Oxime Reactivation of Erythrocyte Cholinesterase Inhibited by Ethyl *p*-Nitrophenyl Ethylphosphonate

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1. Reactivation of erythrocyte cholinesterase inhibited by ethyl *p*-nitrophenyl ethylphosphonate (armine) was studied with *NN'*-dimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (C₂-oxime), *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (C₃-oxime), *NN'*-tetramethylene-(4-hydroxyiminomethylpyridinium bromide) (C₄-oxime) and *NN'*-pentamethylenebis-(4-hydroxyiminomethylpyridinium bromide) (C₅-oxime) as reactivators. The kinetics of reactivation were consistent with a reaction of the type:

and bimolecular rate constants for reactivation were calculated from the corresponding differential equations. 2. Of the four oximes studied C₂-oxime was least effective and the other three oximes were about equally effective reactivators. 3. Reactivation of armine-inhibited cholinesterase by C₃-oxime was also studied in the presence of substrate. This reaction was first-order with respect to inhibited enzyme, and slower than in the absence of substrate.

Armine† is an asymmetric organophosphorus compound that markedly inhibits cholinesterase activity (Razumov, Marković & Muhačeva, 1957; Fukuto & Metcalf, 1959). In the present paper a study of the kinetics of reactivation of armine-inhibited erythrocyte cholinesterase by some bispyridinium 4-aldoxime derivatives is presented. In contrast with most reactivation studies performed so far on other types of inhibited enzyme, in this work reactivation was followed over a long period of time. To establish the degree of interference of substrate with the reactivation process, reactivation was carried out in the presence and in the absence of substrate.

In the absence of substrate, reactivation of armine-inhibited cholinesterase proceeded as if phosphorylation of the free enzyme by the phospho-

rylated oxime had occurred simultaneously with the reactivation process. In the presence of substrate, inhibition by the phosphorylated oxime did not seem to occur. Second-order rate constants were derived for reactivations both in the presence and absence of substrate.

MATERIALS AND METHODS

Sheep erythrocytes were used as the enzyme source. The erythrocytes from 5 ml. of blood were washed twice with 10 ml. of bicarbonate buffer, and then diluted with bicarbonate buffer to the concentration in blood. Bicarbonate buffer contained NaHCO₃ (14.7 mm), NaCl (0.15 M) and MgCl₂ (35 mm); calculated from the Henderson-Hasselbach equation the pH of the buffer was 7.4 when gassed with CO₂ + N₂ (5:95) at 37°. Inhibited cholinesterase samples were prepared immediately before use by incubating 5 ml. of washed erythrocytes for 1 hr. at 37° with armine (final concn. 0.03 μM). Incubation was followed by washing the erythrocytes four times with 15 ml. of bicarbonate buffer.

Stock solutions of armine prepared in water were kept at 4° for not longer than 2 weeks. Further dilutions in bicarbonate buffer were made immediately before use.

The reactivators C₂-oxime, C₃-oxime, C₄-oxime and C₅-oxime were synthesized in this Institute as described by Hobbiger & Sadler (1959). Stock solutions prepared in water were kept at 4° for not longer than 2 weeks. Further dilutions in bicarbonate buffer were made immediately before use.

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† Abbreviations: armine, ethyl *p*-nitrophenyl ethylphosphonate; C₂-oxime, *NN'*-dimethylenebis-(4-hydroxyiminomethylpyridinium bromide); C₃-oxime, *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide); C₄-oxime, *NN'*-tetramethylene-(4-hydroxyiminomethylpyridinium bromide); C₅-oxime, *NN'*-pentamethylenebis-(4-hydroxyiminomethylpyridinium bromide); ACh, acetylcholine chloride.

All experiments were carried out in a Warburg apparatus at 37° in bicarbonate buffer, in an atmosphere of N₂+CO₂ (95:5), at pH 7.4 (Aldridge, 1950). The total volume of the solution phase in the Warburg flask was 2.0 ml. ACh was used as the substrate.

In reactivation studies in the presence of ACh, substrate and oxime were added to the enzyme at the same time. The final concentration of ACh was either 10 or 50 mM, and that of the oxime varied from 10 to 0.3 μ M. Readings of the CO₂ output were taken at 10 min. intervals for about 3 hr., the first reading being taken 5 min. after the addition of substrate and oxime. Since the activity of the controls deviated from linearity after more than 250 μ l. of CO₂ had been produced/Warburg flask, the readings in the experimental flasks were always stopped before this amount was reached.

In reactivation studies in the absence of ACh, the oxime was added to the enzyme at different intervals before the addition of substrate. The final concentration of ACh was 10 mM, and that of the oxime varied from 10 to 0.3 μ M. The activity of inhibited enzyme samples at the beginning of the reaction was always zero. Readings of the CO₂ output were taken between 10 and 35 min. after adding the substrate. The degree of reactivation obtained therefore included the reactivation that occurred before the addition of substrate, and the reactivation that took place from the time of the addition of substrate until 35 min. later. The time of reactivation was taken to be the interval beginning with the addition of oxime, and lasting until 10 min. after the addition of substrate.

No spontaneous reactivation of inhibited enzyme was found after a 3 hr. incubation at 37°.

Cholinesterases inhibited by some organophosphorus compounds become, on storage, progressively non-reactivable by oximes (Hobbiger, 1956; Wilson, 1955; Berends, Posthumus, Sluys & Deierkauf, 1959). Sheep-erythrocyte cholinesterase inhibited by armine showed no such properties during 12 hr. storage at 37°; after 12 hr. 100% reactivation was obtained by incubating the inhibited enzyme with 10 μ M-C₃-oxime.

RESULTS

Reactivation in the presence of substrate. Reactivation of armine-inhibited cholinesterase was studied in the presence of two different substrate concentrations, 10 and 50 mM, with C₃-oxime as reactivator. The concentration of C₃-oxime varied from 10 to 0.3 μ M, and the time-course of reactivation was followed for 2–3 hr. The results obtained are presented as percentages of the inhibition remaining and were calculated from the carbon dioxide output curves (Fig. 1). The activities of the reactivating enzyme obtained from differences between every two successive readings were subtracted from the activity of the controls, giving the inhibition remaining. The initial amount of the inhibited enzyme corresponded to the activity of the controls, as the activity of the inhibited enzyme samples at the beginning of the reaction was always zero.

The kinetics of the reactivation process was first-order, and showed the characteristic of a

Fig. 1. Reactivation by C₃-oxime, in the presence of (a) 10 mM-ACh and (b) 50 mM-ACh, of erythrocyte cholinesterase inhibited by armine. Conc. of C₃-oxime: ○, 0.3 μ M; ●, 1.0 μ M; △, 3.0 μ M; ▲, 10.0 μ M. Each point is the mean of five independent experiments. The lines drawn are the calculated regression lines.

bimolecular reaction with one component in excess (Aldridge, 1950):

$$k_r c_r t = \ln \left(\frac{100}{100-a} \right) \quad (1)$$

where k_r is the bimolecular rate constant, c_r is the molar concentration of the reactivator, a is the activity of the enzyme expressed as percentage of the control and t is the time of reactivation (min.).

Bimolecular rate constants obtained from the slopes of the lines in Fig. 1 are listed in Table 1 separately for each oxime concentration used. The rate of reactivation was lower in the presence of 50 mM-ACh than in the presence of 10 mM-ACh, the mean values of k_r being 4.9×10^3 and 9.6×10^3 l. mole⁻¹ min.⁻¹ respectively. In the presence of 10 mM-ACh the bimolecular rate constants tended to decrease with increasing oxime concentration: when the oxime concentration was increased 30-fold, the k_r decreased by 25%.

When reactivation was carried out in the presence

Table 1. *Reactivation, in the presence of substrate, of erythrocyte cholinesterase inhibited by armine*

Bimolecular rate constants, k_r , for the reactivation of armine-inhibited erythrocyte cholinesterase by C₃-oxime in the presence of 10 mM- and 50 mM-ACh were calculated from the slopes of the regression lines in Fig. 1.

Concn. of oxime (μM)	$10^{-3} k_r$ (l. mole ⁻¹ min. ⁻¹)	
	Substrate...10 mM-ACh	50 mM-ACh
0.3	11.6	5.3
1.0	9.3	4.0
3.0	9.0	4.0
10.0	8.7	6.2
Mean	9.7	4.9

of 10 mM-ACh and 1.0 μM - or 3.0 μM -oxime, deviations from first-order kinetics were observed during the second or third hour of the reaction. The kinetics of these deviations were studied in more detail in the absence of substrate, as described below.

Reactivation in the absence of substrate. In reactivation studies in the absence of substrate, besides C₃-oxime three other oximes were used as reactivators: C₂-oxime, C₄-oxime and C₅-oxime. The results obtained with different concentrations of C₃-oxime are given in Fig. 2. Except when 10 μM -C₃-oxime was used, where practically complete reactivation was obtained, reactivation with lower C₃-oxime concentrations approached an equilibrium, the degree of reactivation at equilibrium depending on the concentration of C₃-oxime. Qualitatively the same results were obtained when reactivation was studied with C₂-oxime, C₄-oxime and C₅-oxime.

The reactivation process did not follow first-order kinetics from the beginning (Fig. 2), and therefore a reversible reaction of the type:

was considered to occur. EI and EH stand for the phosphorylated and free enzyme respectively, and RH and RI for the oxime and phosphorylated oxime respectively.

Further evidence for this type of reaction mechanism was obtained from an additional experiment. The inhibited enzyme samples were incubated for 2 hr. at 37° in the presence of 1 μM -C₃-oxime, which resulted in a 50% reactivation. More reactivator was then added to give a final concentration of 10 μM -C₃-oxime, and this caused 100% reactivation.

If the concentration of oxime is regarded as

Fig. 2. Reactivation by C₃-oxime, in the absence of substrate, of erythrocyte cholinesterase inhibited by armine. Concn. of C₃-oxime: ○, 0.3 μM ; ●, 1.0 μM ; ▲, 3.0 μM ; △, 10.0 μM . Each point is the mean of three independent experiments.

constant, the decrease in the concentration of EI in eqn. (2) is given by:

$$-\frac{d[\text{EI}]}{dt} = k_r[\text{RH}][\text{EI}] - k_i([\text{EI}]_0 - [\text{EI}])^2 \quad (3)$$

where $[\text{EI}]_0$ stands for the concentration of phosphorylated enzyme at the beginning of the reaction. At equilibrium:

$$k_r[\text{RH}][\text{EI}]_e = k_i([\text{EI}]_0 - [\text{EI}]_e)^2 \quad (4)$$

where $[\text{EI}]_e$ stands for the concentration of phosphorylated enzyme at equilibrium. From eqns. (3) and (4) it follows that:

$$-\frac{d[\text{EI}]}{dt} = k_r[\text{RH}] \left\{ [\text{EI}] - \frac{[\text{EI}]_e}{([\text{EI}]_0 - [\text{EI}]_e)^2} ([\text{EI}]_0 [\text{EI}])^2 \right\} \quad (5)$$

and after integration:

$$k_r t [\text{RH}] = \frac{[\text{EI}]_0 - [\text{EI}]_e}{[\text{EI}]_0 + [\text{EI}]_e} \cdot \ln \left\{ \frac{[\text{EI}]_0^2 - [\text{EI}][\text{EI}]_e}{[\text{EI}]_0([\text{EI}] - [\text{EI}]_e)} \right\} \quad (6)$$

$[\text{EI}]_0$, $[\text{EI}]$ and $[\text{EI}]_e$ were calculated from the activity of the controls, from the activity of the reactivating enzyme at t min. reactivation and from the activity of the reactivating enzyme at equilibrium respectively. $[\text{RH}]$ is the molar concentration of the reactivator.

When the right-hand side of eqn. (6) was plotted as a function of the time of reactivation, straight lines were obtained for the reactivation of armine-inhibited cholinesterase by the four oximes studied

(Fig. 3). Results obtained with 10 μM -oxime concentration were not plotted, because of uncertainty about the value of $[\text{EI}]_0$, since with that oxime concentration reactivation proceeded nearly to completion. Not all of the experimental lines in Fig. 3 pass through the origin. This inadequacy might exist in the kinetic treatment, but another reason may be the uncertainty about the time of reactivation. As mentioned in the Materials and Methods section, reactivation of the inhibited enzyme proceeded even after the addition of substrate, whereas the time of reactivation was

taken to be the interval beginning with the addition of oxime to the inhibited enzyme samples and lasting until 10 min. after addition of substrate, when readings of the carbon dioxide output were started.

Second-order rate constants were obtained from the slopes of the lines in Fig. 3 for each oxime concentration separately, and are listed in Table 2. With increasing oxime concentration k_r values tended to decrease. The decrease was only slight for reactivation with C_2 -oxime, but it was particularly pronounced with C_4 -oxime used as reactivator.

Of the four oximes studied C_2 -oxime was the least effective, whereas the other three oximes were about equally effective reactivators.

DISCUSSION

In studies of the kinetics of reactivation of phosphorylated cholinesterase by nucleophilic substances incomplete reactivation and deviation from first-order kinetics were found by other workers, e.g. by Hobbiger, Pitman & Sadler (1960) in studies of di-isopropyl phosphorofluoridate- and tetraethyl pyrophosphate-inhibited cholinesterase by some bispyridinium aldoximes, by Scaife (1959) in the reactivation of isopropyl methylphosphonofluoridate- and ethyl *NN*-dimethylphosphoramidocyanidate-inhibited cholinesterase by C_3 -oxime and pyridinium 2-aldoxime methiodide, and by Heilbronn (1963*a*) who studied the reactivation of ethyl *NN*-dimethylphosphoramidocyanidate-inhibited cholinesterase by C_3 -oxime and *N*-methylpyridinium 2-aldoxime methanesulphonate. Hobbiger *et al.* (1960) as well as Scaife (1959) suggested that this phenomenon may be due to the inhibition of the free enzyme by the phosphorylated oxime. Scaife (1959) calculated equilibrium constants assuming a certain turnover number of cholinesterase, but did not get constant values over a wide range of oxime concentration.

I have found that the mechanism of reactivation of armine-inhibited cholinesterase by the four bispyridinium 4-aldoxime derivatives (C_2 -oxime, C_3 -oxime, C_4 -oxime and C_5 -oxime) studied in the absence of substrate may be expressed in the form of eqn. (2). This mechanism is supported by findings that some phosphorylated bispyridinium aldoxime derivatives are potent cholinesterase inhibitors (Heilbronn, 1963*b*; Lamb, Steinberg & Hackley, 1964). Thus, for instance, the rate constant for inhibition of eel cholinesterase by a compound formed in the reaction of C_3 -oxime and isopropyl methylphosphonofluoridate amounted to $4.28 \times 10^8 \text{ l.mole}^{-1} \text{ min.}^{-1}$ (Lamb *et al.* 1964). Bimolecular rate constants for reactivation calculated from the differential equation corresponding to eqn. (2) were not strictly constant, but decreased

Fig. 3. Reactivation by (a) C_2 -oxime, (b) C_3 -oxime, (c) C_4 -oxime and (d) C_5 -oxime of erythrocyte cholinesterase inhibited by armine. The ordinate is calculated according to eqn. (6). Concn. of oximes: $\circ$, 0.3 μM ; $\bullet$, 1.0 μM ; $\triangle$, 3.0 μM . Each point is the mean of three independent experiments. The lines drawn are the calculated regression lines.

Table 2. Reactivation, in the absence of substrate, of erythrocyte cholinesterase inhibited by armine

Bimolecular rate constants, k_r , for the reactivation of armine-inhibited erythrocyte cholinesterase by the oximes listed below were calculated from the slopes of the regression lines in Fig. 3.

Oxime	Concn. of oxime (μM)	$10^{-3} k_r$ ($\text{l.mole}^{-1} \text{ min.}^{-1}$)
C_2 -oxime	0.3	7.4
	1.0	7.5
	3.0	5.8
C_3 -oxime	0.3	12.2
	1.0	11.7
	3.0	7.5
C_4 -oxime	0.3	18.7
	1.0	12.8
	3.0	6.1
C_5 -oxime	0.3	11.4
	1.0	11.0
	3.0	5.8

with increasing oxime concentration. This could mean that the observed rate constants, i.e. $k_{\text{r.c.}}$, tended towards a saturation value with increasing oxime concentration (Wilson, Ginsburg & Meislich, 1955).

Equilibrium constants were not calculated for the mechanism of reactivation proposed in eqn. (2) because of the unknown molar concentration of the enzyme. For the same reason rate constants for the inhibition of free enzyme by the phosphorylated oxime (k_i in eqn. 2) were not evaluated.

There are differences between the kinetics of reactivation studied in the absence of substrate and that studied in the presence of substrate. The rate of reactivation in the absence of ACh did not follow first-order kinetics from the very beginning of the reaction, whereas in the presence of substrate first-order kinetics was maintained throughout. This effect of substrate was also found by Hobbiger *et al.* (1960) in reactivation studies of tetraethyl pyrophosphate-inhibited cholinesterase by C₃-oxime. The other difference is that in the presence of 50mM-ACh the rate of reactivation was smaller than in the absence of substrate. The rate constants of reactivation were about the same in the presence of 10mM-ACh as in its absence. This similarity might have arisen from inadequate corrections in the rate constants in Table 2 for reactivation occurring when ACh was added. This effect has been referred to above.

Both effects of substrate found in the present experiments may be explained by a competition

for the active centre in the enzyme molecule between phosphorylated oxime and substrate, and oxime and substrate respectively.

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