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EFFECT OF 1-(AR)ALKYL-2-HYDROXYIMINOMETHYL-PYRIDINIUM SALTS ON REACTIVATION AND AGING OF ACETYLCHOLINESTERASE INHIBITED BY ETHYL DIMETHYLPHOSPHORAMIDOCYANIDATE (TABUN)

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Abstract—The reactivation of bovine erythrocyte acetylcholinesterase inhibited by ethyl dimethylphosphoramidocyanidate (tabun) was studied with 1-benzyl- and some 1-alkyl-2-hydroxyiminomethylpyridinium salts. The enzyme activity was not completely restored with these oximes. The reactions are consistent with a reaction scheme involving simultaneous aging of the inhibited enzyme, and reactivation and aging of the inhibited enzyme–oxime complex. The dissociation constants of the complexes and the rate constants of reactivation and aging were evaluated. The value of the dissociation constant decreases with increasing lipophilicity of the oxime. The *n*-propyl compound is more powerful than the other 1-alkyl-pyridinium-oximes in reactivation at high concentrations, the dodecyl compound is the most effective reactivator when used at low concentrations. The benzyl compound is the most potent reactivator at all concentrations. The oximes containing a longer alkyl group accelerate the aging of tabun-inhibited acetylcholinesterase; corresponding 1-alkyl-pyridinium iodides have a retarding effect.

Certain organophosphates inhibit acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by phosphorylating the enzyme at the active site. The activity of the inhibited enzyme can be restored by nucleophilic agents, which displace the organophosphate moiety from the enzyme. The most effective reactivators are found among the aldoximes, like P2S (2-hydroxyiminomethyl-1-methyl-pyridinium methanesulphonate) and trimedoxime (1,1'-(1,3-propanediyl) bis (4-hydroxyiminomethyl-pyridinium) dibromide) [1]. The efficacy of the reactivation depends on the structure of the organophosphate moiety bound to the enzyme. A rather stable phosphorylated enzyme, and, hence, rather resistant to reactivation by oximes, is formed upon inhibition of acetylcholinesterase with the nerve agent ethyl dimethylphosphoramidocyanidate (tabun).

Although many reactivation studies on inhibited acetylcholinesterase which are readily reactivated by oximes have been reported, only a few investigations have been performed with tabun-inhibited enzyme [2–5]. In this paper a detailed study on the reactivation of tabun-inhibited acetylcholinesterase with P2S and some 1-(ar)alkyl analogues is described. The dissociation constants of the complex of inhibited enzyme with oxime, which is initially formed during reactivation, and the rate constants of reactivation were evaluated. In the course of the investigation it turned out that aging of the inhibited enzyme, i.e. conversion into a non-reactivatable form, played a substantial role during reactivation with some of the oximes. An attempt to evaluate the effect of these oximes on aging is also described.

MATERIALS AND METHODS

Materials. Bovine erythrocyte acetylcholinesterase was obtained from Sigma Chemical Co., St. Louis, MO., U.S.A., and had a sp. act. of 27 nkat/mg of protein at 25° in 0.6 mM phosphate buffer, pH 7.5, containing 3.2 mM acetylcholine perchlorate and 0.1 M potassium chloride. P2S was purchased from Dr. F. Raschig GmbH, West Germany. The other pyridinium compounds and tabun were prepared in this laboratory according to methods known from literature and had satisfactory elemental analysis. The structure of the oximes was confirmed by infrared and NMR spectroscopy. All other reagents were commercial products of an analytical grade.

Reactivation experiments. Inhibited acetylcholinesterase was obtained by incubating a mixture of a solution of 2.2 mg enzyme/ml (approx. 10 nM of active sites) of 6.6 mM veronal buffer, pH 7.5, with an equal volume of 0.1 μM tabun in the same buffer for 1 hr at 25°. Under these conditions over 95 per cent of the enzyme was phosphorylated. Excess of inhibitor was hydrolyzed by keeping the solution at pH 10 and 25° for 1 hr. In a control experiment this treatment did not influence the enzyme activity. Incubation of acetylcholinesterase (1.1 mg/ml) in a sample of the inhibited enzyme solution for 24 hr (pH 7.5, 25°) gave less than 5 per cent inhibition of the enzyme indicating a complete hydrolysis of the excess inhibitor. Next, the pH of the inhibited enzyme solution was adjusted to 7.5 and reactivation was started by the addition of 1 volume of an oxime solution in 40 mM phosphate buffer, pH 7.5,

to 4 vol. of the inhibited enzyme solution. The enzyme was allowed to reactivate at pH 7.5 and 25° for 24–48 hr. The restored enzyme activity was measured with time intervals of 12–30 min during the first 4–5 hr, and next, with intervals of 1–2 hr in the following manner. One-ml samples of the reactivation mixture were automatically assayed for enzyme activity at a previously selected time interval (12–30 min) by using an apparatus developed by Keijer [6] for the automatic performance of pH stat titrations. In the Radiometer titration equipment of the apparatus the burette unit SBU 1a was exchanged for an ABU 13 with a 0.25-ml burette assembly. The programmer of the apparatus was used to control also a Braun perfusor type Unit IIb, in which a 50-ml syringe containing a 40 mM phosphate buffer, pH 7.5, was mounted. The perfusor was energized at each movement of the turntable to its next position delivering 0.17 ml of the buffer to the assay mixture. The activity assay was performed at pH 7.5 and 25° with 23 ml of a 3.2 mM acetylcholine perchlorate solution in 0.1 M potassium chloride. The titrant was 0.05 N NaOH. Enzyme activities were corrected for spontaneous hydrolysis of the substrate. Blancs for the activity of the enzyme (A), the enzyme incubated with oxime (AR) and the inhibited enzyme (AI) were determined at the start of the reactivation experiment. Values of A and AR were also determined in samples incubated at pH 7.5 and 25° for approximately the same time period as required to obtain the maximum activity in the reactivation mixture. Incubation for time periods up to 48 hr affects very slightly the values of A and AR.

The percentage of reactivation at time t (% react$_t$) was calculated according to

$$\% \text{ react}_t = \frac{\text{AIR}_t \frac{\text{A}}{\text{AR}} - \text{AI}}{\text{A} - \text{AI}} 100 \qquad (1)$$

where AIR$_t$ is the activity of inhibited enzyme after incubation with oxime for time t. The percentage of maximum reactivation (% react$_\infty$) was obtained as the mean of at least five values of % react calculated by using A and AR values of the blancs which were run for approximately the same time period. Values of % react$_t$ as used for the evaluation of rate constants were calculated with A and AR values measured at the start of the experiment.

Aging experiments. Aging of the inhibited enzyme was determined from the decrease of attainable reactivation. Inhibition of the enzyme and removal of the excess of inhibitor were carried out as described for the reactivation experiments. Aging was started by adjusting the pH of the inhibited enzyme solution to 7.5 and by subsequently mixing of 4 vol. of this solution with 1 vol. of 40 mM phosphate buffer, pH 7.5, without or with added 1-alkyl-pyridinium iodide. During the first 5.5 hr of the aging reaction and, after 20 hr, during the next 9.5 hr, 2.5 ml samples were taken at time intervals of 0.5–1 hr and added to 0.13 ml of a 61 mM trimedoxime solution in 40 mM phosphate buffer, pH 7.5, and allowed to reactivate for 24 hr at 25°. Then, 1 ml samples were assayed for enzyme activity as described for the reactivation experiments. Blancs of A and AR were determined after 24 hr and 48 hr of incubation.

Percentages of reactivation were calculated by using the appropriate blancs of A and AR according to equation (1), where AIR$_t$ is the activity of inhibited enzyme after incubation without or with added 1-alkyl-pyridinium iodide for time t and a subsequent incubation with trimedoxime for 24 hr. The enzyme activity of samples taken at zero time of aging was restored to 98 per cent. Rate constants of aging were calculated from plots of ln (% react$_t$) vs t by means of the method of least squares.

Aging of tabun-inhibited enzyme in the presence of an oxime was followed in a similar manner. Samples of 2.5 ml of the reactivation mixture (see Reactivation experiments) were taken at various times and added to 0.13 ml of a 0.2 M P2S solution in 40 mM phosphate buffer, pH 7.5, and allowed to reactivate for 24 hr at 25°. Next, 1 ml samples were assayed for enzyme activity.

Direct reaction of the oximes with tabun. The direct reaction of the oximes with tabun was followed spectrophotometrically from the decrease of the oxime concentration according to the method given by Hagedorn *et al.* [7]. The reaction was started by addition of 50μl of a 0.123 M tabun solution in isopropanol to 2.5 ml of a 0.2 mM oxime solution in 25 mM veronal buffer, pH 7.5, containing 0.1 M potassium chloride. The decrease in absorbance of this solution at 25° was followed in a Beckman Acta III spectrophotometer at 335 nm. Readings of absorbance were taken during three half lives of the reaction and corrected for the infinity value measured after 10 half lives. Plots of ln (absorbance) vs time were made from which the slope was calculated by means of the method of least squares. The bimolecular rate constants determined in duplicate were obtained as the slope divided by the concentration of tabun.

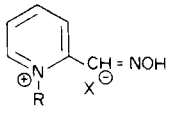
RESULTS

Maximum reactivation. Incubation of tabun-inhibited acetylcholinesterase with the oximes did not result in a complete reactivation. In general, the percentages of maximum reactivation decreased with decreasing concentration of the oxime. No further reactivation was observed after addition of P2S to a final concentration of 10 mM and incubation for 24 hr. The results are summarized in Table 1.

Treatment with 10 mM trimedoxime restored 98 per cent of the enzyme activity. With the same concentration of the oxime complete reactivation was attained of the inhibited enzyme which was formed after inhibition at pH 8.0 and 0° for 1 hr. On the basis of these results it was assumed that at the start of the reactivation experiments 98 per cent of the inhibited enzyme was able to be reactivated.

To study the effect of the time of incubation with an oxime on the ability of the inhibited enzyme to be reactivated the following experiments were carried out. At various times samples of an incubation

Table 1. Percentages of maximum reactivation (% react$_\infty$) of tabun-inhibited acetylcholinesterase obtained after incubation with some 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts at pH 7.5 and 25°



R	X	Range of oxime concentrations used (mM)	% React$_\infty$
CH_3	CH_3SO_3	0.5–3 (6)*	91–96
C_2H_5	I	0.15–0.7 (6)	92–95
C_3H_7	I	0.15–0.7 (6)	92–96
i-C_3H_7	I	0.15–3 (9)	84–96
C_5H_{11}	I	0.05–0.4 (6)	79–83
C_7H_{15}	I	0.06–0.75 (8)	65–72
$C_{12}H_{25}$	I	0.003–0.05 (8)	68–78
$CH_2C_6H_5$	Br	0.003–0.1 (8)	82–97

* Values in parentheses give the number of oxime concentrations used.

mixture were added to P2S (final concentration 10 mM), incubated for 24 hr and assayed for enzyme activity. The results of an experiment carried out with 8 μM l-dodecyl-pyridinium-oxime is shown in Fig. 1. The results indicate that a conversion of the inhibited enzyme occurs in the presence of the oxime into a form which cannot be reactivated (aging). So, a part of the inhibited enzyme has been reactivated after 48 hr of incubation and it is assumed that the rest of it has aged. Similar results were obtained with the l-i-propyl- (0.3 mM), the l-pentyl- (0.1 mM) and the l-heptyl-pyridinium-oxime (0.2 mM).

Kinetic analysis. The reaction of the phosphorylated acetylcholinesterase (EI) with an oxime (OX) forming the reactivated enzyme (E) may be described by [3, 8, 9].

$$EI + OX \rightleftarrows EI \cdot OX \rightarrow E + \text{products} \qquad (2)$$

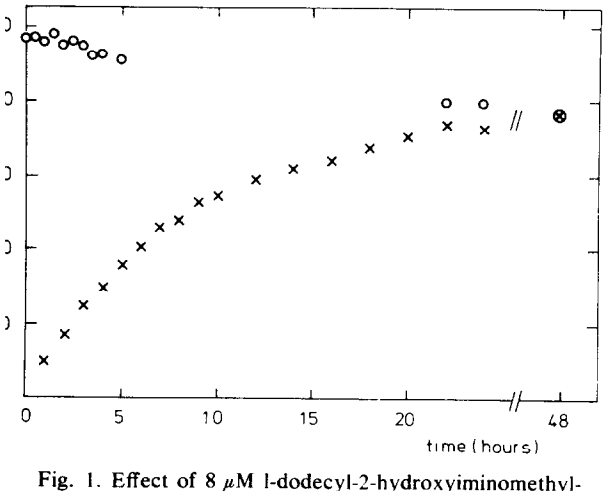
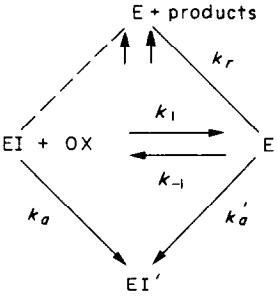


Fig. 1. Effect of 8 μM l-dodecyl-2-hydroxyiminomethyl-pyridinium iodide on reactivation (×) and on the decrease of reactivatability (○) of tabun-inhibited acetylcholinesterase at pH 7.5 and 25°. Maximally attainable reactivation was achieved after 24 hr of incubation with 10 mM P2S (pH 7.5, 25°).

To describe our experiments in which reactivation and aging occur simultaneously, we extended this mechanism and adopted the following scheme:



(3)

where EI' represents the aged enzyme. Reactivation of the inhibited enzyme in the absence of oxime, spontaneous reactivation, was not noticed. In the experiments the oxime concentration used was in at least 750-fold excess over that of the enzyme and was considered to be constant during the reactions.

The dissociation constant of the complex of inhibited enzyme with oxime (K_d) equals

$$K_d = \frac{k_{-1}}{k_1} = \frac{[EI][OX]}{[EI \cdot OX]} \qquad (4)$$

By defining:

$$[EI_{tot}] = [EI] + [EI \cdot OX] \qquad (5)$$

it follows that:

$$[EI_{tot}] = [EI]\left(1 + \frac{[OX]}{K_d}\right) = [EI \cdot OX]\left(1 + \frac{K_d}{[OX]}\right) \qquad (6)$$

The following differential equations can be derived for Scheme 3:

$$-\frac{d[EI_{tot}]}{dt} = -\frac{d[EI]}{dt} - \frac{d[EI \cdot OX]}{dt} = k_a[EI] + k_a'[EI \cdot OX] + k_r[EI \cdot OX]$$

or:

$$-\frac{d[EI_{tot}]}{dt} = \frac{k_a K_d + k_a'[OX] + k_r[OX]}{K_d + [OX]}[EI_{tot}] \qquad (7)$$

$$\frac{d[EI']}{dt} = \frac{k_a K_d}{K_d + [OX]}[EI_{tot}] + \frac{k_a'[OX]}{K_d + [OX]}[EI_{tot}] \qquad (8)$$

$$\frac{d[E]}{dt} = \frac{k_r[OX]}{K_d + [OX]}[EI_{tot}] \qquad (9)$$

Integration of equation 7 leads to:

$$[EI_{tot}]_t = [EI_{tot}]_0 e^{-k_{obs} t} \qquad (10)$$

where:

$$k_{obs} = \frac{k_a K_d + k_a'[OX] + k_r[OX]}{K_d + [OX]} \qquad (11)$$

Integration of equations 8 and 9 after substitution for $[EI_{tot}]$ according to equation 10 leads to:

$$[EI']_t = \frac{k_a K_d + k_a'[OX]}{k_{obs}(K_d + [OX])}[EI_{tot}]_0(1 - e^{-k_{obs} t}) \qquad (12)$$

and:

$$[E]_t = \frac{k_r[OX]}{k_{obs}(K_d + [OX])}[EI_{tot}]_0(1 - e^{-k_{obs} t}) \qquad (13)$$

When $t = \infty$, $[E]_\infty = k_r[OX][EI_{tot}]_0/k_{obs}(K_d + [OX])$.

Hence, equation 13 can be rewritten to:

$$\ln([E]_\infty - [E]_t) = \ln[E]_\infty - k_{obs}t \quad (14)$$

From equations 12 and 13 it follows that:

$$\frac{[EI']_t}{[E]_t} = \frac{[EI']_\infty}{[E]_\infty} = \frac{k_a K_d + k_a'[OX]}{k_r[OX]} \quad (15)$$

Equations 11, 14 and 15 were used for the evaluation of the kinetic parameters K_d, k_r and k_a' from the reactivation data after rewriting these equations in the following manner. By expressing the relative concentrations of reactivated enzyme as % react equation 14 can be represented by

$$\ln(\%\ \text{react}_\infty - \%\ \text{react}_t) = \ln(\%\ \text{react}_\infty) - k_{obs}t \quad (16)$$

Equation 11 can be rearranged to

$$k_{obs} - k_a = \frac{k_{max}[OX]}{K_d + [OX]} \quad (17)$$

where:

$$k_{max} = k_a' + k_r - k_a \quad (18)$$

From the equations 15 and 18 it can be derived that:

$$k_a' = (k_{max} + k_a)\left\{\frac{[EI']_\infty}{[E]_\infty} - \frac{k_a K_d}{[OX]}\right\} \Big/ \left\{1 + \frac{[EI']_\infty}{[E]_\infty}\right\} \quad (19)$$

Kinetic constants. The rate constant of aging in the absence of oxime (k_a) is 2.5×10^{-4} min^{-1}.

A least squares analysis of plots of ln(% react$_\infty$ − % react$_t$) vs t (equation 16) give straight lines with intercept ln(% react$_\infty$) and slope k_{obs}. Two examples are given in Fig. 2. The results of two reactivation experiments carried out with twice the inhibited enzyme concentration are also given and show that the rate of the reactivation process is independent of the initial concentration of the inhibited enzyme in agreement with the proposed reaction scheme.

Values of k_{obs} were determined at various concentrations of the oximes (see Table 1). From these data the parameters K_d and k_{max} were evaluated by fitting equation 17 to a set of $k_{obs} - k_a$, [OX] data by means of the non-linear regression method of

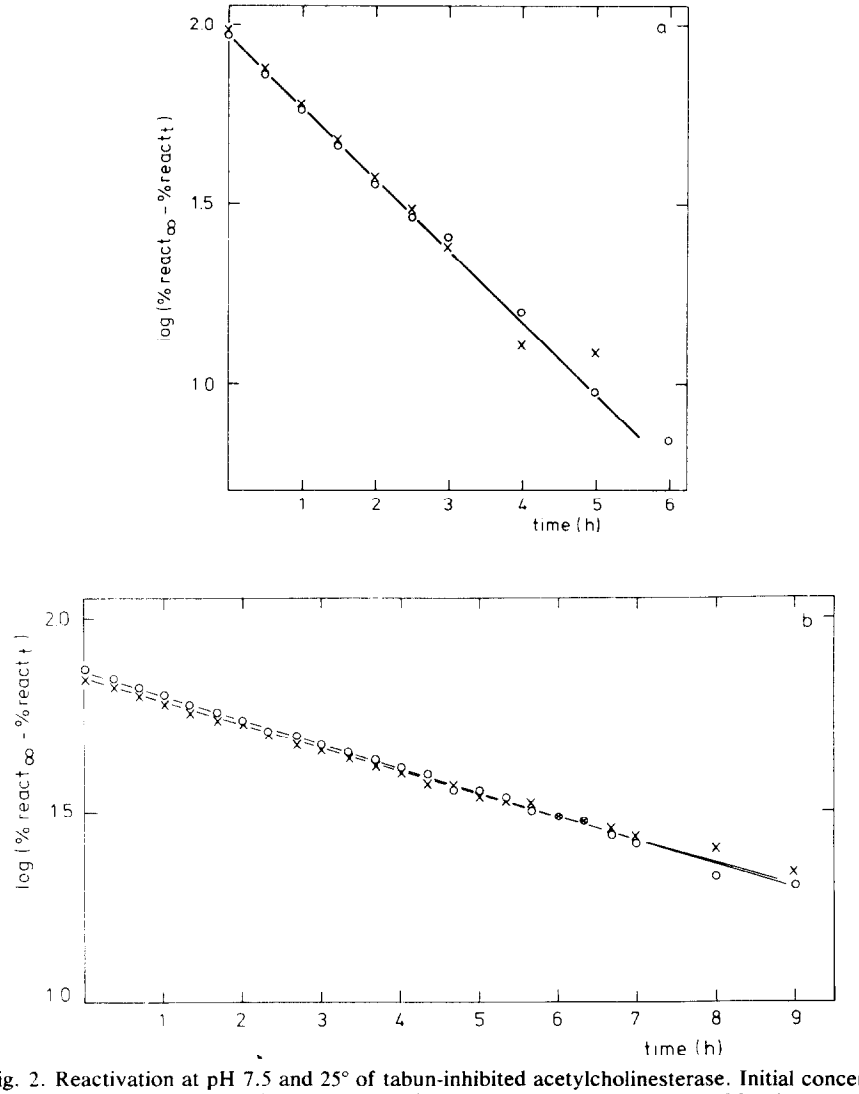
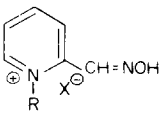


Fig. 2. Reactivation at pH 7.5 and 25° of tabun-inhibited acetylcholinesterase. Initial concentrations of inhibited enzyme: 0.88 mg/ml (×), 1.76 mg/ml (○). (a) Reactivation with 3 mM P2S; (b) reactivation with 0.2 mM 1-heptyl-2-hydroxyiminomethyl-pyridinium iodide.

Table 2. Kinetic constants and their S.E. of reactivation and aging of tabun-inhibited acetylcholinesterase in the presence of some 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts at pH 7.5 and 25°



R	k_{max} (10^{-3} min^{-1})	K_d (mM)	k_r (10^{-3} min^{-1})	k_r/K_d (M^{-1} min^{-1})	k_a' (10^{-4} min^{-1})
CH_3	8.3 ± 0.3	0.54 ± 0.06	8.3 ± 0.3	15 ± 2	2.5 ± 1.0
C_2H_5	13.8 ± 0.9	0.44 ± 0.06	13.8 ± 0.9	31 ± 4	ND
C_3H_7	18.7 ± 1.9	0.34 ± 0.08	18.4 ± 1.9	54 ± 12	5.3 ± 2.4
i-C_3H_7	12.3 ± 1.3	1.8 ± 0.4	12.1 ± 1.4	6.7 ± 1.6	ND
C_5H_{11}	14.1 ± 0.7	0.17 ± 0.02	12.2 ± 0.8	72 ± 10	21 ± 4
C_7H_{15}	3.1 ± 0.1	0.089 ± 0.014	2.4 ± 0.2	27 ± 6	9.0 ± 1.8
$C_{12}H_{25}$	3.7 ± 0.1	0.0077 ± 0.0008	3.2 ± 0.2	420 ± 50	7.7 ± 0.8
$CH_2C_6H_5$	70 ± 5	0.11 ± 0.01	69 ± 5	630 ± 100	12 ± 7

ND—Not determined (see text).

Wilkinson [10]. Next, the value of k_a' was calculated according to equation 19 by using the values obtained for K_d and k_{max} and those measured for % react$_\infty$ from which $[EI']_\infty/[E]_\infty$ was calculated as (98 − % react$_\infty$)/(% react$_\infty$) (see Maximum reactivation). The value of k_a' was obtained as the mean of values calculated for the individual oxime concentrations. Subsequently, k_r was obtained with equation 18. The results together with the values calculated for the bimolecular rate constant of reactivation, k_r/K_d, are given in Table 2. Standard errors of K_d, k_{max}, k_r and k_r/K_d were calculated by means of the methods described by Wilkinson [10].

Aging contributes slightly to the overall rate of the reactions carried out in the presence of the benzyl compounds or of the oximes containing a small alkyl group. Consequently, accurate values of k_a' could not be determined, especially in the case of the ethyl and the *i*-propyl compound. Since reliable values of k_a' were not obtained for these compounds, the values of k_r were evaluated as the mean of values calculated according to equation 15 which was rewritten to an expression of k_r analogous to that given for k_a' (equation 19).

Direct reaction of the oximes with tabun. In the direct reaction of the oximes with tabun a very unstable phosphorylated oxime is formed, as was concluded from the ultra violet (u.v.) spectra of the reaction mixtures after complete reaction [7]. The final spectrum of the P2S-tabun mixture is identical to that of 2-cyano-1-methyl-pyridinium iodide. Similar spectra were obtained after reaction of the other oximes with tabun. Usually, the cyanide moiety is the leaving group in these nucleophilic substitution reactions with tabun. Formation of dimethylamine could not be demonstrated by analyzing the reaction mixture colorimetrically after 10 half lives according to the method described by Dowden [11]. The rate constants given in Table 3 are not corrected for the partial dissociation of the oximes at pH 7.5.

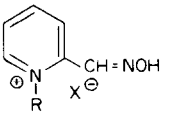
Effect of 1-alkyl-pyridinium iodides on aging. Some 1-alkyl-pyridinium iodides studied exhibit a retarding effect on aging of tabun-inhibited acetylcholinesterase. The rate constants of aging in the presence of 2 mM 1-methyl-pyridinium iodide, 2 mM 1-heptyl-pyridinium iodide or 0.1 mM 1-decyl-pyridinium iodide are 1.7×10^{-4}, 1.1×10^{-4} and 0.7×10^{-4} min^{-1}, respectively.

DISCUSSION

Aging of human erythrocyte acetylcholinesterase inhibited by tabun was previously reported [3], to proceed at a discernible rate in the presence of P2S or trimedoxime. A similar phenomenon is observed in the present experiments with tabun-inhibited bovine erythrocyte acetylcholinesterase and 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts. The reactions can be described by Scheme 3. In addition to aging of the inhibited enzyme, two simultaneous reactions, reactivation and aging, occur from the inhibited enzyme–oxime complex.

In Fig. 3 the dissociation constants of inhibited enzyme–oxime complex are plotted against the hydrophobic fragmental constants of the (ar)alkyl residues as given by Rekker [12]. The affinity of the inhibited enzyme to the oximes increases with the increasing lipophilicity of the oximes. The inhibited enzyme shows a decreased affinity to the oxime containing the branched *i*-propyl group. A similar relationship is found between the dissociation constants of free enzyme-oxime complex and the lipo-

Table 3. Bimolecular rate constants (k_1) of the direct reaction of some 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts with tabun at pH 7.5 and 25°



R	pK_a	k_1 (M^{-1} min^{-1})
CH_3	7.9	83
C_2H_5	8.0	82
C_3H_7	8.0	81
i-C_3H_7	8.2	70
C_5H_{11}	8.1	75
C_7H_{15}	8.0	86
$C_{12}H_{25}$	7.8	91
$CH_2C_6H_5$	8.0	115

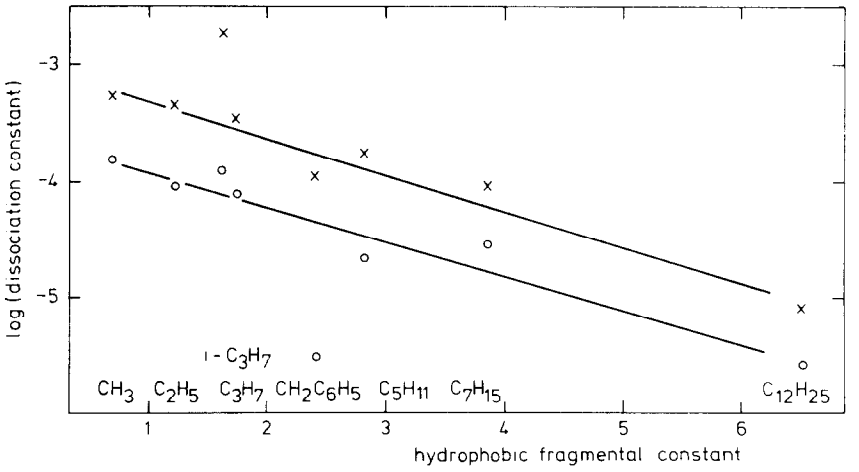


Fig. 3. Plot of the logarithm of the dissociation constants at 25° of the complex between acetylcholinesterase (pH 7.2, O) or tabun-inhibited acetylcholinesterase (pH 7.5, ×) with some 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts against the hydrophobic fragmental constants of the (ar)alkyl residues.

philicity of the oximes (Fig. 3). The dissociation constants were taken from a previous paper [13] or determined as described in the paper (pentyl and benzyl compound). Phosphorylation of the enzyme decreases its affinity to the oximes considerably. Nevertheless, it seems reasonable to assume that the hydrophobic area near the anionic binding site as proposed for the binding of *N*-alkyltrimethylammonium ions [14–16], 1-alkyl-pyridinium and 1-alkyl-2-hydroxyiminomethyl-pyridinium salts [13] to the free enzyme, is involved in the binding of the (ar)alkyl moiety of the oximes to the inhibited enzyme. Patočka [17] observed a different effect of enlargement of the alkyl group on the affinity of bovine erythrocyte acetylcholinesterase inhibited by *i*-propyl methylphosphonofluoridate (sarin) to a series of 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts. Oximes in which the alkyl chain varied from methyl to hexyl, and the benzyl compound were studied. The inhibited enzyme exhibits the highest affinity to the propyl and the benzyl compound.

The reactivity of the oximes towards tabun varies only slightly, as should be expected on the basis of the small variation in their pK_a values (Table 3). After binding to the tabun-inhibited enzyme the oximes show somewhat larger differences in reactivity in the more complicated reactivation reaction (k_r, Table 2). Phosphorylation of the enzyme has the largest effect on its affinity to the 1-benzyl-pyridinium-oxime. It is remarkable that this oxime is by far the most reactive in the reactivation reaction. The value of k_r represents the reactivating potency of the oximes when used in high concentrations. The bimolecular rate constant, k_r/K_d, is a measure of the potency of reactivators used in low concentrations. So, the propyl compound is more powerful than the other 1-alkyl-pyridinium-oximes in reactivation at high concentrations, but the dodecyl compound is the most effective reactivator when used in low concentrations. At all concentrations, however, the benzyl compound is by far the most potent reactivator of tabun-inhibited acetylcholinesterase. Patočka [17] investigating the reactivation of sarin-inhibited acetylcholinesterase found that the k_r and k_r/K_d values of the benzyl compound are also larger than the corresponding values of the 1-alkyl-pyridinium-oximes studied. The effect of enlargement of the alkyl group on k_r and k_r/K_d, however, differs largely from the present findings for the tabun-inhibited enzyme. Although the tabun-inhibited enzyme has a much higher stability towards reactivation with oximes than the sarin-inhibited enzyme, the different effect of enlargement of the alkyl group of the pyridinium-oximes on the rate constants of reactivation of the two enzymes is hard to explain.

The oximes containing a longer alkyl group accelerate the aging of tabun-inhibited acetylcholinesterase. In general, quaternary ammonium compounds, including pyridinium-oximes, have a retarding effect on aging [18–22]. The reverse effect of the 1-alkyl-pyridinium-oximes is not due to the additional binding of these compounds to the hydrophobic area only, as follows from the results obtained with 1-alkyl-pyridinium iodides, which also retard aging. It is not clear how the oxime moiety is involved in the acceleration of the aging. It is generally accepted that aging of phosphorylated or phosphonylated acetylcholinesterase proceeds via the release of an alkyl group from the phosphorus moiety [18, 23, 24]. It is very unlikely that the dealkylation is accelerated by a direct attack of the oxime anion, a hard nucleophile, on the soft electrophilic carbon center of the ethyl group of the phosphoryl moiety in the tabun-inhibited enzyme. Investigations by Keijer *et al.* [25] indicate that a protonated group in the enzyme is involved in the catalysis of the aging of phosphonylated acetylcholinesterases. It might be possible that the undissociated form of the oximes accelerates the aging of tabun-inhibited acetylcholinesterase in an analogous manner. An alternative mechanism for the aging of tabun-inhibited acetylcholinesterase can not be excluded in which the ethoxy group or even the dimethylamino group is split off instead of the

ethyl group. The pyridinium-oximes might accelerate the aging proceeding according to such a mechanism either indirectly due to a conformational change induced by the binding of the oximes or directly by an interaction of the oxime moiety with the phosphoryl group of the inhibited enzyme. Investigations along these lines including a study on the effect of the l-alkyl-pyridinium-oximes on the aging of acetylcholinesterase inhibited by other organophosphates are in progress.

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