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PROBLEMS IN THE TREATMENT WITH OXIMES AND ATROPINE OF RATS POISONED BY ORGANOPHOSPHATES

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Abstract—Some of the factors contributing to the failure of TMB-4 (1,3-di(4-hydroxyiminomethylpyridinium) propane dichloride) and atropine to treat rats poisoned with MBPF (3-methylbutyl-2 methylphosphonofluoridate) have been investigated. The largest doses of oxime that the animal could tolerate produced in the diaphragm concentrations which, according to studies *in vitro*, would be expected to produce barely enough reactivation of the acetylcholinesterase to restore normal function. Greater reactivation *in vitro* could be achieved by using much higher concentrations of oxime. The "ageing" of inhibited diaphragm acetylcholinesterase was found to be relatively slow *in vivo*. The importance of ageing in the therapy of organophosphate poisoning is discussed, with particular reference to poisoning by Soman (3,3-dimethylbutyl-2 methylphosphonofluoridate).

THE EFFECT of treatment with oxime and atropine in rats poisoned by organophosphates depends on the structure of the organophosphate. For example, pretreatment with PAM (2-hydroxyiminomethyl-N-methylpyridinium iodide) and atropine raised the LD_{50} of tetraethyl pyrophosphate twenty-three times, of diisopropyl phosphorofluoridate 16 times, but of isopropyl methylphosphonofluoridate (Sarin) only 2·3 times.[1] It has recently been found that poisoning by 3,3-dimethylbutyl-2 methylphosphonofluoridate (Soman) is resistant to such treatment, the effect of oxime plus atropine being no better than would be expected from atropine alone.

Since reactivation of acetylcholinesterase is a fundamental requirement for successful treatment, the fact that Soman-inactivated acetylcholinesterase cannot be reactivated under physiological conditions[3] can account for the failure of treatment in poisoning by Soman. This failure may be due to the great speed at which the Soman-inactivated acetylcholinesterase is converted spontaneously to a form which does not react with oximes, i.e. to extremely rapid ageing.[3]

An attempt has therefore been made to assess the significance of ageing *in vivo* using, not Soman, but the homologue 3-methylbutyl-2 methylphosphonofluoridate (MBPF). Poisoning by MBPF is difficult to treat, but reactivation of the inactivated enzyme can be achieved *in vitro* by the use of a high concentration (10^{-3}M) of oxime.

MATERIALS AND METHODS

Animals

Female albino rats (170–230 g) were used.

Materials

Atropine sulphate, B.P., and TMB-4 were obtained from commercial sources. Sarin and MBPF were synthesized in this Establishment and were 99–100 per cent pure.

Determination of LD_{50}

Sarin or MBPF was given subcutaneously in 0·9% (w/v) NaCl in a volume of 1 ml/kg. Doses in geometrical progression were given to groups of five animals and the 24-hr mortalities were observed. LD_{50} values and 95 per cent confidence limits were calculated by the method of Irwin and Cheeseman.[4]

LD_{50} values were also determined on animals given atropine (17·4 mg/kg), or TMB-4 (50 mg/kg) or both, intramuscularly 10 min before injection of the organophosphate.

Preparation of the diaphragm

The animals were killed by breaking the neck. The diaphragm was quickly removed and immersed in ice-cold 0·9% NaCl while fatty tissue, blood, connective tissue and the thick dorsal piece were dissected off. This left a U-shaped piece which could be divided into right and left halves. Surplus saline was drained and blotted, and the tissue quickly weighed.

When the experiment involved estimation of the TMB-4 content, the diaphragm was not immersed in saline. Blood was removed by blotting on hard filter paper, and small clots were picked off with fine forceps.

Diaphragm acetylcholinesterase was determined by the Warburg manometric technique. The diaphragm was homogenized in 0·038M-$NaHCO_3$, to a volume of 20 ml/g, with the mortar of the Potter–Elvehjem homogenizer immersed in ice-water unless otherwise stated. 2 ml of this homogenate plus 0·5 ml of water were placed in the main compartment and 0·5 ml of 0·036M-acetylcholine chloride in the side-arm. The flasks were gassed with 5% CO_2 in N_2 and the enzyme activity determined at 38°.

Acetylcholinesterase activities are expressed as μl CO_2 evolved in 30 min by 100 mg wet weight of tissue.

Inhibition and reactivation of diaphragm acetylcholinesterase in vitro

Diaphragm acetylcholinesterase was inhibited *in vitro* by immersing the trimmed weighed pieces of tissue in aqueous organophosphate solution at 0° for 30 min and then washing off surplus organophosphate with 0·9% (w/v) NaCl solution. The objective was to attain at least 90 per cent inhibition and leave no surplus organophosphate. To test that washing was adequate, a portion of homogenate was mixed with an equal volume of homogenate of normal diaphragm and the mixture allowed to stand on the bench for 30 min. In the absence of free inhibitor the activity of the mixture should not be less than the mean of the normal and inhibited preparation when the latter was measured immediately after washing.

Reactivation was carried out in double-side-arm flasks. The main compartment contained 2 ml of homogenate and 0·6 ml of water. One side arm contained 0·2 ml of 0·11 M (2% w/v) acetylcholine chloride and the other 0·2 ml of TMB-4 solution,

pH 7·4. Preparation and gassing of the flasks was carried out in the cold, after which the flasks were brought to 38°. The TMB-4 solution was tipped into the main compartment and allowed to react for a predetermined time before adding acetylcholine to measure the enzyme activity.

Measurement of ageing in vivo

Animals were poisoned with varying doses of organophosphate with the object of inhibiting the diaphragm acetylcholinesterase as much as possible while keeping the animals alive for varying periods. In most cases they had to be protected with atropine to enable them to survive for a sufficient period of time.

The amount of reactivatable acetylcholinesterase was measured as follows: one half of the diaphragm was homogenized in 0·038M $NaHCO_3$ containing 10^{-3}M TMB-4 at pH 7·4, and the other half in bicarbonate solution using a water jacket at room temperature to minimise overheating. The homogenates were left at room temperature (18–20°) for 30 min including the time required for homogenizing. Further manipulation in the bath at 38° occupied 12 min before the acetylcholine was tipped. The measurement required was the difference in activity between the treated and untreated halves of the diaphragm.

Estimation of TMB-4 *in the tissues*

The TMB-4 content of blood, diaphragm and skeletal muscle was estimated by measuring the absorption at 340mμ of alkaline extracts of these tissues after deproteinisation by zinc sulphate and barium hydroxide.[5]

RESULTS

The effects of TMB-4 *and atropine on the toxicity of Sarin and* MBPF

In preliminary experiments TMB-4 was given with atropine to normal rats, and it was found that they could tolerate 100 mg/kg intramuscularly, although some animals showed signs of poisoning by the oxime. When this does of TMB-4 was given without atropine, some of the animals died. In subsequent experiments, 50 mg/kg of TMB-4 was used.

The effects of atropine (17·4 mg/kg), TMB-4, and both together on the toxicity of Sarin or MBPF are shown in Table 1. Atropine alone had a small but barely significant

TABLE 1. THE EFFECTS OF ATROPINE AND TMB-4 ON THE TOXICITY OF SARIN OR MBPF TO FEMALE ALBINO RATS

Treatment	LD_{50} (95% limits) (μg/kg)	
	Sarin	MBPF
None	150 (135–165)	124 (115–135)
Atropine	222 (201–244)	146 (134–159)
TMB-4	184 (169–201)	133 (119–150)
Atropine + TMB-4	No deaths at 1000	209 (189–231)

Atropine sulphate (17·4 mg/kg) or TMB-4 (50 mg/kg) or both, were given intramuscularly 10 min before graded doses of organophosphate, subcutaneously. 24 hr mortality used for calculating LD_{50}'s.

effect on the toxicity of either organophosphate, as had TMB-4 alone. Treatment by both drugs was superior to that by either alone. Whereas the response to treatment in poisoning by MBPF was small, this combination of drugs had a very marked effect in poisoning by Sarin.

The ageing of diaphragm acetylcholinesterase in vivo

Since measurement of ageing in vivo depended on the difference in activity between halves of the same diaphragm, it was first necessary to show that the activities of the two halves did not differ significantly in normal animals. This is shown in Table 2.

TABLE 2. THE ACETYLCHOLINESTERASE ACTIVITIES OF THE TWO HALVES OF THE DIAPHRAGM OF FEMALE RATS

Animal No.	Acetylcholinesterase activity (μl CO_2/100 mg/30 min)	
	Right half	Left half
1	50	51
2	53	53
3	56	50
4	57	58
5	55	53
6	62	64

P that activities of both halves are identical = 0·53; mean value $\pm$S.E.M. $55 \pm 1{\cdot}2 \equiv 0{\cdot}82 \pm 0{\cdot}0018$ μmole ACh/min/g at 7·3 mM ACh.

Two series of experiments were carried out in animals poisoned with MBFP to determine the interval during which the inactivated acetylcholinesterase of the diaphragm remained reactivatable. In the first series the diaphragms were removed within 3 min of an intramuscular injection, and at death which occurred 17 min after injection. Although in each case the acetylcholinesterase had been inhibited to less than 10 per cent, more than 40 per cent of the inactivated enzyme could be reactivated *in vitro* by 10^{-3}M TMB-4. Even so at least 30 per cent of the enzyme could not be reactivated.

At the dose of MBPF used in this experiment, $1{\cdot}2 \times LD_{50}$, survival was not more than 20 min. In order to prolong survival time and to determine the rate of ageing of the inactivated enzyme *in vivo*, atropine was used in a second series of experiments (Table 3). Even after 4 hr, 18 per cent of the enzyme could still be reactivated.

The concentration of TMB-4 *in the tissues*

Reactivation of inactivated acetylcholinesterase in the previous experiments was carried out with a concentration of 10^{-3}M TMB-4. In order to attain this concentration *in vivo* a dose of 375 mg/kg would be required, assuming that it was evenly distributed throughout the animal body without loss. By contrast the maximum intramuscular dose which did not produce symptoms of oxime poisoning was only about

50 mg/kg. Consequently to assess the effects of therapeutic doses of TMB-4 on the inactivated acetylcholinesterase of various tissues, the concentrations of oxime in these tissues have been determined.

In one series of experiments 100 mg/kg of TMB-4 was given to enable the concentration to be followed for as long a period as possible, despite the fact that such a high

TABLE 3. THE PROPORTION OF REACTIVATABLE ACETYLCHOLINESTERASE IN RAT DIAPHRAGM AT INTERVALS AFTER POISONING BY MBPF

Time (min)	Acetylcholinesterase activity (% of normal mean)			Remarks
	Before reactivation	After reactivation	Difference	
3	7	68	61	Dose 150 μg/kg no atropine
17 (died)	5	49	44	
15	5	68	63	Dose 112 μg/kg 2 injections of atropine totalling 26 mg/kg
100	15	56	41	
120	4	42	38	
240	9	27	18	

dose was toxic. Figure 1 shows plots of the log of conc. of oxime vs. time for blood, diaphragm and the muscle of the contralateral leg to the one which was injected. Individual variation was considerable, the range in groups of three animals sometimes being as high as 2 : 1. Peak concentrations were reached in 15 min or less, and corresponded on average to: blood, $3{\cdot}4 \times 10^{-4}$ mole/l.; diaphragm, $6{\cdot}1 \times 10^{-5}$ mole/kg; and skeletal muscle, $2{\cdot}1 \times 10^{-5}$ mole/kg.

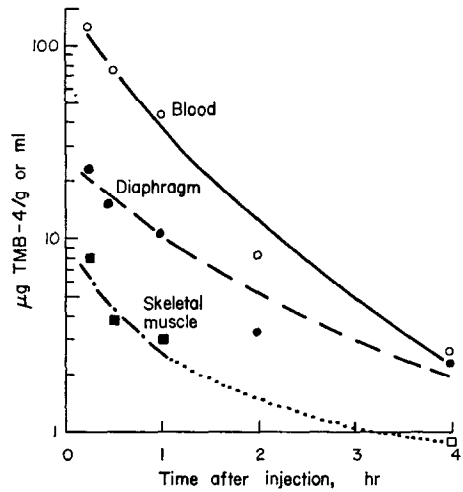

FIG. 1. Concentration of TMB-4 in rat tissues after intramuscular injection of 100 mg/kg. Means of three observations (□—one observation only).

In another experiment various doses of TMB-4 were injected intramuscularly, and the concentrations in the diaphragm 15 min later were measured. Figure 2 shows that there is a linear relationship between dose and concentration, and confirms that there was considerable individual variation, which is especially noticeable after the toxic dose of 100 mg/kg, at which most measurements were made. The dose of 50 mg/kg, used in the toxicological study, produced a concentration of 14 μg/g, i.e. $3{\cdot}7 \times 10^{-5}$ mole/kg. Concentrations in this region are those which must be considered in attempting to assess the therapeutic effects of TMB-4.

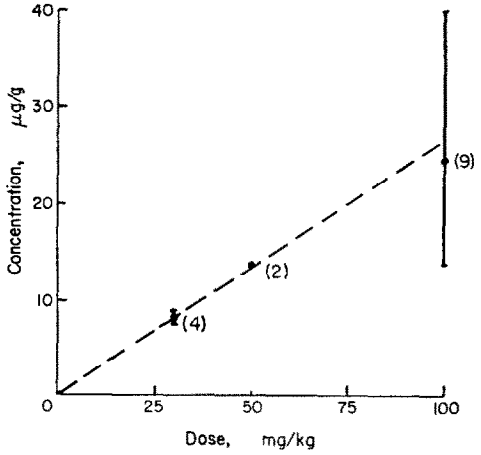

FIG. 2. Concentration of TMB-4 in rat diaphragm 15 min after intramuscular injection of various doses. Vertical line shows range and figures in brackets, No. of observations.

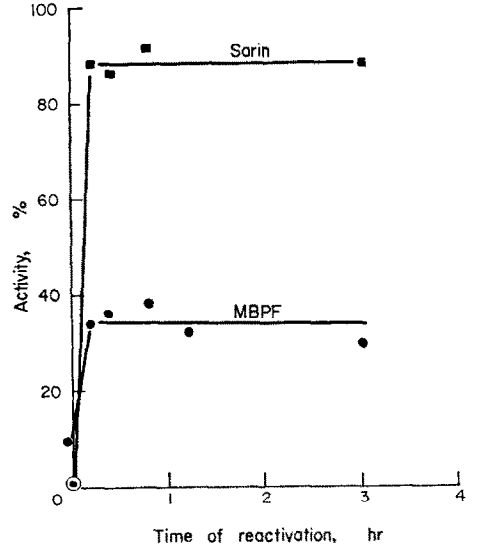

FIG. 3. Rates of reactivation of rat diaphragm acetylcholinesterase *in vitro* by $8{\cdot}6 \times 10^{-5}$M TMB-4.

Reactivation of diaphragm acetylcholinesterase in vitro

As a preliminary to determining the relationship between concentration of TMB-4 and percentage reactivation of diaphragm acetylcholinesterase *in vitro*, some observations on the rate of reactivation were made. Figure 3 shows the rates of reactivation by a concentration of TMB-4 in the range produced by an injection of 100 mg/kg After inhibition by MBPF the activity rose from 10 to 35 per cent in about 10 min and remained unchanged during the next 2 hr or so. After inhibition by Sarin however, 90 per cent restoration of activity occurred rapidly, and this again remained constant. An arbitrary time of 30 min was therefore used in the next experiment, since this would give the maximum extent of reactivation.

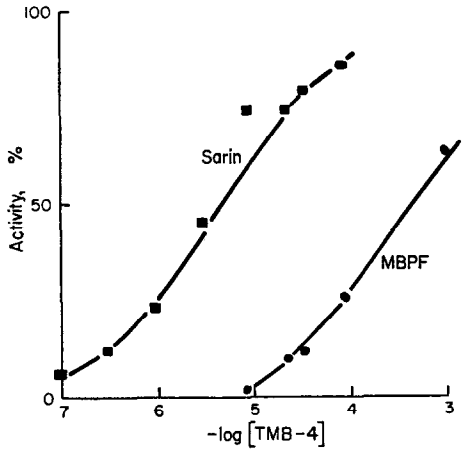

FIG. 4. Relationship between concentration of TMB-4 and percent reactivation of rat diaphragm acetylcholinesterase in 30 min at 38° *in vitro*.

In Fig. 4 the logarithm of the concentration of oxime has been plotted against the percentage restoration of activity in 30 min following inhibition by Sarin or MBPF. From these curves and from the data in Fig. 2 it may be deduced that a dose of 50 mg/kg would produce about 15 per cent reactivation after inhibition by MBPF, and nearly 80 per cent after inhibition by Sarin. A dose of 100 mg/kg would produce 23 and 88 per cent respectively.

DISCUSSION

The experiments just described show that Sarin poisoning is more easily treated by oxime (and atropine) than is poisoning of MBPF. They also show that Sarin-inactivated acetylcholinesterase is more easily reactivated than is MBPF-inactivated acetylcholinesterase. This is not due to rapid ageing of the latter, since four hours after poisoning by MBPF there is still enough reactivatable enzyme left in the diaphragm to restore normal function,[6, 7] provided that it could be reactivated *in vivo*.

The toxicity of TMB-4 limits the maximum concentration which can be attained *in vivo*, and it has been shown here that after a therapeutically acceptable dose of oxime, some 80 per cent reactivation of the diaphragm acetylcholinesterase could be expected after poisoning by Sarin, but only about 15 per cent after poisoning by

MBPF. This latter is very near the critical minimum level required for normal functioning of the organ. Since previous studies[3] had shown that Soman-inhibited acetylcholinesterase was even more difficult to reactivate, it is probable that therapeutic doses of oxime would not even reactivate enough enzyme initially to enable normal function to be restored. This is supported by the observation that even if oxime is given before poisoning, treatment still fails.[2, 8] If very rapid ageing were the cause of ultimate failure to reactivate the inactivated enzyme *in vivo*, some evidence of initial reactivation or of therapeutic effect would be expected; but none has been reported.

Eighty per cent reactivation of the MBPF-inhibited diaphragm enzyme can be achieved *in vitro* if a sufficiently high concentration of oxime is used, but to obtain this *in vivo* would require about 100 times the maximum therapeutic dose. However, once the enzyme has aged, it cannot be reactivated by any known means, since ageing is due to dealkylation of the alkyl methylphosphonoacetylcholinesterase,[9, 10] producing an acid form of the inactivated enzyme. This cannot react with the anion which is the active form of the oxime.[11]

If the oxime cannot initially produce enough reactivation to restore function, ageing is not important. It is also unimportant when therapeutic doses of oxime would produce extensive initial reactivation. It seems to be most important when therapeutic doses of oxime can produce little more than enough acetylcholinesterase to ensure function. In these cases even relatively slow ageing could soon diminish the reactivatable enzyme to such proportions that the fraction reactivated *in vivo* would no longer be able to sustain life. The significance of ageing in therapy cannot be considered in isolation, but only in relation to the ability of therapeutic doses of oxime to produce reactivation in the earliest stages of poisoning.

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