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PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING BY QUATERNARY PYRIDINE ALDOXIMES

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The effect of 18 pyridinium aldoximes on diethylphosphoryl-acetocholinesterase *in vitro* and the protection against lethal poisoning by ethyl pyrophosphate (TEPP) in mice pretreated with 0.095 m.mole/kg. of these oximes was investigated. Monoximes and dioximes of polymethylene-bispyridinium compounds were studied in greater detail since they were up to 22 times more potent than pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide) in reactivating diethylphosphoryl-acetocholinesterase *in vitro* and protected mice against lethal poisoning by up to 15 LD100 of ethyl pyrophosphate. These oximes were also up to 52 times more potent than pyridine-2-aldoxime methiodide in reactivating di-isopropylphosphoryl-acetocholinesterase *in vitro* and were effective in preventing lethal poisoning by dyflos (di-isopropyl phosphorofluoridate). The antidotal action against diethyl phosphostigmine (Ro 3-0340) was even greater than that against ethyl pyrophosphate. Some of the most effective oximes had antidotal actions in poisoning by ethyl pyrophosphate, diethyl phosphostigmine and dyflos when given in 0.0095 m.mole/kg. and this effect was enhanced by 1 mg./kg. atropine sulphate. *In vivo* reactivation of diethylphosphoryl-acetocholinesterases by 0.0095 or 0.095 m.mole/kg. of oximes of polymethylenebispyridinium compounds was demonstrated in blood but not in brain. Atropine-like and neuromuscular blocking activities were studied on isolated organs and protection against lethal doses of neostigmine and related anticholinesterases were also investigated. Some of the oximes of polymethylenebispyridinium compounds have, relative to pyridine-2-aldoxime methiodide, a higher therapeutic ratio in mice and considerably greater water-solubility. The possible advantages to be gained from their use in preference to pyridine-2-aldoxime methiodide are discussed.

The interaction between anticholinesterases of the organophosphate type and acetocholinesterase (also often referred to as true cholinesterase or acetylcholinesterase) yields phosphorylated aceto-cholinesterase which is enzymatically inactive and in many instances has a half-life ranging from several days to weeks. While cholinesterase is in a phosphorylated form acetylcholine accumulates and thus the administration of organophosphates is followed by muscarinic and nicotinic symptoms of acetylcholine poisoning.

Causative treatment of poisoning by certain organophosphates has recently become available as a result of the finding that some oximes, especially pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide; P2AM), restore the activity of phosphorylated acetocholinesterase by dephosphorylation (Childs, Davies, Green, and Rutland, 1955; Wilson and Ginsburg, 1955). The successful treatment of parathion poisoning of man with pyridine-2-aldoxime methiodide has been reported by Namba and Hiraki (1958) and Namba (1958). From biochemical findings (Hobbiger, 1957a, b) it is clear, however, that the effectiveness of pyridine-2-aldoxime methiodide depends on the type of phosphoryl group attached to the enzyme. For equal degrees of enzyme reactivation the quantity of pyridine-2-aldoxime methiodide required when a di-isopropylphosphoryl group is involved, as for instance in poisoning with dyflos (di-isopropyl phosphorofluoridate), is 20 to 30 times higher than that necessary for the treatment of poisoning with parathion, ethyl pyrophosphate (TEPP) or paraoxon where a diethylphosphoryl group is involved. The dose of pyridine-2-aldoxime methiodide used and recommended by Namba and Hiraki (1958) for the treatment of

parathion poisoning is 1 g. or more, given intravenously. Because pyridine-2-aldoxime methiodide is only soluble in water to an extent of 4 to 5% at room temperature this means that at least 20 ml. of a hypertonic solution is required for reactivation of diethylphosphoryl-acetocholinesterase, and this treatment becomes impracticable where a di-isopropylphosphoryl group or another similarly strongly bound group is involved. It has also to be remembered that parathion itself does not phosphorylate acetocholinesterase but is converted into an active phosphorylating agent *in vivo* (Diggle and Gage, 1951), and thus produces symptoms more slowly than other organophosphates, such as ethyl pyrophosphate, paraoxon, or dyflos, which themselves are phosphorylating agents.

Since organophosphorus insecticides are used widely and cases of poisoning have frequently been reported (Pribilla, 1955; Holmes and Gaon, 1956; Conley, 1957; Maresch, 1957; Erdmann and Lendle, 1958; Namba and Hiraki, 1958; Quinby and Lemmon, 1958), reactivators of phosphorylated acetocholinesterase which have a low toxicity and which are more water-soluble and more potent than pyridine - 2 - aldoxime methiodide are urgently needed.

Methods and Materials

Potency of Oximes as Reactivators.—The potency of oximes as reactivators was determined by measuring their effect on diethylphosphoryl- and di-isopropylphosphoryl-acetocholinesterase at 37° in a medium of 0.025 M-$NaHCO_3$ (*p*H 7.4). Human red cells were used as source of acetocholinesterase. The two types of phosphorylated acetocholinesterase were obtained as described previously (Hobbiger, 1957b). Reactivation was studied in the Warburg apparatus, allowing 30 min. incubation of phosphorylated acetocholinesterase with the oxime in the centre compartment before addition of the substrate (acetylcholine chloride; final concentration 0.01 M) from the sidearm. The degree of reactivation was calculated from the CO_2 output between 5 and 35 min. after tipping in the substrate.

For toxicity tests and *in vivo* protection experiments, male white mice of an inbred strain and weighing 18 to 20 g. were used. For simplicity, the weight of each mouse was assumed to be 20 g. All injections were given in a volume of 0.2 ml. and normal saline was substituted when necessary to keep the volume of injected fluid and the number of injections constant between groups. All substances were dissolved in normal saline.

Determination of the LD50.—Oximes were given intraperitoneally using 6 mice for each dose and solutions which represented a geometrical progression of oxime concentrations with a difference of 1.5 between any given dose and the next higher dose. All dose/mortality curves were steep, and, in a certain range, mortality increased by at least 66% when the dose of the oxime was doubled. The point of intersection of the dose/mortality curve with the line representing death of half the mice was taken as the LD50.

In vivo *Protection Against Organophosphate Poisoning.* — The organophosphates and other anticholinesterases were injected subcutaneously in the middle of the back. The expression "complete protection" is used when all mice in a group survived for 24 hr., whereas the expression "partial protection" means that some mice in a group died within 24 hr.

Activity of Acetocholinesterase in Blood and Brain. —This was measured manometrically with 0.03 M-methacholine chloride as substrate (see Hobbiger, 1957a).

Experiments on Isolated Organs.—Antiacetylcholine and antihistamine actions were determined on isolated segments (3 to 4 cm.) of guinea-pig ileum suspended in oxygenated Tyrode solution at 37°. Oximes were added to the organ bath 30 sec. before acetylcholine or histamine and the concentration of the oximes required to reduce the sensitivity of the ileum by 50% was calculated from dose/antagonism curves.

The isolated rat phrenic-nerve diaphragm was set up in the conventional manner (37°; oxygenated Tyrode solution) and the effect of the oximes on the response of the muscle to indirect stimulation by supramaximal rectangular pulses (duration, 0.25 msec.; frequency 1/10 sec.) was recorded. The effects obtained 10 min. after addition of an oxime to the organ bath were used in order to compare activities.

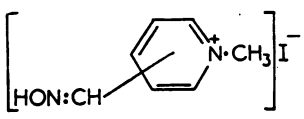
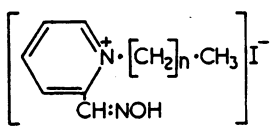
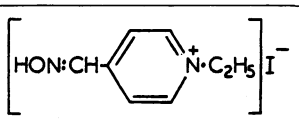
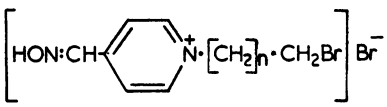
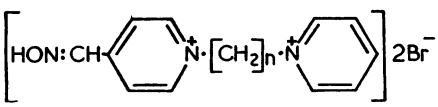
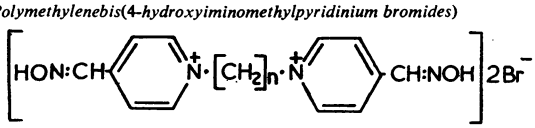
Organophosphates and Other Anticholinesterases.— Those used were (1) ethyl pyrophosphate (TEPP); (2) diethyl phosphostigmine {3-(diethoxyphosphinyloxy)-*NNN*-trimethylanilinium methosulphate, Ro 3-0340}; (3) dyflos (di-isopropyl phosphorofluoridate); (4) paraoxon (diethyl *p*-nitrophenyl phosphate, E 600); (5) neostigmine methylsulphate; (6) a bisneostigmine [*NN'* - decamethylenebis{3-(*N*-methylcarbamoyloxy)-*NNN*-trimethylanilinium bromide}, BC-48]; and (7) a bispyridostigmine [*NN'* - hexamethylenebis{3 - (*N* - methylcarbamoyloxy)-*N*-methylpyridinium bromide}, BC-51].

Oximes.—The oximes used were all pyridinium aldoximes synthesized in this laboratory. They are listed in Table I together with analytical information and solubilities in water and chloroform. The latter were determined spectroscopically in a Hilger-Uvispek using matched quartz cells. Absorption curves were determined in 0.01 N-NaOH.

Methods of Preparation: N - Alkyl - 2 - and - 4 - hydroxyiminomethylpyridinium Iodides.—The appropriate pyridine aldoxime (0.04 M) was dissolved in ethanol and heated under reflux for 24 hr. with an excess (0.08 M) of alkyl iodide. The ethanol and

TABLE I

CHARACTERISTICS OF NEW OXIMES SYNTHESIZED

Compound No.	Structure and Name	Required (%) C	Required (%) H	Found (%) C	Found (%) H	Molar Solubility in Water $\times 10^{-1}$	Molar Solubility in Chloroform $\times 10^{-5}$
	Pyridine aldoxime methiodides 						
—	Pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide)					1·8	2·8
2	Pyridine-3-aldoxime methiodide			(Known compounds)		>10	4·6
3	Pyridine-4-aldoxime methiodide					>10	5·4
	N-*Alkyl-2-hydroxyiminomethylpyridinium iodides* 						
4	$n=1$. *N*-Ethyl-2-hydroxyiminomethylpyridinium iodide					3·5	11·0
5	$n=2$. 2-Hydroxyiminomethyl-*N*-propylpyridinium iodide	37	4·5	37·3	4·7	0·7	12·8
6	$n=3$. *N*-Butyl-2-hydroxyiminomethylpyridinium iodide	39·2	4·9	39·3	5·2	0·3	24·0
							
7	Pyridine-4-aldoxime ethiodide (*N*-ethyl-4-hydroxyiminomethylpyridinium iodide)	34·5	3·9	34·4	3·9	>10	30
	N-ω-*Bromoalkyl-4-hydroxyiminomethylpyridinium bromides* 						
8	$n=2$. *N*-(3-Bromopropyl)-4-hydroxyiminomethylpyridinium bromide	33·3	3·7	33·3	3·8	0·7	2·2
9	$n=3$. *N*-(4-Bromobutyl)-4-hydroxyiminomethylpyridinium bromide	35·5	4·1	35·6	4·3	>10	11·0
10	$n=4$. *N*-(5-Bromopentyl)-4-hydroxyiminomethylpyridinium bromide	37·5	4·5	37·3	4·5	0·1	5·1
	Monoximes of NN′-*polymethylenebis(pyridinium bromides)* 						
11	$n=3$. Trimethylene-1-(4-hydroxyiminomethylpyridinium)-3-pyridinium dibromide	41·7	4·2	41·3	4·4	>10	0·53
12	$n=4$. Tetramethylene-1-(4-hydroxyiminomethylpyridinium)-4-pyridinium dibromide	43·2	4·5	43·5	4·7	>10	0·09
13	$n=5$. Pentamethylene-1-(4-hydroxyiminomethylpyridinium)-5-pyridinium dibromide	44·5	4·9	44·2	4·8	>10	0·32
	NN′-*Polymethylenebis(4-hydroxyiminomethylpyridinium bromides)* 						
14	$n=1$. *NN′*-Methylenebis(4-hydroxyiminomethylpyridinium bromide)	37·3	3·4	36·9	3·5	Decomposes in 0·01 N-NaOH	
15	$n=2$. *NN′*-Ethylenebis(4-hydroxyiminomethylpyridinium bromide)	38·9	3·7	38·8	3·9	0·3	0·06
16	$n=3$. *NN′*-Trimethylenebis(4-hydroxyiminomethylpyridinium bromide)	40·4	4·0	39·8	4·2	>10	0·08
17	$n=4$. *NN′*-Tetramethylenebis(4-hydroxyiminomethylpyridinium bromide)	41·7	4·4	41·8	4·6	2·8	0·54
18	$n=5$. *NN′*-Pentamethylenebis(4-hydroxyiminomethylpyridinium bromide)	43·0	4·6	42·8	4·8	>10	3·2

unreacted alkyl iodide were removed by distillation under reduced pressure and the products crystallized from ethanol or ethanol/ether.

N-ω-Bromoalkyl-4-hydroxyiminomethylpyridinium Bromides.—Pyridine-4-aldoxime (0.05 M) was dissolved in ethanol (50 ml.) and heated under reflux for 18 hr. with excess polymethylene dibromide (0.15 M). The mixture was cooled and filtered to remove any dioxime formed, then the ethanol and unreacted polymethylene dibromide were removed by distillation under reduced pressure. The residual crystals were triturated with ether and recrystallized from ethanol.

Monoximes of NN'-Polymethylenebis(pyridinium Bromides). — N-ω-Bromoalkyl-4-hydroxyiminomethylpyridinium bromide (0.02 M) was dissolved in ethanol (50 ml.) and heated under reflux for 18 hr. with excess pyridine. The ethanol and unreacted pyridine were removed by distillation under reduced pressure. The residual oil was triturated with ether and crystallized from ethanol.

NN' - Polymethylenebis(4-hydroxyiminomethylpyridinium Bromides).—Pyridine-4-aldoxime (0.1 M) was dissolved in ethanol and heated under reflux for 18 hr. with polymethylene dibromide (0.06 M). The product separated on cooling and was recrystallized from methanol. Compound 14 was an exception as it was the only product obtained from the attempted preparation of *N*-bromomethyl-4-hydroxyiminomethylpyridinium bromide.

A full account of biochemical studies on compounds 2 to 18 is in preparation.

Throughout the text, the oximes will be referred to by the compound numbers in Table I. Doses and concentrations are expressed in m.mole/kg. and molarities respectively. In earlier work (Hobbiger, 1957a) a dose of 25 mg./kg. pyridine-2-aldoxime methiodide (0.095 m.mole/kg.) was used and, to make the earlier results directly comparable with those of the present investigation, the unusual dose of 0.095 m.mole/kg. was retained for *in vivo* studies.

Two short preliminary accounts of some of the work presented here have been published (Hobbiger, O'Sullivan and Sadler, 1958; Hobbiger and Sadler, 1958). In the first of these, *NN'*-methylenebis(4-hydroxyiminomethylpyridinium bromide) (compound 14, Table I) was erroneously listed as *N*-bromomethyl-4-hydroxyiminomethylpyridinium bromide.

RESULTS

Reactivation of Phosphorylated Acetocholinesterase in vitro.—Table II shows the potency of the 18 derivatives of pyridinium aldoximes as reactivators of diethylphosphoryl-acetocholinesterase at *p*H 7.4. As can be seen, the hydroxyiminomethyl group is required in the 2-position for highest activity in the case of the methiodides of the pyridine aldoximes, and an increase in size of the alkyl group (compare pyridine-2-aldoxime methiodide and compound 3 with compounds 4 to 6 and 7 respectively) is associated with some loss of activity. The introduction of a terminal bromine atom in the alkyl chain (compounds 8 to 10) greatly enhanced activity in comparison with the parent compound: the monoximes (compounds 11 to 13) of polymethylenebispyridinium compounds were 6.7 to 8 times, and the dioximes (compounds 14 to 18) 2.8 to 22 times, more active than pyridine-2-aldoxime methiodide.

The high potency of compounds 11 to 13 and 15 to 18 as reactivators was not limited to diethylphosphoryl-acetocholinesterase but is even slightly greater with di-isopropylphosphoryl-acetocholinesterase (Table II).

TABLE II

IN VITRO REACTIVATION OF PHOSPHORYLATED ACETOCHOLINESTERASE AND TOXICITY IN MICE

The potency as reactivator was determined from rates of reactivation of (1) diethylphosphoryl and (2) di-isopropylphosphoryl-acetocholinesterase using $4–12\times10^{-6}$ and $5–15\times10^{-5}$ M pyridine-2-aldoxime methiodide respectively as reference (=100). See Methods section for details. Relative toxicities were calculated from the LD50 which in the case of pyridine-2-aldoxime methiodide was 1 m.mole/kg. Oximes were injected intraperitoneally. All comparisons are on a molar basis.

Compound	Potency as Reactivator		Relative Toxicity
	(1)	(2)	
2	0·03		•
3	6		
4	54		
5	63		
6	87		
7	3		
8	190		
9	25		
10	94		
11	800	1,100	200
12	670	1,000	280
13	750	1,100	620
14	282		
15	1,700	2,200	160
16	2,200	5,200	350
17	1,800	3,800	530
18	1,600	3,600	1,500

Effects in vivo

Toxicity.—Since it was our aim to find compounds which were superior to pyridine-2-aldoxime methiodide as antidotes of organophosphate poisoning, the only toxicities investigated were those of oximes which were at least six times more potent than pyridine-2-aldoxime methiodide as reactivators of diethylphosphoryl-acetocholinesterase *in vitro*. Table II shows that, on a molar basis, compounds 11 to 13 and 15 to 18 were 1.6 to 15 times more toxic than pyridine-2-aldoxime methiodide. However, using as therapeutic index the ratio LD50/concentration required for reactivation, most of the new oximes were safer to use than equi-effective doses of pyridine-2-aldoxime methiodide. Particularly

favourable therapeutic indices (2.4 to 15 higher than that of pyridine-2-aldoxime methiodide) were seen with compounds 11, 12, and 15 to 17.

Symptoms produced by lethal doses of compounds 11 to 13 and 15 to 18 were identical with those produced by pyridine-2-aldoxime methiodide, namely dyspnoea followed by convulsions and respiratory arrest. The latter always preceded cessation of the heart beat. With the exception of compound 18, all oximes were well tolerated when given intraperitoneally in a dose of 0.095 m.mole/kg. Since such a dose of compound 18 was lethal its antidotal action was not investigated.

Protection against Lethal Poisoning by Ethyl Pyrophosphate.—Compounds 1 to 17 were investigated for a protective action against lethal poisoning by 0.8 mg./kg. of ethyl pyrophosphate. This particular dose was chosen because it produced with certainty a lethal effect in otherwise untreated mice whereas more than 75% of the mice injected with half that dose survived. Complete protection by 0.095 m.mole/kg. of oxime against lethal poisoning by 0.8 mg./kg. of ethyl pyrophosphate was obtained only with oximes of polymethylenebispyridinium compounds. The latter, with the exception of compound 14, also gave either complete or partial protection against lethal poisoning by 1.6 to 3.2 mg./kg. of ethyl pyrophosphate. Compounds 13, 16, and 17 were the best antidotes, and partial protection against the lethal effect of 12.8 mg./kg. of ethyl pyrophosphate was obtained in mice pretreated with compound 16. These results are summarized in Table III.

TABLE III

PROTECTION AGAINST LETHAL POISONING BY ETHYL PYROPHOSPHATE

Mice received 0·095 m.mole/kg. of oxime intraperitoneally 5 to 10 min. before ethyl pyrophosphate (given subcutaneously). Results are number of mice surviving for 24 hr./number of mice injected.

Oxime	Ethyl Pyrophosphate (mg./kg.)						
	0·4	0·8	1·6	3·2	6·4	12·8	25·6
None ..	11/12	0/12	0/6	0/6	0/6	0/6	
Pyridine-2-aldoxime methiodide		0/6	0/18	0/12			
Compound 2		0/6	0/6				
,, 3		0/6	0/6				
,, 4		0/6	0/6				
,, 5		0/6	0/6				
,, 6		1/6	0/6				
,, 7		0/6	0/6				
,, 8		4/6	0/6				
,, 9		2/6	0/6				
,, 10		1/6	0/6				
,, 11		6/6	3/6	3/6	1/7		
,, 12		6/6	5/6	3/6	1/7		
,, 13		6/6	5/6	6/6	5/9	2/9	
,, 14		6/6	1/6	0/6			
,, 15		6/6	2/6	1/6	0/7		
,, 16		6/6	6/6	6/6	9/11	7/15	0/6
,, 17		6/6	6/6	5/6	8/9	1/9	

Death induced by organophosphates in mice pretreated with an oxime was always associated with all the symptoms of acetylcholine poisoning. In groups with partial protection and in groups receiving the next lower dose of ethyl pyrophosphate, the survivors generally also showed symptoms of acetylcholine poisoning, particularly of the nicotinic type which lasted for several hours. Mice pretreated with compounds 13, 16, and 17 and receiving 0.8 or 1.6 mg./kg. of ethyl pyrophosphate had only very transient and mild symptoms.

The degree of protection against ethyl pyrophosphate given by 0.095 m.mole/kg. of oxime is determined by the interval between the injection of the latter and the administration of the organophosphate. When 0.095 m.mole/kg. of compounds 11 to 13 and 15 to 17 were injected 60 min. earlier none of the mice survived 1.6 mg./kg. of ethyl pyrophosphate, and the interval between injection of the organophosphate and death was the same as that recorded in a group of mice which received 1.6 mg./kg. of ethyl pyrophosphate only.

Two of the most effective antidotes, compounds 13 and 16, were tried at lower doses; 0.0095 m.mole/kg. of either oxime gave definite protection (Table IV). However, mice injected with 0.00095 m.mole/kg. of compounds 13 and 16 were not protected against 0.7 mg./kg. ethyl pyrophosphate; they died at the same time as the mice in the control group.

Protection against Lethal Poisoning by Diethyl Phosphostigmine.—Both diethyl phosphostigmine and ethyl pyrophosphate form diethylphosphoryl-acetocholinesterase, but only the latter is hydrolysed by mammalian esterases with phosphatase activity (Burgen and Hobbiger, 1951; Hobbiger, 1956). Lethal doses of diethyl phosphostigmine cause death after a longer interval than corresponding doses of ethyl pyrophosphate. Thus the oxime can act over a longer period, and it has been suggested that for this reason 0.095 m.mole/kg. of pyridine-2-aldoxime methiodide is far more effective in protecting against diethyl phosphostigmine than against ethyl pyrophosphate (Hobbiger, 1957a). In agreement with such an interpretation was the finding that oximes of polymethylenebispyridinium compounds were also more effective antidotes of diethyl phosphostigmine than of ethyl pyrophosphate. Compounds 13 and 16 were given in a dose of 0.0095 m.mole/kg., and

TABLE IV

PROTECTION AGAINST LETHAL POISONING BY ORGANOPHOSPHATES

Mice received 0·0095 m.mole/kg. of oxime intraperitoneally 5 to 10 min. before the organophosphate (given subcutaneously). Atropine sulphate (1 mg./kg.) was given intraperitoneally 15 min. before the oxime. Results are number of mice surviving for 24 hr./number of mice injected. Dose of organophosphate in mg./kg.

Oxime	Ethyl Pyrophosphate					Diethyl Phosphostigmine							Dyflos				
	0·35	0·7	1·4	2·8	5·6	5	10	20	40	80	160	320	3·8	5	7·5	10	20
(1) Without atropine:																	
None	6/6	1/6	0/6			5/6	0/6	0/6					6/6	0/6	0/6		
Pyridine-2-aldoxime methiodide		1/6	0/6				0/6	0/6						0/6			
Compound 11														3/6	0/6		
,, 13		6/6	0/6					6/6	6/6	5/6	1/6	0/6					
,, 16		5/6	0/6					6/6	6/6	6/6	3/6	0/6		5/6	3/6	0/6	
(2) With atropine:																	
None	6/6	2/6	0/6			6/6	1/6	0/6					6/6	0/6			
Pyridine-2-aldoxime methiodide		5/6	0/6				6/6	3/6	0/6					0/6			
Compound 11		6/6	5/6	0/6										4/6	3/6	0/6	
,, 13		6/6	4/6	0/6						6/6	4/6	0/6					
,, 16		6/6	6/6	3/6	0/6					6/6	6/6	1/6		6/6	6/6	5/6	1/6

Table IV summarizes the remarkable degree of protection against lethal poisoning by diethyl phosphostigmine obtained with the two oximes.

Protection against Lethal Poisoning by Dyflos.—Only pyridine-2-aldoxime methiodide and oximes of polymethylenebispyridinium compounds were used. All mice injected with 0.095 m.mole/kg. of compounds 11 to 13 and 15 to 17 survived a dose of dyflos which produced a 100% mortality in the control group (Table V). Compounds 11, 16, and 17 were the most effective antidotes; they gave partial protection against 3 to 6 LD100 of dyflos.

Cholinergic symptoms of oxime-treated mice surviving 10 to 20 mg./kg. of dyflos were always marked and long-lasting but delayed in onset by comparison with the symptoms in mice of a control group receiving only dyflos.

TABLE V

PROTECTION AGAINST LETHAL POISONING BY DYFLOS

Mice received 0·095 m.mole/kg. oxime intraperitoneally 5 to 10 min. before dyflos (given subcutaneously). Results are number of mice surviving for 24 hr./number of mice injected. The stock solution of dyflos used in these experiments had undergone approximately 40% hydrolysis and for this reason the toxicity of dyflos is lower than that observed in experiments presented in Table IV and in a previous paper (Hobbiger, 1957a). Repetition of the experiment using a limited number of mice and a fresh stock solution of dyflos established the validity of the observations presented in this Table.

Oxime	Dyflos (mg./kg.)					
	5	7·5	10	20	40	80
None	6/6	2/6	0/6	0/6	0/6	
Pyridine-2-aldoxime methiodide		6/6	2/6	0/6	0/6	
Compound 11			6/6	6/6	2/6	0/6
,, 12			6/6	4/6	0/6	
,, 13			6/6	5/6	0/6	
,, 15			6/6	3/6	0/6	
,, 16			6/6	6/6	3/6	0/6
,, 17			6/6	5/6	1/6	0/6

Two of the most effective oximes, compounds 11 and 16, were given in doses of 0.0095 m.mole/kg. Some protection against lethal poisoning by dyflos was also obtained with this lower dose (Table IV).

The Effect of Atropine on the Antidotal Action of Oximes.—Marked enhancement of the antidotal effect of pyridine-2-aldoxime methiodide by high doses of atropine sulphate (10 mg./kg. or more) has been repeatedly demonstrated (Kewitz, Wilson, and Nachmansohn, 1956; Askew, 1957; Hobbiger, 1957a). Since mice tolerate weight for weight more than 100 times the amount of atropine fatal to adult man (Gordon and Frye, 1955) experiments with low doses of atropine should be more important than experiments with large doses of atropine. For this reason mice were given only 1 mg./kg. of atropine sulphate 15 min. before the injection of 0.0095 m.mole/kg. of an oxime. Such a dose of atropine markedly reduced the intensity of muscarinic symptoms after injection of organophosphates but had no antidotal effect on its own. Mice pretreated with atropine and compounds 11, 13, or 16, however, survived larger doses of ethyl pyrophosphate, diethyl phosphostigmine or dyflos than mice pretreated with the oximes only (Table IV).

In vivo *Reactivation of Phosphorylated Acetocholinesterase.* — Speedy reactivation of diethylphosphoryl-acetocholinesterase in blood could be demonstrated in mice which were given 0.0095 or 0.095 m.mole/kg. of compounds 11 to 13 or 15 to 17, 30 min. after a sublethal dose of ethyl pyrophosphate (Table VI). Similarly, enzymatic activity was always high in the blood of mice which as the result of pretreatment with oximes had survived doses of ethyl pyrophosphate

greatly in excess of the LD100. For example, 24 hr. after the injection of ethyl pyrophosphate mice which received first 0.095 m.mole/kg. of compounds 13, 16, or 17, and 5 to 10 min. later 6.4 or 12.8 mg./kg. of ethyl pyrophosphate (survivors of experiments summarized in Table III), had an enzymatic activity in blood which varied between 70 and 81% of that of untreated control mice.

TABLE VI

IN VIVO REACTIVATION OF DIETHYLPHOSPHORYL-ACETOCHOLINESTERASE IN BLOOD

Mice were injected subcutaneously with 0·4 mg./kg. of ethyl pyrophosphate and 30 min. later the oxime was given intraperitoneally. Each result represents the enzyme activity of a pooled sample obtained from 3 mice 1 or 3 hr. after the injection of the oxime. Activities at 1 hr. after the injection are suspected of being slightly above their true value because of reactivation *in vitro* (Hobbiger, 1957a).

Oxime	Dose (m.mole/kg.)	Activity of Acetocholinesterase as % of Control	
		1 hr.	3 hr.
None	—	12	13
Pyridine-2-aldoxime methiodide	0·0095	29	—
	0·095	49	53
Compound 11	0·0095	53	56
	0·095	54	54
,, 12	0·095	56	60
,, 16	0·00095	32	—
	0·0095	54	52
	0·095	55	59
,, 17	0·095	47	56

On the other hand, no reactivation of phosphorylated acetocholinesterase in brain could be demonstrated with certainty in mice which received a sublethal dose of 0.4 mg./kg. of paraoxon or 1.25 to 2.5 mg./kg. of dyflos followed 30 min. later by 0.095 m.mole/kg. of compound 16.

Protection against Lethal Poisoning by Neostigmine and Related Anticholinesterases.— Neostigmine, the bisneostigmine (BC-48) and the bispyridostigmine (BC-51) are potent anticholinesterases which do not inhibit cholinesterase by phosphorylation. The biochemical and pharmacological actions of the last two have been described in detail by Kraupp, Stumpf, Herzfeld, and Pillat (1955) and Herzfeld, Kraupp, Pateisky, and Stumpf (1957). To test the specificity of the antidotal action of oximes of polymethylenebispyridinium compounds in organophosphate poisoning 0.095 m.mole/kg. of compound 16 were given to mice 5 to 10 min. before neostigmine, BC-48 or BC-51. Protection was most marked when neostigmine was used, but very little protection was obtained against BC-51 and none against BC-48. With neostigmine, 0.095 m.mole/kg. of compound 16 raised the LD100 approximately three-fold. These results are summarized in Table VII.

No protection against lethal neostigmine poisoning was found with 0.0095 m.mole/kg. of compound 16.

TABLE VII

EFFECT OF OXIMES ON LETHAL POISONING BY NEOSTIGMINE AND RELATED ANTICHOLINESTERASES

Mice were given the oxime intraperitoneally 5 to 10 min. before the anticholinesterase subcutaneously. Results are number of mice surviving for 24 hr./number of mice injected. The doses of neostigmine, BC-48 and BC-51 are in mg./kg.

Oxime	Dose (m.mole/kg.)	Neostigmine Methylsulphate				
		0·5	0·75	1	2	3
None	—	5/6	0/6	0/6		
Pyridine-2-aldoxime methiodide	0·095		0/6	0/6		
Compound 16	0·095		6/6	6/6	0/6	
,, 16	0·0095		0/6			
		Bisneostigmine (BC-48)				
		0·025	0·05	0·1		
None ..	—	6/6	1/6	0/6		
Compound 16	0·095		1/6	0/6		
		Bispyridostigmine (BC-51)				
		0·2	0·4	0·6		
None	—	6/6	3/6	0/6		
Compound 16	0·095		6/6	1/6	0/6	

Actions on Isolated Organs. — Since both atropine and tubocurarine give some protection against lethal organophosphate poisoning (Parkes and Sacra, 1954), the effect of compounds 11 to 13 and 15 to 17 on the sensitivity of the isolated ileum to acetylcholine and the response to indirect stimulation of the isolated rat phrenic-nerve diaphragm preparation were investigated.

Oximes reduced the sensitivity of the guinea-pig ileum to acetylcholine. The most effective compound *in vivo*, namely compound 16, was the most potent antagonist of acetylcholine on the ileum (Table VIII). Antagonism to histamine could also be demonstrated, but the concentrations required to obtain the same effect on equiactive doses of histamine and acetylcholine were at least 10 times greater for the former than the latter (Table VIII). In each case speedy recovery followed the removal of oximes from the organ bath.

In suitable concentrations the oximes reduced the twitch tension of the indirectly stimulated diaphragm (Table VIII). With the first dose of an oxime a just noticeable and transient increase of twitch tension often preceded paralysis. After

TABLE VIII
EFFECTS OF OXIMES ON ISOLATED ORGANS

Oxime	Guinea-pig Ileum Concentrations (mM) giving 50% Reduction in Sensitivity		Rat Phrenic-nerve Diaphragm Concentrations (mM) giving 70% Paralysis in 10 min.
	Acetylcholine	Histamine	
Pyridine-2-aldoxime methiodide ..	0·11		4·0
Compound 11 ..	0·06		1·7
,, 12 ..	0·16		1·2
,, 13 ..	0·054	>0·5	0·59
,, 15 ..	0·32		2·00
,, 16 ..	0·013	0·12	0·85
,, 17 ..	0·068	>0·5	0·98
,, 18 ..	0·035	>0·5	0·44

removal of the oxime from the organ bath full recovery of twitch tension was always rapid. Edrophonium (0.025 mM), which has a marked anti-curare action on the isolated rat phrenic-nerve diaphragm preparation (Hobbiger, 1952), increased twitch tension transiently by only 7% when added to the organ bath after compound 16 (within 6 min.) had reduced the twitch tension by 40%.

Effect on Alkylated "Receptors" and Energy-rich Phosphates.—Segments of guinea-pig ileum were bathed for 10 min. in 0.7 mM phenoxybenzamine. This resulted in a long-lasting reduction of their sensitivity to histamine as is typical for the effect of β-haloalkylamines (Nickerson, 1956). Addition of compound 16 in a final concentration of 2 mM to the organ bath for 10 min. failed to increase the sensitivity to histamine of the ileum treated with phenoxybenzamine.

Hydrolysis of adenosine triphosphate by pyridine-2-aldoxime methiodide, compound 3 and compound 16 was determined spectroscopically by incubating the oximes with adenosine triphosphate in barbitone buffer (*p*H 7.6) at 37° and developing with Fiske-Subbarow reagent. With a concentration of 2 mM of compounds 3 and 16 hydrolysis of adenosine triphosphate, as judged by the amount of inorganic phosphate present, amounted to 0 and 5% respectively after 15 min. incubation. Similarly pyridine-2-aldoxime methiodide and compound 16 in a concentration of 20 mM produced 1 and 6% hydrolysis respectively after 30 min. incubation.

DISCUSSION

A study of derivatives of pyridine aldoxime has led to oximes more potent than pyridine-2-aldoxime methiodide as reactivators of diethylphosphoryl and di-isopropylphosphoryl-acetocholinesterases. Particularly outstanding as reactivators are certain monoximes and dioximes of polymethylenebispyridinium compounds; for example, compounds 11 to 13 and 15 to 18 (Table II). Compounds 11, 12, 16, and 17 are more water-soluble, and in mice have a greater therapeutic ratio than pyridine-2-aldoxime methiodide; they are thus preferable to compounds 13, 18, and 15, which have the highest toxicity and lowest water-solubility respectively amongst the oximes of polymethylenebispyridinium compounds investigated. Should the toxicity in man of compounds 11, 12, 16, and 17, relative to pyridine-2-aldoxime methiodide, be similar to that found in mice (Table II) rates of reactivation of diethylphosphoryl-acetocholinesterase greatly in excess of those obtainable with pyridine-2-aldoxime methiodide could be accomplished by their administration to man. It might also be possible to reactivate freshly formed human di-isopropylphosphoryl-acetocholinesterase *in vivo*. Since compounds 11, 12, and 16 can be dissolved rapidly in a small volume of water they could be self-administered, in conjunction with atropine.

Oximes of polymethylenebispyridinium compounds, like pyridine-2-aldoxime methiodide, are relatively insoluble in oil (Table I); thus their effect will be exerted mainly outside the central nervous system and there particularly on cell surfaces. The first of these conclusions is supported by the finding that large doses of compound 16, like pyridine - 2 - aldoxime methiodide (Hobbiger, 1957a; Kewitz and Nachmansohn, 1957; Rutland, 1958), have no measurable effect on phosphorylated acetocholinesterase in the brain of mice although speedy reactivation takes place in blood (Table VI). Since these results were obtained by measuring the acetocholinesterase activity of the whole brain it is possible that compound 16 and related oximes nevertheless reactivate phosphorylated acetocholinesterase in a few limited areas of the brain which might play a vital part in survival. Preferential action on cell surfaces has been shown with pyridine-2-aldoxime methiodide (Fleisher, Corrigan, and Howard, 1958), and is characteristic of compounds which have a quaternary nitrogen atom (Schoffeniels, Wilson, and Nachmansohn, 1958). This limitation does not discredit reactivation of phosphorylated acetocholinesterase as the major or sole component of the mechanism by which the oximes act as antidotes, since the functional acetocholinesterase is located on cell surfaces (Koelle and Steiner, 1956; Koelle, 1957).

Whereas there is at present no evidence that the rate of reactivation *in vivo*, by a given oxime, of phosphorlyated acetocholinesterase at different sites depends upon the species studied, species differences in the antidotal effects of certain oximes have been clearly established (Askew, 1956). For this reason, it is advisable not to generalize on the degree of protection obtained with the oximes of polymethylenebispyridinium compounds described. In mice complete protection against the lethal effect of approximately two LD100 or higher doses of ethyl pyrophosphate by 0.095 m.mole/kg. of oxime was obtained only with those pyridine aldoximes which were more than twice as effective as pyridine-2-aldoxime methiodide in reactivating phosphorylated acetocholinesterase, namely the oximes of polymethylenebispyridinium compounds (compounds 11 to 17). Some of the latter in a dose of 0.095 m.mole/kg. protected mice against the lethal effect of 10 to 15 LD100 of ethyl pyrophosphate (Table III), and, as with pyridine-2-aldoxime methiodide (Hobbiger, 1957a), protection against lethal doses of diethyl phosphostigmine was even more marked (Table IV). Compounds 11, 16 and 17 were the most effective against lethal poisoning by dyflos and 0.095 m.mole/kg. protected some mice against 3 to 6 LD100 of dyflos (Table V). The antidotal effect of prophylactic doses of 0.0095 m.mole/kg. of oximes of polymethylenebispyridinium compounds was enhanced by 1 mg./kg. of atropine sulphate regardless of the type of organophosphate used (Table IV).

These results are encouraging and warrant an investigation, in species which are closely related to man, of the most effective oximes of polymethylenebispyridinium compounds (preferably in conjunction with atropine) as prophylactic agents against lethal doses of those organophosphates which form diethylphosphoryl- or di-isopropylphosphoryl-acetocholinesterases. If such experiments are carried out, attention must be paid to the rapidity with which the effect of the oximes wanes, a property which they share with pyridine-2-aldoxime methiodide (Kewitz, Wilson and Nachmansohn, 1956).

While this work was in progress Poziomek, Hackley, and Steinberg (1958) published the synthesis of compounds 4, 15, 16, 17, and 18 and reported that compound 16 is 5 times more potent *in vitro* than pyridine-2-aldoxime methiodide in reactivating isopropylmethylphosphoryl - aceto - cholinesterase. Bay, Krop, and Yates (1958) carried out experiments on a small number of mice, rabbits, cats and dogs giving compound 16 and pyridine-2-aldoxime methiodide, generally in conjunction with atropine, either before or shortly after sarin (isopropyl methylphosphonofluoridate) and found that the former was superior in all species except the dog.

Since atropine-like and neuromuscular blocking activities of pyridine-2-aldoxime methiodide have been demonstrated on isolated organs (Bethe, Erdmann, Lendle and Schmidt, 1957 ; Holmes and Robins, 1955), all oximes of polymethylenebispyridinium compounds were investigated for such properties. No relationship was found between the atropine-like activity of the oximes and their ability to paralyse indirectly stimulated striped muscle on the one hand and their effectiveness as antidotes on the other. An atropine-like action was most pronounced with compound 16 (Table VIII) and when 0.095 m.mole/kg. of this oxime is used the concentration reached *in vivo* might be sufficient to reduce the sensitivity of receptors for muscarinic actions of acetylcholine. It is unlikely that the other oximes have an atropine-like action *in vivo* at this concentration.

With regard to the significance of the effect on striped muscle for the antidotal action of some of the oximes, no valid conclusion can be drawn from the experiments on the isolated diaphragm since compounds containing a quaternary nitrogen atom are much less effective on this preparation than they are *in vivo* (Paton and Zaimis, 1952). However, there is a reasonably good relationship between the toxicity of oximes and their paralysing activity on indirectly stimulated striped muscle (compare Tables II and VIII).

Protection by 0.095 m.mole/kg. of compound 16 against lethal poisoning by neostigmine (Table VII) was much less than against lethal doses of ethyl pyrophosphate or diethyl phosphostigmine. The way in which the former was achieved is unknown, but it is possible that the atropine-like action of compound 16 accounts for it or is a contributory factor. No protection or only very little was obtained with 0.095 m.mole/kg. of compound 16 against lethal poisoning by the bisneostigmine (BC-48) and the bispyridostigmine (BC-51) respectively. The latter, like organophosphates, inhibit cholinesterase for much longer periods than does neostigmine (Kraupp, Stumpf, Herzfeld and Pillat, 1955 ; Herzfeld, Kraupp, Pateisky, and Stumpf, 1957), and thus it is questionable whether the mechanism responsible for protection against lethal neostigmine poisoning contributes significantly to protection against lethal organophosphate poisoning. In this connexion the findings of Grob and Johns (1958a and b) must

be mentioned. They observed that 1 to 2 g. of pyridine-2-aldoxime methiodide ameliorate generalized muscular weakness in man produced by neostigmine and the bispyridostigmine (BC-51) as well as by organophosphates. This indicates that in man improvements obtained by the treatment of non-lethal organophosphate poisoning with pyridine-2-aldoxime methiodide are not always entirely due to dephosphorylation. Our experiments with pyridine-2-aldoxime methiodide (Table VII) show that 0.095 m.mole/kg. of this oxime do not protect mice against lethal neostigmine poisoning.

In view of the high reactivity of oximes of polymethylenebispyridinium compounds, additional experiments were carried out to investigate if they could also restore the sensitivity of "receptors" which had been inactivated in an "irreversible" manner by alkylation (Nickerson, 1949) or if they interacted with energy-rich phosphates. Compound 16 was used for this purpose and found to be devoid of such properties.

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