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THE REVERSAL BY OXIMES OF NEUROMUSCULAR BLOCK PRODUCED BY ANTICHOLINESTERASES

BY

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The reactivation by hydroxamic acids ($R—\overset{\overset{O}{\parallel}}{C}—NHOH$) of acetylcholinesterase (ChE) inhibited by organo-phosphorus anticholinesterases has been described by Wilson and Meislich (1953), Wilson and Ginsburg (1955) and Jandorf, Crowell, and Levin (1955). It has recently been found that the oximes ($\begin{matrix}R\\R'\end{matrix}\!\!>\!C=NOH$) are more potent reactivators of inhibited ChE than the hydroxamic acids and, like the latter, also react with the inhibitor (Childs, Davies, Green, and Rutland, 1955).

Most work on reactivators of inhibited ChE has been done with isolated enzyme preparations, and little on organized mammalian systems *in vitro* or on the whole animal. Roy and Kuperman (1955) have described reversal of the actions of TEPP by nicotinhydroxamic acid methiodide in isolated rabbit auricles and in strips of rabbit duodenum.

In the experiments described below, oximes have been tested for their ability to reverse the actions of organo-phosphorus anticholinesterase compounds at the neuromuscular junction in three preparations—the isolated rat phrenic nerve-diaphragm preparation, the rat gracilis muscle with electrical recording of muscle activity, and the cat tibialis anterior muscle prepared for close-arterial injection.

METHODS

Phrenic nerve-diaphragm preparations were obtained from 200 g. rats as described by Bülbring (1946). The nerve was stimulated with supramaximal square-wave pulses of 12.5 μsec. duration. Rates of stimulation varying from 12/min. to 200/sec. were used, so that both potentiation of response to single stimuli and Wedensky block of the higher rates of stimulation could be seen after addition of the ChE inhibitor.

For the experiments on the rat gracilis muscle, rats weighing between 250 and 400 g. were anaesthetized with urethane (0.5 ml. of 25% sol./100 g. intraperitoneally) and, after insertion of a tracheal cannula and an external jugular venous cannula, the right gracilis was prepared as described by Jarcho, Eyzaguirre, Talbot, and Lilienthal (1950). The obturator nerve twig, cut centrally, was stimulated with single shocks of 0.5 msec. duration from a "square-wave" stimulator at a rate of 10/min. and the voltage adjusted to supramaximal. The action potentials of the gracilis were recorded by means of one pair of fine platinum wire electrodes whose tips were beaded and adjusted to 2 mm. separation. The electrodes were held in a manipulator so that they could be traversed along the surface of the muscle in parallel with the muscle fibres. Action potentials were amplified by a capacity coupled amplifier and fed to one beam of a double-beam cathode ray tube, the other beam being used for msec. time marks. Single sweeps were recorded photographically on 35 mm. film, which was enlarged about 10 times and intervals measured to the nearest mm. One msec. was usually equivalent to 24 mm.

Cats were anaesthetized with pentobarbitone (32 mg./kg. intraperitoneally) and a tibialis anterior muscle was prepared for isometric tension recording and close-arterial injection of drugs as described by Brown (1938). The peroneal nerve was stimulated with single square-wave pulses of 0.5 msec. duration at supramaximal voltage, and the response of the muscle to tetanic stimulation of the nerve at 50/sec. was also tested periodically.

In the experiments *in vivo* on rats and cats the animals were atropinized (1 mg./kg. i.v.) and artificially ventilated throughout the experiment to prevent the lethal muscarinic and central actions of the anticholinesterases. The inhibitors used were tetraethyl pyrophosphate (TEPP), di*iso*propyl phosphorofluoridate (DFP) and *iso*propyl methylphosphonofluoridate (sarin). Di*iso*nitrosoacetone (DINA) and mono*iso*nitrosoacetone (MINA) were the oximes generally used, although a few experiments have been done using tri*iso*nitrosopropane (TINP) and pyridine-2-aldoxime methiodide (PAM).

RESULTS

Rat Phrenic Nerve-Diaphragm Preparation

Addition of anticholinesterase drugs to the bath containing the preparation results in potentiation of the response to single shocks and block to

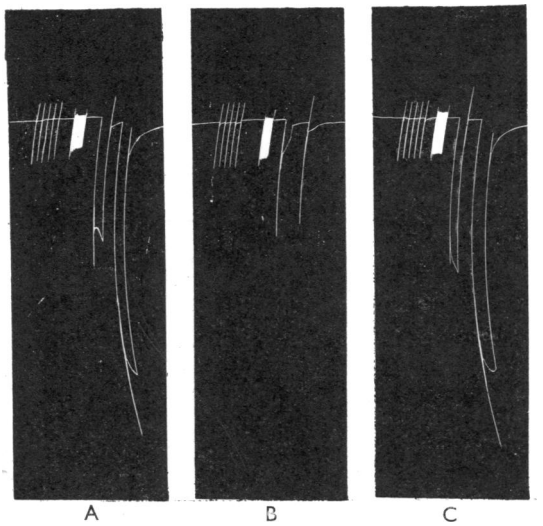

FIG. 1.—Contractions of isolated rat diaphragm to indirect stimulation at 0·5, 5, 50 and 100/sec. A, Control. B, 25 min. after TEPP, 2×10^{-7}M. C, 3 min. after MINA, 10^{-2}M. The preparation was not washed between records.

tetanic stimulation of the nerve (Wedensky block) —where the muscle responds only to the first of a train of stimuli applied to the nerve (Evans, 1951). The general conduct of an experiment was as follows. After the preparation had been set up and left for approximately 30 min. to reach equilibrium, the anticholinesterase was added in a concentration sufficient to cause block of the higher rates of stimulation as shown by a subsequent test. Oxime was then added and the responses to the various rates of stimulation again tested at intervals, usually 2 min. after addition of the oxime, and then at 5 min. intervals. A complete series is shown in Fig. 1, where reversal by the oxime of block at the higher rates of stimulation is seen. The results for a number of anticholinesterases and for the oximes used are given in Table I.

In the experiments in which the response to tetanic stimulation returned to normal, the preparations could be washed and the process of block by anticholinesterase, and of reversal by oxime, repeated with the same doses, and similar recovery times obtained.

Most of the experiments described above were done with short periods of contact between the anticholinesterase and the preparation (10 to 20 min.), but other experiments showed that duration of contact was not important in the reversal by oxime. In one experiment the preparation was left in contact with TEPP 2×10^{-7}M for 5 hr. before addition of oxime (MINA, 10^{-2}M) when complete recovery occurred in 3 min. Similarly with sarin (3.57×10^{-7}M), neuromuscular conduction returned to normal in 4 min. when MINA (10^{-2}M) was added 6 hr. after the anticholinesterase.

That the oximes act at the motor end-plates after addition of anticholinesterase has been demonstrated directly by recording the end-plate potential in a strip of isolated curarized rat diaphragm with the nerve attached as described by Jeffries (1953). The end-plate potential was markedly increased in size and duration by anticholinesterase (TEPP 10^{-7}M), but addition of oxime (DINA 10^{-2}M) to the bath resulted in a prompt return to normal.

One experiment has been done on the isolated phrenic nerve-diaphragm preparation of a kitten (197 g.). Block induced by sarin 3.57×10^{-7}M was reversed in 8 min. by the addition of DINA 10^{-2}M.

To test the direct action of oximes on muscle fibres rat phrenic nerve-diaphragm preparations were set up and oximes added to the bath without previous addition of inhibitor. The muscle was stimulated indirectly at a slow rate (approximately 5/min.) and periodically tested for its response to indirect tetanic stimulation and to direct stimulation. With DINA (10^{-2}M) no response to single stimuli was obtained after 2 hr. and the tetanic response was slight. Direct muscle stimulation resulted in only a feeble response and no recovery occurred after washing. MINA (10^{-2}M) appeared much less toxic, causing only slight reduction in contractions to single stimuli after 4 hr. With TINP (10^{-3}M), the response to single stimuli was almost completely abolished after 3 hr., but at this time a well-maintained tetanus could be obtained.

TABLE I

REVERSAL BY OXIMES OF NEUROMUSCULAR BLOCK PRODUCED BY ANTICHOLINESTERASES

Blocking Agent	Oxime	Time (min.) Necessary for Complete Recovery of Response to Stimulation at 100/sec.	
TEPP 2×10^{-7}M	DINA 10^{-2}M	5	
,, ,,	,, 10^{-3}M	20	
,, ,,	,, 10^{-4}M	—	Slight recovery in 30 min.
,,	TINP 10^{-3}M	—	No recovery in 90 min.
	then DINA 10^{-2}M	15	
,, ,,	MINA 10^{-2}M	3	
,, ,,	,, 10^{-3}M	7	
,, ,,	,, 10^{-4}M	—	Slight recovery in 65 min.
DFP 10^{-6}M	,, 10^{-2}M	2	
,, 10^{-5}M	,, ,,	45	30% recovery in 1 min., 50% in 3 min.
Sarin 3.57×10^{-7}M	DINA 10^{-2}M	2	
,, ,,	,, 10^{-3}M	15	
,, ,,	MINA 10^{-2}M	4	

As with DINA, these effects of MINA and TINP were not reversed by washing and appear to be a direct toxic action on the muscle fibres.

Pyridine-2-aldoxime Methiodide

This compound is particularly interesting, since it is the most potent *in vitro* reactivator of inhibited ChE so far obtained. The second order rate constants for reactivation of TEPP-inhibited red-cell ChE by MINA and PAM are 6.7 and approximately 500 respectively (Davies, personal communication). For sarin, the figures are 22.2 and approximately 200. Early experiments in which this oxime was tested on the rat diaphragm were disappointing in that recovery from anticholinesterase block was not obtained even with 10^{-2}M oxime, but the reason for this was later discovered to be a neuromuscular blocking action of the oxime itself. PAM does inhibit red-cell ChE to a certain extent particularly at the higher concentrations (45% inhibition at 10^{-2}M), but at 10^{-3}M, when neuromuscular block still occurs, its anticholinesterase activity causes only 4% inhibition. This neuromuscular blocking action of PAM is, however, reversed by washing the preparation, when recovery occurs immediately. In later experiments, therefore, the reversal of anticholinesterase block in the rat phrenic nerve-diaphragm by PAM was demonstrated by washing after the oxime had been added and left in contact with the preparation for a short time.

With 10^{-2} and 10^{-3}M-PAM, complete recovery from block by 3.57×10^{-7}M-sarin occurred after $3\frac{1}{2}$ min. contact between the oxime and the preparation, which was then washed and tested. 5×10^{-4}M-PAM when added after block by sarin 3.57×10^{-7}M resulted in only partial recovery as shown by a test after washing. Further oxime was added to the same concentration, and conduction was restored to normal in $3\frac{1}{2}$ min. At this low concentration the neuromuscular blocking action of the oxime seems to be insignificant, since after recovery there was no difference in response before and after washing.

In order to relate the reversal by oximes of anticholinesterase block to reactivation of the inhibited ChE, four experiments were done in which the ChE activity of the hemi-diaphragm used in the Bülbring preparation was estimated by the Warburg technique, after block by TEPP (2×10^{-7}M) and reversal by MINA (10^{-2}M). The remaining half of the diaphragm was divided into two, one part being used to obtain the normal ChE activity, while the other part was placed in normal saline containing TEPP (2×10^{-7}M) to provide a figure for the ChE activity after inhibition. The results are given in Table II.

TABLE II
ChE ACTIVITIES OF RAT DIAPHRAGMS
(μl. CO_2/30 MIN./100 MG.)

Expt.	Controls	Treated with TEPP	Treated with TEPP and MINA
1	50·5	(Not measured)	19·2 (38%)
2	56	11 (19%)	36 (65%)
3	61	11 (18%)	23 (38%)
4	57	19 (33%)	32 (56%)

Rat Gracilis

The gracilis contains two discrete motor end-plate zones, one close to the insertion of the muscle at the knee and the other nearer to the pelvic attachment of the muscle. With a pair of recording electrodes on the surface of the muscle between the two end-plate zones, two muscle spikes are obtained after stimulation of the nerve, originating in each zone and of opposite sign. If the electrodes are placed very close to one end-plate zone (usually that nearer the pelvis) the time interval between the two muscle spikes will approximate to the time taken for a muscle action potential to travel between the two zones. The interval will deviate

TABLE III
EFFECT OF DFP ON CONDUCTION VELOCITY OF RAT GRACILIS

Rat	Interval Between Spikes (msec.) Control	Interval Between Spikes (msec.) Minimum after DFP	Differences (msec.)	% Decrease in Conduction Time
1	2·70	2·08	0·64	23·7
2	2·82	2·25	0·57	20·2
3	2·42	1·97	0·45	18·6
4	2·62	2·01	0·61	23·2
5	3·05	2·58	0·47	15·4
6	2·52	2·12	0·42	16·7
Means	2·69	2·16	0·53	19·6

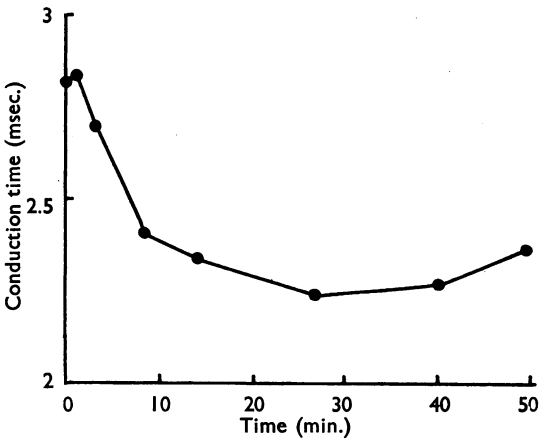

FIG. 2.—Effect of DFP (25 mg./kg. i.v.) on muscle conduction time in rat gracilis muscle. DFP given at zero time.

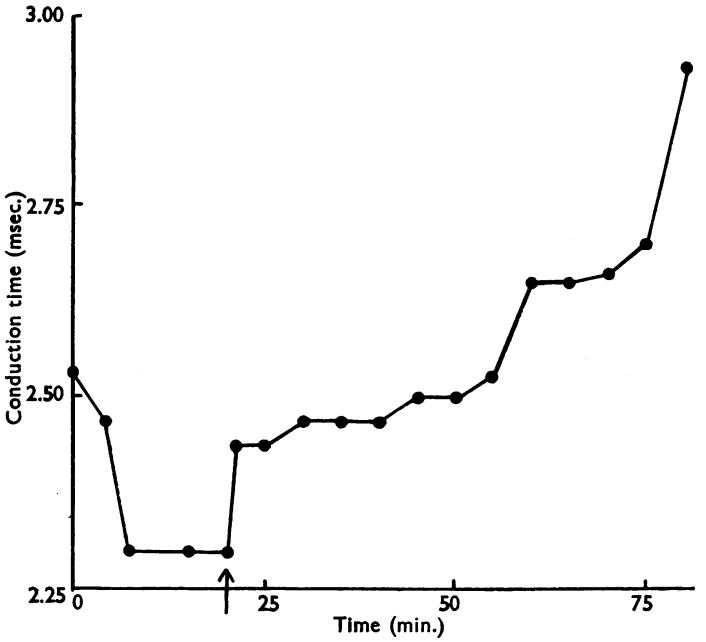

FIG. 3.—Conduction time in rat gracilis muscle. DFP (5 mg./kg. i.v.) given at zero time. MINA (50 mg./kg. i.v.) at arrow. Rat died at 80 min.

from this owing, firstly, to the additional nerve conduction time to the distal end-plate zone, and, secondly, to the muscle conduction time between the recording electrodes and the nearest end-plate zone. The positions of the end-plate zones can be found by traversing the electrodes across the muscle, when the crossing of an end-plate region is shown by reversal of the recorded action potential.

It has been found that DFP 2.5 mg./kg. i.v. causes a slowly developing reduction in the interval between the two spikes (conduction time), reaching a minimum between 10 and 30 min. after the injection. The results are given in detail in Table III, while Fig. 2 shows the time course of the change.

With a mean conduction distance of 11 mm. and the mean % difference of 19.6%, the injection of DFP 2.5 mg./kg. results in an apparent increase in conduction velocity from 4.1 to 5.1 m./sec. However this change is probably not due to a direct action of the DFP on the muscle fibre. Thus the occurrence of Wedensky block shows that the drug was acting at the motor end-plate, indirectly by potentiation of ACh. Single shock stimulation of the nerve would therefore cause a prolonged end-plate potential which would involve depolarization of the adjacent muscle membrane (Fatt and Katz, 1951), consequently reducing the effective conduction distance between the two end-plate zones. Muscular fasciculations started about 1 min. after the injection of DFP and continued throughout the experiment (up to 1 hr. duration). Repetitive firing occurred in the recorded muscle spikes in response to single shock stimulation of the nerve.

Injection of MINA 50 mg./kg. i.v. after the reduction in conduction time by DFP resulted not only in a rapid return towards normal but also in a further progressive increase in conduction time (Fig. 3). Experiments in which MINA alone was given showed that it had a direct effect on conduction time, causing a progressive increase in the interval reaching 10% above the control in 30 min. That there is, however, an action against the DFP-induced changes in addition to this direct effect acting in the same direction is shown by the initial rapid change in conduction time after the injection of oxime (Fig. 3) and also by the fact that repetitive firing due to DFP is promptly abolished (Fig. 4).

Cat Tibialis Anterior

It was found that neuromuscular block in the tibialis anterior due to sarin or TEPP could not be reversed by administration of oxime when both drugs were given by close-arterial injection. Thus block was produced by two injections of 2.5 μg. sarin followed by a further 5 μg., and was not relieved by injection of 250 μg. of DINA. When oxime was given close-arterially before sarin, it did not prevent the blocking action of the sarin. Since the oximes are readily washed out of isolated tissues (that is, before the prolonged slow toxic action occurs), it is concluded that,

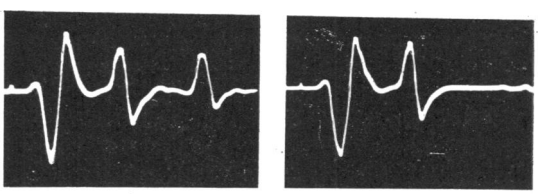

FIG. 4.—Rat gracilis muscle spikes to indirect stimulation. A, After DFP 5 mg./kg. i.v.; B, 1 min. after MINA 50 mg./kg. i.v.

after close-arterial injection, they are not "fixed" in the muscle, and thus a high local concentration of oxime would not be maintained for a sufficient time to obtain reversal of anticholinesterase block. Accordingly, in two experiments, oxime was given intravenously after neuromuscular block of the tibialis anterior had been induced by intravenous or close-arterial TEPP. In one experiment, 25 mg./kg. of MINA was given after 1 mg./kg. TEPP, both intravenously. The ability to maintain a tetanus returned fully after 25 min. Neuromuscular block was again produced by the injection of TEPP 1 mg./kg., and after the injection of 100 mg./kg. of MINA the recovery time was reduced to 11 min. In another experiment, TEPP (40 μg.) was given by close-arterial injection and produced only partial Wedensky block of the tibialis anterior. Injection of MINA 50 mg./kg. resulted in a maintained response to tetanic stimulation of the nerve after 8 min. In a control experiment in which only TEPP (1 mg./kg. i.v.) was given, the ability of the tibialis anterior to maintain a tetanus to indirect stimulation was only 21.5% of the pre-injection control after 60 min.

TABLE IV

EFFECT OF MINA (100 MG./KG. I.V.) ON CONTRACTIONS OF CAT TIBIALIS ANTERIOR TO INDIRECT STIMULATION AT 50/SEC. AFTER SARIN (300 μG./KG. I.V.)

Expt.	% Control Response after Sarin	Maximum % Control Response after MINA	Time (min.) from Injection of MINA to Maximum Recovery
1	1·5	82	20
2	0	100	17
3	1·0	98·5	20
4	0	88	34
5	0	72	45
6	0	91	26

A further series of experiments on cats was done with sarin, from which spontaneous recovery occurs more slowly than with TEPP, and also using a higher dose of oxime (100 mg./kg.). The results are given in Table IV. In two control experiments the recoveries which occurred from neuromuscular block by sarin (300 μg./kg. i.v.) were 2% in 60 min. from the injection of sarin in one experiment and 11% in 30 min. in the other.

DISCUSSION

The reversal by oxime of neuromuscular block due to anticholinesterase in the isolated rat phrenic nerve-diaphragm preparation is very rapid. With most of the anticholinesterases used, previous experiments have shown that prolonged washing is required to obtain recovery from block without addition of oxime. The time taken for recovery is related to the duration of contact of the anticholinesterase with the preparation; thus, for example, recovery after a 5 min. exposure to TEPP (2×10^{-7}M) occurs in 20 min., but with a 60 min. exposure to the same concentration 5 hr. washing is necessary for full recovery (Lovatt Evans, personal communication). However, experiments described above have shown that the time taken for reversal of block by oxime is the same whether the anticholinesterase has been in contact with the preparation for a few minutes or for many hours.

The *in vivo* experiments on the neuromuscular actions of the oximes have perhaps had less dramatic but nevertheless positive results. In the rat gracilis experiments, in addition to the change in conduction time and abolition of the additional spikes, it was noted that the generalized muscle fasciculations due to DFP poisoning disappeared after the injection of oxime.

In the experiments on cat muscle the results have been least encouraging in view of the time taken for recovery. Douglas and Matthews (1952) have shown that after 0.45 mg./kg. TEPP injected intravenously, the ability of the cat diaphragm to maintain a tetanus returns spontaneously to near normal in 50 min. With the tibialis anterior, which is blocked more rapidly after cholinesterase than muscles such as the soleus and diaphragm (similar to the action of decamethonium as described by Paton and Zaimis, 1951), it was found that MINA 25 mg./kg. i.v. caused a return of tetanic contraction in 25 min. after injection of 1 mg./kg. of TEPP. In a control experiment in which only TEPP (1 mg./kg. i.v.) was given, no recovery had occurred in 25 min. The series of experiments in which sarin was used also showed that injection of MINA resulted in a slow return of the ability of the tibialis anterior to maintain a tetanus. The slow rate of the recovery is thought to be due to the local concentration of the oxime being too low. That there is no species difference has been shown by the rapid reversal of sarin block by oxime in the isolated kitten diaphragm.

The mechanism of action of the oximes in reversing neuromuscular block appears to be entirely due to the reactivation of inhibited ChE. Thus in the experiments in which the ChE activities of phrenic nerve-diaphragm preparations were measured, the addition of oximes after TEPP caused an increase in ChE activity which accompanied the return of normal function. Furthermore, the oximes had no effect on block of phrenic nerve-diaphragm conduction produced by (+)-tubocurarine (1.1×10^{-6}M), succinylcholine

chloride (3×10^{-5}M) and decamethonium bromide (2×10^{-5}M). The direct toxic action of the oximes on muscle, however, seems to be an action on the fibre, since the response to direct stimulation is reduced (isolated rat diaphragm) and the conduction velocity is progressively slowed (rat gracilis *in vivo*).

The experiments described above have shown that the oximes have considerable potentiality against neuromuscular block from anticholinesterases. However, the required concentrations of oximes *in vitro* are too high and the *in vivo* dose necessary to reverse block from an anticholinesterase is close to the toxic dose. PAM, while very effective as an *in vitro* reactivator, suffers from the disadvantage of having neuromuscular blocking actions of its own.

Summary

1. The actions of oximes on neuromuscular block due to anticholinesterases have been studied.

2. Wedensky block in the isolated rat phrenic nerve-diaphragm preparation induced by TEPP, DFP or sarin was rapidly reversed by di*iso*nitrosoacetone and mono*iso*nitrosoacetone.

3. Pyridine-2-aldoxime methiodide reversed block due to anticholinesterases, but this could only be demonstrated after washing, since the oxime itself caused neuromuscular block at higher concentrations.

4. In the rat gracilis muscle *in vivo*, DFP caused an apparent increase in conduction velocity which rapidly returned to normal after injection of oxime.

5. Block in the cat tibialis anterior due to intravenous or close-arterial TEPP or intravenous sarin was slowly reversed by intravenous injection of oxime.

6. The oximes have a direct toxic action on muscle, causing a reduction of contraction height and a slowing of conduction velocity.

7. The reversal by oximes of neuromuscular block appears to be due to reactivation of ChE. Block by (+)-tubocurarine, succinylcholine or decamethonium was unaffected.

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