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# The effects of atropine and dyflos on tremor and increase in whole brain acetylcholine produced by injection of oxotremorine in the rat

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1. Injection of oxotremorine in the rat results in tremor and an increase in brain acetylcholine. The effects of atropine and dyflos have been investigated on both these actions.

2. Atropine decreased brain acetylcholine concentration and inhibited oxotremorine-tremor. It did this in doses which did not prevent the oxotremorine-induced increase in whole brain acetylcholine.

3. Dyflos increased brain acetylcholine concentration, but it was without effect on oxotremorine tremor. Some mutual antagonism was observed between the actions of oxotremorine and dyflos on rat brain acetylcholine concentration.

4. These results do not support a causal relationship between the increase in whole brain acetylcholine and the tremor produced by oxotremorine.

It has been suggested that the tremorgenic action of oxotremorine (1,-(2-oxo-pyrrolidino)-4-pyrrolidinobutyne-2) is due to its ability to mobilize brain acetylcholine (Holmstedt & Lundgren, 1966). Cox & Potkonjak (1969), however, found that after injection of oxotremorine in rats, the time course of the tremor and the time course of the increase in whole brain acetylcholine concentration differed markedly. They also noted that doses of oxotremorine which had no significant effect on whole brain acetylcholine could produce tremor. Oxotremorine is a potent direct-acting muscarinic agonist (Cho, Haslett & Jenden, 1962 ; Lévy & Michel-Ber, 1965, 1967). It is therefore possible that oxotremorine acts on the central nervous system either directly or indirectly through the release of acetylcholine. It was decided to investigate the interactions between atropine and oxotremorine, and dyflos and oxotremorine in an attempt to provide more information on the mode of action of oxotremorine as a tremor-producing drug.

## Methods

Male Wistar rats weighing 190–210 g were used for the tremor recording ; a wider range (190–250 g) was used in the brain acetylcholine experiments.

*Brain acetylcholine estimation*

Acetylcholine was extracted from individual rat brains by a method previously described (Cox & Potkonjak, 1967). Estimations were performed on guinea-pig ileum pretreated with mipafox (N,N[1]di-isopropylphosphorodiamidic fluoride) using a 2+2, 4 point assay, with doses given in a 4 block latin square arrangement.

After the assay a sample of each brain extract was boiled for 1 min with 1 N sodium hydroxide, neutralized with 1 N hydrochloric acid, and an aliquot was tested for activity. The results for the few extracts that showed activity after boiling were discounted. A solution of oxotremorine was extracted in the same way as was brain tissue. Before boiling this extract produced a contraction of guinea-pig ileum equivalent to an ED80 dose of acetylcholine. After boiling it gave a contraction equivalent to an ED50 dose of acetylcholine. Thus, lack of activity in the brain extracts after boiling showed that they did not contain a significant amount of oxotremorine.

Extracts from the brains of rats pretreated with atropine did not reduce the sensitivity of the ileum to subsequent doses of acetylcholine, indicating that atropine was not present in these extracts in sufficient concentration to affect the assay.

*Quantitative estimation of tremor*

Tremor was determined by a method described previously (Cox & Potkonjak, 1969). After injection a rat was placed in a Perspex box attached to a gramophone pick-up head. The output produced by the pick-up in response to movements of the rat was connected to a wide band preamplifier (7P3A) of a Grass Polygraph (Model 7) and to a polygraph integrator (7P10A) to give an integrated value of the tremor recording. The tremor was recorded continuously for a 5 min period (from 30 sec to 5 min 30 sec after injection of oxotremorine). One complete deflection of the integrator pen was given an arbitrary value of 100 tremor units. The pen returned to baseline immediately after reaching full deflection and was also automatically zeroed every 15 sec. The tremor is always presented as the mean of the twenty 15-sec samples recorded during the standard 5 min.

Tremor was recorded 15 min after dyflos pretreatment and brain acetylcholine was determined at both 15 and 30 min after the same pretreatment dose. The pretreatment time for atropine was 30 min in all experiments.

The dose/response curves plotted for tremor are the corrected curves obtained by subtracting the mean tremor value relating to the rats receiving only the pretreatment drug, from the mean tremor value of the rats receiving both the pretreatment and oxotremorine.

*Statistical evaluation of results*

Differences between mean brain acetylcholine concentrations were assessed by Student's *t* test (two-tailed). The Mann-Whitney U test (Siegel, 1956) was used to determine the statistical significance between the groups of rats in the tremor recording. Unless otherwise stated the criterion of a significant difference between means was $P \leqslant 0.05$.

### *Drugs*

Acetylcholine chloride, atropine sulphate, and morphine hydrochloride (B.D.H. Ltd.); Mipafox (L. Light & Co. Ltd.); Dyflos (Koch Light Ltd.). Oxotremorine as the free base. Drug injections were freshly prepared in 0.9% saline and administered intraperitoneally in a volume of 0.1 ml./100 g rat.

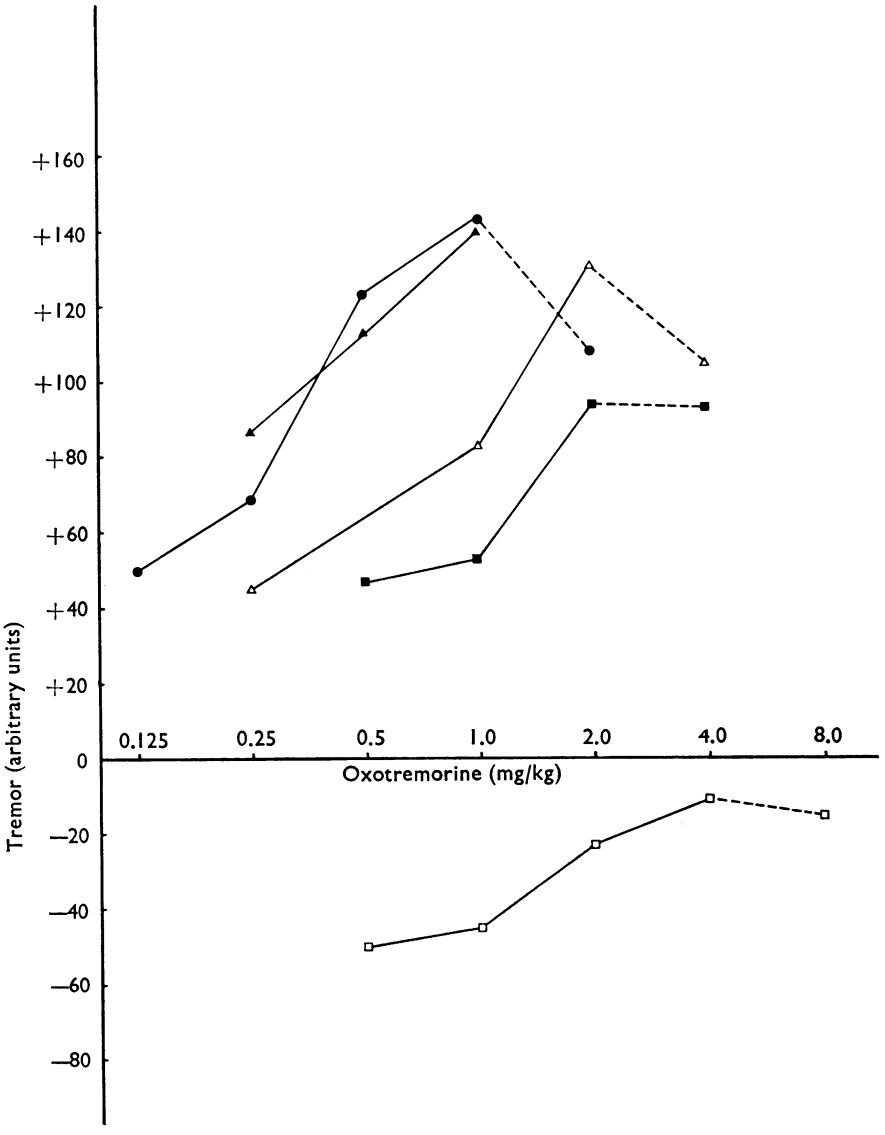


FIG. 1. Effect of atropine on oxotremorine-induced tremor. The corrected mean tremor for oxotremorine is plotted against dose of oxotremorine on a log scale. Curves for: oxotremorine (●) and oxotremorine plus atropine 2.5 (▲), 5 (△), 10 (■) and 40 (□) mg/kg. Each point is the mean of at least five determinations. Mean tremor was corrected by subtraction of the mean tremor value recorded from rats receiving only the pretreatment dose.

## Results

### *Oxotremorine tremor log dose/response curve*

The dose/response curve for oxotremorine-induced tremor is shown in Fig. 1 and repeated for comparison purposes in Fig. 2. The graph shows that the mean tremor increased as the dose of oxotremorine increased from 0.125 to 1 mg/kg. With oxotremorine (2 mg/kg) there was a decrease in the tremor produced. The curve shown is the corrected curve obtained by subtracting the mean background activity recorded from rats receiving only saline injection. Five such rats gave a mean value of 42 arbitrary units. All the doses of oxotremorine gave mean tremor values significantly higher than this control reading.

### *Effect of atropine on oxotremorine-tremor*

The effect of atropine on oxotremorine-tremor is shown in Fig. 1.

Atropine (2.5 mg/kg) produced a mean background activity of 39 units not significantly different from saline controls.

This dose was ineffective against oxotremorine-tremor, the corrected curve being not significantly different from the corrected curve for oxotremorine alone.

Atropine (5 mg/kg) increased the mean background activity to 50 units. This was not significantly different from saline controls. It also produced a significant rightward shift of the oxotremorine log dose/response curve, with a slight depression of the maximum tremor recorded. This maximum occurred with oxotremorine (2 mg/kg), the mean tremor being reduced after oxotremorine (4 mg/kg).

Atropine (10 mg/kg) shifted the oxotremorine dose/response curve even further to the right and also produced a significant increase in the background activity to 73 units.

A higher dose of atropine (40 mg/kg) was also tried, but this dose produced a marked behavioural change. The rats became restless and excited and showed fine tremor. The mean tremor value from these animals was 159 units, a value similar to that obtained with oxotremorine (0.5 mg/kg). After injection of oxotremorine into rats pretreated with atropine (40 mg/kg) the final tremor reading was always less than the reading after atropine on its own. Therefore the corrected tremor obtained by subtraction had a negative sign.

### *Effect of dyflos on oxotremorine-tremor*

Rats receiving dyflos (1 mg/kg) showed no sign of serious untoward effects during the pretreatment period, and no sign of tremor. The mean background activity recorded from these rats was 46 units, a value not significantly different from the saline control value. The corrected values for the tremor produced by oxotremorine after dyflos were similar to those for oxotremorine alone. The dose/response curve produced showed only a slight non-significant rightward shift (Fig. 2).

### *Effect of atropine and oxotremorine on whole brain acetylcholine concentration*

The effect of atropine pretreatment on whole brain acetylcholine concentration is shown in Fig. 3. Pretreatment with atropine (2.5 mg/kg for 30 min) had no effect on whole brain acetylcholine concentration. Atropine (5 and 10 mg/kg) produced

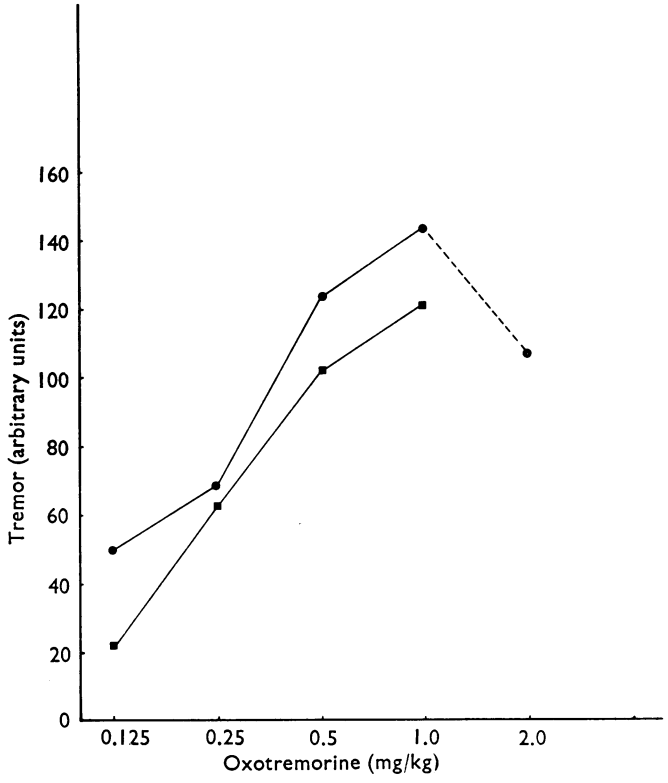


FIG. 2. Effect of dyflos on oxotremorine-induced tremor. The corrected mean tremor for oxotremorine plotted against dose of oxotremorine on a log scale. Curves for oxotremorine (●) and oxotremorine plus dyflos 1 mg/kg (■). Each point is the mean of at least five determinations. Mean tremor was corrected by subtraction of the mean tremor value recorded from rats receiving only dyflos 1 mg/kg.

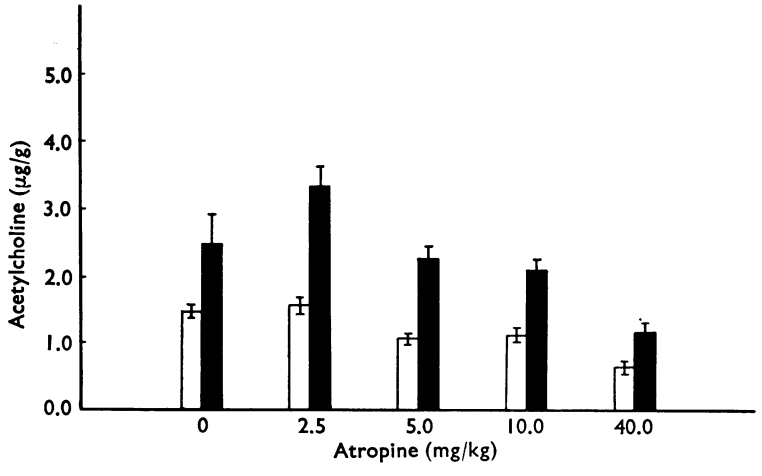


FIG. 3. Effect of atropine pretreatment on the oxotremorine-induced increase in rat brain acetylcholine concentration (μg/g whole brain ± standard error). White columns represent rats pretreated with atropine in the dose stated for 30 min. Black columns represent rats pretreated with atropine in the dose stated for 30 min and oxotremorine 2 mg/kg for 5 min. Each column is the mean from five brains.

small, significant decreases ($P<0.02$) in brain acetylcholine concentration, while atropine (40 mg/kg) produced a larger decrease ($P<0.001$). When oxotremorine (2 mg/kg) was administered to rats pretreated with atropine (2.5 mg/kg) there was an increase in whole brain acetylcholine concentration. This concentration was significantly higher than in either saline-pretreated controls or atropine-pretreated rats. It was not significantly different from the concentration recorded when oxotremorine was administered on its own.

Oxotremorine (2 mg/kg) also produced a significant increase in the brain acetylcholine concentration of rats pretreated with atropine (5 and 10 mg/kg). This effect was not significantly different from the effect of oxotremorine alone. The concentration of acetylcholine in rat brain after injection of atropine 2.5 mg/kg and oxotremorine 2 mg/kg was significantly higher than the concentration after injection of oxotremorine 2 mg/kg into rats pretreated with atropine either 5 or 10 mg/kg. When oxotremorine was injected into rats pretreated with atropine (40 mg/kg) there was only a small non-significant increase in whole brain acetylcholine concentration. The brain concentration in these rats was significantly lower than the concentration in any of the other groups injected with oxotremorine (2 mg/kg).

*Effect of dyflos and oxotremorine on whole brain acetylcholine concentration*

The effect of pretreatment with dyflos on the oxotremorine-induced increase in rat brain acetylcholine concentration is shown in Table 1.

Pretreatment with dyflos (1 mg/kg) for 15 and 30 min produced a significant increase in the concentration of rat brain acetylcholine when compared with the concentration in the saline control group. The concentration after 15 min was significantly higher than the concentration after 30 min. When rats pretreated with dyflos for 15 min received oxotremorine (0.5 mg/kg) 5 min before the end of the pretreatment period, the brain acetylcholine concentration was significantly higher than the concentration in rats receiving oxotremorine alone. It was, however, significantly lower than the concentration recorded after dyflos pretreatment for 15 min. Oxotremorine (2 mg/kg) injected into rats pretreated with dyflos produced an increase in brain acetylcholine concentration which was not significantly different

TABLE 1. *Interaction between dyflos and oxotremorine on rat brain acetylcholine*

| Drug and dose (mg/kg) | Pretreatment time (min) | Brain acetylcholine ($\mu$g/g$\pm$S.E.) | $n$ |
|---|---|---|---|
| 1. Saline | — | 1·49±0·09 | 5 |
| 2. Dyflos 1 | 15 | 3·33±0·39 | 4 |
| 3. Dyflos 1 | 30 | 2·17±0·22 | 9 |
| 4. Dyflos 1 | 15 | | |
| +oxotremorine 0·5 | 5 | 1·96±0·09 | 5 |
| 5. Oxotremorine 0·5 | 5 | 1·29±0·15 | 5 |
| 6. Dyflos 1 | 15 | | |
| +oxotremorine 0·5 | 15 | 1·32±0·11 | 5 |
| 7. Oxotremorine 0·5 | 15 | 1·92±0·16 | 5 |
| 8. Dyflos 1 | 15 | | |
| +oxotremorine 2 | 5 | 2·84±0·18 | 5 |
| 9. Oxotremorine 2 | 5 | 2·48±0·45 | 5 |
| 10. Dyflos 1 | 15 | | |
| +oxotremorine 2 | 15 | 1·54±0·17 | 5 |
| 11. Oxotremorine 2 | 15 | 2·78±0·49 | 7 |

$n$, Number of determinations for each mean.
S.E., Standard error of the mean.

from the increase produced by oxotremorine injected on its own, or dyflos pretreatment on its own.

The brain acetylcholine concentration was also determined 15 min after simultaneous administration of dyflos (1 mg/kg) and oxotremorine (0.5 mg/kg). The final concentration was significantly lower than when either drug was administered alone. Similarly, when dyflos and oxotremorine (2 mg/kg) were administered together the brain acetylcholine concentration was significantly lower than the concentration in rats pretreated with either dyflos (1 mg/kg) or with oxotremorine (2 mg/kg).

**Discussion**

Oxotremorine-tremor in the rat reaches a maximum in the first 5 min after injection (Cox & Potkonjak, 1969). In the present study the effect of oxotremorine on whole brain acetylcholine was therefore investigated 5 min after the injection. Comparison between drug effects on tremor and on brain acetylcholine was also made at this time. Atropine, in doses greater than 2.5 mg/kg, reduced the concentration of rat brain acetylcholine, the highest dose tested (40 mg/kg) being the most effective. These findings do not agree with those of Holmstedt & Lundgren (1966), who found doses of atropine between 5 and 100 mg/kg to be equally effective in reducing rat brain acetylcholine. Atropine in doses of 5 and 10 mg/kg inhibited oxotremorine tremor (Fig. 1), but did not prevent the increase in whole brain acetylcholine concentration (Fig. 3). Only a higher dose of atropine (40 mg/kg), which on its own produced tremor and a marked decrease in brain acetylcholine, prevented the oxotremorine-induced rise in brain acetylcholine. Therefore the ability of atropine to inhibit oxotremorine tremor does not seem to be related to an inhibitory effect on the increase in whole brain acetylcholine concentration. It seems more likely that the antagonism by atropine is related to an effect on some central muscarinic type of receptor. In these circumstances the tremor would be inhibited whether oxotremorine was acting directly on such a receptor or indirectly through the release of acetylcholine. In this context oxotremorine has been shown to be a potent directly acting muscarinic agonist (Cho, Haslett & Jenden, 1962 ; Lévy & Michel-Ber, 1965 ; 1967). Moreover Hammer, Karlen & Sjöqvist (1968) have shown that mice pretreated with atropine have a lower brain oxotremorine concentration than non-pretreated mice and suggested that, in this species, increased clearance from the brain could play a part in the tremor antagonism.

Although the present results with atropine do not support a causal relationship between the increase in whole brain acetylcholine and tremor, they do not exclude the possibility that the tremor is mediated by acetylcholine. If this were the case a cholinesterase inhibitor might be expected to potentiate oxotremorine tremor. Dyflos 1 mg/kg was used as the cholinesterase inhibitor because it has been shown to penetrate the central nervous system (Paulet, Marsol & Coq, 1957), and to produce few symptoms and little evidence of toxicity, while causing marked inhibition of brain cholinesterase (Frawley, Hagan & Fitzhugh, 1952). In our experiments dyflos produced an increase in whole brain acetylcholine without evidence of tremor or any obvious toxic effect. This dose failed to potentiate the tremorgenic action of oxotremorine. Previous attempts to potentiate the effects of tremorine (the precursor of oxotremorine) have also failed. Keranen, Zaratzian & Coleman (1961) observed only summation of the tremorgenic effects of tremorine and physostigmine, and

some antagonism between neostigmine and tremorine. Also physostigmine did not potentiate the hypothermic effect of tremorine.

Holmstedt & Lundgren (1966), using brain homogenates, showed that when dyflos and oxotremorine were added to the same homogenate, the final increase in the acetylcholine concentration was not greater than the sum of the two individual increases. If oxotremorine was causing a release of acetylcholine into the homogenate then one would have expected that the anticholinesterase would have had more than a simple additive effect. Therefore, in their *in vitro* studies, there is not good evidence for an action of oxotremorine involving a release of acetylcholine. In our experiments the *in vivo* interaction between dyflos and oxotremorine was different from that reported by Holmstedt & Lundgren *in vitro*. Oxotremorine 0.5 mg/kg, which on its own did not affect the brain acetylcholine concentration, reduced the increase in whole brain acetylcholine produced by dyflos. When oxotremorine and dyflos were injected together a mutual inhibition of both their effects occurred. At present we have no evidence concerning the nature of the dyflos-oxotremorine interaction. It could be that oxotremorine is protecting cholinesterase from dyflos without interfering with its ability to hydrolyse acetylcholine, or oxotremorine (or a metabolite) might be capable of regenerating cholinesterase in a way similar to that described for the aldoximes (Feldberg & Sherwood, 1954; Hobbiger & Sadler, 1959; Edery, 1962; Bieger & Wassermann, 1967). Both these alternatives are being tested.

If, as has been suggested, oxotremorine-tremor and oxotremorine-induced increase in brain acetylcholine are causally related, then a drug which prevents the increase might be expected to antagonize the tremor. Dyflos prevented the oxotremorine-induced increase in brain acetylcholine (an action which would seem not to be related to an effect on cholinesterase) but was without effect on the tremor.

Therefore, these findings do not support the hypothetical indirect action of oxotremorine but suggest that tremor is probably due to a direct action of oxotremorine on a central nervous system receptor.

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