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Reactivation of Phosphorylated Acetocholinesterases by Pyridinium Aldoximes and Related Compounds

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Certain organophosphates, e.g. tetraethyl pyrophosphate, di*iso*propyl phosphorofluoridate and insecticides of the organophosphate type, inhibit acetocholinesterase by a phosphorylation process. The phosphorylated enzyme is devoid of esterase activity, but this can be restored by substances which displace the phosphoryl group from the enzyme. The best known reactivators are oximes and the most potent oxime is 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine 2-aldoxime methiodide).

Because of the need for an antidote in organophosphate poisoning which is more effective and of greater water-solubility than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (Hobbiger & Sadler, 1959) we have investigated a series of pyridinium aldoximes and related compounds. In each case the reaction with tetraethyl pyrophosphate, the reactivation of diethyl phosphorylacetocholinesterase and the affinity for acetocholinesterase were investigated. In some instances the reaction with di*iso*propyl phosphorofluoridate and the reactivation of di*iso*propyl phosphorylacetocholinesterase were also studied.

It was found that some of the pyridinium aldoximes investigated are much more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as reactivators of both diethyl and di*iso*propyl phosphorylacetocholinesterase and that their potency as reactivators cannot be predicted solely from their reactivity with organophosphates or from their affinity for acetocholinesterase, as measured by the anticholinesterase action.

EXPERIMENTAL

Methods

All experiments were carried out in the Warburg apparatus at 37° in a medium of 0·025 M-$NaHCO_3$ and an atmosphere of N_2+CO_2 (95:5), pH 7·45. All vessels were gassed while being shaken with the mixture of N_2+CO_2 for 5 min. after their transfer to the water bath. All incubation periods mentioned in the text include this period.

Reactivity of oximes with organophosphates. The reaction between oximes and organophosphates leads to the release of acids; this reaction was followed by measuring the displacement of CO_2 from the $NaHCO_3$ medium and its rate constant k, expressed as $l.mole^{-1}min.^{-1}$, was calculated from the formula

$$k = \frac{2{\cdot}303}{[\text{Reactivator}]\,(t_2-t_1)} \log \frac{a}{a-b}.$$

2 mM-Tetraethyl pyrophosphate or di*iso*propyl phosphorofluoridate was used in these experiments; a represents μl. of CO_2 obtained when 2 moles of acid/mole of organophosphate are released. Ten minutes were allowed for equilibration in the water bath and t_1 is the time of addition of the organophosphate from the side arm to the oxime in the main compartment. Only measurements of initial rates were taken and t_2-t_1 are 10 and 20 min. in experiments with tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate respectively; b is μl. of CO_2 produced during this period. Whenever possible concentrations of each oxime were used which on interaction with the organophosphate produced a CO_2 output differing by not more than 10% from that obtained with one of two standard concentrations of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. In all experiments spontaneous hydrolysis of the organophosphate contributed at the most 20% to the observed hydrolysis and no correction was made for this when calculating rate constants.

Reactivation of phosphorylated acetocholinesterases. Washed human erythrocytes or purified bovine-erythrocyte cholinesterase (Winthrop-Stearns Inc.) were used as enzyme source and acetocholinesterase activity was determined by the rate of hydrolysis of 0·01 M-acetylcholine chloride. Final concentrations for the assay of activity of the two enzyme preparations, which are referred to below as human and bovine acetocholinesterase, were 1·7 ml. of washed human erythrocytes/100 ml. and 40 μg. of purified enzyme/ml. respectively in a volume of 3 ml. per Warburg vessel.

Preparations of human diethyl and di*iso*propyl phosphorylacetocholinesterase were obtained by a technique described previously (Hobbiger, 1957). The corresponding phosphorylated bovine acetocholinesterases were prepared in the same way, except that after incubation of the enzyme with the organophosphate and removal of excess of the latter by incubation with rabbit plasma a tenfold dilution was substituted for the procedure of multiple washings.

To assess their relative potencies as reactivators the oximes were incubated with the phosphorylated enzyme in the main compartment of the manometric vessels for 30 min. before addition of substrate; the degree of reactivation obtained was calculated from the CO_2 production during 5–35 min. after the addition of substrate from the expression: Percentage reactivation $= 100\,(A-B)/(C-B)$, where A is the activity of reactivated enzyme, B the activity of phosphorylated enzyme and C the activity obtained by maximum reactivation of phosphorylated enzyme. Under these conditions $(A-B)$ is the sum of (i) the reactivation which occurs before addition of substrate, (ii) the reactivation which takes place from the time of addition of substrate until 35 min. later. Since the latter was found to have a first-order dependence on the concentration of phosphorylated enzyme the calculated percentage reactivation represents the reactivation which was obtained at 20 min. after the addition of substrate. For this reason certain lines in Figs. 1 and 2 do not pass through the origin.

Relative potencies were calculated from the concentrations of reactivators which gave similar percentage reactivations. For studies of the time course of reactivation the same procedure as that employed for assessment of the relative potencies of reactivators was used except that the period of incubation without substrate was varied.

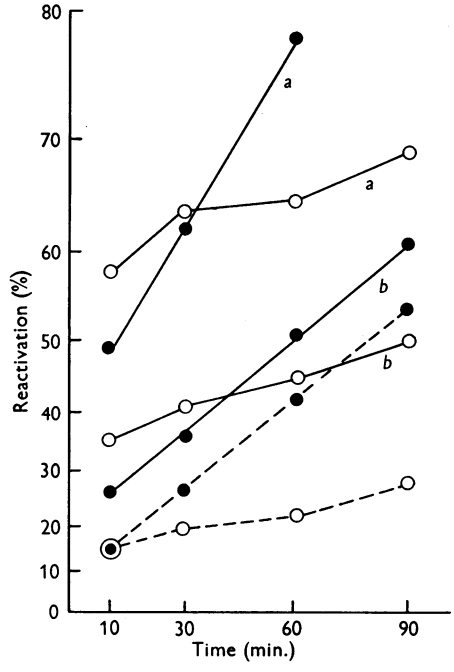


Fig. 1. Reactivation of human diethyl phosphorylacetocholinesterase obtained when 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●—●; *a* 0·012 mM, *b* 0·006 mM) or *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○—○; *a* 0·0005 mM, *b* 0·00025 mM) was incubated with the phosphorylated enzyme in the main compartment of the manometric vessel for various periods (abscissa) before addition of substrate. Under these conditions reactivation takes place during measurement of enzyme activity for assessment of reactivation. Broken lines represent reactivation when 0·006 mM-2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●- - -●) and 0·00025 mM-*NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○- - -○) were incubated for various periods (abscissa) in the side arm of the manometric vessels before transfer to the substrate in the main compartment. Only negligible reactivation takes place during measurement of enzyme activity.

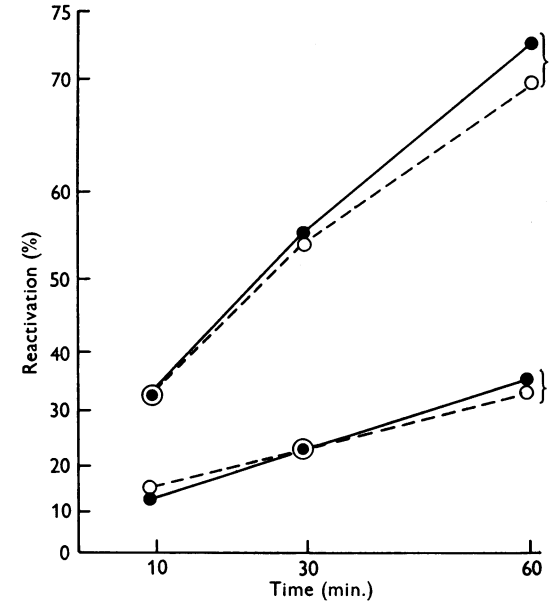


Fig. 2. Reactivation of bovine diethyl phosphorylacetocholinesterase obtained when 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●; *a* 0·006 mM; *b* 0·018 mM) or *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○; *a* 0·0002 mM; *b* 0·0006 mM) were incubated for various periods (abscissa) with the phosphorylated enzyme in the main compartment of the manometric vessel before addition of substrate from the side arm.

Anticholinesterase activity. Fresh human acetocholinesterase and oximes were incubated in the main compartment for 20 min. and I_{50} values were calculated from enzyme activities recorded between 5 and 35 min. after addition of substrate from the side arm.

Relative potencies. The highest concentration of oximes used in any experiment was 0·01 M and all results were expressed relative to those obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (activity of 1). The lowest relative potencies which could be recorded with accuracy were 0·05 for anticholinesterase activity and reactivity with organophosphates and 0·0003 and 0·005 for reactivation of human diethyl and di*iso*propyl phosphorylacetocholinesterase respectively.

The pK_a values listed are the pH values at the half-titration point of 0·03 m-moles in 5 ml. of water at room temperature. A continuous titrator (titrigraph), model SBR 2a, was used for all titrations.

Reactivators

Oximes and related substances were all synthesized in this Laboratory. The method of synthesis, analytical data and solubilities in chloroform and water of oximes listed in Table 1 have been reported in a previous publication (Hobbiger & Sadler, 1959). Oximes listed in Table 3 and 2-hydroxyiminomethyl-*N*-*iso*propylpyridinium iodide were synthesized by the following methods.

β-Pyridinium acetoxime chloride. Excess of pyridine was added to a solution of β-chloroacetoxime in ethanol, and allowed to stand for several hours. The excess of pyridine and ethanol was removed by distillation *in vacuo*. The residue was triturated with acetone and then crystallized from ethanol–acetone, giving white plates, m.p. 193°. (Found: Cl, 20·5. Calc.: Cl, 20·6%).

Betaine aldoxime chloride. This was prepared similarly rom trimethylamine and β-chloroacetoxime in ethanol. White plates, m.p. 81°, were obtained with 1 mol. of water of crystallization from aqueous acetone (Found: C, 35·4; H, 8·2; N, 17·1; Cl, 21·2. Calc.: C, 35·3; H, 8·8; N, 16·5; Cl, 20·9%).

2-*Hydroxyiminomethylpyridinium acetoxime chloride.* This was prepared as described above from 2-hydroxyiminomethylpyridine and β-chloroacetoxime in ethanol. Crystallization from ethanol–acetone afforded cream needles, m.p. 196–197° (Found: Cl, 16·6. Calc.: Cl, 16·5%).

4-*Hydroxyiminomethyl*-N-*methylquinolinium methiodide.* This was obtained as orange needles from ethanol, m.p. 242° (Ginsburg & Wilson, 1957).

4-*Hydroxyiminomethyl*-6-*methoxy*-N-*methylquinolinium iodide.* This was prepared as described above from 4-formyl-6-methoxyquinoline; orange needles from methanol, m.p. 225°, were obtained (Found: I, 36·8. Calc.: I, 36·9%).

2-*Hydroxyiminomethyl*-N-iso*propylpyridinium iodide.* Equimolar quantities of 2-hydroxyiminomethylpyridine and *iso*propyl iodide in ethanol were heated under reflux for 8 hr. The solid obtained on the addition of ether to the cooled reaction mixture gave lemon-yellow needles, m.p. 208° after crystallization from methanol (Found: I, 43·7. Calc.: I, 43·5%).

2-*Hydroxyiminomethyl*-3:3-*dimethyl*-N-*methylindoleninium iodide.* Reaction of nitrous acid with 2:3:3-trimethylindolenine (obtained by a Fischer indole synthesis) gave 2-hydroxyiminomethyl-3:3-dimethylindolenine m.p. 156° (Found: C, 70·0; H, 6·4. Calc. C, 70·2; H, 6·4%). The quaternary compound was prepared by heating the product with excess of methyl iodide in nitrobenzene. The product which separated on cooling afforded deep-yellow plates from methanol, m.p. 192° (Found: C, 43·7; H, 4·8; N, 8·5; I, 38·2. Calc.: C, 43·6; H, 4·5; N, 8·5; I, 38·5%).

RESULTS

Hydrolysis of organophosphates

Hydrolysis of tetraethyl pyrophosphate. The hydrolysis of tetraethyl pyrophosphate by 19 pyridinium aldoximes was investigated at pH 7·45 and 37° (see Methods section) and the results obtained are summarized in Table 1. As can be seen the reactivity of hydroxyiminomethyl-*N*-alkylpyridinium iodides with tetraethyl pyrophosphate was greatly affected by the position of the oxime group relative to the ring nitrogen, but was independent of the size of the *N*-alkyl group. This also applied to derivatives with a terminal bromo substituent in the *N*-alkyl group.

4-Monoximes of *NN'*-polymethylenebis(pyridinium bromide) were 1·5–1·8 times as reactive as their parent compound 4-hydroxyiminomethyl-*N*-methylpyridinium iodide and 4:4'-dioximes had approximately twice the reactivity of their corresponding monoximes. In the dioximes with a polymethylene chain of 1–3 carbon atoms an inverse relationship existed between reactivity and length of the *NN'*-polymethylene group.

The amount of the oxime determined the extent to which tetraethyl pyrophosphate was hydrolysed if the latter was used in excess. This indicates that the oximes undergo a chemical change during hydrolysis. In agreement with this is the finding that mixtures of tetraethyl pyrophosphate and oximes when kept at 37° in 0·1 M-phosphate buffer, pH 7·4, gave a positive cyanide reaction, the intensity of which increased during incubation. Control solutions containing only tetraethyl pyrophosphate or oximes failed to give such a positive cyanide reaction.

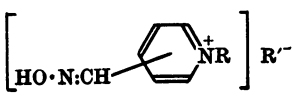
*Hydrolysis of di*iso*propyl phosphorofluoridate.* Differences in reactivity between the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides were less marked when di*iso*propyl phosphorofluoridate was used instead of tetraethyl pyrophosphate (compare Tables 1 and 2). On the other hand, the reactivities of 4-monoximes and 4:4'-dioximes of *NN'*-polymethylenebis(pyridinium bromide) relative to 4-hydroxyiminomethyl-*N*-methylpyridinium iodide were very similar for both organophosphates.

Reactivation of freshly phosphorylated acetocholinesterases

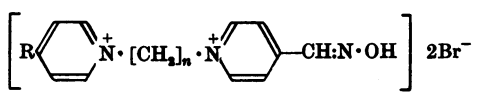
Human diethyl phosphorylacetocholinesterase. Amongst the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides the 2-isomer was the

Table 1. *Biochemical properties of pyridinium and* bis*pyridinium aldoximes*

Potencies of individual oximes are expressed relative to that of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (potency of 1) and each figure was derived from the mean of several experiments, each of which was a parallel assay of the activities of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and several other oximes. 2-Hydroxyiminomethyl-*N*-methylpyridinium iodide had under the experimental conditions described in the Methods section the following potencies: (1) Reactivity: k 29·3 and 26·6 l. mole^{-1} min.$^{-1}$ for the hydrolysis of 2 mM-tetraethyl pyrophosphate by 1 and 2 mM-oxime respectively. (2) 50% reactivation of human and bovine diethyl phosphorylacetocholinesterases were obtained when the phosphorylated enzymes were incubated with 0·012 mM-oxime for 20 and 58 min. respectively before addition of substrate. (3) The oxime concentration required for 50% inhibition of acetylcholine hydrolysis was 4·2 mM.



Position of hydroxyiminomethyl group	R	R′	pK_a	Reactivity with tetraethyl pyrophosphate	Potency as reactivator of diethyl phosphorylacetocholinesterase: Human	Potency as reactivator of diethyl phosphorylacetocholinesterase: Bovine	Potency as inhibitor of acetylcholine hydrolysis by human acetocholinesterase
2	CH_3	I	8·1	1	1	1	1
3	CH_3	I	9·2	0·09	0·0003	—	1·2
4	CH_3	I	8·3	0·36	0·06	—	0·75
2	$CH_2·CH_3$	I	8·2	1	0·54	—	2·1
2	$[CH_2]_2·CH_3$	I	8·3	0·94	0·63	—	5·6
2	$[CH_2]_3·CH_3$	I	8·3	1·10	0·87	—	64
2	$CH(CH_3)_2$	I	8·0	0·95	0·46	—	1·6
4	$CH_2·CH_3$	I	8·2	0·34	0·03	—	0·53
4	$[CH_2]_2·CH_2·Br$	Br	7·9	0·44	1·9	—	17
4	$[CH_2]_3·CH_2·Br$	Br	8·2	0·36	0·25	—	22
4	$[CH_2]_4·CH_2·Br$	Br	8·2	0·27	0·94	—	6



R	n					
H	3	8·0	0·56	8	5	0·75
H	4	8·0	0·53	6·7	4·2	0·40
H	5	8·2	0·47	7·5	4·4	2·7
CH:N·OH	1	7·4	2·60	2·8	—	<0·1
CH:N·OH	2	8·0	1·50	17	20	0·78
CH:N·OH	3	8·0	1·10	22	30	0·90
CH:N·OH	4	7·9	0·99	18	16	1·0
CH:N·OH	5	8·2	0·91	16	13	8·1

most potent reactivator and the 4- and 3-isomers had only 6·0 and 0·03 % respectively of the activity of the former. Enlargement of the *N*-alkyl group caused a slight loss of activity that was most marked with the ethyl and *iso*propyl derivatives, which had approximately 50 % of the activity of the corresponding methyl derivatives.

The introduction of a terminal bromine atom into the *N*-alkyl group markedly enhanced the activity of 4-hydroxyiminomethyl-*N*-alkylpyridinium halides. The most potent reactivators were the 4-monoximes and 4:4′-dioximes of *NN′*-polymethylenebis(pyridinium bromide), which were 2·8–22 times as potent as reactivators as was 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. In the largest homologous series available, namely the 4:4′-dioximes of *NN′*-polymethylenebis(pyridinium bromide), a marked increase in potency resulted from lengthening the *NN′*-polymethylene chain from 1 to 3 carbon atoms but activity then fell gradually when the polymethylene chain was lengthened still further.

These results are summarized in Table 1.

Factors influencing reactivation. The rate of reactivation of human diethyl phosphorylacetocholinesterase by 2-hydroxyiminomethyl-*N*-methylpyridinium iodide in the presence or absence of 0·01 mM-acetylcholine is a function of the oxime concentration, and with a fixed concentration of the oxime shows a first-order dependence on the concentration of phosphorylated enzyme (Hobbiger 1956). This also applied to reactivation by 4-hydroxyimino-*N*-methylpyridinium iodide. When oximes of *NN′*-polymethylenebis(pyridinium bromide) were used as reactivators, however, the observed time course of reactivation was decremental with respect to that obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. The decrement was more marked with the 4:4′-dioximes than with the 4-monoximes, unaffected by 0·1–1·0 mM-disodium ethylenediaminetetra-acetate, and of a similar order when different oximes of a given group were compared. The time courses of reactivation obtained with concentrations of *NN′*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide, which produced similar degrees of reactivation when the oximes were added to the phosphorylated enzyme 30 min. before acetylcholine, are illustrated in Fig. 1.

When substrate was added simultaneously with oximes of *NN′*-polymethylenebis(pyridinium bromide) to the phosphorylated enzyme instead of at a later stage (as was the case when the time course of reactivation was studied) the rate of reactivation showed a first-order dependence on the concentration of phosphorylated enzyme. This was also true for the reactivation which occurred after addition of substrate to a sample containing phosphorylated enzyme exposed previously for some time to any of the oximes.

Thus the cause of the observed decremental time course of reactivation by oximes of *NN′*-polymethylenebis(pyridinium bromide) lies in an interference with reactivation which occurs during the period while the phosphorylated enzyme is incubated with the oxime in the absence of substrate and which is prevented by substrate. Since it is known that acetylcholine protects acetocholinesterase against phosphorylation (Burgen, 1949) and free organophosphates can interfere with reactivation (Hobbiger, 1955, 1956), it was necessary to show that the observed interference was not due to free tetraethyl pyrophosphate.

For this purpose preparations of human diethyl phosphorylacetocholinesterase were incubated for 60 min. with fresh human acetocholinesterase as control enzyme. Although the former was used in a concentration which was 10 times as high as that used in reactivation studies no significant inhibition of the control enzyme could be demonstrated. Similarly a tenfold increase in the concentration of tetraethyl pyrophosphate during preparation of the phosphorylated enzyme did not affect results significantly. Thus it seems unlikely that residual free tetraethyl pyrophosphate could to any significant extent have been responsible for the anomalous time course of reactivation obtained with oximes of *NN′*-polymethylenebis(pyridinium bromide) in the absence of substrate.

Differences between the time courses of reactivation obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and oximes of *NN′*-polymethylenebis(pyridinium bromide) were pH-dependent and became negligible when reactivation was carried out at pH 8·45 instead of at pH 7·45.

The relative potencies of oximes of *NN′*-polymethylenebis(pyridinium bromide) calculated from results obtained in experiments in which oximes and substrate were added simultaneously to the phosphorylated enzyme were 1·2–1·5 times as great as those obtained when the substrate was added to the phosphorylated enzyme 30 min. after the oxime.

Bovine diethyl phosphorylacetocholinesterase. Oximes of *NN′*-polymethylenebis(pyridinium bromide) are more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as reactivators not only of diethyl phosphorylacetocholinesterase of lysed human erythrocytes but also of the diethyl phosphoryl derivative of purified bovine-erythrocyte cholinesterase. The similarity between results obtained under identical experimental conditions with the two types of diethyl phosphorylacetocholinesterase can be seen from the results presented in Table 1.

Differences between time courses of reactivation of bovine diethyl phosphorylacetocholinesterase obtained with oximes of *NN'*-polymethylenebis-(pyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were, on the whole, quite small (see Fig. 2).

Human diisopropyl phosphorylacetocholinesterase. Oximes of *NN'*-polymethylenebis(pyridinium bromide) were also potent reactivators of human di*iso*propyl phosphorylacetocholinesterase when incubated with the phosphorylated enzyme for 30 min. before addition of substrate (Table 2). *NN'*-Trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) was the most potent reactivator, being 52 times as effective as 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. Differences in potencies between the oximes of *NN'*-polymethylenebis-(pyridinium bromide) were in line with those encountered in experiments with human diethyl phosphorylacetocholinesterase. Differences between the time courses of reactivation obtained with oximes of *NN'*-polymethylenebis(pyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were small and of a similar order to those observed in experiments with bovine diethyl phosphorylacetocholinesterase.

Reactivation of 'aged' phosphorylated acetocholinesterase

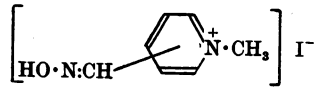
The maximum degree of reactivation of phosphorylated human cholinesterases obtainable with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide or related compounds is inversely related to the 'age' of the phosphorylated enzyme (Hobbiger, 1955, 1956; Davies & Green, 1956). It was found that this was also the case when oximes of *NN'*-polymethylenebis(pyridinium bromide) were used as reactivators of human di*iso*propyl phosphorylacetocholinesterase. Similar results were obtained by Michel (1958), who studied the effect of 'age' on reactivation of methyl*iso*propyl phosphorylacetocholinesterase by *NN'*-tetramethylenebis-(4-hydroxyiminomethylpyridinium bromide).

Inhibition of human acetocholinesterase by oximes

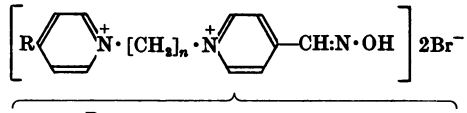
The anticholinesterase action of oximes was assessed by their effect on the rate of hydrolysis of acetylcholine by human acetocholinesterase. Differences in inhibitory potency between the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides were small, and Table 1 shows that the anticholinesterase activities of 2-hydroxyiminomethyl-

Table 2. *Biochemical properties of pyridinium and* bis*pyridinium aldoximes*

All results are expressed relative to those obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (see legend of Table 1). 2-Hydroxyiminomethyl-*N*-methylpyridinium iodide had under the experimental conditions described in the Methods section the following potencies: (1) reactivity with di*iso*propyl phosphorofluoridate: k 3·56 l. mole^{-1} min.$^{-1}$ for the hydrolysis of 2 mM-di*iso*propyl phosphorofluoridate by 2 mM-oxime. (2) 24 min. incubation of human di*iso*propyl phosphorylacetocholinesterase with 0·15 mM solutions of the oxime before addition of substrate was required to give 50% reactivation.



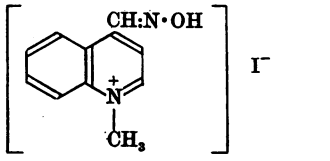
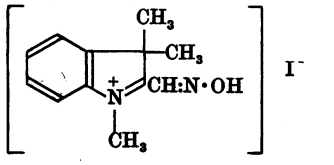
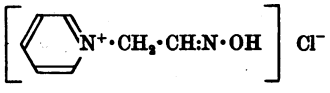
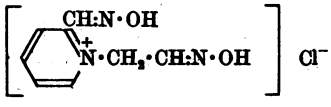
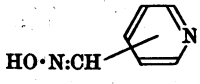
		Reactivity with di*iso*propyl phosphorofluoridate	Potency as reactivator of human di*iso*propyl phosphorylacetocholinesterase
position of oxime group	2	1	1
	3	0·2	—
	4	0·65	—



R	n		
H	3	1	11
H	4	0·94	10
H	5	0·92	11
CH:N·OH	1	3·5	—
CH:N·OH	2	3·1	22
CH:N·OH	3	2	52
CH:N·OH	4	1·9	38
CH:N·OH	5	1·8	36

Table 3. *Biochemical properties of related oximes*

Potencies are expressed relative to those of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (potency of 1). For further details see legend to Table 1.

	pK_a	Reactivity with tetraethyl pyrophosphate	Potency as reactivator of human diethyl phosphorylacetocholinesterase	Potency as inhibitor of acetylcholine hydrolysis by human acetocholinesterase
	8·2	0·78	0·035	10
its 6-methoxy derivative	8·4	0·37	0·008	14
	5·8	0·63	0·004	<0·1
$[(CH_3)_3\overset{+}{N}\cdot CH_2\cdot CH{:}N\cdot OH]\ Cl^-$	9·0	0·05	0·0005	<0·1
	—	0·04	<0·0004	<0·1
	7·9	0·83	0·2	2·8
	—	<0·02	<0·0004	<0·1

N-alkylpyridinium iodides, ω-bromo derivatives of 4-hydroxyiminomethyl-*N*-alkylpyridinium bromides and oximes of *NN'*-polymethylenebis(pyridinium bromide) were, on a whole, directly related to the size of the *N*-alkyl group and the length of the *NN'*-polymethylene chain. 4-Hydroxyiminomethyl-*N*-ethylpyridinium iodide, the ω-bromo derivative of 4-hydroxyiminomethyl-*N*-amylpyridinium bromide and *NN'*-tetramethylene-1-(4-hydroxyiminomethylpyridinium)-4-pyridinium dibromide were the only exceptions.

Experiments with reactivators closely related to pyridinium aldoximes

Additional experiments in which the effects of reactivators, listed in Table 3, on tetraethyl pyrophosphate, human diethyl phosphorylacetocholinesterase and human acetocholinesterase were investigated gave the following results.

4-Hydroxyiminomethyl-*N*-methylquinolinium iodide, its 6-methoxy derivative and 2-hydroxyiminomethyl-3:3-dimethyl-*N*-methylindoleninium iodide had 78, 37 and 63% respectively of the reactivity of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. The first two oximes were much more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as inhibitors of acetocholinesterase, but their potencies as reactivators relative to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were only 3·5 and 0·8% respectively. The indoleninium derivative also was only a very weak reactivator.

β-Pyridinium acetoxime chloride and betaine aldoxime chloride, which, like 3-hydroxyiminomethyl-*N*-methylpyridinium iodide, lack a fully resonating structure but have (as far as complementariness for the phosphorylated enzyme is concerned) the same structural configuration as 2-hydroxyiminomethyl-*N*-methylpyridinium iodide, had very low reactivities and their potencies as reactivators were too small to be recorded with accuracy. The same seems to be true for β-dimethylanilinium acetoxime chloride, although it was not possible to get accurate results

with this compound because of its ready decomposition under the experimental conditions.

Replacement of the *N*-methyl group of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide by $N\text{-}CH_2\cdot CH{:}N\cdot OH$ yielded an oxime with lower reactivity and lower potency as reactivator than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide.

Only trace activities were observed with the three isomers of hydroxyiminomethylpyridine when they were used in 0·01 M concentrations.

These results are summarized in Table 3.

2-Hydroxyiminomethylthiophen, gramine methiodide, Girard's P and T reagents (Girard & Sandulesco, 1936), *p*-nitrosodimethylaniline and *p*-hydroxyiminomethyltrimethylanilinium bromide were devoid of action on both tetraethyl pyrophosphate and human diethyl phosphorylacetocholinesterase when used in 0·01 M concentrations. Similarly ineffective were saturated solutions of the following compounds: 2- and 4-hydroxyiminomethylquinoline and the 6-methoxy derivative of the latter, 4-carboxyl-2-methylquinoline *N*-oxide, 2-methylcinchoninic acid hydrazide and its 6-fluoro derivative, 2-hydroxyiminomethyl-3:3-dimethylindolenine, 3-hydroxyiminomethylindole, 4:4′-di*iso*nitrosoacetaminodiphenyl sulphone, 2:6-dihydroxyiminomethylpyridine, 1-methyl-3-hydroxyiminomethyloxindole and a large series of halogen-, alkyl- and alkoxy-substituted isatin β-oximes and their corresponding *iso*nitrosoacetanilides.

DISCUSSION

Results presented in this paper show that the activity of diethyl and di*iso*propyl phosphorylacetocholinesterase can be restored by a variety of pyridinium aldoximes and bispyridinium aldoximes. Wilson, Ginsburg & Quan (1958), who carried out an extensive study of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and of related compounds, came to the conclusion that the exceptional activity of the former as a reactivator of diethyl phosphorylacetocholinesterase is a consequence of its very high degree of complementariness with phosphorylated acetocholinesterase. They envisage that bonding of the quaternary ammonium group of this oxime to the anionic site of the acetocholinesterase enhances the activity of the oxime as a reactivator by facilitating alignment between the oxime and phosphoryl groups. Furthermore, they believe that the same type of complementariness is responsible for the high activity of 4-hydroxyiminomethyl-*N*-methylpyridinium iodide, whereas the 3-isomer, which is stated to have less than a millionth of the activity of the 2-isomer, is inactive because it lacks complementariness. According to our results the 3-isomer still has 0·5 % of the activity of the 2-isomer as a reactivator if allowance is made for differences in reactivity of the two oximes with organophosphates.

To assess the extent to which the activities of isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides as reactivators also reflected mesomeric properties, we investigated betaine aldoxime chloride and β-pyridinium acetoxime chloride, which structurally resemble 2-hydroxyiminomethyl *N*-methylpyridinium iodide but lack a fully conjugated structure. Both these oximes have reactivities with organophosphates similar to that of 3-hydroxyiminomethyl-*N*-methylpyridinium iodide and share its low activity as a reactivator. Unfortunately their affinity for acetocholinesterase, as measured by the anticholinesterase activity, is less than 10 % of that of 3-hydroxyiminomethyl-*N*-methylpyridinium iodide. Thus no definite conclusion about the contribution towards reactivation by mesomeric and inductive effects can be drawn from our experiments. However, all known potent reactivators possess a conjugated structure.

The effectiveness of 2- and 4-hydroxyiminomethyl-*N*-alkylpyridinium iodides as reactivators can be explained satisfactorily by their complementariness, as proposed by Wilson *et al.* (1958). Although the affinity of these oximes for acetocholinesterase increases with increasing length of *N*-alkyl group, spatial limitations at the anionic site of the phosphorylated enzyme (Wilson, 1955) would tend to reduce their affinity for the anionic site of the phosphorylated enzyme and this could account for the superiority of the methyl derivatives as reactivators. The low activity of 4-hydroxyiminomethyl-*N*-methylquinolinium iodide, its 6-methoxy derivative and 2-hydroxyiminomethyl-3:3-dimethyl-*N*-methylindoleninium iodide as reactivators requires the postulate that spatial limitations at the anionic site are considerable. In support of this interpretation is the low affinity of the indoleninium derivative for acetocholinesterase.

The ω-bromo derivatives of 4-hydroxyiminomethyl-*N*-alkylpyridinium bromides are 4–25 times as effective as reactivators as their parent compound 4-hydroxyiminomethyl-*N*-methylpyridinium iodide, as judged by the ratio (potency as reactivator:reactivity with organophosphates). If we assume that the quaternary-ammonium group of these oximes is bonded to the anionic site of the phosphorylated enzyme, their superiority to 4-hydroxyiminomethyl-*N*-methylpyridinium iodide could be accomplished only by a bonding of the terminal ω-bromo group to a second site on the enzyme surface. Such additional bonding would then be able to exert a directive influence for the orientation of the oxime. In view of the spatial restrictions of the anionic site outlined above the

second bonding site could not be part of the anionic site but would have to be adjacent to it.

The oximes of *NN'*-polymethylenebis(pyridinium bromide) are, according to the results summarized in Tables 1 and 2, at least 6·5–120 and 1·1–26 times as effective as reactivators as are 4-hydroxyiminomethyl-*N*-methylpyridinium iodide and its 2-isomer respectively, if allowance is made for differences in reactivity with organophosphates. Corresponding pairs of monoximes and dioximes have ratios (potency as reactivator:reactivity with organophosphates) which differ by a factor of approximately 2. Again there is no correlation between anticholinesterase activity and activity as a reactivator. To explain the high activity of oximes of *NN'*-polymethylenebis(pyridinium bromide) as reactivators we should have to postulate bonding of the quaternary-ammonium groups to two sites of the enzyme surface, and the finding that the trimethylene derivative has the highest activity then indicates that these two points of attachment are separated by a distance not exceeding 5 Å. The finding of Berry, Davies & Green (1959) that *NN'*-trimethylenebis(2-hydroxyiminomethylpyridinium bromide) is a less potent reactivator than its hexamethylene-derivative does not contradict this, because of the steric differences between these oximes and those which we have investigated.

Our findings are also in agreement with the interpretation that both oxime groups are functional during reactivation, and the possibility must therefore be considered that pyridinium aldoximes with a single quaternary-ammonium group might be bonded to a site which is not identical with the anionic site but which represents the second bonding site discussed above. Nothing is known about the nature of such a second site for attachment of quaternary-ammonium groups of pyridinium aldoximes. However, strong bonding between imidazoles and quaternary-pyridinium derivatives has been demonstrated by van Eys (1958). Receptors for acetylcholine, which must share at least certain features with the active surface of acetocholinesterase, are most readily stimulated by alkylammonium derivatives which have a small cationic head and a second nucleophilic group, e.g. a sulphur atom, between the third and fourth carbon atom of the appendix structure (Lands & Cavalitto, 1954).

Reactivation of human diethyl phosphorylacetocholinesterase (of lysed human erythrocytes) by oximes of *NN'*-polymethylenebis(pyridinium bromide) in the absence of substrate deviates markedly from that obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. This deviation is much less in experiments with human di*iso*propyl phosphorylacetocholinesterase and bovine diethyl phosphorylacetocholinesterase. Several likely sources of interference with reactivation could be disproved. Since Somers & Bay (1959) have shown that an oxime phosphonate derived from 4-hydroxyiminomethyl-*N*-methylpyridinium iodide phosphorylates acetocholinesterase, it is possible that an oxime phosphonate is responsible for at least part of the interference with reactivation observed in our experiments. That interference with reactivation of human diethyl phosphorylacetocholinesterase was far greater than with bovine diethyl phosphorylacetocholinesterase does not necessarily contradict this interpretation, since the former preparation was a crude enzyme preparation whereas the latter represented a highly purified enzyme preparation. The interference with reactivation does not seem to be of practical importance, since oximes of *NN'*-polymethylenebis(pyridinium bromide) have a short duration of action *in vivo* (Hobbiger & Sadler, 1959) and are greatly superior to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide both as reactivators (Hobbiger & Sadler, 1959) and as antidotes to poisoning by a variety of organophosphates (Bay, Krop & Yates, 1958; Hobbiger, O'Sullivan & Sadler, 1958; Hobbiger & Sadler, 1958, 1959; Berry *et al.* 1959).

The finding that the hydrolysis of organophosphates by pyridinium aldoximes and bispyridinium aldoximes yields cyanide needs further investigation in view of its possible clinical importance. The production of cyanide from aliphatic 2-oxo-oximes during their metabolic degradation *in vivo* has already been demonstrated by Askew, Davies, Green & Holmes (1956).

SUMMARY

1. The reactivation of diethyl and di*iso*propyl phosphorylacetocholinesterases by aldoximes of pyridinium and bispyridinium and related compounds was investigated. The anticholinesterase activity of these oximes and the hydrolysis of tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate by them were also studied. All experiments were carried out at pH 7·45 and 37°.

2. The most potent reactivators among the oximes studied were 4-monoximes and 4:4'-dioximes of *NN'*-polymethylenebis(pyridinium bromide), which were greatly superior as reactivators to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine 2-aldoxime methiodide). Reactivation of human diethyl phosphorylacetocholinesterase by oximes of *NN'*-polymethylenebis(pyridinium bromide) was faster in the presence of acetylcholine than without it. The hypothesis is put forward that an oxime phosphonate might be formed during reactivation and interfere with reactivation in the absence of acetylcholine.

3. There is no correlation between anticholinesterase activity and effectiveness as reactivator. The ratios (potency as reactivator:reactivity with organophosphates), obtained with different oximes, support the interpretation that attachment to the phosphorylated enzyme during reactivation enhances activity. Possible sites of attachment are discussed.

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The Enzymic Condensation of Porphobilinogen to Porphyrins

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The ability to catalyse the condensation of porphobilinogen to uroporphyrin has been reported for several tissues and extracts. It now appears that the primary product of the enzyme action is uroporphyrinogen, which differs from the porphyrin in having six additional hydrogen atoms. When the reaction is carried out in the presence of oxygen, uroporphyrin is formed by the oxidation of the porphyrinogen.

Although some of the preparations are capable of producing uroporphyrin III from porphobilinogen, all can be so modified that they produce uroporphyrin I only. Bogorad & Granick (1953) obtained *Chlorella* extracts capable of forming uroporphyrin III, together with coproporphyrin III; after heating, the extracts produced uroporphyrin I only. Hoare & Heath (1958) and Heath & Hoare (1959) showed that suspensions of frozen and thawed *Rhodopseudomonas spheroides* as well as the acetone-dried powders from them condense porphobilinogen to uroporphyrin III and coproporphyrin III, but the cell-free supernatants, after centrifuging, and also the heated acetone-dried powders gave uroporphyrin I only. Booij & Rimington (1957) heated vertebrate haemolysates (65° for fowl and 55° for human) which then catalysed the formation of series I porphyrins only. Granick & Mauzerall (1958) found the same for partially purified preparations from rabbit and from fowl erythrocytes. Bogorad (1958*a*, *b*) prepared a preparation from spinach acetone-dried powders which converted porphobilinogen into uroporphyrinogen I. The addition to this of a fraction obtained from wheat-germ extracts, which alone had no action on porphobilinogen, produced a system which formed uroporphyrin III from porphobilinogen.

In this paper, a partially purified preparation from avian erythrocytes, briefly described by Lockwood & Rimington (1957), has been used in a study designed to test proposed mechanisms of the enzymic condensation of porphobilinogen to uroporphyrinogen.

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