Bull. Environ. Contam. Toxicol. (1997) 58:128-134

Environmental Contamination and Toxicology

Lipid Peroxidation and Histopathological Changes in the Digestive Gland of a Freshwater Snail *Planorbarius corneus* L. (Gastropoda, Pulmonata) Exposed to Chronic and Sub-Chronic Concentrations of PCP

G. I. V. Klobučar, J. Lajtner, R. Erben

Department of Zoology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-1OOOO Zagreb, Croatia

Received: 15 April 1996/Accepted: 2 September 1996

The ubiquitous presence of pentachlorophenol (PCP) in our environment, as well as in living organisms, stimulates the need to understand the precise mechanism of its toxicity. PCP and its salts have been extensively used for almost 60 years as algicides, bactericide, fungicides, herbicides, insecticides, and molluscicides with a variety of applications in the industrial , agricultural and domestic fields (WHO 1987). Despite reduction in world production PCP remains in the environment. Particularly high amounts have been found in runoff waters from wood treatment plants and pulp and paper mills (Roy and Hänninen 1994). It has been shown that metabolic breakdown of other pesticides such as hexachlorocyclohexane, hexachlorobenzene, pentachlorobenzene and pentachloronitrobenzene is also a source of PCP (Feind et al. 1988).

When cells are stressed by a xenobiotic or extreme environmental conditions, they undergo a series of often irreversible biochemical and cellular changes and their study can give indications of the degree of a stress of the organism (Viarengo and Canesi 1991). The lipid-rich digestive gland of molluscs plays a central role in digestion and resorption of food. It is also a main site of detoxification, and therefore most exposed to xenobiotics (Winston et al. 1990), especially lipophilic ones such as PCP. Digestive gland is also rich in polyunsaturated fatty acids (PUFA) and therefore very susceptible to oxidative damage from free radical reactions in biological membranes which results in lipid peroxidation (LP) (Halliwell and Gutteridge 1986). Peroxidation of lipids is considered to be one of the primary mechanisms of cell injury by xenobiotics (Ribera et al. 1991: Thomas and Wofford 1993). Watanabe (1993) and his coworkers reported that the reactive oxygen species superoxide and hydroxyl radicals were produced in microsomes of mouse livers and that the LP was related with the hepatic toxicity of PCP *in vivo* and *in vitro.* PCP also induced an increase in the activity of antioxidative system in an aquatic plant *Eichornia crassipes* (Roy and Hänninen 1994). Wenning and Di Giulio (1988) include phenolics in compounds that can induce stronger production of free radicals. LP can also be a consequence of lysosome and cell damage. Damaged cells and tissues are known to undergo increased LP, presumably secondary to membrane disruption by enzymes released from lysosomes and failure of antioxidant mechanisms (Halliwell and Gutteridge 1986).

The purpose of this study was to examine the histopathological effects of PCP on the digestive gland of *Planorbarius corneus,* to determine whether PCP stimulates LP and to distinguish whether the LP precedes or accompanies the cell damage.

Correspondence to: G. I. V. Klobučar

MATERIALS AND METHODS

Adult specimens of the freshwater pulmonate snail *Planorbarius corneus* L. were collected from the Odra River (16° 08' E, 45°41' W). and acclimatized to laboratory conditions in dechlorinated tap water for 48 hr. The specimens used for the study averaged 22±3mm in height. PCP (sodium salt) was obtained from BDH Chemicals Ltd, Poole, England, Stock solutions of PCP were prepared in distilled water.

The experiment was carried out in six aerated glass dishes, each containing five litres of dechlorinated tap water. Based on the results of the preliminary mortality tests two concentrations were chosen: 450 and 800 µg/L. The LC 50 value for 96 hr is 1420 µg/L. The experiment was repeated three times, with parallel controls, for histological and biochemical assays. One dish was used as a control and received dechlorinated tap water only. There were 30 snails per dish. The test solutions and control water were renewed daily (semi-static test). The experiment lasted for 13 d. Temperature and pH of the test solutions and control water were determined every 24 hr by routine procedures (APHA 1985). Physico-chemical characteristics of the test water were as follows: temperature 20-21°C; pH 7.6-8.0: dissolved oxygen 7.0-8.2: hardness 288mg $CaC0_3$/L, The snails were not fed through the assay to avoid any interference derived from possible cyclic changes in cell structure due to intracellular digestion of endocytosed food.

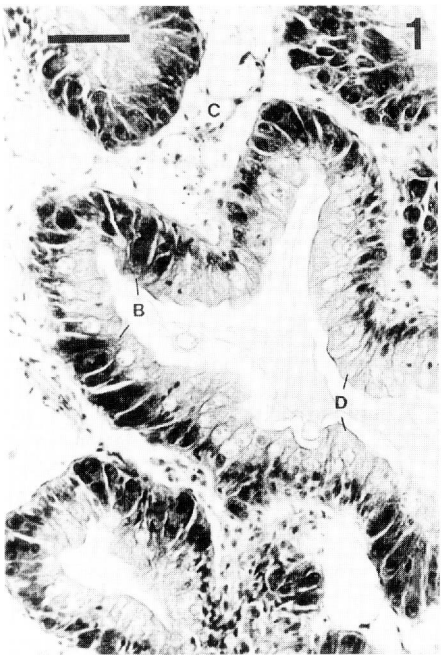
For histological analyses, live specimens were removed from each exposure concentration and control every day. After the shells were removed animals were placed in Bouin's fixative for 24 hr. After fixation, the snails were embedded in paraffin and cut with a microtome into 6 to 8 µm thick slices. The sections were then stained with haematoxylin and eosin.

For the LP measurements digestive glands (on 2, 8 and 13 d) were removed from the visceral mass. damp-dried and aliquots of pooled samples (3-4 animals) were weighed and homogenized using a teflon pestle Potter Elvehjem homogeniser in 100 mM phosphate buffer at pH 7.4 (1:4 w:v). All procedures were carried out at 4°C. The homogenate was centrifuged at 9000 x g for 30 min. A microsomal fraction was separated from the supernatant by ultracentrifuging at 103000 x g for 60 min and made up with 1.5 ml of phosphate buffer (pH 7.4). The samples were analyzed immediately. LP was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in term of malondialdehyde equivalent as described by Ohkawa et al (1979).

Proteins were analyzed by the Lowry method (Lowry et al 1951).
Statistical analysis was performed by using non-parametric Mann-Whitney U test.

RESULTS AND DISCUSSION

A digestive gland of *P. corneus* consists of two differently sized lobules, built of a great number of tubules. The tubules are separated by a thin layer of loose connective tissue and hemolymphatic spaces. The tubule epithelium contains three cell types (Franchini and Ottaviani 1993): the digestive cell, the basophil cell and the mucous cell (Figure l). Digestive cells form the main cell type of the epithelium. They are elongated and their shape varies depending on the stage of digestion. The cytoplasm of digestive cells is lightly vacuolated in distal portions, Vacuoles often contain yellow granules as the result of the terminal stage of lysosomal digestion. The basophil cells are short, triangular in shape, with a broad



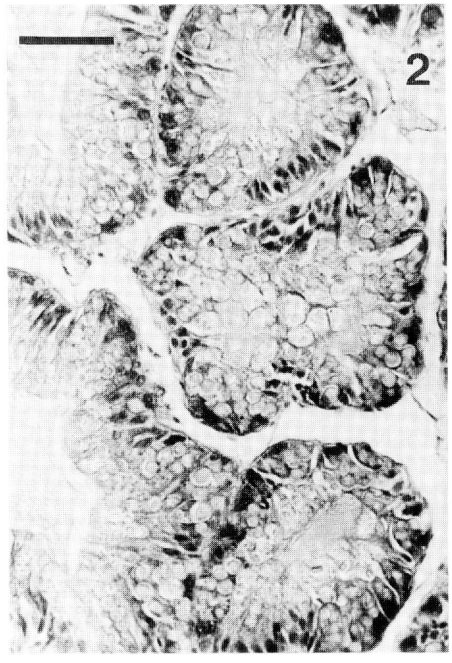


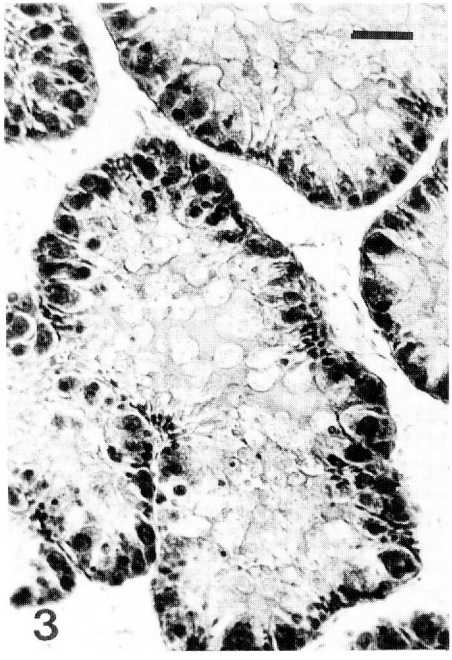
Figure 1. A digestive gland of an untreated snail. Tubules are separated by a thin layer of connective tissue (C) that contains hemolymphatic spaces. The epithelium of tubules consists of digestive cells (D). basophil cells (B) and mucous cells. Scale bar = 50 µm,

Figure 2. A digestive gland of a snail treated with 450 µg/L PCP for 13 d. Increased vacuolation of the epithelium cells is evident. Scale bar = 50 µm.

base. These cells contain large protein granules. There are few elongated mucous cells inserted between the other two cell types. Digestive phases of a digestive gland reflect sequential changes within tubule cells during the dynamic process of intracellular digestion (Saez et al. 1990).

Digestive diverticula of *P. corneus* showed changes first in the 800 µg/L PCP group on the fourth day of the experiment. Increased vacuolisation and granulation of the tubule epithelium cells followed by swelling of digestive cell apices became apparent. Vacuolar degeneration of fish and rat hepatocytes was also observed when these animals were exposed to phenol (Bucher and Hofer 1993) and PCP (Villena et al . 1992).

After six days of exposure to 800 µg/L of PCP, cell borders became less clearly visible. Distortion of the cell membrane, due to the swelling, occasionally resulted in loss of integrity of the apical cytoplasm followed by rupture of epithelial cell apices (Figure 3). Rao and Doughtie (1984) also observed rupture of grass shrimp midgut epithelial cells exposed to PCP. Furthermore, some cells became necrotic and extrusion of cellular contents into the tubule lumen was observed (Figure 4). Dislocation of



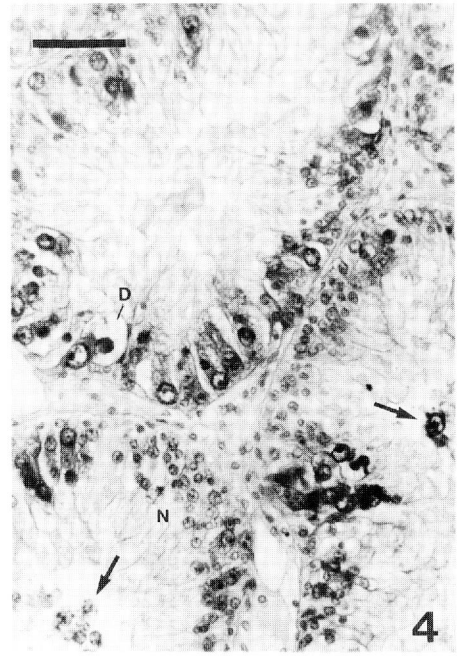


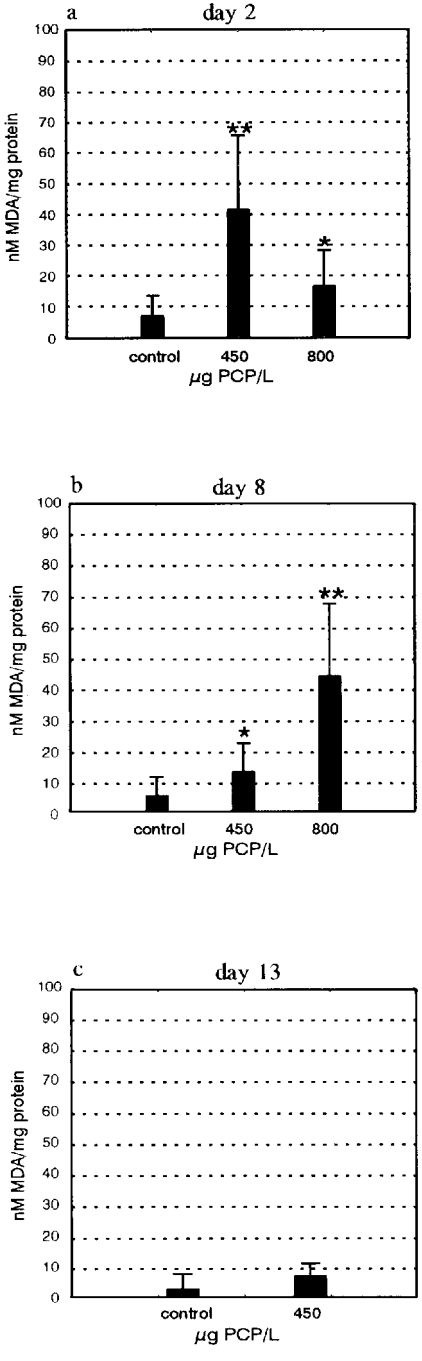
Figure 3. A digestive gland of a snail treated with 800 µg/L PCP for 6 d. Increased vacuolation and swelling is followed by rupture of epithelial cell apices. Scale bar = 50 µm.

Figure 4. A digestive gland of a snail treated with 800 µg/l PCP for 9 d. Breakdown of cell membranes of digestive cells and dislocation of the nucleus (N) is evident. Cell fragmentation are apparent in the lumen of tubules (arrow). Dilated round cells with pyknotic nuclei and general loss of cytoplasmic organization is prominent (D). Scale bar = 50 µm.

digestive cell nucleus, which is an indicator of cell death, was also noticed. Increased number of degenerated, dilated. round cells with pyknotic nuclei and general loss of cytoplasmic organization was also observed (Figure 4). Although it is known that during the digestive process the digestive cell apices are occasionally pinched off in the tubule lumen, (Franchini and Ottaviani 1993) this process was significantly enhanced in the digestive gland of snails treated with 800 µg/L PCP.

Lower concentration (450 µg/1) of PCP induced only slightly enhanced vacuolation on the tenth day of exposure (Figure 2). No additional changes were observed.

Behaviour of the animals exposed to 450 µg/L was unchanged during the whole experiment. Higher concentrations (800 µg/L) were associated with an increased activity of snails. Activity, in time, gradually decreased and complete immobilization was evident prior to death. Egg laying was completely suppressed in all snails treated with PCP.



The content of TBARS was significantly higher in microsomes from the digestive gland of PCP treated snails (Figure 5). TBARS content in the higher concentration of PCP (800 µg/L) was significantly elevated after 2 d (Figure 5a) but was highest when histopathological changes were already evident (Figure 5b), impliying that in this case LP is a late event accompanying cell and tissue destruction. It is well known that PCP causes uncoupling of oxidative phosphorylation by binding to mitochondrial membrane proteins (Weinbach and Garbus 1965) which causes a reduction in the level of adenosine triphosphate (ATP), which in turn stimulates the release of inorganic phosphate. This elicits further glycolysis which results in the accumulation of lactic acid and a concomitant shift toward acidosis (Tjeerdema et al. 1991). PCP becomes more toxic with decrease in pH (Fisher 1990). Deficiency in ATP also inhibits the cation pump of a cell allowing an influx of sodium, chloride, calcium and water into the cell (Fawthrop et al. 1991). Rao et al. (1979) noted inhibition of Ca^{++}-ATPase in the blue crab, *Callinectes sapidus*, hepatopancreas by Na-PCP and DNP under *in vivo* and *in vitro* conditions. In this study, influxes of ions and water caused cellular swelling and damaged cell membranes (Figure 3), which leak intracellular ions, enzymes and other proteins. Low pH inside the cell also stimulates release of lysosomal enzymes causing other histopathological changes (Meyers and Hendricks 1985; Sunila 1987) and subsequently increase in LP (Figure 5b).

Highest concentration of TBARS in lower concentration (450 µM/L) of PCP was observed on the second day of exposure (Figure 5a). Absence of histopathological changes indicate that high LP was not a result of cell damage. Study of Weinbach (1954)

Figure 5. TBA-reactive substances content in the microsomes of digestive gland cells of the freshwater snail *P. corneus* exposed to 450 and 800 µg/L PCP for 2(a), 8(b) and 13(c) days. Statistically significant values from respective control indicated by * P<=0.05 and ** P<=0.01.

showed that PCP exerts biphasic effect in the snail *Lymnea stagnalis* depending on its concentration in the tissue. Higher concentrations of PCP completely inhibit, while lower doses increase oxygen consumption. Increased oxygen consumption, as an initial response of PCP exposed aquatic animals, was also noted in experiments of other researchers (Holmberg et al. 1972; Cantelmo et al. 1978; Rao et al. 1979). Higher oxygen consumption causes a higher rate of oxyradical production. The loss of respiratory control in mitochondria caused by uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol (DNP) leads to an increased oxygen consumption (Stryer 1988). Watanabe (1993) and his co-workers also noticed that the liver of the rats treated with lower concentrations of PCP (less than 100 μM/kg) had the highest TBARS content. Aerobiosis, following a period of anoxia, may also increase oxyradical generation and redox cycling (Wenning and Di Giulio 1988: Winston et al. 1990). It seems that in animals exposed to lower concentrations of PCP the LP is only an initial response, which, in time, is reduced (Figure 5c) by the activation of compensatory adaptive mechanisms (antioxidant) (Regoli and Principato 1995).

In summary, the results demonstrate that the chronic and sub-chronic exposure of snail *P. corneus* to the chronic concentrations of PCP results in histopathological changes and induction of LP in the microsomes of a digestive gland, suggesting that reactive oxygen species are involved in the toxic manifestation of this toxicant.

Acknowledgments. The authors gratefully acknowledge the collaboration of Prof. Dr. R. Klepac, Department of Biology. School of Medicine, University of Zagreb. The study was supported in part by Grant No 1-08-334 from the Croatian Ministry of Science and Technology.

REFERENCES

APHA (1985) Standard methods for the examination of water and wastewater, 16th ed. Amer Pub Health Assoc. New York

Bucher F, Hofer R (1993) Histopathological effects of sublethal exposure to phenol on two variously pre-stressed populations of bullhead *(Cottus gobio* L.) Bull Environ Contam Toxicol 51:309-316

Cantelmo AC, Conklin PJ, Fox FR, Rao KR (1978) Effects of sodium pentachlorophenate and 2,4-dinitrophenol on respiration in crustaceans. In: Rao KR (ed) Pentachlorophenol: Chemistry, pharmacology and environmental toxicology, Plenum Press, New York p 307

Fawthrop DJ. Boobis AR, Davies DS (1991) Mechanisms of cell death. Arch Toxicol 65:437-444

Feind D, Zieris FJ, Huber W (1988) Effects of sodium pentachlorophenate on the ecology of a freshwater model ecosystem. Environ Pollut 50:211-223

Fisher SW (1990) The pH dependent accumulation of PCP in aquatic microcosms with sediment. Aquat Toxicol 18:199-218

Franchini A, Ottaviani E (1993) Histochemical and ultrastructural study of the digestive gland of the freshwater snail *Planorbarius corneus* L. (Gastropoda, Pulmonata). Anim Biol 2:191-198

Halliwell B, Gutteridge JMC (1986) Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. Arch Biochem Biophys 246:501-514

Holmberg B, Jensen S, Larsson A. Lewander K. Olsson M (1972): Metabolic effects of technical pentachlorophenol (PCP) on the eel *Anguilla anguilla* L. Comp Biochem Physiol 43:171-183

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Meyers TR, Hendricks JD (1985) Histopathology. In: Rand GM, Petrocelli SR (eds) Fundamentals of aquatic toxicology. Hemisphere Publishing

Corporation, Washington, p 283
Ohkawa H, Ohishi N. Yagi K (1979) Assay for lipid peroxidases in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358
Rao KR, Fox FR, Conklin PJ, Cantelmo AC, Brannon AC (1979) Physiological and biochemical investigations of the toxicity of pentachlorophenol to crustaceans. In: Vernberg WB, Thurberg FP, Calabrese A, Vernberg FJ (eds) Marine pollution: Functional responses. Academic Press. New York, p 307
Rao KR, Doughtie DG (1984) Histopathological changes in grass shrimp exposed to chromium, pentachlorophenol and dithiocarbamates. Mar Environ Res 14:371-395
Regoli F, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis* exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat Toxicol 31:143-164
Ribera D, Narbonne JF, Michel X, Livingstone DR, O'Hara S (1991) Responses of antioxidants and lipid peroxidation in mussels to oxidative damage exposure. Comp Biochem Physiol 100C:177-181
Roy S, Hänninen O (1994) Pentachlorophenol: Uptake/elimination kinetics and metabolism in an aquatic plant, *Eichhornia crassipes.* Environ Toxicol Chem 13:763-773
Saez V. Marigomez JA. Angulo E, Moya J (1990) Histomorphology and histochemistry of the digestive gland of *Littorina littorea* L. in relation to experimental tidal conditions, food availability, and digestion. Zool Jb Anat 120:185-196
Stryer L (1988). Biochemistry. WH Freeman and Company, New York
Sunila I (1987): Histopathology of mussels *(Mytilus edulis* L.) from the Tvärminne area. the Gulf of Finland (Baltic sea). Ann Zool Fenn 24:55-69
Thomas P, Wofford HW (1993) Effects of cadmium and Aroclor 1254 on lipid peroxidation, glutathione peroxidase activity, and selected antioxidants in Atlantic croaker tissues. Aquat Toxicol 27:159-178
Tjeerdema RS, Fan TW-M, Higashi RM, Crosby DG (1991) Sublethal effects of pentachlorophenol in the Abalone *(Haliotis rufescens)* as measured by in vivo ^{31}P NMR spectroscopy. J Biochem Toxicol 6:45-56
Viarengo A, Canesi L (1991) Mussels as biological indicators of pollution. Aquaculture 94:225-243
Villena F, Montoya G, Klaasen R, Fleckenstein R, Suwalsky M (1992) Morphological changes on nerves and histopathological effects on liver and kidney of rats by pentachlorophenol (PCP). Comp Biochem Physiol 101C:353-363
Watanabe S, Miyasaka K, Yamamoto K, Kawauchi S (1993) Production of active oxygen species and lipid peroxidation by the metabolism of pentachlorophenol. Jpn J Toxicol Environ Health 39:534-542
Weinbach EC (1954) The effect of pentachlorophenol on oxidative phosphorylation. J Biol Chem 210:545-564
Weinbach EC, Garbus J (1965) The interaction of uncoupling phenols with mitochondria and with mitochondrial protein. J Biol Chem 240:1811-1819
Wenning RJ, Di Giulio RT (1988) Microsomal enzyme activities, superoxide production, and antioxidant defenses in ribbed mussels *(Geukensia demissa)* and wedge clams *(Rangia cuneata).* Comp Biochem Physiol 90C:21-28
WHO (1987) Environmental Health Criteria 71: Pentachlorophenol. World Health Organization, Geneva
Winston GW. Livingstone DR. Lips F (1990): Oxygen reduction metabolism by the digestive gland of the common marine mussel, *Mytilus edulis* L. J Exp Zool 255:296-308