Pharmacology Biochemistry & Behavior, Vol. 34, pp. 473–481. © Pergamon Press plc, 1989. Printed in the U.S.A. 0091-3057/89 $3.00 + .00

On the Development of Behavioral Tolerance to Organophosphates I: Behavioral and Biochemical Aspects

CORNELIA J. VAN DONGEN AND OTTO L. WOLTHUIS

Medical Biological Laboratory TNO, P.O. Box 45, 2280 AA Rijswijk, The Netherlands

Received 2 March 1989

VAN DONGEN, C. J. AND O. L. WOLTHUIS. *On the development of behavioral tolerance to organophosphates I: Behavioral and biochemical aspects.* PHARMACOL BIOCHEM BEHAV **34**(3) 473–481, 1989.—The development of tolerance to organophosphates (OPs) was investigated by SC injections of saline and sublethal doses of DFP or soman three times per week or every other day for at least 4 weeks. Shuttlebox performance was tested 1 hr and 24 hr after the injections. Notwithstanding a progressive inhibition of AChE to very low values in various organs, shuttlebox performance was virtually normal 24 hr after the OP injections. However, whereas the performance decrements measured 1 hr after the injection of DFP practically disappeared in the course of weeks, the decrements 1 hr after soman remained approximately the same. These differences between the effects of DFP and soman cannot be explained: 1) by differences in inhibition or de novo synthesis of AChE in various regions of the CNS, the striated muscle or blood, 2) by differences in the reductions of the muscarinic receptors in various regions of the CNS, 3) by differences in the number of nicotinic receptors in the diaphragm muscle, or 4) by differences in phosphorylphosphatase (DFP-ase or somanase) activity in blood plasma or liver.

Tolerance Organophosphates Soman DFP Behavior Biochemistry

THE development of tolerance or adaptation after administration of an irreversibly cholinesterase inhibiting organophosphate (OP) has been described by several authors, not only after chronic administration (8, 9, 14, 46), but also in a matter of hours after a single OP injection (8, 31, 37, 38, 52). Since it is not certain whether "acute" and "chronic" tolerance result from the same underlying mechanism, for the moment these two forms should be considered as separate entities. Chronic tolerance is defined here as the phenomenon that upon repeated OP injections the effects observed become progressively smaller. Most investigators have used behavioral parameters, and DFP or paraoxon as OPs.

In the development of "chronic" tolerance following repeated OP administration, a number of factors may play a role, such as:

1) *Compensatory synthesis of enzymes that metabolize or bind OPs.* The repeated OP injections might induce the synthesis of large amounts of phosphorylphosphatases, carboxylesterases or even of cholinesterase isoenzymes (16, 25, 39), resulting in increased degradation or scavenging of incoming OP. It is unlikely that this is a major factor in the development of tolerance, since cholinesterase becomes progressively inhibited to low values during the development of tolerance as a result of the repeated OP injections. However, it is noteworthy that notwithstanding the repeated administration of these irreversible inhibitors cholinesterase activity is never completely abolished.

2) *Presynaptic effects.* The accumulation of acetylcholine (ACh) in the synapse might lead to a reduced release of ACh by a presynaptic feedback. Although ACh levels tend to become normal a few hours after a single injection of DFP (22,36), presumably by a negative feedback mechanism (1, 41, 42), these levels are generally found to be increased after repeated injections. The results obtained by Russell and Overstreet (46) in animals tolerant to DFP on choline uptake, ACh synthesis and (resting and evoked) release suggest that tolerance is not due to the end-product inhibition. On the other hand, the results of Thomsen and Wilson (49) with paraoxon suggest that a depression of quantal release of this transmitter could account for the tolerance found. Lim *et al.* (33,34) reported a decrease of high-affinity choline uptake in certain brain structures. They suggest that subsensitivity of the presynaptic functions develops during subacute administration of DFP.

3) *Synaptic effects.* A factor hardly considered is that tolerance during chronic treatment with an OP may result from an increasing ACh leakage from the synapse over time, resulting in a progressively faster relief of the depolarization block by ACh. Such a progressively increased leakage might be caused by damage at the synaptic site, similar to that reported for OPs at the neuromuscular junction (4,18) and for carbamates (29) and may at these sublethal doses also occur at the early stages of OP-induced neuropathy (32,44).

4) *Postsynaptic effects.* Following acetylcholinesterase (AChE) inhibition the accumulation of ACh may cause desensitization and a rise in threshold of the postsynaptic membrane. The postsynaptic membrane repolarizes notwithstanding the "continuously" high levels of transmitter and is only triggered by stimulus-released ACh. There is ample evidence that such a reduction of sensitivity may play a role in the development of tolerance, most likely caused by a down-regulation of muscarinic and nicotinic ACh receptors (14, 15, 43, 46, 47), and can even be found in the

offspring of female rats chronically treated with DFP during pregnancy (40). An additional loss of ACh receptors might be due to the OP-induced neuropathy and myopathy (see point 3). Moreover, the biochemical sequela of receptor activation may be another mechanism whereby tolerance develops; whether this is a factor to be reckoned with remains to be determined.

5) *Behavioral factors.* An important point to consider is how much of the tolerance observed is behaviorally augmented, i.e., to what degree does the animal behaviorally "learns to handle" low AChE levels or how much of the tolerance process is a state-dependent phenomenon. For example, it has been shown in two parallel groups of rats chronically treated with paraoxon (group A: treated and behaviorally tested and group B: treated, but not tested) that, when group B was tested behaviorally for the first time after tolerance had developed in group A, instead of being tolerant to OPs, the animals in group B showed a clear decrement in their performance (8). These and other experiments, e.g., on food consumption (10), indicate that behavioral processes can be a contributing factor and should be taken into account.

6) *Other transmitter systems.* It is imaginable that adaptive phenomena in other transmitter systems may play a role in the ultimate symptomatology of the tolerance phenomenon. However, conclusive evidence that changes in other transmitter systems are more than secondary effects is lacking.

It was decided to start an investigation of several aspects of tolerance, since understanding the mechanisms of its development might lead to new avenues to the treatment of OP intoxication. In a direct comparison of two OPs, behavioral and biochemical changes found in rats following chronic administration of DFP and soman, both organophosphates and both irreversible cholinesterase inhibitors, are reported here.

METHOD

Animals

Male Small Wistar rats were used with a starting body weight of 150–170 g. They were bred in our laboratory under SPF conditions, i.e., hysterectomy derived, bacteriologically controlled and kept under sterile conditions. All animals were experimentally naive.

General Procedure

In the first two experiments the animals were injected three times a week (Monday, Wednesday and Friday) and in the third experiment every other day. All injections were subcutaneous; the injection volume was always 1 ml/kg. All animals were tested 1 hour after injection and in most cases also 24 hours later, except during weekends. Experiment 1 contained two treatment groups (saline, soman), Experiment 2 also two treatment groups (saline, DFP) and Experiment 3 consisted of three treatment groups (saline, DFP, soman, each $n = 9$ at the start of the experiment). On the basis of preliminary dose-response studies the dose of DFP selected was invariably 600 μg/kg. For reasons mentioned under the Results section, the dose of soman in Experiment 1 was lowered from 80 μg/kg to 60 μg/kg on day 2 of testing, whereas in Experiment 3, the soman dose was lowered from 65 μg/kg to 60 μg/kg on day 8 of testing.

In all three experiments identically treated parallel groups of animals were present that were not tested behaviorally. Batches of three OP-treated and one saline-treated animals out of these groups, 1 and 24 hour after injection, were used to follow the decrease of AChE in blood, diaphragm and brain during the period of behavioral testing. At the end of the experiments five animals from each treatment group were sacrificed one hour after the last injection for the other biochemical determinations.

Behavioral Method

The shuttlebox apparatus and the procedures used were the same as those used before (55), except that administration of saline or organophosphates and testing was repeated over a period of at least four weeks. Briefly, the method was as follows. Animals that received 20 trials a day at intervals of 1 min (±20% random) were trained to avoid footshock (250 μA, constant current principle) by moving into the other compartment within 10 sec after a light stimulus was presented. It usually took 4–6 days of training to reach the criterion, which was 80% or more correct avoidance responses (CARs). The injections started the day after they had reached criterion and were repeated as indicated under the General Procedures section.

Biochemical Procedures

Tissue preparation. After anaesthesia with diethyl ether rats were decapitated. Blood (3 ml) was collected and heparin (150 IE) was added to the blood samples. Plasma was prepared by centrifugation for 20 min at $6500 \times g$. The diaphragm was dissected and a nontendinous part (about 70 mg) was homogenized with a glass-glass homogeniser in 50 mM Tris/HCl, 1 mM NaCl, 5 mM EDTA and 1% Triton X-100 [1:10 w/v; (21)]. The brain was removed and five brain regions were isolated, i.e., the cerebellum, brain stem, cortex, hippocampus and the remainder (region of nuclei thalami). The brain regions were homogenized in 0.32 M sucrose (1:10 w/v). In some experiments part of the homogenates was centrifuged at $1000 \times g$ for 10 min and the supernatants were used for the measurements. After dissecting the liver, the tissue was homogenized in 50 mM sodium phosphate buffer, pH 7.4 (1:10 w/v). Blood, plasma, homogenates and supernatants were stored at −20°C for maximal 8 weeks.

AChE activity. At the end of the experiment AChE activities were measured in the stored blood samples and homogenates. In blood samples and diaphragm homogenates the AChE activity was determined radiometrically according to Johnson and Russell (30). In homogenates of brain tissue AChE activity was determined according to a modification of the procedure described by Ellman *et al.* (20). Briefly, 2 μl of the homogenate (approximately 15 μg protein) was incubated with 0.2 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 1 mM acetylthiocholine iodide (ASCh) in 50 mM sodium phosphate buffer, pH 7.4 at room temperature. The final volume was 250 μl. The determination was performed using 96-well microtiter plates (Flow Laboratories). The extinction at 405 nm was measured before ($t = 0$) and 15 min ($t = 15$) after the addition of the substrate ASCh. The difference in extinctions at $t = 0$ and $t = 15$ was taken as a measure of enzyme activity. The enzyme activity proceeded linearly with time during the incubation. In some experiments before the addition of DTNB and ASCh the homogenates were preincubated with 1,5-bis-(4-allyldimethyl-ammoniumphenyl) penton-3-one dibromide (BW284c51) or ethopropazine (both 10^{-5} M) for 60 min at 0–4°C to distinguish between true- and pseudocholinesterase.

In the radiometric assays as well as in the spectrophotometric assays the determinations of enzyme activities were performed in duplicate. The enzyme activity was expressed as a percentage of the mean of the values found in blood or homogenates of the tissues from rats treated with saline.

De novo synthesis of AChE. De novo synthesis of AChE in 23 hours was calculated from the difference between enzyme activities found in blood and homogenates of tissues from rats sacrificed 1 hr and 24 hr after injection with soman or DFP.

Determination of muscarinic receptors. [^{3}H]Quinuclidinyl ben-

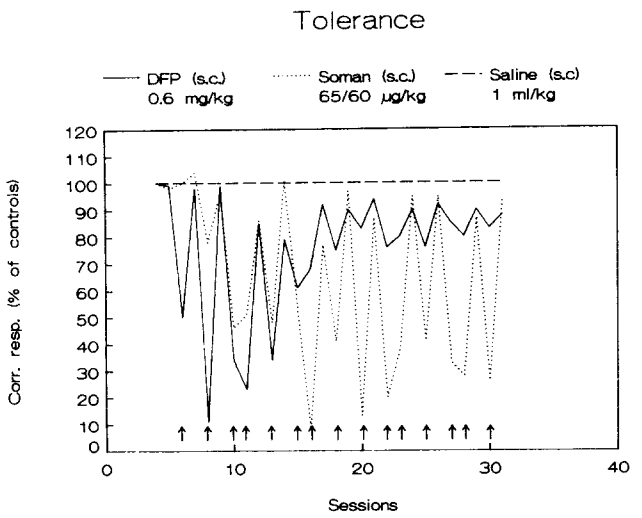

FIG. 1. The effects of repeated injections of either saline (1 ml/kg SC), DFP (600 µg/kg SC) or soman (first 8 injections: 65 µg/kg, rest: 60 µg/kg SC) on shuttlebox performance of rats. Injections (see arrows) were given every other day and tested 1 hour and 24 hours after the injections; on weekends the test 24 hours after the injections were omitted. The results were expressed as percentage of the control group (100%). Number of animals; saline (n = 9), DFP (n = 9) or soman (n = 7). Testing started on Tuesday: almost without exception all sharp downward deflections represent test results obtained one hour after injection. Twenty-four hours after the injections performance approached (saline-injected) control values.

zilate ([^{3}H]QNB) was obtained from New England Nuclear Corp., Boston, MA. The specific activity was 35–45 Ci/mmol. The binding assays were performed using a modification of the method of Yamamura and Snyder (57). Aliquots of the supernatants of the brain regions (approximately 200 µg protein) were incubated with 10 nM [^{3}H]QNB in the presence or absence of 10 µM atropine in 50 mM sodium phosphate buffer, pH 7.4. The final volume was 1 ml. After an incubation period of 60 min with gentle shaking at room temperature, the reaction mixtures were filtered through Whatman GF/C glass fibre filters. Subsequently, the filters were washed three times with 3-ml aliquots of ice-cold 50 mM sodium phosphate buffer, pH 7.4 and were counted for radioactivity [see also Van Dongen and De Lange (51)]. Every determination of binding was performed in duplicate. Specific binding was defined as the difference between binding with and without atropine. The binding was expressed as the percentage of the mean of the values found in the brain regions from rats treated with saline.

Determination of nicotinic receptors. The number of nicotinic receptors was determined according to the procedure described by Lindstrom *et al.* (35). Briefly, strips (the width was approximately 1 cm) of diaphragm, obtained immediately after decapitation with ribs attached, were incubated with 2 µg ^{125}I-αbungarotoxin (^{125}I-αBGT) for 2 hr in 2 ml Krebs solution containing 135 mM NaCl, 4.5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 10 mM glucose. Subsequently, the strips were washed overnight in several changes of the Krebs solution. Then the ribs were removed. To trim away fibers that had been damaged before incubation, about 1.5 mm of both sides of the diaphragms was removed. The muscle was then divided into five regions containing endplates (+EP) and regions lacking endplates (−EP). The −EP regions consisted of peripheral strips nerve branches. The strips were weighed and counted for radioactivity. Specific binding to the +EP regions was calculated by subtracting the nonspecific binding measured in −EP regions from the total binding measured in +EP regions.

Phosphorylphosphatase activity. The hydrolysis of DFP and soman were assayed by incubating plasma (500 and 50 µl, respectively) or liver homogenate (2 ml and 100 µl, respectively) with 11.7 µM DFP or 8.7 µM soman in 0.154 M NaCl/NaOH at pH 7.4 and 37°C. The final volume was 10 ml. During incubation the pH was kept constant using pH stat equipment (Radiometer). During 30 min of incubation 200 µl samples were taken at various time intervals, which were added to 1 ml hexane containing [U-^{2}H]-DFP or [U^2-H]-soman as internal standard. After stirring the mixture, the organic layer was removed and analysed by gas chromatography using a Carbowax column. Two µl of the samples were injected cold on-column. For DFP chromatography was performed at 150°C. The temperature of the nitrogen-phosphorus detector was 275°C. Helium was used as a carrier gas at a flow of 2.5 ml/min. Soman chromatography was performed as described by Benschop *et al.* (7). The diastereoisomers of soman were only separated on the Carbowax column. The first peak was composed of C(−)P(+)- and C(+)P(−)-isomers, and the second peak contained C(+)P(+)- and C(−)P(−)-isomers.

Amounts of DFP and soman in the 200 µl samples were calculated from the ratios of peak heights of the OPs and the internal standards using calibration curves with various concentrations of OPs with addition of known amounts of the internal standards.

First-order rate constants of hydrolysis were determined from the plot of the logarithm of the percentage remaining OP versus time, using linear least square curve fitting. For soman two biphasic hydrolysis curves were obtained, due to the different hydrolysis rates of epimers. The hydrolysis rate constants were calculated using graphical method of residuals used extensively in pharmacokinetics (17).

Protein concentrations. The protein concentrations were determined by a dye binding method (11) as modified by Bio-Rad, using bovine serum albumin as a standard.

Statistics

All relevant comparisons were analyzed with the Student's *t*-test and were tested two-tailed. The term "significant" indicates $p_2 < 0.05$.

RESULTS

Behavior

The combined behavioral results of Experiments 1 and 2 are essentially similar to those of Experiment 3 (Fig. 1), albeit that in Experiments 1 and 2 the tolerance to DFP and performance decrements after soman took longer to develop than in Experiment 3. These differences are most likely due to differences in doses and injection schedules. In Fig. 1 it can be seen that, after the start of the injections on day 6, the performance decrements observable 1 hour after DFP appear more rapidly than those 1 hour after soman. Whereas these decrements subsequently become progressively smaller when measured 1 hour after the repeated injections of DFP, the effects measured 1 hour after soman remain approximately the same. However, when measured 24 hours after their injection, performance after both inhibitors is practically normal.

The doses of soman were lowered to 60 µg/kg in both experiments. The reason was that the condition of one animal in each experiment deteriorated rather abruptly. They died the following day. This was peculiar, since none of the other animals showed signs of intoxication upon close observation. In current series of experiments the dose of soman is kept constant at 60 µg/kg from the beginning; essentially the same results are obtained.

Biochemistry

The biochemical results given are from in the third experiment

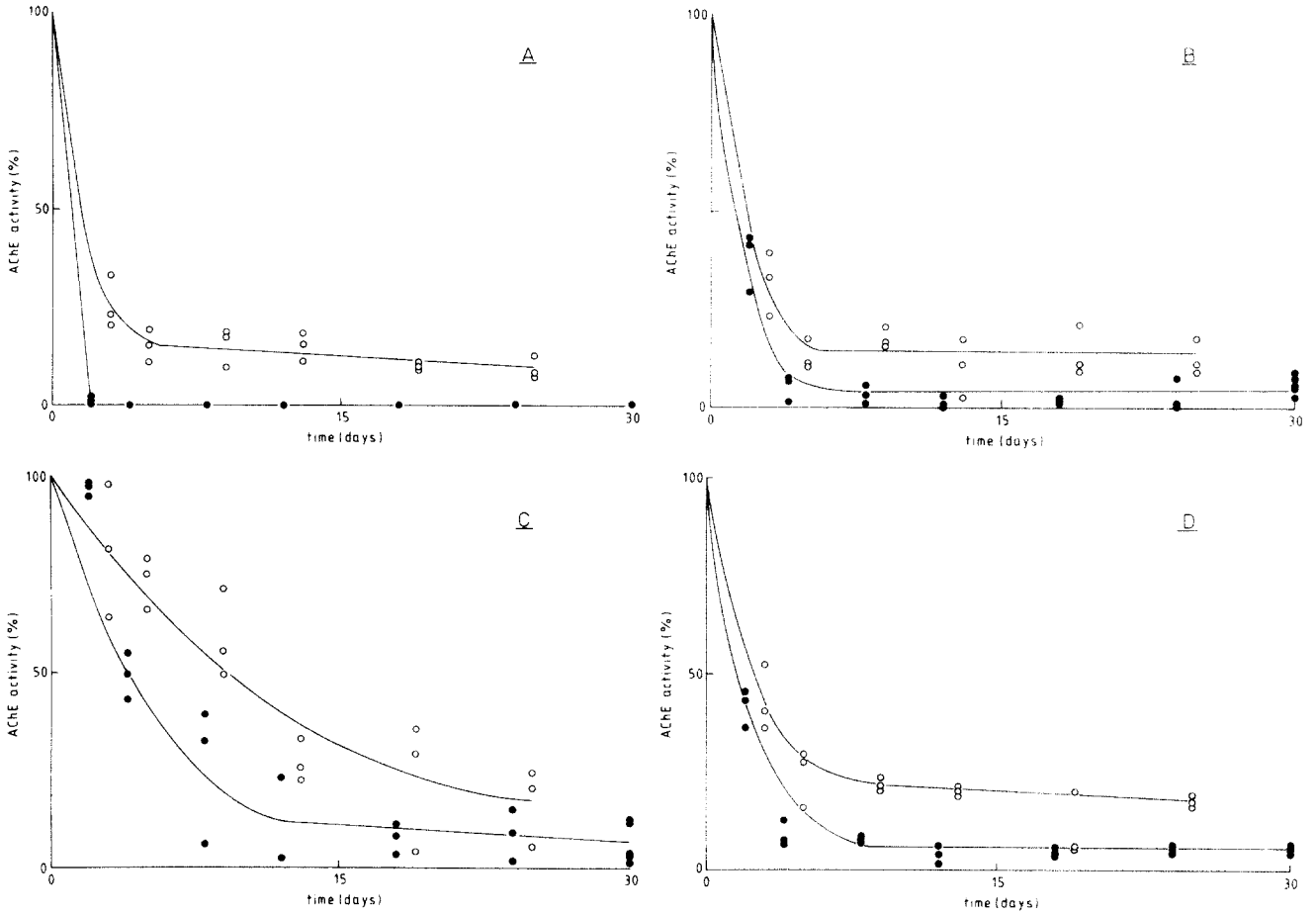

FIG. 2. AChE activity in blood (A,B) and brain stem (C,D) of rats chronically treated with soman (A,C, 65/60 μg/kg, SC; see legend Fig. 1) or DFP (B,D, 600 μg/kg, SC). The rats were treated every other day with the organophosphates starting on day 0. One hour (●) and 24 hours (○) after injection the rats were sacrificed and blood and brain stem were taken for determination of AChE activity. AChE activities are expressed as percentage of the mean value found in blood and brain stem from rats treated with saline (n = 17). Each point represents the mean of AChE activity found in a sample, determined in duplicate.

and are similar to the results found in the first two experiments. The AChE activity was determined by using a radiometric or a spectrophotometic assay. By using BW284c51 and ethopropazine, inhibitors of true- and pseudocholinesterase, respectively, it was found that at least 90% of the enzyme activity measured in blood, brain regions and diaphragm was attributable to true cholinesterase. At the end of the experiment AChE activities of the samples stored at −20°C were determined. The enzyme activity in the samples from control, saline-treated rats was not affected during the storage. As the enzyme activities in blood and tissues did not vary in the samples of the saline-treated rats taken during the experiment, the mean values of the enzyme activities in these samples were taken as the 100% value.

The AChE activity of blood and the tissues from rats, isolated 1 hr after injection of soman or DFP, decreased after successive injections until a certain minimal activity was reached. In Fig. 2 the decreases of AChE activity in blood and in homogenate of brain stem are shown. The AChE activity in the other tissues, i.e., cerebellum, cortex, hippocampus, nuclei thalami and diaphragm behaved similarly. In blood samples taken 1 hr after injection with soman, AChE activity could not be detected any more after the second injection, whereas in blood samples taken 1 hr after injection with DFP, during the whole experiment some enzyme activity could be detected (approximately 6% after the second injection). In brain regions and diaphragm isolated from rats 1 hr after treatment with DFP, AChE activities decreased more rapidly than in those isolated from rats 1 hr after treatment with soman. The minimal activity which was reached after the successive injections varied in blood and the tissues; after 16 injections the following activities were found: 3–9% in the brain regions, 11–21% in diaphragms and 0–6% in blood (Table 1). AChE activities determined after 16 injections are similar in the brain regions from soman- and DFP-treated animals. However, the enzyme activities in diaphragms from rats treated with soman are less inhibited than those from rats treated with DFP, whereas the enzyme activity in blood from rats treated with soman is more inhibited than that from rats treated with DFP.

Interestingly, all AChE activities found in blood and tissue homogenates isolated 24 hr after injection are higher than the activities of those isolated 1 hr after injection. Apparently, some new enzyme had been synthesized during the 23-hour period. The amount of newly synthesized enzyme was calculated. During the successive injections the calculated de novo synthesis of AChE were similar: the means of the values of the de novo synthesis

TABLE 1

ACHE ACTIVITY IN VARIOUS BRAIN REGIONS, DIAPHRAGM AND BLOOD FROM RATS CHRONICALLY TREATED WITH SOMAN (65/60 μg/kg, SC)* OR DFP (600 μg/kg, SC)

Tissue	AChE Activity on Day 30 (% of control, 1 hr after injection) Soman	DFP
Cerebellum	8.7 ± 2.1	7.2 ± 0.5
Brain stem	6.9 ± 2.1	5.9 ± 0.5
Cortex	5.0 ± 2.4	3.7 ± 0.9
Hippocampus	3.0 ± 0.7	3.8 ± 0.2
Nuclei thalami	6.5 ± 1.6	6.6 ± 1.0
Diaphragm	20.6 ± 3.4	11.5 ± 0.6
Blood	0.0 ± 0.0	6.0 ± 1.3

*See legend to Fig. 1.

The rats were injected every other day with the organophosphates and 1 hr after the sixteenth injection (on day 30) the rats were sacrificed and tissues and blood were taken for determination of AChE activity. AChE activity is expressed as a percentage of the mean value found in rats treated with saline (n = 17). The values given represent the means ± standard error of mean (SEM; n = 5).

calculated from five time points are given in Table 2. De novo synthesis in cortex and hippocampus were lowest (7–14%). Hardly any differences in de novo synthesis in blood and homogenates of tissues from soman and DFP treated animals were found.

The maximal binding of [^{3}H]QNB in brain regions was determined by incubating brain tissue with 10 nM [^{3}H]QNB. In preliminary experiments in which the concentration of [^{3}H]QNB was varied from 0.06 to 10 nM, it was found that the chosen concentration of 10 nM allowed the determination of the maximal binding in the 200 μg samples.

TABLE 2

DE NOVO SYNTHESIS OF AChE ACTIVITY IN VARIOUS BRAIN REGIONS, DIAPHRAGM AND BLOOD FROM RATS CHRONICALLY TREATED WITH SOMAN (65/60 μg/kg, SC)* OR DFP (600 μg/kg, SC)

Tissue	De Novo Synthesis of AChE (% of control per 23 hr) Soman	DFP
Cerebellum	19.9 ± 2.3	16.9 ± 0.4
Brain stem	19.2 ± 2.7	14.2 ± 0.5
Cortex	11.3 ± 1.9	7.5 ± 0.4
Hippocampus	13.8 ± 2.1	6.8 ± 0.3
Nuclei thalami	18.1 ± 2.5	12.6 ± 0.2
Diaphragm	21.7 ± 3.0	20.9 ± 1.0
Blood	9.5 ± 0.2	10.0 ± 1.1

*See legend to Fig. 1.

One hour and 24 hours after the second, third, fifth, seventh, tenth and thirteenth injection the rats were sacrificed and the tissues and blood were taken for determination of AChE activity. At each time point three rats of the soman- and DFP-treated groups were taken. AChE activity was expressed as a percentage of the mean value found in rats treated with saline (n = 17). De novo synthesis in 23 hours was calculated from the differences in enzyme activities in the samples obtained from rats 1 and 24 hours after injection. The values for the different time points did not differ significantly and were pooled per (part of each) organ. The values given represent the means ± SEM.

TABLE 3

MAXIMAL BINDING OF [^{3}H]QNB IN VARIOUS BRAIN REGIONS FROM RATS INJECTED WITH SOMAN (65/60 μg/kg, SC)[1] OR DFP (600 μg/kg, SC)

Tissue	Maximal Binding of [^{3}H]QNB (% of control) Soman	DFP
Cerebellum	80 ± 10*	60 ± 10*
Brain stem	68 ± 5*	78 ± 2*
Cortex	53 ± 3*	59 ± 2*
Hippocampus	52 ± 1*	62 ± 2*
Nuclei thalami	73 ± 2*	76 ± 6*

[1]See legend to Fig. 1.

The rats were injected every other day. One hour after the sixteenth injection the rats were decapitated and the brain regions were isolated. The maximal binding of [^{3}H]QNB was determined by incubating the supernatant of the brain homogenates obtained after centrifugation of the homogenates at 1000 × g, with an excess of [^{3}H]QNB. Maximal binding is expressed as percentage of the mean value found in rats treated with saline (n = 5). The given values represent means ± SEM, n = 5.

*Significantly different from control (p_2<0.05).

For the maximal binding of [^{3}H]QNB the following values were found: in cerebellum: 0.16 ± 0.01, in brain stem: 0.63 ± 0.08, in cortex: 1.9 ± 0.2, in hippocampus: 1.8 ± 0.2, and in nuclei thalami: 1.0 ± 0.1 pmol/mg protein. After chronic treatment with soman or DFP, the maximal binding of [^{3}H]QNB was significantly (p_2<0.05) decreased in the brain regions (Table 3). The decreases were highest in cortex and hippocampus (38–48%). In brain stem, cortex and hippocampus from soman-treated rats, less [^{3}H]QNB was bound than in those from DFP-treated rats.

In diaphragms from saline-treated rats the maximal binding of ^{125}I-αBGT was found to be 1.9 ± 0.2 pmol/mg tissue. This value was not significantly (p_2>0.05) affected by the chronic injections of soman or DFP (Table 4). In addition, in the first two experiments it was found that chronic injections of soman or DFP did also not affect the maximal binding of ^{125}I-αBGT in extensor

TABLE 4

MAXIMAL BINDING OF ^{125}I-αBGT IN DIAPHRAGMS FROM RATS CHRONICALLY TREATED WITH SOMAN (65/60 μg/kg, SC)* OR DFP (600 μg/kg, SC)

Injections	Maximal Binding of ^{125}I-αBGT (fmol/mg tissue)
Saline	1.9 ± 0.2
Soman	2.0 ± 0.1
DFP	2.5 ± 0.2

*See legend to Fig. 1.

The rats were treated every other day. One hour after the sixteenth injection the rats were decapitated and the diaphragms were isolated. Subsequently, the diaphragms were incubated with an excess of ^{125}I-αBGT. The given values represent means ± SEM, n = 5. The differences are not significant (p_2>0.05).

TABLE 5

THE EFFECT OF CHRONIC TREATMENT WITH DFP ON PHOSPHORYLPHOSPHATASE ACTIVITY IN PLASMA AND LIVER HOMOGENATE

Injections	Rate Constants of DFP Hydrolysis* (10^{-3}·min^{-1}) Plasma	Liver Homogenate
Saline	17 ± 1	26 ± 3
DFP	12 ± 1	17 ± 3

*Spontaneous hydrolysis rate constant for DFP determined in 0.154 M NaCl, pH 7.4 at 37°C was (4 ± 2)·10^{-3}·min^{-1}.

Rats were injected every other day with saline or DFP (600 μg/kg, SC). One hour after the sixteenth injection they were sacrificed and the rate constants of hydrolysis of DFP were determined in plasma and liver homogenate. The results are expressed as means ± SEM of 5 animals per treatment group.

digitorum longus muscle.

Concerning the phosphorylphosphatase activity, the rate constants of DFP hydrolysis in plasma and liver homogenate from saline-treated animals were 0.017 and 0.026 min^{-1}, respectively. These values were not increased—even insignificantly decreased (see Table 5)—in plasma and liver homogenate from chronically DFP-treated animals. Thus, the repeated DFP injections did not significantly affect the DFP-ase activity.

The results of the somanase activities are shown in Table 6. In plasma three hydrolysis rate constants were calculated, whereas in liver homogenate four reaction rate constants were calculated. The first one or two hydrolysis rate constant (k1 and k2) originated from the hydrolysis of C(−)P(+)- and C(+)P(−)-isomers. The values found in plasma and liver homogenate from animals treated with saline (plasma: 0.009 min^{-1}; liver homogenate 0.227 and 0.006 min^{-1}) hardly differed from the values found in plasma from those treated with soman (plasma: 0.005 min^{-1}; liver homogenate 0.155 and 0.006 min^{-1}). The third (k3) and fourth (k4) hydrolysis rate constants originated from the hydrolysis of C(+)P(+) and C(−)P(−)-isomers. In plasma and liver homogenate from saline-treated animals k3 is much higher than k4 (in plasma: 0.067 versus 0.021 min^{-1}; in liver homogenate (0.147 versus 0.005 min^{-1}). The values do not differ significantly (p_2>0.05) from those found in plasma and liver homogenate from rats treated with soman.

TABLE 6

THE EFFECT OF CHRONIC TREATMENT WITH SOMAN ON PHOSPHORYLPHOSPHATASE ACTIVITY IN PLASMA AND LIVER HOMOGENATE

Injections		Rate Constants of Soman Hydrolysis* (10^{-3}·min^{-1}) Plasma	Liver Homogenate
Saline	k1†	—	227 ± 63
	k2	8 ± 2	6 ± 1
	k3	67 ± 26	147 ± 27
	k4	21 ± 29	5 ± 1
Soman	k1	—	155 ± 17
	k2	6 ± 1	6 ± 1
	k3	40 ± 7	153 ± 21
	k4	9 ± 5	5 ± 1

*Spontaneous hydrolysis rate constants for soman determined in 0.154 M NaCl, pH 7.4 at 37°C was (2 ± 1)·10^{-3}·min^{-1}. No difference was found between the hydrolysis of the diastereoisomers and epimers.

†Hydrolysis rate constants k1 and k2 origined from the hydrolysis of both the C(+)P(−)- and C(−)P(+)-isomers (first peak after GC analysis), and hydrolysis rate constants k3 and k4 originated from the hydrolysis of C(+)P(+)- and C(−)P(−)-isomers (second peak after GC analysis).

Rats were injected every other day with saline or soman (65/60 μg/kg, SC). One hour after the sixteenth injection they were sacrificed and the rate constants of hydrolysis of soman were determined in plasma and liver homogenate. The results are expressed as means ± SEM of 5 animals per treatment group.

DISCUSSION

In the present experiments the effects of repeated injections of saline, DFP or soman on shuttlebox performance and several biochemical parameters were compared.

Behaviorally, the most striking phenomenon was that, when tested 1 hour after injection, tolerance to DFP developed, whereas after soman this was not the case. Yet performance tested 24 hours after injection was practically normal with both organophosphorus cholinesterase inhibitors. Previously, Haggerty *et al.* (27) and Raffaele *et al.* (45) have reported that a single exposure to sublethal doses of soman may cause long-lasting changes in behavior. However, the tests used by these authors are different from those applied here and may represent effects on different functional domains of the CNS. Since behavioral tolerance of the type reported here has been shown to develop not only after DFP, but also after several other organophosphorus and carbamate cholinesterase inhibitors (46), it would seem that soman, and not DFP, is the exception. Since experiments with a series of serine-esterases indicated that soman is a far more specific inhibitor of AChE than DFP (F. Berends, personal communication), the question can be raised whether AChE inhibition is the only initiating factor in the development of tolerance and if so, whether in the case of soman other factors play a role.

To be able to answer this question one has to investigate first whether the differences in the development of behavioral tolerance between DFP and soman cannot be attributed to differences in effects on carboxylesterases, phosphorylphosphatases, AChE or its isoenzymes, nerve terminals, synapses, etc., which are mentioned in the introduction. The present paper describes the first results of experiments in which the effects of DFP and soman are directly compared, in an attempt to answer some of these questions.

At the start of the injection period the inhibition of AChE activities in brain and diaphragm progressed more rapidly after injecting DFP than after soman, a phenomenon that may be due to differences in lipophilicity, degradation or distribution. In this early stage the decrease of AChE activity in brain correlated better than that in blood with the decreases in performance, measured one hour after the injections. At a later stage of the experiment AChE inhibition was different in different organs such as blood, various brain regions and diaphragms. However, both organophosphates reduced the AChE activity to approximately the same levels. The cortex and hippocampus appear to be brain structures that are most affected by DFP and soman, a phenomenon that is in agreement with earlier findings (6, 23, 25, 47, 50).

Since AChE is irreversibly inhibited by DFP as well as by soman, the increase of enzymatic activity between 1 and 24 hours after injection is almost certainly due to the synthesis of new enzyme. The return of AChE in blood, brain regions and diaphragm in the present experiments is faster than those found by Austin and James (5), Grubic *et al.* (24) and Michalek *et al.* (39), but is similar to that found by Fernandez and Stiles (21), Harris *et*

al. (28), Stitcher *et al.* (48) and Yaksh *et al.* (56). Grubic *et al.* (24) have found that the recovery of AChE activity in brain and diaphragm was biphasic in the course of time and Walker and Wilson (54) have reported that high ACh levels may enhance the recovery of AChE.

In the present experiments the recovery of AChE activity was slightly faster after soman than after DFP. This agrees with the findings of Stitcher *et al.* (48). De novo synthesis of AChE in cortex and hippocampus proceeded more slowly than in other brain regions. Similar results have been obtained by Glow *et al.* (23). Gupta *et al.* (26) have found that the uptake of [^{14}C]-valine into protein, a measure of the protein synthesis, is enhanced after chronic DFP treatment. These investigators (25) have suggested that tolerance to DFP toxicity may be due to the recovery of carboxylesterases, AChE and pseudocholinesterase. However, we and also others (39) have not found an enhancement of de novo synthesis of AChE during the successive injections of DFP or soman. It might be that the increase of other OP-binding proteins are involved in development of tolerance.

In this study the effect of chronic treatment of OPs on the binding of the muscarinic antagonist [^{3}H]QNB was investigated. Since many investigators (6, 13, 47, 50, 53) have not found any change in affinity of the [^{3}H]QNB binding to brains from rats treated chronically with OPs, only the effect on the maximal binding of [^{3}H]QNB was investigated. After chronic treatment with DFP or soman, the maximal binding of [^{3}H]QNB, which appears to be a measure of the number of muscarinic receptors found in cortex and hippocampus was affected more than that found in the other brain regions. Other investigators (6, 13, 47, 50) have also found that the reduction of the number of muscarinic receptors is most pronounced in these two regions. A reason for the different responses in the different CNS regions might be the substantially larger duration of the ACh elevation in cortex and hippocampus, since AChE activities were lowest (Table 1).

The difference in reduction of [^{3}H]QNB binding in cortex and hippocampus observed after soman and DFP treatment (Table 3) could not explain the difference in the behavioral effects of both OPs: if the reduction of the number of muscarinic receptors could influence the development of tolerance, one would expect an even larger reduction of [^{3}H]QNB after DFP.

The observations that after both DFP and soman treatment a decrease in number of muscarinic receptors in CNS was found, whereas only after DFP tolerance developed, are very remarkable. Several investigators (46) have found that after repeated injections with a cholinesterase inhibitor the number of muscarinic receptors decreased. They have suggested that this reduction would play a role in the development of tolerance. However, our results suggest that besides the reduction of the number of muscarinic receptors, also other factors, probably presynaptic ones, are important for the development of behavioral tolerance.

In striated muscles, i.e., diaphragm and extensor digitorum longus muscle, it was found that chronic treatment with DFP or soman did not reduce the maximal binding of ^{125}I-αBGT. In the past, only Chang *et al.* (12) and Gupta *et al.* (25) have shown that chronic treatment with an anticholinesterase compound may reduce the number of nicotinic receptors in diaphragm. In the CNS, however, reduction of the binding of [^{3}H]nicotine or [^{3}H]ACh has been observed by several investigators (46).

After the determination of somanase activity, more than one reaction rate constants were obtained (see Table 6). Other investigators have found that the hydrolysis of the C(±)P(+)-isomers proceeds much more rapidly than that of the C(±)P(−)-isomers (17,19). From this, it can be concluded that k1 is attributed mostly to the hydrolysis of C(−)P(+)-isomer and k2 is attributed to the hydrolysis of both, C(+)P(−)- and C(−)P(+)-isomers. Furthermore, k3 can be probably attributed to the hydrolysis of C(+)P(+)-isomers and k4 to that of the hydrolysis of C(+)P(+)- and C(−)P(−)-isomers.

Phosphorylphosphatase activity was tested in vitro in two organs known for their capacity to hydrolyse DFP and soman, viz. blood and liver. The activity found in the in vitro assay resembles probably the activity present in vivo. We did not find any effect of the repeated DFP and soman injections on DFP-ase- and somanase activity, which agrees with observations from others (46).

In summary, in this study it was found that after repeated injections with DFP behavioral tolerance had developed, whereas after repeated injections with soman this had not been the case. This difference could not be attributed to differences in inhibition of de novo synthesis of AChE in blood, various brain regions and diaphragm; to differences in reduction of the number of muscarinic receptors in various brain regions; to differences in the number of nicotinic receptors in striated muscles; or to differences in phosphorylphosphatase activity in blood and liver. Yet, it is possible that some of these biochemical parameters are involved in development of behavioral tolerance for both OPs, but that soman has other actions which prevent the development of tolerance for this compound. One of these actions could be its interaction with the postsynaptic membrane (2). However, this is not likely, since other OPs, for instance DFP, also interact with the postsynaptic membrane (3). The development of tolerance for DFP is probably caused by other factors, for example presynaptic alterations, increased de novo synthesis of OP-binding proteins, or interactions with other neurochemical systems, which will be further investigated.

ACKNOWLEDGEMENTS

The excellent technical assistance of Ingrid Philippens, Bert Fokkema, Raymond Vanwersch, Ton van der Laaken and Jan de Lange is gratefully acknowledged.

REFERENCES

1. Aas, P.; Fonnum, F. Presynaptic inhibition of acetylcholine release. Acta Physiol. Scand. 127:335–342; 1986.
2. Albuquerque, E. X.; Akaike, A.; Shaw, K. P.; Rickett, D. I. The interaction of anticholinesterase agents with the acetylcholine receptor-ionic channel complex. Fundam. Appl. Toxicol. 4:S27–S33; 1984.
3. Albuquerque, E. X.; Deshpande, S. S.; Kawabuchi, M.; Aracava, Y.; Idriss, M.; Rickett, D. L.; Boyne, A. F. Multiple actions of anticholinesterase agents on chemosensitive synapses: molecular basis for prophylaxis and treatment of organophosphate poisoning. Fundam. Appl. Toxicol. 5:S182–S203; 1985.
4. Ariens, A. T.; Meeter, E.; Wolthuis, O. L.; van Benthem, R. M. J. Reversible necrosis at the end-plate region in striated muscle of the rat poisoned with cholinesterase inhibitors. Experientia 25:57–59; 1969.
5. Austin, L.; James, K. A. C. Rates of regeneration of acetylcholinesterase in rat brain; subcellular fractions following DFP-inhibition. J. Neurochem. 17:705–707; 1970.
6. Bartholomew, P. M.; Gianutsos, G.; Cohen, S. D. Differential ChE inhibition and muscarinic receptor changes in CD-1 mice made tolerant to malathion. Toxicol. Appl. Pharmacol. 81:147–155; 1985.
7. Benschop, H. P.; Berends, F.; de Jong, L. P. A. GLC-analysis and pharmacokinetics of the four stereoisomers of soman. Fundam. Appl. Toxicol. 1:177–182; 1981.
8. Bignami, G.; Rosic, N.; Michalek, H.; Milosevic, M.; Gatti, G. L. Behavioral toxicity of anticholinesterase agents: methodological, neurochemical and neurophysiological aspects. In: Weiss, B.; Laties, V. G., eds. Behavioral toxicology. New York: Plenum Press; 1975: 155–215.

9. Bignami, G. Methodological problems in the analysis of behavioral tolerance in toxicology. Neurobehav. Toxicol. 1(Suppl. 1):179–186; 1979.
10. Bignami, G.; Guardini, V.; Scorrano, M. Behaviorally augmented versus other components in organophosphate tolerance: the role of reinforcement and response factors. Fundam. Appl. Toxicol. 5: S213–S224; 1985.
11. Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
12. Chang, C. C.; Chen, T. F.; Chang, S. T. Influence of chronic neostigmine treatment on the number of acetylcholine receptors and the release of acetylcholine from rat diaphragm. J. Physiol. 230: 613–618; 1973.
13. Churchill, L.; Pazdernik, T. L.; Jackson, J. T.; Nelson, S. R.; Samson, F. E.; McDonough, J. H. Topographical distribution of decrements and recovery in muscarinic receptors from rat brains repeatedly exposed to sublethal doses of soman. J. Neurosci. 4: 2069–2079; 1984.
14. Costa, L. G.; Schwab, B. W.; Murphy, S. D. Tolerance to anticholinesterase compounds in mammals. Toxicology 25:79–97; 1982.
15. Costa, L. G.; Murphy, S. D. ^{3}H-nicotine binding in rat brain; alteration after chronic acetylcholinesterase inhibition. J. Pharmacol. Exp. Ther. 226:392–397; 1983.
16. Davis, G. A.; Agranoff, B. W. Metabolic behavior of isoenzymes of acetylcholinesterase. Nature 220:277–280; 1968.
17. De Bisschop, H. C. J. V. Biodegradation of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman). Thesis, 1986.
18. Dettbarn, W. D. Pesticide-induced muscle necrosis; mechanisms and prevention. Fundam. Appl. Toxicol. 4:S18–S26; 1984.
19. De Jong, L. P. A.; van Dijk, C.; Benschop, H. P. Hydrolysis of the four stereoisomers of soman catalyzed by liver homogenate and plasma from rat, guinea pig and marmoset, and by human plasma. Biochem. Pharmacol. 37:2939–2948; 1988.
20. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95; 1961.
21. Fernandez, H. L.; Stiles, J. R. Intra- versus extra-cellular recovery of 16S AChE following organophosphate inactivation in the rat. Neurosci. Lett. 49:117–122; 1984.
22. Fonnum, F.; Guttormsen, D. M. Changes in acetylcholine content of rat brain by toxic doses of DFP. Experientia 25:505–506; 1969.
23. Glow, P. H.; Rose, S.; Richardson, A. The effect of acute and chronic treatment with DFP on ChE activities of some tissues of the rat. Aust. J. Exp. Biol. Med. Sci. 44:73–86; 1966.
24. Grubic, Z.; Sketelj, J.; Klinar, B.; Brzin, M. Recovery of acetylcholinesterase in the diaphragm, brain and plasma of the rat after irreversible inhibition by soman: a study of cytochemical localization and molecular forms of the enzyme in the motor end-plate. J. Neurochem. 37:909–916; 1981.
25. Gupta, R. C.; Patterson, G. T.; Dettbarn, W. D. Mechanisms involved in the development of tolerance to DFP toxicity. Fundam. Appl. Toxicol. 5:S17–S28; 1985.
26. Gupta, R. C.; Dettbarn, W. D. Role of uptake of [^{14}C]valine into protein in the development of tolerance to DFP toxicity. Toxicol. Appl. Pharmacol. 84:551–560; 1986.
27. Haggerty, G. C.; Kurtz, P. J.; Amstrong, R. D. Duration and intensity of behavioral change after sublethal exposure to soman in rats. Neurobehav. Toxicol. Teratol. 8:695–702; 1986.
28. Harris, L. W.; Yamamura, H. I.; Fleisher, J. H. De novo synthesis of acetylcholinesterase in guinea pig retina after inhibition by pinacolyl methylphosphonofluoridate. Biochem. Pharmacol. 20:2927–2930; 1971.
29. Hudson, C. S.; Foster, R. E.; Kangh, M. W. Neuromuscular toxicity of pyridostigmine-bromide in the diaphragm, extensor digitorum longus and soleus muscles of the rat. Fundam. Appl. Toxicol. 5:S260–S269; 1985.
30. Johnson, C. D.; Russell, R. L. A rapid simple radiometric assay for cholinesterase, suitable for multiple determinations. Anal. Biochem. 64:229–238; 1975.
31. Kirby, A. W.; Harding, T. H.; Wiley, R. W. Recovery of the visual evoked response in the cat following administration of DFP, an irreversible cholinesterase inhibitor. Life Sci. 41:2669–2677; 1987.
32. Lemercier, G.; Carpentier, P.; Sentenac-Roumanou, H.; Morelis, P. Histological and histochemical changes in the central nervous system of the rat poisoned by an irreversible anticholinesterase organophosphorus compound. Acta Neuropathol. 61:123–129; 1983.
33. Lim, D. K.; Hoskins, B.; Ho, I. K. Evidence for the involvement of presynaptic cholinergic functions in tolerance to DFP. Toxicol. Appl. Pharmacol. 90:465–476; 1987.
34. Lim, D. K.; Porter, A. B.; Hoskins, B.; Ho, I. K. Changes in ACh levels in the during subacute administration of DFP. Toxicol. Appl. Pharmacol. 90:477–489; 1987.
35. Lindstrom, J. M.; Lennon, V. A.; Seybold, M. E.; Whittingham, S. Experimental autoimmune myasthenia gravis and myasthenia gravis: biochemical and immunochemical aspects. Ann. NY Acad. Sci. 274:254–274; 1976.
36. Mayer, O.; Michalek, H. Effects of DFP and obidoxime on brain acetylcholine levels and on brain and peripheral cholinesterases. Biochem. Pharmacol. 20:3029–3037; 1971.
37. Meeter, E. Desensitization of the end-plate membrane following cholinesterase inhibition, an adjustment to a new working situation. Acta Physiol. Pharmacol. Neerl. 15:243–258; 1969.
38. Meeter, E.; Wolthuis, O. L. The spontaneous recovery of respiration and neuromuscular transmission in the rat after anticholinesterase poisoning. Eur. J. Pharmacol. 2:377–386; 1968.
39. Michalek, H.; Menguz, A.; Bisso, G. M. Mechanisms of recovery of brain acetylcholinesterase in rats during chronic intoxication by isofluorophate. Arch. Toxicol. Suppl. 5:116–119; 1982.
40. Michalek, H.; Pintor, A.; Fortuna, S.; Bisso, G. M. Effects of DFP on brain cholinergic systems of rats at early developmental stages. Fundam. Appl. Toxicol. 5:S204–S212; 1985.
41. Molenaar, P. C.; Polak, R. L. Stimulation of atropine on acetylcholine release and synthesis in cortical slices from rat brain. Br. J. Pharmacol. 40:406–417; 1970.
42. Molenaar, P. C.; Polak, R. L. Inhibition of acetylcholine release by activation of acetylcholine receptors. Prog. Pharmacol. 3/4:39–44; 1980.
43. Overstreet, D. H.; Yamamura, H. I. Receptor alterations and drug tolerance. Life Sci. 25:1865–1878; 1979.
44. Petras, J. M. Soman neurotoxicity. Fundam. Appl. Toxicol. 1:242; 1981.
45. Raffaele, K.; Hughey, D.; Wenk, G.; Olton, D.; Modrow, H.; McDonough, J. Long-term behavioral changes in rats following organophosphonate exposure. Pharmacol. Biochem. Behav. 27:407–412; 1987.
46. Russell, R. W.; Overstreet, D. H. Mechanisms underlying sensitivity to organophophorous anticholinesterase compounds. Prog. Neurobiol. 28:97–129; 1987.
47. Schiller, G. D. Reduced binding of ^{3}H-quinuclidinyl benzilate associated with chronically low acetylcholinesterase activity. Life Sci. 24:1159–1164; 1979.
48. Stitcher, D. L.; Harris, L. W.; Moore, R. D.; Heyl, W. C. Synthesis of cholinesterase following poisoning with anticholinesterase: effects of theophylline and N^{6}, O^{2}-dibutyryl adenosine 3,5-monophosphate on synthesis and survival. Toxicol. Appl. Pharmacol. 41:79–90; 1977.
49. Thomsen, R. H.; Wilson, D. F. Chronic effects of paraoxon on transmitter release and the synaptic contribution to tolerance. J. Pharmacol. Exp. Ther. 237:689–694; 1986.
50. Upchurch, M.; Wehne, J. M. Effects of chronic DFP treatment on spatial learning in mice. Pharmacol. Biochem. Behav. 27:143–151; 1987.
51. Van Dongen, C. J.; De Lange, J. Influence of pinacolyl dimethylphosphonate on soman storage in rats. J. Pharm. Pharmacol. 39:609–613; 1987.
52. Van Dongen, C. J.; Valkenburg, P. W.; van Helden, H. P. M. Contribution of de novo synthesis of acetylcholinesterase to spontaneous recovery of neuromuscular transmission following soman intoxication. Eur. J. Pharmacol. 149:381–384; 1988.
53. Volpe, L. S.; Biagioni, T. M.; Marquis, J. K. In vitro modulation of bovine caudate muscarinic receptor number by organophosphates and carbamates. Toxicol. Appl. Pharmacol. 78:226–234; 1985.
54. Walker, C. R.; Wilson, B. W. Regulation of acetylcholinesterase in muscle cultures after treatment with DFP: ribonucleic acid and protein synthesis. Neuroscience 1:509–513; 1976.
55. Wolthuis, O. L.; Vanwersch, R. A. P. Behavioral changes in the rat

after low doses of cholinesterase inhibitors. Fundam. Appl. Toxicol. 4:S195–S208; 1984.
56. Yaksh, T. C.; Filbert, M. G.; Harris, L. W.; Yamamura, H. I. Acetylcholinesterase turnover in brain, cerebrospinal fluid and plasma. Neurochemistry 25:853–860; 1975.
57. Yamamura, H. I.; Snyder, S. H. Muscarinic cholinergic binding in rat brain. Proc. Natl. Acad. Sci. USA 71:1725–1729; 1974.