# The Reactivation of Cholinesterase Inhibited with Organophosphorus Compounds

## 2. REACTIVATION BY PYRIDINEALDOXIME METHIODIDES

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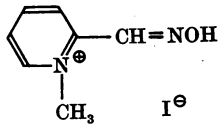
Pyridine-2-aldoxime methiodide (I) is by far the most effective compound yet found in restoring the activity of the enzyme cholinesterase (ChE) after inhibition with organophosphorus compounds (Davies & Green, 1955; Wilson & Ginsburg, 1955).




(I)

In the previous paper (Green & Smith, 1958) it was shown that the formation of complexes between the reactivating agent and the inhibited enzyme could assist reactivation very significantly. In the present paper we have examined the contribution of complex formation to the efficacy of pyridinealdoxime methiodides as reactivators of inhibited ChE.

## EXPERIMENTAL

### *Materials*

The pyridinealdoxime methiodides were obtained by boiling the oximes with methyl iodide in ethanol (Green & Saville, 1956). Quinoline-4-aldoxime methiodide, m.p. 232–233° (Found: I 40·2. $C_{11}H_{11}N_2OI$ requires I 40·4%) and *pyridine-2-aldoxime ethiodide*, m.p. 186–188° (Found: I 45·3. $C_8H_{11}N_2OI$ requires I 45·6%) were prepared similarly.

### *Reactivation experiments*

These were carried out on human erythrocytes at 25° and pH 7·4 as described earlier (Davies & Green, 1956), whereby samples from a reactivation mixture containing inhibited enzyme and reactivator were taken at intervals and analysed for ChE activity by the electrometric method. With the higher concentrations of oxime, reactivation continued to an appreciable extent in the assay vessel despite the presence of excess (0·01 M) of acetylcholine, which gives the appearance of exceptionally rapid reactivation during the first few seconds of contact between the oxime and inhibited enzyme. For pyridine-2-aldoxime methiodide and ChE inhibited with tetraethylpyrophosphate (TEPP) or *iso*propyl methylphosphonofluoridate (Sarin) this effect is still noticeable even with a concentration of reactivator in the assay vessel of $10^{-5}$ M. The effect can be readily compensated for if the extent of the apparent rapid reactivation is taken as the enzyme activity at zero time of contact between inhibited enzyme and reactivator. However, since this reduces the difference between the measured enzyme activities at zero and infinite time, the percentage error is increased and the calculated rates are less accurate.

## RESULTS

If complex formation is the first step in reactivation then the overall process may be described by the scheme:

$$EI + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EIA \xrightarrow{k_3} E + IA,$$

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

Provided that the rates of formation of the complex and its dissociation into its components are large compared with the rate of breakdown of the complex to give free enzyme, the overall rate of reactivation is given by

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

where $K = [EI] [A]/[EIA] = k_2/k_1$

and $[EI_{total}] = [EI] + [EIA]$.

If the reactivator is in large excess, as is always the case in these experiments, the observed velocity is given by

$$v = k_{observed} [EI_{total}].$$

So that $$k_{observed} = k_3 [A]/K + [A]. \quad (2)$$

In order to test the validity of this equation over a wide range of [A], it is best rearranged as originally proposed by Eadie (1942), namely

$$k_{observed} = k_3 - k'K, \quad (3)$$

where $k'$ is a parameter obtained by dividing $k_{observed}$ by [A].

Fig. 1 shows the relationship between $k_{observed}$ and the apparent second-order-rate constant $k'$ for reactivation by pyridine-2-aldoxime methiodide and pyridine-4-aldoxime methiodide of ChE inhibited with TEPP. The agreement with equation (3) is within the experimental error. The values of $k_3$ and $K$ obtained from the intercept and slope are given in Table 1.

Table 1. *Equilibrium constants K (moles/l.) and breakdown rates $k_3$ (min.$^{-1}$) of the intermediate complexes formed in the reactivation of erythrocyte cholinesterase*

| Reactivator | | ChE inhibited by TEPP | ChE inhibited by Sarin | ChE inhibited by DFP | p$K_a$ | Rate of† reaction with Sarin |
|---|---|---|---|---|---|---|
| Pyridine-2-aldoxime methiodide | $K$ | 0·00014 | — | 0·0008 | 8·0 | 33 |
| | $k_3$ | 0·081 | — | 0·015 | | |
| Pyridine-4-aldoxime methiodide | $K$ | 0·0031 | 0·0008 | — | 8·5 | 47 |
| | $k_3$ | 0·034 | 0·031 | — | | |
| *iso*Nitrosoacetone* | $K$ | 0·02 | 0·01 | — | 8·3 | 28 |
| | $k_3$ | 0·16 | 0·24 | — | | |

* $K$ and $k_3$ taken from Green & Smith (1958).
† Rate constant (l./mole/min.) taken from Green & Saville (1956).

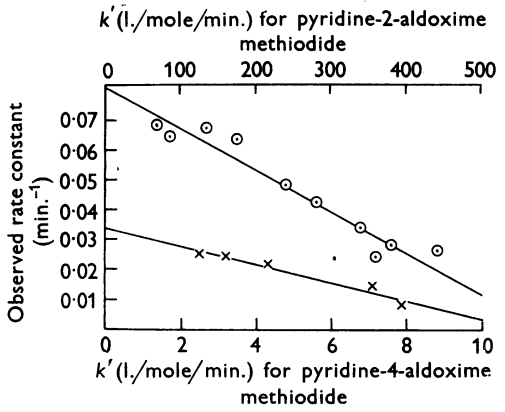



Fig. 1. Plot of the observed first-order-rate constant against the calculated second-order-rate constant ($k'$) for reactivation of erythrocyte ChE inhibited with TEPP, by varied concentrations of pyridine-2-(⊙) and pyridine-4-(×)-aldoxime methiodides.

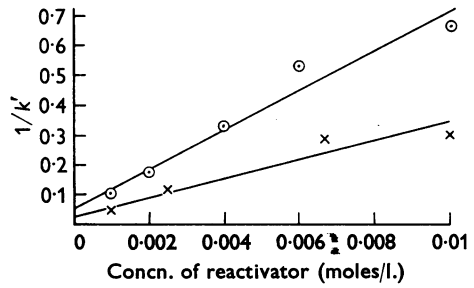



Fig. 2. Reactivation by pyridine-2-aldoxime methiodide (⊙) of erythrocyte ChE inhibited with DFP, and by pyridine-4-aldoxime methiodide (×) of erythrocyte ChE inhibited with Sarin. $k'$ is the calculated second-order-rate constant (l./mole/min.).

With other inhibitors the experimental scatter was rather greater and it was impossible to prove adherence of the rate to equation (1) over a wide concentration range. Nevertheless, for ChE inhibited with either Sarin or di*iso*propyl phosphorofluoridate (DFP) the results were consistent with complex formation as shown below.

Equation (2) may be rearranged into the form

$$1/k' = K/k_3 + [A]/k_3. \quad (4)$$

In Fig. 2, $1/k'$ is plotted against [A] for reactivation by pyridine-2-aldoxime methiodide of ChE inhibited with DFP and by pyridine-4-aldoxime methiodide of ChE inhibited with Sarin. Both plots are consistent with equation (4). The values of $k_3$ and $K$ obtained from the slopes and intercepts are included in Table 1; but, since reactivation in more dilute solution was too slow to enable satisfactory rate constants to be measured, these values of $k_3$ and $K$ must be regarded as tentative.

To confirm the above values for $k_3$ and $K$ some competitive studies were carried out with mixtures of reactivators. For a mixture of two reactivating agents, A and B, both of which form complexes, the observed first-order-rate constant is given by

$$k_{observed} = \frac{k_A K_B [A] + k_B K_A [B]}{K_A K_B + K_B [A] + K_A [B]}, \quad (5)$$

where $k_A$ and $k_B$ are the respective values of $k_3$, and $K_A$ and $K_B$ are those of $K$. Now, if [B] is kept constant and [A] is varied, then, provided that the values of $K_A$, $K_B$, [A] and [B] are such that $K_B$ [A] is negligible compared with $K_A K_B + K_A$[B],

$$k_{observed} = \frac{k_A}{K_A}\left(\frac{K_B}{K_B + [B]}\right)[A] + \frac{k_B [B]}{K_B + [B]}. \quad (6)$$

In Fig. 3, the observed rate of reactivation is plotted against the concentration of *iso*nitrosoacetone (A) for the reactivation by mixtures of *iso*nitrosoacetone and pyridine-4-aldoxime methiodide of ChE inhibited with Sarin. From the slope, intercept and values of $k_A$ and $K_A$ for *iso*nitrosoacetone given in Table 1, $K_B = 0{\cdot}0012$ mole/l. and $k_B = 0{\cdot}037$ min.$^{-1}$, in fair agreement with the previous values.

In order to check equation (5) when $K_B$ [A] is no longer negligible, mixtures of pyridine-2-aldoxime methiodide and *iso*nitrosoacetone were used to reactivate ChE inhibited with TEPP. The observed

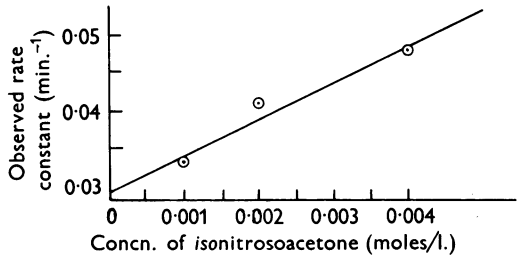



Fig. 3. Reactivation of erythrocyte ChE inhibited with Sarin by pyridine-4-aldoxime methiodide (0·005 M) plus varied concentrations of *iso*nitrosoacetone.

Table 2. *Rate of reactivation of erythrocyte cholinesterase inhibited with tetraethylpyrophosphate by mixtures of pyridine-2-aldoxime methiodide* (0·0001 M) *and varied concentrations of* isonitrosoacetone

Rate constants are in min.$^{-1}$.

| Concn. of *iso*nitrosoacetone (M) | $k_{observed}$ | |
|---|---|---|
| | Experimental | Calc. from equation (5) |
| 0 | 0·0340 | 0·0338 |
| 0·001 | 0·0375 | 0·0372 |
| 0·002 | 0·0447 | 0·0408 |
| 0·004 | 0·0463 | 0·0470 |
| 0·005 | 0·0447 | 0·0498 |
| 0·007 | 0·0554 | 0·0552 |

rates are compared in Table 2 with those calculated from equation (5).

A few experiments have been carried out with other quaternized heterocyclic aldoximes. Pyridine-3-aldoxime methiodide was very much less active than either of its isomers, and at a concentration of 0·01 M would reactivate ChE inhibited with TEPP or with Sarin only slowly. Quinoline-4-aldoxime methiodide at 0·001 M was about as effective as pyridine-4-aldoxime methiodide against ChE inhibited with TEPP, and pyridine-2-aldoxime ethiodide showed activity comparable with that of the corresponding methiodide.

## DISCUSSION

Kinetic data cannot in general be used to prove that a mechanism for a reaction is correct although they can show that a particular mechanism is incorrect. Normally they can be used only as confirmatory evidence for a mechanism which has been postulated on other grounds. The results given above do not show that the complex formation mechanism for reactivation is necessarily unique or correct, but they are consistent with this mechanism. It has also been necessary to assume that $k_3$ is much smaller than $k_1$ or $k_2$. Both the mechanism and the assumption about $k_3$ are reasonable since they are closely analogous to the situation in the more widely studied ChE–substrate action (see Bernhard, 1955). These reservations must be borne in mind in the following discussion.

The many hydroxylamine derivatives which have been found to reactivate inhibited ChE may be divided into two groups: those with positively-charged nitrogen atoms and those without. Wilson (1955) has pointed out a significant difference between these two groups, namely, that the members of the former group are about 20–100 times more effective in reactivating the diethylphosphoryl enzyme (e.g. ChE inhibited by TEPP) than in reactivating the di*iso*propylphosphoryl enzyme (e.g. ChE inhibited by DFP), whereas those in the latter group are only two to nine times more effective. This difference was attributed to 'promotion' of the intrinsic activity of the cationic compounds by interaction with the anionic site in the diethylphosphoryl enzyme.

The nature of this 'promotion' effect may be seen from equation (1). In dilute solution where $[A] \ll K$, (1) becomes

$$v = k_3 [EI] [A]/K. \quad (7)$$

If the strength of the complex is increased by electrostatic attraction between the cationic site in the reactivator and the anionic site in the inhibited enzyme, then the dissociation constant $K$ will decrease, and the rate of reactivation $v$ will correspondingly increase. The importance of this effect will depend on the distance apart of the two charged groups. This will depend on the extent to which the anionic site is sterically shielded by the dialkylphosphoryl group of the inhibitor. This shielding will be greater for the larger di*iso*propylphosphoryl group than for the diethylphosphoryl group, so that $K$ will tend to be smaller for the diethylphosphoryl enzyme than for the di*iso*propylphosphoryl enzyme.

This effect is illustrated by the data for pyridine-2-aldoxime methiodide in Table 1. The intrinsic activity of the compound in detaching the dialkylphosphoryl group from the enzyme is measured by $k_3$, which is about five times as great for the diethylphosphoryl enzyme as for the di*iso*propylphosphoryl enzyme. This ratio is about the same as the ratio of the activities of non-cationic compounds (Wilson, 1955) which, in fact, is roughly the same as the ratio of the reactivities of nucleophilic reagents generally towards diethylphosphoryl and di*iso*propylphosphoryl compounds (Dostrovsky & Halmann, 1953). The 'promotion' effect is measured by the ratio of $1/K$ for the two phosphorylated enzymes. $1/K$ is about six times greater for the diethylphosphoryl enzyme than for the di*iso*propylphosphoryl enzyme, so that in dilute solution pyridine-2-aldoxime methiodide will reactivate the diethylphosphoryl enzyme about 30 times as fast as the di-

*iso*propylphosphoryl enzyme, as found by Wilson for other cationic compounds.

The combination of intrinsic activity and 'promotion' will account for many of the variations in activity observed in hydroxylamine derivatives. Thus nicotinhydroxamic acid methiodide, which shows the 'promotion' effect, is a superior reactivator of the diethylphosphoryl enzyme to nicotinhydroxamic acid (Wilson, Ginsburg & Meislich, 1955), although the latter will react about three times as rapidly with simple organophosphorus compounds at pH 7·4. Pyridine-2-aldoxime methiodide possesses both high reactivity and the ability to form strong complexes and is accordingly very effective as a reactivating agent.

The intrinsic activity of any reactivator will depend both on the ability of the reactivating agent to react directly with organophosphorus compounds and on the orientation and position of the reactivator in the complex. This latter factor may in some instances be critical and may account for the unexpectedly low reactivating power of pyridine-3-aldoxime methiodide.

The way in which the reactivator fits on to the enzyme surface will depend both on the geometry of the reactivator and inhibited enzyme and on the attraction between the cationic centre in the reactivator and the anionic site on the enzyme. Since the values of $k_3$ for reactivation of ChE inhibited with TEPP by pyridine-2- and -4-aldoxime methiodides differ by a factor of only about 2, and since the reactivities of these oximes with organophosphorus compounds are similar (see Table 1), it seems probable that the two oximes are orientated in a similar manner on the enzyme surface. If this is so, the quaternary nitrogen atom in the 4-isomer may be appreciably further from the anionic site on the enzyme surface than is the quaternary nitrogen atom in the 2-isomer, resulting in weaker binding and a much higher value for $K$ as found experimentally.

Pyridine-2-aldoxime methiodide and *iso*nitrosoacetone are about equally reactive with organophosphorus compounds at pH 7·4, and it is of interest that the $k_3$ values also differ only by a factor of about 2, which is consistent with the complexes formed by both compounds being suitably orientated to facilitate reactivation. For ChE inhibited with Sarin, $k_3$ for pyridine-4-aldoxime methiodide is considerably less than that for *iso*nitrosoacetone, which would suggest that the pyridine compound is forced by the strong binding into a less satisfactory orientation for reactivation to occur.

## SUMMARY

1. Kinetic studies of reactivation by pyridine-2- and -4-aldoxime methiodides of erythrocyte cholinesterase inhibited with organophosphorus compounds indicate that a preliminary step is the formation of a complex between the inhibited enzyme and the reactivator.

2. The reactivating powers of the isomeric pyridinealdoxime methiodides are compared in terms of their intrinsic reactivities with organophosphorus inhibitors, the strength of the complexes and the orientation of the reactivator in the complex.

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## REFERENCES

Bernhard, S. A. (1955). *J. Amer. chem. Soc.* **77**, 1973.
Davies, D. R. & Green, A. L. (1955). *Disc. Faraday Soc.* **20**, 269.
Davies, D. R. & Green, A. L. (1956). *Biochem. J.* **63**, 529.
Dostrovsky, I. & Halmann, M. (1953). *J. chem. Soc.* p. 502.
Eadie, G. S. (1942). *J. biol. Chem.* **146**, 85.
Green, A. L. & Saville, B. (1956). *J. chem. Soc.* p. 3887.
Green, A. L. & Smith, H. J. (1958). *Biochem. J.* **68**, 28.
Wilson, I. B. (1955). *Disc. Faraday Soc.* **20**, 119.
Wilson, I. B. & Ginsburg, S. (1955). *Biochim. biophys. Acta*, **18**, 168.
Wilson, I. B., Ginsburg, S. & Meislich, E. K. (1955). *J. Amer. chem. Soc.* **77**, 4286.

# The *N*-Terminal Groups of Calf-Thymus Histones

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It is well established that the histones of calf-thymus nucleoprotein consist of a mixture of proteins, and partial fractionations of them have been achieved (e.g. Stedman & Stedman, 1951; Davison & Butler, 1954; Daly & Mirsky, 1955; Crampton, Moore & Stein, 1955). As a continuation of the work in this Laboratory, the study of the *N*-terminal groups of the mixed histones from calf-thymus glands was undertaken by the fluorodinitrobenzene method (Sanger, 1945). It was expected that the results would be helpful in controlling the fractionation of the histones. While this work was in progress a dissertation on this topic appeared (Haley, 1955) and the work described has since been