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THE EFFECT OF THE PESTICIDE, ENDOSULFAN,
ON HEPATIC CYTOCHROME P-450, LIVER AND GILL ULTRASTRUCTURE,
AND SELECTED HAEMATOLOGICAL PARAMETERS OF
OREOCHROMIS MOSSAMBICUS (PETERS, 1852) (PISCES : CICHLIDAE)

by

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ABSTRACT

Two possible ways in which fish may acclimate to prolonged exposure to sub-lethal levels of endosulfan were investigated: induction of the hepatic mono-oxygenase system and changes in haematology. These studies also involved an examination of the ultrastructure of liver and gill tissue. Endosulfan caused none of the characteristic phenobarbital-type inductive effects on the liver of Oreochromis mossambicus. There was no increase in cytochrome P-450 levels, microsomal protein or hepatosomatic index. The reasons for the lack of induction are discussed. The effect of endosulfan on the following haematological parameters was determined: haemoglobin, erythrocyte count, microhaematocrit, mean erythrocyte volume and mean erythrocyte haemoglobin concentration. The only effect was an increase in erythrocyte count. The value of changes in haematological parameters as indicators of pollution-induced anemia in the field is discussed. No changes in gill ultrastructure were noted although electron micrographs of liver tissue showed lesions in hepatocytes. The diagnostic value of pollution-induced pathological changes on cell ultrastructure are discussed. Recommendations are made with regard to future large scale application of pesticides on or near large water bodies.

CHAPTER 1 GENERAL INTRODUCTION

The insecticide endosulfan is generally grouped with the cyclodiene organochlorines (Edwards, 1974; Goebel *et al.*, 1982). As a cyclic sulfite ester it differs from the cyclodiene organochlorines in its chemical, physical and toxicological properties (Maier-Bode, 1968; Goebel *et al.*, 1982). The technically active substance consists of two isomers, A- and B-endosulfan with similar insecticidal properties (Goebel *et al.*, 1982). The insecticidal action of endosulfan is mainly as a contact and stomach poison (Knauf, 1982). Endosulfan is marketed in a number of different formulations which include: technical endosulfan, dusts, granules and emulsifiable concentrates (Maier-Bode, 1968; Goebel *et al.*, 1982).

In contrast to other organochlorine pesticides, endosulfan is readily metabolised by micro-organisms (fungi, bacteria and algae), plants, insects, fish and homeothermic animals (Gorbach, 1982). Accumulation in the fatty tissues of animals is therefore limited. A comparative study with DDT and Dieldrin showed endosulfan to have a concentration factor of below 10^{-3} , whereas that of DDT and Dieldrin was in the order of 12 (Bressau, 1975, in Goebel *et al.*, 1982). Shoettger (1970) showed that accumulation of endosulfan in fish reached a plateau within less than 12 hours. Endosulfan does not give rise to persistent residues in the animal body, in fact residue levels decrease rapidly in insects, fish and mammals when these animals are no longer exposed to the toxin (Maier-Bode, 1968; Shoettger, 1970; Gorbach, 1982).

The persistence of pesticides in soil and water depends on factors such as pH, temperature, chemical composition and biota, and therefore, differs markedly in different localities (Edwards, 1974). A relatively short persistence of 100 to 120 days for endosulfan in soil was reported by Rao & Murty (1980).

Analysis of soil samples from 1500 cultivation areas in the USA indicated that endosulfan does not pose a problem as regards persistence in soil at present dosing levels (Crockett et al., 1974).

Endosulfan also has a low persistence in water. Levels in contaminated water usually decrease to levels below detection in a month or less (Eichelberger & Lichenberg, 1971; Fox & Matthiessen, 1982; Gorbach, 1982). Endosulfan does not, therefore, show a tendency to accumulate in terrestrial or aquatic ecosystems and does not appear to become concentrated in the food chain (Gorbach, 1982). Matthiessen et al. (1982) found residue levels in top predators to be no higher than in the animals they consumed.

Endosulfan, at low dosages (12g ha^{-1}), is relatively non-toxic to non-target insects, mammals and birds (Maier-Bode, 1968; Knauf, 1982). If, however, high levels of endosulfan are sprayed ($500\text{--}1000\text{g ha}^{-1}$) then direct mortality of birds, mammals, reptiles, fish and arthropods results (Koeman et al., 1978). The main concern about endosulfan is that it is toxic to aquatic invertebrates and extremely toxic to fish. Twenty-four hour LC50 values for different species of fish range from $0,09\text{--}11,2\text{ ug l}^{-1}$ (Maier-Bode, 1968; Shoettger, 1970; Fox & Matthiessen, 1982; Knauf, 1982). Even in the Okavango Delta where ultra low volumes ($6\text{--}12\text{ g ha}^{-1}$) are sprayed in an attempt to eradicate tsetse fly, endosulfan-related fish kills have been reported (Douthwaite et al., 1981). The lethal and sub-lethal effects of endosulfan on fish are not well understood, therefore, this study investigates selected toxicological effects of sub-lethal levels of endosulfan on fish.

Matthiessen (1983, pers. comm.) noted that fish appear to become more efficient at metabolizing endosulfan after prolonged exposure. Blood parameters were disturbed at the beginning of the spraying season but began

returning to normal before spraying ceased (Matthiessen, 1981). Likewise, liver lesions lessened in intensity and fish breeding, although delayed, still took place after fish had acclimated to sub-lethal levels (Matthiessen & Roberts, 1982; Matthiessen & Logan, 1983). Matthiessen et al. (1982) found that relative concentrations of A- and B-endosulfan and endosulfan sulphate changed during the spraying season. The ratio of endosulfan sulphate (the first metabolite) to A- and B-endosulfan increased six-fold. This suggests that there may be an increase in the activity, or levels, of the hepatic enzymes involved in endosulfan metabolism.

In mammals the hepatic cytochrome P-450 mono-oxygenase system has received much attention and the role of these systems in the biotransformation of drugs and other xenobiotics (including pesticides) is well established (Mannering, 1981). The hepatic cytochrome P-450 dependent mono-oxygenases occur in lower vertebrates, but have received little attention although they undoubtedly play a similar role in the detoxification of xenobiotics (Schwen & Mannering, 1982a).

In general, the reaction rates in fish hepatic microsomes are much slower than in mammals (Schwen & Mannering, 1982b). Although endosulfan, and other organochlorines, have been shown to induce the mixed function oxidase system in rats (Mannering, 1981; Tyagi et al., 1984), there are inconsistent reports about the effects of different inducing agents on the cytochrome P-450 systems of fish (Schwen & Mannering, 1982c). Therefore, one of the aims of this study was to determine whether endosulfan induces the hepatic cytochrome P-450 mono-oxygenase system of Oreochromis mossambicus (Peters, 1852) and thereby the rate at which endosulfan is metabolised. The implication of inducing the mixed-function oxidase system is that this effectively increases the lethal dose of the pesticide. Therefore, an initial low dose of endosulfan could be

sprayed to induce the mixed-function oxidase system. Thereafter higher doses could be sprayed with a reduction in fish mortality.

The liver is the main organ of detoxification and is the region in which pesticides are concentrated (Couch, 1975; Walsh & Ribelin, 1975).

Ultrastructural changes in liver tissue may occur either as a result of pesticide damage to hepatocytes (Couch, 1975) or may be associated with the induction of hepatic enzymes (Claude, 1969). As the fine structure of the liver reflects the degree to which the hepatocytes may be impaired and also if ultrastructural changes characteristic of hepatic mono-oxygenase induction are occurring, liver ultrastructure was examined.

Endosulfan is known to affect oxygen consumption (Rao et al., 1980, 1981). Fish in advanced stages of poisoning appear to suffer respiratory stress and "gasp" at the surface. These symptoms are similar to those noted by Powers (1980) for hypoxia. Drewett & Abel (1983) noted similar behaviour in trout exposed to the chlorinated hydrocarbon, Lindane, and hypoxic conditions. Haematological parameters are sensitive indicators of changes in ecological conditions, including pollutants and hypoxia (Silbergeld, 1974; Bansal et al., 1979; Hille, 1982; Natarajan, 1984; Zbanyszek & Smith, 1984). Fish adapt to prolonged hypoxic conditions by increasing the oxygen carrying capacity of their blood: increased haematocrit, increased haemoglobin content, increased blood buffering capacity and an increase in haemoglobin oxygen affinity (Powers, 1980). Associated with these changes are increases in serum lactate levels and a decrease in pH (Powers, 1980). Endosulfan has been shown to cause an increase in erythrocyte counts in the field (Matthiessen, 1981). Therefore, a second aim of this study was to investigate the effect of endosulfan on selected haematological parameters. This would enable us to determine if endosulfan causes similar changes in haematology to

those evolved by fish to acclimate to hypoxic conditions. Pollutants can cause hypoxia in fish by damaging gill tissue, therefore gill histology was also examined. The possible effects of pathological changes in gill tissue on oxygen consumption and osmoregulation are discussed.

This thesis investigates two possible ways in which fish may acclimate to prolonged exposure to low levels of endosulfan: induction of the hepatic mono-oxygenase system, and changes in haematology. These studies involve ultrastructural examination of liver and gill tissue; therefore, the diagnostic importance of these tissues in fish toxicology will also be discussed. Detailed literature reviews on the hepatic cytochrome P-450 dependent mono-oxygenase system, and the effects of endosulfan on fish, are also included in this study.

CHAPTER 2 THE EFFECTS OF ENDOSULFAN ON FISH (A REVIEW)

INTRODUCTION

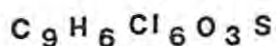
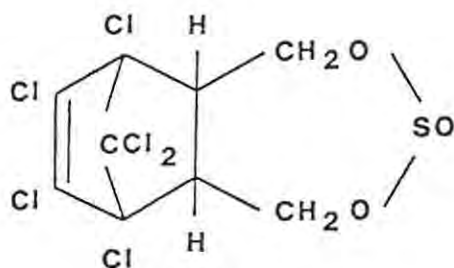
Fish are sensitive to a wide range of pesticides (Edwards, 1974; Brown, 1978). The nature of these chemical effects on fish is variable: apart from causing death, many pesticides have been shown to affect growth rate, reproduction and behaviour (Edwards, 1974; Brown, 1978). Under experimental conditions fish may survive sublethal doses, but in the wild these fish would be more vulnerable to predation, less able to compete with other fish and less able to withstand normal environmental stresses such as seasonal temperature variations, reproduction or temporary starvation (Edwards, 1974). The early life history stages of fish are particularly susceptible to some pesticides and, as survival at this point in the life cycle is minimal for most species, a further decrease in the survival rate may be disastrous for future stocks (Edwards, 1974).

Some pesticides (such as the organochlorines) are very persistent in the environment (Edwards, 1974). This results in their accumulation in body tissues and in food chains with ecologically detrimental effects. This does, however, not appear to be a problem with endosulfan which is a relatively non-persistent organochlorine (Goebel *et al.*, 1982). Although endosulfan is known to be extremely toxic to fish (Maier-Bode, 1968), there is little information available on the effects of sublethal levels on their physiology, pathology and behaviour. In addition the exact mode of action of endosulfan and its isomers is not known. This review groups the research findings to date into seven sections: (1) chemical and physical properties; (2) toxicity; (3) environmental accumulation; (4) accumulation and metabolism in fish; (5) physiological effects; (6) pathological effects, and (7) behavioural

effects.

CHEMICAL AND PHYSICAL PROPERTIES

The structure and chemical formula of endosulfan(I) are given below (Maierbode, 1968; Goebel et al., 1962):



5-norbomene-2,3-dimethanol-1,4,5,6,7,7,-hexachloro-cyclic sulphite (Chemical Abstracts)

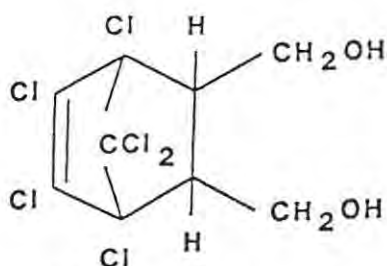
α,β -1,2,3,4,7,7-hexachlorobicyclo-[2,2,1]-heptene-(2)-bis-hydroxymethylene-(5,6)-sulphite

6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a,-hexahydro-6,9,methano-2,4,3-benzodioxathiepin-3-oxide (IUPAC)

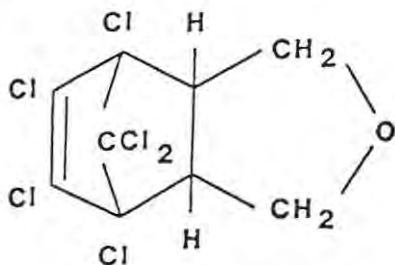
Endosulfan is prepared by the diene synthesis of hexachlorocyclopentadiene and cis-butene-(2)-diol, or by hydrolysis of the Diels-Alder adduct from hexachlorocyclopentadiene and cis-1,4-diacetoxybutene-(2) to endosulfan diol and its subsequent conversion with thionyl chloride to the cyclic sulfite ester. This results in a mixture of seventy percent A-endosulfan and thirty percent B-endosulfan (technical endosulfan). Both these isomers have similar insecticidal properties.

Pure endosulfan consists of colourless and almost odourless crystals. A-endosulfan has a melting point 109°C and B-endosulfan a melting point of 213°C. At 80°C A-endosulfan has a vapour pressure of 10 mm Hg. A-endosulfan has a specific weight of $d^{20}_4 1.745$ and is virtually insoluble in water but soluble in organic solvents. Technical endosulfan has a melting point of 80°-90°C. It is stable in storage and against sunlight. Aqueous or alcoholic alkaline solutions and acids hydrolyse endosulfan to endosulfan diol II.

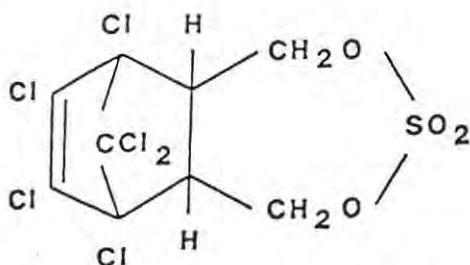
Technical endosulfan is marketed in formulations as an emulsifiable concentrate (EC), a wettable powder (WP), dust, granules and ultra low volume (ULV) formulations with varying concentrations of active substance. The transformation products and metabolites are described by Goebel *et al.* (1982) and are illustrated below:



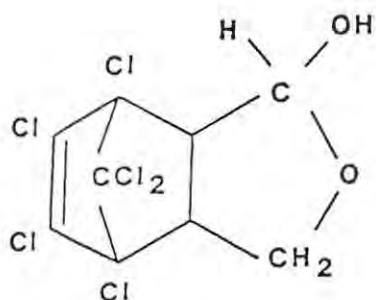
$C_9H_8Cl_6O_2$ Endosulfandiols (II) (endosulfan alcohol)



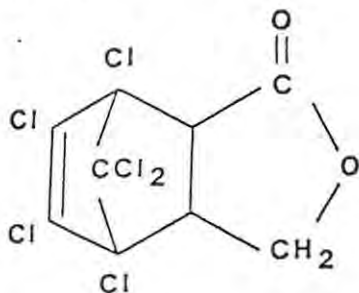
$C_9H_6Cl_6O$ Endosulfan ether (III)



$C_9H_6Cl_6O_4S$ Endosulfan sulphate (IV)



$C_9H_6Cl_6O_2$ Hydroxyendosulfan ether (V)



$C_9H_4Cl_6O_2$ Endosulfan lactone (VI)

Goebel et al. (1982) describe the gas chromatographic methods used for determining the concentration of endosulfan and its metabolites in water and tissue.

TOXICITY

The symptoms of endosulfan poisoning in trout (Salmo gairdneri) and white suckers (Catostomus commersoni) described by Shoettger (1970) are similar to those described by Holden (1974) for fish exposed to lethal levels of other organochlorine insecticides. The fish seem very excitable at first and swim rapidly about the aquaria. Later they surface, lose equilibrium and move with spasmodic jerks. With time most fish sink to the bottom where their opercular movements become erratic. Many of the trout become darker and the suckers mottled. Death follows after a variable period, depending on the concentration of the pesticide and the water temperature. Trout which

survived a 120 h bioassay were placed into fresh aerated water, but they did not survive longer than 5-7 days (Shoettger, 1970). Ludemann & Neumann (1960, in Shoettger, 1970) found similar results with carp (Cyprinus carpio).

Loeb (1963, in Shoettger, 1970) determined the oral toxicity of endosulfan to carp (C. carpio). Technical endosulfan was not toxic in 120 h doses of 119 and 234 mg kg⁻¹, but doses of 49 and 195 mg kg⁻¹ of 24% miscible endosulfan were lethal within 118 and 22 h respectively. A dust form of the insecticide was found to be twice as toxic as the miscible formulation. The lethal oral levels for carp are similar to the lethal doses for rats. Although the doses for carp cannot be compared directly with lethal concentrations in water (discussed later), they suggest that endosulfan is more easily absorbed across gill membranes (the main region of pesticide absorption; Holden, 1974) and oral membranes than by the intestine.

The 24 h LC50 values for different species of fish range from 0.09-11,2 ug l⁻¹ (Fox & Matthiessen, 1982). Fox & Matthiessen (1982) conducted laboratory experiments which showed that the 24 h LC50 values for fish from the Okavango Delta ranged between 1,2-7,4 ug l⁻¹ depending on species (Table 1; also included are the estimated safe levels of endosulfan in the water).

Rao et al. (1980) measured the relative toxicity of technical endosulfan, the two isomers (A & B) and the formulated products (35% emulsifiable concentrate and 4% dust). Their results are shown in Table 2. The toxicity of the compounds to Labeo rohita are given in order of decreasing toxicity : isomer A, 35% EC, technical endosulfan, 4% dust, and isomer B. Devi et al. (1981) found similar toxicity of these chemicals to Channa punctatus. Isomer A is about 20 times more toxic than isomer B. Isomer A poses a greater threat to aquatic systems as it is the most toxic isomer and is less strongly bound to

Table 1. Acute toxicity of endosulfan to various species of Okavango fish (from Fox & Matthiessen, 1982).

	Mean weight (g)	Logarithms of slope and intercept of median lethal time/concentration regression		24h LC50 + 95% confidence limits of regression line at 24 h ($\mu\text{g litre}^{-1}$)	Estimated safe level ($\mu\text{g litre}^{-1}$)
		a	b		
<u>Schilbe mystus</u>	141	3,75	-0,83	5,11 4,17-6,02	0,22
<u>Synodontis</u> spp.	72	3,64	-0,64	5,57 2,14-10,23	0,19
<u>Tilapia sparrmanii</u>	62	3,67	-0,60	7,35 4,07 13,49	0,23
<u>Barbus</u> spp.	0,76	3,22	-0,71	1,22 0,48 2,88	0,05
<u>Aplocheilichthys johnstonii</u>	0,42	3,50	-0,83	2,57 1,90 3,39	0,11

Estimated safe level = 48h LC50 x 0.1 (Sprague, 1971).

Table 2. Relative toxicity of technical endosulfan, the isomers of endosulfan, and two formulated products to Labeo rohita; 95% confidence limits in parentheses (from Rao et al., 1980).

Compound	96h LC50 ($\mu\text{g l}^{-1}$)	Toxicity in relation to technical material
Endosulfan-A	0,33 (0,32-0,34)	3,33 times more toxic
35% EC	1,0 (0,98-1,02)	1,1 times more toxic
Technical endosulfan	1,1 (1,07-1,12)	-
4% Dust	1,25 (1,21-1,30)	0,85 times as toxic
Endosulfan-B	7,1 (7,02-7,23)	0,16 times as toxic

the soil. It is therefore incorporated with runoff water and accumulates in the aquatic environment to a greater extent (Rao et al., 1980).

Temperature

Generally the toxicity of pesticides increases with increasing temperature, although several exceptions in which the reverse is true have been reported (Holden, 1974). The general hypothesis is that respiration, and therefore pesticide intake via the gills, will be more rapid at higher temperatures due to an increased oxygen demand and decreased oxygen solubility (Holden, 1974).

Shoettger (1970) found that the toxicity of endosulfan to rainbow trout (Salmo gairdneri) and white suckers (Catostomus commersoni) is influenced by temperature and length of exposure. The 24 and 120 h TLM's (total mortalities) for trout at 1,5° and 10°C range from 5,9-0,7 ugl^{-1} and 2,1-0,3 ugl^{-1} respectively. The TLM ranges for suckers at 10° and 14°C are 8,1-2,5 ugl^{-1} and 6,6-1,8 ugl^{-1} respectively. In general, the toxicity of endosulfan to these species increases with higher temperatures and longer exposure. The relationship between toxicity and temperature is shown in Figure 1. Toxicity increases significantly up to approximately 72-96 h. This suggests that endosulfan toxicity is associated with an accumulation in excess of the amounts that may be metabolised or stored in lipids. Macek et al. (1969) showed that the effect of temperature decreases with prolonged exposure, indicating that temperature influences the rate of reaction.

Water quality

Shoettger (1970) showed that calcium and magnesium salts do not alter the toxicity of endosulfan to suckers. pH also has no immediate effect on toxicity, but if endosulfan solutions are aged in an alkaline solution then toxicity is decreased. Endosulfan aged in a solution of pH 8,4 is only

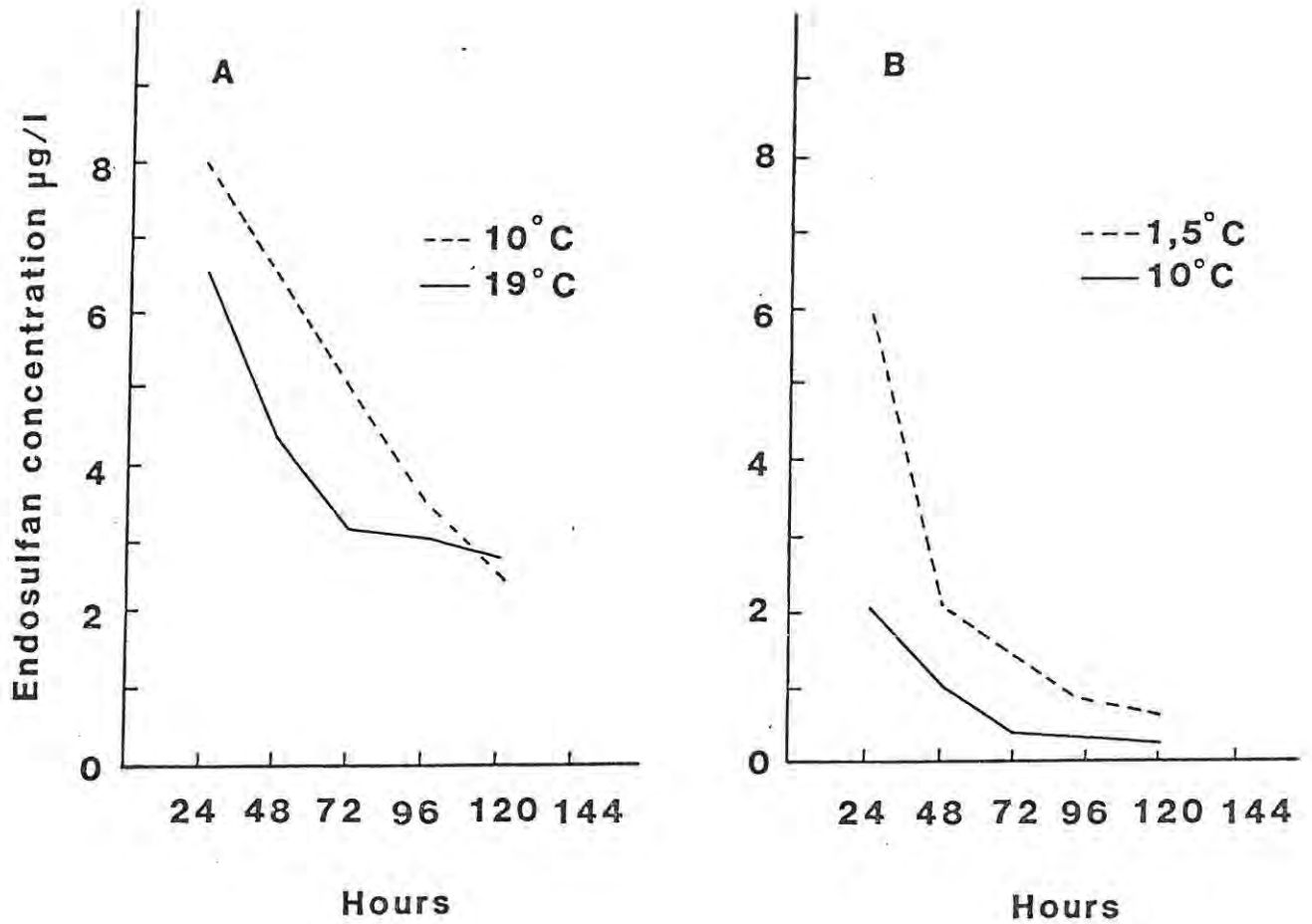


Figure 1. Effects of time and temperature on TLm's of (A) white sucker (*Catostomus commersoni*) and (B) rainbow trout (*Salmo gairdneri*) to endosulfan (from Shoettger, 1970).

slightly toxic after 72 h. Nearly all the toxicant degrades within 24 h at pH 9,4. Ageing endosulfan in an acidic solution has no effect on toxicity.

Fish size

Holden (1974) stated that most investigations on the effect of pesticides on different sizes of fish suggested that susceptibility decreases with increasing size. Holden (1974) also pointed out that increasing the numbers of fish per tank usually increases the LC50 values. This density dependent effect should be taken into account when the susceptibility of different size fish are compared.

Shoettger (1970) found that the susceptibility of goldfish (Carassius auratus) to repeated sublethal concentrations of endosulfan (7 ug l^{-1}) was strongly related to mass. The lightest individuals were the most susceptible. The correlation of fish mass with the number of treatments before death gave a coefficient of 0,89 which suggested an accumulation of endosulfan with time. Larger fish, because of their greater mass, require a longer exposure to attain the same concentration of endosulfan in their tissues as smaller fish. Shoettger (1970) found a further correlation of 0,77 between the lipid content and the number of treatments before death occurred. This indicates that individuals with higher levels of muscle lipids survived the longest. Muscle lipids probably aid in the detoxification of endosulfan by providing a reservoir for its storage. This effectively helps keep pesticide levels below the lethal threshold and allows the chemical to be metabolised slowly (Yarbrough & Coons, 1975). Shoettger (1970) also found a high endosulfan level in the abdominal fat, suggesting that this may also be an important storage region.

Effect of flow

The simplest form of testing toxicity is under static water conditions. However, according to Holden (1974) this technique has a number of disadvantages: some pesticides, particularly the organochlorines, are liable to be lost from the water surface as a result of aeration. Volatile constituents of pesticide formulations are lost, which may change the toxicity of the formulation. A further disadvantage of static conditions is that waste products accumulate in the water, and metabolites (such as ammonia) may contribute to the toxic conditions in the test aquaria. Some pesticides, particularly organochlorines, are also adsorbed to tank surfaces or taken up by bacterial films. Lastly, unless aquaria are very large, and the fish small, oxygen concentrations fall and carbon dioxide concentrations rise, creating further toxic conditions.

In contrast, continuous flow systems allow for the maintenance of near constant concentrations and avoid most of the disadvantages of the static systems. Such systems, however, are more complicated and more expensive to use (Holden, 1974). Most of the published data has been carried out in static systems (Holden, 1974).

Holden (1974) reviewed a number of studies which compared LC50 values in static and continuous flow systems. Generally, the mortality of fish was greater and more rapid in flowing water. Mortality in the static systems was lower because concentrations of pesticides decrease as a result of their being metabolised by fish and adsorbed onto various surfaces in the aquaria.

Toxicity to eggs, fry and fingerlings

According to Holden (1974) the eggs of fish are usually very resistant to toxic substances. However fry of several species have been found to be much

more sensitive than the fingerlings and adults.

Shoettger (1970) found that fertilized rainbow trout eggs were extremely resistant to endosulfan. This resistance may be related to the selective permeability of the egg chorion or to under-development of endosulfan sensitive structures at the time of treatment, or both (Shoettger, 1970). A fish toxicant, Antimycin B (which inhibits oxidative phosphorylation), was tested on eggs from the same batch. At a concentration of $10 \mu\text{g l}^{-1}$ total mortality of eggs occurred indicating that the chorion is permeable to Antimycin B, or that it has a different mode of action (Shoettger, 1970).

Shoettger (1970) found that exposure of rainbow trout eggs (in the 32 cell stage) to various concentrations of endosulfan for 30 or 120 minutes appeared to have no effect on their hatching success. Mortalities of eggs hatched 29 days after fertilization were similar to those observed in control groups.

Berger (pers. comm., in Shoettger, 1970) also found that trout eggs were unaffected by endosulfan, although hatched fry mortalities were high. He found that levels of up to $50\,000 \mu\text{g l}^{-1}$ were not lethal over the 25 day incubation period. However, fry became more susceptible as they grew and absorbed their yolk sacs. Concentrations of 750, 500 and $250 \mu\text{g l}^{-1}$ caused 100% mortality of trout fry after 7, 12 and 20 days respectively.

ENVIRONMENTAL ACCUMULATION OF ENDOSULFAN

Residues in soil

Pesticides reach the soil, through direct applications, or indirectly in rain or dust, or from plant or animal remains which become incorporated with the soil (Edwards, 1974; Brown, 1978). The soil is an environmental reservoir for

these residues from which they move into the atmosphere, water or living organisms. A number of factors influence the persistence of insecticides in soil: these include the chemical stability of the insecticide, its volatility, solubility, concentration and formulation. The persistence of insecticides is further influenced by properties of the soil and its microflora and fauna (Edwards, 1974; Matsumura and Murti, 1982). These properties are discussed in detail below:

(1) The type of soil: in general, insecticides are retained longer in heavier soils and in those with a high organic content (Edwards, 1974). Edwards et al. (1957, in Edwards, 1974) showed that organochlorine insecticides were adsorbed least in sand and by increasing amounts in silty clay loam, light sandy clay loam, coarse silt, silty clay, sandy loam, clay loam and muck. The main influence of soil structure on insecticide persistence is the mechanical composition of the soil.

(2) Organic matter content: this seems to be the single most important factor influencing the persistence of insecticides in soil. Edwards (1974) stated that pesticides are likely to persist much longer in soil with a high organic content.

(3) Clay content: soils which contain clay have a much higher internal surface area than sandy soils. Therefore one would expect insecticides in clay soils to be retained longer as a result of the increased surface area. Evidence available seems to indicate that this is a valid assumption (Edwards, 1974).

(4) pH: the breakdown of organochlorine compounds is not generally affected by pH, although the organophosphate compounds are (Edwards, 1974). Endosulfan has been shown to break down faster in an alkaline solution, but is unaffected

by acidic conditions (Shoettger, 1970).

(5) Mineral ion content: the amounts and kinds of minerals in the soil influence both its type and structure and, therefore, the persistence of insecticides (Edwards, 1974). Adsorption occurs very rapidly in soils with iron (Edwards, 1974). Downs (1951, in Edwards, 1974) showed that those soils which catalysed the decomposition of DDT most effectively had a high iron and aluminium content. Minerals are important in influencing the breakdown of insecticides in soil but there is insufficient evidence to fully assess their role.

(6) Temperature: insecticides are lost from the soil mainly by chemical degradation, bacterial decomposition and volatilisation (Edwards, 1974); all of these processes are influenced by temperature. At low temperatures these processes slow down with a concomitant reduction in insecticide loss. Temperature also influences the adsorption of insecticides in soils because adsorption tends to be exothermic, so that increased temperatures decrease adsorption and release insecticides (Edwards, 1974). Although raised temperatures increase the rate of chemical degradation of pesticides, warm soils are usually also dry ones which hold pesticides much more firmly than wet ones.

(7) Moisture content: the main influence of soil moisture on the persistence of insecticides is by its effect on the adsorption of the insecticides onto various soil fractions (Edwards, 1974). Water can compete for adsorption sites with insecticides as it is a polar molecule which is strongly adsorbed by the soil colloids. In dryer soils there are fewer water molecules to compete with the insecticide molecules for adsorption sites. Soil humidity (directly related to soil moisture), can influence the persistence of

insecticides in three ways. Firstly, it can influence the adsorption of an insecticide; secondly, it can affect the rate at which it diffuses into the soil; and thirdly, it can affect the availability of the adsorbed toxicant (Edwards, 1974). The rate of adsorption from the particulate state decreases as humidity increases. For example, a 10% increase in humidity can double the toxicity of an insecticide in the soil (Edwards, 1974). Harris & Mazurek (1966, in Edwards, 1974) tested the persistence of 34 insecticides and concluded that not all were affected the same way by soil moisture. Some competed strongly with water molecules for adsorption sites whilst others did not. Finally, in soils that are consistently wet, insecticides will persist for a much shorter time than in dry soils (Edwards, 1974).

Gorbach (1982) reviewed the literature on endosulfan residues in soil and concluded that endosulfan has a low persistence in soil. Rao & Murty (1980) showed endosulfan to have a persistence of 100-120 days; this would change subject to the conditions discussed above. Pesticides are also degraded by micro-organisms and sunlight, this is discussed in detail in the next section.

Residues in water

Even the more persistent insecticides, such as the organochlorines, do not remain long in water unless they are carried in suspension adsorbed to particulate matter (Edwards, 1974). Within hours or days of contamination (depending on the factors discussed below), the amounts fall to low background levels (Edwards, 1974). Gorbach (1982) found that in general residue levels of endosulfan had decreased to concentrations below detection within 1 month. Several factors influence the persistence of pesticides in water:

(1) Solubility: the organochlorine insecticides are all relatively insoluble, although they differ in their solubilities, which are all temperature

dependent. Even the more soluble insecticides disappear from water quite rapidly. Evidence suggests that they are gradually taken up by the bottom mud and organic matter (Edwards, 1974).

(2) Mud bottom: laboratory and field studies have shown that organochlorine insecticides (DDT, Toxaphene & Lindane) are quickly concentrated in the bottom mud and vegetation. Different types of mud bottom, like different soil types, can bind more or less insecticide (Edwards 1974). Baily & Hannum (1967, in Edwards, 1974) showed that pesticide concentrations in water are related to the particle size of the sediments, the highest concentrations appearing in the sediments composed of smaller particles. Lotse *et al.* (1968, in Edwards, 1974) supported this conclusion, although little is known about the actual mechanism by which pesticides bind with mud.

(3) Organic matter: insecticides show an affinity for both living and dead organic matter, particularly the lipid portion of such material (Edwards, 1974). Gorbach (1982) found that endosulfan residues decreased faster in water containing organic matter. If the organic matter is floating, the pesticide tends to remain in suspension in the water, whereas if the organic material is in the bottom mud it tends to remove the pesticide from the water above (Edwards, 1974). The relative importance of organic matter in adsorbing and inactivating pesticides in water is not well understood.

(4) Temperature and pH: an increase in temperature increases the rate of insecticide breakdown (Edwards, 1974; Gorbach, 1982). Generally, organochlorine insecticides are little affected by pH, although endosulfan is broken down rapidly in an alkaline solution.

(5) Micro-organisms and sunlight: the two most important degradation forces

operating on pesticides in the environment are micro-organisms and sunlight (Matsumura, 1982). Microbial degradation of pesticides differs from higher animals (Matsumura, 1982). The purpose of metabolic reactions on xenobiotics in higher animals is to convert them to more polar and therefore excretable forms (Mannering, 1981). The mixed-function oxidase system in the liver is the main region of detoxification (Mannering, 1981). In microbes metabolic activities are associated with the production of energy. The metabolic activities of micro-organisms encompass many different types of biological processes. These may be classified in several categories (Table 3) and are covered in detail by Matsumura (1982). The ultra violet portion of sunlight also makes an important contribution to the degradation of pesticides (Matsumura, 1982).

Bacteria, fungi and algae show a high tolerance for endosulfan. No biomass declines were noted even at concentrations of 100 mg kg^{-1} endosulfan (Martens, 1972, in Goebel *et al.*, 1982). Gorbach and Knauf (1971, in Goebel *et al.*, 1982) noted that endosulfan is readily taken up by micro-organisms and metabolised to endosulfan diol (II). A complete degradation scheme of endosulfan using a mixed culture of soil micro-organisms, as compared against degradation in a sterile medium, is given in Figure 2. The half-life periods of the individual metabolites are given in Table 4.

A case study in the Okavango Delta

In the Okavango Delta pesticides reach the water in four ways: (1) direct application by aerial spraying, (2) spray drift from nearby aerial spraying operations, (3) ground spraying operations near water bodies, and (4) surface run-off (Davies & Bowles, 1979; Douthwaite *et al.*, 1981). Direct application of endosulfan spray over water bodies is the main source of pesticide residue

Table 3. General classification of microbial metabolism of pesticides (from Matsumura, 1982).

I. Enzymatic

- A. Incidental metabolism: Pesticides themselves cannot serve as energy sources
1. Metabolism by generally available enzymes
 - a. Metabolism due to generally-present broad-spectrum enzymes (hydrolases, oxidases, etc.)
 - b. Metabolism due to specific enzymes present in many microbe species
 2. Analog-induced metabolism (cometabolism)
 - c. Metabolism by enzymes utilizing substrates structurally similar to pesticides
- B. Catabolism: Pesticides serve as energy sources
- d. Pesticides or a part of the molecule are the readily available source of energy for microbes
 - e. Pesticides are not readily utilized. Some specific enzymes must be induced.
- C. Detoxification metabolism
- f. Metabolism by resistant microbes

II. Nonenzymatic

- A. Participation in photochemical reactions
 - B. Contribution through pH changes
 - C. Through production of organic and inorganic reactants
 - D. Through production of cofactors
-

Table 4. Stability of endosulfan metabolites in sterile nutrient (control) medium, and in medium inoculated with mixed culture of soil micro-organisms (from Miles & Moy, 1979).

	Time for 50% degradation	
	Control	Inoculated
Endosulfan lactone	5,5 h	5,5 h
A-endosulfan	12,5 wk	1,1 wk
B-endosulfan	5,7 wk	2,2 wk
Endosulfan ether	> 20 wk	6 wk
Endosulfan a-hydroxyether	> 20 wk	8 wk
Endosulfan sulphate	> 20 wk	11 wk
Endosulfan diol	> 20 wk	14 wk

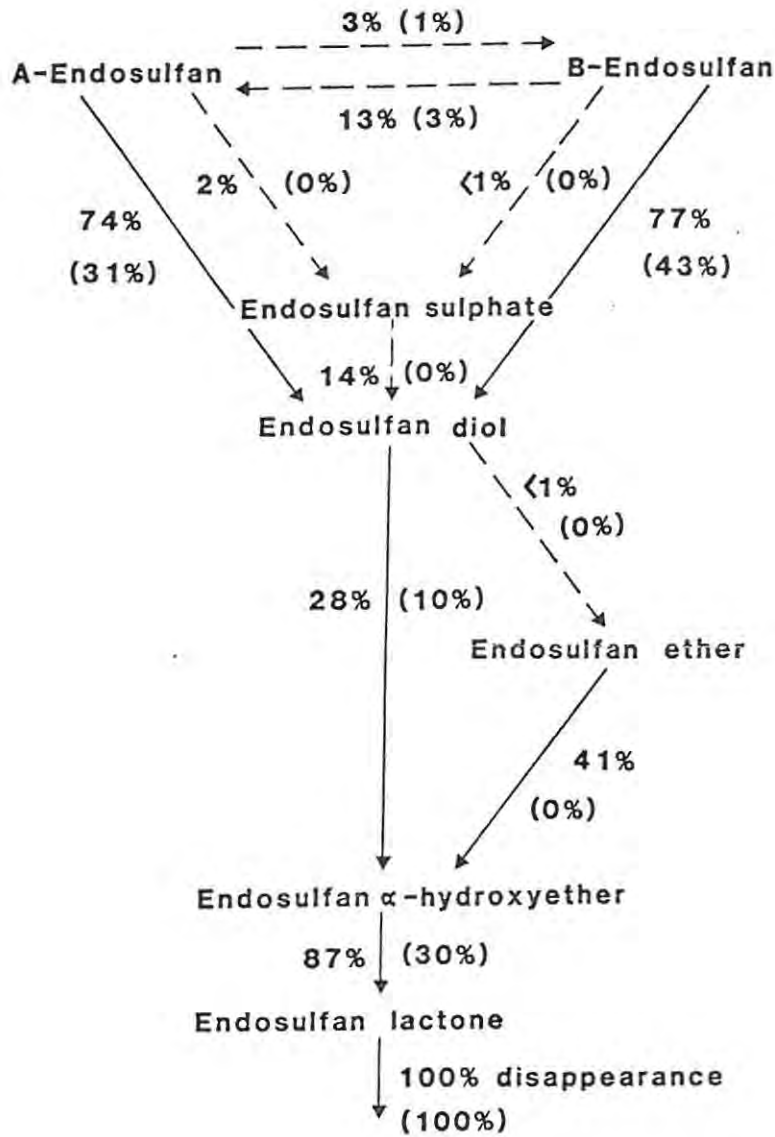


Figure 2. Conversion of A- and B-endosulfan and metabolites in aqueous nutrient medium inoculated with a mixed culture of soil micro-organisms and in sterile medium (bracketed numbers) (from Miles & Moy, 1979).

in the Okavango Delta (Douthwaite et al., 1981).

In the sprayed areas of the Okavango Delta, concentrations of endosulfan in the water (measured 30 cm below the surface) 6-9 h after spraying 9,5 g ha⁻¹ ranged between 0,2-4,2 ug l⁻¹ (Fox & Matthiessen, 1982). Endosulfan levels declined rapidly, although there was some evidence that the rate at which concentrations declined varied in different habitats. Endosulfan was undetectable after 5 days in well-vegetated pools that had been sprayed. Whereas in less well-vegetated channels and lagoons detectable quantities remained for 10-20 days after spraying (Fox & Matthiessen, 1982).

Fox & Matthiessen (1982) investigated the influence of submerged vegetation and silt on endosulfan concentration using tanks and cages in the field (Figure 3). Their results correspond well with those mentioned earlier in this section (page 19). In the absence of submerged materials endosulfan concentrations were relatively high after 9 h and remained detectable over a longer period. Greve & Wit (1971, in Fox & Matthiessen, 1982) found that endosulfan is readily adsorbed by a variety of submerged organic materials. With the evidence presented in this section it seems clear that the rapid drop in endosulfan concentration in habitats with much submerged material is due to endosulfan adsorption to suspended organic material and the bottom sediments. However, the pesticide can still enter the food chain of organisms which ingest suspended organic materials or the bottom sediment.

ACCUMULATION AND METABOLISM IN FISH

Accumulation

Investigations by Shoettger (1970) using ¹⁴C labelled endosulfan indicated that the insecticide is taken up and deposited differentially in different

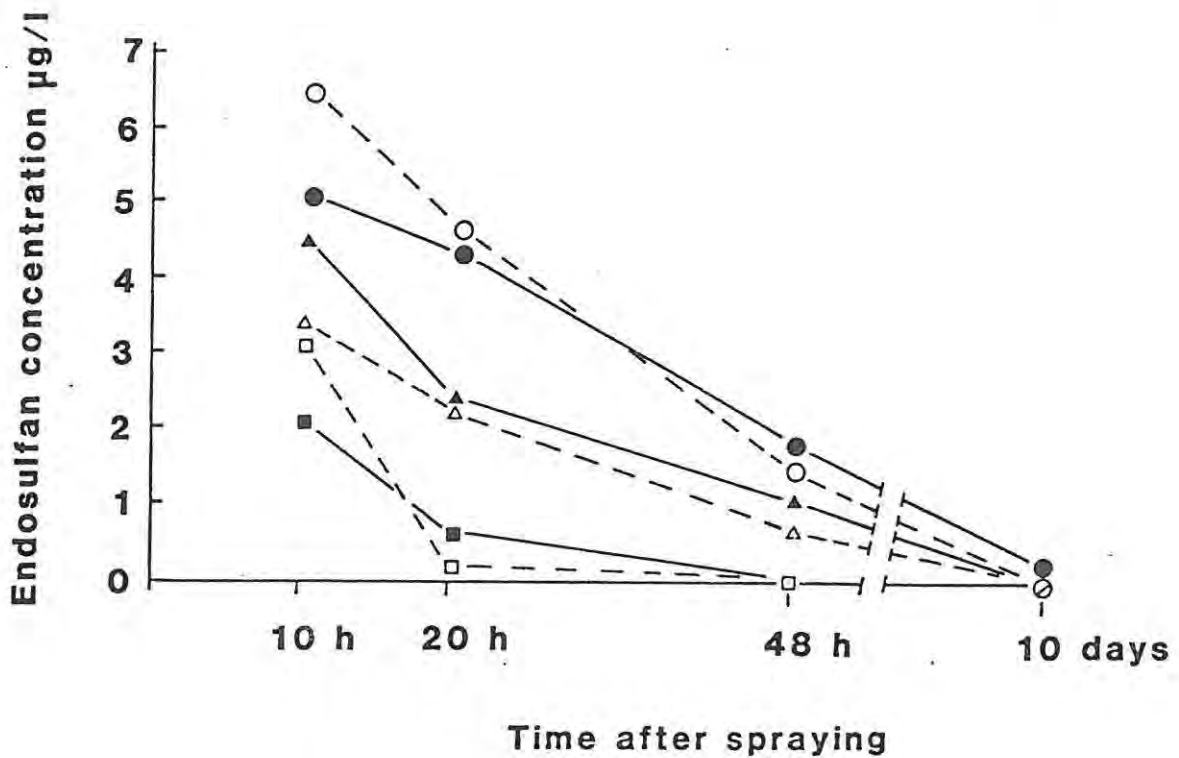


Figure 3. Concentration of endosulfan in water 200 mm deep after an aerial application of $9,5\text{g ha}^{-1}$. Treatment A. (circles), tanks containing swamp water only. Treatment B. (triangles), tanks containing water and uprooted vegetation. Treatment C. (squares), cages containing undisturbed silt and vegetation in pool adjacent to tanks. Open and shaded points represent replicates of each treatment (from Fox & Matthiessen, 1982).

tissues of fish. The highest accumulation after 12 h (in ug/g dry tissue) was in the liver (22,9) and in the gut and faeces (14,5). Accumulation was lower in the blood (8,9), brain (6,5), kidney (6,0), heart (5,6), empty gut (5,3), skin (2,6) and muscle (1,8). All tissues mentioned except the brain showed either an increase, or were highly variable, in activity levels when treated with high concentrations of ^{14}C endosulfan.

Muscle tissue, because of its mass, is probably one of the largest reservoirs for endosulfan deposition. The residues in muscles and skin did not increase markedly when fish were exposed to higher ^{14}C endosulfan concentrations. This suggests that the insecticide may attain saturation levels in the lipids as has been shown to occur with aldrin in rats (Ludwig et al., 1964 in Shoettger, 1970).

Matthiessen et al. (1982) measured the accumulation of endosulfan residues in fish and their predators in the Okavango Delta. They found that although endosulfan is detoxified by fish it does accumulate while they are exposed. The viscera contained higher residue concentrations (max. 2,83 mg kg⁻¹ in Hepsetus odoe) than the caudal muscles (max. 0,19 mg kg⁻¹ in Clarias gariepinus). The mean concentration in the water was 0,5 ug l⁻¹ (range 0,2-4,2 ug l⁻¹, dropping to < 0,025 ug l⁻¹ after 5-20 days). This implies that the maximum concentration factors were 380 for muscle and 5600 for viscera (Matthiessen et al., 1982). Residue levels returned to near normal within 3 months after the cessation of spraying, although they were still detectable after 12 months (< 0,005 mg kg⁻¹).

The residues in dead fish were higher than in live fish. Residues in dead fish, expressed as a proportion of lipid content, were up to 15 times higher than in live fish (Matthiessen et al., 1982). Table 5 shows the results of a

Table 5. Endosulfan residues (A, B & sulphate) and lipid content of adult *Hepsetus odoe* exposed for up to three days to endosulfan 35% EC at 1 μ g a.i. per litre in 700 litre tanks (from Matthiessen *et al.*, 1982).

Tissue	Living fish		Dead fish	
	Endosulfan residue (mg kg ⁻¹ wet wt)	Lipid (%)	Endosulfan residue* (mg kg ⁻¹ wet wt)	Lipid (%)
Caudal muscle	0,05	0,46	0,13	0,34
Total viscera	6,73	28,14	0,74	1,95
Liver	3,34	17,40	3,10	7,36
Brain	0,15	8,99	0,51	6,38

* The values refer to the total residue concentration (mg kg⁻¹ wet wt) in pooled samples from two-four fish.

3 day laboratory experiment in which Hepsetus odoe was exposed to endosulfan (35% EC) at 1 ug a.i. (active ingredient) per litre. The group which died of endosulfan poisoning contained higher residues in muscle and brain than survivors, but lower concentrations in viscera and liver. A notable feature about the tissue of the dead fish is that it contained less lipid than the tissues of survivors.

Table 6 shows that species with higher percentages of body lipids tend to contain higher levels of endosulfan. This indicates that tissue lipids may act as a reservoir to absorb excess endosulfan. In Table 7 the fatty tissues such as visceral adipose and the ovary had the highest residues. The bile, involved in excretion, also had high levels (mainly the first metabolite, endosulfan sulphate). The lean tissues such as the muscle and the gut had low levels. An exception to this trend is the gills, which have a high lipid content and low endosulfan residue levels. The most important factor governing the accumulation of endosulfan residue in fish appears to be the concentration of lipid in their tissues. Very fatty fish such as Marcusenius macrolepidotus were not killed by spraying, and in laboratory experiments lean Hepsetus odoe died more rapidly (Matthiessen et al., 1982). Adipose tissue may therefore protect fish to some extent by diverting residues away from sensitive organs. In the field the overriding factor seemed to be size of fish, smaller individuals being most vulnerable (Matthiessen et al., 1982).

Table 8 shows the relative concentrations of A-endosulfan, B-endosulfan and endosulfan sulphate residues in Clarias species. Endosulfan sulphate was present in higher concentrations than the A or B isomers. The ratio of endosulfan sulphate to the A and B isomers increased 6-fold during the spraying season, suggesting that the enzymes catalyzing this intermediate step

Table 6. Relationship between the lipid concentration in viscera and the endosulfan residue concentration (A, B & sulphate). Samples were taken from living fish 2 to 3 weeks after termination of 1978 spray season (from Matthiessen et al., 1982).

Species	Mean body wt (g)	Lipid in viscera (%) Mean (SE)	Mean endosulfan residue in viscera (mg kg ⁻¹ wet wt)*
<u>Marcusenius macrolepidotus</u> (insectivore)	99	49,4 (1,3)	1,00 (0,13)
<u>Hepsetus odoe</u> (piscivore)	467	16,6 (2,6)	0,80 (0,40)
<u>Oreochromis macrochir</u> (planktivore)	250	5,0	0,16
<u>Schilbe mystus</u> (insectivore+piscivore)	103	3,7 (0,6)	0,14 (0,07)
<u>Clarias gariepinus</u> and <u>C. ngamensis</u> (omnivorous carnivores)	351	2,7 (0,9)	0,14 (0,04)

* The values represent the mean of up to five fish: no standard error of mean is given for Oreochromis for which n = 1.

Table 7. Distribution of endosulfan residues (A, B & sulphate) in tissues of *Oreochromis macrochir*, 3 weeks after termination of the 1978 spray season (from Matthiessen *et al.*, 1982).

Tissue	Lipid in tissue (%)	Endosulfan residue* (mg kg ⁻¹ wet wt)	Calculated endosulfan residue* in extracted lipids, assuming all residues in lipid fraction (mg kg ⁻¹ lipids)
Caudal muscle	0,81	< 0,010	< 1,234
Total viscera	5,00	0,158	3,167
Ovary	6,43	0,389	6,057
Liver	4,21	0,190	4,539
Gut	1,88	< 0,010	< 0,532
Bile	4,76	0,362	7,612
Visceral adipose	42,10	1,606	3,815
Gill	6,78	0,182	2,685

* Each value represents a pooled sample of seven adult fish, except for total viscera for which n = 1.

Table 8. The changing ratios of A and B endosulfan and endosulfan sulphate in *Clarias* spp. viscera during the 1978 spray season (from Matthiessen et al., 1982).

Date	Mean endosulfan residue* (mg kg ⁻¹ wet wt)			Total residue (mg kg ⁻¹)	Ratio sulphate/ (A & B)
	A-	B-	'sulphate		
5 days after cycle 1	0,027	0,026	0,058	0,111	1,09
1 week after cycle 4	0,035	0,029	0,225	0,289	3,51
2 weeks after cycle 6	0,003	0,016	0,124	0,143	6,53

* Samples in which no residues were detected have not been included. Means are derived from samples of two-five fish. When comparing mean residue concentrations after cycles 1 and 6, those for A-endosulfan differ significantly (t test: $P < 0.05$), whereas those for B-endosulfan and endosulfan sulphate do not ($P > 0.1$).

may become more active with prolonged exposure (Matthiessen et al., 1982).

Finally, Matthiessen et al. (1982) showed that fish contamination by the present rates of endosulfan application in the Okavango Delta is unlikely to cause sublethal poisoning to humans. The USA has set a tolerance limit of $0,2 \text{ mg kg}^{-1}$ endosulfan in meat, and the levels of endosulfan in the muscle tissue of fish in the Okavango Delta are below this (Matthiessen et al., 1982).

Further support that the present levels are not persistent is that residue levels in animals at the top of the food chains (crocodiles and fish-eating birds) were no higher than in the animals they consumed (Matthiessen et al., 1982).

Shoettger (1970) studied the rate of uptake of ^{14}C labelled endosulfan in different tissues of fish and found this to vary according to the tissue tested (Figure 4). The levels of endosulfan in the liver were considerably higher than those in the blood, indicating that endosulfan may be removed from the blood and stored in the liver (Shoettger, 1970). The lower levels recorded in other tissues (except gut and faeces) may be as a result of this function of the liver (Shoettger, 1970). The majority of the radio activity in the gut is contained in the faeces and not in the tissue itself, thereby showing that the high level of endosulfan and its metabolites is not as a result of absorption from the blood, but is the result of ^{14}C endosulfan excreted via the bile duct (Shoettger, 1970; Rao et al., 1980; Rao et al., 1981; Matthiessen et al., 1982).

Metabolism

Shoettger (1970) postulated that the liver removes endosulfan from the bloodstream, changes it into an aromatic metabolite and conjugates it with glucuronic acid. Glucuronide formation is one of the most common routes of

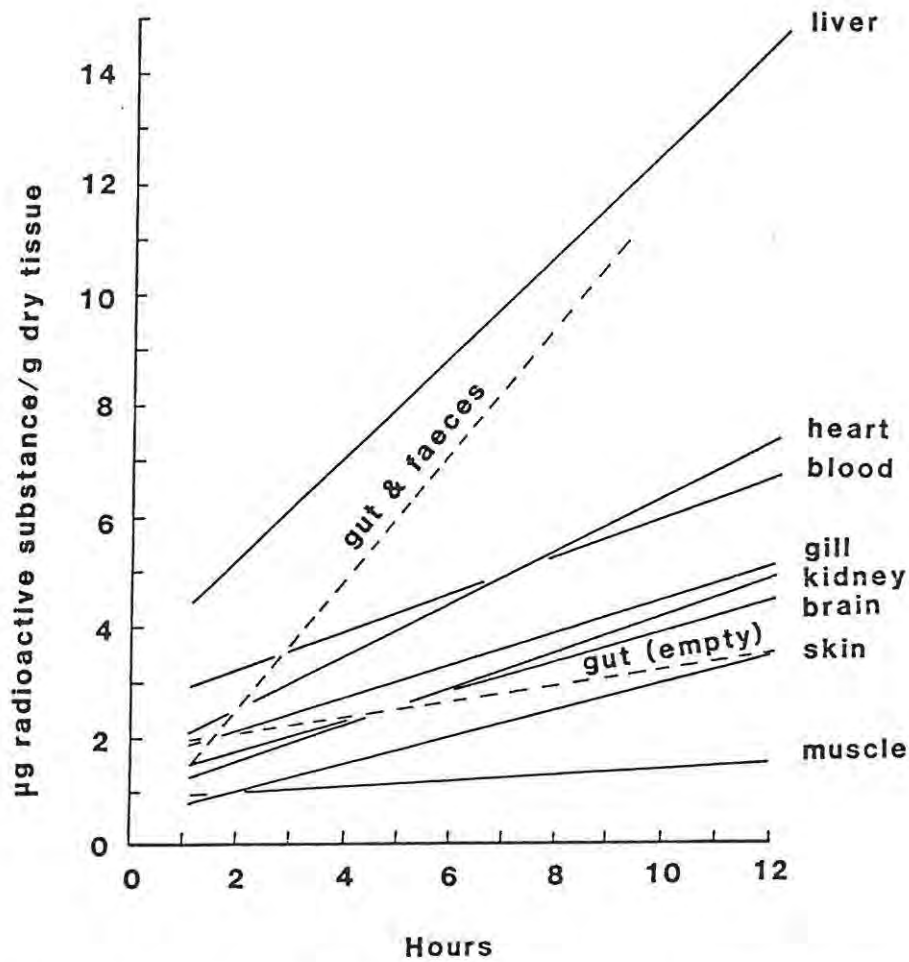
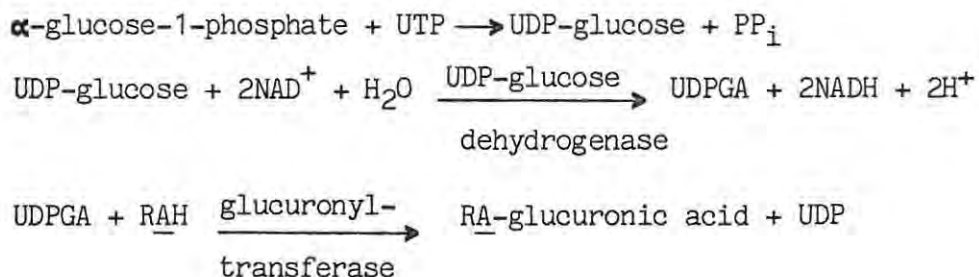


Figure 4. Uptake of radioactive substances in tissues of white suckers (*Catostomus commersoni*) during their exposure to $20\mu\text{g l}^{-1}$ of ^{14}C -labelled endosulfan at 19°C (from Shoettger, 1970).

metabolism of many drugs and xenobiotics and occurs in a variety of birds and mammals, in some reptiles, adult terrestrial amphibia, marine arthropods, insects and fish (Shoettger, 1970; Mandel, 1972; Jondorf, 1981). In fish the conjugate is then discharged with the bile into the gall bladder from which it is subsequently released into the gut. This is similar to the metabolism and excretion of endosulfan in rats and rabbits (Shoettger, 1970; Dorough *et al.*, 1978).

The conjugation reaction involves the condensation of the xenobiotic or drug, or their biotransformation products, with D-glucuronic acid (Mandel, 1972). This reaction does not take place directly but requires the activation of glucuronic acid by the synthesis of uridine diphosphate glucuronic acid (UDPGA), which serves as a donor of the glucuronic acid to the various types of acceptors in the transfer reaction: phenolic, alcoholic, carboxylic, aromatic amides and sulfhydryl compounds (Mandel, 1972; Jondorf, 1981). This transfer reaction is mediated by microsomal transfer enzymes located mainly in the liver (Jondorf, 1981), but also in other tissues, eg. kidney (Mandel, 1972). The general sequence of reactions with a substrate \underline{RAH} is given by Jondorf (1981) as:



(\underline{A} is part of the acceptor group and in the context here can be O, COO, NH, or S.)

Shoettger (1970) reported that the end product of endosulfan metabolism was endosulfan alcohol, but later studies (Rao & Murty, 1980; Rao *et al.*, 1980 & Rao *et al.*, 1981, on fish, and Dorough *et al.*, 1978, on rats) have shown that endosulfan sulphate, endosulfan hydroxyether, endosulfan alcohol (diol), endosulfan lactone and endosulfan ether are concentrated in the liver (mainly) and kidney and eliminated with the faeces and urine. Endosulfan is oxidized to endosulfan sulphate (an intermediate step as this chemical is also toxic), which is further metabolised to the non-toxic alcohol or ether. Rao *et al.* (1981) isolated further metabolites which they were unable to identify.

Elzner (1973, in Goebel *et al.*, 1982) investigated the metabolites occurring in rat urine and proposed two pathways of endosulfan degradation:

(1) Endosulfan was oxidised and hydrolysed to hydrophylic sulphur-containing metabolites, sulphuric acid esters of endosulfan diol (II) and their oxidation products. As hydrolysates of the sulphur-containing metabolites, endosulfan diol (II), hydroxyendosulfan ether (V), and endosulfan lactone (VI) could be identified by gas chromatography and thin-layer chromatography. Endosulfan sulphate (IV) was excreted as a lipophilic metabolite (Figure 5).

(2) Endosulfan (I) was hydrolysed to endosulfan diol (II), partly oxidised to hydroxyendosulfan ether (V), and endosulfan lactone (VI). These substances were conjugated to form hydrophylic excretion products (Figure 6). Endosulfan lactone (VI) and endosulfan sulphate (IV) were excreted as lipophilic metabolites.

PHYSIOLOGICAL EFFECTS

Endosulfan differs so markedly from other chlorinated hydrocarbons of the cyclodiene group in its chemical properties, physiological effects and behaviour on the surface of live animals and plants that it cannot be grouped

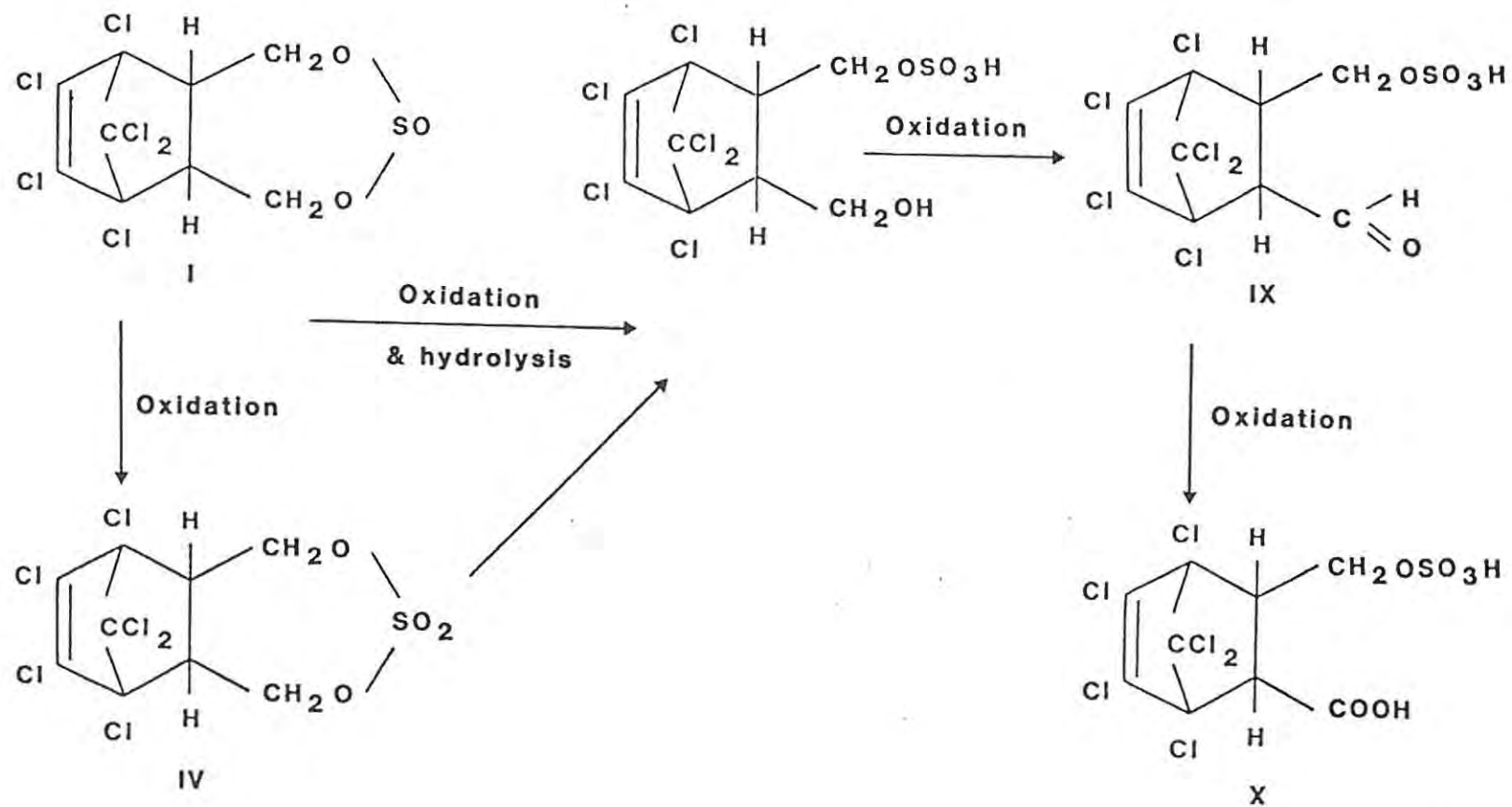


Figure 5. Degradation of endosulfan (from Elzner, 1973, in Goebel *et al.*, 1982).

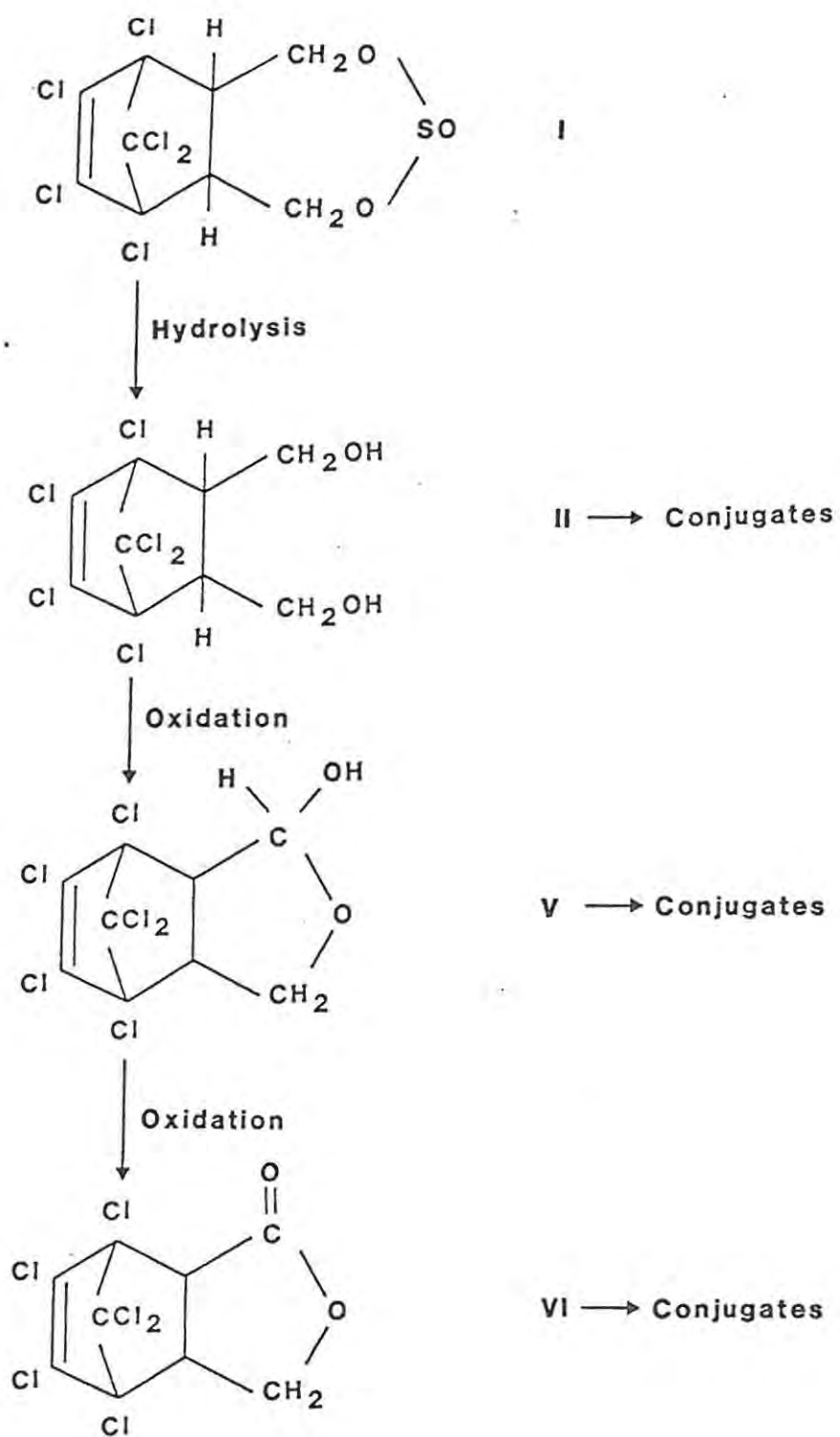


Figure 6. Degradation of endosulfan (from Elzner, 1973, in Goebel et al., 1982).

among them (Maier-Bode, 1968).

Generally, the cyclodiene insecticides bring about death by initially stimulating and then exhausting the nervous system. In the chlorinated hydrocarbon, DDT, this is achieved by the insecticide inhibiting the production of cholinesterase (Albert, 1979). Römer (1959, in Maier-Bode, 1968) could not detect cholinesterase inhibition after insects were treated with endosulfan. Experiments by Farberwerke Hoechst (in Maier-Bode, 1968) indicate that in insects a neurotropic action seems to predominate. More recently Knauf (1982) reported that endosulfan is primarily a contact and stomach poison. However, the precise mode of action of endosulfan is not known.

Only a limited number of studies have been carried out on the physiological effects of endosulfan. However, endosulfan has been shown to affect respiration, nitrogen excretion, ATPase activity in some tissues, cause haematological changes and to cause biochemical changes in the liver, kidney, muscle, brain and gills.

Respiration

Pesticides in general cause respiratory stress, and usually oxygen consumption increases with the onset of symptoms, although it may decrease again shortly before death (Holden, 1974).

Rao et al. (1980) carried out experiments on Labeo rohita which showed that oxygen consumption and carbon dioxide liberation increased at all concentrations of endosulfan below the LC50 value. At concentrations above the LC50 value ($5-7 \text{ ug l}^{-1}$), oxygen consumption and carbon dioxide liberation decreased. According to Corbett (1974, in Rao et al., 1980) an initial

increase and then a subsequent decrease in oxygen consumption indicates that the toxicant acts as an uncoupler. Further studies by Rao et al. (1981) on the effect of endosulfan on oxygen consumption in Macrornathus aculeatum showed different results. There was no increase in oxygen consumption as reported for L. rohita (Rao et al., 1980), but a decrease in oxygen consumption and progressive inactivity ending in death without convulsions. Rao et al. (1981) postulate that this may be because the action of endosulfan on M. aculeatum is more like a respiratory poison than a neurotoxicant, and the primary cause of death may be respiratory failure.

Nitrogen excretion

Measurement of changes in total nitrogen excreted, as a result of exposure to endosulfan, reflects the effect of this pesticide on metabolism. This is because energy for maintenance and activity comes from the catabolism of food, and in fish, protein is the main source of energy (Jrueger et al., 1968). Rao et al. (1981) showed that endosulfan caused a steady decrease in nitrogen excretion and therefore in metabolism in Macrornathus aculeatum. This may be related to reduced oxygen availability for oxidative metabolic processes.

ATPase activity

Dalela et al., (1978) postulated that the ATPase enzyme system was a possible sight for the toxic action of endosulfan. They therefore investigated the effect of endosulfan on the ATPase activity of the brain, gill, liver and kidney tissues. These tissues were exposed to various sublethal concentrations of endosulfan (0,00534; 0,00355; 0,00213 and 0,00174 mg^l⁻¹ at 25°C ± 4°C) for 30 and 60 days. The greatest inhibitive effect was on the oligomycin-insensitive Mg²⁺ ATPase in the brain, gill and liver tissues (highest inhibition occurred at highest concentrations after 60 days). However, in the kidney the most marked inhibition was of oligomycin sensitive

(mitochondrial) Mg^{2+} ATPase.

Endosulfan interferes with energy metabolism in vivo and therefore may impair normal organ function.

Haematological changes

The haematology of fish is often sensitive to pollution-induced stress (Silbergeld, 1974). Matthiessen (1981) measured the effect of endosulfan in the Okavango Delta on four haematological characters: erythrocyte count, leucocyte count, haemoglobin concentration and plasma protein concentration. In all cases blood cell counts were significantly raised, while plasma protein levels were often disturbed. Total haemoglobin concentration did not change, but the mean weight of haemoglobin per erythrocyte declined markedly in Tilapia species. It is worth mentioning here that synchronized studies by Matthiessen & Roberts (1982) showed significant pathological lesions in the liver and brain of several species at the same time as the haematological characters were disturbed. In most cases these disturbed levels declined to near the limits of detection within three months, indicating the absence of long term effects on these haematological characters.

Biochemical changes in some tissues

Murty & Devi (1982) demonstrated that endosulfan and its isomers caused biochemical changes in the liver, kidney, muscle, brain and gills. Protein, glycogen and lipid concentration in the liver, and glycogen of muscle were significantly decreased (Figures 7, 8 & 9), whilst the protein and glycogen of the kidney, and protein content of the brain were significantly increased. The two organs which were affected most markedly were the liver and kidney. This is probably because they are the regions of detoxification. Both isomers A and B caused biochemical changes, but those caused by the more toxic A

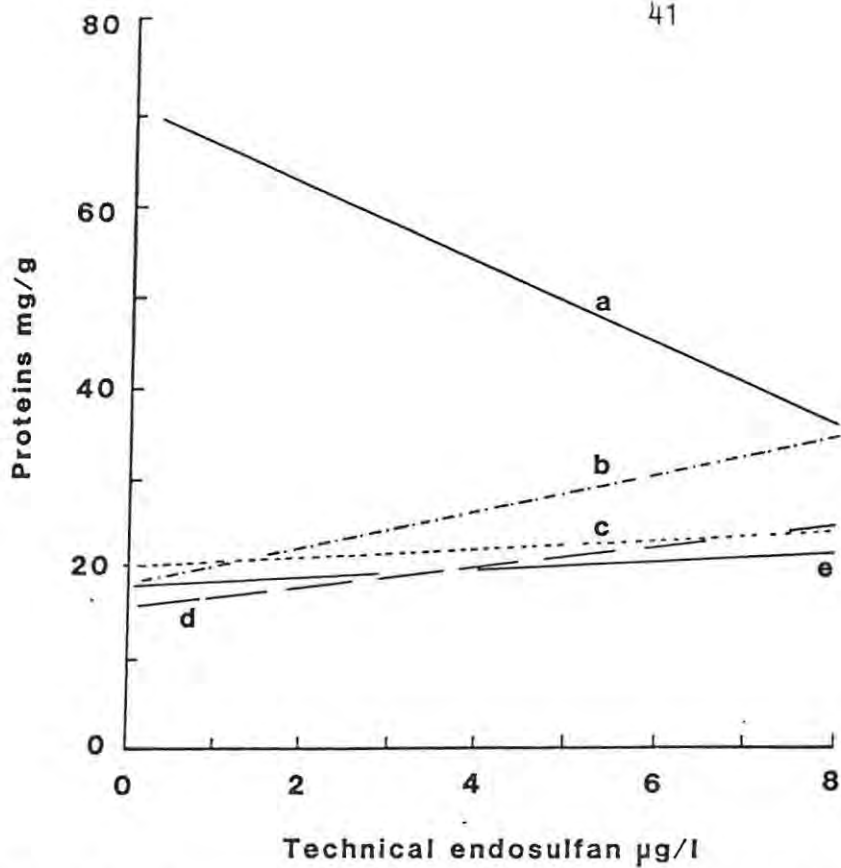


Figure 7. Regression lines of the effect of technical grade endosulfan on the protein content of (a) liver, (b) kidney, (c) muscle, (d) brain, and (e) gills (from Murty & Devi, 1982).

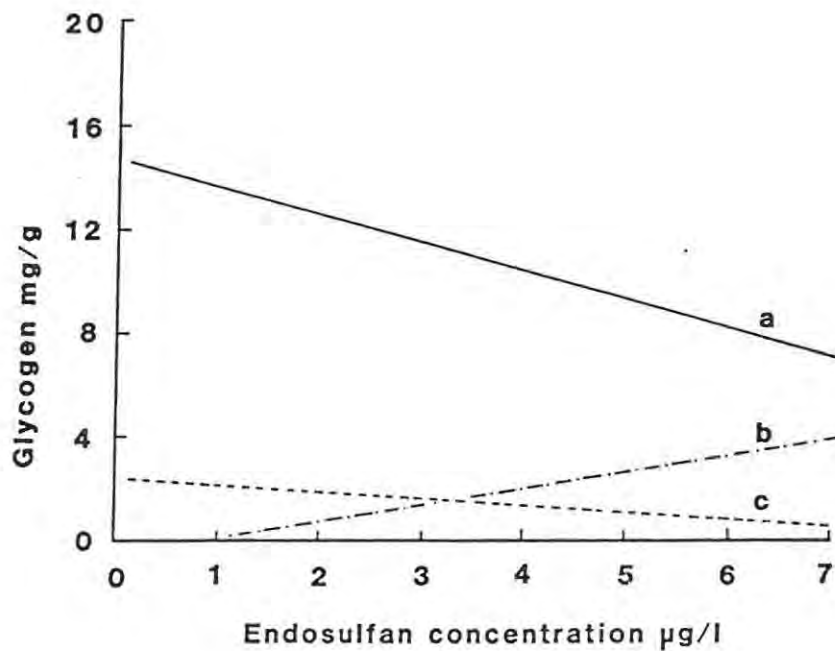


Figure 8. Regression lines of the effect of technical grade endosulfan on the glycogen content of (a) liver, (b) kidney and (c) muscle (from Murty & Devi, 1982).

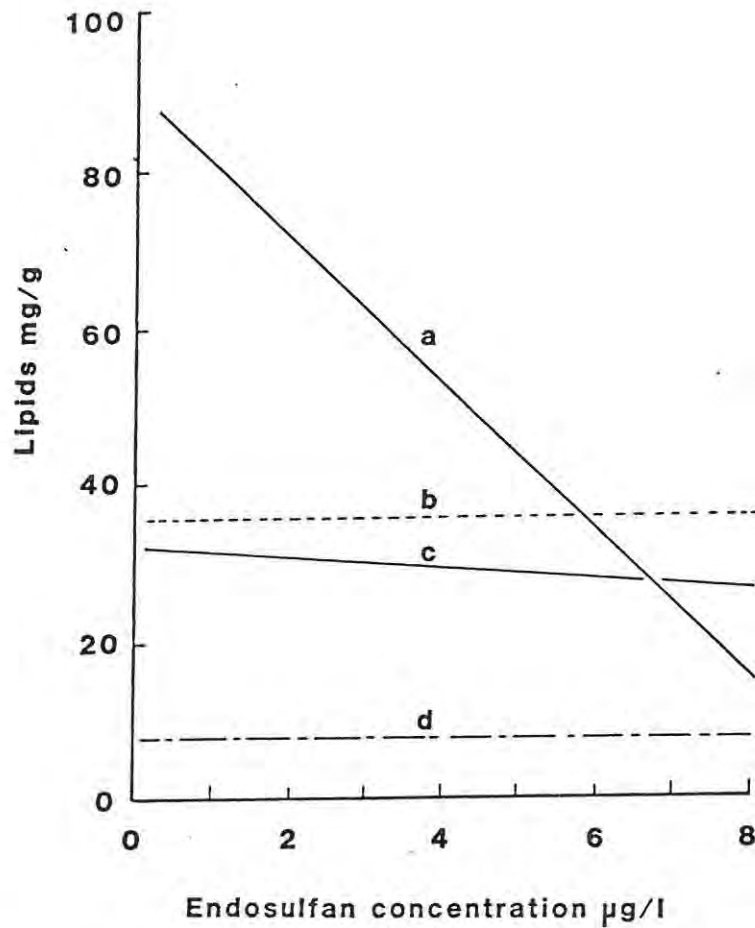


Figure 9. Regression lines of the effect of technical grade endosulfan on the lipid content of (a) liver, (b) brain, (c) muscle and (d) gills (from Murty & Devi, 1982).

isomer were greater.

PATHOLOGICAL EFFECTS

Walsh (1975, in Goebel et al., 1982) studied the effect of endosulfan on the pathology of Onchorynchus kitsuch and Salvelinus namaycush over a period of seven days. No histopathological or pathological alterations were noted.

Other studies have, however, shown such alterations: histological studies by Rao et al. (1980) on Labeo rohita showed that the only tissue with any histopathological changes was the liver (unfortunately they do not mention what other tissues were investigated). The changes observed were: (1) the cell boundaries became indistinct, (2) the cytoplasm became hyaline and less dense, (3) nuclei were vacuolated and chromatin appeared to be scattered with one or two large dots, and (4) there was some indication of the presence of vacuolated cells.

A more detailed study was conducted by Matthiessen & Roberts (1982) on the effects of endosulfan on the brain and liver of fishes in the Okavango Delta. They found that endosulfan definitely caused pathological lesions in the liver and caused changes in brain tissue which occurred simultaneously with the onset of spraying and persisted until the spraying season ended.

They found that the severity of the liver lesions varied between species: e.g. lesions in the piscivore Hepsetus odoe were relatively mild, whereas those in the omnivore Clarias gariepinus and herbivore Tilapia rendalli were more serious. Initially, hepatic lesions in Clarias gariepinus were characterized by generalized toxic necrosis, focal necrosis and subscapular oedema which gradually lessened in intensity and disappeared before the spraying season ended. This shows that the detoxification processes may improve in efficiency

during the spraying season.

Liver damage was particularly severe in the peribiliary tissue, supporting Shoettger's (1970) idea that endosulfan and its isomers are excreted in the bile. Evidence for biliary excretion was further strengthened by Matthiessen & Roberts (1982) noting that the gall bladders of Tilapia rendalli and Oreochromis andersonii were enlarged during the spraying season.

During the spraying season large amounts of lipid accumulated in the liver of C. gariepinus. This may have been caused by inhibition of metabolic processes or may be a strategy for coping with influxes of a fat soluble toxin as was shown in organochlorine resistant Gambusia species by Yarbrough & Coons (1975).

Another change in the C. gariepinus liver is that melano-macrophage centres and perivascular haemopoietic elements were lost from poisoned livers and not replaced until six months later.

In Tilapia rendalli brain tissue, endosulfan related changes included encephalitis, meningitis and oedema, with an associated inflammatory infiltrate of eosinophilic granule cells. These changes demonstrate that endosulfan has a neurotoxic effect. Soon after endosulfan spraying these brain tissue changes were severe enough to affect behaviour (Matthiessen & Roberts, 1982). Most brain damage disappeared during the month after the spraying season ended. The behaviour-related effects are discussed in the next section.

Shoettger (1970) investigated the effect of endosulfan on egg pathology. He exposed trout eggs to concentrations of endosulfan as high as $50\ 000\ \mu\text{g l}^{-1}$,

and neither gross nor microscopical examination of eggs revealed any abnormal development that was attributable to endosulfan.

BEHAVIOURAL EFFECTS

Because endosulfan is a neurotoxin, at least in part, it seems obvious that it should affect behaviour. The behavioural symptoms of endosulfan poisoning are discussed in the section on "Toxicity" (page 9). This section is confined to some limited observations of fish in the Okavango Delta in which the fish were hyperactive and reproductive behaviour appeared to be disturbed.

Fox (pers. comm., in Matthiessen & Roberts, 1982) noted that nests of Tilapia rendalli were 75% less frequent in sprayed areas and postulated that this may result from endosulfan affecting its complex reproductive behaviour. These results would be extremely difficult to verify unless nest counts over several seasons had been conducted so the variation was known. Matthiessen & Logan (1983) carried out replicated laboratory experiments on the effect of endosulfan on the breeding behaviour of O. mossambicus. They found that endosulfan ($0,5 \text{ ug l}^{-1}$) delayed the onset of breeding behaviour although exposed fish still bred successfully. All the larvae, however, died. Fox also noted that gill net catches increased immediately after spraying; this was because the fish were hyperactive and slightly unco-ordinated. Fish in this state would be vulnerable to predators such as birds and crocodiles.

CHAPTER 3 HEPATIC CYTOCHROME P-450 (A REVIEW)

INTRODUCTION

Terrestrial and aquatic animals are exposed to a wide variety of foreign chemicals, or xenobiotics, many of which are toxic (Guthrie & Perry, 1980). In fish and higher vertebrates most of these chemicals are converted into more polar derivatives in the liver, thereby facilitating their excretion (Bend et al., 1980; Paine, 1981). The most common routes of xenobiotic metabolism involve oxidation, reduction, hydrolysis and conjugation (La Du et al., 1972). The method by which endosulfan is metabolized is described in detail in the review on the effects of endosulfan on fish.

The enzymes affecting many of these biotransformations are located in the endoplasmic reticulum (Brodie et al., 1955; Claude, 1969). The presence of microsomal pigments was reported by Klingenberg (1958) and Garfinkel (1958, in Mannering, 1981), and Omura & Sato (1962) demonstrated their haemoprotein nature. Omura & Sato (1964a & b) showed that the pigments were capable of combining with carbon monoxide to give a characteristic absorbance at 450 nm when reduced by sodium dithionate. They also showed that detergent solubilized the pigment and converted it to a carbon monoxide - combining derivative with a maximum absorbance at 420 nm.

Mueller & Miller (1953) reported the oxidative N-demethylation of aminoazo dye by hepatic microsomes. A few years later Brodie et al. (1955) showed that microsomal enzymes catalyse the side chain oxidation of barbituates, the N-dealkylation of amines, the deamination of alkylamines, the cleavage of alkyl and aryl ethers and aromatic hydroxylation. Conney et al. (1957) demonstrated that carbon monoxide inhibited microsomal N-demethylation. Studies in the

early 1960's showed that the administration of phenobarbital to rats increased the cytochrome P-450 content of liver microsomes and the rate of xenobiotic/drug metabolism. These increased activities were also inhibited by carbon monoxide (Mannering, 1981; Paine, 1981). Microsomal preparations from fish livers have been shown to catalyse oxidation reactions: hydroxylation, O- and N- dealkylation, N- and S- oxidation, epoxidation, epoxide hydration and desulphuration (Schwen & Mannering, 1982b). But in general, reaction rates are considerably lower than in mammals (Schwen & Mannering, 1982b). In vivo drug metabolism has also been demonstrated, including that of aromatic hydrocarbons (Stegman, 1981), 2-acetylaminofluorene (Lotlikar et al., 1967), aflatoxins (Scarpelli, 1976) and parathion (Ludke et al., 1972). The results of these studies led to the realization that cytochrome P-450 was intimately involved in drug/xenobiotic oxidations.

This microsomal enzyme system was initially shown to consist of a NADPH dependent flavoprotein reductase and an unidentifiable terminal oxidase (Strittmatter & Velick, 1956; Phillips & Langdon, 1962; Williams & Kamin, 1962). Cytochrome P-450 can now simplistically be envisaged as the site where oxygen, electrons from NADPH and drugs/xenobiotics all interact (Figure 10). Oxidation at this site transforms the drug/xenobiotic into a polar metabolite which can then be excreted (Bend et al., 1980; Paine, 1981).

Cytochrome P-450 is now known to be responsible for the oxidative conversion, in mammals, of many foreign chemicals such as drugs, pesticides, carcinogens and environmental pollutants (La Du et al., 1972; Guthrie & Perry, 1980; Jenner & Testa, 1981). The role of cytochrome P-450 in fish has not been clearly established although the mono-oxygenase system undoubtedly plays a similar role in detoxification of xenobiotics (Chambers & Yarbrough, 1976; Schwen & Mannering, 1982a). Unlike most enzyme systems, which are substrate

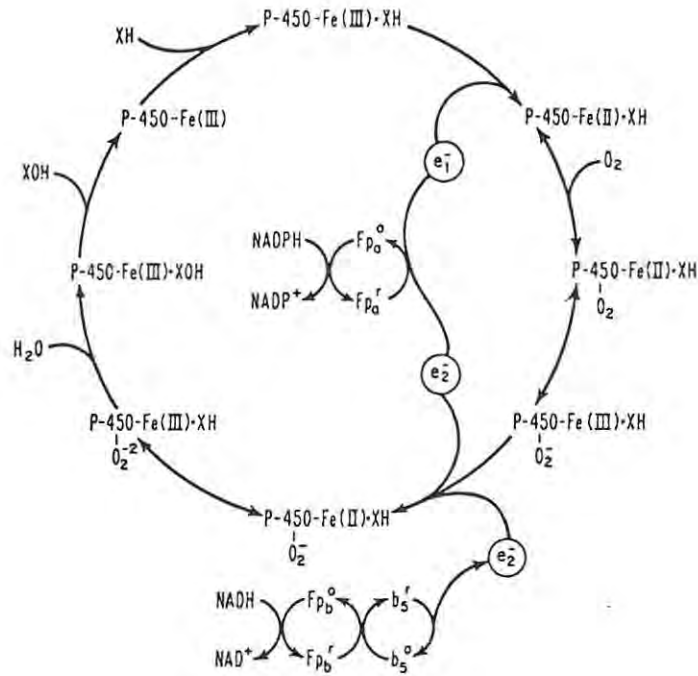


Figure 10. Cytochrome P-450- and cytochrome b₅-linked hepatic mono-oxygenase system. P-450(III) = ferricytochrome P-450; P-450(II) = ferrocycytochrome P-450; P-450-Fe(III) = oxy-anionferricytochrome P-450; b₅ = cytochrome b₅; Fp_a = NADPH-cytochrome P-450 reductase; Fp_b = NADH-cytochrome b₅ reductase; XH = substrate. (from Mannering, 1981).

specific the hepatic cytochrome P-450-linked metabolizing system has a broad substrate selectivity (Mannering, 1981).

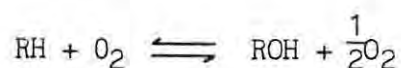
Hepatic cytochrome P-450 was originally thought to be a single molecular entity. However, evidence began to accumulate indicating more than one species of P-450. Rates of oxidation of a variety of substrates did not always parallel one another when animals were treated with different inducing agents (Mannering, 1981). Additionally the relative rates of oxidation of selected substrates by animals of different species differed widely (Mannering, 1981). It is now known that there is multiplicity of cytochrome P-450 (Gustafsson et al., 1980; Mannering, 1981; Paine, 1981). These systems may represent heterogeneity of molecular species, or heterogeneity of physical forms of a given species of P-450, or they may reflect heterogeneity due to the diverse locations of the haemoprotein in the membrane (Mannering, 1981).

Cytochrome P-450 enzymes occur widely in nature in most animal tissues and organelles, plants and in micro-organisms (White & Coon, 1980). In mammals cytochrome P-450 occurs in highest concentrations in the liver and adrenals, but also occurs in the microsomes of the kidney, intestinal mucosa, lung, skin, testis, placenta and brain (Mannering, 1981). The location of cytochrome P-450 is not limited to microsomes. It is also found in mitochondria (Akhrem et al., 1980; Mannering, 1981). Cytochrome P-450 has been observed in the liver of fish, amphibia, reptiles and birds (Mannering, 1981; Schwen & Mannering, 1982a; Noshiro & Omura, 1984). Payne et al. (1984) reported cytochrome P-450 in the kidneys of fish. The mono-oxygenase systems of fish, frogs and snakes were identified as cytochrome P-450 systems by the inhibitory effects of carbon monoxide, SKF 525-A, A naphthoflavone, and by the requirement for NADPH (Schwen & Mannering, 1982b). Cytochrome P-450 has also been shown to play an important role in the metabolism of pesticides by

insects (Jondorf, 1981). Most work has been carried out on mammals; this review will therefore rely heavily on mammalian literature for demonstrating the general principles of the hepatic cytochrome P-450-linked drug/xenobiotic metabolizing system.

MECHANISM OF CYTOCHROME P-450-LINKED OXIDATIONS

Although a wide variety of oxidative reactions are catalysed by cytochrome P-450 in microsomes, these reactions can essentially be envisaged as hydroxylations (Table 9). *In vitro* investigations have shown that fish metabolize xenobiotics by mechanisms similar to those found in mammals: oxidation, reduction, hydrolysis and conjugation (Schwen & Mannering, 1982b). Molecular oxygen has been shown to be the source of the hydroxyl group for hydroxylation (Paine, 1981):

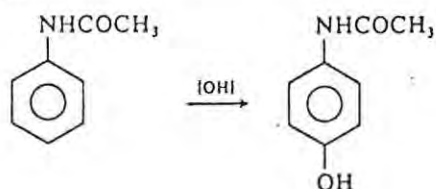


A hydroxylation reaction in which one atom of each oxygen molecule is transferred to the substrate is termed a "mono-oxygenase" (Paine, 1981). If both atoms of the oxygen molecule are inserted into the substrate it is known as a "di-oxygenase" (Paine, 1981).

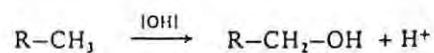
The microsomal P-450 linked mono-oxygenases require an external source of electrons such as NADH or NADPH for the insertion of oxygen into the substrate (Paine, 1981). The reaction is thought of as a mechanism whereby NADPH reduces a component (A) in microsomes, which reacts with molecular oxygen to form an "active oxygen" intermediate which is then transferred to the substrate (Mannering, 1972, in Mannering, 1981). Gillette (1963) has envisaged the reaction as follows:

Table 9. Some hydroxylation reactions catalysed by cytochrome P-450 (from Paine, 1981).

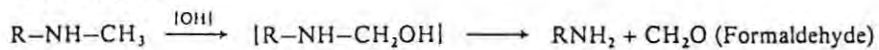
Aromatic hydroxylation



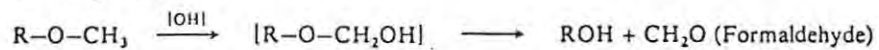
Aliphatic hydroxylation



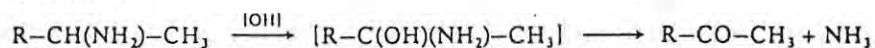
N-Dealkylation



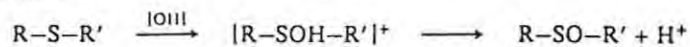
O-Dealkylation



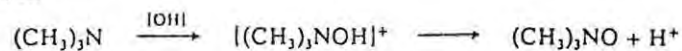
Deamination

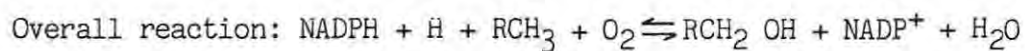
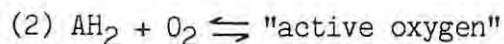
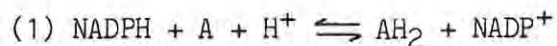


Sulphoxidation



N-Oxidation





The above enzymic reaction involves the oxidation of the substrate (RCH_3) and the oxidation of NADPH to NADP^+ ; the reaction has therefore been termed a mixed-function oxidase (Paine, 1981). The cytochrome P-450-linked drug/xenobiotic metabolizing systems are therefore also known as mixed-function oxidases or mono-oxygenases.

Two cytochrome P-450-linked mono-oxygenase systems occur in hepatic microsomes (Mannering, 1981): the first, a system consisting of an NADPH reductase and any of several species of cytochrome P-450. The reductase is called NADPH-cytochrome P-450 reductase when it transfers electrons to cytochrome P-450 (the natural electron acceptor), and NADPH-cytochrome c reductase when cytochrome c is used experimentally as an electron acceptor. The second system consists of NADH-cytochrome b5 reductase, cytochrome b5, and cytochrome P-450. The most important role of this system appears to be that of synergising the NADPH-linked system (Mannering, 1981). The microsomal membrane can be considered a facultative component as it is indirectly responsible for the interaction of the components of the system (Mannering, 1981).

Electron transfer pathways to cytochrome P-450 and stoichiometry

Omura et al. (1966) fractionated the adrenal cortex mitochondrial electron transfer system involving cytochrome P-450. This produced a particulate

fraction containing cytochrome P-450 free of other haemoproteins, and a soluble fraction which retained the activity of cytochrome P-450 reductase. The soluble fraction contained a non-haem iron protein called adrenodoxin, and an FAD flavo-protein (NADPH-cytochrome c reductase); both of these were needed for the reconstitution of NADPH-cytochrome P-450 reductase activity. From their findings Omura et al. (1966) proposed the electron transport system shown in Figure 11. However, direct evidence that the adrenal cortex mitochondrial system is the same type as that found in the microsomes, is lacking (Paine, 1981). Additionally, the hepatic microsome system is not inhibited by anti-adrenodoxin antibody indicating that the non haem-iron protein, if present, is not adrenodoxin (Masters et al., 1973).

The electron transport scheme shown in Figure 11 requires that equivalent amounts of NADPH, oxygen and substrate be utilized in the reaction. Experiments with rat hepatic microsomes have given variable results: Ernster & Orrenius (1965) demonstrated a 1:1:1 stoichiometry of oxygen utilization, NADPH disappearance and substrate formation. Beuning & Franklin (1974) similarly showed a 1:1 relationship of oxygen utilization and product formation. However, a number of studies showed that considerably more NADPH was oxidized than could be accounted for by product formation, although in at least one other study this stoichiometry was reversed (Mannering, 1981). The determination of the stoichiometry of NADPH utilization and product formation by microsomes is complicated by endogenous NADPH oxidation. Endogenous enzyme activity (NADPH oxidase activity) may consume as much or more NADPH than is required for the oxidation of added substrate (Mannering, 1981). According to Mannering (1981) a stoichiometry of NADPH utilization to product formation of less than one might be explained if endogenous NADPH oxidase is suppressed by the presence of substrate. Jeffery & Mannering (1979) revealed that a stoichiometry of greater than one can be explained by the presence of

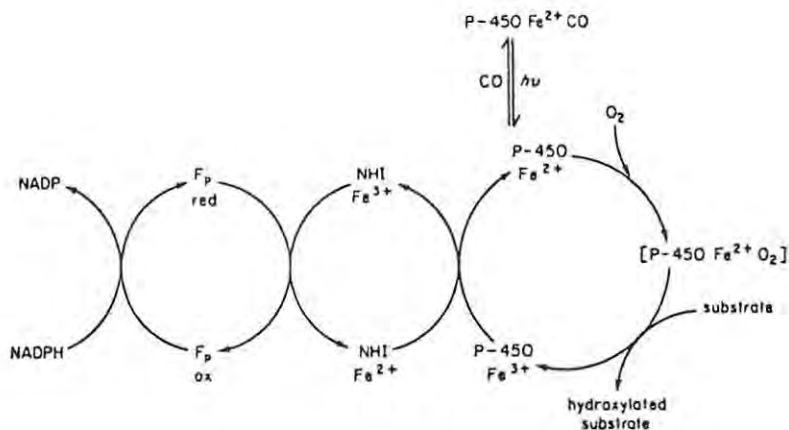


Figure 11. Electron transfer system to cytochrome P-450.
 Fp = Flavoprotein (in the liver cytochrome c (P-450) reductase, in the adrenal, adrenodoxin reductase);
 NHI = non-haem iron protein (in the adrenal adrenodoxin);
 hv = light of 450 nm (from Omura *et al.*, 1966).

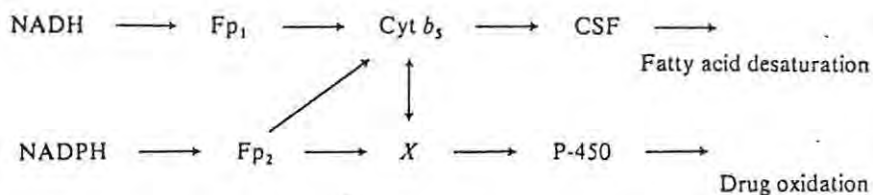


Figure 12. Electron transfer pathways in liver microsomes indicating their relationships with functions. Fp₁ = NADH cytochrome b₅ reductase flavoprotein; Fp₂ = NADPH-specific flavoprotein usually thought of as NADPH cytochrome c reductase; X = non-haem iron or lipid?; CSF = cyanide sensitive factor (from Paine, 1981).

nucleotide pyrophosphate in microsomes.

NADH Synergism

NADH is somehow involved in cytochrome P-450 linked mono-oxygenase reactions. Estabrook & Cohen (1969) demonstrated that NADH on its own was unable to support microsomal aminopyrine demethylation, but when both NADH and NADPH were present a synergistic response occurred. Mannering (1981) reported that in general most hepatic mono-oxygenase systems are supported by NADH at only 10-20% of the rate obtained with NADPH.

Estabrook & Cohen (1969) carried out experiments to show the interaction of the two nucleotides. This was examined by measuring fluorescence and optical changes associated with the reduction of the microsomal flavoproteins, NADPH-cytochrome-c reductase and NADH cytochrome-b5-reductase. The addition of NADH caused a decrease in absorbance and fluorescence, indicating flavoprotein reduction. A significant change in fluorescence and absorbance was not noted when NADPH was added; either prior to, or after the addition of NADH.

Estabrook & Cohen (1969) concluded that cross reactivity between the two flavoprotein species must occur, permitting them to function with either reduced pyridine nucleotide when bound to the microsomal membrane (Figure 12).

Hildebrand & Estabrook (1971, in Mannering, 1981) and Estabrook *et al.* (1971, in Mannering, 1981) also implicated cytochrome b5 in mono-oxygenase activities. They demonstrated that the steady state of reduced cytochrome b5 in the presence of NADH and NADPH was decreased by the addition of substrate (ethyl-morphine). An ethylmorphine-enhanced rate of oxygen utilization and product formation linked the oxidation of ethylmorphine to the alteration in the steady-state reduction of cytochrome b5.

Jansson & Schenkman (1973) and Schenkman *et al.* (1976, in Mannering, 1981) proposed that cytochrome b5 acts as a "drain" for electrons from NADPH-cytochrome P-450 reductase. Jansson & Schenkman (1973) reported a progressive inhibition of NADPH-supported aminopyrine demethylation with an increase in concentration of incorporated cytochrome b5. This observation was used to support their hypothesis that the cytochrome b5 does not participate directly in the transport of the second electron but diverts electrons from NADPH away from cytochrome P-450. If the above hypothesis were sound, the addition of an antibody against cytochrome b5 should stimulate mono-oxygenase activity in microsomes that have not been enriched with cytochrome b5, not inhibit it slightly as is the case (Mannering, 1981). Cinti & Ozols (1975, in Mannering, 1981) repeated Jansson & Schenkman's (1973) experiments and found different results - incorporation of cytochrome b5 did not inhibit mono-oxygenase activity. They explained Jansson & Schenkman's (1973) results by saying the presence of detergent was probably responsible for inhibition of mono-oxygenase activity noted.

In the fatty acid desaturation enzyme system, electrons from NADH are transferred to the fatty acyl CoA substrate via cytochrome b5 and a "cyanide-sensitive factor" (Conn & Stumpf, 1976). If NADH and cytochrome b5 are involved in the transfer of electrons to cytochrome P-450 one would expect that any diversion of electrons from NADH to the fatty acid desaturation system would cause a cessation of the synergistic effect of NADH on the cytochrome P-450 system (Paine, 1981). Correia & Mannering (1973) used Stearoyl CoA to divert electrons to the fatty acid desaturation pathway; this removed the NADH synergism. Correia & Mannering (1973) also investigated the possibility that the inhibition of the flow of electrons to the desaturation pathway (after the addition of Stearoyl CoA) might restore the synergistic effect of NADH. Cyanide was used to divert electrons away from the

desaturation pathway and NADH synergism was restored. Correia & Mannering (1973) explained NADH synergism as follows (Figure 13):

- (1) The first electron for the reduction of cytochrome P-450 substrate complex must come from NADPH since NADH will not support oxidation in the absence of NADPH.
- (2) The second electron required for the reduction of reduced cytochrome P-450 substrate complex may come from either NADPH or NADH.
- (3) When the second electron is derived from NADH it is transferred to cytochrome P-450 via cytochrome b₅. It is not known if the second electron can be transferred from NADPH via cytochrome b₅ to cytochrome P-450 (Paine, 1981).

In the scheme shown in Figure 13, NADH will exert a synergistic effect because it can maintain a higher steady-state level of reduced cytochrome b₅ (Paine, 1981). Therefore, cyanide is more effective at diverting electrons to the cytochrome P-450 system when NADH is present because there are more electrons to divert (Paine, 1981). The addition of NADH to NADPH shifts the rate limiting step from the site of entry of the second electron to the entry of the first electron. The reason for this is only NADPH provides the first electron whereas both NADPH and NADH provide the second electron.

Mannering *et al.* (1974) carried out an immunological study which provided further support for the involvement of cytochrome b₅. They showed that anti-(cytochrome b₅)-immunoglobulin inhibited the synergistic effect of NADH with NADPH. However, when anti-(cytochrome b₅)-immunoglobulin was added with sufficient pure cytochrome b₅ to neutralize the antibody, inhibition did not occur. Control gamaglobulin from rabbits that had not received b₅ did not prevent synergism. This further supports the involvement of cytochrome b₅ in NADH synergism.

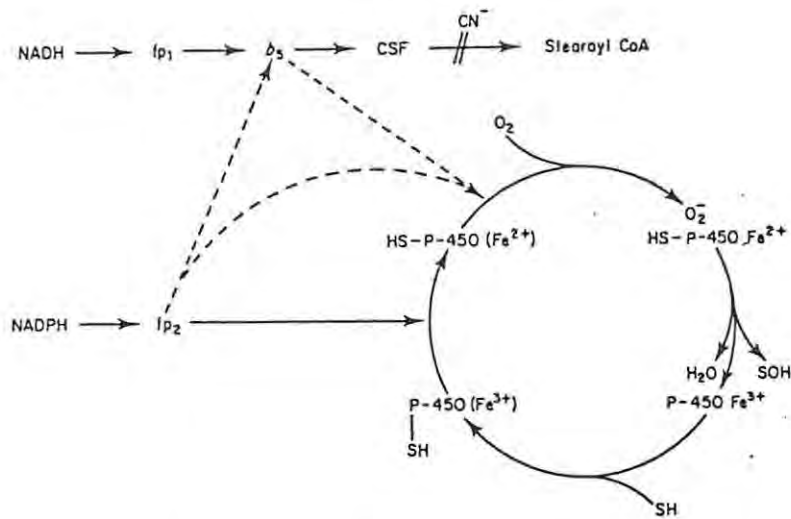


Figure 13. Scheme showing how cytochrome b_5 might transfer electrons from NADPH and NADH to cytochrome P-450. fp_1 = cytochrome b_5 reductase flavoprotein; fp_2 = cytochrome c reductase flavoprotein; CSF = cyanide sensitive factor; SH = substrate (from Paine, 1981).

NADH synergism of NADPH-supported mono-oxygenase activities of hepatic microsomes of the trout, frog and snake suggests the participation of cytochrome-b5 in these reactions. For the trout p- nitrophenetole metabolism was supported almost as well by NADH as NADPH indicating that cytochrome-b5 may play an important role in certain mono-oxygenase reactions (Schwen & Mannering, 1982b).

COMPONENTS OF THE HEPATIC MONO-OXYGENASE SYSTEM.

Cytochrome P-450

The structural bases of the properties of cytochrome P-450 are poorly understood due to difficulties in the isolation and purification of the enzyme (Mannering, 1981; Paine, 1981).

The molecular weight of electrophoretically homogenous haemoproteins obtained from livers of untreated and induced rabbits, rats and mice are known to fall roughly into two groups: those with a molecular weight of approximately 48 000 and those approximating 54 000 (Mannering, 1981).

The amino acid composition of the seven cytochrome P-450's isolated from rabbit, rat and bacteria, listed in Table 10 are fairly similar. The amino acid histidine and a thiolate anion have been implicated as ligands for the iron of cytochrome P-450 (Mannering, 1981).

Using the alkaline pyridine-haemochromogen spectrum, the haem present in cytochrome P-450 has been shown to be a protohaem (Mannering, 1981). It is therefore accepted that the haem of the hepatic cytochrome P-450 is Ferriprotoporphyrin IX (Maines & Anders, 1973, in Mannering, 1981). The spectral, magnetic, chemical and functional characteristics of individual

Table 10. Amino acid composition of cytochrome P-450 from the rabbit, rat and bacteria (from Mannering, 1981).

Amino acid	Rabbit		Rat			<u>P. putida</u>
	P-450LM ₂	P-450LM ₄	PB-450B	PB-450D	3MC-450B	P-450 _{cam}
HaH cystine	6	7	7	7	6	6
Asx	36	38	42	41	43	36
Met	7	10	11	15	10	9
Thr	23	24	29	25	29	19
Ser	30	31	30	34	38	21
GLx	42	46	52	52	43	55
Pro	24	28	27	30	37	27
Gly	32	38	33	54	32	26
Ala	23	29	29	17	26	34
Val	26	32	27	20	28	24
Ile	19	22	27	25	24	24
Leu	54	58	59	40	54	40
Tyr	9	11	14	14	14	9
Phe	31	28	32	27	28	17
His	11	11	13	10	13	12
Lys	19	26	27	38	31	13
Trp	1	7	2	1	6	1
Arg	<u>29</u>	<u>30</u>	<u>25</u>	<u>22</u>	<u>25</u>	<u>24</u>
Totals:	422	476	486	472	487	397
NH ₂ -terminal	Met	Multiple	Asx-Glx-Asx	His	His-Ala-Asx-Tyr	Thr
COOH-terminal	Arg	Lys	Gly-Ala	-	Val	Val

protoporphyrin haemoproteins result from the specific protein imposed liganding of the haem iron (Mannering, 1981). The ligand field of a haemoprotein that can function both as an electron transferase and as an oxidase would be expected to undergo a series of changes during the overall mono-oxygenase reaction; an understanding of these changes is essential for the elucidation of the mechanism of cytochrome P-450-linked mono-oxygenase reactions (Mannering, 1981).

Figure 14 shows that four of the six ligands of the iron of cytochrome P-450 are co-ordinated with pyrrole nitrogens. The fifth appears to be a sulphur ligand and the exact nature of the sixth has not been determined (Mannering, 1981).

Because of the difficulties experienced in solubilizing cytochrome P-450, many investigators have endeavoured to gain insight into the mechanism of cytochrome P-450-linked mono-oxygenase reactions by spectral studies; the optical properties of cytochrome P-450 are quite similar despite their widely differing sources (different species and organs) and substrate selectivities (Mannering, 1981). Many of the major differences in the function of different species of cytochrome P-450 are therefore not revealed by their spectra (Mannering, 1981). The molecular extinction coefficient of $91 \text{ nM}^{-1}\text{cm}^{-1}$ determined by Omura & Sato (1962) for the reduced CO spectrum of cytochrome P-450 between 450 nm and 490 nm, is still considered to be an accurate index of the cytochrome P-450 content of microsomes (Mannering, 1981).

When a substrate binds to cytochrome P-450 it causes a change in the spin state of the iron as well as the conformation of the cytochrome (Mannering, 1981). Substrates are divided into groups according to the type of change they produce as observed by difference and absolute spectra.

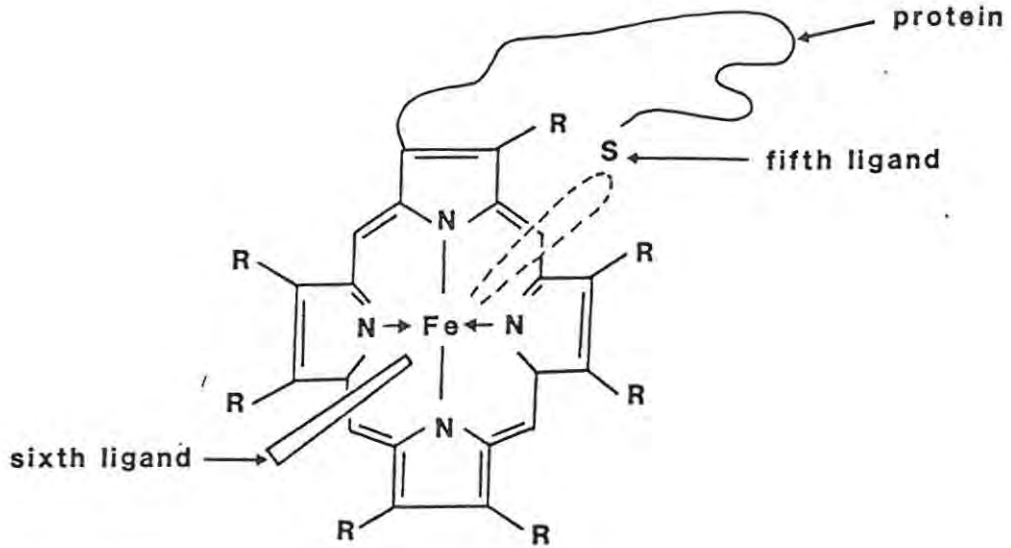


Figure 14. Haem moiety showing bonds of fifth and sixth ligands (from Gorrod & Temple, 1973).

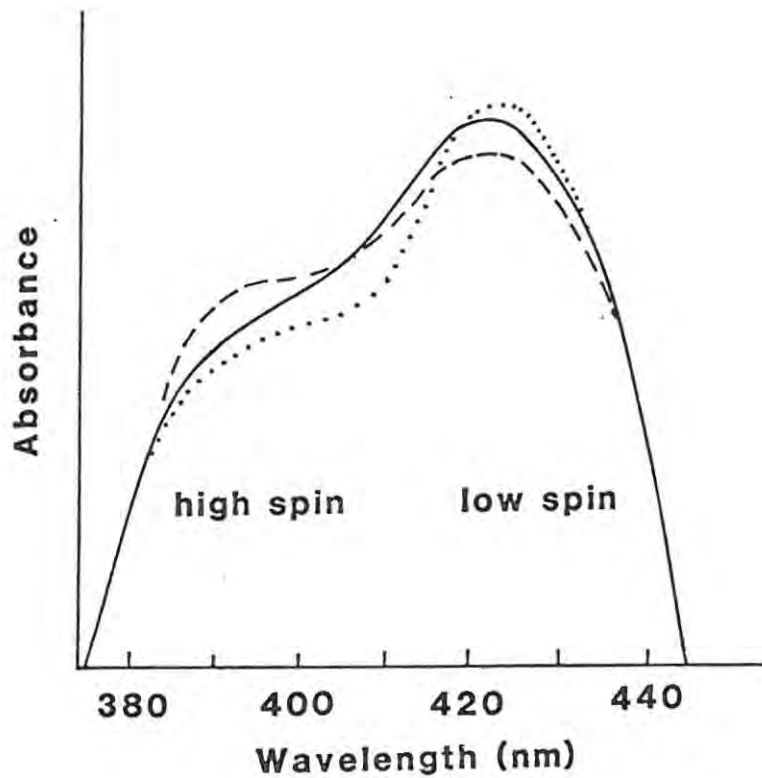


Figure 15. A stylized diagram of the absolute spectrum (continuous line) of cytochrome P-450 showing the effect of Type-I (dashed line) and Type-II substrates (dotted line) (after Peisach *et al.*, 1972, in Ho Yeun, 1977).

An absolute spectrum (Figure 15) is obtained in two ways:

(1) Inducing the enzyme system to increase the levels of cytochrome P-450 and thereby reduce interference by cytochrome b5 (cytochrome b5 levels are not markedly affected by induction (La Du et al., 1972; Mannering, 1981). Non-induced microsomes are used as a control to balance the spectrophotometer on the basis of protein concentration.

(2) The problem of cytochrome b5 interference has been solved by removing cytochrome b5 from microsomes. This is achieved by treating microsomes with nagrase (a bacterial proteinase), or with the non-ionic detergent, Luberol (Paine, 1981).

Figure 15 also shows the effect of Type I and Type II substrates on the spectrum of cytochrome P-450. Basically there are three types of spectra: Type I, Type II and reverse Type I. Kumaki & Nebert (1978, in Mannering, 1981) have classified the compounds causing these spectral changes in accordance with their structures: Type I substrates (peak at about 385-390 nm, trough at about 420 nm; examples: cyclohexane, chloroform, n-hexane, carbon tetrachloride, carbon disulphide) are distinctly hydrophobic, which allows them to interact with a hydrophobic portion of the protein moiety of the haemoprotein. Type II compounds (peak at 425-430 nm, trough at 390-410 nm; examples: actylamine, pyridine) are amines which can form a nitrogen ligand with the haem iron. Reverse type I compounds (peak at 420 nm and trough at 385-390 nm; examples: ethanol, 1-butanol, acetone, N, N-dimethylformamide, cyclohexane oxide, demethyl sulphoxide, tetrahydrofusam) form an oxygen ligand with the haem iron because they contain lone-pair electrons on sterically accessible oxygen atoms. A type I substrate (benzphetamine) and a type II substrate (aniline) were less reactive with cytochrome P-450 from the trout than from the rat according to Schwen & Mannering (1982a; Table 11).

Table 11. Type I and type II substrate binding of hepatic cytochrome P-450 (from Schwen & Mannering, 1982a).

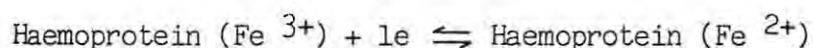
	Benzphetamine*	Aniline**
Rat	0,034 (\mp 0,003)	0,031 (\mp 0,002)
Trout	0,015 (\mp 0,006)	0,025 (\mp 0,005)
Frog	0,009 (\mp 0,002)	0,015 (\mp 0,003)
Snake	0,010 (\mp 0,003)	0,020 (\mp 0,004)

Concentrations of benzphetamine and aniline were 0,28 and 10,0 mM, respectively. Values are the mean and SE. N = 3.

* Type I binding (Δ OD/nmol P-450);
 Δ OD was measured between 390 and 420 nm.

** Type II binding (Δ OD/nmol P-450);
 Δ OD was measured between 395 and 425 nm.

The difference spectra obtained by the binding of drugs to cytochrome P-450 has not yielded any clear insight into the actual binding processes for the following reasons (Paine, 1981): Cytochrome P-450, in common with other haemoproteins which participate in electron transport, can undergo reversible oxidation and reduction:



These haemoproteins have in common a tetrapyrrole prosthetic group containing iron, in either the ferrous (Fe^{2+}), or ferric (Fe^{3+}), state which forms coordinate bonds by sharing electrons with the nitrogen atoms of the tetrapyrrole ring (Paine, 1981). Because the iron in haem is a hexavalent octahedral complex, the iron in the planar tetrapyrrole structure can form ligands with two other species at right-angles to the plane of the tetrapyrrole (Figure 16A). The physical characteristics of haemoproteins will be dependent on the nature of the 5th and 6th ligands linked to the haem prosthetic group (Paine, 1981). The physical properties of these haemoproteins is a function of the "ligand field strength", which is dependent on the structure of the substrate (atom, ion or molecule) which is the donor partner (Paine, 1981). The difference spectra produced can be understood by considering the electronic configuration of the haem iron.

According to Paine (1981), the ferrous ion has six electrons distributed about five 3d orbitals, one of the outermost orbitals contains two electrons, which according to Hund's rule will be of opposite spin. The ferric ion has five electrons distributed about five orbitals (one electron in each orbital). In an octahedral environment the five d orbitals are split into two groups of differing energy.

Three of the five orbitals are of lower energy and are called " t_{2g} " orbitals. The remaining two of higher energy are called " e_g " orbitals. In a strong ligand field (i.e. for a nucleophile which is strongly bound), the donation of electrons from the ligand will force electrons into the orbitals of lower energy (t_{2g}). This will reduce the number of unpaired electrons on the haem iron (Fe^{2+} or Fe^{3+}) and gives rise to the "low spin state". If the ligand field is weak the electrons will occupy all the available molecular orbitals (the Fe^{2+} complex will have four unpaired electrons and the Fe^{3+} five). This is known as the "high spin state". Cytochrome P-450 exists in both the high spin and low spin states, transition between these two states is caused by properties of the substrate. The high and low spin states are expressed spectrally as Type I and Type II binding spectra, respectively (Figure 15).

Yu et al. (1974) and Lipscomb & Gunsalus (1973) obtained information on the nature of the binding and active site of cytochrome P-450_{cam} from the camphor-grown organism Pseudomonas putida. Cytochrome P-450_{cam} is present in P. putida in a soluble state. Despite the fact that the known substrates of P-450_{cam} are restricted to D- or C-camphor, and these oxidations are NADH- rather than NADPH-linked, it is hoped that the information gained will improve our knowledge of the "membrane bound" cytochrome P-450 systems (Paine, 1981). Reducing equivalents from NADH are transferred to cytochrome P-450_{cam} via the non-haem iron putidaredoxin (this is analogous to the adrenal mitochondrial system described earlier). Cytochrome P-450_{cam} when reduced will react with CO to give a Soret band peak at 446 nm (Yu & Gunsalus, 1974).

Yu et al. (1974) reported that cytochrome P-450_{cam} binds in a 1:1 mole ratio with camphor and produces a characteristic spectrum. Lipscomb & Gunsalus (1973) proposed a model of the active site of cytochrome P-450_{cam} (Figure 16). They proposed that the ferriprotoporphyryn IX molecule is held in the protein

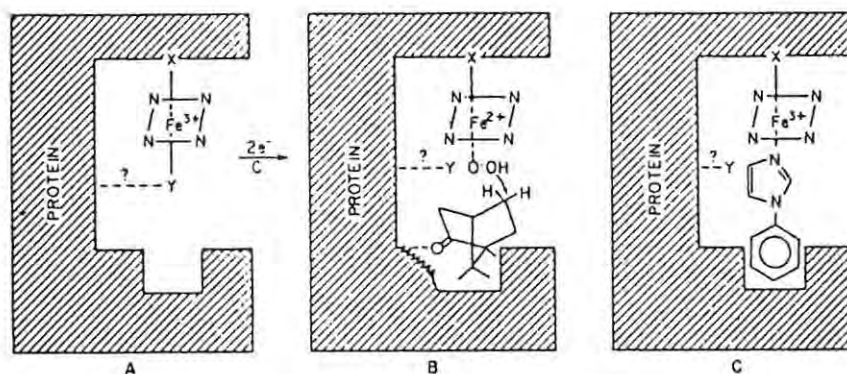


Figure 16. Model of the active site of cytochrome P-450_{cam}. X and Y are protein ligands. A = postulated structure of oxidized P-450_{cam}; B = postulated structure of oxycytochrome P-450_{cam}-camphor complex; C = postulated structure of N-phenylimidazole complex (from Lipscomb & Gunsalus, 1973).

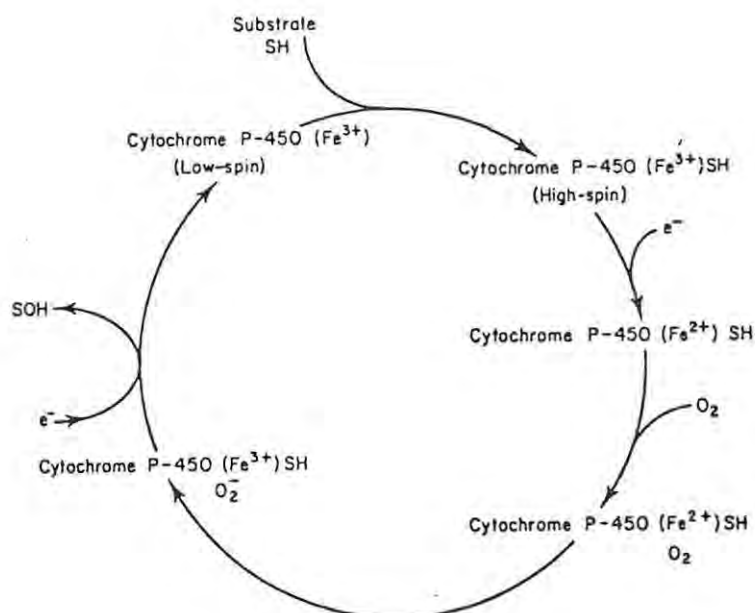


Figure 17. Proposed mechanism, involving the superoxide ion for hydroxylation reactions catalysed by cytochrome P-450 (from Paine, 1981).

by two strong ligands X and Y, which might be histidine and cysteine. These ligands do not prevent removal of the haem prosthetic group and the resultant apoprotein can be reconstituted to an active P-450_{cam} with bovine haematin (Paine, 1981).

The molecule with both X and Y bound to the iron is probably a low spin species, the addition of camphor alters the spin state. The camphor is postulated to bind near the haem in a hydrophobic region displacing, or weakening one of the ligands to the haem. This postulate is supported by the extremely strong binding of P-450_{cam} for N-phenylimidazole (this is a competitive inhibitor of camphor hydroxylation and unlike camphor produces a low spin complex). This suggests that phenylimidazole binds in a similar way to the haem moiety as ligand Y. Ligand Y on this basis may be the imidazole ring of histidine (Paine, 1981).

Unlike the adrenodoxin-requiring system of the adrenal cortex and the putidaredoxin-requiring system of *P. putida*, purified hepatic cytochrome P-450 contained no measurable non-haem iron (Paine, 1981) i.e. the mechanism of electron transfer is different in the hepatic cytochrome P-450. It is possible that the role of the non-haem iron is carried out by phosphatidylcholine in the hepatic system (Paine, 1981). Gunsalus *et al.* (1973) demonstrated that in the absence of putidaredoxin, NADH will reduce the P-450_{cam} complex from the ferric to ferrous state. It will not, however, supply the reducing equivalents to cleave the substrate and regenerate the ferric form of P-450_{cam}. In addition, neither adrenodoxin or phosphatidylcholine will substitute for the role of putidaredoxin in transferring the second electron to the P-450_{cam} substrate complex despite the similar redox potentials of adrenodoxin and putidaredoxin.

Although there are many unresolved questions, evidence from experiments on the bacterial P-450_{cam} (Gunsalus *et al.*, 1973) and resolved hepatic systems (Strobel & Coon, 1971) indicates the involvement of the superoxide (O_2^-) ion as the "active oxygen species" formed during substrate oxidation (Figure 17).

Figure 17 shows the interaction of the substrate (SH) with the ferric form of P-450. This is followed by electron transfer from NADPH resulting in the ferrous P-450 substrate complex. The following step involves the interaction of this complex with molecular oxygen to produce the oxyferrocytochrome substrate complex. Next, the haem iron of the cytochrome is oxidised from the ferrous back to the ferric state, resulting in electron transfer from the haem iron to the oxygen molecule. This produces the activated form of the oxygen molecule which will be inserted into the substrate. The next step is the transfer of the second electron from NADPH via the reductase. This results in the oxygenation of the substrate and the regeneration of the ferric form of the now substrate-free P-450, which is then able to re-enter the catalytic cycle. The precise nature of the activated form of oxygen produced is still unknown (Paine, 1981). The reader is referred to Mannering (1981) for more detail on the chemical reactions occurring in Figure 17.

Multiple Forms of Cytochrome P-450

Different forms of cytochrome P-450 have been purified and identified primarily by differences in apparent molecular weight (Coon *et al.*, 1977). The nomenclature of these different cytochrome P-450s is based on two factors (Coon *et al.*, 1977):

- (1) their origin i.e. P-450_{Lm} indicates that this cytochrome was isolated from liver microsomes, and
- 2) their relative mobilities when subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, e.g. P-450_{Lm1} to P-450_{Lm7}.

Two of the above seven cytochrome P-450_{Lm}'s have been studied in detail: the phenobarbital-inducible P-450_{Lm2} and the polycyclic hydrocarbon-inducible P-450_{Lm4} (Table 12). P-450_{Lm2} and P-450_{Lm4} differ in minimum molecular weights, L- and N-terminal amino acids, and spectral characteristics (Table 12).

However, they both appear to be glycoproteins and have the same carbohydrate content (two manose and one glucosamine per polypeptide chain). Dean & Coon (1977) demonstrated using antibodies that these two cytochromes were dissimilar. They found that antibodies prepared against purified P-450_{Lm2} do not cross-react with P-450_{Lm4}, and vice versa.

The amino acid sequence of cytochrome P-450_{Lm2} is similar to that found in pre-proteins such as pancreatic zymogens and myeloma immunoglobulin light chains (Paine, 1981). In this context Nebert (1979, in Mannering, 1981) has hypothesized that as many species of cytochrome P-450 may exist as there are chemicals capable of inducing this enzyme system. He proposed that specific cytochrome P-450s are formed by individual hepatocytes in the same manner that immunoglobulins are formed by individual B-lymphocytes i.e. in theory hepatocytes would possess an "antibody type system" for producing specific species of cytochrome P-450 in response to specific chemicals.

Mannering (1981) mentions that catalytic properties of cytochrome P-450 systems are determined in part by the membrane. The composition of this membrane differs with age, sex and environment of the same species; the substrate selectivity may be altered accordingly (Mannering, 1981).

Alternatively, a given species of P-450 may be located in more than one environment of the membrane, and the degree of reactivity with substrates will depend on the peculiarities of each environment (Mannering, 1981).

Table 12. Properties of the two major forms of liver microsomal cytochrome P-450 (from Coon et al., 1978).

Property studied	Values obtained	
	P-450 _{LM2}	P-450 _{LM4} *
Polypeptide molecular weight	48 700	55 300
Apparent molecular weight	300 000	500 000
Haem content, per polypeptide chain	1	1
C-Terminal amino acid residue	Arginine	Lysine
N-Terminal amino acid residue	Methionine	?
Carbohydrate residues per polypeptide chain	3	3
Phospholipid residues per polypeptide chain	0,3	0,5
Absorption maxima:		
oxidized form	418 nm	394 nm
reduced CO complex	451 nm	448 nm
Maximum electron uptake per haem-containing polypeptide	1	1

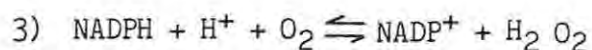
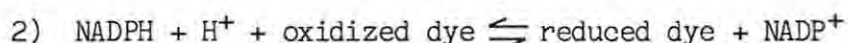
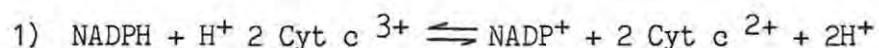
* Previously named cytochrome P-448.

Ullrich & Kremmers (1977, in Mannering, 1981) suggested an evolutionary role of multiple P-450 systems. They proposed that a single enzyme system with a broad substrate selectivity would necessarily exhibit low substrate affinity and would therefore be of limited value in the detoxification of many potent toxins. These toxins only reach very low concentrations in the tissues. A limited number of more selective enzymes would remove low concentrations of toxins from tissues more effectively than a single, relatively non-selective enzyme.

The variety of catalytic activities attributed to cytochrome P-450 are now accounted for by the existence of multiple forms of cytochrome P-450 which exhibit different but overlapping substrate affinities (Table 13). For an extensive review on the multiplicity of cytochrome P-450 the reader is referred to Lu & West (1980).

Reductases

An NADPH-linked reductase capable of reducing cytochrome c was discovered in rat liver by Horecker (1950). This enzyme was shown to be of microsomal origin (Phillips & Langdon, 1962; Williams & Kamin, 1962). These authors showed that microsomal cytochrome c reductase would catalyse the following reactions:



Gillette (1963) showed that cytochrome c inhibited microsomal drug metabolism.

Table 13. Substrate specificity of different forms of cytochrome P-450
(from Coon et al., 1978).

Substrate tested	Activity of forms of P-450 _{LM}
Benzphetamine	LM2 most active
Testosterone	LM1,7 most active in 6- β -hydroxylation LM2 most active in 16- α -hydroxylation
Biphenyl	LM2 most active in 4-hydroxylation
Benzo(a)pyrene	LM2 most active, followed by LM1
Benzo(a)pyrene(-)trans- 7,8-diol	LM4* most active, followed by LM7 and LM1 LM4 most selective in formation of diol- epoxide I over diol-epoxide II**
Warfarin	LM2 regioselective in formation of (R)-4-and (S)-6-hydroxy warfarin LM4* in formation of (R)-6- and (S)-6- hydroxywarfarin ***

* Previously named cytochrome P-448.

** Benzo(a)pyrene 7,8-diol-9,10-epoxides exist as a pair of stereoisomers in which the 7-hydroxyl group is either cis (diolepoxide-1) or trans (diolepoxide-2) to the 9,10-epoxide.

*** The terms R and S specify the absolute configuration of the optical enantiomers of hydroxywarfarins.

This suggested that NADPH-cytochrome c reductase might be a component of this system. As more was learned about cytochrome P-450-linked mono-oxygenase systems it became apparent that cytochrome P-450 is the natural acceptor of electrons from the reductase (Mannering, 1981).

NADPH cytochrome P-450 reductase is now envisaged as reducing cytochrome P-450 directly or indirectly through a non-haem iron protein or some other unidentified carrier (Paine, 1981). A non-haem iron was not present in the catalytically active purified hepatic cytochrome P-450 system of Ballon *et al.* (1974). The reductases associated with bacterial and mitochondrial cytochrome P-450 differ from the hepatic microsomal NADPH cytochrome P-450 reductase in that they contain only flavin mononucleotide (FMN) and transfer electrons to cytochrome P-450 via the non-haem iron proteins putidaredoxin and adrenodoxin, respectively (Mannering, 1981).

The precise role of NADPH-cytochrome c reductase is unknown (Paine, 1981). Even less is known about NADPH cytochrome-P-450 reductase (Paine, 1981). The current assumption, in spite of little evidence, is that NADPH cytochrome c reductase reflects the activity of NADPH cytochrome P-450-reductase.

Rat liver cytochrome P-450-reductase seems to comprise a single polypeptide chain with a molecular weight of 74 000 (Coon *et al.*, 1978). This chain undergoes aggregation under non-denaturing conditions to a species with an apparent molecular weight of 450 000 (Coon *et al.*, 1978). Each polypeptide chain contains one molecule of FMN and one molecule of flavine adenine dinucleotide (FAD) (Coon *et al.*, 1978). Coon *et al.* (1978) showed that the catalytic activities of FMN-depleted reductase retained the ability to accept electrons from NADPH and to catalyse the reduction of ferricyanide. It did, however, lose the ability to reduce cytochrome P-450. This suggested that the

mechanism of catalysis by the hepatic mono-oxygenase system involves separate roles for the two flavin molecules. It has been suggested that FAD is the flavin which accepts electrons from NADPH, and that FMN participates in the subsequent transfer of electrons to cytochrome P-450 (Coon et al., 1978).

Cytochrome P-450 and reductase activity in fish

Schwen & Mannering (1982a) showed that the levels of components of the hepatic mono-oxygenase system of the trout were considerably lower than those found in the rat: levels of cytochrome P-450 were 34% of that of the rat. Levels of cytochrome-b5 in microsomes were 11% of that of the rat; NADPH-cytochrome P-450 reductase and cytochrome c reductase activities were 14% and 23% of the activity of the rat respectively (Figure 18). When reductase activity is calculated relative to the amount of cytochrome P-450, the value of the trout was about half that of the rat (Figure 19). The reactivity of trout NADPH cytochrome P-450 reductase (0,014) was lower than that of the rat (0,022).

FACTORS AFFECTING CYTOCHROME P-450 ACTIVITY.

According to Williams (1972), Mannering (1981) and Paine (1981) the major factors affecting the activity of the cytochrome P-450-linked enzyme system are:

- (1) species and strain differences
- (2) age
- (3) hormonal differences (e.g. sex differences)
- (4) nutritional status
- (5) environmental factors (e.g. temperature, time of day, season, etc.)

The general pattern of xenobiotic metabolism is common to all species (Williams, 1972). However, the enzymes involved in these reactions may vary

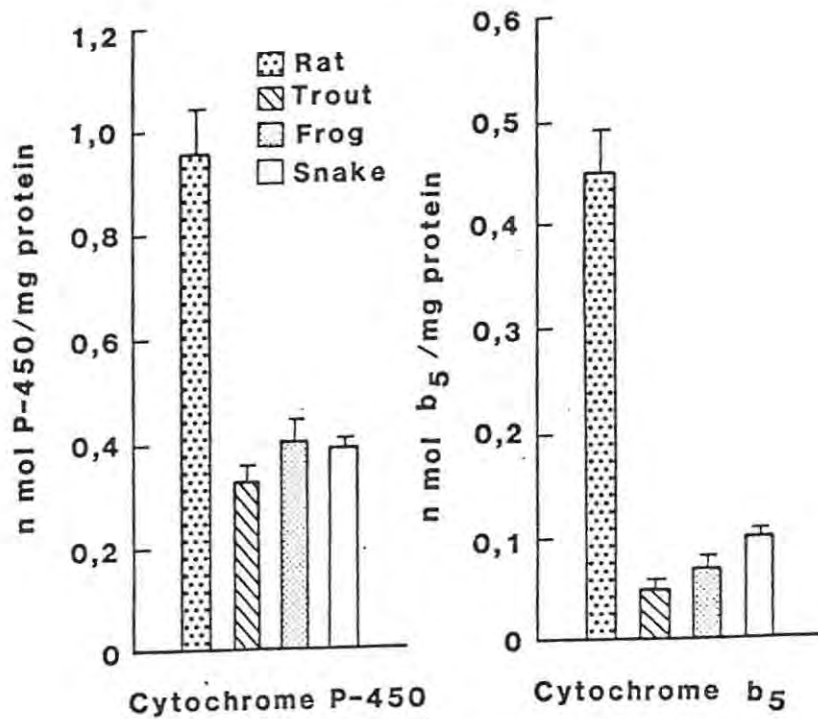


Figure 18. Concentrations of cytochrome P-450 and cytochrome b₅ in hepatic microsomes of the rat, trout, frog and snake. Values are the mean and SE. N = 5 or more (from Schwen & Mannering, 1982a).

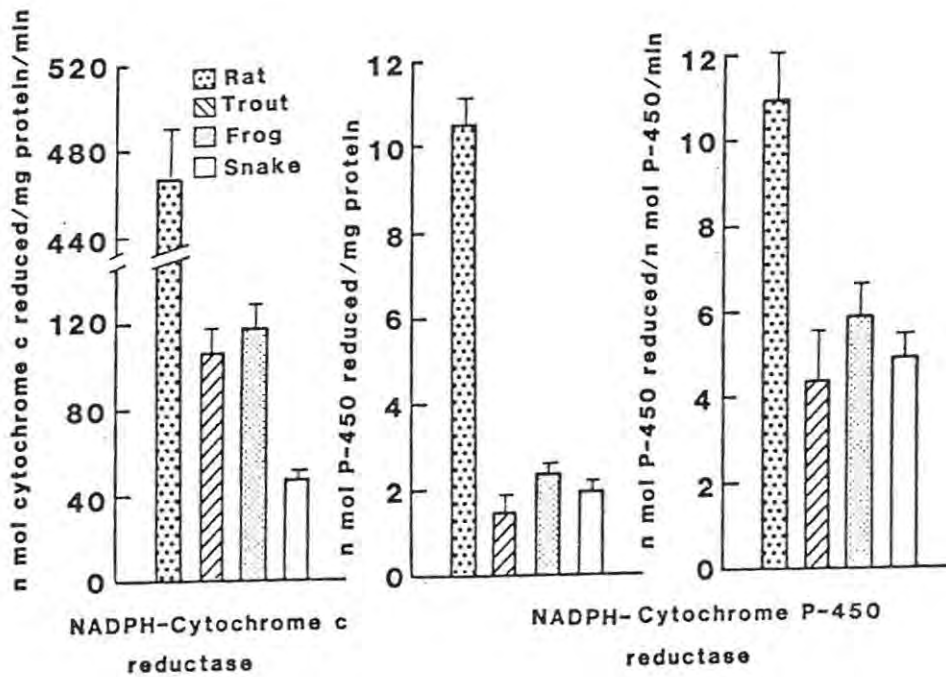


Figure 19. NADPH-cytochrome c- and NADPH-cytochrome P-450 reductase activities of hepatic microsomes from the rat, trout, frog and snake. Values are the mean and SE. N = 4 or more (from Schwen & Mannering, 1982a).

quantitatively and qualitatively from one species to another (Williams, 1972; Mannering, 1981). Qualitative differences in cytochrome P-450's of trout, frog and snake were indicated by the differences in inhibitory effects of SKF 525-A and A-naphthoflavone on mono-oxygenase activities (Schwen & Mannering, 1982a). Strain differences have been reported in mice, rats and rabbits (Paine, 1981). Paine (1981) mentioned that in rabbits two-fold and four-fold strain differences have been observed, not only in basal levels of microsomal P-450-linked enzymes, but also in their responsiveness to induction by phenobarbital. He also mentioned that work by Vessel & Page (1968) demonstrated that genetic factors were the major cause of variation in drug metabolism between human individuals. Further work by Vessel et al. (1971) showed that in addition to the basal levels of microsomal P-450-linked enzymes being under genetic control, the response to the inducer phenobarbital was found to be the same in identical twins, but different in non-identical twins.

Foetal and new-born animals exhibit very low levels of cytochrome P-450-linked enzymes (Paine, 1981). Responsiveness to phenobarbital is also absent during early foetal life in rabbits, but appears in the last 4-5 days of pregnancy (Fouts & Hart, 1965).

Although androgenic hormones increase microsomal enzyme activity, not all hepatic metabolic enzymes reach their peak activity at sexual maturity, as the cytochrome P-450 dependent activities have developmental patterns unrelated to one another (Gram et al., 1969). Little attention has been paid to drug metabolism in old animals (Paine, 1981). Two-year old rats have been shown to have lower rates of drug metabolism than younger animals (Kato & Takanaka, 1968).

The livers of male rats have a greater hydroxylating activity towards certain

substrates than do the livers of females and immature males (Paine, 1981). This differential response can be annulled by the castration of males or by the administration of oestradiol (Gillette, 1963). Conversely the activity in females can be increased by dosing with testosterone. These findings indicated to Conney (1967) that the balance between male and female sex hormones was important in determining the activity of cytochrome P-450-linked enzymes. This relationship between microsomal enzyme activity and sex hormones was complicated by the finding that adrenalectomy of male rats decreased the activity of hexobarbital oxidase and annulled the sex difference (Gillette, 1963; Kato & Gillette, 1965). The "male activity" could be restored by the injection of cortisone, but not testosterone - indicating differing roles for the two hormones. The role of cortisone is not clear as adrenalectomy had no effect on microsomal enzyme activities of female rats (Kato & Gillette, 1965). Sex related differences have also been noted in fish (Stegman & Chevion, 1980), but not in the mouse, rabbit or guinea pig (Mannering, 1981).

Vitamin deficiency or protein deficiency markedly alters the activity of the hepatic microsomal P-450-linked enzymes (Paine, 1981). Many adaptive changes in biochemical activity have been shown to occur when diet is altered (Schimke & Doyle, 1970). The important dietary factors with regard to the microsomal cytochrome P-450-linked enzymes have been shown to be the protein content of the diet, the unsaturated fat content and its state of oxidation, and the presence of non-nutrient inducing components such as flavones (Paine, 1981).

The little that is known about factors affecting cytochrome P-450-linked enzyme activity indicates that the cytochrome P-450 mediated drug/xenobiotic metabolism is complex. Although these studies have been carried out on mammals the same factors are likely to affect fish cytochrome P-450 activity.

Therefore it is important to keep those factors, known to affect cytochrome P-450 activity, constant during experiments.

INDUCTION OF CYTOCHROME P-450 BY FOREIGN COMPOUNDS

When hepatic microsomes of animals treated with an inducer are isolated their drug/xenobiotic activity is enhanced for a variety of substrates (Greim, 1981). This enhancement is due to increased enzyme content and not increased enzyme activity (Greim, 1981). There is strong evidence that induction represents de novo synthesis of enzymatic proteins rather than the activation of pre-existing enzyme forms or a decrease in the rate of protein degradation (Greim, 1981).

The induction of P-450 systems involves DNA-dependent RNA synthesis and requires protein synthesis; inhibitors of either process, e.g. actinomycin D or cycloheximide, prevent these increased activities (Conney & Gilman, 1963; Gelboin & Blackburn, 1964). During the last twenty years more than two hundred pharmacologically and chemically unrelated compounds have been shown to enhance microsomal drug hydroxylating enzymes (Paine, 1981). Examples of these compounds include barbiturates, non-barbiturate sedatives, insecticides, analgesics, antihistamines and polycyclic hydrocarbons (Paine, 1981). Based on the biological effects produced, most of these agents can be placed in one of two groups (Conney, 1967):

- (1) the phenobarbital-type, or
- (2) the polycyclic hydrocarbon-type.

The phenobarbital-type increases the ability of the microsomes to metabolize a large number of drugs and hormones (Paine, 1981). It causes an increase in P-450 content, NADPH-cytochrome c and cytochrome P-450 reductases, microsomal

protein and phospholipid as well as liver weight (Conney, 1967). The phenobarbital-type inducers include the chlorinated hydrocarbon pesticides and endosulfan (Mannering, 1981; Schwen & Mannering, 1982c; Tyagi *et al.*, 1984).

The polycyclic hydrocarbon-type inducer (e.g. 3-methylcholanthrene, or 3, 4 benzpyrene) produces a more selective stimulation of hepatic enzymes, and increases the metabolism of fewer substrates (Table 14). Induction by polycyclic hydrocarbons also produced an altered form of cytochrome P-450, originally cytochrome P-448 and now called P-450_{Lm4} (Mannering, 1981).

A third type of inducing compound has been reported, the administration of which induces enzymes in a pattern similar to the co-administration of phenobarbital and 3-methylcholanthrene (Paine, 1981). This third type of inducer comprises a mixture of polychlorinated biphenyls (an insecticide Arochlor TM) or polybrominated biphenyls (Firemaster BP 6TM, hexachlorobenzine and the synthetic steroid pregnenolone 16- α -carbonitril). It is not clear whether these compounds comprise a new class of inducer or if the mixed induction is due to these agents being mixtures of phenobarbital-type and 3-methylcholanthrene-type inducers (Paine, 1981).

Fish appear to be resistant to the phenobarbital-type inducers (Schwen & Mannering, 1982c). Although results have not been consistent as Gutman & Kidron (1971) reported induction by phenobarbital, and Burns (1976) induction by phenylbutazone. Fish mono-oxygenase systems have been induced by 3-methylcholanthrene-type inducers (Schwen & Mannering, 1982c).

Turnover studies

The phenobarbital and 3-methylcholanthrene-type inducers increase the microsomal P-450-linked activities by the same basic sequence of events : DNA-

Table 14. Hepatic parameters altered by phenobarbital and 3-methylcholanthrene induction (from Conney, 1967).

Hepatic activity	Effect of inducer*	
	Phenobarbital	3-methylcholanthrene
Cytochrome P-450 (P-450 _{LM2})	+	?
Cytochrome P-448 (P-450 _{LM4})	?	+
NADPH-cytochrome c reductase	+	0
NADPH-cytochrome P-450 reductase	+	0
Benzamphetamine N-demethylation	+	0
Hexobarbital hydroxylation	+	0
Steroid metabolism	+	0
3,4-benzpyrene hydroxylation	+	+
Microsomal protein	+	±
Liver weight	+	±

* Key: + = increase; ± = slight increase; 0 = no change.

dependent RNA synthesis, followed by protein synthesis (Gelboin & Blackburn, 1964). Gelboin & Blackburn (1964) mention that 3-methylcholanthrene induction does not appear to be due to an inhibition of the rate of P-450 degradation.

Schimke et al. (1968) administered radioactive labelled amino acids to control and phenobarbital treated rats. Their results indicated that phenobarbital increases the rate of the synthesis of some, but not all, of the proteins of the endoplasmic reticulum. Jick & Shuster (1966) measured the level of NADPH-cytochrome c reductase in phenobarbital treated mice. Their results suggested that increased levels of activity resulted from increased synthesis and decreased breakdown. NADPH cytochrome c reductase levels were studied because its level in the liver is also increased by phenobarbital treatment and, unlike cytochrome P-450, it can easily be purified (Paine, 1981).

Kuriyama et al. (1969) reported that a single dose of phenobarbital produced a rapid increase in the rate of NADPH cytochrome c reductase synthesis.

Repeated doses caused much larger increases in the rate of reductase synthesis. A reduction in the rate of degradation occurred at the same time as the increased reductase synthesis. After the cessation of phenobarbital treatment the rate of degradation increased whilst synthesis continued.

Inducers increase the rate of synthesis of cytochrome P-450, but whether the rate of degradation is also affected, as in NADPH cytochrome c reductase, remains an open question (Paine, 1981).

Stimulatory effects of inducers on protein synthesis.

When cytochrome P-450 is induced there is a corresponding increase in the incorporation of amino acids into microsomal proteins, but not into other subcellular fractions (Kato et al., 1966; Paine, 1981). This increased incorporation could not be attributed to changes in cofactor levels, amino

acid concentration, or factors present in cell sap (Paine, 1981). Inducers also stimulate the incorporation of t RNA-bound amino acids (Paine, 1981). This suggests that the increased incorporation was not as a result of an increased rate of amino acid activation, but was dependant on steps between t-RNA and peptide formation on ribosomes (Paine, 1981).

Inducers cause increases in both transcription and translation of RNA (Gelboin, 1964; Kato *et al.*, 1966). Kumar & Padmanaban (1980) and Bar-Nun *et al.* (1980) showed that the levels of translatable hepatic cytochrome P-450 mRNA are substantially increased during induction. The mechanism of induction appears to be similar to the lac operon of bacteria proposed by Jacob & Monod (1961). However, Paine (1978; 1981) pointed out that some common denominator exists between the different and diverse inducers. He suggested that induction by many diverse compounds is mediated through a common endogenous inducer.

Stimulatory effects of inducers on haem synthesis

Haem is a component of cytochrome P-450, and cytochrome P-450 accounts for more than half the haem requirement of the liver (Paine, 1981). Therefore, any increase in cytochrome P-450 levels should cause an increased demand for haem. This would be reflected by an increase in the activity of 5-aminolaevulinic acid synthetase, the rate limiting enzyme which controls the overall activity of the haem biosynthetic pathway (Tait, 1978). Paine (1981) reported that there is evidence that a stimulation of both the activity of 5-aminolaevulinic acid (5-ALA) synthetase and the rate of hepatic haem synthesis precede the increase in cytochrome P-450 following induction.

The increase in cytochrome P-450 caused by phenobarbital can be prevented by amino triazole, an inhibitor of hepatic haem synthesis (Baron & Tephly, 1969).

This suggests that an increased supply of haem is a primary event in the induction of cytochrome P-450 (Paine, 1981). However, an increase in haem synthesis alone is not sufficient to increase cytochrome P-450: administration of 5 ALA, which bypasses the rate-limiting step in haem synthesis, does not increase hepatic cytochrome P-450 (Druyen & Kelly, 1972). According to Paine (1981) the current concensus is that haem synthesis is not rate-limiting in the induction of hepatic cytochrome P-450. The main controlling factor is the synthesis of the apoprotein.

Topography of cytochrome P-450 and the enzymes involved in its synthesis.

Haem synthesis must in some way be co-ordinated with the formation of the cytochrome P-450 apoprotein moiety (Paine, 1981). The first reaction leading to the synthesis of haem (the formation of 5 ALA) as well as the last two steps to protoporphyrin and then to haem, take place inside the mitochondria, while the intermediary steps take place in the cytosol (Sano & Cranick, 1961). The synthesis of apocytochrome P-450 takes place on the ribosomes of the rough endoplasmic reticulum (Paine, 1981). It is not known whether apocytochrome P-450 is synthesised on those parts of the rough endoplasmic reticulum in close association with the mitochondria or is just generally associated with rough endoplasmic reticulum (Paine, 1981). Meier *et al.* (1978) isolated a subfraction of the endoplasmic reticulum associated with mitochondria which was shown to be involved in cytochrome P-450 synthesis. However, it is not clear whether this was a true cellular entity or an artefact of fractionation (Paine, 1981).

The mechanism of haem insertion into apocytochrome P-450 is not known (Paine, 1981). Neither is the question of whether cytochrome P-450 is synthesised together with other components of the microsomal membrane, or if cytochrome P-450 is synthesised and inserted into pre-existing membranes (Paine, 1981).

Studies in vitro suggest that newly synthesised cytochrome P-450 is inserted into pre-existing membranes (Paine, 1981).

Phenobarbital induction of cytochrome P-450 has been shown to cause a proliferation of rough and smooth endoplasmic reticulum (Conney, 1967; Massey & Butler, 1979). Even after chronic administration of phenobarbital only the cells of the centrilobular region of the liver responded (Massey & Butler, 1979). 3-methylcholanthrene, however, does not seem to cause significant proliferation of the endoplasmic reticulum (Conney, 1967).

Yang & Strickhart (1975) and Yang (1977) demonstrated that purified cytochrome P-450 or P-448 added to a microsomal membrane enhances the rate of drug metabolism. This could result from the incorporation of purified cytochrome P-450 into the membrane, or alternatively it reflects the ability of P-450 reductase to couple with exogenous cytochrome P-450 (Paine, 1981).

Genetics of induction: the Ah locus

The cytochrome P-450-linked enzyme called arylhydrocarbon hydroxylase, or benzpyrene-3-mono-oxygenase, is highly inducible by polycyclic hydrocarbons, drugs and natural products in the mammal liver and other organs (Paine, 1981). In common with other P-450-linked enzymes, induction requires transcription followed by translation (Gelboin et al., 1972). The basal and induced levels of arylhydrocarbon hydroxylase vary in different mouse strains: some strains are inducible and others non-inducible (Nebert & Gelboin, 1969). This suggested that induction may be under genetic control. Therefore Gielen et al. (1972) carried out various crosses of the C 57, DBA and N2W mouse strains (Table 15). The results of these crosses showed that the induction of arylhydrocarbon hydroxylase by 3-methylcholanthrene was under the genetic control of the Ah locus.

Table 15. Genetics of hepatic arylhydrocarbon hydroxylase (AHH) induction by 3-methylcholanthrene (3MC) in mice (from Gielen *et al.*, 1972).

Parents	Theoretical genotype *	AHH activity		
		Control	3MC treated	
C57	AhAh	630	2700	
DBA	ahah	570	550	
NZW	ahah	770	760	
F1 generation				
ahah x ahah	[ahah	780	660	
AhAh x ahah	[AhAh	590	2500	
Backcross				
(F ₁ gen x parent)				
AhAh x ahah	[AhAh	620	580(20)	2600(23)
	[ahah			
AhAh x AhAh	[AhAh	670	none	2700(10)
	[AhAh			
F2 generation from				
F1 x F1 of C57 x DBA				
	[AhAh	770	730(4)	3090(12)
	[AhAh			
	[ahah			

* Ah = dominant expression of aromatic hydrocarbon responsiveness.
 ah = Recessive.

** Numbers in parenthesis = number of mice.

CHAPTER 4 MATERIALS AND METHODS

COLLECTION OF TEST ORGANISMS

Hatchery-reared fish are preferable for toxicity studies (Greenberg *et al.*, 1981), but these were not available in sufficient numbers in the correct size range. Sub-adult Oreochromis mossambicus ranging in size from 80-100 mm standard length (SL) were therefore collected in the Kowie estuary (33°36'S, 26°54'E) using a thrownet. Sub-adults were used because fish, like rats, show sex differences in cytochrome P-450 activity in mature individuals (Stegman & Chevion, 1980; Mannering, 1981). The fish were placed immediately into large aerated drums and transported back to the laboratory where they were acclimated from saline (35 ppt) to fresh water over two days. Mortality during transportation was less than one percent. On arrival the fish were examined and found to be in good condition, free of disease and with low levels of parasites. Subsequent microscopical examination of liver and gill tissue of control fish did not show lesions from previous exposure to toxicants or diseases.

ACCLIMATION AND EXPERIMENTAL DESIGN

Figure 20 shows the design of the through-flow experimental system. All the components of the system were non-toxic materials and the entire system was housed in a constant environment room, as recommended by Greenberg *et al.* (1981). The header tanks were topped up, as required, with tap water, which then gravity fed into 100 litre glass aquaria. The flow rate into the aquaria was controlled with clamps. Water left the aquaria via an overflow to the floor drain.

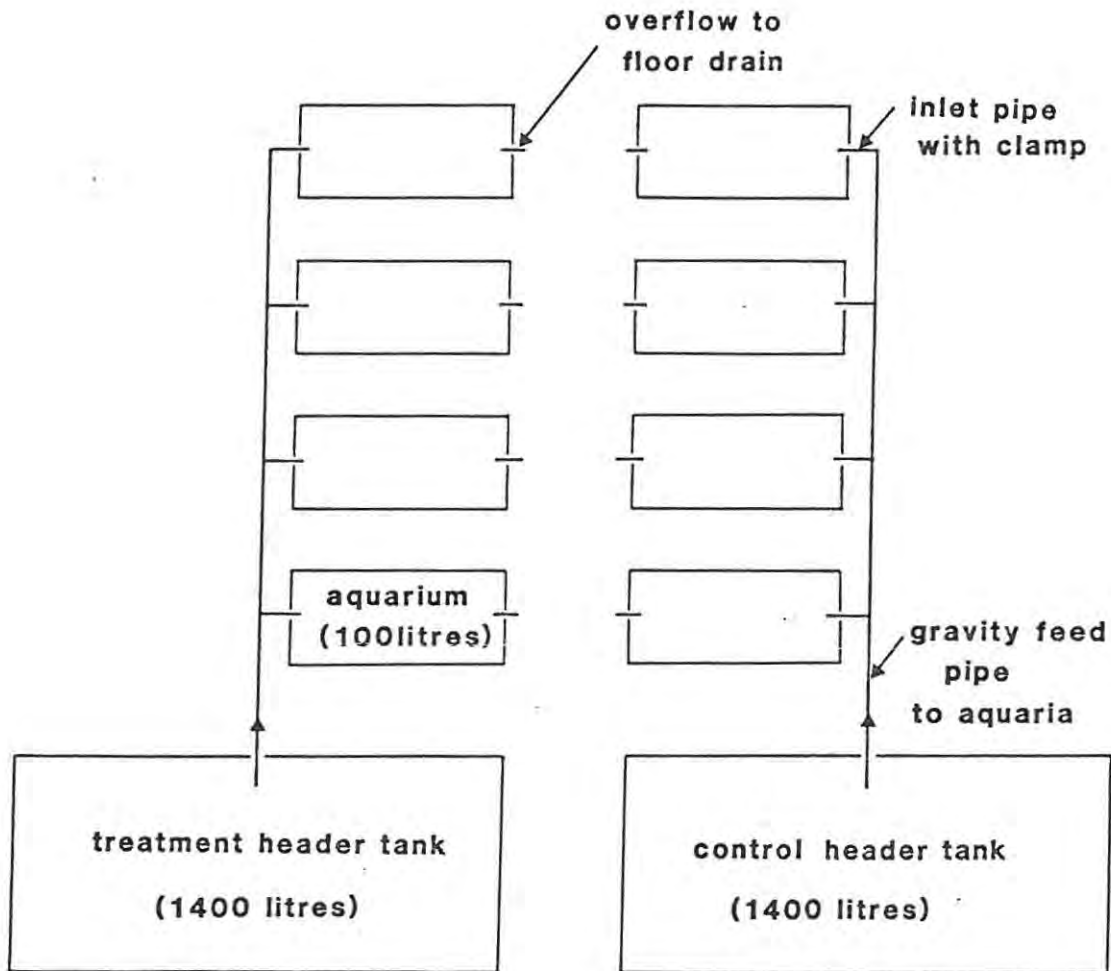


Figure 20. Stylized diagram of the experimental through-flow system used in this study.

Twenty fish of similar size (range 80-100 mm SL) were placed randomly in each aquarium. They were acclimated for three to four weeks. Mortalities only occurred within the first 24 hours after capture. The photoperiod was 13:11 hours (light: dark). The water quality parameters during the acclimation and toxicity studies are shown in Table 16. Fish were fed Epol commercial trout pellets at 3-4% of their body mass in dry weight of pellets daily. Faeces and uneaten food were syphoned out daily.

TWENTY-FOUR AND FORTY-EIGHT HOUR LC50 VALUES

It was necessary to determine the 24 and 48 hour LC50 values of the test animals to enable us to establish what dose levels to use. Groups of ten fish were placed randomly in 100 litre tanks. Two control tanks were used. Fish in the remaining tanks were exposed to different levels of endosulfan. These tests were carried out under static conditions. The dosing water was replaced every twelve hours to limit accumulation of metabolites and degradation and adsorption of endosulfan. The times to 50% mortality were noted for different concentrations. These were log-transformed and plotted against log concentration ($\log Y = \log 346,74 - 1,21 \log X$; $r^2 = 0,95$). The 24 and 48 hour LC50 values were calculated to be 9,1 and 5,1 $\mu\text{g l}^{-1}$ endosulfan respectively (Table 17). The 95% confidence limits were calculated using the method of Sokal & Rohlf (1973) for calculating the standard error for any estimated value \hat{Y} along the regression line. This value is then multiplied by the t value for $n - 2$ degrees of freedom. The LC50 values are shown in Table 17 together with the estimated safe levels after Sprague (1971).

TOXICITY STUDIES

Fish were dosed at four different levels : (1) below the estimated safe

Table 16. Water quality parameters during acclimation and toxicity studies

Water quality parameter	Mean value with standard deviation in brackets	Method used
Flow rate (ml min ⁻¹)	362 (89,3)	measuring cylinder
Temperature (°C)	19 + 2	mercury thermometer
pH	7 - 7,3	pH meter
Oxygen (% saturation)	> 60%	oxygen meter
Total ammonia (mg ml ⁻¹)	0,14 (0,07)	titration
Chlorine (mg ml ⁻¹)	0,075 (0,025)	Hach kit

Table 17. LC50 values for *O. mossambicus* sub-adults exposed to different concentrations of endosulfan (95% confidence limits in brackets).

24 hour LC50	48 hour LC50	Estimated safe level (after Sprague, 1971 : 48 h LC50 x 0,1)
9,1 µg l ⁻¹ (8,1 - 10,1)	5,1 µg l ⁻¹ (4,1 - 6,1)	0,51 µg l ⁻¹

concentration ($0,1 \text{ ug l}^{-1}$); (2) at the safe concentration ($0,5 \text{ ug l}^{-1}$) (this level is similar to the mean concentration of endosulfan reported in the Okavango Delta soon after aerial spraying by Fox & Matthiesson; 1982); (3) above the safe concentration and below the lethal concentration ($1,5 \text{ ug l}^{-1}$); (4) at the 48 hour LC50 concentration ($5,1 \text{ ug l}^{-1}$).

The most common methods of dosing in toxicity studies involves the use of mariotte bottles or peristaltic pumps (Mount & Brungs, 1967; Freeman, 1971; Bengtsson, 1972; McAllister *et al.*, 1972; Abram, 1973; Knauf, 1975). In this study a stock solution of endosulfan was made up daily in forty litres of water. A portion of the stock solution was diluted in the header tank to give the required concentration. This was mixed thoroughly through two baffles using a submersible pump, pumping at 60 l min^{-1} for 15 minutes. The treated water was then gravity fed through the treatment aquaria. The levels of endosulfan were checked at the African Explosives and Chemical Industries (Pty) Ltd laboratory using gas chromatography.

The intervals between spraying cycles in the Okavango Delta have been approximately two weeks (Davies & Bowles, 1979) and induction usually occurs in 10 days or less (Mannering, 1981), therefore, fish were exposed to endosulfan for two weeks before being sacrificed for histological examination, measurement of hepatic cytochrome P-450 activity and measurement of haematological parameters.

MEASUREMENT OF CYTOCHROME P-450 ACTIVITY

Preparation of the liver microsomal fraction

Fifteen fish in each of the control and treatment tanks were sacrificed after two weeks by being pierced through the brain with a dissecting needle. This

was carried out at the same time each day (09h00-09h30) to avoid possible inaccuracies caused by variations in enzyme levels due to diurnal rhythms (Mannering, 1981). Length (mm SL) and mass (g) were recorded. The liver was then carefully dissected out to avoid damage or contamination, weighed and perfused in chilled 0,9% saline solution for thirty minutes. Perfusion in saline removes some of the haemoglobin which interferes with cytochrome P-450 activity (Mannering, 1981). The method used for the preparation of the fish liver microsomal fraction was a modification of the method used by Cinti et al. (1972).

All glassware was washed in detergent and then soaked in chromic acid overnight, rinsed in tap and distilled water and oven dried at 60°C. This care was taken to avoid any possible contamination of the microsomal fraction.

The chemicals used were of analytical grade and were accurately weighed to the nearest 0,001g and made up to volume in volumetric flasks with freshly distilled water, and stored in a refrigerator at 4°C. If the time lapse between experiments was more than four days the solutions were freshly made up. The chemicals used included; 0,9% NaCl (sodium chloride), 0,25M C₆H₁₂O₆ (sucrose), 1,15% KCl (potassium chloride), Ca Cl₂.2 H₂O (calcium chloride) and sodium phosphate buffer pH 7,4 (this was made up from sodium dihydrogen phosphate (Na H₂ PO₄ H₂O) and disodium hydrogen phosphate (Na₂ HPO₄) and the pH was checked on a pH meter (Maron & Ames, 1983)).

After the liver had been excised, all operations were carried out at 4°C by placing the glassware in a crushed ice bath. After perfusion of the liver in 0,9% NaCl, a 25% W/V liver to sucrose homogenate was prepared (1 g liver : 3 volumes 0,25 M sucrose). The livers were minced into small pieces, with a pair of scissors, and then homogenised with the required volume of sucrose in

a tissue homogeniser. The pestle was rotated and moved up and down in the homogenising vessel 8-10 times by hand. Homogenisation causes the disruption of many cells and results in a homogenate of intracellular particles, cell sap and some unbroken cells and the different components can be separated by centrifugation (De Duve, 1983). The homogenate was transferred to a teflon centrifuge tube and placed in a Sorval RC-5 superspeed refrigerated centrifuge set at a temperature of 3-5°C. The tubes were centrifuged at 4 000 g ($5,8 \times 10^3$ revolutions per minute) for 10 minutes to allow cell debris and nuclei to sediment out (Ho Yuen, 1977). This was followed by centrifugation at 10 000 g ($9,2 \times 10^3$ rpm) for 20 minutes to allow the mitochondria to settle out (Cinti *et al.*, 1972; Ho Yuen, 1977).

The supernatant was poured into a 20 ml flask which contained 23,52 mg $\text{Ca Cl}_2 \cdot 2\text{H}_2\text{O}$. This resulted in a supernatant with a concentration of 8 mM $\text{Ca Cl}_2 \cdot 2\text{H}_2\text{O}$. Cinti *et al.* (1972) found that Ca^{2+} caused the endoplasmic reticulum to aggregate and therefore to sediment out at much lower centrifuge speeds : 15-20 minutes at a force of 27 000 g (15 500 rpm) instead of a force of 105 000 g for one hour. In this study the 20 ml sample was centrifuged for 30 minutes. The supernatant was poured out and the pellet washed by resuspending the pellet, using a glass rod, in 1,15% KCl solution. It is important to remove excess Ca^{2+} which may interfere with cytochrome P-450 activity (Ho Yuen, 1977). The washed pellet was resedimented by centrifugation at 27 000 g for a further 30 minutes. The supernatant was then poured off and the microsomal pellet homogenised in sodium phosphate buffer (pH 7,4). The homogenate was made up to 10 ml with buffer in a volumetric flask.

Microsomal protein determination

The method of Bradford (1976) was used in preference to the biuret (Mokrasch & McGilvery, 1956), Lowry (Lowry *et al.*, 1951), and modified Lowry methods

(Hartree, 1972) because it is a sensitive and rapid method of measuring microgram quantities of protein. This method of protein determination involves the binding of Coomassie Brilliant Blue G-250 to protein with little or no interference from cations such as sodium or potassium nor from carbohydrates such as sucrose (Bradford, 1976).

Coomassie Brilliant Blue G-250 (50 mg) was dissolved in 25 ml of 95% ethanol. To this solution 50 ml of 85% W/V phosphoric acid was added. The resulting solution was diluted to a volume of 500 ml. Bovine serum in 0,9% saline solution was used as the standard. 0,1 ml of protein was pipetted into a test tube and 5 ml of protein reagent was added. This was mixed by inverting the test tube. The absorbance in 4 ml cuvettes at 595 nm (> 2 minutes < 60 minutes) was measured and compared against a reagent blank.

Determination of cytochrome P-450

Cytochrome P-450 levels were determined using the methods of Omura & Sato (1964a). Six ml of the microsomal suspension was reduced with two milligrams of sodium dithionite ($\text{Na}_2 \text{S}_2\text{P}_4$). Three ml was then pipetted into a reference cuvette. Carbon monoxide was bubbled through the remaining 3 ml for one minute. The carbon monoxide compound of reduced cytochrome P-450 has an intense absorption at 450 nm. The difference spectra between the reference and sample cuvettes was measured from 380 nm to 540 nm and recorded. The cytochrome P-450 values were calculated from an average of three peaks. The amount of cytochrome P-450 can be calculated from the optical density difference between 450 and 490 nm, and the molar extinction coefficient of $91 \text{ nM}^{-1} \text{ cm}^{-1}$ using the Lambert-Beer equation (Lehninger, 1975):

$$\text{Log } \frac{I_0}{I} = E c l$$

I

where: I_0 is the intensity of incident light
 I is the intensity of transmitted light
 E is the molar absorption coefficient
(in units of litres per molecentimeter)
 c is the concentration of the absorbing species in
moles per litre
 l is the thickness of the light absorbing sample in
centimeters.

HISTOLOGICAL AND ULTRASTRUCTURAL EXAMINATION

Preparation for transmission electron microscopy (TEM)

Three fish from each of the control and treated aquaria were sacrificed for histological and ultrastructural examination of liver and gill tissue. The preparation of these tissues for ultrastructural examination follow closely those outlined by Cross (1980).

Liver and gill tissue were dissected out rapidly and placed in chilled 5% buffered glutaraldehyde. The liver tissue was dissected from the centrilobular region and gill tissue from the central region of the second gill arch. Alterations in the fine structure of tissues takes place rapidly after death, hence the speed and the chilled primary fixative (Glauert, 1975). The gill arches were cut into sections 1 mm thick and the liver into cubes of approximately 1 mm and left to fix for 18 hours at 4°C.

Glutaraldehyde stabilizes the fine structure of tissues by forming crosslinks between the protein molecules (Hayat, 1970). It has rapid specimen penetration and increases the permeability of tissue to embedding media (Hayat, 1970). Glutaraldehyde has no staining properties and does not fix

lipids. A second fixative, 1% buffered osmium tetroxide, was therefore used to fix the lipid fraction. The tissue was then washed in phosphate buffer (pH 7.3) and fixed in osmium tetroxide for 120 minutes.

The embedding medium used was a mixture of Araldite and Epon 812. Important properties of embedding media are: (1) they should completely and uniformly impregnate the tissue and preserve the ultrastructure of cells, (2) they should polymerise evenly, avoiding damage to cells from shrinkage or expansion, and give good cutting characteristics, and (3) they should allow the specimen to be stained and be stable under electron bombardment (Pease, 1964; Wischnitzer, 1967; Glauert, 1975).

These resins are not soluble in water and the fixed tissue was therefore dehydrated in a series of alcohol baths. As the resins are not miscible with alcohol, a transitional solvent, propylene oxide, with dehydrating properties, was used prior to embedding with resin. The fixed, dehydrated tissue was embedded in a series of propylene oxide and embedding medium mixtures - 75 : 25, 50 : 50 and 25 : 75. The tissue was left in each mixture for an hour and in pure resin overnight. The material was then placed in resin containing moulds and left to polymerise at 60°C for 36 hours.

As glass is a supercooled liquid which is subject to slow flow at room temperature and therefore a loss in sharpness with time (Wischnitzer, 1967), glass knives (45°) were made on an LKB knifemaker shortly before use. The block was trimmed crudely using a valet razor blade. Sections of 2-3 µm were then cut using a L.K.B. 8800 Ultratome III, stained with toluidine blue and viewed under a light microscope. The orientation of the desired area of tissue was determined and the necessary adjustments in the block orientation on the microtome were made.

The block was trimmed to form a trapezoidal face (approximately 0,2 mm x 0,2 mm). The face was then polished with a fresh glass knife and ultrathin sections (60-90 nm) were cut, picked up on a 400 mesh grid, stained with uranyl acetate for 35 to 60 minutes, washed, and then stained in Reynold's lead citrate for 3 minutes. Uranyl acetate stains nucleic acids and proteins, while lead stains have an affinity for cell membranes, glycogen and nucleoproteins in the presence of reduced osmium (Hayat, 1970).

The stained ultrathin sections were then viewed and selected photographs taken on a Jeol JEM 100 CX III transmission electron microscope.

Preparation for scanning electron microscopy (SEM)

Gill tissue was fixed and dehydrated in alcohol as for transmission electron microscopy. The tissue was then placed in a series of baths of increasing amyl acetate in alcohol (25 : 75, 50 : 50, 75 : 25) and then into 100% amyl acetate. Dehydrated tissue was placed in a Polaron E 3000 critical point drier and dried using carbon dioxide. The specimen was then sputter-coated with gold in a Hitachi Hus 3B vacuum evaporator and observed using a Jeol JSM U3 scanning electron microscope.

HAEMATOLOGICAL METHODS

The haematological methods of Blaxhall (1972) and Blaxhall & Daisley (1973) were followed. Seven fish per tank were used in this study.

The fish were caught gently in a hand net to minimise stress. A 1 ml plastic syringe and 27,5 g x 12.7 mm needle were used for taking blood samples. Fish were stunned with a blow to the head. The needle was then inserted directly

into the sinous venosus, after a ventral incision (anus to the pectoral fin base) had been made exposing the heart. This precaution was taken because of the small size of the fish and to avoid contamination of the blood with body fluids.

0,1 - 0,2 mls of blood were taken under gentle aspiration. The needle was withdrawn, detached and the blood was mixed well in a vial containing anti-coagulant (heparin). Blood analysis was completed within three hours of taking samples. Three different haematological parameters were measured: haemoglobin concentration, haematocrit (packed cell volume) and total erythrocyte count.

Haemoglobin estimation: 20 ul of blood was sucked up in a sysmex autodiluter and mixed with 9.94 ml of sysmex diluent (active ingredients sodium chloride and phosphate buffer). Three drops of sysmex lyser (active ingredients: potassium ferricyanide and organic quaternary ammonium salt) were added. This was then centrifuged at 10 000 rpm for five minutes to remove the turbidity caused by the high number of leucocytes in fish blood (Blaxhall & Daisley, 1973). Haemoglobin was then estimated on a sysmex cc 150 microcellcounter.

Erythrocyte count: An electronic cell counter cannot be used for fish blood as erythrocyte nuclei cannot be lysed to facilitate leukocyte counts (Blaxhall & Daisley, 1973). Therefore a 1 in 50 dilution of the blood in formal citrate was made using a bulb-type diluting pipette. This was well mixed and introduced into an improved Neubauer counting chamber and the cells counted by eye. One mm^2 of a 5 mm^2 grid were counted and total erythrocyte count estimated.

Microhaematocrit: Blood was drawn into heparin coated microhaematocrit tubes

(75 mm long x 1,1 mm internal diameter). One end was sealed using a bunsen flame. The tubes were then centrifuged in a microhaematocrit centrifuge for 5 minutes at 10 000 rpm. Readings were made with the aid of a MSE microhaematocrit reader.

CHAPTER 5 RESULTS AND DISCUSSION

BEHAVIOUR

Dosing at a concentration of $0,1 \text{ ug l}^{-1}$ endosulfan did not have any noticeable effect on fish behaviour. However at a concentration of $0,5 \text{ ug l}^{-1}$ the fish moved to the far corner of the tank (away from disturbance) and became more sensitive to auditory and visual stimuli. The dosed fish did not feed as well as the control fish. Dosing at $1,5 \text{ ug l}^{-1}$ resulted in similar but more pronounced behavioural responses.

The first signs of poisoning at lethal levels are that the fish move up higher in the water column and occasionally, when disturbed, swim with bursts of energy and vibrations down their body. These bursts become more frequent as poisoning progresses and the fish often bump into the side of the tank. This is interspersed with periods when the fish swim around slowly, orientated at 45° to the surface "gasping" for air. The fish at this stage often exhibit choking movements of the operculum. The symptoms during the final hours include; disorientated swimming with fish occasionally making darts for the surface where they "hung" for a while with their bodies suspended vertically. These efforts gradually weaken and the fish sink to the bottom where they die.

Sixteen fish which survived the 48 hour LD50 were placed in fresh water and after a week 63% had recovered completely and were feeding. Kleiner *et al.* (1984), similarly, found no delayed mortality with fathead minnows (Pimephales promelas) which survived 24 hour LD50 concentrations of endosulfan.

HEPATIC CYTOCHROME P-450

Results

Table 18 shows the length, mass and liver mass of control and treated fish. Table 19 summarises the liver mass as a percentage of body mass, the microsomal protein levels in the liver and cytochrome P-450 activity per mg microsomal protein. The only statistically significant change (using a t-test: p values given in Table 19) in the parameters measured was a decrease in the liver mass of fish dosed at 0,5 and 1,5 $\mu\text{g l}^{-1}$.

Discussion

Dosing with endosulfan at 0,5 and 1,5 $\mu\text{g l}^{-1}$ caused a decrease in liver mass as a percentage of body mass. This is probably an indirect effect of endosulfan, as dosing affected the appetite of fish. Furthermore, Walsh & Ribelin (1975) reported that endosulfan causes hyperemia of the intestine, which may impair digestion. Swallow & Flemming (1969) showed that O. mossambicus respond to starvation by reducing liver glycogen levels and the resorption of liver tissue. There is an associated decrease in the liver mass to body mass ratio. The ultrastructural implications of this decrease are discussed later.

The levels of cytochrome P-450 reported in this study (0,09-0,13 n moles/mg microsomal protein) are low but never the less within the range reported for fish in the literature (Table 20). Hepatic cytochrome P-450 values measured in other lower vertebrates and selected mammals are also summarised in Table 20. Cytochrome P-450 levels cannot accurately be compared in different studies as the methods of microsomal preparation differ slightly. Arinc & Adali (1983) showed that cytochrome P-450 activity in different preparations of trout microsomes varied from 0,07-2,7 n moles/mg microsomal protein. Furthermore there are marked differences between individual fish in

Table 18. Length, mass and liver mass of control and treated Oreochromis mossambicus (standard deviation in parentheses; n = 15 for each treatment).

	length	mass	liver mass
Control	87,53 (3,6)	25,56 (2,95)	1,64 (0,52)
Treated (0,1 $\mu\text{g l}^{-1}$)	87,80 (5,6)	25,06 (4,71)	1,51 (0,46)
Control	90,07 (5,16)	27,90 (4,34)	1,64 (0,48)
Treated (0,5 $\mu\text{g l}^{-1}$)	88,67 (7,28)	24,88 (5,84)	1,21 (0,45)
Control	91,4 (5,40)	28,21 (4,60)	1,31 (0,47)
Treated (1,5 $\mu\text{g l}^{-1}$)	88,2 (5,99)	24,6 (4,67)	0,91 (0,36)

Table 19. Liver mass as percent of body mass, hepatic microsomal protein and cytochrome P-450 levels in control and treated Oreochromis mossambicus (standard deviation in parentheses; n = 15 for each treatment).

	Liver x 100 Body mass (g)	Microsomal protein*1 (mg microsomal protein/g of liver)	Cytochrome P-450*1 (n moles/mg microsomal protein)
Control	6,34 (1,54)	6,95 (1,24)	0,12 (0,029)
Treated (0,1 $\mu\text{g l}^{-1}$)	6,03 (1,16)*	6,24 (0,69)*	0,10 (0,012)*
Control	5,83 (1,28)	5,57 (0,34)	0,14 (0,012)
Treated (0,5 $\mu\text{g l}^{-1}$)	4,79 (1,16)**	5,96 (0,79)*	0,11 (0,015)*
Control	4,60 (1,25)	6,76 (0,69)	0,10(0,021)
Treated (1,5 $\mu\text{g l}^{-1}$)	3,64 (0,96)**	5,11 (0,78)*	0,09 (0,015)*

* = not significant (p = 0,1)

** = significant (p = 0,05)

*1 = five livers were pooled for one sample (n = 3)

Table 20. Cytochrome P-450 levels reported for different animal species.

Species	Normal Cytochrome P-450 levels (nmoles/mg microsomal protein)	References
<u>Salmo trutta</u> (brown trout)	0,32	Schwen & Mannering (1982a)
<u>S. gairdneri</u> (rainbow trout)	0,1-0,4	Ahokas <u>et al.</u> (1975) Elcombe & Lech (1979) Seppa <u>et al.</u> (1981) Arinc & Adali (1983) Eisele <u>et al.</u> (1984)
<u>S. trutta lacustris</u> (rainbow trout)	0,40	Ahokas <u>et al.</u> (1977)
<u>Pseudopleuronectes americanus</u> (winter flounder)	0,17-0,23	James <u>et al.</u> (1979); Foureman <u>et al.</u> (1983);
<u>Mugil cephalus</u> (grey mullet)	0,47	James <u>et al.</u> (1979)
<u>Dasyatis sabina</u> (stingray)	0,43	James <u>et al.</u> (1979)
<u>Raja erinacea</u> (little skate)	0,32	James <u>et al.</u> (1979)
<u>Oreochromis mossambicus</u> (bream)	0,12	This study
<u>Xenopus laevis</u> (platanna)	0,10	Noshiro & Omura (1984)
<u>Rana spp</u> (frogs)	0,43-0,77	Noshiro & Omura (1984)
<u>Thamnophis</u> (garter snake)	0,39	Schwen & Mannering (1982a)
rat	0,67-1,04	Bend <u>et al.</u> (1980); Noshiro & Omura (1984)
rabbit	0,80-1,52	Bend <u>et al.</u> (1980); Arinc & Adali (1983)
sheep	0,60	Arinc & Adali (1983)

populations. For example, Foureman et al. (1983) showed a range of 0,12 to 0,6 nm cytochrome P-450/mg microsomal protein in wild populations of winter flounder. Another complication is that Sakai et al. (1983) demonstrated different pH and temperature optima for the mixed-function oxidases in different species of fish.

Table 21 summarises the chemicals known to induce or not induce hepatic cytochrome P-450 in fish. The reports are conflicting as both DDT and phenobarbital have been claimed to be inducing and non-inducing agents. However, most evidence indicates that fish are insensitive to the phenobarbital inducers (for more detail on induction see review page 79) but are induced by the polycyclic aromatic hydrocarbons (PAH); these include 3-methylcholanthrene, B-naphthoflavone and polychlorinated biphenyl insecticides.

Induction by phenobarbital in mammals results in increased microsomal protein, an increase in the hepatosomatic index and an associated increase in metabolism of many drugs xenobiotics and hormones. Polycyclic aromatic hydrocarbon (PAH) inducers usually cause the formation of novel forms of cytochrome P450 and an elevation in enzyme rates such as 7-ethoxyresorufin de-ethylase (7-ERD) activity and benzo(a)pyrene hydroxylase (AHH) activity (Foureman et al., 1983). The typical response of mammalian livers to PAH inducers are dramatic increases in AHH activity and a hypsochromic shift in the wave-length of maximal absorption of cytochrome(s) P-450 in its co-ligated and reduced form from 450 to 448 nm. Although PAH substances cause induction many workers have not observed the typical shift to 448 nm (Schwen & Mannering, 1982c; Foureman et al., 1983). However, recent work by James & Bend (1980), Williams & Buhler (1982) and Eisele et al. (1984) have demonstrated that fish are capable of synthesizing P-448 and P-449 under the appropriate conditions. There are complications to the clear cut idea that

Table 21. Reported inducers and non-inducers of cytochrome P-450 in fish liver.

Inducers	Reference
Phenobarbital	Gutman & Kidron (1971)
DDT	Gutman & Kidron (1971)
Phenylbutazone	Burns (1976)
3-methylcholanthrene	Schwen & Mannering (1982a)
Benzo (a) pyrene	Gerhart & Carlson (1978)
Benzanthracene	Elcombe <u>et al.</u> (1979)
Dimethylbenzanthracene	James & Bend (1978)
Dibenzanthracene	Pohl <u>et al.</u> (1976)
Tetrachlorodibenzo - p - dioxin	Bend <u>et al.</u> (1979)
B - naphthoflavone	Chevion <u>et al.</u> (1977); Eisele <u>et al.</u> (1984)
Polychlorinated biphenyls	Hill <u>et al.</u> (1976); Lidman <u>et al.</u> (1976); Addison <u>et al.</u> (1978); James & Weatherby (1978); Elcombe & Lech (1979); Elcombe <u>et al.</u> (1979)
Polybrominated biphenyls	James & Weatherby (1978)
Crude oil	Elcombe <u>et al.</u> (1979) Payne & Penrose (1975) Yarbrough & Chambers (1977) Payne <u>et al.</u> (1984)
Non-inducers	Reference
Phenobarbital	Schwen & Mannering (1982c); Eisele <u>et al.</u> (1984)
DDT & DDE	Addison <u>et al.</u> (1977)
Phenylbutazone	Bend & James (1978)
Non-planer isomers of polychlorinated biphenyls	James & Bend (1978); Elcombe <u>et al.</u> (1979)

the PAH inducers induce mixed-function oxidases, as Fisher et al. (1983, in Foureman et al., 1983) reported that induction in wild fish exposed to PAH inducers only occurred in a few species.

Arinc & Adali (1983) solubilized and partially purified two forms of cytochrome P-450 from trout liver. Foureman et al. (1983) reported wide and significant fluctuations in 7-ERD and AHH activity in the winter flounder. These fluctuations were not in synchrony, indicating at least two forms of cytochrome(s) P-450. Foureman et al. (1983) did not show a clear relationship between AHH metabolism and the level of cytochrome P-450, lending further support to the idea that relative proportions of sub-populations of cytochrome may be the key factor in the rate of metabolism. These studies demonstrate that there are different forms of cytochrome P-450 in fish liver and that one mechanism of PAH induction is the increase of a particular form. This is usually reflected in an increase in total cytochrome P-450 levels. (Elcombe & Lech, 1979; Schwen & Mannering, 1982c; Eisele et al., 1984).

Endosulfan caused none of the characteristic inductive effects on the liver of O. mossambicus. There was no increase in cytochrome P-450 levels, microsomal protein or hepatosomatic index.

Schwen & Mannering (1982c) found the lack of induction by phenobarbital was not caused by the failure of phenobarbital to accumulate in the liver. The same applies for endosulfan which is also known to accumulate in the liver (Shoettger, 1970). It also seems that the lack of phenobarbital and endosulfan induction was not due to rapid metabolism of the inducing agent as mono-oxygenase activity in fish is low (Chambers & Yarbrough, 1976; Schwen & Mannering, 1982c). Bend et al. (1973) suggested that the lack of phenobarbital induction in fish may be due to the inhibition of mono-oxygenase

activity by phenobarbital. However, Elcombe & Lech (1979) showed that phenobarbital does not inhibit mono-oxygenase activity in fish.

The reason for the lack of induction by phenobarbital-type inducing agents in lower vertebrates is difficult to determine because the mechanism of phenobarbital induction in mammals is not known. More recent information suggests that cytochrome P-450 exists, at least partly, in the microsomal membrane in an inactive or apoprotein form (Seidel et al., 1984). According to Schwen & Mannering (1982c), one possibility to be considered is that phenobarbital induces mono-oxygenase systems indirectly by modulating concentrations or activities of endogenous components that normally regulate the steady state levels of cytochrome P-450. If this assumption is valid, then the failure of phenobarbital type inducers to induce mono-oxygenase systems in lower vertebrates would indicate either that the steady states of their mono-oxygenase systems are controlled differently to mammals, or that the endogenous mediators in lower vertebrates are not affected by phenobarbital (Schwen & Mannering, 1982c). Alternatively, fish do not have suitable forms of cytochrome P-450 for detoxifying phenobarbital-type inducers; this seems unlikely as metabolites of endosulfan have been reported to occur in the bile of fish (Shoettger, 1970; Rao et al., 1981)

Induction of mammalian mono-oxygenase systems by 3MC and certain other polycyclic hydrocarbons is mediated through cytosolic receptors (Nebert et al., 1975). Both mammals and lower vertebrates are induced by 3MC, which suggests that cytosolic receptors may be involved in both groups.

The reported increase in endosulfan metabolism after exposure of fish to sub-lethal levels is not explained by the induction of the mixed-function oxidase system. The lack of induction of this system by chlorinated hydrocarbon

insecticides (e.g. Chlordane, DDT, Dieldrin, Lindane, Toxaphene and Endosulfan) does explain why these chemicals are so toxic to fish. The implications of this are dealt with in the conclusion.

GILL ULTRASTRUCTURE

Results

Figure 21A shows the general morphology of the primary gill filaments (pl) and the secondary lamellae (sl) of control O. mossambicus. A higher magnification view of this tissue in Figure 22A shows the efferent blood canal (c) running through the primary filament. Nucleated blood cells (bc) are visible in this canal and within the capillaries of the secondary lamellae. Sections of gills from fish treated with the 24 hour LD50 dose of $5,1 \text{ ug l}^{-1}$ endosulfan for two days are shown in figures 21B and 22B. There is no evidence of lesions in the treated fish.

Scanning electron micrographs (Figure 23) of control and treated gill tissue show no differences. Both have well developed secondary lamellae and microridge structures (mr) on the primary filaments and secondary lamellae. The gill epithelium is covered with a thin layer of mucus. Figure 24 shows scanning electron micrographs of the chloride cells of control and treated fish. There is no visible damage to the treated fish. Gill tissue taken from fish exposed to lower doses of endosulfan similarly showed no tissue lesions.

Discussion

The 14 main types of gill lesion reported in the literature are illustrated in Figure 25. The most commonly reported lesion (1) is a lifting of epithelial cells from the gill lamellae and the interlamellar zones of the gill filament, and associated oedema (Christie & Battle, 1963; Andrews et al., 1966; Van Valin et al., 1968; Eller, 1971, 1975; Mallatt, 1985). However Walsh & Ribelin (1975) did not find this lesion occurring in fish exposed to a wide range of pesticides. They pointed out that lamellar epithelium separates from the underlying endothelium soon after death. Other common lesions reported

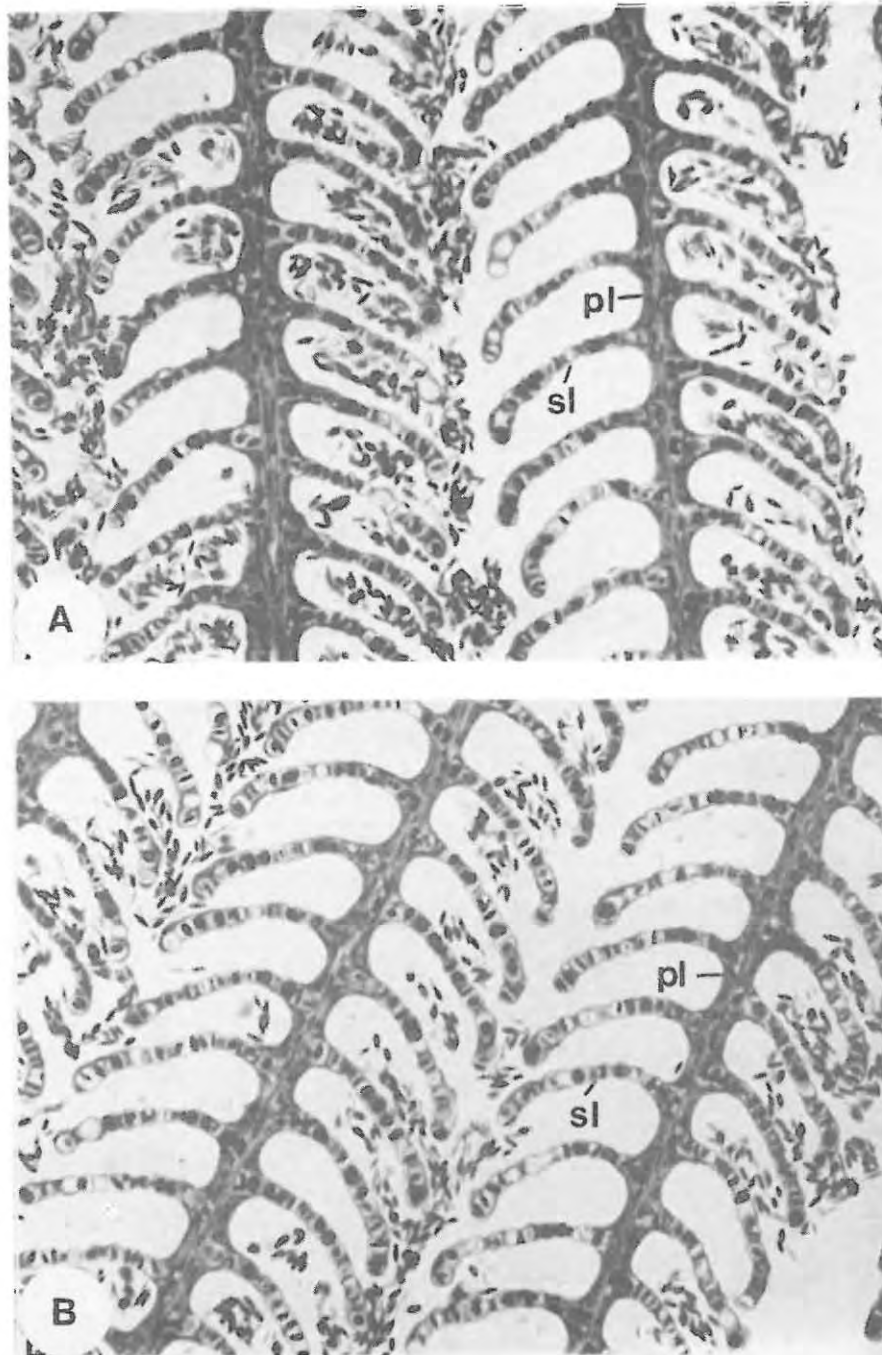


Figure 21. (A) Longitudinal section through the primary filaments (pl) of control fish showing well developed secondary lamellae (sl). (B) Longitudinal section through the primary filament of a fish dosed with 5.1 ug l^{-1} endosulfan. There are no differences from the control above. (x 270)

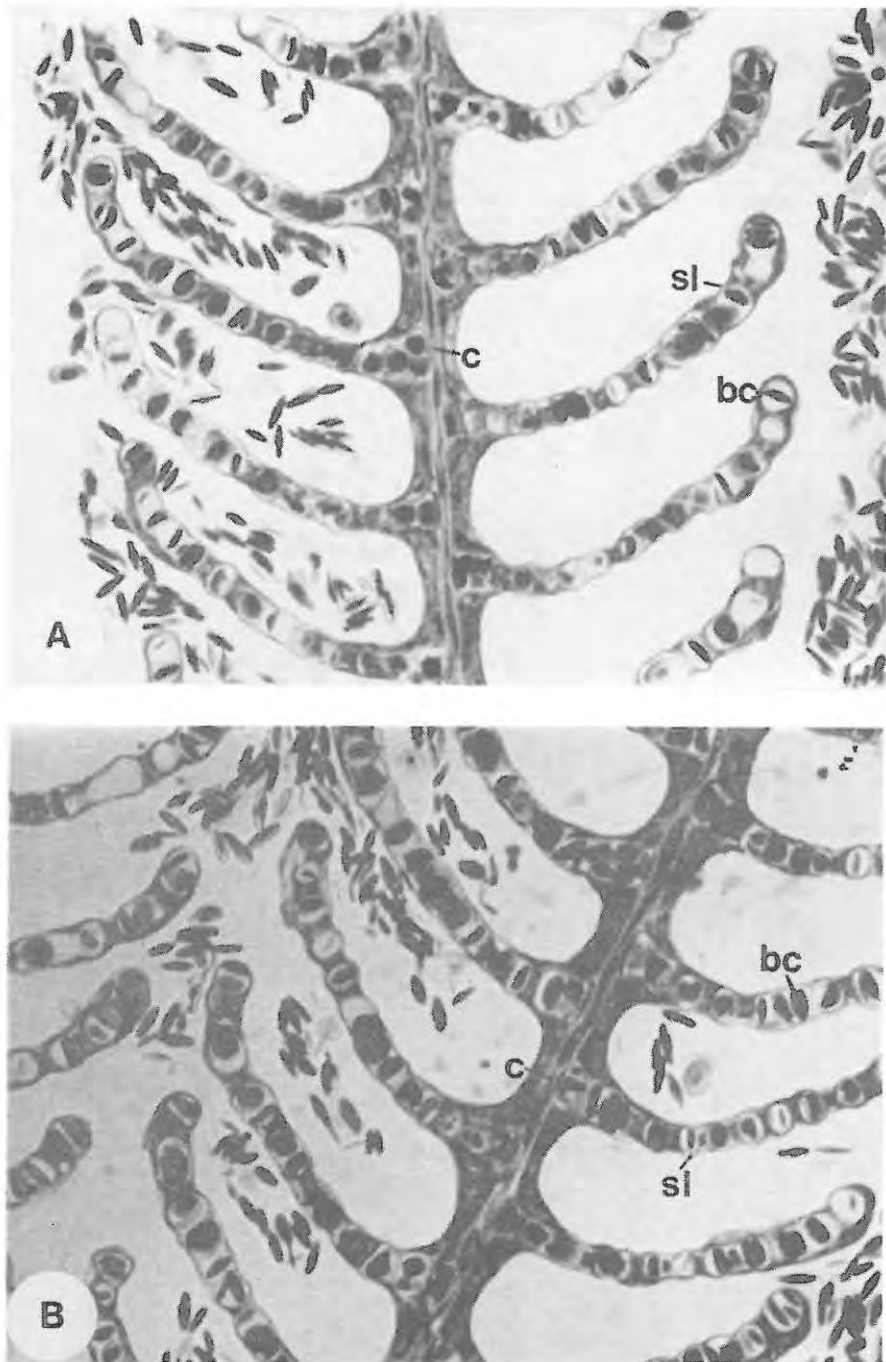


Figure 22. Longitudinal sections of control (A) and treated (B) gills showing well developed secondary lamellae (sl). The nucleated blood cells (bc) are present in the efferent blood canal (c) and the capillaries within the secondary lamellae. There were no differences between control and treated fish. (x 540)

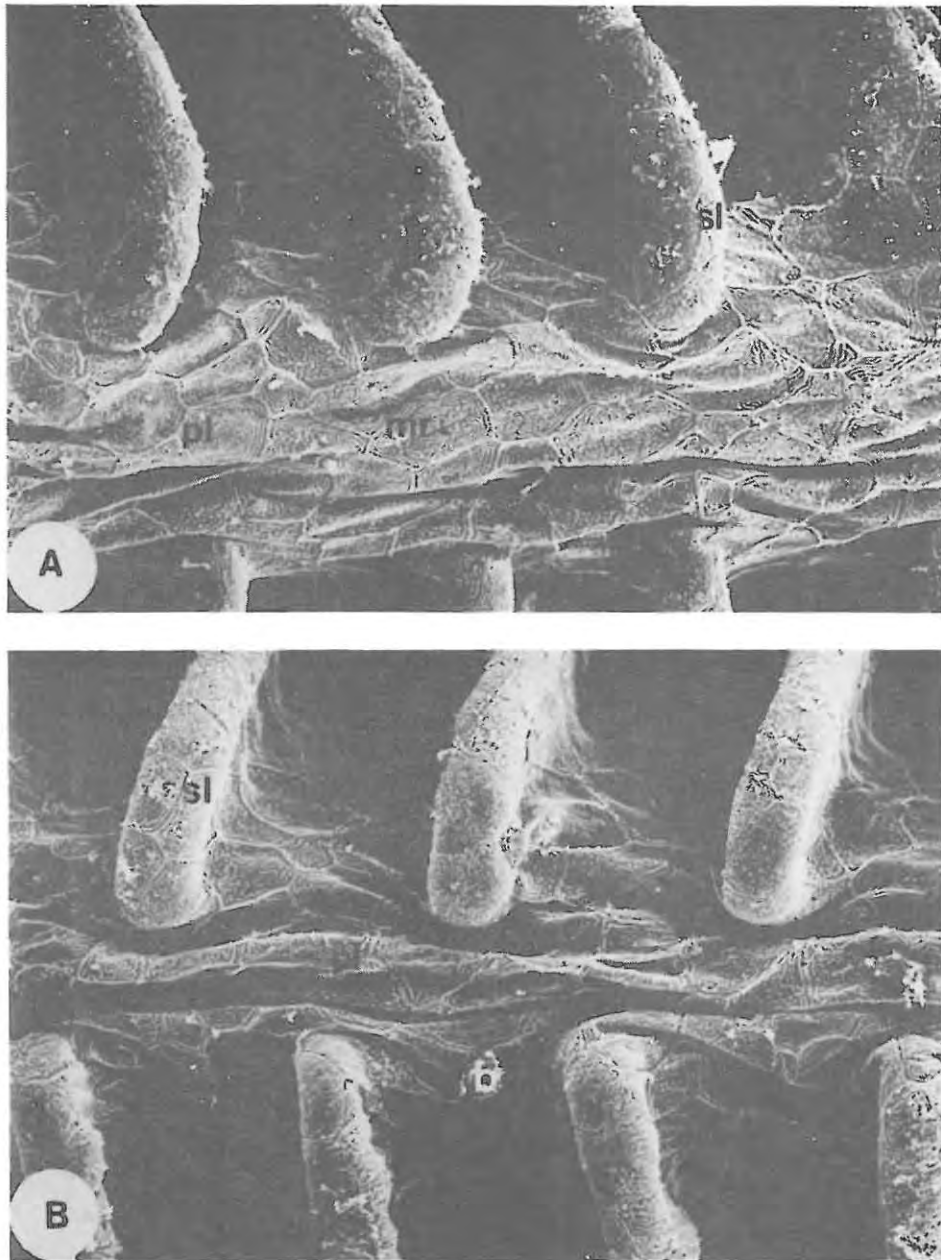


Figure 23. (A) Scanning electron micrograph of primary filaments (pl) of control fish showing well developed secondary lamellae (sl) and microridge structures (mr). (B) Longitudinal section through the primary filament of a fish dosed with $5,1 \text{ ug l}^{-1}$ endosulfan; b = blood cell. There are no differences from the control above. (x 1000)

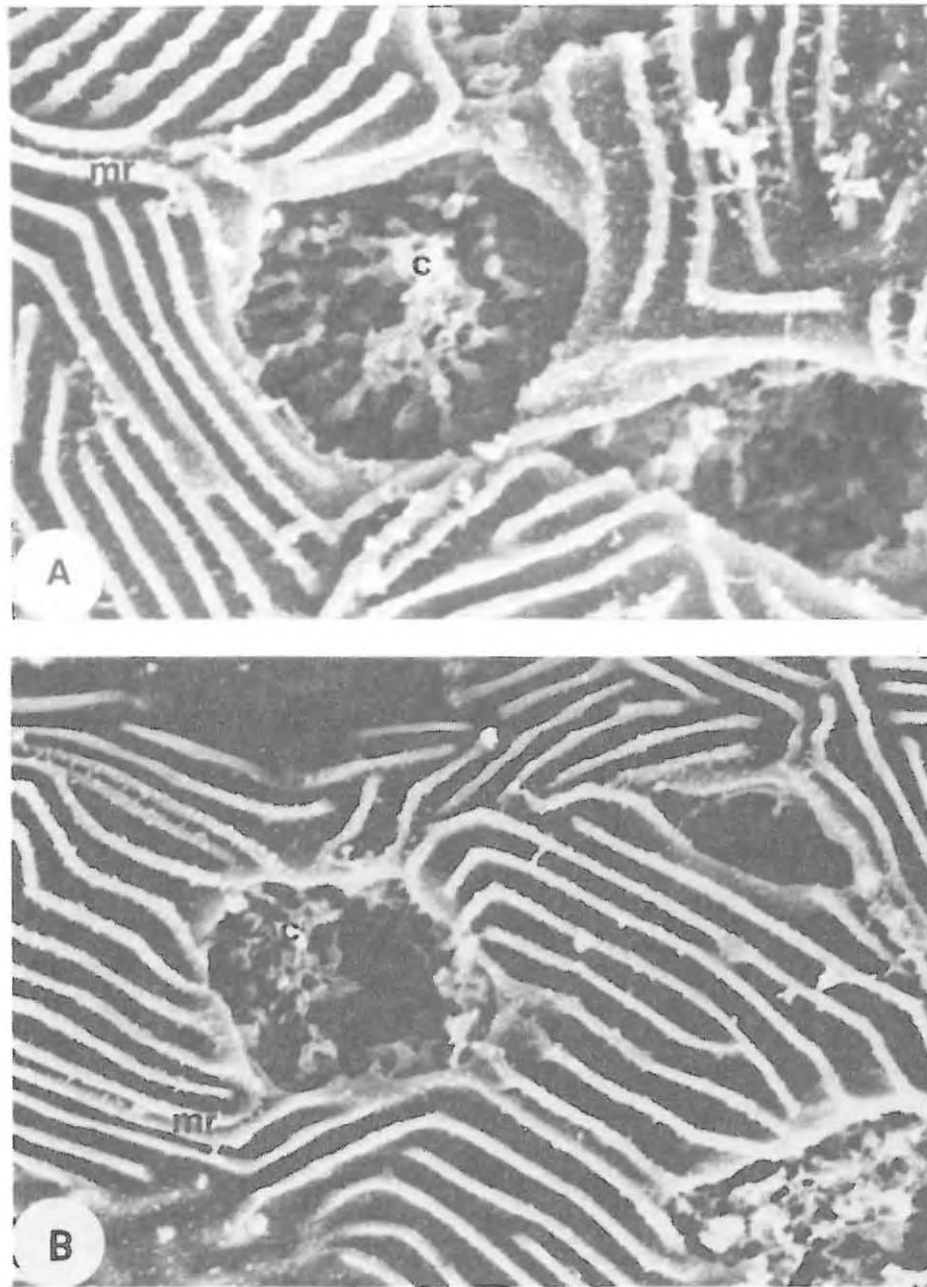


Figure 24. High power scanning electron micrograph of the chloride cells (c) and microridges (mr) on the gills of control (A) and treated (B) fish. There appears to be no damage to these structures in fish exposed to endosulfan. (A x 10 000; B x 8 000).

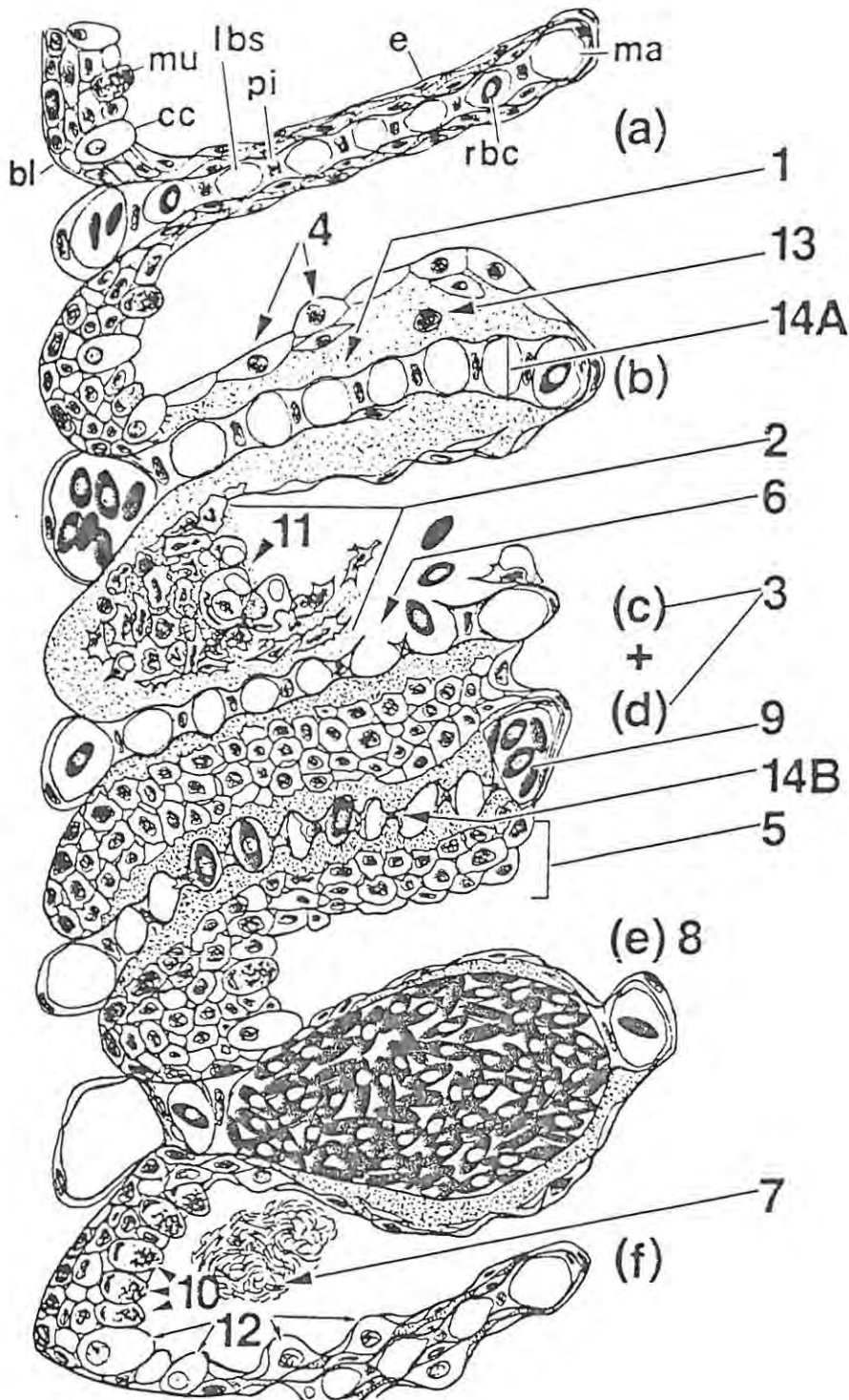


Figure 25. Composite diagram of the common irritant-induced gill lesions. Six respiratory lamellae are shown (a-f), the top one of which is normal. The lesions are numbered as follows: (1) epithelial lifting; (2) necrosis; (3c & d) lamellar fusion; (4) hyperthrophy; (5) hyperplasia; (6) epithelial rupture and bleeding into pharynx; (7) mucous secretion; (8) clavate lamella, or lamellar aneurism (e); (9) vascular congestion; (10) mucous cell proliferation; (11) chloride cell damage; (12) chloride cell proliferation; (13) leukocyte infiltration of epithelium; (14A) dilation of lamellar blood sinus; (14B) constriction of lamellar blood sinus. For photomicrographs of some of these lesions, see Eller (1975). Abbreviations: bl = basal lamina; cc = chloride cell; e = typical lamellar epithelial cells; lbs = lamellar blood sinus; ma = marginal blood channel; mu = mucous cell; pi = pillar cell; rbc = erythrocyte (from Mallatt, 1985).

include the following: necrosis; hypertrophy; hyperplasia and rupture of branchial epithelium; lamellar fusion; bulbing of lamellae; hypersecretion and proliferation of mucus cells; and changes in chloride cells and gill vasculature (Lowe, 1964; Gilderhus, 1966; Van Valin et al., 1968; Eller, 1969; McCraren et al., 1969; Eller, 1975; Drewett & Abel, 1983; Mallatt, 1985).

Mallatt (1985) reviewed the literature on the changes induced in gills by toxicants and other irritants. He made the following conclusions: (1) toxicant-induced alterations are largely nonspecific although certain lesions are associated more frequently with some toxicants than others. Heavy metals, for example, most frequently caused necrosis of the branchial epithelium and hypersecretion of branchial mucus cells. (2) Many of the commonly reported gill alterations seem to reflect physiological adaptation to stress. Therefore, future studies should be more concerned with investigating the physiological mechanisms behind branchial responses. (3) Most kinds of gill lesion are reported in studies where fish are exposed to lethal levels of toxicant. Eller (1975), in a review on the effects of pesticides on gills, also noted that gill damage is easily detectable in studies designed to measure acute toxicity within one or two weeks; but, in long term, subacute studies, gill damage is subtle and is not always observed.

In this study no lesions in the gills were observed. Walsh & Ribelin (1975) and Rao et al. (1980) similarly noted no lesions in the gills of fish exposed to endosulfan. Therefore, it is unlikely that endosulfan interferes with oxygen transfer or osmoregulation by damaging gill tissue.

LIVER ULTRASTRUCTURE

Results

Light microscope observations revealed the general histological features of the liver tissue (Figure 26A). The liver lobe is surrounded by a thin connective tissue capsule which extends as trabeculae into the body of the lobes dividing them into lobules. The connective tissue capsule consists of simple squamous epithelium (mesothelium) and a thin layer of connective tissue.

The hepatocytes are apparent as anastomosing cords, clumps or rosettes of several cells arranged around a central vein or sinusoid (S) or as anastomosing two cell thick laminae (Figure 26A). Each hepatocyte has an irregular polygonal form and contains a single spherical nucleus. The staining of the cells causes light and dense regions. The light regions represent areas of glycogen accumulation and the denser regions represent lipid droplets, endoplasmic reticulum and other cellular organelles. No further differentiation of cells was possible using light microscopy.

Observation of ultrathin sections of control liver using electron microscopy revealed three types of cells: hepatocytes, endothelial cells and Kupffer cells.

The general appearance of a hepatocyte is shown in Figure 27. The shape of hepatocytes ranges from oval to polygonal. They have a centrally located nucleus (N) with widely dispersed and marginal heterochromatin (He) and a nucleolus (Nu). The nucleus has several nuclear pores (arrow). Mitochondria (Mt) are generally more concentrated around the nucleus and the region of the cell adjacent to the space of Disse (SD). Large regions of the cell contain

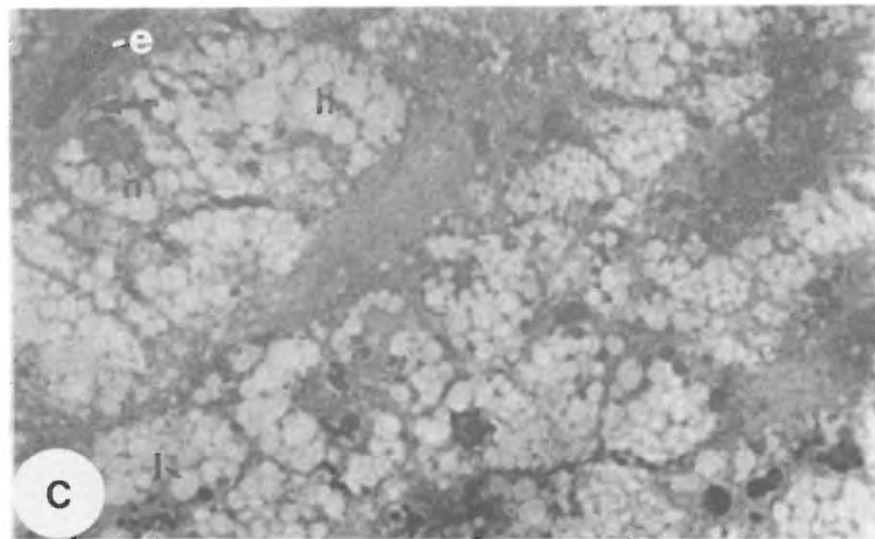
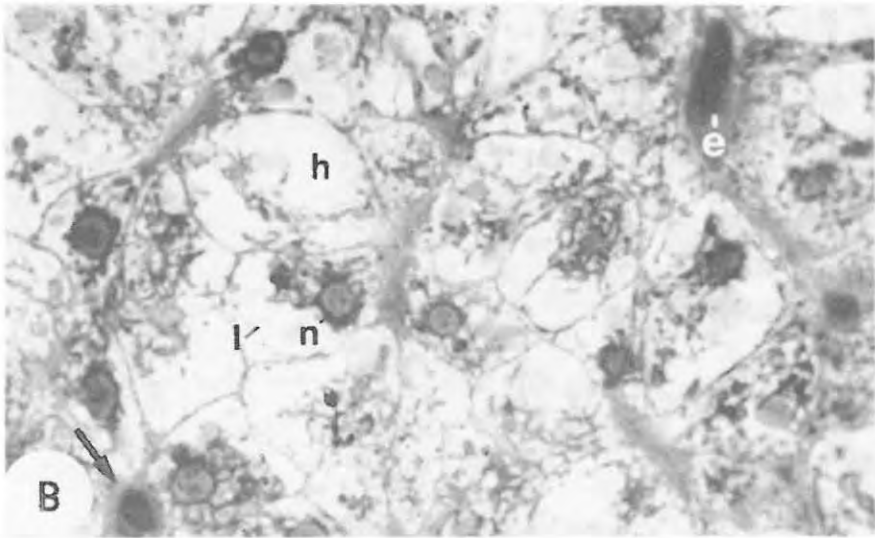
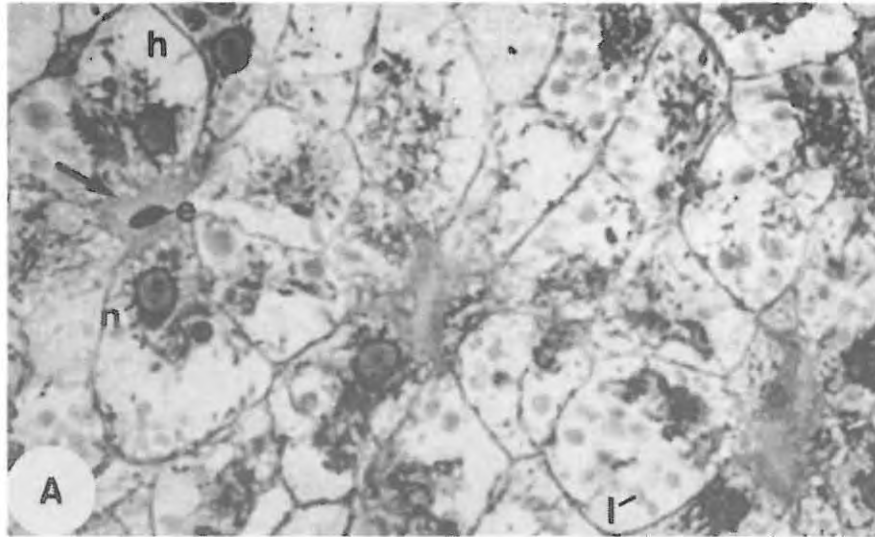


Figure 26. Sections through the liver of control (A) and *O. mossambicus* dosed with 1,5 $\mu\text{g l}^{-1}$ (B) and 5,1 $\mu\text{g l}^{-1}$ endosulfan (C). The hepatocytes (h) contain nuclei (n), lipid vacuoles (l) and indistinct cell organelles are arranged around sinusoids (arrow) which contain erythrocytes (e). Note the shrinkage of hepatocytes in (B) and extensive vacuolation in (C). (x 540)

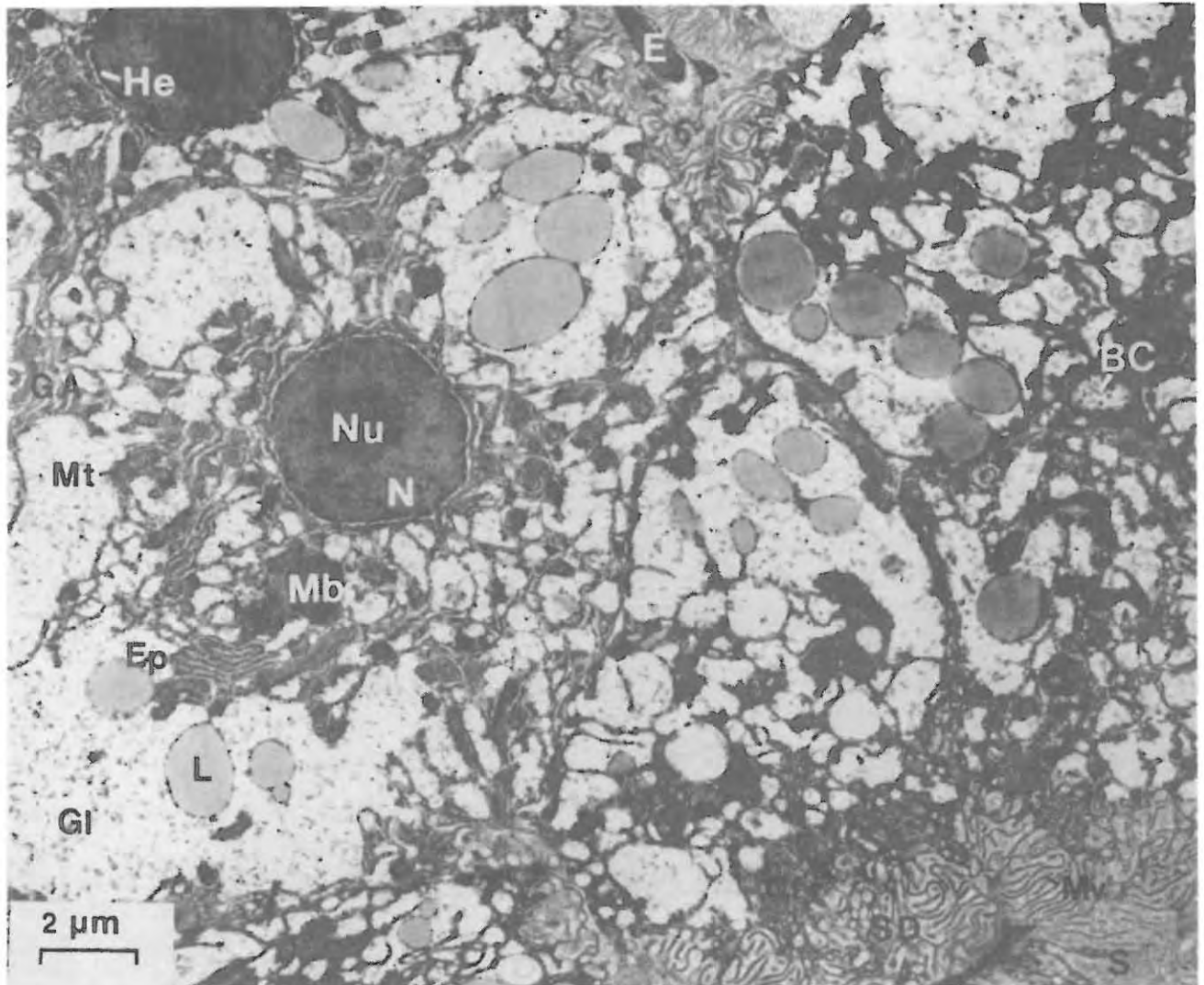


Figure 27. Transmission electron micrograph of a typical *O. mossambicus* hepatocyte abutting on a sinusoid (S). N = nucleus; Nu = nucleolus; Ep = endoplasmic reticulum; Mt = mitochondria; L = lipid; Mb = microbody; GA = golgi apparatus; Np = nuclear pore; SD = space of Disse; Gl = glycogen; Cf = collagen fibres; Mv = microvilli; BC = bile canaliculus; He = heterochromatin; E = erythrocyte.

glycogen (Gl) and lipid droplets (L) were present in these glycogen pools. Collagen fibres (Cf) were present in the space of Disse. Hepatocytes contain the other usual cellular organelles; rough endoplasmic reticulum (Ep) and Golgi apparatus (GA).

Dense microbodies (Mb) were often present in hepatocytes. These microbodies are thought to contain enzymes such as uricase, catalase and A-amino acid oxidase (Beaufay & Berthet, 1963, in Chapman, 1981). Lysosomes were also encountered in some cells (Figure 28). These bodies were surrounded by either one or two trilaminar membranes (Tm) and appeared as round or polygonal shapes. The interior of these lysosomes was characterized by highly ordered fibrillar networks (Fn) which suggested an ordered continuum within. Dense granular material (Gm) was also present in these bodies.

Bile canaliculi (BC) are usually formed by the close apposition of two to four cells (Figure 29A) and involve cell to cell contact by junctional complexes (Figure 29B). At the region of the bile canaliculus numerous microvilli (Mv) extend into the lumen. A bile canaliculus is a specialized site of cell to cell apposition involving a modification of the adjacent plasma membrane of the apposed cells so as to form an intercellular conduit (Chapman, 1981).

The two other types of cell viewed, endothelial and Kupffer cells, both occurred in the sinusoidal lumen. Endothelial cells have irregular nuclei and greatly attenuated cytoplasm which formed a thin often discontinuous barrier between the sinusoidal lumen and the space of Disse (Figure 30A). Cytoplasmic constituents of endothelial cells include numerous free ribosomes (r), sparse rough endoplasmic reticulum (Ep) and abundant pinocytotic vesicles (PV). Endothelial cells contained numerous pits (P) and were held in close apposition with other endothelial cells by means of interdigitating membranes

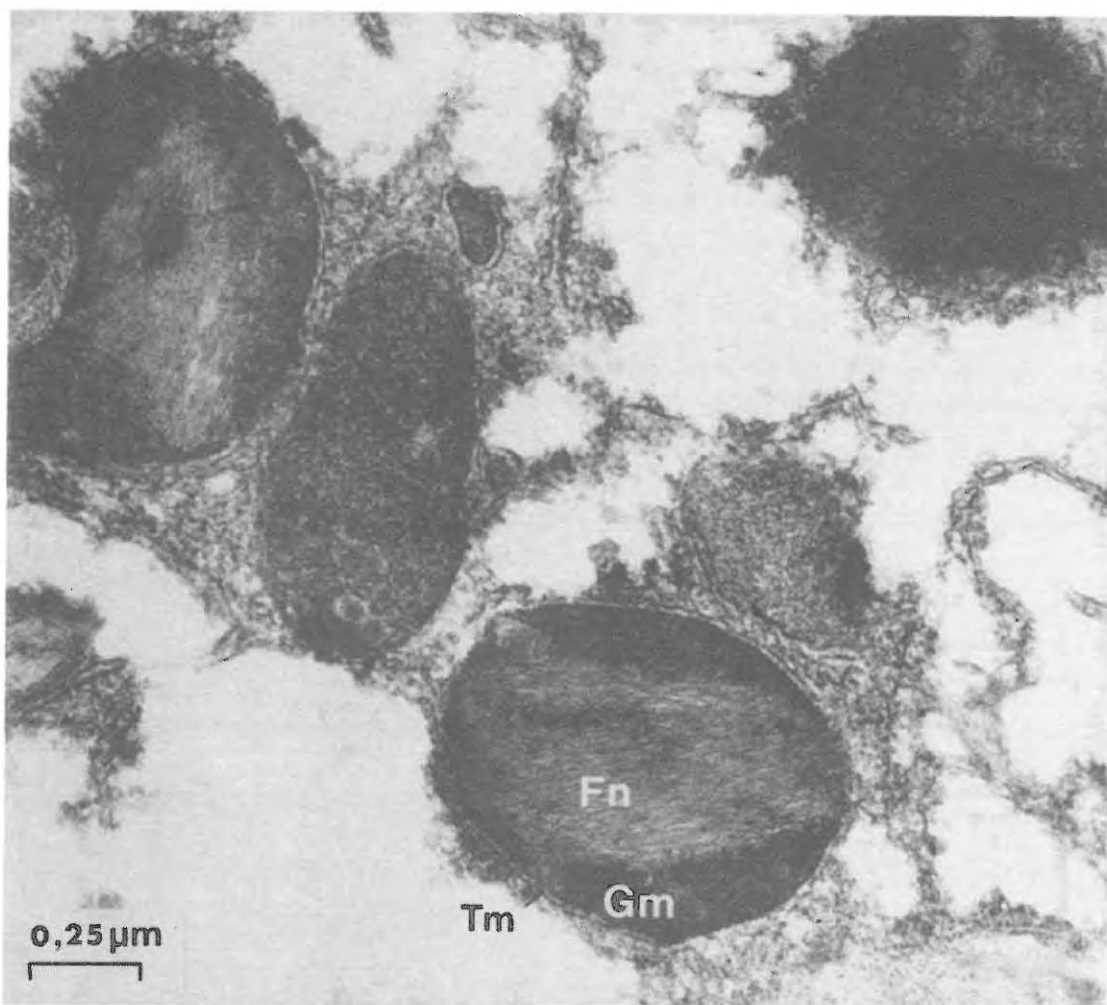


Figure 28. Electron micrograph of lysosomes in a hepatocyte. Note the trilaminar membrane (Tm), ordered Fibrillar network (Fn) and granular material (Gm).

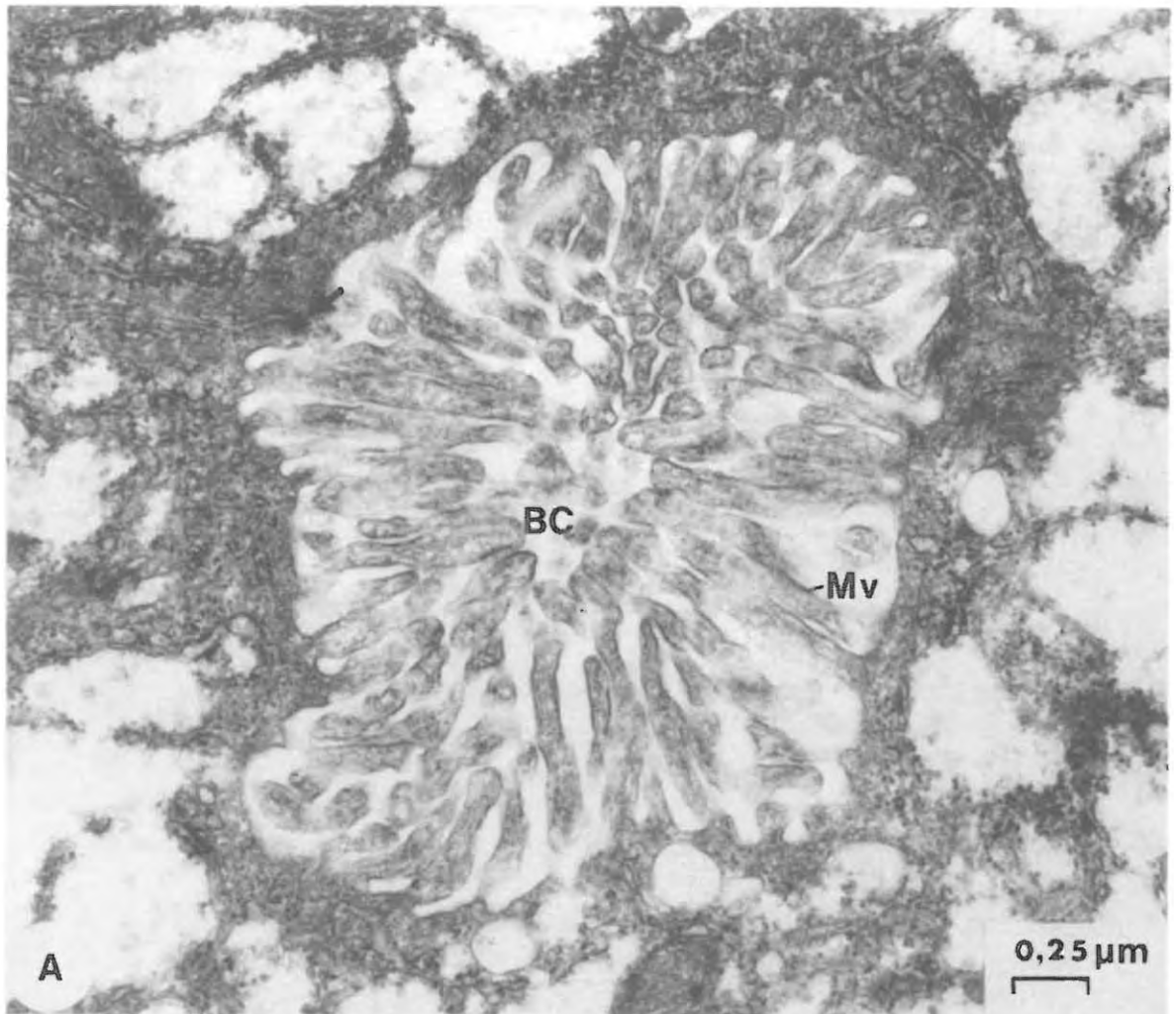


Figure 29. (A) Electron micrograph of a bile canaliculus (BC) formed by the apposition of at least four hepatocytes. Note the junctional complexes (arrow) and microvilli (Mv). (B) High power electron micrograph of a bile canaliculus junctional complex (arrow).

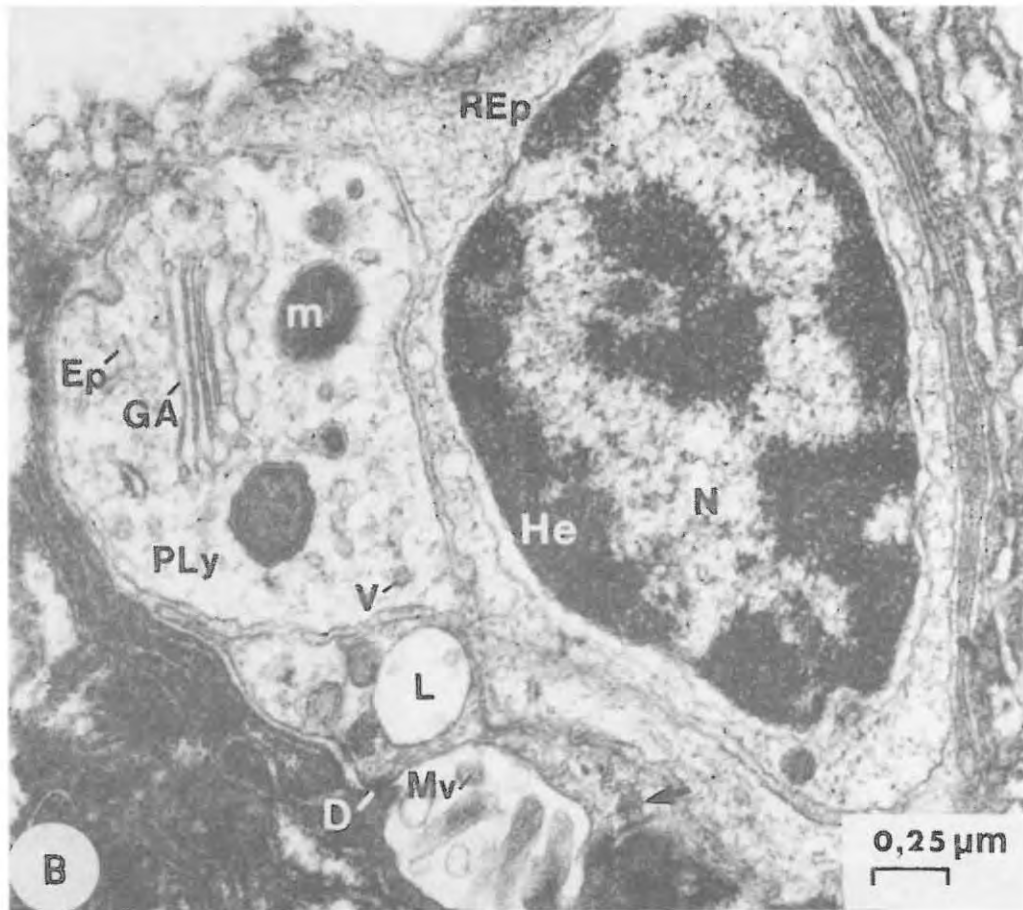
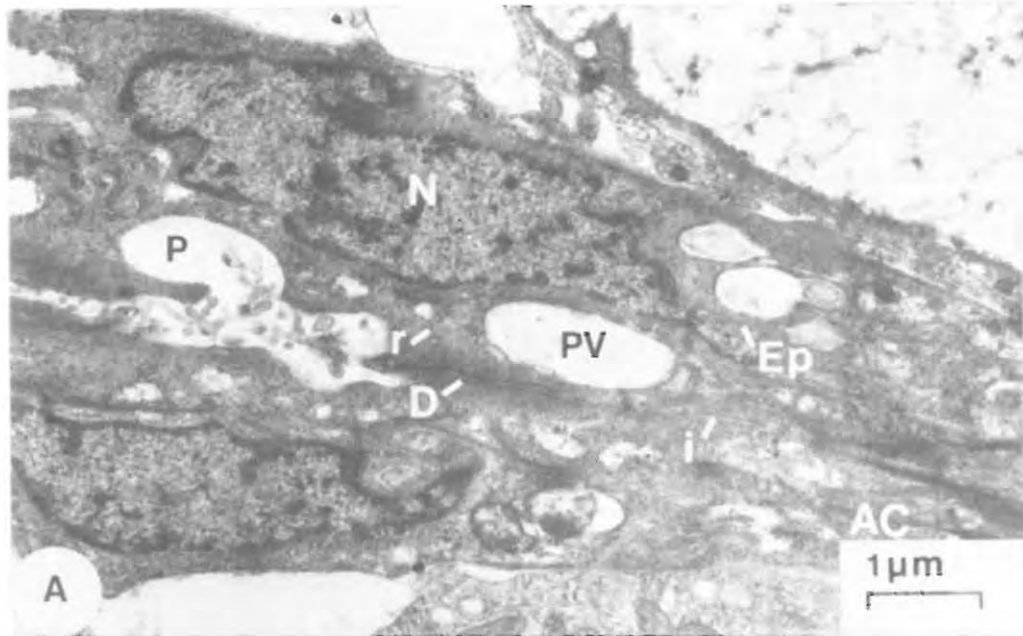


Figure 30. (A) Endothelial cells in the sinusoidal lining. Note the attenuated cytoplasm (AC). N = nucleus; Ep = endoplasmic reticulum; P = pit; PV = pinocytotic vesicle; i = interdigitating membrane; D = desmosome; r = ribosomes. (B) Kupffer cell in the sinusoidal lining. Ep = endoplasmic reticulum; REp = rough Ep; m = lysosome-like micro body; PLY = phagolysosome; N = nucleus; He = heterochromatin; L = lipid; V = vesicle; Mv = microvilli; D = desmosome; GA = golgi apparatus; arrow = electron dense particles.

(i) and desmosomes (D).

An electron micrograph of a Kupffer cell is shown in Figure 30B. The nuclei of these cells are irregular with clumps of heterochromatin at the nuclear margin and in the centre. Surface irregularities were found on the luminal border of the Kupffer cells. These usually took the form of microvilli-like projections of cytoplasm (Mv). Kupffer cells contained a well developed Golgi apparatus, rough endoplasmic reticulum (REp) and numerous cytoplasmic vesicles of varying electron density and size. These included large lysosome-like microbodies (m), small electron dense granules (V), clumps of small electron dense particles (arrow) and lipid droplets. Mitochondria were present but in low numbers. The Kupffer cells are in close apposition with endothelial cells, and desmosomes (D) are present on the abutting membranes.

Liver sections of treated fish showed distinct lesions. Treatment at 1,5 $\mu\text{g l}^{-1}$ endosulfan caused a decrease in cell size and a decrease in the proportion of glycogen in hepatocytes (Figure 26B). At 5,1 $\mu\text{g l}^{-1}$ endosulfan caused extensive vacuolation of hepatocytes. Cellular changes in fish dosed at 1,5 and 5,1 $\mu\text{g l}^{-1}$ caused a slight disarray of liver cords and the sinusoidal lumen became more indistinct (Figure 26). No noticeable changes occurred in fish dosed at lower levels.

Electron micrographs of liver tissue from fish treated at 1,5 $\mu\text{g l}^{-1}$ endosulfan showed no marked differences in cellular organelles from the controls, although there was a slight increase in vacuoles, and as reported for the light microscope sections a decrease in glycogen reserves. Similarly, in the fish treated at 5,1 $\mu\text{g l}^{-1}$ there was no obvious damage (such as irregular nuclei, nuclear inclusions or enlarged mitochondria). However, the hepatocytes were packed with lipid-like vacuoles (Figure 31). These vacuoles

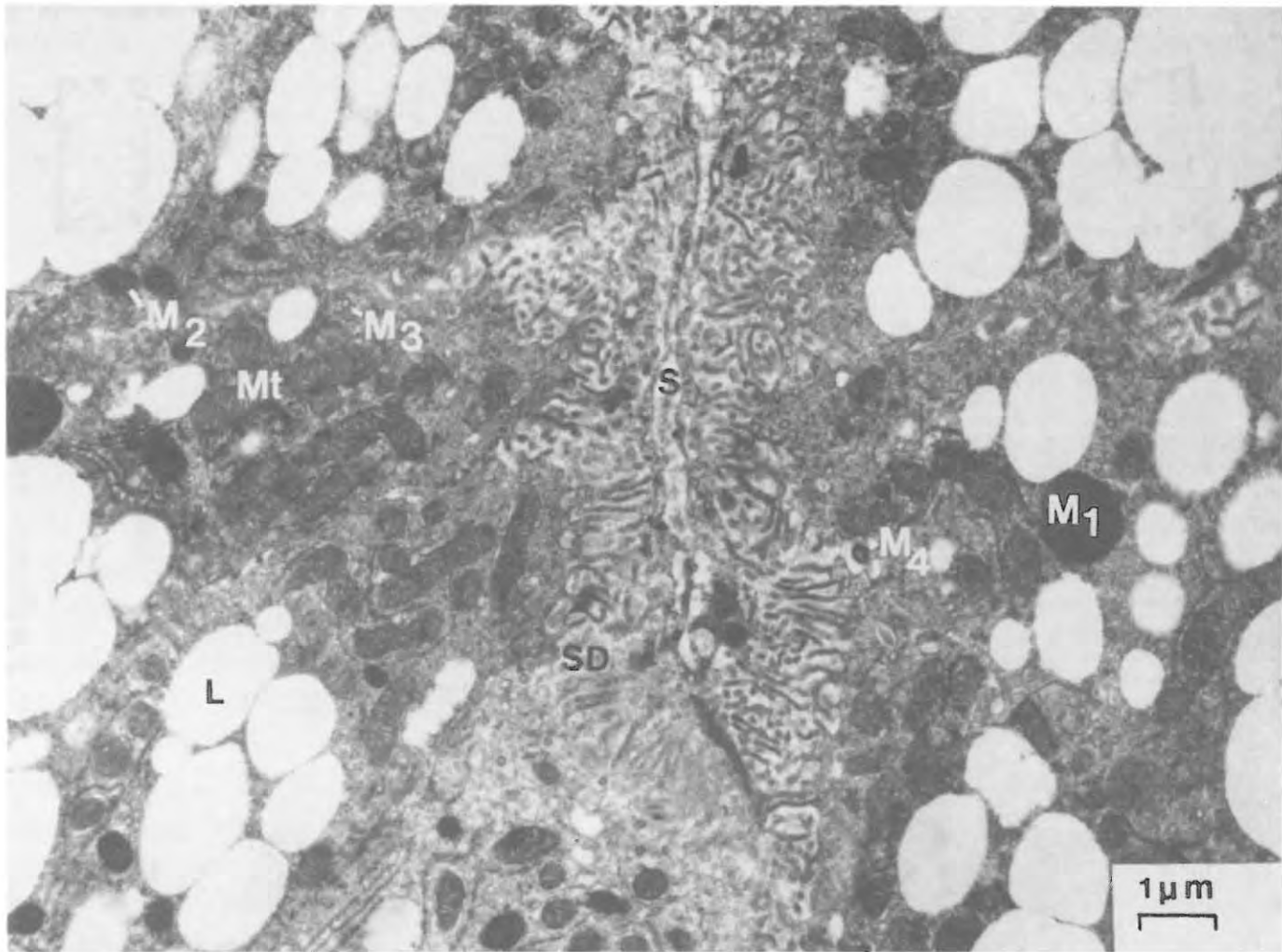


Figure 31. Electron micrograph of hepatocytes from *O. mossambicus* dosed with $5,1 \text{ ug l}^{-1}$ endosulfan. Note the high concentration of lipid vacuoles (L) and mitochondria (Mt) near the space of Disse (SD). There are numerous microbodies (M_{1-4}) of differing sizes and electron density.

were more variable in size and were of lower electron density than the lipid droplets encountered in control hepatocytes. Another difference from the control hepatocytes was that there appeared to be intense activity in the region of the hepatocyte adjacent to the space of Disse (Figure 31). There was a high concentration of mitochondria, lipid-like vacuoles and numerous microbodies of various sizes and electron densities. An electron dense microbody can be seen in the sinusoidal lumen.

There was no proliferation of endoplasmic reticulum or increase in the number of lysosomes in treated fish.

Discussion

Reports describing liver ultrastructure of normal fish and fish exposed to environmental stress are few. The ultrastructural features described for O. mossambicus are similar to those described for rainbow trout, Salmo gairdneri, channel catfish, Ictalurus punctatus and the atlantic croaker Micropogon undulatus (Hinton & Pool, 1976; Chapman, 1981; Eurell & Haensly, 1982). Eurell & Haensly (1982), however, did not see any Kupffer cells.

Other reports have been concerned with the changes in the ultrastructure of hepatocytes in relation to hyperplasia, adenoma and hepatoma (Scarpelli et al., 1963), temperature acclimation (Berlin & Dean, 1967), administration of the polychlorinated biphenyl Aroclor 1254 (Hacking et al., 1978; Sivarajah et al., 1978), the effect of diet (Leatherland, 1982; Storch & Juario, 1983; Segner et al., 1984), DDT (Weis, 1974), sodium arsenate (Sorenson, 1976), and the effect of mercury (Studnicka, 1983).

Endosulfan caused a decrease in fish appetite, therefore the effect of diet on hepatocyte ultrastructure is of interest. Storch & Juario (1983) reported

that starvation of the milkfish, Chanos chanos, caused ultrastructural changes in the liver. The prominent features characterizing the hepatocytes of starved fish were a reduction in cell and nucleus size, an apparent loss of nucleoli, condensation of chromatin material in fry, loss of stored glycogen, reduction of endoplasmic reticulum profiles, an increase in the number of electron dense bodies containing large amounts of iron, and an increase in mitochondrial size. These changes were reversible following short periods of feeding. Leatherland (1982) noted a decrease in the hepatosomatic index of fasted Coho salmon (Onchorhynchus kisutch). There was an associated increase in endoplasmic reticulum and decrease in glycogen. Murty & Devi (1982) carried out biochemical analysis of fish treated with chronic levels of endosulfan. They showed a decrease in liver glycogen, protein and lipid (see review page 40). These reports may explain the decrease in glycogen, cell size and hepatosomatic index observed in this study in fish treated with $1,5 \text{ ug l}^{-1}$ endosulfan. Although none of the other ultrastructural changes were noted.

Although light microscope observations are important in higher vertebrates, they seem to be of limited diagnostic value in fish (Couch, 1975; Ribelin & Walsh, 1975). Most liver lesions in fish exposed to pesticides were general or non-specific. For example, the organochlorines caused vacuolation of hepatocytes, hepatocyte necrosis, liver cord disarray, loss of glycogen and fat, cellular shrinkage, presence of mitotic cells, binucleate cells, swollen cells, pleomorphic cells, bizarre cells with enlarged nuclei and acidophilic pigmented inflammatory foci (Couch, 1975). The most commonly encountered change following pesticide exposure was vacuolation with lipid material (Couch, 1975). Pesticides within the same group (e.g. chlorinated hydrocarbons) may cause different or non-comparative changes in the livers of fish. Similarly, comparisons between the different groups of pesticides is

not feasible (Couch, 1975).

Endosulfan caused some changes which are similar to those reported for other chlorinated hydrocarbons. In this study there was cell shrinkage as a result of the loss of glycogen at $1,5 \text{ ug l}^{-1}$ endosulfan. This shrinkage caused slight liver cord disarray and the sinusoids had a more flattened appearance in cross-section. At $5,1 \text{ ug l}^{-1}$ endosulfan there was characteristic vacuolation of hepatocytes.

Couch (1975) recommended that in future studies on the effects of pesticides on fish livers emphasis should be placed on histochemical and electron microscopical studies in order to understand the actual mechanism of pesticide-induced injury. Few ultrastructural studies have, however, been carried out.

Weis (1974) investigated the ultrastructural changes induced by low concentrations of DDT in the livers of zebra fish (Brachydarnio rerio) and guppies (Poecilia reticulata). After 24 hours in $1,0 \text{ ug l}^{-1}$ or 72 hours in $0,3 \text{ ug l}^{-1}$ zebra fish had a complete loss of glycogen and a decrease in cell size. Guppies exposed for up to 28 days showed only gradual accumulation of smooth endoplasmic reticulum and no change in glycogen, lipid, rough endoplasmic reticulum or cell size. This result is interesting in that it shows a different response in the livers of two species of fish which had differing tolerances to DDT. The zebra fish were DDT sensitive, a concentration of 3 ug l^{-1} was toxic to most zebra fish within 24 hours whereas this concentration was not toxic to guppies after 28 days. The most likely reason for the tolerance of the guppy is the greater amount of lipid in the liver.

Yarbrough & Coons (1975) studied the difference in the fine structure of

insecticide-resistant and insecticide-susceptible mosquito fish (Gambusia affinis). They found enlarged hepatocytes with lipid inclusions occurred in resistant fish, giving further support to the hypothesis that organochlorine-resistance may be related to increased lipid deposition in the liver.

Yarbrough & Coons (1975) found no evidence of any other cellular changes, such as proliferation of endoplasmic reticulum, in insecticide-resistant fish.

Two studies have been carried out on the effects of the polychlorinated biphenyl, Aroclor 1254, on the livers of fish. Sivarajah et al. (1978) showed general vacuolation of hepatocytes in trout but not in carp injected with 25 mg Aroclor per kg body mass. Once again different species respond differently to the same pesticide. Electron microscope observations showed enlargement of rough endoplasmic reticulum in the trout and carp. Hacking et al. (1978) showed ultrastructural changes in trout exposed to dietary levels of 10 and 100 $\mu\text{g l}^{-1}$ Aroclor 1254 for 229 to 330 days. The most common changes encountered included irregular and bizarre nuclear outlines, separation of nucleolar components and large nuclear pseudo-inclusions. Other changes included increases in smooth endoplasmic reticulum, altered rough endoplasmic reticulum, reduced and altered glycogen, increased lipid and hypoxic vacuoles. At sublethal doses ($<1,5 \mu\text{g l}^{-1}$ endosulfan) endosulfan did not cause any major ultrastructural changes, and those which did occur were too generalized to be distinguished from nutritionally-induced changes or changes resulting from other pollutants. At present, too few ultrastructural studies have been carried out on the livers of fish for us to know the potential of ultrastructural examination as a diagnostic tool. Furthermore, the liver ultrastructure is affected differently in different species of fish exposed to the same pesticide. This ultrastructural examination was most useful because it enabled us to determine whether there was proliferation of endoplasmic reticulum. This is normally associated with the induction of hepatic mixed-

function oxidases (Mannering, 1981; Paine, 1981). Endosulfan did not cause proliferation of endoplasmic reticulum, whereas Aroclor 1254, a known inducer of mixed-function oxidases in fish (see cytochrome P-450 discussion), did cause proliferation of endoplasmic reticulum (Hacking et al., 1978; Sivarajah et al., 1978). Ultrastructural examination also showed that even at lethal concentrations there was no irreversible damage to the organelles of the hepatocyte. This explains why many of the fish removed from the LD50 concentration were able to recover. The main area of detoxification of endosulfan appears to be in the region of the hepatocyte adjacent to the space of Disse as there was a high concentration of mitochondria and numerous vacuoles of differing size and electron density.

HAEMATOLOGY

Results

The effects of endosulfan on various haematological parameters are compared with control values in Table 22. There was no statistical change (using a t-test; p values are given in Table 22) in any of the parameters except for an increase in erythrocyte count at a dosing level of $1,5 \text{ ug l}^{-1}$ endosulfan.

Discussion

Hypoxic conditions (defined by Wood (1980) as inadequate uptake of oxygen by tissues) have been shown to have a definite effect on haematological parameters of fish. The immediate response is an increase in heart rate and ventilation volume (Prosser, 1973). In addition fish will often gulp air and/or utilize primarily the water at the air:water interface which has the highest oxygen content (Powers, 1980). These are symptoms shown by fish stressed by environmental pollutants (Sellers *et al.*, 1975; Bansal *et al.*, 1979; Drewet & Abel, 1983; Natarajan, 1984; Zbanyszek & Smith, 1984; this study).

When fish are exposed to low oxygen for several days they increase the oxygen carrying capacity of their blood. There is an increased haematocrit resulting from the retention of serum in muscle tissues (Cameron, 1970) or the release of stored erythrocytes from the spleen and the synthesis of more erythrocytes (Powers, 1980). This results in an increase in erythrocytes and haemoglobin (Powers, 1980). These fish also improve their blood buffering capacity and increase oxygen-haemoglobin affinity which appears to be regulated by a combination of intraerythrocyte organic phosphates, ATP, GTP and inositol-P5 (Isaaks & Harkenness, 1980). Associated with hypoxia are an increase in serum lactate levels and a decrease in pH (Powers, 1980).

Table 22. Haematological values for control and endosulfan dosed *O. mossambicus*. Values are means of six observations; standard deviation is shown in brackets (MEV = mean erythrocyte volume, MEHE = mean erythrocyte haemoglobin concentration).

Blood parameters					
	Haemoglobin (g%)	Erythrocytes ($\times 10^6 \text{mm}^{-3}$)	Microhaematocrit (%)	MEV (μm)	MEHC (%)
control	7,1 (0,57)	1,37 (0,20)	21,5 (4,0)	163,88 (39,39)	34,00 (6,99)
endosulfan (0,1 $\mu\text{g l}^{-1}$)	6,9 (0,78)*	1,38 (0,13)*	22,1 (4,3)*	161,97 (39,90)*	31,78 (5,66)*
endosulfan (0,5 $\mu\text{g l}^{-1}$)	7,0 (0,73)*	1,41 (0,15)*	22,2 (5,0)*	156,91 (32,66)*	32,67 (6,25)*
endosulfan (1,5 $\mu\text{g l}^{-1}$)	6,5 (0,62)*	1,67 (0,14)**	23,8 (6,1)*	142,45 (32,40)*	28,83 (7,75)*
endosulfan (5,1 $\mu\text{g l}^{-1}$)	6,9 (0,65)*	1,45 (0,14)*	27,3 (3,4)*	157,46 (37,00)*	31,23 (4,6)*

* not significantly different from control (t-test, $p = 0,1$)

** significantly different from control (t-test, $p = 0,05$)

The effects of pollutants on haematology have shown no clear trends although some of them undoubtedly cause respiratory stress (hypoxia). Zinc, for example, is known to cause tissue hypoxia as a result of severe gill damage (Skidmore, 1970; Burton et al., 1972; Skidmore & Tovell, 1972). Drewet & Abel (1983) carried out a comparative study on the effect of Lindane (a representative chlorinated hydrocarbon) poisoning and hypoxia in trout. They found the symptoms of both causes of death are similar and pathological examination of liver and gills showed that hypoxia and Lindane poisoning could only be distinguished by small gaps in the gill lamellar epithelium in the latter.

Because pesticides and other pollutants may cause hypoxia as a result of gill damage (Burton et al., 1972), erythrocyte damage (Crandall & Goodnight, 1963; Kodama et al., 1982a; Kodama et al., 1982b), and impaired function of enzymes in metabolic pathways. For example: (1) energy metabolism (Dalela et al., 1978; Felts & Heath, 1984), (2) carbohydrate metabolism (Koundinya & Ramamurthi, 1979; Singh & Srivasthava, 1981; Sastry & Siddiqui 1982, 1984), (3) cellular metabolism, lactate dehydrogenase and succinic dehydrogenase activities (McCorckle & Yarbrough, 1974; Anderson et al., 1978; Hendrickson & Bowden, 1978; Natarajan, 1984; Sastry & Siddiqui, 1984), and (4) acetyl choline esterase activity (Edwards, 1974). Pesticides may also cause increased oxygen consumption as a result of behavioural changes, for example increased activity (Holden, 1974).

It is reasonable to assume that fish may acclimate to some pesticides in a similar way to hypoxia. However, little literature is available on the effects of pollutants on haematology and no clear patterns have yet emerged (Table 23). In general the organophosphate insecticides seem to cause a decrease in haematocrit, haemoglobin and erythrocyte count, whereas the

Table 23. The effect of different pollutants on various haematological parameters of fish. (MEV = mean erythrocyte volume; MEHC = mean erythrocyte haemoglobin concentration).

	Haematocrit	Erythrocyte count	Haemoglobin	MEV	MEHC	Reference
Hypoxia	↑	↑	↑			Powers (1980)
Zinc		↓				Mcleay (1975)
Copper		—				Dick & Dixon (1985)
Nitrite			↓			Scarano <u>et al.</u> (1984)
Aromatic hydrocarbons	↑	↑	↑			Zbanyszczek & Smith (1984)
Quinalphos*			↓			Sastry & Siddiqui (1984)
Sumithion*	↓	↓	↓			Koundinya & Ramamurthi (1979)*1
Metasystox*	↓	↓	↓			Natarajan (1981)
Sevin	↓		↓			Koundinya & Ramamurthi (1979)*1
Malathion*	↓	↓	↓			Mishra & Srivastava (1983)
Chlordane**	—	↑	↑	↓		Bansal <u>et al.</u> (1979)
Chlordane**	↑	↑	↑	↓	↓	Verma <u>et al.</u> (1979)*2
DDT **	—	—				Mustafad & Murad (1984)
Heptachlor**	↑					Andrews <u>et al.</u> (1966)
Endosulfan**		↑	—			Matthiessen (1981)
Endosulfan**	—	↑	—	—	—	This study

Sevin is a carbamate insecticide

* Organophosphate insecticides

** Chlorinated hydrocarbon insecticides

*1 From Sastry & Siddiqui (1984)

*2 From Bansal et al. (1979)

— No change

↑ Increase

↓ Decrease

chlorinated hydrocarbon insecticides tend to have no effect or cause an increase in the mentioned parameters.

Although many routine methods already exist in human medicine to assist in providing evidence of an abnormality or disease process, there are problems associated with using these methods to assess fish health. There is considerable variation in haematological parameters between individuals and populations of fishes (Blaxhall, 1972; Denton & Yousef, 1975; Van Vuren & Hattingh, 1978). There is also variation with season, age, nutritional state, temperature and handling (Barnhart, 1969; Blaxhall, 1972; Powers, 1980; Hille, 1982). It is difficult, therefore, to establish normal values. The effect of pollutants on blood is obviously not a simple acclimation process as occurs with hypoxia. The variations in haematological effects indicate that although these toxins may cause hypoxia, the physiological and pathological effects may interfere with acclimation processes. For example, zinc is known to cause hypoxia due to gill damage but fish are unable to acclimate to this condition as one of the other effects of zinc and other heavy metals is blood cell damage (Crandall & Goodnight, 1963; Kodama *et al.*, 1982a, 1982b).

Ultrastructural observations of sections through gill and liver tissue showed no obvious damage to blood cells (see previous section on gill and liver ultrastructure)

The evidence available indicates that haematological parameters have limited use in indicating pollution-induced anemia in the field. However, if the pollution history of a body of water is known and the effects of the various pollutants on haematology have been documented in laboratory experiments, then haematology may have some diagnostic value, particularly when used in conjunction with pathological, physiological and behavioural observations. White blood cell counts and leucocrit may be valuable for indicating a general

stress condition in fish - characteristically there is a lowering of the white blood cell thrombocyte count and leucocrit (Iwana *et al.*, 1976; McLeay & Gordon 1977; Mustafa & Murad, 1984; Dick & Dixon, 1985).

In this study endosulfan caused an increase in erythrocytes whilst other parameters remained unchanged. This change shows a similar trend to the changes caused by other chlorinated hydrocarbons (Table 23). Matthiessen (1981) found a similar increase in erythrocyte count in fish exposed to endosulfan in the Okavango Delta. This change was associated with a decrease in mean weight of haemoglobin per erythrocyte. The changes reported in both studies are characteristic of acclimation to hypoxic conditions, although such acclimation is usually associated with increased haemoglobin and haematocrit (Powers, 1980). The evidence presented indicates that one of the ways fish may acclimate to endosulfan is to increase the number of erythrocytes and thereby the oxygen carrying capacity of the blood.

CHAPTER 6 CONCLUSIONS

Endosulfan had no effect on gill histology and, therefore, there was no morphological impairment of oxygen transfer or osmoregulation. There were only generalized changes in liver ultrastructure which were of little diagnostic importance, although notably there was no proliferation of endoplasmic reticulum. Haematological studies showed that haematocrit and haemoglobin were unchanged and there was an increase in erythrocyte count. The above parameters taken individually are of little diagnostic value. However, for pollution-induced pathological changes to be of diagnostic value a number of different tissues and organs should be examined and the results pooled. If this is done then undamaged tissues are also of diagnostic value: for example, endosulfan causes no damage to the gills whereas other pesticides have been reported to cause lesions in gill tissue (Walsh & Ribelin, 1975). A complicating factor here is the interaction of different pollutants which may result in synergism or antagonism. Therefore, if possible, the pollution history of a particular water body should be known, and tissue and water analysis carried out for the suspected toxins.

Endosulfan, in common with other phenobarbital-type inducers, did not induce the hepatic mixed-function oxidase system in O. mossambicus. It seems therefore that the apparent short-term acclimation of O. mossambicus to sub-lethal levels of endosulfan may be partly due to an increase in the fatty content of the liver. Lipid soluble chlorinated hydrocarbons are thought to be sequestered by the lipid in the body (particularly the liver), this keeps the levels of toxin below the lethal threshold and allows it to be broken down slowly (Yarbrough & Coons, 1975). When the concentration of the pesticide is

so high that the buffering effect of the lipid pools, and enzymatic breakdown of endosulfan cannot keep pesticide concentrations below the lethal threshold, then death results. The liver does not seem to be involved in acclimation to long-term doses of sub-lethal levels of endosulfan, as the only changes were a decrease in hepatocyte size and glycogen reserves. However, if large numbers of fish are exposed to endosulfan (or other organochlorines) there may be selection for fatty fish, with livers containing a high proportion of lipids, which are better able to survive exposure to these toxins. The increase in erythrocyte numbers is not of sufficient magnitude for it to be considered as an important mechanism of acclimation to endosulfan.

Because of the lack of obvious endosulfan-induced pathological lesions shown in this study, and others, it seems likely that endosulfan interferes with some essential biochemical process. This interference appears to be reversible as more than fifty percent of fish surviving 48 hour LD50 treatment recovered when placed in fresh water.

The knowledge that some pesticides induce mixed-function oxidases in the livers of fish, and others do not, is of importance as it is preferable to use pesticides which cause induction. Therefore a serious consideration for future large scale application of pesticides, which will enter water bodies at between the "safe" and lethal concentration to fish, should be whether the pesticide causes induction of the hepatic mixed-function oxidase system. An initial low dose could be sprayed to induce the enzyme system and thereafter higher doses could be sprayed with reduced fish mortality. Further work should be carried out on the effects of organophosphate, carbamate, pyrethroid and other, specialised, groups of pesticides on the mixed-function oxidases of fish.

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