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Effect of Chlorpyrifos on Hepatic Gamma-Glutamyl Transferase, Serum Cholinesterase and Xenobiotic Metabolizing Enzyme Activities in Rats

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Chlorpyrifos (O,O- diethyl-O-[3-5-6-trichloro-2-pyridyl] phosphorothioate) (CAS Registry No. 2921-88-2) is the active ingredient in Dursban and Lorsban insecticides. These insecticides were introduced in 1963 as an active ingredient in organophosphate pesticide formulations for agriculture and household application (Abou-Donia 1995). Chlorpyrifos is metabolized in the liver by microsomal xenobiotic-metabolizing enzymes. These enzymes catalyze the oxidative desulfuration of chlorpyrifos to chlorpyrifos oxon. Chlorpyrifos oxon elicits its toxic effects by phosphorylating acetylcholinesterase, ultimately resulting in failure to hydrolyze acetylcholine neurotransmitter. This cascade of reactions results in accumulation of acetylcholine at both muscarinic and cholinergic receptors (Abou-Donia 1995). Both chlorpyrifos and chlorpyrifos oxon are rapidly hydrolyzed to 3,5,6 trichloro-2-pyridinol by hepatic mixed function oxidases (Sultatos and Murphy 1983). The induction or inhibition of these microsomal xenobiotic enzymes may increase or decrease the toxicity of organophosphorus insecticides depending on the structure of the compound. The hepatotoxicity and hepatocarcinogenic activity of test substances were assessed by the status of gamma-glutamyl transferase (GGT) enzyme (Shukla et al. 1989). Gamma-glutamyl transferase catalyzes the transfer of the gamma-glutamyl group from a gamma-glutamyl peptide to an amino acid or another peptide. This enzyme is widely used as a biomarker in preneoplastic lesions of the liver during chemical carcinogenesis (Peraino et al. 1983).

In the present study, male Sprague Dawley rats were fed chlorpyrifos at 100, 200 and 300 ppm dose levels for 14 days, to determine the effect of this insecticide on liver, liver enzymes and serum enzymes.

MATERIALS AND METHODS

Chlorpyrifos (99% pure) was obtained from Dow Chemical Co, Midland, MI. Dithioerythritol, glycylglycine and gamma-glutamyl-p-nitroanilide were procured from Sigma Chemical Co, St. Louis, MO. Glucose-6-phosphate dehydrogenase, glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (NADP)

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were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals used in these studies were of analytical or reagent grade.

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc. Indianapolis, IN) weighing 150-170 g were housed in standard stainless-steel cages placed in a temperature controlled room (25 ± 5°C) with access to commercial rat feed and water *ad libitum.* Animals were weighed at the beginning and end of the 14 day experimental period. The dietary experiment consisted of thirty-six rats randomly assigned to one control group and three experimental groups. Each group had nine rats. Rats from the control group received chlorpyrifos-free diet, whereas the three experimental groups received chlorpyrifos blended with powdered feed at concentrations of 100, 200 and 300 ppm, for a period of 14 days. At the end of the experiment, feed efficiency was determined by measuring daily feed intake and weight gain ratio. Animals were sacrificed, blood samples were collected and hepatic microsomes were isolated as described earlier (Vodela and Dalvi 1995). The concentration of cytochrome P-450, activities of aminopyrine N-demethylase, aniline hydroxylase and cytosolic glutathione S-transferase, and liver glutathione content were determined as described by Gawai et al. (1992). Serum cholinesterase and serum GGT activity were determined using Sigma diagnostic kit procedures # 418 and # 421, respectively. Microsomal protein concentrations were measured by the biuret method modified to include 0.1 ml of 1% deoxycholate in each sample (Dalvi and Dalvi 1991).

Hepatic GGT activity in homogenate, cytosol, supernatant and microsomes was determined following procedures described previously (Roomi and Goldberg 1981). In brief, the sample (0.5 ml) was incubated with 1 ml of the substrate mixture (4 mM gamma-glutamyl-p-nitroanilide, 40 mM glycylglycine and 11 mM $MgCl_2$ in 185 mM Tris buffer, pH 8.25) at 37° C. After 10 min, 1 ml of 25% trichloroacetic acid was added and mixed. The sample was centrifuged at 3000 x g for 15 minutes, and the absorbance of the clear supernatant fraction was estimated at 405 nm. The enzyme activity was determined using the molar extinction coefficient 9.6 mM^{-1} cm^{-1}. The GGT activity was expressed as nmoles of p-nitroaniline formed/min/mg of protein.

Statistical analysis: Results were statistically analyzed and tested for significance ($P \leq 0.05$) using Student's "t" test.

RESULTS AND DISCUSSION

The calculated average consumption of chlorpyrifos was 89, 57 and 40 mg/kg/d at 300, 200 and 100 ppm dietary levels, respectively. Dietary substitution of chlorpyrifos in rats at 200 and 300 ppm resulted in 100% mortality within a week, whereas at 100 ppm a significant decrease in liver weight (38%), cytochrome

P-450 (39%) and serum cholinesterase (84%) activity was observed; however, there was no significant difference in serum GGT, glutathione S-transferase, aniline hydroxylase and aminopyrine N-demethylase activities as compared to the untreated control values (Table 1).

Clinical signs observed in all treated rats at 100 ppm were weight loss and decreased feed efficiency (24%); however, there were no clinical signs of poisoning or gross pathological changes. The decrease in cytochrome P-450 content in liver of rats in the present study, which was also noticed in single dose studies (Vodela and Dalvi 1995), suggests a chlorpyrifos-induced inhibitory effect on the mixed-function oxidase system. The inhibition of cytochrome P-450 may be due to direct binding of the chlorpyrifos metabolites to cytochrome P-450 or due to liver damage which might have resulted from the toxic burden of this insecticide. Serum GGT, the biochemical marker of hepatotoxicity (Lum and Gambion 1972) was not altered. Hence it is confirmed that the inhibition of cytochrome P-450 was not due

Table 1. Effect of dietary chlorpyrifos on hepatic xenobiotic-metabolizing enzymes and serum GGT and cholinesterase activity in rats

Parameter	**Control**	**100 ppm**
Cytochrome P-450[a]	0.574 ± 0.02	0.352 ± 0.02*
Aminopyrine N-demethylase[b]	5.25 ± 0.49	5.47 ± 0.03
Aniline hydroxylase[C]	0.45 ± 0.03	0.43 ± 0.11
Glutathione S-transferase[d]	2.04 ± 0.18	2.34 ± 0.09
Serum cholinesterase[e]	369 ± 13	60 ± 2*
Serum GGT[e]	4.97 ± 0.55	6.53 ± 1.21

*Mean ± SEM of 9 animals ($P \leq 0.05$)
[a]Expressed as nmoles/mg protein
[b]Expressed as nmoles of formaldehyde formed/min/mg protein
[c]Expressed as nmoles of p-aminophenol formed/min/mg protein
[d]Expressed as umoles of product formed/min/mg protein
[e]Expressed as Sigma Units/liter

to hepatic disease or liver injury. It is likely that the binding of chlorpyrifos metabolites to cytochrome P-450 resulted in the chlorpyrifos-induced inhibition of this hemoprotein.

Gamma-glutamyl transferase activity was measured in homogenate, post-mitochondrial supernatant (9000 g), cytosol(105,000 g) and microsomal fractions of liver to identify the effect of chlorpyrifos on this enzyme on different cellular subfractions. As GGT in liver is localized in the plasma membranes of hepatocytes rather than endoplasmic reticulum (Roomi and Goldberg 1981) we observed higher levels of GGT in liver homogenate, followed by microsomes, supernatant (Table 2), serum (Table 1) and cytosol (Table 2) in rats exposed to control diet. There was no significant difference in the GGT activity in liver homogenate and cytosol between chlorpyrifos-fed rats and control rats; whereas, there was a significant decrease in the post-mitochondrial supernatant and microsomal fractions in the chlorpyrifos-fed rats (Table 2).

Table 2. Effect of dietary chlorpyrifos on gamma-glutamyl transferase activity in rats

Parameters	**Control**	**100 ppm**
Liver homogenate	10.32 ± 0.88	11.97 ± 1.29
Supernatant (9000 g fraction)	5.65 ± 0.74	2.74 ± 0.13*
Cytosol	2.76 ± 0.13	2.12 ± 0.21
Microsomes	7.71 ± 0.28	2.73 ± 0.08*

*Mean ± SEM of 9 animals ($P \leq 0.05$)
GGT activity expressed as nmol of p-nitroaniline formed/min/mg protein

This decrease in microsomal GGT activity is correlated to cytochrome P-450 inhibition. Roomi and Goldberg (1981) also observed a direct correlation between microsomal GGT and cytochrome P-450 levels. Abnormally high levels of GGT were observed in tumors of a variety of tissues including hepatocellular carcinomas (Henigan and Pitot 1985) and malignant squamous cell carcinomas of the skin (Rosalski 1975) in experimental animals. High serum GGT activity has also been

found in cases of cholestasis and acute viral hepatitis (Boelsterili 1979). The decreased cholinesterase activity is the classical indication of organophosphate toxicity, which was also noticed in subsequent studies (Vodela and Dalvi 1995; Pence et al. 1991). The long-term significance of serum cholinesterase depression in the absence of clinical toxicity, however, remains unknown.

These results cannot be directly extrapolated to other organophosphates or other animal species. Whether or not the hepatic metabolism results in net activation or net detoxification of chemicals such as chlorpyrifos depends on the relative rates of the activation and detoxification of the toxic chemical, transit times within the liver and the anatomical localization of enzymes catalyzing these sequential reactions. The factors may vary significantly with different compounds and among different species. Although chlorpyrifos would normally not be fed to rats, the possibility of poisoning may occur due to contamination of feed or litter while dusting or when used as an insecticide. These dietary studies and earlier single dose studies in rats (Vodela and Dalvi 1995) also prove chlorpyrifos to be a mixed-function oxidase system inhibitor, however, it is neither hepatotoxic nor a tumorigenic insecticide in rats fed at 100 ppm for 14 days.

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