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THE EFFECT OF OXIMES ON ISOLATED ORGANS INTOXICATED WITH ORGANOPHOSPHORUS ANTICHOLINESTERASES

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Abstract—The oxime concentration needed for "complete functional recovery" of the isolated diaphragm treated with organophosphorus anticholinesterases, varies considerably with the inhibitor used. After Soman "aging" of the inhibitor–cholinesterase combination product is completed in about 60 min. With the other anticholinesterases used, "aging" cannot be observed within 4–6 hr. The degree of functional recovery observed can be correlated with the percentage reactivation of the acetylcholinesterase in the diaphragm. The marked variation in the oxime concentration needed to counteract the effect of different anticholinesterases, was confirmed using the isolated rat hindquarter preparation perfused with blood and using the isolated rat ileum. In contrast to this the dose of atropin needed to counteract various inhibitors in the rat ileum shows very little variation. It is concluded that two factors are important in the oxime treatment of anticholinesterase poisoning, i.e. the effective oxime concentration and the "aging" rate. The relative preponderance of these factors varies with the anticholinesterase used.

INTRODUCTION

THE EFFICACY of the treatment of poisoning with organophosphorus anticholinesterases using atropin in combination with oximes, has been established in experimental animals as well as in man in a substantial number of cases.[1–8]

However a number of authors have described experiments in which this form of treatment appeared to be relatively ineffective against intoxication with Tabun,[2, 7, 8] Soman[9] and OMPA.[1] Loomis and Salafsky[9] try to explain the low efficacy of treatment in the case of Soman poisoning. They show that reactivation of Soman inhibited acetylcholinesterase is less complete than of Sarin inactivated enzyme. They propose rapid "aging" of the enzyme-inhibitor combination as an explanation for this finding.

In order to elucidate the cause of the failure of the treatment of anticholinesterase poisoning under certain circumstances, we studied the effect of the oximes PAM and MINA on three different preparations intoxicated with a number of different organophosphorus anticholinesterases. The preparations used were the phrenic nerve-diaphragm preparation, the rat hindquarter preparation perfused with blood and the isolated rat ileum.

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MATERIALS AND METHODS

Phrenic nerve-diaphragm preparation

This is essentially the method described by Bülbring,[10] except for a somewhat modified muscle holder. The preparation is set up in a vessel containing 100 ml of Krebs–Ringer-bicarbonate-glucose solution[11] at 37° aerated with 5% CO_2 in O_2. The contractions are recorded on a smoked drum. The "standard test procedure" is as follows (Fig. 1).

1. Application of a sequence of tetanic stimuli of 25, 50, 100 and 200 stimuli per sec for 10 sec, followed by 3 min rest.

2. Addition (under stimulation with a frequency of 1 per 10 sec) of anticholinesterase at such a dose that the phase of repetitive response is passed within 5 min. This indicates a complete inhibition of cholinesterase.[12] As an extra safety margin washing of the preparation is delayed until 30 min after the addition.

3. Repetition of the test mentioned (1). If cholinesterase inhibition is complete a single contraction will be seen at all stimulation frequencies.

4. Addition of oxime and removal by washing after 30 min.

5. Repetition of the test mentioned (1). If the cholinesterase has been reactivated sufficiently, the normal tetanic contractions will be observed at all four stimulation frequencies.

The objective was to determine the concentration of oxime just producing "complete functional recovery" of the diaphragm, i.e. restoring normal tetanic contractions at all stimulation frequencies used. For this purpose a number of different oxime doses were added to different diaphragms treated with the same dose of anticholinesterase. The oxime concentration was increased until either complete functional recovery was obtained or a toxic dose was reached. All experiments were repeated at least three times. Allowance should be made for errors of at least a factor of 2 in the determination of limiting oxime concentrations.

Determination of cholinesterase activity in the intact diaphragm: both the right and left hemidiaphragms of rats fasted for 24 hr, are used concurrently and treated according to a pre-determined scheme. After the performance of the tests the muscles are separated from the ribs, weighed and placed into Warburg-vessels containing 2·5 ml Krebs–Ringer-bicarbonate without glucose. The side vessel contains 0·25 ml Krebs–Ringer solution with acetylcholine chloride in a concentration of 0·1 M. After gassing for 10 min with 5% CO_2 in N_2, the taps are closed and the contents of the side arm tipped into the main vessel. The reading is started after another 10 min. The evolution of CO_2 is measured for 60 min. The values obtained between 15 and 60 min are expressed as μl CO_2 per 100 mg muscle (wet weight). In the experiments of Table 5 the following doses were used.

Exp. 1: 10 μg Sarin + PAM $2{\cdot}5 \times 10^{-4}$ M
Exp. 2: 10 μg Sarin + MINA 6×10^{-5} M
Exp. 3: 10 μg Tabun + PAM 2×10^{-3} M
Exp. 4: 10 μg Tabun + MINA $1{\cdot}2 \times 10^{-1}$ M, 30 min
Exp. 5: 10 μg Tabun + MINA $1{\cdot}2 \times 10^{-1}$ M, 90 min
Exp. 6: 500 μg DFP + PAM 2×10^{-5} M

The average value of 23 controls with uninhibited cholinesterase was $76 \pm 2{\cdot}0$ μl CO_2/45 min, the average of 22 controls with completely inhibited cholinesterase was $17 \pm 0{\cdot}8$ μl CO_2/45 min.

Isolated rat hindquarter perfused with blood

A detailed description of the method will be published elsewhere. A rat hindquarter is prepared with a canula in the aorta and one in the vena cava. It is put in a chamber which is kept at 37° and saturated with water vapour. The heparinized rat blood, freed from all white elements, is pumped through a glass cylinder containing glass beads (glasspowder as used for the construction of sintered glass filters no. 0*) before entering into the preparation. This is a reasonably effective trap for circulating leucocytes and trombocytes which are constantly released from the tissues.

The blood passes through an oxygenator and is collected in a beaker of 100 ml from which it is sucked up by the pump. The entire system contains about 40 ml blood. The preparation is "washed" by removing the blood (about 30 ml) from the collecting beaker and replacing it by fresh, treated blood. Stimulating electrodes are put on the sciatic nerve in the upper leg and the contractions of (mainly) the tibialis anterior muscle are recorded on a smoked drum. In most of these preparations muscular contractions are maintained for over 7 hr. The perfusion pressure is maintained at an average value of 120 mm Hg by adjusting the pumping speed. The perfusion rate was 20 ml/min in the average. Anticholinesterases and oximes are added to the blood contained in the beaker.

The test procedure is identical to that for the diaphragm except that the "washing" is less extensive because otherwise very large volumes of blood would be needed. Also the effective dose of anticholinesterase is much higher than in the diaphragm, possibly because of extensive aspecific binding to the much larger tissue masses. The effective dose of anticholinesterase cannot be determined by using the phase of repetitive response as a guide, since in this preparation the enhanced contractions persist even at very high doses during the entire period the anticholinesterase is effective. Therefore, in most cases the dose has been determined which produces a complete abolition of the tetanic contractions and this dose, multiplied by a factor of 4 as a safety margin, has been taken as the standard dose.

Isolated intestine

The isolated rat ileum is put up in a vessel of 20 ml containing Krebs–Ringer-bicarbonate plus 0·2% (w/v) glucose at 37°, oxygenated with 5% CO_2 in O_2. The pieces of ileum are loaded with 1 g. The contractions are recorded on a smoked drum. In order to facilitate the diffusion of anticholinesterase and antidotes, the pieces of ileum are cut lengthwise. Provided they are agitated sufficiently by the passing gas bubbles, they react exactly as normal pieces of intestine. Two adjacent pieces of ileum are run in parallel. The test procedure used on the intestine is in principle comparable to that used on the muscle preparations but differs in practical execution. In the atropin experiments the sequence is as follows.

1. The reactivity of the ileum is tested by addition of 10 μg of acetylcholine. Pieces that do not react to this dose with a contraction of at least 10 mm (magnification 3 times), are usually discarded.

2. Either the "effective dose", i.e. the dose producing maximum contraction in 5–10 min, or 25 times the effective dose of anticholinesterase is added and washed out after 20 min.

* We are grateful to the N.V. Leerdam glass works, Leerdam, The Netherlands, for supplying us with this powder.

3. Five minutes after washing, 1 μg of atropin sulphate is added and this is repeated (sometimes with 0·5 μg doses) every 3 min without washing, until the contraction is abolished completely. Complete abolition is assumed when further addition of 10 μg of atropin sulphate remains without effect.

In the oxime experiments the following sequence is used.

1. The reaction of the ileum is tested as above.
2. Anticholinesterase is added and washed out after 20 min.
3. Oxime is added and washed out after 10 min.
4. The contraction height 15 min after washing, is noted and a high (20–100 μg) dose of atropin is added in order to establish the base line.

The contraction height is measured just before addition of the oxime and again 15 min after the oxime has been washed out. These two values are referred to the base line obtained after the final dose of atropin. By dividing the two values obtained in this way, a "percentage functional recovery" can be calculated. A functional recovery of 90 per cent and over is considered as "complete functional recovery".

Anticholinesterases and oximes

The following anticholinesterases were used: Sarin (isopropyl methylphosphonofluoridate), Soman (1,2,2-trimethylpropyl methylphosphonofluoridate), DFP (diisopropyl phosphorofluoridate), Tabun (ethyl N-dimethylphosphoramidocyanidate). We are grateful to Dr. H. L. Boter, Chemical Laboratory, National Defence Research Organization, T.N.O. for synthetizing these compounds.

The oximes used were PAM (pyridine aldoxime methiodide, in the highest concentrations the chloride was used) and MINA (mono-iso-nitroso-acetone).

RESULTS

I. *Phrenic nerve-diaphragm preparation*

A. *Determination of oxime concentrations needed for complete functional recovery from poisoning with a number of organophosphorus compounds.*

Using the test procedure described under methods the concentrations of PAM and MINA were determined just producing a complete functional recovery (maintenance of a tetanic contraction at 200 stimuli per sec for 10 sec). These limiting concentrations are presented in columns 3 and 4 of Table 1. This table also contains, in columns 5 and 6 the values, relative to the concentration needed to abolish the effect of Sarin. In column 7 the ratios of the effective concentrations of MINA and PAM are calculated.

TABLE 1. FINAL OXIME CONCENTRATIONS JUST PRODUCING "COMPLETE FUNCTIONAL RECOVERY" OF THE ISOLATED DIAPHRAGM INTOXICATED WITH A NUMBER OF ORGANOPHOSPHORUS COMPOUNDS
(Bath volume 100 ml)

Compound	Dose μg/100 ml	[PAM] mole per l.	[MINA] mole per l.	Rel. [PAM]	Rel. [MINA]	MINA PAM
Sarin	10	$2{\cdot}5 \times 10^{-4}$	6×10^{-5}	1	1	0·24
Soman	10	$>1{\cdot}6 \times 10^{-2}$	$>6{\cdot}4 \times 10^{-2}$	>64	>1066	—
DFP	500	2×10^{-1}	4×10^{-3}	0·8	67	20
Tabun	10	2×10^{-3}	$>1{\cdot}2 \times 10^{-1}$	8	>2000	>60

Table 1 shows the following results. The oxime concentrations needed for complete functional recovery vary over a wide range. This is especially marked for MINA (range 1–2000) but is also evident in the case of PAM (range 1–64). However, a complete functional recovery cannot be obtained in all cases. After Soman a certain degree of recovery is found, but both PAM and MINA fail to produce a complete recovery (Fig. 2). The concentration of $1{\cdot}6 \times 10^{-2}$ M PAM mentioned in Table 1, had a distinctly better effect than $0{\cdot}8 \times 10^{-2}$ M and $6{\cdot}4 \times 10^{-2}$ M MINA was still somewhat better than $1{\cdot}6 \times 10^{-2}$ M. This indicates that these concentrations, as presented in Table 1, are probably not to a significant degree determined by an interference of the "aging" process (section C, page 1305). After Tabun a complete recovery is found with PAM, but with MINA a complete recovery cannot be obtained at the highest possible concentration. Here again the concentration of $1{\cdot}2 \times 10^{-1}$ M MINA is more active than $0{\cdot}6 \times 10^{-1}$ M. These doses are within the "toxic" range of MINA, but according to Table 5 reactivation of cholinesterase still occurs. The functional recovery after Tabun followed by MINA is better than after Soman followed by either PAM or MINA, since in the former case a tetanus at 200 stimuli per sec can be maintained for a few seconds whereas in the latter case only a twitch-like contraction is seen (Fig. 2). The ratio [MINA]/[PAM] which for Sarin and Soman (section C, page 1305) is probably not essentially different from 1, is very much greater than 1 for DFP and Tabun.

B. *Evaluation of the test procedure*

A number of experiments were performed in order to evaluate the test procedure adopted.

1. *Relation between the dose of anticholinesterase and the oxime concentration needed for complete functional recovery.* If the dose of anticholinesterase is chosen in such a way that the cholinesterase is inhibited completely, a further increase in the dose must not affect the result obtained with a certain dose of oxime, provided sufficient anticholinesterase has been removed by the washing process. That this is indeed the case has been shown in an experiment with Sarin. Using 10 μg and 100 μg of Sarin, final concentrations of PAM of 2×10^{-4} M and 4×10^{-4} M were needed respectively for complete functional recovery.

2. *Spontaneous recovery.* After administration of most organophosphorus anticholinesterases and removal by washing, a spontaneous recovery occurs which in most cases is only very slight and is probably caused by the gradual diffusion of excess acetylcholine accumulated during the phase of repetitive response. In some cases however a considerable, progressive, recovery is noted which in all probability is due to spontaneous reactivation of the inhibited cholinesterase. As it is conceivable that the differences observed in the oxime concentrations needed for complete functional recovery (Table 1) might be caused by differences in rate of spontaneous recovery, this recovery was investigated more in detail in experiments in which distilled water was added instead of oxime. At each stimulation frequency the extent of functional recovery was evaluated, using an arbitrary scale from 0 (single, twitch-like contraction) to 5 (normal tetanic contraction). The results obtained with a number of anticholinesterases are summarized in Table 2. This table shows that spontaneous recovery is most outspoken with Sarin, a compound which according to Table 1 is most sensitive to oximes. Therefore experiments were performed in order to

determine in how far a previous functional recovery (grade 5) at 100 stimuli per sec (a grade of recovery which surpasses the spontaneous recovery found with Sarin) affects the oxime concentration needed for further, "complete", functional recovery at 200 stimuli per sec (our test criterium).

TABLE 2. SPONTANEOUS RECOVERY OF THE FUNCTION OF A DIAPHRAGM TREATED WITH DIFFERENT ANTICHOLINESTERASES ACCORDING TO THE TEST PROCEDURE DESCRIBED ON PAGE 1300, WITH THE EXCEPTION THAT DISTILLED WATER IS ADDED INSTEAD OF OXIME

(Grading according to an arbitary scale)

Compound	dose, μg	Grading at different stimulation frequencies			
		25/sec	50/sec	100/sec	200/sec
Sarin	10	5	4	2	0
Soman	10	2	1	0	0
DFP	500	2	1	0	0
Tabun	10	2	2	0	0

The results summarized in Table 3 show that the concentration needed for recovery at 100/sec is at most 1/6 of the concentration needed for recovery at 200/sec. Therefore a spontaneous recovery which is not even complete at 100/sec can have very little if any effect on the concentration ultimately needed for complete recovery at 200/sec.

3. *The "toxicity" of the oximes used.* The experiments described in this paper are limited by the "toxic" effects that oximes might have on the isolated diaphragm.

The "toxic" effects noted under our experimental conditions (stimulation frequency 1 per 10 sec) are at first a decrease in contraction height. At still higher concentrations a contracture is seen which at first is reversible but eventually becomes irreversible.

With PAM the "toxic" effects start at about 4×10^{-3} M; the highest concentration that can be used lies around 2×10^{-2} M. With MINA the "toxic" effects appear at 2×10^{-2} M; the highest concentration is about 10^{-1} M.

C. *Experiments on "aging"*

The lack of complete recovery after Soman might be caused by "aging" of the

TABLE 3. COMPARISON OF FINAL CONCENTRATIONS OF OXIME NEEDED FOR COMPLETE FUNCTIONAL RECOVERY AT 100 STIM. PER SECOND VERSUS THOSE NEEDED FOR RECOVERY AT 200 STIM. PER SECOND

Compound	Oxime	Oxime concentration needed for recovery at		Ratio conc. at 200/sec / conc. at 100/sec
		100/sec	200/sec	
Sarin	PAM	1×10^{-5}	$2{\cdot}5 \times 10^{-4}$	25
DFP	PAM	2×10^{-5}	2×10^{-4}	10
	MINA	2×10^{-4}	4×10^{-3}	20
Tabun	MINA	2×10^{-2}	$> 1{\cdot}2 \times 10^{-1}$	> 6

enzyme-inhibitor combination product which restricts the reactivatability of the inhibited enzyme.[13] In the case of Tabun followed by MINA, aging cannot explain the incomplete recovery since complete recovery can be obtained with PAM and also because, according to Table 5 a progressive reactivation of cholinesterase is found. Aging was studied by varying the period of time between the addition of anticholinesterase and the addition of oxime. Since the diaphragm can be kept functioning for about 6 hr in our setup, aging can be studied only during this period. A number of diaphragms were inhibited with either DFP or Tabun and the diaphragms were kept twitching for 4–6 hr after removal of the inhibitor. After this period it was established that still "complete functional impairment" was present and oximes were added in the same optimum concentration as in the experiments mentioned in Table 1 (in the experiments with Tabun only PAM was used). A complete functional recovery was still obtained in this way, indicating that with DFP and Tabun no significant aging had occurred within 4–6 hr. Aging after addition of Sarin has not been studied because of the considerable spontaneous recovery. In order to investigate a possible effect of aging in the case of Soman, the dose of anticholinesterase was increased threefold and the preparation washed after 5 min instead of 30 min. Oxime was added either immediately after the washing (indicated as $t = 0$) or after a certain period of time (indicated as $t = 15$ [min], etc.). At $t = 0$ complete recovery can now be obtained with MINA. It is apparent from Fig. 3, that, as the interval between addition of anticholinesterase and oxime is increased, the recovery progressively decreases. This may be due to "aging", but might conceivably also be caused by an increasing inhibition of cholinesterase, if it is assumed that the 5 min period is insufficient for complete cholinesterase inhibition. Therefore parallel with each experiment a control was run in which distilled water was added instead of oxime. In none of these controls any indication was observed of incomplete inhibition at $t = 0$ or of a progressive inhibition at later times. Figure 3 indicates that the Soman-enzyme reaction product is aged completely in about 60 min.

Now that it is known that aging causes the incomplete recovery after intoxication with Soman in the experiments described (section A), an estimate can be made of the oxime concentration that would reactivate completely if aging could be eliminated. This estimate can be based on the concentration of oxime just producing complete recovery at $t = 0$.

For MINA it can be estimated at 4×10^{-2} M, still a very high concentration.

D. *Cholinesterase activity*

Cholinesterase activity was determined in the intact diaphragm in order to avoid the difficulty of a release of unspecifically bound anticholinesterase during the process of homogenization. Since the oxygen consumption of an isolated diaphragm, kept under nitrogen for 60 min at 37°, is practically reduced to zero, the question arises whether the cholinesterase activities measured in the intact diaphragm under nitrogen, correspond to the activities present in the intact diaphragm kept under oxygen during our testing procedure. This question cannot be answered with absolute certainty but an estimate of the percentage reactivation can be made in the following way. It was shown that the oxygen consumption of the diaphragm was not affected by addition of 660 μg DFP per 100 ml for 40 min. Now two Warburg manometers were selected having practically equal volumes. A hemidiaphragm was brought in each of these

flasks under 95% O_2 + 5% CO_2 and with 0·2% (w/v) glucose added. To one of the flasks acetylcholine was added. In 4 experiments the difference in pressure between the two flasks was 21 ± 0·9 mm Brodie solution per 30 min. After addition of 15 μg DFP to the flasks containing acetylcholine, the pressure changes in the two vessels became identical. Now the following experiments were performed (Table 4). Both halves of a diaphragm were set up in the ordinary 100 ml vessels. In all, 12 diaphragms were used. To all left halves 660 μg DFP was added and removed after 30 min.

TABLE 4. DIFFERENCES IN PRESSURE CHANGES (MM BRODIE SOLUTION) PRODUCED IN 30 MIN BY HEMIDIAPHRAGMS TREATED AS INDICATED

(Each figure in the last column represents the average of 3 diaphragms)

Exp. no.	Left or right hemidiaphragm	DFP added	PAM added	Ac. chol. added	Δ(r − l) mm Brodie
1	l	+	−	+	− 1
	r	−	−	−	
2	l	+	−	+	+26
	r	−	−	+	
3	l	+	+	+	−12
	r	−	+	−	
4	l	+	+	+	−8·5
	r	−	+	+	

Thereafter either 1 ml PAM (final concentration $1{\cdot}75 \times 10^{-3}$ M) or 1 ml distilled water was added for 10 min. The muscles were then placed in each of the two matched Warburg flasks. Acetylcholine was added according to Table 4. Pressure changes were read and the values for the two hemidiaphragms subtracted.

From the results of Table 4 percentage reactivation may be calculated in two different ways, i.e. (12/26) × 100 = 46 per cent and (12/[12 + 8·5] × 100 = 59 per cent. This is in good agreement with the 58 per cent reactivation that was found in comparable experiments under nitrogen.

After it had been shown, by comparing the hydrolysis of acetylcholine with that of butyrylcholine, that practically all cholinesterase present in the diaphragm is acetylcholinesterase, the percentage reactivation was determined with a number of anticholinesterases using PAM or MINA as reactivators. The results of the reactivation experiments are summarized in Table 5. According to this table a complete recovery at 200 stimuli per second corresponds roughly with a reactivation of around 50 per cent and recovery at 100 stimuli per sec with a reactivation of about 17 per cent. This correlation between function and cholinesterase activity confirms previous findings by Barstad.[14]

II. *Isolated hindquarter preparation*

A. "*Toxicity*" *of the oximes*

In order to determine the maximum oxime concentration that can be tolerated by

TABLE 5. REACTIVATION OF CHOLINESTERASE IN THE INTACT DIAPHRAGM BY ADDITION OF PAM OR MINA AFTER COMPLETE INHIBITION OF CHOLINESTERASE WITH A NUMBER OF ORGANOPHOSPHORUS COMPOUNDS

(Number of determinations in brackets in column 3. Percentage reactivation was calculated by using the average of all control values. For added doses, see under methods.)

Exp. no.	Additions		Average μl. CO_2/45 min	% reactivation	functional recovery at
1	Sarin + PAM		54 (4)		
	Sarin + aq. dest		18 (2)	63	200/sec
	Aq. dest. + PAM		76 (2)		
2	Sarin + MINA		48 (4)		
	Sarin + aq. dest		20 (2)	53	200/sec
	Aq. dest + MINA		67 (2)		
3	Tabun + PAM		41 (4)		
	Tabun + aq. dest		16 (2)	41	200/sec
	Aq. dest + PAM		91 (2)		
4	Tabun + MINA		25 (4)		
	Tabun + aq. dest	30′	20 (2)	14	100/sec
	Aq. dest + MINA		77 (2)		
5	Tabun + MINA		41 (4)		cannot be measured
	Tabun + aq. dest	90′	26 (2)	41	due to toxic effect of
	Aq. dest + MINA		86 (2)		MINA
6	DFP + PAM		29 (6)		
	DFP + aq. dest		17 (3)	20	100/sec
	Aq. dest + PAM		75 (3)		

the hindquarter preparation, PAM and MINA were added without anticholinesterase.

The effect on the twitch height in the presence of the oxime and the effect on the tetanic contractions, after the oxime was "washed out", was noted. The maximum tolerated doses were PAM: 2×10^{-5} mole and MINA: 1×10^{-3} mole.

B. *Functional recovery by addition of oximes after organophosphorus intoxication*

Experiments comparable to the experiments mentioned (section IA) on the isolated diaphragm were performed on the hindquarter preparation. The only important difference was a much higher dose of anticholinesterase and a less extensive washing. The course of an experiment is shown in Fig. 4. Sarin, DFP and Tabun have been used in the following doses: Sarin 300 μg, DFP 10 mg and Tabun 1000 μg.

The results of these experiments are summarized in Table 6. Since expression of the oxime dose as a concentration is difficult in this preparation, the amounts of oxime in moles added to the blood is given in this table. For comparison with the phrenic nerve-diaphragm preparation the amounts of oxime added to the diaphragm in 100 ml fluid, are also presented in this table. Considering the differences in technique, Table 6 shows a satisfactory agreement between the two preparations. The marked differences in oxime concentration needed for complete functional recovery found in the diaphragm, are also evident in the hindleg preparation. The remarkably high MINA/PAM ratios after DFP and Tabun are also present in both preparations.

TABLE 6. THE AMOUNT OF OXIME (IN MOLES ADDED) NEEDED FOR COMPLETE FUNCTIONAL RECOVERY (AFTER INTOXICATION WITH A NUMBER OF ANTICHOLINESTERASES) OF THE RAT HINDQUARTER PREPARATION AS COMPARED WITH THE PHRENIC NERVE-DIAPHRAGM PREPARATION

(L: hindquarter preparation; D: diaphragm)

Compound	PAM		MINA		$\frac{\text{MINA}}{\text{PAM}}$	
	L	D	L	D	L	D
Sarin	1×10^{-6}	$2{\cdot}5 \times 10^{-5}$	1×10^{-6}	6×10^{-6}	1	0·24
DFP	2×10^{-6}	2×10^{-5}	1×10^{-4}	4×10^{-4}	50	20
Tabun	5×10^{-5}*	2×10^{-4}	2×10^{-3}	$>1{\cdot}2 \times 10^{-2}$	40*	>60

* Inaccurate because of the 'toxic' effect of PAM at this dose.

III. *The isolated rat ileum*

A. *Evaluation of the method used*

1. *The effect of anticholinesterases alone on the isolated rat ileum.* Addition of an anticholinesterase to the rat ileum causes a contraction starting after a latent period which becomes shorter as the dose of anticholinesterase is increased. The ileum contracts at an increasing rate with increasing doses of anticholinesterase. When the maximum contraction is reached, a fairly constant level is maintained with minor, irregular fluctuations for many hours, even after washing of the preparation. Only with Sarin, which also in the diaphragm shows a rapid spontaneous reversibility, the contraction height diminishes fairly rapidly. The average decrease in contraction level, 45 min after addition of Sarin, was in 10 experiments 20·4 $\pm$ 5·0% of the initial contraction height. On renewed addition of Sarin the contractions return to the initial level.

2. *The effect of atropin.* Atropin presumably acts by antagonizing the effect of (accumulated) acetylcholine, but does not reactivate inhibited cholinesterase.

As is shown in Fig. 5, the contractions caused by DFP are abolished by atropin but return progressively during washing for 1 hr. Renewed addition of atropin again abolishes the contractions. If anticholinesterase is added again, immediately after the atropin has been washed out, it produces no effect (compare with the oxime experiments, Fig. 6). Even after a dose of atropin, 20-fold the effective dose, complete re-establishment of the contractions occurs after prolonged washing (compare with the oxime experiments, Fig. 7). This confirms the notion that the effect of atropin in anticholinesterase poisoning is completely "symptomatic".

3. *The effect of oximes.* If a sufficient dose of oxime is added to a piece of ileum, contracted by previous addition of an anticholinesterase, the intestine relaxes and remains relaxed after washing (Fig. 6). A second dose of anticholinesterase, added immediately after removal of an oxime, again produces a contraction of the intestine (Fig. 6) exactly similar to the contraction produced in a fresh preparation.

If oxime is added in a suboptimal concentration the relaxation ("functional recovery") is incomplete or absent. After certain anticholinesterases very high doses of oxime must be added in order to obtain complete relaxation. Under these circumstances contractions may be partly re-established after the oxime has been washed

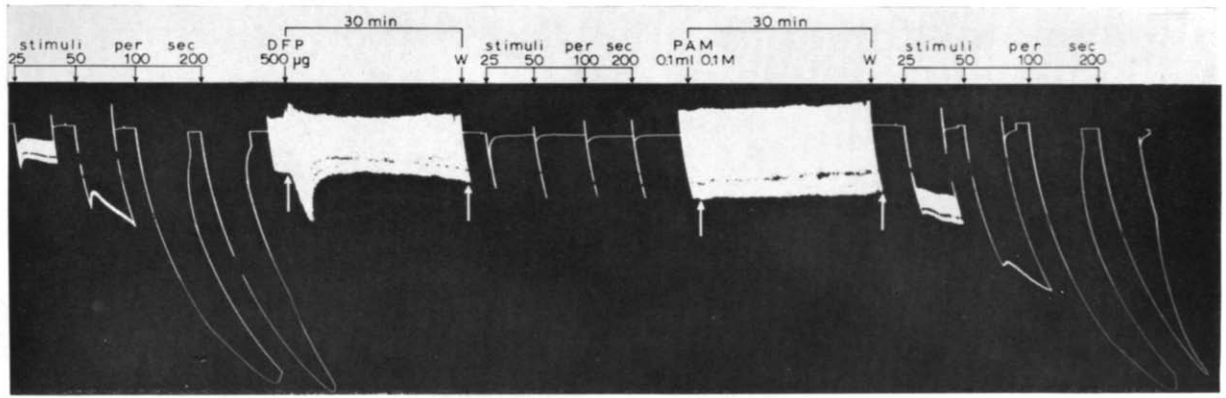

FIG. 1. Test method used for determining the effective oxime concentration in the isolated diaphragm.

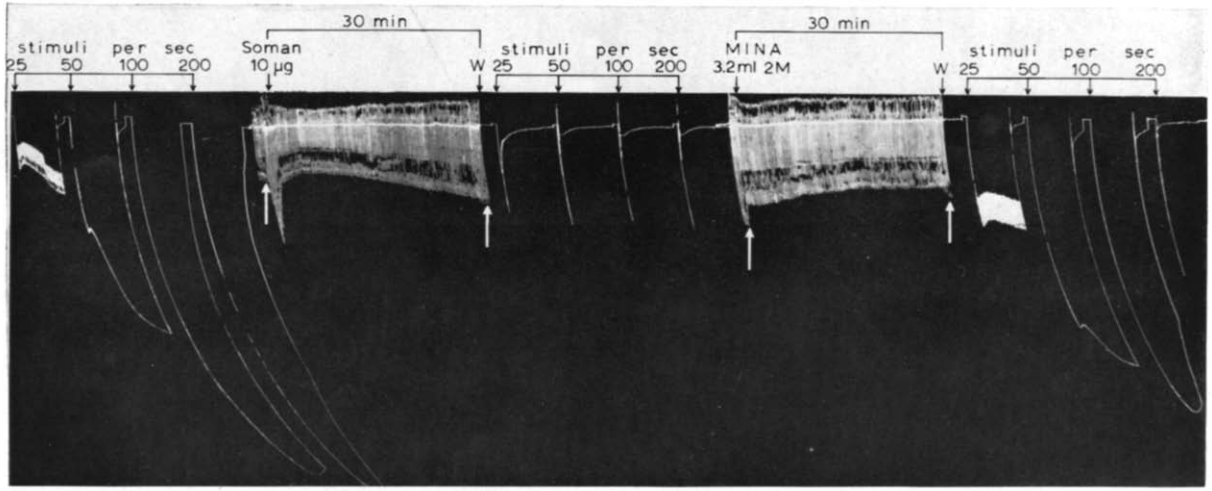

FIG. 2. Incomplete recovery of the diaphragm treated with Soman after addition of MINA to a final concentration of 6·4 $\times 10^{-2}$ M.

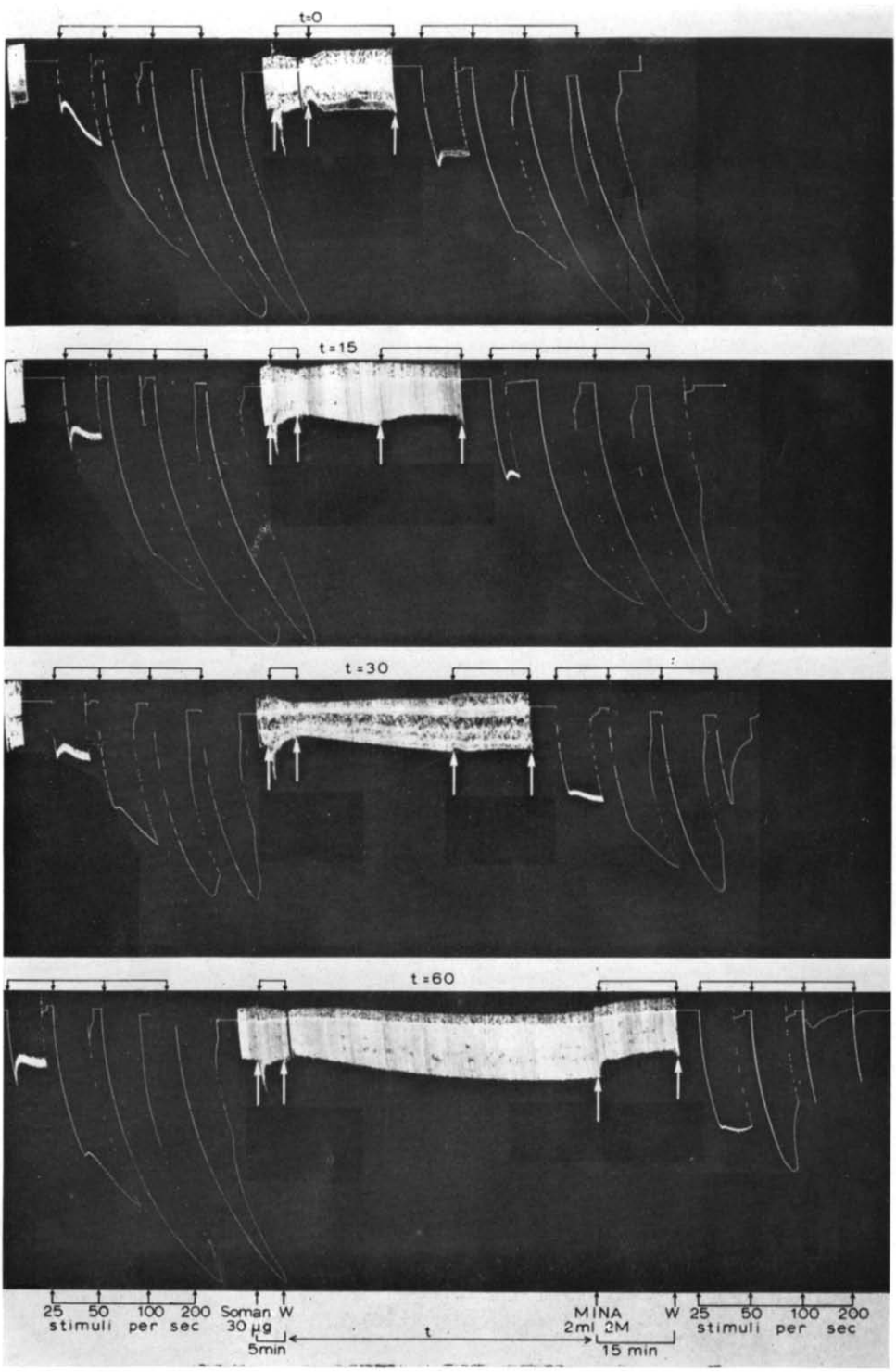

FIG. 3. Progressive failure of oxime treatment with increasing period of time between addition of Soman and addition of MINA.

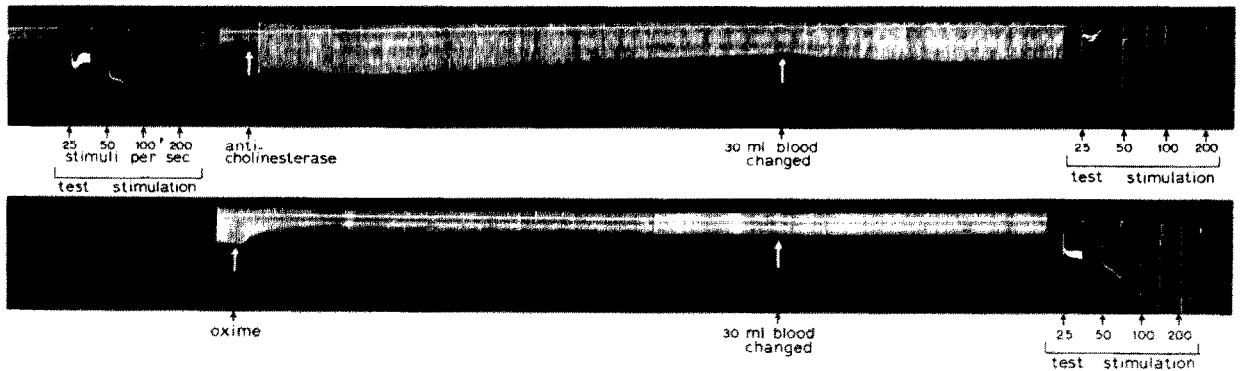

FIG. 4. Test method used for determining the effective oxime concentration in the isolated rat hindquarter perfused with rat blood. Note persistence of repetitive response after addition of anticholinesterase until oxime is added.

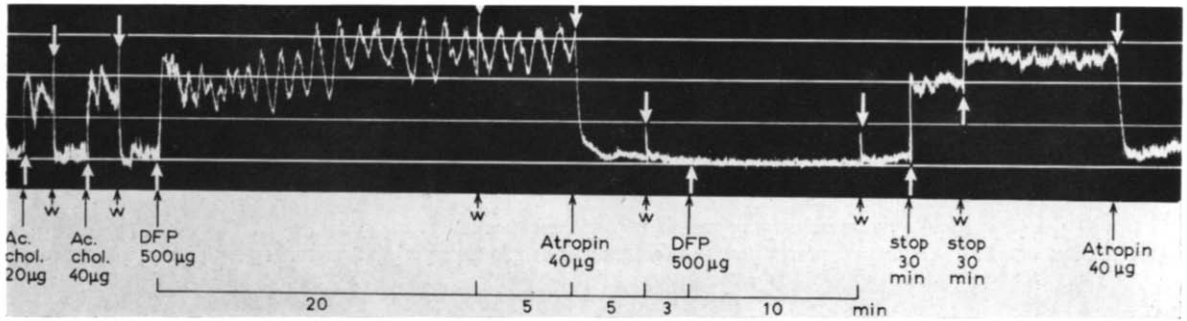

FIG. 5. The effect of atropin on the isolated rat ileum treated with DFP.

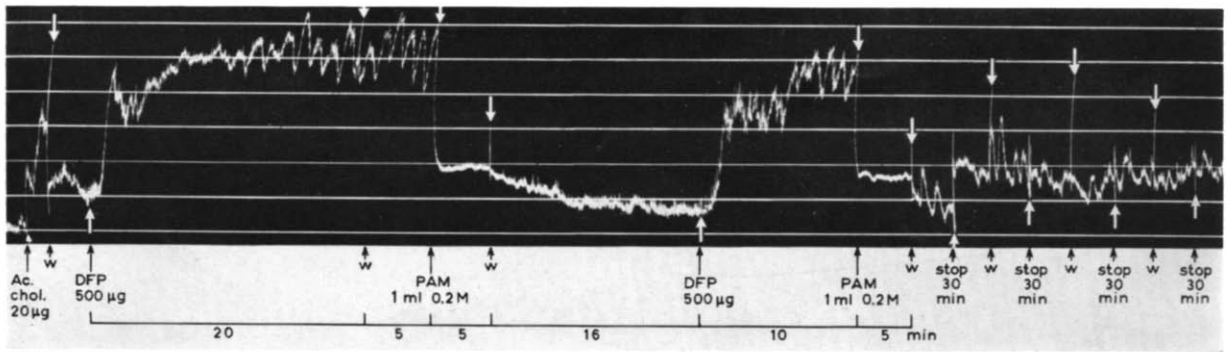

FIG. 6. The effect of PAM on the isolated rat ileum treated with DFP.

away. A further increase in the oxime dose may then in most cases abolish the contractions completely and now they do not return after washing (Fig. 7). Subsequent renewed addition either of an anticholinesterase or of acetylcholine then produces a normal contraction again, showing that the intestine has not been damaged irreversibly. This atropin-like or antispasmodic effect is found for PAM from $0{\cdot}25 \times 10^{-3}$ M upward and for MINA from 1×10^{-3} M upward. A similar effect was noted by Lindgren and Sundwall[15] and Loomis *et al.*[16]

In order to study the reactivating effect independent of this antispasmodic effect (which appeared to be readily reversible), the height of contraction was always noted 15 min after removal of the oxime.

B. *The effect of atropin on the intestine intoxicated with different anticholinesterases*

Anticholinesterases were added in two doses, the "effective dose" (see Methods) and 25 times the effective dose. The dose of atropin needed for complete reduction of the contractions, caused by a number of anticholinesterases, was determined. The results are presented in Table 7. This table shows that, irrespective of the dose and the nature of the anticholinesterase used, the dose of atropin needed to abolish the contractions lies in 24 out of 33 experiments between 2 and 4 μg atropin and in all experiments between 1 and 6 μg.

TABLE 7. DETERMINATION OF THE DOSE OF ATROPIN NEEDED TO PRODUCE A COMPLETE RELAXATION OF THE RAT ILEUM CONTRACTED BY AN ANTICHOLINESTERASE. EACH VALUE REPRESENTS A SEPARATE EXPERIMENT

(Bath volume 20 ml)

Compound	Dose (μg)	Dose of atropin (μg) needed for complete relaxation	Average
Sarin	2	2, 2·5, 3, 3	2·6
	50	2, 2, 2·5, 3, 4	2·7
Soman	2	1, 1·5, 1·5, 2·5	1·6
	50	1·5, 1·5, 2	1·7
DFP	20	1, 2·5, 4	2·5
	500	1, 2·5, 4	2·5
Tabun	2	3, 4, 4, 6	4·3
	50	2, 3, 3, 4, 4, 4, 5	3·6

C. *The effect of oximes on the intestine intoxicated with different anticholinesterases*

The effect of PAM and MINA on the functional recovery after intoxication with a number of organophosphorus anticholinesterases, is shown in Table 8. The results show that, especially for MINA, the concentration needed for complete functional recovery varies considerably with the anticholinesterase used. After Soman complete functional recovery cannot be obtained. Furthermore the ratio MINA/PAM which is 1 for Sarin, is 50 for DFP and more than 40 for Tabun. This is in agreement with the results obtained with the phrenic nerve-diaphragm preparation and the hindquarter preparation and therefore lend these results a more general importance.

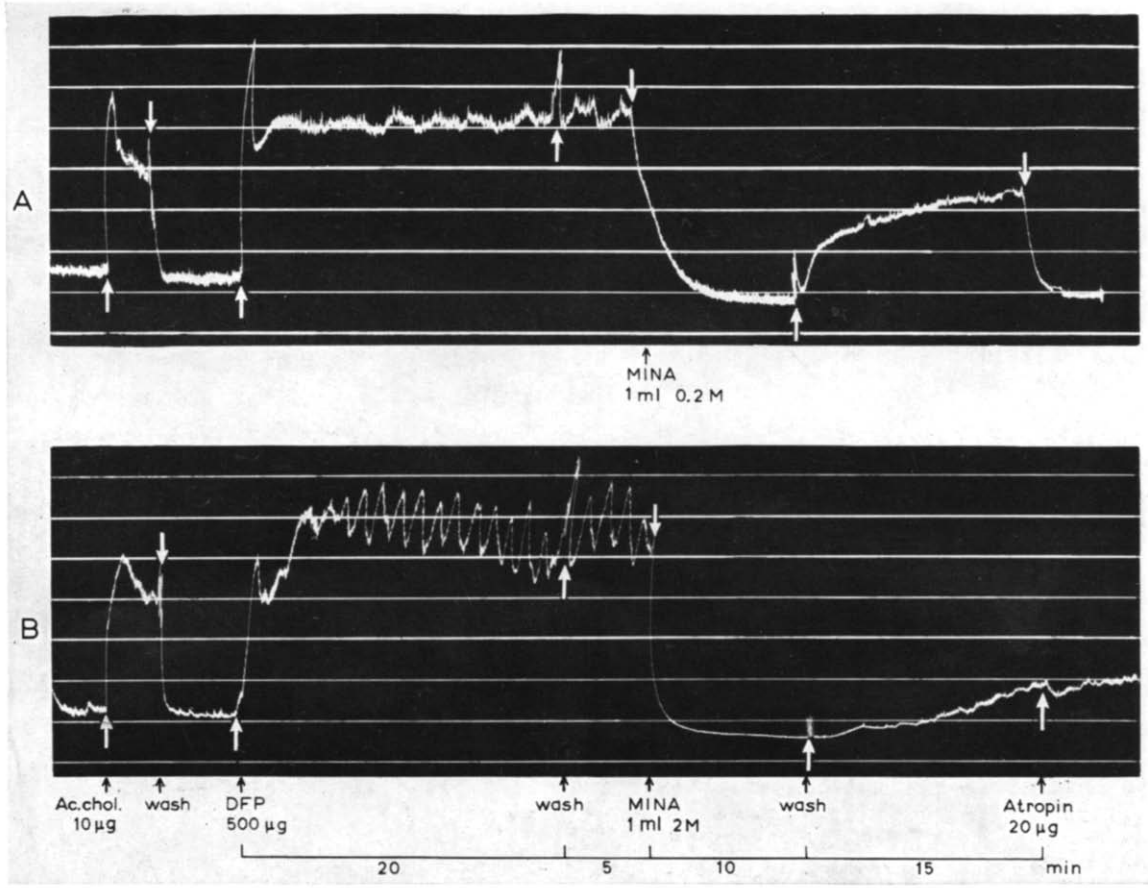

FIG. 7. Upper curve: The effect of MINA on the isolated rat ileum treated with DFP. The initial abolition of the contraction is due to partial reactivation of cholinesterase combined with a 'spasmolytic' effect, as is apparent after washing and subsequent addition of atropin.

Lower curve: At a higher dose of MINA the relaxation is caused by complete reactivation of cholinesterase probably combined with the 'spasmolytic' effect. After washing, a slight increase in tone is observed which however cannot be abolished by atropin.

TABLE 8. "FUNCTIONAL RECOVERY" OF THE ISOLATED RAT ILEUM TREATED WITH A NUMBER OF ANTICHOLINESTERASES FOLLOWED BY EITHER PAM OR MINA

Compound	Dose μg	Oxime concentration producing 50% recovery		Oxime concentration producing "complete" recovery		
		PAM	MINA	PAM	MINA	MINA / PAM
Sarin	50	$1{\cdot}2 \times 10^{-4}$	$3{\cdot}5 \times 10^{-4}$	$2{\cdot}5 \times 10^{-3}$	$2{\cdot}5 \times 10^{-3}$	1
Soman	50	4×10^{-2}*	$>2 \times 10^{-1}$	$>4 \times 10^{-2}$	$>2 \times 10^{-1}$	—
DFP	500	$3{\cdot}5 \times 10^{-4}$	7×10^{-3}	1×10^{-3}	5×10^{-2}	50
Tabun	50	5×10^{-4}	2×10^{-1}†	5×10^{-3}	$>2 \times 10^{-1}$	>40

* 43% recovery.
† 39% recovery.

DISCUSSION

Using three different preparations, i.e. the isolated phrenic nerve-diaphragm preparation, the isolated rat hindquarter preparation perfused with rat blood, and the isolated rat ileum, the efficacy of the oximes PAM and MINA in counteracting different anticholinesterases, was studied. Very similar effects were observed in all three preparations, indicating that these effects are expressions of basic mechanisms acting in a similar way in different parts of the body.

The conclusions drawn from these observations will therefore have a bearing on a number of vital processes in the living animal and in man.

The efficacy of oximes in anticholinesterase poisoning is governed by two factors acting independently, i.e. the effective oxime concentration and the aging rate of the cholinesterase-inhibitor combination product.

Effective oxime concentration: Large differences in effective concentration were found, depending on the one hand on the anticholinesterase used, on the other hand (but to a smaller degree) on the oxime used.

The question arises how the concentrations used in these experiments compare with the concentrations likely to occur in man and animals during oxime treatment. Sundwall[17] observed in man, after intramuscular injection of P_2S, a maximum plasma concentration of 25 μg per ml (1×10^{-4} M). Similarly a dose of 53 mg/kg injected into a rat[7] corresponds to a concentration of 2×10^{-4} M if distributed uniformly over the body and a dose of 150 mg/kg of MINA[7] corresponds in this way to a concentration of $1{\cdot}7 \times 10^{-3}$ M.

As both PAM and MINA are excreted rapidly, these high concentrations ordinarily cannot be maintained for more than a few minutes. As some of the concentrations of Tables 1 and 8 are considerably above these values, it is conceivable that the concentration factor may be of decisive importance in the treatment of man against anticholinesterase poisoning. The objection could be made that our tests for "complete functional recovery" are too severe and that the effective oxime concentrations found in our experiments are therefore much higher than those needed in the treatment of anticholinesterase poisoning.

Our test at 200 stimuli per sec during 10 sec is undoubtedly too severe since stimulation frequencies of this magnitude are unlikely to occur in the body for such a long time. The test at frequencies between 50–100 per sec corresponds better with the situation *in vivo*.[18]

Our results presented in Table 3 show that at a stimulation frequency of 100/sec (corresponding according to Table 5 to about 20 per cent cholinesterase activity) the differences between the oxime concentrations remain and that, e.g. in the case of Tabun treated with MINA, still an extremely high concentration of MINA (2×10^{-2} M) must be maintained for 30 minutes in order to restore function. Therefore, although the absolute concentrations determined in our experiments may be higher than the concentrations that will be needed *in vivo*, we feel that the differences in concentration observed, remain important in any situation and that these differences will undoubtedly affect the therapeutic value of a given oxime. The practical importance of the high concentrations needed in certain cases can only be evaluated in animal experiments.

The observation that the concentration differences are more marked with MINA than with PAM, may be of some importance. It is conceivable that other reactivators might be found in which these concentration differences are smaller or even absent.

Aging rate of the cholinesterase-inhibitor combination product: In our experiments it was demonstrated that for Soman aging is complete in about 60 min, whereas with DFP and Tabun no aging can be observed within 4–6 hr. The importance of the aging process in the case of an acutely fatal intoxication is determined by the time passing between the moment the intoxication starts and the moment at which death would occur without treatment and the effect of aging need only be considered for those compounds with which aging is extremely rapid.

In case of repeated or chronic intoxication, aging might well become of importance in the treatment of poisoning with all anticholinesterases.

According to the results described in this paper the organophosphorus anticholinesterases may in principle be divided in 4 groups as presented in Table 9. In evaluating this table it should be kept in mind that the effective oxime concentrations might perhaps have been different if other reactivators had been used.

TABLE 9. CLASSIFICATION OF ORGANOPHOSPHORUS ANTICHOLINESTERASES WITH RESPECT TO OXIME TREATMENT

Group no.	Effective oxime concentration	Aging	Example
1	low	slow	DFP
2	high	slow	Tabun
3	low	rapid	unknown
4	high	rapid	Soman

The higher the effective oxime concentration, and the higher the aging rate, the less effective will be the treatment with oximes.

Failure of oxime treatment against Tabun and Soman must primarily be ascribed to the high oxime concentration needed. In the case of Soman the aging process may have a slight additional effect.

At the moment it seems unlikely that the aging rate of a given compound can be substantially affected in the body although effects *in vitro* on aging have been described.[19, 20] Therefore improvement in the treatment of anticholinesterase poisoning

may be expected mainly from improving the efficacy of reactivators, either by producing compounds active at lower concentrations, or by developing methods for increasing the concentration in the body and prolonging the action of the oximes known at present. However, in this respect the possibilities are limited by the toxicity of these oximes.

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