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Effects of Low Doses of Cholinesterase Inhibitors on Behavioral Performance of Robot-Tested Marmosets

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WOLTHUIS, O. L., B. GROEN, R. W. BUSKER AND H. P. M. VAN HELDEN. *Effects of low doses of cholinesterase inhibitors on behavioral performance of robot-tested marmosets.* PHARMACOL BIOCHEM BEHAV 51(2/3) 443–456, 1995.—To investigate at which dose levels undesirable effects started, behavioural performance and several physiological parameters were measured in marmosets (*Callithrix jacchus*) after soman (1.75 and 3.5 μg/kg), sarin (3 and 6 μg/kg), physostigmine (10 and 20 μg/kg), and pyridostigmine (200 and 400 μg/kg). Effects on performance were investigated with a discrete-trial, two-choice visual discrimination task and a hand–eye coordination task. The former test appeared more sensitive to disruption than the hand–eye coordination task. "Motor speed" was not disrupted by any of the four compounds. However, "choice time" as well as "no attempts" increased and were clearly more disturbed by soman and physostigmine than by sarin and pyridostigmine. All effects had disappeared after 24 h. Except for a small effect of sarin on heart rate and blood pressure, none of the cholinesterase (ChE) inhibitors affected a number of physiological parameters at behavioural effective doses that caused a profound ChE inhibition in blood. Take together, these results strongly suggest that both soman and physostigmine may interfere with higher CNS functions at low dose levels. These effects may go undetected because physical signs are absent.

Marmosets Performance Organophosphates Carbamates Cholinesterase Pretreatment

OXIME therapy appears not equally effective against intoxication with different nerve agents. Notably, oxime therapy after intoxication by soman leaves much to be desired. As a result, the search for a sign-free pretreatment with reversible ChE inhibitors, such as the carbamates pyridostigmine and physostigmine, has been the subject of many studies during the past decades (12,16,37). Several NATO countries have now adopted a pretreatment with pyridostigmine, to be taken whenever there is a threat that nerve agents will be used, such as has been the case in the Iraq war. It will be clear that any pretreatment, which might have to be taken during several weeks, should be devoid of side effects.

Although the details of the kinetics are not fully understood, the rationale is to reversibly inhibit 30–40% of the available AChE by a carbamate and thereby protect this AChE fraction from inhibiton by the nerve agent. Upon irreversible inhibiton of the remaining AChE by the nerve agent, the (increased?) decarbamoylation creates a situation in which apparently a few percents of reactivated AChE remain available, which appear sufficient for survival, provided therapy with atropine and an oxime is rapidly administered. Although a sign-free dose schedule with pyridostigmine—also in man—is well established, little is known about the dose levels of pyridostigmine, and paticularly those of physostigmine that start to cause undesirable side effects. In contrast to pyridostigmine, which carries a quaternary *N*-atom, physostigmine contains a tertiary *N*-atom and will, therefore, more easily pass the blood–brain barrier.

Similarly, little is known about the dose levels of sarin and soman that start to cause undesirable effects. Because especially physostigmine, and also soman (18), will easily pass the blood–brain barrier, effects on CNS function are to be expected; hence, the emphasis in the present study on behavioural performance.

In an earlier study with rats (34), it was found that at dose levels of 30% of the LD_{50} neither soman, sarin, tetraethyl pyrophosphate (TEPP), physostigmine, nor pyridostigmine had an effect on simple motor tasks such as maximum running

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speed, or on a number of stepping parameters when the animal walked in a hollow, rotating wheel. In contrast, at lower dose levels, soman, physostigmine, and, to a small extent, pyridostigmine dose-dependently disrupted performance of a recently acquired shuttlebox task, an acquired motor coordination task, and open field behaviour. Sarin and TEPP were ineffective, even at doses of 30% of the LD_{50}. The dose levels at which these inhibitors caused overt symptoms were much higher. It was concluded that after exposure to low doses of cholinesterase inhibitors, different types of behaviour, particularly those involving higher central nervous system (CNS) functions, may be disrupted at dose levels that do not cause physical incapacitation.

The absence of detectable effects after a dose of 30% of the LD_{50} of sarin and TEPP, as well as the higher doses of pyridostigmine than physostigmine needed to obtain an effect, did not come as a surprise because these agents have predominantly, but not exclusively, peripheral effects (18,32). It was expected that soman, which acts preferentially on the CNS, and physostigmine, which easily passes the blood-brain barrier, cause behavioural disruptive effects at low dose levels. However, it was surprising that pyridostigmine also caused behavioural decrements at dose levels below 30% of its LD_{50} value, albeit at a dose that was 2.5–3 times higher than that of physostigmine on a molar basis. This suggests that more pyridostigmine reaches the brain than was hitherto assumed. In turn, this may explain why pretreatment with this carbamate, which is considered to act peripherally, protects against the lethal—although not against the incapacitating—effects of a preferentially centrally acting inhibitor like soman.

To increase the likelihood that these findings may be extrapolated to man, it is imperative that they are substantiated by similar findings in at least one other species, preferably one that is closer to man. For this, the marmoset was chosen for a number of reasons. First, the marmoset is considered ideal for behavioural laboratory studies (13,28), it has become a much-used experimental animal in recent years (1,3,4,11,15), and its behaviour is sensitive to cholinergic manipulation (4,5,9,22,23). Directly relevant for the present investigation are the results of the latter authors (9), who found that 35–55% of the LD_{50} of sarin disrupted a visually guided reaching task, an effect that could not be explained by indirect effects on motivation or gross mobility. In essence, their elegant and simple technique formed the basis for the hand-eye coordination task used in the present experiments. Second, marmosets are sensitive to carbamate prophylaxis (8), similar to rhesus monkeys and considerably more than rodents. Third, in vivo and in vitro studies showed that the marmoset responded to oxime therapy differently from mice, rats, guinea pigs, and dogs, but in vitro their neuromuscular preparations responded in a way similar to those of rhesus and man (27,30,33). Other reasons, such as the low levels of scavenging blood carboxylesterases (similar to man, different from rodents), the reproduction rate of marmosets in captivity, as well as the size and manageability of the animal, led to the choice of the marmoset as the experimental animal for the present study.

On the basis of the above-mentioned earlier investigations in rats (34), a number of working hypotheses were formulated: 1) soman and physostigmine, due to their actions on the CNS, would cause a disruption of performance at a lower dose (% of LD_{50}) than after sarin or pyridostigmine, respectively; 2) the effects detectable 30 min after injection, even after the irreversible organophosphate ChE inhibitors, would have disappeared 24 h later; and 3) at those dose levels that begin to disrupt behaviour, the blood ChE will be only moderately inhibited and neuromuscular transmission will be hardly affected.

The objective of this study is to contribute to an assessment of the risk that exposure to ChE inhibitors may cause subtle disruptions of "higher" CNS functions that may go unnoticed because physical signs are absent. Particularly suspect are compounds such as physostigmine and soman, which easily penetrate the blood-brain barrier and may act on the CNS at low dose levels. Extrapolated to man, it may mean that such subtle disruptions of CNS functions affect decision making, logic, memory, etc., which are all vital for complex operations.

Before this study was started, approval was obtained from The Animal Care Committee of TNO Health Research Organization to use this type and number of animals.

METHOD

Animals

For testing at the relevant dose levels, as well as for the pharmacological experiments in anaesthetized animals, 120 marmosets (*Callithrix jacchus*) were minimally required. Performance tests consisted of 2 (tests) × 4 (compounds) × 2 (doses) × 6 (n/group) + 12 saline controls (108 marmosets) and pharmacological tests consisted of 4 (compounds) × 3 (animals per treatment) (12 marmosets).

Animal Histories

Except for five animals used in the visual discrimination test (see below), all animals were experimentally naive. These five animals had been trained on hand-eye coordination and had been injected once, at least 2 months earlier, with a low dose of physostigmine or pyridostigmine (both highly reversible ChE inhibitors). These five animals took the place of five other animals that did not reach an acceptable level of performance, not even after 3 months of training.

All animals were obtained from the Primate Center TNO. It appeared impossible to obtain sufficient quantities of marmosets of the desired age and sex. When tested their ages were 1.5–3 years (n = 136), 3–5 years (n = 9), and 6–8.5 years (n = 9). The total population tested was 154 animals and consisted of 99 males and 55 females. The extra animals were used for dose range finding purposes, three died (see below), some animals were used for additional ChE determinations, and several animals did not reach an acceptable performance level, notwithstanding prolonged training. Usually five or six animals were tested as a group when they had reached an acceptable level of performance. Because it was desirable to have at least one saline-treated control animal in such a test group, extra marmosets were used as saline-treated control animals.

In the test lab the animals were kept one to a home cage. These home cages (60 × 60 × 45 cm) were made of stainless steel mesh wire and contained a sitting shelf, a climbing pole, and usually some plastic toys. A continuously playing CD player provided "soft listening music" during the day. Light was on from 0700 to 1900 h. Temperature was regulated at 25 ± 1°C and humidity was kept between 50% and 80%. The cages were changed once every 2 weeks for cleaning purposes. The plastic boxes with bedding under each home cage were changed once per week.

Apart from the terminal pharmacological experiments, three additional animals died; in the dose range finding experiments, one animal received a lethal dose of soman, a second animal escaped and was bitten to death by another marmoset,

and a third animal appeared healthy upon arrival from the breeding centre (where they had suffered from a colibacillosis epidemic), but died from a coli infection before it could be tested. All other animals (n = 139) were found healthy by regular veterinary inspection and could be returned to the breeding centre after testing with low doses of ChE inhibitors. They appear to breed well.

Apparatus

In essence, the apparatus consists of a programmable robot, centrally and symmetrically situated between two identical and vertical test panels. The robot ran on rails in such a way that the test panels, in a cage-by-cage manner, were guided along a row of cages situated on both sides of the rails (Fig. 1). Each cage contained a marmoset. The robot and test panel were connected on-line with a hard disk containing IBM-compatible AT-PC computer. Two colour TV cameras, one vertically and one horizontally directed, allowed observation of the performance of the animals during testing.

The robot held an 8.5-cm-long stainless steel suction tube (diam. 4 mm) that contained optic fibres. For each trial the robot turned to a plateau with little wells, each containing a small (diam. approx. 5 mm) marshmallow-like reward. By a connection to a vacuum line, the robot sucked one reward onto the tip of the tube and moved it into the starting position behind the test panel. One optic fibre in the tube guided infrared light, which reflected against the reward sucked onto the tube. Via another optic fibre, the reflected light was used to detect the presence or absence of the reward at the end of the tube, and was instrumental in registering the time of removal. The test panel that slid in front of the steel rods of the cage (see below) at the beginning of each test session contained a small loudspeaker, right and left alphanumerical displays, and, below each display, a window that could be opened and closed by a pneumatically driven and vertically sliding door. The rod that drove the sliding window door was interrupted by a piece of cannula that (by bending) prevented the animal from being hurt if its paw was caught in the closing door.

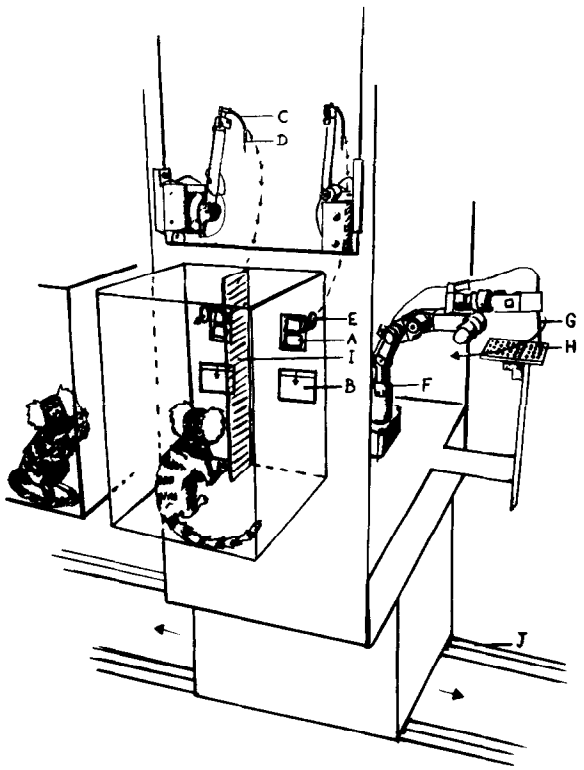

FIG. 1. The robot-assisted behavioural test apparatus. A = α-numerical display, B = window, C = handles, D = small chain attached to the handle, E = holes through which the handles are introduced into the cage, F = robot arm, G = suction tube, H = tray with marshmallow-like rewards (diam. 5 mm), I = partition screen, and J = rails.

On the right and left, near the top outer side of the displays, a small hole was present in the test panel through which motor-driven handles could be introduced into the cage when discrimination performance (see below) was tested. At the beginning of the training period, a small 1-2-cm chain was attached to the tip of the handle, making it tempting for the animal to pull at the chain and thereby the handle. By pulling the handle, microswitches were activated that led to opening of the window on that side (if a correct response was made in the visual discrimination test; see below).

On the inner side of the test panel, a photocell-monitored trough was constructed. When the animal did not properly retrieve the reward through the window into the test cage, the reward dropped into the trough and was detected by interrupting a light beam.

The test cages (l × w × h = 24 × 24 × 32.5 cm) had a bottom of mesh wire, sidewalls of dark Plexiglas (which prevented the animals from seeing each other during testing), and a transparent ceiling for TV monitoring. The side of the test cage directly in front of the panel consisted of horizontal stainless steel rods, spaced wide enough apart so that the animal could stick its arm at full length through the window of the test panel and grab the reward through the window when it was presented. Perpendicular to and at the middle of the rods a vertical mesh wire partitioning screen (from top to bottom) projected 7 cm into the cage; this screen prevented the animal from sitting in the middle in front of the test panel and not making a clear choice between the right or left window (in the discrimination test; see below).

Training and Testing

The effects of a single IM injection of each of four compounds (i.e., two carbamates and two organophosphates, in addition to saline), were tested on the performance of two behavioural tests: 1) a discrete-trial, two-choice visual discrimination task, and 2) a hand–eye coordination task. Although it was initially attempted to teach each animal both tasks (20 trials for each task), this seemed to confuse the animals and was omitted.

The discrete-trial, two-choice visual discrimination task (hereafter called visual discrimination task). Five days per week the animals were subjected to one session of 40 successive trials per day. A session started when the test panel was in place. At the beginning of each trial a nondirectional sound signal (piezo-generated, 3 KHz) was presented, intended to alert the animal. Immediately thereafter both handles were introduced in the test cage and the right *or* left alphanumerical display was switched "on" (in a quasirandom order). At that point in time the "choice time" started, which stopped when a handle was pulled.

If the animal pulled the "correct" handle (i.e., on the side where the alphanumerical display was illuminated) the window opened. The animal, facing the window, could then grab

the reward presented in the open window directly in front of it. Because the animal then directly faced the reward, the time it took to grab the reward from the suction tube was not so much a matter of choice, but rather a question of motor efficacy. Hence, the time that elapsed between pulling of the handle and removal of the reward from the suction tube was taken as a parameter of "motor speed." After removal of the reward, the window closed, the illumination went "off," and the handles were retracted, whereupon the intertrial period (approx 12 s) started. If the animal had not removed the reward from the suction tube within 10 s, the window closed, the handles retracted, and the illumination of the alphanumerical display went "off." This was registered as "correct handle, no hit" (see below). The number of times that the animal pulled the correct handle and did not retrieve the reward was negligible; this hardly ever occurred. An intertrial period then started, triggered by the closure of the window. The duration of the intertrial interval was 8 s when the reward stayed in place or 10 s when the quasirandom program determined that it had to go to the other window. If the animal pulled the wrong handle (i.e., on the side where the alphanumerical display was not illuminated) the window remained closed, the handles were retracted, the alphanumerical display went "off," and a new trial—correct handle on the same side—was started after 8 s. If the animal did not pull a (correct or incorrect) handle at all during 20 s, a new trial was also started after an intertrial interval of 8 s.

The following parameters were registered: N = number of trials; *choice time* = time elapsing between "light on, handle available" and actually pulling the handle and thereby operating the connected microswitch; *motor speed* = time elapsing between operating the microswitch and removal of the reward from the suction tube; A = attempts (i.e., the animal sticks its paw through the window); F = the number of failures (i.e., the animal removes the reward from the suction tube, but drops it into the trough); H = number of "hits" (i.e., the animal successfully retrieves the reward from the suction tube, and eats it in almost 100% of the cases, as has been checked repeatedly via the TV monitors). The percentage "hits," expressed as $H/N \times 100\%$, is taken as the *score* to judge the performance of the animal.

The hand-eye coordination test. This test also started after the test panel was in position; one session per day, each consisting of 40 trials in succession. In case of a successful removal of the reward, the intertrial interval was 15 s; when not successful it was 2 s. Only the right window was used. Each trial started with a sound signal, immediately followed by illumination of the alphanumerical display and opening of the window. At that point in time, the suction tube with the reward attached was in the "ready" position, 7.5 cm to the right of the window and out of sight for the animal. When the window opened, the reward was guided horizontally (midline viewed from the window) from right to left at a speed of 8 cm/s. Because the window is 8 cm wide, the animal had approximately 1 s to retrieve the moving reward. A "hit" (H) was registered if the animal successfully retrieved the moving reward from the suction tube. As in the discrimination test, the attempts (A) and failures (F) were also registered. The percentage "hits," expressed as $H/N \times 100\%$, is taken as the *score* and used as criterion to judge the performance of the animal.

General procedures for both behavioural tests. Once the animals had reached an acceptable level of performance (i.e., when the animals had at least once reached a score of 70% "hits" or more), they were injected IM with saline (0.5 ml/kg) and 30 min later their performance was tested. If performance dropped more than 10%, apparently as a result of this saline injection, this procedure with saline was repeated. When the drop in performance was absent or negligible, a test session without injection was performed on day 3, and the following day (day 4) the animals were injected with one of the four ChE inhibitors, or again with saline. Performance (session 4) after soman, sarin, pyridostigmine, or saline was tested 30 min after the injection, whereas this took place 20 min after the physostigmine injection. For all compounds, performance and recovery were tested again on the 4 days following the day of the injections. Blood was obtained by a heel-prick method for the determination of total blood ChE activity; that is, 2–5 days before injection of the test compounds (to test whether a heel-prick affected subsequent performance), and immediately before and 1 h after the injection of the ChE inhibitors (or saline). The blood sample 1 h after injection was obtained after the animals had been tested for their performance.

All results were expressed as percentage change of the animals' own control value. In the case of performance, the control scores were those obtained when testing took place 30 min after the saline injection (2 days before actually testing the effects of the ChE inhibitors), and in the case of total blood ChE activity, the control values were those obtained from the blood drawn just before the ChE inhibitors were injected.

The saline injections preceding session 2 were always given on a Tuesday; the injections with compound or saline preceding test session 4 were always administered on a Thursday. The sessions (one session each day) 5, 6, 7, and 8 were performed on the following Friday, Monday, Tuesday, and Wednesday, respectively. No testing took place during the weekend.

Total Blood ChE Activity and Blood AChE Activity

The heel of the marmoset was punctured using an Autoclix lancet (Boehringer, Mannheim, Germany). Blood samples (5 μl) were taken and immediately mixed with 1% saponin (BDH, Poole, UK), frozen in liquid nitrogen, and stored at -70°C for a maximum of 2 weeks. After appropriate dilution, samples were assayed for ChE activity in microtiter plates, using a modification of the Ellman method (10). For the determination of AChE and butyrylcholinesterase (BuChE), ethopropazine (2.5 μM; Sigma, St. Louis, MO) and BW284C51 (10 μM; Sigma) were used as specific inhibitors of BuChE and AChE, respectively. AChE from electric eel (Sigma) was used as a reference. Although it was originally intended to measure only ChE, it was later decided to measure AChE and BuChE as well, as suggested by other findings in the meantime (31)

Pharmacological Experiments

The lowest dose of each of the four ChE inhibitors that caused behavioural changes was tested in ketamine-anaesthetized animals (n = 3 per inhibitor) for their effects on the ECG (lead II), heart rate (HR), average blood pressure (avBP), neuromuscular transmission (NMT), and respiratory minute volume (RMV). The techniques have been reported before (30). Briefly, silver electrodes were used to monitor the ECG for which we used an AC amplifier built by the electronic department in the lab. HR was monitored by registration of the R-tops of the ECG. A tracheal cannula with a anemometer [see (18)] registered the RMV. Blood pressure was registered by a pressure transducer connected to the carotid artery. Silver electrodes were inserted around the sciatic nerve, the leg of

the animal was fixed, and neuromuscular transmission was assessed by registration of the contractions of the gastrocnemius soleus muscles following tetanic stimulation. Just before, and 5, 10, 15, 20, 30, 45, and 60 min after the injection of the inhibitors blood was drawn from the carotid arteries from these animals to determine total ChE activity and in most animals also AChE and BuChE activity.

Chemicals

All injections were performed with sterile solutions after disinfecting the skin with 70% ethyl alcohol. Soman and sarin were synthesized at the Prins Maurits Laboratory TNO and were at least 99% pure. Pyridostigmine bromide (Mestinon) was obtained from Hoffmann-La Roche BV (Mijdrecht, The Netherlands) and physostigmine (eserine sulphate) from Nutritional Biochemical Corporation (Cleveland, OH).

Statistics

The nonparametric Friedman test (26) was applied to determine whether significant treatment effects could be detected during the 8 days of testing. The Wilcoxon Signed Rank test (26) was used to compare the mean effects—within animals—on day 2 (saline) and day 4 (saline or ChE inhibitor). Pearson's r (26) was calculated to test for a possible correlation between ChE inhibition and behavioural decrements.

The multiple t-test of Welch (20), including Bonferroni's correction, as well as the Mann-Whitney U-test (26), was applied to test for possible differences between the mean effects in animals treated with ChE inhibitors and the mean effects in animals treated two times with saline (see Fig. 6). When the term "significant" is used, this indicates a $p < 0.05$.

RESULTS

General

Motivational factors. During preliminary experiments it was established during 5 days that the animals voraciously ate 80 of the rewards and still finished their daily diet. Hence, sessions of 40 trials were chosen to be on the safe side. During training each animal could maximally obtain 40 rewards if 100% of its responses were correct; saturation effects during training were not observed. During testing ChE inhibitors might induce loss of appetite. Although this motivational factor during testing is not excluded, it appears unlikely because when the highest dose of each of the four ChE inhibitors was injected into animals (n = 2 per dose of compound) and when 40 rewards were placed into their home cage, the animals consumed all 40 without delay. A second reason that seems to exclude effects on food motivation is the observation that during the whole course of the investigations, there were no leftovers from the normal amount of food in the evening of the same day when the animals were tested after administration of the ChE inhibitors, as might be expected when their appetite was affected by these compounds.

Sex differences. It was not possible to obtain an equal number of female and male marmosets. Therefore, it was not possible to distribute the females equally over the treatment

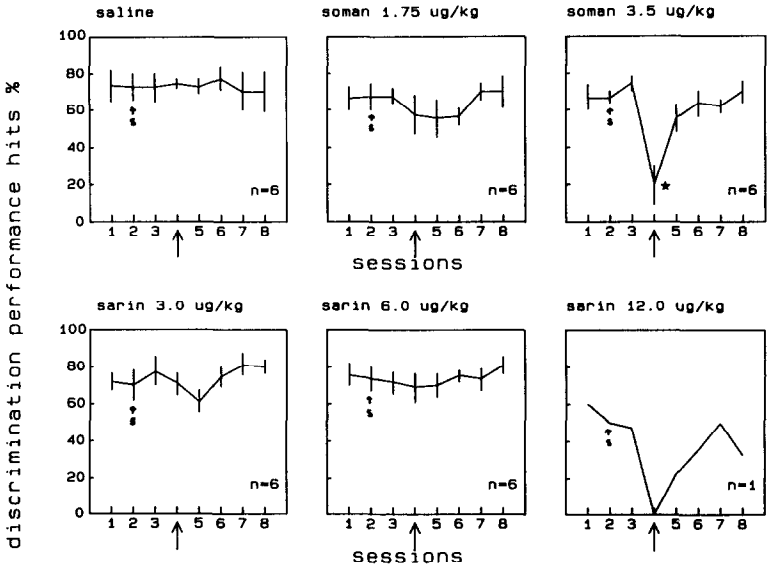

FIG. 2. The effects on visual discrimination performance of IM saline (0.5 ml/kg), soman (1.75 or 3.5 μg/kg), or sarin (3.0, 6.0, or 12 μg/kg). Correct behavioural responses are scored as "hits" (i.e., a successful retrieval of the reward). After a training period (not shown) testing began on session 1. On session 2 of the test period, each animal was injected IM with saline (see s↑) and on session 4 with the above-mentioned compounds or again with saline (see arrow at bottom). Performance testing started always 30 min after the injections. In contrast with soman, these low doses of sarin had no effect on performance of this task, except in a single animal injected with 12 μg/kg sarin (bottom right). Means ± SEM. ★Significantly different from saline.

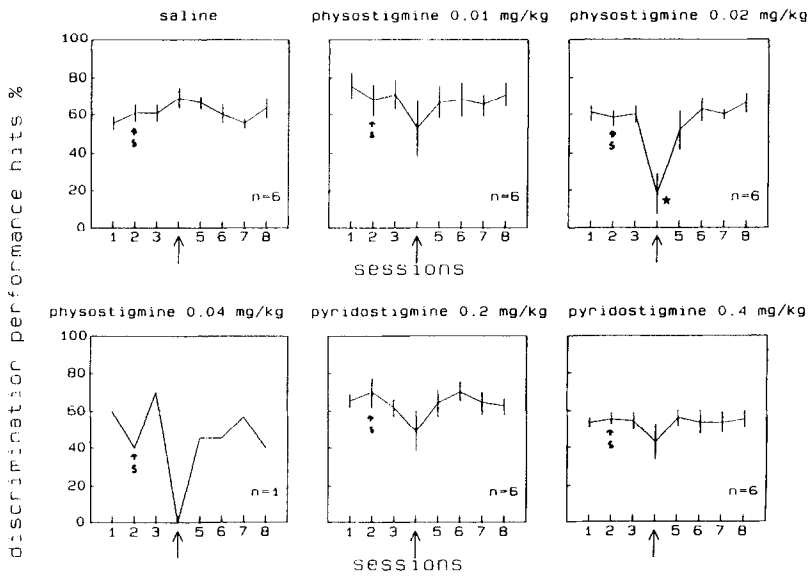

FIG. 3. The effects on visual discrimination performance of IM saline (0.5 ml/kg), physostigmine (10, 20, or 40 μg/kg), or pyridostigmine (200 or 400 μg/kg). Behavioural testing started 30 min after the injections, except after physostigmine, where the time interval was 20 min. An additional animal received a dose of 40 μg/kg physostigmine. For further details see Fig. 2.

groups. Nevertheless, each treatment group contains animals of both sexes. When compared at the same dose levels, the decreases of ChE activities and decrements of behaviour were in the same range; trends or significant differences between sexes were not found.

Behavioural Experiments

In Figs. 2-5 the effects of saline and two dose levels of soman, sarin, physostigmine, and pyridostigmine on the performance of the visual discrimination or hand-eye coordination tests are shown, not counting the single animals that were tested after a higher dose of sarin and physostigmine. The number of animals in each key group is $n = 6$. In addition, the results of some animals from the dose range finding experiments treated with a higher dose of physostigmine or sarin were added as an illustration. Effects of a higher dose of soman (7 μg/kg) are not shown because the animal became very ill and died. Moreover, two animals treated with a higher dose of pyridostigmine (0.8 mg/kg) developed overt symptoms (heavy salivation, tremors), and behavioural testing made no sense.

The variability of the results is large, which is not surprising considering the fact that these were not inbred animals. This variability is most clearly visible at the lowest doses of each of the compounds. For example, at the dose level of 1.75 μg/kg soman, performance decrements in two of the six animals in the hand-eye coordination test (Fig. 4) was fairly large (45% and 53%), was only slightly decreased in two others, and slightly increased in two animals. A dose of 3.5 μg/kg caused substantial performance decrement in all six animals. In the same test, physostigmine (Fig. 5), at a dose of 0.01 mg/kg, caused a substantial decrement in two out of six animals (57%, 73% reduction), whereas the reduction in two animals was smaller and in two other animals performance was even slightly improved. A dose of 0.02 mg/kg physostigmine induced substantial decrements in performance of hand-eye coordination in three animals (45%, 88%, and 90% reduction), smaller reductions (8% and 12%) in two animals, and a 10% improvement of performance in one animal. Similar findings in the hand-eye coordination test were seen after sarin (Fig. 4). However, after a dose of pyridostigmine (Fig. 5) of 0.2 mg/kg, three out of six animals showed a substantial decrement of hand-eye coordination performance (40%, 40%, and 42% reduction), whereas after a dose of 0.4 mg/kg, also three out of six animals showed a performance decrement, but the reductions were smaller (20%, 25%, and 30%). Hence, after pyridostigmine, a dose-response effect on hand-eye coordination performance was not found.

Figure 6 is an overview showing the mean performance reduction per treatment group as well as the mean reduction in total blood ChE activity. The performance changes are obtained by a simple subtraction (within each animal) of the performance score after saline in session 2 from the performance after the test compound in session 4 (Figs. 2-5). The changes in ChE activity in blood are similarly obtained by subtracting the values before injection (= 100%) from those obtained 1 h after injection of the test compounds. In Fig. 6 it can be seen that dose-dependent decrements on performance are evident following soman, sarin, or physostigmine. However, following pyridostigmine a dose-response effect on performance was not observed, notwithstanding the fact that blood ChE became more inhibited upon doubling the dose of pyridostimine. Figure 6 indicates that, at roughly the same level of blood ChE inhibition, soman causes a larger performance decrement than sarin, whereas physostigmine—even at a lower level of blood ChE inhibition—causes a larger performance decrement than pyridostigmine. On a μmol/kg basis the differences between the performance effects of these ChE inhibitors are even larger because, in that case, the two doses

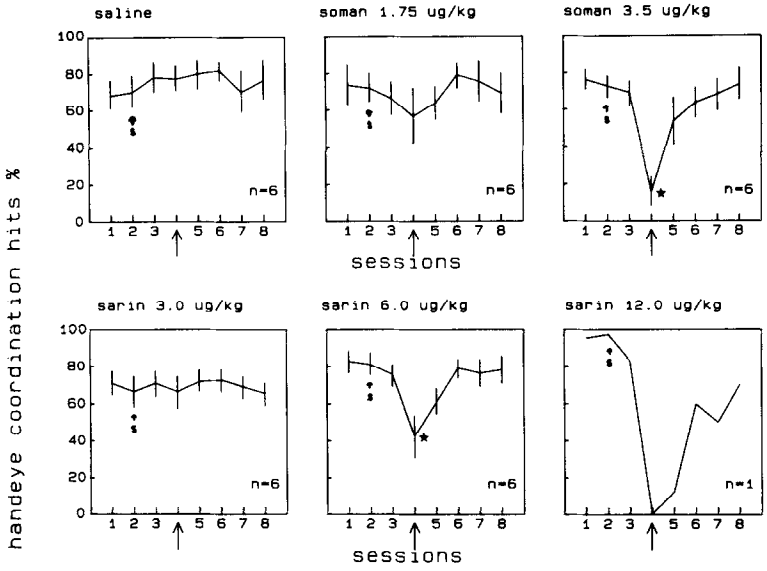

FIG. 4. The effects on hand-eye coordination performance of the same doses of saline, soman, or sarin as in Fig. 2. In this test a dose of 6 μg/kg sarin caused a significant performance deficit, in contrast with the absence of such an effect in the visual coordination test in Fig. 2. For further details see Fig. 2.

of sarin are at least 2.2 times higher than those of soman, whereas the two doses of pyridostigmine are 28 times higher than those of physostigmine.

It is interesting to note that the performance decrements caused by sarin (6 μg/kg) in the visual discrimination test are absent, whereas a significant effect is observed in the hand-eye coordination test. For the other compounds, the differences in the size of the effects in the two tests are negligible.

In the visual discrimination test a number of "choice" parameters as well as the "motor speed" were assessed. A relevant "choice" parameter (Fig. 7), [i.e., the number of correct choices expressed as a percentage of the correct responses following an injection with saline (2 days earlier)] showed a significant decrease of correct choices upon doubling the dose of soman. With physostigmine, the average decrease upon doubling the dose was not significant due to one aberrant animal that exhibited a large performance increase. After sarin the decrease was negligible, and after pyridostigmine there was an overall decrease of performance that did not further decrease at a dose that was two times higher.

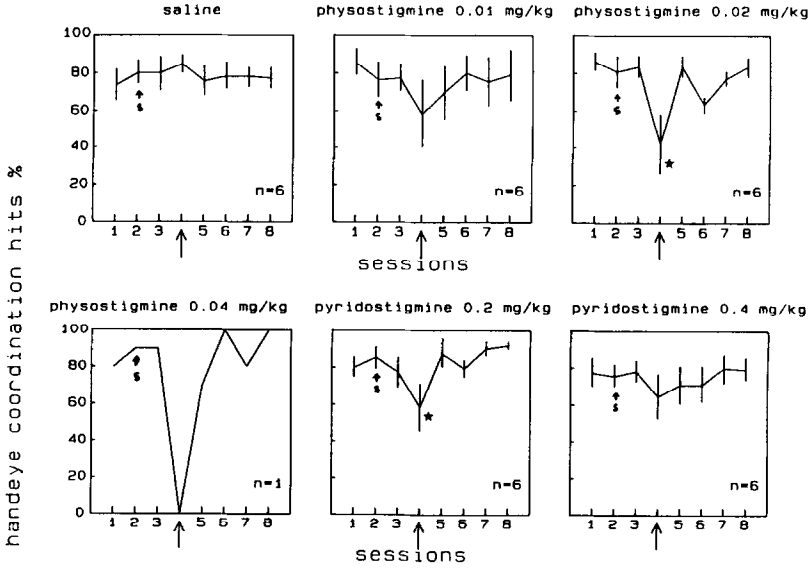

FIG. 5. The effects on hand-eye coordination performance of the same doses of saline, physostigmine, or pyridostigmine as in Fig. 3. Note that here also a dose-response effect of pyridostigmine is absent. For details see Fig. 2.

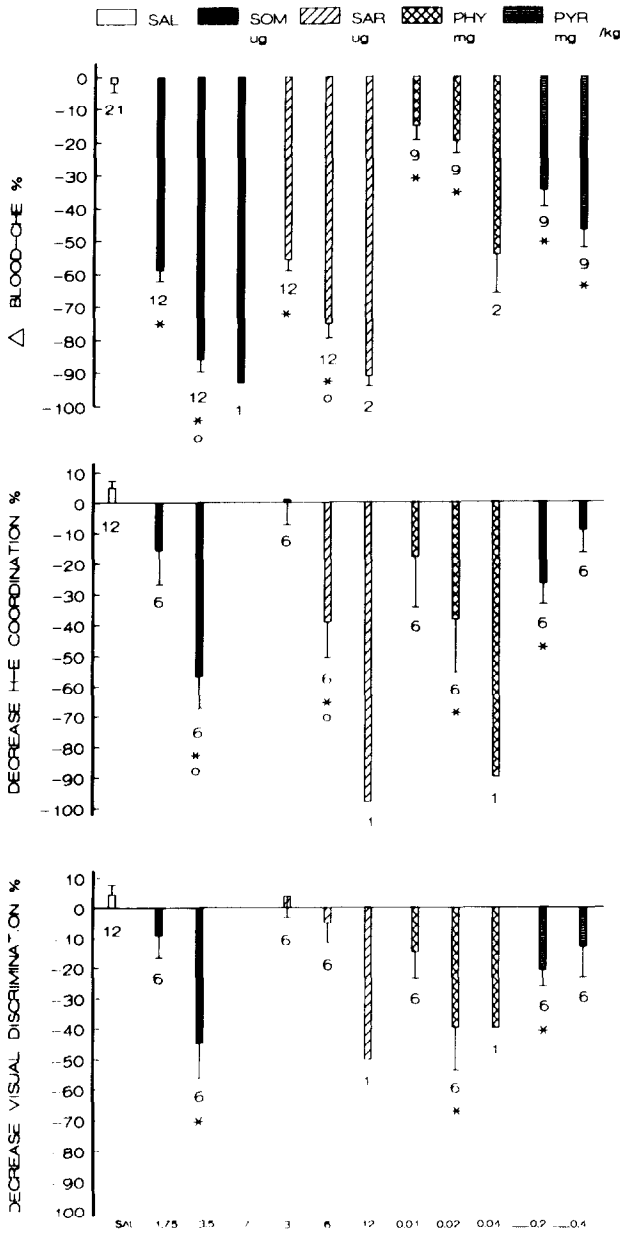

FIG. 6. Overview of the effects of saline, soman, sarin, physostigmine, or pyridostigmine (doses at the bottom) on total blood CHE activity (top), on hand-eye (H-E) coordination performance (middle), and on visual discrimination performance (bottom). The numbers below the bars refer to the number of animals used. Doses of each compound are at the bottom of the graph. The performance changes are obtained by a subtraction (within each animal) of the performance score (% hits) after saline at test session 2 from the performance score at session 4 (see Figs. 2-5). The changes in blood CHE activity are similarly obtained by subtracting the values before injection (= 100%) from those obtained 1 h after the injection of the test compounds. Means ± SEM. *Significantly different from the saline-treated control group. °Significantly different from the lower dose of the same compound.

Analysis of the distribution of the "choice times" (example in Fig. 8) suggested that the decrease of the number of choices in the time allotted was the result of an increase in the number of "no attempts." Indeed, further analysis showed that when both the correct and incorrect choices were taken into account, the decrease in correct choices shown in Fig. 7 could not be attributed to an increase in incorrect responses, but was mainly due to an increase in the number of "no attempts". This increase in "no attempts" was largest after soman and physostigmine, as can be seen in Table 1.

Motor speed could only be measured when a correct choice was made; the window only opened after the animal had pulled the correct handle. When the data in Fig. 7 and those in Table 1 are compared with those in Fig. 9, it can be seen, in particular after soman and physostigmine, that the number of correct choices decreased whereas motor speed (Fig. 9) was hardly affected. This indicates that after both compounds the animals have difficulty in making a choice, but once they have made their choice, they have no problems in retrieving their nonmoving reward as fast or sometimes even faster than before.

After sarin and pyridostigmine, motor speed was also hardly changed. Because after sarin the changes in the percentage of "correct choices" (Fig. 7) as well as the number/percentage of "no attempts" (Table 1) remained practically unchanged and because the changes in these parameters after pyridostimine appeared to be rather limited, effects of these two compounds in the visual discrimination test remain absent or relatively small (Figs. 2 and 3). In contrast, sarin (6 μg/kg) caused a significant decrement in the hand-eye coordination test. The finding in the hand-eye coordination test that pyridostigmine causes a significant decrement at 200 μg/kg and a smaller effect (not significantly different from saline controls) at twice that dose level is poorly understood.

A correlation between performance decrements and ChE inhibition in blood was absent in most cases; there were only weak intraindividual correlations in the hand-eye coordination test between these two parameters following soman 3.5 μg/kg (r = 0.61) and following pyridostimine 0.4 mg/kg (r = 0.75)

Cholinesterase Inhibition

It can be seen in Fig. 6 that all ChE inhibitors, upon doubling the dose, cause a dose-dependent decrease of ChE activ-

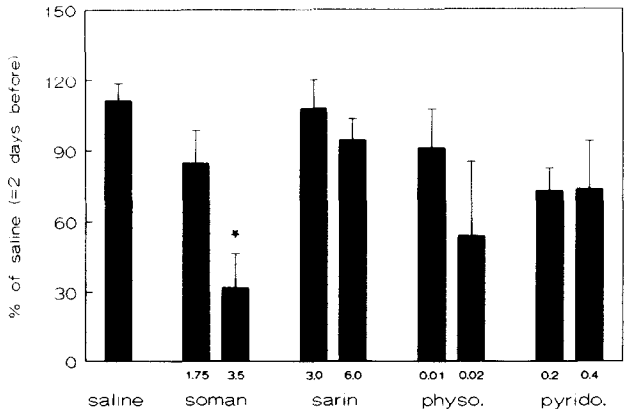

FIG. 7. The changes in the number of correct choices within 20 s after the injection of the test compounds on session 4, expressed as a percentage of the number of correct choices made after the injection of saline on session 2 within the same animal. A significant (★) decrease in the percentage of correct choices occurs after the higher dose of 3.5 μg/kg soman. A decrease after physostigmine can also be observed but the difference with the saline control group is not significant (due to a large value in one animal).

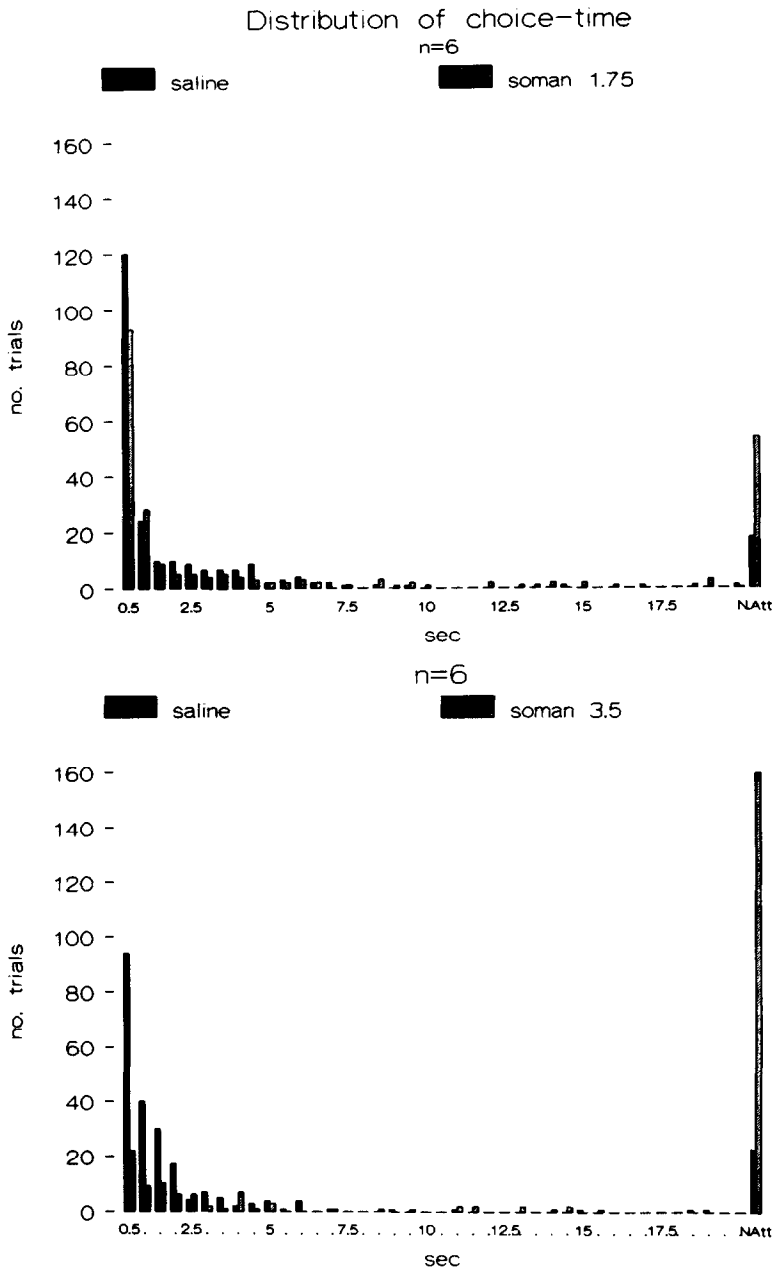

FIG. 8. An example of the graphs used to analyze the distribution of choice time during the 20-s periods, plotted in a cumulative manner for a group of animals treated with 1.75 μg/kg soman and its concomitant saline-treated control (top), as well as a group treated with 3.5 μg/kg soman and its concomitant saline-treated control (bottom). Natt = no attempts.

ity in blood, albeit that in the case of the carbamates this effect is rather weak upon doubling the dose. Following IM injection, on a μmol/kg basis, the ChE-inhibiting efficacy appears to decrease in the order soman > sarin > physostigmine > pyridostigmine.

In the pharmacological experiments (see below) repeated blood samples were taken following the injection of a single dose of each compound into ketamine-anaesthetized animals (n = 3 per ChE inhibitor). The changes in total blood ChE, AChE, and BuChE activity in these animals are shown in Fig. 10 within the same animals. Although the number of animals is small, it seems reasonable to conclude that: 1) the reduction in total blood ChE activity at these dose levels is mainly due to a reduction in AChE activity, and 2) physostigmine, at a dose level that causes performance decrements, causes only a relatively mild inhibition of total ChE and AChE in blood.

Pharmacological Experiments

In Table 2 the results are shown of experiments in which the changes in a variety of physiological parameters were re-

TABLE 1

THE ABSOLUTE NUMBER OF "NO ATTEMPTS" OUT OF 40 POSSIBLE ATTEMPTS AS WELL AS THE PERCENTAGE CHANGE OF "NO ATTEMPTS" (COMPARED WITHIN ANIMALS WITH THE "NO ATTEMPTS" AFTER SALINE)

Compound/Dose (μg/kg)	NUMBER OF "NO ATTEMPTS"	
	Absolute Number of No Attempts	As % of "No Attempts" After Saline (2 Days Before)
Saline (0.5 ml)	2.3 ± 0.89	−3 ± 4.9%
Soman (1.75)	9.2 ± 4.94	18 ± 12.6%
Soman (3.5)	27.7 ± 4.25*	62 ± 14.1%*
Sarin (3.0)	6.5 ± 2.45	−7 ± 8.3%
Sarin (6.0)	3.0 ± 2.61	4 ± 7.0%
Physostigmine (10)	12.3 ± 6.19	21 ± 15.3%
Physostigmine (20)	26.4 ± 6.73*	56 ± 23.0%
Pyridostigmine (200)	11.4 ± 5.75	21 ± 11.9%
Pyridostigmine (400)	17.2 ± 5.57*	26 ± 17.3%

The largest increases in the "no attempts" are seen with soman and physostigmine. The increase in "no attempts" after pyridostigmine does not grow after doubling the dose. The changes after sarin are negligible.

*Significant increase in the number of "no attempts". Means ± SEM, n = 6 per treatment group.

corded. The changes after 30 min are presented as a percentage of the control values within each animal, which are subsequently averaged. Thirty minutes is chosen because, in the behavioural experiments, testing starts at 30 min after injection, except in the case of physostigmine. However, in this case the effects of physostigmine were also assessed 30 min after injection. Apart from a small effect of pyridostigmine on heart rate and on the average blood pressure, hardly any effects were found. The heart rates were quite variable [control values obtained before injection were 244 ± 20.4 (n = 12)] whereas blood pressure was fairly constant [control values before injection were 76 ± 3.1 (n = 12)].

DISCUSSION

The picture that emerges from these experiments is the following. In general, the effects on hand-eye coordination performance were slightly larger than those on visual discrimination (Fig. 6), which was most clearly evident in those groups of animals that were injected with 6 μg/kg sarin. As in rats (34), the dose levels of soman and physostigmine used cause predominantly CNS effects; in the visual discrimination test these two compounds caused a decrease in the parameter "choices" (Fig. 7) and an increase in the parameter "no attempts" (Table 1), whereas the parameter "motor speed" (Fig. 9) was not significantly changed. Together this indicates that after both compounds, the animals have difficulty in making a choice or initiating rapid actions, but once they have made their choice or started their movements they have no problems in retrieving their reward as fast, or individually sometimes even faster, than before. At these dose levels, sarin has a preferentially peripheral effect (as in rats). This agent does not change performance in the visual discrimination test; it affects neither the "choice" parameters nor the "motor speed" parameter. Yet, at a dose of 6 μg/kg, it causes a significant and fairly large decrement on hand-eye coordination. Therefore, it is likely that this effect of sarin is mainly due to the disturbances of the peripherally determined components of the responses in the hand-eye coordination test.

The *first* working hypothesis was that a disruption of CNS functions following soman would occur at a lower dose than after sarin. Moreover, physostigmine was expected to cause a disruptive effect at a lower dose than pyridostigmine. The IV LD_{50} of soman is approximately 8 μg/kg (8), the IM LD_{50} is approximately 9 μg/kg, and the IM LD_{50} of sarin is approximately 22.5 μg/kg (9). Because four out of a group of six animals showed a decrease (in two cases, substantial: 45% and 53%) of their hand-eye coordination performance after a dose of 1.75 μg/kg soman, this dose was considered as a borderline effective (behavioural disrupting) dose, although the group average did not significantly differ from saline-treated controls. The lowest dose of sarin (3 μg/kg) led to a relatively small decrease of hand-eye coordination in one animal, whereas three out of six animals showed an improvement of performance, in one animal even as much as 35%. Hence, sarin 3 μg/kg was not and 6 μg/kg was considered to be a behaviourally disruptive dose. As a fraction of the LD_{50}, the behaviourally effective dose of soman is approximately 19% of the LD_{50}, whereas for sarin it is approximately 27% of its LD_{50}. The latter value seems somewhat lower than the values of 33–55% found by other investigators (9).

When expressed on a μmol/kg basis, the effective dose of soman was 9.62×10^{-3} μmol/kg, whereas for sarin it was much higher (i.e., 42.8×10^{-3} μmol/kg). In earlier experiments in inbred rats (34) with a much lower response variability, the differences in the behavioural disrupting doses between soman and sarin were larger, which was ascribed to the earlier finding that soman has a more predominant effect on the central nervous system than sarin (18).

The LD_{50} values of physostigmine and pyridostigmine in the marmoset are not known. The effective (behavioural disrupting) IM dose of physostigmine is 0.02 mg/kg. At a dose level of 0.01 mg/kg, physostigmine has a borderline effect, because two out of six animals showed a substantial (57%, 73%) decrement of hand-eye performance. Pyridostigmine, at a dose of 0.2 mg/kg IM, caused a smaller but significant performance decrement of hand-eye coordination. For poorly understood reasons, twice that dose had no significant effect. However, it is clear that on a μmol/kg basis, one has to administer approximately 25 times more pyridostigmine than physostigmine to induce a significant behavioural decrement.

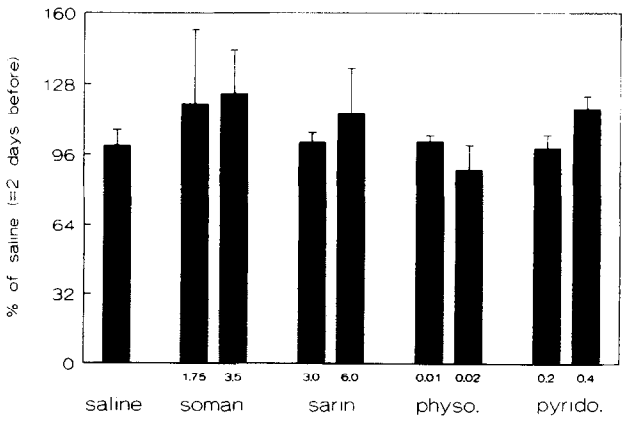

FIG. 9. The "motor speed" after injection of saline, soman, sarin, physostigmine (physo), or pyridostigmine (pyrido). It can be seen that none of the inhibitors significantly affect this parameter.

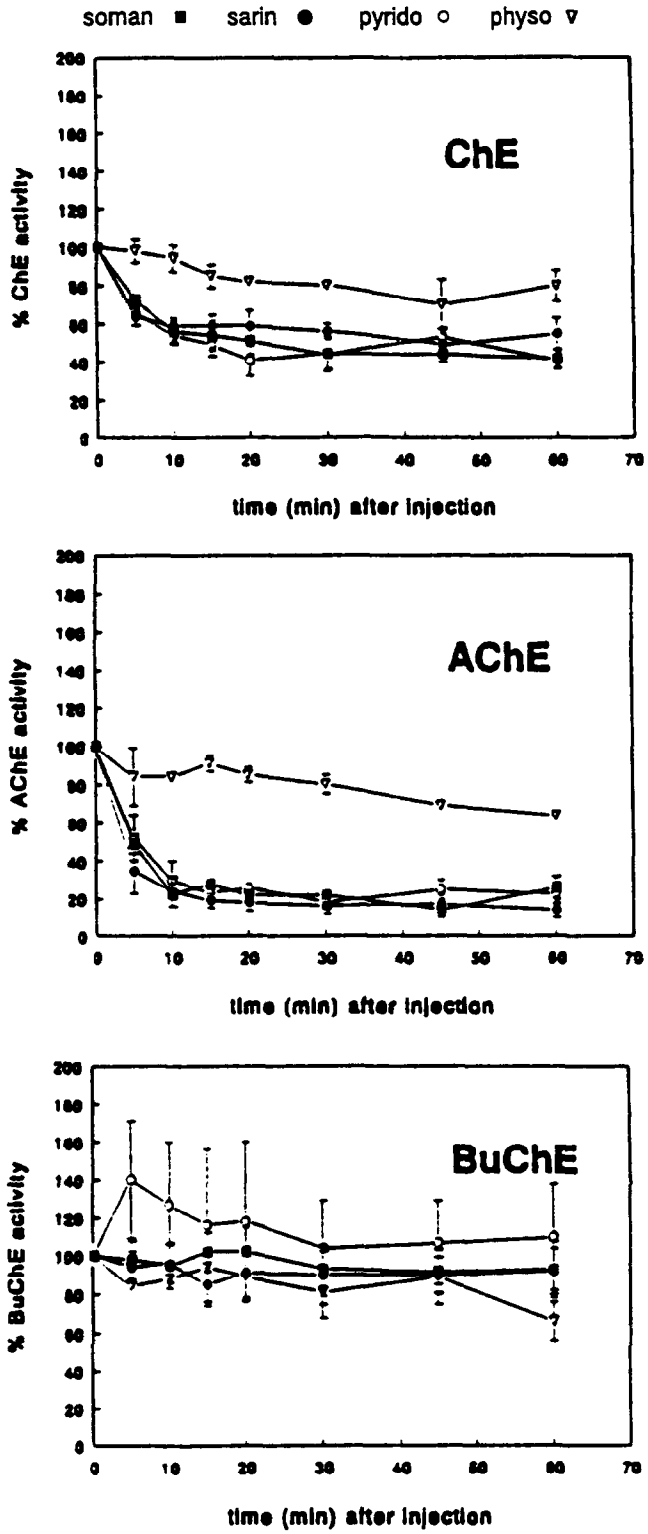

FIG. 10. The changes in total ChE, AChE, and BuChE activity in blood of ketamine-anaesthesized marmosets after IM injection of soman (1.75 μg/kg), sarin (6.0 μg/kg), physostigmine (20 μg/kg), or pyridostigmine (200 μg/kg). The blood samples were obtained from animals in the pharmacological experiments. Note that at this dose level physostigmine causes a much smaller reduction of ChE activity than the low doses of the other inhibitors, yet in the behavioural experiments this dose of physostigmine causes a significant performance deficit. These results suggest that the changes in total blood ChE activity are due to inhibition of AChE and not to BuChE activity. Statistical calculation of significant differences is not justfiable due to the small treatment group size ($n = 3$, and for sarin and physostigmine AChE activity was only measured in two animals, see also Table 2).

In conclusion, the results indicate that the second working hypothesis is valid.

The *second* working hypothesis was that the behavioural effects detected shortly after the administration of the four ChE inhibitors will have disappeared after 24 h, even those following administration of the irreversibly inhibiting organophosphates. This appeared to be the case. Although ChE activity later than 60 min after injection of these compounds (see also pharmacological experiments) was not determined, it is likely that the disappearance of the behavioural decrements 24 h after injection of the reversibly inhibiting carbamates is mainly due to recovery of enzyme activity. In the case of the organophosphates, it is likely that the decrements have disappeared due to the combined effects of recovery of enzyme activity [by de novo synthesis and, in the case of sarin, also by partial spontaneous recovery (18)] and the occurrence of tolerance (6,7,25). Because little was known about the behavioural tolerance to soman, a study was started in our laboratory to investigate the development of tolerance to this agent in rats (19,29,35,36). It was considered likely that tolerance to soman does develop, but that secondary effects of soman may obscure the detection of this phenomenon.

Whatever the underlying mechanism may be, on the basis of the results, it is concluded that the second working hypothesis is valid.

The *third* working hypothesis was that neuromuscular function will be hardly affected at doses that disrupt behaviour and that these doses will only moderately inhibit blood ChE activity. The first part of this working hypothesis is valid; of all the physiological parameters measured—including those on neuromuscular transmission (see Table 2)—only a small effect of pyridostigmine on heart rate and blood pressure was found. The second part of this working hypothesis is not valid; both organophosphates cause >50% inhibition of blood ChE at minimal doses that induce behavioural decrements. The inhibition levels induced by the carbamates are lower; note that 0.02 mg/kg physostigmine caused a highly significant decrement of hand-eye coordination performance at a level of inhibition of blood ChE of only approximately 23%.

Again, these values indicate that blood ChE activity measurements do not provide an adequate indicator to assess whether the amount of ChE inhibitor that has been administered (pretreatment) or to which one has been exposed (nerve agent) is "safe" or will cause undesirable side effects. For example, physostigmine at a dose of 0.02 mg/kg causes an inhibition of blood ChE of approximately 23% and induces a highly significant disruption of performance, whereas sarin at a dose of 3 μg/kg causes an inhibition of blood ChE of approximately 55% and does not affect performance in either behavioural test. Measurements of AChE activity are equally inadequate, because the results of the present experiments show that at these dose levels of the four ChE inhibitors the decrease of total blood ChE activity is almost totally due to inhibition of AChE (see Table 1).

TABLE 2

PERCENTAGE CHANGE IN PHYSIOLOGICAL PARAMETERS OF KETAMINE-ANAESTHETIZED MARMOSETS, MEASURED 30 MIN AFTER AN I.M. INJECTION OF A SINGLE DOSE OF A CHE-INHIBITOR

Compound/Dose (μg/kg)	HR (%)	avBP (%)	NMT (%)	RMV (%)	Blood ChE	
					Total	AChE
Soman (1.75)	−17 ± 3.0	−3 ± 3.4	+7 ± 12.0	0	−56 ± 6.3	−79 ± 2.2
Sarin (6.0)	−6 ± 9.7	−3 ± 5.7	+7 ± 5.5	−2 ± 1.7	−45 ± 8.8	−84 (2) (86, 81)
Physostigmine (20)	+9 ± 4.9	−14 ± 10.7	−4 ± 5.2	−2 ± 1.7	−19 ± 2.4	−16 (2) (21, 10)
Pyridostigmine (200)	−25 ± 12.8	−22 ± 3.5	−7 ± 3.0	+2 ± 1.7	−55 ± 13.7	−86 ± 9.5

n = 3 for each compound. However, blood AChE-inhibition after sarin and physostigmine was only assessed in two animals per treatment; individual data are shown in parentheses. HR = heart rate, avBP = average blood pressure, NMT = neuromuscular transmission, RMV = respiratory minute volume. Means ± SEM These data are merely indicative for the absence of important physiological changes (except enzyme inhibition). Statistical evaluation is not justifiable due to small group sizes.

Concerning the extrapolation of these data to humans is the following. In the Chinese Medical Encyclopedia (38), the LD_{50} for humans, both for sarin and soman, is estimated to be 10 mg per person; the exposure route is not mentioned. Extrapolated to men of 70 kg this would mean that for soman 1.2% and for sarin 4.2% of their respective LD_{50} values would cause a decrement in performance. However, most likely this estimated LD_{50} is too high and might be derived from the LD_{50} in rodents. In contrast to, for example, rats, the blood of primates such as marmosets and humans does not contain large amounts of scavenging carboxylesterases, one of the main reasons for the differences in the LD_{50} values between these species. The LD_{50} in primates is much lower than in rodents, and Western experts estimate the LD_{50} of soman and sarin in men to be around 1 mg. Accordingly, doses that may cause performance deficits in man would be 12% of the LD_{50} for soman and 42% of the LD_{50} for sarin. In view of the low dose of soman, it is entirely possible that disturbances in higher brain functions may occur that might go unnoticed because physical signs are absent. Following exposure to sarin, the likelihood that physical symptoms are noticed is greater.

An interesting aspect of the effects of soman, and to a certain extent also those of physostigmine, is the finding that the number of "no attempts" (see Table 1) increase drastically. Extrapolated to humans, this could mean that a soldier exposed to a low dose of soman or receiving physostigmine pretreatment during combat would have difficulty in initiating rapid actions. Certainly an undesirable side effect.

No human LD_{50} estimates are available for the carbamates. In handbooks of medicine a dose of 6 mg physostigmine per individual is reported to cause grave symptoms. Three daily oral doses of 30 mg pyridostimine, as a pretreatment against organophosphate poisoning, have been introduced in several armies. This dosage schedule seems to cause only minor symptoms or none at all. Pyridostigmine has been used in the treatment of myasthenia gravis, for which disease doses up to 720 mg per day (in divided doses) have been prescribed according to handbooks of medicine.

It is possible that in marmosets, like in rats (34), more pyridostigmine enters the CNS than hitherto suspected. However, the absence of an expected dose-response effect after administration of pyridostigmine in the present experiments is then hard to understand. Nevertheless, a dose of 0.2 mg/kg caused a significant blood ChE inhibition (42 ± 6.9%) and a significant decrement (27 ± 6.7%) of hand-eye coordination when compared with saline-treated control animals. Based on a (human) weight basis of 70 kg, one might expect that a dose of 14 mg would cause a significant performance decrement. A dose of 0.4 mg/kg caused a higher degree of blood ChE inhibition (56 ± 4.5%), but this only resulted in an insignificant performance decrement of hand-eye coordination of 9 ± 7.4%. Although in the visual discrimination test a similar trend is detected (i.e., the performance decrements after 0.2 mg/kg were more pronounced than after 0.4 mg/kg pyridostigmine), the values after 0.2 mg/kg did not differ significantly from those of saline-treated control animals. It might be that the lack of a dose-response effect in the case of pyridostigmine is due to an effect other than ChE inhibition (2).

Physostigmine, at a dose level of 0.01 mg/kg, induces in marmosets a highly variable effect on hand-eye coordination performance; in two out of six animals a substantial reduction (57%, 73%) was observed (versus effect of saline within the same animals). As in the case of soman (see above), this dose of physostigmine might be called a borderline effective dose, although here also the average effect of physostigmine on the performance of the group did not significantly differ from that in saline-treated control animals. A dose of 0.02 mg/kg physostigmine caused a highly significant performance decrement. This dose causes a smaller than 20% decrease in ChE activity in blood and might not offer a significant protection against organophosphate intoxication. If a direct extrapolation on a weight basis to a human of 70 kg would be considered valid, this would mean that a dose of 0.70 mg might induce performance decrements in a number of individuals, whereas 1.4 mg might cause a highly significant effect in practically all subjects. However, this is speculative and does not take the route of administration into account. On the other hand, speculations of this type may provide at least some dose indications when physostigmine would be considered as a pretreatment drug and its effects would be tested in human volunteers. Results of experiments with guinea pigs suggest that physostigmine in that case will most likely be combined with a cholinolytic (16,21).

Like marmosets, humans may be expected to show a high variability. As a consequence, one has to be careful to conclude that a certain dose (e.g., of a pretreatment drug) is "safe" because the effects in a treatment group do not differ significantly from those in a control group. A pretreatment that would cause a substantial decrement of performance in, for example, 20–50% of the individuals most certainly would not be acceptable.

In conclusion, the present results show that ChE inhibitors

and in particular soman and physostigmine may cause incapacitation (performance deficits) at dose levels that are much lower than those that cause overt signs of intoxication. Most likely these effects are predominantly due to effects on the CNS. Sarin and pyridostigmine also cause performance deficits at doses lower than those that cause overt signs. The nature of these effects is most likely more peripheral and the doses of these compounds needed to cause performance decrements are higher than those of soman and physostigmine, respectively. The doses of these two latter compounds are so low and the CNS effects are so uncharacteristic of the classical intoxication picture that these subtle incapacitating effects may go undetected. Because it appears likely that soman and physostigmine may affect choice and decision-making processes, one should be aware of these effects when exposure to low doses of this organophosphate are likely to have occurred or when physostigmine is considered as a pretreatment agent.

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