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Comparative investigation of behavioral, neurotoxicological, and immunotoxicological indices in detection of subacute combined exposure with methyl parathion and propoxur in rats

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Abstract

The effects of 6 weeks of oral exposure to propoxur (PR; at doses of 0.851 and 8.51 mg/kg body wt.), methylparathion (MP; at doses of 0.218 and 0.872 mg/kg body wt.), and their combinations were investigated in male Wistar rats. Measurement endpoints of the investigation were certain general toxicological parameters (body weight gain, organ weights), plaque-forming cell (PFC) count from the spleen, open field (OF) behavior, auditory startle response (ASR), prepulse inhibition (PPI), rotarod performance, somatosensory and auditory cortical evoked potentials, and peripheral nerve conduction velocity. The treated rats did not show any sign of acute intoxication during the 6 weeks of exposure. The higher dose of PR, but not of MP, significantly decreased the relative liver weight. Both agents produced a significant dose-dependent increase of OF activity, with larger expression after 2 weeks than after 6 weeks. The number of ASR responses and the ASR amplitude increased. The amplitude after PPI was increased by MP but only minimally altered by PR and the combinations. There was a small, but with high-dose PR significant, increase in the latency of the somatosensory evoked potentials. Neither of the two substances alone had any effect on the PFC response. The effect of the combination of high-dose PR and low-dose MP was significantly different from that of high-dose PR alone on the liver weight, on the ASR amplitude, and on the PFC/10^6 cell and PFC/spleen counts. With high-dose MP and low-dose PR, no such interaction was observed. According to the results, the noneffective dose of MP can influence the toxicity of the effective dose of PR in a combined exposure situation.

Keywords: Behavioral toxicity; Neurotoxicity; Immunotoxicity; Methyl parathion; Propoxur; Rat

1. Introduction

Anticholinesterase agents of the organophosphate and carbamate groups have been widely used in the agricultural and hygienic control of insects. They are highly toxic to a number of species, including humans, by various routes of exposure (Agarwal, 1993; Tsao et al., 1990). Their main target is the cholinesterase enzyme but, besides and partly beyond enzyme inhibition, some organophosphates can cause delayed neuropathy, behavioral and electrophysiological changes, and alterations in the immune system (WHO, 1986a). In our previous studies done in rats with dimethoate, methyl parathion, and dichlorvos, altered spontaneous cortical activity and modified latency and duration of sensory evoked potentials (Dési et al., 1994, 1998; Dési and Nagymajtényi, 1988, 1999; Nagymajtényi et al., 1992, 1994), as well as changes in the behavior (Schulz et al., 1990) and in certain immune functions (Institóris et al., 1995a, b) of the animals, have been observed following subacute administration or in three-generation studies.

Methyl parathion (MP) is used as an insecticide and acaricide in agriculture. It is highly toxic to mammals and humans by inhalation and ingestion and moderately toxic by dermal adsorption (ATSDR, 1990). Prolonged exposure of workers causes neurological and behavioral symptoms (OHS, 1991). Volunteers receiving 11 mg/kg for 30 days per os had a 15% decrease in plasma cholinesterase activity but no effect on blood cell count

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Table 1
Single and combined doses of the two pesticide agents used for subacute per os treatment of the rats

Substance		Dose	
Methyl parathion (MP)	Low dose (L)	1/100 LD_{50}	0.218 mg/kg
	High dose (H)	1/25 LD_{50}	0.872 mg/kg
Propoxur (PR)	Low dose (L)	1/100 LD_{50}	0.851 mg/kg
	High dose (H)	1/10 LD_{50}	8.51 mg/kg
Combinations	MP(H) + PR(L)		0.872 mg/kg MP + 0.851 mg/kg PR
	MP(L) + PR(H)		0.218 mg/kg PR + 8.51 mg/kg

(Rider et al., 1969). Other results indicate that MP can have an immunomodulating effect as it decreases mitogen-induced T-cell proliferation (Park and Lee, 1978) and inhibits neutrophyl chemotaxis of human peripheral blood leukocytes (Lee et al., 1979) in vitro.

Propoxur (PR) is used mainly for household pest control and for residual spraying in malaria eradication programs (WHO, 1986b). Like other carbamates, PR reversibly blocks acetylcholinesterase activity (Alvares, 1992). In rats, a single dose of ca. 1/10 LD_{50} caused a 60% drop in cholinesterase activity and marked disturbances in higher nervous functions (decreased open field exploration, active avoidance task performance; Thiesen et al., 1999). In our earlier studies, the immunomodulating effects of PR (8.51 mg/kg administered to rats per os for 4 weeks) have been described (Institóris et al., 2001; Siroki et al., 2001).

Combination toxicology has been gaining importance with the recognition that real-life exposure is, nearly exclusively, multiple. In the case of organophosphates and carbamates, the common target enzyme increases the chance of observing interactions, both additive and antagonostic. With PR and the organophosphate azinphosmethyl, an additive toxic effect was observed, while the interaction of PR and the pyrethroid cyfluthrin was found to be potentiating (WHO/FAO, 1989). Prevention of intoxication and early detection of poisoning in humans requires sensitive methods of recognition, possibly utilizing indices based on behavioral, neurological, and immunological outcomes. Therefore, it seemed worthwhile to perform a comparative investigation of the effects on the nervous and immune systems resulting from subchronic low-dose exposure of rats to PR and MP or their combination.

2. Materials and methods

2.1. Substances

Methyl parathion (*O*-dimethyl-*O*-4-nitrophenyl-phosphorothioate) of 98% purity was purchased from Claus Huth GmbH (Hamburg, Germany) and propoxur (2-1-methylethoxy-phenol methyl carbamate) of 99.4% purity was the generous gift of Bayer AG (LeverKusen, Germany). Lyophilized complement and sheep red blood cells (SRBC) were supplied by HUMAN Serum Production and Medicine Manufacturing Co. Ltd., (Budapest, Hungary) and RPMI 1640, by Chemical Co. (St. Louis, MO, Sigma USA). The other materials were from REANAL Factory of Laboratory Chemicals (Budapest, Hungary).

2.2. Animals and treatment

Four-week-old male Wistar rats (obtained from Research Institute of Laboratory Animals, Gödöllő, Hungary) were used for the experiments. The animals were kept under standard conditions (22°C, 80% humidity, 12 h light/dark cycle). Rodent food and water were accessible ad libitum. Two groups of 10 animals each [one for Plaque-forming cell (PFC) assay—see below—and one for behavioral and electrophysiological tests and organ weight determination] were assigned for each treatment variation (Table 1). Methyl parathion was dissolved in distilled water and propoxur in sunflower oil and were given separately (within 10 min) via gavage, for 6 weeks, in a 5 days per week schedule at a volume of 1.0 mL/kg.

2.3. Behavioral investigations

The animals' behavior was tested after 2 and 6 weeks of treatment. Open field (OF) activity was investigated in 10-min sessions. Horizontal, vertical, and local motility and speed of movements were detected by arrays of infrared LEDs and sensors (ACTIFRAME, Gerb Electronic, Berlin, Germany). The sessions were between 9.00 AM and 2.00 PM, before the daily treatment. Illumination at the floor of the OF was 10 lux, with ca. 30 dB white background noise.

The acoustic startle response (ASR) and prepulse inhibition were measured following the OF test, on the same day, using ResponderX (Columbus Instruments, Columbus, OH, USA) equipment. The parameters of the eliciting stimulus were 5000 Hz, 110 dB, and 200 ms; for the prestimulus, they were 1000 Hz, 50 dB, and 500 ms. Ten stimuli per session were applied in a random sequence with intervals between 10 and 15 s.

2.3.1. Rotarod

For the evaluation of motor performance, a standard commercial rotarod for rats (Rota-Rod L 8500, LSI LETICA, Barcelona, Spain) was used. The tests were performed at the 2nd and 6th weeks in maximally 10-min durations.

2.4. Neurotoxicological investigations

Cortical electrical activity was recorded 1–3 days after the last behavioral test. The rats were anesthetized with urethane (1000 mg/kg i.p.) and placed in a stereotaxic frame. The skull over the left hemisphere was opened and silver electrodes were placed on the primary somatosensory and auditory centers (Par1 and Tel areas, respectively; Zilles, 1982). Wounds were sprayed with 10% lidocaine and the exposed dura was covered with warm paraffin oil. Thirty minutes later, sensory evoked potentials were recorded using 50–50 stimuli at a repetition rate of 1 Hz. Somatosensory electric stimulation was performed by electrodes pricked into the whiskery part of the nasal skin, delivering 3- to 4-V, 0.05-ms square pulses. For auditory stimulation, clicks (40 dB, 2 ms) of a small earphone put into the ear of the animals were used (for a complete description of the electrohysiological methods, see Dési and Nagymajtényi, 1999).

The conduction velocity of a peripheral nerve (the tail nerve) was measured according to Miyoshi and Goto (1973) with the modification that the recording was made at room temperature (21–22°C) instead of 37°C. The relative and absolute refractory periods were measured according to Anda et al. (1984). All electrophysiological recording and analysis were carried out with NEUROSYS 1.11 software (EXPERIMETRIA, Budapest, Hungary). The latency and duration of the averaged sensory evoked potentials were measured off-line (see Fig. 4). Once the above electrophysiological investigations, were completed, the rats were sacrificed with an overdose of urethane. The Weights of the brain, liver, heart, lung, kidneys, spleen, thymus, and adrenal glands were determined.

2.5. PFC assay

The animals were immunized with 2×10^9 SRBC in 0.2 mL PBS ip on the 38th day of treatment. Four days later, the spleen was removed and the PFC number (calculated for 10^6 cells and for the whole spleen) was determined (Institóris et al., 1995a).

2.6. Statistics

To check the normality of distribution the Kolmogoroff–Smirnov test was used. Equality of variance was checked by Bartlett's test. Univariate ANOVA ($n = 10$) was performed following square root or $\log_x$ transformation; in the case of nonnormal data distribution the Kruskal–Wallis ANOVA was used. Intergroup differences were checked by subsequent Dunnett's test.

3. Results

3.1. General toxicology

Treatment with MP and PR had no statistically significant ($P < 0.05$) effect on the body weight gain of the animals; all deviations from the control were below 5%. The high PR dose significantly decreased the relative liver weight (g/100 g body weight) versus the control ($P < 0.05$), while its combination with MP(L) (low MP dose) resulted in a significant increase in the liver weight versus that with PR(H) (high PR dose) (Fig. 1, top). The combination PR(H) + MP(L) also increased the relative kidney weight vs control ($P < 0.05$) (Fig. 1, bottom).

3.2. Behavioral effects

3.2.1. Open field

Most of the OF effects of the agents were more expressed after 2 than after 6 weeks of treatment (Fig. 2). Rats treated with PR exhibited a dose-dependent significant hyperactivity (increased horizontal ambulation) in the OF on the 2nd week (Fig. 2, top). In case of MP, hyperactivity induced by the high dose was less than that by the low dose (U-shaped relationship). The effect of the combinations was dominated by the high dose component; no synergism was seen. The changes of the OF central area exploration were very similar (Fig. 2, middle). Vertical exploration (rearing) was increased nearly twofold (Fig. 2, bottom) and was also more expressed after 2 than after 6 weeks of treatment. In the latter two parameters, MP(L) seemed to intensify the effect of PR(H), but this effect was below significance. The effect on defecation, another variable of OF behavior, was inconclusive.

3.3. Acoustic startle response

The change in the number of positive motor responses (i.e., startle reactions) was more expressed after 6 than after 2 weeks of treatment (Fig. 3, top). PR dose-dependently increased the number of positive responses, but the PR(H) + MP(L) combination caused a great decrease. The effect of MP was an increase (with inverse dose-dependence), and the effects of MP(H) and MP(H) + PR(L) were nearly identical. The amplitude of ASR (proportionate to muscle force exerted) was, after 6 weeks, increased in nearly all treated groups (Fig. 3, middle), but none of the changes was significant.

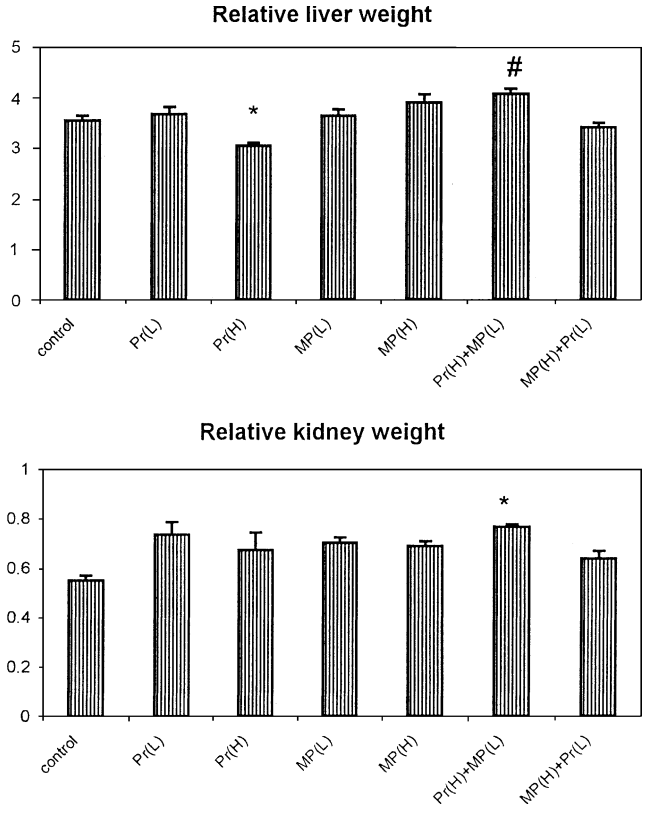

Fig. 1. Effect of propoxur (PR), methyl parathion (MP), and the combinations on the relative weight of the liver (top) and the kidneys (bottom) ($X \pm$SE, $n = 10$). Groups are denoted as stated in the text. $^{*}P < 0.05$ vs untreated control; $\#P < 0.05$ vs high-dose internal control. Treatments were for 6 weeks.

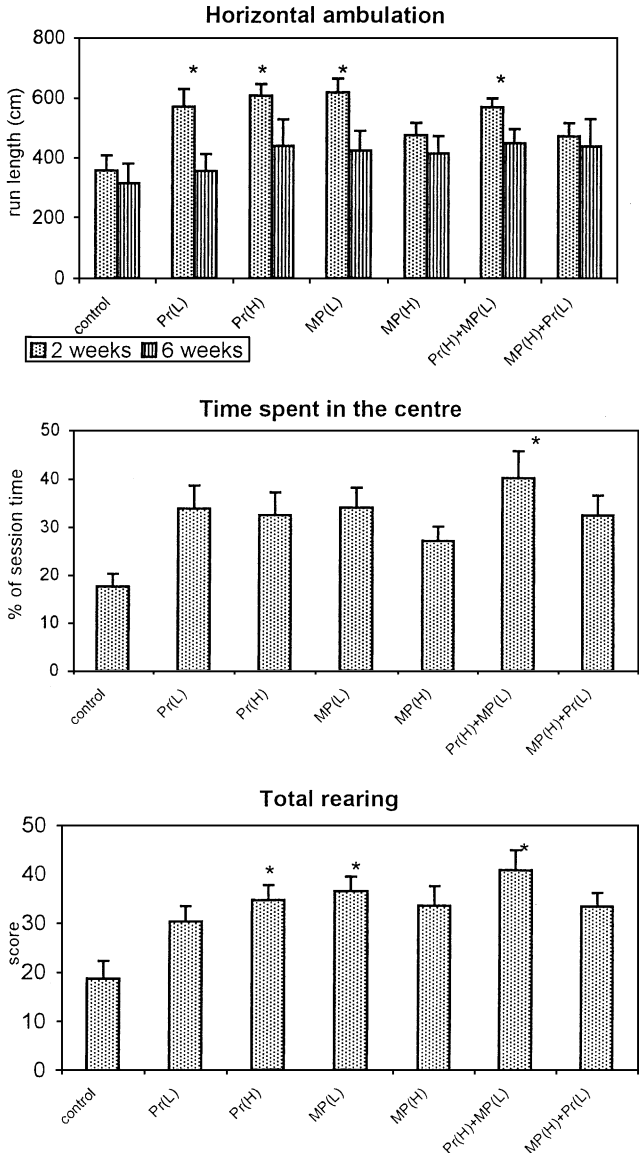

Fig. 2. Effect of propoxur (PR), methyl parathion (MP), and the combinations on parameters of the open field activity: distance run in horizontal ambulation (top), time spent in the center (middle), and total number of rearings (bottom). Significance is as in the legend to Fig. 1; treatment times are indicated by the bar patterns.

There was no clear dose-dependence, the effect of MP(H) + PR(L) was equal to that of MP(H), and the PR(H) + MP(L) combination had no visible effect vs that of the control. The effect of prepulse inhibition was moderate and was not uniform in the different groups: compared to the amplitudes without prepulse (Fig 3, bottom vs middle) the effect was minimal in the controls, and the response amplitude decreased in the PR groups and increased in the MP groups. The change among the animals with combination treatment was similar to that in the MP-treated animals. Within the prepulse results (Fig. 3, bottom) the ASR amplitude in both MP-treated groups was significantly higher than that in the controls.

There were no consistent changes in the rotarod performance of the animals following 2 or 6 weeks of treatment with any of the agents or combinations.

3.4. Neurophysiological effects

3.4.1. Evoked potentials

The somatosensory cortical evoked potential showed, after averaging, an onset latency of 4.96 ± 0.54 ms (Fig. 4, top). Treatment with both agents caused an increase of the latency (Fig. 4, bottom), whereby the effect of PR was stronger and, with the high dose, significant ($P < 0.05$). The effect of MP was less strong (below significance) and had inverse dose-dependence. The effect of the combinations was approximately equal to that of the lower doses, indicating an absence of synergism. The changes of the auditory cortical evoked potential were insignificant.

3.5. Tail nerve action potential

PR, MP, and the combinations had no significant effect on the conduction velocity and refractory periods.

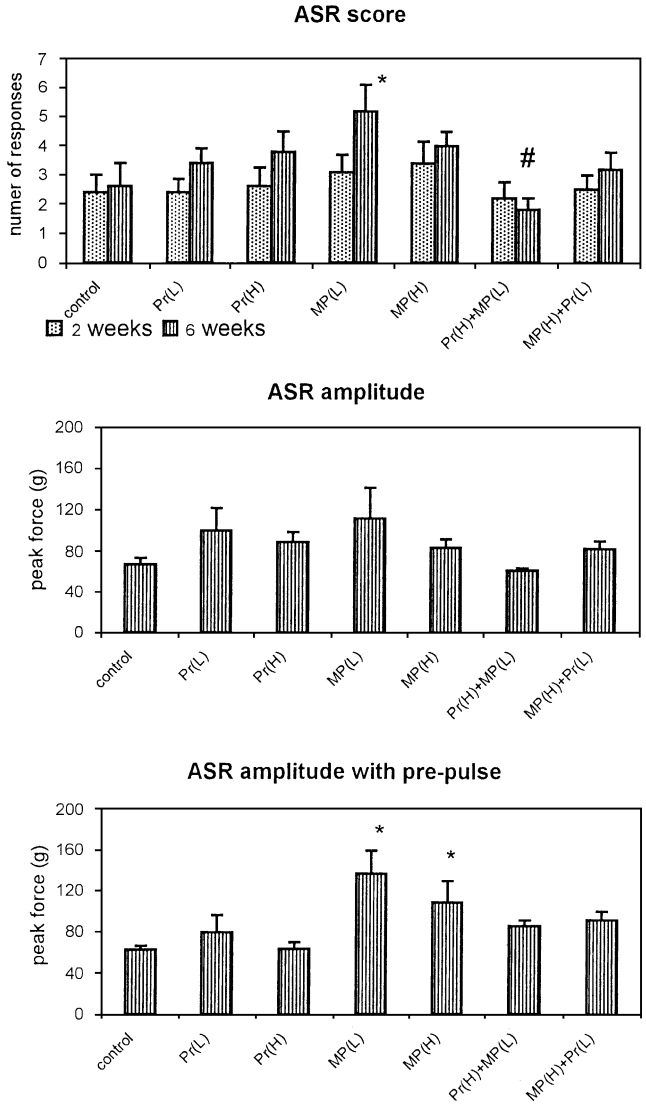

Fig. 3. Effect of propoxur (PR), methyl parathion (MP), and the combinations on parameters of the acoustic startle response: number of responses to a series of 10 stimuli (top), and amplitude of the motor response without (middle) and with (bottom) prepulse inhibition. The levels of significance are the same as those in the legend to Fig. 1.

3.5.1. PFC assay

Similar to those used for behavioral and neurophysiological tests, there were no noteworthy group differences in the body weight of the animals used for PFC assay during the 6 weeks of treatment. A significant decrease was observed in the number of PFCs related to 10^6 spleen cells in the PR(H) + MP(L)- and MP(H) + PR(L)-treated groups ($P<0.05$ vs control), and the former group also differed from the PR(H) internal control ($P<0.05$). When the PFC count was related to the whole spleen, a marked and significant decrease was found in the PR(H) + MP(L) combination ($P<0.05$ both vs control and vs PR(H) dose) (Fig. 5).

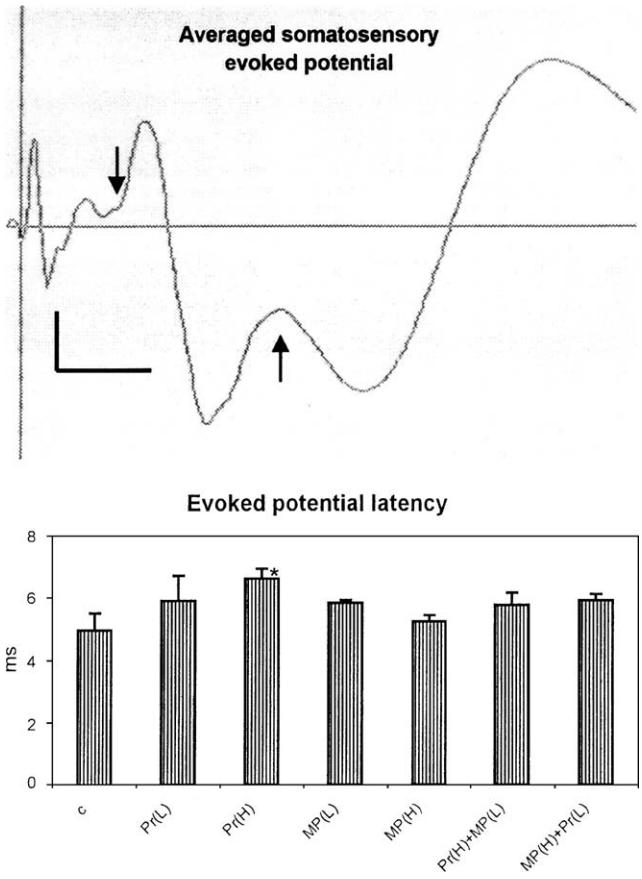

Fig. 4. Top: shape of the averaged somatosensory evoked potential. The onset latency of the fast wave component (the wave between the arrows) was measured between the first arrow and the stimulus artifact. Calibration: 1 mV, 5 ms. Bottom: effect of propoxur (PR), methyl parathion (MP), and the combinations on the latency of the somatosensory cortical evoked potential. The treatment period was 6 weeks; $^{*}P<0.05$ vs untreated control.

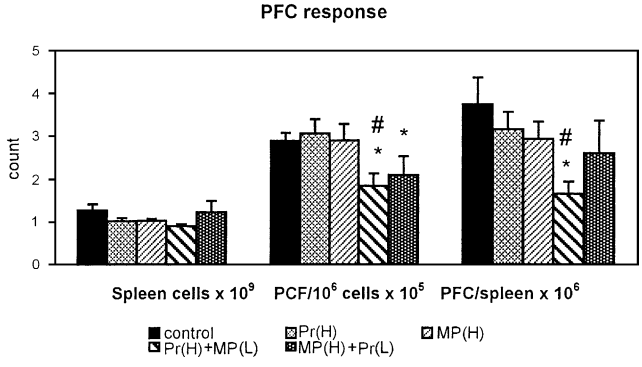

Fig. 5. Effect of propoxur (PR), methyl parathion (MP), and the combinations on the PFC content of the spleen. $^{*}P<0.05$ vs untreated control; $^{\#}P<0.05$ vs high-dose internal control. (Note different scaling factors for each bar group.)

4. Discussion

Methyl parathion is known to cause, beyond cholinesterase inhibition in the blood (Venkataraman et al.,

Table 2
Time dependence of the effects of the two substances on relative liver weight and PFC count, as revealed by comparisons of the results of the present work with those obtained in other studies

	Dose (mg/kg)	Relative liver weight (per 100 g body)		PFC count/10^6 cells ($\times 10^5$)		PCF content of the spleen ($\times 10^6$)	
		This study; 6 weeks of treatment	Siroki et al. (2001), 4 weeks treatment	This study	Siroki et al. (2001)	This study	Siroki et al. (2001)
Propoxur	8.51	3.04±0.06	4.93±0.01	3.06±0.33	2.00±0.19	3.15±0.41	1.52±0.01
	0.851	3.68±0.14	4.43±0.08	3.10±0.35	3.26±0.44	3.36±0.06	2.65±0.05
Control		3.55±0.1	4.34±0.07	2.88±0.19	4.17±0.35	3.74±0.63	2.79±0.02
		This study; 6 weeks of treatment	Ûndeger et al. (2000), 4 weeks treatment				
Methyl parathion	0.872	3.65±0.12	4.25±0.064				
	0.218	3.91±0.16	4.06±0.066				
Control		3.55±0.10	3.88±0.070				

1994) of the central nervous system (Hahn et al., 1991; Kumar and Desiraju, 1992), or after prenatal exposure in the brain of the offspring (Gupta et al., 1986), a number of alterations in various nervous system functions of experimental animals.

In a previous experiment with MP at a largely similar dosing (1/100 and 1/50 LD_{50}, 6 weeks), Schulz et al. (1990) described increased first-minute OF activity and an increased tendency of the treated rats to stay in the center of the field, both effects similar to those described above. Generally, organophosphates (OPs) were found to influence several other elements of behavior in experimental animals, like emotional reactions to stressful stimuli (Gralewicz and Socko, 1997), a novelty response in the open field (Socko et al., 1999), or conditioned taste aversion (Roney et al., 1986).

Propoxur, a member of the carbamate family of insecticide agents, has been described as causing behavioral alterations following intraperitoneal administration. At doses in rats of 2–8 mg/kg (Ruppert et al., 1983) and in mice of 2 mg/kg (Kobayashi et al., 1985), i.e., in the dose range used in the present work, PR influenced motility in general and the frequency of rearing in particular. According to some reports (Sheets et al., 1997) the OF hyperactivity seen with both substances can be due to a causal relationship with the level of cholinesterase inhibition. A decrease in cortical cholinesterase activity after subchronic oral exposure (1/50 LD_{50}, 6 weeks) of rats to OPs, including MP, was reported by Nagymajtényi et al. (1988).

The finding that some of the effects were more pronounced after 2 than after 6 weeks may reflect some kind of adaptation. Such a phenomenon has been mentioned by several authors and various mechanisms have been proposed (Gupta et al., 1986; Milatovic and Dettbarn, 1996).

The acoustic startle response (and prepulse inhibition) is considered a measure of sensorimotor information processing—a mechanism with significant cholinergic modulation (Fendt and Koch, 1999; Sipos et al., 2001). Researchers have found that increased cholinergic activity (as expected, e.g., under the influence of enzyme inhibitors) reduces ASR (Fendt and Koch, 1999). The lesion of cholinergic neurons in the nucleus basalis was found to impair prepulse inhibition (Ballmaier et al., 2001). From our results, the effect of PR (ASR amplitude reduced by the prepulse in PR-treated animals) was in accord with the above (Fig. 3, middle). In the cases of MP and the combinations, no such effect was seen, which may have been due to a direct effect of the metabolite of MP, methyl paraoxon, on the receptors involved (Rocha et al., 1995, 1996).

The general trend of changes in the somatosensory evoked potentials were in line with previous findings of our laboratory (Dési and Nagymajtényi, 1999; Nagymajtényi et al., 1992, 1994), in which the depression (increased latency and decreased amplitude) of cortical evoked potentials was found concomitant with increased spontaneous activity. The latter is under cholinergic modulation, as described, e.g., by Metherate et al. (1992).

In the present study PR(H) decreased the relative liver weight after 6 weeks. In an earlier experiment, 4 weeks of treatment with the same doses of PR (Siroki et al., 2001) resulted in an increased liver weight (Table 2). With MP(H), the relative liver weight increased minimally in the present study, while Ûndeger et al. (2000) reported a significant increase after 4 weeks of treatment. This indicates a possible time-dependent effect of PR and MP treatment on the liver. Similar time dependence was observed in the PFC response: both PFC/10^6 spleen cells and the PFC content of the

spleen decreased significantly following a 4-week (Siroki et al., 2001) but not after a 6-week administration of PR(H) in the present study. In the dose range applied, MP had no effect on the PFC content of the spleen, which is in accord with data in the literature (Crittenden et al., 1998; Institóris et al., 1995a; Street and Sharma, 1975; Ündeger et al., 2000).

Both liver weight and PFC response indicated an interaction between PR(H) and MP(L) (Figs. 1 and 5). When given alone, a higher MP dose (1 to 6 mg/kg) administered to mice for 28 days per os did not modify the humoral immune response (Crittenden et al., 1998). In respect of the liver weight, no histological changes were detected in the livers of rabbits following 8 weeks of oral treatment with 0.04–1.5 mg/kg MP (Street and Sharma, 1975). This indicates that the interaction present in our results is probably a kinetic one (i.e., metabolic interference of the two agents) and, except for the cholinesterase enzyme, does not involve a common target in the liver or in the immune system. In some of our previous works, insecticide agents were combined with various heavy metals (another group of major environmental contaminants). In those studies (Institóris et al., 1999, 2000, 2001) interactions were detected in several toxicological outcomes. This time it was found that the noneffective dose of MP can influence the toxicity of the effective dose of PR in combined exposure. A possible common conclusion is that in combined exposure the minimum effective level (the smallest dose causing a detectable effect in the test system used) of the single components—a parameter heavily relied upon in regulatory toxicology—can be altered.

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