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# ESTERASES ACTIVITY AND PARAOXON TOXICITY IN RAT
# AKTIVNOST ESTERAZA I TOKSIČNOST PARAOKSONA KOD PACOVA

**Abstract**

The purpose of the folowing investigation was to investigate the degree of inhibition of acetylcholinesterase, butyrylcholin-esterase and carboxylesterase, as posible indication of the relative importance of these enzymes as a potential protective mechanism during prolonged paraoxon exposure of rats.

**Key words:** Acetylcholinesterase, Butyrylcholinesterase, Carboxylesterase, Paraoxon.

**Izvod**

Cilj ovog istraživanja je da se odredi stepen inhibicije enzima acetilholinesteraze, butirilhilinesteraze i karboksilesteraze za vrijeme produženog tretiranja pacova paraoksonom i ispita važnost ovih enzima kao zaštitnih mehanizama toksičnosti.

**Ključne riječi:** Acetylcholinesterasa, Butyrylcholinesterasa, Carboxylesterasa, Paraoxon.

## INTRODUCTION

One of the most important reasons for the long-standing popularity of the organophosphorus (OP) compounds as insecticides is the fact that, unlike the organochlorine insecticides which preceded them, the OP insecticides are usually nonpersistant in the environment and they do not typically bioaccumulate. Their efficancy as insecticides has afforded them great utility even today after over 30

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years of use. Frequently, however they have high acute toxicity levels, and therefore do pose a threat of accidental poisoning during occupational handling (**Savage** et al., 1988).

The acute toxicity of organophosphorus compounds in mammals is usually attributed to their irreversibile inhibition of acetylcholinesterase (AChE; EC3.1.1.7), an enzyme that terminates the action of acetylcholine in the nervous system. The increase of acetylcholine at holinergic synapses resulting from the inhibition of AChE, paticulary in brain and diaphragm, produces a variety of pharmacological effects that culminate in death by respiratory failure (**Holmstedt**, 1959).

Other serine active site enzymes such as the carboxylesterase (EC3.1.1.1) and butyrylcholinesterase (BuChE; EC 3.1.1.8) can also react with OP compounds. Binding to and the resulting inhibition of CarbE and BuChE, which in the short-terme is not life treatening, may serve as a means of detoxifying in vivo by reducing the free concentration of OP compound, available to inhibit AChE (**Chambers and Chambers**, 1990).

Although the function role of these enzymes is unknown, they can play important part in detoxication processes of OP compounds. This is supported by studies showing that the toxicity of organophosphates can be potentiated by inhibition of carboxylesterase (**Bošković,** 1979; **Clement**, 1984) and tolerance to soman develops, when plasma carboxylesterase recovers during chronic exposure (**Sterri** et al., 1981).

Parathion (diethyl-p-nitrophenyl phosphorothioate), a widely use organophosphorus insecticide, is involved in large number of acute poisonings of agricultural workers every year (**Murphy**, 1986). Parathion is bioactivated in vivo via oxidative desulfuration to forme its oxygene analogs and active metabilite paraoxon (diethyl-p-nitrophenyl phosphate) (Neal, 1967) which is potent inhibitor of the enzymes and is responsible for the cholinergic crisis typical of parathion poisonings.

The purpose of the following investigation was to investigate the contribution of CarbE and BuChE, as so-colled false target, to OP toxicity. Finally, the degree of inhibition of brain and diaphragm AChE in comparison to, plasma CarbE and BuChE was expected to give an indication of the relative importance of these enzymes as a potential protective mechanisms during prolonged paraoxon exposure of rats.

## MATERIALS AND METHODS

### Materials

Paraoxon (diethyl-p-nitrophenyl phosphate), iso-OMPA (tetraisoprophyl pyrophosphoroamide), and acetylthiocholine iodide were purchased from Sigma

Chemicals Company, St. Louis, MO, USA. All chemicals were either analytical or reagent grade.

**Animals**

Male Spraque-Dawley rats weighing 200-250 g were used in this study. They were haused four per cage in a temperature (22+1 C), humidity (50-10%), and light (12h light/12h dark cycle) controlled room with free access to pellet food (Rodent Laboratory Chow 5001, Purina Mills, Inc., St. Louis, MO, USA) and were acclimatized to our laboratory conditions for at least 48 hours before being used in these experiments.

**Treatment**

Paraoxon was dissolved in deionized water and injected daily at doses of 0.12 mg/kg body weight, subcutaneously (sc) in the back of the nack. The volume of injections was kept constant, 100um/100 g body weight for all treatments. Groups of animals (consisted of 3-5 rats) were injected for a period of 5, 10 or 20 consecutive days and sacrificed 24 hours after the last treatment. Two groups of animals were injected only once and sacrificed 1 or 24 hours after treatment. Animals were observed for cholinergic hyperactivity, salivation, diarrhea, fasciculations, and tremors. Body weight of all animals was recorded prior to each daily injection. The mean body weight changes (+SD) was determined from all rats in a given group.

**Enzyme preparation**

Animals were sacrificed by decapitation. The diaphragm muscle was removed and freed from extraneous tissues. Muscles were minced with a razor and homogenized in 50 vol. (w/v) 50 mM phosphate buffer (pH 8.0) with a Politron for 30s, followed by sonication with a Branson cell-disrupting Sonifier for 15s. The brain without cerebellum was quickly isoleted on ice after sacrifice and homogenized with a Potter-Elvehjem type homogenizer using a glass receptacle and Teflon pestle. Brain was homogenized in 20 vol. (w/v) of ice-cold 50 mM sodium phosphate buffer (pH 8). The muscle and brain homogenates were passed through double cheese-chloth to remove connective tissues and AChE was solubilized with 0.5% Triton X-100. At the time of decapitation, blood was collected in heparinized tubes. Plasma was separated by centrifugation and stored at -70° C.

**Determination of enzyme activity**

Acetylcholinesterase activity was determined by measuring the rate of hydrolisis of acetylthiocholine iodide ($3x10^{-4}$ M) in 0.1 M sodium phosphate buffer (pH 8) according to the method of Ellman et al. (1961). Substrate was incubated with brain (2.5 mg wet tissue) or muscle (5 mg wet tissue) preparation in total volume of 3.2 ml. The rate of increase of absorbance (410 nm) was recorded for 5

min after equilibration in the temperature-controled (25 $^0$C) cuvette compartment of a Beckman DU-8 Spectrophotometer. Correction was made for non enzymatic hydrolisis of substrate.

Butyrylcholinesterase (BuChE, EC 3.1.1.8) activity was measured in plasma using a similar procedure used for AChE activity determination exept that the butyrylthiocholine iodide ($3x10^{-2}$ M) was the substrate used.

Carboxylesterase (EC 3.1.1.1) activity was measured in plasma using the method by Ljungquist and Augustinsson (1971). The assay mixture contained enzyme preparation (about 0.5 mg/ml plasma protein) in 0.1 M Tris-HCl buffer (pH 7.8) and 0.5 mM p-nitrophenylacetate (NpAc) in total volume of 3.0 ml. The liberation of p-nitrophenol was followed spectrofotometricaly at 400 nm (25 C) with destilled water in the reference cuvette. The molar extinction coefficient of 17000 $M^{-1}$ $cm^{-1}$ was used to calculate the specific activity.

Protein content was determined in brain, diaphragm and plasma of rats using the method by Bradford (1976). Bio-Rad protein kit was used, and was consisted of colored complex Coomassie briliant blue and protein standard, liofilized albumine from bovine plasma. The absorbance (595 nm) was recorded at a Beckman DU-8 Spectrofotometer.

### Statistical analysis

Means and standard deviations of values, determined for tissues from groups of three to five animals were calculated. The Student t-test was applied to determine statistical significance between means. Values were considered significantly different when $p<0.05$. Computer program was used to perform linear regression on the absorbance change to generate values for plots and determine protein contents of samples.

## RESULTS

### Signs of poisoning.

The subcutaneous administration of paraoxon (0.12 mg.kg) to rats induced slight gross behavioral changes like grooming and chewing following the third injection. Animals showed onset of signs of cholinergic hyperactivity such as hypersalivation, muscular fasciculation and tremors following the fourth consecutive dose. The severity of the signs was increased during days 6-9. During further application of the same amount of paraoxon, however, the toxicity signs became weaker and were absent by day 12. Rats appeared normal throughout the remainder of the injection period (20 days), having received an accumulated dose of 2.4 mg of paraoxon.

**Growth rates.**

Animals in paraoxon-treated groups demonstrated significantly reduced growth rates when compared with control animals (Fig. 1).

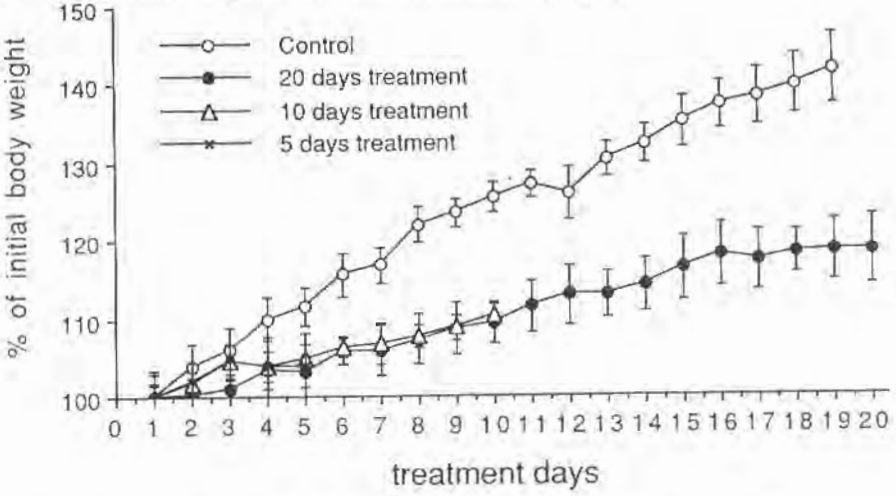


Fig. 1 Effect of subacute administration of Paraoxon (0.12 mg/kg)on growth rate in rats
Sl. 1 Uticaj subakutnog tretmana paraoksona (0,12 mg/kg) na brzinu rasta pacova

During the first five days treated animals grew 93% as fast as did the control animals. During the next five days, paraoxon treated animals grew at slower rate, 87% of control. After the 19th day, these animals grew at rates which were only 84% of the control rate.

**Biochemical data.**

Protein concentrations and acetylcholin-esterase activity of brain and diaphragm homogenates are summarized in Table 1 and Table 2.

Tab.1-Protein Content and In Vivo Effect of Acute and Subacute Paraoxon Treatment on Acetylcholinesterase Actovity from Rat Brain

*Tab. 1-Sadržaj proteina i aktivnost acetilholinesteraze mozga pacova akutno i subakutno tretiranih paraoksonom*

| Treatment | Protein (mg/ml) | Activity (umolACTh/g/h) | Spec. activity nmolACTh/mg pr./h |
|---|---|---|---|
| Control | 104.00 ± 5.4 | 685.41 ± 68.63 | 109.84 ± 10.46 |
| 1 hour | 101.6 ± 4.2 | 557.93 ± 38.38* | 91.52 ± 6.05*** |
| 24 hour | 109.6 ± 4.0 | 555.67 ± 33.07* | 84.50 ± 4.85*** |
| 5 days | 97.0 ± 4.8 | 205.55 ± 10.61*** | 35.32 ± 1.74*** |
| 10 days | 117.6 ± 7.6 | 144.56 ± 8.57*** | 20.49 ± 1.14*** |
| 20 days | 104.6 ± 5.4 | 141.18 ± 7.98*** | 22.50 ± 1.21*** |

Tab.2-Protein Content and In Vivo Effect of Acute and Subacute Paraoxon Treatment on Acetylcholinesterase Activity from Rat Diaphragm

*Tab.2-Sadrzaj proteina i aktivnost acetilholinesterase dijafragme pacova akutno i subakutno tretiranih paraoksonom*

| Treatment | Protein (mg/ml) | Activity (umolACTh/g/h) | Spec. activity nmolACTh/mg pr./h |
|---|---|---|---|
| Control | 115.20 ± 11.2 | 116.82 ± 11.78 | 11.90 ± 1.21 |
| 1 hour | 103.6 ± 9.6 | 96.56 ± 2.37 | 15.53 ± 1.82 |
| 24 hour | 105.6 ± 20.0 | 91.48 ± 6.50* | 14.40 ± 0.86* |
| 5 days | 119.6 ± 1.2 | 68.33 ± 9.45** | 9.52 ± 1.30*** |
| 10 days | 130.8 ± 7.2 | 50.26 ± 5.79** | 6.40 ± 0.70*** |
| 20 days | 127.6 ± 2.8 | 47.44 ± 3.68*** | 6.20 ± 0.47*** |

Protein content of diaphragm preparations was 10% higher than brain. However, specific activity was 6.5 times greater in brain than in diaphragm of the control rats. Paraoxon administered in single dose caused a significant change in activity for brain and diaphragm AChE. After 5 days of treatment, AChE activity were reduced to 30% and 58% for brain and diaphragm, respectivly. Maximum reduction of AChE activity was observed on day 20 and the decrease in activity of brain AChE was 80% while in diapharagm it was 60%.

Effects of prolonged treatment with paraoxon and acute treatment with iso-OMPA on BuChE activity from rat plasma are shown in Table 3.

Tab.3-Protein Content and Effect of Acute and Subacute Paraoxon and iso-OMPA Treatment on Butyrylcholinesterase Activity from Rat Plasma

*Tab.3-Sadržaj proteina i aktivnost butirilholinesteraze plazme pacova akutno i subakutno tretiranih paraoksonom i iso-OMPA*

| Treatment | Protein mg/ml | Activity umolACTh°/g/h | Specif. activity nmolACTh°/mg pr./h |
|---|---|---|---|
| Control | 50.31 ± 3.41 | 2.57 ± 0.19 | 100 |
| 1 hour | 45.87 ± 1.23 | 2.43 ± 0.29 | 94.67 |
| 24 hour | 48.3 ± 0.83 | 2.62 ± 0.29 | 101.97 |
| 5 days | 51.7 ± 1.50 | 1.75 ± 0.28 | 68.22 |
| 10 days | 54.53 ± 1.10 | 1.59 ± 0.93 | 62.07 |
| 20 days | 47.68 ± 1.91 | 1.60 ± 0.15 | 62.27 |
| iso - OMPA | 49.28 ± 3.50 | 0.46 ± 0.34 | 18.01 |

Xdose about eight fold the acute

3 * p <0.050 **p<0.01 ***p<0.001

Acute paraoxon treatment did not cause a significant change in BuChE activity when examined 1h or 24h after the injection. After 5 days of treatment, BuChE activity was reduced to 68% of control and further changes were not seen during the treatment. Single application of iso-OMPA, which is known as a selective inhibitor of BuChE, caused more than 80% inhibition of plasma BuChE.

Table 4 summarizes the data which present CarbE activities from rat plasma after paraoxon and iso-OMPA treatment. Single paraoxon treatment caused a significant change in CarbE activity when examined 1h but not 24h after the injection. A progressive inhibition of this enzyme activity was seen when administration was continued for 5 and 10 days. During 20 days exposure to paraoxon, CarbE activity recovered to some extent and significant change was not found. Acute treatment with iso-OMPA produced significant inhibition of CarbE activity.

Tab.4-Protein Content and Effect of Acute and Subacute Paraoxon and iso-OMPA Treatment on Carboxylesterase Activity from Rat Plasma
*Tab.4-Sadržaj proteina i aktivnost karboksilesteraze plazme pacova akutno i subakutno tretiranih paraoksonom i iso-OMPA*

| Treatment | Protein mg/ml | Activity umolACTh°/g/h | Specif. activity nmolACTh°/mg pr./h |
|---|---|---|---|
| Control | 50.31 ± 3.41 | 38.70 ± 3.91 | 100 |
| 1 hour | 45.87 ± 1.23 | 31.78 ± 2.49 | 82.11 |
| 24 hour | 48.3 ± 0.83 | 36.27 ± 0.80 | 93.72 |
| 5 days | 51.7 ± 1.50 | 30.80 ± 1.31 | 79.60 |
| 10 days | 54.53 ± 1.10 | 27.31 ± 2.99 | 70.57 |
| 20 days | 47.68 ± 1.91 | 36.94 ± 2.71 | 95.45 |
| iso - OMPA | 49.28 ± 3.50 | 24.40 ± 4.24 | 63.05 |

## DISCUSSION

Acetylcholinesterase at the neuromuscular junction plays an assential role in the removal of acetylcholine from the synaptic cleft. Inhibition of this enzyme modifies neuromuscular transmission, as seen in twich potentiation, fasciculation, muscular weakness and acute necrosis of the muscle fibers (**Dettbarn**, 1984).

The present study demonstrated that prolonged use of paraoxon, which targets AChE, has resulted in tolerance to the typical signs of cholinergic hyperactivity. Rats could tolerate an accumulative dose about eight fold the acute LD50 value when nonlethal dose of paraoxon was given daily over a 20-day

period. The animals sustained apperent normal activity with significantly reduced AChE activity (Table 1-2). The data also demostrate that brain AChE was much more sensitive to inhibition by paraoxon than was diaphragm AChE, during a prolonged treatment. During the initial 5 days of treatment there was a significant decrease in AChE activity in brain and diaphragm as well as CarbE and BuChE activity in plasma. Further administration of paraoxon led to increase of inhibition of AChE but not of CarbE and BuChE activity. Administration of iso-OMPA significantly ($p<0.05$) inhibited CarbE and BuChE activity, reducing plasma activity of these enzymes to 63% and 18% of control, respectivly. From the data presented in Table 4 it is evident that in 20-day treated rats, CarbE activity recovered to some extent compared with the initial inhibition observed on day 5. This suggest that the renewed availability of nonspecificic binding sites for paraoxon may have aforded protection against further inhibition of AChE and contributed to the tolerance development similar to observations confirming the contribution of CarbE to detoxication and to the development of tolerance seen with DFP and other organophosphates (Sterri et all 1981; Clement,1984; Gupta at all. 1985).

The efficasy for detoxication is a function of the relative affinities of the organophosphorus compounds for CarbE and AChE but also of the molar concentration of the enzymes. However, the quantitative importance of BuChE in the irreversibile binding of OP compounds is negligible with the respect to detoxication because the concentration of BuChE binding sites is very low (1-2 nmol). In contrast, estimates of the total number of CarbE binding sites have shown to be over 2000 nmol (**Maxwell** et all., 1987).

The importance of CarbE in tolerance development was established by the administration of CarbE inhibitors (i.e., iso-OMPA or mipafox) which completely abolished tolerance development to DFP (**Gupta** et all., 1985). Pretreatment with iso-OMPA may protect unspecific binding sites without affecting AChE, and therefore more OP compound is available to inhibite AChE activity, representing the role of CarbE as false targer or scavengers in regard to organophosphate (**Dettbarn** and **Gupta**, 1989).In summary, CarbE represents a significant alternative phosphorylation site for OP compounds and are capable of appreciably reducing the concentration of OP available to inhibit the primary critical target, AChE. Efficancy of detoxication is a function of relative affinities of the OP for CarbE and AChE and, since CarbE is a saturable system, the total acquired OP dosage.

## CONCLUSION

The present study demonstrated that use of paraoxon has resulted in significant inhibition of AChE, BuChE and CarbE enzymes. Prolonged administration of paraoxon led to increase of inhibition of AChE but not of CarbE and BuChE activity. In 20-day treated rats, CarbE activity recovered to

some extent confirming to the contribution of CarbE to detoxication and to the development of the tolerance.

Among the diverce biochemical reactions involved in detoxication of OP compounds, CarbE performs the role of a high affinity-low capacity detoxication process. Carboxylesterase detoxication contrasts with other detoxication enzymes, such as OP hydrolase, that are high capacity-low affinity enzymes that can detoxify higher concentrations of OP moleculs, but have Km for OP compounds in the mM concentration range. Consequently, CarbE is important for detoxication of highly toxic OP compounds, in which affinity for the detoxication enzyme is more important than detoxication capacity.

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## AKTIVNOST ESTERAZA I TOKSIČNOST PARAOKSONA KOD PACOVA

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### REZIME

Organofosforni insekticidi mogu izazvati razvoj tolerancije na njihovu toksičnost, što se primjećuje kao smanjenje ili nestanak znaka i simptoma toksičnosti za vrijeme subakutne ekspozicije. U ovom radu je ispitan stepen inhibicije acetilholinesteraze, butirilholinesteraze i karboksilesteraze pacova subakutno tretiranih paraoksonom. Akutni tretman pacova paraoksonom uzrokovao je značajno smanjenje aktivnosti ovih enzima. Višednevnim tretmanima ovim organofosfornim jedinjenjem se značajno smanjila aktivnost AChE, ali ne i BuChE i CarbE. Nakon 20 dana tretmana, primjećuje se reaktivacija CarbE, što potvrđuje značajnu ulogu ovog enzima u procesu detoksikacije OP insekticida, kao i moguću važnu ulogu u procesu razvoja tolerancije.