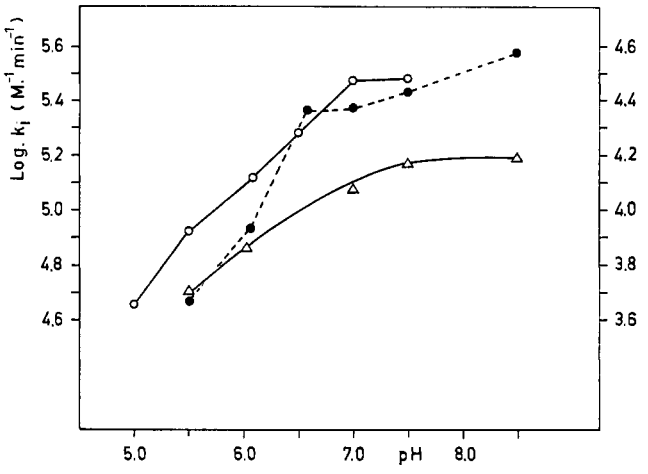


Figure 5. S-enzyme. Effect of pH on bimolecular rate constant (k_i) of inhibition by methylphenylcarbamyl-fluoride ●, -chloride ○ (righthand site y-axis) and paraoxon △

Pyrophosphate-HCl buffer + NaCl, ionic strength at pH = 7.0, μ = 0.4; temp. 27 °C.

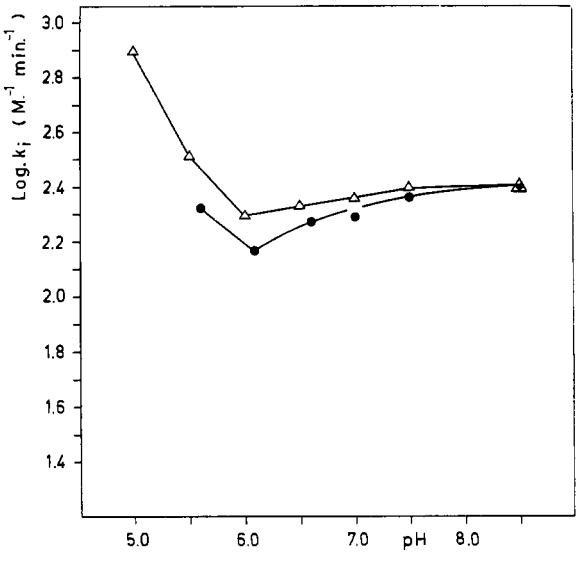


Figure 6. R-enzyme. Effect of pH on the bimolecular rate constant (k_i)

Conditions and legend as in Figure 5

tion is likely to be the formation of a tetrahedral addition intermediate, the k-fluoride/k-chloride ratio as high as 5.4, provided evidence for the contribution of an electrophilic mechanism. An important argument had been the knowledge that the hydrolysis of benzoyl-fluorides is acid catalyzed whereas that of the -chlorides is not (Metzger and Wilson, 1964).

Because of the instability of the carbamyl-chloride, a constant preincubation period of 2 minutes was applied and only the inhibitor concentration was varied with these two carbamates. The data of Table II (10 + 11) show that the -fluoride is the better inhibitor for both enzymes although the k_i values for the R-enzyme are decreased about 1000- and 500-fold. The -fluoride/-chloride reactivities are about 8. and 3. for the S- and R-enzyme, respectively. In the experiments with the R-enzyme, the solubility of the -chloride became a limiting factor which may have caused an error in the last value. Therefore the experiments with both enzymes and inhibitors were repeated with 10% acetone in the incubation

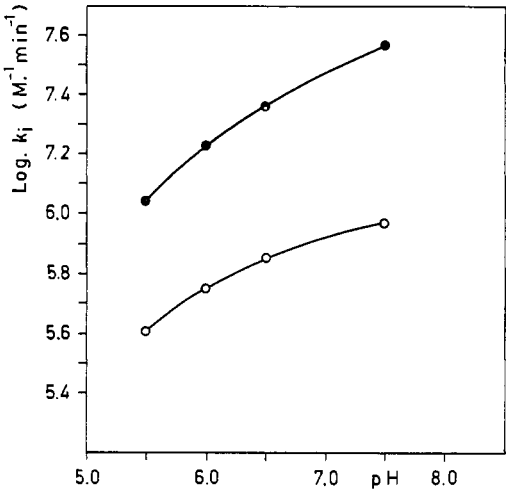


Figure 7. Bovine erythrocyte AChE. Effect of pH on bimolecular rate constant (k_i)

Conditions and legend as in Figure 5

medium. The presence of acetone decreases k_i values about 2.6 fold in the S- and hardly in the R-enzyme. No doubt, the decrease of k_i in the R-enzyme is significantly more pronounced with the carbamyl-fluoride. However, as compared with the at least 170-fold interstrain ratio in k_i, the change in relative reactivities for the two carbamates is rather small. Following Wilson's argumentation, the results suggest acid catalysis of carbamylation in both enzymes.

Decarbamylation Rate. The rate of decarbamylation of the *N*-methylcarbamyl enzyme was estimated in the following ways. First, the enzymes were preincubated with physostigmine to reach roughly 90% steady-state inhibition (Wilson *et al.*, 1960).

1. The inhibited enzyme-physostigmine mixture was added to a column of Sephadex G 25 (1 × 10 cm.) and slowly eluted with the pyrophosphate buffer. The fraction with enzyme was then assayed at varying time intervals as described.

2. After separation of enzyme and inhibitor by the column, the enzyme was continuously assayed for a period up to 1 hour. The record of O.D. *vs.* time showed the increase in the rate of hydrolysis due to reactivation by decarbamylation.

3. No column was applied; instead the enzyme-inhibitor mixture was diluted 350 times with the assay medium and treated as in procedure 2. The decarbamylation rate constants (k_3) were estimated by a method which is essentially an application of the first-order equation:

$$\log \text{fraction carbamylated enzyme} = -k_3 t/2{,}3$$

Similar results were obtained by the three procedures while the values of k_3 obtained were independent from the carbamate used to obtain the *N*-methylcarbamyl enzymes.

The decarbamylation rate constants were about 5×10^{-2} and 6×10^{-3} min.$^{-1}$ for the *N*-methylcarbamylated S- and R-enzyme, respectively, at pH 7.5, 33 °C, and ionic strength μ = 0.4. Due to its low value, that for the R-enzyme is rather rough. There is no doubt, however, that it is significantly lower than that for the S-enzyme. The k_i and k_3 values obtained are consistent with estimated steady state inhibitions for the two enzymes.

pH Effects on Irreversible Inhibition. To obtain possible information on the cause of the decreased inhibition rates in the R-enzyme, preliminary experiments on the effect of pH on the reaction with the two carbamates discussed and with paraoxon were performed.

It is beyond the scope of this paper to discuss pH effects on irreversible inhibition of AChE in general and the following notes should suffice. The interested reader is referred to some points in the discussions following a paper read by Krupka (1967a).

Although the reaction of the acid-transferring inhibitors (Wilson, 1964) is formally analogous to the acylation by substrates, this of course does not necessarily imply that the detailed mechanistics of acylation and (or) rate limiting steps are identical.

If a comparison between rates of substrate hydrolysis and inhibition is made, the k_i values of the latter should be compared with V_m/K_m for substrates (Metzger and Wilson, 1964). As with V_m/K_m, also a second-order rate constant, pH effects on k_i may be due to those on the dissociation constant of the enzyme-inhibitor complex and/or on the first-order rate constant of acylation. Further, neutral inhibitors should be compared with neutral substrates.

To avoid complications pyrophosphate-HCl has been used in the whole range in spite of the limited buffer capacity at the higher pH's. The pH was measured in the complete reaction mixture and checked to be constant. After a two-minute preincubation period with varying concentrations of the inhibitor, the reaction mixture was diluted for assay to pH 7.0 with the buffered substrate solution. No inactivation occurs in the controls. Similar experiments were performed with bovine erythrocyte AChE. Results are shown in Figures 5, 6, and 7. Assuming a single ionizing group affects k_i in the acid range, $k_i = k_{i\,max}/(1 + [H^+]/K_a)$. Apparent K_a values were obtained from the intercept with the x-axis of a line fitted to the experimental points in a $1/k_i$ *vs.* $[H^+]$ plot.

Several characteristics should be pointed out. In none of these cases do the results fit a linear relationship in a $1/k_i$ *vs.* $[H^+]$ plot as expected if a single ionizing group affects k_i. Apparent pK_a's are lower if estimated at lower pH's.

With the erythrocyte enzyme, the two carbamates studied show a rather similar pH dependence of k_i. With the S-enzyme, this also holds for paraoxon.

The R-enzyme shows a unique dependence of k_i as particularly shown with paraoxon. At pH values higher than 6, apparent pK_a's are about 5.9 and 5.3 with the carbamylfluoride and paraoxon, respectively. However, decrease of the pH to 5.0 results, with paraoxon, in a significant increase in k_i to a value 3 times the estimated maximum. The latter increase also occurred when HEPES was used as a buffer.

The pH dependence of the reaction of the two carbamates with the erythrocyte enzyme can be compared with that of V_m/K_m for the hydrolysis of phenylacetate by the same enzyme as reported by Krupka (1966). The conditions are rather different, notably the ionic strength was lower in Krupka's experiments. The pK_a values with the two carbamates are similar to that of V_m/K_m with phenylacetate (pK = 5,6) at lower pH values and up to 0.7 pK unit higher at higher pH's. Although there are differences, the data are not inconsistent with the idea that an identical group is involved in a rate determining step in acylation by phenylacetate and these carbamates. The results with the S-enzyme also show that k_i is primarily affected by an ionizing group with a pK_a around 6.0.

Unfortunately pH studies on AChE with irreversible inhibitors are rather scarce. A similar pH dependence in the acid range has been reported by Wilson and Bergmann (1950a,b), while recently Reiner and Aldridge (1967) reported the absence of any pH effect between pH 5.5 and pH 8.0 with two neutral carbamates and slight effects with another one and the dimethyl analog of paraoxon. It is clear that generalizations cannot be made yet. The increase in k_i at low pH for the reaction of the R-enzyme with paraoxon can be explained in several ways. It may suffice to mention that the effect is not necessarily specific for the R-enzyme. It may be that it is not shown in the S-enzyme to this extent because of the 600-fold higher value of k_i and moreover protonation of a group with pK 6.3 is blocking the inhibition reaction in this enzyme. The effect may even be due to catalysis by protons from the medium (specific acid catalysis). A more urgent question seems whether the pK_a of the basic group essential for inhibition in the S-enzyme and erythrocyte AChE is really decreased in R as suggested by the log k_i — pH profile above pH 6.0. The slight pH effect between pH 6.0 and pH 7.5 may be caused by compensation through the mechanism which affects the increase in k_i at lower pH's. It seems, however, improbable that the profile for the R-enzyme is composed in part by a curve similar to that of S. Thus the conclusion is that the pK_a of the basic group mentioned is indeed decreased and probably is in the order of 5.8. This conclusion implies that the basic group is assumed to be still functional in the R-enzyme although possibly to a lesser degree or in a not strictly rate-limiting step. No data about pH effects on neutral substrates are available, for the mite enzymes. However, pK_a of V_m for ASCh-hydrolysis is 6.5 and 6.2 for the S- and R-enzyme, respectively (Smissaert, 1969).

GENERAL DISCUSSION

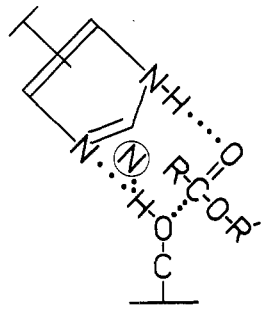
In this section, the results will be generalized as far as possible and a hypothesis for the cause of the insensitivity of the R-enzyme to irreversible inhibitors will be discussed. First it can be noticed that both enzymes are inhibited by high ASCh concentrations and are to be classified as true cholinesterases. Variation of *N*-alkyl substituents and *S-N* distance in thioacetates does not affect V_m seriously. It seems, therefore, that deacetylation is more or less rate limiting and/or the specificity of the acetylation reaction is rather low. The effect of the substitution of acetyl by propionyl is opposite in the two enzymes, and the thiocholine ester of the latter is a better substrate for the S-enzyme.

The k_i values of a rather diverse series of irreversible inhibitors were given and two pairs of compounds discussed in some detail. Here attention is directed to a possible generalization. The higher ratios of k_i values for S/R (Table II) are obtained with the better inhibitors of the S-enzyme, arbitrarily taken as those with k_i values higher than $10^4 M^{-1}$ $min.^{-1}$. This may be expected since with these inhibitors enzyme catalysis is probably most pronounced. However, some of these inhibitors show much lower ratios (Nos. 8 and 9). In these cases the acyl residues, to be covalently bound with the enzyme, are of the smallest within this series while the leaving group is likely to be anionic site directed. On the other hand, high S/R ratios (>500) are always obtained with inhibitors having a single bulky group (*O*-isopropyl, *N*-phenyl, or *o-p*-nitrophenyl) which is not likely to be anionic site directed (Nos. 2−, 3, 6, and 11). The reason this phenomenon is explicitly mentioned to occur with a single bulky group is that a second one is in general also interfering with acylation of normal AChE. Two compounds with ethoxy groups, of intermediate size, show consistent S/R ratios (Nos. 4, 5). It seems that the bigger the not-ionic-site-directed groups, the more serious in general is interference with inhibition of the R-enzyme. A serious exception is provided by compound 1, a mixture of two optical isomers

with ethoxy, ethyl residues, showing the extreme S/R ratio of 10,000. Either the fact that a mixture of two compounds is concerned or, better, the consideration that the S/R ratio is exceptional because k_i of the S-enzyme is so, may provide an explanation. It seems, however, more realistic to admit that we do not understand and that the size of the acyl residue is probably not the only factor determining the ratio of k_i values.

The effect of the size of the acyl group discussed is consistent with the decreased V_m value for the propionyl ester with the R-enzyme. The generalization made is, of course, purely descriptive and does not serve as an explanation.

A most intriguing question is the cause of the decreased sensitivity of the R-enzyme to these acid transferring inhibitors. We can at the moment only speculate about the alteration in the R-enzyme responsible for the decreased reactivity. Starting with some reasonable assumptions it is, however, possible to build a picture which is consistent with the data and may serve as a working hypothesis. Unfortunately, nothing definite is to be said about the detailed mechanism of acylation in any AChE in general. The best and furthest reaching assumptions possible are to be derived from knowledge of catalytic mechanisms in chymotrypsin and from the catalysis of nonenzymic reactions (Bender, 1964; Bruice and Benkovic, 1966; Johnson, 1967). An attractive proposal (Bender, 1964) may be described by concerted general acid–general base catalysis by the two nitrogen atoms of one or two imidazole residues of histidine (Scheme 2). The mechanism is essentially identical to an early pro-



Scheme 2. Possible position in the S-enzyme of an imidazole side chain relative to that of serine hydroxyl showing bonds in the transition state of the formation of the tetrahedral addition-intermediate

For simplicity, the structural impossibility of the colinear bonds drawn is neglected. In the circle the altered relative position of the basic nitrogen in the R-enzyme is shown. The rest of this imidazole residue is not drawn

posal by Bergmann *et al.*, (1950) for electric eel AChE and has recently been adapted by Krupka (1967a,b) for the erythrocyte enzyme. The pK_a of the inhibition reaction for paraoxon and the methylphenylcarbamyl halogens, the similarity of the pH dependence of k_i with the two latter compounds, the pK_a of V_m for ASCh hydrolysis, the fluoride/chloride reactivities, the absence of an effect of 10% acetone on V_m of ASCh hydrolysis and the finding (Smissaert, unpublished) that photooxidation by methylene blue inactivates both enzymes are all consistent with the operation of this mechanism.

Pertinent to the following discussion is the finding that the first-order rate constant of decarbamylation (k_3) of the methylcarbamyl enzyme is considerably decreased in the R-enzyme. Since the k_i values are shown to be decreased, this provides evidence that also the first-order rate constant of acylation (k_2) in the R-enzyme is decreased. Further there is some evidence (Smissaert, 1969) that the decreased V_m of ASCh hydrolysis in the R-enzyme is due to an alteration in the conformation of the active site. The cause of the change in conformation in turn should be due to some amino acid replacement, or deletion, somewhere else in the enzyme molecule. Because the active site is composed only of a small fraction of the amino acids of the enzyme molecule, the difference is also statistically much more likely to be due to an altered conformation than to an altered chemical composition of the active site. This will therefore be assumed to be true and essentially the cause of the decreased acylation rates. The finding that the carbamylfluoride is still a better inhibitor than the corresponding chloride suggests that the formation of the tetrahedral addition intermediate is still the rate-determining reaction in carbamylation of the R-enzyme. The apparent pK_a of k_i in the R-enzyme, although decreased as compared with S, is presumably still that of the basic nitrogen of an imidazole residue involved in general base catalysis of this step (Scheme 2). Other things being equal, the lower pK_a would mean that proton transfer from serine hydroxyl to the basic nitrogen, in other words general base catalysis, is less favorable. This would provide, at least qualitatively, an easy explanation for the decreased k_i values of the R-enzyme.

An interesting possibility effecting both the decreased pK_a and diminished general base catalysis would be a closer approach of the nitrogen to the hydrogen of serine hydroxyl (Scheme 2). The decreased pK_a would be explained by repulsion of protons from the nitrogen by the hydroxyl dipole (Webb, 1963), which is assumed to occur in the free enzyme and perhaps in the ground state of the initial enzyme inhibitor (substrate) complex. The cause of less frequent or weaker hydrogen bonding between the nitrogen and the serine oxygen, although they are closer together, would be a wrong angle between the two atoms disturbing colinearity of transfer, which would occur in the transition state of the addition intermediate (Scheme 2). A closer approach of the imidazole residue to the serine hydroxyl may also provide an explanation of the effect of bulky groups in the acyl moiety.

This picture is of course highly speculative and will mainly serve as a working hypothesis. It is, however, consistent with the results described in this paper and with work about the pH effects on substrate hydrolysis and reversible inhibition which will be published elsewhere.

A main source of uncertainty in the discussion is the fact that it has not been possible so far to obtain convincing values for the first-order rate constants of acylation. Hopefully an improvement of the technique made recently will provide us with these constants.

Results and conclusions of this work deviate at several points from those of Voss and Matsumura (1964, 1965). Briefly the most basic comments are as follows:

(1) Hestrin's technique, in which the ACh remaining after incubation with the enzyme is determined, has been used throughout their work. The results have been treated as initial velocities instead of using the integrated Michaelis equation (Dixon and Webb).

(2) The use of this method in experiments where the substrate and the irreversible inhibitor are simultaneously mixed with the enzyme leads to complications in the kinetics neglected by the authors.

(3) The significance of the so called "enzyme-inhibitor-affinity constant" for paraoxon reported for both enzymes (Voss and Matsumura, 1965) is quite obscure while the method applied is not explained. Anyhow, the values given ($5.1 \times 10^{-5} M$ and $2.6 \times 10^{-6} M$ paraoxon for R- and S, respectively) show that the reciprocal

of the affinity constant is meant and they are likely to be a hundredfold too low.

(4) The results are obviously influenced by the action of more than one enzyme. It is also uncertain whether the R-strains were homozygous for the ChE-gene.

(5) Voss and Matsumura (1964) suggested that inhibition of the enzyme in the presence of $2 \times 10^{-3}M$ of ACh is natural and "close to the actual fact." There are, however, good reasons to doubt this seriously.

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