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DIFFERENTIAL ALTERATIONS OF CHOLINERGIC MUSCARINIC RECEPTORS DURING CHRONIC AND ACUTE TOLERANCE TO ORGANOPHOSPHORUS INSECTICIDES

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Abstract—Male mice treated for 2 weeks with the anticholinesterase insecticide disulfoton (*O,O*-diethyl *S*-[2-(ethylthio)-ethyl] phosphorodithioate; 10 mg per kg per day) became tolerant to the hypothermic and antinociceptive effects of disulfoton itself and of oxotremorine, a muscarinic cholinergic agonist. Homogenates of brain and ileum from tolerant animals exhibited reduced binding of the specific muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB). In forebrains of tolerant animals, the number of receptors (B_{max}) was decreased 40% with no change in the affinity constant. Acetylcholinesterase (AChE) activity was 15% of control. Forty-eight hours after a single injection of disulfoton (10 mg/kg) mice were more resistant than their controls to the hypothermic and antinociceptive effects of a second administration of the same insecticide and of oxotremorine. Tolerance was not present 96 hr after a single administration of disulfoton. A single injection of disulfoton produced 74, 65 and 27% inhibition of AChE activity after 4, 48 and 96 hr respectively. Four hours after a second injection at 48 or 96 hr, 73 or 72% inhibition was found. [^{3}H]QNB binding of animals treated with a single injection of disulfoton and of controls did not differ at either time point. An increase in the K_i for inhibition of [^{3}H]QNB binding by unlabeled oxotremorine was observed in forebrain from mice killed 48 hr after a single injection of disulfoton, indicating a decreased affinity of the muscarinic receptor for agonists. Binding of [^{3}H]oxotremorine-M was decreased significantly 48 hr after a single injection of disulfoton and after chronic treatment. It is suggested that a differential down-regulation of muscarinic receptors occurs in acute and chronic tolerance, involving agonist and antagonist binding sites and depending on duration of exposure.

Administration of repeated sublethal doses of an anticholinesterase organophosphorus insecticide leads to the development of tolerance to its toxicity, as shown first by Rider *et al.* [1], using octamethylpyrophosphoramide. Subsequently, Brodeur and DuBois [2] reported that rats tolerant to the insecticide disulfoton were subsensitive to a lethal dose of the cholinergic agonist carbachol. Furthermore, isolated tissue preparations from tolerant animals were found to be subsensitive to the effects of different cholinergic agonists [3–5]. These results have led to the hypothesis [6] that tolerance is, at least partially, mediated by a decreased sensitivity of cholinergic receptors.

Recent studies have shown that, when both rats and mice are chronically treated with various organosphosphorus compounds, there is reduced binding of the specific muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to their central and peripheral tissues [7–14]. Similar results were also found with the carbamate anticholinesterase neostigmine [15, 16].

Two papers by Overstreet *et al.* [17, 18] showed that tolerance to the hypothermic effect of diisopropylfluorophosphate (DFP) can develop after a single injection of the same compound. These authors also reported that, following a single injection of DFP to rats, animals were subsensitive to the hypothermic effect of the cholinergic agonist pilocarpine and to its inhibitory action on water intake. They suggested that the mechanisms underlying the recovery of animals following acute administration and those underlying tolerance development may be similar if not identical [17].

The aim of the present study was to investigate if similar mechanisms could explain both acute and chronic tolerance to organophosphates by measuring the resistance to the hypothermic and antinociceptive effects induced by these compounds and by cholinergic muscarinic agonists. In particular, since alterations of cholinergic receptors have been found after chronic treatment with organophosphates, we tested the possibility of an involvement of muscarinic receptors in acute tolerance.

MATERIALS AND METHODS

Animals and treatments. Male Charles River CD-1 mice (25–35 g) were housed five per cage in air-conditioned rooms under a constant temperature and light schedule (ambient temperature 25°; 12 hr dark). Food and water were available *ad lib.* In the chronic experiments, animals were treated daily for 14 days with the insecticide disulfoton (*O,O*-diethyl *S*-[2-(ethylthio)-ethyl] phosphorodithioate; technical

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grade, 97%; Di-Syston, Mobay Chemical Corp., Kansas City, MO; 10 mg per kg per day, i.p.). The insecticide was dissolved in corn oil in concentrations such that the proper dose was contained in 5 ml/kg body weight. Oxotremorine (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water and injected s.c. in a final volume of 1 ml/kg body weight. Animals were decapitated, and brain and ileum were rapidly removed for assays.

Hypothermia. Rectal temperature was measured, as an index of body temperature, by a thermistor mounted in a rectal probe connected to a Tele-thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). The flexible thermistor probe was inserted 25 mm into the rectum. Two or three control measurements were taken for each mouse during an interval of 30 min before the injection of disulfoton or oxotremorine. The average value of these measurements was taken as the initial temperature at 0 time. During temperature measurements, mice were kept in a plastic restrainer, and the thermistor probe was retained in the rectum until a constant temperature reading was obtained.

Antinociception. Antinociception was measured by the tail-immersion technique as described by Sewell and Spencer [19]. Briefly, the animals were restrained in a plastic container, and the nociceptive reaction time, in seconds, was determined when the tail was immersed in a constant temperature a set at 50° (± 0.5). The nociceptive end-point was a violent jerk of the tail. A 30-sec "cut-off" time was imposed for all animals that failed to respond to the stimulus. The control nociceptive sensitivity ranged from 4 to 8 sec in all the experiments.

[^{3}H]QNB binding. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB; 40.2 Ci/mmole; New England Nuclear, Boston, MA) binding was assessed essentially according to the method of Yamamura and Snyder [20]. Tissue homogenates (5%) were prepared in Na_2HPO_4/KH_2PO_4 buffer (0.05 M; pH 7.4) with a Brinkmann Polytron homogenizer and centrifuged three times at 50,000 *g* for 10 min. Each time the supernatant fraction was discarded, and the pellet was resuspended in phosphate buffer. Assays of [^{3}H]QNB binding were carried out by incubating a quantity of resuspended pellet (representing 30–60 μg of protein) with [^{3}H]QNB at 27° for 1 hr in 2 ml of phosphate buffer. Bound [^{3}H]QNB was separated from free by vacuum filtration through Whatman GF/C filters, washed three times with 3 ml of ice-cold buffer. Atropine sulphate (10^{-5} M; Nutritional Biochemicals Corp., Cleveland, OH) was added to half of the tubes for estimation of specific binding defined as the difference between binding in the presence and absence of atropine. The filters were placed in 10 ml Liquiscint (National Diagnostic, Somerville, NJ) and counted in a Packard Tricarb scintillation spectrometer at an efficiency of 40%. Each determination was done in triplicate.

Proteins were determined by the method of Lowry *et al.* [21] with bovine serum albumin as a standard.

Saturation binding experiments were carried out in the same manner with the exception that a range (0.02 to 2 nM) of [^{3}H]QNB concentrations was used. Saturation binding data were transformed according to the method of Scatchard [22].

For measurement of the competitive displacement of [^{3}H]QNB binding by oxotremorine, a final concentration of 0.08 nM of total [^{3}H]QNB was used, and tissue homogenates were diluted 1 : 10 with phosphate buffer prior to use. At these concentrations only 5% of the total [^{3}H]QNB was bound. The log of the concentration of oxotremorine (M) was plotted against the percentage of [^{3}H]QNB displaced by a given concentration. K_i values for oxotremorine were calculated following the equation

$$K_i = IC_{50} / \left(1 + \frac{[[^3H]QNB]}{K_{QNB}}\right)$$

where IC_{50} is the concentration of oxotremorine which caused 50% displacement of [^{3}H]QNB binding; [[^{3}H]QNB] is the concentration of [^{3}H]QNB used in the experiment and K_{QNB} is the equilibrium dissociation constant for [^{3}H]QNB determined from separate experiments. Hill coefficients for [^{3}H]QNB and oxotremorine were calculated by plotting log [I/(100 − I)] vs log C where I is the percent inhibition of specific binding and C the concentration of ligand (oxotremorine or [^{3}H]QNB).

Acetylcholinesterase activity determination. Acetylcholinesterase (AChE) activity was assayed by the colorimetric method of Ellman *et al.* [23] as modified by Benke *et al.* [24]. An aliquot of tissue homogenate (equivalent to approximately 0.8 mg tissue), 5 μl of 1.0 M acetylthiocholine (Sigma Chemical Co.) and 50 μl of 0.1 M 5,5-dithiobis (2-nitrobenzoic) acid (DTNB, Sigma Chemical Co.) were added to an appropriate volume of sodium phosphate buffer (0.1 M; pH 8 at 25°) to make a final volume of 5 ml. The absorbance (at 412 nm) was read immediately after the addition of the substrate acetylthiocholine and after 30 min of incubation at 27°. The initial absorbance, as well as reagent blank absorbance was subtracted from the final reading. The change in absorbance during the incubation is due to formation of 5-thio-2-nitrobenzoate from DTNB and thiocholine, the hydrolytic product of acetylthiocholine. AChE activity is usually expressed as percentage of control activity.

[^{3}H]Oxotremorine-M binding. For [^{3}H]oxotremorine-M (83.3 Ci/mmole, New England Nuclear) binding, tissues were homogenized in 10 vol. of 0.32 M sucrose and centrifuged for 10 min at 1,000 *g*. The supernatant fraction was recentrifuged at 17,500 *g* for 20 min, and the pellet was resuspended in 0.32 sucrose and centrifuged at 17,000 *g* for 20 min. The final pellet was resuspended in sufficient Na_2HPO_4/KH_2PO_4 buffer (0.05 M; pH 7.4) so that there was the equivalent of 50 mg tissue/ml. The incubation system consisted of the phosphate buffer, [^{3}H]oxotremorine-M, and an aliquot of tissue homogenate corresponding to approximately 0.8 mg protein. Atropine sulphate (10^{-5} M) was added to half of the tubes for estimation of specific binding. After incubation for 15 min at 30°, tubes were placed in ice for 15 min and then centrifuged at 27,000 *g* for 15 min at 0°. Preliminary experiments indicated that centrifugation was preferable to filtration because [^{3}H]oxotremorine-M binds to the filters which yield, therefore, erroneously low ratios of specific/non-specific binding. Furthermore, there is a rapid rate of dissociation of the [^{3}H]oxotremorine-M receptor

complex at the room temperature at which filtration is conducted. After centrifugation, the supernatant fraction was discarded and the pellet was washed rapidly three times with ice-cold 0.9% NaCl. The internal walls of the tubes were carefully dried and the pellet was solubilized with 1 ml Protosol (New England Nuclear). Econofluor (10 ml; New England Nuclear) was added, and the radioactivity was counted in a Packard Tricarb scintillation spectrometer with an efficiency of 40%. Each determination was done in triplicate.

Statistical analysis. Data were evaluated using a one-way analysis of variance or two-tailed Student's *t*-test. Analysis of covariance on the regression lines [25] was used in comparing Scatchard plots for [^{3}H]QNB binding and χ^2 test for comparing proportional mortalities after challenges with carbachol. Parameters of oxotremorine binding were calculated by the NONLIN computer program [26].

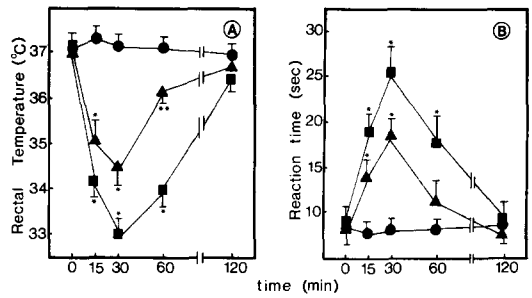

Fig. 2. Hypothermic (A) and antinociceptive (B) effects of oxotremorine in mice. Oxotremorine was dissoved in distilled water and administered subcutaneously. Control animals were injected with distilled water (1 ml/kg). Each point is the mean (± S.E.M.) of eight mice. Key: (●——● control; (▲——▲) oxotremorine, 0.05 mg//kg; (■——■) oxotremorine, 0.1 mg/kg; (★) significantly different from control, $P < 0.01$; and (★★) significantly different from control, $P < 0.05$.

RESULTS

The organophosphorus insecticide disulfoton and the cholinergic muscarinic agonist oxotremorine induced a dose-dependent hypothermic and antinociceptive effect (Figs. 1 and 2). Animals treated for 14 days with disulfoton (10 mg per kg per day) became tolerant to the hypothermic and antinociceptive effect of a further injection of disulfoton (10 mg/kg) administered 24 hr after the end of the treatment (Fig. 3). Disulfoton-tolerant animals were also significantly less sensitive than controls to oxotremorine (0.1 mg/kg, s.c.)-induced hypothermia (ΔT at 30 min: $-4.92 \pm 0.15°$ in controls and $-1.44 \pm 0.19°$ in disulfoton-treated; $N = 6$; $P < 0.001$) and antinociception (reaction time at 30 min: 28.9 ± 0.9 sec for control and 14.6 ± 2.8 in disulfoton-treated; $N = 6$; $P < 0.002$).

As shown in Table 1, [^{3}H]QNB binding was decreased in forebrain, hindbrain and ileum of tolerant animals; the change was due to a decrease in the number of receptors (B_{max}) in the brain and in both B_{max} and affinity (measured as the reciprocal of the dissociation constant K_d) in the ileum. AChE activity was inhibited 70–85% in all tissues. However, as has been reported previously [13, 14], the gradual production of this degree of inhibition did not result in obvious cholinergic signs of toxicity.

Subsequent experiments showed that mice given a single injection of disulfoton (10 mg/kg) were tolerant to the hypothermic and antinociceptive effect of a second injection of disulfoton given 48 hr after the first (Fig. 4). Pretreatment with a single injection of disulfoton (10 mg/kg) also rendered the animals more resistant than controls to the hypothermic and analgesic effects of oxotremorine (0.1 mg/kg) administered 48 hr after disulfoton (Fig. 5). No resistance to disulfoton- and oxotremorine-induced antinociception and hypothermia was present 96 hr after the first injection of disulfoton (Figs. 4 and 5).

[^{3}H]QNB binding in forebrain never differed between treated and control animals from 4 to 96 hr after the administration of a single dose of 10 mg/kg

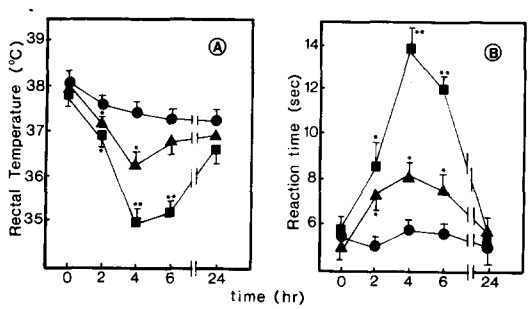

Fig. 1. Hypothermic (A) and antinociceptive (B) effects of disulfoton in mice. Disulfoton was administered by i.p. injection in corn oil. Control animals were injected with vehicle only. Each point is the mean (± S.E.M.) of eight mice. Key: (●——●) corn oil; (▲——▲) disulfoton, 7.5 mg/kg; (■——■) disulfoton, 10 mg/kg; (★) significantly different from control, $P < 0.05$; and (★★) significantly different from control, $P < 0.01$.

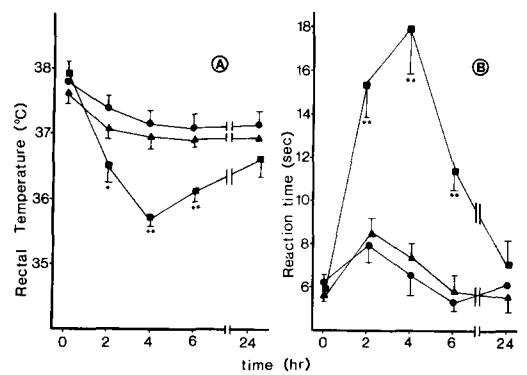

Fig. 3. Hypothermic (A) and antinociceptive (B) effects of disulfoton in chronically disulfoton-treated mice. Mice were treated with disulfoton for 14 days (10 mg per kg per day). Control animals were injected with corn oil for the same period. Disulfoton was administered i.p. at the dose of 10 mg/kg 24 hr after the end of the treatment to corn oil and disulfoton-pretreated mice. Each point is the mean (± S.E.M.) of six to eight animals. Key: (●——●) control; (■——■) control + disulfoton; (▲——▲) disulfoton (chronic) + disulfoton; (★) significantly different from control, $P < 0.05$; and (★★) significantly different from control, $P < 0.01$.

Table 1. [^{3}H]QNB binding and AChE activity in tissues from mice chronically treated with disulfoton*

	Control		Treated		
	[^{3}H]QNB binding		[^{3}H]QNB binding		AChE activity†
	B_{max} (pmoles/mg protein)	K_d (nM)	B_{max} (pmoles/mg protein)	K_d (nM)	% Control
Forebrain	1.58	0.15	0.97‡	0.10	14.7‡
Hindbrain	0.33	0.07	0.23§	0.08	29.6‡
Ileum	0.19	0.17	0.10‡	0.32§	19.3‡

* Mice were treated with disulfoton for 14 days (10 mg per kg per day). Control animals received daily injections of corn oil (5 ml/kg). All animals were killed 24 hr after the end of the treatment. Results are representative of a typical experiment.

† AChE activity in control was 13.2 ± 0.8, 5.9 ± 0.4 and 4.3 ± 0.6 μmoles ATC per min per g tissue for forebrain, hindbrain and ileum respectively.

‡ Significantly different from control, $P < 0.01$.

§ Significantly different from control, $P < 0.05$.

of disulfoton (Fig. 6). AChE activity was maximally inhibited (74%) 4 hr after disulfoton and was still 63% inhibited 48 hr later; at 96 hr AChE activity was still significantly lower than control but inhibition was decreased to 26% (Fig. 6). A second injection of disulfoton, 48 or 96 hr after the first administration, further inhibited AChE activity to 72 and 73% comparable to that found after the first injection (Fig. 6).

Saturation binding experiments of [^{3}H]QNB confirmed the results obtained using a single concentration of ligand. There was no difference in [^{3}H]QNB binding between control and treated animals 48 hr after disulfoton when either B_{max} (1.67 vs 1.60 pmoles/mg protein) or K_d (0.18 vs 0.18 nM) was compared.

Displacement of [^{3}H]QNB binding in forebrain from control animals with unlabeled oxotremorine showed a K_i value of 0.41 ± 0.04 and a Hill coefficient of 0.56 ± 0.02 (Table 2). In mice killed 48 hr after a single administration of disulfoton, the K_i value for oxotremorine and the Hill coefficient were increased (Table 2).

Scatchard analysis of oxotremorine binding showed two classes of receptors with different affinities designated by their dissociation constants K_H and K_L. While the relative percentage of high (n_H) and low (n_L) affinity receptors did not appear to be affected by treatment with disulfoton, the values of K_H were increased (Table 2). Similar results, in addition to a decrease in the binding of [^{3}H]QNB, were obtained in animals treated for 14 days with disulfoton (Table 2).

Binding of [^{3}H]oxotremorine-M was decreased significantly after chronic treatment with disulfoton and 48 hr but not 4 hr or 96 hr after a single injection of the insecticide (Table 3). The incubation was carried out at a concentration of [^{3}H]oxotremorine-M of 4.12 nM which is thought to bind almost exclusively to high affinity receptors [27].

Resistance to the hypothermic and antinociceptive effect of disulfoton and oxotremorine which had disappeared 96 hr after a single injection of 10 mg/kg of disulfoton (Figs. 4 and 5) was still present 96 hr

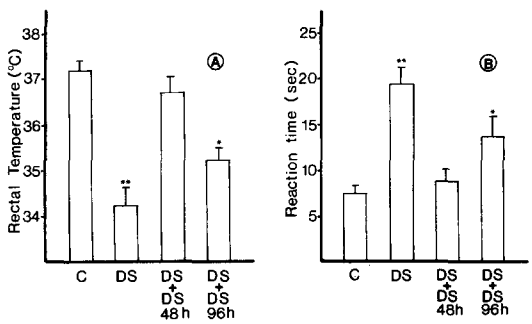

Fig. 4. Acute tolerance to the hypothermic (A) and antinociceptive (B) effects of disulfoton (DS) in mice. Animals were injected at 0 time with 10 mg/kg disulfoton in corn oil. Hypothermia and antinociception were measured 4 hr after the initial administration of disulfoton (second bar) or 4 hr after a second injection at 48 or 96 hr (third and fourth bars). Each bar represents the mean (± S.E.M.) of eight to ten mice. Key: (★) significantly different from control, $P < 0.05$; and (★★) significantly different from control, $P < 0.01$.

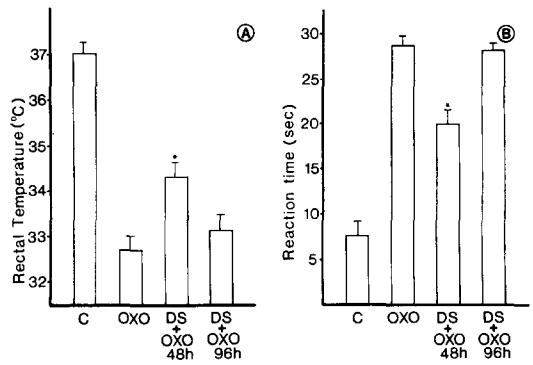

Fig. 5. Acute tolerance to the hypothermic (A) and antinociceptive (B) effects of oxotremorine (OXO) in mice. Animals were injected at 0 time with 10 mg/kg disulfoton (DS) in corn oil. Hypothermia and antinociception were measured 30 min after administration of oxotremorine to control animals (second bar) and to animals injected 48 and 96 hr earlier with disulfoton (third and fourth bars). Each bar represents the mean (± S.E.M.) of eight to twelve mice. All the figures are significantly different from control ($P < 0.01$). Key: (★) significantly different from control animals given oxotremorine, $P < 0.01$.

Table 2. Binding parameters of oxotremorine in mouse forebrain*

	K_i (μM)	Hill coefficient	n_H (%)	K_H (μM)	n_L (%)	K_L (μM)	N†
Control	0.41 ± 0.04	0.56 ± 0.02	43 ± 4	0.065 ± 0.014	57 ± 4	0.975 ± 0.068	3
Disulfoton‡ (acute)	1.05 ± 0.12§	0.91 ± 0.06§	45 ± 1	0.320 ± 0.075§	55 ± 2	1.220 ± 0.080	3
Disulfoton‖ (chronic)	0.94§	0.86§	44	0.293§	56	1.09	2

* Binding of oxotremorine was measured by displacement of [^{3}H]QNB binding. The concentration of [^{3}H]QNB was 0.08 nM. Ten to twelve concentrations of oxotremorine (10^{-9} to 10^{-3} M) were used for each determination.

† Number of experiments performed in triplicate using forebrains pooled from five to six mice.

‡ Mice were given a single injection of disulfoton (10 mg/kg) and killed after 48 hr.

§ Significantly different from control, $P < 0.05$.

‖ Mice were treated with disulfoton for 14 days (10 mg per kg per day) and killed 24 hr after the end of the treatment.

after the last injection of the 14 days treatment with disulfoton. Reaction time after oxotremorine (0.1 mg/kg) was 28.1 ± 0.9 sec in control animals and 10.5 ± 1.3 sec in disulfoton-treated mice (N = 6; $P < 0.01$), and 10.6 ± 0.6 sec vs 5.9 ± 0.3 sec after disulfoton (10 mg/kg) in control and chronically disulfoton-treated animals respectively (N = 6; $P < 0.05$).

DISCUSSION

The investigation showed that tolerance to the hypothermic and antinociceptive effects of the organophosphorus insecticide disulfoton, and to the muscarinic cholinergic agonist oxotremorine, developed after chronic treatment with disulfoton as well as after a single injection. Furthermore, the data suggest that the mechanism underlying both the acute and chronic tolerance was a subsensitivity of the muscarinic cholinergic receptors and that a decrease of muscarinic receptor density, as measured by antagonist binding, was present in chronic tolerance whereas only agonist binding was altered in acute tolerance.

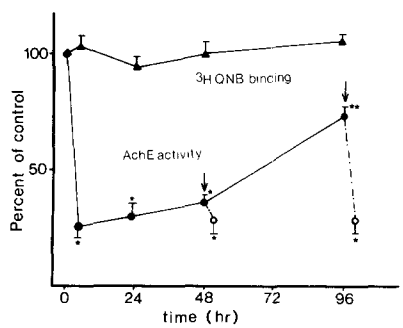

Fig. 6. [^{3}H]QNB binding and AChE activity in mice forebrain after a single injection of disulfoton. Animals were administered disulfoton (10 mg/kg) at 0 time. [^{3}H]QNB binding and AChE are expressed as percentage of control. [^{3}H]QNB binding in control was 0.91 ± 0.04 pmoles/mg protein measured at a concentration of 0.15 nM. Control AChE activity was 11.35 ± 1.13 μmoles ATC hydrolyzed per min per g wet tissue. Arrows indicate a second injection of disulfoton 48 or 96 hr after the first one, and broken lines show the consequent inhibition of AChE activity, measured after 4 hr. Each point is the mean of six to eight values. Key: (★) significantly different from control, $P < 0.01$; and (★★) significantly different from controls, $P < 0.05$.

The finding that chronic treatment with disulfoton rendered the animals subsensitive to hypothermia induced by the same insecticide as well as by oxotremorine is consistent with the results obtained by Overstreet *et al.* [28], who found that rats chronically treated with diisopropylfluorophosphate (DFP) became tolerant to its hypothermic effect and to that of pilocarpine and carbachol. We also found that tolerance was extended to the antinociceptive effect of disulfoton and oxotremorine. Chronically treated animals presented a reduced binding of [^{3}H]QNB in central and peripheral tissues (Table 1), thus confirming the results obtained by different laboratories [7–14]. Particularly in the forebrain, the change in [^{3}H]QNB binding was associated with a decrease of the number of muscarinic receptors (B_{max}) without any change in the affinity constant (K_d).

When disulfoton or oxotremorine was administered 48 hr after a single injection of disulfoton (10 mg/kg), mice were tolerant to their hypothermic and antinociceptive effects. This result confirms the development of acute tolerance after a single injection of an organophosphorus compound as first reported by Overstreet *et al.* [17, 18]. The phenomenon appears to have been relatively rapidly reversible since, when disulfoton or oxotremorine was administered 96 hr after the first injection of disulfoton, mice did not show any resistance to their hypothermic or antinociceptive effects. Measurements of AChE activity in forebrain showed that it was only 26.4% of control 4 hr after disulfoton administration, 37.9% at 48 hr, but had increased to 73.5% at 96 hr (Fig. 6). It is interesting to note that Overstreet *et al.* [17] found that resistance to pilocarpine was lost at 20 days after a single injection of DFP when brain AChE activity was 75% of control. Whether the return of AChE to some critical value (e.g. 75%) can account for the disappearance of receptor subsensitivity needs further investigation. The different time (96 hr vs 20 days) required to reach this AChE value was probably due to the fact

Table 3. [^{3}H]Oxotremorine-M binding in mouse forebrain*

	[^{3}H]Oxotremorine-M bound† (fmoles/mg protein)		P
Acute experiments			
Control	57.74 ± 4.75	(10)	
Disulfoton (10 mg/kg)			
48 hr	36.79 ± 6.34	(5)	<0.02
96 hr	64.20 ± 3.00	(5)	NS‡
Chronic experiments			
Control	64.14 ± 0.98	(5)	
(for 14 days)	38.56 ± 3.27	(5)	<0.001

* In the acute experiments, mice were killed 48 or 96 hr after a single injection of disulfoton (10 mg/kg in corn oil). Control animals were injected with corn oil only (5 ml/kg). In the chronic experiments, mice were treated for 14 days with disulfoton (10 mg per kg per day) while control animals received an equal number of corn oil injections. Animals were killed 24 hr after the end of the treatment. Results are the means (± S.E.M.) of the number of animals given in parentheses.

† [^{3}H]Oxotremorine-M binding was measured as described in Materials and Methods, and the final concentration of [^{3}H]oxotremorine-M was 4.12 nM.

‡ NS = not significant.

that DFP is essentially an irreversible inhibitor of AChE and *de novo* synthesis of the enzyme is required for recovery of activity [29] while with disulfoton some spontaneous reactivation of the phosphorylated enzyme appeared to occur within a few days. Tolerance to a second injection of disulfoton could also be due to a different degree of AChE inhibition after the second injection. Administration of disulfoton 48 hr after a previous injection of disulfoton reduced AChE to 27.1% of control, a value almost identical to that found after the first injection. These results suggest that altered metabolism of disulfoton (activation to the oxygen analog or detoxification) cannot explain the development of tolerance after a single injection. Furthermore, animals were tolerant to a direct acting cholinergic compound, oxotremorine, which is not affected by AChE. This suggests the direct involvement of muscarinic receptors in acute tolerance. [^{3}H]QNB binding in forebrain did not differ from control when measured 48 hr after a single injection of disulfoton. It appears, then, that a decrease in muscarinic receptors, as measured by antagonist binding, is not responsible for the observed subsensitivity to disulfoton and oxotremorine after a single injection of disulfoton. However, the binding of oxotremorine, measured by displacement of [^{3}H]QNB binding, was different in control and experimental animals. The displacement of [^{3}H]QNB binding by oxotremorine deviated from single site mass-action kinetics, and the binding isotherm had a Hill coefficient of less than 1. The most likely explanation for this is the existence of two classes of muscarinic receptors for agonist, which have equal affinity for antagonist [27, 30]. In forebrain from animals given a single injection of disulfoton, the K_i for inhibition of [^{3}H]QNB binding by oxotremorine was increased 2.5-fold, indicating a decreased affinity of the agonist for the muscarinic receptor. The relative numbers of high (n_H) and low (n_L) affinity binding sites were comparable in control and animals treated with a single dose of disulfoton; however, the equilibrium dissociation constants for the high (K_H) and, to a non-significant extent, the low (K_L) affinity sites increased in treated animals, indicating that the affinity for the receptor had decreased. The increase in K_H was greater than that of K_L, so that the ratio K_L/K_H decreased causing a steepening of the binding isotherm for oxotremorine and an increase in the Hill coefficient.

Direct binding of [^{3}H]oxotremorine-M was decreased in tolerant animals 48 hr after a single injection of disulfoton but not at 4 or 96 hr. The concentration used was in the nanomolar range, a concentration at which [^{3}H]oxotremorine-M is considered to bind almost exclusively to the high affinity binding sites [27]. Similar alterations of oxotremorine and [^{3}H]oxotremorine-M binding were found also in animals chronically treated with disulfoton when the binding of the antagonist [^{3}H]QNB also was decreased (Tables 2 and 3).

The phenomenon of acute tolerance after administration of an organophosphate has not been studied in detail. There are, however, many reports on the desensitization of muscarinic receptors in cultured cells. Although comparisons between *in vitro* and *in vivo* systems are rather difficult, our results bear some similarity to those obtained by other authors with mouse neuroblastoma cells. Incubation with the cholinergic agonist carbachol causes a rapid decrease in carbachol-stimulated cycle GMP formation without any change in [^{3}H]QNB binding [31, 32]. If preincubation time with carbachol is increased there is a progressive loss of [^{3}H]QNB binding [31–35].

The initial phenomenon has been defined as short-term desensitization [32], and it is very rapidly reversible by removal of the agonist [31, 32]. This rapid desensitization could result from a change in an agonist binding site of the receptor and/or from inactivation of the calcium channels that couple the receptor with guanylate cyclase [36, 37]. The involvement of calcium channels in short-term desensitization has been reported recently [36]. Young [38] showed a decreased binding of carbachol to desensitized muscarinic receptors from guinea pig intestinal smooth muscle without any change in antagonist

binding. A decrease in high affinity agonist binding has been shown also to occur in beta-adrenergic receptors from turkey erythrocytes after catecholamine-induced desensitization [39]. In our study, stimulation of muscarinic cholinergic receptors after administration of disulfoton was due to accumulation of acetylcholine in the synapses, caused by inhibition of AChE. Different durations of exposure of the muscarinic receptors to acetylcholine after acute and chronic treatment with disulfoton could possibly explain the differential alterations of agonist and antagonist binding.

Resistance to the hypothermic and antinociceptive effects of disulfoton was similar after acute and chronic treatment. Animals were, however, more resistant to oxotremorine after chronic treatment with disulfoton than after acute administration.

When all the data obtained in the hypothermia and antinociception experiments after acute and chronic disulfoton treatment were compared, an analysis of variance showed a significant difference ($P < 0.01$) between chronically and acutely disulfoton-treated animals in their antinociceptive responses and a slight difference ($0.1 > P > 0.05$) when comparing the hypothermic effects. Further, chronically treated mice were also more resistant than controls to a lethal dose (4.2 mg/kg, an LD_{80}) of carbachol while at 48 hr after a single injection of disulfoton such resistance was not observed. Mortality was 90% in controls, 20% in chronically disulfoton-treated ($P < 0.01$) and 80% in acutely treated mice (not significantly different from control but significantly different from chronically treated mice, $P < 0.01$). It appears that, after chronic administration of disulfoton, the loss of muscarinic receptors, as revealed by decreased binding of [^{3}H]QNB, can account for this difference.

In summary, tolerance to the hypothermic and antinociceptive effects of organophosphorus compounds, AChE inhibitors, developed after chronic and acute administration. In both cases, although to different degrees, tolerance to the same effects induced by muscarinic cholinergic agonists was present. Our results suggest that a differential down-regulation of muscarinic receptor occurred in these two phenomena, similar to the suggested short-term and long-term desensitization of muscarinic receptors observed *in vitro* [33].

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