Brit. J. Pharmacol. (1955), **10**, 462.

THE REACTIVATION BY OXIMES AND HYDROXAMIC ACIDS OF CHOLINESTERASE INHIBITED BY ORGANO-PHOSPHORUS COMPOUNDS

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(RECEIVED AUGUST 27, 1955)

Many organo-P-compounds, such as dialkylphosphorofluoridates, tetraalkylpyrophosphates and dialkyl *p*-nitrophenylphosphates, are potent inhibitors of cholinesterase (ChE). In contrast to other specific inhibitors such as eserine and neostigmine, their action is non-competitive and not readily reversible. It is now generally believed that these inhibitors phosphorylate some vital group at the active centre of the enzyme (Aldridge, 1954; Wilson, 1954) and that reversal of inhibition requires the rupture of the phosphorus-enzyme bond.

Inhibition by compounds containing a dimethoxyphosphoryl group is reversed moderately quickly by water (Aldridge and Davison, 1953), whereas inhibition by compounds containing a diethoxyphosphoryl group is reversed only slowly by water (Aldridge and Davison, 1953) but more readily by other nucleophilic reagents such as choline and, more particularly, hydroxylamine (Wilson, 1952). It was later shown that hydroxamic acids were considerably more effective than hydroxylamine in reactivating TEPP-inhibited ChE and would also reactivate ChE inhibited by di*iso*propylphosphorofluoridate (dyflos), which is resistant to reactivation by either water or hydroxylamine (Wilson and Meislich, 1953; Wilson and Ginsburg, 1955).

These results suggested that other compounds containing an ionizable NO—H group might be effective reactivating agents. Simple oximes are only weakly acidic, but many *iso*nitroso compounds are known which are much stronger acids and which have now been shown to be very effective reactivating agents for ChE inhibited by TEPP, dyflos and *iso*propyl methylphosphonofluoridate (Sarin). A number of hydroxamic acids have also been studied for comparison, but they are generally less potent than the *iso*nitroso compounds.

METHODS

Red-cell Experiments.—Washed human red cells were suspended in an equal volume of saline and allowed to stand in contact with a dilute solution of the inhibitor (for concentration see Tables) for 10 min. at 25° C. The cells were then twice washed with ice-cold saline to remove excess of the inhibitor and stored at 0° C. until immediately before use. One volume of the cell suspension was then mixed with one volume of the reactivating agent in "barbitone buffer"* (as used later in the enzyme assay procedure) and stored at 25° C. At intervals, 1.0 ml. of this mixture was added to 10.0 ml. acetylcholine (0.01M) in barbitone buffer. ChE determinations were by the electrometric method (Michel, 1949).

Rat Brain Experiments.—Rats were killed by cervical dislocation. The brain was removed immediately and the adhering membranes were dissected off. Each g. of tissue was dispersed in 4 ml. saline (0.85%) by means of a Potter-Elvehjem homogenizer. The homogenate was used within 1 hr. of preparation. The dilute aqueous inhibitor (0.05 ml.—for concentration see Tables) was added to the enzyme dispersion (2.0 ml.) and the mixture was allowed to stand for 2 hr. at 25° C. One volume of homogenate was then mixed with one volume of reactivating agent in barbitone buffer, and after either 1 or 24 hr. an aliquot was withdrawn and assayed for ChE activity by the electrometric method.

With both types of experiment controls were run upon the enzyme alone, upon the enzyme in the presence of the reactivator, and upon the inhibited enzyme.

The percentage reactivation was calculated from the following expression:

$$\frac{\text{Activity of Reactivated Enzyme} - \text{Activity of Inhibited Enzyme}}{\text{Activity of Enzyme Control} - \text{Activity of Inhibited Enzyme}} \times 100$$

In general, at 0.01M, the reactivating agent did not depress the activity of the enzyme control by more than about 10%, so that the maximum possible percentage reactivation was over ninety. If the reactivation be calculated from the enzyme activity in the presence of the reactivator, the numbers given in the Tables would generally be a little higher.

* Barbitone Na, 0.01M; KH_2PO_4, 0.002M; KCl, 0.3M.

Materials.—The oximes and hydroxamic acids used in this investigation were synthesized by two of us (A. F. C. and A. L. G.). They were characterized by melting-point determinations and nitrogen analyses.

RESULTS

Reactivation of TEPP-inhibited ChE

Table I shows the effect of a number of *iso*-nitroso compounds on human red cell ChE inhibited by TEPP. Picolinhydroxamic acid and nicotinhydroxamic acid methiodide—the most effective hydroxamic acids reported by Wilson and Ginsburg (1955)—are included for comparison together with pyrimidine-2-hydroxamic acid, a new compound, which appears to be more active than either of these.

TABLE I

THE REACTIVATION OF ChE INHIBITED BY TEPP

The ChE of washed human red cells was inhibited by contact with 10^{-6}M-TEPP for 10 min. at 25° C. Excess TEPP was removed with ice-cold saline. The inhibited cells were incubated at 25° C. with solutions of reactivating agent in barbitone buffer at *p*H *circa* 7.4. Aliquots were withdrawn at intervals and the ChE activity determined by the Michel electrometric method.

Agent	Concn. (M)	% Reactivation in ¼ hr.	1 hr.	6 hr.
Di*iso*nitrosoacetone (DINA) ..	·01	58	78	—
Mono*iso*nitrosoacetone (MINA) ..	·01	53	97	—
*iso*Nitrosoacetophenone (INAP) ..	·01	49	83	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	11	24	84
Glyoxime	·01	1	8	23
Diacetylmonoxime	·01	—	11	40
Pyrimidine-2-hydroxamic acid ..	·01	50	92	100
Picolinhydroxamic acid	·01	37	83	100
Nicotinhydroxamic acid methiodide	·05	10	33	76

Reactivation of Dyflos-inhibited ChE

A wide range of oximes and hydroxamic acids has been tested against ChE inhibited by dyflos. Table II gives the results of screening tests on red blood cells.

TABLE II

THE REACTIVATION OF ChE INHIBITED BY DYFLOS

Method as in caption to Table I. Inhibitor, 10^{-6}M-dyflos.

Agent	Concn. (M)	% Reactivation in 1 hr.	3 hr.	6 hr.
Phenyl glyoxime	·01	50	64	67
*iso*Nitrosoacetophenone (INAP) ..	·01	45	61	66
Di*iso*nitrosoacetone (DINA) ..	·01	32	58	64
Mono*iso*nitrosoacetone (MINA) ..	·01	15	40	56
p-Methoxy*iso*nitrosoacetophenone	·005	7	15	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	4	4	8
Glyoxime	·01	1	2	3
Diacetylmonoxime	·01	0	0	0
Acetoxime	·01	0	0	0
Picolinhydroxamic acid	·05	44	56	—
Nicotinhydroxamic acid	·01	2	6	11
Nicotinhydroxamic acid methiodide	·01	3	8	15
Salicylhydroxamic acid	·01	2	11	17

A few other compounds were tested in a preliminary way against dyflos-inhibited rat brain ChE. Some of these at concentrations between 0.01M and 0.1M showed about 50% reactivation after 24 hr.: these were salicylaldoxime, benzhydroxamic acid and furohydroxamic acid. The following compounds showed no activity even after contact for 24 hr.: α- and β-furfuraldoximes, *p*-hydroxybenzaldoxime, *p*-aminobenzhydroxamic acid and hippurohydroxamic acid.

Di*iso*nitrosoacetone (DINA) showed similar activity towards both rat brain and red cell cholinesterase inhibited by dyflos.

Reactivation of Sarin-inhibited ChE

Table III shows results for several oximes and hydroxamic acids against sarin-inhibited red blood cell ChE. The *iso*nitroso compounds are generally more effective against ChE inhibited by sarin than against ChE inhibited by TEPP. This is in marked contrast to water and hydroxylamine, which will reactivate ChE inhibited by TEPP but not by sarin, and picolinhydroxamic acid, which is far more effective against ChE inhibited by TEPP than against ChE inhibited by sarin.

TABLE III

THE REACTIVATION OF ChE INHIBITED BY SARIN

Method as in caption to Table I. Inhibitor, 10^{-7}M-sarin.

Agent	Concn. (M)	% Reactivation in ¼ hr.	1 hr.	6 hr.
Di*iso*nitrosoacetone (DINA) ..	·01	94	100	—
Mono*iso*nitrosoacetone (MINA) ..	·01	89	100	—
*iso*Nitrosoacetophenone (INAP) ..	·01	30	65	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	8	33	69
Glyoxime	·01	2	7	23
Nicotinhydroxamic acid methiodide	·05	11	39	54
Picolinhydroxamic acid	·05	5	20	55

Several compounds have been tested against both red blood cell and rat brain ChE inhibited by sarin. As with dyflos the results were similar irrespective of enzyme preparation.

A wide range of compounds were initially examined for ability to reactivate rat brain ChE inhibited with sarin. All the compounds in the four groups below were tested at 0.01M, at 25° C. and at a *p*H of approximately 7.4.

Group 1. Over 50% reactivation in 1 hr.: DINA, *iso*nitrosoacetophenone (INAP), mono*iso*nitrosoacetone (MINA), phenylglyoxime, pyridine-2-aldoxime methiodide, and pyridine-4-aldoxime methiodide.

Group 2. About 50% reactivation in 24 hr.: (*a*) *Oximes.* Di*iso*nitroso*cyclo*hexanone, glyoxime, *iso*nitrosoacetylacetone (INAA), *iso*nitrosoacetylfuran, isatin-β-oxime, pyridine-2-aldoxime, pyridine-4-aldoxime, salicylaldoxime, and tri*iso*nitrosopropane.

(*b*) *Hydroxamic acids.* Nicotinhydroxamic methiodide, picolinhydroxamic acid, and pyrimidine-2-hydroxamic acid.

Group 3. About 20% reactivation in 24 hr.: (*a*) *Oximes.* *o*-Chlorobenzaldoxime, diacetylmonoxime, methylglyoxime, pyridine-3-aldoxime methiodide, and pyrogallaldoxime. (*b*) *Hydroxamic acids.* Furohydroxamic acid and salicylhydroxamic acid.

Group 4. Less than 10% reactivation in 24 hr.: (*a*) *Oximes.* Acetoxime, cyclohexane-1:2-dionedioxime, gallacetophenone oxime, glucose oxime, *o*-hydroxyacetophenone oxime, *m*- and *p*-hydroxybenzaldoximes, *iso*nitrosodimedone, pentane-2:3:4-trione-2:3-dioxime, pyridine-3-aldoxime, and pyrrole-2-aldoxime. (*b*) *Hydroxamic acids.* Benzhydroxamic acid, hippurohydroxamic acid, *iso*nicotinhydroxamic acid, *p*-methylbenzhydroxamic acid, and tropohydroxamic acid.

Discussion

Early work on the mechanism of ChE inhibition by phosphorus anticholinesterases suggested that the process of inhibition was irreversible. That this was not entirely true followed from the fact that ChE inhibited by TEPP did slowly recover some of its activity spontaneously, and later work by Aldridge and Davison (1953) on the spontaneous recovery of ChE after inhibition by diethoxy- and, more particularly, dimethoxyphosphoryl compounds showed that the reversal was a first order chemical reaction with an activation energy corresponding to that of the direct hydrolysis of the P-compounds themselves. These results agreed with the theory of ChE action developed by Wilson (1954) in which the inhibition was considered to occur by direct phosphorylation of some group at the active centre of the enzyme. Reversal occurred by displacement of the enzymic group from the phosphorus atom by another nucleophilic compound. A wide range of nucleophilic reagents was found by Wilson (1951) to be moderately effective in reversing inhibition by TEPP, but ineffective in reversing inhibition by dyflos. The later finding that hydroxamic acids could reverse inhibition by dyflos as well as by TEPP (Wilson and Meislich, 1953; Wilson and Ginsburg, 1955) supported the phosphorylation theory of the inhibitory process. The compounds described in the present paper are generally more effective than the hydroxamic acids in reactivating ChE inhibited by TEPP or dyflos, and markedly more effective in reactivating ChE inhibited by sarin—which, as with dyflos, is not reactivated by either water or hydroxylamine.

There is considerable variation in activity in compounds of a given chemical type against different inhibitors. The *iso*nitroso compounds are most effective against sarin, slightly less effective against TEPP, and least effective against dyflos. On the other hand, whereas picolinhydroxamic acid is nearly as effective as the oximes against TEPP and dyflos, it is only weakly effective against sarin, whilst nicotinhydroxamic acid methiodide shows similar activity to picolinhydroxamic acid against sarin, but is much less potent against either TEPP or dyflos.

When the same group of compounds is considered against different inhibitors the order of effectiveness does not remain the same. Thus from the results in Tables II and III it may be seen that the order of activity with dyflos is INAP> DINA>MINA whereas with sarin it is DINA $\simeq$ MINA>INAP. It is difficult, at present, to see any reason for this. Similarly with the hydroxamic acids, listed for their reactivation of sarin-inhibited rat brain ChE, there is considerable difference between the activity of those in sub-groups 2, 3 and 4, yet chemically and structurally there is apparently no more in common between the members of individual groups than between those of different groups. In the wide range of compounds tested, the only common factor among the most effective members is a pK_a value of around 8. It is probable that the reactivation, being a nucleophilic reaction, occurs through the anion of the oxime, so that, since the tests are carried out at *p*H 7.4, if the pK_a of the oxime is much higher than 7.4 the fraction of oxime ionized will be too small for reactivation to occur at a significant rate, whereas if the pK_a is much lower than 7.4—as with *iso*nitrosodimedone which has a *p*K of 5.7—although all the material will be ionized, the anion will be too weakly nucleophilic to be an efficient reactivator. From the screening results on sarin-inhibited rat brain ChE, and from the variation of activity among the same group of compounds with different inhibitors, it is obvious that, although this factor is concerned, it is not the decisive one. This is particularly so where the hydroxamic acids are concerned. Steric factors are, no doubt, also important and may account for the significantly lower activity of INAA compared with MINA and INAP, but it is difficult to see a reason why picolin- and pyrimidine-2-hydroxamic acids are more active than nicotin-, *iso*nicotin-, and benzhydroxamic acids, since sterically the compounds are similar—except for the presence of the α-nitrogen atom in the two active compounds—and the dissociation constants of all five compounds are of the same order.

SUMMARY

1. Compounds of a new series—the oximes—have been shown to be potent reactivators of both red cell and rat brain ChE inhibited by TEPP, sarin or dyflos.

2. These compounds are generally superior to the hydroxamic acids, particularly against sarin-inhibited ChE.

3. Factors influencing the relative activity of different compounds against different inhibitors have been discussed.

We wish to thank Miss June Petts for assistance with the biochemical work and Messrs. G. L. Sainsbury and C. Stratford for help with the preparation of many of the compounds examined.

REFERENCES

Aldridge, W. N. (1954). *Chem. & Ind.*, 473.
—— and Davison, A. N. (1953). *Biochem. J.*, **55**, 763.
Michel, H. O. (1949). *J. Lab. clin. Med.*, **34**, 1564.
Wilson, I. B. (1951). *J. biol. Chem.*, **190**, 110.
—— (1952). Ibid., **199**, 113.
—— (1954). *Mechanism of Enzyme Action*, ed. McElroy and Glass, p. 642. Baltimore: Johns Hopkins Press.
—— and Ginsburg, S. (1955). *Arch. Biochem. Biophys.*, **54**, 569.
—— and Meislich, E. K. (1953). *J. Amer. chem. Soc.*, **75**, 4628.