Br. J. Pharmac. (1972), **46**, 714–724.

Actions of the selective inhibitor of cholinesterase tetramono*iso*propyl pyrophosphortetramide on the rat phrenic nerve-diaphragm preparation

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Summary

1. Tetramono*iso*propyl pyrophosphortetramide (*iso*-OMPA) added for 15 min to the rat isolated phrenic nerve-diaphragm in a concentration of 30 μM, produced a complete selective and stable inhibition of cholinesterase. A concentration of 3 μM produced near complete inhibition of cholinesterase, and a concentration of 300 μM also inhibited acetylcholinesterase marginally.

2. Inhibition of cholinesterase was associated with a sustained increase in the neuromuscular blocking action of exogenous butyrylcholine but not of exogenous acetylcholine. *Iso*-OMPA, 300 μM, in addition caused transient increases in the sensitivity of the rat diaphragm to exogenous acetylcholine and butyrylcholine. In the same concentration, it had a curare-like action on the frog rectus abdominis muscle preparation.

3. *Iso*-OMPA, 30 μM, caused reversible increases in the amplitude of the twitch response and tetanic responses, which were of a similar magnitude in the indirectly stimulated preparation and the directly stimulated curarized preparation. Caffeine had a similar effect on the twitch response and its effectiveness was increased by *iso*-OMPA, and *vice-versa*. Amongst anticholinesterases, octamethyl pyrophosphortetramide and tetraethylpyrophosphate also enhanced the amplitude of the tetanic response, but paraoxon, dyflos, and mipafox did not.

4. It is concluded that *iso*-OMPA, in concentrations (3 and 30 μM) which in 15 min give near maximal or maximal selective inhibition of cholinesterase, has no effect on the transmission of nerve impulses at the neuromuscular junction, but enhances reversibly the amplitude of the contractile response to stimulation by a direct action upon the muscle fibre, which involves a mechanism related to but not identical with that by which caffeine potentiates twitch tension. In higher concentrations, *iso*-OMPA has a curare-like action at the neuromuscular junction.

Introduction

The rat isolated phrenic nerve–diaphragm preparation has been widely used for the study of neuromuscular transmission and of drugs which act upon this process. The junctional region contains a high concentration of acetylcholinesterase (acetylcholine acetylhydrolase; E.C. 3.1.1.7) (Koelle, 1950, 1951) whose importance in the rapid destruction of acetylcholine after its release from the nerve terminals is well established. In addition, it has been shown by Denz (1953) that cholinesterase

acylcholine acylhydrolase ; E.C. 3.1.1.8) is present in the synaptic gutters of the neuromuscular junctions in the rat diaphragm, and, therefore, the possibility that this enzyme may play a contributory role in the transmission process needs investigation.

Most anticholinesterase agents in concentrations required for marked inhibition of one type of cholinesterase inhibit both types of cholinesterase, but some, in a certain range of concentrations selectively inhibit only one type. Tetramono*iso*-propyl pyrophosphortetramide (*iso*-OMPA) belongs to the latter category. It phosphorylates cholinesterase about 600 times faster than acetylcholinesterase and the phosphorylated enzyme formed by it is very stable (Austin & Berry, 1953 ; Aldridge, 1953 ; Davison, 1953). *Iso*-OMPA was used, therefore, to elucidate the role of cholinesterase at the neuromuscular junction in the rat isolated phrenic nerve–diaphragm preparation, and the results obtained in these and related studies are described in this paper.

Methods

Male Wistar rats (200–300 g) were used. The left hemi-diaphragm with its phrenic nerve and rib cage insertion was suspended in an organ bath containing 40 ml Tyrode solution of the following composition (mM): NaCl, 138 ; KCl, 2·7 ; $CaCl_2$, 3·6 ; $MgCl_2$, 2·1 ; $NaHCO_3$, 11·9 ; glucose, 11. The bath fluid was maintained at 37° C and was gassed with 95% O_2:5% CO_2. The resulting pH was 7·15.

Muscle tension recordings

Muscle tension unless otherwise stated was recorded on a smoked drum by a spring-loaded lever giving an 18-fold magnification. In some experiments, muscle tension was recorded isometrically (resting load=3 g) by a strain gauge (Statham G 1/32/350 ; range $\pm$91 g) connected to a recorder with a heated stylus (Devices).

Indirect stimulation

The phrenic nerve was stimulated with a circular electrode or an open ' Palmer phrenic nerve electrode ' connected to an electronic square wave generator (Palmer) with a constant voltage output. All stimuli were supramaximal and their parameters were: 0·1 ms duration and 1–4 V. To produce twitch responses the phrenic nerve was stimulated at a frequency of 0·2 Hz. For tetanic responses, the nerve was stimulated for 10 s with 20, 50 or 100 Hz every 5 min ; only one frequency was used per diaphragm. Twenty Hz produced a partial tetanus, whilst 50 and 100 Hz produced a full tetanus. The maximum amplitude of contraction at the end of the 10 s period of stimulation was used as the measure of the tetanic response.

Direct stimulation

In experiments in which the curarized muscle was stimulated directly, the stimuli were of 20–60 V and 1 ms duration, and the same frequencies were used as for indirect stimulation. The bath fluid level was kept at the level of the line formed by the motor end plates. To avoid variable short-circuiting of the applied current

in the organ bath by gas bubbles on the surface of the bath fluid, the gas was switched off during tetanic stimulation, and in experiments involving low frequencies of stimulation, at those points where the record was needed for measurements.

Determination of cholinesterase activity

The whole diaphragm was perfused *in situ* with Tyrode solution until the effluent was clear. The left hemidiaphragm was then suspended in the organ bath, and to obtain enough material for determination of cholinesterase activity, the remainder of the diaphragm was also excised and placed in the organ bath. *Iso*-OMPA was added to the organ bath for 15 min and then washed out. As shown by Taugner & Fleckenstein (1950) inhibition of cholinesterases in the diaphragm by carbamates proceeds at the same rate in the presence and absence of stimulation. In the work described in this paper, it was found that inhibition of cholinesterase in the isolated diaphragm by *iso*-OMPA also proceeded at the same rate in the presence and absence of stimulation.

Cholinesterase activity of *iso*-OMPA-treated and control diaphragms was determined in both intact diaphragms and homogenates made from them. In experiments with an intact diaphragm the rib cage and fatty tissue were removed, and after blotting and weighing, the diaphragm muscle (600–800 mg) was placed in a vessel containing Tyrode solution, 1 ml/100 mg tissue, and either butyrylcholine in a concentration of 10 μM or acetylcholine in a concentration of 50 μM. The vessel was placed in a water bath maintained at 37° C, and continuously gassed with 95% O_2:5% CO_2. A sample (0·3 ml) of the incubation medium was immediately withdrawn for assay, and thereafter at intervals (not more than 4 samples in all). In experiments with a homogenized diaphragm, the diaphragm was dried by blotting with filter paper, weighed and then homogenized by grinding with washed sand in a mortar using Tyrode solution. The final volume of Tyrode solution was 10 ml/100 mg tissue when acetylcholine (50 μM) (which is hydrolyzed by acetylcholinesterase and cholinesterase) was the substrate and 2 ml/100 tissue when butyrylcholine (10 μM) (which is hydrolyzed predominantly by cholinesterase) was the substrate. Sampling was the same as in experiments with intact diaphragms.

The frog isolated rectus abdominis muscle preparation, not sensitized with anticholinesterases, was used to determine the concentration of ester in the samples collected by comparing the responses produced by them with those produced by known concentrations of the same ester. Since homogenates had been diluted with respect to intact preparations, the half-lives obtained from them were divided by the dilution factor. Control experiments showed that the sensitivity of the preparations to acetylcholine and butyrylcholine was not altered by the presence of an equal concentration of choline which would be present when half the ester had been metabolized. The K^+ concentrations of the diluted homogenates were determined by flame photometry, and further control experiments showed that the sensitivity of the preparation to the two esters was not altered by the addition of 0·3 ml samples of Tyrode solution containing the increased K^+ concentrations present in the homogenates. In the case of hydrolysis by the intact diaphragm, corrections were made to take into account the amount of ester withdrawn in previous samples since this had not been metabolized by the diaphragm. No correction was made for spontaneous hydrolysis since this was negligible under the experimental conditions. Plots of the log of the amount of ester not hydrolyzed against time were

linear for at least 50% hydrolysis, and the half-life of the ester was determined from such plots.

Materials

Acetylcholine chloride (British Drug Houses); butyrylcholine iodide, tetramono-*iso*propyl pyrophosphortetramide (*iso*-OMPA), octamethyl pyrophosphortetramide (OMPA), di*iso*propyl phosphorofluoridate (Dyflos), *N,N'*-di*iso*propylphosphorodiamidic fluoride (Mipafox), diethyl 4-nitrophenyl phosphate (Paraoxon), (+)-tubocurarine chloride, and decamethonium iodide (Koch-Light Laboratories); caffeine (Hopkin & Williams); tetraethyl pyrophosphate (Albright & Wilson).

Where appropriate, results are expressed as means ± S.E.M. Statistical significance of difference was calculated by Student's *t* test, a probability of 5% being taken as significant. All concentrations are expressed as final concentrations in the organ bath. Unless otherwise stated, *iso*-OMPA was present in the organ bath for 15 min at a time, and then washed out.

Results

Inhibition of cholinesterases in the rat isolated phrenic nerve–diaphragm preparation by iso-*OMPA*

Intact diaphragms which had been exposed for 15 min to 30 μM *iso*-OMPA hydrolyzed acetylcholine at the same rate as did control diaphragms. With diaphragms exposed to 300 μM *iso*-OMPA, the hydrolysis of acetylcholine was inhibited by 23%. In contrast, the hydrolysis of butyrylcholine was inhibited by 68% and 71% respectively.

Inhibition calculated from the enzymic activities of homogenates made from diaphragms exposed to *iso*-OMPA was as follows. *Iso*-OMPA, 30 μM, was without effect upon the rate of hydrolysis of acetylcholine, whilst 300 μM reduced it by 14%. The values for inhibition of the hydrolysis of butyrylcholine by 3 μM, 30 μM, and 300 μM *iso*-OMPA were 78%, 85% and 86%, respectively. These results are presented in Table 1.

Effect of iso-*OMPA upon neuromuscular block in the rat isolated phrenic nevre–diaphragm preparation by acetylcholine and butyrylcholine added to the organ bath*

Acetylcholine and butyrylcholine, added to the organ bath, reduce the twitch tension of the indirectly stimulated rat diaphragm and their effectiveness in this respect will depend on the rate of their enzymic hydrolysis. In a series of experi-

TABLE 1. *Effect of exposing the rat diaphragm preparation to* iso-*OMPA for 15 min upon the rates of hydrolysis of acetylcholine (ACh) and butyrylcholine (BuCh) by the intact preparations and by homogenates made from them*

Substrate (M)	Mean half-life in min ± S.E.M. (n=4) Control		*iso*-OMPA, 3 μM		*iso*-OMPA, 30 μM		*iso*-OMPA, 300 μM	
	Intact	Homogenized	Homogenized		Intact	Homogenized	Intact	Homogenized
ACh, 50 μM	15·7±0·4	1·3	—		15·9±1·6	1·2	20·5	1·5
BuCh, 10 μM	46·0±6·0	8·2±0·3	36·9		147±16·7	53·2±7·1	157·0	58·0

Where no standard error is given, the result is the mean of two observations, both within 10% of the mean.

ments, the concentration of acetylcholine and butyrylcholine required for 50% reduction in twitch tension of the indirectly stimulated phrenic nerve-diaphragm preparation (IC50) was calculated from the effects of graded concentrations of the esters added at intervals to the organ bath for 5 min and then washed out. *Iso*-OMPA was then added to the organ bath for 15 min and the sensitivity of the preparation to both esters was tested at intervals whilst *iso*-OMPA was present and after its removal from the bath. Increases in sensitivity were calculated by the ratio of concentrations required to produce the same degree of neuromuscular block and are called the sensitization factors.

Control experiments showed that without the addition of *iso*-OMPA to the organ bath the sensitivity of the preparation increased with time (Table 2), and all sensitization factors in experiments with *iso*-OMPA were corrected for this.

A concentration of 30 μM *iso*-OMPA was without effect upon the sensitivity to acetylcholine, whether this was tested in the presence of the *iso*-OMPA or after its removal. A concentration of 300 μM *iso*-OMPA while in the organ bath increased the sensitivity to acetylcholine by a factor of $2{\cdot}1 \pm 0{\cdot}2$ ($n=4$) but this factor decreased speedily and approached 1/10 min after the removal of the *iso*-OMPA.

By contrast, 30 μM *iso*-OMPA increased the sensitivity to butyrylcholine by a factor of about 3 whilst *iso*-OMPA was in the organ bath and this increase was sustained for at least 90 min after changing the bath fluid. When 300 μM *iso*-OMPA was employed, the sensitivity to butyrylcholine while *iso*-OMPA was present increased by a factor of $11{\cdot}2 \pm 0{\cdot}4$ ($n=4$) and on removing the *iso*-OMPA, this fell within 10 min to a value of $8{\cdot}5 \pm 0{\cdot}2$, and remained at this level for the succeeding 90 minutes.

Since these experiments indicate that *iso*-OMPA, 300 μM, had reversible effects upon the neuromuscular junction, its effects on the frog isolated rectus abdominis muscle preparation were also investigated. In concentrations up to 1 mM, *iso*-OMPA was without agonistic effects, and in concentrations up to 30 μM the action of acetylcholine was neither enhanced nor antagonized. *Iso*-OMPA, 300 μM, approximately halved the sensitivity to acetylcholine and this effect was maximal in 30 minutes. The antagonism could be overcome by increasing the concentration of acetylcholine.

Effect of iso-*OMPA upon responses to indirect stimulation*

Twitch response

When stimuli were applied to the phrenic nerve at 0·2 Hz, the amplitude of the twitch response was not altered by 30 μM *iso*-OMPA. However, when a single twitch response was elicited at 5 min intervals in otherwise identical conditions, an

TABLE 2. *Increase in the sensitivity of the rat diaphragm preparation to exogenous acetylcholine (ACh) and butyrylcholine (BuCh) with the passage of time*

Time (min)	0	30	75	100	125	150
ACh	1	$1{\cdot}1 \pm 0{\cdot}03$	$1{\cdot}4 \pm 0{\cdot}1$	$1{\cdot}5 \pm 0{\cdot}03$	$1{\cdot}7 \pm 0{\cdot}1$	$2{\cdot}2 \pm 0{\cdot}2$
BuCh	1	1·2	1·5	1·8	2·2	2·3

The table shows sensitization factors $\pm$S.E.M., $n=5$ for ACh. With BuCh each figure is the mean of two observations, both within 10% of the mean.

increase in the amplitude of about 10% was observed. With 300 μM *iso*-OMPA, the amplitude of the twitch response (0·2 Hz) gradually increased by 27 $\pm$ 5·1% (n=7) in 10 min and then remained at this level. After washing out the *iso*-OMPA, the twitch response returned to control levels within 5 minutes. The increase of twitch tension could be obtained repeatedly in the same preparation.

Recordings made on a fast drum (Fig. 1) showed that *iso*-OMPA, 300 μM prolonged the twitch response from 103·4 $\pm$ 0·3 ms to 110·7 $\pm$ 0·3 ms (n=7). This was caused almost entirely by a prolongation of the phase of contraction from 54·6 $\pm$ 0·1 ms to 60·4 $\pm$ 0·2 ms (P<0·001). The phase of relaxation showed a much smaller increase from 48·8 $\pm$ 0·3 ms to 50·3 $\pm$ 0·2 ms (P<0·01).

Tetanic response

3 μM *iso*-OMPA marginally increased the tetanic response to 20 Hz. In contrast, *iso*-OMPA, 30 μM, had marked effects. With 20 Hz, a pronounced increase in tension was seen, which reached a maximum in 10 to 15 min (Fig. 2). With 50 Hz the increase was smaller and with 100 Hz it was marginal. These findings are summarized in Table 3, which also shows that the increases were readily reversible on removing *iso*-OMPA from the organ bath.

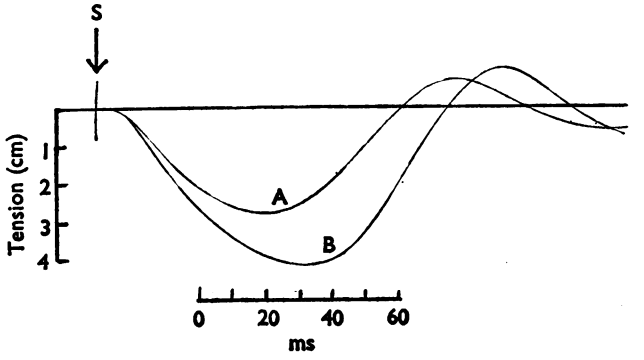

FIG. 1. Effect of 300 μM *iso*-OMPA, present for 15 min in the organ bath, upon the twitch response of the indirectly stimulated rat diaphragm preparation, recorded on a fast moving drum. S: stimulus applied. A: control. B: in the presence of *iso*-OMPA.

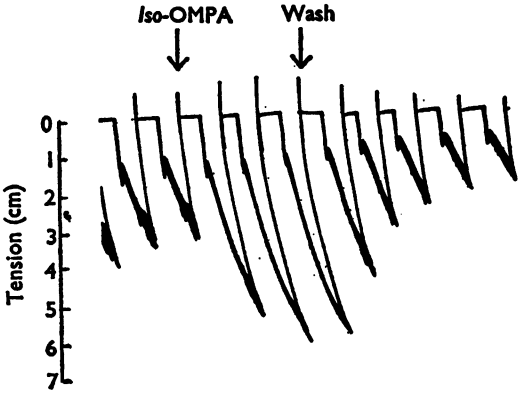

FIG. 2. Effect of 30 μM *iso*-OMPA upon the partial tetanus elicited by indirect stimulation (20 Hz) of the rat diaphragm preparation for 10 s every 5 minutes. Drum stopped after each period of stimulation.

Experiments in which 50 Hz was used and *iso*-OMPA was added to the organ bath for 30 min showed that the increase of the tetanic response was maintained for as long as *iso*-OMPA was present in the organ bath, and that washing reversed the increase as readily as that obtained with a 15 min exposure. Increasing the concentration of *iso*-OMPA from 30 to 300 μM, produced no further increase of tetanic response to 50 and 100 Hz, but further increased the response to 20 Hz (see Table 3). A fast drum record of the effect of 300 μM *iso*-OMPA on tetanic response to 20 Hz is shown in Fig. 3.

Effect of iso-*OMPA upon responses to direct stimulation*

In preparations which had been rendered completely unresponsive to stimulation of the phrenic nerve by 5 μM (+)-tubocurarine and then were stimulated directly, the following results were obtained.

Twitch response

A concentration of 30 μM *iso*-OMPA was without effect upon the amplitude of the twitch response produced at 0·2 Hz, but caused an increase of about 10% when single twitch responses were elicited at 5 min intervals. In a concentration of 300

TABLE 3. *Percentage increase in the amplitude of the tetanic response of the indirectly stimulated rat diaphragm preparation (isotonic recording with spring-loaded lever) produced by* iso-*OMPA added to the organ bath*

Conc. (μM) *iso*-OMPA	Frequency of stimulation	Time (min) after adding *iso*-OMPA to organ bath 5	10	15		20	25	30	35	40	45
30	20 Hz	79	110	121	W	52	29	12	7	2	0
30	50 Hz	26	30	30	A	20	16	11	7	3	0
30	100 Hz	2	3	3	S	0	0	0	0	0	0
300	20 Hz	230	250	197	H	31	28	22	10	0	0

Each figure is the mean of two observations (individual results within $\pm 20\%$ of the mean).

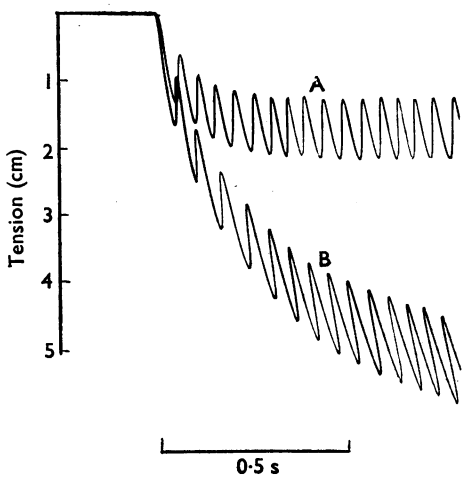

FIG. 3. Effect of 300 μM *iso*-OMPA present in the organ bath for 15 min upon the partial tetanus evoked by indirect stimulation of the rat diaphragm preparation at 20 Hz. A: control. B: in the presence of *iso*-OMPA.

μM *iso*-OMPA increased the amplitude by 26±4·1% (n=7). After washing out the *iso*-OMPA, the twitch response returned to control levels within 5 min, and the increase could be obtained repeatedly in the same preparation.

Tetanic response

The effects of *iso*-OMPA upon the responses to direct stimulation were qualitatively the same but less pronounced than those upon responses to indirect stimulation. Since it is possible that this difference was due to greater short-circuiting of the stimulation current as the muscle contracted, isometric recording was used to compare the effects of *iso*-OMPA upon both directly and indirectly elicited tetanic responses. Under these conditions the increase in tension which occurred in the presence of *iso*-OMPA when diaphragms were stimulated indirectly did not differ significantly ($P>0·05$) from those produced by direct stimulation. Thus, 30 μM *iso*-OMPA increased the responses to indirect and direct stimulation with 20 Hz by 69±9% and 66±13% ($P>0·4$) respectively, and the corresponding figures for 300 μM *iso*-OMPA were 173±28% and 193±31% ($P>0·05$) respectively.

Effects of caffeine on the twitch response of the rat isolated phrenic nerve–diaphragm preparation

In the indirectly stimulated rat isolated phrenic nerve–diaphragm preparation caffeine produced effects on the twitch response (0·2 Hz) which were comparable to those produced by *iso*-OMPA. One mM caffeine caused a 10% and 2 mM a 25% increase in the twitch tension. These effects were or became maximal in 10 minutes. In addition, unlike *iso*-OMPA, it produced contractures which developed rapidly and reached a maximum in about 1 minute. If 300 μM *iso*-OMPA was added to the organ bath and allowed to remain, a sustained increase in twitch tension occurred as already noted. Using this increased value as the control level, 0·5 mM caffeine now produced 11% and 1 mM a 24% increase in twitch tension (Fig. 4). In the presence of caffeine, the sensitivity of the preparation to *iso*-OMPA

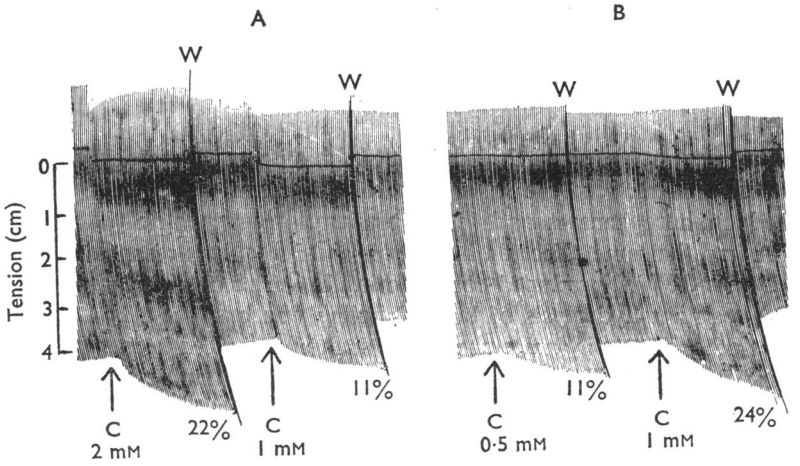

FIG. 4. Effect of *iso*-OMPA upon the action of caffeine on the rat diaphragm preparation. A: Effects in the absence of *iso*-OMPA; B: effects in the presence of 300 μM *iso*-OMPA. The percentages are increases in the amplitude of the twitch response 5 min after addition of caffeine (C). W: Wash.

TABLE 4 *Summary of the principal effects in the rat diaphragm of* iso-*OMPA, present in the bath for 15 min, upon amplitude of the tetanic response to 20 Hz and upon the levels of acetylcholinesterase and cholinesterase activity in intact and homogenized preparations*

		30 μM *iso*-OMPA	300 μM *iso*-OMPA
A.	Indirect	69%	173%
	Direct	66%	193%
B.	Intact	0	23%
	Homogenized	0	14%
C.	Intact	68%	71%
	Homogenized	85%	86%

A: percentage increase in tetanic response. Indirect: indirect stimulation. Direct: direct stimulation. B: percentage inhibition of acetylcholinesterase. C: percentage inhibition of cholinesterase.

was likewise increased by a factor of about two. The same results were obtained in directly stimulated curarized preparations. The caffeine-induced contracture was not affected by *iso*-OMPA.

Effect of compounds structurally related to iso-*OMPA*

To investigate whether the action of *iso*-OMPA was shared by other organophosphate anticholinesterases, and in particular those which had some structural resemblance to *iso*-OMPA, studies were undertaken of the effect of several organophosphate anticholinesterases on the response of the rat isolated phrenic nerve–diaphragm preparation to direct tetanic stimulation at 20 Hz in the presence of (+)-tubocurarine. The potent anticholinesterases, dyflos, paraoxon, and mipafox were devoid of any effect upon the tetanic response in concentrations up to 300 μM or more. On the other hand, octamethyl pyrophosphortetramide (OMPA), 1 mM, which is not an anticholinesterase *per se* but converted to an anticholinesterase *in vivo* produced a sustained increase of the tetanic response of 125% and 3 mM produced an increase of 440% in 10 minutes. Tetraethyl pyrophosphate (TEPP), 300 μM–1 mM, produced a variable increase in the tetanic response of up to 50% after 5 minutes. This increase was not sustained and declined to near control levels in 15 minutes. Three mM TEPP reduced the responsiveness of the preparation to direct stimulation.

Discussion

The 15 min exposure of the isolated rat phrenic nerve–diaphragm preparation to 30 μM *iso*-OMPA reduced the rate of hydrolysis of butyrylcholine by 85 and 70% as assessed on homogenates and intact preparations respectively. Since 300 μM *iso*-OMPA had the same effect on hydrolysis as 30 μM, it is reasonable to conclude that 30 μM *iso*-OMPA produced complete inhibition of cholinesterase and that the *iso*-OMPA-resistant hydrolysis of butyrylcholine was brought about by an enzyme or enzymes other than cholinesterase. One such enzyme has been reported recently by Berry & Rutland (1971) who demonstrated a physostigmine-resistant enzyme in the rat diaphragm capable of metabolizing butyrylcholine. Differences in the concentration of this enzyme in extracellular and intracellular compartments could account for the differences observed between intact diaphragms and homogenates with regard to the level of inhibition of hydrolysis of butyrylcholine that could be attained with *iso*-OMPA. Another pathway for the hydrolysis of butyrylcholine is

via acetylcholinesterase which hydrolyzes butyrylcholine at 5% of the rate of acetylcholine (Hobbiger, unpublished observations).

Iso-OMPA, 30 μM, although producing a stable phosphorylated cholinesterase (Austin & Berry, 1953 ; Aldridge, 1953), had no irreversible effects upon the responses of the preparation to indirect stimulation, neither did it alter the sensitivity of the diaphragm to externally applied acetylcholine nor the rate of hydrolysis of this ester. On the other hand, it produced a sustained increase in the sensitivity of the diaphragm to externally applied butyrylcholine. Therefore, it can be concluded that cholinesterase plays no significant role in neuromuscular transmission in the rat isolated phrenic nerve–diaphragm for stimulation rates up to 100 Hz.

Iso-OMPA, 300 μM, caused an increase in the sensitivity to externally applied butyrylcholine, which was partly reversible. It also caused a reversible increase in the sensitivity to externally applied acetylcholine. On the frog rectus abdominis muscle, the same concentration of *iso*-OMPA had a curare-like effect. It would seem therefore that in both preparations, *iso*-OMPA in this concentration binds reversibly to receptors for acetylcholine. The greater sustained increase in sensitivity to externally applied butyrylcholine caused by 300 μM compared to 300 μM *iso*-OMPA is curious in view of the fact the lower concentration produced maximum or near maximum inhibition of cholinesterase. It may be that 300 μM *iso*-OMPA altered the distribution of the ester, for example by preventing its access to an intracellular fraction of the physostigmine-resistant enzyme.

In concentrations required for extensive or complete inhibition of cholinesterase, *iso*-OMPA caused comparable reversible increases in the response of the muscle to indirect and direct stimulation. The greatest effect was seen with 20 Hz (120% and 250% increase with 30 and 300 μM *iso*-OMPA respectively) and the least with 100 Hz (3% with both 30 and 300 μM *iso*-OMPA). Twitch responses showed increases of up to 27% with 300 μM *iso*-OMPA. Since the effects on responses to direct stimulation were unaffected by (+)-tubocurarine, the most likely cause of this is an activation of the contractile mechanism inversely related to the frequency of stimulation and arising from an action of *iso*-OMPA on a component of the muscle fibre itself.

Caffeine had a similar effect, and the observation that *iso*-OMPA potentiates the action of caffeine and *vice versa* might provide a useful clue to the site of action of *iso*-OMPA. The caffeine-induced potentiation of the response of striped muscle to stimulation can be explained in terms of increased energy supply due to inhibition of phosphodiesterase (Butcher & Sutherland, 1962). Pyrophosphate, like the methylxanthines, is an inhibitor of phosphodiesterase (Cheung, 1966), and *iso*-OMPA might well share this property. However, if *iso*-OMPA was indeed acting on the same receptor site (phosphodiesterase) as caffeine, it would be expected that their combined effects would be simply additive. Since the two drugs potentiate each other, they are more likely to act at different though closely related sites. Alternative possibilities for the site of action of *iso*-OMPA are as follows. Phosphorylase *b* is converted to phosphorylase *a* by phosphorylation of a serine residue in each of four subunits. The ability to phosphorylate serine residues in cholinesterases is a property of organophosphate anticholinesterases (Jarndorf, Michel, Schaffer, Egan, and Summerson, 1955) and thus *iso*-OMPA could act by phosphorylating phosphorylase *b*. This phosphorylation should be fairly stable, however, whereas the effect of *iso*-OMPA is easily reversible. Another possibility is

that *iso*-OMPA increases the concentration of phosphorylase *a* by inhibiting phosphorylase phosphatase.

The activity of anticholinesterase compounds structurally related to *iso*-OMPA allows certain tentative conclusions to be made concerning the structural requirements for potentiation of the response of diaphragm muscle to stimulation. Alkoxy and alkylamino derivatives of phosphoric acid such as paraoxon, dyflos, and mipafox were devoid of this action. OMPA and *iso*-OMPA which are tetramide derivatives of pyrophosphorus acid were active. TEPP, a derivative of pyrophosphorus acid but devoid of amide groups, exhibited weak activity, but its activity was counteracted by a depressant action on the muscle. It would appear, therefore, that the pyrophosphorus moiety together with tetramide substituent groups are required for potentiation of the response of the diaphragm muscle to stimulation.

Caffeine, unlike *iso*-OMPA, produces contractures. Lüttgau & Oetliker (1968) investigated the nature of the caffeine-induced contractures in the semi-tendinosus and iliofibularis muscles of the frog, and came to the conclusion that they could be explained by a displacement of calcium ions in the tubular walls of the T-system. The T-system does not appear to be affected by *iso*-OMPA in the same way as caffeine.

I am indebted to Professor F. Hobbiger for advice during the course of this work, and for his help in the preparation of the manuscript. It is also a pleasure to record my gratitude to Mrs. D. A. F. Gettins for technical assistance, and Miss R. J. Turner for secretarial assistance.

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(*Received March* 20, 1972)