

# CHEMICAL REACTIVATION OF PHOSPHORYLATED HUMAN AND BOVINE TRUE CHOLINESTERASES

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Organophosphates, such as tetraethyl pyrophosphate (TEPP) and diisopropyl phosphorofluoridate (DFP), which have a dialkylphosphato group, form with human plasma cholinesterase (also called pseudo- or butyro-cholinesterase) two types of phosphorylated enzyme which possess different stabilities of the enzyme-phosphorus linkage (Hobbiger, 1955). Only phosphorylated enzyme I, which is formed initially, can be readily reactivated by nucleophilic reagents such as nicotinhydroxamic acid methiodide (NHAM). Phosphorylated enzyme I then changes into phosphorylated enzyme II, most probably by transphosphorylation. With DFP or diisopropyl *p*-nitrophenylphosphate (D 600) the rate of transphosphorylation is faster than with TEPP or diethyl *p*-nitrophenylphosphate (E 600) and at 37° C. and a pH of 7.45 approximately 90% of the inhibited enzyme is converted into phosphorylated enzyme II within 1 hour.

Since the hydrolysis of acetylcholine *in vivo* is mainly accomplished by true cholinesterase (also called aceto-cholinesterase) the formation of a phosphorylated true cholinesterase which cannot be reactivated by nucleophilic reagents could seriously affect the therapeutic usefulness of NHAM and other more potent substances. The experiments on human plasma cholinesterase were therefore extended to human and bovine true cholinesterases which had been inhibited by an organophosphate containing either a diethyl- or diisopropylphosphato group and the findings are reported in this paper. In addition to NHAM, ammonium molybdate and pyridine-2-aldoxime methiodide, a potent reactivator of phosphorylated electric eel cholinesterase and phosphorylated human and rat true cholinesterases (Wilson and Ginsburg, 1955; and Childs, Davies, Green, and Rutland, 1955), were used for enzyme reactivation. The results obtained with all these reactivators show that the organophosphates form two types of phosphorylated enzyme with human and bovine true cholinesterases which thus behave like plasma cholinesterase. Only the initial

form (phosphorylated enzyme I) can readily be reactivated by the nucleophilic reagents. Transphosphorylation—formation of the truly irreversible phosphorylated enzyme II—occurs both *in vitro* and *in vivo*, and the rate of transphosphorylation is dependent upon pH.

## METHODS

All experiments were carried out in the Warburg apparatus.

Cholinesterase activity was determined by the manometric technique using 0.01 M-acetylcholine chloride as substrate for human and bovine true cholinesterases and 0.02 M-(±)-acetyl-β-methylcholine chloride as substrate for the true cholinesterases of whole rabbit blood. The procedures adopted for determination of enzyme activity and reactivation were the same as those described in an earlier publication (Hobbiger, 1955).

Except when otherwise stated, the gas phase consisted of 95% N<sub>2</sub>+5% CO<sub>2</sub> and a solution containing 0.025 M-NaHCO<sub>3</sub>, 0.075 M-NaCl, 0.075 M-KCl, 0.04 M-MgCl<sub>2</sub> and 0.1% bovine plasma albumin (Armour Laboratories) was used for all dilutions and as the medium during inhibition, reactivation and determination of enzyme activity.

The enzyme preparations used were human red cells which had been twice washed with 0.9% NaCl, purified bovine erythrocyte cholinesterase (Winthrop-Stearns Inc.) and heparinized whole rabbit blood.

The organophosphates used as inhibitors had either a diethyl- or diisopropylphosphato group in common; they were:

- (a) organophosphates containing a diethylphosphato group:
  - tetraethyl pyrophosphate (TEPP),
  - diethyl *p*-nitrophenylphosphate (E 600),
  - 3-(diethoxyphosphinyloxy)-*N*-trimethylanilinium methyl sulphate (Ro 3-0340),
  - 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3-0422).
- (b) organophosphates containing a diisopropylphosphato group:
  - diisopropyl phosphorofluoridate (DFP),
  - diisopropyl *p*-nitrophenylphosphate (D 600).

The substances used for reactivation of the inhibited enzyme were:

nicotinhydroxamic acid methiodide (NHAM)\*  
ammonium molybdate (AM) and  
pyridine-2-aldoxime methiodide (P<sub>2</sub>AM).

Solutions of these reactivators were freshly made before use. Solutions of NHAM, AM, and trimethylamine (TrMA) were adjusted with NaOH to pH 7.45 at which all the experiments were carried out.

Throughout the text the inhibitors and reactivators are referred to by the abbreviations given above.

## RESULTS

### Interaction of Organophosphates and Reactivators

NHAM, AM and P<sub>2</sub>AM react with all the organophosphates which were used for enzyme inhibition to give products which are without anticholinesterase activity. With the reactivator in excess, the inactivation of the organophosphates by reactivators shows the kinetics of a first order reaction (Fig. 1) and no evidence of an equilibrium is detectable. The rate of inactivation of the organophosphates is inversely related to their stability in aqueous solutions, and the differences in potency between the three reactivators are shown in Table I.

The nature of the interaction of organophosphates and reactivators was studied by the manometric technique. Under the same conditions as were used for reactivation of the inhibited enzyme all three reactivators promote the hydrolysis of the organophosphates (Fig. 2). The total amount of acid produced in experiments in which the reaction was allowed to go to completeness was identical with that expected from the appearance of two acidic radicals for each molecule of organophosphate.

### Experiments with Human True Cholinesterase

**Affinity of Reactivators for Human True Cholinesterase.**—In the presence of 0.01 M-acetylcholine chloride only NHAM and P<sub>2</sub>AM inhibit the enzyme to any significant extent in concentrations below 10<sup>-1</sup> M (Table II). The inhibition is readily reversed by dilution and is independent of the sequence in which enzyme, substrate and reactivator are mixed.

**Effect of Reactivators on Human True Cholinesterase Inhibited by Organophosphates Containing a Dialkylphosphato Group.**—The inhibition of human true cholinesterase by TEPP, Ro 3-0340, Ro 3-0422, or DFP cannot be reversed by addition of substrate, and after a dilution which is sufficient to reduce the organophosphate concentration to ineffective levels

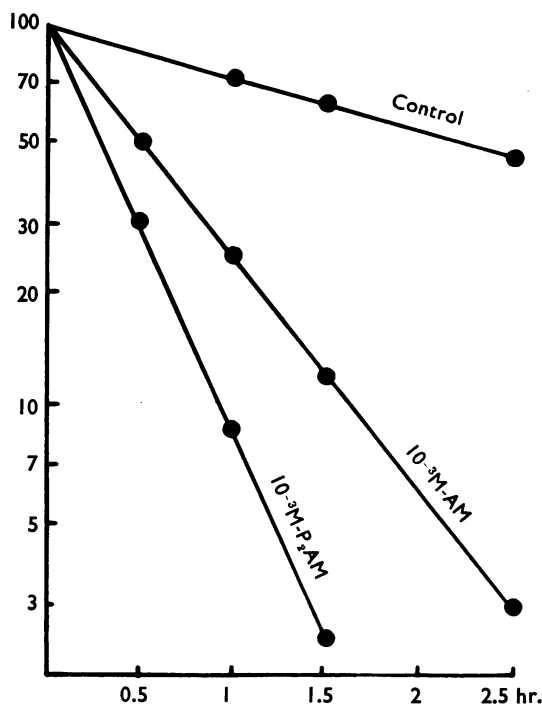


FIG. 1.—Time course of inactivation of 10<sup>-4</sup>M-TEPP by 10<sup>-3</sup>M-AM and 10<sup>-3</sup>M-P<sub>2</sub>AM at 37° C. and pH 7.45. Experimental conditions as described in legend of Table I. Abscissa: duration of incubation. Ordinate: residual activity in % of the activity of freshly made solutions of TEPP (logarithmic scale).

enzyme recovery never exceeds 5% during the first hour (Burgen and Hobbiger, 1951; and Hobbiger, 1954). This also applies to inhibition produced by E 600 or D 600.

Usually the addition of NHAM, AM or P<sub>2</sub>AM to human true cholinesterase, which has been inhibited by one of the above-mentioned six organophosphates, markedly increases enzyme activity

TABLE I  
INACTIVATION OF ORGANOPHOSPHATES BY REACTIVATORS AT 37° C. AND pH 7.45

All incubations were carried out in the Warburg apparatus using the same medium and gas phase as for reactivation and determination of enzyme activity. The loss in anticholinesterase potency was calculated from the changes in the molar concentration of the organophosphates required for 50 % inhibition of human plasma cholinesterase under standard conditions.

Organo- phosphate (10 <sup>-4</sup> M)	% Reduction in Activity after 1 Hr. Incubation with						
	—	NHAM		AM		P <sub>2</sub> AM	
		10 <sup>-2</sup> M	10 <sup>-3</sup> M	10 <sup>-2</sup> M	10 <sup>-3</sup> M	10 <sup>-2</sup> M	10 <sup>-3</sup> M
TEPP .. ..	28	>99	53	>99	75	>99	91
E 600 .. ..	<1	3	—	6	—	7	—
DFP .. ..	18	46	21	>99	53	>99	44
D 600 .. ..	<1	5	—	6	—	6	—

\* In a previous publication (Hobbiger, 1955) NHA was used as the abbreviation; to avoid confusion with nicotinhydroxamic acid (Wilson; 1955, and Wilson, Ginsburg and Meislich, 1955) the abbreviation NHAM is preferred in this paper.

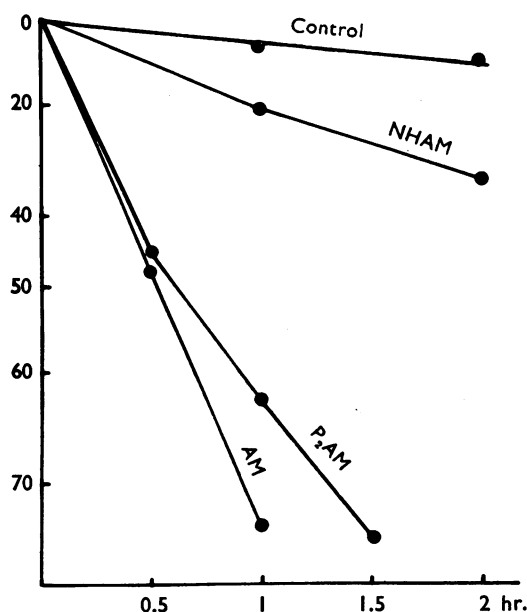


FIG. 2.—Hydrolysis of  $2.5 \times 10^{-3}$ M-Ro 3-0422 by reactivators at  $37^\circ$  C. and pH 7.45. Ordinate: % hydrolysis of Ro 3-0422 as calculated by the ratio:  $100 \times$  amount of  $\text{CO}_2$  released/amount of  $\text{CO}_2$  that would be released by the liberation of two acidic radicals per molecule Ro 3-0422 (logarithmic scale). Abscissa: time after addition of reactivator (final concentration  $2.5 \times 10^{-3}$ M) to Ro 3-0422.

within 1 hour. As with human plasma cholinesterase (Hobbiger, 1955), speed and degree of reactivation of inhibited human true cholinesterase at  $37^\circ$  C. and pH 7.45 depend on the type of organophosphate and reactivator, the concentration of free organophosphate, and the duration of enzyme inhibition.

**Reactivation after Short Inhibition.**—The greatest degree of enzyme reactivation is obtained after short exposures of human true cholinesterase to an organophosphate. The following standard conditions were therefore chosen for studying the effect of the reactivators:

Human red cells were incubated for 20 min. with an organophosphate in a concentration required to produce between 80 and 90% inhibition. The solution containing enzyme plus inhibitor was then diluted with an equal volume of reactivator and 0.3

ml. of this mixture was added to the side-arms of the manometric vessels for further incubation. Enzyme activity was determined by adding the contents of the side-arms to the centre of the manometric vessels which contained 2.7 ml. of substrate dissolved in buffer. The percentage reactivation was calculated from the expression:

$$100 \times \frac{(\text{activity of reactivated enzyme} - \text{activity of inhibited enzyme})}{\text{activity of control enzyme} - \text{activity of inhibited enzyme}}$$

Reactivations up to 75 to 80% were used for evaluation of the time course of reactivation and comparison of the activity of the three reactivators.

Under these conditions the following results were obtained:

If the enzyme is inhibited by TEPP or Ro 3-0422, NHAM in a concentration of  $5 \cdot 10^{-3}$ M or higher readily restores a considerable amount of enzyme activity within 1 hour and the time course of reactivation closely approaches that of a first order reaction (Fig. 3). The same concentrations of

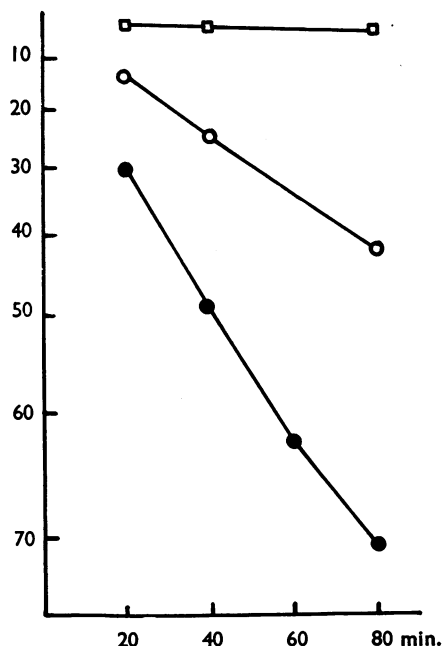


FIG. 3.—Enzyme reactivation by NHAM. The enzyme was first incubated for 20 min. with  $2 \cdot 10^{-3}$ M-TEPP or  $10^{-4}$ M-DFP. For reactivation equal volumes of reactivator and the mixture of enzyme plus inhibitor were used. ●—●: inhibition by TEPP; reactivator,  $1 \cdot 10^{-3}$ M-NHAM. ○—○: inhibition by DFP; reactivator,  $5 \cdot 10^{-3}$ M-NHAM. □—□: inhibition by DFP; reactivator,  $1 \cdot 10^{-3}$ M-NHAM. Abscissa: duration of exposure of the inhibited enzyme to NHAM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

TABLE II

Reactivator	Molar Concentration Producing 50% Enzyme Inhibition in the Presence of 0.01M-acetylcholine
P <sub>3</sub> AM	$3 \cdot 5 \cdot 10^{-3}$
NHAM	$5 \cdot 10^{-2}$
AM	$\geq 10^{-1}$

NHAM also reduce inhibition by Ro 3-0340 or E 600, but the rate of enzyme reactivation becomes progressively slower with time. NHAM has only a very small effect on the inhibition produced by DFP or D 600, and enzyme recovery amounts to less than 10% during the first hour after addition of 0.1 M-NHAM to the inhibited enzyme (Fig. 3).

AM is more effective than NHAM in restoring enzyme activity after inhibition by TEPP, Ro 3-0422, or DFP, and the rate of reactivation always closely approaches that of a first order reaction (Fig. 4). If the enzyme is inhibited by DFP, the concentration of AM needs to be 22 times higher to obtain the same effect as after inhibition by TEPP or Ro 3-0422. With either Ro 3-0340, E 600 or D 600 as inhibitor, less than 25% of the enzyme activity is restored by AM in concentrations which within 1 hour restore 60% of the activity of an enzyme inhibited by a more labile organophosphate with the same dialkylphosphato group (Fig. 4).

P<sub>2</sub>AM is the most potent reactivator, but like AM its effectiveness depends on the organophosphate which is used for enzyme inhibition. With TEPP

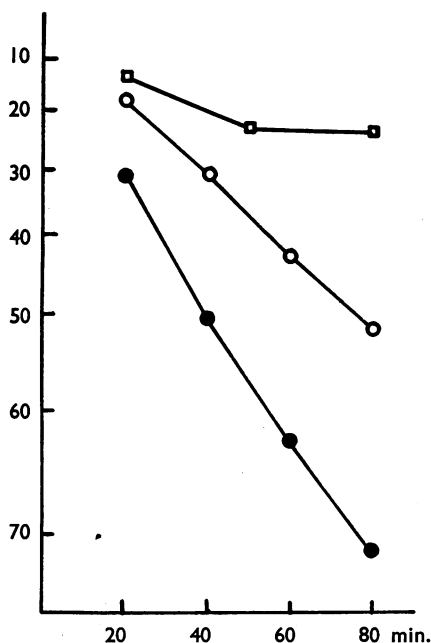


FIG. 4.—Enzyme reactivation by AM. The enzyme was first incubated for 20 min. with  $1.10^{-5}$ M-Ro 3-0422,  $5 \times 10^{-5}$ M-E 600 or  $10^{-5}$ M-DFP. For reactivation equal volumes of reactivator and the mixture of enzyme plus inhibitor were used. ●—●: inhibition by Ro 3-0422; reactivator,  $3.10^{-5}$ M-AM. □—□: inhibition by E 600; reactivator,  $3.10^{-5}$ M-AM. ○—○: inhibition by DFP; reactivator,  $4.10^{-2}$ M-AM. Abscissa: duration of exposure of the inhibited enzyme to AM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

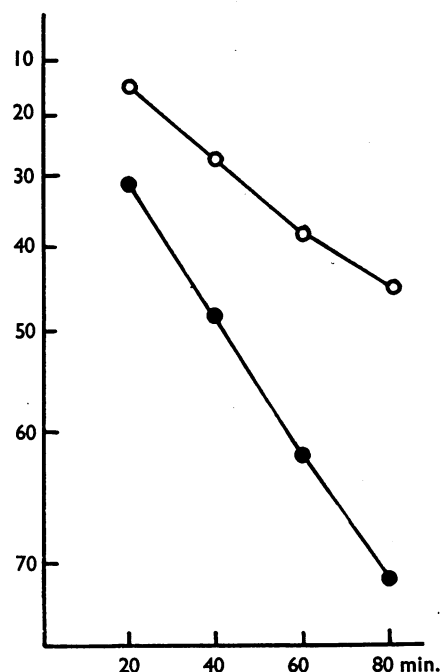


FIG. 5.—Enzyme reactivation by P<sub>2</sub>AM: The enzyme was first incubated with  $2.10^{-7}$ M-TEPP for 20 min. Equal volumes of reactivator and the mixture of enzyme plus inhibitor were used for reactivation. ●—●: reactivation by  $3.10^{-5}$ M-P<sub>2</sub>AM. ○—○: reactivation by  $3.10^{-5}$ M-P<sub>2</sub>AM in the presence of  $2.10^{-3}$ M-TrMA. Abscissa: duration of exposure of the inhibited enzyme to P<sub>2</sub>AM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

as the inhibitor P<sub>2</sub>AM in a concentration of  $2.10^{-5}$ M or higher restores a considerable amount of enzyme activity within 1 hour (Fig. 5). A similar effect is obtained after inhibition by DFP with P<sub>2</sub>AM in a concentration of  $5.10^{-4}$ M or higher. The rate of enzyme reactivation approaches that of first order reaction in each instance. Concentrations of P<sub>2</sub>AM which reactivate an enzyme inhibited by TEPP are less effective if E 600, Ro 3-0340 or Ro 3-0422 are the inhibitors and the rate of recovery deviates from first order kinetics. Similar results are obtained if one compares the reactivation after inhibition by D 600 with the reactivation after inhibition by DFP.

The differences in the rate of reactivation of enzymes inhibited by organophosphates with a common dialkylphosphato group are the result of interference of free inhibitor with the reactivation process.

If enzyme reactivation is allowed to proceed in the centre of the manometric vessels—which is, however, not possible with NHAM since this reactivator markedly inhibits cholinesterase in the concentra-

TABLE III

MOLAR CONCENTRATION OF REACTIVATOR REQUIRED TO PRODUCE 50% REACTIVATION OF COMPLETELY INHIBITED HUMAN TRUE CHOLINESTERASE IN 60 MIN. The enzyme is incubated with the inhibitor for 20 min. before reactivation and any interference of free inhibitor with the reactivation process is eliminated by dilution.

Reactivator	Inhibition Produced by an Organophosphate Containing a	
	Diethylphosphato Group	Diisopropylphosphato Group
NHAM	7.5 . $10^{-2}$	> $10^{-1}$
AM	2.3 . $10^{-3}$	5.1 . $10^{-2}$
P <sub>2</sub> AM	2.7 . $10^{-5}$	6.1 . $10^{-4}$

tions required for reactivation (see Tables II and III)—the free inhibitor is 20 times diluted and thus cannot significantly interfere with the reactivation process. Under such conditions enzyme reactivation shows the characteristics of a first order reaction, and identical results are obtained with equimolar concentrations of a given reactivator as long as organophosphates with the same dialkylphosphato group are used. With sufficiently high concentrations of the reactivator more than 90% of enzyme activity can be restored, and the time required to achieve this is inversely related to the concentration of the reactivating agent.

Table III gives the molar concentrations of the reactivators which in the absence of interference by free organophosphate produce 50% enzyme reactivation within 1 hour.

*Effect of Trimethylamine (TrMA) on Enzyme Reactivation.*—During reactivation NHAM and P<sub>2</sub>AM, but not AM, become attached to the anionic site of an enzyme inhibited by an organophosphate with a diethylphosphato group. This is shown by the effect of TrMA on reactivation. TrMA is a competitive inhibitor of human true cholinesterase with an I 50 of  $2.10^{-2}$ M in the presence of 0.01 M-acetylcholine chloride. When  $2.10^{-1}$  M-TrMA is present during reactivation in the side-arms of the manometric vessels (see conditions described above) the following results are obtained:

After inhibition by TEPP, TrMA slows down the rate of enzyme reactivation by NHAM or P<sub>2</sub>AM (Fig. 5). TrMA also cancels out the interference by free inhibitor (Ro 3-0340, Ro 3-0422 or E 600) with the reactivation process by preventing further inhibition after reactivation has taken place. Thus in the presence of TrMA very similar rates of enzyme reactivation are obtained no matter which of the four organophosphates containing a diethylphosphato group is used as the inhibitor.

TrMA does not interfere with enzyme reactivation by AM and it has only a negligible effect on the reactivation of DFP inhibited enzyme by P<sub>2</sub>AM.

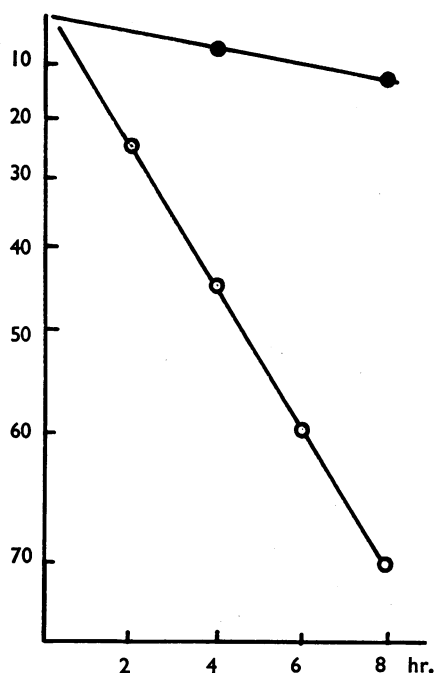


FIG. 6.—Enzyme reactivation after prolonged inhibition. Fresh human red cells were first incubated with the inhibitor. Small samples were taken after various periods of incubation and exposed for 1 hr. to  $10^{-2}$ M-P<sub>2</sub>AM. Enzyme reactivation was then determined after a 10-fold dilution. Similar results were obtained when high concentrations of AM were used for enzyme reactivation. ●—●: enzyme inhibited by an organophosphate containing a diethylphosphato group. ○—○: enzyme inhibited by an organophosphate containing a diisopropylphosphato group. Abscissa: duration of inhibition before addition of reactivator. Ordinate: % enzyme activity which cannot be restored by the reactivator (logarithmic scale).

*Reactivation after Prolonged Inhibition.*—The reactivating power of all three reactivators is inversely related to the duration of enzyme inhibition (Fig. 6) and after prolonged inhibition all three nucleophilic reagents are less effective in restoring enzyme activity. Identical findings are obtained with organophosphates which have the same dialkylphosphato group.

The truly irreversible stage of enzyme inhibition, which will be referred to as phosphorylated enzyme II, is not the result of enzyme deterioration during prolonged incubation at 37° C. Enzyme solutions which are kept at 37° C. for 24 hours and are then incubated with an organophosphate for 20 min. can be reactivated to the same extent as solutions of fresh enzyme.

Similarly phosphorylated enzyme II is also not the result of secondary reactions of the organophosphates with cholinesterase. If this were the case its rate of formation should be proportional to the

lability of the ester link of the organophosphate used and to the concentration of free organophosphate. Identical results are, however, obtained using organophosphates which have a common dialkylphosphato group but which differ widely in their stability; and if free inhibitor is removed by addition of 10% rabbit plasma (final concentration) to red cells which have been in contact with TEPP, E 600, Ro 3-0422 or DFP for 20 min. (in a medium of 0.025 M-NaHCO<sub>3</sub>) the results of reactivation are similar to those observed with controls. Furthermore, a hundredfold increase in the concentration of TEPP or DFP is devoid of any accelerating effect.

Phosphorylated enzyme II thus seems to be the result of transphosphorylation, and its rate of formation will be referred to as "rate of transphosphorylation."

Transphosphorylation is to some extent governed by the incubation medium and proceeds at a faster rate if NaHCO<sub>3</sub> is used as the medium instead of a solution which contains NaHCO<sub>3</sub> + salt. Results with 95% O<sub>2</sub> + 5% CO<sub>2</sub> did not differ from those with 95% N<sub>2</sub> + 5% CO<sub>2</sub>.

All the findings described so far were obtained with fresh red cells as the source of enzyme. The cholinesterase of red cells which have been kept in the deep freeze or over dry ice (in stoppered containers) behaves differently from the cholinesterase of fresh red cells. Whereas fresh red cells lose less than 10% of their cholinesterase activity during 12 hours' incubation at 37° C. under aerobic or anaerobic conditions, the cholinesterase of red cells which have been kept at low temperatures before use is less stable during incubation and shows a greater rate of transphosphorylation under anaerobic conditions (Table IV). Both these effects are far less under aerobic conditions (95% O<sub>2</sub> + 5% CO<sub>2</sub> as gas phase) or under anaerobic conditions (95% N<sub>2</sub> + 5% CO<sub>2</sub> as gas phase) when 10<sup>-3</sup>M-

potassium ferricyanide is present during incubation of the enzyme with an organophosphate. Attempts to make the cholinesterase of fresh red cells behave like the cholinesterase of stored cells by adding a 10<sup>-3</sup>M concentration of glutathione, cysteine, sodium cyanide or potassium ferrocyanide were unsuccessful.

#### *Experiments with Purified Bovine True Cholinesterase*

Purified bovine true cholinesterase which has been inhibited by an organophosphate with a dialkylphosphato group can also be reactivated by NHAM, AM and P<sub>2</sub>AM and, on a whole, the results are identical with those obtained with human true cholinesterase. However, one exception was noticed. The reactivation of TEPP-inhibited bovine true cholinesterase by low concentrations of P<sub>2</sub>AM, e.g., 3.10<sup>-6</sup>M, approaches a maximum during the first 20 min. after addition of the reactivator to the inhibited enzyme and then continues only at a low speed. This discrepancy in the behaviour of the two true cholinesterases is the result of interference by free inhibitor. With the red cell preparation it could be shown that no free TEPP was present 30 minutes after the addition of TEPP to the enzyme; this was not so with bovine true cholinesterase. Removal of free TEPP from bovine true cholinesterase by addition of rabbit plasma to the inhibited enzyme before reactivation (Davison, 1954) revealed that the true time course of enzyme reactivation by P<sub>2</sub>AM is identical for both enzymes, i.e., reactivation is a first order reaction.

Purified bovine true cholinesterase which has been inhibited for long periods also behaves like human true cholinesterase. On prolonged inhibition two types of phosphorylated enzyme are formed. The time course of transphosphorylation has the characteristics of a first order reaction (Fig. 7) and depends on the medium used during incubation. If Mg<sup>2+</sup> is replaced by an equivalent amount of Na<sup>+</sup> or if the experiments are carried out in 0.025 M-NaHCO<sub>3</sub> transphosphorylation proceeds faster than in the usual buffer medium (see Methods). The removal of free inhibitor (achieved by the addition of rabbit plasma to an enzyme which had been in contact with the inhibitor for 20 min.) does not prevent the formation of phosphorylated enzyme II, and its rate of formation under such conditions is intermediate between that found with 0.025 M-NaHCO<sub>3</sub> and 0.025 M-NaHCO<sub>3</sub> + salt as incubation media.

Experiments in which 95% O<sub>2</sub> + 5% CO<sub>2</sub> were used as the gas phase instead of 95% N<sub>2</sub> + 5% CO<sub>2</sub> gave identical results, and storage of the enzyme at

TABLE IV  
EFFECT OF STORAGE AT LOW TEMPERATURES ON THE REVERSIBILITY OF ENZYME INHIBITION

1 hr. contact of the inhibited enzyme with 10<sup>-3</sup>M-P<sub>2</sub>AM was used for reactivation. Inhibition, reactivation and determination of enzyme activity were all carried out in 95% N<sub>2</sub> + 5% CO<sub>2</sub> and with the buffer (described under Methods) as medium.

Source of Enzyme	% Enzyme Activity of Controls after 4 Hr. Incubation	After 4 Hr. Incubation with 2 × 10 <sup>-2</sup> M-TEPP: % Reactivation
(1) Fresh red cells . . . . .	98	93
(2) Red cells which have been kept on dry ice for 30 min. before use . . . . .	81	63
(3) as (2), but after thawing the red cells were kept on dry ice for another 30 min. before use . . . . .	68	24

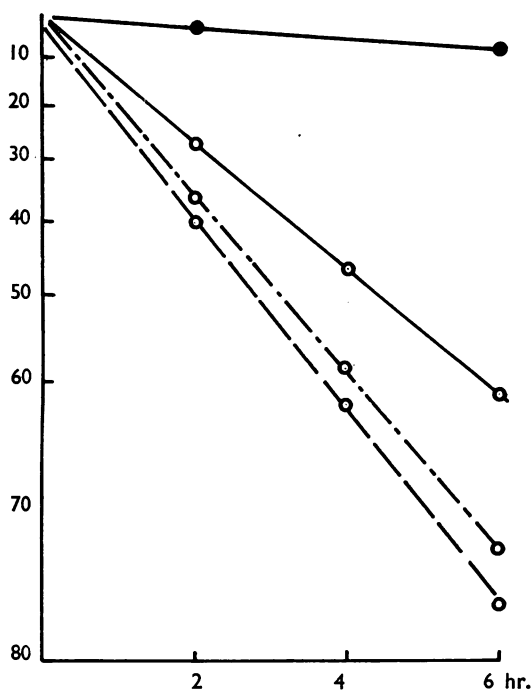


FIG. 7.—Reactivation of purified bovine true cholinesterase after prolonged incubation with organophosphates. Experimental conditions as described in the legend to Fig. 6. Inhibition by TEPP (●—●) or DFP (○—○) using the buffer as medium (described under Methods). ○—○: inhibition by DFP using as medium the same buffer but with  $Mg^{2+}$  replaced by  $Na^+$ . ○—○: inhibition by DFP using 0.025 M- $NaHCO_3$  as medium. Abscissa: duration of inhibition before addition of reactivator. Ordinate: % enzyme activity which cannot be restored by the reactivator (logarithmic scale).

low temperatures before use had no effect on enzyme stability or on the rate of transphosphorylation.

To study the effect of pH on transphosphorylation the enzyme was incubated with the inhibitor in solutions containing different concentrations of  $NaHCO_3$  while retaining the gas phase of 95%  $N_2$

TABLE V

EFFECT OF pH ON THE RATE OF FORMATION OF PHOSPHORYLATED ENZYME II

Purified bovine true cholinesterase was incubated with  $1 \times 10^{-7}M$ -TEPP for 16 hr. or  $1 \times 10^{-6}M$ -DFP for 2 hr. The inhibited enzyme was then incubated with  $P_2AM$  for 1 hr. and enzyme activity was determined after a 10-fold dilution.

pH	% Enzyme Activity Restored by $10^{-6}M$ - $P_2AM$ after Inhibition by	
	TEPP	DFP
8.0	86	75
7.5	77	59
7.0	61	39
6.5	55	33
6.0	52	33

TABLE VI

IN VITRO REACTIVATION OF THE TRUE CHOLINESTERASES OF A RABBIT TREATED WITH DFP *IN VIVO*

Blood samples were taken before and after an i.m. injection of 1 mg. DFP (in arachis oil)/kg. A part of each blood sample was incubated for 1 hr. with  $10^{-3}M$ - $P_2AM$  or  $10^{-3}M$ -AM before addition of substrate. Enzyme activity was determined with 0.02 M-(±)-acetyl-β-methylcholine chloride. All enzyme activities are expressed in % of enzyme activity before the injection of DFP.

Hours After the Injection of 1 mg. DFP/kg.	Enzyme Activity of	
	Untreated Blood	Blood Treated with the Reactivator
0	100*	100
4	36	62
29	21	21
53	35	34
76	53	53

\* = 41  $\mu$ l.  $CO_2$ /10 min. Similar results were obtained in 5 other rabbits injected with DFP.

and 5%  $CO_2$ . After a fixed period of incubation the bicarbonate concentration of all solutions was adjusted to 0.025 M and enzyme reactivation was then carried out at pH 7.45. The results of these experiments, which are summarized in Table V, show that transphosphorylation is catalysed by acid. An adjustment in the ionic strength of the different media by addition of NaCl had no effect on the results.

#### Reactivation of True Cholinesterases of Rabbit's Blood after Inhibition *in vivo*

The true cholinesterases of rabbit blood behave *in vitro* and *in vivo* like human and bovine true cholinesterases, i.e., they form two types of phosphorylated enzyme. If rabbits are injected intramuscularly with DFP and the activity of the true cholinesterases of whole blood is determined at various intervals afterwards, pre-incubation of the blood samples with  $P_2AM$  or AM restores some of the enzyme activity during the early stages of inhibition, but is without any effect when blood samples which have been taken 24 hours after the injection of DFP are treated in the same way (Table VI).

#### DISCUSSION

The findings reported in this paper indicate that the inhibition of human and bovine true cholinesterases by organophosphates which contain a dialkylphosphato group involves the following stages:

Enzyme + organophosphate → addition complex → phosphorylated enzyme I → phosphorylated enzyme II.

If human and bovine true cholinesterases are exposed to an organophosphate for 20 min. more

than 90% of the enzyme activity can be restored by the nucleophilic reagents NHAM, AM or P<sub>2</sub>AM at 37° C., pH 7.45, with a bicarbonate-salt solution as medium and in the absence of free organophosphate. Identical rates of enzyme reactivation are obtained with a fixed concentration of a given reactivator if organophosphates which have a common dialkylphosphato group are used as inhibitors and the time course of reactivation has the characteristics of a first order reaction. Since the rates of hydrolysis of organophosphates by nucleophilic reagents vary considerably (Table I), reactivation of the inhibited enzymes must be the result of an action of the nucleophilic reagents on a phosphorylated enzyme.

Trimethylamine reduces the effect of P<sub>2</sub>AM and NHAM on an enzyme which has been inhibited by an organophosphate with a diethylphosphato group but has little effect on the reactivation by AM. Thus it seems that the anionic site of the active enzyme surface contributes to the reactivation process by binding and orientating substituted ammonium structures such as P<sub>2</sub>AM and NHAM. Such a promoting effect is absent (or negligible) if the organophosphate has a diisopropylphosphato group.

In the presence of free organophosphate, enzyme reactivation is governed by the effect of the nucleophilic reagent on phosphorylated and active enzyme and the type and concentration of free organophosphate. Enzyme reactivation is only obtained if the reactivated enzyme is protected by the nucleophilic reagent against further inhibition or if the free organophosphate is hydrolysed by the nucleophilic reagent during enzyme reactivation.

As with human plasma cholinesterase (Hobbiger, 1955), the degree of reactivation of phosphorylated human and bovine true cholinesterases is inversely related to the duration of inhibition. It seems that the dialkylphosphato group becomes initially attached to one reactive group of the esteratic site (to form phosphorylated enzyme I) and then is slowly transferred to another reactive group with which it forms a bond of higher stability that cannot be affected by nucleophilic reagents (phosphorylated enzyme II).

Cohen, Oosterbaan and Warringa (1955) recently obtained from mammalian true cholinesterase, inhibited by DFP, a polypeptide which contained an intact diisopropylphosphato group and which gave serine phosphate on acid hydrolysis. Cohen *et al.* concluded from this work that serine is the original point of attack of DFP on the protein. The results presented here make such an interpretation

unlikely and support the hypothesis put forward by Wagner-Jauregg and Hackley (1953) that not serine but another amino acid acts as the primary acceptor and forms a labile linkage with the dialkylphosphato group.

The experiments, in which the effect of pH on transphosphorylation was investigated, indicate that a group with a pH of 7.3 is involved. It is reasonable to assume that the rate of transphosphorylation is determined by the stability of phosphorylated enzyme I. If this is so, the results indicate that a P-N linkage between phosphorus and histidine is initially formed during phosphorylation. The dialkylphosphato group might then be transferred to serine (phosphorylated enzyme II), but the isolation of serine phosphate does not necessarily prove it (Wagner-Jauregg and Hackley, 1953).

The rate of transphosphorylation, which shows the characteristics of a first order reaction, is reduced by Mg<sup>2+</sup> and depends on the nature of the dialkylphosphato group of the organophosphate. If the organophosphate contains a diisopropylphosphato group transphosphorylation is accomplished at a far greater speed than with an organophosphate with a diethylphosphato group (Figs. 6 and 7).

Phosphorylated enzyme II is not the result of enzyme deterioration during prolonged incubation or of secondary reactions of the organophosphates with the enzyme. It has also been demonstrated *in vivo* in rabbits and seems to occur in man. The activity of the true cholinesterase of red cells of a patient with myasthenia gravis who has taken TEPP twice daily ever since he was put on this drug eight years ago (case report No. 2; Burgen, Keele and McAlpine, 1948) was only 3% of the mean activity of true cholinesterase of red cells of 6 control subjects. Incubation of the red cells with 10<sup>-2</sup> M-P<sub>2</sub>AM for 1 hour increased enzyme activity to 7%. Since no case of such a low activity of true cholinesterase of red cells has been reported (for variations of enzyme activity in normal subjects see Davies, 1954), these findings indicate that most of the enzyme was present as phosphorylated enzyme II.

Grob and Harvey (1949) observed that after TEPP the recovery of cholinesterase activity of human red cells *in vivo* was far more rapid during the first 24 to 48 hours than after DFP. After 48 hours the rates of enzyme recovery were similar for both organophosphates—approximately 2%/day of the original enzyme activity. These results can be explained by the transphosphorylation theory, and thus they provide additional indirect evidence for it.



## SUMMARY

1. The effects of the nucleophilic reagents nicotinhydroxamic acid methiodide (NHAM), ammonium molybdate (AM) and pyridine-2-aldoxime methiodide ( $P_2$ AM) on human and bovine true cholinesterases which have been inhibited by an organophosphate containing a dialkylphosphato group were studied.

2. The results indicate that inhibition of human and bovine true cholinesterases by an organophosphate containing either a diethylphosphato group (TEPP, E 600, Ro 3-0340 or Ro 3-0422) or a diisopropylphosphato group (DFP or D 600) is a phosphorylation process which yields two types of phosphorylated enzyme. Only phosphorylated enzyme I, which is formed initially, can be reactivated by the nucleophilic reagents. In the absence of free inhibitor identical rates of enzyme reactivation are obtained if organophosphates with a common dialkylphosphato group are used for inhibition. The time course of reactivation shows the characteristics of a first order reaction.

On prolonged inhibition phosphorylated enzyme I is converted into phosphorylated enzyme II which cannot be reactivated by the nucleophilic reagents. This conversion seems to be the result of a transphosphorylation process which has the characteristics of a first order reaction. If the organophosphate has a diisopropylphosphato group transphosphorylation proceeds at a far greater speed than with organophosphates which have a diethylphosphato group.

Transphosphorylation is influenced by the medium used for incubation and occurs also *in vivo*. The pH dependence of transphosphorylation indicates that the dialkylphosphato group becomes

attached first to the amino group of histidine (phosphorylated enzyme I).

3. All organophosphates are hydrolysed by nucleophilic reagents and the rate of hydrolysis is inversely related to the stability of the organophosphates.

4. Enzyme reactivation in the presence of an effective concentration of free organophosphate is determined by (a) hydrolysis of free organophosphate by the nucleophilic reagent, (b) protection of the enzyme against phosphorylation by the nucleophilic reagent, and (c) reactivation of the phosphorylated enzyme.

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