EQUILIBRIUM KINETICS OF REACTIVATION OF PHOSPHONYLATED ACETYLCHOLINESTERASE BY OXIMES

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A kinetic equation was derived for the calculation of constants characterizing the reactivation of phosphonylated cholinesterases by oximes in equilibrium conditions. Using the equation, the reactivation efficiency of 17 oximes on O-ethylmethylphosphonylated and O-isopropylmethylphosphonylated mouse brain acetylcholinesterase was tested.

The high toxicity of organophosphorus compounds is attributed to an irreversible inhibition of acetylcholinesterase in the nervous system of animals[1]. The mechanism of inhibition consists in the phosphorylation of the active site of this enzyme which is formed by the serine hydroxyl[1,2]. Spontaneous dephosphorylation by water is very slow and can be accelerated by some nucleophilic agents, *e.g.* oximes[3]. The most efficient compounds of this type which are described as reactivators of cholinesterase are oximes of the pyridine series with a quaternary nitrogen in the molecule[4,5]. The reactivation effect of these compounds depends on the chemical structure of the oxime and on the character of the alkylphosphoryl group bound at the active centre of cholinesterase[6] as well as on the source of the enzyme used[7–9].

The present paper deals with the reactivation effect of 17 different oximes on O-ethylmethylphosphonylated and O-isopropylmethylphosphonylated mouse brain acetylcholinesterase *in vitro* under equilibrium conditions. To allow a subsequent correlation of the results with those of therapeutical treatment of mice the reactivations were done at pH 7·4 and 38°C.

EXPERIMENTAL

Chemicals. The reactivators used were prepared by Dr Bielavsky of this institute; only 1-methyl-2-pyridiniumaldoxime iodide (pralidoxime, 2-PAM) and N,N'-trimethylene-bis-(4-pyridiniumaldoxime) dibromide (trimedoxime, TMB-4) were commercial products of Léčiva, Prague. The chemical structure of the reactivators is shown in Table I. O-Ethyl-S-(2-dimethylaminoethyl)-methylphosphonothioate (EDEM) and O-isopropylmethylphosphonofluoridate (IMPF) were kindly provided by Dr J. Vachek of this institute. All other chemicals were commercial products.

Acetylcholinesterase. The source of the enzyme was a 10% (w/v) homogenate of whole mouse brains using animals of either sex (from the Mezno stock), in a veronal–phosphate buffer made isotonic with sodium chloride[10]. The animals were killed by severing the carotids, their brains

were excised, rinsed in physiological saline and homogenized in an Ultra-Turrax. The homogenates were maintained at 4°C for not more than two days.

Preparation of phosphonylated acetylcholinesterase. O-Ethylmethylphosphonylated acetylcholinesterase was prepared by combining 1 ml of the 10% brain homogenate with 1 ml 10^{-8}M EDEM and the mixture was incubated at 25°C for 30 min. Likewise, O-isopropylmethylphosphonylated acetylcholinesterase was prepared by combining 1 ml brain homogenate with 1 ml 10^{-8}M IMPF and a 30 min incubation at 25°C.

Reactivation of phosphonylated acetylcholinesterase. After a 30 min incubation the mixture of enzyme and inhibitor (2 ml) was added to 1 ml reactivator solution of known molarity and after t min of incubation at 25°C the mixture was combined with veronal-phosphate buffer[10] to a total volume of 20 ml. One ml 0·1M acetylcholine iodide was then added and residual activity was measured. The acetylcholinesterase activity was estimated electrometrically[10] in a semiautomatic recording device[11]. The measurement was conducted at 25°C, acetylcholine iodide being used as substrate at a final concentration of $4{\cdot}76 \cdot 10^{-3}$M. With each series of estimation of activity of the reactivated enzyme (a_r), the activity of the uninhibited enzyme (a_0) was also estimated, when, instead of the solution of the organophosphorus inhibitor, the enzyme solution was mixed with 1 ml buffer; and the activity of the inhibited enzyme (a_i) when 1 ml buffer was added instead of the reactivator solution. The values thus obtained were used for computing

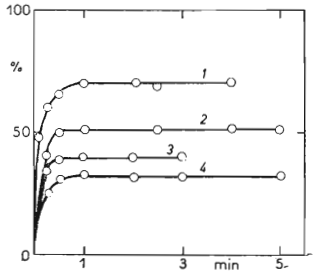


FIG. 1

Time Dependence of Reactivation of O-Ethylmethylphosphonylated and O-Isopropylmethylphosphonylated Mouse Brain Acetylcholinesterase by Oximes at pH 7·4 and 38°C

Reactivation r of the O-ethylmethylphosphonylated enzyme with 1 mM *IV* (*1*) and 0·1 mM *I* (*3*) and reactivation of O-isopropylmethylphosphonylated enzyme with 0·1 mM *VI* (*2*) and 0·1 mM *IV* (*4*) expressed as %.

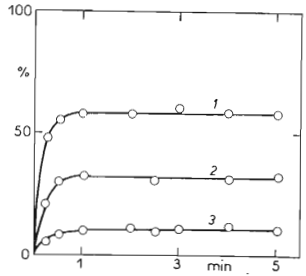


FIG. 2

Time Dependence of Reactivation r of O-Isopropylmethylphosphonylated Mouse Brain Acetylcholinesterase by 1-Methyl-2-pyridiniumaldoxime Iodide (*I*) at pH 7·4) and 38°C

Oxime concentration: *1* 1 mM, *2* 0·25 mM, *3* 0·05 mM.

the percentage of reactivated enzyme according to

$$\% \text{ reactivation} = 100(a_r - a_i)/(a_0 - a_i) .$$

The fractional reactivation r is obtained by omitting the factor 100.

RESULTS AND DISCUSSION

With most reactivators the time course of reactivation was followed, *i.e.* the dependence of the degree of reactivation on the period of incubation of the phosphonylated enzyme with the reactivator. The rate reactivation is rather high, the half-times being of the order of 10^1 s, the final level of reactivation being reached within 1–2 min (Fig. 1 and 2). The rate of reactivation is practically independent of the reactivator used (Fig. 1) and of its concentration (Fig. 2). The reactivator concentration affects only the degree of reactivation.

The dependence of reactivation on reactivator concentration was studied with all the 17 compounds. The period of incubation of the phosphonylated enzyme with the reactivator was always 5 min which is sufficient for reaching a reaction equilibrium. The dependence of the degree of reactivation expressed as fractional reactivation r on reactivator concentration is sigmoid (Fig. 3) and can be rectified by a probit-logarithmic transformation[8,9].

The reactivation kinetics is expressed by the general scheme[12]

$$\mathrm{EI} + n\,\mathrm{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EIR_n} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}, \qquad (1)$$

where EI is the phosphonylated enzyme, R is the reactivator, $\mathrm{EIR_n}$ the transition complex with n molecules of the bound reactivator, E is the regenerated enzyme and P is the reaction product; k_1, k_{-1} and k_2 are the appropriate rate constants.

During the first reaction phase a labile complex $\mathrm{EIR_n}$ is formed. This part of the reaction is reversible and equilibrium is attained rapidly. In the subsequent phase the $\mathrm{EIR_n}$ complex is degraded to the regenerated enzyme and the reaction product represented by the phosphonylated oxime[13]. This part of the reaction is irreversible and is characterized by a rate constant k_2 which appears to be the rate-limiting factor of the whole scheme.

If the rate of decomposition of the $\mathrm{EIR_n}$ complex is great ($k_{-1} \approx k_2$) and equilibrium between the inhibited and the reactivated enzyme is attained rapidly. The free fraction of the enzyme actually represents r so that $(1 - r)$ is that fraction of the enzyme which is bound in a complex. The rate of reaction from left to right (v_1) is proportional to the amount of free enzyme, *i.e.* r, as well as to the concentration of the reactivator R so that $v_1 = k_1 r[\mathrm{R}]^n$. The rate of reaction from right to left is proportional to the complex-bound enzyme only $(1 - r)$ so that $v_{-1} = (1_{-1} + k_2) . (1 - r)$.

At equilibrium the two rates are equal so that

$$\frac{1 - r}{r} = \frac{k_1[\mathrm{R}]^n}{k_{-1} + k_2} . \qquad (2)$$

If K_r is substituted into equation (2) for $(k_{-1} + k_2)/k_1$ and the logarithm is taken we obtain

$$\log [(1 - r)/r] = n \log \mathrm{R} - \log K_r , \qquad (3)$$

which is a straight-line equation of the slope form and is formally identical with Hill's empirical equation[14] which became popular in enzymology for characterizing enzyme interactions with substrates and inhibitors[15–18]. The quantity n, the so-called Hill or interaction coefficient, expresses the apparent number of ligands bound to the enzyme.

By plotting $\log [(1 - r)/r)]$ against log R a straight line is obtained with a slope equal to n with an intercept on the abscissa equal to the negative logarithm of reactivator concentration bringing about 50% reactivation (pR_{50}) and with an intercept

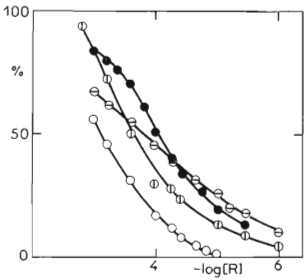


FIG. 3

Dependence of the Percentage of Reactivation r of O-Ethylmethylphosphonylated Mouse Brain Acetylcholinesterase on Concentration (R) of *IV* (⊖) and *V* (⊙) and Dependence of the Percentage of Reactivation of O-Isopropylmethylphosphonylated Mouse Brain Acetylcholinesterase on the Concentration of *I* (○) and *VII* (●)

Reactivation took place at pH 7·4 and 38°C.

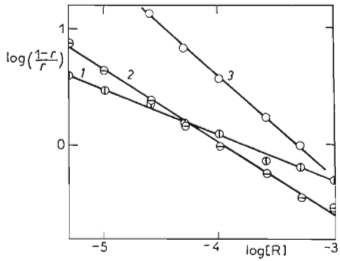
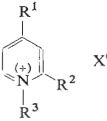


FIG. 4

Dependence of the Degree of Reactivation of Phosphonylated Mouse Brain Acetylcholinesterase on the Concentration of Oxime in Hill's Plot

1 Reactivation of O-ethylmethylphosphonylated enzyme by *IV* (n 0·42); 2 reactivation of O-isopropylmethylphosphonylated enzyme by *VI* (n 0·65) and reactivation of the O-isopropylmethylphosphonylated enzyme by *XV* (n 0·87).

TABLE I
Chemical Structure of the Reactivators Used



Compound	R^1	R^2	R^3	$X^{(-)}$
I	—H	—CH=NOH	—CH_3	I
II	—CH=NOH	—CH=NOH	—CH_3	I
III	—CH=NOH	—H	—CH_2—$N^{(+)}$(pyridine)—CH=NOH	2 Cl
IV	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}$(pyridine)—CH=NOH	2 Br
V	—CH=NOH	—H	—CH_2—O—CH_2—$N^{(+)}$(pyridine)—CH=NOH	2 Cl
VI	—CH=NOH	—H	—CH_2—CH=CH—CH_2—$N^{(+)}$(pyridine)—CH=NOH	2 Br
VII	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}$(pyridine)	2 Br
VIII	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}$(pyridine, 1-C_2H_5)	2 Br
IX	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}$(pyridine, 1-CH_3)	2 Br
X	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}$(morpholine, 4-CH_3)	2 Br
XI	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}(CH_3)_3$	2 Br
XII	—CH=NOH	—H	—CH_2—CH_2—CH_2—CH_2—$N^{(+)}(CH_3)_3$	2 Br
XIII	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}(CH_3)_2C_2H_5OH$	2 Br
XIV	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}(C_2H_5)_2CH_3$	2 Br
XV	—CH=NOH	—H	—CH_2—CH_2—CH_2—$N^{(+)}(C_2H_5)_3$	2 Br
XVI	—CH=NOH	—H	—CH_2—O—CH_2—$N^{(+)}(CH_3)_3$	2 Cl
XVII	—CH=NOH	—H	—CH_2—O—CH_2—$N^{(+)}$(morpholine, 4-CH_3)	2 Cl

TABLE II

Kinetic Constants Characterizing the Reactivation of Phosphonylated Mouse Brain Acetylcholinesterase by Oximes

The values of the constants are shown as arithmetic means ± the standard deviation of the mean. The number of values from which the means were taken varied from 6 to 10. *n* Hill's interaction coefficient; *r* correlation coefficient characterizing the dependence between reactivation efficiency and reactivator concentration according to equation (*3*).

Compound	$pK_r \pm$ S.D.	$pR_{50} \pm$ S.D.	n ± S.D.	r_b
	O-Ethylmethylphosphonylated Acetylcholinesterase			
I	2·77 ± 0·12	3·75 ± 0·15	0·74 ± 0·07	0·9791
II	2·50 ± 0·15	2·44 ± 0·16	1·02 ± 0·08	0·9820
III	4·35 ± 0·10	3·57 ± 0·08	1·22 ± 0·07	0·9933
IV	1·61 ± 0·04	3·85 ± 0·09	0·42 ± 0·02	0·9959
V	3·87 ± 0·20	4·02 ± 0·23	0·96 ± 0·09	0·9720
VI	2·25 ± 0·07	3·82 ± 0·12	0·59 ± 0·05	0·9827
VII	3·25 ± 0·04	3·53 ± 0·04	0·92 ± 0·02	0·9982
VIII	1·99 ± 0·05	3·26 ± 0·09	0·61 ± 0·05	0·9895
IX	1·29 ± 0·02	2·30 ± 0·04	0·56 ± 0·02	0·9975
X	2·51 ± 0·02	3·40 ± 0·03	0·74 ± 0·02	0·9993
XI	3·09 ± 0·09	3·72 ± 0·12	0·83 ± 0·05	0·9915
XII	3·14 ± 0·03	3·26 ± 0·05	0·96 ± 0·03	0·9989
XIII	2·55 ± 0·06	3·15 ± 0·07	0·81 ± 0·06	0·9895
XIV	3·58 ± 0·07	4·31 ± 0·08	0·83 ± 0·06	0·9883
XV	1·23 ± 0·05	2·28 ± 0·09	0·54 ± 0·04	0·9909
	O-Isopropylmethylphosphonylated Acetylcholinesterase			
I	3·43 ± 0·19	3·24 ± 0·18	1·06 ± 0·10	0·9727
III	2·56 ± 0·07	3·20 ± 0·08	0·80 ± 0·04	0·9932
IV	4·24 ± 0·04	4·65 ± 0·05	0·91 ± 0·05	0·9978
V	1·94 ± 0·04	3·40 ± 0·08	0·57 ± 0·03	0·9955
VI	2·45 ± 0·06	3·95 ± 0·10	0·62 ± 0·03	0·9942
VII	2·66 ± 0·04	3·34 ± 0·05	0·80 ± 0·03	0·9968
VIII	1·65 ± 0·02	2·90 ± 0·03	0·57 ± 0·02	0·9986
IX	2·73 ± 0·05	2·68 ± 0·05	1·02 ± 0·04	0·9972
X	2·63 ± 0·05	3·20 ± 0·07	0·82 ± 0·05	0·9956
XI	2·48 ± 0·02	3·06 ± 0·03	0·81 ± 0·02	0·9991
XII	3·34 ± 0·14	3·67 ± 0·15	0·91 ± 0·10	0·9756
XIII	2·51 ± 0·10	3·50 ± 0·12	0·72 ± 0·07	0·9812
XIV	3·20 ± 0·06	4·15 ± 0·09	0·77 ± 0·03	0·9959
XV	2·88 ± 0·03	3·31 ± 0·04	0·87 ± 0·02	0·9989
XVI	2·68 ± 0·04	2·85 ± 0·05	0·94 ± 0·04	0·9975
XVII	3·70 ± 0·14	4·16 ± 0·17	0·89 ± 0·12	0·9023

on the ordinate equal to the negative logarithm of the dissociation constant (pK_r). pR_{50} and pK_r are related by $pK_r = n\,pR_{50}$ and the two are equal for $n = 1$.

A typical example of this type of plot is shown in Fig. 4. The straight lines were drawn through the experimental points by a computer using a least-squares procedure, the constants and their standard deviations being shown in Table II. The high values of the correlation coefficient indicate that the experimental values fit relationship (3) very well.

Hill's coefficient in the present work was usually less than unity so that the interaction of the phosphonylated acetylcholinesterase with the reactivators can be characterized as a negative cooperative reaction[19]. Similar values of Hill's coefficient were obtained in studying the interaction of O-isopropylmethylphosphonylated human[8] as well as rat[9] brain acetylcholinesterase with the reactivators.

The stoichiometry of this interaction corresponds to a situation where one molecule of the phosphonylated enzyme reacts with one molecule of the reactivator. The apparent number of reacting molecules of the reactivator expressed by Hill's coefficient is mostly less than one, apparently due to the fact that the reactivation is not complete even at the highest concentrations of the reactivator[20]. This may be caused by the inhibitory effect of the reactivators on acetylcholinesterase at higher concentrations[21]. This explains at the same time the frequently reported deviations from first-order kinetics[22–24].

To evaluate the reactivator from the point of view of its effect on inhibited acetylcholinesterase the reactivation kinetics has a special significance under equilibrium conditions. In view of the rapid attainment of this equilibrium the resulting reactivation effect depends only on the reactivator concentration.

The time factor can be neglected which becomes particularly clear if one realizes the conditions under which the reactivation takes place in the living organism. The time required to build up a sufficient concentration of the reactivator for a measurable effect is substantially longer than the time required for reactivation. The resulting reactivation effect thus depends only on the actual concentration of the reactivator attained at the site of damaged enzyme. This, on the other hand, depends on a number of factors influencing the permeability of the membranes to the reactivator and one thus cannot expect a simple relationship between the reactivation constants measured *in vitro* and the therapeutical activity of the compounds *in vivo*. It appears that even a comparison of the efficiency of the reactivators on two types of inhibited acetylcholinesterase is not simple. An attempt at a mutual correlation of the constants shown here was unsuccessful due to nonlinearity of the regression lines. Evaluation of linearity by the χ^2-test showed that deviations from linearity were significant in all cases tested.

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