Bull Philipp Biochem Soc 9:11-15 (1990)

Superoxide Generation in Catfish Liver Microsomes Treated with Menadione

Armando A. Andaya

Department of Biology, De La Salle University
2401 Taft Avenue, Manila, Philippines 1004

The study investigated the possible involvement of oxygen free radicals in mediating the toxicity of menadione (2-methyl-1,4-naphthoquinone) in channel catfish *Ictalurus punctatus* L. under laboratory exposure conditions. It was hypothesized that menadione is metabolized via a flavoenzyme NADPH-cytochrome P-450 reductase resulting in the production of semiquinones which under aerobic condition generates toxic oxygen species including superoxide anions, hydrogen peroxide, and singlet oxygen. The probable existence of this flavoenzyme in catfish liver microsomes was indicated and its possible involvement in the metabolism of menadione was demonstrated. The concentration-dependent rates of menadione-induced cytochrome c reduction and oxygen consumption provided evidences for the presence of superoxide generating system in catfish liver microsomes. These observations validate the claim that oxygen free radicals could be involved, at least in part, in the mode of toxic action of menadione in channel catfish.

Keywords: superoxide, oxygen free radicals, catfish *Ictalurus punctutus* (L)., menadione.

Fish, as a group, is believed to employ the mixed-function oxygenase (MFO) system in the metabolism of endogenous substrates or detoxication of environmental chemicals [5]. The MFO system is found to be practically associated with the microsomal fractions of piscine liver cells [12]. The mono-oxygenase reaction involves the participation of an NADPH-dependent flavoenzyme cytochrome P-450 reductase.

Although the mono-oxygenase reactions constitute important detoxication pathways, the MFO system can be induced by environmental chemicals to form reactive intermediates or electrophilic metabolites that are more toxic than the parent compounds [7]. The stimulation of the flavoenzyme reductase activity could lead to the generation of superoxide anion radical ($O_2^{\cdot -}$) and the production of hydroxyl anion (OH.), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) [6]. These oxygen free radical species are known to cause widespread cytotoxic effects including lesions of DNA, inactivation of enzymes, alteration of cell redox status, and disruption of membrane permeability [8].

Quinones constitute a large group of naturally occurring and synthetic compounds amenable to one-electron reduction via the NADPH-dependent cytochrome P-450 reductase pathway resulting in the generation of $O_2^{\cdot -}$ and production of H_2O_2 [24]. Certain quinone anti-cancer drugs owe their therapeutic effects on the free radical-mediated cytotoxicity of their basic quinone constituent [4,9]. However, several quinone compounds have been shown to possess pesticidal properties [15]. In fact, dichlone (2,3-dichloro-1,4-naphthoquinone) has gained wide acceptance as an effective fungicide [27]. But, much information gaps exist about the toxicity of dichlone and other quinone pesticides and their environmental effects have not been fully elucidated at present. The methylated naphthoquinone menadione (2-methyl-1,4-naphthoquinone) is also known to possess pesticidal properties [19] but it is of relatively low toxicity to mammals including man [22]. Menadione is commonly used for the treatment of hypoprothrombinemia in humans [23]. It had also been demonstrated that the metabolism of menadione involves the generation of $O_2^{\cdot -}$ and the production of H_2O_2 [24].

The present study investigated the probability of implicating free radical mechanisms in mediating the toxic action of quinone pesticides and other quinones on non-target fish species when exposed to ambient levels of these pesticides in contaminated natural waters. More specifically, it was attempted to demonstrate the presence of a superoxide generating system and to estimate the extent of superoxide generation in the microsomal system of the fish liver following interaction with menadione.

MATERIALS AND METHODS

Test compounds. Analytical grade menadione was obtained from Sigma Chemical Corp. (St. Louis, MO). On the day of use, known amount of menadione was dissolved in double-distilled water and added to the reaction mixture to achieve the nominal concentrations tested.

Microsome preparation. Yearling channel catfish (*Ictalurus punctatus L.*) 30-60 g body weight were obtained

from commercial hatcheries. On the day of sacrifice, whole livers were excised from each fish, blotted dry, and weighed. These organs were homogenized in 1:4 w/v ice-cold 0.05 M Tris/0.20 M sucrose buffer by passing 4-5 times a mechanically driven Teflon pestle into a glass homogenizing tube. The homogenate was centrifuged at 4°C for 20 min at 10,000 x g. The supernatant collected were subsequently centrifuged at O°C for 60 min at 105,000 x g to yield a microsomal pellet. The pellet was washed and resuspended in ice-cold 1.15% KCl equal to the original weight of the liver. The microsomal protein was determined by the method of Lowry et al. [14]. Microsomes were stored at -70°C in 0.5 ml portions for later use.

Superoxide-generating system. To test the possibility of superoxide generation, it would require the specific demonstration of the existence of a superoxide generating system in the liver microsome of channel catfish. To achieve this purpose, the activity of the microsomal flavoenzyme cytochrome P-450 reductase was measured according to the method of Phillips and Langdon [20]. This flavoenzyme mediates one-electron reduction of quinones with concomitant release of superoxide. Therefore, it is reasonable to assume that the detection of such flavoenzyme may provide an indirect evidence of the existence of a superoxide generating system in the microsomal fraction of catfish liver. A specific test for the presence of the flavoenzyme-mediated superoxide generating system involves the induction of the reductase activity of the enzyme in the presence of required electron source NADPH which could be inactivated subsequently by the addition of its competitive inhibitor $NADP^+$ [26].

Superoxide generation. The involvement of menadione in stimulating $O_2^{\cdot -}$ generation was evaluated by measuring the activity of the flavoenzyme in the reduction of exogenous cytochrome c [18] and in the augmentation of oxygen consumption in catfish liver microsomes [17]. The difference in the rates of cytochrome c reduction before and after addition of superoxide dismutase (SOD) was taken as an index of $O_2^{\cdot -}$ generation in the presence of menadione. Polarographic measurement of O_2 was made using the YSI oxygen meter provided with a clark-type oxygen electrode (Yellow Spring Instrument Corp., Yellow Spring, OH). Values were reported in nmoles per min per mg protein.

RESULTS AND DISCUSSION

The quinone pesticides including dichlone have been found to be acutely toxic to fish [13], amphibians [27], and several invertebrate species [16]. Recently, Andaya and DiGiulio [3] estimated from probits of kill of yearling channel catfish, LC_{50} values of 720 ug/L and 42 ug/L for menadione and dichlone, respectively. However, the mechanism of toxic action of these quinone pesticides to aquatic species has not received attention.

Earlier investigations indicated that the pesticidal properties of quinone pesticides involves direct enzyme inactivation [19]. Recent studies revealed that dichlone [21] and menadione [17] undergo reduction via the NADPH-cytochrome P-450 reductase pathway to auto-1 oxidizable semiquinones which subsequently generate superoxide anion that dismutates to produce hydrogen peroxide. The cytotoxicity of oxygen free radical species generated during the metabolism of quinones has been documented already. This free radical generating property has led to the assumption that quinone pesticides may initiate similar reactions in fish following environmental exposures to these chemicals. The present study attempted to test the validity of this hypothesis.

Superoxide generating system. To implicate oxygen free radical mediated toxicity of menadione it was first attempted to demonstrate the presence of an NADPH-cytochrome P-450 reductase system in the microsomal fraction of the catfish liver. The presence of this flavoenzyme was indicated by the stimulation of its cytochrome c reduction potential with the addition of NADPH, its electron source, or by the inactivation of its activity in the presence of $NADP^+$, the competitive inhibitor of NADPH.

As shown in Fig. 1, the addition of NADPH to the microsomal fraction of catfish liver induced cytochrome c reduction as indicated by an increased rate of change in optical density of the reaction mixture (0.0663 per min). However, the addition of 50 and 100 uM $NADP^+$ caused a significant progressive decay in cytochrome c reduction

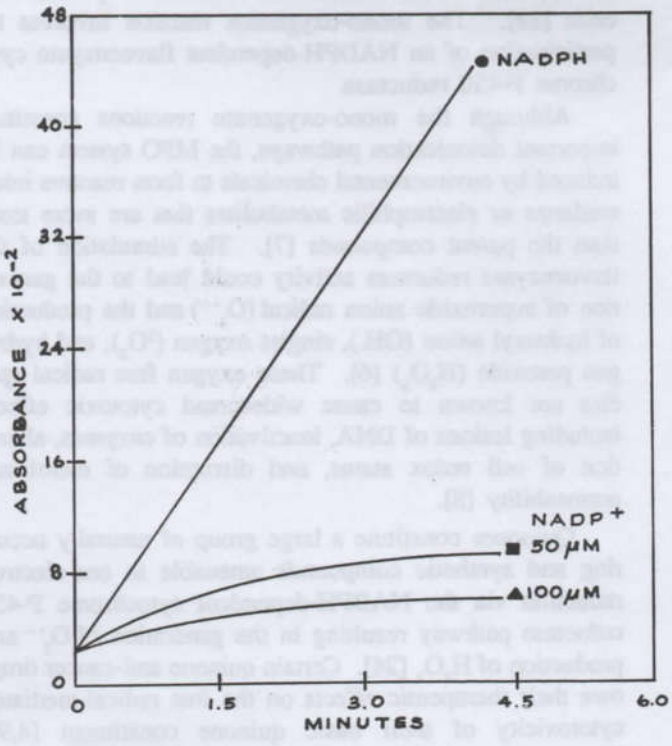

Fig. 1. Effect of $NADP^+$ on the rates of liver microsomal cytochrome C reduction in yearling channel catfish *Ictalurus punctatus* L.

as indicated by decreased optical densities estimated at 0.0251 and 0.0115 per min, respectively. In fact, the reductase activity of the flavoenzyme was reduced by as much as 82.65% of the control value after the addition of 100 uM $NADP^+$. It seems logical to suggest that the allosteric effect of the end product inhibitor is exerted by interfering with the abstraction of reducing equivalent from the substrate decreasing the capacity of the enzyme to channel electroms to cytochrome c.

The specific activity of the microsomal flavoenzyme NADPH-cytochrome P-450 reductase in catfish liver ranged from 17.1-18.9 nmoles per min per mg protein. These values approximated the reductase activity of sexually mature rainbow trout, 14.7 nmoles [25] but underestimated those recorded for the marine species: sheephead, 94.9 nmoles; flounder, 34.0 nmoles; stingray, 49.3 nmoles; and skates, 60.0 nmoles [11]. However, compared to the spiny lobster, 4.3 nmoles and blue crab, 5.2 nmoles [12], the activity of catfish liver flavoenzyme was relatively much higher. In general, mammalian species such as man (78 nmoles) and rat (96 nmoles) have greater activity than aquatic species [2]. It was postulated that the pattern of flavoenzyme activity may reflect the evolutionary development of fish, as a group, compared with their mammalian counterparts [1].

Menadione-induced superoxide generation. Having demonstrated the probable existence of a flavoenzyme which possesses an NADPH-cytochrome P-450 reductase activity it was assumed that the flavoenzyme can catalyze the reduction of menadione to semiquinone intermediates and cause the generation of superoxide anion as semiquinone auto-oxidizes to hydroquinone. Furthermore, the superoxide anion released can in turn lead to the stimulated reduction of cytochrome c when present in the reaction mixture.

Experimental data presented in Table 1 support the assumption. An estimated 50% increase in cytochrome c reduction was realized when menadione was added to the reaction mixture. This observation suggests the occurrence of superoxide-mediated reduction of cytochrome c concommitant to the flavoenzyme-catalyzed metabolism of menadione. Since SOD is a specific scavenger of superoxide anion radical, its addition to the reaction mixture should cause a concentration-dependent inhibition of cytochrome c reduction. By causing the dismutation of $O_2^{\cdot -}$ to $H_2O_2 + O_2$, SOD was able to abstract $O_2^{\cdot -}$ away from its involvement with the reduction of cytochrome c. Since the rate of reduction of cytochrome c by $O_2^{\cdot -}$ occurred at stoichiometric ratio of 1:1, it can be estimated that the amount of $O_2^{\cdot -}$ generated by the microsomal system in the presence of menadione would be equivalent to the degree of inhibition of the reduction of cytochrome c when SOD was added to the reaction mixture. Conversely, an SOD-dependent increase in superoxide anion was obtained at levels ranging from 4.07 to 16.00 nmoles per min per mg protein (Fig. 2).

Table 1. Changes in the rates of cytochrome c reduction in hepatic microsomes of channel catfish treated with menadione. Values are means ± standard deviations of (n) number of determinations.

Additions	Cytochrome c reduction (nmole/min/mg protein)		Percent change over control
cyt c	17.09 ± 2.11	(3)	0.0
MNQ + cyt c	25.64 ± 1.56	(3)	50.03
MNQ + cyt c + SOD			
15 uM/L	21.57 ± 1.27	(5)	15.87
30	17.23 ± 0.34	(5)	32.80
90	13.37 ± 0.81	(5)	47.85
150	9.38 ± 0.74	(5)	63.42
240	9.64 ± 1.27	(5)	62.40

The reaction mixture consisted of 200 uM cytochrome c and 50 uM menadione (MNQ) and specified amounts of superoxide dismutase (SOD) in 0.3 M Na/K phosphate buffer, pH 7.0 in which 0.2 mg microsomal protein was incubated at 24°C. Cytochrome c reduction was calculated from absorbance values recorded at 550 nm and a molar extinction coefficient of 1.9 x 10-4/mM/cm.

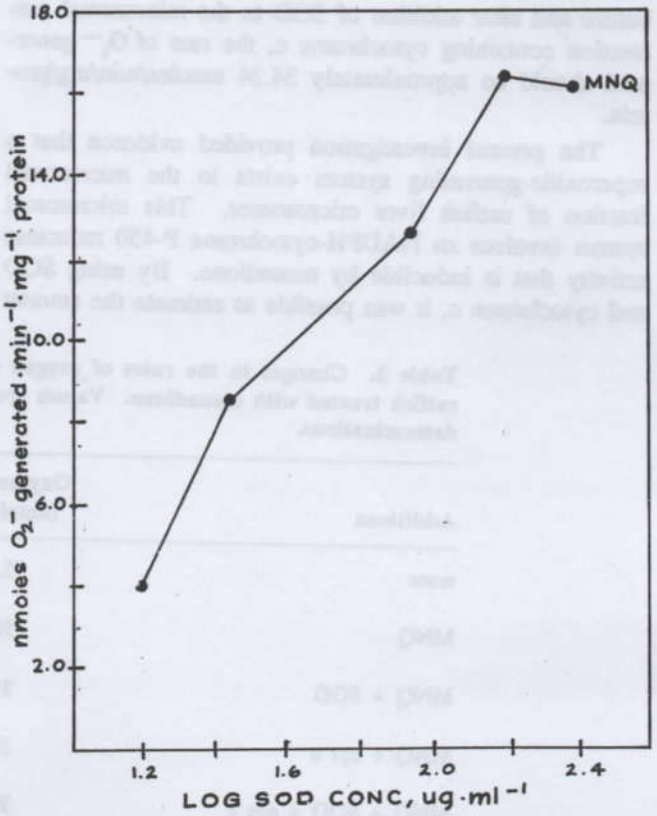

Fig. 2. Superoxide generation in liver microsomes of yearling channel catfish *Ictalurus punctutus* L. following treatment with menadione.

Menadione-induced respiratory burst. Since molecular oxygen is an electron acceptor in the oxidation-reduction reaction involving menadione, it is consequently consumed in the process. As shown in Fig. 3, there was a significant increase in the amount of oxygen taken up by the microsomal system when menadione was added to the reaction mixture.

The consumption of oxygen in the aerobic oxidation of the semiquinone intermediates is revealed by the generation of superoxide anion when menadione was added to the liver microsomal system. The experimental data presented in Table 2 supported this assertion as the addition of either SOD or cytochrome c caused significant decrease in the respiratory rate of menadione-treated microsomes. However, the data suggest that cytochrome c was relatively more effective in inhibiting the respiratory rate of menadione-treated microsomes.

The observed difference in the rates of oxygen consumption may be theoretically expected. Hassan [10] explained that SOD-mediated dismutation of $O_2^{\cdot -}$ utilizes half of O_2 consumed as shown in the equation: $2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$. However, in the superoxide-mediated reduction of cytochrome c, no O_2 consumption occurs: $O_2^{\cdot -} + Fe(III) \longrightarrow Fe(II) + O_2$. Based on these reactions, the amount of $O_2^{\cdot -}$ generated via SOD-inhibitable reduction of cytochrome c should be approximately twice the amount of O_2 consumed. From the data, it can be estimated that with a calculated difference of 17.12 nmoles O_2 consumed before and after addition of SOD to the microsomal suspension containing cytochrome c, the rate of $O_2^{\cdot -}$ generated should be approximately 34.24 nmoles/min/mg/protein.

The present investigation provided evidence that a superoxide-generating system exists in the microsomal fraction of catfish liver microsomes. This microsomal system involves an NADPH-cytochrome P-450 reductase activity that is inducible by menadione. By using SOD and cytochrome c, it was possible to estimate the amount of superoxide anion generation in the menadione-treated microsomes. Thus, it may be concluded that menadione could potentially implicate oxygen free radical-mediated reactions in exerting its toxic effects on yearling channel catfish. This toxicological property may have important ramifications in evaluating the toxicity of pesticides and other environmental chemicals which possess a basic quinone structure.

Acknowledgments. This study was conducted under the supervision of Dr. R. T. DiGiulio of the School of Forestry and Environmental Studies, Duke University, North Carolina. Research funds were provided in part from

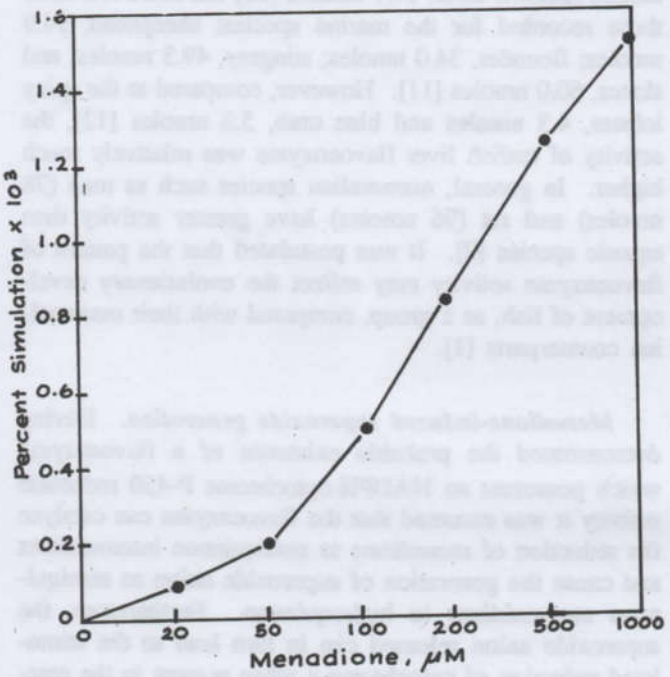

Fig. 3. Effect of menadione (2-methyl-1,4-naphtoquinone) on the rates of oxygen uptake in liver microsomes of yearling channel catfish *Ictalurus punctutus* L.

Table 2. Changes in the rates of oxygen consumption of hepatic microsomes of channel catfish treated with menadione. Values are means ± standard deviations of (n) number of determinations.

Additions	Oxygen consumption rate (nmole/min/mg protein)	Percent Change over control
none	5.66 ± 0.06 (4)	0.0
MNQ	90.06 ± 9.67 (4)	1591.17
MNQ + SOD	38.97 ± 12.15 (4)	56.73
MNQ + cyt c	22.02 ± 0.64 (4)	75.55
MNQ + SOD + cyt c	39.14 ± 11.59 (4)	56.54

Reaction mixture consisted of 10mM KCN, 10mM NADPH, 200 uM cytochrome c, 90 ug/mL SOD, 100 uM menadione (MNQ) in 0.3 M Na/K phosphate buffer, pH 7.0 in which 0.2 mg microsomal protein was incubated at 24oC.

the U.S. Environmental Protection Agency, the University of North Carolina Water Resources Research Institute, and the North Carolina Board of Science and Technology. The author was the recipient of a Fulbright-Hayes Fellowship during the conduct of this study.

LITERATURE CITED

1. Adamson RH. 1967. Drug metabolism in marine vertebrates. Fedr. Proc. Fedn. Am. Socs. exp. Biol. 26:1047-1055.
2. Akohas JT, NT Karki, A Oikari and A Soivio. 1976. Mixed function monooxygenease of fish as an indicator of pollution of aquatic environment by industrial effluent. Bull. Environ, Contam. Toxicol. 16:270-274.
3. Andaya AA and RT DiGiulio. 1987. Acute toxicities and hematological effects of two substituted naphthoquinones in channel catfish. Arch. Environ. Contam. Toxicol. 16:233-238.
4. Bachur NR, SL Gordon and MV Gee. 1978. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res. 38:1745-1750.
5. Chambers JF and JD Yardbrough. 1976. Xenobiotic biotransformation systems in fishes. Comp. Biochem. Physiol. 55:77-84.
6. Ernster L and H Thor. 1982. NADPH-cytochrome P-450 reductase as a superoxide-radical generator. In: Oxygenase and oxygen metabolism. Academic Press, Inc.
7. Fingermann SW, IA Brown, M Lynn and EC Short. 1983. Responses of channel catfish to xenobiotics: induction and partial characterization of a mixed function oxygenase. Arch. Environ. Contam. Toxicol. 12:195-201.
8. Freeman BA and JD Crapo. 1982. Biology of disease. Free radicals and tissue injury. Lab. Invest. 17:412-426.
9. Handa K and S Sato. 1975. Generation of free radicals of quinone group-containing anti-cancer chemicals in NADPH-microsome system as evidenced by initiation of sulfite oxidation. Gann. 66:43-47.
10. Hassan HM. 1984. Exacerbation of superoxide radical formation by paraquat. In Packer L. (ed) Oxygen Radicals in Biological Systems. Methods in Enzymology. Academic Press 105:523-532.
11. James MO and JR Bend. 1980. Polycyclic aromatic hydrocarbon induction of cytochrome P-450 dependent mixed-function oxidase in marine fish. Toxicol. Appl. Pharmacol. 57:117-133.
12. James, MO, MAQ Khan and JR Bend. 1979. Hepatic microsomal mixed-function oxidase activities in several marine species common to coastal Florida. Comp. Biochem. Physiol. 62c:155-164.
13. Johnson WW and MT Finley. 1980. Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. United States Department of Interior. Fish and Wildlife Service. Resource Public. 137. Washington, D.C.
14. Lowry O, NM Rosenbrough, AL Farr and RJ Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
15. McNew GL and HP Burchfield. 1951. Fungitoxicity and biological activity of quinones. Contrib. Boyce Thompson Inst. 16:357-374.
16. Metelev NV, AI Kanaev and NG Dzasokhova. 1983. Water Toxicology. Kolos Publ. Moscow.
17. Misra HP and I Fridovich. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Bio. Chem. 247:3164-3170.
18. Omura T and R Sato. 1964. The carbon monoxide-binding pigment of liver microsomes - I. The evidence for its hemoprotein nature. J. Biol. Chem. 239:2370-2378.
19. Owens RG. 1953. Studies on the nature of fungicidal action. I. Inhibition of sulfhydryl-, amino-, iron-, and copper-dependent enzymes *in vitro* by fungicides and related compounds. Contrib. Boyce. Thompson Inst. 17:221-242.
20. Phillips AH and RG Langdon. 1962. Hepatic triphosphopyridine nucleotide-cyctochrome c reductase: isolation, characterization, and kinetic studies. J. Biol. Chem. 237:2652-2660.
21. Pritsos CA, DF Jensen, D Pisani and RS Pardini. 1982. Involvement of superoxide in the interaction of 2,3- dichloro-1,4-naphthoquinone with mitochondrial membranes. Arch. Biochem. Biophys. 217:98-109.
22. Saxena J, HC Sikka and G Zweig. 1974. Structural and functional changes in mouse fibroblast cells treated with 2,3-dichloro-1,4-naphtohoquinone (dichlone). Pest. Biochem. Physiol. 4:185-200.
23. Sittig M. 1981. Handbook of toxic and hazardous chemicals. Noyes Publications, Park Ridge. N.J.
24. Smith MT, H Thor and S Orrenius. 1981. Formation and inactivation of active oxygen species during drug metabolism in isolated hepatocytes. In: R. Sato and R. Kato. Microsomes, Drug Oxidations and Drug Toxicity. Japan Scientific Press, Tokyo.
25. Stegeman JJ and M Chevron. 1980. Sex differences in cytochrome P-450 and mixed function oxygenase activity in gonadally mature trout. Biochem. Pharmacol. 29:553-558.
26. Strobel HW and Digham JD. 1978. Purification and properties of NADPH-cytochrome P-450 reductase. Methods Enzymol. 5289-96.
27. USEPA. 1981. Pesticide Registration Standard - 2,3-dichloro-1,4-napthoquinone (dichlone). USEPA 540/RS 00-81-001. Washington, D.C.