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STUDIES ON THE MECHANISM OF TOXICITY OF THE ORGANOPHOSPHORUS PESTICIDE TRIAMIPHOS

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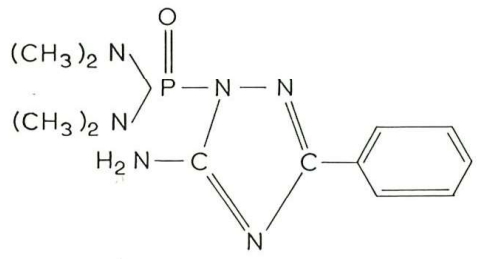
The i.p. LD_{50} of the organophosphorus ester triamiphos to adult male rat is about 15 mg/kg. The symptoms are typical of peripheral cholinesterase inhibition. Thus death is due to respiratory paralysis; there is no stimulation of the central nervous system. Measurement of the cholinesterase activity of several tissues after i.p. administration of various doses of triamiphos indicates that: (1) the rapidity of true cholinesterase inhibition at the myoneural junction is a major factor in the outcome of the acute intoxication; (2) brain true cholinesterase is not significantly inhibited; (3) in vivo, pseudocholinesterase is more inhibited than true cholinesterase; (4) except in the intestine, no return of cholinesterase activity was observed within 18 hr following the injection. Triamiphos is not a direct cholinesterase inhibitor but conversion by the liver to an anticholinesterase agent has been demonstrated by incubation of triamiphos with a fortified liver preparation. The active metabolite is very labile and does not readily cross the blood brain-barrier.

Anticholinesterase Triamiphos Organophosphorus pesticide

1. INTRODUCTION

Triamiphos is the common name for the organophosphorus compound 5-amino-1-(bisdimethylamido) phosphoryl 3-phenyl-1,2,4-triazole which has the following chemical structure:



It was first synthesized by Van den Bos et al. (1960). It is a white odourless powder. Its solubility in water at 20°C is 0.025 g/100 ml and it is easily soluble in most organic solvents. It exhibits fungicide, insecticide and acaricide activities but is mostly used for powdery mildew control on apples and ornamental plants (Martin, 1968; Tilemans, 1968).

The acute oral toxicity in mice is between 10 and 30 mg/kg (Van den Bos et al., 1960) but to our knowledge no detailed information on its toxicity has been published.

The toxicity of organophosphorus pesticides is usually ascribed to inhibition of acetylcholinesterase. Comparing the LD_{50} of various 3-substituted 5-amino-1-(bisdimethylamido)phosphoryl 1,2,4-triazoles, Tolkmith (1966) concluded that it is improbable that the toxicity of triamiphos arises from direct esterase inhibition.

This paper reports the results of our investigation on the mechanism of the acute toxicity of triamiphos in rats.

2. MATERIALS AND METHODS

Male Sprague–Dawley rats, body weight 150–250 g, were used. They were maintained on the usual diet ad libitum except that they were fasted for 16 hr before the experiment.

For the in vivo experiments, triamiphos was dissolved in propylene glycol–dimethylformamide (50:50, v/v) and administered i.p. The strength of the solution was adjusted so that the animals received volumes equivalent to 0.05% of their body weight. The control animals received equal volume of solvent.

Blood samples were collected in heparinized tubes, kept at 0°C, either from the tail at different time intervals after treatment or from the severed neck of animals killed by decapitation. Hematocrit was directly determined with an International micro-capillary centrifuge. A blood fraction was then diluted 60-fold with cold 0.05 M phosphate buffer, pH 7.4, for whole blood cholinesterase measurement. The remainder was centrifuged at 2500 rpm for 10 min in a refrigerated Sorvall RC_2 centrifuge to separate the plasma. Heart, intestine, brain and diaphragm were rapidly removed,washed with cold buffer, blotted on a dry filter paper and weighed. Heart, 2% (w/v), intestine, 2% (w/v) and diaphragm, 10% (w/v) were homogenised in 0.05 M phosphate buffer, pH 7.4, with an Ultra-Turrax (Janke and Kunkel K.G., Germany). The brains, 1% (w/v), were homogenised in the same buffer with a Potter–Elvehjem type homogeniser fitted with a teflon pestle (Braun, Germany). The heart and diaphragm homogenates were degassed under slight vacuum and kept at 0°C until used. The intestine homogenates were centrifuged at 1000 rpm for 5 min at 0°C. The top fat layer and the cell debris were discarded; the supernatant was used for incubation. The plasma was diluted 5-fold with buffer. The protein content of the homogenates and plasma was measured by the method of Lowry et al. (1951).

Cholinesterase activity was determined by a slight modification of the spectrophotometric method of Ellman et al. (1961). Stock acetylhiocholine iodide (Sigma) solution in water, 30 mM, was prepared daily. Stock solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), (U.C.B., Belgium), 45 mM, was prepared in Na_2HPO_4–KH_2PO_4 buffer, 0.05 M, pH 7, just before use. Substrate thiol reagent mixture contained 8.8 ml phosphate buffer, 0.05 M, pH 7.4, 1 ml stock acetylthiocholine solution and 0.2 ml DTNB solution. 1 ml of the substrate thiol reagent solution was added to a test tube at 37°C containing 1.5 ml of phosphate buffer, 0.05 M, pH 7.4, and 0.5 ml of homogenate or plasma or whole blood. After rapid mixing the assay mixture was transferred into a photocell (1 cm path length) kept at 37°C in the temperature controlled compartment of a PMQ II Zeiss spectrophotometer. Absorbance readings were taken at 30 sec intervals for 2 min, the first reading being taken 1 min after the substrate thiol reagent had been added. A substrate blank was included in each assay and correction was made for spontaneous hydrolysis of acetylthiocholine. Since the hematocrit of each blood sample was measured, red blood cell cholinesterase activity could be calculated by substracting the plasma activity from the whole blood activity (Voss and Sachsse, 1970).

Results were calculated in nmoles thiocholine released per min per mg tissue, plasma protein or per ml red blood cell and were expressed in per cent of control values.

Experiments designed to study triamiphos activation and cholinesterase inhibition in vitro are described in the Results section.

3. RESULTS

3.1. *In vivo acute toxicity*

Triamiphos administered by the i.p. route is highly toxic to rats. After administration of 20 mg/kg, all the animals die within 15 min with typical peripheral cholinergic signs: salivation, lachrimation, muscular tremor followed by muscular weakness, laboured breathing and cyanosis. The animals die from anoxia resulting from respiratory failure due to paralysis of the respiratory muscles which is probably aggravated by the bronchoconstriction and the obstruction of the airways by hypersecretion. The animals never develop typical convulsions as for example after parathion. The acute LD_{50} by the i.p. route is about 15 mg/kg. The animals always develop clinical manifestations within 15 min after the injection and the animals which do not die within 25 min always survive. Among 28 animals receiving 10 mg/kg, i.p., 6 developed cholinergic signs and 3 died 10 min after the injection. No clinical signs were observed after i.p.

administration of 2.5 or 5 mg/kg. Animals receiving lethal doses could be maintained by repeated administration of atropine.

The symptoms observed in intoxicated animals and the chemical structure of triamiphos suggest that like schradan, another phosphoramidate, triamiphos is not a potent inhibitor of the central nervous system cholinesterase, but acts preferentially on peripheral cholinesterase. It should, however, be pointed out that, following triamiphos injection, the symptoms appear much faster that after schradan poisoning (Dubois et al., 1950). The mainly peripheral action of triamiphos was confirmed by measuring the degree of true cholinesterase (brain, red blood cell, diaphragm) inhibition and pseudocholinesterase (heart, intestine, plasma) inhibition produced 3 hr after i.p. injection of various doses of triamiphos (fig. 1). After administration of low doses of triamiphos, pseudocholinesterase is more inhibited than true cholinesterase. More than 80% inhibition of plasma and heart cholinesterase is produced by a dose (5 mg/kg, i.p.) which causes no clinical manifestation. Brain true cholinesterase is not inhibited. Doses which kill all non-atropinized animals (20 mg/kg) cause only 15% inhibition of brain cholinesterase. Peripheral true cholinesterase (red blood and cell diaphragm) are inhibited following triamiphos administration in vivo. However the degree of inhibition produced by 20 mg/kg is not significantly different from the degree of inhibition produced by doses which cause no or moderate cholinergic signs (fig. 1).

The absence of a relationship between the degree of inhibition and the doses of triamiphos is not due to reactivation of the inhibited enzymes during the homogenate preparation. True cholinesterase and pseudocholinesterase, inhibited in vivo, does not reactivate in vitro since incubation of diaphragm and intestine homogenate at 37°C for 5 hr does not modify the percentage of remaining cholinesterase activity.

These results suggested that the degree of peripheral true cholinesterase inhibition was not the only factor responsible for the severity of the cholinergic signs produced by triamiphos. We first wondered whether triamiphos, or its product of metabolic transformation by the liver, had any direct action on the cholinergic receptor. Triamiphos alone, 10^{-4} M, or a mixture containing triamiphos, rat liver, NADP and glucose-6-phosphate (see below) were added to a

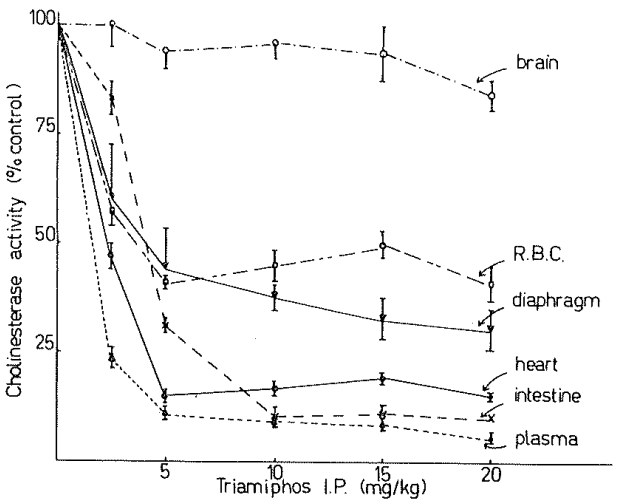


Fig. 1. Brain, red blood cell (R.B.C.), diaphragm, heart, intestine and plasma cholinesterase activity after i.p. administration of triamiphos to male rats. The animals were sacrificed 3 hr after the treatment. The animals dosed with 15 or 20 mg/kg triamiphos received atropine, 20 mg/kg, s.c. Each point is the mean ± S.E.M. of 8 animals.

guinea pig ileum preparation and the muscular contractions were recorded. No direct effect was observed; only potentiation of the acetylcholine response due to cholinesterase inhibition by the active metabolite produced after addition of the liver-triamiphos preparation could be evidenced. Experiments were then performed to test the influence of the rate of peripheral true cholinesterase inhibition on the acute toxicity of triamiphos. Since the initial results (fig. 1) suggested that after in vivo administration of triamiphos the degree of diaphragm true cholinesterase inhibition parallels that of red blood cell cholinesterase, this last activity was measured on the same animals before and at different time intervals after the injection of various doses of triamiphos. The cholinesterase activity was followed until death or for 50 min in surviving animals (fig. 2). No difference in the rate of red blood cell cholinesterase inhibition was found among animals receiving lethal and non-lethal doses of triamiphos. It could, of course, be objected that red blood cell and diaphragm true cholinesterase differ in their accessibility to the inhibitor and although they exhibit the same degree of inhibition 3 hr after the treatment (fig. 1) they may be inhibited at different rates during the critical period following the treatment. Another experiment was

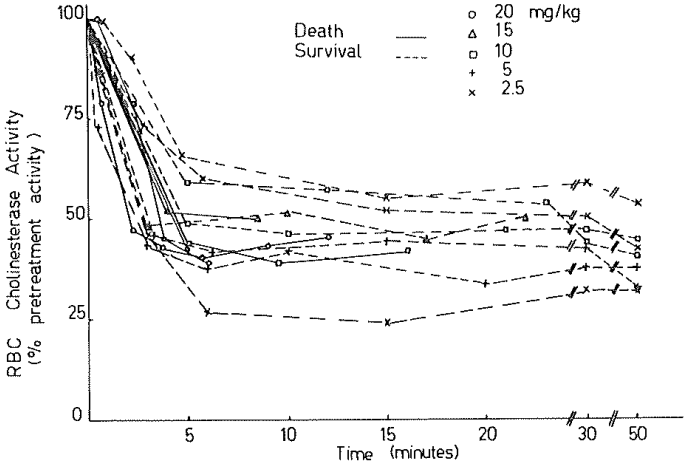


Fig. 2. Time course of red blood cell cholinesterase activity after i.p. administration of various doses of triamiphos to male rats. Each line represents the results obtained with the same animal.

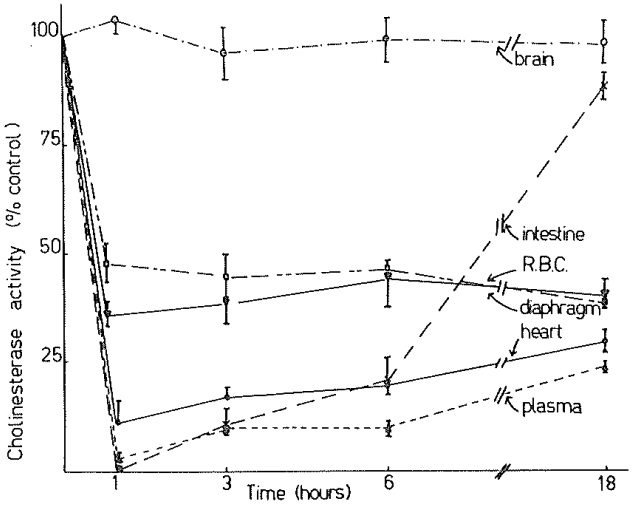


Fig. 4. Time–cholinesterase activity relationship after the i.p. administration of triamiphos, 10 mg/kg, to male rats. Each point is the mean ± S.E.M. of 3 animals.

therefore planned to test this point. Some animals received triamiphos, 2.5, 5 or 20 mg/kg, i.p., and were sacrified at different time intervals following the injection. The time–response relationship observed (fig. 4) suggests that the rate of diaphragm cholinesterase inhibition is different according to the outcome of intoxication.

The degree of cholinesterase inhibition present at different time intervals after i.p. administration of triamiphos, 10 mg/kg, was also determined (fig. 4). Except in intestine, no significant return of cholinesterase activity was observed in vivo during 18 hr.

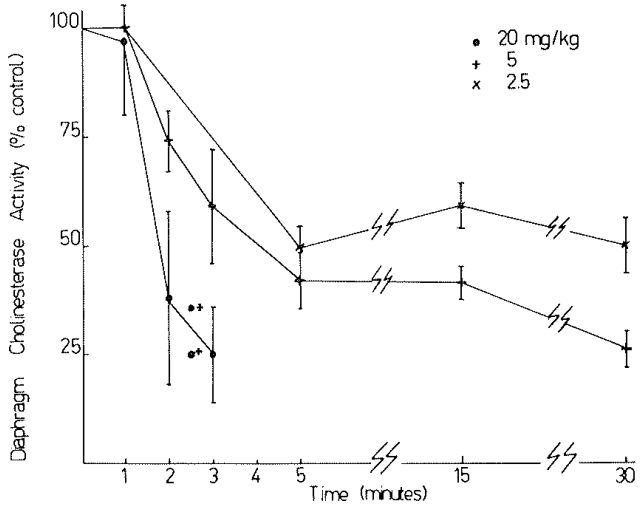


Fig. 3. Diaphragm cholinesterase activity at different time intervals after the i.p. administration of various doses of triamiphos to male rats. For the surviving animals each point is the mean ± S.E.M. of 3 animals. +: animal died.

3.2. *In vitro experiments: conversion of triamiphos to an anticholinesterase agent*

Preincubation of brain homogenate diaphragm homogenate or plasma with triamiphos (final concn. 10^{-4} M) at 37°C for 20 min did not reduce their cholinesterase activities. This indicated that the inhibition observed in vivo was the result of the metabolic transformation of triamiphos by the organism into a cholinesterase inhibitor. Therefore, experiments were conducted to demonstrate the metabolic conversion of triamiphos to an anticholinesterase agent in vitro.

In a first series of experiments triamiphos was incubated (final concn. 10^{-4} M) with a liver preparation (equivalent to 100 mg of liver) containing the microsomes and the cellular sap (obtained by centrifuging the whole liver homogenate at 700 000 *g*) in a final volume of 3 ml phosphate buffer, pH 7.4, containing nicotinamide (final concn. 1.4×10^{-2} M), $MgCl_2$ (final concn. 10^{-2} M), NADP (final concn. 2.6×10^{-6} M) and glucose-6-phosphate (final concn. 0.7×10^{-6} M). The incubation was performed at 37°C under air with shaking. At different time intervals (0, 5, 15, 30, 60 min) 0.5 ml of the mixture was pipetted off and added to a test tube kept at 37°C containing 1.5 ml of 0.33% control brain homoge-

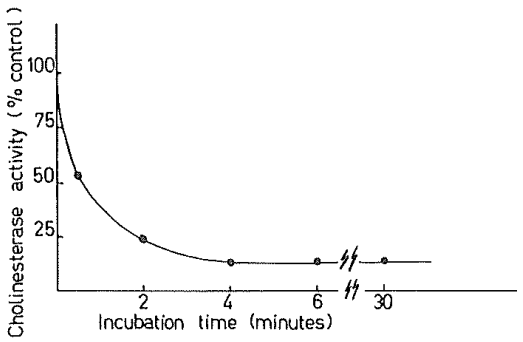


Fig. 5. Activation of triamiphos in vitro: effect of incubation time on brain cholinesterase incubated in vitro with triamiphos, 10^{-4} M and a fortified liver preparation (see text).

nate. After 20 min preincubation, cholinesterase activity was measured as described under Materials and Methods. Under these conditions no anticholinesterase activity could be demonstrated in the aliquot removed from the liver–triamiphos incubation system.

These results led us to suspect that either the liver incubation system was not adequate to activate triamiphos or the active metabolite was very labile and rapidly destroyed by liver enzymes or in aqueous media. To test this last hypothesis the following experiment was performed: in a beaker were added together the liver preparation (equivalent to 100 mg of liver), the same cofactors as listed above and also brain homogenate (50 mg); triamiphos (final concn. 10^{-4} M was then added and the mixture (final volume 3 ml) was incubated at 37°C. At different time intervals, 0.2 ml aliquots were removed and their cholinesterase activity was directly measured and compared to the cholinesterase activity of a similar preparation in which triamiphos was replaced by an equivalent volume of buffer. A very rapid inhibition of the brain cholinesterase present during the metabolic transformation of triamiphos by liver was now evident (fig. 5).

4. DISCUSSION

This investigation demonstrates that the acute toxicity of the organophosphorus pesticide triamiphos results from inhibition of true cholinesterase in peripheral tissues. Triamiphos is not the direct inhibitor. In the organism it is quickly metabolised into an active esterase inhibitor which is responsible for its acute toxic effect. The active metabolite is very labile. Its formation by the liver in vitro can nevertheless be demonstrated when cholinesterase is present during liver activation of triamiphos. The high rate of triamiphos activation by liver (fig. 5) may explain the rapid onset of the symptoms after in vivo administration.

Results of the in vivo experiments demonstrate that the active metabolite does not readily enter the brain (fig. 1). Therefore only muscarinic and nicotinic signs are observed after administration of a toxic dose of triamiphos. Death is primarily due to paralysis of the respiratory muscles.

This clinical sign and the study of the degree and rate of diaphragm true cholinesterase inhibition after administration of different doses of triamiphos indicate that an important factor responsible for its acute toxicity is the rate of cholinesterase inhibition at the myoneural junction.

When diaphragm cholinesterase activity is reduced by at least 70% within the first minutes, the animals die although they survive when the same degree of inhibition is produced more slowly (fig. 3).

The time–cholinesterase inhibition relationship after administration of triamiphos, 10 mg/kg, suggests that inhibited enzyme is not reactivated (fig. 4). Return of activity is probably due to resynthesis of new enzyme molecules; after 18 hr this is only evident in intestine, tissue known for its high rate of protein synthesis.

In vivo, pseudocholinesterase is more sensitive to inhibition than true cholinesterase. Plasma cholinesterase measurement is therefore an adequate test to control workers exposed to this pesticide.

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