REACTIVATION STUDIES ON ORGANOPHOSPHATE INHIBITED HUMAN CHOLINESTERASES BY PRALIDOXIME (P-2-AM)

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INTRODUCTION

Pralidoxime iodide (2-formyl-l-methylpyridinium iodide oxime; Pyridine-2-Aldoxime Methiodide; P-2-AM) was first developed by Wilson and Ginsburg (1955), as a specific reactivator of organophosphate inhibited acetylcholinesterase. They demonstrated that purified electric eel acetylcholinesterase inhibited by tetraethylpyrophosphate (T.E.E. P.) and diisopropylfluorophosphate (D.F.P.) could be reactivated in the presence of P-2-AM *in vitro*. Subsequently Hobbiger (1956) demonstrated the reactivation of phosphorylated human and bovine acetylcholinesterase by P-2-AM. The mode of reactivation is believed to be due to a transfer of the substituted phosphate group from the phosphorylated enzyme to the oxime to produce a phosphorylated oxime and the free enzyme. This is only possible provided the enzyme does not undergo 'aging' (Hobbiger, 1955; Davis and Green, 1956).

The first *in vivo* success in humans using P-2-AM as a reactivator of phosphorylated acetylcholinesterase was reported by Namba and Hiraki (1958) who demonstrated its prompt antidotal effect in five patients suffering from accidental Parathion poisoning. These pioneering studies laid the foundation for the use of P-2-AM as an antidote, in organophosphate pesticide poisoning.

However, during the management of acute intentional severe organophosphate pesticide poisoning, there were no clinical or biochemical evidences of reactivation of the phosphorylated AChE (Ganendran and Balabaskaran, 1976). Similar observations have been reported by other workers in patients who had been poisoned by a number of organophosphate pesticides (Barr, 1964; 1966).

In order to ascertain the reactivation, if any of the commonly used insecticide Malathion [0, 0-dimethyl-S-(1, 2 bis (ethoxycarboxy ethyl) phosphorothioate] and its more potent analogue Malaoxon [0, 0-dimethyl-S (1, 2 bis ethoxycarboxyl ethyl) phosphate], *in vitro* experiments were conducted using fresh human blood as source of acetylcholinesterase (AChE, E.C.3.1.1.7.) and butyrylcholinesterase (BuChE, E.C.3.1.1.8.).

MATERIALS AND METHODS

Malathion and Maloxon (technical grade, Cyanamide Co., Ltd.), were used as anticholinesterases without further purification. Pralidoxime iodide (Sigma Chemical Co., St. Louis, M.O., U.S.A.) was used as the reactivator. Acetylthiocholine iodide and butyrylthiocholine iodide (Sigma Chemical Co., St. Louis, M.O., U.S.A.) were used as substrates to estimate AChE and BuChE activities respectively in whole blood.

Enzyme Preparation: Fresh human whole blood was obtained by venepuncture from a volunteer using a disposable syringe with a 18 G needle to avoid haemolysis, and was used as enzyme source. Five millilitre aliquots of blood were dispensed into EDTA treated sterile plastic containers and were gently shaken to avoid coagulation. Inhibition and reactivation studies were carried out on AChE from whole blood as well as from haemolysed

erythrocytes. Similar studies on BuChE were conducted on whole blood only.

Whole blood that had been treated with the inhibitor and or reactivator was haemolysed in 5 times its volume by an 0.3% Saponin solution at pH 7.4 and shaken manually for 5 minutes to complete haemolysis. Haematocrits were estimated by centrifuging a microcapillary tube filled with a sample of blood in an International microcapillary centrifuge (Model M.B.) and values read out using an International microcapillary reader.

Erythrocytes were isolated by centrifuging whole blood that had been treated with inhibitor and or reactivator at 3000 rpm for 15 minutes on a bench centrifuge and the plasma decanted off. The erythrocytes were washed twice in physiological saline and adjusted to pH 7.4. The washed erythrocytes were suspended in 5 times its volume of an 0.3% Saponin solution, freshly prepared in distilled water at pH. 7.4, and shaken manually for 5 minutes to complete haemolysis.

The haemolysed solution of whole blood and erythrocytes were then diluted to 1:1800 with 0.1M phosphate buffer and 3.0 ml samples of their solution were used as enzyme source.

Assay Method: A modified spectrophotometric method of Ellman *et al.*, (1961), was used to estimate AChE and BuChE. All studies were conducted in 0.1 M phosphate buffer at pH 7.4 and at 37°C. A typical run used for estimating enzyme activites consisted of 3.0 ml of enzyme solution (either treated or untreated with inhibitor) 0.02 ml of a 75 mM substrate and 0.05 ml of 10 mM DTNB solutions.

The reaction rates were measured during the initial linear period of the reaction which always included the first minute of the reaction as recorded. All inhibition and reactivation experiments were done in duplicates and included appropriate controls.

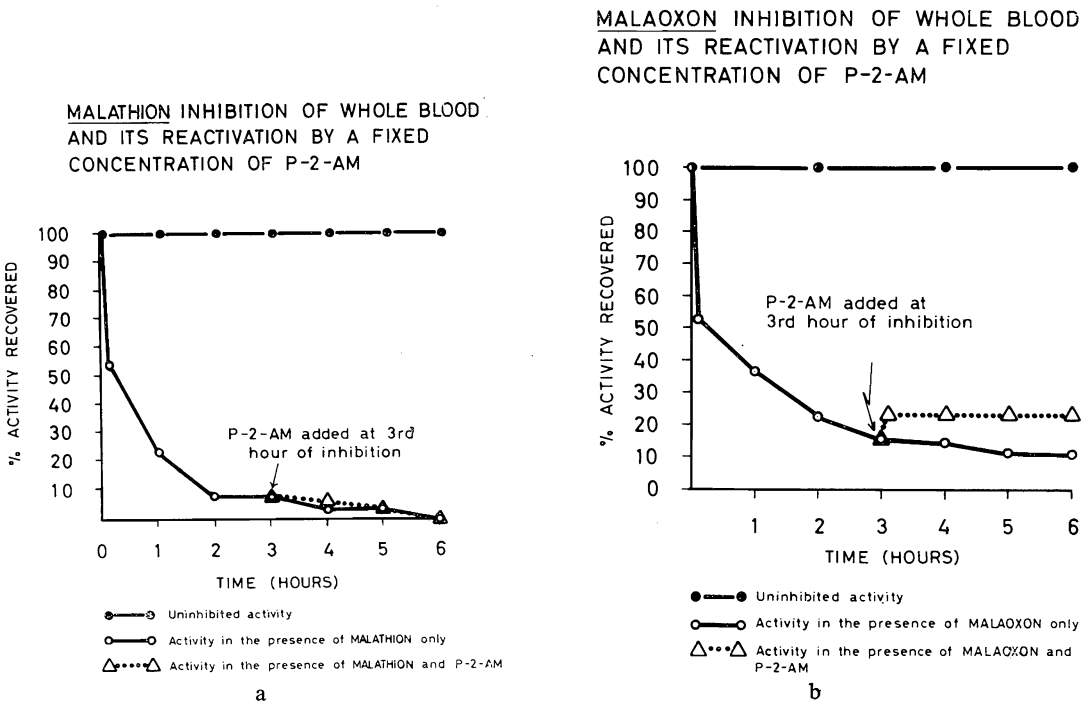
RESULTS

There were no variations in the AChE and BuChE activities of whole blood in 0.1 M phosphate buffer at pH 7.4 and 37°C for up to 6 hours.

Similarly there were no variations in the erythrocyte AChE as well as in AChE and BuChE activities of whole blood that had been incubated at 37°C for 3 hours followed by a further three hours incubation with fixed concentrations of P-2-AM (0.1 mg/ml of whole blood).

When haemolysed whole blood was used to assay acetylcholinesterase activity, the contribution of butrylcholinesterase towards the hydrolysis of acetylthiocholine (which was used as a substrate to assay the former) varied from 13.5% to 8.0% for dilutions of whole blood having haematocrits of 40 - 50 %. As each experiment was done on the same sample of blood obtained from one volunteer the contribution made by butrylcholinesterase was constant and regarded as insignificant.

Both Malathion and Malaoxon gave 50% inhibition of whole blood AChE activity at zero time at concentrations of 9500 µg/ml and 6 µg/ml of whole blood respectively (Fig. 1 a and 1 b). At the third hour about 80%-90% of the AChE activity could be inhibited. Reactivation of Malaoxon inhibited whole blood at the third hour by P-2-AM (0.1 mg/ml of whole blood) produced about 8% at zero time and 12% when incubated with P-2-AM for a further 3 hours. With Malathion, whole blood AChE activity inhibited for 2 hours when incubated with varying concentrations of P-2-AM for 3 hours could not be reactivated at all (Fig. 2) while with Malaoxon, the reactivation of AChE activity increased up to 10% at ten times the therapeutic concentration of P-2-AM which was calculated to be equivalent to 1.0 mg/ml of blood, based on the I.V. administration of



Figs. 1a, b—Whole blood was inhibited with (a) Malathion or (b) Malaoxon at a concentration of 9,500 µg/ml or 6 µg/ml respectively of whole blood at 37°C and assayed at zero time, and hourly for 6 hours. At ten end of the 3rd hour, similarly inhibited samples where incubated with P-2-AM at a fixed concentration of 0.1 mg/ml of whole blood and assayed at zero time, and hourly for 3 hours. The blood was haemolysed, diluted 1:1800 and 3.0 ml of it were assayed for AChE activity in 0.1 M phosphate buffer at 37°C and pH 7.4 using 500 µM Acetylthiocholine as substrate as described in the text.

Activity of inhibited blood assayed at various intervals was expressed as a percentage of the activity measured at zero time (0-0). Residual inhibited activity (0-0) and reactivated activity have been expressed as a percentage of the activity measured at zero time for inhibited blood.

1 gm to a 50 kg man. With both inhibitors however, no reactivation was observed for BuChE activity even at ten times the therapeutic concentrations of P-2-AM.

When Malaoxon was premixed with P-2-AM for 0, 1, 1.5 and 3 hours and then exposed to whole blood at zero time, it was found that the potency of Malaoxon decreased with increasing concentrations of P-2-AM in the premixed samples, regardless of the premixing and exposure times. Besides, the decrease in potency was more marked with longer premixing times and at higher P-2-AM concentrations (Fig. 3).

When reactivation for 3 hours with P-2-AM was attempted on whole blood inhibited with Malaoxon, it was found that maximum reactivation was obtained with blood inhibited with it for 3 hours. Reactivation attempted on blood inhibited for a longer or shorter period than 3 hours produced decreased reactivation. Further reactivation could not be achieved with blood inhibited for 24 hours by both the therapeutic concentrations as well as by ten times this strength. Although at the therapeutic concentration of P-2-AM, reactivation was not possible after 16 hours of inhibition with Malaoxon, reactivation could be obtained for a further 8 hours by increas-

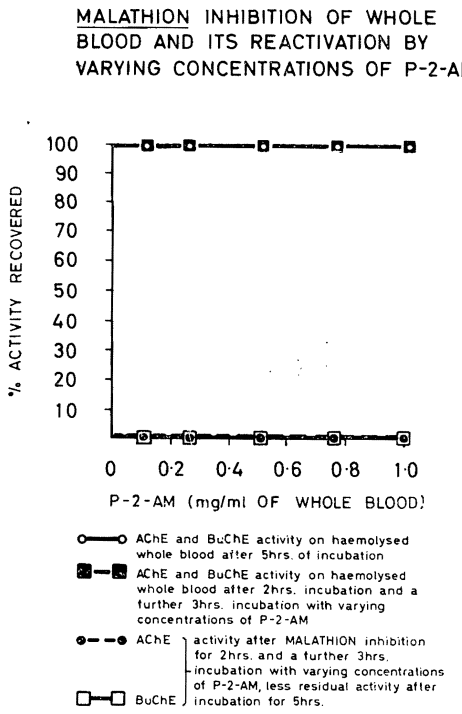


Fig. 2—Whole blood was inhibited with Malathion at a concentration of 9500 µg/ml of 37°C and assayed at zero time, and hourly for 5 hours. At the end of the second hour, a similarly inhibited sample was incubated with P-2-AM at varying concentrations ranging from 0.1 mg/ml to 1.0 mg/ml of whole blood for a further 3 hours and assayed. The blood was haemolysed, diluted 1:1800 and 3.0 ml of it were assayed for AChE and BuChE activities in 0.1 M phosphate buffer at 37°C and pH 7.4 using 500 µM acetylthiocholine and butrylthiocholine as substrates as described in the text.

ing the P-2-AM concentration ten fold (Fig. 4).

DISCUSSION

Most patients with acute severe organophosphate pesticide poisoning are treated within 2-3 hours after being in contact with the poison, hence all *in vitro* studies were conducted by exposing blood to the organophosphate for the same duration. Since there were no differences in the reactivation by P-2-AM of Malathion inhibited erythrocytes or whole blood AChE, all subsequent studies were conducted with whole blood.

In all reactivation studies a maximum period of 3 hours was chosen to expose the inhibited cholinesterase to P-2-AM as the latter has been shown to be excreted within three hours of infusion into a patient with Malathion poisoning (Quinby *et al.*, 1963).

Malaoxon is an oxidation product of Malathion metabolism produced largely by the action of liver microsomal enzymes. It is a well known fact that Malathion is less toxic than Malaoxon to both vertebrates and insects (O'Brien, 1967). Their potencies are also reflected by their inhibitory effect on cholinesterases in the blood as can be seen from this study. Malaoxon was found to be approximately 1500 times more potent as an inhibitor of whole blood AChE. Thus in Malathion poisoning, it is possible that the concentration of Malaoxon in the body could increase with time as a result of the action of liver microsomal enzymes on the Malathion circulating in the body.

Although P-2-AM was found to be an effective rectivator *in vivo* of Parathion inhibited cholinesterases by Namba and Hiraki (1958) as well as *in vitro* by Hobbiger (1956), it does not appear to be so in this work. It is possible that in the case of Malathion and Malaoxon poisoning 'aging' of the enzymes occurs so rapidly that reactivation is not significant even with 10 times the therapeutic concentration of P-2-AM. However, P-2-AM did inactivate Malaoxon to an extent of 60 - 70% at ten times the therapeutic concentration in the *in vitro* premixing studies.

Unfortunately it has been shown by a number of workers that when P-2-AM is given in high doses to volunteers (up to ten times the recommended therapeutic dose) it produces dizziness, blurred vision, diplopia, tachycardia, fatigue in the jaw, a bitter taste and rhini-

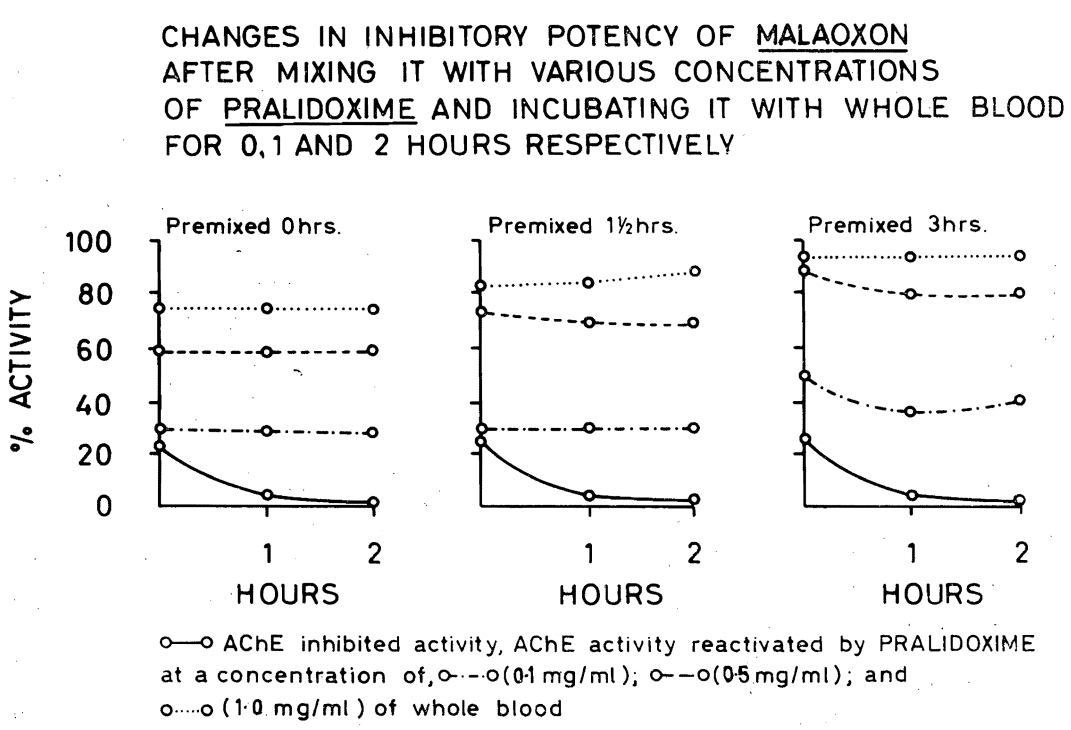


Fig. 3—A fixed concentration of Malaoxon and varying concentrations of P-2-AM were mixed and incubated at 0.1 M phosphate buffer at pH 7.4 and 37°C for (a) 0 hours (b) 1.5 hours and (c) 3.0 hours. A constant volume of this solution was pipetted into 1 ml of whole blood, so that the concentration of Malaoxon was 6 µg/ml of whole blood, while that of P-2-AM was from 0.1 mg/ml to 1.0 mg/ml of whole blood, and incubated at pH 7.4 and 37°C for (i) 0 hour (ii) 1 hour and (iii) 2 hours. The blood was haemolysed and diluted to 1:1800 and the residual AChE activity was estimated as described in the text.

tis lasting for about 2 hours (Namba, 1971; Jager and Stagg, 1958; and Sundwall, 1961).

Such being the case too much reliance should not be placed on the therapeutic value of P-2-AM for at least in the case of Malathion poisoning and certainly should not replace large doses of atropine and ventilation care in the management of Malathion poisoning.

SUMMARY

There is biochemical and clinical evidence that P-2-AM (Pyridine-2-Aldoxime Methiodide, Pralidoxime) does not reactivate human acetylcholinesterase inhibited by either Malathion or Malaoxon. *In vitro* studies using Pralidoxime iodide up to ten times the recommended concentrations, produced insignificant reactivation of cholinesterases inhibited by Malathion or Malaoxon. This was observed inspite of prolonged exposure of the inhibited cholinesterases to the oxime. The value of Pralidoxime as a reactivator of phosphorylated cholinesterases is therefore in doubt, and should not be used in preference to large doses of atropine and other supportive treatment in poisoning by organophosphate pesticides.

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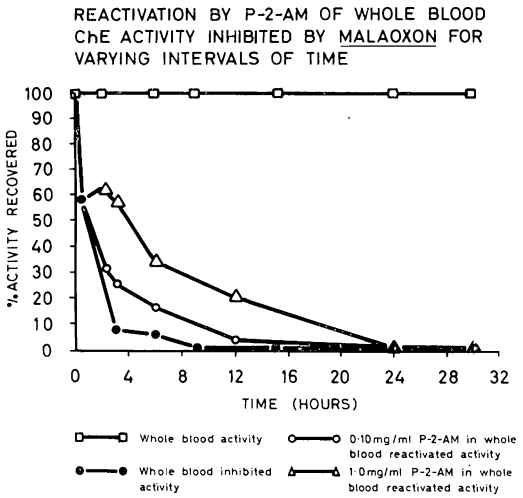

Fig. 4—Whole blood was incubated with Malaoxon at a concentration of 6 μg/ml and at an appropriate intervals of time, 1.0 ml aliquots were withdrawn for (a) assay and (b) reactivation with P-2-AM at a concentration of 0.1 mg/ml or 1.0 mg/ml of whole blood for 3 hours. At the end of this period the blood was haemolysed, diluted 1:1800 and the residual activity in 3.0 ml of it was estimated using 500 μM Acetylthiocholine as substrate in 0.1 M phosphate buffer at pH 7.4 at 37°C. The residual activity in organophosphate inhibited whole blood as well as in P-2-AM reactivated whole blood were expressed as a percentage of the activity of inhibited whole blood incubated for the same duration of time as the inhibited blood but further incubated for 3 hours with P-2-AM.

N.B. The points on the graph represent only the duration of exposure of whole blood to the organophosphate and do not include the reactivation time of 3 hours with P-2-AM.

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