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The Reactivation of Cholinesterase Inhibited with Organophosphorus Compounds

1. REACTIVATION BY 2-OXOALDOXIMES

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The ability of many chemical compounds to restore the activity of cholinesterase (ChE) after inhibition by organophosphorus compounds is now well established (Childs, Davies, Green & Rutland, 1955; Wilson, 1955). It has also been shown that recovery of enzymic activity is due to chemical displacement of the phosphoryl group of the inhibitor from the group to which it is attached at or near the active centre of the enzyme (Davies & Green, 1956; Jandorf, Crowell & Levin, 1955). The most powerful reactivators, hydroxamic acids and oximes, will react rapidly with many organophosphorus inhibitors themselves (Hackley, Plapinger, Stolberg & Wagner-Jauregg, 1955; Green & Saville, 1956), but there is little correlation between these reactivities

and the ability of hydroxamic acids and oximes to reactivate the inhibited enzyme (Childs *et al.* 1955). It has been suggested (Davies & Green, 1956) that this lack of correlation may be due to the formation of a preliminary complex between the reactivator and the inhibited enzyme. In this and the subsequent paper, this possibility has been tested kinetically for the two most potent groups of reactivating agents, 2-oxoaldoximes (I) and quaternized pyridine aldoximes.

(I)

(R = methyl, ethyl, *n*-propyl, *isopropyl*, *tert.*-butyl, phenyl and cyclohexyl).

EXPERIMENTAL

Materials

The oximes were prepared by the action of nitrous acid on compounds possessing a suitably activated methylene group. *iso*Nitrosoacetophenone (I, R = phenyl) was obtained from acetophenone (Claisen & Manasse, 1887); the remaining compounds were obtained from β -oxo esters ($R \cdot CO \cdot CH_2 \cdot CO_2 \cdot Et$) by Freon's method (1939) for *iso*-nitrosoacetone. 2-Oxo-n-valeraldoxime (I, R = n-propyl), m.p. 49–50° (Found: N, 12.8. $C_5H_9O_2N$ requires N, 12.2%), 2-oxo-isovaleraldoxime (I, R = isopropyl), m.p. 32–33° (Found: N, 12.2%), 2-oxo-2-cyclohexylacetaldoxime (I, R = cyclohexyl), m.p. 72–74° (Found: N, 9.2. $C_7H_{11}O_2N$ requires N, 9.0%) and 2-oxo-3,3-dimethylbutyraldoxime (I, R = *tert.*-butyl), m.p. 52–53° (Found: N, 10.7. $C_6H_{11}O_2N$ requires N, 10.9%), have not previously been described.

Methods

Reactivation experiments. The ChE of human erythrocytes was inhibited by treatment with a dilute aqueous solution of tetraethylpyrophosphate (TEPP) or isopropyl methylphosphonofluoridate (Sarin). Saline suspensions of the inhibited cells were mixed with the oxime in barbitone buffer, pH 7.4, at 25°. At suitable time intervals samples were withdrawn and the ChE activity was measured by the electrometric method (Michel, 1949). The experimental procedure and the method of calculating the rate constants have been described fully (Davies & Green, 1956).

Reaction rates. During the reaction between 2-oxoaloximes and Sarin, 3 moles of acid are liberated. The reaction rates were measured by continuous titration of this acid with alkali as described in detail elsewhere (Green & Saville, 1956).

Dissociation constants. These were given by the pH of an aqueous solution of the oxime half-neutralized with alkali.

RESULTS

Complex formation in reactivation by isonitrosoacetone (I, R = methyl)

If the formation of a complex between the reactivating agent and the inhibited enzyme is an essential preliminary step in reactivation, then this

Fig. 1. Reactivation by isonitrosoacetone of erythrocyte cholinesterase inhibited with TEPP (⊙) or Sarin (×). [A] is the concentration (mole/l.) of isonitrosoacetone and k is the observed rate constant (min.^{-1}).

process may be represented by a set of equations similar to those already familiar in the Michaelis-Menten scheme for enzyme-substrate interaction,

where EI is the inhibited enzyme, A the reactivator and EI.A the intermediate complex.

If the formation of the complex and its dissociation back into its components are rapid compared with its breakdown to give the free enzyme, then the overall rate of reactivation will be given by

$$v = dE/dt = k_3 [EI \cdot A] = k_3 [EI_{\text{total}}] [A]/[A] + K, \quad (1)$$

where $K = [EI] [A]/[EI \cdot A] = k_2/k_1$

and $[EI_{\text{total}}] = [EI] + [EI \cdot A]$.

In each experiment the rate of reactivation by isonitrosoacetone, which was in vast excess over the inhibited enzyme, was first-order with respect to the reactivator, i.e.

$$v = k_{\text{observed}} [EI_{\text{total}}]. \quad (2)$$

But, if complex-formation occurred, the rate would be given by equation (1) above, so that

$$k_{\text{observed}} = k_3 [A]/K + [A], \quad (3)$$

which may be rearranged in the form

$$1/k_{\text{observed}} = K/k_3 [A] + 1/k_3. \quad (4)$$

In Fig. 1, $1/k_{\text{observed}}$ is plotted against $1/[A]$ for the reactivation by isonitrosoacetone of ChE inhibited with TEPP or with Sarin. A linear relationship, consistent with (4), is found in both cases. The values of K and k_3 , obtained from the slopes and intercepts, are 0.02 mole/l. and 0.16 min.^{-1} for ChE inhibited with TEPP, and 0.01 mole/l. and 0.24 min.^{-1} for ChE inhibited with Sarin.

Reactivation by other 2-oxoaloximes

A number of 2-oxoaloximes (I) have been studied which have similar dissociation constants and reactivities with Sarin, but in which the alkyl group R was varied extensively. Table 1 shows the rates of reactivation by these compounds of ChE inhibited with TEPP or with Sarin. Preliminary experiments showed that, at the concentrations of reactivator selected, the observed rate of reactivation was roughly proportional to the concentration. This will only occur in dilute solution where $K \gg [A]$ and when (1) becomes

$$v = k_3 [EI_{\text{total}}] [A]/K \quad \text{and} \quad k_{\text{observed}} = k_3 [A]/K.$$

DISCUSSION

The reactivities of hydroxamic acids and oximes with organophosphorus compounds are fairly closely related to the dissociation constants of the hydroxamic acids or oximes, although other factors do

Table 1. *Reactivation of erythrocyte cholinesterase inhibited with tetraethylpyrophosphate or with Sarin by 2-oxoaldoximes (R·CO·CH:N·OH) at pH 7.4 and 25°*

Concentration of the reactivator is 0.005M for the TEPP-inhibited enzyme and 0.002M for the Sarin-inhibited enzyme, except where otherwise stated. The rate constant given for the reaction between Sarin and the *tert.*-butyl compound is less certain than those for the other compounds, since with this particular compound the acid was not liberated at a first-order rate throughout the whole course of the experiment.

R	Second-order-rate constant (l./mole/min.) for reactivation of ChE inhibited with		Second-order-rate constant (l./mole/min.) for reaction with Sarin at pH 7.4 and 25°	pK _a
	TEPP	Sarin		
CH ₃	7	21	28	8.3
C ₂ H ₅	11	20	24	8.4
<i>n</i> -C ₃ H ₇	—	24*	28	8.3
<i>iso</i> -C ₃ H ₇	—	24†	26	8.3
<i>cyclo</i> Hexyl	8.3‡	11	23	8.4
<i>tert.</i> -C ₄ H ₉	2.4	5	(10)	8.6
Phenyl	11	6	18	8.3

* Rate at 0.0004M.

† Rate at 0.0013M.

‡ Rate at 0.002M.

have some influence, particularly with oximes (Green & Saville, 1956). The correlation is sufficient to enable the relative reactivity of any hydroxamic acid or oxime with a particular organophosphorus compound to be predicted roughly from the structure and dissociation constant of the hydroxamic acid or oxime. But, when the abilities of these compounds to reactivate inhibited ChE are compared, marked differences are found. For example, picolinhydroxamic acid and pyrimidine-2-hydroxamic acid are much more effective in reactivating ChE inhibited with TEPP than are other hydroxamic acids of comparable reactivity with organophosphorus compounds (Childs *et al.* 1955). These differences are not explicable on an electronic basis, but suggest rather that there is a difference in mechanism between reaction with simple organophosphates and reactivation. This difference could be the formation of a complex between the reactivator and the inhibited enzyme before fission of the phosphorus-enzyme bond.

Fig. 1 shows that reactivation by *isonitroso*-acetone at low concentration is consistent with this theory. The further consequence of the theory, that when the concentration of the reactivator is large ($[A] \gg K$) the rate becomes $k_3[E]_{\text{total}}$, that is, independent of the reactivator concentration, could not be demonstrated directly since at higher concentrations of the reactivator than those shown in Fig. 1 the rate of reactivation was too fast to measure with any precision.

Previously (Davies & Green, 1956) it has been shown that the rates of reaction of nucleophilic reagents with organophosphorus compounds (II),

when R' and R'' were varied, were in the same ratio as the rates of reactivation by *isonitroso*acetone of the corresponding inhibited enzyme (III, where E is some group in the enzyme).

From this parallelism it was argued that reactivation by *isonitroso*acetone was a simple chemical displacement reaction and that no complex formation occurred. However, this argument is valid only if the strength or orientation of the complex formed between the reactivator and the phosphorylated enzyme depends on the alkyl groups associated with the phosphorus atom. If the site on the enzyme to which the reactivator is bound is not sterically affected by these alkyl groups then the complex may not be influenced by them, in which case the above parallelism would not disprove the existence of an intermediate complex.

It is not obvious which part of the 2-oxoaldoxime molecule is responsible for the formation of the complex, but since these compounds do not possess a positive charge they are unlikely to be held by electrostatic attraction to the anionic site on the inhibited enzyme, which is believed to be shielded by the dialkylphosphoryl group (Wilson, 1955). The nucleophilic oximino group may contribute something to the binding force, although it will be shielded from direct contact with the enzyme surface by the phosphoryl group of the inhibitor and it is more likely that the reactivator is held by a hydrogen bond on to the carbonyl oxygen atom, or by van der Waals interaction between the enzyme and the alkyl group R.

The reactivation rates for the 2-oxoaldoximes given in Table 1 were measured under conditions where the observed rate constant (k_{observed}) is roughly k_3/K . Now k_3 measures the intrinsic ability of the oxime to displace the enzyme from the phosphoryl group so that, provided the orientation of the oximino group relative to the phosphoryl

group is not seriously changed by alteration in other parts of the oxime molecule, k_3 for a series of closely related oximes might be expected to run parallel to the intrinsic reactivity (as measured by the second-order-rate constant k') of these oximes with any other organophosphorus compound possessing the same dialkylphosphoryl group as the inhibited enzyme. For example, the values of k_3 for reactivation of the *isopropyl* methylphosphonyl enzyme by the oximes in Table 1 might be expected to be related to the rates of reaction of these oximes with Sarin. If this is true, any variations in K will be reflected by the ratio $k_{\text{observed}}:k'$.

Table 1 shows that variation of R from methyl to *isopropyl* has little effect on this ratio for ChE inhibited with Sarin, which suggests that the oxime is bound at the carbonyl group rather than by van der Waals interaction with the alkyl group. But, when the size of R is increased still further, to *cyclohexyl* and *tert.*-butyl, the ratio $k_{\text{observed}}:k'$ begins to drop. When R is phenyl the relative ease of reactivation of ChE inhibited with TEPP or with Sarin becomes inverted. This would suggest that the steric requirement for hydrogen bonding on to the carbonyl oxygen atom is more stringent for the *isopropyl* methylphosphonyl enzyme than for the diethylphosphoryl enzyme.

Some indirect support for this concept is given by the variation in reactivating power of the isomeric pyridine hydroxamic acids. The most active, picolinhydroxamic acid (IV) (Childs *et al.* 1955; Jandorf *et al.* 1955; and Wilson & Ginsburg, 1955), possesses a nitrogen atom in a position analogous to that of the carbonyl oxygen atom in 2-oxoalldoximes (I) and could form complexes in the same way.

Nicotinhydroxamic acid and *isonicotin*hydroxamic acid, in which the nitrogen atom is in the β - or γ -positions in the pyridine ring, could also be attached to the enzyme by hydrogen bonds to their heterocyclic nitrogen atoms, but these complexes would no longer have the nucleophilic oxygen atom in the hydroxyl group at the optimum position for reactivation to occur. Wilson, Ginsburg & Meislich (1955) have in fact shown that the dependence on

concentration of the reactivation by nicotinhydroxamic acid of ChE inhibited with TEPP can be expressed by an equation of the form (1) consistent with the formation of such a complex. ChE inhibited with Sarin is reactivated by picolinhydroxamic acid (IV) much more slowly than is ChE inhibited with TEPP (Davies & Green, 1956), which is consistent with a reduced ability of the heterocyclic nitrogen atom to be held to the *isopropyl* methylphosphonyl enzyme by a hydrogen bond, owing to greater steric repulsion by the inhibited enzyme of adjacent parts of the pyridine ring in the hydroxamic acid.

SUMMARY

1. Kinetic studies on the reactivation by 2-oxoalldoximes ($\text{R}\cdot\text{CO}\cdot\text{CH:N}\cdot\text{OH}$) of cholinesterase inhibited with organophosphorus compounds show that the formation of a complex between the reactivator and the inhibited enzyme is a likely step in the reactivation process.

2. Variation of the alkyl group (R) has only a minor effect on the reactivating power of 2-oxoalldoximes, which indicates that it is probably the carbonyl group in the oxoalldoxime which is linked to the enzyme in the complex.

3. An explanation is given for the marked differences in reactivating powers of the isomeric pyridine hydroxamic acids.

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