Brit. J. Pharmacol. (1957), **12**, 438.

PROTECTION AGAINST THE LETHAL EFFECTS OF ORGANOPHOSPHATES BY PYRIDINE-2-ALDOXIME METHIODIDE

BY

F. HOBBIGER

From the Department of Pharmacology, Middlesex Hospital Medical School, London

(RECEIVED MAY 7, 1957)

The mechanism responsible for the protection against lethal organophosphate poisoning by pyridine-2-aldoxime methiodide (P-2-AM) was studied in the mouse. Two types of organophosphates were used: ethyl pyrophosphate (TEPP), E 600, Ro 3-0340, and Ro 3-0422 which form with true cholinesterase a diethylphosphoryl enzyme (1) and DFP, D 600, and Ro 3-0351 which form with true cholinesterase a di*iso*propylphosphoryl enzyme (2).

In vitro and under the experimental conditions used more than 50% reactivation of (1) was obtained within 1 hr. by concentrations of P-2-AM ranging from 0.5 to 1×10^{-5} M; 30 times higher concentrations of the oxime were required to achieve the same effect with (2). *In vivo* reactivation of phosphorylated true cholinesterases in blood amounted to 10 to 24% within the first 30 min. if 25 mg./kg. P-2-AM was injected (i.p.) 5 min. before a sublethal dose of TEPP, E 600, Ro 3-0340, or Ro 3-0422 and reactivation reached a maximum within 1 to 2 hr. after the injection of the oxime. P-2-AM was more effective when given 30 min. after the organophosphate. The effect of 25 mg./kg. P-2-AM on the phosphorylated true cholinesterase in brain (experiments with TEPP and E 600) was negligible. A dose of 25 mg./kg. P-2-AM had no consistent effect on the phosphorylated true cholinesterases in blood and brain of mice injected with sublethal doses of DFP, D 600, or Ro 3-0351.

The protection by 25 mg./kg. P-2-AM against lethal doses of TEPP, E 600, Ro 3-0422, and Ro 3-0340 was greater than that obtained with 50 mg./kg. atropine sulphate, but the degree of protection was determined by the organophosphate itself and not its dialkylphosphoryl group. Protection by 25 mg./kg. P-2-AM against lethal doses of DFP, D 600, and Ro 3-0351 was negligible. The antidotal effect of P-2-AM was potentiated by atropine. Mice which were injected with atropine and P-2-AM were protected to a greater extent against DFP than against Ro 3-0422, and protection against DFP was only slightly less than protection against TEPP. This is difficult to reconcile with a specific action of P-2-AM on phosphorylated cholinesterases.

The interaction between cholinesterase and organophosphates which have a common dialkylphosphoryl group leads to the formation of an inhibited enzyme with identical properties (Burgen and Hobbiger, 1951 ; Aldridge and Davison, 1953 ; Hobbiger, 1954, 1955 and 1956). The reason for this is that cholinesterase attempts to hydrolyse the organophosphates, but the hydrolysis process is incomplete and the dialkylphosphoryl group of the organophosphate—unlike the acyl group of a true substrate—remains attached to the enzyme for a considerable period. Reactivation of phosphorylated cholinesterase in aqueous solution is determined by the dialkylphosphoryl group and can be markedly enhanced *in vitro* by nucleophilic reagents which remove the dialkylphosphoryl group from the enzyme by virtue of their high affinity for the phosphorus. One of the most powerful reactivators of phosphorylated true cholinesterase is pyridine-2-aldoxime methiodide (P-2-AM) (Childs, Davies, Green, and Rutland, 1955 ; Wilson and Ginsburg, 1955). The concentrations of P-2-AM which are required to restore 50% of the activity of phosphorylated human true cholinesterase within 1 hr. at 37° and *p*H 7.4 (medium: 0.025 M $NaHCO_3$, 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin) are 2.7×10^{-5} M for the diethylphosphoryl enzyme and 6.1×10^{-4} M for the di*iso*propylphosphoryl enzyme (Hobbiger, 1956). Thus it seems possible that P-2-AM and (or) other oximes might be of therapeutic value in organophosphate poisoning. To study this, experiments were carried out in mice, and the following account gives the results obtained on the reactivation *in vitro* of phosphorylated true cholinesterases

in blood and brain by P-2-AM, the effect of P-2-AM *in vivo* on the same enzymes and the protective action of P-2-AM against lethal organophosphate poisoning. Additional results show that in some cases atropine markedly potentiates the antidotal effect of P-2-AM in organophosphate poisoning. The degree of protection achieved by P-2-AM and by the combined use of atropine and P-2-AM is determined by the organophosphate itself and not its dialkylphosphoryl group, and results obtained with an individual organophosphate (Kewitz, Wilson, and Nachmansohn, 1956) are, therefore, not applicable to all organophosphates which form the same type of phosphorylated enzyme.

METHODS

Male white mice weighing between 18 and 20 g. were used in all experiments. The organophosphates were injected subcutaneously in a volume of 0.1 ml./20 g. mouse and all other drugs were administered intraperitoneally in a volume of 0.2 ml./20 g. mouse. In any individual experiment, the total volume of fluid injected was kept constant by using aliquots of 0.9% NaCl as substitute for a drug.

The organophosphates used were: (1) organophosphates with a diethylphosphoryl group: ethyl pyrophosphate (TEPP), diethyl *p*-nitrophenylphosphate (E 600), 3-(diethoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3-0340), 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3-0422), and (2) organophosphates with a di*iso*propyl phosphoryl group: dyflos or di*iso*propyl phosphorofluoridate (DFP), di*iso*propyl *p*-nitrophenylphosphate (D 600), 3-(di*iso*propoxyphosphinyloxy)-pyridine (Ro 3-0351).

The organophosphates will be referred to throughout the text by the abbreviations given in brackets. TEPP, DFP, and Ro 3-0351 were made up as 10% w/v and E 600 as 1% w/v stock solutions in ethanol, and 0.9% NaCl was used for all subsequent dilutions. Ro 3-0340 and Ro 3-0422 were always dissolved freshly in 0.9% NaCl. The poor water-solubility of D 600 made it necessary to use ethanolic solutions at all dose levels.

Atropine was used as the sulphate and references in the text apply to the salt.

For pyridine-2-aldoxime methiodide the abbreviation P-2-AM is used in preference to P_2AM (Hobbiger, 1956).

In vitro *Reactivation by P-2-AM.*—Since TEPP, E 600, Ro 3-0422 and DFP are hydrolysed enzymatically by liver and other tissues, the following technique was used to reduce interference by free organophosphates with the reactivation process (Hobbiger, 1955 and 1956). A group of several mice was injected with 1 mg./kg. atropine to avoid changes in haemoconcentration resulting from excessive fluid loss following cholinesterase inhibition. Thirty minutes later a sublethal dose of an organophosphate was injected, and after a further interval of not less than 30 min. blood was collected by cardiac puncture under ether anaesthesia. The individual blood samples were heparinized and pooled. The brains were then removed and homogenized in 10 parts of 0.025 M $NaHCO_3$. The effect of different concentrations of P-2-AM on the inhibited enzymes was investigated in the Warburg apparatus under the same conditions as used for the estimation of cholinesterase activity. 0.3 ml. of a solution containing 2 vol. blood or brain homogenate and 1 vol. P-2-AM (dissolved in 0.025 M $NaHCO_3$) were added to the side arm of each manometric vessel and tipped into the main compartment after various periods of incubation; the latter contained 2.7 ml. of substrate dissolved in 0.025 M $NaHCO_3$. Cholinesterase activity was recorded over three consecutive periods of 20 min. The activities of P-2-AM treated blood or brain and of controls for inhibition and normal enzyme activity were determined concurrently and % reactivation was calculated from the expression: 100 × (activity of reactivated enzyme − activity of inhibited enzyme): (activity of control enzyme obtained from untreated mice − activity of inhibited enzyme).

In vivo *Effect of P-2-AM on Phosphorylated True Cholinesterases.*—All mice were injected with 1 mg./kg. atropine. One group served as controls for normal cholinesterase activity, another group was injected with a sublethal dose of an organophosphate 30 min. after the atropine had been given, and the remaining mice had in addition to the organophosphate an injection of 25 mg./kg. P-2-AM either 5 min. before or 30 min. after the organophosphate. Two mice of each group were sacrificed at various intervals and cholinesterase activities of whole blood and brain in each group were determined on pooled samples. Blood was collected by the technique described previously and brains were homogenized for 2 min. in 40 parts of 0.025 M $NaHCO_3$ immediately after their removal from the body. 0.3 ml. of blood and 1 ml. of brain homogenate respectively were then added without delay to the main compartment of each manometric vessel, and the total volume of fluid was adjusted to 2.7 ml. by addition of 0.025 M $NaHCO_3$. The manometric vessels were at once transferred to the Warburg bath and gassed for 5 min. with a mixture of 95% N_2+5% CO_2. After allowing for temperature equilibration, the substrate (0.3 ml.) was added to the enzyme from a sidearm and enzyme activity was recorded over 3 consecutive periods of 15 min.

Experiments in which the activity of aliquots of the same blood sample was determined after different periods of incubation in the Warburg bath showed that the 1:9 dilution of the blood which was used in these experiments was not always sufficient to arrest *in vitro* reactivation of diethylphosphoryl cholinesterase. This applied particularly to blood samples taken at 30 or 35 min. after the injection of P-2-AM and to a smaller extent to blood samples taken 1 hr. after the injection of P-2-AM (Fig. 1). To

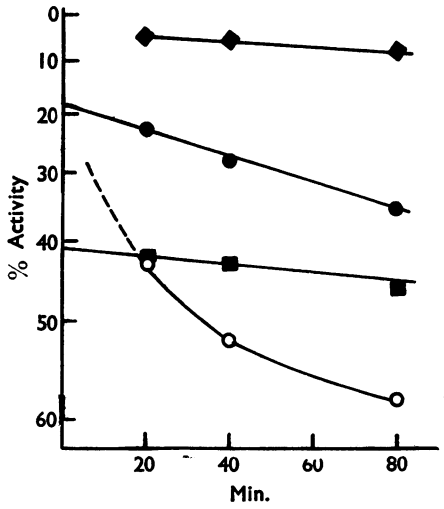

FIG. 1.—*In vitro* reactivation of phosphorylated true cholinesterases in blood samples taken from mice which have been injected with P-2-AM and an organophosphate. The mice received either 25 mg./kg. P-2-AM 5 min. before the injection of 0.4 mg./kg. TEPP (●, ○, ■) or TEPP alone (◆). 0.3 ml. blood was used in each Warburg vessel and incubated either together with 8 parts 0.025 M $NaHCO_3$ in the main compartment (●, ■, ◆) or without dilution in the sidearm (○). ●, ○, ◆: blood collected 30 min. after the injection of TEPP; ■: blood collected 60 min. after the injection of TEPP. The intercepts with the ordinate are taken as the true enzyme activity at the time when the blood was collected. Abscissa: min. of incubation before addition of substrate to the enzyme (●, ■, ◆) or enzyme to substrate (○). Ordinate: activity as % of controls (logarithmic scale).

minimize the error resulting from *in vitro* reactivation the activities of aliquots of each blood sample were determined after different periods of incubation in the Warburg bath; the activities of these blood samples were then used to obtain the true enzyme activity by extrapolation, as shown in Fig. 1. The activities of individual blood samples shown in Fig. 3 and Table I were all obtained by this procedure.

No *in vitro* reactivation was observed when brain homogenates were used.

In vivo *Protection against Organophosphate Poisoning.*—The lethal dose of each organophosphate was determined in otherwise untreated mice and in mice which were injected with 25 mg./kg. P-2-AM, 50 mg./kg. atropine, and 25 mg./kg. P-2-AM+50 mg./kg. atropine. With each individual organophosphate all observations were made concurrently on the same batch of mice. Atropine was injected 30 min. before and P-2-AM 5 min. before the organophosphate; the same time interval was adhered to in mice which received both drugs. The amounts of organophosphate which were injected were arranged in a geometric progression. In each group, 4 mice received the same treatment and a period of 6 hr. was used as the criterion of survival. By this technique only twofold changes in the lethal dose of an organophosphate or multiples of it could be recorded.

Measurement of Cholinesterase Activity.—Cholinesterase activity was measured by the manometric technique at 37° in a 0.025 M $NaHCO_3$ solution and in an atmosphere of 95% N_2+5% CO_2. The hydrolysis of 0.03 M (±)-acetyl-β-methylcholine chloride was used as a measure of the activity of the true cholinesterases in blood and brain.

Mouse blood, unlike human blood, contains true cholinesterase in both the red cells and the plasma. In a pooled sample of blood which was obtained from 6 mice and which had a packed cell volume of 45%, the red cells contributed 71% and plasma 29% towards the hydrolysis of (±)-acetyl-β-methylcholine. Experiments in which P-2-AM was used failed to disclose any difference in behaviour between the phosphorylated true cholinesterases of red cells and plasma, and thus it seems likely that they are either identical or closely related enzymes.

RESULTS

In vitro *Reactivation of Phosphorylated True Cholinesterases in Mouse Blood and Brain by P-2-AM*

True Cholinesterases in Blood.—Thirty minutes after the subcutaneous injection of 50 to 75% of the lethal dose of an organophosphate the activity of the true cholinesterases in blood was reduced by at least 75%. If blood samples were collected at this time and incubated with suitable concentrations of P-2-AM, more than 80% of the activity of the inhibited enzymes was restored by the oxime. Under the experimental conditions (see Methods) identical rates of reactivation were obtained by a fixed concentration of P-2-AM if inhibition was produced by organophosphates which have a common dialkylphosphoryl group. Within the observed range, namely up to 80% reactivation, reactivation proceeded at a rate characteristic for a first order reaction (Fig. 2).

It has been shown previously that the rate of interaction between P-2-AM and the organophosphates used in this work is determined by the organophosphates themselves and not their dialkylphosphoryl group (Hobbiger, 1956). The results with P-2-AM thus indicate that the inhibition of true cholinesterases in mouse blood by organophosphates is the result of enzyme phosphorylation, and the inhibited enzymes will, therefore, be referred to in the text as phosphorylated true cholinesterases or phosphorylated enzymes.

The concentrations of P-2-AM which were required under the experimental conditions to restore 50% of the activity of the phosphorylated true cholinesterases in mouse blood within 1 hr. were 4.2×10^{-6} M for the diethylphosphoryl

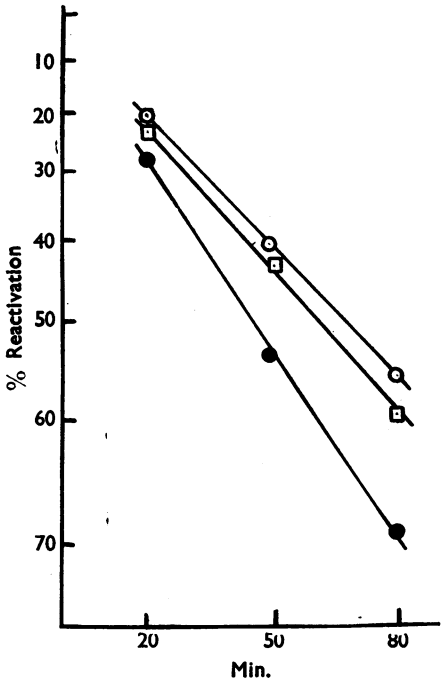

FIG. 2.—*In vitro* reactivation of phosphorylated true cholinesterases in blood. Blood was collected 30 min. after the injection of 0.4 mg./kg. E 600 and 2.5 mg./kg. DFP respectively. The true cholinesterases in blood of the mice injected with E 600 were inhibited to an extent of 91% and for reactivation 7.5×10^{-6} M P-2-AM (●—●) and 2.5×10^{-6} M P-2-AM (○—○) were used. The inhibition in the DFP injected mice amounted to 95% and for reactivation 1×10^{-4} M P-2-AM (□——□) was used. Abscissa: duration of reactivation. Ordinate: % reactivation of inhibited (phosphorylated) enzyme (logarithmic scale).

enzymes and 1.4×10^{-4} M for the di*iso*propylphosphoryl enzymes.

If reactivation was carried out in a medium which contained 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin in addition to 0.025 M $NaHCO_3$, such as in the medium used in earlier work with phosphorylated human true cholinesterase (Hobbiger, 1956), higher concentrations of P-2-AM were required to achieve an effect equal to that obtained with 0.025 M $NaHCO_3$ as medium. Experiments in which a mixture consisting of equal parts of phosphorylated true cholinesterases of the mouse and human was incubated with P-2-AM failed to show any difference in the sensitivity to P-2-AM between corresponding phosphorylated enzymes obtained from the two species. The reduction in the effectiveness of P-2-AM when used in a medium of 0.025 M $NaHCO_3$, 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin was mainly due to the presence of $MgCl_2$ (unpublished results).

True Cholinesterase in Brain.—P-2-AM also reactivated the phosphorylated true cholinesterase in brain homogenates (obtained from the brains of mice which had been injected with TEPP, E 600, DFP or D 600), and the results obtained in these experiments are consistent with the interpretation that inhibition is the result of enzyme phosphorylation. However, to achieve equal rates of reactivation of corresponding phosphorylated enzymes in blood and brain the concentration of P-2-AM had to be approximately doubled if the latter were used. This does not necessarily mean that the phosphorylated true cholinesterases in blood and brain have different sensitivities to P-2-AM, since crude enzyme preparations were used and a variety of factors, such as differences in *p*H, salts, proteins, etc., could easily account for the observed discrepancies.

Reactivation After Prolonged Inhibition.—The inhibition of human, bovine and rabbit true cholinesterases by organophosphates involves the following stages: cholinesterase + organophosphate→addition complex→phosphorylated cholinesterase which can be reactivated by nucleophilic reagents→phosphorylated cholinesterase which cannot be reactivated by nucleophilic reagents (Hobbiger, 1956; Davies and Green, 1956).

The same sequence of reactions also applies to the phosphorylation of the true cholinesterases of the mouse. If P-2-AM was added to mouse blood which had been collected at different intervals after the injection of an organophosphate it was found that the effectiveness of P-2-AM was inversely related to the duration of enzyme inhibition. The phosphorylated true cholinesterase in brain behaved like the corresponding enzymes in blood. The rate of formation *in vivo* of a phosphorylated true cholinesterase which cannot be reactivated by P-2-AM showed the characteristics of a first order reaction and was determined by the dialkylphosphoryl group of the organophosphate. The time required to convert 50% of the phosphorylated enzyme which can be reactivated by P-2-AM into a phosphorylated enzyme which cannot be reactivated by P-2-AM was approximately 36 hr. for the diethylphosphoryl enzyme and 4 hr. for the di*iso*propylphosphoryl enzyme.

In vivo *Effect of P-2-AM on the Phosphorylated True Cholinesterases of Mouse Blood and Brain*

Experiments with Organophosphates which Form a Diethylphosphoryl Enzyme.—The activity of the true cholinesterases in blood of atropinized

mice was reduced by at least 75% within 30 min. after the injection of 0.4 mg./kg. TEPP or E 600, 5 mg./kg. Ro 3-0340 or 0.02 mg./kg. Ro 3-0422. Spontaneous reactivation of the phosphorylated true cholinesterase *in vivo* was slow, and only a small increase in enzyme activity took place during a period of 2 hr. following the injection of the organophosphate.

Mice which were injected with 25 mg./kg. P-2-AM either 5 min. before or 30 min. after the administration of TEPP, E 600, Ro 3-0340, or Ro 3-0422 showed consistently higher activities of the true cholinesterases in blood than the mice of a control group which were injected with the organophosphate alone. P-2-AM was most effective when given at a time when inhibition had reached a maximum and the higher enzyme activities in P-2-AM treated mice could only be the result of a reactivation process. Differences in the degree of reactivation between groups of mice injected with TEPP, E 600, Ro 3-0340 or Ro 3-0422 were small and of an order similar to those observed in different experiments with the same organophosphate. Fig. 3 shows a plot of the reactivation in an experiment with TEPP and Table I gives the range over which individual results are spread.

If mice were injected with P-2-AM and an organophosphate all blood samples taken within 1 hr. after the injection of the oxime showed reactivation *in vitro* on incubation at 37° in an atmosphere of 95% N_2+5% CO_2; this reactivation *in vitro* was greatest in undiluted blood samples and under the experimental conditions always incomplete (Fig. 1).

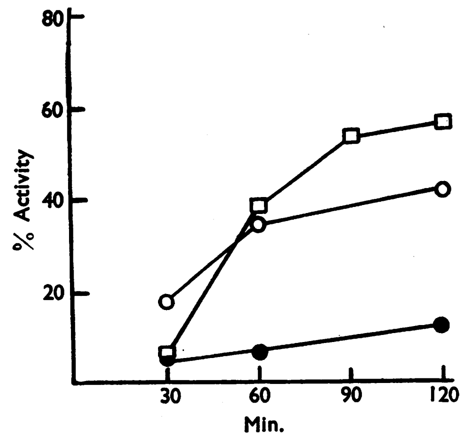

FIG. 3.—*In vivo* effect of P-2-AM on phosphorylated true cholinesterases in blood. Activity of true cholinesterases in blood samples taken from mice injected with 0.4 mg./kg. TEPP (●) and mice which had 25 mg./kg. P-2-AM either 5 min. before (○) or 30 min. after (□) the organophosphate. All enzyme activities are corrected for *in vitro* reactivation by the procedure shown in Fig. 1. Abscissa: Interval between injection of TEPP and collection of blood. Ordinate: Enzyme activity as % of control.

TABLE I

IN VIVO EFFECT OF P-2-AM ON THE PHOSPHORYLATED TRUE CHOLINESTERASES IN BLOOD OF MICE INJECTED WITH A SUBLETHAL DOSE OF TEPP, E 600, Ro 3-0340 OR Ro 3-0422

The degree of reactivation was calculated as follows: % reactivation of phosphorylated enzyme $=100\times\frac{a-b}{c-b}$ where a, b, and c represent the activities of the true cholinesterases in blood samples. a, refers to mice injected with P-2-AM and an organophosphate; b, to mice injected with an organophosphate alone; and c, to mice used as controls. All enzyme activities are corrected for *in vitro* reactivation (see Methods and Fig. 1).

Min. after Injection of P-2-AM	% Reactivation if 25 mg./kg. P-2-AM is Injected	
	5 Min. Before Organophosphate	30 Min. After Organophosphate
30–35	10–24	31–39
60–65	27–35	45–52
120–125	33–40	49–56

The true cholinesterase of brain was only inhibited in those mice which were injected with 0.4 mg./kg. TEPP or E 600; 0.02 mg./kg. Ro 3-0422 or 5 mg./kg. Ro 3-0340 had no effect on it. Experiments in which 25 mg./kg. P-2-AM was injected either 5 min. before or 30 min. after 0.4 mg./kg. TEPP or E 600 was given showed that the oxime had only a small effect on the phosphorylated cholinesterase of the brain (Table II) and the highest degree of reactivation obtained in any individual experiment was 7%. Control experiments in which the brains of mice which had been injected with either E 600 or E 600 and P-2-AM were homogenized together with the brains taken from normal mice showed that no measurable inhibition took place during homogenization and incubation in the Warburg bath; thus it is unlikely that *in vitro* artifacts obscured any reactivation which had taken place *in vivo*.

TABLE II

EFFECT OF 25 MG./KG. P-2-AM ON THE ACTIVITY OF TRUE CHOLINESTERASE IN BRAIN OF MICE INJECTED WITH 0·4 MG./KG. E 600

	Activity as % of Controls After	
	3 Hr.	6 Hr.
Experiment 1:		
E 600 only	14	17
P-2-AM 5 min. before E 600 ..	16	23
P-2-AM 30 min. after E 600 ..	15	16
Experiment 2:		
E 600 only	16	16
P-2-AM 5 min. before E 600 ..	14	20
P-2-AM 30 min. after E 600 ..	18	19

Experiments with Organophosphates which Form a Diisopropylphosphoryl Enzyme.—An

injection of 25 mg./kg. P-2-AM had no consistent effect on the phosphorylated true cholinesterases of blood or brain if the mice were injected with DFP, D 600 or Ro 3-0351.

Protection against Lethal Organophosphate Poisoning by P-2-AM, Atropine and a Combination of Atropine and P-2-AM

If mice were injected with 25 mg./kg. P-2-AM 5 min. before the administration of TEPP, E 600, Ro 3-0422 or Ro 3-0340 different degrees of protection were obtained against lethal doses of these organophosphates. Table III shows that the protection is greatest in those mice which were injected with Ro 3-0340, less in the mice which were injected with E 600, and smallest if TEPP or Ro 3-0422 were used.

TABLE III

PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING

Column a gives the lethal dose for mice in mg./kg. Protection is shown in columns b, c, and d and expressed in multiples of the lethal dose (given in column a) required to cause death under the experimental conditions. Whenever a range is given partial survival was obtained with the lower dose and all mice died with the higher dose. Columns b, c, and d refer to the following conditions: b, 25 mg./kg. P-2-AM was given 5 min. before the organophosphate. c, 50 mg./kg. atropine was given 30 min. before the organophosphate. d, 50 mg./kg. atropine and 25 mg./kg. P-2-AM were given 30 min. and 5 min. respectively before the organophosphate.

Organophosphate	Lethal Dose in mg./kg. (a)	Multiples of Lethal Dose		
		(b)	(c)	(d)
TEPP	0·7	2	1–2	32
Ro 3-0422 ..	0·04	2	1–2	8–16
E 600	0·6	2–4	2	>128
Ro 3-0340 ..	10	8	1–2	32–64
DFP	5	1–2	2	16–32
Ro 3-0351 ..	25	1–2	1–2	8

The protection by P-2-AM against lethal doses of organophosphates which form a di*iso*propylphosphoryl enzyme was far smaller than that against organophosphates which form a diethylphosphoryl enzyme. A dose of 25 mg./kg. P-2-AM saved the life of less than 50% of the mice which were injected with one lethal dose of DFP or Ro 3-0351 and no protection was observed in experiments with D 600.

If P-2-AM treated mice were injected with a dose of an organophosphate which was lethal under these conditions all the characteristic sequelae of cholinesterase inhibition, like salivation, diarrhoea and overactivity of striped muscle, were seen. This suggested that the protection by P-2-AM against lethal doses of organophosphates might be enhanced by atropine.

Mice which were injected with 50 mg./kg. atropine 30 min. before a lethal dose of the organophosphate was given showed only nicotinic but no muscarinic symptoms of acetylcholine poisoning. The antidotal effect of 50 mg./kg. atropine was smaller than that of 25 mg./kg. P-2-AM if TEPP, E 600, Ro 3-0340 or Ro 3-0422 were used, but for DFP, D 600 and Ro 3-0351 the reverse was true (Table III).

Mice which were injected with 50 mg./kg. atropine and 25 mg./kg. P-2-AM were protected to a greater extent than would have been expected from an additive effect of the two antidotes, and the degree of protection was determined by the organophosphate itself and not by its dialkylphosphoryl group (Table III). The majority of the mice died within 20 min. of the injection of a dose of an organophosphate which was lethal under these conditions, and all mice which survived the first hour remained alive during the period of observation of 6 hr. The findings with E 600 and DFP were in agreement with the results obtained by Kewitz *et al.* (1956).

No further protection was obtained if, in addition to atropine and P-2-AM, 100 mg./kg. nikethamide, 7 mg./kg. picrotoxin, or 60 mg./kg. leptazol were injected intraperitoneally together with the oxime.

Mice which were injected with 25 mg./kg. l-hyoscine and 25 mg./kg. P-2-AM showed the same degree of protection against TEPP as those mice which received 50 mg./kg. atropine and 25 mg./kg. P-2-AM. On the other hand, the combination of hyoscine and P-2-AM was approximately twice as effective as the combination of atropine and P-2-AM in Ro 3-0422 poisoning.

Effect of Ethanol.—The results obtained with D 600 are not included in Table III, since its low solubility in water necessitated the use of ethanolic solutions with all doses and ethanol itself modified the results of toxicity tests to a considerable extent.

Mice which had been injected subcutaneously with 0.1 ml. ethanol became less lively, swayed and walked unsteadily when roused; this effect lasted from 2 to 4 hr. If ethanolic solutions of the organophosphates were injected, the lethal dose was approximately double that found when aqueous solutions of the organophosphates were used; this also applied to mice which had been pretreated with either P-2-AM or atropine. Mice which had received both atropine and P-2-AM and were then injected with higher doses of ethanolic solutions of the organophosphates became unconscious within a short time after the injection of the organophosphate and showed a considerable reduction in rate and depth of respiration. Under these conditions it became exceedingly difficult to establish the exact time of

death, especially since the incidence of death was spread over the entire period of observation (6 hr.) and the mice just " faded away " without terminal convulsions or other noticeable symptoms. Experiments in which TEPP and Ro 3-0340 were injected into mice which had received both

TABLE IV

EFFECT OF ETHANOL ON THE PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING

For explanation of cols. a, b, c, and d, see Table III.

Organophosphate	Lethal Dose in mg./kg. (a)	Multiples of Lethal Dose		
		(b)	(c)	(d)
TEPP :				
Aqueous ..	0·7	2	1–2	32
Ethanolic ..	1·4	2	2	8
Ro 3-0340 :				
Aqueous ..	10	8	1–2	32–64
Ethanolic ..	20	2	1	16

atropine and P-2-AM showed that the protection obtained under these conditions was less than that observed when aqueous solutions of the organophosphates were used (Table IV).

DISCUSSION

The interaction between true cholinesterase and an organophosphate leads to the formation of a phosphorylated enzyme which is enzymically inactive. Thus the injection of an organophosphate is followed by an accumulation of acetylcholine (which is constantly released from its store at various sites) and corresponding symptoms (Douglas and Paton, 1951 ; Stewart, 1952 ; Barnes and Duff, 1954). Acute death from organophosphate poisoning always appears to be the result of respiratory failure, although its character varies from one species to another (Modell and Krop, 1946 ; Candole, Douglas, Evans, Holmes, Spencer, Torrance, and Wilson, 1953). If acetylcholine is indeed responsible for the acute lethal effects of organophosphate poisoning one would expect that antagonists of its actions should be useful antidotes. During the past 20 years a number of competitive antagonists of the muscarinic actions of acetylcholine have been tested in this respect and found to give only a limited degree of protection, even when used in high doses. This also applies to antagonists of the nicotinic actions of acetylcholine. On the other hand, animals which receive positive pressure ventilation in addition to atropine (Barnes, 1953) or which are pretreated with antagonists of the muscarinic as well as the nicotinic actions of acetylcholine show a far greater degree of protection (Candole and McPhail, 1954 ; Parkes and Sacra, 1954 ; Lewis, McKeon, and Lands, 1955). This seems to indicate that it is not the accumulation of acetylcholine at any particular site which is responsible for death in acute organophosphate poisoning, but that some mechanism is involved which requires the accumulation of acetylcholine at various sites and which is also linked to respiration.

The danger connected with the combined use of several antidotes, each of which might produce lethal effects on its own, necessitates an entirely new approach, for example reactivation of phosphorylated cholinesterases or enzyme protection, for the treatment of organophosphate poisoning.

It has recently been found that relatively small amounts of certain oximes speedily restore the activity of freshly phosphorylated true cholinesterases *in vitro* (Childs *et al.*, 1955 ; Wilson and Ginsburg, 1955). The results presented in this paper show that in the mouse pyridine-2-aldoxime methiodide (P-2-AM), which was the most active oxime found so far, was less effective in reactivating phosphorylated true cholinesterases *in vivo* than was indicated by the *in vitro* experiments. According to the findings of Askew, Davies, Green, and Holmes (1956) with related oximes one would expect that, after an intraperitoneal injection of 25 mg./kg. P-2-AM, a concentration of 1 to 4×10^{-4} M of the oxime would be maintained in the blood for at least 15 min. In *in vitro* experiments, such concentrations were sufficient to restore within a few minutes more than 90% of the activity of true cholinesterases if they were inhibited by organophosphates which formed a diethylphosphoryl enzyme. However, the *in vivo* reactivation during 30 min. amounted to only 10 to 24% if 25 mg./kg. P-2-AM was injected 5 min. before TEPP, E 600, Ro 3-0340, or Ro 3-0422, and varied from 31 to 39% if the oxime was injected 30 min. after the organophosphate. Since the oxime was given intraperitoneally in a volume of 0.2 ml./20 g. mouse, it was possible that the rate of absorption was unfavourable and the concentrations of P-2-AM in blood have to be measured before the *in vivo* results can be fully correlated with the *in vitro* results. Davies and Green (1956) have shown that the active factor in the reactivation process is the anion of the oxime, and differences in *p*H could also be partly responsible for the observed differences between *in vitro* and *in vivo* effectiveness of P-2-AM. The finding that blood samples which were collected 30 min. after the injection of TEPP or other organophosphates in P-2-AM treated mice contained an effective

concentration of the oxime but could only be partly reactivated during incubation at 37° in the Warburg cannot be interpreted at the present moment, since nothing is known about protein binding and the metabolism of P-2-AM under the experimental conditions; accelerated transphosphorylation (Hobbiger, 1956; Davies and Green, 1956) has also to be considered in this connexion.

The time course of reactivation of phosphorylated true cholinesterases indicates that the concentration of the oxime in blood falls below an effective level within 1 to 2 hr. after a single injection of 25 mg./kg. P-2-AM. This is in agreement with the findings of Kewitz *et al.* (1956), who studied the protection against E 600 in mice which had received 40 mg./kg. P-2-AM intraperitoneally at various intervals before the injection of the organophosphate and found that the antidotal effect of the oxime was greatly reduced within 2 hr.

A dose of 25 mg./kg. P-2-AM had only a negligible effect on the phosphorylated true cholinesterases in the brain of mice injected with TEPP or E 600, although the oxime was a highly effective reactivator when allowed to act on brain homogenates. P-2-AM behaved in this respect like other quaternary ammonium compounds, such as organophosphates (Burgen and Hobbiger, 1951; Hobbiger, 1954; Koelle and Steiner, 1956). Mayer and Bain (1956) have shown that the chief barrier to the entrance of quaternary ammonium compounds into the brain lies between the outer wall of the capillary endothelium and the glial plasma membrane and that this barrier can be overcome by higher doses. Thus the failure of P-2-AM to reach the brain is not absolute, and with higher doses of the oxime a greater degree of reactivation has been obtained (Kewitz and Nachmansohn, 1957).

25 mg./kg. P-2-AM was without any *in vivo* effect if the true cholinesterases were inhibited by organophosphates which formed a di*iso*propylphosphoryl enzyme (DFP, Ro 3-0351 or D 600). This was to be expected from the effect of P-2-AM on the inhibition produced by organophosphates which form a diethylphosphoryl enzyme since the *in vitro* experiments showed that the di*iso*propylphosphoryl enzyme was approximately 30 times less sensitive to P-2-AM than the diethylphosphoryl enzyme.

If one compares the *in vivo* effect of P-2-AM on phosphorylated true cholinesterases with its antidotal effect in lethal organophosphate poisoning it is difficult to interpret the latter entirely in terms of cholinesterase reactivation as has been postulated by Kewitz and Wilson (1956), Kewitz *et al.* (1956), Kewitz (1957) and Kewitz and Nachmansohn (1957). Progressive enzyme reactivation took place during a period of 1 to 2 hr. following the injection of the organophosphate if mice were injected with 25 mg./kg. P-2-AM 5 min. before a sublethal dose of an organophosphate which forms a diethylphosphoryl enzyme (TEPP, E 600, Ro 3-0340 or Ro 3-0422). However, both *in vitro* and *in vivo* experiments failed to show that P-2-AM was more effective in restoring enzyme activity if inhibition was produced by Ro 3-0340 or E 600 than if inhibition was produced by TEPP or Ro 3-0422. It is thus difficult to understand why protection by P-2-AM decreased in the order: Ro 3-0340 > E 600 > TEPP ≡ Ro 3-0422. Different degrees of protection were also found if the mice received atropine as well as P-2-AM before the injection of the organophosphate. E 600 and Ro 3-0340 are far more stable than TEPP or Ro 3-0422, and one is tempted to speculate that differences in the rates of *in vivo* phosphorylation of true cholinesterases account for the observed differences in protection. Experiments with organophosphates which form a di*iso*propylphosphoryl enzyme (DFP and Ro 3-0351) speak against such an interpretation. Mice which were pretreated with atropine and P-2-AM were protected to a greater extent against DFP than against Ro 3-0422, and protection against TEPP was only slightly greater than protection against DFP. Since P-2-AM had no measurable effect on the inhibition produced by DFP it is difficult to see how the protection obtained by the combined use of atropine and P-2-AM could be entirely the result of enzyme reactivation. Askew (1956), who studied the antidotal properties of a series of oximes, was also unable to find any direct correlation between their potency as reactivators of sarin-inhibited cholinesterase *in vitro* and their effectiveness as antidotes of sarin poisoning of rats *in vivo*.

It is possible that the actions of P-2-AM are not restricted to phosphorylated cholinesterases and that other actions play some part in the protection against organophosphates under certain conditions, for example, when mice are injected with atropine and P-2-AM. Holmes and Robins (1955) have shown that P-2-AM blocks neuromuscular transmission in the isolated phrenic nerve diaphragm of the rat in concentrations which are required for enzyme reactivation. Furthermore, P-2-AM is an anticholinesterase in its own right (Hobbiger, 1956). Both neuromuscular blocking

agents and combinations of atropine and eserine are antidotes of lethal organophosphate poisoning. The protection obtained by the combined use of atropine and eserine is particularly effective, although eserine on its own is only a very feeble antidote (Koster, 1946; DuBois, Doull, Okinaka and Coon, 1953). Thus eserine shares with P-2-AM the property of greatly potentiating the effect of atropine.

It is widely believed that organophosphates possess other actions in addition to those mediated by cholinesterase inhibition (for references, see Murtha, McNamara, Edberg, Bergner, and Wills, 1955; Cohen and Posthumus, 1955; Groblewski, McNamara, and Wills, 1956; Naess, 1956). Perhaps some of the differences observed in the experiments reported here are caused by them.

Askew (1956) has recently shown that the antidotal potency of a number of oximes varies between different species. This has to be borne in mind before the results in the mouse, especially the antidotal effects obtained by the combined use of atropine and P-2-AM, are generalized.

The present work shows that the results obtained with one particular organophosphate (Kewitz *et al.*, 1956; Askew, 1956) are not applicable to other organophosphates which form the same type of phosphorylated enzyme. Screening of new oximes for their antidotal properties should, therefore, include as many organophosphates as possible and by doing so organophosphate insecticides could be selected which are less dangerous than some of those which are widely used nowadays.

I wish to thank Dr. A. L. Morrison, of Roche Products Ltd., Welwyn Garden City, for a generous supply of the organophosphates of the Roche series and Dr. D. R. Davies, of the Chemical Defence Establishment, Porton, for a gift of pyridine-2-aldoxime methiodide.

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