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REACTIVATION BY IMIDAZO-PYRIDINIUM OXIMES OF ACETYLCHOLINESTERASE INHIBITED BY ORGANOPHOSPHATES

A STUDY WITH AN IMMOBILIZED ENZYME METHOD

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Abstract—A new procedure is described for studying inhibition and reactivation of acetylcholinesterase (AChE). The enzyme, electric eel AChE, is immobilized on fiberglass and the enzymatic activity is continuously monitored in an open reactor by an assay adapted from the Ellman's method. The use of immobilized AChE permits independent inhibition and reactivation of the enzyme. Side-reactions between substrate, inhibitor and reactivator are avoided. This method is used to determine the reactivating efficiency of a new series of imidazo-pyridinium oximes for the enzyme inhibited by different organophosphorous compounds. Kinetic parameters of reactivation were determined after AChE inhibition by sarin, VX and paraoxon. The more efficient reactivators have a short methylene bridge (C3 to C6) between imidazolium and pyridinium rings.

Against soman inhibition, the pyrimidoxime or 1-(1-methyl-imidazolinium) 3-(4-carbaldoxime-pyridinium) propane dibromide, introduced immediately after the inhibitor, gives the same result as TMB-4 (37% reactivation). 1-benzyl 2-carbaldoxime pyridinium bromide was found to be more potent in reactivating tabun inhibited AChE than pyrimidoxime.

Imidazo-pyridinium oximes with a long methylene bridge (C8 to C10) are good reversible inhibitors of free AChE ($K_i < 1\ \mu M$).

Organophosphates inhibit acetylcholinesterase (AChE) by phosphorylation of a serine residue in the active site. The inhibited enzyme can be reactivated by a variety of oximes, such as 2-PAM, TMB-4, and HI-6 [1].

These reactivators are usually tested *in vitro* using purified AChE inhibited by an organophosphate. The restored activity is usually measured by the pH-stat [2] method or by Ellman's method [3]. This batch assay is often disturbed by undesirable side-reactions such as reversible inhibition of AChE by oximes, retroinhibition by the phosphorylated oximes, a potent inhibitor formed during the reactivating process, and nonenzymatic hydrolysis of the substrate by oximes. These inconviences can be counteracted by gel filtration or dialysis of the various molecular species but these methods are not well adapted for systematic investigations.

To overcome these problems we have recently described a continuous flow method adapted from Ellman's technique [4]. In this paper the reactivation potency of some oximes was evaluated with rat brain slices as a model of AChE in its biological membrane. Previous *in vitro* and *in vivo* studies [4, 5] with rat brain AChE have shown that imidazo-pyridinium oximes 1a and 1b are reactivators almost as potent as TMB-4, after the inhibition by VX and paraoxon; moreover these oximes had weak acute toxicity in the rat.

Here we report the use of electric eel AChE immobilized by adsorption on fiberglass paper for continuous flow assay, enabling us to follow the inhibition and the reactivation of the enzyme in the presence (or not) of the substrate without chromogen (DTNB) and without excess of any inhibitor or reactivator species. For the same purpose, Trammel *et al.* [6] used a continuous flow method with electric eel AChE covalently linked on polyethylene beads layered on a column.

The efficiency of a series of imidazo-pyridinium oximes was tested with this asssay against some well-known chemical warfare organophosphorous compounds before being tested on animals.

MATERIALS AND METHODS

Apparatus. The reactor which contains the immobilized enzyme is a support for the filter disk (swinny, type, Millipore). The internal volume is approximately 0.4 ml. The apparatus is the same one that has been used with rat brain slices [4]. The use of a reactor with a small internal volume minimizes the transit time of the reactants and enables a short response time to be obtained. The temperature of the reaction was kept at 25° by a thermostated jacket.

Reagents. Electric eel AChE type VI-S, 5-5′-dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine bromide were obtained from Sigma Chemical Co., St Louis, MO. The buffer was Tris–HCl 25 mM (pH 7.8) and DTNB was 0.2 mM in Tris–HCl buffer. Organophosphorous agents (OP), diethyl *p*-nitrophenylphosphate (paraoxon), ethyl *S*-

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diisopropyl aminoethyl methylthiophosphonate (VX), isopropyl methylfluorophosphonate (sarin), (1,2,2-trimethylpropyl a methylfluorophosphonate (soman) and ethyl dimethylphosphoramidocyanidate (tabun) were prepared in our laboratory according to known methods from the literature and have a satisfactory analysis. OP were distilled just before utilization (purity: 96–99%) and stock solutions in 2-propanol were kept one week at +5° (only one day for soman and paraoxon). Imidazopyridinium 4-oximes [7], TMB-4 [8], and 1-benzyl 2-carbaldoxime pyridinium bromide [9] were also prepared according to published procedures. Microanalysis and NMR revealed that compounds **1g** to **1i** (Fig. 1) are complexed with a pyridine 4-aldoxime molecule. Compound 1f (n = 7) is crystallized with half a molecule of this oxime. pK_a of these oximes was determined by potentiometry with a Tacussel pH meter Isis 2000 at 25°.

Preparation of immobilized AChE. The enzyme was immobilized by adsorption on glassfiber filter. Disks (10 mm diameter) were cut from Schleicher and Schull (N3362) filter paper 0.5 mm thick. Each disk received 20 μl of a fresh solution of enzyme in distilled water (usually 0.65 mg protein/ml). They were dried in a vacuum desiccator at room temperature for 72 hr and then stored at −20°. During storage the activity of the enzyme disks was stable for up to three months. The enzyme disk was doubled with a disk without enzyme inside a toric joint and inserted in the reactor between two stainless steel grids and two flat joints.

Activity determination. The acetylthiocholine hydrolysis was continuously monitored by the optical density at 412 nm. At a constant flow rate and 25°, the activity of the immobilized enzyme is directly proportional to the optical density: Activity = Flow rate × O.D./($E \times l$), where E is the molar absorptivity (13,600 $cm^{-1} M^{-1}$) and l the path length of the cell (1 cm).

The flow rate, usually 72 ml/hr, was measured for each assay. It was chosen as a compromise between short transit time and moderated consumption of

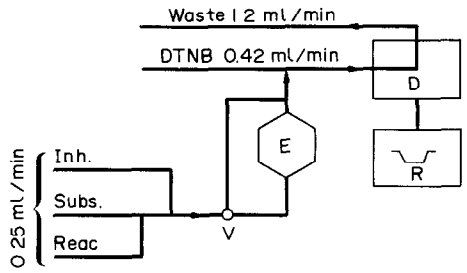
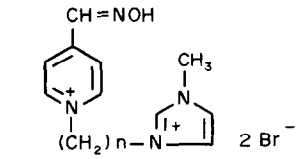


Fig. 2. Scheme of the apparatus for determination of AChE activity by continuous flow method: V, three-way valve; E, enzymatic reactor; D, colorimeter; R, recorder. DTNB = 5-5′-dithio-(2-nitrobenzoic acid); Inh = inhibitor; Subs. = acetylthiocholine bromide; Reac. = reactivator.

reactants. The residence time of these reactants in the reactor is about 20 sec. The base line was determined using the by-pass derivation. A stabilized activity was obtained after 3 hr of washing with Tris buffer. To avoid this time-consuming step, each reactor was rinsed with buffer in a separate device before use. The stable activity was 5% of the activity initially deposed on the disk.

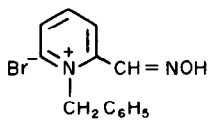
In the apparatus (Fig. 2), substrate, inhibitor or reactivator were diluted three times by the other inputs; this was taken into account in the calculations. The substrate concentration routinely used in the assay was 1.67 mM (5 mM before dilution). Under assay conditions the spontaneous loss of activity did not usually exceed 5% per day. A new enzyme disk is used every day but several assays can be easily performed with the same enzyme disk in a few hours. When AChE cannot be completely reactivated (soman, tabun) a new enzyme disk is used for each assay.

Determination of the kinetic parameters of the immobilized enzyme. The kinetic parameters of free and immobilized enzyme were determined. The substrate concentration taken into account for immobilized enzyme is the concentration in steady-state



1

Ia : n = 2 (7,98)
Ib : n = 3 (8,16)
Ic : n = 4 (8,20)
Id : n = 5 (8,27)
Ie : n = 6 (8,34)
If : n = 7 (8,00)*
Ig : n = 8 (8,17)**
Ih : n = 9 (8,21)**
Ii : n = 10(8,32)**



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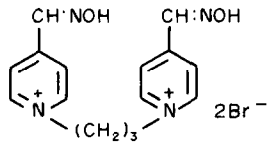

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Fig. 1. Chemical structure of reactivators. pKa for compounds 1a–i, indicated between brackets, were determined potentiometrically at 25°. *This compound is crystallized with one-half a molecule of pyridine-4-carbaldoxime. **These compounds are crystallized with one molecule of pyridine-4-carbaldoxime.

conditions. For lower substrate concentration the fraction of substrate hydrolysis is then about 50%. This substrate concentration was calculated as follows: (S)r = (S)o − (P)r, where (S)r and (P)r are the concentrations of substrate and product in the reactor and (S)o is the input substrate concentration. (P)r was calculated from the product concentration measured in the output. A correction factor was used to adjust the dilution of the product introduced by the Ellman's reagent.

Reversible inhibition due to the thiocholine was negligible even with the lowest substrate concentration.

The kinetic parameters of the enzyme in solution were determined by measuring the initial velocities at various substrate concentrations. The assay mixture contained: Tris–HCl buffer (pH 7.8, 25 mM), DTNB (0.2 mM) and substrate (2.10^{-5}–10^{-2} mM). Total volume was 3 ml. The reaction was initiated by addition of the enzyme.

Inhibition by organophosphorous compounds. Inhibition of immobilized AChE was measured after a fixed reaction time (usually 5 min) with a solution of inhibitor. The inhibition was performed without substrate. The observed first order rate constants k_i(obs) were calculated according to: $\mathrm{Ln}(A_i/A_o) = -k_i(\mathrm{obs}) \times t$, where A_o is the activity before inhibition and A_i is the activity after inhibition. The inhibition was also continuously monitored in the presence of the substrate. With all the tested organophosphates the activity decreased exponentially, indicating that inhibition of immobilized AChE, at a fixed inhibitor concentration, was actually a first order process.

Reactivation. After inhibition and washing with buffer, oxime solution was introduced for a fixed time (usually 5–15 min). The observed first order reactivation constant $k_{r(obs)}$ was calculated according to:

$$\mathrm{Ln}(A_o - A_r) = -(k_{r(obs)} \times t) + \ln(A_o - A_i)$$

where A_r is the activity after reactivation. The spontaneous reactivation of inhibited enzyme was negligible. The reactivation assays were generally conducted in the presence of the substrate at 1.67 mM. A typical recording of reactivation experiment is shown in Fig. 3.

Reversible inhibition. The reversible inhibition induced by oximes was evaluated by the ratio between initial activity and activity measured under continuous injection of oxime. At the concentrations used in these measurements, the oxime-induced acetylthiocholine hydrolysis was negligible. The fraction of activity inhibited was a hyperbolic function of oxime concentration. Apparent inhibition constants for 1.67 mM substrate concentration were calculated by double-reciprocal plot method and linear regression.

RESULTS

Immobilization procedure

The efficiency of the immobilization procedure is quite low (5%) but for such an application, this is not a limiting factor: for immobilization each "enzyme disk" needs less than 20 μg of commercial enzyme (100 IU/mg of protein). This method is extremely simple and convenient for screening studies which require preparation of immobilized enzyme in large series. The interactions between adsorbed enzyme and support are strong enough to avoid possible desorption induced by ionic strength or detergent. In control experiments the immobilized enzyme was not eluted by washing for two hours (flow-rate: 60 ml/H) with NaCl (2 M) or with Triton X100 (0.6%).

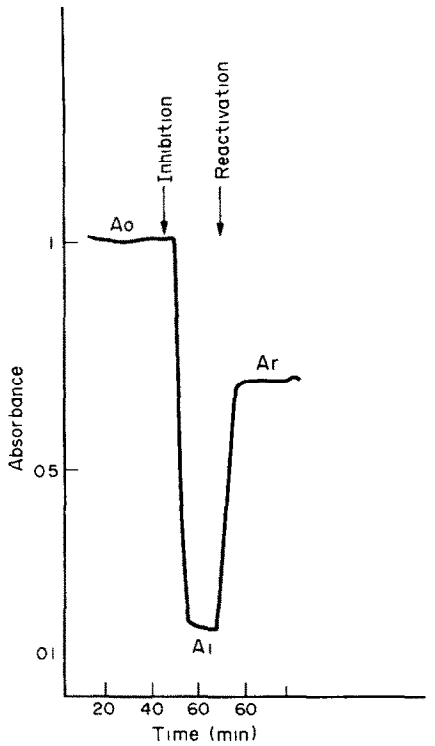

Fig. 3. Time course of inhibition of immobilized electric eel AChE by sarin ($6.7 \cdot 10^{-7}$ M) followed by reactivation with oxime 1b ($5 \cdot 10$–5 M). The inhibitor is injected for 1 min; after rinse, the AChE activity had reached a new stable level Ai. Injection of the reactivator for 5 min gives Ar which is the level after reactivation. Ar–Ai gives the restored activity.

Kinetic parameters of immobilized AChE

It is of great importance with immobilized enzyme to take into account possible effects of mass transfer limitations on the observed kinetic results. A simple method to evaluate these effects is to compare the kinetic parameters of the soluble enzyme with those obtained for the immobilized one. Data shown (Fig. 4) indicate that the Michaelis constants are identical. So the effects of diffusion on the activity of the immobilized enzyme can be neglected.

Inhibition by organophosphates

The observed inhibition constants $k_{i(obs)}$ were proportional to the inhibitor concentration. The second order rate constants of inhibition k_i were calculated (Table 1). The dissociation constant between enzyme and inhibitor cannot be determined by this method. It is not possible to use inhibitor concentrations close

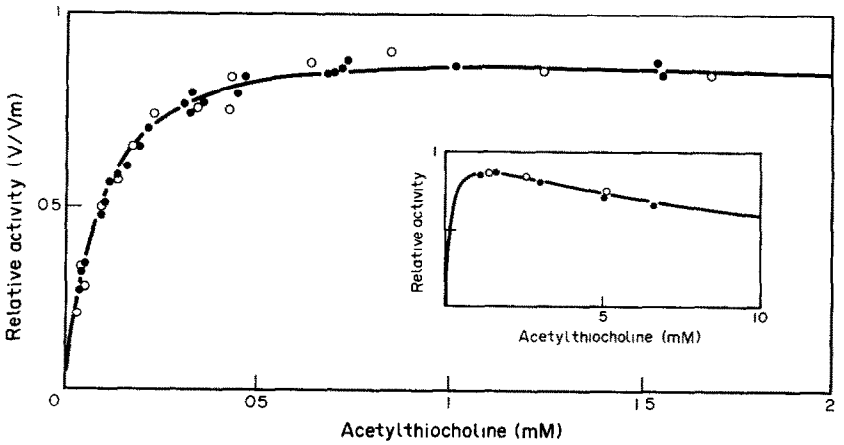

Fig. 4. Substrate-dependence of soluble (○) and immobilized (●) AChE. The curve is calculated according to $V = V_m \cdot S/(S + K_m + S/K_{ss})$ with $K_m = 0.09$ mM and $K_{ss} = 15$ mM.

to K_I value. The enzyme would be almost completely inhibited even with the shorter inhibition time permitted by the method. The order of inhibitory power (soman > VX > sarin > tabun > paraoxon) is in good agreement with the results of Forsberg and Puu [10]. The difference in inhibitory power was reported by these authors to be essentially due to differences in affinity rather than to an increase in chemical reactivity.

Reactivation

The efficiency of the reactivation *in vitro* depends on several factors. One of these is the structure of the organophosphate group bound to the enzyme. The organophosphates inhibitors were selected to give phosphorylated-AChE (E-OR) with different phosphorous substituents

R = —P(O)(OEt)$_2$ (paraoxon),
—P(O)CH$_3$ (OEt) (VX),
—P(O)CH$_3$(O—iC$_3$H$_7$) (sarin),
—P(O)CH$_3$—OCH(CH$_3$)C(CH$_3$)$_3$ (soman),
—P(O)—N(CH$_3$)$_2$(OEt) (tabun).

The enzyme inhibited by paraoxon, VX or sarin, can be completely reactivated by imidazo-pyridinium oximes and TMB-4.

The reactivation of inhibited enzyme proceeds according to the equation:

$$EP + RH \underset{K_r}{\longleftrightarrow} (EP - RH) \xrightarrow[k_{r(max)}]{} EH + RP$$

where K_r is the dissociation constant for the equilibrium between phosphorylated enzyme and reactivator, and $k_{r(max)}$ is the rate constant for nucleophilic displacement of the phosphoryl group. For concentrations much lower than the K_r value, $k_{r(obs)}$ increases proportionately to the reactivator concentration.

With higher concentrations the observed first order reactivation constant reaches the "maximal" rate constant $k_{r(max)}$. The reactivation rates were determined and data are shown in Fig. 5. These reactivation rates are not modified by the absence of the substrate.

The imidazopyridinium oximes with a short methylene bridge (**1a–e**) have a high chemical reactivity while those with a long methylene bridge (**1g–i**) have a low chemical reactivity but a high affinity for the inhibited enzyme. Structural data indicate that **1g–i** behave as complexing agents of pyridine 4-aldoxime. The high affinity of **1g–i** for the sarin and VX inhibited enzyme is well correlated with the affinity for the native enzyme as judged from their reversible inhibitory power (Table 2). The marked increases of affinity between **1e** and **1f** then between **1f** and **1i** may be related to the presence of pyridine-4-aldoxime. The exact effects of the associated oxime molecule on the properties of these compounds are not well understood.

The reactivations by oximes are less efficient for enzyme inhibited by soman or tabun (Table 3). The more efficient reactivator against tabun is benzyl-P2A, compound **2**, an oxime described by de Jong

Table 1. Bimolecular rate constants for inhibition of immobilized AChE by organophosphate inhibitors with substrate 1.67 M (k_{is}) or without substrate (k_i). Each inhibitor was tested at various concentrations (8–10 plots) for 1, 2 or 5 min

Inhibitor	k_i (min$^{-1}\cdot$M^{-1})	Conc. range (M)	k_{is} (min$^{-1}\cdot$M^{-1})	Conc. range (M)	k_{is}/k_i
Soman	$1.4\cdot10^8 \pm 0.03$	$1–15\cdot10^{-9}$	$9.15\cdot10^6 \pm 0.07$	$0.5–10\cdot10^{-8}$	15
VX	$7.9\cdot10^7 \pm 0.5$	$0.5–15\cdot10^{-9}$	$3.00\cdot10^6 \pm 0.22$	$1.6–10\cdot10^{-8}$	23
Sarin	$6.8\cdot10^7 \pm 0.28$	$0.7–3\cdot10^{-8}$	$3.10\cdot10^6 \pm 0.30$	$3–30\cdot10^{-8}$	22
Tabun	$2.2\cdot10^6 \pm 0.13$	$1–7\cdot10^{-7}$	$2.40\cdot10^5 \pm 0.16$	$1–16\cdot10^{-7}$	9.2
Paraoxon	$1.5\cdot10^5 \pm 0.10$	$3–20\cdot10^{-6}$	$2.75\cdot10^4 \pm 0.1$	$3–20\cdot10^{-7}$	5.5

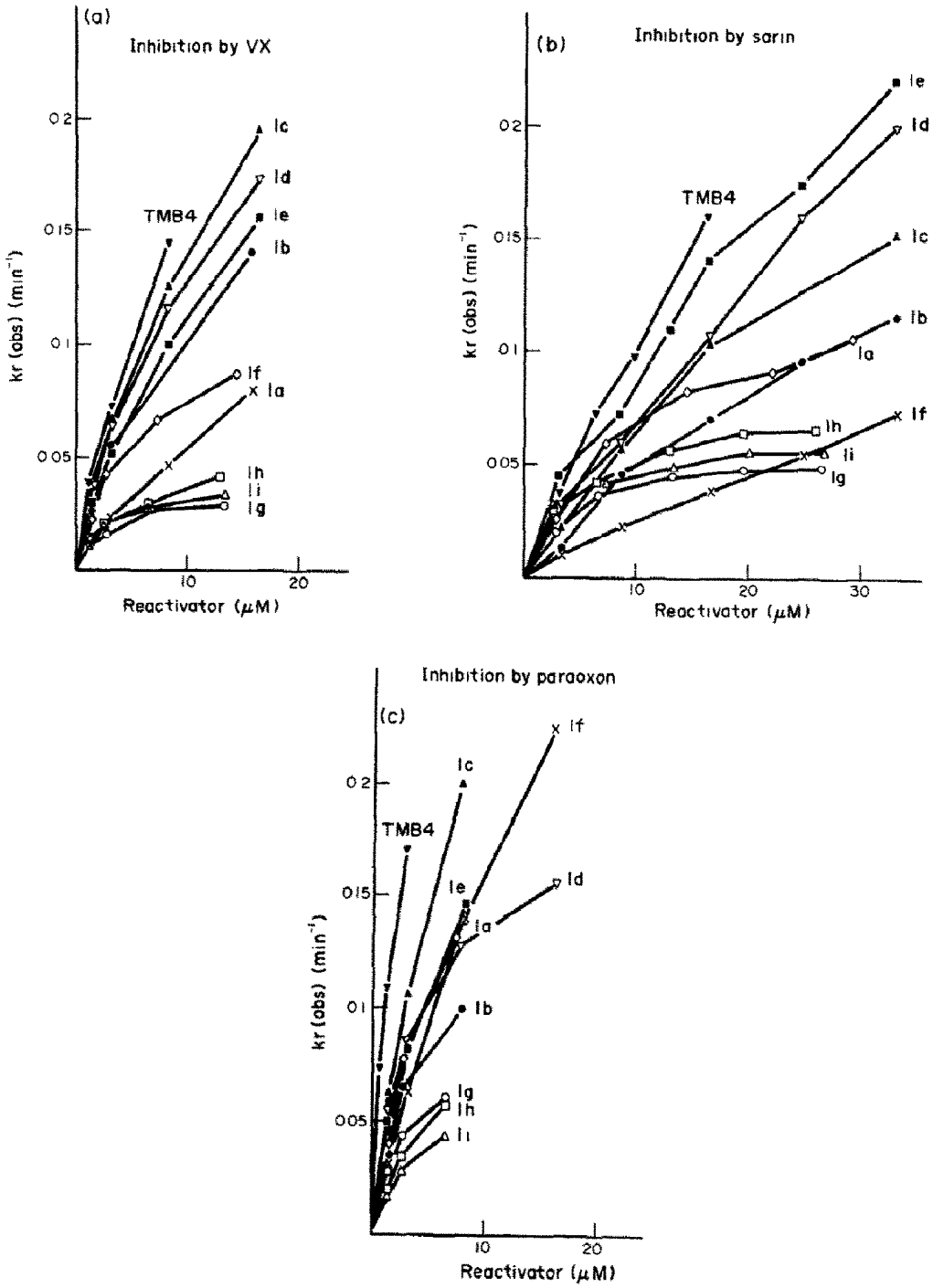

Fig. 5. Observed first-order reactivation constants vs reactivator concentrations. The enzyme was previously all inhibited by (a) VX, (b) sarin, and (c) paraoxon.

et al. [9]. This compound gives an overall restoration of the initial activity (see Tables 3 and 4). Imidazo-pyridinium oximes are slightly reactivator of tabun inhibited AChE. The high affinity of oximes **1f–i** for the phosphorylated AChE can be confirmed by competition experiments (Table 4). Introduced simultaneously with 1b (n = 3), the imidazo-pyridinium oxime 1h (n = 9) competes for binding to the VX phosphorylated AChE and reduces the reactivation reaction. In the same way the reactivation of tabun inhibited AChE with the 1-benzyl-2-carbaldoxime pyridinium bromide **2** is hindered by the oxime **1h**.

It is well known that enzyme inhibited with soman rapidly loses its ability to be reactivated by an oxime. This conversion in a non-reactivatable form, known

Table 2. Kinetic parameters for inhibition of intact AChE and for reactivation of sarin and inhibited AChE by oximes **1e–i**

Reactivator	K_i* (μM)	K_r (μM) after sarin inhibition	$k_{r(max)}$ (min^{-1})	K_r (μM) after VX inhibition	$k_{r(max)}$ (min^{-1})
1e	†	>20		17	
1f	8.4	11.5	0.14	5.7	0.12
1g	1.4	4.4	0.058	1.8	0.034
1h	0.9	4.2	0.075	4.4	0.052
1i	0.75	2.4	0.048	5.3	0.044

* K_i is the apparent inhibition constant of oximes in the presence of the substrate (1.67 mM).
† No inhibition at 15 μM. $k_{r(max)}$ and K_r values were determined by double reciprocal plot and linear regression. For oxime **1e** K_r cannot be significantly determined, a lower limit was estimated.

Table 3. Maximum of AChE reactivation reached with oximes

Inhibitor*	Reactivator†	% React.
Soman	TMB-4	37
Soman	1b	37
Soman	2	26
Tabun	TMB-4	73
Tabun	1b	30
Tabun	1d	22
Tabun	1e	10
Tabun	P2S	15
Tabun	2	100

* AChE was 100% inhibited without substrate by $1.67 \cdot 10^{-8}$ M soman for 2 min or 1.10^{-6} M tabun for 3 min.
† The reactivators were injected for 30 min at 10^{-3} M. The reactivators were injected against soman immediately after inhibition.

as aging, is also encountered with other inhibitors [11] but at a lower rate than for soman. With all the reactivators studied, there is no more than 5% of the initial activity, if the reactivation is initiated more than 30 min after inhibition.

In order to control the rate of aging, a short inhibition time (2 min) was used and the reactivator was injected immediately after the inhibitor. Under these conditions AChE activity was partly restored; oxime **1b** and TMB-4 induced the greatest reactivation (see Table 3).

DISCUSSION

Method

The immobilization procedure without any covalent binding is useful to avoid chemical modifications of the protein structure and to minimize the effects of the diffusion. The microscopic observations of the immobilized preparation, after histochemical revelation of cholinesterase activity by Koelle's method [12], show that the activity is localized at the surface of the fibers. The porosity of the fiberglass paper is sufficient to permit the use of relatively high flow-rates. In these conditions the thickness of the unstirred layers at the enzyme surface is minimized. The kinetic of the immobilized enzyme is essentially diffusion-free. For analytical assays, the adsorption process of AChE at the surface of fiberglass seems more convenient than covalent binding inside porous carrier materials. The apparent K_m of AChE immobilized in polyethylene beads [5], polyacrylamide membrane or nylon tubing [13] is flow-rate dependent and significantly increased in comparison with soluble enzyme. The interpretation of inhibition-reactivation results would be rather complicated under diffusion controlled conditions.

In addition to the classical Ellman's method with free AChE, the use of immobilized enzyme in a continuous flow apparatus for reactivation studies has the following advantages: the activity after reactivation is measured after washing off the excess of the inhibitor and of the oxime. Observed values need no correction due to the interactions of the reaction products with oximes itself or phosphorylated oximes [14]. For the same reason the chemical hydrolysis of acetylthiocholine induced by oximes cannot interfere with the enzymatic activity measured.

The assay is efficient for the rapid determination of reversible or irreversible inhibitions and for the screening of AChE reactivators or protectors.

Table 4. Effect of oxime **1h** on reactivation of phosphorylated AChE by oximes **1b** and **2**

Inhibitor	Oximes	Oxime concentration (time of reaction)	Reactivation (%)
VX	1b	33 μM (5 min)	55
VX	1h		11
VX	1b + 1h		21
Tabun	2	1 mM (30 min)	100
Tabun	1h		5
Tabun	2 + 1h		10

Interaction of inhibitors and reactivators with acetylcholinesterase

The comparison of the kinetic parameters of enzyme inhibition by organophosphates in the presence or the absence of substrate (Table 1) shows that the substrate gives a protective effect which is not the same for all the inhibitors. The results obtained with paraoxon and tabun are not in accordance with a strict competition between substrate and inhibitors for binding to the active site of AChE. The theoretical protective effect, for this substrate concentration, assuming a strict competition would be: $1 + (S/k_m) = 19.5$.

The observed reactivation constants $k_{r(obs)}$ of inhibited-AChE by oximes were not modified by the absence of the substrate. This indicates that the substrate has no affinity for the binding site of the reactivator on the inhibited enzyme.

Oximes **1f–i** are poor reactivators for inhibited-AChE but good reversible inhibitors for free immobilized enzyme. The reversible inhibition by oximes **1f–i** protects AChE against phosphorylation by any OP agents tested (not shown here). This protective effect could be interesting in a prophylactic perspective.

It is interesting to point out that oxime **1h** prevents the reactivation of VX-inhibited-enzyme by a more efficient oxime. It seems likely that this compound shares the same enzyme binding site on the phosphorylated enzyme than pyrimidoxime **1b**, but with a greater affinity and a slight reactivation potency. The same observation is valid for tabun inhibited AChE: the binding of **1h** with the inhibited enzyme prevents the reactivation by compound 2 (Table 4).

The affinity of a compound for the phosphorylated AChE can be estimated by this method, even if this compound is not a reactivator. For example, the inefficiency of oxime **1h** against tabun is not due to its low affinity for the tabun-inhibited AChE but to its lack of activity (see Table 4). These competition experiments can be performed by this method more easily than by the conventional one.

This 1-benzylpyridinium 2-oxime (compound 2) is able to reactivate completely the tabun-inhibited enzyme several hours (6 and more) after inhibition. No aging was detected. De Jong *et al.* [15] showed that the spontaneous rate of aging of enzyme inhibited by tabun (half-life: 46 hr) can be modulated by various oximes. After partial reactivation by oximes **1b** and **1h**, compound 2 also gives a total reactivation, while after partial reactivation by TMB-4, the enzyme cannot be completely reactivated by the compounds **2** (maximal reactivation: 85%). These observations suggest that TMB-4 favours the aging more than oximes **1b** and **1h**.

TMB-4 is the best reactivator for immobilized electric eel AChE inhibited by the main organophosphorous inhibitors tested. Against tabun, the 1-benzyl 2-pyridinealdoxime salt is the more efficient. The imidazopyridinium-oximes **1a–e** were found to have an excellent reactivation potency *in vitro*. The low acute toxicity of pyrimidoxime **1b** [6] makes it an attractive alternative to existing therapeutic by oximes. The prophylactic effect of oximes **1g–i** requires further investigations of this imidazolinium family.

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