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THE REACTIVATING AND ANTIDOTAL ACTIONS OF N,N'-TRIMETHYLENEBIS (PYRIDINIUM-4-ALDOXIME) (TMB-4) AND N,N'-OXYDIMETHYLENEBIS (PYRIDINUM-4-ALDOXIME) (TOXOGONIN), WITH PARTICULAR REFERENCE TO THEIR EFFECT ON PHOSPHORYLATED ACETYLCHOLINESTERASE IN THE BRAIN

FRANZ HOBBIGER and VLADIMIR VOJVODIĆ*

Department of Pharmacology, Middlesex Hospital Medical School, London, W.1.

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Abstract—TMB-4 is 1·15 and 1·4 times more active than Toxogonin as a reactivator of diethylphosphoryl and diisopropylphosphoryl-acetylcholinesterase, respectively, when experiments are carried out at a pH of 7·45 and the phosphorylated enzyme is prepared from washed human red cells. The anion, i.e. the active species, of TMB-4 has approximately double the reactivating activity of the Toxogonin anion. This is not attributable to differences between nucleophilic activities. Toxogonin and TMB-4 are reasonably stable in solutions at pH 6 to 7, but at an alkaline pH Toxogonin is considerably less stable than TMB-4.

Both oximes, given prophylactically in a dose of 0·01 m-mole/kg i.p., raise the LD_{50} of Diethylphosphostigmine to the same extent. The protective effect of Toxogonin against Paraoxon is also comparable to that of TMB-4.

TMB-4 and Toxogonin, in equimolar doses, produce comparable degrees of reactivation of diethylphosphoryl acetylcholinesterase *in vivo*. At a dose level of 0·001 m-mole/kg, i.p., both oximes partly reactivate diethylphosphoryl acetylcholinesterase in the blood of mice. Reactivation in the brain of mice, as calculated from the acetylcholinesterase activity of homogenates of the whole brain, requires much higher doses of the oximes and with 0·1 m-mole oxime/kg, i.p., reactivation still amounts to less than 10 per cent. There is evidence that this is an overassessment of the reactivation which occurs in brain *in vivo*.

Results in rats are similar to those in mice. They also show that reactivation in the cerebellum is greater than in the whole brain, as assessed from the acetylcholinesterase activity of homogenates. This limits any conclusions drawn from work with homogenates of the whole brain. There is no evidence that Toxogonin is either more lipoid-soluble or crosses the blood brain barrier more easily than does TMB-4. It is concluded that these experiments fail to show that there is any substantial difference between the reactivating and antidotal effects of TMB-4 and Toxogonin.

ORGANOPHOSPHATES such as tetraethyl pyrophosphate (TEPP), diethyl 4-nitrophenyl phosphate (E 600; Paraoxon) diisopropyl phosphorofluoridate (DFP), and many other dialkyl phosphates are potent inhibitors of acetylcholinesterase because the enzyme in attempting to hydrolyse these compounds forms a dialkylphosphoryl derivative

* Permanent address: Institute of Toxicology, Military Medical Academy, Belgrade, Yugoslavia.

which is very stable relative to the acylated enzyme formed during the hydrolysis of acetylcholine. The dialkylphosphoryl moiety occupies part of the active surface of acetylcholinesterase, and while the enzyme is in a phosphorylated form it is unable to hydrolyse a substrate. Consequently, the administration of a dialkyl phosphate leads in mammals to the accumulation of acetylcholine, the physiological substrate of acetylcholinesterase, and when sufficient acetylcholine has accumulated at certain sites death ensues.

The dialkylphosphoryl group can be removed from the enzyme by certain nucleophilic substances which are generally referred to as reactivators. The most potent reactivators are pyridinium aldoximes. Their effectiveness in restoring enzyme activity depends on the dialkylphosphoryl moiety and is approximately twenty times greater for diethylphosphoryl acetylcholinesterase [$(EtO)_2PO$-AChE; formed by TEPP, Paraoxon and other diethyl phosphates] than for diisopropylphosphoryl acetylcholinesterase [$(Pr^iO)_2PO$-AChE; formed by DFP and other diisopropyl phosphates].[1] The first pyridinium aldoxime synthesized and studied in detail was N-methyl pyridinium-2-aldoxime iodide (Pralidoxime; 2-hydroxyimino-methyl methylpyridinium iodide; PAM; 2-PAM; P-2-AM).[2, 3] The reactivating potency of this oxime is such that it can be used in man to reverse the symptoms of poisoning by dialkyl phosphates which produce diethylphosphoryl acetylcholinesterase. However, to obtain sufficient reactivation of diethylphosphoryl acetylcholinesterase in man, a dose of 0·06–0·12 m-mole/kg, i.e. 1–2 g, should be injected i.v.[4] Work on oximes related to P-2-AM has led to the synthesis of N,N′-trimethylenebis (pyridinium-4-aldoxime) dibromide (TMB-4)[5, 6] which is approximately twenty times more potent than P-2-AM as a reactivator of both diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase.[5, 7, 8] This also applies to the antidotal effects of the two oximes against dialkyl phosphates, as shown in experiments on animals.[9] In spite of this, TMB-4 has never replaced P-2-AM for use in man, probably because of a report that TMB-4 in doses of 0·043 m-mole/kg produced marked hypotension and anuria.[10] These effects are absent with lower doses of TMB-4,[11] e.g. 0·009 m-mole/kg, which ought to produce a greater therapeutic effect than that obtainable with P-2-AM. Recently N,N′-oxydimethylenebis (pyridinium-4-aldoxime) dichloride (Toxogonin) which is closely related to TMB-4 has been synthesized and it has been reported that this oxime is superior to TMB-4 as a reactivator of dialkylphosphoryl acetylcholinesterases.[12, 13] Toxogonin in a dose of 0·01 m-mole/kg, given i.v., has also been used successfully in man for the treatment of poisoning by Paraoxon,[14] and it is thought that this oxime is a better antidote than P-2-AM, partly because it has a higher reactivating potency, and partly because it is capable of reactivating diethylphosphoryl acetylcholinesterase in the central nervous system.[15] P-2-AM and TMB-4, on the other hand, produce little or no reactivation in the central nervous system when given in doses which are sufficient to produce a marked reactivation at peripheral sites;[9, 16–21] large doses of these oximes, however, do produce some reactivation.[17, 22, 23] In view of the need for a drug which can be used therapeutically and perhaps even prophylactically against dialkyl phosphates and other anticholinesterases of the organophosphate type, and to gain information on the significance of the reactivation of phosphorylated acetylcholinesterase in the central nervous system, we carried out a detailed comparison between TMB-4 and Toxogonin. In this paper we wish to present our results concerning the reactivation *in vitro* and the protective effect against lethal doses of organophosphates

in mice, and report on the effect *in vivo* of the oximes on diethylphosphoryl acetylcholinesterase in the blood and central nervous system of mice and rats.

MATERIALS AND METHODS

Oximes

In the majority of experiments N,N'-trimethylenebis (pyridinium-4-aldoxime) (TMB-4) was used as the dibromide. It was synthesized by the method of Hobbiger and Sadler.[9] In a few experiments with the corresponding dichloride, kindly provided by Dr. D. R. Davies, C.D.E.E. Porton, the results were identical to those obtained with the dibromide. N,N'-oxydimethylenebis (pyridinium-4-aldoxime) (Toxogonin) was used as the dichloride. It was kindly provided by Merck, Darmstadt.

The pK_a values of the oximes were determined with a continuous titrator (Radiometer, type TTT Ic), and represent the pH values at the half-titration point of 0·02 m-mole oxime in 25 ml of water at 37°.

Oxime concentrations (in studies concerning the stability and the uptake into the brain) were determined from the u.v. absorption of the oxime anions.[24] For this the test solutions were made alkaline and the optical densities were measured with a Hilger Uvispek at 345 mμ in the case of TMB-4 and at 355 mμ in the case of Toxogonin.

Organophosphates

The organophosphates used were diethyl 4-nitrophenyl phosphate (E 600; Paraoxon) 3-(diethoxyphosphinyloxy)-N-trimethylanilinium sulphate (Ro 3-0340; Diethylphosphostigmine), tetraethyl pyrophosphate (TEPP) and diisopropyl phosphorofluoridate (DFP). They were all kindly provided by Dr. D. R. Davies, C.D.E.E. Porton, except TEPP which was obtained from Albright and Wilson, Ltd.

Hydrolysis of organophosphates by oximes

Determinations of the hydrolysis of organophosphates by oximes were carried out in the Warburg apparatus at 37° in a medium of 0·025 M $NaHCO_3$ and an atmosphere of $N_2 + CO_2$ (95:5), pH 7·45, fluid volume 3 ml. The amount of CO_2 released within the first 20 min after addition of the oxime, final concentration 2mM, to the organophosphate, final concentration 2mM, was used for comparisons between oximes.[8]

Acetylcholinesterase activity

Acetylcholinesterase activity was also measured in the Warburg apparatus at 37° in a medium of 0·025 M $NaHCO_3$ and an atmosphere of $N_2 + CO_2$ (95:5), pH 7·45. The total volume of fluid per vessel was 3 ml. Acetylcholine chloride, in a final concentration of 0·01 M, was used as substrate in experiments with washed human red cells and acetyl-β-methylcholine chloride, in a final concentration of 0·03 M, was the substrate in experiments with whole blood of mice or rats and with brain homogenates.

Reactivation in vitro *of human diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase*

Human diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase were prepared by incubating washed human red cells with TEPP and DFP, respectively

and then removing the excess of organophosphate as described previously.[25] A volume of 0·05 ml phosphorylated packed red cells was used in each Warburg vessel and the CO_2 output after addition of substrate was recorded over several consecutive 10-min periods.

Reactivation was calculated as follows:

$$\text{Reactivation} = 100 \times \frac{A - A'}{A'' - A'}\,\%$$

where A is the acetylcholinesterase activity in the presence of a given concentration of oxime, A′ the acetylcholinesterase activity in the absence of oxime and A″ the acetylcholinesterase activity obtained when TMB-4, in a final concentration of 0·1 mM, is present for 30 min before the addition of acetylcholine. A″ represents the maximum reactivation obtainable. The relative reactivating potencies of TMB-4 and Toxogonin were calculated from the percentage reactivation obtained with several concentrations of each of the two oximes.

Reactivation in vivo *of diethylphosphoryl acetylcholinesterase in mice and rats*

Mice. Male white mice weighing between 18 and 25 g were used. In any individual experiment the mice were of the same age and the weight difference between them did not exceed 2 g. All mice received an i.p. injection of 0·015 m-mole atropine/kg, given as atropine sulphate, to protect them against excessive muscarinic actions of accumulating acetylcholine; 10 min later a sublethal dose of Paraoxon was given s.c. (in the middle of the back) and after a further interval of 30 min one group were injected i.p. with 0·9 % NaCl, a second group with TMB-4, and a third group with Toxogonin. The mice were killed with ether 2 hr after the injection of the oxime or NaCl, and blood was collected by cardiac puncture. A volume of 0·2 ml blood was used per Warburg vessel and the CO_2 production from 10 to 70 min after the addition of substrate to the blood was taken as a measure of acetylcholinesterase activity. Reactivation was calculated as follows:

$$\text{Reactivation} = 100 \times \frac{\frac{B}{B'} - \frac{A}{A'}}{1 - \frac{A}{A'}}\,\%$$

where A is the acetylcholinesterase activity of the blood of mice injected with Paraoxon and NaCl, and A′ the acetylcholinesterase activity of the same blood incubated with 0·1 mM TMB-4 for 30 min at 37° before addition of substrate. B and B′ are acetylcholinesterase activities corresponding to A and A′ for the blood of mice injected with Paraoxon and an oxime. A′ and B′ represent the maximum reactivation obtainable.

To measure reactivation in the central nervous system, atropine, Paraoxon and oximes or NaCl were injected as described above. The mice were killed with ether at various intervals after the injection of oxime or NaCl, as stated in Table 3, and bled by opening the right ventricle before the brain was removed. The whole brain was homogenized in 0·025 M $NaHCO_3$ in an analytical mixer mill and the final concentration of brain tissue in each homogenate was adjusted to 25 mg/ml. For determinations

of acetylcholinesterase activity, 1 ml of the homogenate was used in each Warburg vessel and reactivation was calculated as described for blood.

Rats. Male Wistar rats weighing between 200 and 250 g were used. Atropine, Paraoxon and oximes or 0·9% NaCl were injected in volumes of 0·1 ml/100 g. The routes of injection and intervals between injections were as in mice. Two hours after the injection of the oximes or NaCl the rats were killed with ether and blood was collected by cardiac puncture. The brain vessels were then washed free of blood by perfusing them with 0·9% NaCl before removal of the brain from the cranial cavity. For determination of acetylcholinesterase activity 0·3 ml of blood, homogenates of whole brain containing 25 mg tissue/ml and homogenates of the cerebellum containing 100 mg tissue/ml were used; reactivation was calculated as in the experiments on mice.

Protection against lethal organophosphate poisoning

Male white mice weighing between 18 and 20 g were used. All drugs were injected in a volume of 0·1 ml/10 g and the total volume of fluid injected was kept constant by injecting when necessary 0·9% NaCl as a substitute for a drug. Subcutaneous injections were given in the middle of the back.

RESULTS

Stability and lipid solubility

TMB-4 and Toxogonin are readily soluble in water and reasonably stable in solution when the pH is near neutrality. At a pH of 6–8 the loss of oxime during storage at 2° for 1 week amounted to less than 10 per cent. The half-lives of 0·02 mM solutions at room temperature, i.e. 18–20°, were:

	pH 6·5	pH 9	pH 10·5
TMB-4	19 days	14 days	11 days
Toxogonin	18 days	7 days	1 hr

Both oximes have a very low lipoid solubility as shown by the result of the following experiment. Ten ml of a 0·01 M solution of TMB-4 or Toxogonin, adjusted to pH 2, were shaken with 10 ml chloroform. Five ml of the chloroform phase were removed and evaporated, and the residue was redissolved in 5 ml of 0·025 M $NaHCO_3$. One ml of this $NaHCO_3$ solution (in a total volume of 3 ml) failed to reactivate human diethylphosphoryl acetylcholinesterase *in vitro*, whereas TMB-4 and Toxogonin in a concentration of 0·2 μM produced a measurable degree of reactivation. This means that the concentration of TMB-4 and Toxogonin in the chloroform phase was less than 0·6 μM. The same result was obtained with the chloroform extract of strongly alkaline solutions of TMB-4, but because of the instability of Toxogonin at a high alkaline pH, it was not possible to carry out similar experiments with Toxogonin.

Reactivation in vitro *of human diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase*

TMB-4 and Toxogonin are potent reactivators of human diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase. Both oximes reactivate diethylphosphoryl acetylcholinesterase in concentrations of 0·1 μM or higher. Reactivation of diisopropylphosphoryl acetylcholinesterase requires higher concentrations of the oximes

and the ratio of the concentrations which produce equal degrees of reactivation of diisopropylphosphoryl and diethylphosphoryl acetylcholinesterase is about 20:1. The characteristics of the reactivation by Toxogonin are identical with those previously reported for TMB-4.[8] With both oximes the rate of reactivation is a function of the oxime concentration, and with a fixed concentration of oxime and in the presence of substrate, reactivation shows a first-order dependence on the concentration of phosphorylated enzyme. In the absence of substrate the time course of reactivation gradually deviates more and more from that of a first-order reaction and finally reactivation proceeds only at a very slow rate. The level of reactivation obtained under these conditions is also directly related to the oxime concentration.

Comparisons of the rates of reactivation obtained in the presence of 0·01 M acetylcholine, show that at pH 7·45 TMB-4 is slightly more potent than Toxogonin as a reactivator. Expressing the activity of Toxogonin as a percentage of that of TMB-4, the means (± S.D.), calculated from eight experiments were:

	Activity of Toxogonin relative to TMB-4 (taken as 100)
Diethylphosphoryl acetylcholinesterase	87 ± 4·5
Diisopropylphosphoryl acetylcholinesterase	72 ± 8

The pK_a values for TMB-4 and Toxogonin, at 37° were 8 and 7·7, respectively. From consideration of the dissociation curve this means that at pH 7·45, i.e. the pH used for determining rates of reactivation, 22 and 36% of TMB-4 and Toxogonin, respectively, are present in form of the anion which is the active, i.e. reactivating species. The reactivating potencies of the Toxogonin anion, expressed as a percentage of that of the TMB-4 anion, therefore, are 53 and 44 per cent for diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase, respectively.

TMB-4 and Toxogonin both hydrolyse TEPP and DFP. At a pH of 7·45 and at 37° the rates of hydrolysis of TEPP and DFP by Toxogonin were 1·57 and 1·23 times, respectively, faster than those by TMB-4. Since the rate of hydrolysis of an organophosphate by closely related oximes appears to be a reasonable measure of the nucleophilic activity of the latter,[26] it follows that the nucleophilic activity [as represented by the product (rate of hydrolysis) × (concentration of anion ion at pH 7·45)] of the Toxogonin anion relative to that of TMB-4 anion is 96 per cent, as assessed from the hydrolysis of TEPP, and 75 per cent, as assessed from the hydrolysis of DFP.

Reactivation is a function of the nucleophilic activity of the oxime anion and its alignment and bonding to the phosphorylated acetylcholinesterase.[27] Since the nucleophilic activities of the anions of TMB-4 and Toxogonin are very similar, the small difference between their reactivating potencies is probably mainly due to a difference in their alignment and bonding to the phosphorylated enzyme. In support of this conclusion is the finding that when nonphosphorylated acetylcholinesterase was incubated with the oxime for 10 min before the addition of acetylcholine, final concentration 0·01 M, a concentration of 6·6 mM TMB-4 produced 50 per cent inhibition, whereas 9·3 mM Toxogonin was required to produce the same effect.

Protection of mice against lethal organophosphate poisoning

The i.p. LD_{50}'s of TMB-4 and Toxogonin, calculated from the percentage mortality produced by graded doses of the oximes in groups of six mice, were 0·21 and 0·29 m-mole/kg, respectively.

To study the protective effect of the oximes against lethal doses of organophosphates, a dose of 0·01 m-mole/kg which on its own does not produce any visible changes in the conditions of the animals, was chosen. The oximes were injected i.p. 10 min before an organophosphate was given s.c. The organophosphates used were Paraoxon and Diethylphosphostigmine. They were chosen because both form diethylphosphoryl acetylcholinesterase, but the former is lipoid soluble and phosphorylates acetylcholinesterase at both peripheral and central sites, whereas the latter has a very low lipoid solubility and thus phosphorylates acetylcholinesterase mainly at peripheral sites.[28]

As can be seen from Tables 1 and 2 TMB-4 and Toxogonin raise the LD_{50} of both Paraoxon and Diethylphosphostigmine. In the case of Diethylphosphostigmine the two oximes are equiactive. With Paraoxon the protective effect of Toxogonin appears to be slightly greater than that of TMB-4. In order to get more information about this difference ($P > 0·9$), the protective effects of TMB-4 and Toxogonin were investigated in an additional experiment. In this experiment, in which groups of thirteen mice were used for each dose of oxime, 0·01 m-mole TMB-4/kg protected nine and six mice against death from 0·0048 and 0·0096 m-mole Paraoxon/kg respectively, whereas with 0·01 m-mole Toxogonin/kg the number of survivors in the corresponding groups were eleven and six (P for the difference between the effects of the two oximes is 0·9).

TABLE 1. PROTECTIVE EFFECT IN MICE OF TMB-4, TOXOGONIN AND ATROPINE AGAINST PARAOXON

Antidote	m-mole Paraoxon/kg								
	0·0012	0·0024	0·0048	0·0096	0·019	0·038	0·077	0·154	0·307
Control	10/10	0/10							
Atropine		10/10	4/10						
TMB-4			8/10	2/10	2/10				
Toxogonin			8/10	4/10	3/10				
TMB-4 + Atropine								8/10	3/10
Toxogonin + Atropine								9/10	3/10

The oximes, 0·01 m-mole/kg, and atropine, 0·02 m-mole/kg, were injected i.p. 10 min before Paraoxon was given s.c. The ratios represent: number of mice surviving for 24 hr/number of mice injected.

Tables 1 and 2 also show that 0·01 m-mole/kg of the two oximes give a greater protection than is obtained with 0·02 m-mole atropine/kg, and that a combination of atropine and an oxime is much more effective against Paraoxon than would be expected from an additive behaviour of the two. No difference exists between the protective effect of a combination of atropine and TMB-4 and that of a combination of atropine and Toxogonin.

TABLE 2. PROTECTIVE EFFECT IN MICE OF TMB-4, TOXOGONIN AND ATROPINE AGAINST DIETHYLPHOSPHOSTIGMINE

Antidote	m-mole Diethylphosphostigmine/kg							
	0·0125	0·025	0·05	0·1	0·2	0·4	0·8	1·6
Control	11/12	0/12						
Atropine			4/6	0/6				
TMB-4						6/6	4/6	0/6
Toxogonin						6/6	4/6	0/6
TMB-4 + atropine							5/6	0/6
Toxogonin + atropine							4/6	0/6

Experimental conditions as in experiments with Paraoxon (Table 1).

Reactivation in vivo *in the blood and the central nervous system*

Experiments on mice. Both oximes, in doses of 0·001 m-mole/kg and higher, reactivate diethylphosphoryl acetylcholinesterase at peripheral sites as shown by the difference between the acetylcholinesterase activity in the blood of mice which had been injected s.c. with 0·0024 m-mole Paraoxon/kg and the acetylcholinesterase activity in the blood of mice which had been injected s.c. with 0·0024 m-mole Paraoxon/kg and 30 min later with 0·001 or 0·01 m-mole TMB-4 or Toxogonin/kg, i.p. Two hours after the injection of the oximes the reactivation in blood, calculated from the activities of pooled samples, each of which contained equal volumes of blood from six mice which had received the same treatment, was as follows.

	% Reactivation in the blood of mice injected with	
Dose of oxime in m-mole/kg	TMB-4	Toxogonin
0·001	28	29
0·01	59	62

This is in marked contrast to the effect of the two oximes on diethylphosphoryl acetylcholinesterase in the brain, as assessed from the acetylcholinesterase activity of of homogenates of the whole brain. Table 3 shows that 0·01 m-mole TMB-4 or Toxogonin/kg had very little reactivating effect, and even after a dose of 0·1 m-mole oxime/kg the mean reactivation calculated from eight individual experiments was still less than 10 per cent. Again, there was no difference between the effects of TMB-4 and Toxogonin.

The discrepancy between the reactivation in blood and that in brain, however, must be even greater than is indicated by the results above. In most of the experiments summarized in Table 3 the CO_2 output increased gradually with time in the case of brain homogenates from oxime treated mice, whereas this did not apply to brain homogenates from mice which had received Paraoxon only. For example, in the experiments listed under the numbers 6–9 in Table 3 the mean reactivations calculated from the CO_2 output between 10 and 70 min after the addition of substrate were 10·2 per cent and 10·3 per cent for TMB-4 and Toxogonin treated mice, respectively

TABLE 3. REACTIVATION OF DIETHYLPHOSPHORYL ACETYLCHOLINESTERASE IN THE BRAIN OF MICE *IN VIVO*

Experiment No.	% Reactivation in mice injected with Paraoxon and 30 min later with:		Acetyl cholinesterase activity (% of control) of mice injected with Paraoxon only	Dose of Paraoxon (m-mole/kg)	Time between injection of oxime and removal of brain (hr)	Dose of oxime in m-mole/kg
	TMB-4	Toxogonin				
1	3·9	2·6	14	0·0024	1	0·01
2	4·4	3·3	10			
3	0	1·2	15			
Mean	2·8	2·4				
4	13	14	10	0·0024	1	0·1
5	6·8	10	12			
6	10·5	11	18	0·0018	1	
7	6	6	16		2	
8	15	13	15		3	
9	9·4	11	15		4	
10	4·2	2·8	29	0·0012	2	
11	1·4	1·4	27			
Mean	8·3	8·7				
12	14	11	62	0·001	26	
13	3·1	(activity below control)	65			
14	8·3	17	64			

In each case reactivation was calculated from the acetylcholinesterase activities of pooled homogenates of three whole brains.

Using for calculations the CO_2 output during the periods 10 to 40 and 40 to 70 min after addition of substrate, the reactivation in both TMB-4 and Toxogonin treated mice was 9·2 per cent for the 10 to 40 min period, whereas the corresponding values for the 40- to 70- min periods were 11·1 for the TMB-4 group and 11·3 per cent for the Toxogonin group.

Furthermore, when brain homogenates containing 25 mg brain tissue in 1 ml of 0·025 M $NaHCO_3$ were stored at 4° the activities of homogenates from oxime treated mice increased much more than those of homogenates from mice which had received Paraoxon only. For example, in the experiments listed under the numbers 6 to 9 in Table 3 the mean acetylcholinesterase activity of homogenates from mice treated with Paraoxon only, increased on storage at 4° for 4 days from 16 to 23 per cent, whereas those from mice treated with Paraoxon and TMB-4 or Toxogonin increased from 25 to 55 per cent and 25 to 58 per cent, respectively.

Experiments on rats. Experiments were carried out also in rats under conditions identical to those used for mice, except that in studies on the reactivation in the central nervous system, the blood vessels of the brain were washed free of blood by perfusing them with 0·9 per cent NaCl before the brain was removed from the cranial cavity.

The reactivations in blood, calculated from the acetylcholinesterase activities of pooled samples of equal volumes of blood collected from six rats 2 hr after the i.p, injection of 0·001 m-mole oxime/kg (given 30 min after 0·0018 m-mole Paraoxon/kg. s.c.) were 45 and 46 per cent in rats injected with TMB-4 and Toxogonin, respectively.

With a dose of 0·01 m-mole oxime/kg the reactivations were 86 per cent and 89 per cent for TMB-4 and Toxogonin, respectively.

Reactivation of diethylphosphoryl acetylcholinesterase in the brain, as assessed from the acetylcholinesterase activity of whole brain, required much higher doses of the oximes and as Table 4 shows, after doses of 0·1 m-mole oxime/kg injected i.p.

TABLE 4. REACTIVATION OF DIETHYLPHOSPHORYL ACETYLCHOLINESTERASE IN THE BRAIN OF RATS *IN VIVO*

Experiment No.	% Reactivation in rats injected with Paraoxon and 30 min later with:		Dose of oxime (m-mole/kg)	Reactivation assessed from the acetylcholinesterase activity of:
	TMB-4	Toxogonin		
1	13	13	0·1	homogenates of the whole brain
2	11	13		
3	11	10		
4	34	35	0·1	homogenates of the cerebellum
5	27	27		
6	26	29		
7	24	21		
8	30	29		
Mean (Expts. 4–8)	28	28		
9	7	7	0·01	
10	6	7		

Reactivation was calculated from the acetylcholinesterase activity of pooled homogenates of the whole brain or cerebellum of two rats which had received the same treatment. Paraoxon was given in a dose of 0·0018 m-mole/kg s.c. and reduced acetylcholinesterase activity by 85 to 90 per cent in individual experiments. The interval between injection of oxime and removal of the brain was 2 hr.

30 min after 0·0018 m-mole Paraoxon/kg, s.c., the reactivation in the brain was much less than the reactivation obtained in blood with 0·001 m-mole oxime/kg. These results closely resemble those obtained with mice and as can be seen from Table 4, there is again no difference between the effects of TMB-4 and Toxogonin.

No consistant gain in the acetylcholinesterase activity of brain homogenates from oxime treated rats occurred during measurements of acetylcholinesterase activity, i.e. during 10 to 70 min after addition of substrate to homogenates, but, as in mice, during storage at 4° the activity of homogenates from oxime treated rats increased to a much greater extent than that of homogenates from rats which had received Paraoxon only.

In view of the report by Erdmann[15] that Toxogonin produced a marked reactivation in the cerebellum of rats previously injected with Paraoxon, we also investigated the effect of TMB-4 and Toxogonin on diethylphosphoryl acetylcholinesterase in the cerebellum. Table 4 summarizes these results and shows that the reactivation calculated from the activity of cerebellar homogenates of rats injected with 0·1 m-mole oxime/kg, was considerably greater than that observed in the experiments in which homogenates of the whole brain were used. Table 4 further shows that in the cerebellum reactivation was still detectable when rats were injected with 0·01 m-mole oxime/kg, and that there was no difference between TMB-4 and Toxogonin.

The reactivation observed in the experiments with homogenates of the cerebellum is not an *in vitro* artifact, since no consistant gain of acetylcholinesterase activity could be detected during the period of 60 min used for recording the acetylcholinesterase activity of homogenates obtained from oxime-treated rats.

Concentrations of oximes in the brain

Erdmann[15] also reported that Toxogonin could be demonstrated in the brain of rats which had been injected i.v. with 0·14 m-mole Toxogonin/kg. The method used by Erdmann consisted of homogenizing the brain with five parts of water, shaking the homogenate with chloroform, separating the aqueous phase from cell debris and chloroform by centrifugation, and determining the concentration of oxime in the aqueous phase spectrophotometrically at an alkaline pH. Under these conditions, the concentration of oxime present in brain 20 min after injection of 0·14 m-mole Toxogonin/kg, was found to be 0·015 μmole/g brain.

Using brains which had been washed blood-free with 0·9% NaCl we were unable to detect by the above method TMB-4 or Toxogonin in the brain of rats 20 min after the injection of 0·1 m-mole oxime/kg i.p. We therefore used the following approach. The brain was homogenized in five parts of 0·025 M $NaHCO_3$ and centrifuged at 6200 *g* for 10 min. The supernatant obtained in this way was heated in a boiling water-bath for 5 min, and centrifuged once more. One ml of the final supernatant was incubated with diethylphosphoryl acetylcholinesterase, and the reactivation obtained with it was compared with that obtained with known amounts of the oximes which had also been heated for 5 min. Using this method the amounts of TMB-4 and Toxogonin present in brain 30 min after the i.p. injection of 0·1 m-mole oxime/kg was found to be of the order of 0·0015–0·003 μmole/g brain.

DISCUSSION

Our comparisons between TMB-4 and Toxogonin show that at pH 7·45 and with phosphorylated acetylcholinesterase prepared from human red cells TMB-4 is 1·15 and 1·4 times more potent than Toxogonin as a reactivator of diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase, respectively. Since the pK_a values of Toxogonin and TMB-4 are 7·7 and 8 respectively, this means that the anion, i.e. the active species, of Toxogonin has only approximately 50 per cent of the reactivating potency of the anion of TMB-4. Studies of the nucleophilic activity of the oximes indicate that the anion of TMB-4 is a better reactivator than the Toxogonin anion because of a better alignment and bonding to phosphorylated acetylcholinesterase.

Engelhard and Erdmann[12, 13] reported that at pH 7·5 Toxogonin was approximately twice as potent as TMB-4 as a reactivator of both diethylphosphoryl and diisopropylphosphoryl acetylcholinesterase. These authors studied reactivation in the presence of free organophosphate and since under these conditions Toxogonin hydrolyses organophosphates faster than does TMB-4 it is very likely that the reactivation recorded by them was not the best measure of the true reactivating potency of the two oximes. In addition, the data presented in one of their tables (*Table* 2)[13] seem to contradict that Toxogonin is more potent than TMB-4 as a reactivator. Our conclusion that *in vitro* and at pH 7·45 TMB-4 is slightly more potent than Toxogonin as a reactivator is in agreement with the findings of Kitz *et al.*[29] and Heilbronn and Tolagen.[30] The former reported that TMB-4 was slightly more active than Toxogonin

as a reactivator of diethylphosphoryl acetylcholinesterase, and Heilbronn and Tolagen found that the same applied to the reactivation of the phosphorylated acetylcholinesterase formed by Tabun (ethyl N-dimethylphosphoramidocyanidate) or Sarin (isopropyl methylphosphonofluoridate), i.e. of (EtO)(Me_2N)PO-AChE and (Pr^iO)(Me)PO-AChE, respectively.

The similarity between the *in vitro* reactivating potencies of TMB-4 and Toxogonin has a parallel in their protective action against lethal doses of organophosphates in mice. The two oximes give the same protection against Diethylphosphostigmine, an organophosphate which forms diethylphosphoryl acetylcholinesterase and acts predominantly at peripheral sites.[28] In the case of Paraoxon, which acts at both peripheral and central sites, Toxogonin appears to be slightly better than TMB-4 as an antidote, but the difference in our experiments is not significant (P 0·9).

Our results also show that in mice Toxogonin does not pass the blood brain barrier more easily than does TMB-4. The reactivation of diethylphosphoryl acetylcholinesterase in the brain of mice *in vivo*, as assessed from the acetylcholinesterase activity of homogenates of the whole brain, is negligible by comparison with the reactivation obtained at peripheral sites and is comparable with both oximes. Even with large doses such as 0·1 m-mole oxime/kg the average reactivation amounts to less than 10 per cent and part of this is probably an artifact, caused by the release of oxime during homogenizing, since acetylcholinesterase activity in homogenates of brains from oxime treated mice increases during measurements and on storage. Using a small number of animals, calculating reactivation without using each group of mice as its own control, and allowing for possible artifacts, the conclusion might be reached that reactivation can not be demonstrated with certainty.[9] In rats, the species used by Erdmann,[15] reactivation of diethylphosphoryl acetylcholinesterase in the CNS by TMB-4 and Toxogonin, as determined from the acetylcholinesterase activity of homogenates of the whole brain, is slightly greater than in mice and less likely to be distorted by *in vitro* artifacts. Again the reactivation in the brain of rats is comparable with both oximes and measurements of the oxime concentration reached in the brain of rats not treated with an organophosphate fail to show any difference between the two oximes. The latter observations are in contrast with Erdmann's findings. The reason for this is unknown. Our conclusion that there is no major difference between the two oximes as far as their entry in the brain is concerned is supported by the observation that there is very little difference between the effects of Toxogonin and TMB-4 on Paraoxon induced abnormalities of the encephalogram.[31]

Reactivation in the cerebellum is much more pronounced than reactivation in the rest of the brain, as assessed by the acetylcholinesterase activity of homogenates. A detailed study of this (to be published shortly) has shown that this difference arises from variations between the amounts of functional and non-functional[32] acetylcholinesterase in different parts of the brain. Our finding that reactivation occurs in the cerebellum of rats injected with 0·1 m-mole/kg Toxogonin confirms the findings of Erdmann,[15] but our experiments also show that TMB-4 produces the same effect as Toxogonin.

Since reactivation, as assessed by the acetylcholinesterase activity of homogenates of the whole brain, is, therefore, misleading our interpretation of what part reactivation of phosphorylated acetylcholinesterase plays in the antidotal action of pyridinium aldoximes must wait until reactivation at vital sites can be measured

accurately. Studies of this kind will probably give more information than studies of the uptake of oximes into the central nervous system,[33] since the latter can tell us nothing about the concentration of oximes at sites of the phosphorylated functional acetylcholinesterase.

Evidence supporting the conclusion that there is little to choose between TMB-4 and Toxogonin is provided by the finding that Toxogonin is not more effective than TMB-4 as an antidote in poisoning by the two organophosphates Sarin and Tabun.[30] However, before a final conclusion can be reached on which oxime, if any, is better for use in man, it is necessary to consider also other factors such as the toxicity of the oximes and their pharmacological effects, i.e. actions not attributable to reactivation of phosphorylated acetylcholinesterase or hydrolysis of organophosphates. The toxicity of Toxogonin is lower than that of TMB-4,[15, 30] but the difference is probably too small to be of any importance with the doses used at present in man.[14] Comparisons between the pharmacological actions of the oximes, comparable to those which have been carried out with TMB-4 and P-2-AM,[34] have not yet been undertaken. Should such studies be undertaken it is important to remember that the purity of the oxime is an important factor, since byproducts can arise during the synthesis of bisquaternary pyridinium aldoximes[35] and with large doses of the oximes the byproducts might mask the pharmacological actions of the former. This might also be of importance for studies in man.

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