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# EFFECT OF pH, TEMPERATURE AND IONIC STRENGTH ON THE AGING OF PHOSPHONYLATED CHOLINESTERASES

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## SUMMARY

The rates of aging of cycloheptyl methylphosphonylated bovine erythrocyte acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) and horse serum butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) have been investigated as function of pH, temperature and ionic strength.

From the pH–rate profiles at 25 °C and $I = 0.100$, it was concluded that a protonated group with p$K$ 5.84 is involved in the aging of the phosphonylated acetylcholinesterase and a protonated group with p$K$ 7.35 in the aging of the phosphonylated butyrylcholinesterase. The influence of temperature and ionic strength on the pH–rate profile of aging of the phosphonylated acetylcholinesterase suggests the participation of an undissociated carboxyl group of the enzyme catalyzing the formation of a carbonium ion in the rate-determining step.

The mechanism of aging of phosphonylated butyrylcholinesterase turned out to be more complicated.

## INTRODUCTION

Organophosphorus compounds inactivate cholinesterases by phosphorylating the enzyme. The inhibited cholinesterases can be reactivated by reaction with powerful nucleophiles as certain oximes and hydroxamic acids [1]. In 1955 Hobbiger [2] observed that phosphorylated cholinesterases are converted spontaneously into a form which cannot be reactivated. This conversion, called aging, implies the release of an alkyl or alkoxy group from the phosphorus moiety in a first-order reaction [3–5].

$$R_1O(R_2)P(O)O\text{–Enzyme} \rightarrow {}^{-}O(R_2)P(O)O\text{–Enzyme} + R_1{}^{+} \begin{matrix} \nearrow \text{alcohol} \\ \searrow \text{alkene} \end{matrix} \quad (1)$$

($R_1$ = alkyl and $R_2$ = alkyl or alkoxy)

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Indications concerning the mechanism of aging of alkyl methylphosphonylated cholinesterases were obtained by comparing rates of aging with rates of unimolecular solvolysis of corresponding alkyl tosylates, brosylates and halides [6].

The qualitative correlation observed suggests that the rate-determining step in the aging reaction is an unimolecular fission of the C–O bond in the alkoxy group $R_1O$ (Eqn 1). Substantial evidence was further presented by Michel et al. [7] who demonstrated that a carbonium ion is formed in the aging of pinacolyl methylphosphonylated acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7).

Data reported in the literature on the influence of pH on aging are scanty and conflicting; Michel et al. [7] and Hobbiger [8] suggested that a protonated group of the enzyme is involved in aging, Berends [9] observed no effect of pH on the rate of aging and Berry and Davies [10] found that the rate of aging is directly proportional to the $H^+$ activity.

In the present investigations the influence of pH on the rate of aging of cycloheptyl methylphosphonylated acetylcholinesterase and butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) was studied over a large pH range, varying temperature and ionic strength. The mechanism of aging will be discussed and suggestions will be made on the nature of amino acid residue(s) which are probably involved.

## MATERIALS AND METHODS

### *Materials*

Bovine erythrocyte acetylcholinesterase was obtained from Winthrop Laboratories Inc. and had a spec. act. of 1.5 units/mg protein at 25 °C in a 0.5 mM phosphate buffer, pH 7.5, containing 3 mM acetylcholine chloride.

Butyrylcholinesterase, partially purified from horse serum according to a modified Strelitz [11] procedure, was purchased from Organon and had a spec. act. of 3.5 units/mg protein at 25 °C in 0.5 mM phosphate buffer, pH 7.5, containing 20 mM acetylcholine chloride. Cycloheptyl methylphosphonofluoridate was prepared in this laboratory as published previously [6]. All other reagents were of analytical grade.

### *Choice of inhibitor*

Preliminary investigations on the influence of pH on the aging of some phosphonylated cholinesterases suggested that the reaction might be catalyzed by a protonated group with an ionization constant of about 6.5. The rate constant of aging of a number of alkyl methylphosphonylated acetylcholinesterases and butyrylcholinesterases were already determined at pH 7.5 [6]. If we assume that the above mentioned protonated group catalyzes aging, the rate constants of aging of cycloheptyl methylphosphonylated cholinesterases between pH 4 and 9 would fall in the range $1.5 \cdot 10^{-1}$–$1 \cdot 10^{-4}$ $min^{-1}$ making their determination possible with a S.D. of 4% or less. The good reactivatability of the phosphonylated enzymes and the short half-life time of the inhibitor at pH 10 were additional advantages.

### *Methods*

Bimolecular reaction constants for inhibition were determined according to Qoms and Boter [12].

For the determination of rate constants of aging stock solutions of acetylcholinesterase (9.8 mg/ml) and of butyrylcholinesterase (7 mg/ml) were prepared in a 35.8 mM sodium diethylbarbiturate–sodium acetate buffer, pH 9.0, and were stored at 2 °C. No loss of activity was observed within a month. 4 ml of a solution of NaCl or a chloride of another monovalent cation were added to 10 ml stock solution to obtain the desired ionic strength.

Phosphonylation of the enzymes was performed at 25 °C and pH 9.0 during 10 min. 11 ml of the enzyme solution were added to an equal volume of a solution of cycloheptyl methylphosphonofluoridate in water (3.2 $\mu$M in case of acetylcholinesterase and 41.2 $\mu$M in case of butyrylcholinesterase). Under these conditions over 99% phosphonylation was attained without aging.

Removal of the excess inhibitor was achieved by alkaline hydrolysis. Storing the solution of the phosphonylated enzyme during 1 h at pH 10 and 25 °C results in a complete hydrolysis of the excess inhibitor since its half-life at pH 10 in 0.1 M KCl is about 5 min. The conditions used for phosphonylation and for hydrolysis of the inhibitor do not affect the activity of the enzymes.

Incubation of a 1.0-ml sample of this solution with 0.25 ml of a 15 mM solution of *N*-methylpyridinium-2-aldoxime methanesulphonate in a 40 mM phosphate buffer, pH 7.5, gave 99% reactivation within 21 h. 20 ml of the phosphonylated enzyme solution were allowed to attain the desired temperature while the pH meter of a Radiometer titration stand was calibrated with standard phthalate, phosphate and carbonate buffers according to Alner et al. [13]. The temperature was kept constant within 0.1 °C. A Radiometer pH meter PHM 26 equipped with glass electrode G202c and calomel electrode K4018 with salt bridge B530 was used. The solution was transferred to the thermostated titration stand. Aging was started by admixture of a previously estimated amount of 0.1 M HCl to bring the pH near the desired value, followed immediately by the addition of water making the total vol. 35.7 ml. The reaction was performed in an atmosphere of $CO_2$-free $N_2$. The pH at which the reaction was carried out was determined during the aging. Changes in pH were less than 0.005 pH unit for more than 24 h. 25 samples of 1.0 ml were taken after various periods of aging and incubated with 0.25 ml of a 15 mM solution of *N*-methylpyridinium-2-aldoxime methanesulphonate in a 40 mM phosphate buffer, pH 7.5, at 25 °C and allowed to reactivate for 24 h. Aging was determined by the decrease in attainable reactivation. Then, samples of 1.25 ml were transferred with 23 ml acetylcholine chloride (3.2 mM in 0.1 M KCl in the case of acetylcholinesterase and 9.6 mM in water in case of butyrylcholinesterase) to a titration vessel. The substrate hydrolysis was followed at 25 °C and pH 7.5 by using an apparatus for the performance of a great number of pH-stat titrations as described before [14]. Blancs for enzyme, enzyme incubated with reactivator and phosphonylated enzyme were run simultaneously.

The aging reactions were followed over 90% completion. When aging did not proceed to 90% within 8 h, a mechanized system was used to add samples of the phosphonylated enzyme to a reactivator solution at pre-selected times. The phosphonylated enzyme solution was transferred to a thermostatted 50-ml syringe, equipped with a 0.8-mm bore stainless steel needle, mounted in a Braun perfusor type Unita IIb. Test tubes containing 0.25 ml of a 15 mM *N*-methylpyridinium-2-aldoxime methanesulphonate solution in 40 mM phosphate buffer, pH 7.5, were

placed in a LKB Radi Rac turntable with thermostatted bath to ensure reactivation at 25 °C. Magnetic stirring discs were placed in these tubes. Efficient mixing was achieved by fast rotation of a magnet underneath the tubes. With an infusion rate of 750 $\mu$l/min adjusted on the perfusor reproducible samples of 1.0 ml were delivered by energizing the apparatus during 81 s. Mixing was performed during addition of the sample. After a selected time, adjusted in seconds on a pulse-counter (Kübler EVs 15.13/1), the next tube is moved under the perfusor and another 1-ml sample is delivered. Programming of perfusor, turntable and stirring device are controlled by a Sodeco pulse-sender type KN 551 and two Kübler pulse-counters.

Percentages reactivation were calculated according to

$$\% \text{ reactivation} = \frac{EIR\frac{E}{ER} - EI}{E - EI} \times 100\% \qquad (2)$$

where EIR, E, ER and EI are activities of aged enzyme incubated with reactivator for 24 h, of enzyme, of enzyme incubated with reactivator and of phosphonylated enzyme, respectively. Rate constants of aging ($k_{obs}$) were calculated by the method of least-squares from the first-order decrease of the percentage reactivation with time ($2.303\log(\%\text{ reactivation}) = -k_{obs}t$).

## RESULTS

### *Stereospecificity of aging*

Since cycloheptyl methylphosphonofluoridate is a racemic mixture, it is possible to obtain two phosphonylated enzymes differing in configuration around the phosphorus atom. In general, only one of these enzymes shows aging while the other does not age over at least 24 h [15]. The composition of the phosphonylated enzyme solution was determined with the aid of inhibition experiments with an equimolar amount of cycloheptyl methylphosphonofluoridate at 25 °C and pH 8.5. The rate constants of inhibition calculated from three experiments performed with acetylcholinesterase are $7.9 \cdot 10^7\ M^{-1} \cdot min^{-1}$ for the fast reacting isomer and $>10^5\ M^{-1} \cdot min^{-1}$ for its optical antipode. From similar experiments performed with butyrylcholinesterase the following bimolecular reaction constants were calculated: fast reacting isomer $3.8 \cdot 10^7\ M^{-1} \cdot min^{-1}$, optical antipode $3.2 \cdot 10^6\ M^{-1} \cdot min^{-1}$.

The large difference in rate constants indicates that only one isomer phosphonylates acetylcholinesterase. In agreement with the inhibition data straight lines were obtained over 90% for the aging of acetylcholinesterase by plotting the logarithm of the percentage reactivation versus time (Fig. 1a). Phosphonylation of butyrylcholinesterase on incubation with an excess of cycloheptyl methylphosphonofluoridate, however, leads to the formation of two phosphonylated enzymes ($38 \cdot 10^6 : 3.2 \cdot 10^6$). The plots of logarithm of percentage reactivation versus time are consistent with the prediction derived from the inhibition experiments (Fig. 1b). The first-order rate constants of aging were obtained by applying the Guggenheim method [16] or by subtracting the proper percentage non-aging phosphonylated enzyme prior to calculation by the least-squares method (Fig. 1c).

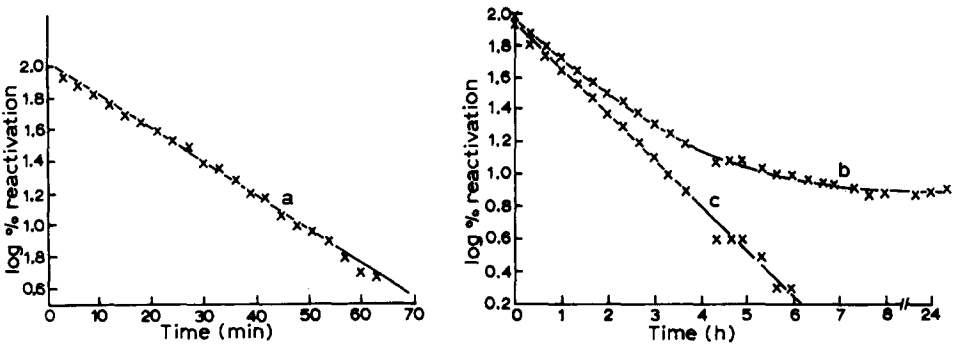


Fig. 1. Decrease in reactivatability at 25 °C and $I = 0.100$ of (a) cycloheptyl methylphosphonylated acetylcholinesterase at pH 6.11, (b) cycloheptyl methylphosphonylated butyrylcholinesterase at pH 7.48 and (c) results obtained after correcting the data obtained from (b) for the amount of nonaging phosphonylated butyrylcholinesterase.

*pH–rate profiles*

The effect of the pH on the rate of aging of the phosphonylated cholinesterases at 25.0 °C and $I = 0.100$ is illustrated by plots of log $k_{obs}$ versus pH (Fig. 2). These plots suggest that the undissociated form of an acidic group is involved in the aging reaction.

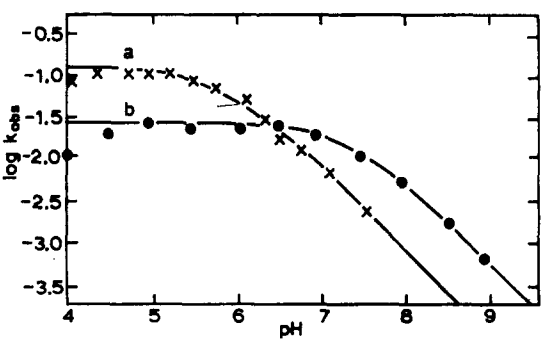


Fig. 2. Influence of pH on the rate of aging of cycloheptyl methylphosphonylated cholinesterase at 25.0 °C and $I = 0.100$. (a) acetylcholinesterase; (b) butyrylcholinesterase. Experimental points represent log $k_{obs}$ values. Dissociation curves of acids with p$K$ 5.84 (a) and 7.35 (b) are drawn.

The dependence of the rate constant of aging on pH can be described by

$$k_{obs} = k_{max} \frac{[XH]}{[X^-] + [H^+]} \tag{3}$$

where $k_{max}$ is the rate constant of aging of the protonated enzyme species XH and $[XH]/([X^-] + [H^+])$ the fraction of the phosphonylated enzyme in the undissociated form. After introduction of the equilibrium constant for the dissociation of this group

$$K = a_H \frac{[X^-]}{[XH]} \tag{4}$$

in which $a_{H+}$ equals the $H^+$ activity, the above mentioned equation can be transformed into

$$k_{obs} = k_{max} \frac{a_{H+}}{a_{H+} + K} \tag{5}$$

The values of $k_{max}$ and $K$ were evaluated from

$$\frac{1}{k_{obs}} = \frac{1}{k_{max}} + \frac{K}{k_{max}\, a_{H+}} \tag{6}$$

by a weighted least-squares method. Eqn 6 is obtained by rearranging Eqn 3. Weights which were inversely proportional to the standard deviations of the individual $k_{obs}$ values, were applied to the $k_{obs}$ values assuming that errors in these values are mutually independent and are independent of the pH at which they were determined. Errors in $a_{H+}$ values were assumed to be negligible with respect to those in the $k_{obs}$ values.

The results are given in Table I.

TABLE I

INFLUENCE OF pH, TEMPERATURE AND IONIC STRENGTH ON THE AGING OF CYCLOHEPTYL METHYLPHOSPHONYLATED ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

p$K$ and $k_{max}$ values were calculated by a weighted least-squares method from rate constants of aging of the phosphonylated enzymes measured in a 7.2 mM sodium diethylbarbiturate–sodium acetate buffer between pH 4.75 and 7.5 and between pH 4.9 and 9.0 for acetylcholinesterase and butyrylcholinesterase, respectively. The ionic strengths (0.100 and 0.020) were realized by addition of calculated amounts of NaCl. Constants are presented with standard deviation.

| Ionic strength | Temp. | Ionization | | | Aging | | |
|---|---|---|---|---|---|---|---|
| | | p$K$ | $\Delta H_i$ (kcal/mole[1]) | $\Delta S_i$ (cal/degree per mole) | $k_{max} \cdot 10^3$ (min$^{-1}$) | $\Delta H^*$ (kcal/mole[1]) | $\Delta S^*$ (cal/degree per mole) |
| Acetylcholinesterase | | | | | | | |
| 0.100 | 15.0 | 5.91 ± 0.03 | 1.2 ± 0.9 | −23 ± 3 | 41 ± 2 | 17.3 ± 0.8 | −13 ± 3 |
| | 25.0 | 5.84 ± 0.04 | | | 127 ± 9 | | |
| | 35.0 | 5.85 ± 0.02 | | | 312 ± 11 | | |
| 0.020 | 25.0 | 6.17 ± 0.02 | | | 97 ± 3 | | |
| Butyrylcholinesterase | | | | | | | |
| 0.100 | 15.0 | 7.57 ± 0.06 | 9.0 ± 0.2 | −3.6±0.5 | 10.2 ± 0.6 | 14.7 ± 0.6 | −25 ± 2 |
| | 25.0 | 7.35 ± 0.01 | | | 26.4 ± 0.2 | | |
| | 35.0 | 7.13 ± 0.01 | | | 57.5 ± 0.8 | | |
| 0.020 | 25.0 | 7.67 ± 0.04 | | | 31.4 ± 1.2 | | |

*Temperature dependence*

The influence of temperature on the rate of aging was investigated by performing experiments at 15.0 and 35.0 °C. In all experiments the rate constants observed between pH 4.9 and 9.0 fitted dissociation curves of a weak acid. The p$K$ and $k_{max}$ values with their standard deviations are given in Table I. From the

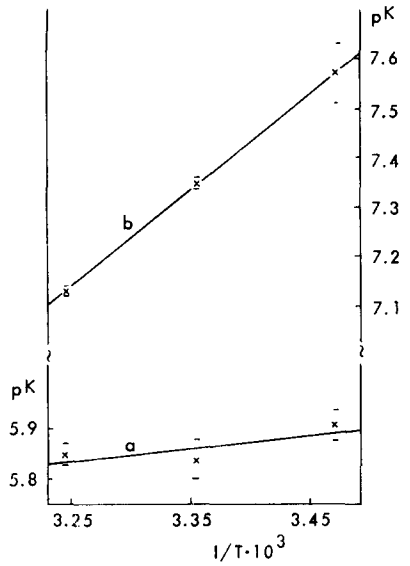


Fig. 3. Influence of temperature on the p$K$ of the group involved in the aging of (a) cycloheptyl methylphosphonylated acetylcholinesterase and (b) cycloheptyl methylphosphonylated butyrylcholinesterase at $I = 0.100$. p$K$ values are presented with standard deviations.

changes of p$K$ with temperature the heats of ionization ($\Delta H_i$) were calculated by applying the Van 't Hoff equation ($\Delta H_i = -R\mathrm{d}\ln K/\mathrm{d}1/T$) (Fig. 3), assuming that the heat capacities of reactants and products are not significantly different. With $\Delta F_i = -\mathrm{RT}\ln K$ and $\Delta F_i = \Delta H_i - T\Delta S_i$ the entropy of ionization at 25 °C was calculated. The influence of temperature on $k_{max}$ is illustrated by Arrhenius plots (Fig. 4). Thermodynamic parameters are given in Table I.

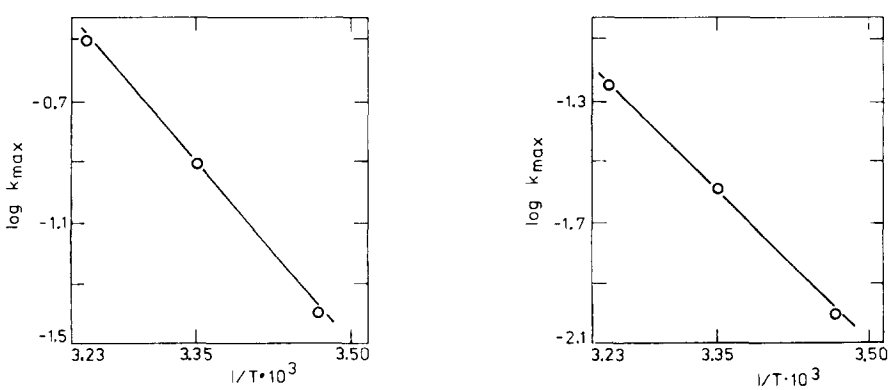


Fig. 4. Influence of temperature on the pH-independent rate constant ($k_{max}$) of aging of (a) cycloheptyl methylphosphonylated acetylcholinesterase and (b) cycloheptyl methylphosphonylated butyrylcholinesterase at $I = 0.100$ presented as Arrhenius plots.

*Effect of ionic strength*

Prior to the determination of pH–rate profiles at different ionic strengths a test was made on the influence of a number of monovalent cations in different

concentrations on the aging of cycloheptyl methylphosphonylated acetylcholinesterase and butyrylcholinesterase at pH 7.5 and 25 °C. Plots of log $k_{obs}$ versus ionic strength reveal a similar effect on the rate constant when ionic strength is made up to 0.020, 0.100, 0.200 and 0.300 with LiCl, NaCl, KCl and $NH_4Cl$. These results suggest the absence of a specific salt effect. The influence of ionic strength on p$K$ and $k_{max}$ is presented in Table I.

## DISCUSSION

From the qualitative correlation of the rate constants of aging of alkyl methylphosphonylated acetylcholinesterase and butyrylcholinesterase with the rate constants of unimolecular solvolysis of corresponding alkyl tosylates, brosylates and halides, Benschop and Keijer [6] suggested that aging of alkyl methylphosphonylated cholinesterases takes place by C–O fission in a unimolecular reaction. This finding was substantiated by Michel et al. [7].

The good fit of the observed rate constants of aging to the curve calculated for the ionization of one dissociable group in the pH region 4.75–9.0 for the phosphonylated butyrylcholinesterase and 4.5–7.5 for the phosphonylated acetylcholinesterase indicates that the degree of protonation of one group dissociating in these regions governs the rate of aging. The rate constant of aging corrected for the degree of protonation is the pH-independent rate constant, $k_{max}$.

The way in which the protonated group is involved may be directly by a participation in the aging reaction or indirectly by stabilization of a conformation of the enzyme, which is able to catalyze aging. If the protonated group is assumed to participate directly in the aging process, a possible mechanism may be the formation of a hydrogen bond by the protonated group with one of the oxygen atoms that are bound to the phosphorus atom. This hydrogen bond would polarize the C–O bond of the alkoxy group thereby facilitating its fission by formation of a carbonium ion.

The variation of $k_{max}$ with temperature obeys the Arrhenius law. It is therefore improbable that the changes in $k_{max}$ with temperature are due to a conformational change by which the regions of the phosphonylated enzymes which are responsible for the aging reaction are changed in such a way that this influences the aging process within this temperature range.

The decrease in $k_{max}$ for the aging of phosphonylated acetylcholinesterase by lowering the ionic strength is typical for a reaction mechanism in which a carbonium ion is formed in the rate-determining step [17].

If the p$K$ value of a certain group, its heat of ionization and the effect of ionic strength on the p$K$ are known, the nature of the group can often be specified provided that the group is not buried in the interior of the molecule or is involved in a system of more ionizing groups. Indications that the active site of acetyl- and butyrylcholinesterase is on or near the surface of the protein, were obtained from ESR studies of the enzymes spin labeled in their active site with a nitroxylorganophosphorus compound [18, 19]. As a consequence, the protonated group involved in aging is probably not a buried one. Then, if we assume that in the present study the thermodynamic parameters of ionization and the influence of ionic strength on the p$K$ can be compared with those generally observed for protonated groups in proteins, data suggest that the protonated group is a undissociated carboxyl group. Generally,

the heat of ionization of carboxyl groups in proteins may fall between −1.5 and +1.5 kcal/mole and the entropy of ionization is between −18 and −21 cal/degree per mole [20]. The observed increase of the p$K$ with decreasing ionic strength is consistent with the participation of a neutral group, since lowering of the ionic strength of the medium results in the enhancement of the electrical free energy needed to create the two charged particles from a neutral species [21]. The rather high p$K$ value of 5.84 compared to the p$K$ values usually observed for carboxyl groups in proteins (3.1–4.6) might be due to a stabilization of the undissociated form by formation of a hydrogen bond and/or a non-polar environment which may enhance the p$K$ value considerably as was found in lysozyme [22] and in ribonuclease $T_1$ [23].

Alternatively the participation of an undissociated sulfhydryl group with a relatively low p$K$ value might be considered. The dissociation of this group would be influenced in the same way by the ionic strength of the medium. Since thermodynamic data concerning the dissociation of the sulfhydryl groups in proteins are not available this alternative cannot be excluded on kinetic evidence. The observation that the rate constants of aging of both phosphonylated cholinesterases did not change when the reactions were performed in presence of *p*-chloromercuribenzoic acid, a compound which reacts readily with sulfhydryl groups in proteins, makes the participation of a sulfhydryl group unlikely (Table II).

TABLE II

EFFECT OF *p*-CHLOROMERCURIBENZOIC ACID ON THE AGING OF CYCLOHEPTYL METHYLPHOSPHONYLATED ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE AT pH 7.5, 25 °C AND $I = 0.100$

Rate constants of aging presented with standard deviation as calculated by least-square analysis of first-order decrease in attainable reactivation with time. Procedure a: Aging performed under standard conditions (see under Materials and Methods); Procedure b: Aging performed under standard conditions in presence of 1 mM *p*-chloromercuribenzoic acid added prior to phosphonylation; Procedure c: Same experiment as mentioned under Procedure b but with the mercurial added just before start of the aging process.

| Procedure | Acetylcholinesterase $k \cdot 10^3$ (min$^{-1}$) | Butyrylcholinesterase $k \cdot 10^3$ (min$^{-1}$) |
|---|---|---|
| a | 2.54 ± 0.03 | 10.9 ± 0.2 |
| b | 2.47 ± 0.07 | 11.7 ± 0.3 |
| c | 2.53 ± 0.04 | 11.2 ± 0.4 |

Although the results obtained by Benschop and Keijer [6] suggest the same mechanism for the aging of both phosphonylated acetylcholinesterase and butyrylcholinesterase, the dependence of $k_{max}$ for aging of butyrylcholinesterase on ionic strength does not agree with a carbonium ion mechanism.

The p$K$ of the protonated group involved in aging of butyrylcholinesterase (7.35) is close to the value expected for an imidazolium group (5.6–7.0). Its heat of ionization (9.0 kcal/mole) lies within the ranges that are found for imidazolium (6.5–7.5 kcal/mole) and ammonium groups (10–12 kcal/mole). However, the change in p$K$ due to a change in ionic strength of the medium does not correspond with the participation of these groups, since the free energy of deprotonation of a cationic acid is hardly influenced by a change in ionic strength of the medium [21]

A straightforward explanation cannot be given for the phenomena observed for the aging of phosphonylated butyrylcholinesterase. The aging may be catalyzed by participation of an imidazolium or ammonium group of the enzyme. However, the influence of ionic strength on p$K$ and $k_{max}$ is conflicting with the participation of such a group and with a unimolecular reaction, respectively. Data may be interpreted by assuming that more than one ionizing group is involved. The results indicate that the mechanism of aging of phosphonylated butyrylcholinesterase is much more complicated than that described for acetylcholinesterase.

REFERENCES


1 Wilson, I. B. and Ginsburg, S. (1955) Biochim. Biophys. Acta 18, 168–170
2 Hobbiger, F. (1955) Br. J. Pharmacol. 10, 356–362
3 Berends, F., Posthumus, C. H., v. d. Sluys, I. and Deierkauf, F. A. (1959) Biochim. Biophys. Acta 34, 576–578
4 Fleisher, J. H. and Harris, L. W. (1965) Biochem. Pharmacol. 14, 641–650
5 Harris, L. W., Fleisher, J. H., Clark, J. and Cliff, W. J. (1966) Science 154, 404–406
6 Benschop, H. P. and Keijer, J. H. (1966) Biochim. Biophys. Acta 128, 586–588
7 Michel, H. O., Hackley, Jr, B. E., Berkowitz, L., List, G., Hackley, E. B., Gillian, W. and Pankau, M. (1967) Arch. Biochem. Biophys. 121, 29–34
8 Hobbiger, F. (1956) Br. J. Pharmacol. 11, 295–303
9 Berends, F. (1964) Reactivering en "veroudering" van esterasen geremd met organische fosforverbindingen, Ph.D. Thesis, University of Leiden
10 Berry, W. K. and Davies, D. R. (1966) Biochem. J. 100, 572–576
11 Strelitz, F. (1944) Biochem. J. 38, 86–88
12 Ooms, A. J. J. and Boter, H. L. (1965) Biochem. Pharmacol. 14, 1839–1846
13 Alner, D. J., Greczek, J. J. and Smeeth, A. G. (1967) J. Chem. Soc. (A) 1205–1211
14 Keijer, J. H. (1970) Anal. Biochem. 37, 439–446
15 Keijer, J. H. and Wolring, G. Z. (1969) Biochim. Biophys. Acta 185, 465–468
16 Guggenheim, E. A. (1926) Phila. Mag. 2, 538–543
17 Frost, A. A. and Pearson, R. G. (1953) Kinetics and Mechanism, pp. 138–142, John Wiley and Sons, London
18 Morrisett, J. D., Broomfield, C. A. and Hackley, Jr, B. E. (1969) J. Biol. Chem. 244, 5758
19 Hsia, J. C., Kosman, D. J. and Piette, L. H. (1969) Biochem. Biophys. Res. Commun. 36, 75
20 Steinhardt, J. and Beychok, S. (1964) in The Proteins (Neurath, H., ed.), Vol. 2, pp. 243–246, Academic Press, New York
21 Steinhardt, J. and Beychok, S. (1964) in The Proteins (Neurath, H., ed.), Vol. 2, p. 172, Academic Press, New York
22 Dahlquist, F. W. and Raftery, M. A. (1968) Biochemistry 7, 3277–3280
23 Ida, S. and Ooi, T. (1969) Biochemistry 8, 3897–3901