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THE EFFECTS OF P_2S, TMB_4 AND $L\ddot{u}H_6$ ON THE RAT PHRENIC NERVE DIAPHRAGM PREPARATION TREATED WITH SOMAN OR TABUN

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Abstract—The oximes P_2S, TMB_4 and $L\ddot{u}H_6$ were applied to the isolated rat phrenic nerve diaphragm either before or after the addition of one of the cholinesterase inhibitors soman and tabun and the effects were studied. Soman and tabun abolished the ability of the preparation to sustain tetanic contractions on indirect stimulation; this is in accordance with the results of earlier experiments. The oximes antagonized this effect partially or completely. P_2S was the most effective of the three oximes tested both when applied before and after soman. The efficacy of $L\ddot{u}H_6$ was about equal to that of TMB_4. When added to the organ bath either before or after tabun P_2S yielded less favourable results than those of TMB_4 and $L\ddot{u}H_6$. In these experiments the functional recovery obtained with either of the three oximes was better when they were administered after tabun than before this agent. When the oximes were added to the bath both before and after tabun the results were about equal to those obtained in the experiments in which they were administered after the anticholinesterase only. The results of experiments in which P_2S and $L\ddot{u}H_6$ were injected into soman or tabun poisoned atropinized rats either 10 min before or $1\frac{1}{2}$ min after the nerve gas show essentially the same trend as those obtained in the experiments *in vitro*.

CHOLINESTERASE inhibitors cause a decrease of the ability of the isolated diaphragm to sustain a tetanus induced by indirect supramaximal electrical stimulation. The degree of prevention or reversal of this decrease has been proved to be a valuable measure of the effectiveness of potential antidotes against the influence of toxic organophosphorus compounds on neuromuscular transmission (Erdmann and Engelhard,[1] and van der Meer and Wolthuis)[3] Using this principle the last named authors showed that the oximes PAM and MINA in low doses could completely restore the tetanic response of isolated rat diaphragms after poisoning with certain organophosphates—e.g. sarin and DFP—whereas, even after higher concentrations of these oximes, recovery was incomplete in diaphragms pretreated with related cholinesterase inhibitors such as soman. Erdmann and Engelhard[1] compared the effects of PAM on the rat phrenic nerve diaphragm preparation treated with parathion

* Throughout this paper the following abbreviations are used: DFP: diisopropyl phosphorofluoridate; PAM: 2-hydroxyiminomethyl-N-methylpyridinium iodide; P_2S: 2-hydroxyiminomethyl-N-methylpyridinium methanesulphonate; MINA: (mono)isonitrosoacetone; TMB-4: 1,3-trimethylenebis(4-hydroxyiminomethylpyridinium bromide); $L\ddot{u}H_6$: (= Toxogonin):1,1′-(oxodimethylene)bis(4-hydroxyiminomethylpyridinium chloride.

Soman and tabun are: 1,2,2-trimethylpropyl methylphosphonofluoridate and ethyl N-dimethylphosphoramido cyanidate respectively.

or DFP with those of the newer oxime $LüH_6$. They found $LüH_6$ to be the more active of the two.

The purpose of the present investigation was two-fold:

(*a*) to compare the effectiveness of the oximes P_2S, TMB_4 and $LüH_6$ in preventing or abolishing blockade of neuromuscular transmission in the isolated rat diaphragm, caused by soman or tabun, and

(*b*) to obtain information about the extent to which mortality of rats poisoned with soman or tabun may be prevented by either P_2S or $LüH_6$.

METHODS

Materials

P_2S, TMB_4, $LüH_6$ and atropine sulphate were obtained commercially; soman and tabun were synthesized in the Chemical Laboratory RVO-TNO at Rijswijk, The Netherlands. All solutions were freshly prepared in demineralized water.

Experiments with the phrenic nerve diaphragm preparation

The phrenic nerve diaphragm preparations were obtained from inbred female albino rats from the laboratory strain weighing 120–140 g. The dissection and mounting methods used were essentially those described by Bülbring.[5] The preparations were incubated at 37°C in Krebs–Ringer solution containing 0·2% glucose and aerated with 95% O_2 + 5% CO_2. The phrenic nerve was stimulated supramaximally with square pulses of 1 V and 75 μsec duration. Stimulation was performed once every 10 sec, except when its ability to sustain a tetanic contraction was tested. This test was performed as shown in Fig. 1. During four periods of 10 sec the phrenic nerve was stimulated with successively 25, 50, 100 and 200 stimuli/sec. During the 3 min preceding and following each tetanic stimulation the preparation received no stimuli. In the course of each experiment three such tests were performed.

The experimental design was the following. After stimulation at the rate of 1 stimulus in 10 sec for 15 min a test was carried out. Fifteen minutes after the completion of this test 10 μg soman or 20 μg tabun were added to the 100 ml Krebs–Ringer solution, in which the isolated diaphragm was incubated, and allowed to act for 30 min. Then the preparation was rinsed and immediately thereafter it was tested again in order to assess the effect of the cholinesterase inhibitor.

Three minutes after this test one of the oximes was administered and left in contact with the isolated diaphragm for 30 min in a final concentration of 10^{-2} M in the soman experiments and of $0{\cdot}5 \times 10^{-4}$ M in the tabun experiments. Thereafter the preparation was rinsed and the effect of the oxime was determined by carrying out a third test immediately.

In other experiments, the described experimental design was followed with the exception that the oxime, instead of being administered after the second test, was added to the organ bath 10 min prior to the soman or the tabun and was washed out together with the cholinesterase inhibitor. No further oxime was administered, unless stated otherwise (Fig. 3).

Control experiments were performed in which either the cholinesterase inhibitor or the oxime or both were omitted.

The contractions of the diaphragm preparation were recorded on a smoked drum; they were quantified by planimetry of the area bounded by the curves obtained. The

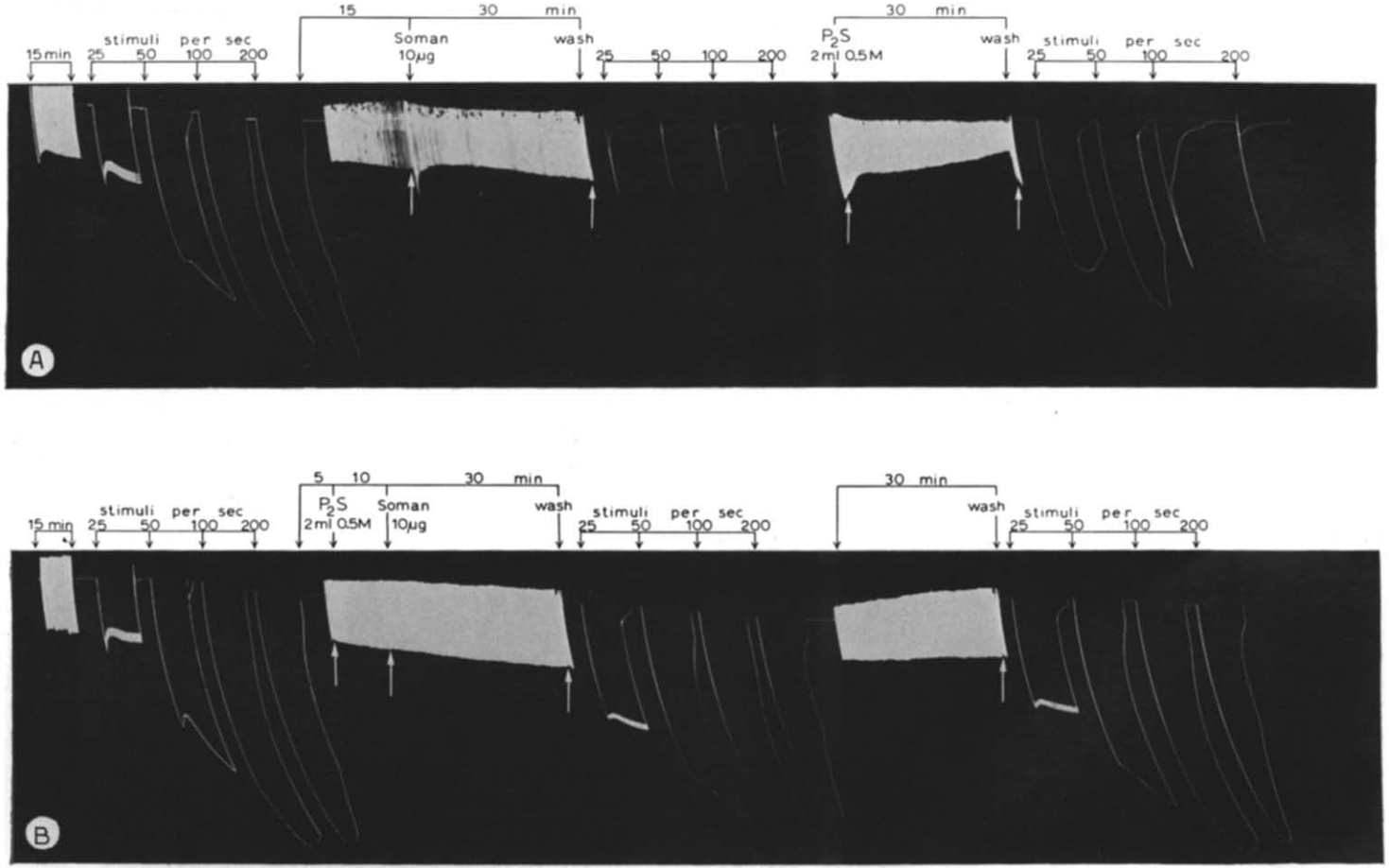


FIG. 1(a). The effects of soman (10 μg/100 ml) on the tetanic performance of the isolated phrenic nerve diaphragm preparation of the rat and the partial restoration of function by the subsequent application of P_2S (10^{-2} M).

(b). The effect of the administration of soman (10 μg/100 ml) preceded by the addition to the organ bath of P_2S (10^{-2} M). No impairment of function.

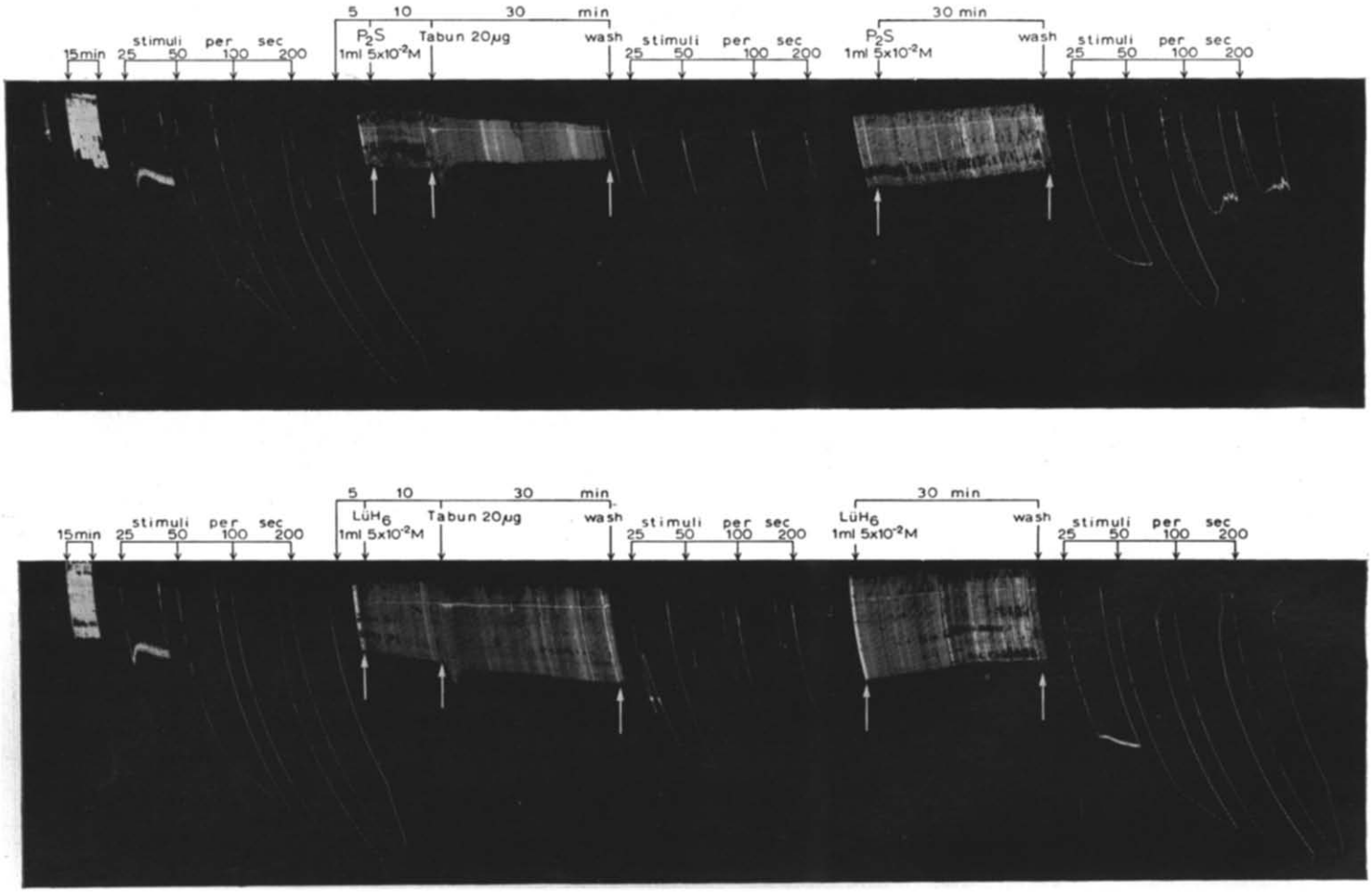
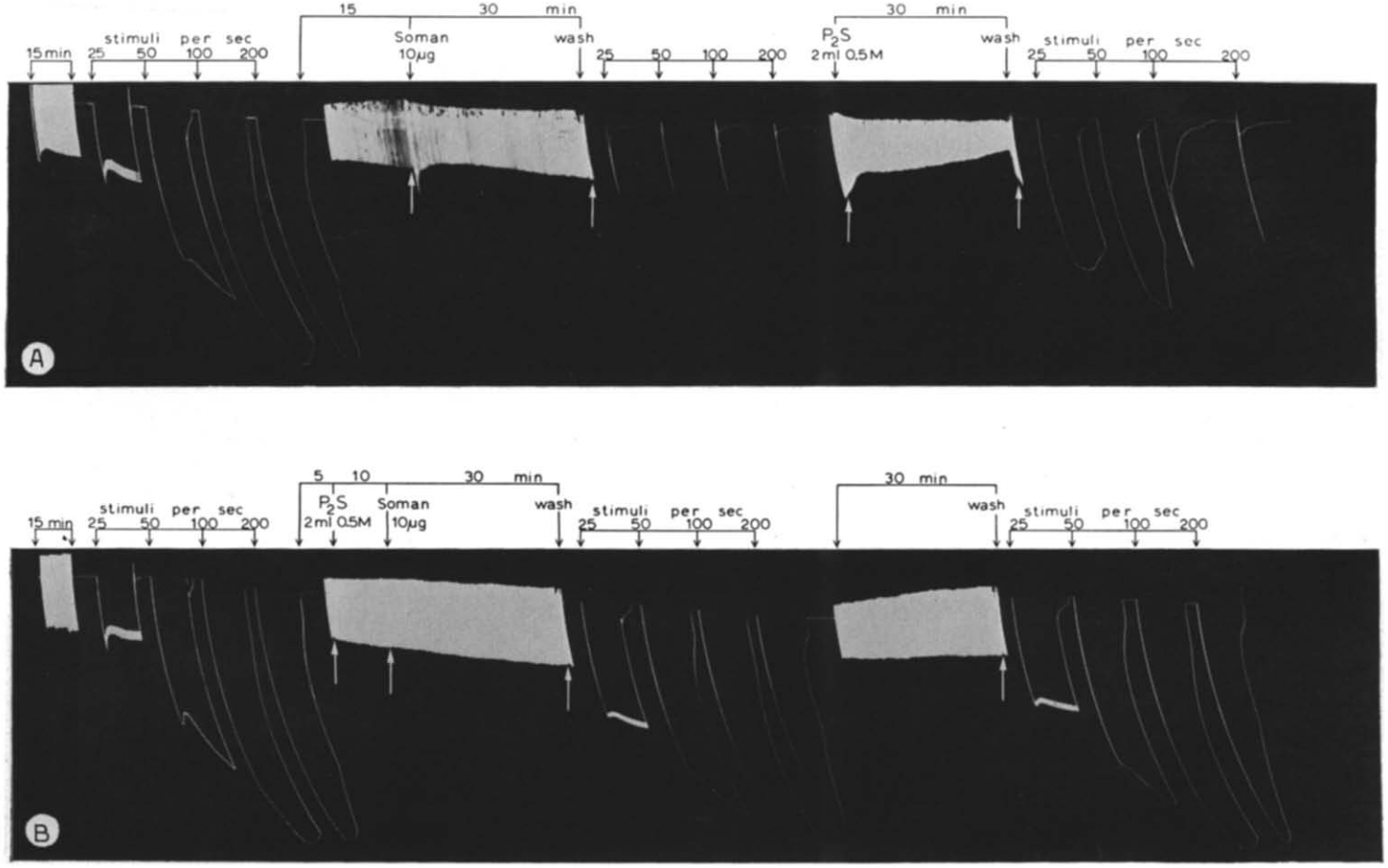


FIG. 3. Isolated phrenic nerve diaphragm preparations of the rat. The presence of P_2S or $L\ddot{u}H_6$ in the organ bath before and during the application of tabun (20 µg/ml) does not prevent or prevents only partially the loss of the tetanic performance. The second administration of the oximes after the removal of the tabun from the bath results in a functional recovery which is incomplete after P_2S and complete after $L\ddot{u}H_6$.



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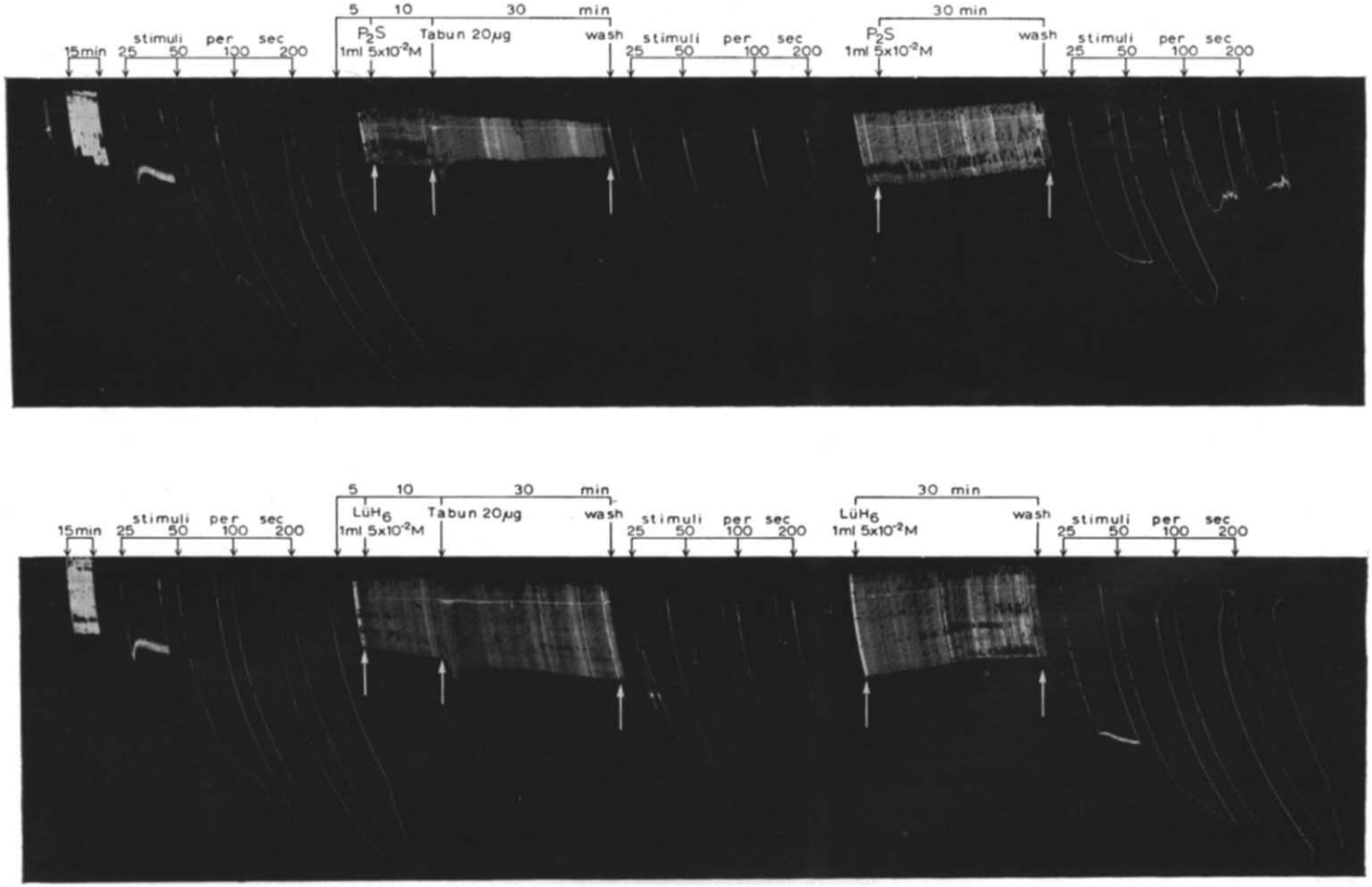

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values given in Table 1 and Fig. 2 represent the data derived from the third or second test expressed as percentages of the areas of the corresponding contractions in the first (control) test of the same preparation at the four different stimulation frequencies applied.

TABLE 1. THE TETANIC PERFORMANCE OF THE PHRENIC NERVE DIAPHRAGM PREPARATION OF THE RAT AT DIFFERENT STIMULATION FREQUENCIES

	Control experiments				
	Tetanic contraction as percentages of the first test at the various stimulation frequencies				No. of diaphragms
	(25/sec)	(50/sec)	(100/sec)	(200/sec)	
No drugs applied	162, 190 193	86, 121 120	77, 94 87	70, 62 61	3
Soman only (10 μg/100 ml)					
2nd test	5, 7	2, 2	1, 1	1, 1	
3rd test	9, 11	5, 4	1, 1	1, 1	2
Tabun only (20 μg/100 ml)					
2nd test	7 ± 3·1	1 ± 0·2	1	1	
3rd test	25 ± 9·9	3 ± 0·8	1 ± 0·2	1	5
Oximes only (1×10^{-2})					
P_2S: after 2nd test	133	101	91	80	1
after 1st test	134	95	96	89	1
$L\ddot{u}H_6$: after 2nd test	108	95	99	82	1
after 1st test	140	113	95	93	1
TMB_4: after 2nd test	120	103	77	94	1
after 1st test	140	96	93	82	1

The values in the various columns represent the areas bounded by the curves obtained in the third or second test, expressed as percentages of the corresponding values derived from the first test of the same preparation. No drugs or only either a cholinesterase inhibitor or an oxime were applied to the organ bath.

Experiments with intact animals

In earlier experiments the optimal therapeutic dose of P_2S and of $L\ddot{u}H_6$ had been established on account of the results of the i.p. administration of various amounts of the oximes together with atropine 1·5 min following the subcutaneous injection of a fixed dose of soman or tabun. After the administration of soman the best survival was obtained with 150 mg/kg of either of the oximes, after tabun a dose of 100 mg/kg offered a slightly better result.

In the experiments to be described the efficacy of each oxime in the mentioned doses was tested in male rats (160–190 g). Application was performed by the i.p. route either 10 min before or 1·5 min after the s.c. injection of the nerve gas. Soman was administered in the doses of 220, 270 and 330 μg/kg; these amounts represent approx. 1·3, 1·6 or 2·0 times the subcutaneous LD_{50} respectively; tabun was given in the doses of approx. 2·0, 2·7, 3·5 or 4·5 times the LD_{50} which amounted to 760, 1070, 1330 or 1710 μg/kg. These doses appeared to allow possible differences in the effects of the two oximes to be demonstrated. All animals received 37·5 μg/kg atropine sulphate i.p. 1·5 min after the injection of the nerve gas. In a few series of experiments this dose of atropine alone did not protect more than two out of a total number of sixty in the soman experiments and none out of sixty animals in the experiments with tabun against the lethal effects of these cholinesterase inhibitors.

RESULTS

1. *Control experiments*

Table 1 summarizes the results of experiments in which either no drugs or one of the cholinesterase inhibitors alone or one of the oximes alone was applied to the isolated phrenic nerve diaphragm preparation.

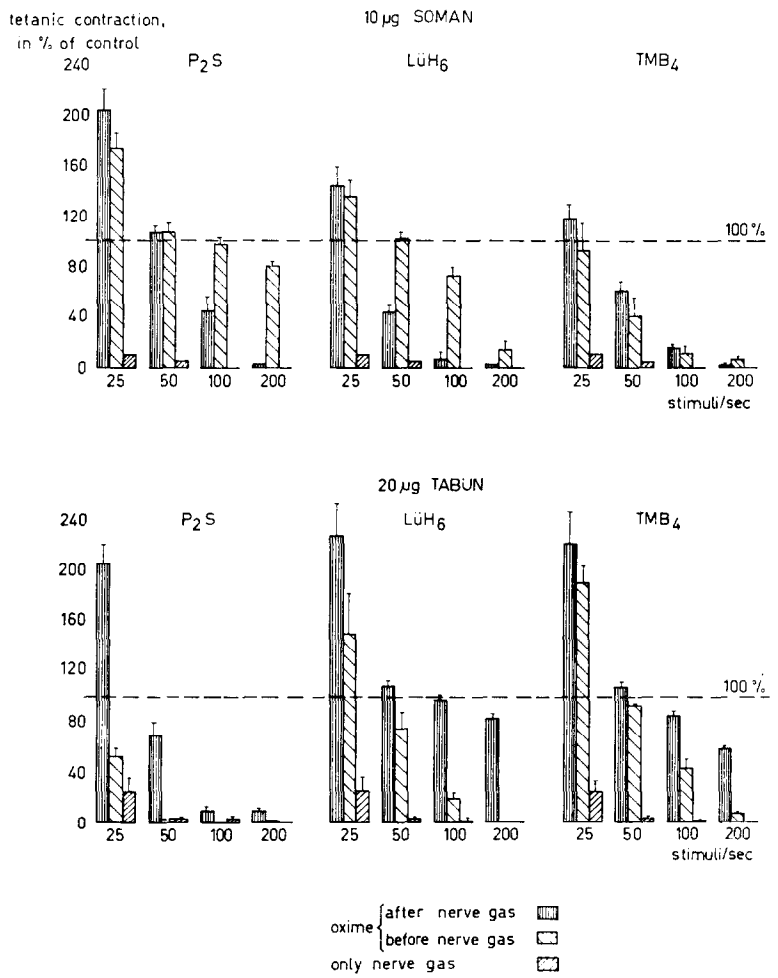

FIG. 2. The tetanus sustaining ability of the phrenic nerve diaphragm preparation after treatment with soman or tabun and the effects of P_2S, TMB_4 and $L\ddot{u}H_6$ applied after or before the cholinesterase inhibitor. The bars in the block diagrams represent the areas bounded by the curves obtained in the third test, expressed as percentages of the corresponding values derived from the first test of the same preparation. Each bar represents the mean result of five experiments. The vertical line on top of each column indicates the standard error.

It appeared that even when no drugs were applied the performance of the preparation changed somewhat with time; at the time of the third test the contractions produced at stimulation frequencies of 25/sec had become larger, those at 100/sec and 200/sec smaller than during the first test.

In experiments in which the oximes were administered alone either before or after the second test the performance of the preparation was not significantly different from that of preparations not exposed to drugs.

The addition of soman (10 μg/100 ml) or tabun (20 μg/100 ml) to the organ bath produced a complete loss of the normal ability of the preparation to sustain a tetanus. Such preparations reacted only with a twitch on tetanic stimulation (Fig. 1a and 3a). Comparison of the results of stimulation with 25 and 50 stimuli/sec during the second test with those obtained during the third test reveals that in course of time there occurred a small unsignificant spontaneous recovery of function after the injection of tabun. There was a slight indication of a similar effect after soman.

2. *The effects of oximes on the performance of the soman or tabun treated phrenic nerve diaphragm preparation during electric stimulation*

The efficacy of the oximes P_2S, TMB_4 and $L\ddot{u}H_6$ in antagonizing the blockade of neuromuscular transmission in the tetanically stimulated phrenic nerve diaphragm preparation, treated with soman or tabun, was investigated in experiments in which these oximes were applied either after or before the cholinesterase inhibitor. The results are represented in Fig. 2. The differences between the effects are most spectacular at the higher stimulation frequencies.

In the experiments in which the oximes were applied to soman pretreated and subsequently washed preparations they produced only a partial recovery of the lost capacity to sustain a tetanus (see Fig. 1a and left hand bars of Fig. 2). P_2S was more effective in this respect than TMB_4 or $L\ddot{u}H_6$. In the tabun pretreated preparations on the other hand the application of TMB_4 or $L\ddot{u}H_6$ after the second test produced a complete functional recovery, whereas the restoring effect of P_2S was only partial.

When the oximes were present in the organ bath before and during the exposure of the diaphragm preparation to the cholinesterase inhibitor (see Fig. 1 and middle bars of Fig. 2) the following results were obtained. P_2S afforded a nearly complete protection against the action of soman, whereas the effects of TMB_4 and of $L\ddot{u}H_6$ were smaller. It appeared that P_2S and $L\ddot{u}H_6$ were more effective against soman when applied before than when applied after this cholinesterase inhibitor. Such a difference was not seen with TMB_4. In contrast to the results with soman treated preparations, the protection against the tabun effect resulting from the presence of the oximes in the organ bath before and at the same time as the tabun was distinctly smaller than the recovery of function, produced by the oximes when applied after washing out the tabun. Actually the effect of the "prophylactic" administration of P_2S was negligable.

In a small number of experiments the oximes P_2S and $L\ddot{u}H_6$ were applied twice, once before and once after the tabun. Two such experiments are illustrated in Fig. 3. The second application of the oxime resulted in a functional recovery of the preparation which was complete in the case of $L\ddot{u}H_6$ and incomplete after P_2S. These results are not different from those, obtained in the experiments in which the oximes had been applied only after the tabun.

3. *Experiments on intact atropinized rats*

The results of experiments in which the influence of prophylactic and therapeutic treatment with P_2S or $L\ddot{u}H_6$ of soman or tabun poisoned rats was investigated are

summarized in Table 2. Although an exact determination of the antidotal ratios between these oximes was not carried out, the results show the same tendency as those obtained with the isolated phrenic nerve diaphragm: against soman intoxication P_2S was more effective than $LüH_6$, but $LüH_6$ was more effective than P_2S against tabun poisoning.

TABLE 2. THE INFLUENCE OF THE PROPHYLACTIC OR THERAPEUTIC TREATMENT WITH P_2S OR $LüH_6$ ON THE MORTALITY OF RATS AFTER INJECTION OF SOMAN OR TABUN

s.c. Dose of soman ($\times LD_{50}$)	Mortality ratio in 24 hr		s.c. Dose of tabun ($\times LD_{50}$)	Mortality ratio in 24 hr		Route and time of oxime injections
	P_2S	$LüH_6$		P_2S	$LüH_6$	
1·3	5/24	24/24	2·0	5/12	3/12	
1·6	8/24	24/24	2·7	5/12	2/12	i.p. 1½ min
			3·5	8/12	6/12	after nerve
			4·5	11/12	6/12	gas
1·3	2/12	9/12	2·0	2/12	2/12	
1·6	4/12	12/12	2·7	3/12	3/12	i.p. 10 min
1·6	4/12	8/12	3·5	11/12	1/12	before nerve
2·0	7/12	12/12	4·5	8/12	5/12	gas
1·3	8/12	11/12	2·0	0/12	0/12	i.m. 10 min
1·6	11/12	12/12	2·7	3/12	0/12	before nerve gas
1·3	5/12	10/12	2·0	3/12	0/12	s.c. 10 min
1·6	11/12	11/12	2·7	2/12	0/12	before nerve gas

All animals received an i.p. injection of 37·5 mg/kg atropine sulphate 1·5 min after the nerve gas. The soman animals received 150 mg/kg of the oximes, the tabun animals 100 mg/kg. In each experiment groups of twelve rats were used.

DISCUSSION

Erdmann and Engelhard[1] concluded from their experiments that $LüH_6$ surpasses PAM in its therapeutic action both on the phrenic nerve diaphragm preparation and on the intact rat after poisoning by the cholinesterase inhibitors paraoxon and DFP. From our present work in which on account of its better solubility P_2S instead of PAM was used, it appears that the same is true when tabun is used as the cholinesterase inhibitor, a finding which is in agreement with the results of Heilbronn and Tolagen.[2] These investigators observed a better reactivation of mouse erythrocyte cholinesterase inhibited by tabun and a more effective protection of mice against tabun and sarin with $LüH_6$ than with P_2S. However from the present work on the isolated phrenic nerve diaphragm and on the intact rat it appears that against soman P_2S is more effective than either TMB_4 or $LüH_6$. This indicates that the effectiveness of the oximes in relation to each other depends on the type of cholinesterase inhibitor involved. This phenomenon, which was demonstrated earlier by van der Meer and Wolthuis[3] working with the oximes PAM and MINA, makes it difficult to think of a particular oxime as being the drug of choice against organophosphorus poisoning in general.

The relative inefficacy of TMB_4 against soman *in vitro* when applied either before or after the cholinesterase inhibitor might well be related to the finding of Berends (personal communication)[4] that the ability of this oxime to reactivate soman inhibited acetylcholinesterase is markedly diminished by the presence of even a trace of free

soman as a result of the formation of a TMB_4–soman complex which itself is a strong inhibitor of acetylcholinesterase.

The observation that the functional protection resulting from the presence of one of the oximes in the organ bath before and during the exposure of the phrenic nerve diaphragm preparation to tabun, was smaller than the recovery of function produced by these oximes when applied after the tabun, may be explained by the assumption that also in this case an oxime inhibitor complex has been formed. It is also possible that there may be a tendency for the oxime, which is ionic, to be washed out before the inhibitor, since a further functional recovery occurred when application of oxime to the preparation was repeated.

In the control experiments in which no drugs or only an oxime were applied, the performance of the preparation changed somewhat in the course of time. The tetanic contraction produced by 25 stimuli/sec increased and that produced by 100 or 200 stimuli/sec decreased. This phenomenon or part of it could also be observed in experiments in which an oxime had produced complete or partial protection against or recovery from the effects of a cholinesterase inhibitor. In the assessment of the oxime effects allowance for this phenomenon was made.

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