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SPONTANEOUS REACTIVATION AND AGING OF DIMETHYLPHOSPHORYLATED ACETYLCHOLINESTERASE AND CHOLINESTERASE

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SUMMARY

Spontaneous reactivation and aging of dimethylphosphorylated acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from human and bovine erythrocytes and rat brain, and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) from human and rat plasma was studied (pH 7.4; 37 °C), after inhibition by *O,O*-dimethyl-4-nitrophenyl phosphate or *O,O*-dimethyl-2,2-dichlorovinyl phosphate.

The first-order rate constants obtained for spontaneous reactivation (k_r) are 0.0136, 0.0092 and 0.00606 min^{-1} for acetylcholinesterase from human and bovine erythrocytes and rat brain, and 0.00504 min^{-1} for rat plasma cholinesterase. For human plasma cholinesterase, k_r is $\geqq$ 0.00014 min^{-1}.

The rate of aging was calculated from the amount of enzyme which could not be reactivated with oximes. Aging of dimethylphosphorylated human erythrocyte and rat brain acetylcholinesterase follows the kinetics of a first-order reaction with rate constants of aging (k_{ag}) of 0.00297 and 0.00172 min^{-1}, respectively. For rat plasma cholinesterase, k_{ag} is about 0.00058 min^{-1}, and this value was obtained from only two different times of aging.

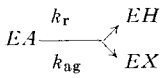
Due to aging, the kinetics of spontaneous reactivation deviates from first order. A theoretical equation is derived for calculating the time course of spontaneous reactivation. The agreement between theory and experiment was satisfactory when the time course was calculated using the experimentally obtained constants k_r and k_{ag}.

INTRODUCTION

Organophosphorus compounds phosphorylate the active site of cholinesterases. The phosphorylated enzyme can undergo two reactions: one is hydrolysis which

Abbreviations: DNPP, *O,O*-dimethyl-4-nitrophenyl phosphate; DDVP, *O,O*-dimethyl-2,2-dichlorovinyl phosphate; TMB-4, *N,N'*-trimethylene-bis-(4-hydroxyiminomethylpyridinium bromide.

leads to formation of the active enzyme (spontaneous reactivation), and the other is dealkylation (aging) which leads to an enzyme incapable of being reactivated:



EA, *EH* and *EX* are the phosphorylated enzyme, active enzyme and enzyme incapable of being reactivated, respectively; k_r and k_{ag} are the first-order rate constants for spontaneous reactivation and aging, respectively.

To our knowledge, only few data are available on the k_r and k_{ag} constants of dimethylphosphorylated cholinesterases[1–7]. This paper describes the evaluation of the k_r and k_{ag} constants for dimethylphosphorylated cholinesterases, and an equation is derived for calculating the time course of spontaneous reactivation. The sources of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) were human and bovine erythrocytes and rat brain, and of cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8), human and rat plasma. To produce the dimethylphosphorylated enzyme, *O,O*-dimethyl-2,2-dichlorovinyl phosphate (DDVP) and *O,O*-dimethyl-4-nitrophenyl phosphate (DNPP) were used as phosphorylating agents.

MATERIALS AND METHODS

All experiments were done at 37 °C in 0.1 M phosphate buffer, pH 7.4. The buffer was prepared by titrating 0.1 M NaH_2PO_4 with 0.1 M Na_2HPO_4 to the required pH. The enzyme activity was determined by the method of Ellman *et al.*[8] using acetylthiocholine iodide as substrate.

Inhibitors

DDVP (93% pure) was obtained from the World Health Organisation, Geneva, Switzerland, and DNPP from Bayer, A.G., Elberfeld, West Germany. Stock solutions (50 mM) of DDVP and DNPP were prepared in dimethylformamide and kept at 4 °C up to 2 months. The necessary dilutions were done in water immediately before use.

Enzyme preparations

Human erythrocytes (from heparinized blood) were washed twice with 0.15 M NaCl and then diluted with buffer. Rat brain was homogenized (in a glass-Teflon homogenizer of the Potter–Elvehjem type) in 0.15 M NaCl (40 mg wet weight brain/ml) and diluted with buffer. Human and rat plasma were obtained from heparinized blood and also diluted with buffer. Bovine erythrocytes (Winthrop Laboratories Inc., New York) were dissolved in buffer immediately before use.

Enzyme inhibition

The inhibitor (50 μl) was added to 3.0 ml of a suitable diluted enzyme preparation, which contained the thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid). The final concentration of the thiol reagent was 0.33 mM. To control samples, 50 μl water were added instead of the inhibitor solution. After a given time, substrate (50 μl) was added (final concentration 1.0 mM), and the absorbance read at 412 nm on a Unicam spectrophotometer (in thermostated cells) at 30-s intervals for 2.0 min, the first

reading being done 30 s after addition of substrate. The absorbance increased linearly with time in both inhibited and control samples.

Spontaneous reactivation

The enzyme preparation (undiluted plasma; 40 mg rat brain/ml homogenate; human erythrocytes diluted with buffer to the volume of blood; 2 mg bovine erythrocytes/ml) was incubated for 10 min with an inhibitor solution (50 μl/3.0 ml enzyme preparation), that produced about 65% (rat brain, human and rat plasma) or 85% enzyme inhibition (human and bovine erythrocytes). Thereafter, the enzyme–inhibitor mixture was diluted with buffer: the dilutions (v/v) were 1:60 for rat plasma, 1:120 for rat brain homogenate, 1:200 for bovine erythrocytes, 1:300 for human plasma, and 1:60 for human erythrocytes. The buffer did not contain the thiol reagent. At suitable time intervals samples (3.0 ml) were withdrawn, the thiol reagent added (100 μl), and the enzyme activity determined as described under Enzyme inhibition. The time interval from enzyme dilution until addition of substrate was taken as the time of spontaneous reactivation. The inhibitor concentrations remaining after dilution would give $\leqq 5\%$ inhibition within 1 h.

Aging of the inhibited enzyme

The enzyme preparation was incubated with an inhibitor concentration that produced about 90% inhibition. At suitable time intervals aliquots were diluted with the buffer containing the thiol reagent and the oxime TMB-4 [*N,N'*-trimethylene-bis-(4-hydroxy-iminomethylpyridinium bromide), 0.1 mM final concentration]. After 5.0 min, substrate was added and the enzyme activity determined as described under Enzyme inhibition.

To avoid spontaneous reactivation during aging, inhibitor solutions were repeatedly added when necessary. The time interval from the first addition of the inhibitor until dilution into buffer (which contained the oxime) was taken as the time of aging.

The enzyme concentrations were the same as given under Spontaneous reactivation.

RESULTS AND DISCUSSION

The inhibitory properties of DDVP and DNPP were determined in order to establish the required conditions in the study of spontaneous reactivation and aging.

TABLE I

SECOND-ORDER RATE CONSTANTS FOR INHIBITION (k_a) OF ACETYLCHOLINESTERASE AND CHOLINESTERASE BY DDVP AND DNPP

Enzyme	$10^{-5} \cdot k_a$ ($M^{-1} \cdot min^{-1}$)	
	DDVP	*DNPP*
Acetylcholinesterase		
Human erythrocytes	1.17	4.55
Rat brain	1.54	16.2
Cholinesterase		
Human plasma	8.70	0.42
Rat plasma	1.83	10.2

The rate of inhibition was measured at 3–4 different concentrations of the inhibitor. At each concentration, the time of inhibition was varied from 0.5 to 5.0 min.

In all reactions studied, the first-order rate constants of inhibition were a linear function of the inhibitor concentrations, and the calculated second-order rate constants of inhibition (k_a) are given in Table I.

Spontaneous reactivation

The time course of spontaneous reactivation of acetylcholinesterase and cholinesterase was the same, irrespective whether the enzyme was inhibited by DDVP or DNPP. This is shown in Fig. 1 for the spontaneous reactivation of dimethylphosphorylated rat plasma cholinesterase and human erythrocyte acetylcholinesterase. Up to a certain time, spontenaous reactivation followed the time course of a first-order reaction, and k_r was calculated from the linear part of the curve according to:

$$\ln \frac{100}{\%\ \text{inhibition}} = k_r \cdot t \qquad (1)$$

or $$\log \%\ \text{inhibition} = \log 100 - 0.4343 \cdot k_r \cdot t \qquad (1a)$$

where % inhibition was calculated from: 100% inhibition = activity of control sample − activity of inhibited sample at zero time, and inhibition = activity of control sample at time t − activity of inhibited sample at time t. Spontaneous reactivation of the inhibited rat brain acetylcholinesterase also deviates from first-order kinetics (after about 30 min), and k_r was calculated as described above. The rate constants obtained are given in Table II.

Dimethylphosphorylated human plasma cholinesterase does not reactivate spontaneously to a measurable extent within 2 h. However, when left for 24 h, 17.5% of the inhibited enzyme reactivated spontaneously (mean of 15 determinations) and this would correspond to a k_r value of $1.4 \cdot 10^{-4}$ min^{-1}. This will be a minimum value for k_r if the inhibited enzyme undergoes aging during that time.

Spontaneous reactivation of dimethylphosphorylated bovine erythrocyte acetylcholinesterase was measured only after inhibition with DDVP and the k_r obtained is given in Table II.

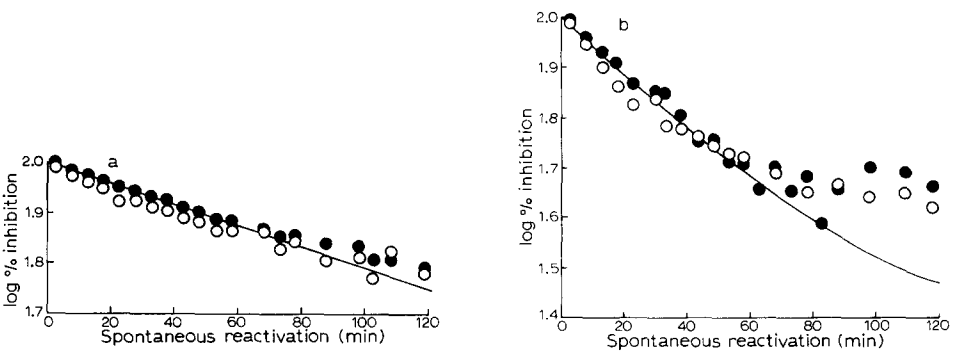


Fig. 1. Spontaneous reactivation of dimethylphosphorylated rat plasma cholinesterase (a) human erythrocyte acetylcholinesterase (b) after inhibition by DDVP (●) and DNPP (○). Each point is the mean of (a), 2–4 and (b), 4–5 individual measurements. The curve is calculated according to Eqn 8.

TABLE II

FIRST-ORDER RATE CONSTANTS FOR SPONTANEOUS REACTIVATION (k_r) AND AGING (k_{ag}) OF DIMETHYLPHOSPHORYLATED ACETYLCHOLINESTERASE AND CHOLINESTERASE

The numbers in parentheses represent the number of points from which the rate constants were calculated.

Enzyme	10^2 ($k_r \pm$ S.E.) (min^{-1})	10^2 ($k_{ag} \pm$ S.E.) (min^{-1})
Acetylcholinesterase		
Human erythrocytes	1.36 ± 0.14 (12)	0.297 ± 0.018 (20)
Bovine erythrocytes	0.92 ± 0.05 (7)	—
Rat brain	0.606 ± 0.057 (12)	0.1720 ± 0.0070 (19)
Cholinesterase		
Human plasma	≧ 0.014	—
Rat plasma	0.504 ± 0.028 (24)	approx. 0.058

The published data on the rates of spontaneous reactivation of cholinesterases are comparable with the k_r constants in Table II. Many of these results have been obtained from *in vivo* recovery of dimethylphosphorylated enzymes. Dimethylphosphorylated acetylcholinesterase from rabbit, rat and sheep erythrocytes reactivate spontaneously at 37 °C with a half-life between 1 and 2 h[1–3,5]. Mouse and rat brain acetylcholinesterase, and horse serum cholinesterase, are also reported to reactivate spontaneously within about 2 h (at 37 °C), but no rate constants have been calculated[4,6].

Aging of the inhibited enzyme

The time course of aging was also the same, irrespective of whether DDVP or DNPP were used as inhibitors.

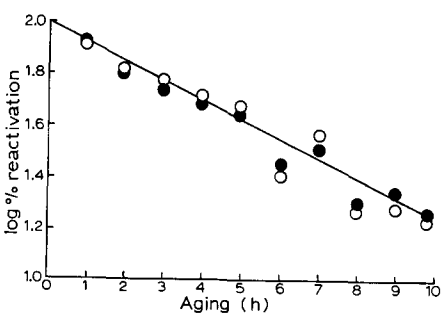


Fig. 2. Aging of dimethylphosphorylated human erythrocyte acetylcholinesterase after inhibition by DDVP (●) and DNPP (○). Each point is the mean of 5–10 individual measurements; the line is calculated from the mean values.

The rate of aging of dimethylphosphorylated acetylcholinesterase (human erythrocytes and rat brain) was calculated from the amount of enzyme which could not be reactivated with TMB-4. Initially all enzyme is reactivatable (*cf.* Fig. 2). The conversion to enzyme incapable of reactivation followed the kinetics of a first-order reaction, and the rate constant k_{ag} was calculated from:

$$\ln \frac{100}{\%\ \text{reactivation}} = k_{ag} \cdot t \qquad (2)$$

or $$\log \%\ \text{reactivation} = \log 100 - 0.4343 \cdot k_{ag} \cdot t \qquad (2a)$$

where % reactivation was calculated from: 100% reactivation = activity of control sample (without TMB-4) − activity of inhibited sample (without TMB-4) and reactivation = (activity of reactivated enzyme/0.85) at time t − activity of inhibited sample (without TMB-4) at time t. The oxime itself is an inhibitor of acetylcholinesterase at the concentrations used in these experiments (0.1 mM). The degree of inhibition of human erythrocyte and rat brain acetylcholinesterase was 15% and the activity of the reactivated enzyme was corrected for that amount. The rate constants obtained are given in Table II.

Rat plasma cholinesterase could not be completely reactivated with 0.1 mM TMB-4, even when the time of aging was short. Higher concentrations of TMB-4 could not be used because TMB-4 is an inhibitor, and it also enhances the non-enzymic hydrolysis of acetylthiocholine. Within 0–10 h of aging, only 48 ± 6% of the inhibited rat plasma is capable of being reactivated with 0.1 mM TMB-4 (mean of 24 determinations). After another 10 h of aging, the enzyme capable of reactivation decreases to 34 ± 10% (mean of 24 determinations); this would correspond to a rate constant $k_{ag} = 0.00058\ \text{min}^{-1}$.

Dimethylphosphorylated human plasma cholinesterase cannot be fully reactivated (time of reactivation up to 30 min) with either 0.1 mM TMB-4 or 0.5 mM pyridinium-2-aldoxime methyliodide, even when the time of aging is short (about 10–20 min). This is not due to very rapid aging, because spontaneous reactivation of the enzyme occurs even after several hours, as described above. Aging of the inhibited human plasma cholinesterase was not studied further.

Blaber and Creasey[5,6] published data on aging *in vivo* of dimethylphosphorylated acetylcholinesterases; sheep erythrocyte acetylcholinesterase becomes incapable of being reactivated after about 1–2 h and rat brain acetylcholinesterase, after about 12 h. The latter value is in agreement with our data (Table II), *i.e.* half-life for aging is 6.6 h. Witter and Gaines[7] obtained a half-life of 2 h for aging *in vitro* and *in vivo* of dimethylphosphorylated chicken brain acetylcholinesterase.

Theoretical treatment

The rates of spontaneous reactivation and aging are both proportional to the concentration of the phosphorylated enzyme:

$$\frac{d\,[EH]}{dt} = k_r\,[EA] \qquad (3)$$

$$\frac{d\,[EX]}{dt} = k_{ag}\,[EA] \qquad (4)$$

It follows from Eqns 3 and 4 that at any time the ratio between active enzyme and enzyme incapable of reactivation formed from EA will equal:

$$\frac{[EH]}{[EX]} = \frac{k_r}{k_{ag}} \qquad (5)$$

The time course of the formation of the active enzyme can be calculated if k_r and k_{ag} are known:

$$\frac{d\,[EH]}{dt} = k_r\,E_0 - (k_r + k_{ag})\,[EH] \tag{6}$$

Eqn 6 is obtained by combining Eqns 3–5, and $[E_0]$ is the total enzyme concentration:

$$[E_0] = [EA] + [EH] + [EX] \tag{7}$$

The solution of the inhomogeneous differential Eqn 6 is:

$$[EH] = \frac{k_r\,[E_0]}{k_r + k_{ag}} \left(1 - e^{-(k_r + k_{ag})\,t}\right) \tag{8}$$

and Eqn 8 defines the concentration of active enzyme as a function of time.

Using the rate constants from Table II, the time course of spontaneous reactivation (*i.e.* formation of active enzyme) has been calculated from Eqn 8 for the dimethylphosphorylated acetylcholinesterases and rat plasma cholinesterase. The agreement between theory and experiment is good, as seen from a comparison between the theoretical and experimental curve on Fig. 1. The maximal discrepancy between the theoretical and experimental curve is 14% enzyme activity for human erythrocytes, and this occurs after 120 min. For rat plasma the maximal discrepancy is 5% (Fig. 1) and for rat brain it is 9% enzyme activity (after 1 h of spontaneous reactivation). These differences are partly due to reinhibition of the enzyme by the inhibitor which is still present during spontaneous reactivation (*cf.* Materials and Methods). Although this does not account for all the difference, the reasons for the remaining difference has not been further investigated.

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