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The Uptake, Tissue Distribution and Metabolism of the Mothproofing Agent Eulan WA New in Species of Freshwater Fish

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Doctor of Philosophy

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To my Mother and Father for their continued support, encouragement and example throughout my years at home and in education.

ACKNOWLEDGEMENTS

"Synergism is the key word in collaboration it connotes that the joint effort is greater than the sum of several contributions made to it ..."

P. B. Medawar
(from - Advice to a Young Scientist)

I am indebted to; my Supervisors, Dr. N. C. Price, Dr. D. E. Wells and Dr. M. J. North, for their advice, support and understanding throughout this work. Dr. D. E. Wells and his family for their memorable hospitality during the periods of my collaboration with the Freshwater Fisheries Laboratory (Pitlochry), Messrs. D. Moore, G. Hanslip and A.E.G. Christie, for their assistance in the execution of research programmes involving fish management and fish tissue 'clean-up' and GLC analysis. Dr. T. E. Tooby for his information on the toxicity of Eulan WA New to freshwater fish; and to my colleague, Dr. J. S. Langdon, for his shared research experiences and philosophical discussion.

Finally, my thanks go to NERC for the funding of this work (Grant No. GR3/4129) of aquatic concern, providing a rare opportunity for xenobiochemical study; and to both Mrs. M. Abrahamson and my sister Hazel for their efficient typing of this thesis.

This collaborative project has involved research in both the Biological Science Department (University of Stirling) under the supervision of Dr. N. C. Price and Dr. M. J. North, and the Environmental Chemistry unit (Freshwater Fisheries Laboratory, Pitlochry) under the supervision of Dr. D. E. Wells.

The mothproofing agent Eulan WA New used in the textile industry is a formulation of PCSD (Polychloro-2-[chloromethyl sulphonamido] diphenyl ether) components (the active ingredients) and PAD (Polychloro-2-aminodiphenyl ether) impurities, plus solvent and surfactant. Throughout this work the term Eulan WA New refers to the chemical mixture of active ingredients plus impurities, minus the solvent and surfactant.

ABBREVIATIONS

ADP	: adenosine 5'-diphosphate
ATP	: adenosine 5'-triphosphate
BSA	: bovine serum albumin
Cyt.b_5	: cytochrome b_5
Cyt.P-450	: cytochrome P-450
2,4-D	: 2,4-dichlorophenoxyacetic acid
DEE	: diethylether
DMF	: dimethylformamide
DTT	: dithiothreitol
p,p'-DDT	: 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane
GLC	: gas liquid chromatography
GSH	: reduced glutathione
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
pHMB	: p-hydroxymercuribenzoate
HPA	: hide powder azure
HPLC	: high performance liquid chromatography
MS222	: ethyl-m-aminobenzoate
MTBE	: methyltertiarybutyl ether
NAD^+	: nicotinamide adenine dinucleotide
NADH	: nicotinamide adenine dinucleotide (reduced)
$NADP^+$	: nicotinamide adenine dinucleotide
NADPH	: nicotinamide adenine dinucleotide (reduced)
PAD(s)	: polychloro-2-aminodiphenyl ether(s)
5-PAD	: pentachloro-2-aminodiphenyl ether
PCB(s)	: polychlorinated biphenyl(s)
PSCD(s)	: polychloro-2-[chloromethyl sulphonamido] diphenyl ether(s)
6-PCSD	: pentachloro-2-[chloromethyl sulphonamido] diphenyl ether
PMSF	: phenylmethylsulphonylfluoride

Abbreviations (cont.)

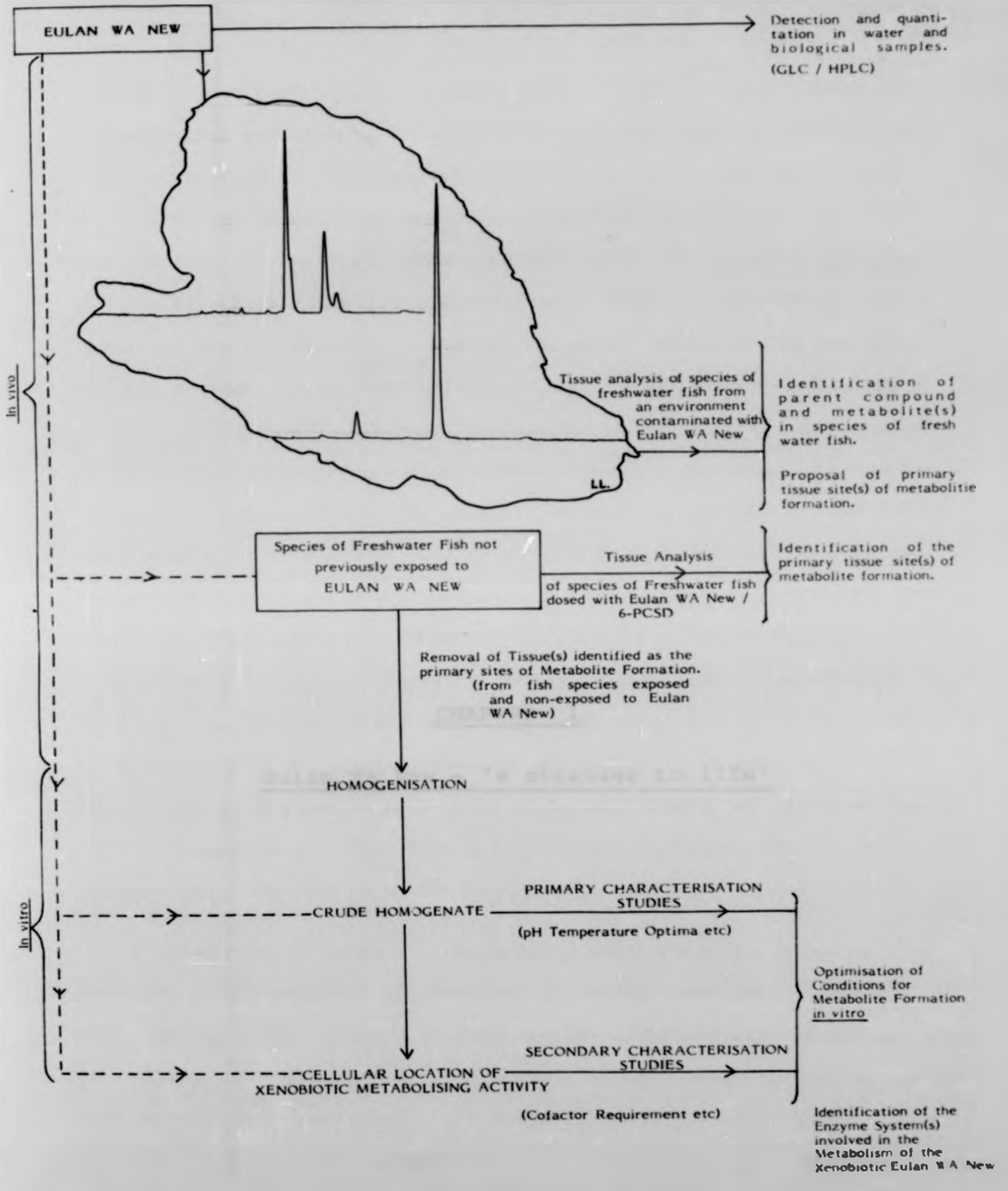
UDPGA	:	uridine-5'-diphospho-α-D-glucuronic acid
2,4,5-T	:	2,4,5-trichlorophenoxyacetic acid
TMA	:	trimethylamine
TRICINE	:	N-[2-hydroxy 1,1-bis(hydroxymethyl)ethyl]-glycine
TRIS	:	2-amino 2(hydroxymethyl)1,3-propandiol

SYNOPSIS

The active ingredients of the mothproofing agent Eulan WA New are polychloro-2-(chloromethyl sulphonamido) diphenyl ether (PCSD) components, differing in their degree of aromatic ring chlorination. It has been shown that direct release of textile effluents has resulted in the contamination of the aquatic environment by Eulan WA New and the exposure of aquatic organisms to this complex xenobiotic.

Dosing studies of this work showed that the accumulation of the major active ingredient of Eulan WA New, 6-PCSD (hexachloro-2-[chloromethyl sulphonamido] diphenyl ether) in freshwater fish tissue occurred mainly by gill and cutaneous routes. It accumulates to high concentrations, especially in the bile of pike *E. lucius* for example, after short periods of dosing. Analysis of pike and goldfish *C. auratus* tissue showed generally high ratios of 5-PAD:6-PCSD providing evidence for the *in vivo* cleavage of the sulphonamido bond of 6-PCSD with the formation of 5-PAD metabolite. The primary locations of the 5-PAD forming activity in these fish were identified as the liver and digestive tissue. *In vitro* studies showed the presence of an activity in goldfish, carp *C. carpio* and pike liver (homogenates and subcellular fractions) which catalysed the formation of 5-PAD from 6-PCSD. The maximum rate of 5-PAD formation was observed when liver homogenate was prepared in phosphate buffer (40 mM) of neutral to alkaline pH, containing dithiothreitol, NADPH and/or NADH, and (from a preliminary study) showing a requirement for oxygen. The activity is characteristic of a microsomal monooxygenase type (cyt.P-450 dependent and/or cyt.P-450 independent). Complementary data of contrasting differences in the ability of species of freshwater fish to metabolise 6-PCSD to 5-PAD was obtained from *in vivo* and *in vitro*

studies. 5-PAD formation occurred in goldfish, carp and pike liver tissue with an apparent absence (or greatly reduced activity) in tissue of rainbow trout *S. gairdneri*, perch *P. fluviatilis* and eel *A. anguilla*. Such apparent qualitative differences in the metabolism of Eulan WA New by species of freshwater fish highlight the importance of a careful choice of fish species as model systems to assess environmental contamination/metabolism of this xenobiotic.



A GENERAL SUMMARY OF THE IN VIVO AND IN VITRO APPROACHES USED TO STUDY THE METABOLISM OF EULAN WA NEW IN SPECIES OF FRESHWATER FISH

L.L. - Loch Leven, an environment contaminated with EULAN WA NEW

CHAPTER 1

Eulan WA New - 'a stranger to life'

Life is a result of a myriad of inter-related chemical processes compartmented within the living cell and yet intimately dependent upon interaction with the surrounding environment. Organisms are exposed to an enormous variety of chemicals in the environment, some of which are essential for cell nutrition and cell function. Many substances of natural origin (to which the organism is exposed) are however non-nutritive and non-functional and are therefore generally defined as foreign. Mason et al (1965) referred to those: "components of the chemical environment foreign to the metabolic network* of the organism, as xenobiotic compounds from the Greek *zenos* and *bios* meaning Stranger to life". Such natural xenobiotics are found as constituents of food; for example, in vegetables, fruits and fungi as glycosides, alkaloids, terpenes and aromatic acids. During ingestion of food, organisms are unable to distinguish and separate nutrients from contaminating xenobiotics. However to prevent these components from accumulating to toxic levels, the organism has developed mechanisms which facilitate their elimination. This process is attributed to enzymes which biotransform xenobiotics, to provide structural modifications resulting in their excretion. The general nature and broad function of these enzymes are discussed in section 3.1.

The enzymes of xenobiotic metabolism most probably evolved in response to the exposure of organisms to natural xenobiotics mainly in food (Parke, 1974). The biotransformation of xenobiotics is the major function of this group of enzymes which also have been shown to metabolise some endogenous substrates. For example, the cyt.P-450 dependent monooxygenases have been shown to participate in both xenobiotic and

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*Metabolic network - 'the systems of enzyme-catalysed reactions essential to the life and function of a cell type' - Mason et al (1965)

steroid metabolism (Conney and Klutch, 1963; Conney et al, 1965). The adaptative capacity of the cell determines the extent to which the foreign compounds are utilised within the organism and consequently the extent to which they remain 'strangers'.

Through the genius of man an enormous spectrum of synthetic products have been produced of industrial, pharmaceutical and cosmetic use. Examples of these substances are given in Table 1.

At the beginning of the 20th century the textile industry recognised the need for protection of their products from textile-feeding pests. In 1967 a report by Bayer stated that 15 million U.S. dollars worth of wool was lost each year in the German Federal Republic due to wool-feeding pests. This necessitated a need to develop mothproofing, involving the chemical treatment of woollen fibres to prevent damage by the larvae of woollen pests. The chemical structures of the active ingredients of the major mothproofing agents are given in Figure 1. A common characteristic feature of these compounds is their high affinity for keratin, binding to the protein in manner similar to that of textile dyes involving hydrophobic, dipolar interactions and hydrogen bonding (Bayer, 1967). The wool-bound mothproofing agents show resistance to both washing and dry-cleaning processes. Keratin treated with the mothproofing agents is not digested by larvae of pests, and so affords protection to the wool.

A major mothproofing agent employed by the textile industry is Eulan WA New. The active ingredients of this are PCSDs (see section 2.1 for chemical structures). PCSDs are also present in other moth-proofing agents (Table 2) which are, together with Eulan WA New, in world-wide use. This and other xenobiotics (Table 1) may be released to the environment through use or during their manufacture, or may be

present in industrial effluents. This results in the exposure of organisms to both the synthetic xenobiotics as well as those of natural design normally encountered. Studies have indicated that fish in polluted waters show an increased frequency of tumours (Brown et al, 1973, 1975). As exemplified here, the potential toxicity of xenobiotics is generally seen through their effects upon exposed organisms of the environment. This leaves the environmental biologist to cure the "disease" rather than curing the symptoms. Westöö and Norén (1977) and Wells and Cowan (1983) have identified the presence of the mothproofing agent Eulan WA New in fish tissues, arising from textile effluents. Consequently such environmental contamination demands an assessment of its impact upon the aquatic organism. This work involves a study of the 'interaction' of Eulan WA New with species of freshwater fish.

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Table 1

Examples of xenobiotics which may be applied purposefully or accidentally to organisms.

		Additives
Food	:	colouring - cochineal, fluorescein dyes
		flavouring - coumarin, vanillin
		sweeteners - sodium cyclamate, saccharin
		Contaminants
		from packing - organotins, butylphthalate
		medicines and growth improvers added to wildstock - antibiotics, oestrogens
		Pesticides - see below
Drugs	:	Sulphonamides - sulphathiazole (2-sulphanilamido-thiazole)
		alkylating agents - Myleran (Busulphan, 1,4-bis-[Methanesulphonoxy]-butane)
		phenol derivatives - salicylic acid
Pesticides	:	Rodenticides - warfarin
		Insecticides - DDT, Dieldrin, captan, malathion
		Herbicides - 2,4-dichlorophenoxyacetic acid
		molluscides - pentachlorophenol
		Fungicides - hexachlorobenzene
Industrial* chemicals	:	Glycols - Ethylene glycol
		Aromatic hydrocarbons - xylene

* used in industry as solvents, fuels and intermediates in the manufacture of pesticides, paints, detergents, plastics, drugs, cosmetics etc.

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oxylate - (active ingredient

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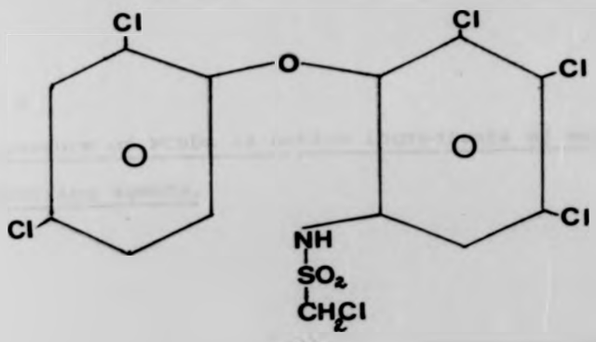

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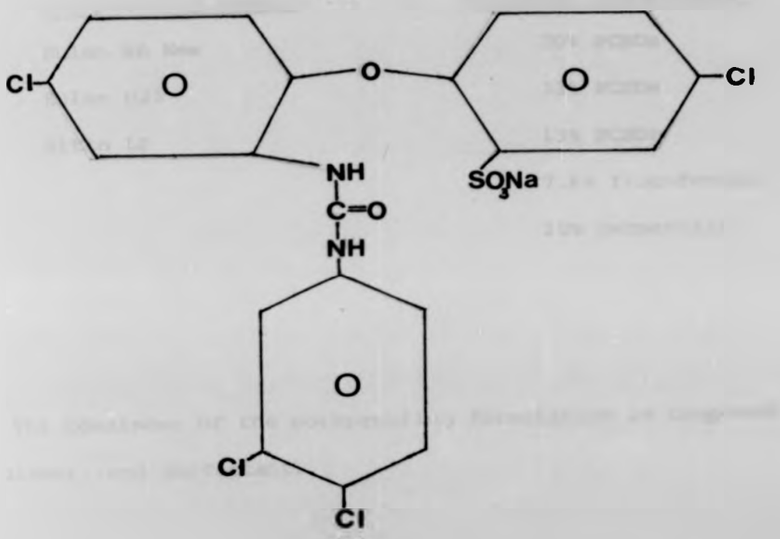

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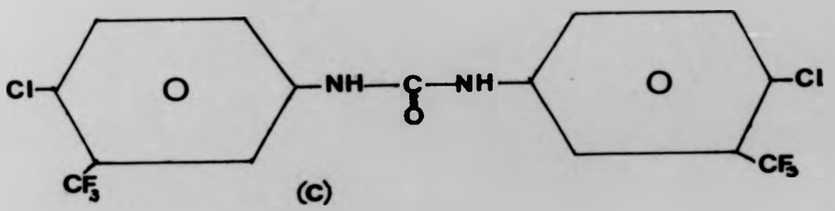

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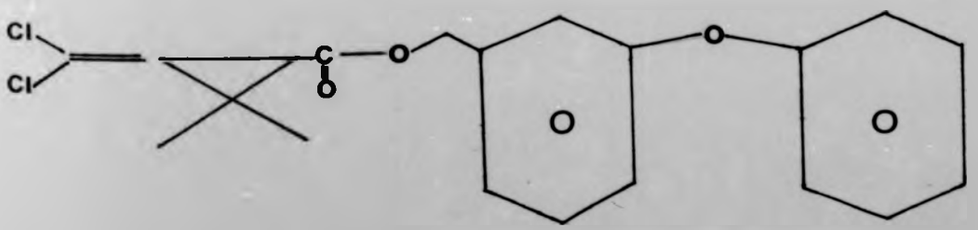

(d)

Aromatic rings

(a) PCSD (6-PCSD,pentachloro-2-[chloromethyl sulphonamido] diphenyl ether) - (active ingredient in mothproofing agents of Table 2)

(b) sulcofenuron - 5-chloro-2(4-chloro-2-[3,4-chlorophenyl] phenoxyureido)-benzene sulphonic acid - (active ingredient of the mothproofing agent Mitin FF HC)

(c) flucofenuron - 3,3'-di-(trifluoromethyl)4,4 dichloro.N,N-diphenyl-urea - (active ingredient of the mothproofing agents Mitin N and Mitin L.P)

(d) Permethrin - 3-phenoxybenzyl-2,2-dimethyl-(1S, 1R, Cis, trans)-3-(2,2-dichlorovinyl)cyclopropane carboxylate - (active ingredient of the mothproofing agents, mothproofing Agent 79 and Perigen)

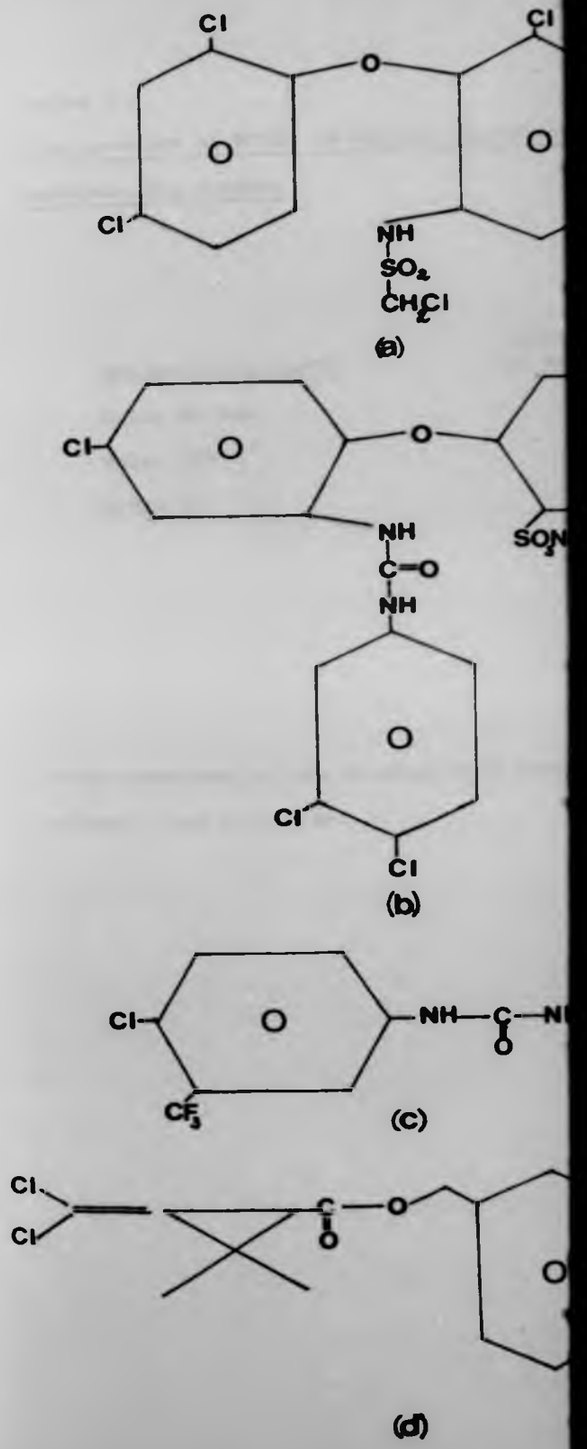

Figure 1

Chemical structure of active ingredients of the major mothproofing agents.

Table 2

The presence of PCSDs as active ingredients of major mothproofing agents.

Mothproofing Agents	Percentage composition of active ingredients*
Eulan WA New	20% PCSDs
Eulan U33	33% PCSDs
Mitin LP	13% PCSDs
	7.6% flucofenuron
	20% permethrin

* The remainder of the mothproofing formulation is composed of solvent, and surfactant.

CHAPTER 2

The Uptake, Tissue Distribution and *in vivo* Metabolism of Eulan WA New and 6-PCSD in species of Freshwater Fish.

2.1 INTRODUCTION

'Science moves but slowly slowly moving on from point to point'

Alfred, Lord Tennyson (1809-1892)

A major 'rate determining step' in the scientific progress of environmental biological research, has been the development of suitable techniques for the identification and quantitative analysis of micro-concentrations of xenobiotics present in the Biosphere. A combination of improved separation techniques and the application of modern physical techniques has contributed greatly to both the characterisation of xenobiotics, and an understanding of their metabolism in living systems. Today's level of sophistication shows the coupling of gas liquid chromatography (GLC) systems with mass spectroscopic (MS) systems, and the application of high performance liquid chromatography (HPLC) to the analysis of xenobiotics. Their complex nature and improved efficiency and sensitivity contrasts with respect to earlier thin layer chromatography (TLC) systems, for example.

Characterisation studies leading to the identification of some of the components of the mothproofing agents Eulan WA New were published by Westöö and Norén (1977). A more detailed study leading to the identification of the major components of Eulan WA New and a reproducible method for their quantitative analysis was published by Wells (1979) and Wells and Cowan (1981). Structures of the identified components of Eulan WA New are given in Figure 1. The major 6-PCSD component was observed to degrade thermally upon GLC analysis resulting in cleavage of the sulphonamido bond and formation of 5-PAD. However the 6-PCSD component was found to be protected from thermal degradation by methylation. This derivatisation of PCSD was subsequently carried out prior to GLC analysis (section 2.3.5.3) [thethermolability of PCSD was not recognised in the earlierwork of Westöö and Norén (1977)

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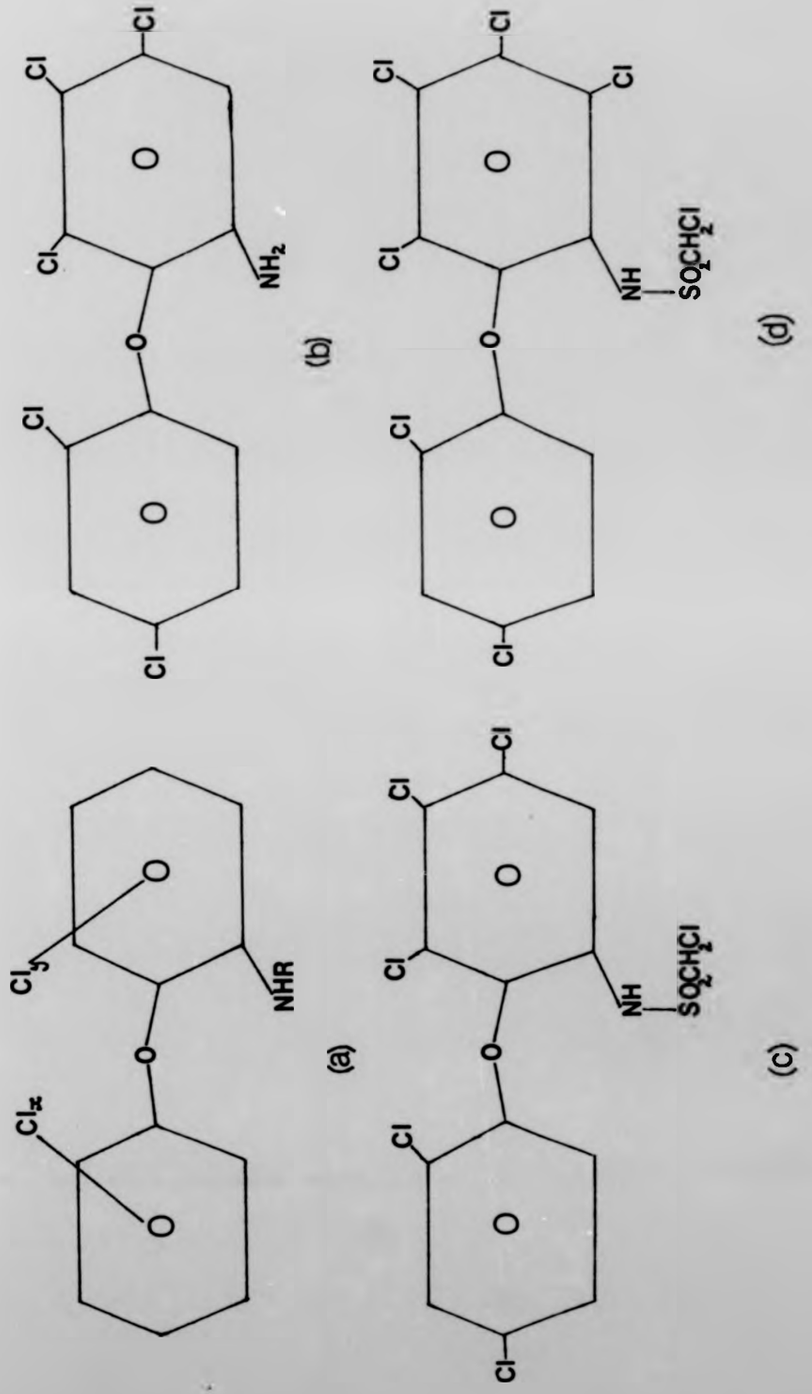

(a) General formula, where R may be H or SO_2CH_2Cl, x+y may be 4, 5, 6 denoting the chlorine atoms in the component.

(b) 5-PAD (2',4',2,3,4-pentachloro-6-aminodiphenyl ether), main impurity.

(c) 6-PCSD (2',4',2,3,4-pentachloro-6-(chloromethyl sulphonamido) diphenyl ehter, the main active ingredient.

(d) 7-PCSD (2',4',2,3,4,5-Hexachloro-6-(chloromethyl sulphonamido) diphenyl ether.

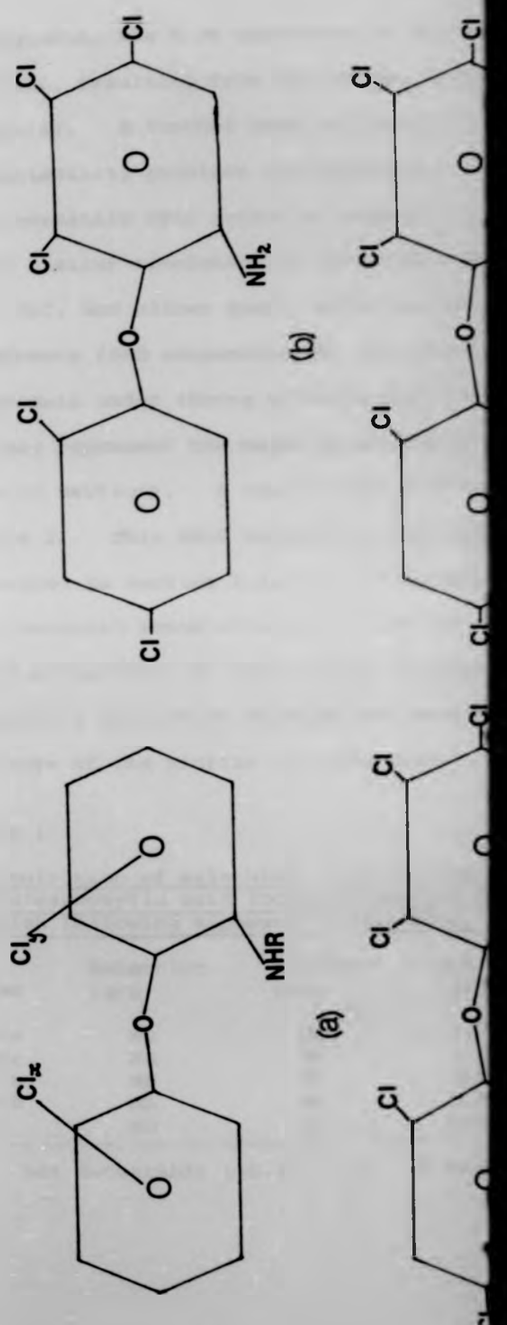

Figure 1

Structures of active ingredients and some minor components of Eulan WA New.

consequently the high concentration of 5-PAD in fish tissues may be an artifact, resulting from the thermal degradation of PCSD upon GLC analysis]. A further means of analysing Eulan WA New, circumventing thermolability problems was introduced by Wells and Johnstone (1981). This versatile HPLC method of analysis is performed at room temperature, shows similar resolution of the components of Eulan WA New as observed with GLC, and allows quantitative analysis of both PCSD and PAD components (PAD components are produced by the cleavage of the PCSD components under strong alkaline conditions (Westöö and Norén, 1977) and may represent the major metabolite of PCSD *in vivo* - discussed later in this section). A sample HPLC profile of Eulan WA New is shown in Figure 2. This HPLC system was employed throughout this work as described in section 2.3.6.3. A suitable system for the analysis of environmental xenobiotics should detect both parent compound and the major metabolites formed *in vivo* (if such metabolism occurs). The importance of this is shown by the data of Table 1 obtained from the exposure of the pinfish (*L. rhomboides*) to malathion.

Table 1

Concentration of malathion, malaoxon, and monocarboxylic acid (MCA) and dicarboxylic acid (DCA) metabolites in various organs of a pinfish following exposure to malathion.

Organ	Malathion µg/g	Malaoxon µg/g	MCA µg/g	DCA µg/g
Brain	ND	ND	1.7	0.22
Liver	ND	ND	6.0	0.25
Gills	ND	ND	2.5	0.36
Flesh	ND	ND	3.9	0.34
Gut	ND	ND	31.4	0.70

ND - not detectable (<0.10 µg/g) From Cook and Moore (1976)

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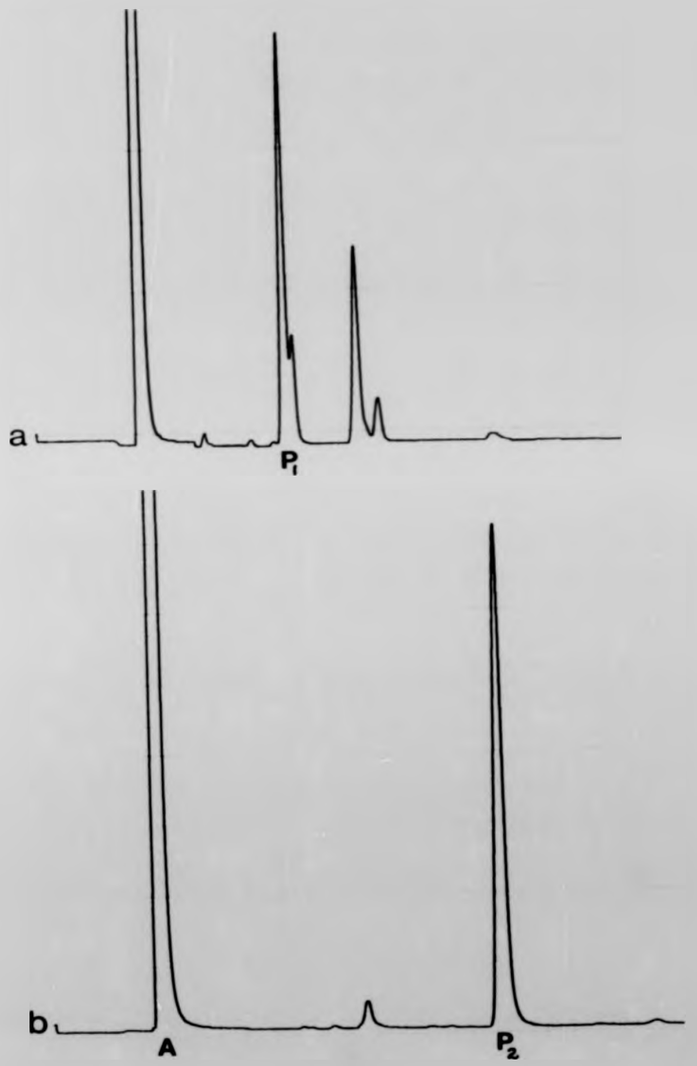


P_1 - 6-PCSD (major active ingredient of Eulan WA New)

P_2 - 5-PAD (major impurity)

The components of Eulan WA New are further described in section 3.4.2.1.

HPLC profiles of:

(a) Eulan WA New

(b) PAD standard

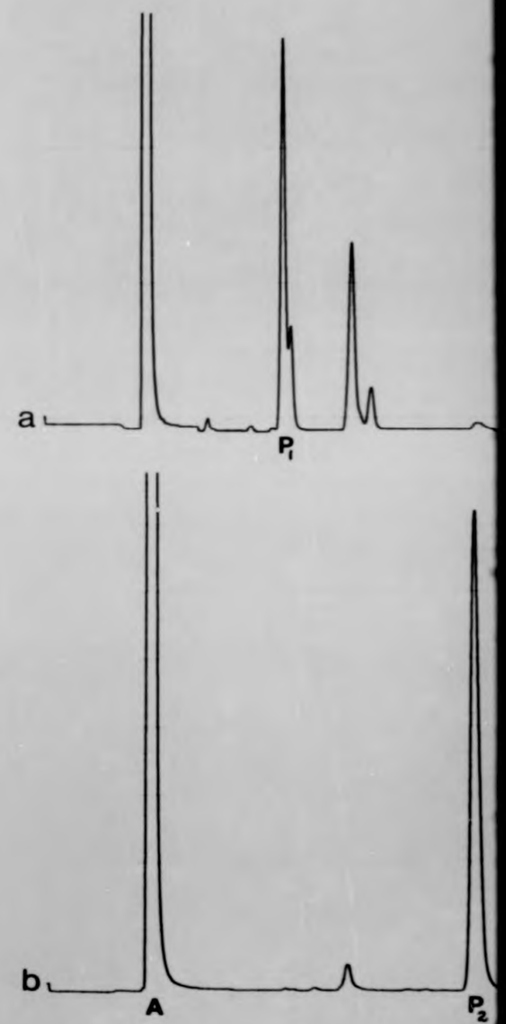


Figure 2

A sample HPLC profile of the mothproofing agent Eulan WA New showing PAD impurity.

If analysis of only the parent compound were possible, then the absence of detectable levels may lead to the erroneous conclusions that the fish was not exposed to malathion. In studies of the analysis and metabolism of xenobiotics, HPLC has been invaluable, particularly when functioning in the reverse phase mode (this is suitable for the detection of xenobiotics with their general lipophilic character). For example, the metabolism of the polycyclic aromatic hydrocarbon benzo(a)pyrene has been studied using HPLC (Holder et al, 1974). Studies in the formation of labile reactive metabolites, e.g. benzo(a)-pyrene-7,8-diol, 9-10-epoxides (not amenable to study by conventional GLC techniques) has been possible using HPLC (Huberman et al, 1976). Further HPLC has enabled the study of the covalent interactions of the reactive metabolites and cellular macromolecules with the identification by HPLC of modified RNA species (Koreeda et al, 1976).

The solubility of PCSD in aqueous environments has been studied (Wells and Cowan, 1983) and ranged between 1.2 µg/l at pH 4.6 to 1304 µg/l at pH 9.5 with the greatest rate of change between pH 6.5 and pH 7.5 (corresponding with the pKa of PCSD of 7.2). Residues of Eulan WA New in aqueous baths following woollen treatment are released as effluents from textile mills. The effluent may be treated at a sewage treatment plant, effectively removing the residual concentrations of Eulan WA New (Kanne et al, 1981). The effect of Eulan WA New in effluents on the microorganisms involved in the sewage treatment process has not been assessed in detail. An alternative to the release of textile effluents to sewage treatment plants is their direct release to the aquatic environment. This was found to be the route of contamination of Loch Leven (Kinross) by Eulan WA New. Open water concentrations taken from Loch Leven in 1980 showed an average concentration of 6-PCSD of 408 ng/l ±74, n + 15 (Wells and Cowan, 1983). The organisms at risk from

contamination with Eulan WA New include (in addition to the enormous variety occurring in the aquatic environment) fish-eating birds and mammals and also ultimately Man himself. Loch Leven is a major centre for sport fishing and also a nature reserve, features attracting a wide range of scientific researchers (IBP. 1972/73). A research programme to monitor the contamination of a variety of fish species taken from the loch with Eulan WA New was conducted from 1964-78 (Wells and Cowan, 1983). An analysis of muscle taken from trout (*Salmo trutta*), pike (*Esox lucius*) and perch (*Perca fluviatilis*) collected over this period from Loch Leven, show the increase in contamination of fish tissues with PCSD over this period (Fig. 3). A comparison of the tissue distribution of Eulan WA New in species of freshwater fish showed contrasting differences in tissue levels of 6-PCSD and 5-PAD. For example the tissues of the brown trout from Loch Leven contained predominantly 6-PCSD residues in contrast to those of the pike which contained mainly 5-PAD. 5-PAD may constitute up to 10% of the level of 6-PCSD in Eulan WA New as an impurity of manufacture (this fraction varies from 1-10% depending on the batch of mothproofing agent supplied). Consequently the high 5-PAD tissue concentrations observed in some freshwater fish taken from an environment contaminated with Eulan WA New, may arise from mechanisms of selective absorption or excretion of components of Eulan WA New. Alternatively, 5-PAD may be formed *in vivo*, through the cleavage of the sulphonamido bond of 6-PCSD (analogous to the alkaline hydrolysis of 6-PCSD to 5-PAD, Westöö and Norén, 1977). In order to determine the pathways accounting for the 5-PAD accumulation in some freshwater fish, a variety of freshwater fish species were dosed with Eulan WA New and purified 6-PCSD (free of PAD). During periods extending from minutes to days, the shorter periods were employed to identify the primary tissues in which 6-PCSD and 5-PAD accumulate.

● - Pike

■ - Perch

▲ - Trout

(All values are for the major active ingredient 6-PCSD except for pike, where the concentration of Eulan is determined as that of the metabolite PAD.)

Data taken from Wells and Cowan, 1983.

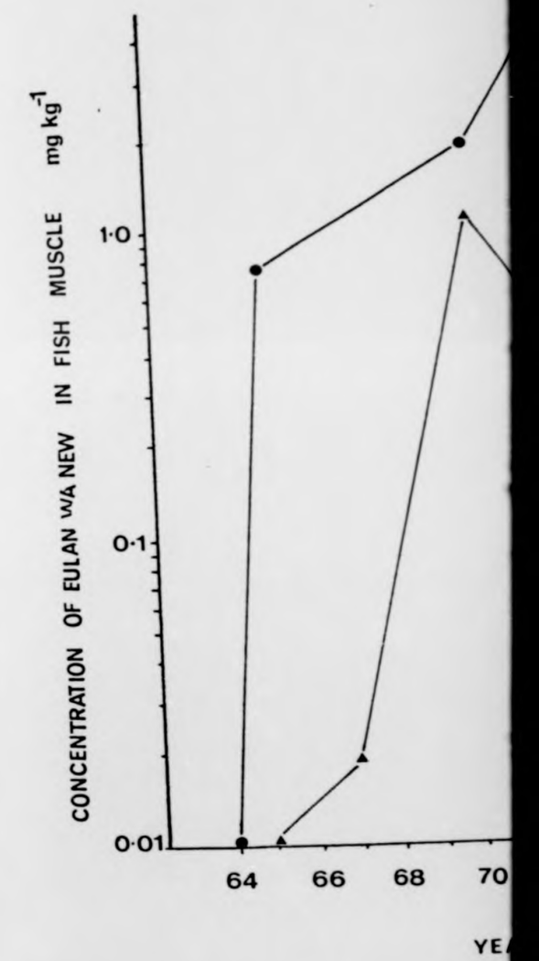


Figure 3

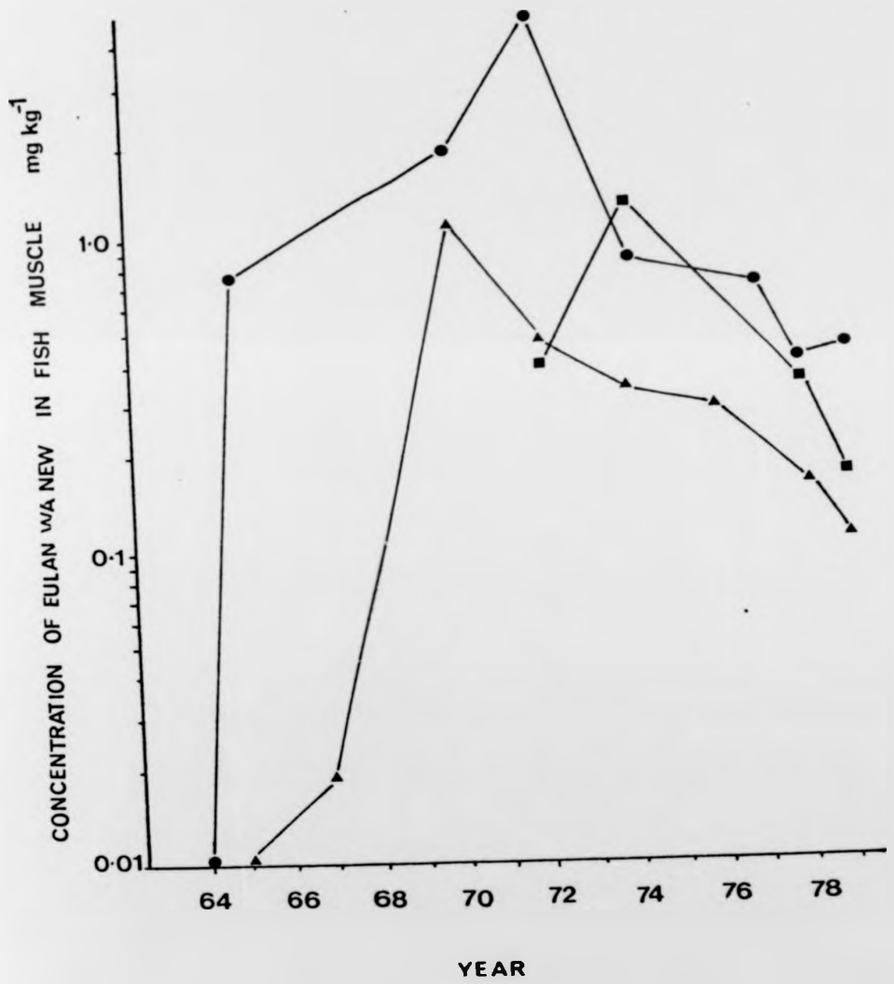
Concentration of Eulan WA New in the muscle tissue of fish taken from Loch Leven, Kinross between 1964 and 1979.

active ingredient 6-PCSD

ncentration of Eulan is

tabolite PAD.)

an, 1983.



in the muscle tissue of fish

between 1964 and 1979.

2.2 MATERIALS

Freshwater fish used in dosing studies

Pike (*Esox lucius*) were caught by nets from Loch Faskally (Pitlochry). Trout (*Salmo gairdneri*) were supplied from a local fish farm (College Mill Trout Farm, Almondbank, Perthshire) and goldfish (*Carassius auratus*) also from a local supplier.

Methyl tertiary butyl ether (MTBE), Methanol, Chloroform, hexane and diethylether (DEE) - were supplied as HPLC grade solvents by Rathburn Chemicals (Walkerburn) Ltd.

Sodium sulphate - anhydrous, granular, was prepared from the analytical reagent grade material by drying at 200°C for 4h.

Basic alumina - Merck 1097, BDH Chemicals Ltd. (Poole, Dorset) was heated to 200°C for 2h and deactivated to 4% v/m (Brockmann Activity II-III) with hexane washed distilled water.

Acidic alumina - was prepared from basic alumina by washing with 1 M hydrochloric acid. The slurry was filtered and dried at 200°C for 4h, cooled and deactivated to 4% v/m with distilled water.

Glacial acetic acid (general purpose reagent), dimethylformamide (DMF, Analar grade) and orthophosphoric acid (analar grade) were supplied by BDH Chemicals Ltd. (Poole, Dorset).

Tetrabutylammonium hydroxide (TBA) solution - (0.05 M) was prepared by dissolving 35.5 ml of ammonia solution (sp. gr. 0.88) in 10 ml TBA and 90 ml distilled water.

Methyl iodide - Fluke, Puriss grade.

Ethyl-m-aminobenzoate - (MS222) - was obtained from Sigma (Poole, Dorset).

Eulan WA New - was supplied by Bayer (Shipley, Great Britain).

2.3 METHODS

2.3.1 Purification of 6-PCSD from Eulan WA New

A semi-preparative separation of the components of Eulan WA New to obtain a pure pentachloro-2-(chloromethyl sulphonamido) diphenyl ether (6-PCSD) was achieved by the semi-preparative use of the HPLC system described in section 2.3.6.3. Eulan WA New, 0.5 mg in 5 µl DMF, was injected on to the HPLC column, and the absorbance of the eluant was monitored at 270 nm. This wavelength was sufficiently higher than the λmax of 230 nm to give an on scale deflection at the maximum absorbance setting of 2.0. The 6-PCSD was separated from other Eulan WA New components and collected at the detector outlet. This was repeated several times and the fractions containing the 6-PCSD pooled. The 6-PCSD solution was acidified and extracted with methyl tertiary butyl ether (4 × 5 ml). The ether extract was evaporated to dryness in a stream of dry air and redissolved in DMF to give a final concentration of 5.6 mg/ml .

2.3.2 Preparation of fish liver lipid extract

0.2 g of fish liver tissue was homogenised with 3 ml chloroform: methanol (2:1, v/v). The organic phase was retained and the extraction procedure repeated. The pooled chloroform:methanol extracts were shaken with 0.88% potassium chloride in water (1/4 volume of the organic phase) and the upper phase discarded. Distilled water:methanol (2:1, v/v, 1/4 of the volume of the organic phase) was added to the lower phase and the washing procedure repeated. The lower phase containing purified lipid (Folch et al, 1957) was concentrated by evaporation of the solvent prior to its subjection to the modified "clean-up" method upon mixed alumina as described in section 2.4.3, and HPLC analysis.

2.3.3 Dosing conditions for exposure of pike to Eulan WA New or purified 6-PCSD in surrounding water

The initial experiments, involving dosing with Eulan WA New were undertaken using a static glass tank system (2 × 2 × 0.5 m) containing 1,600 litres of water. The second experiment involving dosing with 6-PCSD was performed using a glass tank containing 40 litres of water. In each case water was supplied from Loch Faskally by pump and gravity feed.

The concentrated solution of Eulan WA New or 6-PCSD was diluted in methanol and added to dosing tanks to give a final concentration of 5 µg/l . The water concentrations of 6-PCSD and 5-PAD at each stage of the experiment were determined by extraction and GLC analysis.

Sexually immature pike varying between 23 and 31 cm in length and 60 and 180 g in weight were employed in the dosing studies. In the long term dosing experiments with Eulan WA New, six pike were placed in dosed water and sequentially removed after exposure times of 4, 8, 14, 26, 50 h and 10 days. In the short-term dosing experiments with 6-PCSD, three pike were placed in dosed water and sequentially removed after exposure times of 10, 20 and 40 min. Following such exposure to 6-PCSD the pike were anaesthetised with MS222, to allow a blood sample to be taken from the haemal arch. Pike were then killed by severing the spinal cord and completely dissected. All tissues were stored individually at -20°C prior to extraction, "clean up" and analysis.

2.3.4 The dosing of salmon fry with Eulan WA New and their subsequent feeding to pike

Four sexually immature pike varying in length between 19.5 and 23.0 cm, and in weight between 57.0 and 96.0 g were used in this study. Each was placed in a separate mesh cage within a large fibre glass tank (2 × 2 × 0.5 m) containing 1,600 litres of water, supplied from Loch

Faskally by a pump and gravity-feed mechanism. The pike were maintained for three weeks in the cages, during which they developed a regular feeding pattern of 2-3 salmon fry (3.0 - 3.5 cm long) per day.

Salmon fry from the laboratory stock population were placed in a glass tank containing 40 litres of water dosed with Eulan WA New to a concentration of 50 µg/l . Salmon fry were removed from the dosing bath after 2h and were immediately offered to the caged pike. The pike continued to feed on this daily diet of dosed salmon fry over the dosing period of 10 days. The four pike were removed from the cages for dissection and tissue analysis after 7h (ingested 3 fry), 24h (ingested 3 fry), 77h (ingested 5 fry) and 221h (ingested 15 salmon fry) respectively.

2.3.5 Dosing goldfish and rainbow trout with purified 6-PCSD

Two litres of distilled water were placed in each of two glass tanks (of three litres capacity). To one tank was added a solution of purified 6-PCSD in DMF, giving an initial concentration of 0.26 ppm 6-PCSD, and the second (control) tank an equivalent volume of DMF. Eight sexually immature goldfish weighing between 3.3 and 6.6 g and measuring between 6.5 and 8.0 cm in length were employed in this study. Four goldfish were added to each of the tanks. After dosing periods of 10 min., 30 min., 2h and 6.5h, goldfish were removed from each tank and killed by severing of the spinal cord. The goldfish were dissected and the liver plus bile, skin plus muscle, digestive tract and gill tissues were removed. These tissues were stored at -20°C before tissue extraction, "clean up" and analysis.

The dosing of eight sexually immature rainbow trout (weighing between 3.5 and 5.5 g and measuring between 6.5 and 7.5 cm in length) with 6-PCSD (0.5 ppm) was carried out as described above for goldfish.

Samples were removed after 10 and 45 min. dosing with 6-PCSD. After 35 and 45 min. dosing with 6-PCSD, all exposed rainbow trout died.

2.3.6 Extraction, "clean up" and GLC analysis of fish tissues

2.3.6.1 Continuous hexane extraction of fish tissue samples

Fish tissues were weighed and ground with anhydrous sodium sulphate (≃4g) to give a free flowing powder. This was transferred to an extraction thimble, placed in a soxhlet extraction assembly and charged with 100 ml hexane. Continuous extraction with hot hexane was carried out for 2h at a syphon rate of 20 cycles per hour. After cooling the final volume of hexane was adjusted to 100 ml. A suitable aliquot was removed for sample "clean up" and further analysis.

2.3.6.2 "Clean up" of hexane tissue extracts

A diagrammatic representation of the "clean up" method described by Wells and Cowan (1981) is given in Figure 4. A small (hexane washed) cotton wool plug was placed at the base of the glass column to secure the mixed alumina. 2 g of acidic alumina was added, followed by 1 g of basic alumina to the acidic base. The concentrated hexane tissue extract (≃ 200 µl) was added and absorbed onto the head of the mixed alumina. The column was then successively eluted with 35 ml hexane (E3), 25 ml DEE (E4) and finally [following acidification of the column with 1 ml acetic acid solution (15% v/v acetic acid in hexane) over a period of 2-5 min.] with 25 ml DEE solution (30% v/v DEE in hexane, E5). 5-PAD was eluted from the mixed alumina column in the E3 eluate and 6-PCSD in the E5 eluate.

HPLC profiles of the following successive eluates from a mixed alumina column loaded with Eulan WA New and PAD standards:

E3 - 35 ml Hexane

E4 - 25 ml DEE

E5 - 25 ml DEE/Hexane (30/70, v/v) - following column acidification with 1 ml 15% v/v, acetic acid in hexane.

a - cotton-wool plug

b - 2 g acidic alumina

c - 1 g basic alumina

d - eluting solvent

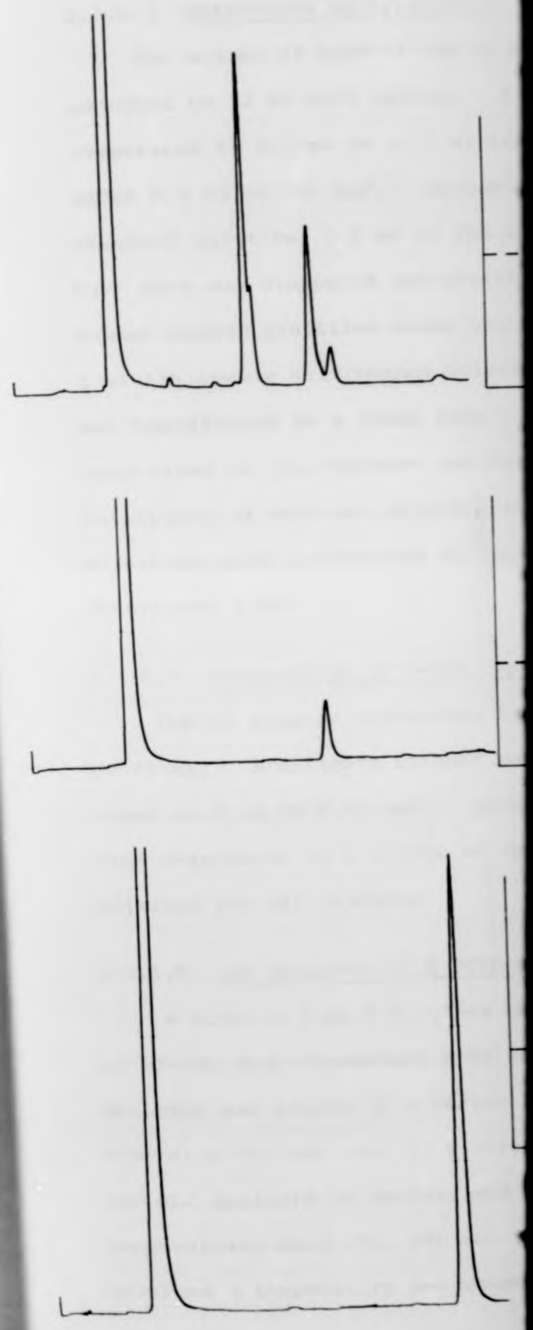


Figure 4

The selective elution of PCSDs and PADs from a mixed alumina column used for "clean up" of fish tissues extracts by the method of Wells and Cowan (1981).

successive eluates from a

th Eulan WA New and PAD standards:

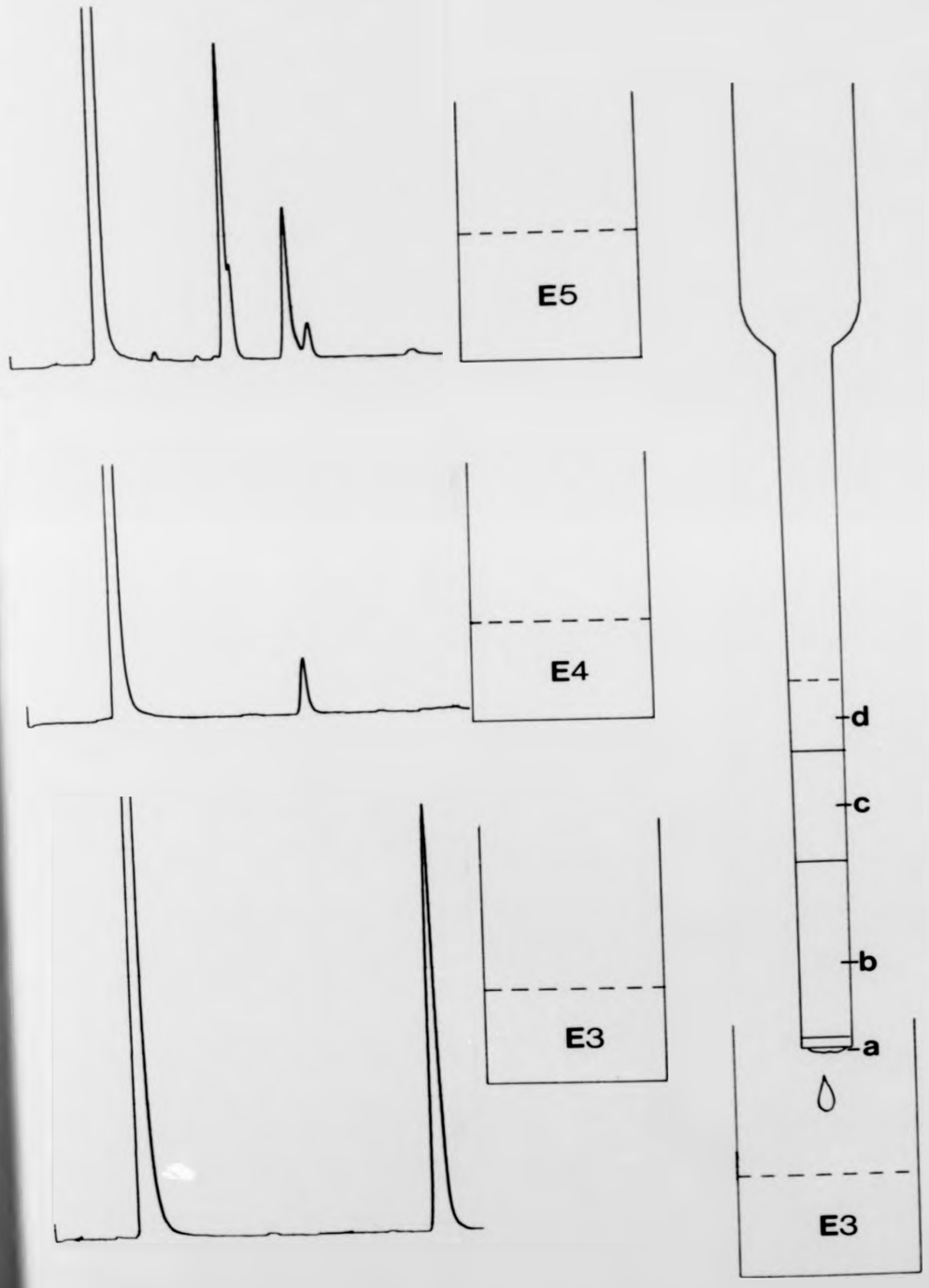
, v/v) - following column

15% v/v, acetic acid in

Os and PADs from a mixed alumina

fish tissues extracts by the

981).



2.3.6.3 Extractive methylation of 6-PCSD prior to GLC analysis

The volume of each of the E3 eluates (containing 6-PCSD) were adjusted to 25 ml with hexane. A suitable aliquot was removed and evaporated to 0.5 ml in a 10 ml tube. To the concentrated sample was added 0.5 ml of 0.1 mg/l decachlorobiphenyl in hexane internal standard solution, 0.5 ml of TMA solution and 100 µl methyl iodide. Each tube was stoppered and shaken mechanically for 30 min. 9 ml of hexane washed distilled water was added to each sample followed by 1 ml 15% acetic acid/hexane solution. After mixing the organic phase was transferred to a clean tube. The organic fractions were each evaporated to just dryness and further reconstituted with 1 ml hexane. An aliquot of each was injected for GLC analysis. PCSD standard solutions were derivatives in parallel to provide GLC standards of methylated PCSD.

2.3.6.4 Preparation of 5-PAD containing eluates for GLC analysis

The E3 eluates containing 5-PAD were each adjusted to a volume of 35 ml. A suitable aliquot was taken and the internal standard added (0.5 ml of 0.02 mg/l decachlorobiphenyl). The samples were each evaporated to a volume of approximately 1 ml. Aliquots were injected for GLC analysis.

2.3.6.5 GLC analysis of 6-PCSD (methylated derivative) and 5-PAD

A 1.8 m × 2 mm i.d. glass column, packed with 1% Dexsil 300 coated on 80-100 mesh Chromosorb W HP and a nickel-63 electron capture detector was placed in a Varian 1400 gas-liquid chromatograph. Oxygen-free nitrogen was used as a carrier gas at a flow rate or 42 ml/min. For GLC analysis of derivatised PCSD, the injector, column and detector temperatures were 250, 240 and 300°C respectively. Analysis of PAD involved a temperature programme with column temperatures increasing

from 170°C to 250°C at 4°C/min. Quantitation of PCSD and PAD components was made using a Vista 401 digital readout system.

2.3.7 Extraction, "clean up" and HPLC analysis of fish tissue extracts

2.3.7.1 MTBE extraction of fish tissues and "clean-up" of extracted samples

Fish tissues were weighed, ground with anhydrous sodium sulphate (≃4 g) to give a free-flowing powder, and then extracted with 3 × 5 ml fractions of MTBE, with a 30 min. period of mechanical mixing after each addition of extraction solvent. The total MTBE extract from each tissue was evaporated to just dryness and the residue redissolved in 100 µl MTBE prior to application to a mixed alumina column for "clean up". [The necessary modification of the published "clean up" procedure (Wells and Cowan, 1981) section 2.3.6.2, to allow HPLC analysis of both 6-PSCD and 5-PAD is described in the results section 2.4.3.]

2.3.7.2 Preparation of 6-PCSD and 5-PAD containing eluates for HPLC analysis

The eluates collected from mixed alumina columns containing 6-PCSD and 5-PAD (section 2.4.3) were taken to just dryness and the residue redissolved in 100 µl DMF. 5 µl or 10 µl aliquots were injected upon the HPLC column for analysis. Quantitative analysis was achieved by comparison with 6-PCSD and 5-PAD standards.

2.3.7.3 Instrumentation for HPLC analysis

A stainless steel analytical column (25 cm × 5 mm internal internal diameter) [Shandon Southern Ltd., Runcorn, U.K.] was packed with hypersil ODS (Shandon Southern Ltd.) under high pressure by a Haskel air amplifier pump (0-6900 psi, Olin Energy Systems Ltd.,

Sutherland U.K.) for the reverse phase mode. A methanol/water mixture (85/15 v/v) was adjusted to a measured pH of 3.6 with orthophosphoric acid (Wells and Johnstone, 1981). This was pumped under pressure through the column by an Altex 110A solvent metering analytical pump (Anachem, Luton, Bedfordshire) with a pulse dampener attachment, at a flow rate of 2 ml min^{-1}. The absorbance of the column eluant was monitored at 230 nm on passage through an analytical flow cell (5 µl volume, 10 mm path length) by a Cecil 272 spectrophotometer. A plan of the components of the HPLC system is given in Figure 5.

Conditions for HPLC analysis:

Flow rate	-	2 ml/min
Solvent	-	methanol/water (85/15, v/v) pH 3.6
Wavelength monitored	-	230 nm
Chart speed	-	2 or 5 mm/min
Fullscale deflection	-	0.2, 0.1 absorbance units at (10 mv or 2.5 mv)

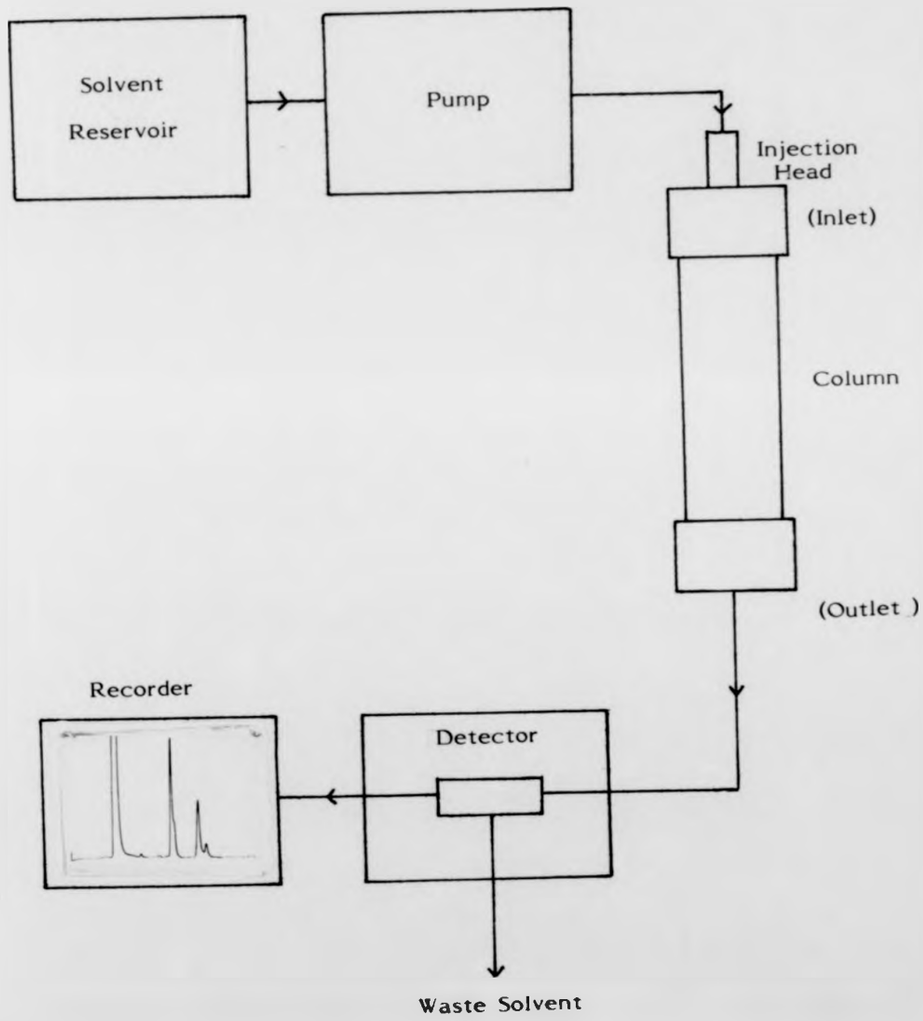


Fig 5. Plan of the Components of the HPLC System

2.4 RESULTS

2.4.1 Purification of 6-PCSD from Eulan WA New and its addition to water for the dosing of fish

HPLC analysis of the pooled 6-PCSD containing eluates collected from the HPLC column outlet following injection of Eulan WA New (section 2.3.1) showed the presence of a single 6-PCSD component (Fig. 6b). A high concentration of the 6-PCSD sample was injected upon the HPLC column to determine the purity. A PAD component was not detectable (Fig. 6d). Consequently the 6-PCSD dosing solution was free of detectable 5-PAD.

A volume of water dosed with 6-PCSD and an equal volume of water taken from a control tank (not dosed with 6-PCSD) was extracted prior to the addition of either goldfish or trout. A single 6-PCSD peak was observed in the HPLC profile of MTBE extracted dosed water and no detectable peaks in the control sample (Fig. 7). The concentration of 6-PCSD in the dosing tank did not vary appreciably when goldfish, trout or pike were dosed with 6-PCSD. The dosing concentration of 6-PCSD was therefore effectively constant over the dosing period.

2.4.2 A comparison of MTBE (cold extraction) and hexane (hot continuous extraction) in the extraction of Eulan WA New and 5-PAD from fish tissues

In the absence of fish tissue both the MTBE and hexane extraction methods showed similar extraction efficiencies for 6-PCSD and 5-PAD, between 90-100% (Table 2). In the presence of fish tissue both methods of extraction (following "clean up" on mixed alumina columns) showed similar extraction efficiencies for 6-PCSD and 5-PAD. 60-70% was extracted from the tissue and 75-88% 5-PAD by both methods (Table 2). The lower extraction efficiencies of 6-PCSD suggest that a non-extractable fraction remains associated with the tissue in both techniques. The variation of extraction efficiencies of 6-PCSD and

HPLC profiles of:

(a) Purified 6-PCSD prepared by the collection of the 6-PCSD component of Eulan WA New from the HPLC column eluate (section

(b) Eulan WA New

(c) a concentrated sample of 6-PCSD

(d) 5-PAD standard

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

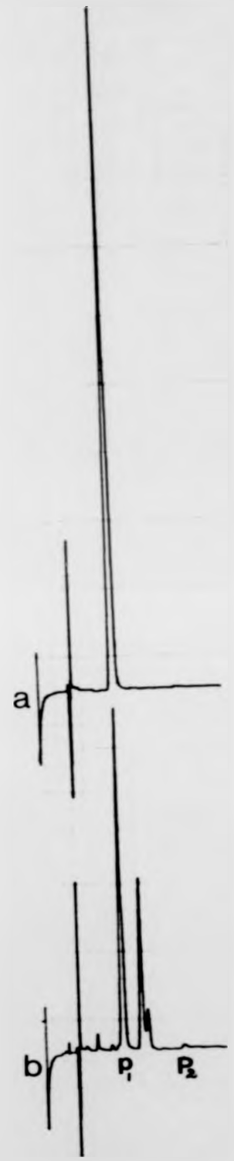


Figure 6

Purified 6-PCSD prepared from Eulan WA New.

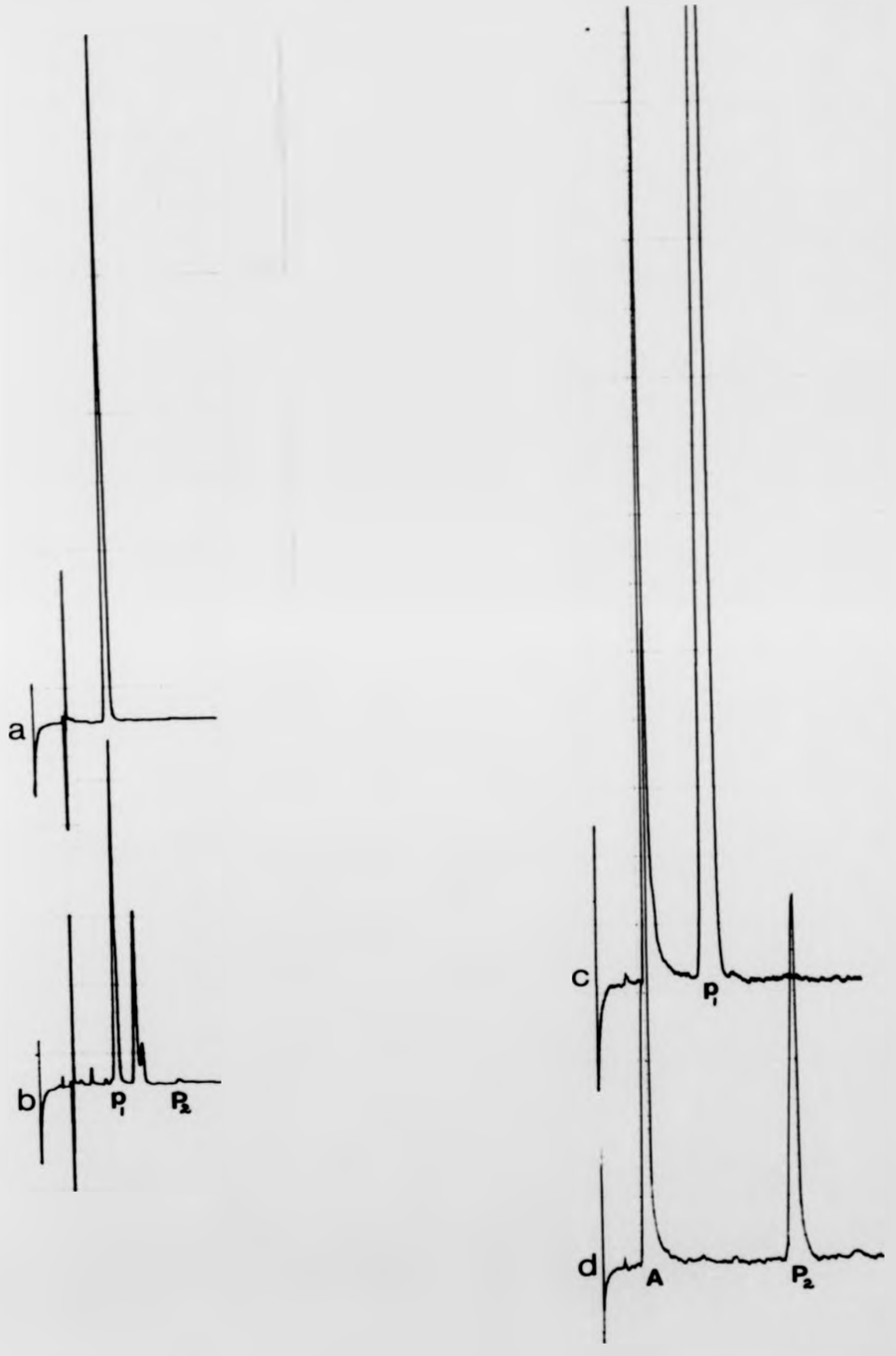
l by the collection of the

an WA New from the HPLC column

f 6-PCSD

olvent

m Eulan WA New.



HPLC profiles of a:

(a) 6-PCSD extracted from water of a tank dosed with 6-PCSD prior to fish dosing studies;

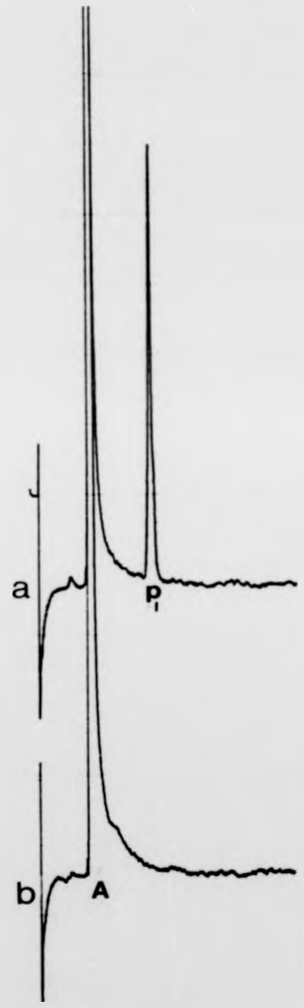
(b) the same volume of water extracted from a control tank, not containing 6-PCSD.

A - DMF—HPLC injection solvent

P_1 - 6-PCSD

Figure 7

The presence of 6-PCSD in the water of tanks used for the dosing of goldfish and rainbow trout.



er of a tank dosed with

ng studies;

extracted from a control

SD.

vent

water of tanks used for the

w trout.

Table 2

The extraction of Eulan WA New and 5-PAD standards spiked with fish tissue.

		MTBE[1]		hexane[2]	
Blank	6-PCSD	91	89	92	92
	5-PAD	98	95	93	94
+ 0.2 g fish liver (after "clean up" on mixed alumina columns)	6-PCSD	60	65	65	68
	5-PAD	80	75	85	80

1 - as described in section 2.3.6.1

2 - as described in section 2.3.5.1

5-PAD between fish tissues, using either of the methods, has not been studied. Also how closely the mixing of tissue homogenates with Eulan WA New and 5-PAD relates to the compartmentation of the xenobiotic in tissues *in vivo*, is unknown. How the extraction efficiencies of 6-PCSD of 5-PAD vary between these conditions cannot be easily evaluated.

The standard soxhlet extraction method was employed for the extraction of fish tissues analysed by GLC. The MTBE extraction method was employed for the extraction of fish tissues analysed by HPLC. The latter method was found to be less time consuming and resulted in the extraction of lower concentrations of tissue coextractants from fish tissues.

2.4.3 Modification of "clean up" procedure for HPLC analysis of tissue extracts

After dosing with 6-PCSD for 2h, the HPLC profile of an MTBE extract of goldfish liver tissue showed a number of components with similar retention times to 6-PCSD (Fig. 8b). These additional components were not completely resolved from 6-PCSD and consequently affect the quantitation of the latter. In order to determine whether those components are products of the metabolism of 6-PCSD or are coextracted with 6-PCSD from tissue samples, a sample of liver from a fish not exposed to 6-PCSD was extracted with MTBE and analysed by HPLC. The resulting profiles showed the presence of components with retention times similar to 6-PCSD (Fig. 8d). Samples of this non-dosed tissue extract mixed with Eulan WA New and 5-PAD standards were subjected to the tissue clean up procedure of Wells and Cowan (1981) (section 2.3.5.2). The tissue coextractants were eluted, together with 6-PCSD, from the mixed alumina column in the final eluate following column acidification (Fig. 9). The replacing of the DEE eluting

HPLC profiles of:

(a) and (c) 6-PCSD standards

(b) a liver extract from a goldfish dosed with 6-PCSD, without prior "clean up" on a mixed alumina column;

(d) a liver extract from a goldfish not dosed with 6-PCSD and not subjected to "clean up" on a mixed alumina column.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

E - tissue coextractants

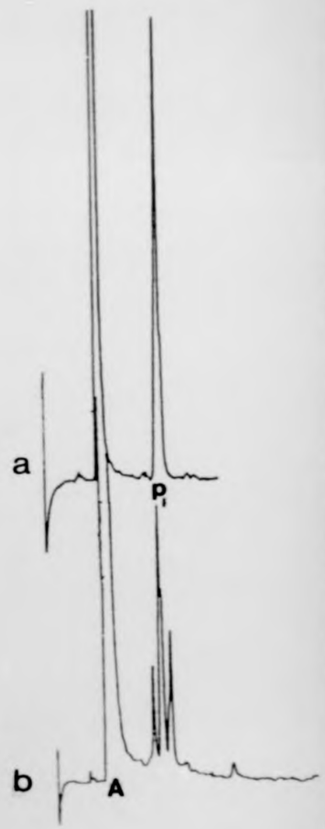


Figure 8

The presence of components absorbing at 230 nm with similar retention times to 6-PCSD, revealed by HPLC analysis of fish tissue extracts.

dosed with 6-PCSD,

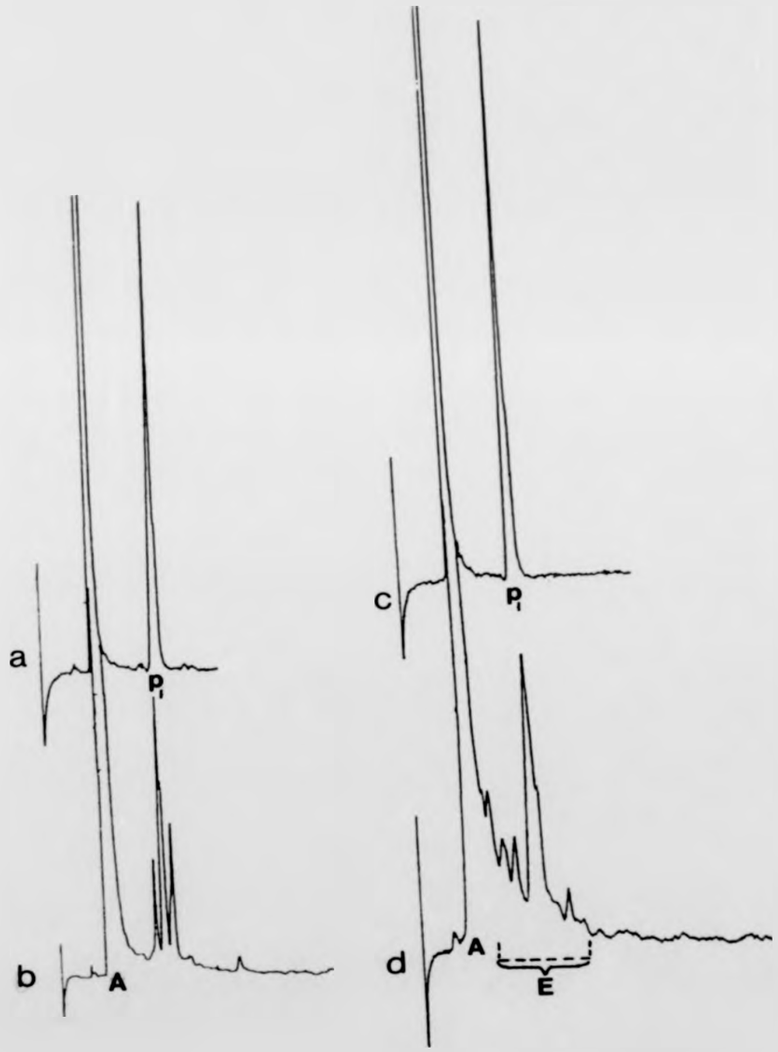
ed alumina column;

ot dosed with 6-PCSD

n a mixed alumina column.

at 230 nm with similar

by HPLC analysis of



HPLC profiles of:

(a) The E3 eluate (hexane) collected from a mixed alumina column loaded with Eulan WA New and PAD (with or without added liver extract).

(b) The E4 eluate (diethyl ether) from a mixed alumina column loaded with samples described in (a).

(c) The E5 eluate (diethyl ether/hexane) collected after acidification of a mixed alumina column loaded with Eulan WA New and PAD standards alone.

(d) as (c) but from a column loaded with Eulan WA New, PAD standards and goldfish liver extract.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

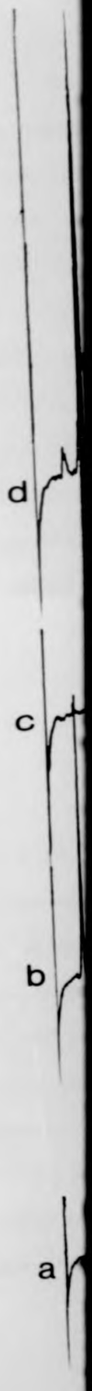
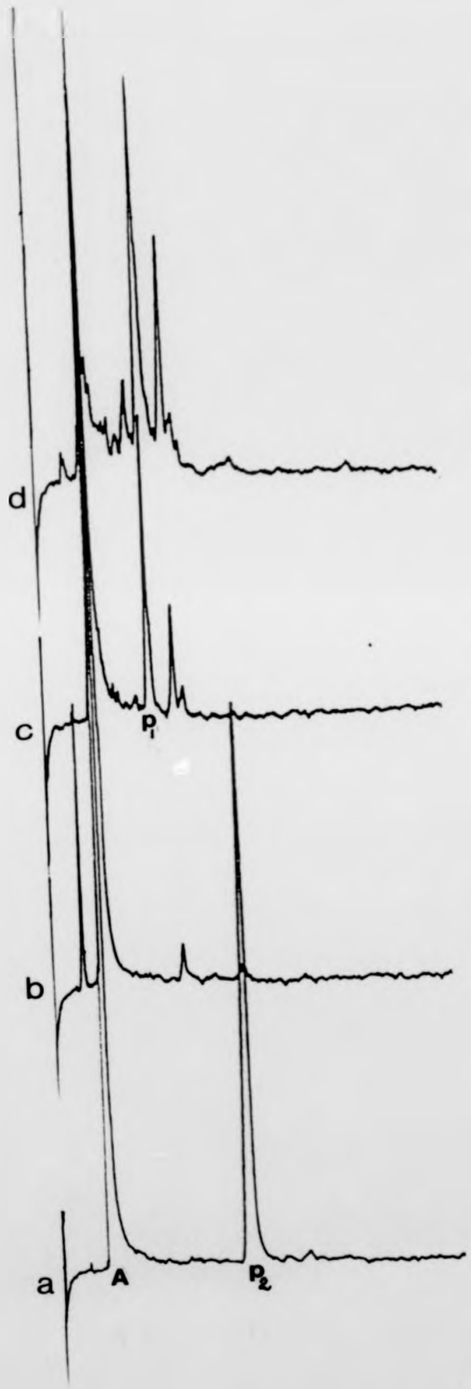


Figure 9

The presence of tissue coextractants together with PCSDs in the HPLC profile of the E5 eluate from a mixed alumina column.



om a mixed alumina
PAD (with or

a mixed alumina
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gether with PCSDs in
a mixed alumina column.

solvent with either MTBE (Fig. 10) or chloroform/methanol (2:1, v/v, Fig. 11) did not result in the separation of the 6-PCSD and tissue coextractants. However selective removal of 6-PCSD from the tissue coextractants was achieved by elution of the mixed alumina column with methanol after the DEE elution step (Fig. 12). The lipid components from fish liver tissue (taken from a pike not exposed to 6-PCSD) were extracted by the method of Folch et al (1957). A sample of the resulting extract was subjected to the modified "clean up" procedure which included the methanol elution step. Components in the tissue extract with similar retention times to 6-PCSD were observed in the final eluate collected from the mixed alumina column (Fig. 13). These components represented approximately 20% of the total tissue extract by weight and were the only components to absorb substantially at 230 nm. The tissue coextractants therefore are lipid components with polar character, shown by their retention upon the mixed alumina column. The diethyl ether eluate from the column showed little absorbance at 230 nm. This eluate has been shown to contain the largest amount of non-polar tissue coextractants (Wells and Cowan, 1981). Subsequently, a single elution step was developed to replace the hexane and DEE successive elution steps, consisting of eluting the column with MTBE. The separation of 5-PAD, 6-PCSD and tissue coextractants was achieved by successive elution of a mixed alumina column with MTBE (25 ml), methanol (25 ml) and MTBE (10 ml) respectively [the latter following column acidification] (Fig. 14). The successful separation of 6-PCSD, 5-PAD and tissue coextractants from tissues of dosed fish, was demonstrated for liver tissue taken from a goldfish dosed with 6-PCSD (Fig. 15); and also separation of 6-PCSD and tissue coextractants from gill tissue of a trout dosed with 6-PCSD (Fig. 16).

HPLC profiles of the following successive eluates collected from a mixed alumina column loaded with a sample of Eulan WA New, 5-PAD and fish liver extract:

(a) Hexane (35 ml)

(b) MTBE (25 ml)

(c) DEE/hexane (30:70, v/v, 25 ml) collected after acidification of the column.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

E - tissue coextractants

Figure 10

HPLC profiles of successive eluates from a mixed alumina column loaded with Eulan WA New, PAD standards and liver extract.

essive eluates collected

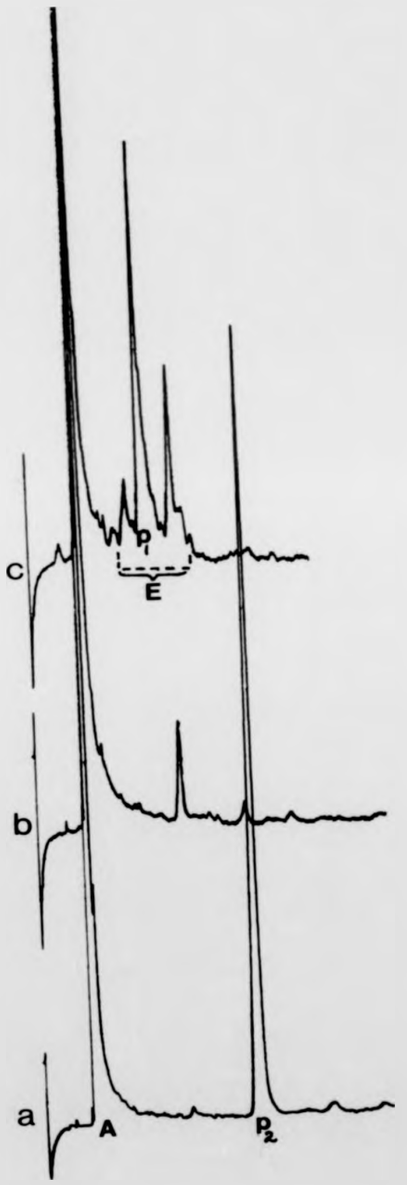
with a sample of

extract:

collected after

from a mixed alumina

D standards and liver



HPLC profiles of the following successive eluates collected from a mixed alumina column loaded with a sample of Eulan WA New, 5-PAD and liver extract:

(a) Hexane (35 ml)

(b) Chloroform/methanol (2:1 v/v, 25 ml)

(c) DEE/hexane (30:70, v/v, 25 ml) collected after acidification of the column.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

E - tissue coextractants

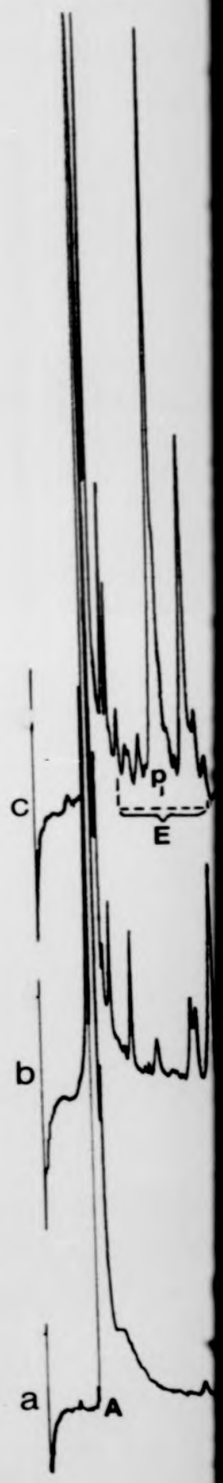


Figure 11

HPLC profiles of successive eluates from a mixed alumina column loaded with Eulan WA New, 5-PAD standards and liver extract.

ssive eluates

n loaded with a

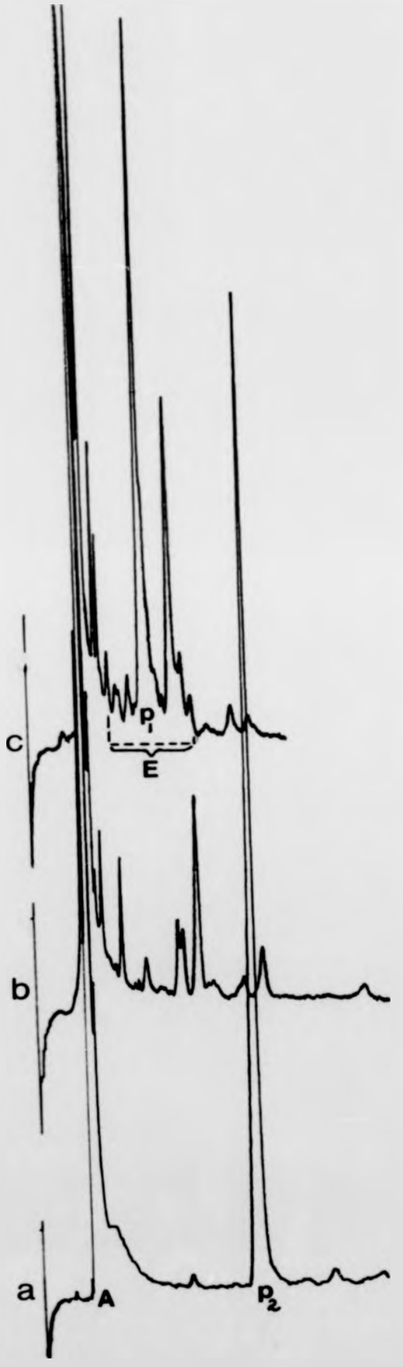
lver extract:

25 ml)

collected after

from a mixed alumina

PAD standards and



HPLC profiles of the following successive eluates collected from a mixed alumina column loaded with a sample of Eulan WA New, 5-PAD standards and liver extract:

(a) Hexane (35 ml)

(b) DEE (25 ml)

(c) Methanol (25 ml)

(d) DEE/hexane (30:70, v/v, 25 ml) after acidification of the column.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

- tissue coextractants

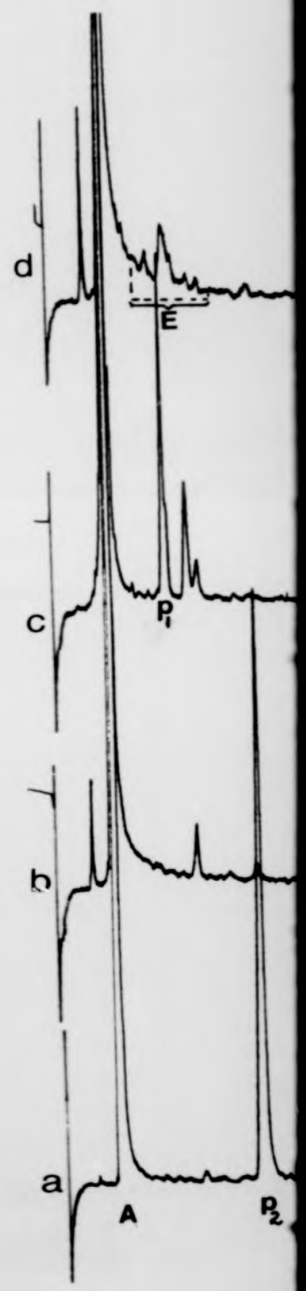


Figure 12

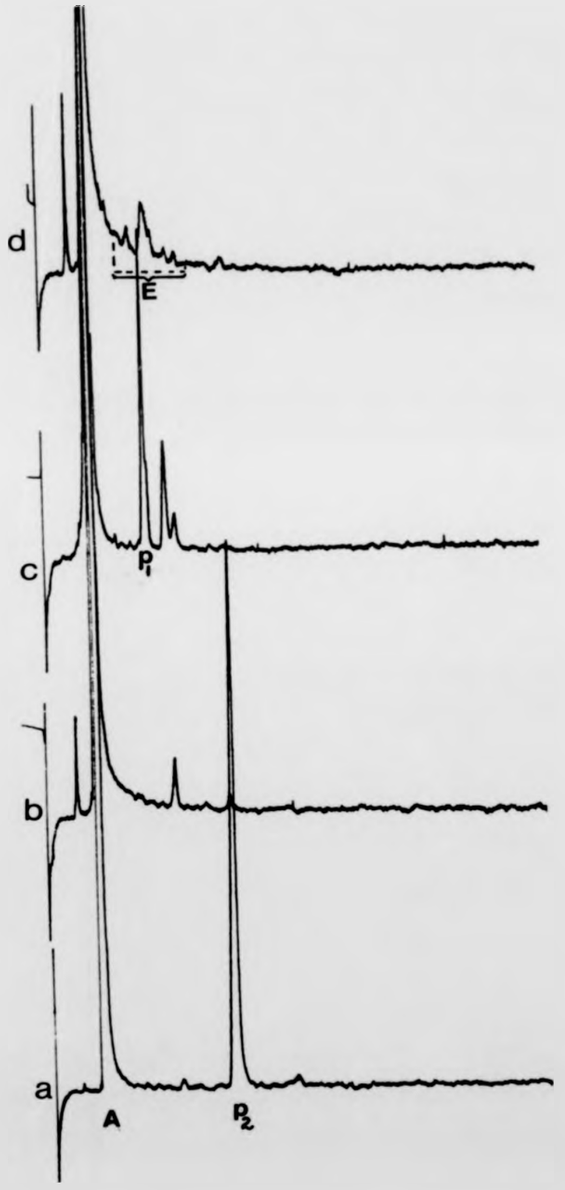
The successful separation of 6-PCSD, 5-PAD and polar tissue coextractants by their successive elution from a mixed alumina column.

ressive eluates collected

with a sample of Eulan WA

ract:

after acidification



3D, 5-PAD and polar tissue

elution from a mixed

HPLC profile of the following successive eluates collected from a mixed alumina column loaded with a sample of liver extract:

(a) Hexane (35 ml)

(b) DEE (25 ml)

(c) Methanol (25 ml)

(d) DEE/hexane (30:70, v/v. 25 ml) after acidification of the column.

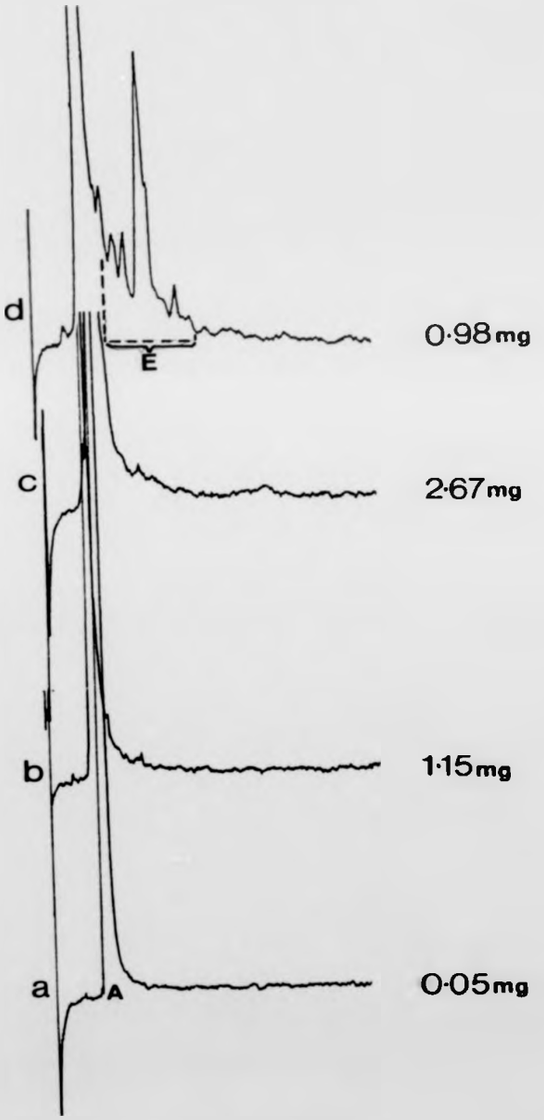
A total of 5 mg of the liver extract obtained by the method of Folch et al (1957) was added to the column and the residues of each eluate weighed prior to HPLC analysis. (The weight of the residues of each eluate are given beside the corresponding HPLC profiles.)

A - DMF, HPLC injection solvent

E - tissue coextractants

Figure 13

Polar lipid coextractants in the final eluate from a mixed alumina column loaded with a sample of fish liver extract.


d
0·98mg
E
c
2·67mg
b
1·15mg
a
A
0·05mg

HPLC profiles of the following successive eluates collected from a mixed alumina column loaded with a sample of Eulan WA New, 5-PAD and liver extract:

(a) MTBE (25 ml)

(b) Methanol (25 ml)

(c) MTBE (10 ml) following acidification of the column

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

E - tissue coextractants

(d) volume of MTBE required to elute 5-PAD

(e) volume of methanol required to elute 6-PCSD

(PCSD components of Eulan WA New)

× - major 6-PCSD and 5-PAD components

• - 7-PCSD

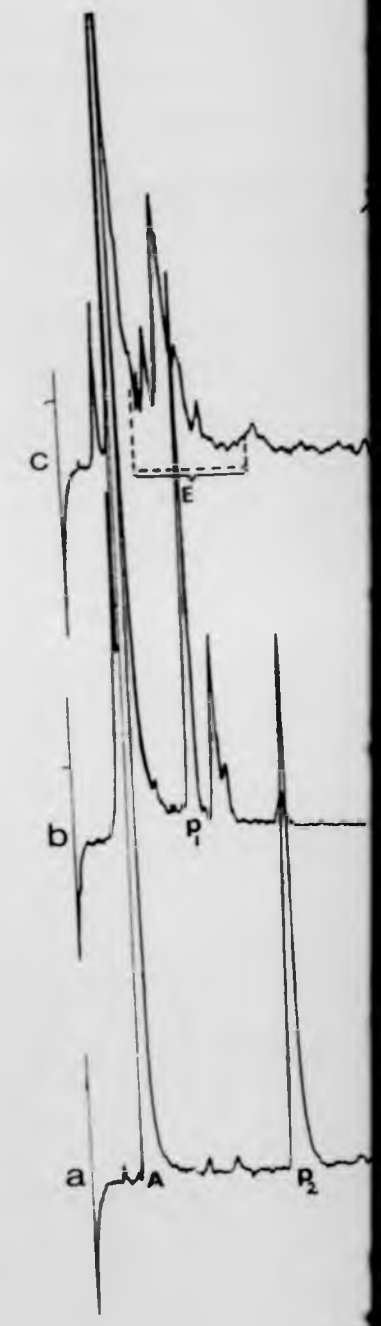


Figure 14

The three step separation of 5-PAD, 6-PCSD and tissue coextractants from a mixed alumina column allowing HPLC quantitative analysis of tissue extracts.

ssive eluates collected from

sample of Eulan WA New,

cation of the column

te 5-PAD

elute 6-PCSD

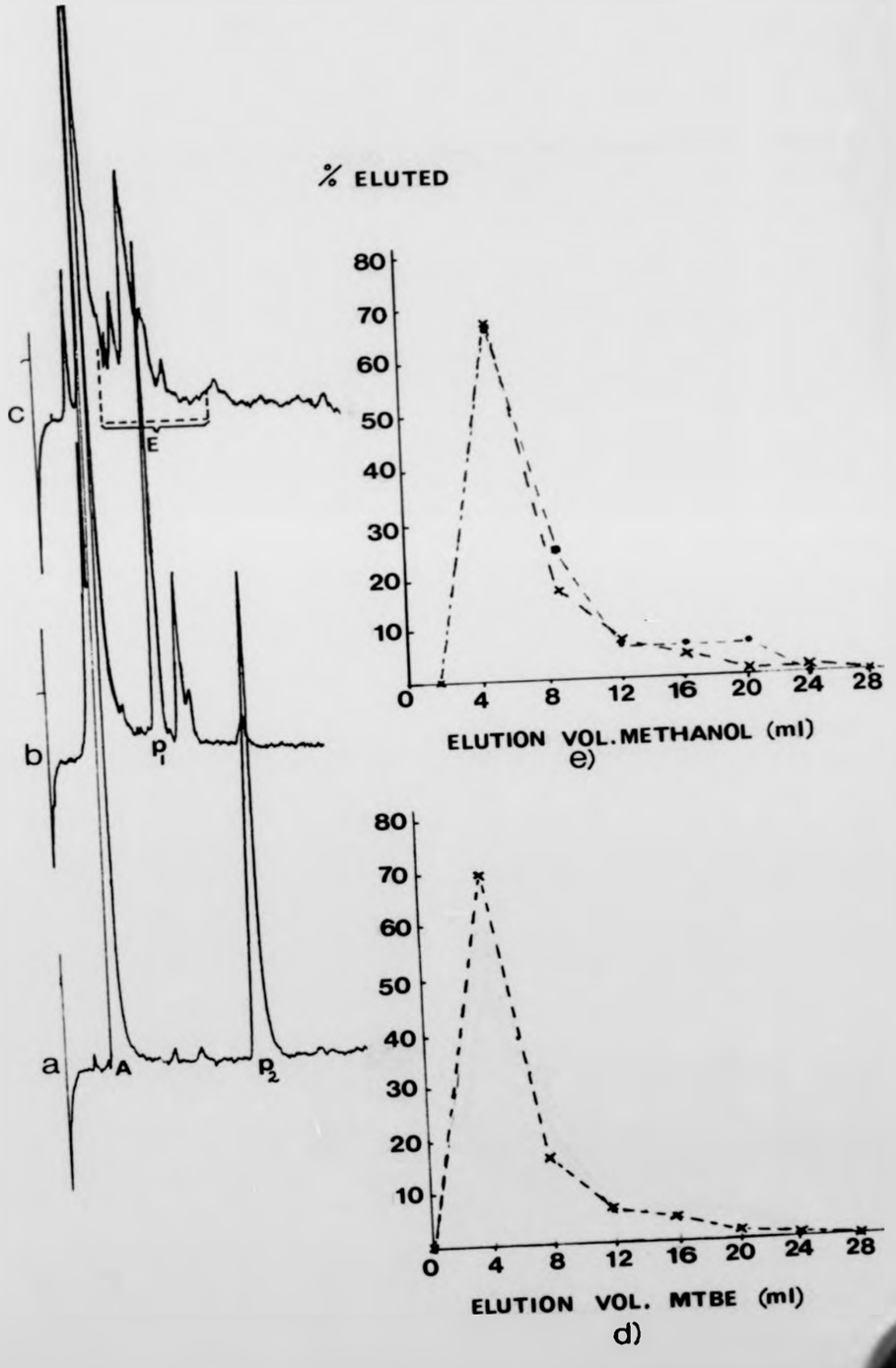
ew)

nents

, 6-PCSD and tissue

column allowing

ue extracts.



HPLC profiles of:

(a) The eluate collected from a mixed alumina column, loaded with a liver extract from a goldfish dosed with 6-PCSD for 2h, obtained by elution with 25 ml MTBE.

(b) The subsequent eluate collected from the column described in (a) by elution with 25 ml methanol.

(f) The next eluate from the column described in (a) and (b) collected after column acidification and elution with 10 ml MTBE.

(c) The eluate collected from a mixed alumina column loaded with a liver extract from a control goldfish (i.e. not dosed with 6-PCSD), obtained by elution with 25 ml MTBE.

(d) The subsequent eluate collected from the column described in (c) by elution with 25 ml methanol.

(e) The next eluate from a column described in (c) and (c) collected after acidification of the column, by elution with 10 ml MTBE.

A - DMF

P_1 - 6-PCSD

P_2 - 5-PAD

E - tissue coextractants

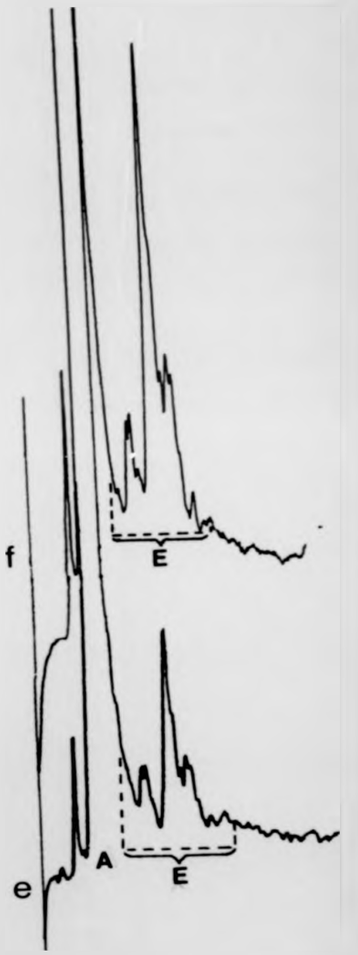


Figure 15

The separation of 6-PCSD, 5-PAD and polar coextractants from goldfish liver tissue extract.

ed alumina column, loaded
dfish dosed with 6-PCSD
h 25 ml MTBE.

from the column described
ethanol.

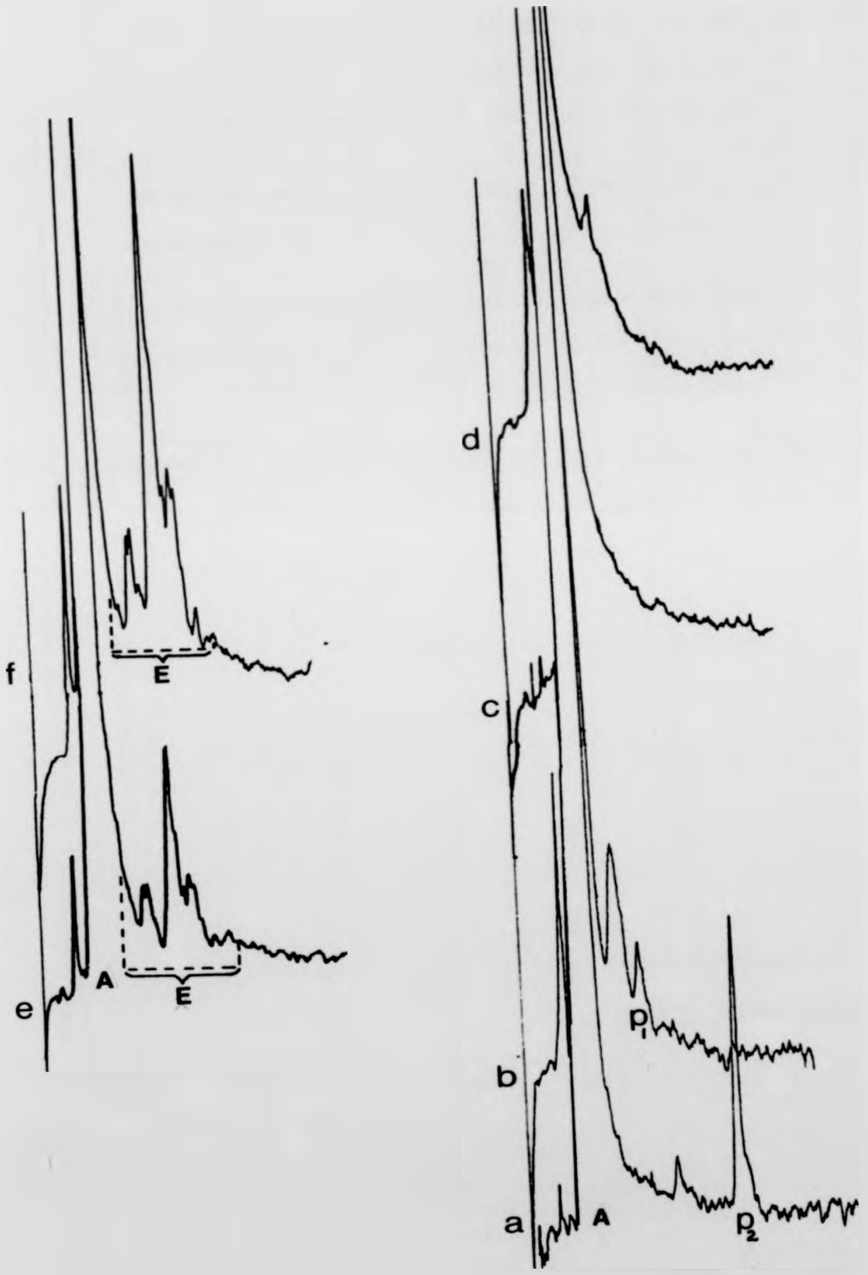
n described in (a) and (b)
cation and elution with

ixed alumina column loaded
ntrol goldfish (i.e. not
by elution with 25 ml MTBE.

ed from the column described
methanol.

described in (c) and (c)
of the column, by

nd polar coextractants from



HPLC profiles of:

(a) The eluate collected from a mixed alumina column loaded with gill extract from a trout dosed with 6-PCSD for 45 min, obtained by elution with 25 ml MTBE.

(b) The subsequent eluate collected from the column described in (a) by elution with 25 ml methanol.

(c) The next eluate from the column described in (a) and (b) after acidification of the column by elution with 10 ml MTBE.

(d), (e) and (f) the eluates as described in (a), (b) and (c) but from a column loaded with gill extract from a control trout (i.e. not dosed with 6-PCSD).

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

E - tissue coextractants

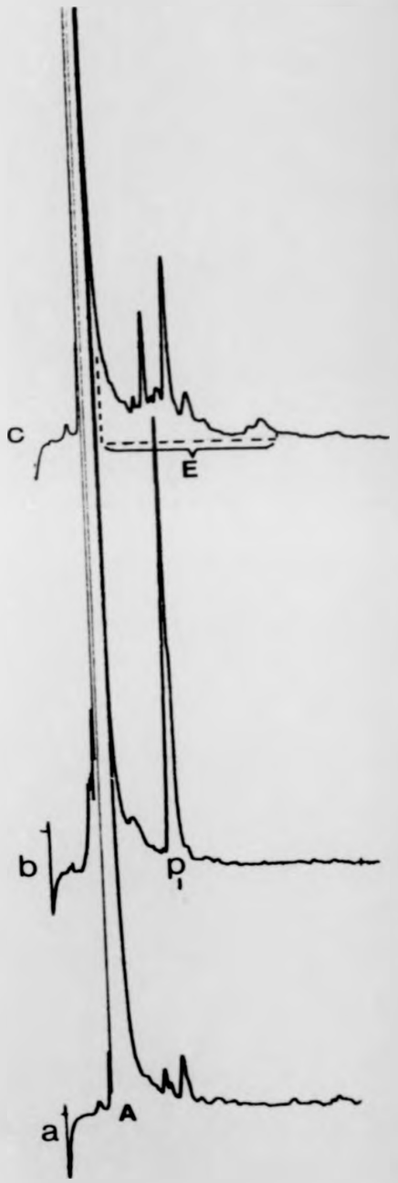


Figure 16

The separation of 6-PCSD from tissue coextractants from trout gill tissue extract.

xed alumina column loaded

dosed with 6-PCSD for

th 25 ml MTBE.

ed from the column

th 25 ml methanol.

n described in (a) and

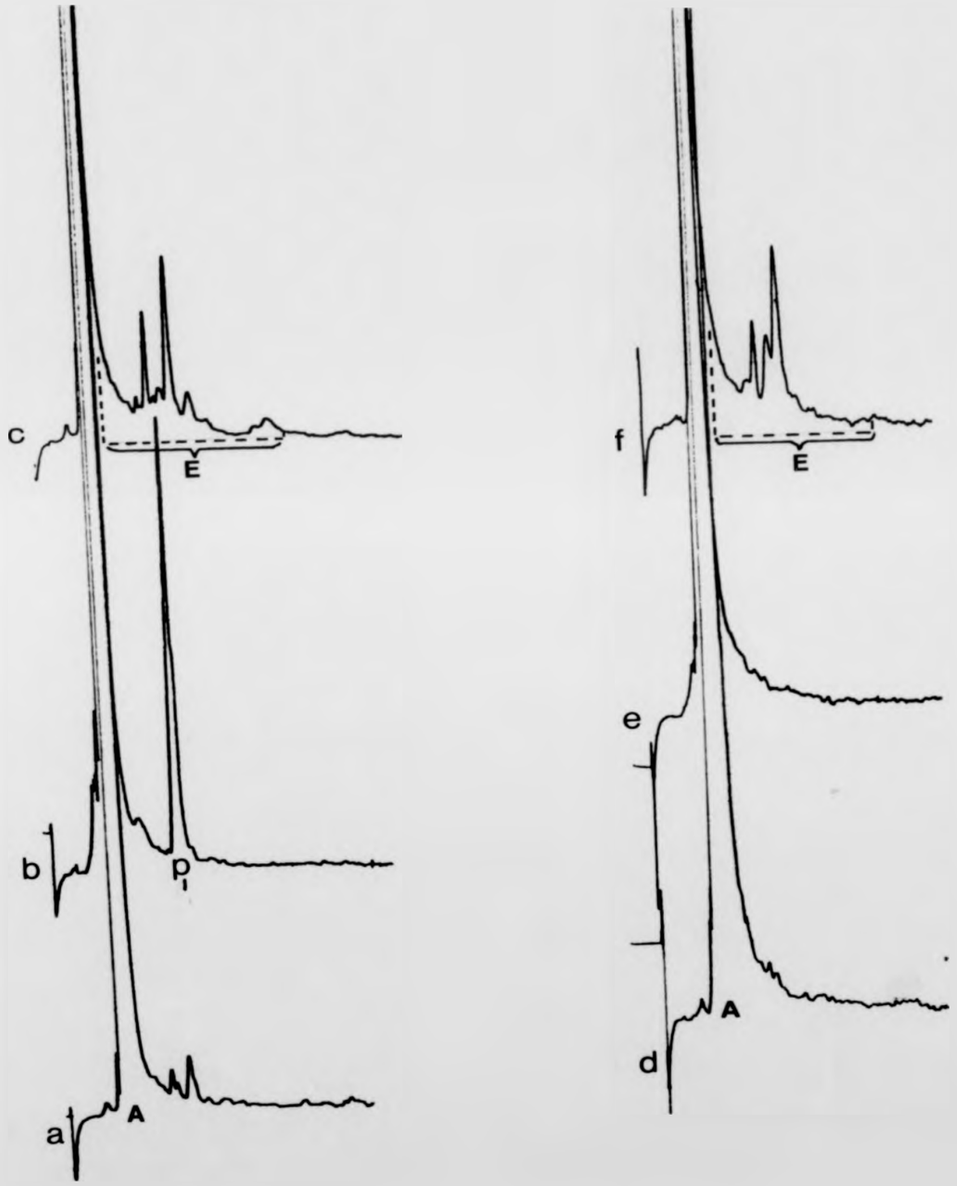
column by elution

escribed in (a), (b) and

with gill extract from

ed with 6-PCSD).

sue coextractants from trout



2.4.4 Dosing pike with Eulan WA New and 6-PCSD in surrounding water

Pike were dosed with Eulan WA New over a period of 240h. The concentrations of 6-PCSD and 5-PAD in fish tissues were determined by GLC analysis of tissue extracts and are given in Table 3. After 4h and 8h of dosing with Eulan WA New, 5-PAD to 6-PCSD ratios were found to be high in most tissues (Table 4), much greater than that observed in the Eulan WA New dosing solution of <0.1. An exception is the bile with a 5-PAD to 6-PCSD ratio of 0.01 after 4h dosing with Eulan WA New. This is a consequence of the accumulation of 6-PCSD in the bile to a very high concentration (66.0 µg/g of tissue). The concentrations of 6-PCSD and 5-PAD in bile are expressed relative to those in the surrounding dosing solution, that is as bioaccumulation factors in Table 5. The concentrations of 6-PCSD and 5-PAD in bile may rise to levels five orders of magnitude greater than that observed in surrounding water. After 4h dosing, 40% of the total amount of 6-PCSD in the fish tissues was found in the bile which represents only 0.15% of the total fish tissue by weight (Table 6).

Another tissue which constitutes only a very small fraction of the total fish tissue by weight and yet accumulates 6-PCSD and 5-PAD to relatively high concentrations is the spleen (Table 3). Processes accounting for these observations are suggested in the discussion of this chapter (section 2.5).

The bioaccumulation factors for 6-PCSD decline during the dosing period whereas that for 5-PAD generally increases (Table 5). This provides evidence for the *in vivo* metabolism of 6-PCSD to 5-PAD. A pointer to the possible sites of 5-PAD formation is to be found from inspection of the data in Table 4. After 4h dosing the liver and the intestine show very high ratios of 5-PAD to 6-PCSD. Indeed the liver

Table 3 Concentrations of 6-PCSD and 5-PAD in Pike tissue after dosing with Eulan WA New from 4 to 240h.

Pike tissues analysed	4 hours		8 hours		14 hours		26 hours		50 hours		240 hours	
	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD
Oesophagus	0.165	0.595	0.679	0.720	0.33	ND	0.982	0.351	0.104	0.590	1.425	1.376
Stomach	0.069	0.345	0.505	0.492	0.166	0.213	0.212	0.486	ND	0.865	0.587	1.948
Intestine	0.046	1.737	0.501	0.178	0.209	0.363	0.499	0.502	0.266	0.892	0.485	0.851
Spleen	1.106	7.044	10.552	11.009	0.817	2.372	5.393	3.660	0.212	1.139	3.633	22.187
Liver	0.168	3.553	0.283	0.909	0.443	3.880	0.197	1.813	0.336	0.425	0.750	5.513
Bile	66.00	0.801	19.847	1.320	0.283	0.511	19.370	0.992	0.064	4.129	1.807	17.634
Kidney	0.335	1.719	1.987	0.326	0.671	ND	1.015	1.522	ND	2.696	2.922	14.450
Gills	ND	0.318	0.981	0.397	0.510	0.484	0.510	0.794	0.777	0.833	ND	2.204
Eye	0.338	0.262	1.566	0.424	0.298	0.082	0.248	0.225	0.481	0.295	0.798	1.740
Brain	2.193	4.010	3.196	2.590	1.057	0.658	1.692	ND	0.077	2.795	3.059	5.623
Muscle	0.143	0.131	0.134	0.084	0.475	ND	LS	LS	0.007	0.504	0.130	0.713
Blood	0.289	0.461	1.34	0.243	1.22	0.263	2.092	0.348	1.312	0.546	2.446	1.190
Skin	0.486	0.267	0.402	0.141	LS	LS	0.312	0.167	ND	0.355	0.644	LS

LS - refers to a lost sample, taken to dryness due to a soxhlet malfunction.

ND - no detection of the molecule in the tissue; if present, its concentration in the tissue will be 0.01 µg.
The data given are tissue concentrations expressed as µg/g tissue.

The data refer to individual fish taken for analysis at each time point; the natural variation between these individual samples probably accounts for the lack of smooth progression in the analytical data.

Table 4

5-PAD:6-PCSD Ratio in Pike Tissues, after exposure to Eulan WA New for 4 and 8 hours.

Pike Tissue Analysed	5-PAD:6-PCSD Ratio in Pike Tissue 4 Hour	8 hour
Oesphagus	3.6	1.10
Stomach	5.0	0.97
Intestine	37.8	0.36
Spleen	6.4	1.04
Liver	21.1	3.21
Bile	0.01	0.07
Kidney	5.13	0.17
Gills	ND	0.40
Eye	0.78	0.27
Brain	1.83	0.81
Muscle	0.92	0.63
Blood	1.60	0.18
Skin	0.55	0.35

N.D. - a calculation of the ratio is not possible, since a 6-PCSD was not detected in this tissue. (If present, there is less than 0.01 µg in this tissue.)

Table 5

Bioaccumulation Factors for 6-PCSD and 5-PAD in Pike Bile exposed to Eulan WA New from 4 to 240 hours.

Dosing time in hours	Bile Bioaccumulation factors 6-PCSD	5-PAD	5-PAD:6-PCSD
4	13,200	1,602	0.1
8	3,969	2,640	0.7
14	57	1,022	18.0
26	3,874	1,984	0.5
50	13	8,258	635.2
240	361	35,268	97.7

Table 6

The amounts of 6-PCSD and 5-PAD in tissues and whole pike, exposed to Eulan WA New for 4 hours.

Pike Tissue Analysed	Weight of Tissue (g)	6-PCSD (µg/tissue)	5-PAD (µg/tissue)	5-PAD:6-PCSD
Oesophagus	0.296	0.049	0.176	3.6
Stomach	0.877	0.060	0.302	5.0
Intestine	0.842	0.039	1.462	37.5
Spleen	0.031	0.031	0.199	6.4
Liver	0.413	0.069	1.468	21.3
Bile	0.162	10.692	0.130	0.01
Kidney	0.238	0.080	0.410	5.1
Gills	0.605	-	0.166	-
Eye	0.934	0.316	0.245	0.8
Brain	0.137	0.300	0.548	1.8
Muscle	98.920	14.140	12.650	0.9
Blood	0.567	0.164	0.261	1.6
Skin	0.984	0.478	0.263	0.6
Total/Fish	105.000	26.418	18.280	0.7

Assumptions made in calculating the total amoung of 6-PCSD and 5-PAD/fish:-

(i) The difference in total fish weight and total samples tissue weight is the weight of muscle tissue, ignoring contribution of skeletal structure.

(ii) The concentration of 6-PCSD and 5-PAD in muscle is that determined in the muscle sampled analysed.

(iii) The tissues sampled are the total amount of those tissues in the fish.

is exceptional in showing a generally high ratio of 5-PAD to 6-PCSD concentrations during the dosing period (data from Table 3), highlighted by comparison with the blood (Fig. 16). To identify more clearly a primary site of 5-PAD formation and to eliminate the possibility of selective accumulation of 5-PAD from Eulan WA New, a second experiment involving short periods of dosing with purified 6-PCSD was performed (section 2.3.2). Of the tissues analysed, - gill, blood, bile and liver - only the liver showed an increase in 5-PAD concentration with exposure time, illustrated by comparison with the blood (Fig. 17).

The absence of 5-PAD from digestive tissue and spleen was shown in a separate experiment in which a single pike was dosed for 20 min. with 6-PCSD (6.6 mg/l), Table 7. These data from Table 7 and from the dosing experiments with purified 6-PCSD strongly suggest that the liver is a primary site of 5-PAD formation in the pike.

2.4.5 Dietary accumulation of Eulan WA New by the pike

The concentrations of 6-PCSD and 5-PAD in pike tissues, as a result of the ingestion of salmon fry dosed with Eulan WA New, are shown in Table 8. These data show particularly high concentrations of 6-PCSD and 5-PAD in the bile and spleen. The spleen constitutes only 0.02% of the total weight of fish tissue and yet accumulate 3% of the total amount of 6-PCSD and 23% of the total amount of 5-PAD present in the tissues of a pike after the ingestion of three dosed salmon fry during a 7h period. The bile taken from a pike orally dosed with salmon fry for 22lh showed particularly high concentrations of 6-PCSD and 5-PAD (empty bile sacs in the earlier pike account for the absence of analytical data). This tissue constituted only 0.03% of the total weight of the fish and contained 36% of the total 5-PAD and 9% of the

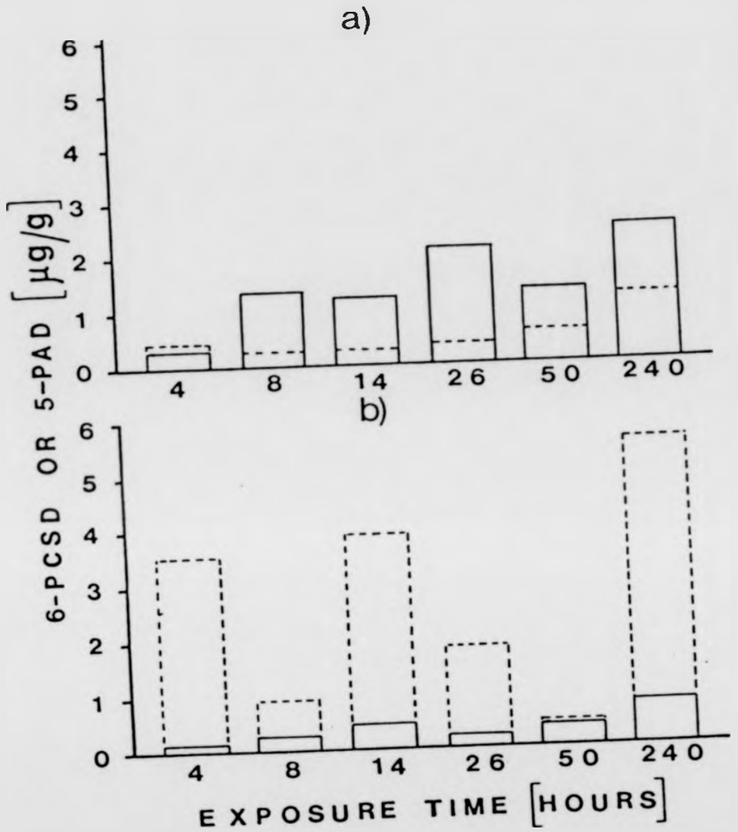


Figure 16

A comparison of the concentration of 6-PCSD (———) and 5-PAD (----) in pike blood (a) and liver (b), over a period of 10 day exposure to Eulan WA New.

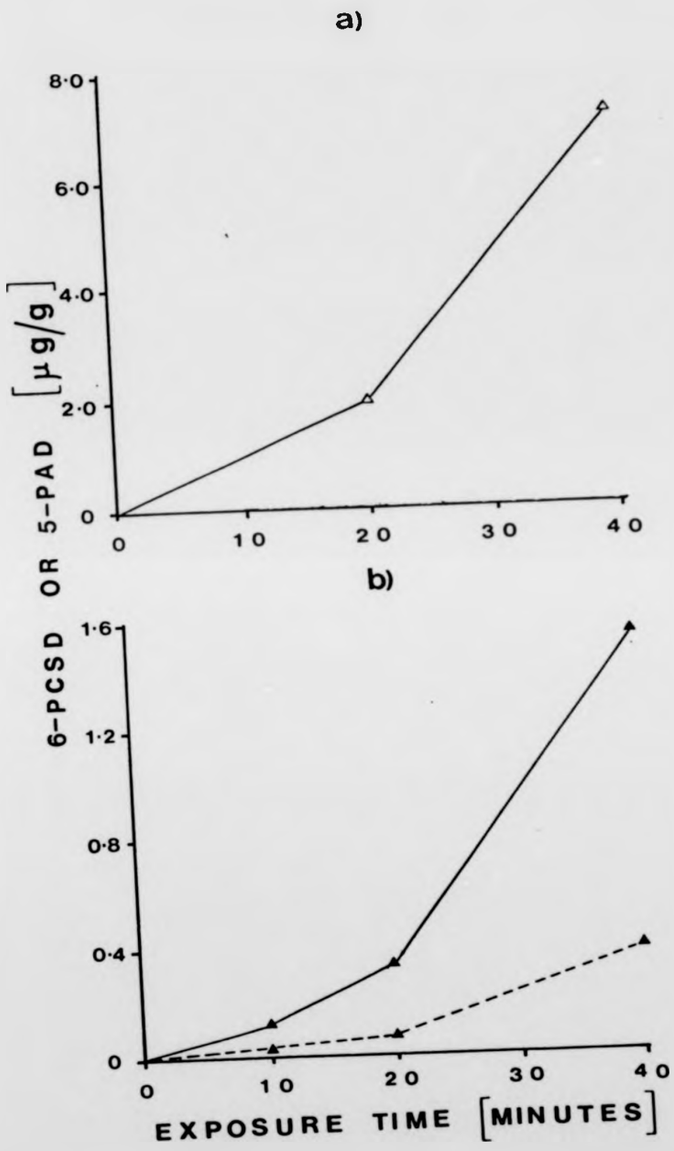


Figure 17

A comparison of the concentrations of 6-PCSD (———) and 5-PAD (- - - - -) in pike blood (a) and liver (b) over a period of 40 mins. exposure to purified 6-PCSD.

Table 7

HPLC Determination of Tissue Concentrations in a Pike dosed with purified PCSD for 20 minutes.

Pike Tissue Analysed	6-PCSD	5-PAD µg/g tissue
Intestine	8.7	ND
Kidney	87.5	ND
Liver	59.5	3.41
Gills	139.4	ND
Blood	52.7	ND
Stomach	2.0	ND
Bile	2.9	2.36
Muscle/skin	2.3	ND
Spleen	ND	ND

For HPLC analysis of tissue concentrations of 6-PCSD and 5-PAD, a pike was dosed for 20 mins. with 6.6 mg/l pure 6-PCSD.

N.D. - the amount of 6-PCSD and 5-PAD if so present in these tissues is less than 0.05 µg/tissue.

Table 8 Concentration (µg/g) of 6-PCSD and 5-PAD in Pike Tissues after Oral Administration of Eulan WA New Bound to a Diet of Salmon Fry.

Exposure time hours	7		24		77		221	
No. Fry Fed	3		3		7		15	
	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD
Muscle	0.010	0.052	0.047	0.030	0.154	0.249	0.068	0.047
Liver	0.084	0.048	0.099	0.479	0.054	1.460	0.210	1.709
Kidney	0.120	0.691	0.130	0.712	0.307	23.670	0.802	2.651
Spleen	6.57	234.500	-	-	-	-	23.209	12.043
Bile	-	-	-	-	-	-	20.916	218.200
Eye	0.052	0.049	0.183	0.116	0.041	0.040	0.074	0.181
Gill	0.121	0.410	LS	LS	0.076	0.389	0.166	0.483
Blood	0.215	0.243	0.861	0.062	0.196	0.079	0.278	0.799
Oesophagus	0.286	0.547	0.489	0.346	-	-	1.947	0.518
Intestine	0.083	0.178	0.398	0.270	0.135	0.488	0.635	0.578
Intestine fat	0.166	1.133	0.518	1.942	0.055	1.001	1.543	3.393
Intestine content	-	-	4.814	0.211	1.579	1.100	-	-
Intestine wash	0.382	0.200	-	-	-	-	-	-
Stomach wall	0.303	14.069	0.646	0.133	0.189	0.115	0.715	0.866
Stomach wash	0.800	0.800	1.348	0.028	0.100	8.275	1.700	1.200
Stomach content	0.850	0.034	-	-	-	-	0.807	0.245
Stomach fat	0.297	0.769	0.571	3.158	0.324	2.754	1.182	3.198
Skin	0.071	0.082	LS	LS	0.057	0.308	0.893	0.174
Brain	0.285	0.760	-	-	-	-	0.411	2.339

- not sampled
LS lost sample, i.e. after extraction (consequence of soxhlet malfunction).

total amount of 6-PCSD in the fish. It is also of interest that adipose tissue of the above pike accumulated both 5-PAD and 6-PCSD to high concentrations with 8 and 19% of the total amount of 5-PAD and 6-PCSD respectively associated with adipose tissue taken from around the digestive tract.

A study of 5-PAD to 6-PCSD ratio in pike tissues is a major pointer to those tissues concerned with the formation of 5-PAD *in situ*. In this study the 5-PAD:6-PCSD ratio in Eulan WA New was 0.015, whereas the overall calculated ratio in pike dosed orally for 7h (ingested 3 dosed salmon fry) was 11.2, providing evidence for the *in vivo* formation of 5-PAD from 6-PCSD in the pike (the assumptions made in calculating the total amount of 6-PCSD and 5-PAD per fish are given in Table 6).

Table 9

5-PAD:6-PCSD Ratio in Tissues of a Pike, 7 hours after Oral Administration.

	5-PAD:6-PCSD
Stomach wall	46.4
Stomach wash	1.0
Stomach content	0.04
Stomach fat	2.59
Intestine	2.14
Intestine wash	0.79
Intestine fat	6.83
Salmon fry	0.02
Eulan WA New	0.015

Table 9 shows the ratios of 5-PAD to 6-PCSD in the digestive tract tissue of a pike 7h after oral dosing with Eulan WA New. The stomach/intestinal wash refers to the distilled water rinse which may remove any Eulan WA New associated with mucus and/or the epithelial cells themselves. These data indicate that the stomach is a probable primary site of 5-PAD formation, by either intracellular metabolism of 6-PCSD within the stomach wall, or, alternatively, through absorption of 5-PAD formed by an extracellular mechanism.

2.4.6 Dosing of goldfish and trout with 6-PCSD in surrounding water

The following results refer to the data of Table 10.

The tissues of the goldfish in which 6-PCSD initially accumulated were the gills and skin/muscle, representing the major routes of uptake of 6-PCSD from the surrounding water. The liver (of those tissues analysed) was the first tissue in which 5-PAD was identified after a 10 min. dosing period with 6-PCSD. A trend of increasing 5-PAD concentration in this tissue was observed for up to 2h dosing with 6-PCSD. After the shortest dosing times, the concentration of 6-PCSD in the goldfish liver was below the detection limit for GLC analysis (approximately 0.01 µg/g tissue), suggesting a high turnover rate to 5-PAD during its *in vivo* metabolism. 6-PCSD and 5-PAD were identified in HPLC profiles of goldfish tissues extracts shown in Figure 18 and Figure 19 respectively.

Gill and liver tissue of rainbow trout showed high concentrations of 6-PCSD after a dosing period of 10 min. with 6-PCSD in surrounding water (Table 11).

Table 10 Concentration (µg/g tissue) of 6-PCSD and 5-PAD in Goldfish Tissues after dosing with 6-PCSD for varying periods.

Tissue	10 min		30 min		2h		6.5h	
	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD
Liver/bile	ND	0.50	ND	2.31	0.59	26.92	49.20	13.92
Digestive tract	ND	ND	ND	0.16	12.17	3.73	44.50	6.64
Skin/Muscle	0.22	ND	0.48	0.06	3.44	1.63	11.13	3.12
Gills	2.18	ND	9.85	ND	19.40	0.30	32.78	3.04

ND - no detection of the compound in the tissue (i.e. a level below 0.01 µg.g tissue, the minimum detectable concentration).

The analysis of 6-PCSD and 5-PAD in tissues of goldfish dosed for 10 min and 30 min were made by GLC (section 2.3.5) and of those dosed for 2h and 6.5h by HPLC (section 2.3.6). The more sensitive GLC method was required to detect the low levels of these compounds present after short periods of dosing.

HPLC profiles of:

(a) 6-PCSD standard

(b) The eluate collected by elution of a mixed alumina column, loaded with liver extract from a goldfish dosed with 6-PCSD for 2h, with 25 ml methanol.

(c) As in (b) but from a column loaded with digestive tissue extract.

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

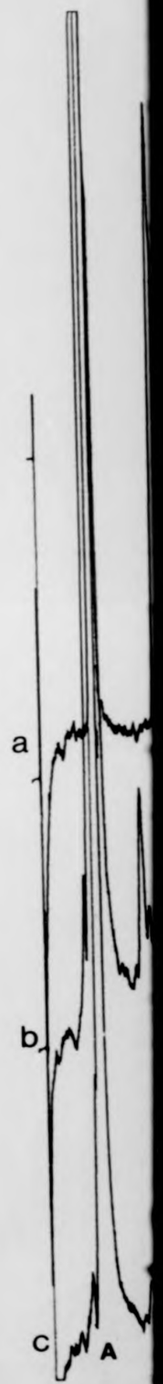


Figure 18

The identification of 6-PCSD in tissues of goldfish dosed with 6-PCSD.

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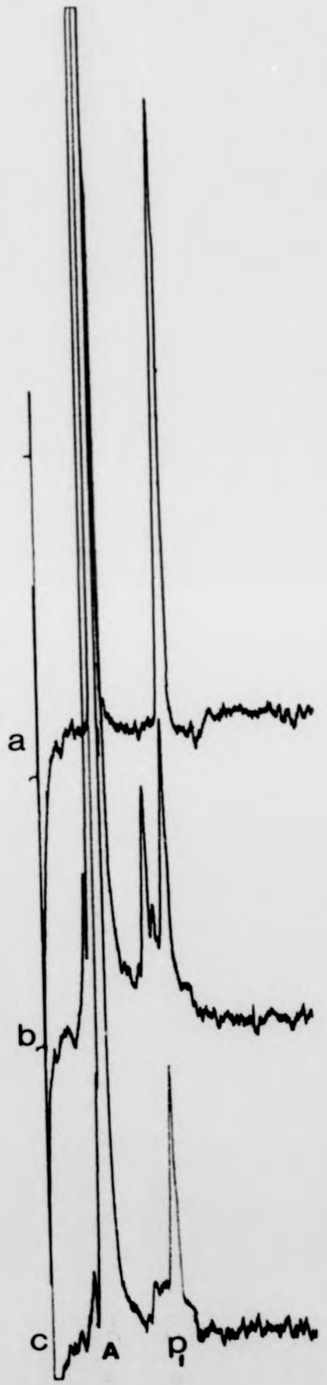

HPLC profiles of:

(a) 5-PAD standard

(b) The eluate collected by elution of a mixed alumina column loaded with liver extract from a goldfish dosed with 6-PCSD for 2h, with 25 ml MTBE.

(c) A blank in which anhydrous sodium sulphate was extracted with MTBE and subjected to column "clean up" on a mixed alumina column. The profile shown refers to the eluate collected by elution with 25 mls MTBE.

A - DMF, HPLC injection solvent

P_2 - 5-PAD

Figure 19

The identification of 5-PAD in the liver of goldfish dosed with 6-PCSD

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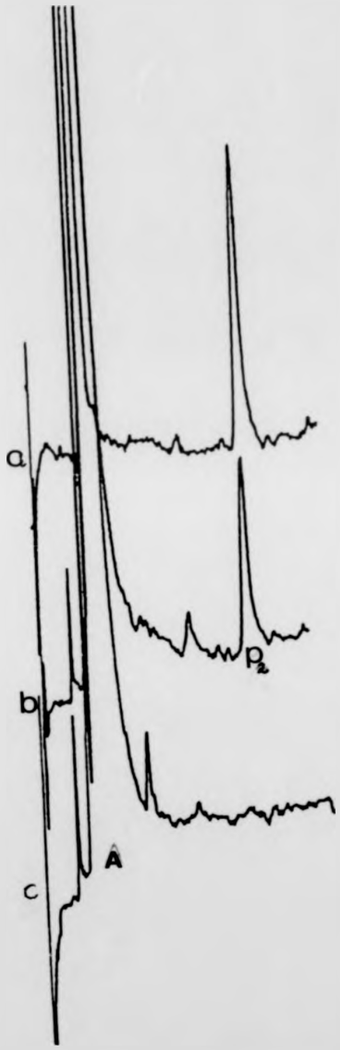


Table 11

HPLC quantitative analysis of 6-PCSD and 5-PAD in tissues of rainbow trout dosed with 6-PCSD.

	Gill		Liver/bile		Skin/muscle		Digest	
Period of dosing 6-PCSD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD	6-PCSD	5-PAD
10 min.	28.6	ND	14.5	ND	3.2	ND	2.9	ND
45 min.	46.0	ND	28.2	ND	8.5	ND	6.2	ND

ND - The amount of 6-PCSD and 5-PAD if present in these tissues is less than 0.05
[values µg/g tissue]

The bioaccumulation factors (which describe the amount of 6-PCSD in tissues relative to that of the surrounding water) for gill and liver/bile, were 36 and 18 respectively, after a 10 min. dosing period. Trout dosed with 6-PCSD died after 35-45 min. exposure. No 5-PAD was identified in any of the tissues sampled from the dosed trout. An HPLC profile of liver tissue extract taken from a trout dosed with 6-PCSD for 45 min. is shown in Figure 20.

2.5 DISCUSSION

The hydrophobic nature of 6-PCSD and its hydrolysis product 5-PAD is shown by their high n-octanol/water partition coefficients (P) of 44,000 and 639,000 respectively (Wells and Cowan, 1983). Those values are similar in magnitude to those of naphthalene (log P, 4.77) and p,p'DDT (log P,5.75) [from Veith et al, 1979] for example. The accumulation of xenobiotics in the tissues of small fish to steady-state concentrations, relative to their concentration in surrounding water, can be predicted from the xenobiotics partition coefficient; since log P and log BCF (Bioconcentration factor - the concentration of the component accumulated from surrounding water in fish tissues relative to the concentration in surrounding water) are linearly related under the conditions reported by Veith et al (1979).

HPLC profiles of the following successive eluates collected from a mixed alumina column loaded with trout liver extract from a trout dosed for 45 min. with 6-PCSD:

(a) MTBE (25 ml)

(b) Methanol (25 ml)

A - DMF, HPLC injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

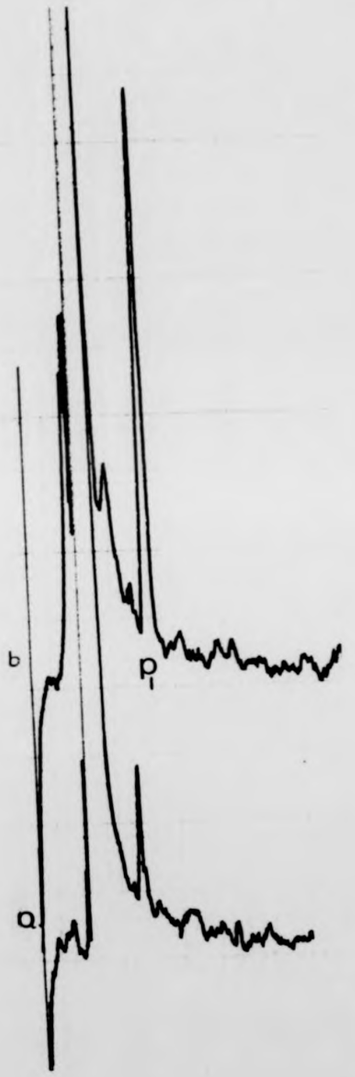
Figure 20

The absence of 5-PAD in trout liver taken from a trout dosed with 6-PCSD for 45 min.

ccessive eluates collected

d with trout liver extract

th 6-PCSD:



iver taken from a trout

Consequently the lipophilic character of 6-PCSD accounts for its bioconcentration in fish tissues to relatively high concentrations. For example, after 10 min. dosing with 6-PCSD (0.5 ppm) the concentration of this xenobiotic in trout gill tissue was thirty-six times that of the surrounding water. The gill tissue of fish provides a major site for the uptake of xenobiotics from the surrounding aqueous environment as demonstrated for DDT (Premdas and Anderson, 1963), dieldrin and lindane (Gackstatter, 1966), petroleum hydrocarbons (Lee et al, 1972) and the butyl ester of 2,4-D (Rogers and Stalling, 1972). The major factor accounting for the gill as a primary route for the uptake of xenobiotics is that this tissue presents such a large surface area (0.75 - 48 times as great as the rest of the body surface) for the diffusion of lipid soluble substances (Gray 1954). Such membrane solubility shown by xenobiotics may result in their interaction with functional proteins of the membrane environment, and may also affect the integrity of the phospholipid structures. The toxicity of DDT has been observed to correlate with the degree of perturbation of membrane phospholipid, measured by differential scanning calorimetry of dipalmitoyl phosphatidylcholine liposomes (Packham et al, 1981). From short term dosing studies involving pike (section 2.4.4), goldfish and rainbow trout (section 2.4.6) the gill and skin/muscle samples analysed represented the primary sites of 6-PCSD accumulation. Studies involving the uptake of the xenobiotic sodium dodecyl sulphate from surrounding water by the goldfish (Tovell et al, 1975) identified gill and the skin as major routes of uptake of this xenobiotic, accounting for 70% and 20% of the body burden of the compound respectively. In addition the inherent "drinking mechanism" of fish accounted for the uptake of approximately 10% of the xenobiotic. The swallowing of water containing

xenobiotic may therefore provide an additional route by which the xenobiotics are transported to digestive tissues, other than through dietary intake or sequestering of xenobiotics from circulation by this tissue. In this work an independent assessment of the contribution of each of these possible xenobiotic uptake routes has not been made, however, as stated previously in this section, the gills and skin/muscle represent the major routes of uptake for 6-PCSD from surrounding water in pike, goldfish and trout.

Pike absorbed 6-PCSD from the digestive tract leading to its appearance in other tissues of the fish, after swallowing salmon fry dosed with Eulan WA New (section 2.4.5). The significance of this route in the accumulation of xenobiotics through biomagnification [the process by which tissue concentrations of bioaccumulated chemical residues increase as the material passes up the food chain through two or more trophic levels (Brungs and Mount, 1978)] has resulted in some controversy. Macek et al (1979) report that a critical evaluation of the relative significance of the food chain in residue accumulation of di(2-ethylhexyl)phthalate, trichlorobenzene, leptophos, cadmium, endrin and kepone indicate that the potential for movement through food chains is relatively insignificant compared with the body burden resulting from the accumulation of these xenobiotics from surrounding water. However such a generalisation cannot be extended to all xenobiotics since Weininger (1978) has concluded that the accumulation of PCB s in Lake Michigan trout from surrounding water constitutes 3.1% of the total residues of the PCBs, the remainder arises from the sediment based food chain.

The analysis of pike tissues following dosing with Eulan WA New and 6-PCSD in surrounding water and also with Eulan WA New in the

diet, highlighted the heterogenous distribution of the xenobiotics in pike tissues. One of the smallest tissues of the pike is the bile which was found to constitute only 0.15% of the total tissue of a pike by weight. However 40% of the total body burden of 6-PCSD was found compartmented in the bile after 4h dosing with Eulan WA New in surrounding water. After longer periods of dosing, the analysis of bile from a pike fed fish (contaminated with Eulan WA New) over a period of 221h, showed the bile to contain 36% and 9% of the total body burden of 5-PAD (the hydrolysis product of 6-PCSD) and 6-PCSD respectively. Therefore both 6-PCSD and 5-PAD accumulate to high concentrations in bile. The features of xenobiotics which account for extensive biliary accumulation have been studied in mammals. Factors of importance include molecular weight, lipid solubility, molecular structure and biotransformation of the xenobiotic (Millburn, 1976). Two major factors which determine extensive biliary accumulation are a relatively high molecular weight and polarity. These structural properties may arise through biomodification of the xenobiotic involving conjugation mechanisms, including glucuronidation (introduces 176 molecular weight units) and glutathione conjugation (introduces 300 molecular weight units). A minimum threshold molecular weight for extensive biliary accumulation has been observed in organisms which are species dependent. For organic anions this value is 325 $\pm$ 50 for the rat, 400 $\pm$ 50 for the rabbit (Hirom et al, 1972a). Xenobiotics with molecular weights below the threshold value tend to be excreted predominantly in the urine. Similar relationships between physiochemical structure of a xenobiotic and biliary accumulation to those observed in mammals have been reported in fish (Adamson and Guarino, 1972). In addition a number of xenobiotics and/or xenobiotic metabolites have been observed to

accumulate to high concentrations in fish bile (Table 12). The majority of

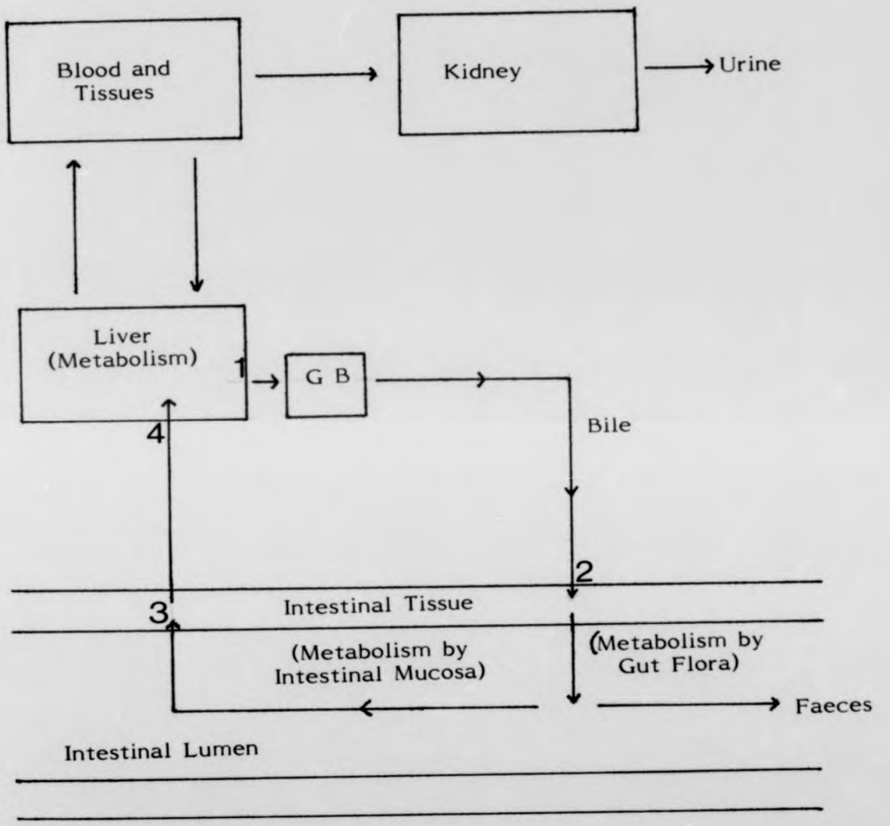
Table 12

The accumulation of xenobiotics and/or their metabolites in fish bile.

Xenobiotic accumulated in fish bile (parent compound and/or metabolite)	Reference
phenol red	Adamson and Guarino (1972)
DDT	Lech et al (1973)
sulphobromophthalein	Schmidt and Weber (1973)
TFM (3-trifluromethyl-4-nitrophenol)	Lech (1973)
Di-2-ethylhexylphthalate	Melancon and Lech (1976a)
TCB (tetrachlorobiphenyl)	Guiney et al (1977)
Naphthalene/ methylnaphthalene	Melancon et al (1979)

xenobiotics accumulating in fish bile are as conjugates, however this is not always the case. Sulphathiazole contains a sulphonamido group, which has the pKa of 7.1. Consequently, in physiological fluids this molecule exists mainly in its unionised form, which contributes the polarity necessary for its accumulation in bile (Hirom et al, 1972b). Analysis of bile taken from pike dosed with Eulan WA New and 6-PCSD identified high concentrations of 6-PCSD and 5-PAD in this fluid suggesting that these components are accumulated in bile without prior conjugation. The sulphonamido bond in 6-PCSD has a pKa of 7.2 and consequently the molecule will show some polar character in physiological fluids. This may together with its relatively high molecular weight account for its accumulation in bile of fish to high concentrations without conjugation. (One

must also bear in mind however that the "clean up" procedure to which each tissue extract was subjected involves exposure to the strong acidic and basic environments of acidic and basic alumina. Such conditions may result in deconjugation of xenobiotics conjugated *in vivo*). The accumulation of a xenobiotic in bile may be extremely high, for example 2-methylnaphthalene was found to be accumulated in the bile of fish to levels 140,000 times the level observed in surrounding water (Melancon and Lech, 1979). Similarly, the dosing of pike with Eulan WA New in surrounding water, led to the accumulation of 6-PCSD in bile to concentrations 13,000 times that of the aqueous environment. Such a localised magnification of xenobiotics in the bile has led to the proposal of sampling fish bile as a useful monitor of environmental contamination (Lech et al, 1973; Statham et al, 1976). In agreement with this proposal, sampling and analysis of pike bile would be a useful environmental index to judge the contamination of the aquatic environment with Eulan WA New (although both 6-PCSD and 5-PAD concentrations should be determined in such bile samples, since it appears that extensive *in vivo* metabolism of 6-PCSD to 5-PAD occurs in pike. Pike taken from an environment contaminated with Eulan WA New show predominantly 5-PAD in their tissues [Wells and Cowan, 1983]). Compartmentation of 6-PCSD in bile does not necessitate its excretion from fish. During feeding bile is secreted into the anterior section of the digestive tract. Consequently the xenobiotic secreted into the digestive tract may be excreted with faeces and/or alternatively absorbed by digestive tissue. The absorption by digestive tissue initiates enterohepatic circulation of the xenobiotic (Fig. 20). This process has rarely been studied in fish but has been implicated for sodium dodecyl sulphate (Tovell et al, 1975) and pentachlorophenol (Kobayashi, 1979)



G B: Gall Bladder (bile)

1, 2, 3, 4 - Route of Enterohepatic Circulation

(Conjugation may occur in Liver and reconjugation in Intestinal Tissues. Deconjugation may occur in the Lumen.)

Fig 20. Enterohepatic circulation of a xenobiotic showing possible sites of metabolism

in goldfish. The microflora of the digestive tract represent the major site of β-glucuronidase, consequently enterohepatic circulation may result in biomodification of the xenobiotic in three distinct sites, the liver, lumen and digestive tissue, the latter representing a site of reconjugation (Hirom et al, 1976). The dietary uptake of 6-PCSD by digestive tissue and its distribution in the tissues of pike suggests that this component may participate in the cyclical process of enterohepatic circulation.

Faecal and urinary excretory routes are difficult to monitor in fish, presenting practical problems in their collection. This accounts for the relatively few studies conducted on the relative importance of these routes in the excretion of xenobiotics and/or their metabolites. Lee et al (1972) have suggested that non-metabolised polycyclic aromatic hydrocarbons may be excreted in faeces following biliary secretion whereas the polar metabolites are excreted in fish urine. In addition the polar metabolite of DDT, DDA was actively excreted in urine (Pritchard et al, 1977) and taurine conjugates of 2,4-D and 2,4,5-T have also been observed in urine of fish (James and Bend, 1976). A further excretory route in fish is the gill. At present, the only xenobiotic which has been reported to be extensively excreted via this tissue is the anaesthetic ethyl m-aminobenzoate-MS222 (Maren et al, 1968).

The spleen is a further fish tissue which is similar to the bile, representing only a very small fraction of the total fish tissue by weight. However, the spleen of pike was observed to accumulate both 6-PCSD and 5-PAD to high concentrations (section 2.4.4, and 2.4.5). When the Atlantic salmon was dosed with the chlorinated hydrocarbon DDT (1 ppm) in surrounding water, the spleen accumulated 1.5 ppm of

the xenobiotic in 5 min. (Premdas and Anderson, 1963). The accumulation of 6-PCSD in the spleen may be in the short term a consequence of erythrocyte trapping and/or the filtering of the blood stream with macrophages which congregate in the melanomacrophage centres of the spleen (Ellis et al, 1976). A longer term response which may in addition to the latter processes account for the accumulation of both 6-PCSD and 5-PAD, is the process of antigen trapping by macrophage ingestion which has been observed for example in the spleen of the mirror carp, *Cyprinus carpio* (Secombes et al, 1980).

Wells and Cowan (1983) observed that 6-PCSD and particularly 5-PAD accumulate to high concentrations in the adipose tissue of fish taken from an environment contaminated with Eulan WA New. Similarly, studies involving the dietary accumulation of Eulan WA New in this work showed the adipose tissue to accumulate 19% and 8% of the total body burden of 6-PCSD and 5-PAD respectively after a dosing period of 221h. The liver of the dogfish, *Squalus acanthias* is an important lipid storage site and may constitute up to 75% lipid. A dietary dose of DDT was observed to be absorbed by the digestive tissue and in addition up to 55% of the dose could be compartmented in the lipoidal liver tissue (Dvorchik et al, 1972). Such sequestering of xenobiotics by partitioning with lipid is of importance in considering the effects of xenobiotics on future fish generations. Wells and Cowan (1983) observed both 6-PCSD and particularly high concentrations of its hydrolysis product 5-PAD in ova of trout during spawning, taken from an environment contaminated with Eulan WA New. Such a transfer of xenobiotic associated with lipid to the ova has been observed in other fish. A transfer of both DDT and PCB in marine fish from the maternal liver to the egg

and finally to the liver of the mature foetus has been implicated (Butler and Schutzmann, 1979). During egg development of the female rainbow trout, 5% of the total body burden of TCB appeared in the ova, and similarly a fraction in the sperm of the male trout (Guiney et al, 1979). Aromatic and chlorinated hydrocarbons are highly toxic to developmental stages of fish (Birge et al, 1979). Schimmel et al (1974) upon dosing fry and juveniles of the sheepshead minnow with Arochlor 1254 in surrounding water (0.1 µg/l), observed that this xenobiotic was thirty times more toxic to fry than to juveniles or adults. Also Nebecker et al (1974) found newly hatched fry of this fathead minnow to be more sensitive to this compound than older fry. Consequently such reports demand an assessment of the relative sensitivity of xenobiotics to different fish developmental stages and also the ability of the different stages to 'deal with' (uptake biotransform and eliminate) xenobiotics.

In the tissue of pike dosed with Eulan WA New and 6-PCSD in oral dosing studies or in surrounding water, high 5-PAD to 6-PCSD ratio were generally observed. After dosing a pike for 4h with Eulan WA New in surrounding water, the calculated whole body tissue ratio of 5-PAD to 6-PCSD was 0.7. A pike dosed orally with Eulan WA New for 221h gave a whole body tissue ratio of 11.2. The 5-PAD: 6-PCSD ratio of Eulan WA New was <0.1. These data provide evidence for the *in vivo* conversion of 6-PCSD to 5-PAD through cleavage of the sulphonamido bond of 6-PCSD. Hamburger et al (1981) also observed a similar trend of increasing 5-PAD to 6-PCSD ratio in whole carp tissue with increasing dosing time. (In a pike or goldfish dosed with 6-PCSD over short dosing periods; the liver was cited as a primary site of 5-PAD formation. In a single pike dosed for 20 min. with 6-PCSD, the only tissue analysed showing the

presence of 5-PAD was the liver (and associated bile). In the goldfish this was also the first tissue to show the presence of 5-PAD and in addition a consistently high 5-PAD to 6-PCSD ratio throughout the dosing period. It was necessary to reduce dosing times to such short periods to identify primary sites of 5-PAD formation (minimally 10 min) since, for example after 30 min. dosing of goldfish with 6-PCSD liver/bile, digestive tissue and skin/muscle each showed the presence of 5-PAD. Dosing studies, particularly those involving the dietary uptake of Eulan WA New in pike, implicated the digestive tissue as a probable site of 5-PAD formation. In contrast to both pike and goldfish, the rainbow trout did not show detectable 5-PAD in any of the tissues analysed, over a 45 min. dosing period. Wells and Cowan (1983) observed particularly low 5-PAD to 6-PCSD ratio in brown trout taken from an environment contaminated with Eulan WA New (this apparent species dependent *in vivo* metabolism of 6-PCSD to 5-PAD is discussed further in Chapter 4).

The dosing of freshwater fish with Eulan WA New and 6-PCSD has allowed the identification of both the pike and goldfish as species metabolising 6-PCSD to 5-PAD, through *in vivo* cleavage of the sulphonamido bond of 6-PCSD. By reducing periods of dosing with 6-PCSD, the liver (primarily) and digestive tissues were sited in these species as major locations of the 5-PAD forming activity. Subsequent *in vitro* studies involving these tissues taken from freshwater fish were carried out to characterise further the 5-PAD forming activity (see Chapter 3).

CHAPTER 3

The *in vitro* Metabolism of Eulan WA New and 6-PCSD in Species of Freshwater Fish.

3.1 INTRODUCTION

A general feature of xenobiotics is their hydrophobic nature. Consequently when these compounds are released to the aquatic environment they preferentially partition to the lipophilic components therein. The homeostasis of the individual biotica is dependent both on their encapsulation from the external environment, and the internal compartmentation of metabolic events; allowing the organised interaction of cellular processes characteristic of a living organism. The most fundamental structure of this is the hydrophobic membrane environment, conferring an exposed lipophilic character and both external and internal sites for xenobiotic accumulation in the living organism. In the absence of cellular mechanisms for the removal of such compounds, accumulation upon continued exposure would elicit a dose-dependent toxic response. However through evolution, mechanisms have evolved in organisms as a consequence of their selective advantage, including the mechanisms of xenobiotic metabolism. Removal of xenobiotics from sites of accumulation *in vivo*, and return to the external environment, requires a bio-modification of their structure resulting in reduced lipophilic character. The result is that they preferentially partition into the physiological excretory fluids. Such biotransformation reactions are catalysed by the enzymes of xenobiotic metabolism.

In general the concept of an enzyme is that of a molecule showing a high specificity both in terms of substrate utilised and the particular bond cleaved. This provides a delicate regulation mechanism with rapid removal of small quantities of substrate upon formation. With the enormous variety of xenobiotic substrates such specificity of design would demand an equally enormous spectrum of specific protein catalysts. In response to such a metabolic challenge economical and efficient cellular design has provided the enzymes of xenobiotic

Table 1 Characteristic features of the xenobiotic metabolising enzymes, cyt.P-450 dependent monooxygenases, UDP-Glucuronosyltransferase and Glutathione transferase of mammals.

Enzyme	Types of Reaction Catalysed	Subcellular Location	Inducers
cyt.P-450 dependent monooxygenase	N-oxidation, sulfoxidation, epoxidation, N,O-dealkylation, desulphuration, deamination	microsomal	Phenobarbital[1] 3-methylcholanthrene[2]
UDP-Glucuronosyl-transferase	Glucuronidation of compounds containing nucleophilic O, N, S and C atoms	microsomal	Phenobarbital[3] 3-methylcholanthrene[4]
Glutathione transferase	Conjugation with glutathione (GSH) of a compound containing an electrophilic C atom, or S or N	cytosolic*	Phenobarbital[5] 3-methylcholanthrene[6]

*microsomal Glutathione transferase activity has been reported (Morgenstern et al, 1979)

References referring to inducers: 1 - Colbert et al, 1979; 2 - Bresnick et al, 1979; 3 and 4 - Bock et al, 1973; 5 - Arias et al, 1976; 6 - Baars et al, 1978.

metabolism. In contrast to the intrisic specificity of the previously described proteins, these enzymes possess a characteristically broad ('loose') specificity and the ability to catalyse a number of inter-related reactions, necessary for their interaction with such a broad spectrum of substrates. The enzyme's hydrophobic character and subcellular location is suited for the sequestering of such hydrophobic xenobiotics. Quantitatively, the activity of these enzymes is regulated in response to the exposure of organisms to xenobiotics through the mechanism of enzyme induction (a feature shown in Table 1). By these means, the cell has successfully achieved a sensitive mechanism for dealing with 'alien-substrates', foreign to the normal metabolism of the cell. Such simplicity of metabolic design results in both low substrate/enzyme affinities and low turnover rates. However these are generally compensated by large cellular concentrations of these enzymes. It is only recently through focussed research upon particular enzymes of xenobiotic metabolism that details of their characteristic functional elements have been explored. This has provided an insight into some of the mechanism by which xenobiotic metabolism proceeds.

The general subcellular location of the major enzymes of xenobiotic metabolism is microsomal (associated with endoplasmic reticular membranes) [Table 1]. The role of the membrane in xenobiotic metabolism has in part been explored. The properties of bound enzymes are modified by the binding matrix. This is evident with artificially immobilised enzymes (Katchalski et al., 1971, Zaborksky, 1973), whose pH optima, affinity for various substrates may be different from those of the soluble enzymes. Consequently the binding matrix can determine the conformation of the bound protein, the local ionisation of bound substrates and even the substrates physical access to the enzyme. These factors determine the catalytic capacity of the enzyme. The

membrane associated UDP-glucuronosyltransferase is inactivated by delipidation. Reactivation was achieved upon addition of glycerophosphatides to the enzyme (Graham et al, 1977). Similarly, with the development of solubilisation techniques the basic components of the cyt.P-450 dependent monooxygenase system were resolved from the microsomal membrane and successfully reconstituted. NADPH cyt.c.(P-450) reductase, cyt.P-450 and a lipid component were required to reconstitute the functional monooxygenase system (Lu and Coon, 1968). The heat-stable lipid fraction was identified as phosphatidylcholine (Strobel et al, 1970). The association with membrane lipids is therefore essential for the xenobiotic metabolising activity of the latter enzymes. Studies involving both cyt.P-450 dependent monooxygenase and UDP-glucuronosyltransferase led to the identification of two distinct substrate pools, one aqueous and the second associated with the membrane environment. In both studies the pool of substrate utilised by these enzymes is that associated with the membrane (Schuster et al, 1975; Zakim and Vessey, 1977). Inhibition of UDP-glucuronosyltransferase dependent glucuronidation of 4-nitrophenol (Hänninen and Alanen, 1966) and cyt.P-450 dependent hydroxylation of p-aniline (Cohen and Mannering, 1973) was observed with alcohols of increasing chain length. This correlation between inhibition and increasing partition coefficient of the alcohols provides evidence for the occurrence of the interaction between enzyme and substrate within the membrane environment. The membrane appears to provide access of the substrate to the active site of the enzyme. The structural modulation of the membrane environment by the xenobiotic substrates and effects imposed upon the membrane associated enzyme by this, suggests a multifunctional nature of the membrane environment in:

(a) the sequestering of xenobiotics within the cell in sites containing enzymes for their modification;

(b) the membrane may establish a restricted plane of diffusion between the substrate and the enzyme;

(c) the membrane may also provide restricted orientations of the substrate that are energetically favourable to catalysis.

A major soluble xenobiotic metabolising enzyme is the glutathione transferase. In contrast to the microsomal enzymes previously discussed, these proteins are important for a number of functions. Immunological studies have shown glutathione transferase and the binding protein 'ligandin' to be the same molecules (Litwack et al, 1971). Consequently glutathione transferase may function in the reversible binding of a wide range of hydrophobic molecules (Levi et al, 1969). Bilirubin may be bound and transported within the hepatocyte by binding with glutathione transferase: bilirubin being both toxic and poorly soluble in physiological solutions. The glutathione transferase may bind irreversibly to highly reactive electrophilic compounds. Such covalent binding was observed between glutathione transferase and the carcinogenic oxidation product of an azo dye (Ketterer et al, 1967). Glutathione transferase can therefore encounter and disarm particular reactive species by a covalent interaction, thought to involve an essential sulphydryl group of the protein (Ketterer and Christodoulides, 1969). This 'suicidal' process provides protection against reactive species that can modify cellular macromolecules (Jakoby and Keen, 1977). The single most characteristic feature of glutathione transferase is its ability to bind an enormous variety of hydrophobic molecules. The enzyme therefore possesses a membrane like lipophilic topography (Ketley et al, 1975). In addition to the role of glutathione transferase in xenobiotic metabolism, the transport and storage functions of this protein demand a degree of mobility within the cell.

Xenobiotic metabolising enzymes catalyse a number of inter-related reactions involving a large number of lipophilic substrates (Table 1). The molecular basis of the broad specificity of these enzymes has been the focus of recent research. Questions have arisen whether such broad specificity is that of a single protein, modulated through the membrane environment, or modified post-transcriptionally to give different functionally active forms of the same protein (functional heterogeneity). Alternatively does protein polymorphism exist, resulting in distinct molecular forms showing differing substrate specificities which together account for the observed broad specificity (structural heterogeneity)? Glutathione transferase was found to be represented by a group of proteins. Six distinct species of glutathione transferase were obtained from an anion exchange column and are named in reverse order of their elution; AA, A, B, C, D, and E. Each of these molecular forms were observed to catalyse the conjugation of 1-chloro-2,4 dinitrobenzene with reduced glutathione. However, the affinity of the different proteins for this substrate differed over four orders of magnitude. This pattern of overlapping substrate specificities is characteristic of the glutathtione transferases (Jakoby et al, 1976). However some individual specificity, related to particular glutathione transferases with some xenobiotic substrates has been observed. For example of the glutathione transferases, A, B, C, D, and E, the demethylation of dimethyl 1-naphthylphosphate resided with the transferase E activity (Hutson, 1977).

Some evidence for the functional heterogeneity of UDP-glucuronosyl-transferase has been obtained from the effect of inducers on the glucuronidation of substrates. Wishart (1978) found that rats pretreated with phenobarbital showed an increased specific activity (160-220%) with respect to the conjugation of six substrates including

bilirubin and testosterone. Pretreatment with 3-methylcholanthrene led to an increase in the specific activity (220-330%) of glucuronidation of six different substrates including 2-aminophenol and 4-nitrophenol. The activities associated with both groups of substrate varied with respect to their time of appearance during development. In similar induction studies, Sladek and Mannering (1966) observed that the treatment of rats with 3-methylcholanthrene resulted in spectrally distinguishable forms of cyt.P-450 (Imai and Sato, 1966a). Studies of the carbon monoxide different spectra of 3-methylcholanthrene treated rats, showed a maximum absorbance at 448 nm, differing from the cyt.P-450 of untreated rats by 2 nm. Therefore the presence of two spectrally distinguishable forms of cyt.P-450 were observed, the normal P-450 (phenobarbital induced) and P-448 (3-methylcholanthrene induced). The questions as to whether these represented distinct molecular forms of cyt.P-450, or different physical states of a single protein was resolved by the introduction of separation techniques. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, has led to the identification of five or six forms of cyt.P-450 in rabbit liver microsomes and between five and seven forms of cyt.P-450 in rat liver microsomes (Lu and West, 1980). Antibodies raised to purified cyt.p-450 have been shown to be monospecific (Thomas et al, 1976) consequently, distinct molecular species (isoenzymes) of cyt.P-450 exist in mammalian microsomes. Nebert (1980) has proposed the possible existence of hundreds or even thousands of different cyt.P-450 species, with only very minor structural differences relating to their differences in substrate specificity. The mechanism of their production and cellular design of such species may be similar to that exhibited by the immunoglobulins, with the polypeptide chain possessing both constant and variable regions. This proposal questions

the sensitivity and limitations of the present techniques of separation and protein structure determination of cyt.P-450. At present there is very little empirical evidence to support this theory.

Purified cyt.P-450 and cyt.P-448 have also been studied in terms of their specificity with respect to monooxygenation of xenobiotic substrates. Cyt.P-450 was found to be highly reactive in the N-demethylation of benzphetamine but almost inactive in the hydroxylation of benzo(a)pyrene (Ryan et al, 1975). Cyt.P-448 showed the reverse substrate specificity with respect to these substrates (Fujita and Mannering, 1973). Also various molecular species of cyt.P-450 have been found to catalyse the N-demethylation of benzphetamine or the hydroxylation of benzo(a)pyrene at rates that can differ by a factor of one hundred (Ryan et al, 1979). As well as showing marked differences in the rates of catalysis of substrates, different forms of cyt.P-450 exhibit positional selectivity and stereoselectivity, shown by the metabolism of benzo(a)pyrene (Deutsch et al, 1978) testosterone (Haugen et al, 1975) and Warfarin (Fasco et al, 1978). The substrate specificity of certain molecular species of cyt.P-450 was also observed in steroid hydroxylation studies. The 7α-hydroxylation of cholesterol is catalysed by a unique species of cyt.P-450 (Boyd et al, 1973) which has a very short half-life of 2h (Gielen, et al, 1975).

Evidence that distinct molecular forms of cyt.P-450 are inducible has been obtained from studies coupling mRNA translation with immunoprecipitation techniques. Cytoplasmic RNA was isolated from control and 3-methylcholanthrene pretreated rat livers, and translated by a rabbit reticulocyte system. Translational products were precipitated with a mono-specific antibody to cyt.P-450 c (defined from SDS-PAGE gels). Only in 3-methylcholanthrene treated rats was cyt.P-450 c

observed (Brenswick et al, 1979). Liver polysomes were isolated from control and phenobarbital pretreated rats. The translational products of RNA from these rat livers were treated with a mono-specific antibody to cyt.P-450b. This was synthesised mostly by polysomes of the phenobarbital treated rat at seven times the rate of that of the control (Colbert et al, 1979). Consequenly, structurally distinct molecular species of cyt.P-450 exist and their presence and activity may be modulated through environmental exposure of the organism to xenobiotics, by induction mechanisms. The distinct molecular forms of cyt.P-450 show over-lapping substrate specificities. However the catalysis of monooxygenation of some substrates by particular molecular species also occurs. The activity of the different forms may be controlled by the availability of reducing equivalents from the reductase as the limiting enzyme in the system, patterns of induction (as described above), and perhaps to varying extents by the phospholipid environment of the membrane.

The mammalian design of xenobiotic metabolising enzymes may represent only a stage of evolutionary development. In contrast, microorganisms possess a greater adaptative capacity with generation times in minutes rather than years. Enrichment techniques (Kluyver and Van Niel, 1956) have allowed the isolation of organisms which can utilise almost any organic carbon substrate as a sole source of carbon. The cyt.P-450 from a Pseudomonad grown on camphor as a sole source of carbon (Gunsalus and Wagner, 1978) has been isolated and studied. The cyt.P-450 monooxygenase exhibits mechanistic features identical with those of the mammalian system and yet shows a very restricted specificity for oxidizable substrates.

In conjunction with studies of the molecular basis of the characteristic broad specificity of xenobiotic metabolising enzymes, the

mechanism by which xenobiotic substrates are modified has received some attention. Quantitatively, the most important group of xenobiotic metabolising enzymes are the microsomal monooxygenases. The mechanism by which monooxygenation proceeds has in part been explored. The general reaction catalysed by these enzymes is as follows:

$$XH + O_2 + NADPH + H^+ \rightarrow XOH + H_2O + NADP^+$$

where XH = xenobiotic substrate and XOH = hydroxylated product.

Gillette (1968) proposed the following mechanism:

$$NADPH + A + H^+ \rightarrow AH_2 + NADP^+$$

$$AH_2 + O_2 \rightarrow \text{"activated oxygen"}$$

$$\text{"activated oxygen} + X \rightarrow X - OH + A + H_2O$$

$$NADPH + O_2 + X \rightarrow NADP^+ + H_2O + X - OH \ .$$

These enzymes were classified as mixed function oxidases (Mason, 1957, 1965) or monooxygenases (Hayaishi, 1969) since they catalyse the uptake of an oxygen molecule per molecule of substrate resulting in a single oxygen atom appearing in the product and the second being reduced to water. From reconstitution studies the essential units required for monooxygenation were found to be the haemoprotein cyt.P-450, NADPH cyt.c(P-450) reductase and a phospholipid fraction (Lu and Coon, 1968). However NADH in the absence of NADPH could catalyse (although at reduced rates) the microsomal monooxygenation of substrates (Krisch and Staudinger, 1961). Also in the presence of both NADPH and NADH microsomal monooxygenase activity was enhanced to values greater than with NADPH alone, a process referred to as "NADH synergism" (Hildebrandt and Estabrook, 1971). It was proposed that an alternative pathway supplying electrons from NADH to cyt.P-450 existed and from immunochemical studies this was finally proven to involve both NADH-cyt.b_5 reductase and cyt.b_5 (Jansson and Schenkman, 1977). The synergistic effect elicited by NADH was attributed to the role of reduced cyt.b_5 as a donor of a second electron to cyt.P-450.

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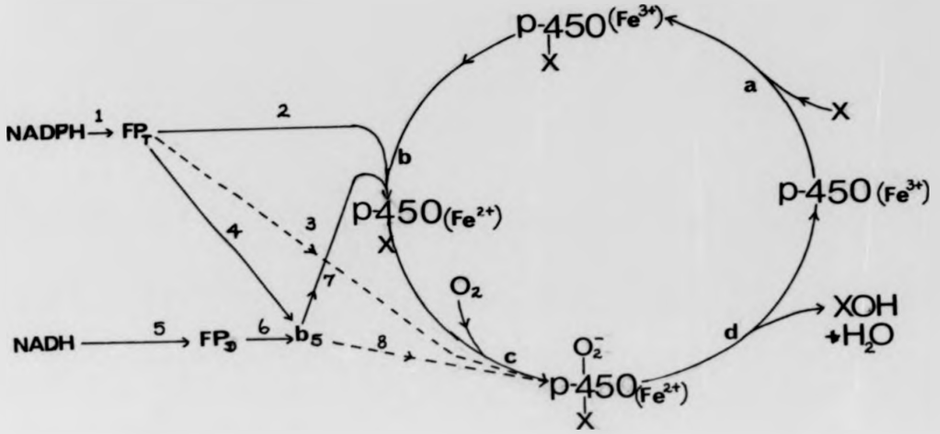
The proposed pathway of electron transfer coupled to monooxygenation by cyt.P-450 is schematically shown in Figure 1. The sequence of



FP_T-NADPH-cytochrome P-450 reductase; FP_D-NADH-cytochrome b_5 reductase; X-substrate; b_5-cytochrome b_5; P-450, cytochrome P-450.

Figure 1 Microsomal electron transfer during monooxygenation (adapted from Jansson and Schenkman, 1977; taken from Bend and James, 1978)

catalytic events leading to monoxygenation of a xenobiotic substrate are:

STEP (a) The formation of a haemoprotein-substrate complex by interaction of the substrate with the oxidised (ferric) form of cyt.P-450.

(b) The cyt.P-450-substrate complex is reduced to give the ferrous form of the haemoprotein, involving the donation of a single electron. The electron may be donated via the pathway (1 and 2) involving NADPH-cyt.c reductase; or alternatively via (5, 6 and 7) involving NADH-cyt.b_5 reductase.

(c) The reduced cyt.P-450-substrate complex then combines with molecular oxygen resulting in the formation of an unstable oxygenated reduced cyt.P-450-substrate complex.

(d) An electron is then supplied from either NADPH or NADH (pathway 3 or 8) which results in the decomposition of the hydroxylated product, oxidised cyt.P-450 and water.

This general scheme represents the present understanding of the basic mechanism of cyt.P-450 dependent monooxygenation. However, many questions remain unanswered including:

(i) What is the nature of the 'active oxygen species' and how does it interact with such a broad spectrum of xenobiotics as are known to be substrates of cyt.P-450?

(ii) What are the features of cyt.P-450 that permit it to react with both oxygen and substrate in a manner resulting in catalysis of xenobiotic modification?

(iii) Is there more than a single mechanism of oxygen activation related to the multiple forms of cyt.P-450 which are selectively induced by xenobiotics?

(iv) What regulates the pattern of electron supply from the reduced pyridine nucleotides and how are these restricted in the metabolism of the substrate?

Recent studies of microsomal monooxygenases has led to the identification in the microsomes of the flavoprotein monooxygenase, dimethylaniline monooxygenase (N-oxide forming) (EC.1.14.13.8) described by Ziegler and Petit (1966). The purified enzyme from hog microsomes was observed to catalyse the monooxygenation of a wide range of 'N' and 'S' containing xenobiotic substrates in the absence of cyt.P-450 (Ziegler and Mitchell, 1972). Studies on the mechanism

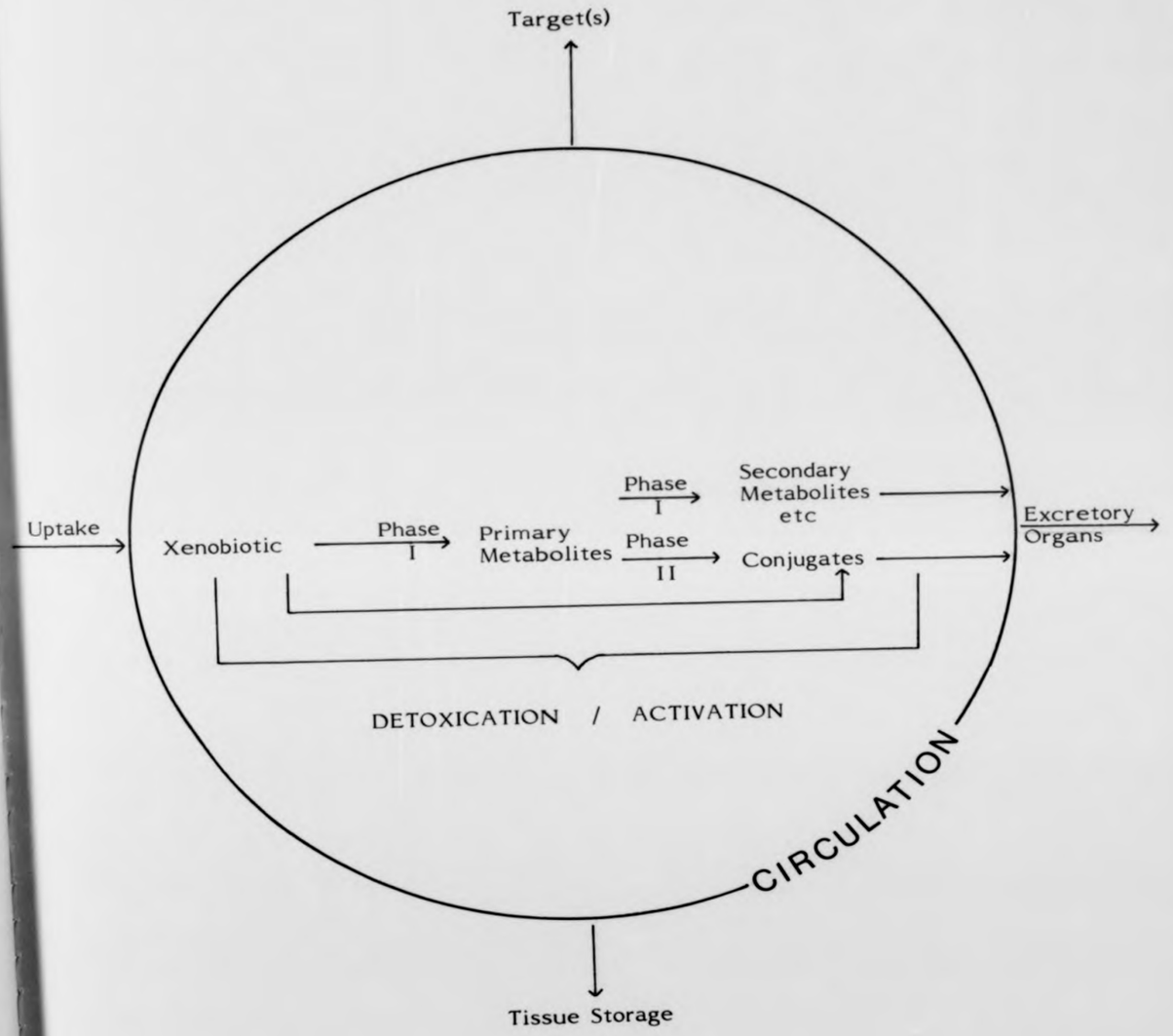
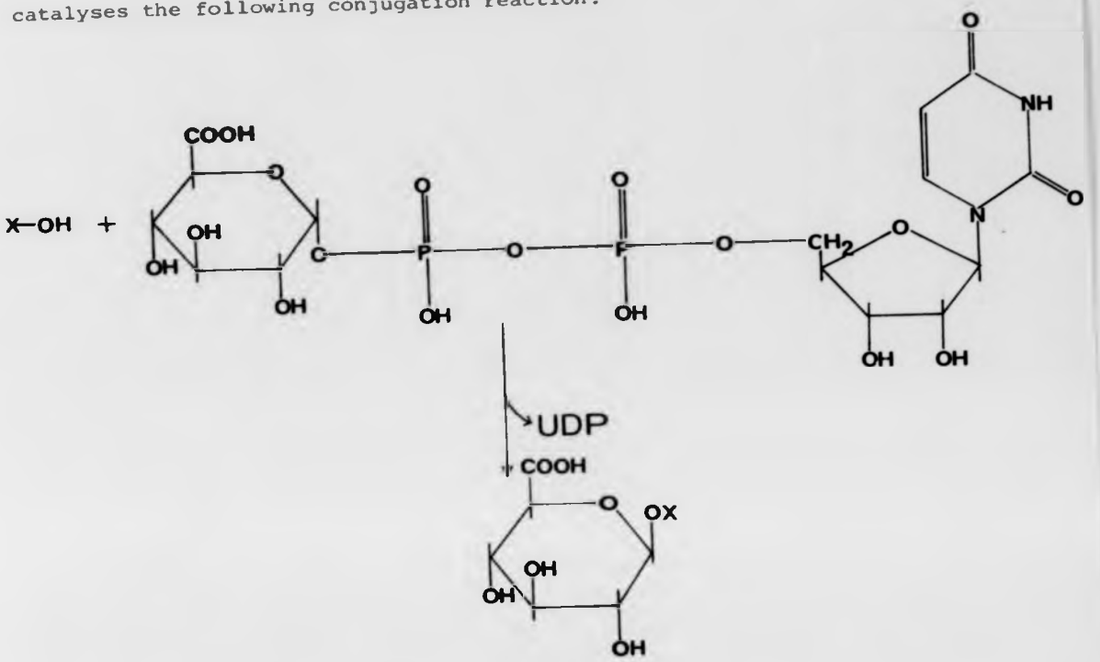


Fig 2. The uptake, metabolism, interaction and excretion of a xenobiotic in an organism

of monooxygenation of this enzyme has suggested that a peroxy-flavin complex is the activated oxygen species reacting with the substrate (Ziegler, 1980). It is interesting that a peroxycytochrome P-450 is implicated as a reactive species interacting with the substrate leading to its oxidation (Estabrook et al, 1979).

In general xenobiotic metabolism is rarely an act of a single enzyme but involves the sequential action of several of them. The types of reactions catalysed by these enzymes are classified into defined groups or phases (Table 1). No sequence is implied by this distinction, and recently the phase I and II reactions have been referred to in more informative terms as functionalisation and conjugation reactions respectively (Jenner and Testa, 1978). A xenobiotic substrate may be directly conjugated if it possesses a functional moiety within its structure which can be so modified. A substrate containing no such 'conjugand accepting groups' is most frequently primarily modified by a phase I type functionalisation reaction, e.g. the introduction of an hydroxyl group by microsomal monooxygenase activity. This modified xenobiotic is then a functionalised substrate which can be conjugated with either sulphate or a glucuronyl residue by either a sulfotransferase or a UDP-glucuronosyltransferase, for example. A diagrammatic representation of the metabolism of a xenobiotic in an organism is shown in Figure 2, illustrating the possible interplay of phase I and II type activities. The phase II, conjugation reactions, are enzyme catalysed energy requiring biosynthetic reactions with the exception of conjugation with glutathione. (Here the energy of the reaction is derived from the mutual reactivity of the nucleophilic sulphur of glutathione with an electrophilic centre of a xenobiotic substrate.) The formation of a xenobiotic conjugate alters the lipophilic character

of the xenobiotic substrate. For example UDP-glucuronosyltransferase catalyses the following conjugation reaction:



X - OH - Xenobiotic substrate

- SH

- NH_2

The introduction of the glucuronyl residue into the xenobiotic substrate increases the polarity of the molecule and also contributes a carboxyl group which exists primarily as an unprotonated species at the pH of most physiological fluids. This allows salt formation and facilitates excretion by both biliary and urinary routes. Similarly, conjugation with glutathione confers polar character to the xenobiotic. However this conjugand is sequentially degraded and modified prior to the excretion of the xenobiotic as a mercapturic acid (Fig. 3). Such modification reflects the cell's economy since it provides aminoacid

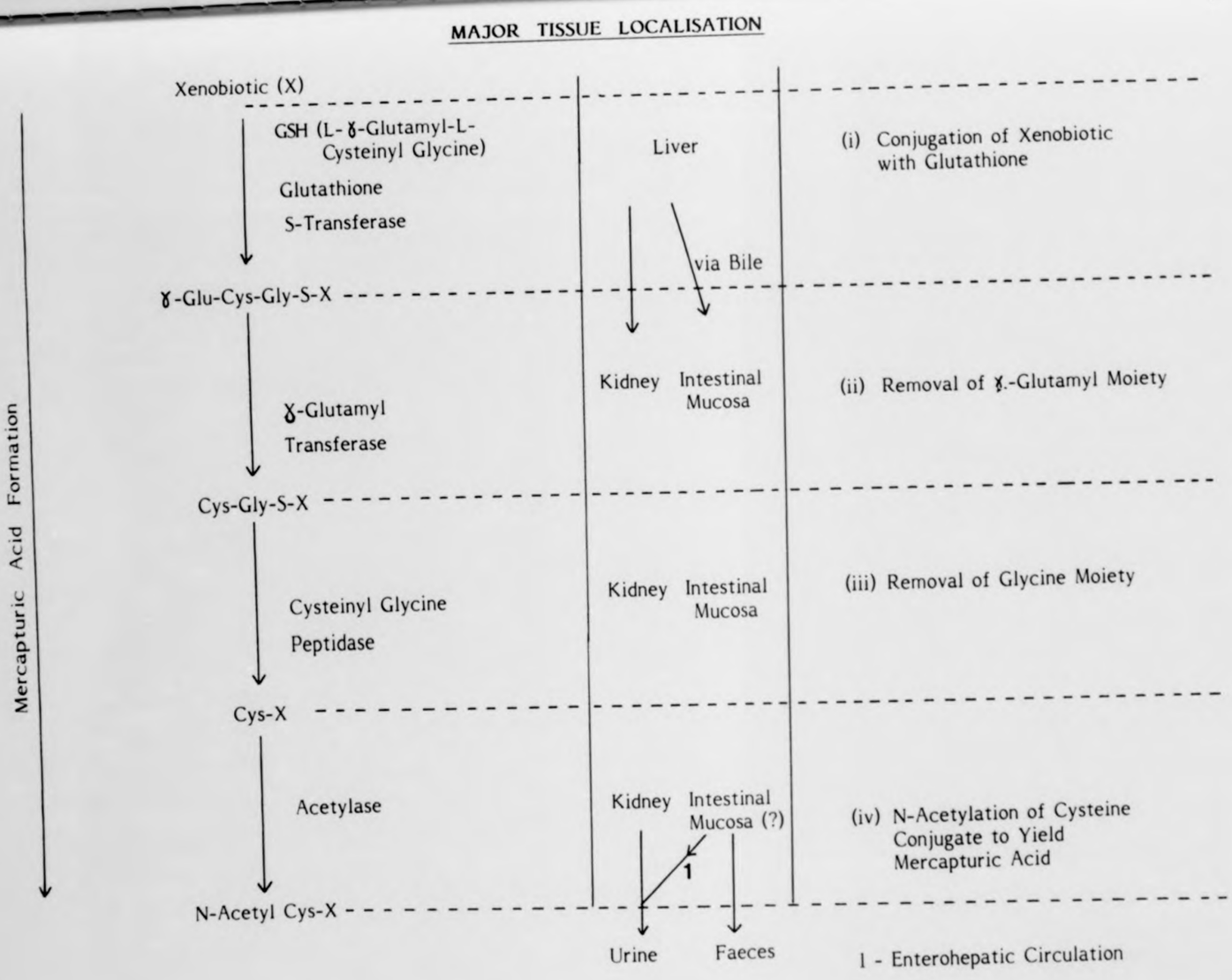


Fig 3. Possible routes of Xenobiotic Excretion as a Mecapturic Acid

precursors for the resynthesis of glutathione via the γ-glutamyl cycle, and may also provide a means of transport of aminoacids to different tissue locations (Orlowski and Meister, 1970). Highlighted in Figure 2 is the dichotomy imposed by xenobiotic metabolism, that of xenobiotic detoxication or activation. In general, the pathways of xenobiotic metabolism result in the excretion from the organism of a deactivated xenobiotic. However, this viewpoint may be somewhat naive since especially functionalisation reactions, (and also in some cases conjugation reactions) may result in an activation of the xenobiotic. Monooxygenation of a number of xenobiotic substrates proceeds via an epoxide intermediate. These species are particularly reactive and may chemically interact with cellular macromolecules, such reactive species have been implicated as mutagens and carcinogens. For example the mycotoxin aflatoxin B_1 is oxidised by a microsomal monooxygenase resulting in the formation of the intermediate aflatoxin B_1-2,3 oxide. The latter is the metabolically activated ultimate hepatocarcinogen in both the rat (Swenson et al, 1977) and the rainbow trout (Ayres et al, 1971). Microsomal monooxygenases also catalyse the formation of the proximate carcinogen N-hydroxy-2-acetylaminofluorene from the xenobiotic 2-acetylaminofluorene. However, the ultimate carcinogen is identified as the sulphated conjugate of this metabolite which binds to cellular macromolecules (Miller, 1970, Miller and Miller, 1976). Conjugation as well as providing a mechanism for leading to the excretion of potentially harmful xenobiotics also in some instances may lead to the transport and accumulation of carcinogenic compounds in target tissues. Some arylamines induce tumour formation in the urinary bladder (Clayson, 1962). The arylamines are oxidised by monooxygenases to hydroxylated intermediates (Clayson and Garner, 1976) which may be further glucuronidated (Kadlubar et al, 1977). The

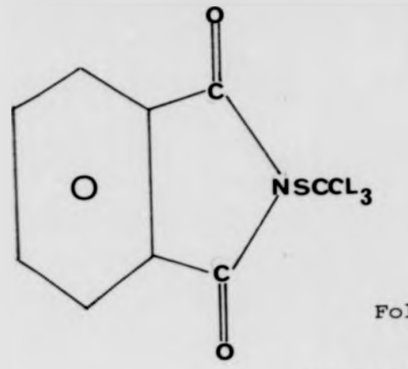
N-glucuronides are transported to the kidney where they enter the urine. Because of the acidic nature of urine (pH 5-6) hydrolysis of the glucuronide occurs. The N-hydroxy metabolites so released may form electrophilic arylnitrenium ions which can conveniently modify DNA and RNA. Consequently glucuronidation generates the proximate carcinogen, transports it to the target organ where the formation of the ultimate carcinogen occurs (Kadlubar et al, 1977). Therefore the metabolism of a xenobiotic may not always result in a deactivation of a toxic component which can be readily excreted from the living organism.

A survey of the complement of xenobiotic metabolising enzymes possessed by the broad diversity of living organisms has not been completed. However of the microorganisms so far studied, fungi do appear to possess a complement of xenobiotic metabolising enzymes similar to those observed in mammals (Chapter 5). Identification of pathways of biotransformation of xenobiotics suggest that fish also contain a similar complement of xenobiotic - metabolising enzymes to those observed in mammals (Table 2). Consequently fish possess mechanisms similar to those found in mammals to deal with xenobiotics (Sieber and Adamson, 1977) released into the aquatic environment.

An important structural feature of the xenobiotic Eulan WA New is the 'N-S' bond. In Eulan WA New this bond is broken under forcing conditions (e.g. upon refluxing with concentrated alkali) (section 3.4.3.1). The strength of this bond is determined by its neighbouring substituents. For example, a contrasting situation is observed with the trichloromethyl sulphenyl fungicides, e.g.:

Table 2 The *in vivo* biotransformation of xenobiotics in fish

Reaction	Species	Compound	Reference
Oxidation	Rainbow trout	Methylnaphthalene	Melancon and Lech (1978)
	Carp	Rotenone	Fukami et al (1969)
O-Dealkylation	Rainbow trout	Pentachloroanisole	Glickman et al (1977)
	Rainbow trout	Fenitrothion	Miyamoto et al (1979)
N-Dealkylation	Carp	Dinitramine	Olson et al (1977)
Hydrolysis	Rainbow trout	Diethylhexyl phthalate	Melancon and Lech (1976a)
Glucuronide conjugation	Rainbow trout	3-trifluoromethyl-4-nitrophenol	Lech (1973)
Glutathione Conjugation	Carp	Molinate	Lay and Menn (1980)
Sulphate conjugation	Goldfish	Pentachlorophenol	Akitake and Kobayashi (1975)
Acetylation	Rainbow trout	Ethyl m-amino benzoate	Hunn et al (1968)

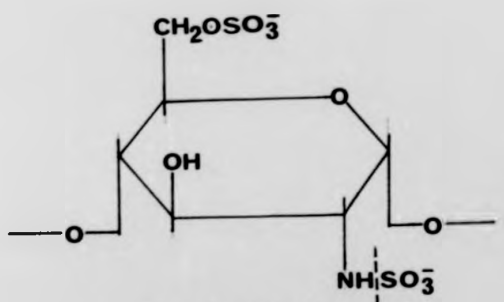


Folpet

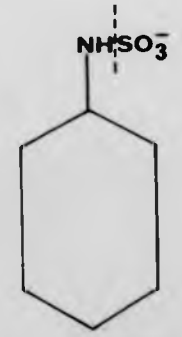
[N-(trichloromethylthio)phthalimide]

Here the 'N-S' bond stability is greatly modified by the unusual substituents on both nitrogen and sulphur atoms. The dicarbonyl attachment to the nitrogen and the trichloromethyl attachment to the sulphur. The result of this is that the 'N-S' bond is readily cleaved non-enzymatically on exposure to physiological fluids by a reaction with cellular thiols both free simple thiols and protein associated sulphydryl groups (Lukens 1969a and b). Consequently enzymes containing essential functional sulphydryl groups are inhibited by the xenobiotic, including glyceraldehyde 3-phosphate dehydrogenase (Siegel, 1971) and aldolase (Montie and Sisler, 1962).

The endogenous molecule heparin and the xenobiotic cyclamate both contain 'N-S' bonds that are enzymatically cleaved:



unit of heparin



cyclamate

---- site of enzymatic cleavage

Table 3 Comparison of reported activities known to cleave the 'N-S' bond.

Organism	Substrate	pH Optima	Subcellular location of activity	Observations
Rat (spleen)	heparin	pH 5.1	Lysosomal	Both sulphate and phosphate were inhibitors. Only 5% of substrate was cleaved.[1]
Pseudomonad	cyclamate	pH 6.5-6.7	? activity present in 20,000g supernatant	Specific for hydrolysis of sulphamates, preferentially aliphatic sulphamates 3-8 carbon atoms. Sulphamates of secondary amines were scarcely hydrolysed. Sulphanilamide and cyclohexyl sulphate were also scarcely hydrolysed. Hg^{2+} inhibited, but pHMB did not show strong inhibition.[2]

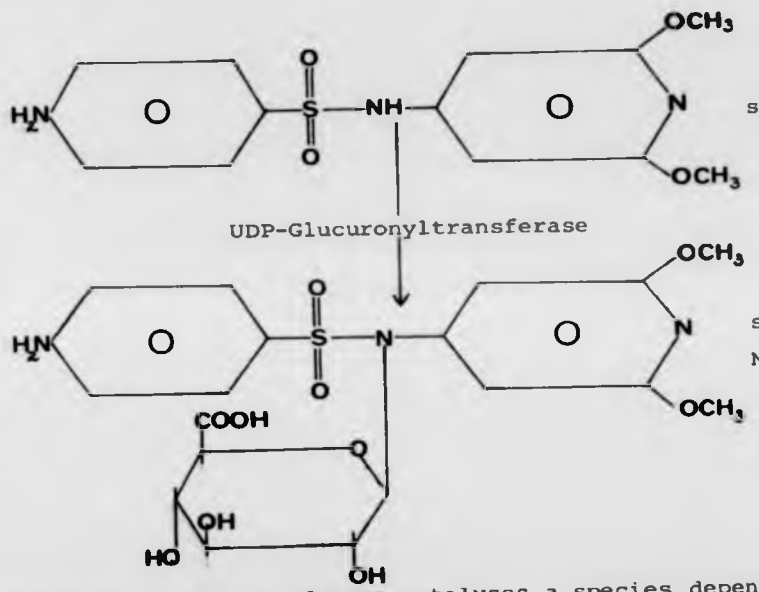
1 - Friedman and Arsenis (1974)
2 - Dietrich et al (1973)

The metabolism of the 'N-S' bond is a very rare metabolic event with few published studies of the 'sulphamatase' type activities. The reaction is not identified as a characteristic reaction of the defined xenobiotic metabolising enzymes. Some properties of 'sulphamatases' have been published (Table 3). The heparin sulphamatase activity has been studied in both the mammalian system (Friedman and Arsenis, 1972 and 1974) and also in the microorganism *Flavobacterium heparinum* (Dietrich et al, 1973), grown in media containing heparin. The sulphamatase metabolising the xenobiotic cyclamate was found to reside with the intestinal flora of mammals. The activity was isolated from guinea pig faeces mainly in strains of *Pseudomonas* (Asahina et al, 1972a and b) and was partially characterised (Niimura et al, 1974). The proposal that the rat liver was a site of cyclamate metabolism (Koijama and Ichibagase, 1968; Ichibagase et al, 1972) was not substantiated by liver perfusion studies (Prosky and O'Dell, 1971) or further in *in vitro* studies involving liver homogenate (Draser et al, 1972). [It is interesting that the food industry have supplied an analogue of cyclamate, 3-methylcyclopentyl sulphamate, that masks the bitter after-taste of sweetners like saccharin, but is safer since it is without the 'metabolic complications of cyclamate' (BIBRA, 1974). However studies with the xenobiotic suggest that it is a substrate of the cyclamate sulphamatase and consequently it follows a similar pathway of metabolism ... (Renwick, 1977).]

The *in vivo* metabolism of Eulan WA New has been observed in some species of freshwater fish from both dosing studies (sections 2.4.4 and 2.4.6) and analysis of fish samples from a contaminated environment (Wells and Cowan, 1983). The major metabolic event is the cleavage of the sulphonamido bond which may occur by (a) a 'sulphamatase' like activity described above; (b) PCSD may be enzymatically modified

providing a substrate in which the 'N-S' bond is further enzymatically cleaved or (c) PCSD may be enzymatically modified to provide an unstable species which degrades with subsequent cleavage of the sulphonamido bond.

The xenobiochemistry of the sulphonamides provides an example of a possible type of enzymatic modification of the 'N-S' bond:



UDP-glucuronosyltransferase catalyses a species dependent glucuronidation of the 'N' atom of the sulphanamido bond (Bridges et al, 1965).

It has been suggested that Eulan WA New results in the death of the larvae of clothes moths by preventing their normal digestion of keratin (Bayer, 1967). Also a reduced feeding habit was observed in tadpoles when dosed with Eulan WA New (Osborn and French, 1981). One interpretation of this data is to propose an inhibitory interaction of Eulan WA New with the hydrolytic enzymes of digestion. Wells and Cowan (1983) observed high concentrations of 5-PAD and corresponding high 5-PAD:6-PCSD ratio in the digestive tissue of the pike taken from an environment contaminated with Eulan WA New. This may reflect the

metabolism of the 'N-S' bond of 6-PCSD within the lumen of the digestive tract by digestive proteinases, and absorption of the amine product by epithelial tissue.

To identify which of the xenobiotic metabolising enzymes is involved in the utilisation of Eulan WA New as a substrate is not an easy task because of characteristic broad specificity of these enzymes. However, suggestions have been made above of some enzymes which metabolise substrates similar to Eulan WA New and also of some implications of other enzymes which may be involved in the cleavage of the sulphonamido bond of PCSD.

This chapter reports the preliminary characterisation of the *in vitro* 5-PAD forming activity observed in some freshwater fish species and proposes the type of xenobiotic metabolising enzyme(s) involved in the biotransformation of Eulan WA New.

Section 3.2 MATERIALS

Freshwater fish

Pike (*Esox lucius*) were caught by hand-line from the Forth and Clyde canal (Kilsyth, Strathclyde) and maintained in tanks prior to study. Perch (*Perca fluviatilis*), carp (*Cyprinus carpio*) and eel (*Anguilla anguilla*) were obtained by gill-netting from Loch Fad (Isle of Bute). Rainbow trout (*Salmo gairdneri*) were supplied from a local fish farm (College Mill Trout Farm, Almond Bank, Perthshire). Goldfish (*Carassius auratus*) were obtained from a local supplier.

Proteinases

Proteinases employed in this study were purchased from Sigma Chemicals Ltd. (Poole, Dorset) and included:- Pepsin (EC.3.4.4.1) from hog stomach mucosa; Trypsin (EC.3.4.21.4) from hog pancreas; α-chymotrypsin (EC.3.4.21.1) from bovine pancreas and Protease V (Pronase), a non-specific protease from *Streptomyces griseus*.

For studies involving the chemical hydrolysis and modification of Eulan WA New, sodium hydroxide (Analar grade), glacial acetic acid (general purpose reagent) acetic anhydride (Analar grade) and dimethylformamide (DMF, Analar grade) were supplied by BDH Chemicals Ltd. (Poole, Dorset).

The following chemicals:- glutathione (GSH, reduced form), uridine-5'-diphospho-glucuronic acid (UDPGA, sodium salt), s-acetyl coenzyme A (sodium salt), β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$, monosodium salt), β-nicotinamide adenine dinucleotide phosphate (β-NADPH, reduced form, tetrasodium salt), β-nicotinamide adenine dinucleotide (β-NAD^+, sodium salt); adenosine-5'-triphosphate (ATP, disodium salt), adenosine-5'-diphosphate (ADP, disodium salt), phenylmethylsulphonylfluoride (PMSF), p-hydroxymecuribenzoate (pHMB, sodium salt), dithiothreitol (DTT, Clelands reagent), bovine serum albumin (BSA, 96-99% albumin), 1-chloro-2,4-dinitrobenzene (CDNB, Analar grade), hide powder azure (HPA) and iodoacetamide were supplied by Sigma (Poole, Dorset). β-nicotinamide adenine dinucleotide (β-NADH, reduced from monosodium salt) was supplied by the Boehringer Corporation Ltd. (London) and glycerol (Analar grade) by BDH Chemicals Ltd. (Poole, Dorset).

Microflora growth Medium A

The composition of the growth medium was as follows:- 2 mg purified 6-PCSD, 0.6 g Na_2SO_4, 2 g NaCl, 0.1 g $MgSO_4.7H_2O$, 5 mg $MnSO_4.4H_2O$, 5 mg $FeSO_4.7H_2O$, 15 mg $CuSO_4.5H_2O$, 0.15 mg $ZnSO_4.7H_2O$, 0.03 mg $Na_2MoO_4.2H_2O$, 60 mg $CaCl_2.2H_2O$, 0.35 g K_2HPO_4, 0.25 g KH_2PO_4, 50 mg yeast extract per litre of distilled water. The phosphate and yeast extracts were sterilised separately and the pH adjusted to 7.2 prior to sterilisation.

Other chemicals not described here but are employed in this section are those previously described in the Materials section of Chapter 2, p.14.

Section 3.3 METHODS

3.3.1 Extraction of Eulan WA New and 5-PAD from buffers of pH 3.0 to 9.0, in the presence and absence of fish tissue homogenates

Eulan WA New (20 µg, 20 µl of a 1 mg/ml 6-PCSD in DMF solution) and 5-PAD (20 µg, 20 µl of 1 mg/ml 5-PAD in DMF solution) were added to buffers of pH 3.0 to 9.0 (0.1M citric acid/sodium citrate buffer, pH 3.0. 4.0, 5.0 and 6.0; 0.1M potassium phosphate buffer, pH 7.0 and 8.0; and 0.1M glycine/sodium hydroxide buffer pH 9.0), giving a total volume in each case of 2 ml. 2 ml of methyl tertiary butyl ether (MTBE) was added to each of the buffers containing Eulan WA New and 5-PAD, and the solutions were each hand-shaken for approximately 2 min.. The aqueous and MTBE phases were allowed to separate, before the upper MTBE extract was carefully removed using a pasteur pipette and placed in a glass vial of capacity 5 ml. 2 × 1 ml portions of MTBE were further added to the aqueous phase, and the extraction procedure repeated. The pooled MTBE extract, from each sample, was evaporated to dryness by placing the vial in a water bath at 35°C, and evaporating the solvent under a stream of dry-air. The resulting residue from

each extract was dissolved in DMF or methanol (50 or 100 µl) and aliquots (5 or 10 µl) were injected for HPLC analysis. The extraction of Eulan WA New and 5-PAD from buffers of pH 3.0 to 9.0 was repeated, with hexane replacing MTBE as extraction solvent.

A frozen sample of pike liver homogenate (0.5 g pike liver/5 ml 50 mM potassium phosphate buffer, pH 7.4) was thawed, and 400 µl aliquots were added to buffers of pH 3.0 to 9.0, containing 6-PCSD (20 µg/tube) and 5-PAD (20 µg/tube). Each tube was extracted with 1 × 2 ml and 2 × 1 ml fractions of MTBE, by the MTBE extraction procedure described in this section. Vigorous shaking with MTBE led to the formation of a single phase matrix structure in the tubes, which was found to be resolved into aqueous and MTBE phases, upon addition of a small volume of methanol. The extraction of Eulan WA New and 5-PAD from buffers containing tissue homogenate was repeated with hexane replacing MTBE as extraction solvent.

Samples of frozen goldfish bile (0.2 ml, diluted to 1 ml with 50 mM potassium phosphate buffer, pH 7.4) and of homogenates of goldfish fat tissue (0.3 g in 3 ml buffer, pH 7.4) and goldfish gill tissue (0.3 g in 3 ml buffer, pH 7.4) were thawed, and 400 µl aliquots were added to 50 mM potassium phosphate buffer pH 7.4, containing Eulan WA New (20 µg) and 5-PAD (20 µg) giving a final volume of 2 ml. The samples were immediately extracted with MTBE as described in this section, and the percentage of 6-PCSD and 5-PAD extracted from each sample was determined from the resulting HPLC profiles.

A reference to 'MTBE extraction' in this chapter refers to the MTBE extraction procedure described above.

3.3.2 The chemical breakdown and modification of Eulan WA New

3.3.2.1 Preparation of individual PCSD components from Eulan WA New

The preparation of the individual PCSD components of Eulan WA New, involves the same methods described previously for the preparation of 6-PCSD from Eulan WA New (section 2.3.1). To obtain samples of each of the individual PCSD components, it was necessary to inject a highly concentrated sample of Eulan WA New in DMF onto the HPLC column. On reaching the column exit, Eulan WA New was resolved into its individual PCSD components. Consequently, as each component was detected upon passage through the flow-cell, the column eluate was collected. By this means eluates containing each of the individual PCSD components of Eulan WA New, were sequentially obtained from the HPLC column outlet. This procedure was repeated, and the final samples of pooled HPLC column eluates extracted with MTBE. The residues from each of the extracted samples were weighed and redissolved in DMF, giving the stock solutions of the individual PCSD components of Eulan WA New of known concentration.

3.3.2.2 The alkaline hydrolysis of individual PCSD components and of Eulan WA New

A method for the alkaline hydrolysis of Eulan WA New to its amine (PAD) products, has been described by Westöö and Norén (1977). The same alkaline conditions were used for the hydrolysis of Eulan WA New as published in the latter method; however, DMF was chosen as a solvent for Eulan WA New and PCSD components replacing the 1-propanol.

500 µl of the individual PCSD stock solutions in DMF (≙ 1 mg PCSD) and 500 µl of Eulan WA New in DMF (1 mg) were placed in pear-shaped pyrex flasks of total volume 5 ml. To each was added an equal volume of 50% w/v aqueous potassium hydroxide. Reflux condensers were

attached to the necks of each of the flasks and the samples heated to 150°C in an oil bath for a 24h period. After this period 50-100 µl aliquots of the cooled samples were removed and added to 2 ml MTBE, hand-shaken, and then further diluted with 2 ml distilled water. The tubes were again hand-shaken for a period of 2 min, and the phases allowed to separate. The MTBE upper phase was removed with a pasteur pipette and the extraction procedure repeated with the addition of 2 × 1 ml MTBE to the aqueous phase. The total MTBE extracts from each sample were taken to dryness by placing the tubes in a water bath at 35°C and evaporating the solvent in a stream of dry air. The residues were redissolved in DMF (50-100 µl) and a suitable aliquot injected for HPLC analysis.

3.3.2.3 The chemical breakdown of Eulan WA New upon heating with reduced glutathione (GSH) and uridine-5'-diphospho-α-D-glucuronic acid (UDPGA)

GSH (5 mg) and UDPGA (5 mg) were each added to a 1 ml sample of Eulan WA New in DMF (1 mg). The samples were placed in pyrex tubes of capacity 10 ml, and heated in an oil bath to 100°C for 24h. The samples were cooled and 5 µl aliquots were injected directly for HPLC analysis. A control sample, of Eulan WA New in DMF (1 mg) in the absence of GSH or UDPGA was heated in parallel with the above samples and analysed by HPLC.

3.3.2.4 Modification of Eulan WA New and 5-PAD under conditions for chemical acetylation

Eulan WA New

Eulan WA New (1 mg) was dissolved in glacial acetic acid (1 ml) and the sample placed in a pear-shaped pyrex flask (capacity 5 ml), with an equal volume of acetic anhydride. A reflux condenser was attached to the neck of the flask, and the sample refluxed at 120°C for 2h. 5 µl aliquots were taken after 30 min., 1h and 2h of refluxing, from the cooled sample, and injected directly for HPLC analysis (no change in column performance was observed upon direct injection of these samples).

5-PAD

The method for the chemical acetylation of PAD was that described by Westöö and Norén (1977). 5-PAD (1 mg) was dissolved in 1 ml glacial acetic acid in a pear-shaped pyrex flask (total vol. 5 ml). To this was added an equal volume of acetic anhydride and immediately upon mixing a 5 µl aliquot was taken for HPLC analysis. The mixture was allowed to stand at room temperature for up to 12h, with 5 µl aliquots taken at intervals during this period and injected for HPLC analysis. After 12h at room temperature a reflux condenser was attached to the neck of the flask and the mixture refluxed at 120°C for 3h. Upon cooling a further aliquot was taken for HPLC analysis.

Two control samples of glacial acetic acid plus Eulan WA New (1 mg) of 5-PAD (1 mg) in the absence of acetic anhydride, were subjected to the same conditions as those containing acetic anhydride and sampled in parallel.

3.3.3.1 Preparation of Pike liver and digestive tissue crude homogenates

The liver and digestive tract were dissected from a Pike immediately after death, and associated fatty-tissue removed. The digestive tract was divided into stomach and intestinal tissue. Liver, stomach and intestinal tissues, were each cut into small pieces and placed in 2 vol. of ice-cooled homogenisation buffer; 50 mM potassium phosphate buffer, pH 7.4, for liver and intestine; and 50 mM citric acid/sodium citrate buffer pH 4.0, for the stomach tissue. Each sample was placed in an ice bucket and homogenised by ten stokes of a motor-driven glass-in-teflon homogeniser, giving liver, stomach and intestinal crude homogenates.

3.3.3.2 Determination of the pH optima of proteinases and proteinase activities in Pike liver and digestive tissue homogenates, using Hide-powder azure (HPA) as proteolytic substrate

The method for the assay of proteolytic activity, with HPA as substrate, is that published by North and Whyte (1984).

HPA was added to distilled water (10 mg/ml) and sonicated to give a homogenous suspension. 0.5 ml of the HPA suspension was added to 0.5 ml aliquots of the following incubation buffers; 100 mM KCl/HCl pH 2.0; 100 mM citric acid/sodium citrate buffer, pH 3.0, 4.0, 5.0 and 6.0; 100 mM potassium phosphate buffer pH 7.0 and 8.0, and 100 mM glycine/sodium hydroxide buffer pH 9.0. 0.1 ml aliquots of each of the proteinases (0.1 mg/incubation mixture), pepsin, chymotrypsin, trypsin and protease V were added to each of the buffered solutions containing HPA, pH 2-9. Similarly, 0.1 ml aliquots of each of the pike liver, stomach and intestinal homogenates were incubated with HPA, pH 2-9. The tubes containing commercial proteinases were incubated at 35°C and those containing pike tissue homogenates at 25°C. The reaction was

stopped by the addition of 0.2 ml, 50% trichloroacetic acid to each of the incubation mixtures. After centrifugation (400 rpm, 5 min.), the absorbance at 595 nm was determined in each of the resulting supernatants.

3.3.3.3 Incubation of commercial proteinases and pike liver and digestive tissue proteinase preparations at their pH optima for proteolytic activity, with Eulan WA New

The pH optima for proteolytic activity (defined using HPA as substrate) for the following proteinases were: Pepsin (pH 2.0), chymotrypsin (pH 7.0), protease V (pH 7.0) and trypsin (pH 8.0). 0.1 ml of each of the proteinases (0.1 mg/tube) were incubated with Eulan WA New (20 µg/tube) in incubation buffers of total vol. 2 ml at the pH values defined for their maximum proteolytic activity. After incubation at 35°C for 3h the incubation mixtures were extracted and analysed by HPLC.

The pH optima for proteolytic activity of the pike liver and digestive tissue homogenates were; liver homogenate (pH 3.0), stomach homogenate (pH 1.5 and 3.0) and intestinal tissue homogenate (pH 7.0 and 8.0). 100 µl aliquots of the homogenates were incubated with Eulan WA New (20 µg/tube) at these pH values and the same procedure followed as described above.

3.3.3.4 The incubation of Eulan WA New with pike liver and digestive tissue homogenates, pH 2→9

Eulan WA New (20 µg/tube) was added to incubation buffers pH 2→9 (the buffers have been previously described in section 3.3.3.2). 100 µl aliquots of pike liver, stomach and intestinal homogenates were each added to the buffers containing Eulan WA New (pH 2→9). The incubation mixtures of total vol. 2 ml, were incubated at 25°C for 3h. After incubation, the mixtures were extracted by MTBE and analysed by HPLC.

3.3.3.5 The incubation of pieces of pike liver, stomach and intestinal tissue in buffers (pH 2–9) containing Eulan WA New

Small pieces of pike liver, stomach and intestinal tissue were taken from a pike immediately after death, and transferred to 2 ml of incubation buffers (pH 2–9) containing Eulan WA New (20 µg/tube). After 1h incubation both tissue pieces and surrounding buffer were extracted and anlysed by HPLC (the extraction of the surrounding buffer involves the standard MTBE extraction procedure). The MTBE extraction of tissue pieces is described in section 2.3.7.1 .

3.3.3.6 Preparation of the 100,000 g supernatant fraction of pike tissues and the liver microsomal pellet

Liver, stomach, intestine, kidney, eye, gills, skin/muscle, spleen, brain and swim-bladder, were quickly dissected from a pike immediately after death. The tissues were cut into small pieces and placed in 2 volumes of homogenisation buffer; 0.25 M sucrose/0.1 M potassium phosphate pH 7.4, and each homogenised by ten strokes of a motor-drive glass-in-teflon homogeniser. The crude homogenates were placed in 10 ml screw-cap centrifuge tubes and centrifuged at 35,000 rpm (100,000 g) in a 10 × 10 ml rotor in the MSE 65 superspeed centrifuge. The clear supernatants were removed (using a pasteur pipette) from both the fatty surface layer and microsomal pellet.

The pike liver microsomal fraction was prepared from pike liver crude homogenate by the insertion of an additional centrifugation step. The crude homogenate was transferred to a 15 ml tube, placed in a 16 × 15 ml rotor and spun at 10,000 rpm (10,000 g) in the MSE 18 centrifuge. The resulting 10,000 g supernatant was placed in a 10 ml screw-cap tube and centrifuged at 100,000 g as described in the previous paragraph. The supernatant was decanted leaving the microsomal (100,000 g) pellet.

3.3.3.7 Assay for glutathione transferase activity in pike tissue 100,000g supernatants and pike liver microsomal pellet

Glutathione transferase activity was assayed by monitoring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) spectrophotometrically as described by Habig et al, (1974). The assay mixtures of total volume 3 ml contained 0.1 M potassium phosphate buffer pH 6.5, 1 mM GSH (200 µl, 15 mM GSH), 1 mM CDNB (50 µl of a 60 mM CDNB in DMF solution) and 50 µl of the 100,000 g pike tissue supernatants. The addition of the 100,000 g supernatant initiated the conjugation reaction which was monitored at 344 nm.

50 µl aliquots of pike liver 100,000 g pellet resuspended in 1 ml 0.1 M potassium phosphate buffer, pH 7.5, were incubated at 20°C for 30 min. in the presence and absence of iodoacetamide (10 mM). Following incubation, 50 µl aliquots of the samples were assayed for glutathione transferase activity as described for the 100,000 g supernatant fractions. An increased concentration of GSH (5 mM) was added to assay mixtures to allow for the reaction of GSH with excess iodoacetamide.

3.3.3.8 Incubation of Eulan WA New with pike tissue 100,000 g supernatants under conditions for the assay of glutathione transferase activity

50 µl aliquots of each of the pike tissue 100,000 g supernatants were added to 0.1 M potassium phosphate buffer (pH 6.5) containing Eulan WA New (20 µg/tube) and GSH (5 mM), giving a final incubation mixture volume of 2 ml. After incubation at 25°C for 3h the samples were extracted with MTBE and analysed by HPLC.

3.3.3.9 Protein determination in fish tissue fractions

The protein concentration in fish tissue fractions was determined by the method of Lowry et al (1951). Aliquots of a bovine serum albumin protein standard (10 mg BSA in 10 ml distilled water) were transferred to glass test tubes giving the range of protein 0, 25, 50, 100, 150 and 200 µg. 20 µl of fish tissue fractions were diluted with distilled water to a volume of 200 µl, and 50 µl aliquots, of each of the samples were transferred to glass test tubes. The volume of each tube was adjusted to 200 µl by the addition of the necessary volumes of distilled water. 250 µl of 1 M sodium hydroxide/0.25% sodium dodecyl sulphate was added to each of the samples, which were allowed to stand at room temperature for a period of 30 min. Reagent D, of the following composition was prepared upon requirement:

50 ml 2% sodium carbonate

0.5 ml 1% copper sulphate ($CuSO_4 \cdot 5H_2O$)

0.5 ml 2% sodium potassium tartrate.

2.5 ml aliquots of Reagent D were added to each of the samples, each sample was mixed and allowed to stand at room temperature for a further 15 min. Additions of 250 µl of freshly prepared Folin-Ciolalteu reagent (diluted 1:1 with distilled water) followed by rapid mixing, resulted in the development of colour over a period of 45 min. The absorbance at 750 nm at the end of this period was determined for each of the samples.

3.3.3.10 Preparation of Rat, Pike and Trout 10,000 g supernatants and their addition to incubation mixtures containing Eulan WA New and 5-PAD

Livers were removed from a rat, pike and trout, immediately after death. 1 g of liver tissue from each was placed in 4 ml of ice-cooled homogenisation buffer, 0.25 M sucrose/0.1 M potassium phosphate pH 7.4. The tissues in buffer were each placed in an ice-bucket and homogenised by ten strokes of a motor-driven glass-in-teflon homogeniser. The resulting crude homogenates were transferred to 15 ml centrifuge tubes and placed in a 16 × 15 ml rotor which was centrifuged at 10,000 rpm (10,000 g) in the MSE 18 centrifuge for 45 mins at 4°C. Aliquots of the resulting 10,000 g supernatants were added to the incubation mixtures described below.

The following incubation mixtures were prepared in 0.1 M potassium phosphate, pH 7.4 incubation buffer, of total volume 2 ml:

i) UDPGA (2 mM), $MgCl_2$ (5 mM) and (a) Eulan WA New (20 µg/tube)
(b) 5-PAD (20 µg/tube)

ii) NADPH (2 mM) and (a) Eulan WA New (20 µg/tube)
(b) 5-PAD (20 µg/tube)

iii) Acetyl CoA (0.5 mM) and (a) Eulan WA New (20 µg/tube)
(b) 5-PAD (20 µg/tube)

100 µl aliquots of the rat 10,000 g supernatant were added to each of the above incubation mixtures, and the procedure repeated for both pike and trout 10,000 g supernatants. The incubation mixtures containing rat 10,000 g supernatant, were incubated at 35°C, and those containing pike and trout 10,000 g supernatants, 25°C for a 3h period. Upon completion of the incubation the incubation mixtures were extracted with MTBE and further analysed by HPLC.

3.3.4.1 The dosing of goldfish with 6-PCSD, and the removal of liver, spleen and digestive tract tissue, and contents, to study the *in vitro* metabolism of 6-PCSD

The dosing of goldfish with 6-PCSD, involves in summary: the dosing of water with 6-PCSD and addition of goldfish, removal and dissection of fish after dosing, tissue extraction, extract 'clean-up' and HPLC or GLC analysis. These methods have been previously described in this work, sections 2.3.3 to the end of 2.3.7.

In this particular study, eight goldfish were employed, seven were dosed with 6-PCSD (0.4 ppm) for a period of 2h, and one placed in a control bowl containing no 6-PCSD for the same period. A sample dosed goldfish and a control goldfish were each dissected and the liver, spleen and digestive tract tissues, and digestive tract contents were extracted and further analysed by HPLC.

3.3.4.2 Incubation of goldfish digestive tract contents with 6-PCSD

Goldfish digestive tract contents were exuded from the digestive tracts of dosed goldfish by gently rolling a Pasteur pipette along the length of the tissue. The contents from each of the six digestive tracts were each placed in tubes containing 10 ml of sterilised microflora growth medium A (described by Tokieda et al, 1979, but with 1 ppm 6-PCSD replacing the reported cyclohexylamine). Three of the six tubes were flushed with carbon dioxide and sealed, so providing an anaerobic environment for incubation. The remaining tubes were incubated aerobically. The samples were incubated at 25°C for a period of up to one week, with sampling of both anaerobic and aerobic incubation mixtures at time zero, 3 days and 1 week. A separation of the digestive contents after incubation, into pellet and supernatant fractions by a low speed centrifugation step (500 g, 3 min) was carried out. The pellet fractions were extracted as described for fish tissues

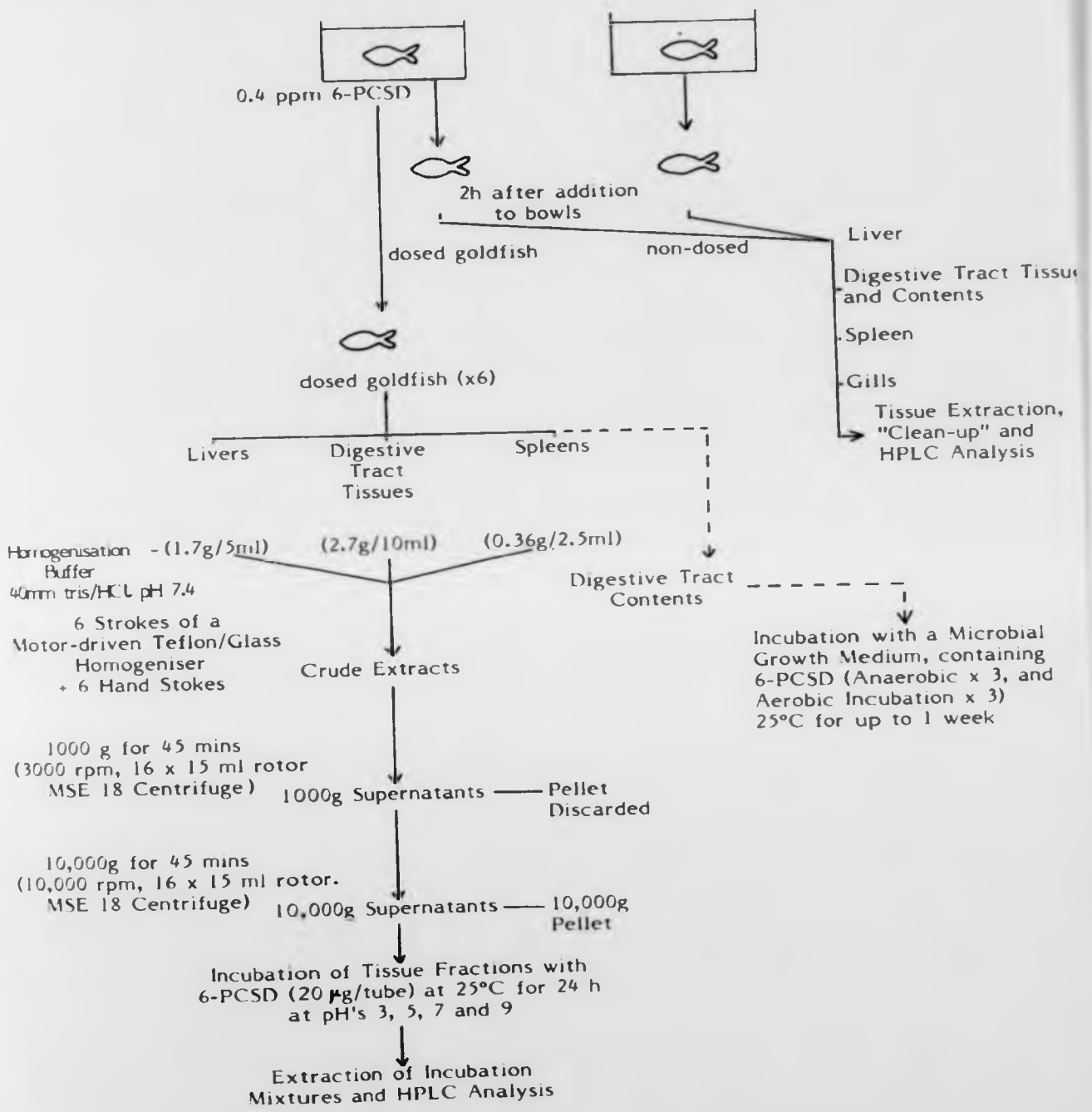


Fig.4 SUMMARY OF THE EXPERIMENTAL PROCEDURE FOR THE STUDY OF THE METABOLISM OF 6 - PCSD BY GOLDFISH LIVERS, SPLEENS AND DIGESTIVE TISSUE FRACTIONS AND DIGESTIVE TRACT CONTENTS FROM GOLDFISH DOSED WITH 6 - PCSD

(section 2.3.7) and the supernatants by the MTBE standard extraction procedure. Unwanted coextractants were removed from pellet extracts by the mixed alumina column clean-up procedure described in section 2.4.3, before HPLC analysis of both the pellet and supernatant samples.

3.3.4.3 Preparation of subcellular fractions from dosed goldfish tissues, and their *in vitro* incubation with 6-PCSD

The procedures described in this section are summarised in Figure 4. Livers, spleens and digestive tract tissues dissected from goldfish dosed with 6-PCSD for 2h, were each added to the homogenisation buffer, 40 mM Tris/HCl pH 7.4. A total of; 1.67 g liver tissue in 5 ml buffer; 0.36 g spleen tissue in 2.5 ml buffer and 2.73 g digestive tract tissue in 10 ml buffer, were homogenised by six rotating strokes and six hand-strokes of a motor-driven teflon-in-glass homogeniser. From the resulting crude homogenates, subcellular fractions were prepared up to the 10,000g supernatants and pellets, as described in Figure 4. 50 µl aliquots of each of the fractions, from each of the tissues, were incubated with 6-PCSD (20 µg/tube; 20 µl of a 1 mg/ml 6-PCSD in DMF solution) in pH 3.0 (40 mM glycine/HCl); pH 5.0 (40 mM acetate/acetic acid); pH 7.4 (40 mM Tris/HCl) and pH 9.0 (40 mM glycine/NaOH) buffers. The final volume of each of the incubation mixtures was 2 ml. After incubation at 25°C for 24h the samples were extracted using MTBE and analysed by HPLC.

3.3.5. Preparation of Goldfish liver homogenate

A goldfish was killed by severing the spinal column and the liver tissue (≏ 0.3 g) removed and placed in ice-cooled homogenisation buffer, 40 mM Tris/HCl pH 7.4 (3 ml). The tissue was homogenised by six strokes of a motor-driven teflon-in-glass homogeniser followed by six hand strokes. A 400 µl aliquot of the resulting goldfish homogenate was incubated with 6-PCSD (20 µg, 20 µl of 1 mg/ml 6-PCSD in DMF solution) in 40 mM Tris/HCl pH 7.4 as incubation buffer, for 8h at 19°C. The final volume of each of the incubation mixtures was 2 ml. Following incubation the tubes were extracted with MTBE and analysed by HPLC.

3.3.6.1 Incubation of Goldfish liver homogenate with 6-PCSD in a range of incubation buffers

400 µl aliquots of goldfish liver homogenate (prepared as described above) were added to the following incubation buffers containing 6-PCSD (20 µg); 40 mM Tris/HCl pH 7.4; 0.25 M sucrose/ 40 mM Tris/HCl pH 7.4; 40 mM potassium phosphate buffer pH 7.4; 40 mM Tricine/NaOH buffer pH 7.4 and 40 mM Hepes/NaOH buffer pH 7.4. The incubation mixtures (final volume 2 ml) were incubated at 19°C for 2h, extracted with MTBE and analysed by HPLC.

3.3.6.2 Preparation of Goldfish liver homogenate in 40 mM Tris/HCl pH 7.4 and 40 mM potassium phosphate pH 7.4

Livers were taken from six goldfish immediately after death and were each individually divided into two equal portions by weight. One half of the liver fractions was placed in 40 mM Tris/HCl pH 7.4 and the second half in 40 mM potassium phosphate homogenisation buffers (0.9 g tissue in 9 ml buffer). The tissue samples were homogenised by the method described previously in 3.3.5.1. 400 µl aliquots of each of the liver homogenate samples were incubated at 19°C for 8h with the respective homogenisation buffers containing 6-PCSD (10 µg/tube).

A sample of the liver homogenate in phosphate buffer was transferred to a 15 ml centrifuge tube, placed in a 16 × 15 ml rotor and centrifuged at 3000 rpm (1000 g) in the MSE 18 centrifuge for 30 min. at 4°C. A 400 µl aliquot of the resulting supernatant was incubated with 6-PCSD as described above for the tissue homogenate.

Incubation studies with Goldfish liver homogenate

3.3.6.3 A study of the time course of incubation of Goldfish liver homogenate with 6-PCSD

Goldfish liver homogenate was prepared in 40 mM potassium phosphate buffer pH 7.4, as described previously in section 3.3.5.1. 400 µl aliquots of the homogenate were incubated with 6-PCSD (10 µg/tube) in phosphate buffer at 19°C giving a final incubation mixture volume of 2 ml. The reaction was stopped by the extraction of incubation mixtures with MTBE after 0, 1, 2, 4 and 8h of incubation. The resulting extracts were prepared for HPLC injection and analysis.

3.3.6.4 The incubation of Goldfish liver homogenate prior to assay for 5-PAD forming activity

Goldfish liver homogenate was incubated at 19°C for up to 8h. 400 µl aliquots were taken after incubation for 0, 1, 2, 4 and 8h and added to 40 mM potassium phosphate buffer pH 7.4 containing 6-PCSD (10 µg/tube), (giving a total incubation mixture volume in each case of 2 ml) and were incubated at 19°C for 8h. After the incubation period with 6-PCSD the samples were extracted with MTBE and analysed by HPLC.

3.3.6.5 Incubation of Goldfish liver homogenate with Bovine serum albumin (BSA), Glycerol, sucrose, phenylmethylsulphonyl fluoride (PMSF), p-hydroxychloromecuribenzoate (pHMB) and Dithiothreitol (DTT); prior to incubation with 6-PCSD

Samples of Goldfish liver homogenate were incubated at 19°C for up to 8h, with each of the following; BSA (1 mg/ml), glycerol (25% w/v), sucrose (0.25 M), PMSF (1 mM), pHMB (1 mM), DTT (5 mM) and in 40 mM potassium phosphate buffer, pH 7.4 alone. At the beginning of the incubation period, and at intervals during this period, 400 µl aliquots were taken from each of the incubated homogenate samples and added to 40 mM potassium phosphate buffer (pH 7.4), containing 6-PCSD (10 µg/tube) and assayed for PAD formation. The same concentration of components in the incubated homogenates were present in the incubation mixtures of the homogenates with 6-PCSD, giving a total volume of incubation mixtures in each case of 2 ml. After incubation with 6-PCSD at 19°C for 8h the incubation mixtures were extracted with MTBE and analysed by HPLC.

3.3.7 Preparation and incubation of Pike liver homogenates with 6-PCSD, in the presence and absence of DTT

DTT (5 mM) was added to one of two 5 ml samples of 40 mM potassium phosphate (pH 7.4), homogenisation buffers. A liver was dissected from a Pike immediately after death and 0.5 g of the tissue transferred to each of the homogenisation buffers (cooled on ice). Homogenisation was carried out as described in section 3.3.5.1 and 400 µl aliquots of the resulting pike liver homogenates were added to incubation mixtures of the respective homogenisation buffers containing 6-PCSD (10 µg/tube) of total volume 2 ml. After incubation with 6-PCSD, at 19°C for 8h, the incubation mixtures were extracted with MTBE and analysed by HPLC.

3.3.8 Preparation and incubation of Goldfish, Carp, Pike, Trout, Perch and Eel liver homogenates with 6-PCSD and Eulan WA New in the presence of DTT

Livers were dissected from goldfish, carp, pike, trout, perch and eel, immediately after death and placed in ice-cooled homogenisation buffer; 40 mM potassium phosphate buffer/5 mM DTT, pH 7.4 (0.1 g tissue/1 ml buffer). Each tissue sample was homogenised as described in 3.3.5.1. 400 µl aliquots of the tissue homogenates were added to the homogenisation buffer containing 6-PCSD (10 µg/tube), giving a total volume of 2 ml for each incubation mixture. After incubation at 19°C for 8h the samples were extracted and analysed by HPLC.

3.3.9.1 The effect of dialysis on the 5-PAD forming activity of Goldfish liver 1000 g supernatant

Goldfish liver homogenate was prepared in 40 mM Tris/HCl pH 7.4 as described in section 3.3.5.1. The resulting crude homogenate was transferred to 15 ml centrifuge tube, placed in a 16 × 15 ml rotor and centrifuged at 3000 rpm (1000 g) (in the MSE 18 centrifuge) for 30 min at 4°C. 2 ml of the resulting 1000 g decanted supernatant was placed in dialysis tubing (18/32) and dialysed against 20 ml of 2 mM Tris/HCl pH 7.4 for a period of 8h at 4°C. After this period, the dialysate was divided into two 10 ml fractions and placed in round-bottom glass flasks of total volume 500 ml. The neck of each flask was covered with cling film and pierced with a needle a number of times. These samples were frozen at -70°C and then quickly transferred to the lyophiliser and placed in an upright position. Following lyophilisation (over a period of 8h) the residues in each flask were redissolved in 0.5 ml distilled water, by carefully rolling the solution around the inner-surface of the flask. Each 0.5 ml sample containing dissolved residues were removed using a Pasteur pipette and their pH measured, and found to be between pH 7.2 - 7.4.

Upon preparation, a sample of goldfish 1000 g supernatant was stored at 4°C. 400 µl aliquots were taken, at the beginning and end of both the dialysis and lyophilisation stages, and incubated with Eulan WA New (10 µg/tube) at 19°C for 3h. This gave a measure of the expected total activity to be found in the dialysed supernatant at these stages. The latter incubation procedure was repeated with 400 µl aliquots of dialysed 1000 g supernatant taken at the completion of both dialysis and lyophilisation stages. A sample of the prepared lyophilised dialysate (0.5 ml) was added to incubation mixtures of both dialysed 1000 g supernatant (400 µl) with Eulan WA New (20 µg/tube), and to Eulan WA New alone. Each of the prepared incubation mixtures described were adjusted to a volume of 2 ml with 40 mM Tris/HCl buffer pH 7.4, and incubated at 19°C for 8h. Following incubation the mixtures were extracted with MTBE and analysed by HPLC.

3.3.9.2 The addition of small molecular weight components to incubation mixtures of dialysed and non-dialysed Goldfish homogenates with 6-PCSD.

Goldfish liver dialysed 1000 g supernatant was prepared in 40 mM potassium phosphate buffer pH 7.4 by the procedure described above. 400 µl aliquots of the dialysed supernatant were incubated with 6-PCSD (20 µg/tube) in 40 mM potassium phosphate pH 7.4 incubation buffer, containing each of the following; NADPH (2 mM), GSH (5 mM), UDPGA (2 mM), ATP (1 mM), ADP (1 mM) and in the phosphate buffer alone. The total volume of each incubation mixture was 2 ml. After incubation at 19°C for 8h, the incubation mixtures were extracted with MTBE and analysed by HPLC.

The above procedure was repeated with the addition of 400 µl aliquots of non-dialysed 1000 g supernatant to incubation mixtures containing 6-PCSD (10 µg/tube) in the phosphate buffer and each of the following;

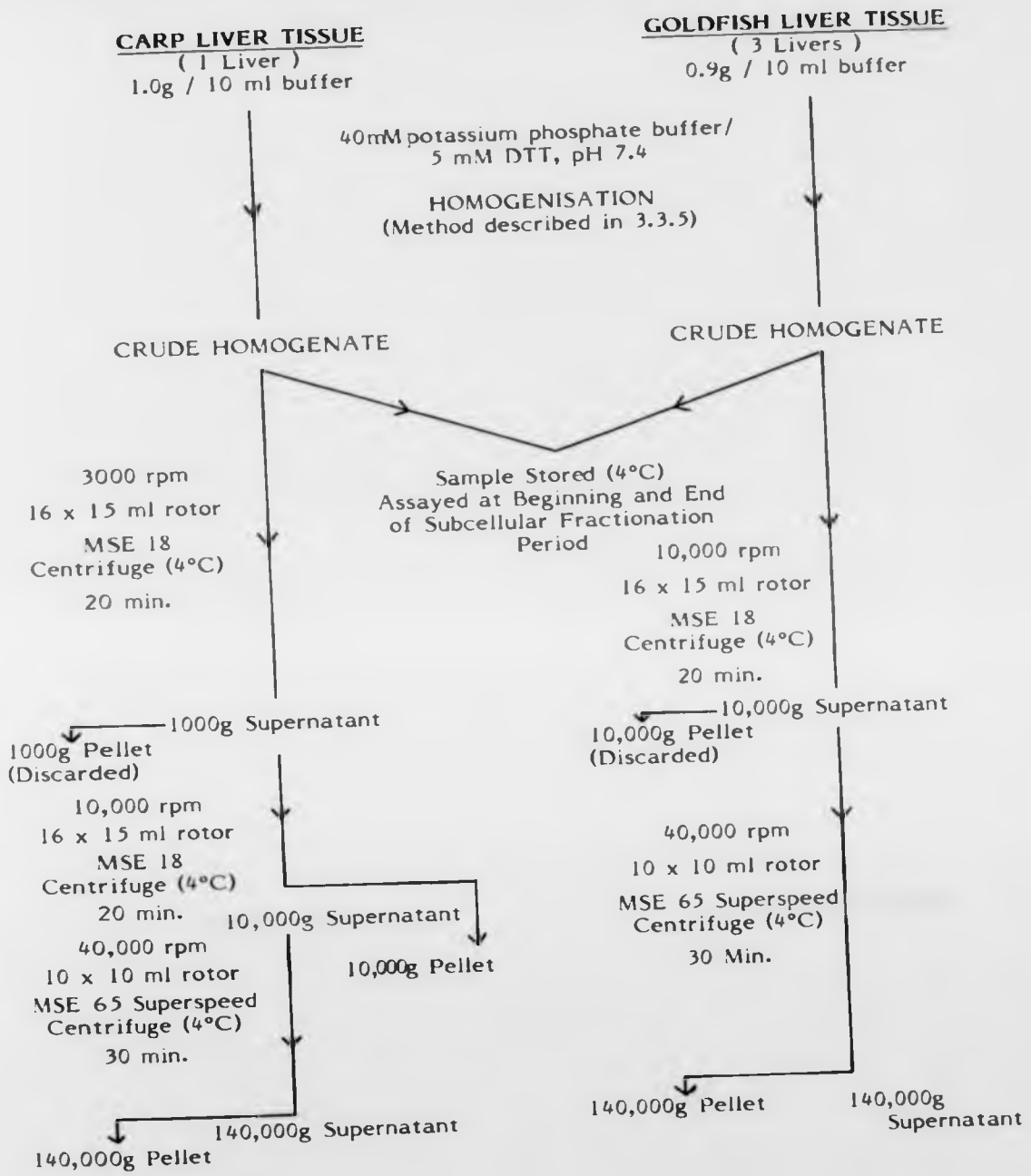


Fig. 5 RAPID SUBCELLULAR FRACTIONATION OF CARP AND GOLDFISH LIVER TISSUE

$NADP^+$ (2 mM), NADH (2 mM), NAD^+ (2 mM), NADPH (2 mM), GSH (5 mM), UDPGA (2 mM), DTT (5 mM); and in the phosphate buffer alone. In addition, a sample of lyophilised dialysate (0.5 ml, prepared as previously described in this section 3.3.9.1) was incubated with non-dialysed liver 1000 g supernatant and 6-PCSD (10 µg/tube).

3.3.9.3 The effect of incubation with NADPH on the 5-PAD forming activity of Goldfish liver homogenate

Goldfish liver homogenate was prepared (as described in section 3.3.5.1) in 40 mM potassium phosphate/5 mM DTT, pH 7.4 homogenisation buffer. The homogenate was divided into two equal samples and to one was added NADPH (2 mM). Both homogenates (in the presence and absence of NADPH) were incubated at 19°C, and 400 µl aliquots removed from each after 0, 1 and 2h of incubation. The aliquots were added to incubation mixtures of 6-PCSD (10 µg/tube) in homogenisation buffer, and in addition NADPH (2 mM) was added to the incubation mixtures containing the homogenate plus NADPH. Following incubation with 6-PCSD at 19°C for 8h, the samples were extracted with MTBE and analysed by HPLC.

3.3.10 Subcellular location of 5-PAD forming activity in Goldfish and Carp livers

3.3.10.1 Carp

A sample of carp liver crude homogenate was stored at 4°C and assayed for 5-PAD forming activity at both the beginning and end of the subcellular fractionation period. This gave a measure of the expected activity to be found in carp liver subcellular fractions.

A rapid subcellular fractionation was carried out as shown in Figure 5. Aliquots of the following subcellular fractions were incubated with 6-PCSD (10 µg/tube) in the incubation buffer; 40 mM potassium phosphate/5 mM DTT, pH 7.4:

(400 µl) 1,000 g supernatant

(400 µl) 10,000 g supernatant

10,000 g pellet (the pellet was not formed after centrifugation so this fraction was not assayed)

(400 µl) 140,000 g supernatant

(400 µl) 140,000 g pellet (of 1 ml resuspended pellet)

(400 µl) 140,000 g supernatant/ (100 µl) 140,000 g pellet (of 1 ml resuspended pellet)

In addition, the 140,000 g supernatants and pellets were individually assayed for 5-PAD forming activity in incubation mixtures containing each of the following NADPH (2 mM), NADH (2 mM), UDPGA (2 mM), GSH (5 mM) and Acetyl CoA (0.5 mM). After incubation at 19°C for 8h the incubation mixtures were extracted and analysed by HPLC.

3.3.10.2 Goldfish

As described in section 3.4.10.1 for the carp, a sample of goldfish liver crude homogenate was stored at 4°C, and assayed at both the beginning and end of the subcellular fractionation period. The rapid subcellular fractionation of goldfish liver homogenate is shown in Figure 5. Aliquots of the following subcellular fractions were incubated with 6-PCSD (10 µg/tube) in the incubation buffer; 40 mM potassium phosphate/5 mM DTT, pH 7.4:

(400 µl) 1,000 g supernatant

(400 µl) 140,000 g supernatant

(100 µl) 140,000 g pellet (of 1 ml resuspended pellet)

(400 µl) 140,000 g supernatant/(100 µl) 140,000 g pellet (of 1 ml resuspended pellet)

In addition, the 140,000 g pellet was incubated with 6-PCSD in incubation mixtures containing NADPH (2 mM) and NADPH (2 mM) plus

NADH (2 mM). Following incubation at 19°C for 8h, the incubation mixtures were extracted and anlysed by HPLC.

3.3.11 Incubation of Goldfish liver homogenate with 6-PCSD in sealed incubation mixtures flushed with oxygen and carbon dioxide

Goldfish liver homogenate was prepared in 40 mM potassium phosphate buffer (pH 7.4) as described in 3.3.5.1. Two 400 µl aliquots were each added to incubation mixtures of 6-PCSD (10 µg/tube) in phosphate buffer, one was immediately flushed with oxygen and sealed, and the second flushed with carbon dioxide and sealed. Both were incubated at 19°C for 8h. After incubation the samples were extracted with MTBE and analysed by HPLC.

Standard conditions for HPLC Analysis

HPLC profiles shown in this section (3.4) were obtained under the following conditions:

Solvent flow rate	-	2 ml/min
Chart speed	-	5 mm/min
HPLC injection volume	-	5 µl or 10 µl of extracted samples redissolved in 50 or 100 µl of Methanol or dimethylformamide
Absorbance setting (230 nm)	-	Either a full scale deflection of 0.2 or 0.1 absorbance units (10 mV maximum deflection).

[Detection of 6-PCSD and 5-PAD in dosed Goldfish tissues extracts (section 3.3.4) required the maximum sensitivity for HPLC analysis. Here, the above standard conditions were changed by altering the absorbance setting giving a maximum deflection of 0.1 absorbance units at 2.5 mV.]

3.4 RESULTS

3.4.1 Extraction efficiencies of 6-PCSD and 5-PAD components from buffers containing Eulan WA New and 5-PAD standards, in the presence and absence of fish tissue homogenates

The extraction of Eulan WA New and 5-PAD standards from buffers of pH 3.0 to 8.0 was studied using both MTBE and hexane as extraction solvents (the method of extraction is described in section 3.3.1). MTBE extracted between 85 and 100% of the major 6-PCSD and major 5-PAD components of Eulan WA New and 5-PAD standards respectively, from buffers of pH 3.0 to 8.0. By comparison, extraction with hexane (by the described extraction procedure) was less efficient, resulting in the extraction of 57-73% of 6-PCSD and 25-35% 5-PAD, from the buffers of pH 3.0 to 8.0 (Fig. 6 a).

Repeated HPLC injections of 10 µl aliquots of Eulan WA New and 5-PAD standards in methanol (50 ppm) were carried out, and the peak heights of the major 6-PCSD and major 5-PAD component of Eulan WA New and 5-PAD standards respectively were measured in the resulting HPLC profiles. A 5% and 6% standard deviation in peak height was observed for 6-PCSD and 5-PAD respectively in the nine injections of the same sample (Table 4). Nine 2 ml samples of 50 mM potassium phosphate buffer, pH 7.4, containing 5 µg of both Eulan WA New and 5-PAD, were extracted with MTBE. The residues from each of the evaporated extracts were resuspended in 100 µl methanol and 10 µl aliquots of each of the samples were injected for HPLC analysis. The peak heights of extracted 6-PCSD and 5-PAD components were measured from the resulting HPLC profiles, and varied by 8% and 13% respectively (Table 5). The variation in peak height upon extraction from the buffer was similar to that observed upon direct injection of the same sample. The amount of 6-PCSD and 5-PAD could therefore be reproducible extracts from the aqueous environment with a variation of approximately 10% in the peak

Table 4

The variation in peak height of the major components 6-PCSD and 5-PAD in repeated HPLC injections of 10 µl aliquots of 50 ppm Eulan WA New and 5-PAD standards in methanol.

10 µl injections	6-PCSD Peak Height (10 units maximum deflection)	5-PAD Peak Height (10 units maximum deflection)
1	5.8	7.4
2	6.3	6.8
3	6.2	7.0
4	6.1	6.5
5	6.3	6.4
6	6.6	6.6
7	6.3	5.9
8	5.8	6.6
9	5.8	6.6
n = 9	$\bar{x}$ = 6.13 $\theta n-1$ = 0.29	$\bar{x}$ = 6.64 $\theta n-1$ = 0.44

Variation in peak height was: 5% and 6% of the mean

Table 5

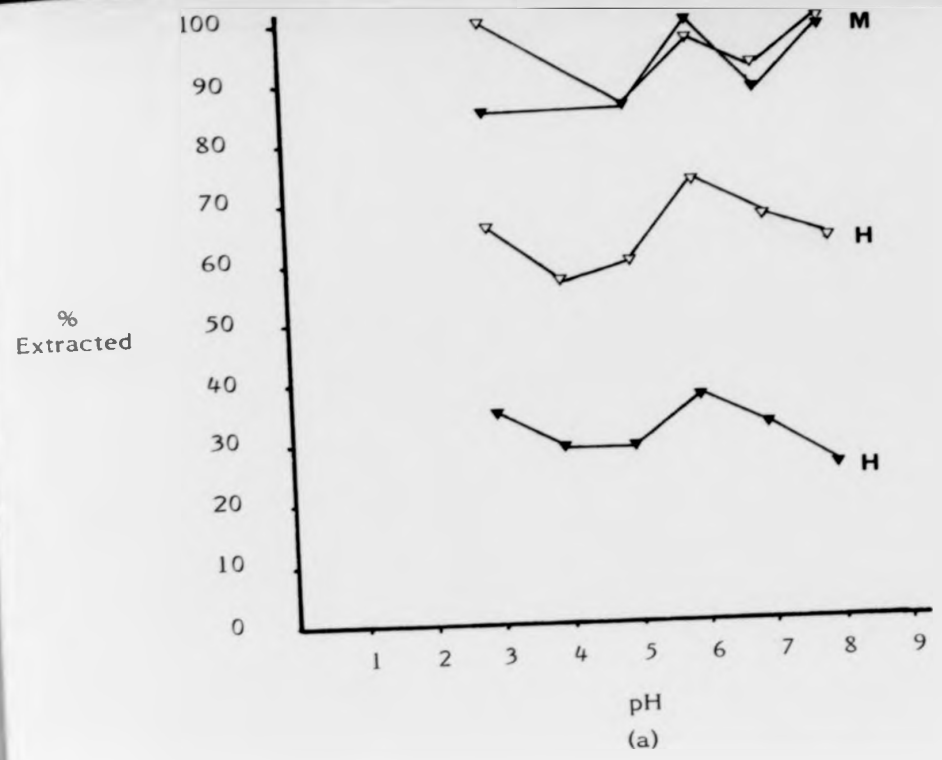
The variation in peak height of the major components 6-PCSD and 5-PAD in HPLC profiles of extracted Eulan WA New and 5-PAD (5 µg each/tube) from buffer pH 7.4.

10 µl injection	6-PCSD Peak Height (10 units maximum deflection)	5-PAD Peak Height (10 units maximum deflection)
1	5.8	6.6
2	7.3	5.9
3	5.7	5.9
4	5.4	6.1
5	4.8	5.9
6	5.7	6.0
7	5.4	5.2
8	5.5	5.2
9	5.8	6.4
n = 9	$\bar{x}$ = 5.71	$\bar{x}$ = 5.91
	θ_{n-1} = 0.65	θ_{n-1} = 0.46

Variation in peak height was: 8% and 13% of the mean

Composition of extracted samples: 5 µg Eulan WA New; 5 µg 5-PAD in 2 ml 50 mM potassium phosphate buffer pH 7.4.

Following extraction with MTBE, the residue was redissolved in 100 µl methanol and 10 µl aliquots injected for HPLC analysis.



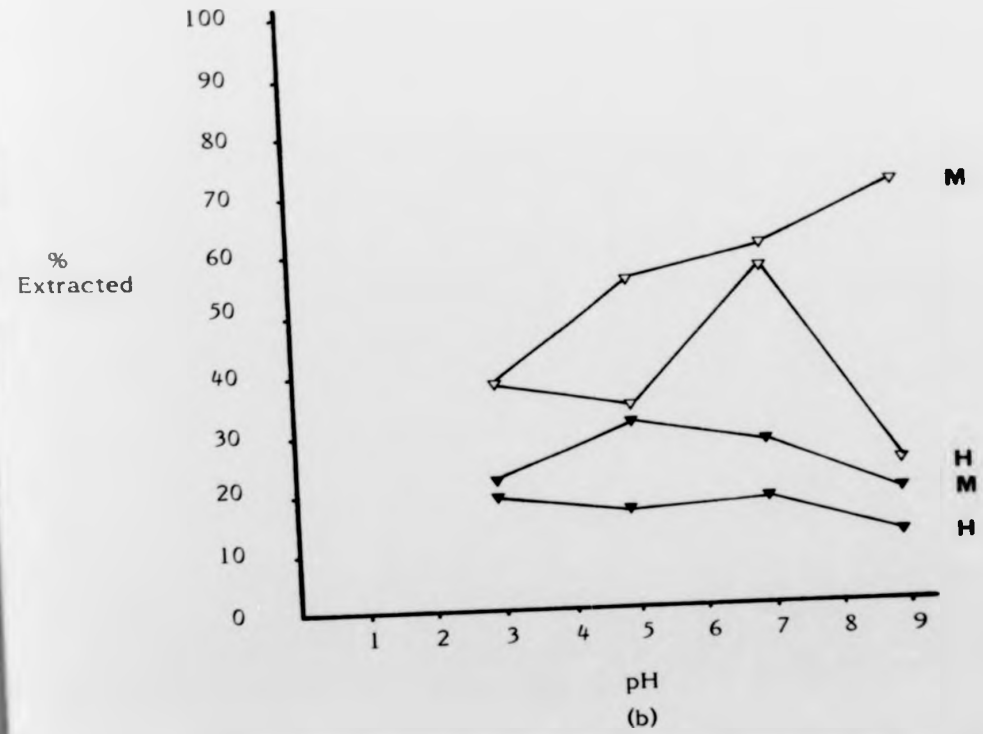


Fig 6. Extraction efficiencies of 6-PCSD and 5-PAD from buffers pH 3 - 9 in the absence (a) and presence (b) of fish tissue homogenates

height of 6-PCSD and 5-PAD components. The injection of 10 µl of the 100 µl methanol sample, from extraction of the buffered solution, is an injection of 0.5 µg Eulan WA New and 5-PAD upon the HPLC column. A 10% variation in peak height is equivalent to a detection of 0.5 µg ± 0.05 µg 6-PCSD and 5-PAD by the HPLC system. The extraction efficiencies of 6-PCSD and 5-PAD from the buffer were calculated to be 93 and 89% respectively (data from Table 1 and 2), this agrees with values plotted in Figure 6a.

400 µl aliquots of pike liver homogenate were added to buffers of pH 3.0 to 9.0 containing Eulan WA New and 5-PAD. Substantially lower extraction efficiencies of 6-PCSD and 5-PAD were observed, compared with the values observed in the absence of the homogenate (Fig. 6b). However the two solvents did not differ widely in their ability to extract these components. Generally a higher percentage extraction of 6-PCSD and 5-PAD was observed by MTBE from these samples. MTBE extracted between 38-70% 6-PCSD and 18-31% 5-PAD over the pH range of 3.0 to 9.0 in the presence of the tissue homogenate.

The addition of different tissue homogenates and a sample of goldfish bile to pH 7.4 buffer containing Eulan WA New and 5-PAD, and their subsequent extraction with MTBE, resulted in varying extraction efficiencies with the differing tissue samples. The percentage of 6-PCSD extracted from the different tissue samples was observed to vary more widely than that of 5-PAD (Table 6).

Table 6

The percentage of 6-PCSD and 5-PAD extracted from pH 7.4 buffer containing Goldfish tissue homogenates

Goldfish Tissue Homogenates	% Extraction 6-PCSD	5-PAD
Gills	69	38
Fat	40	38
Bile	79	33

As well as a variation in the extraction efficiencies of 6-PCSD and 5-PAD from incubation mixtures containing different tissue homogenates; extraction efficiency will probably also depend on the particular tissue subcellular fraction added to incubation mixtures. A later study (sections 3.4.3.4, 5 and 6) involved the addition of 10,000 g supernatants of rat, pike and trout livers to incubation mixtures (pH 7.4) containing Eulan WA New and 5-PAD; extraction efficiencies for 6-PCSD and 5-PAD of between 80-100% using the documented MTBE extraction procedure were obtained.

3.4.2.1 The alkaline hydrolysis of Eulan WA New and its individual PCSD components

The individual PCSD components were prepared from Eulan WA New by a semi-preparative HPLC method described in section 3.3.2.1. The conditions for alkaline hydrolysis of Eulan WA New and PSCD components are described in section 3.3.2.2.

Alkaline hydrolysis of Eulan WA New resulted in the formation of at least three products absorbing at 230 nm in the HPLC profile, and which are resolved from the individual PCSD components of Eulan WA New (Fig. 7b). Alkaline hydrolysis of the individual PCSD components of Eulan WA New assisted in the identification of four new products in HPLC profiles, one of which was not completely resolved from the 7-PCSD

HPLC profiles of:

(a) Eulan WA New

1 - 6-PCSD
2 - 7-PCSD
3 - 5-PCSD

(b) Eulan WA New after alkaline hydrolysis

(c) 5-PCSD after alkaline hydrolysis (Peak II - 4-PAD)

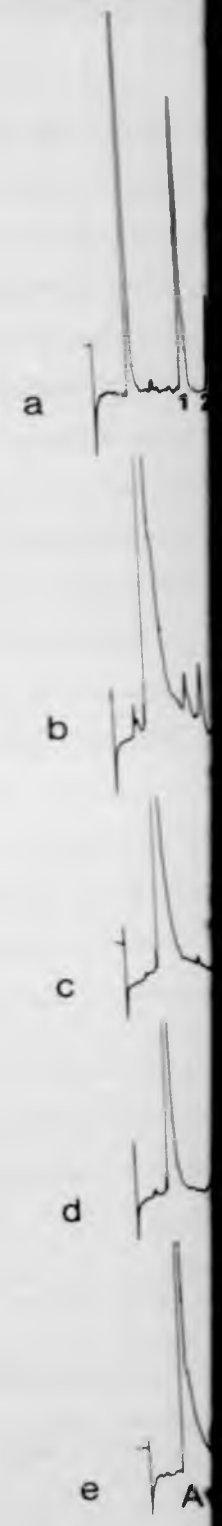
(d) 7-PCSD after alkaline hydrolysis (Peak IV - 6-PAD)

(e) 6-PCSD after alkaline hydrolysis (Peak I - Unknown
III - 5-PAD)

A - This peak on each of the HPLC profiles absorbing strongly at 230 nm, is that of DMF, the solvent used to redissolve the prepared residues prior to HPLC injection.

Figure 7

Alkaline hydrolysis of Eulan WA New and its individual PCSD components.



rolysis

s (Peak II - 4-PAD)

s (Peak IV - 6-PAD)

s (Peak I - Unknown
III - 5-PAD)

profiles absorbing strongly

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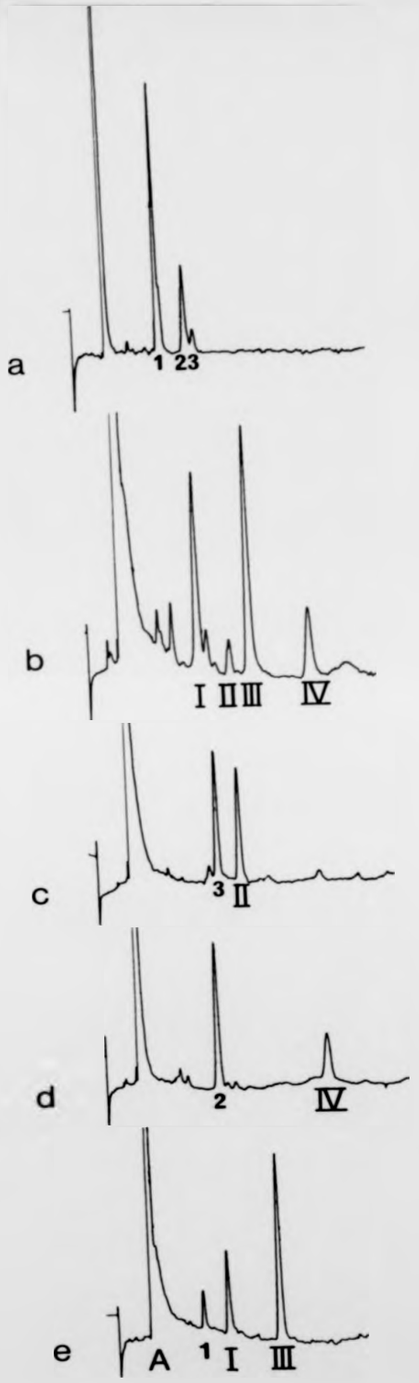


component of Eulan WA New by the HPLC system. The major 6-PCSD component of Eulan WA New is observed upon HPLC analysis to be a doublet peak, the minor component not being completely resolved from the major 6-PCSD component (Fig. 7a, peak 1). The alkaline hydrolysis of the 6-PCSD component resulted in the formation of two new peaks in the HPLC profile, coeluting with peaks I and III of the Eulan WA New alkaline hydrolysis products (Fig. 7e). Peak I is not resolved from the 7-PCSD component of Eulan WA New. The alkaline hydrolysis of the 7-PCSD component of Eulan WA New, resulted in the formation of a single product in the HPLC profile, coeluting with peak IV in the HPLC profile of Eulan WA New alkaline hydrolysis mixture (Fig. 7d). The alkaline hydrolysis of the minor PCSD component of Eulan WA New in HPLC profiles, 5-PCSD, resulted in the formation of a single major peak which coelutes with peak II in the HPLC profile of the Eulan WA New showing alkaline hydrolysis products (Fig. 7c). [This 5-PCSD sample was contaminated with a small concentration of the neighbouring 7-PCSD component, consequently a small concentration of the 7-PCSD alkaline hydrolysis product can be observed in this HPLC profile, Fig. 7c].

This method of the selective alkaline hydrolysis of Eulan WA New and its individual PCSD components, has allowed the identification of the parent PCSD components from which each of the alkaline hydrolysis products arise:

6-PCSD (1) results in the formation of peaks I and III upon alkaline hydrolysis

7-PCSD (2) results in the formation of peak IV upon alkaline hydrolysis

5-PCSD (3) results in the formation of peak II upon alkaline hydrolysis

Westöö and Norén (1977) identified the presence of 6-PCSD and 7-PCSD components of Eulan WA New. These workers observed that alkaline hydrolysis of Eulan WA New gave rise mainly to the 5-PAD product with increasing amounts of 6-PAD upon prolonged alkaline hydrolysis. Wells (1979), in a more detailed study identified the presence of 7, 6 and 5-PCSD components of Eulan WA New and showed that 6-PCSD was hydrolysed under alkaline conditions to the 5-PAD product. Assuming that alkaline hydrolysis results in the cleavage of the sulphonamido bond of the PCSD components, giving rise to the corresponding amine (PAD) product (the relationship reported by Wells above) then the alkaline hydrolysis products observed in the HPLC profiles can be assigned the structures of a:

Peak II - 4-PAD

Peak III - 5-PAD

Peak IV - 6-PAD

(A proposed structure for peak I cannot be assigned since the parent PCSD component has not been identified in the reported studies. Future work will involve its selective elution by the semi-preparative HPLC method and identification by GC/MS, together with the verification of the structures proposed for the alkaline hydrolysis products.)

3.4.2.2 The chemical breakdown of Eulan WA New upon heating with reduced glutathione (GSH) or uridine-5'-diphospho-α D-glucuronic acid (UDPGA)

The HPLC profile of Eulan WA New was unchanged after heating Eulan WA New in DMF for 3h. However, identical treatment of samples of Eulan WA New in DMF containing either UDPGA or GSH (5 mg/ml), resulted in the appearance of new peaks in the HPLC profiles of the heated samples. The resulting products coeluted in HPLC profiles with the alkaline hydrolysis products (PADs) of Eulan WA New (Fig. 8).

HPLC profiles of:

(a) Eulan WA New in DMF after heating to 100°C for 3h.

(b) Eulan WA New in DMF, containing GSH after heating to 100°C for 3h.

(c) Eulan WA New in DMF, containing UDPGA after heating to 100°C for 3h.

(d) Eulan WA New after alkaline hydrolysis showing

Peaks I - Unknown
II - 4-PAD
III - 5-PAD
IV - 6-PAD

A - DMF peak, the HPLC injection solvent.

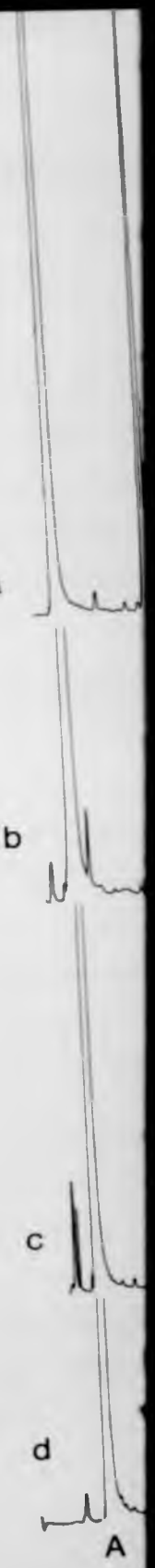


Figure 8

HPLC profiles showing the breakdown of Eulan WA New upon heating with GSH or UDPGA.

to 100°C for 3h.

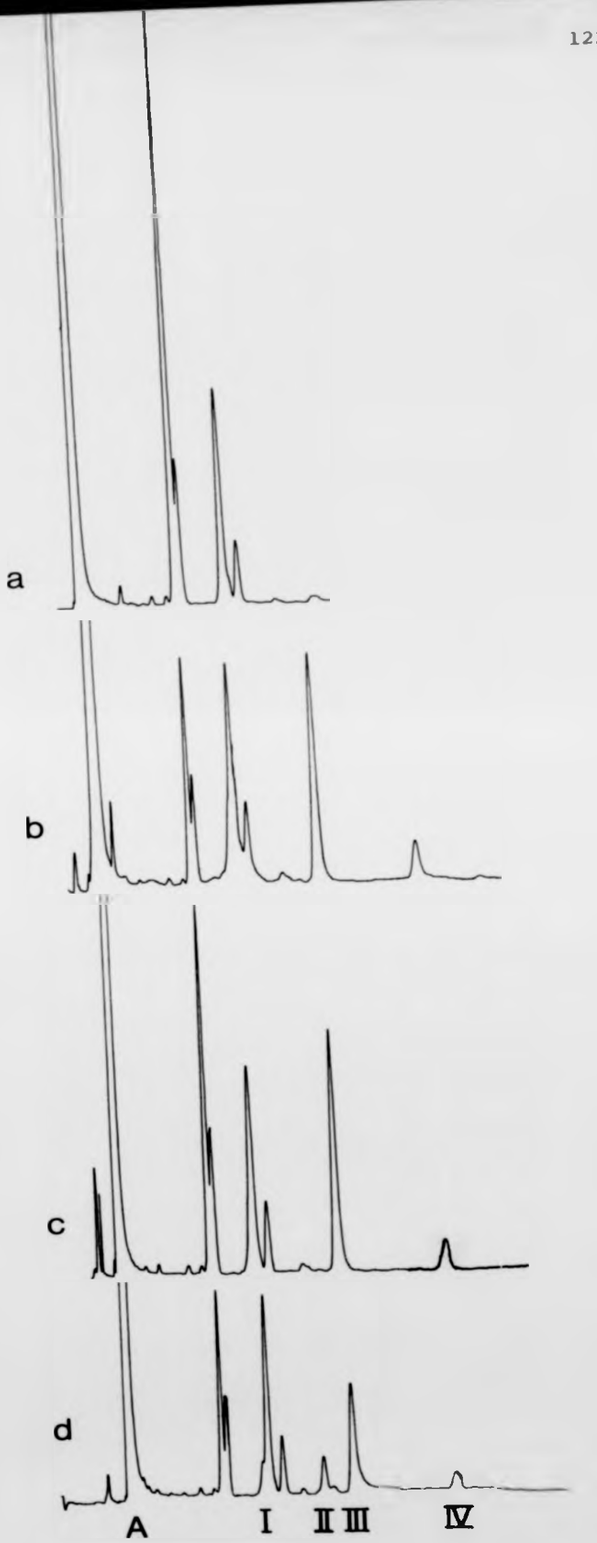
SH after heating to

UDPGA after heating

olysis showing

olvent.

of Eulan WA New upon



3.4.2.3 The chemical modification of Eulan WA New and 5-PAD under acetylating conditions

The conditions for the acetylation of Eulan WA New and 5-PAD are described in section 3.3.2.4.

Eulan WA New

The HPLC profile of Eulan WA New was unchanged after refluxing in the absence of acetic anhydride (Fig. 9a and b). However, after refluxing Eulan WA New for a 30 min.- 2h period, in the presence of acetic anhydride, at least three new peaks were observed upon HPLC analysis (Fig. 9f, peak V, VI and VII).

5-PAD

The HPLC profile of 5-PAD was unchanged after refluxing in the absence of acetic anhydride (Fig. 10a and b). However upon addition of acetic anhydride (at room temperature) the minor 5-PAD component disappeared from HPLC profiles with a corresponding increase of a new peak (Fig. 10c, peak VIII), over a period of 1-12h at room temperature the major 5-PAD component decreased in HPLC profiles with the corresponding increase of a second new peak (Fig. 10d-g, peak IX). Refluxing of the 12h mixture resulted in the further modification of this second peak to form the third new peak (Fig. 10h, peak X).

From the HPLC profiles, showing products of 5-PAD formed under acetyling conditions, it is reasonable to propose the formation of monoacetylated major 5-PAD at room temperature, and the bisacetylated 5-PAD upon refluxing. In agreement with this proposal was the identification by GC/MS analysis of both mono- and bisacetylated amino products, in a sample of PADs refluxed under acetylating conditions by Westöö and Nóren (1977).

HPLC profiles of:

(a) Eulan WA New standard

(b) Eulan WA New after refluxing in the absence of acetic anhydride

(c) Eulan WA New upon addition of acetic anhydride and refluxing for periods of

(d) 30 min

(e) 1h

(f) 2h

New peaks formed under acetylating conditions are labelled V, VI and VII.

A DMF, HPLC injection solvent

(NB. Profile (d) is skewed to the right of profiles (c), (e), (f) and is therefore not totally in alignment with the latter profiles.)

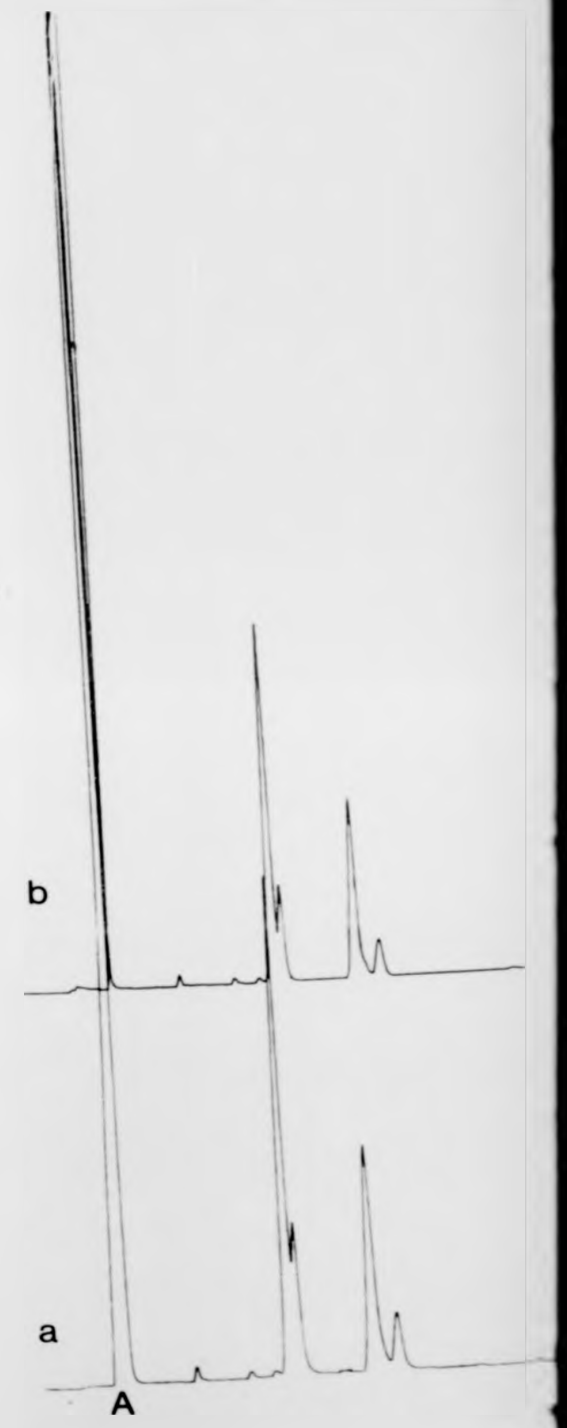


Figure 9

The chemical modification of Eulan WA New under acetylating conditions.

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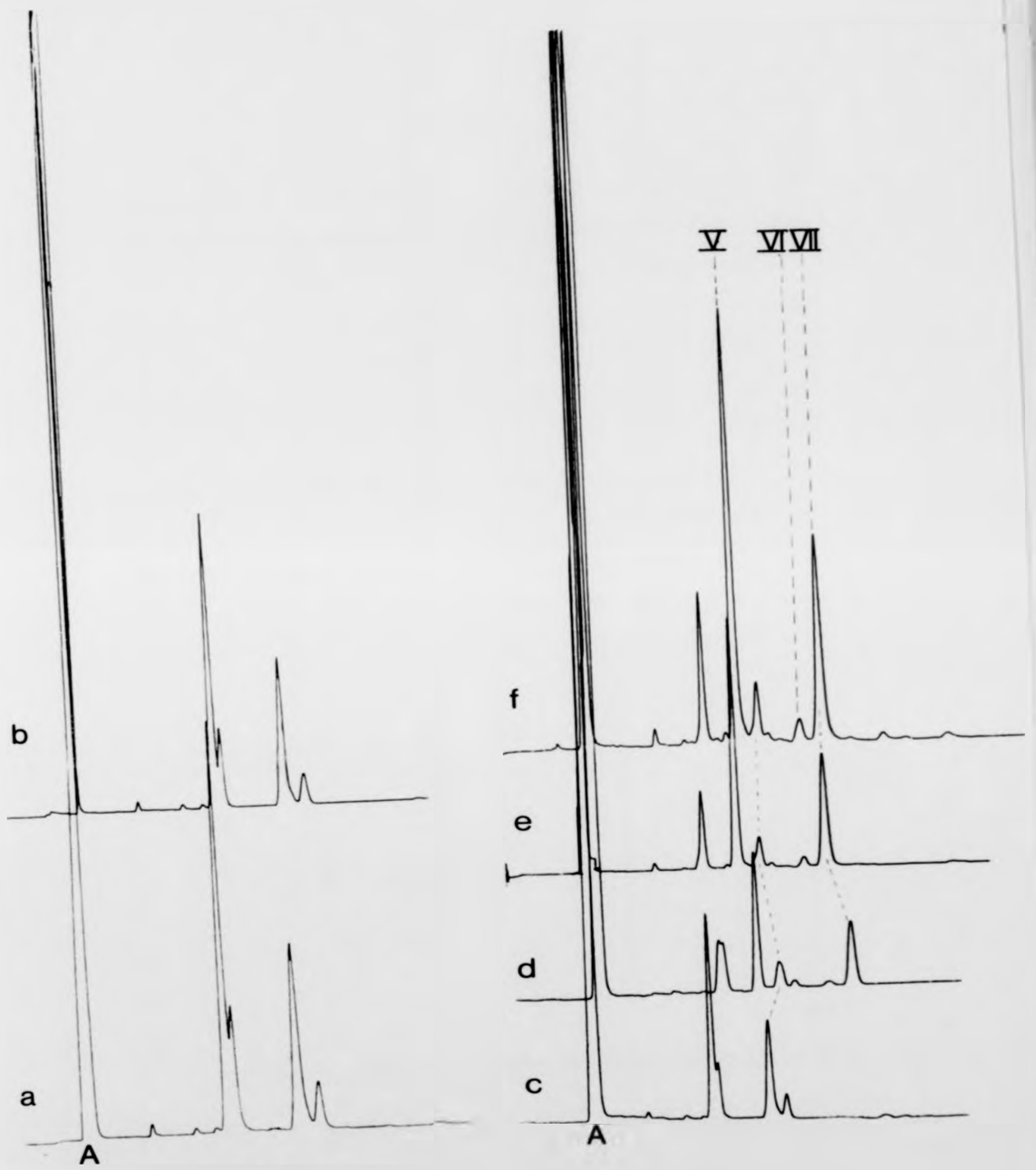
A New under



HPLC profile of:

(a) 5-PAD standard

(b) 5-PAD after refluxinq in the absence of acetic anhydride

(c) 5-PAD upon addition of acetic anhydride and after a period of:

(d) 1h

(e) 2h

(f) 4h

(g) 12h

(h) 12h 5-PAD mixture, refluxed in the presence of acetic anhydride for 3h

New peaks formed under acetylating conditions are labelled VIII, IX and X in HPLC profiles.

A — DMF, HPLC injection solvent

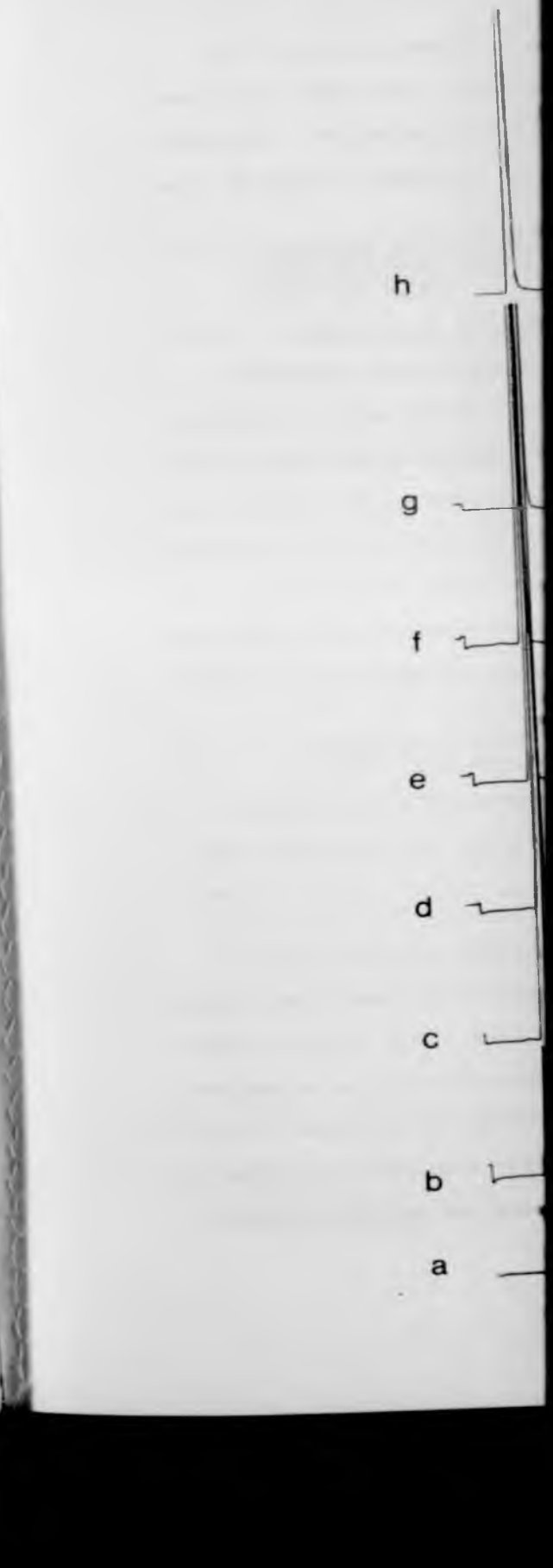


Figure 10

The modification of 5-PAD under acetylating conditions.

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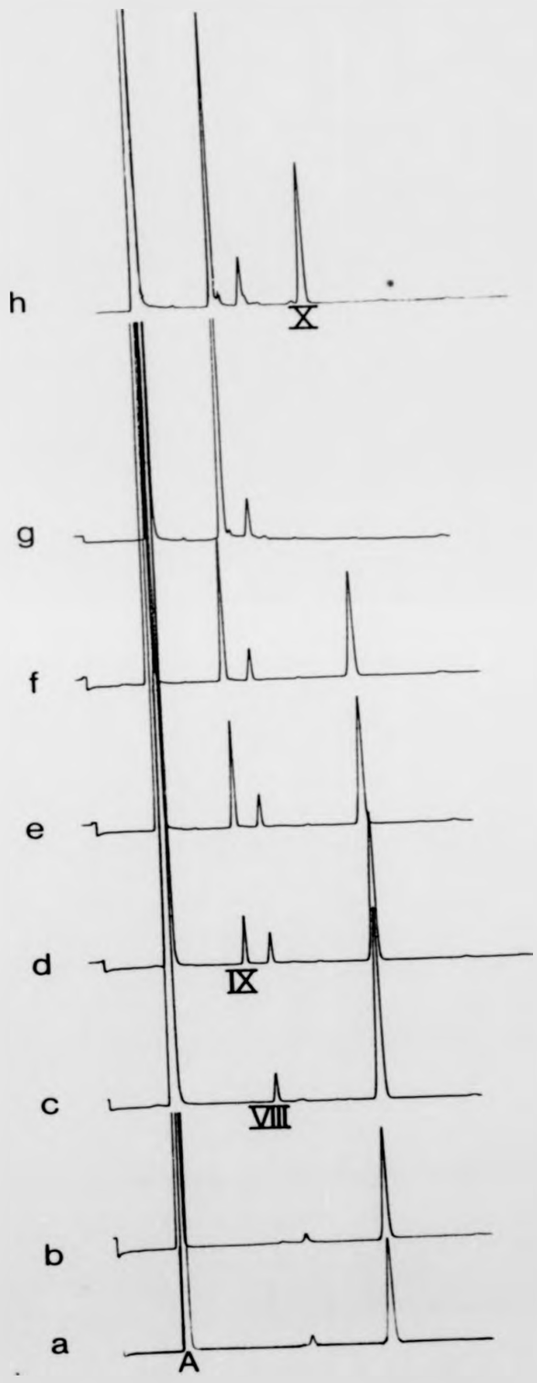
dride and after a

presence of acetic

ditions are labelled

ylating conditions.



The two major products of Eulan WA New which are formed under acetylating conditions, most probably arise from the 6-PCSD doublet component. Preliminary GC/MS analysis of the acetylated products of Eulan WA New has identified the presence of acetylated 6-PCSD.

3.4.3 Incubation of Eulan WA New with proteinases and with crude proteinase preparations from Pike liver and digestive tissue

3.4.3.1 Incubation of Eulan WA New with proteinases

Incubation conditions are described in section 3.3.3.3. The incubation of Eulan WA New with proteinases at their pH optima, defined using HPA as proteolytic substrate (Pepsin, pH 2.0; chymotrypsin, pH 7.0 and Trypsin, pH 8.0), did not result in the formation of PAD in any of the incubation mixtures (Fig. 11). The small concentration of PAD in each of the HPLC profiles coeluting with the major 5-PAD component of the PAD standard, is the small concentration of PAD present in Eulan WA New.

3.4.3.2 Incubation of Eulan WA New with proteinase preparations of Pike liver and digestive tissue

Conditions for the incubation of Eulan WA New with Pike tissue crude homogenates and tissue pieces are described in sections 3.3.3.3, 4 and 5.

The pH optima of proteinase activities in crude homogenates of Pike liver, stomach and intestinal tissue were determined with HPA as substrate (Fig. 12). Eulan WA New was incubated with the tissue homogenates at the defined pH optima, liver pH 3.0, stomach pH 1.5 and 3.0, and intestine pH 7.0 and 8.0. No PAD formation was observed in any of the incubation mixtures. Sample HPLC profiles of extracted incubation mixtures are shown in Figs. 13, 14 and 15.

HPLC profiles of Eulan WA New after incubation with the following proteinases:

(a) Pepsin

(b) Trypsin

(c) Chymotrypsin

(d) Protease V

(e) HPLC profile of PAD standard

A – DMF, HPLC injection solvent

P_1 - 6-PCSD

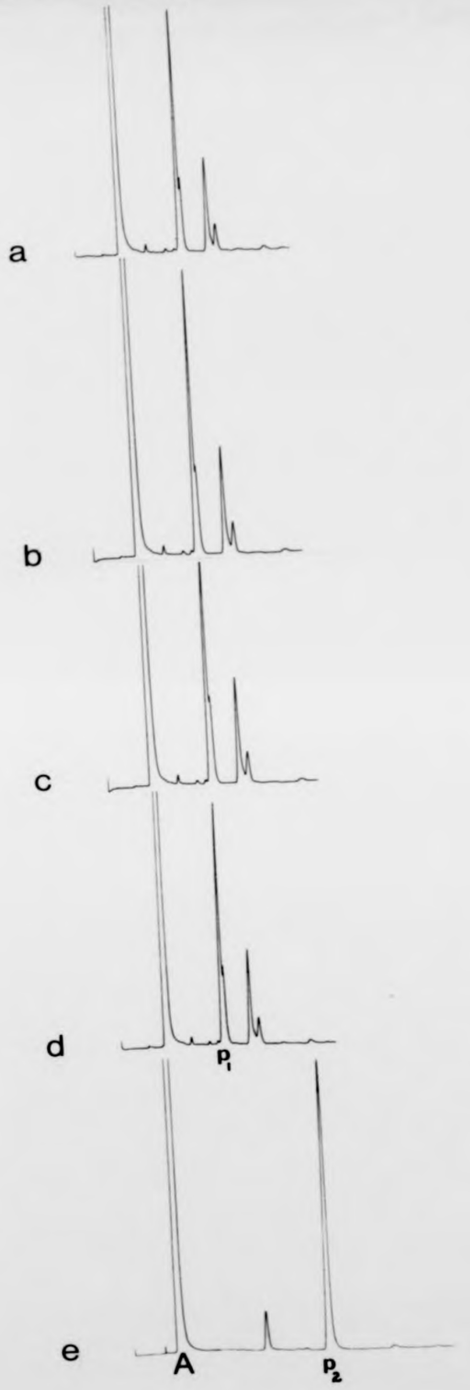
P_2 - 5-PAD

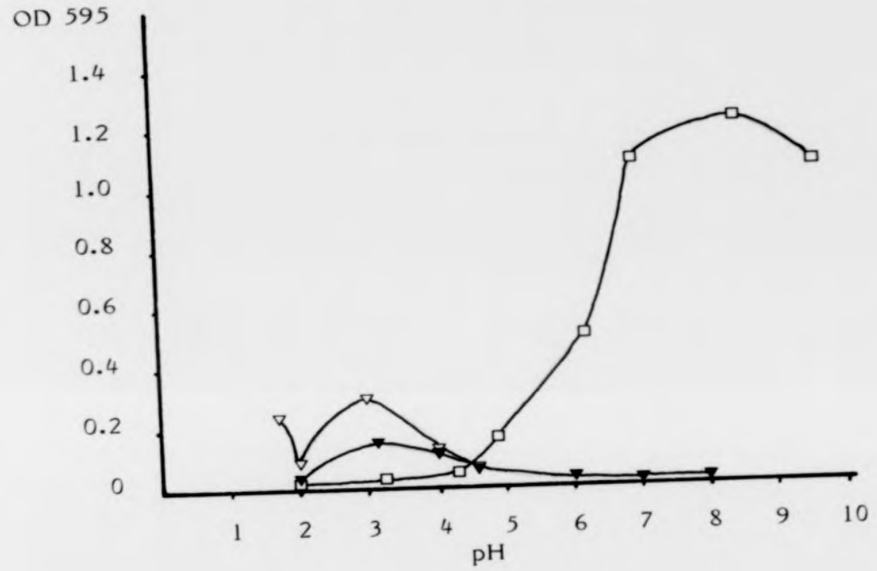
Figure 11

HPLC profile of Eulan WA New after incubation with purified proteinases.

incubation with the

er incubation with





▼ - Liver Homogenate
▽ - Stomach Homogenate
□ - Intestine Homogenate

Fig 12. The pH optima of proteinases in crude homogenates of Pike Liver and digestive tissue, with HPA as substrate

HPLC profile of:

(a) the extracted incubation mixture, of Eulan WA New with pike liver crude homogenate (pH 3.0)

(b) Eulan WA New standard

(c) PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

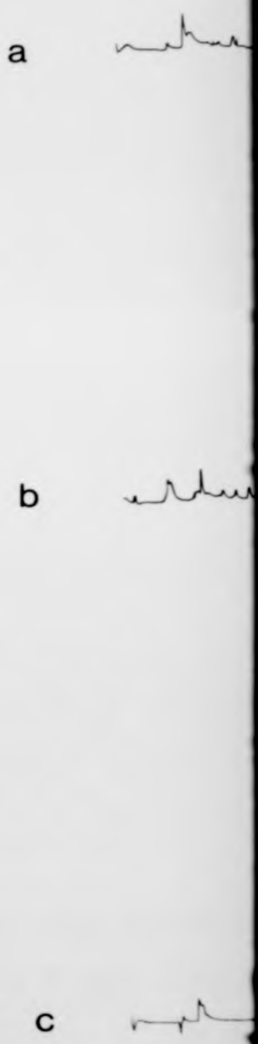
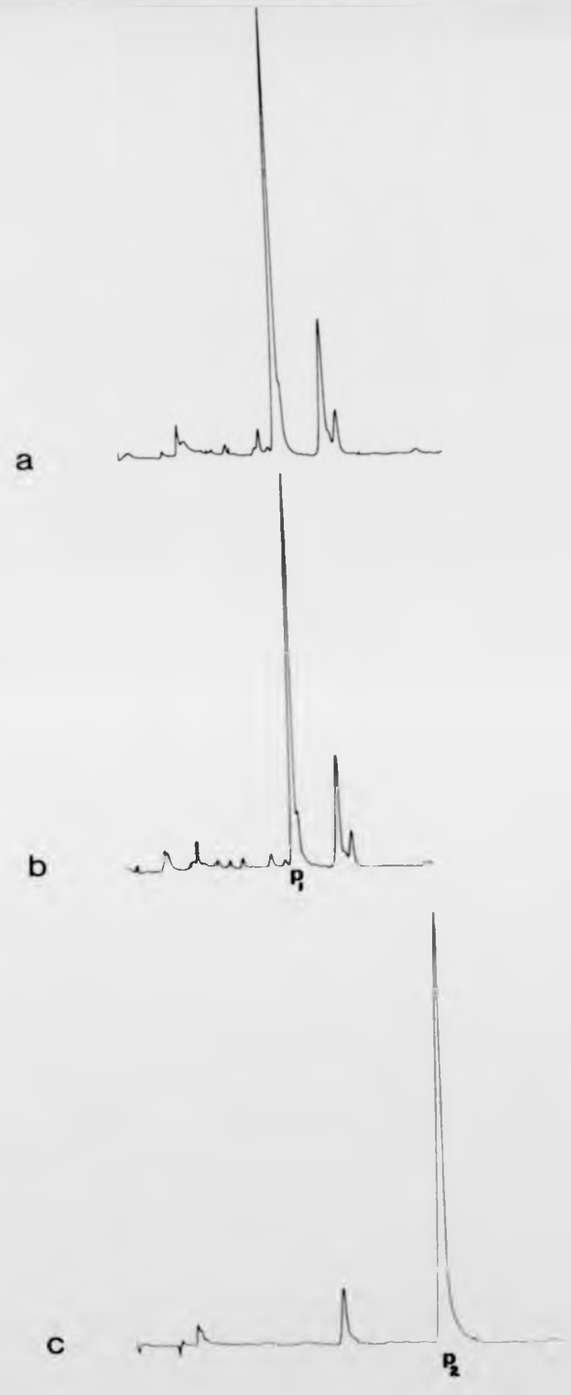


Figure 13

The absence of PAD formation in an incubation mixture of Eulan WA New with pike liver homogenate (pH 3.0).

ure, of Eulan WA New

ate (pH 3.0)



an incubation mixture of

nogenate (pH 3.0).

HPLC profile of:

(a) the extracted incubation mixture, of Eulan WA New with pike stomach crude homogenate (pH 1.5)

(b) Eulan WA New standard

(c) PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

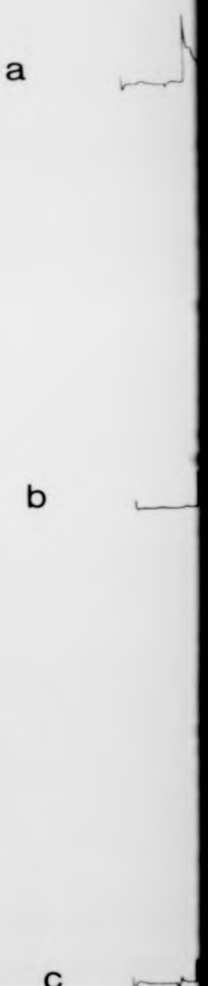
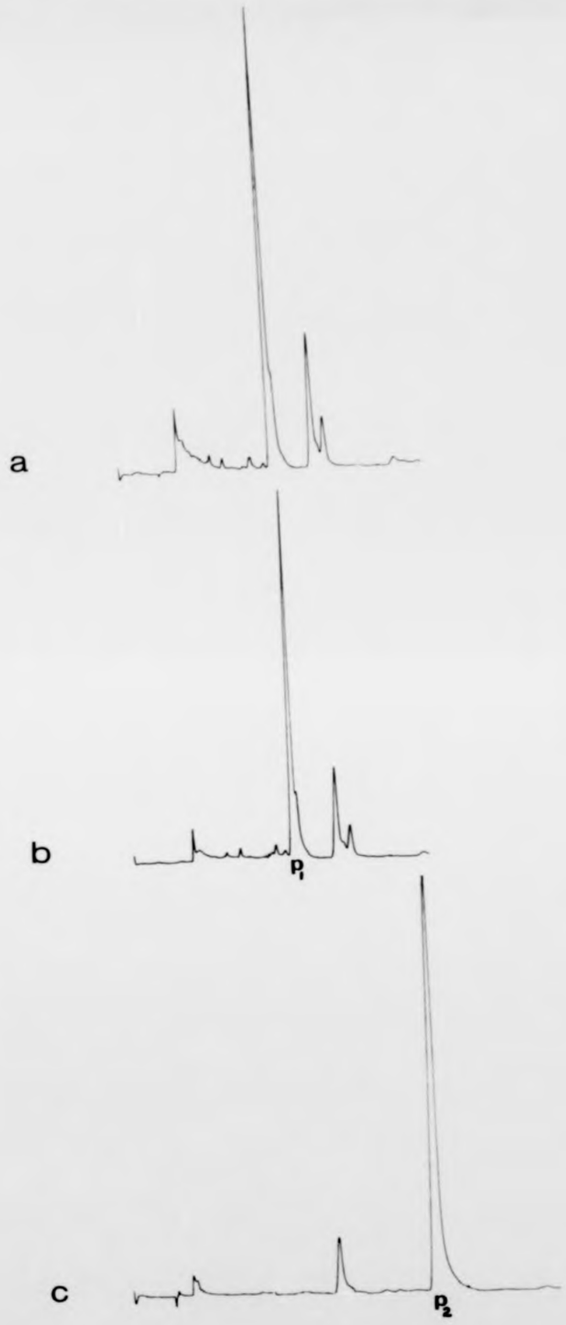


Figure 14

The absence of PAD formation in an incubation mixture of Eulan WA New with pike stomach crude homogenate (pH 1.5).

ure, of Eulan WA New with

(pH 1.5)



an incubation mixture of

crude homogenate (pH 1.5).

HPLC profile of:

(a) the extracted incubation mixture, of Eulan WA New with pike intestine crude homogenate (pH 8.0)

(b) Eulan WA New standard

(c) PAD standard

P_1 - 6-PCSD

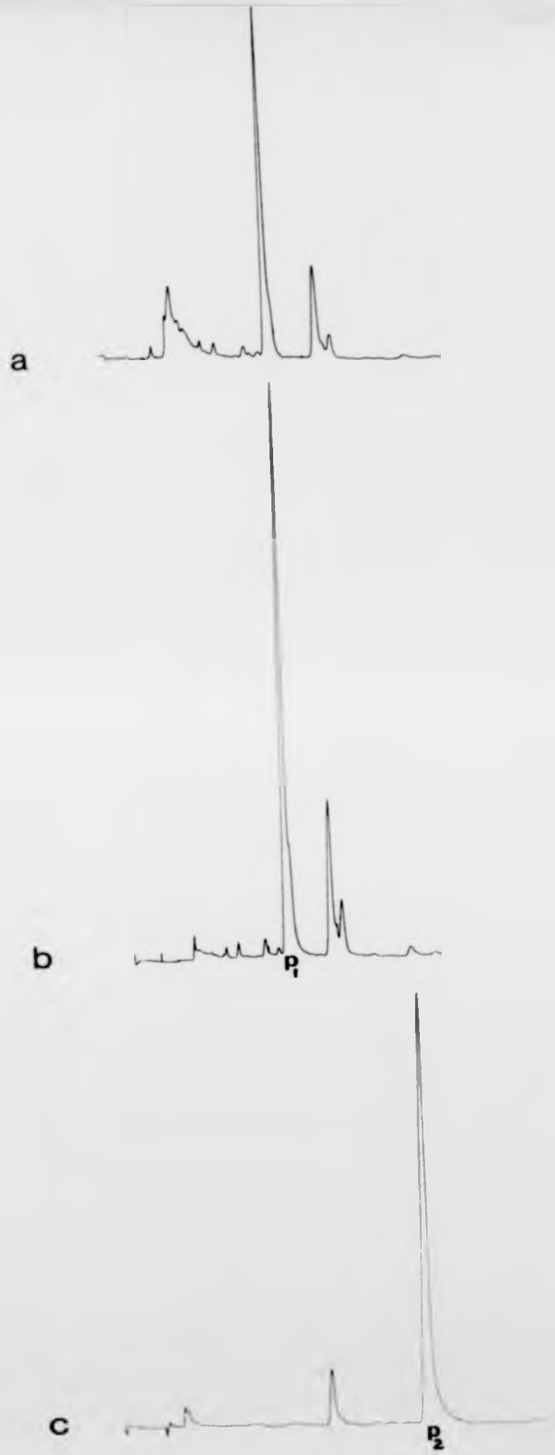
P_2 - 5-PAD

Figure 15

The absence of PAD formation in an incubation mixture of Eulan WA New with pike intestine crude homogenate (pH 8.0).

ture, of Eulan WA New

nogenate (pH 8.0)



an incubation mixture

tine crude homogenate

Pike liver, stomach and intestinal tissue crude homogenates were incubated with Eulan WA New at pH 2-9. HPLC profiles of extracted incubation mixtures of liver, stomach and intestinal crude homogenates, showed no PAD formation at any of the pH values.

The incubation of tissue pieces of pike liver, stomach and intestine in buffers (pH 2-8) containing Eulan WA New did not result in the formation of PAD in HPLC profiles of MTBE of either tissue or surrounding buffer.

3.4.3.3 Incubation of Eulan WA New with Pike tissue 100,000 g supernatants under conditions of detectable Glutathione transferase activity

The specific glutathione transferase activities in pike tissue 100,000 g supernatants are given in Table 7. No detectable glutathione transferase activity was observed in the pike liver 100,000 g pellet in the presence or absence of iodoacetamide.

The incubation of Eulan WA New with the 100,000 g supernatants from the pike tissues named in Table 4, did not show PAD formation under conditions for the assay of glutathione transferase activity. Figure 12 shows the HPLC profile of the extracted liver 100,000 g supernatant (pH 6.5) after incubation with Eulan WA New under conditions for glutathione transferase activity.

Pike liver 100,000 g supernatant was incubated with Eulan WA New and GSH at pH 2-8. No PAD formation was observed in HPLC profiles of these extracted incubation mixtures.

The incubation conditions for acetylase, monooxygenase and glucuronosyltransferase activity are described in 3.3.5.9.

Table 7

Glutathione transferase specific activities in 100,000 g supernatant fractions of pike tissues

Tissues Assayed	Specific activity µmoles of CDNB formed/ min/mg of protein
Liver	0.065
Kidney	0.013
Eye	0.003
Gills	0.047
Skin/muscle	0.003
Spleen	0.003
Intestines	0.010
Brain	0.017
Stomach	0.012
Swim-bladder	0.028

HPLC profiles of:

(a) the extracted incubation mixture of pike liver 100,000 g supernatant (pH 6.5) incubated with Eulan WA New under conditions for glutathione transferase activity.

(b) Eulan WA New standard

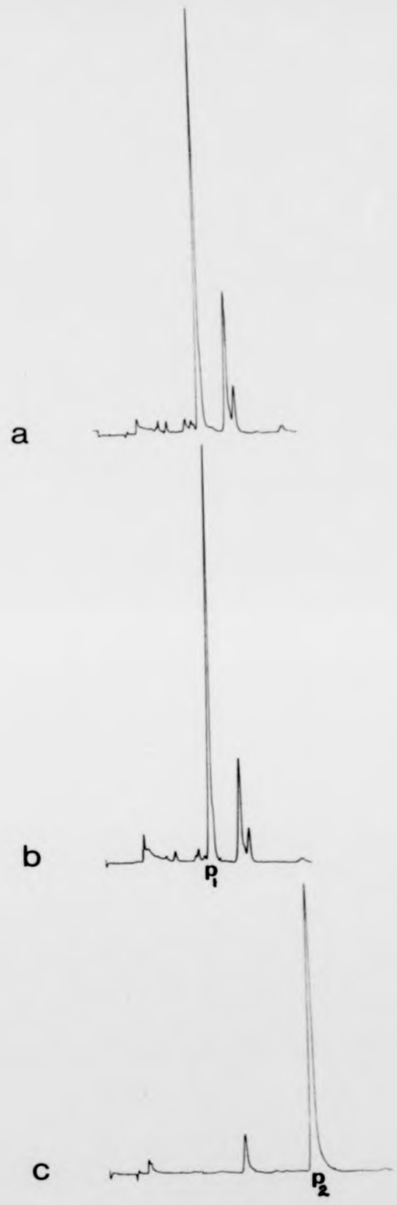
(c) PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 16

The absence of PAD formation in an incubation mixture of pike liver 100,000 g supernatant with Eulan WA New when incubated under conditions for the assay of Glutathione Transferase activity.


a
b
P1
c
P2

3.4.3.4 The incubation of Eulan WA New and 5-PAD with Rat, Pike and Trout liver 10,000 g supernatants, under conditions for Acetylase activity

HPLC profiles of extracted incubation mixtures containing rat, pike and trout liver 10,000 g supernatants, and Eulan WA New, did not show the formation of PADs or the acetylated PCSD products (Fig. 17). The incubation of the 10,000 g supernatants with 5-PAD, under conditions for Acetylase activity, did not result in the formation of the acetylated PAD products observed in the chemical acetylation studies (Fig. 18).

3.4.3.5 The incubation of Eulan WA New and 5-PAD with Rat, Pike and Trout liver 10,000 g supernatants under conditions described for the assay of Monooxygenase activity

No PAD formation was observed in incubation mixtures of rat, pike and trout 10,000 g supernatants with Eulan WA New after incubation under conditions described for the assay of monooxygenase activity (Fig. 19).

Incubation of the 10,000 g supernatants with 5-PAD, under the same conditions, did not result in the identification of new peaks, or observable differences in the peaks of Eulan WA New and PAD in the HPLC profiles of extracted incubation mixtures, compared with those of the standards (Fig. 20).

3.4.3.6 The incubation of Eulan WA New and 5-PAD with Rat, Pike and Trout liver 10,000 g supernatants, under conditions for the assay of Glucuronosyltransferase activity

No PAD formation was observed in incubation mixtures of rat, pike and trout liver 10,000 g supernatants with Eulan WA New, under conditions described for the assay of glucuronosyltransferase activity. No differences were observed between the PCSD components of HPLC profiles, extracted from incubation mixtures and those of the

HPLC profiles of extracted incubation mixtures of Eulan WA New with the following tissue 10,000 g supernatants, under conditions for acetylase activity:

(a) rat liver 10,000 g supernatant

(b) pike liver 10,000 g supernatant

(c) trout liver 10,000 g supernatant

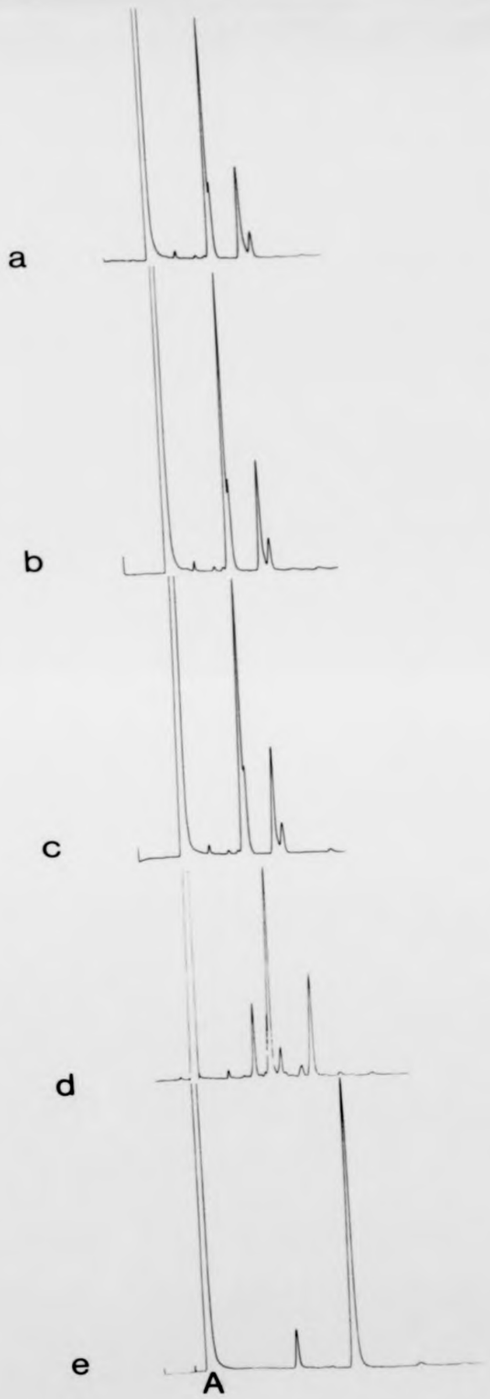
(d) HPLC profile of Eulan WA New after chemical acetylation

(e) HPLC profile of PAD standard

A - DMF peak, HPLC injection solvent

Figure 17

The absence of formation of PAD or acetylated PCSD products in incubation mixtures of Eulan WA New and Rat, Pike and Trout liver 10,000 g supernatants, under conditions for the assay of Acetylase activity.



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AD or acetylated PCSD products
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ants, under conditions for
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HPLC profiles of the extracted incubation mixtures of 5-PAD with the following tissue 10,000 g supernatants, under conditions for acetylase activity:

(a) rat liver 10,000 g supernatants

(b) pike liver 10,000 g supernatant

(c) trout liver 10,000 g supernatant

(d) HPLC profile of 5-PAD after chemical acetylation

A - DMF peak, HPLC injection solvent

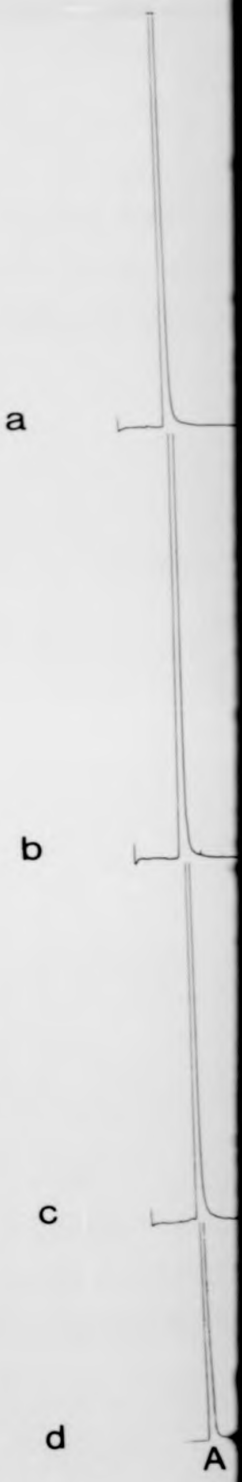

Figure 18

The absence of formation of acetylated PAD products in incubation mixtures of 5-PAD with Rat, Pike and Trout liver 10,000 g supernatants, under conditions for the assay of Acetylase activity.

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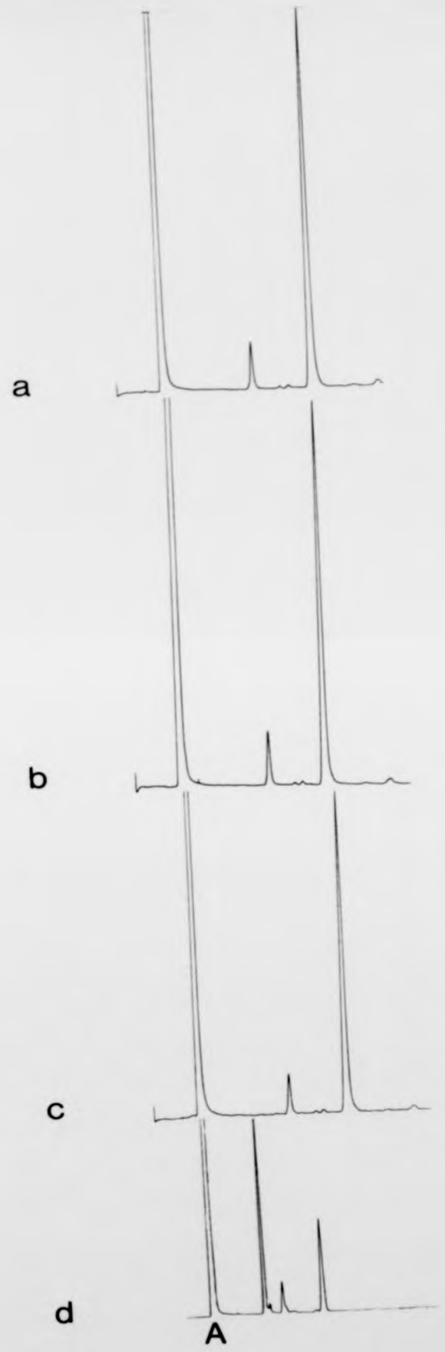


HPLC profiles of the extracted incubation mixtures of Eulan WA New with the following tissue 10,000 g supernatants, under conditions described for the assay of monooxygenase activity:

(a) rat liver 10,000 g supernatant

(b) pike liver 10,000 g supernatant

(c) trout liver 10,000 g supernatant

(d) HPLC profile of Eulan WA New standard

(e) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

A - DMF peak, HPLC injection solvent

Figure 19

The absence of PAD formation in incubation mixtures of Eulan WA New with Rat, Pike and Trout liver 10,000 g supernatants, under conditions described for the assay of monooxygenase activity.

HPLC profiles of the extracted incubation mixtures of Eulan WA New with the following tissue 10,000 g supernatants, under conditions described for the assay of monooxygenase activity:

(a) rat liver 10,000 g supernatant

(b) pike liver 10,000 g supernatant

(c) trout liver 10,000 g supernatant

(d) HPLC profile of Eulan WA New standard

(e) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

A - DMF peak, HPLC injection solvent

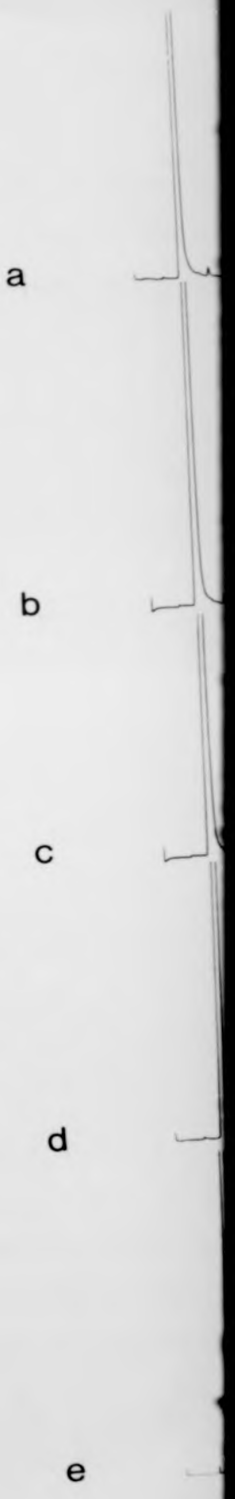
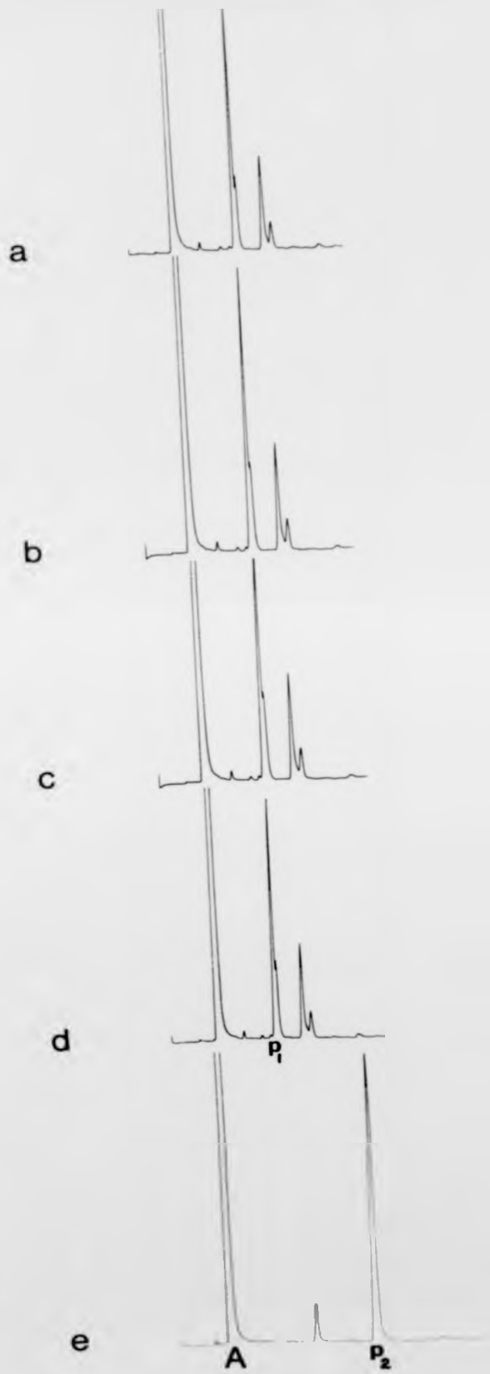


Figure 19

The absence of PAD formation in incubation mixtures of Eulan WA New with Rat, Pike and Trout liver 10,000 g supernatants, under conditions described for the assay of monooxygenase activity.


a
b
c
d
P_1
e
A
P_2

HPLC profiles of the extracted incubation mixtures of 5-PAD with the following tissue 10,000 g supernatants, under conditions described for the assay of monooxygenase activity:

(a) rat liver 10,000 g supernatant

(b) pike liver 10,000 g supernatant

(c) trout liver 10,000 g supernatant

(d) HPLC profile of 5-PAD standard

A - DMF peak,HPLC injection solvent

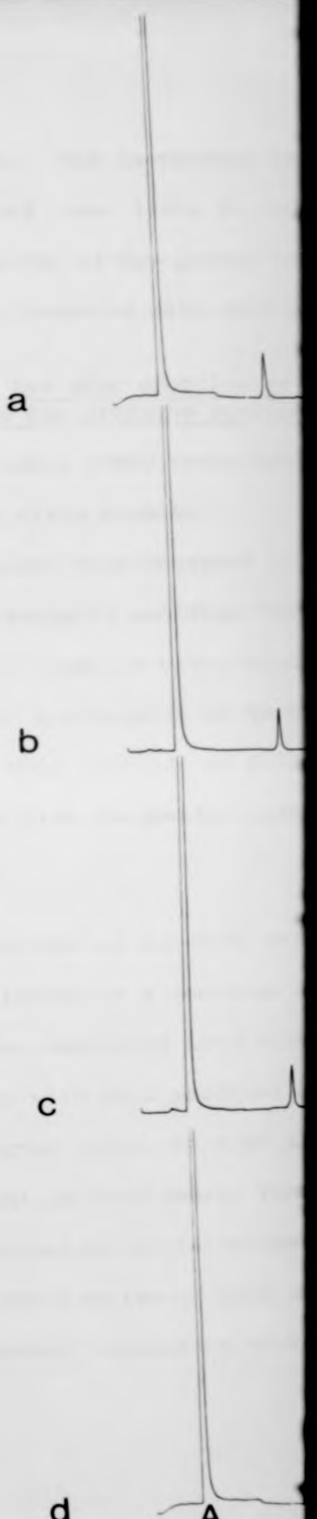


Figure 20

The absence of new products in MTBE extracts of incubation mixtures of 5-PAD with Rat, Pike and Trout liver 10,000 g supernatants, under conditions described for the assay of monooxygenase activity.

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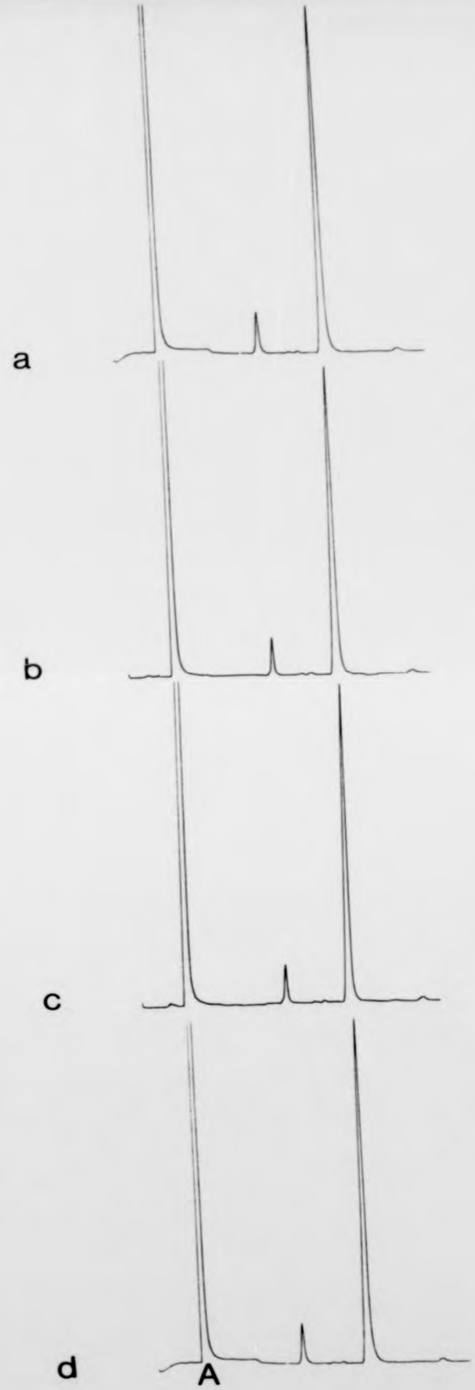
monooxygenase



extracts of incubation

d Trout liver 10,000 g

ribed for the assay of

Eulan standard. The incubation of 5-PAD with 10,000 g supernatants of rat, pike and trout liver 10,000 g supernatants did not result in the identification of new peaks, or observable differences in the HPLC profiles, compared with that of the 5-PAD standard.

Assessment of the HPLC profiles of extracted incubation mixtures, illustrated in the previous section (3.4.3):

(a) No detectable 5-PAD formation was observed in HPLC profiles from these *in vitro* studies.

(b) No new peaks were observed in HPLC profiles suggesting that no MTBE-extractable modified PCSD and/or PAD metabolites were formed in these *in vitro* studies.

(c) No clear qualitative or quantitative differences were observed between HPLC profiles of Eulan WA New and PAD and standard extracted from incubation mixtures and their standard HPLC profiles.

The formation of non-MTBE extractable PCSD and/or PAD metabolites would be reflected in a decrease of the parent component in the HPLC profile of the extracted incubation mixture. This would be observed by comparison with HPLC profiles of the standards. In connection with this latter point, it must also be borne in mind that a variation in peak height of PCSD and/or PAD components may result from their differing extraction efficiencies from an incubation mixture. Extraction efficiencies of PCSD and PAD components may also vary between different incubation mixtures.

3.4.4 The *in vitro* metabolism of 6-PCSD to 5-PAD by liver subcellular fractions prepared from Goldfish dosed with 6-PCSD

The extraction, 'clean-up' and HPLC analysis of fish tissues has been previously described in this work (section 2.3.7). 6-PCSD was detected in the goldfish liver with the HPLC detection system on a high sensitivity setting (Fig. 21). However at this sensitivity the strongly absorbing DMF peak (the HPLC injection solvent) interferes with that of 6-PCSD. To allow a more accurate quantitation (measurement of peak height), the sample was re-extracted and finally redissolved in methanol for HPLC injection. Methanol absorbs only weakly at 230 nm and therefore gave an improved HPLC profile. 6-PCSD was identified in goldfish tissues, Figure 22 shows the coelution of 6-PCSD standard with that of 6-PCSD in goldfish digestive tissue. 5-PAD was identified in both liver and digestive tissue by coelution with the 5-PAD standard (Fig. 23). PAD:PCSD ratios in liver, gill and digestive tissue were calculated to be 3.38, 0.03 and 0.55 respectively. These values are indicative of *in vivo* metabolism of 6-PCSD to 5-PAD, particularly in goldfish liver (section 2.4.6). The goldfish used in this study are therefore behaving as those previously analysed in dosing studies in metabolising 6-PCSD to 5-PAD.

Liver, digestive tract and spleen tissues were taken from dosed goldfish and subcellular fractions prepared as described in section 3.3.4.3. Samples of each of the tissue crude homogenates, of equal volume to those added to incubation mixtures were extracted by MTBE. This small volume of crude homogenate represents only a small fraction of the total tissue homogenised. Also the HPLC analysis of MTBE extracts of incubation mixtures was carried out with the HPLC detection system working at a sensitivity 8× less than that for the detection of 6-PCSD and 5-PAD in dosed goldfish tissue extracts. Consequently

HPLC profile of

(a) 6-PCSD dosing solution

(b) the 'cleaned' digestive tissue extracts taken from a goldfish dosed with 6-PCSD

(c) the 'cleaned' digestive tissue extracts taken from a goldfish not dosed with 6-PCSD

(d) the 'cleaned' liver tissue extract taken from a goldfish dosed with 6-PCSD

(e) the 'cleaned' liver tissue extract taken from a goldfish not dosed with 6-PCSD

A - DMF, HPLC injection solvent

'cleaned' - refers to the removal of the bulk of coextracted materials by the modified mixed alumina "clean-up" method described in section 2.4.3.

The above samples injected for HPLC analysis, were each prepared from the methanol eluates collected from mixed alumina columns. Methanol elutes PCSD components from coextracted material on the mixed alumina column.

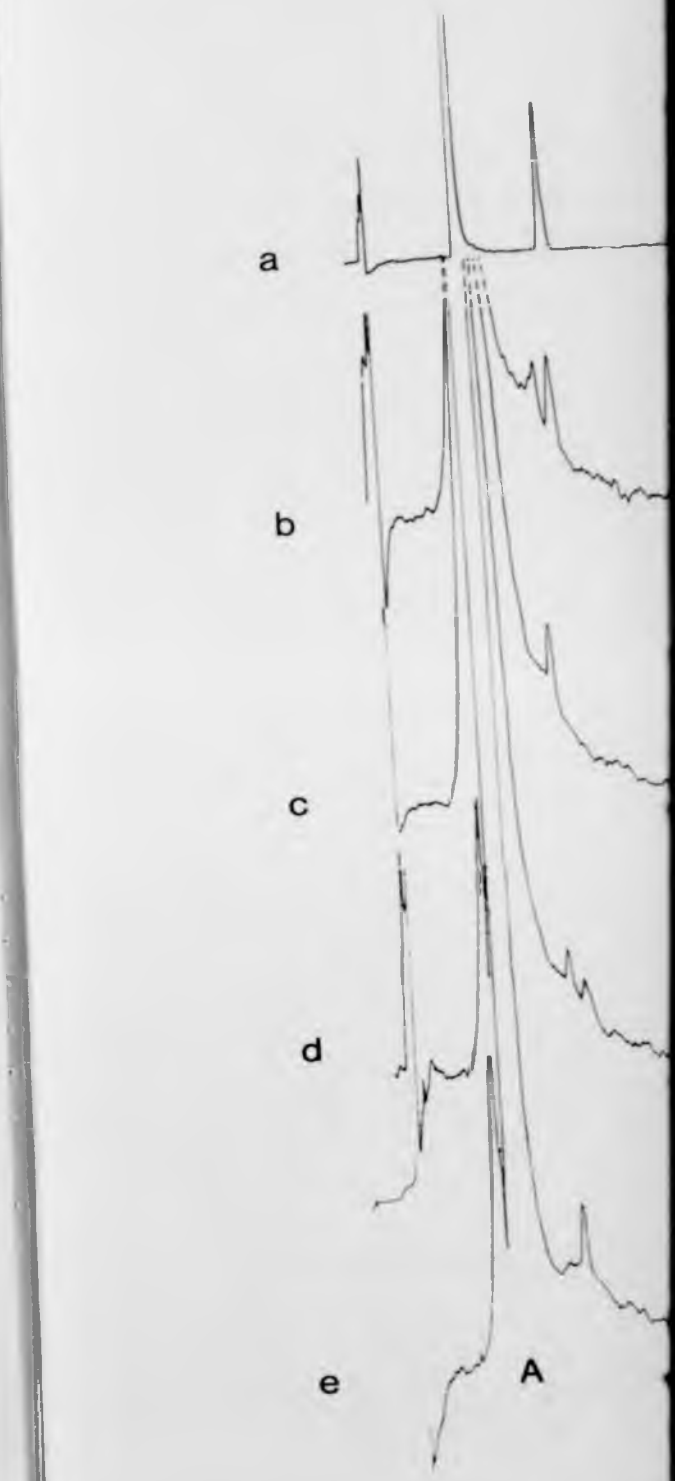


Figure 21

Detectable 6-PCSD in liver and digestive tissue of a sample dosed goldfish used in the *in vitro* studies.

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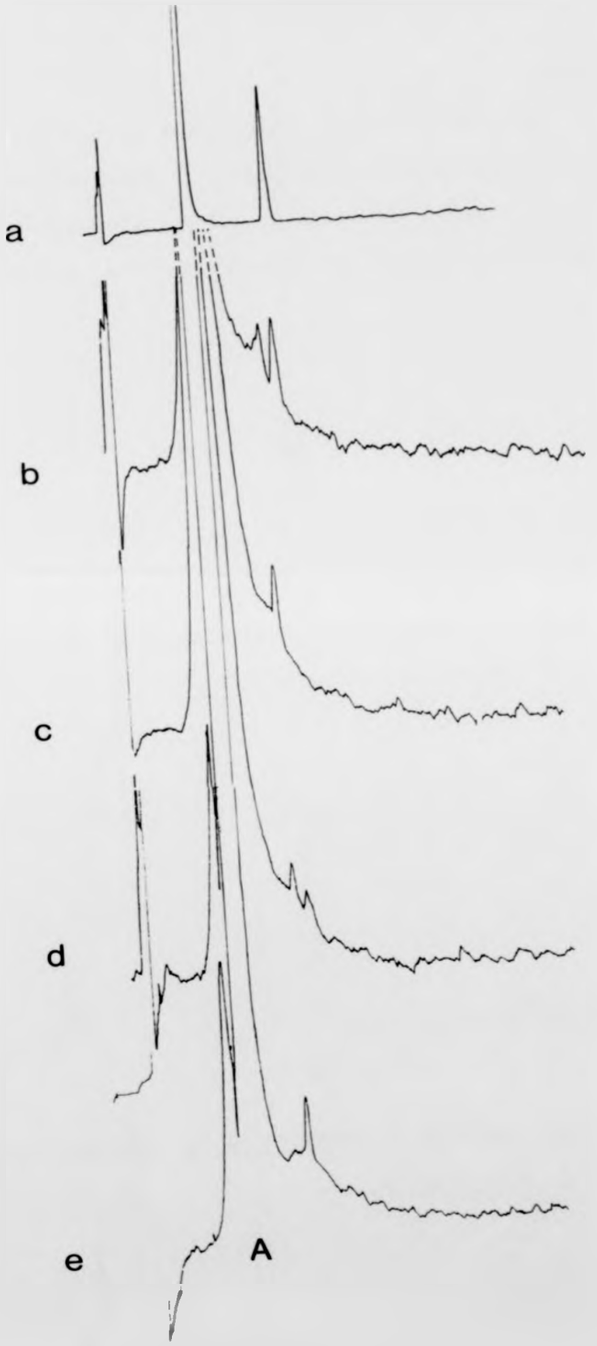

HPLC profile of:

(a) 6-PCSD standard

(b) the 'cleaned' digestive tissue extract from a goldfish dosed with 6-PCSD injected in methanol for HPLC analysis

(c) the 'cleaned' digestive tissue extract from a goldfish not dosed with 6-PCSD, injected in methanol for HPLC analysis

P_1 - 6-PCSD

'cleaned' - refers to the removal of the bulk of coextracted materials by the modified mixed alumina 'clean-up" method described in section 2.4.3.

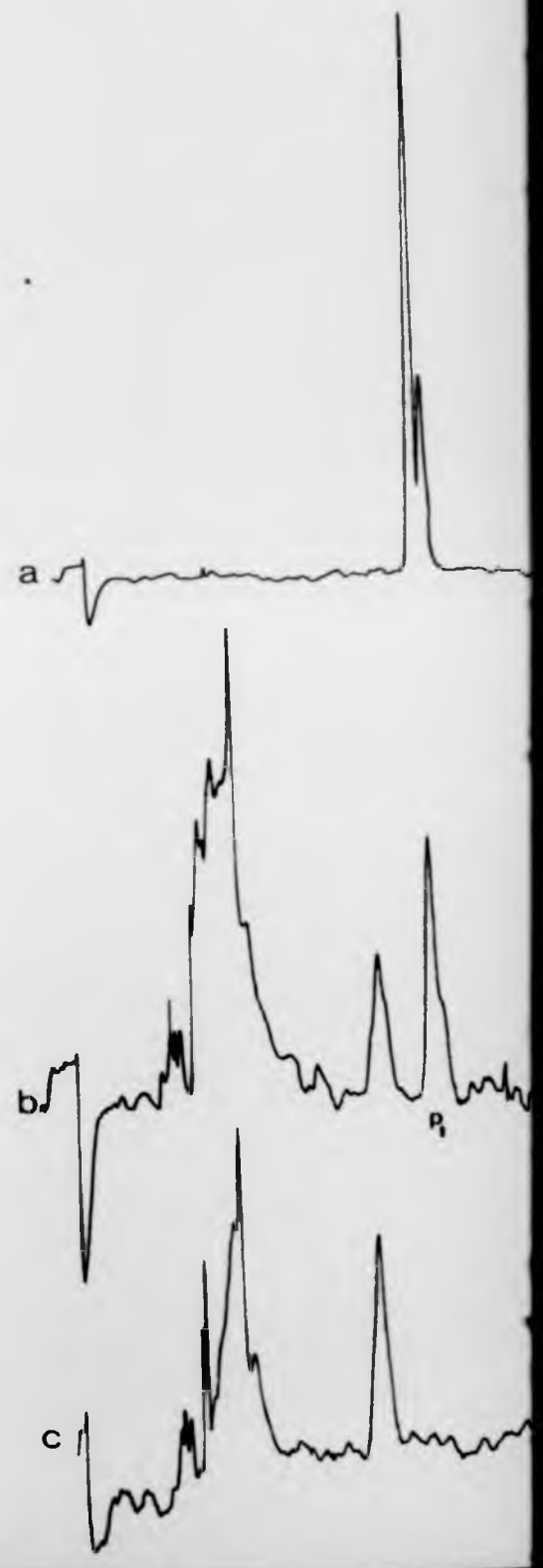

Figure 22

The identification of 6-PCSD in a digestive tissue extract taken from a goldfish dosed with 6-PCSD.

tract from a goldfish

hanol for HPLC analysis

tract from a goldfish not

thanol for HPLC analysis

the bulk of coextracted

mixed alumina "clean-up"

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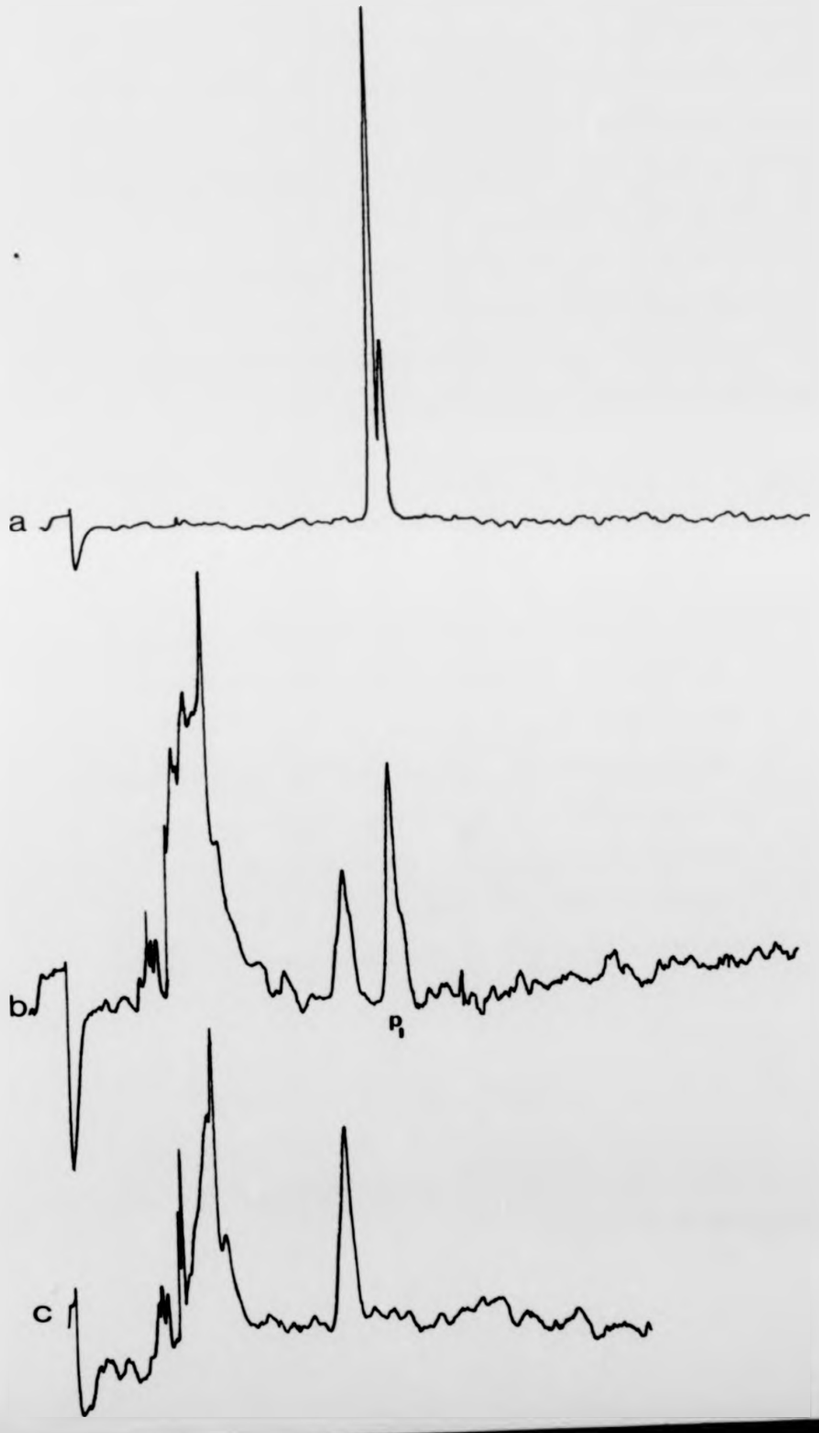


HPLC profile of:

(a) PAD standard

(b) the 'cleaned' liver tissue extract, taken from a goldfish dosed with 6-PCSD

(c) the 'cleaned' liver tissue extract, taken from a goldfish not dosed with 6-PCSD

(d) the 'cleaned' digestive tissue extract, taken from a goldfish dosed with 6-PCSD

(e) the 'cleaned' digestive tissue extracts taken from a goldfish not dosed with 6-PCSD

P_2 - 5-PAD

'cleaned' - refers to the removal of the bulk of coextracted materials by the modified mixed alumina "clean-up" method described in section 2.3.4

The above samples injected for HPLC analysis were each prepared from MTBE eluates collected from mixed alumina columns. MTBE elutes PAD components from PCSD components and coextracted materials on the mixed alumina column

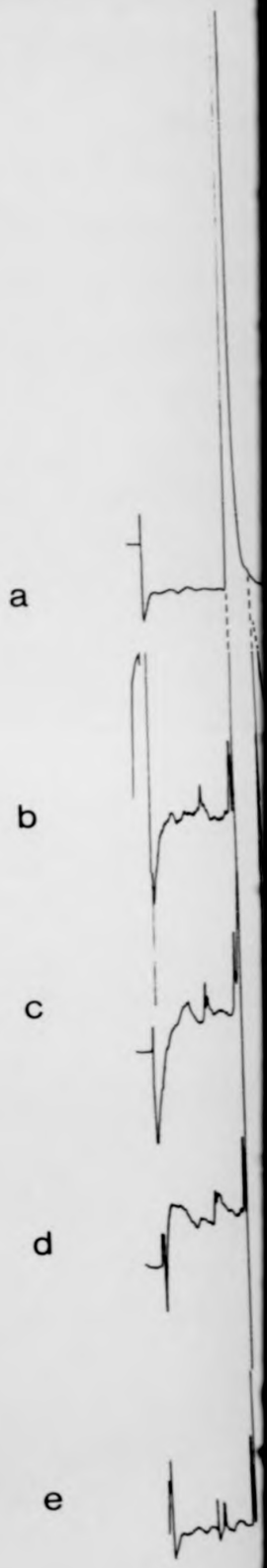
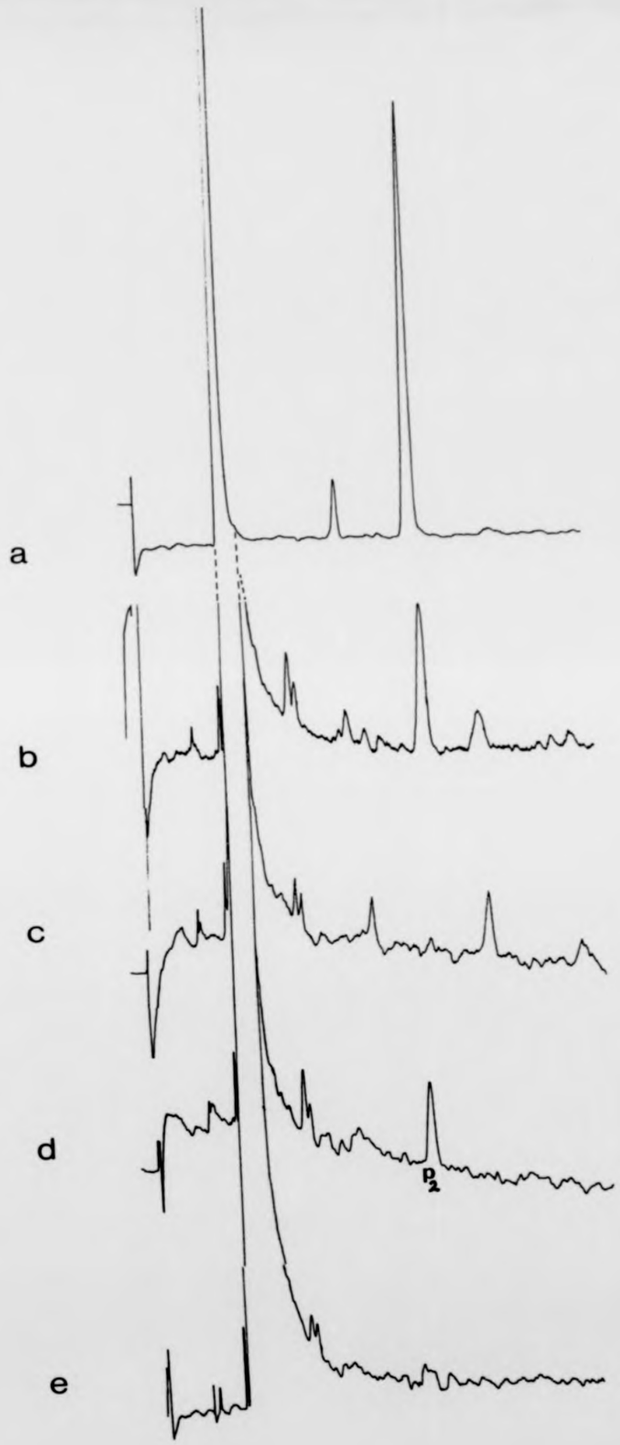


Figure 23

The identification of 5-PAD in liver and digestive tissue of a goldfish dosed with 6-PCSD.


a
b
c
d
e
p2

analysis of tissue crude homogenates at this detection sensitivity showed no detectable 6-PCSD or 5-PAD. The 6-PCSD and 5-PAD in tissues from the dosing of goldfish did not therefore interfere with the analysis of 6-PCSD and 5-PAD components in the *in vitro* studies.

The incubation of dosed goldfish liver subcellular fractions with 6-PCSD resulted in the *in vitro* formation of 5-PAD. The activity was greatest at pH 7.4 with lower activities at pH 3.0, 5.0 and 9.0. Figure 24 shows the variation of 5-PAD formation with pH in each of the liver subcellular fractions incubated with 6-PCSD. 5-PAD forming activity was not observed in the 10,000 g pellet (plus triton X100) but was observed in the liver crude homogenate, 1000 g and 10,000 g supernatants (Fig. 25). The heating of a sample of goldfish liver crude homogenate (70°C, 3 min), prior to its incubation with 6-PCSD, abolished 5-PAD formation (Fig. 26). No 5-PAD formation was observed when goldfish spleen and digestive tissue homogenates were incubated with 6-PCSD at pH 3, 5, 7 and 9. Also anaerobic and aerobic incubation of dosed goldfish digestive contents in a growth medium containing 6-PCSD did not result in 5-PAD formation.

3.4.5 Identification of a 5-PAD forming activity in liver homogenates prepared from livers of Goldfish not previously exposed to Eulan WA New

Liver homogenate (prepared from goldfish not previously exposed to Eulan WA New) when incubated with 6-PCSD at pH 7.4 resulted in the formation of 5-PAD (Fig. 28). Incubation of a sample of homogenate with trypsin (0.5 mg/ml) at 25°C for 30 min, resulted in the loss of 5-PAD forming activity upon incubation with 6-PCSD.

A comparison of the specific activities of 5-PAD formation by goldfish liver homogenates prepared from livers of dosed and non-dosed

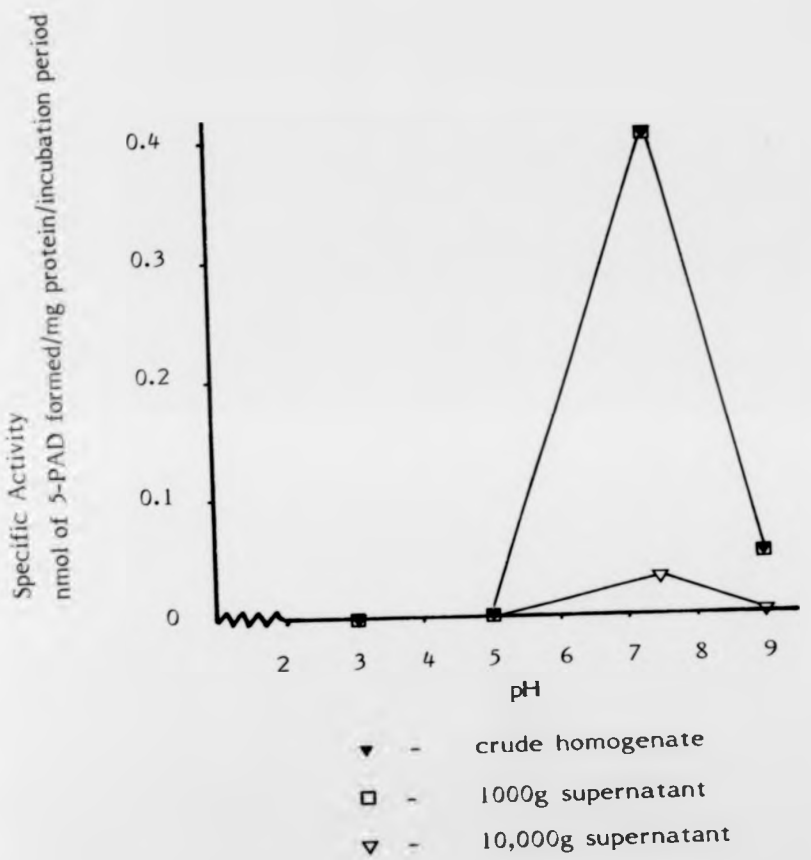


Fig 24. The variation of 5-PAD forming activity with pH in Goldfish liver subcellular fractions

HPLC profiles of extracted incubation mixtures of 6-PCSD with the following subcellular fractions prepared from dosed goldfish liver tissue:

(a) crude homogenate

(b) 1000 g supernatant

(c) 10,000 g supernatant

(d) 10,000 g supernatant + triton X100 (0.1%)

(e) 10,000 g pellet + triton X100 (0.1%)

(f) triton X100 only

(g) HPLC profile of PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

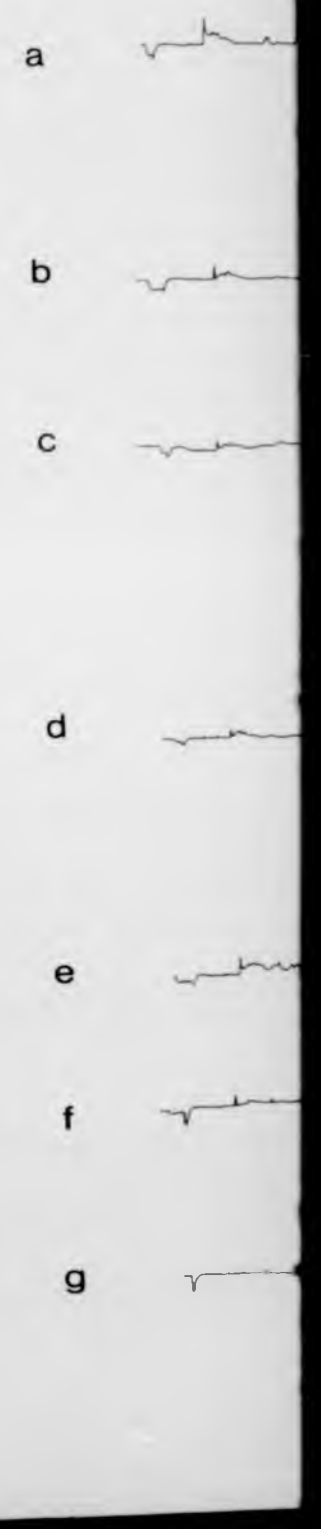
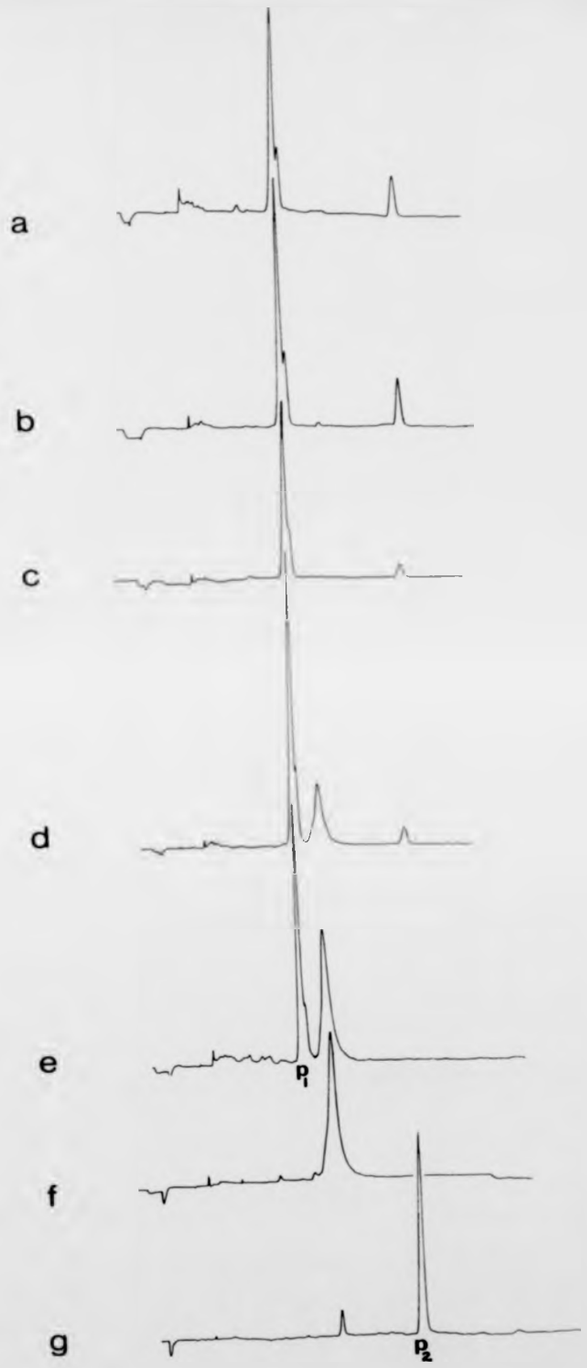


Figure 25

The formation of 5-PAD by the subcellular fractions prepared from dosed goldfish liver tissue.


a
b
c
d
e
P_1
f
g
P_2

HPLC profile of;

(a) standard 6-PCSD

(b) an extracted incubation mixture of 6-PCSD with 'heat-treated' liver crude homogenate

(c) an extracted incubation mixture of liver crude homogenate in the absence of 6-PCSD

(d) an extracted incubation mixture of 6-PCSD with liver crude homogenate

(e) PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

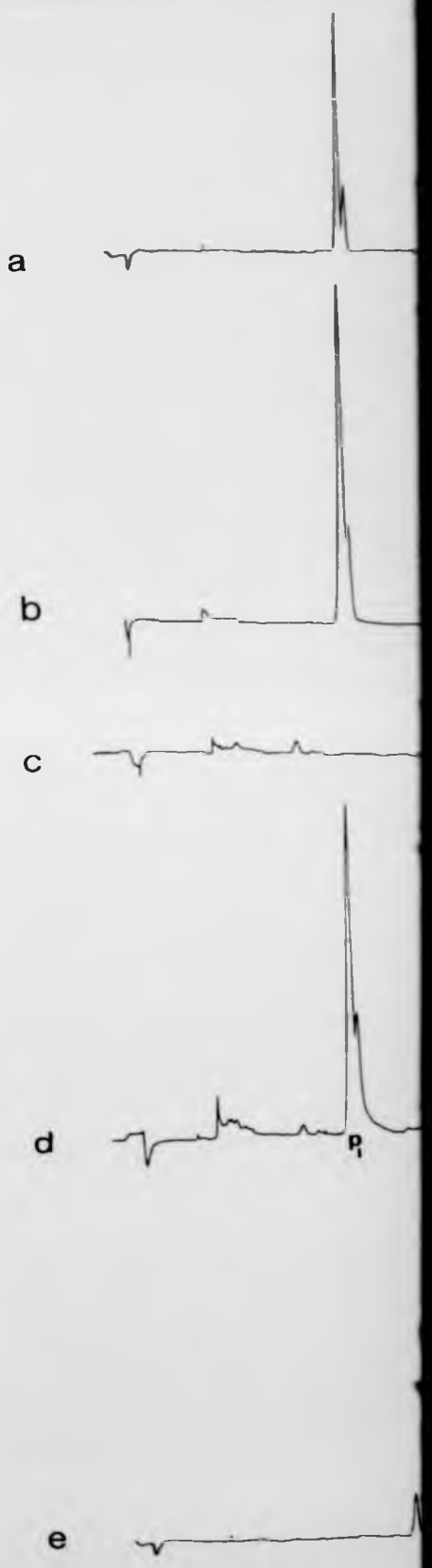
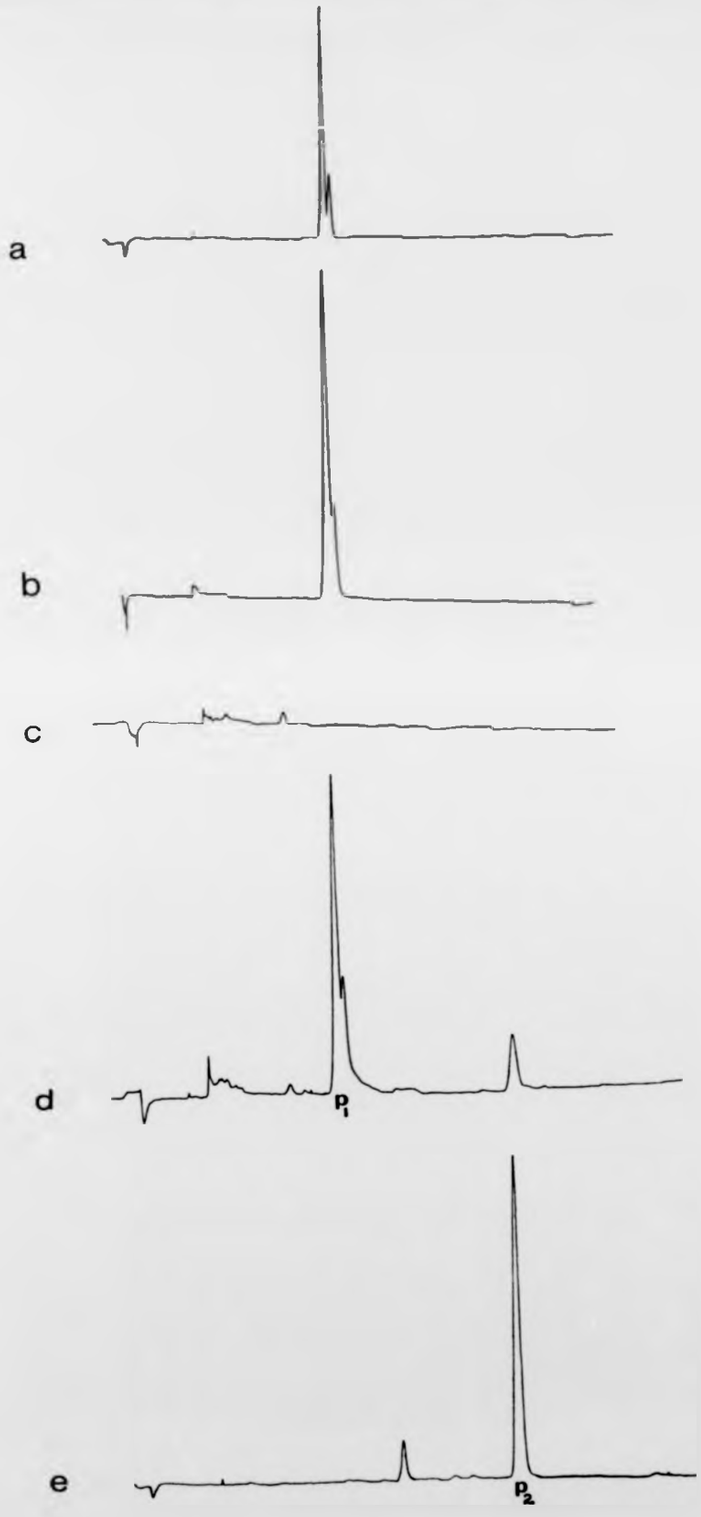


Figure 26

The absence of 5-PAD formation in heat treated liver crude homogenate prepared from a goldfish dosed with 6-PCSD.



ure of 6-PCSD with 'heat-treated'

ure of liver crude homogenate

ure of 6-PCSD with liver

n heat treated liver crude

ish dosed with 6-PCSD.

HPLC profiles of an extracted incubation mixture of Eulan WA New with the following goldfish crude homogenate preparations (pH 7.4):

(a) spleen

(b) digestive tissue

(c) an extracted anaerobic incubation mixture of goldfish digestive tract contents from a growth medium containing 6-PCSD

(d) liver

(e) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5 PAD

Figure 27

The absence of 5-PAD formation in incubation mixtures of spleen and digestive tissue homogenates and digestive tract contents.

bation mixture of Eulan

crude homogenate

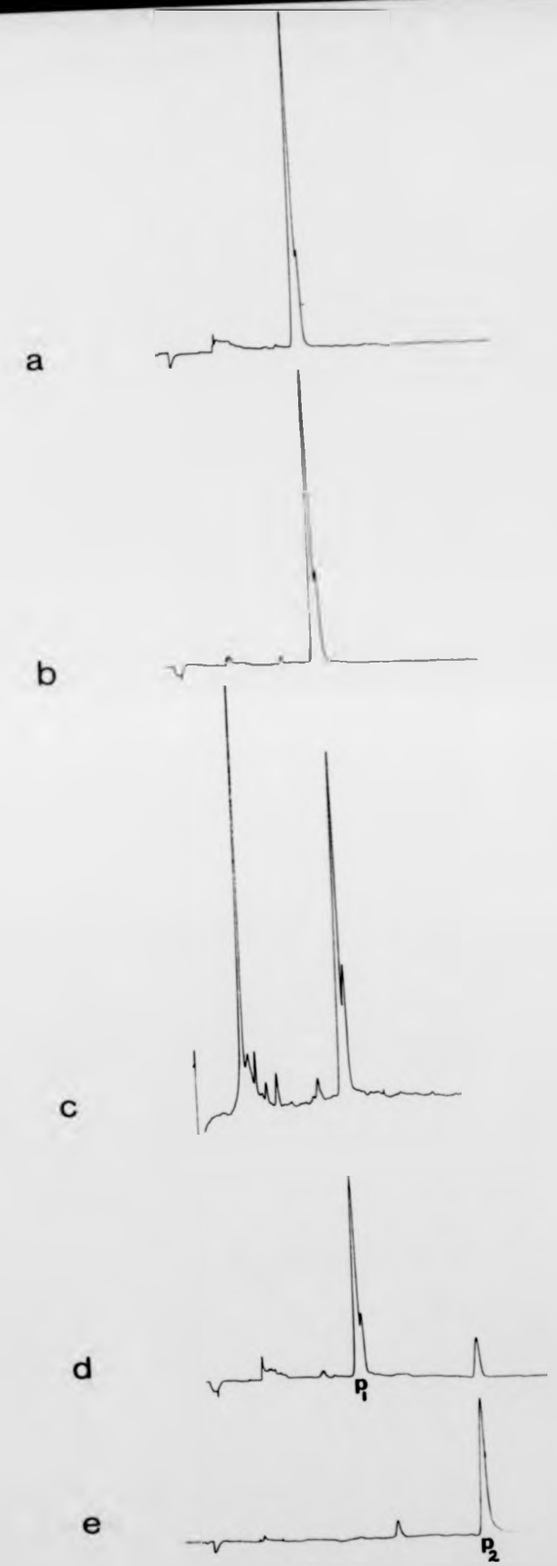
tion mixture of goldfish

a growth medium

d

incubation mixtures of

enates and digestive



HPLC profile of extracted incubation mixture:

(a) liver crude homogenate treated with trypsin, prior to incubation with 6-PCSD

(b) liver crude homogenate incubated with 6-PCSD

(c) liver crude homogenate, after incubation in the absence of 6-PCSD

(d) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

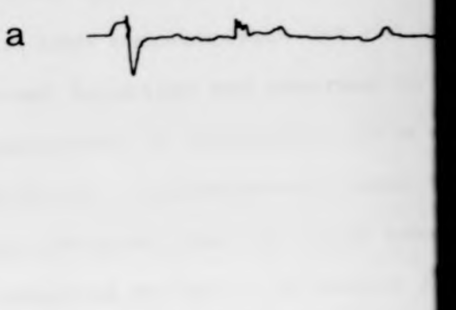
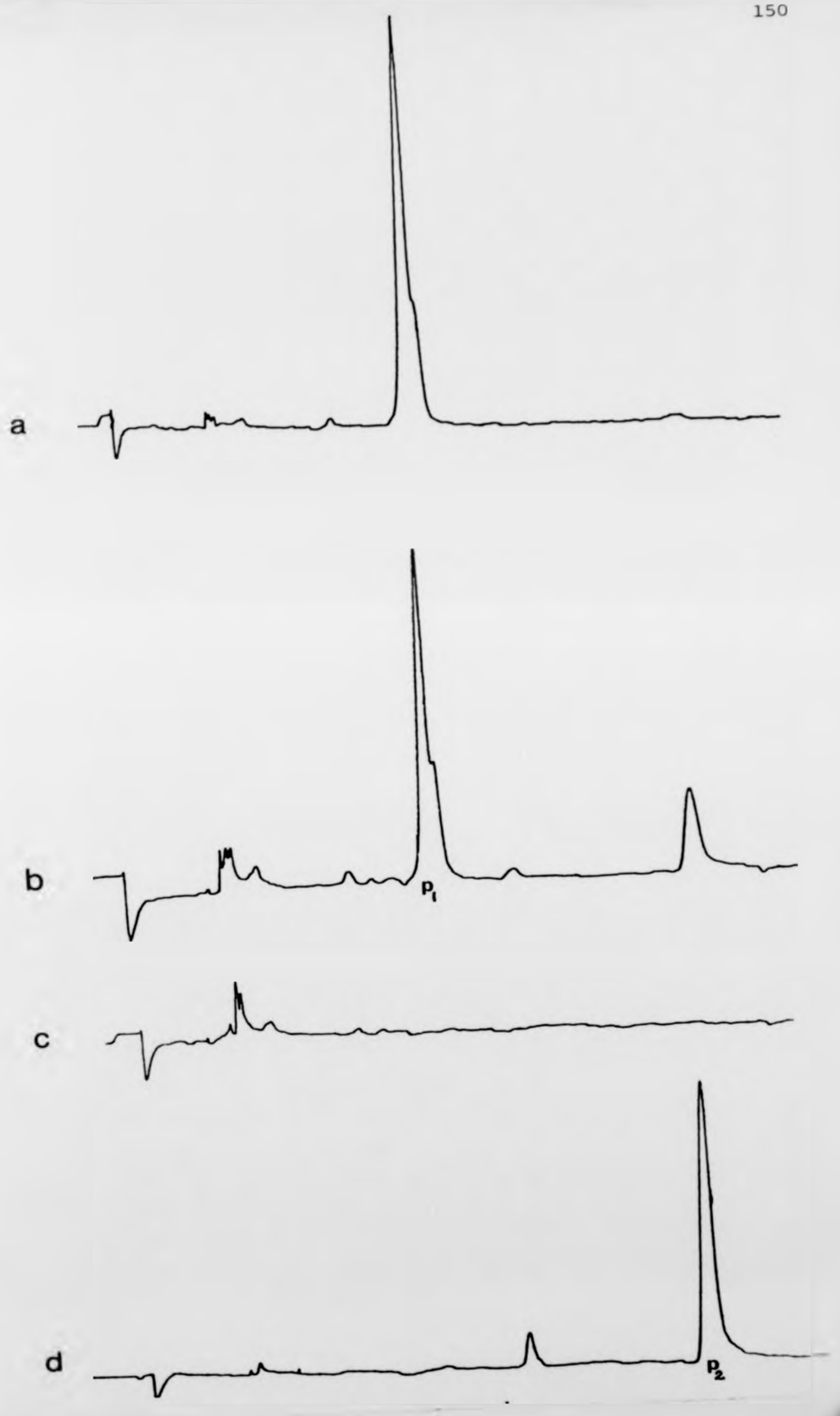


Figure 28

The *in vitro* formation of 5-PAD by liver crude homogenate prepared from a goldfish not previously exposed to 6-PCSD.



ion mixture:

ed with trypsin, prior to

ated with 6-PCSD

r incubation in the

ard

by liver crude homogenate

eviously exposed to 6-PCSD.

goldfish were very similar, 0.42 and 0.40 nmol of 5-PAD formed/mg of protein/incubation period, respectively.

3.4.6 Preliminary characterisation of the 5-PAD forming activity in Goldfish liver homogenates

3.4.6.1 Determination of the 5-PAD forming activity of Goldfish liver homogenate in different incubation buffers

The 5-PAD forming activities of goldfish liver prepared in Tris/HCl buffer (pH 7.4), when added to different incubation buffers (each of the same concentration and pH) are shown in Figure 29. Similar 5-PAD formation was observed in extracted incubation mixtures of liver homogenate in Tris/HCl, 0.25 M sucrose/Tris/HCl, Hepes and Tricine buffers. Approximately three times this value of 5-PAD formation was observed when the liver homogenate was incubated in potassium phosphate buffer. To assist in the detection of small amounts of 5-PAD the amount of 6-PCSD in the incubation mixtures was reduced from 20 µg to 10 µg/tube and the sensitivity of the HPLC detection increased (0.2 to 0.1 absorbance units maximum deflection). Under these conditions of analysis the liver homogenate was added again to Tris/HCl and phosphate buffers. The resulting HPLC profiles are shown in Figure 30. Increased 5-PAD formation was again observed in phosphate buffer.

3.4.6.2 The 5-PAD forming activities of Goldfish liver homogenates prepared in different buffers

The preparation of goldfish liver homogenates in Tris/HCl and phosphate buffers is described in section 3.3.6.2.

HPLC profiles of extracted incubation mixtures of goldfish liver crude homogenates, prepared and incubated in Tris/HCl and in potassium phosphate buffer showed a 2-fold higher 5-PAD formation in the phosphate buffer (Fig. 31). The specific activities for 5-PAD

HPLC profiles of extracted incubation mixtures of 6-PCSD with goldfish liver homogenate in the following incubation buffers:

(a) 40 mM Tris/HCl pH 7.4

(b) 0.25 M sucrose/40 mM Tris/HCl pH 7.4

(c) 40 mM potassium phosphate buffer pH 7.4

(d) 40 mM Tricine/NaOH buffer pH 7.4

(e) 40 mM Hepes/NaOH buffer pH 7.4

P_1 – 6 PCSD

P_2 - 5-PAD

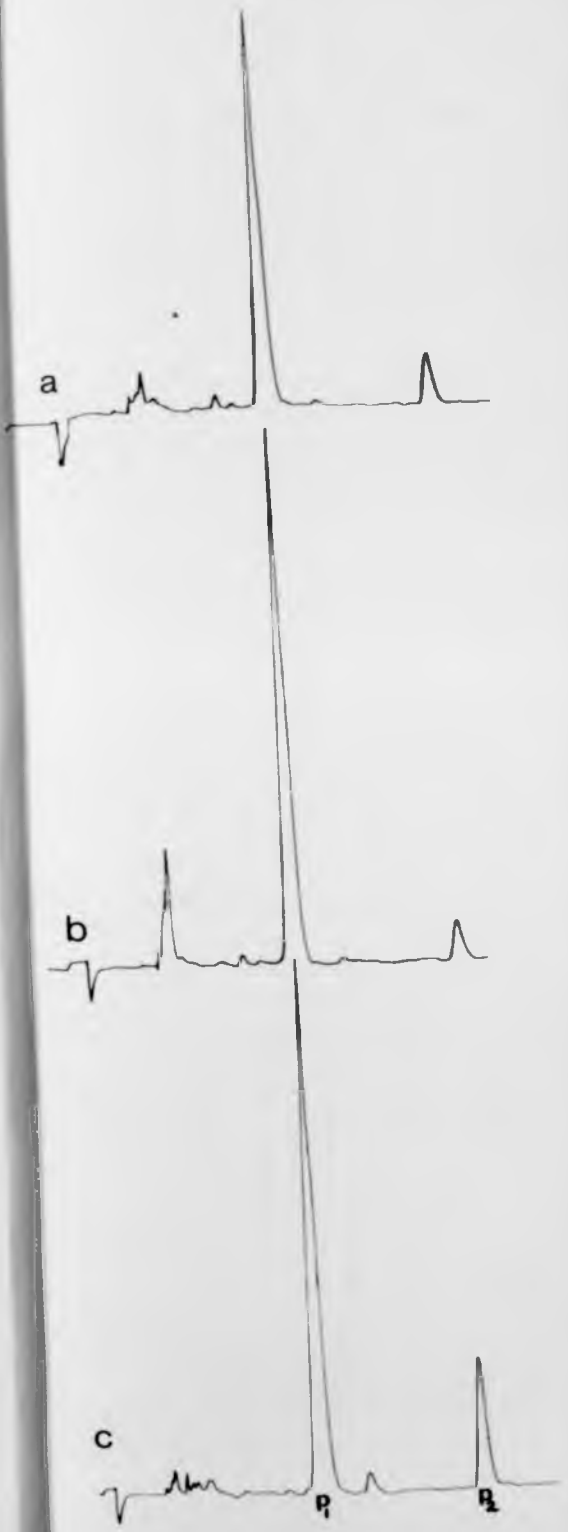


Figure 29

Comparison of 5-PAD formation by Goldfish liver homogenate in a range of incubation buffers.

ion mixtures of 6-PCSD

the following incubation

pH 7.4

fer pH 7.4

7.4

4

Goldfish liver homogenate

HPLC profile of extracted incubation mixtures of 6-PCSD with goldfish liver homogenate in the following incubation buffers:

(a) 40 mM Tris/HCl pH 7.4

(b) 40 mM potassium phosphate buffer pH 7.4

(c) HPLC profile of PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

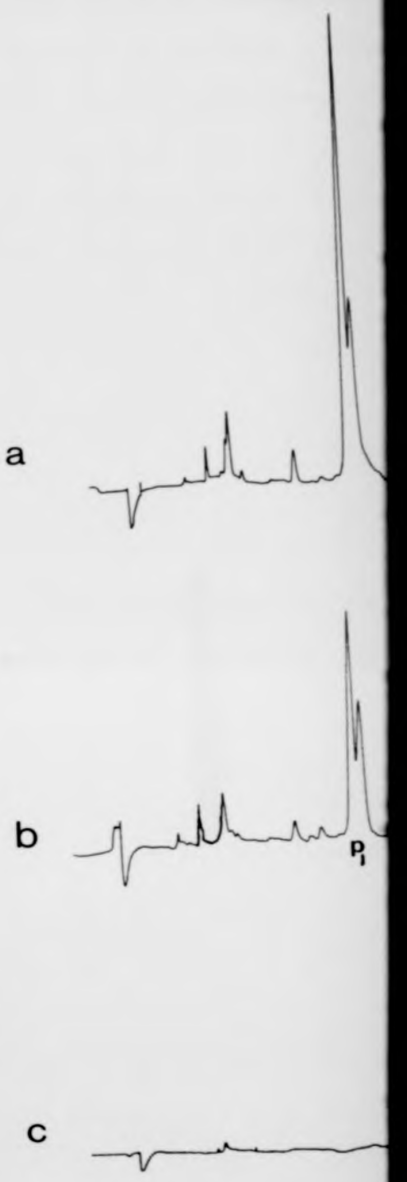


Figure 30

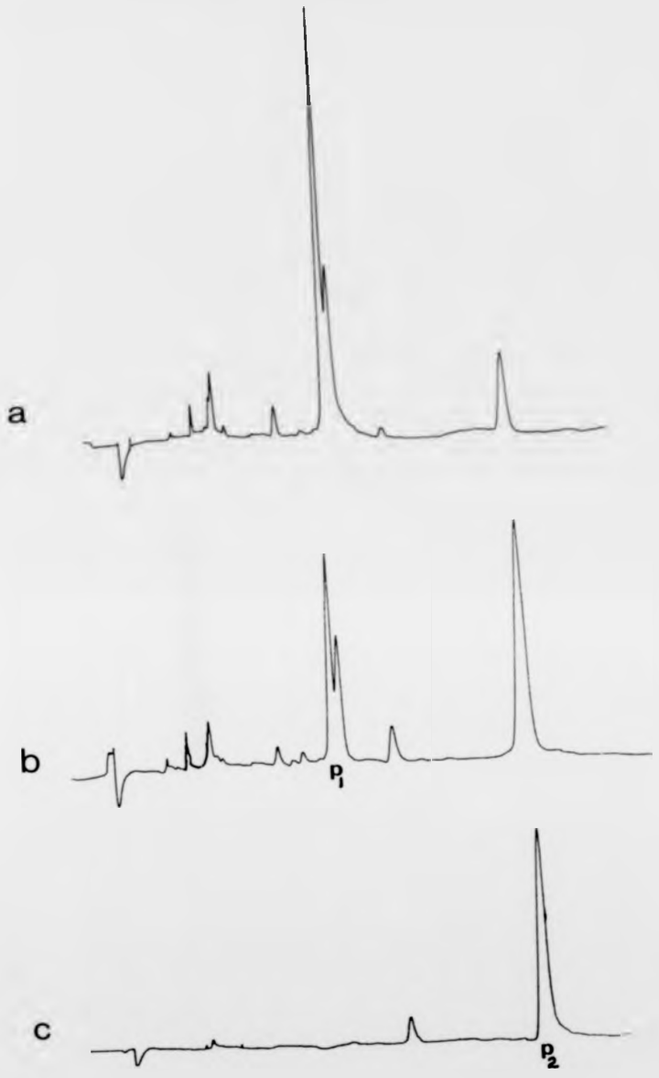
Improved HPLC profiles showing increased 5-PAD formation in potassium phosphate buffer.

ixtures of 6-PCSD with

wing incubation buffers:

pH 7.4

sed 5-PAD formation



HPLC profiles of extracted incubation mixtures of 6-PCSD with the following goldfish liver fractions, prepared and incubated in the latter buffers:

(a) goldfish liver crude homogenate (40 mM Tris/HCl pH 7.4)

(b) goldfish liver crude homogenate (40 mM potassium phosphate buffer pH 7.4)

(c) goldfish liver 1000 g supernatant (40 mM potassium phosphate buffer pH 7.4)

(d) HPLC profile of 5-PAD standard

The peaks in addition to those of 6-PCSD and 5-PAD are tissue homogenate coextractants since they appeared in the HPLC profile of extracted goldfish liver homogenate in the absence of 6-PCSD

P_1 - 6-PCSD

P_2 - 5-PAD

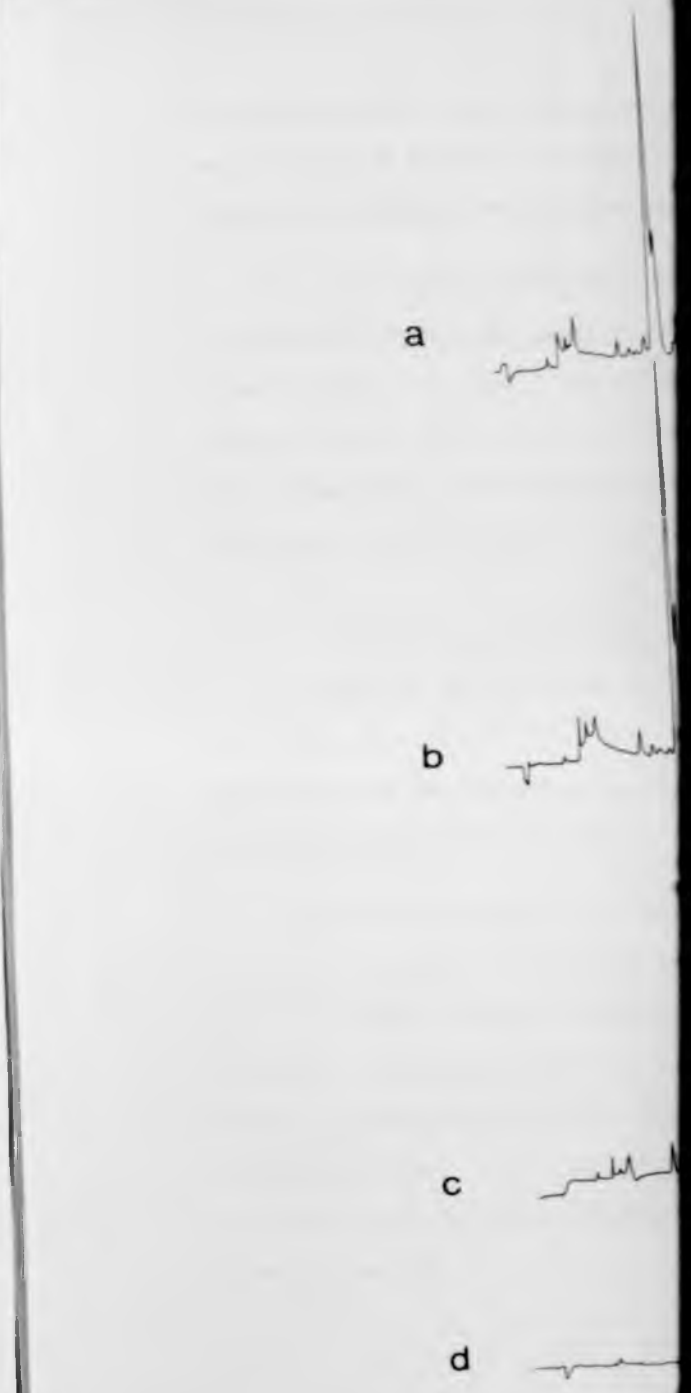


Figure 31

The preparation and incubation of Goldfish liver fractions in phosphate and Tris/HCl buffers.

mixtures of 6-PCSD with
, prepared and incubated

40 mM Tris/HCl pH 7.4)
40 mM potassium phosphate

(40 mM potassium

CSD and 5-PAD are tissue
ppeared in the HPLC
omogenate in the absence

ldfish liver fractions in

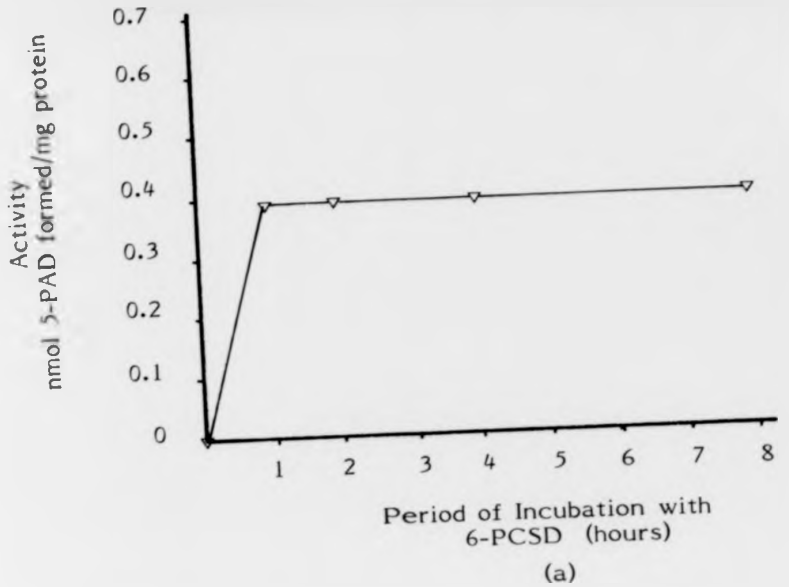
formation of the crude homogenates in phosphate buffer and Tris/HCl were 0.8 and 0.6 nmol of 5-PAD formed/mg of protein incubation period respectively under the same preparation and incubation conditions.

The crude liver homogenate in phosphate buffer was further fractionated by centrifugation and the 1000 g supernatant assayed for 5-PAD forming activity. Of interest was the observation that the 5-PAD forming activity of the 1000 g supernatant was substantially lower than that of the crude homogenate (Fig. 31c), 0.3 nmol of 5-PAD formed/mg protein/incubation period.

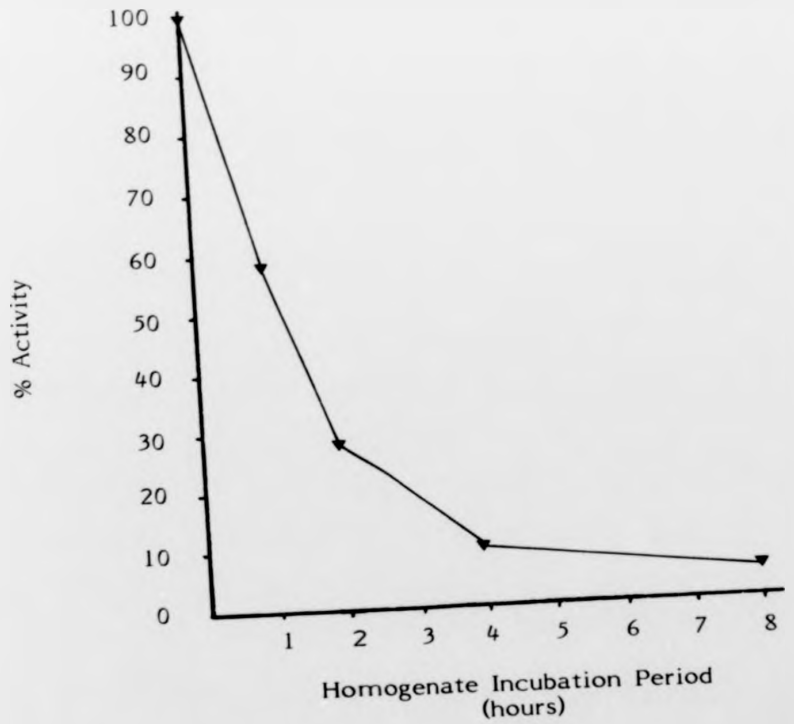
3.4.6.3 The stability of the 5-PAD forming activity of goldfish liver homogenates upon incubation at 19°C

A study of the time course of the formation of 5-PAD involving the incubation of goldfish liver homogenate with 6-PCSD, for periods up to 8h, showed no further 5-PAD formation after a 1h incubation period (Fig. 32a).

The homogenate was incubated at 19°C and aliquots removed after 0, 1, 2, 4 and 8h, for incubation with 6-PCSD (for 8h in each case), and the amount of 5-PAD formed determined. The homogenate showed a loss of 5-PAD forming activity with increasing incubation period. After 2h incubation at 19°C, 70% of the 5-PAD forming activity was lost (Fig. 32b). Only 10% of the total 6-PCSD present was converted to 5-PAD so it is most unlikely that substrate availability was limiting 5-PAD formation.



(a)



(b)

Fig 32. The time course of 5-PAD formation (a) and the loss of 5-PAD forming activity (b) of Goldfish liver homogenate upon incubation at 19°C

3.4.6.4 The activity and stability of 5-PAD formation by Goldfish liver homogenate in incubation mixtures of differing compositions

The addition of either BSA (1 mg/ml) or sucrose (0.25 M) incubation mixtures containing goldfish liver homogenate, did not change the observed trend of decreasing 5-PAD formation upon incubation (Fig. 33a, e). The addition of PMSF (1 mM) did result in a small increase in the stability of 5-PAD formation but the general trend of decreasing 5-PAD formation was still observed over the incubation period (Fig. 33c). Differences in the initial activities of 5-PAD formation were observed upon addition of these and other molecules to the goldfish liver homogenate (Table 8). Both the addition of sucrose and more so glycerol (25% w/v) greatly reduced the 5-PAD forming activity to values substantially lower than those observed in phosphate buffer alone. The addition of pHMB completely inhibited 5-PAD formation by goldfish liver homogenate.

DTT (5 mM) was the only component which when added to incubation mixtures increased both the stability and activity of 5-PAD formation in liver homogenates. A study of the time course of metabolism of 6-PCSD by liver homogenate in the presence of DTT, showed a larger total amount of PAD formed in the presence of DTT than in its absence (Fig. 34a). The loss of 5-PAD forming activity in homogenates upon incubation at 19°C was substantially reduced upon addition of DTT to the liver homogenate in phosphate buffer (Fig. 34b). A sample of HPLC profile showing increased 5-PAD formation by goldfish liver homogenate upon addition of DTT is shown in Fig. 35.

The calculated specific activities of 5-PAD formation by goldfish liver homogenates in phosphate buffer only, varied widely. Six separate preparations, each prepared by an apparently identical

procedure, gave initial specific activities of 5-PAD formation ranging between 0.13 - 0.39 nmol of 5-PAD formed/mg protein/8h incubation period. After incubation of homogenates at 19°C for 2h, (prior to their assay for 5-PAD formation) 0-40% of the initial 5-PAD forming activity was observed. In the presence of DTT, the initial activities of the homogenates ranged between 0.33 - 0.74 nmol of 5-PAD formed/mg protein/8h incubation period, and after the 2h incubation period 13-100% of the initial activity remained. The reasons for these large variations in 5-PAD forming activities in homogenates prepared by the same procedure, are not clear from this study, but these differences do highlight the apparent lability of the 5-PAD forming activity of goldfish liver homogenate.

3.4.7. Identification of 5-PAD forming activity in Pike liver crude homogenate when incubated with 6-PCSD, in the presence of DTT

The absence of 5-PAD forming activity in pike liver crude homogenates when incubated with Eulan WA New was reported previously in this work (section 3.3.3.4). Incubation of pike liver crude homogenate (pH 7.4), with 6-PCSD in the presence of DTT (5 mM), resulted in a detectable 5-PAD formation (Fig. 36).

Table 8

The initial specific activities of 5-PAD formation of goldfish liver homogenates in incubation mixtures of differing compositions

Composition of Goldfish liver homogenate incubation mixture	Initial specific activity nmol 5-PAD formed/mg protein/ incubation period
40 mM phosphate buffer pH 7.4	0.39
[1] 0.25 M sucrose/phosphate buffer pH 7.4	0.25
40 mM phosphate buffer pH 7.4	0.13
[2] 40 mM phosphate buffer pH 7.4 PMSF (1 mM)	0.10
40 mM phosphate buffer pH 7.4 BSA (1 mg ml^{-1})	0.17
40 mM phosphate buffer pH 7.4/ 25% w/v Glycerol	0.04
40 mM phosphate buffer pH 7.4/ 1 mM pHMB	ND

Preparation Buffer and incubation - 40 mM potassium phosphate buffer pH 7.4

1,2 - Two separate samples of liver homogenate prepared from livers of goldfish by the same procedure.

ND - No detectable activity <0.01 n mol 5-PAD formed/ mg protein/incubation period.

Incubation of goldfish liver homogenate at 19°C in incubation mixtures of the following composition, prior to assay for 5-PAD formation:

(a) 0.25 M sucrose/40 mM potassium phosphate buffer, pH 7.4

(b) 40 mM potassium phosphate buffer, pH 7.4

(c) PMSF (1 mM)/40 mM potassium phosphate buffer, pH 7.4

(d) 40 mM potassium phosphate buffer, pH 7.4

(e) Bovine serum albumin (1 mg ml^{-1}) in 40 mM potassium phosphate buffer, pH 7.4

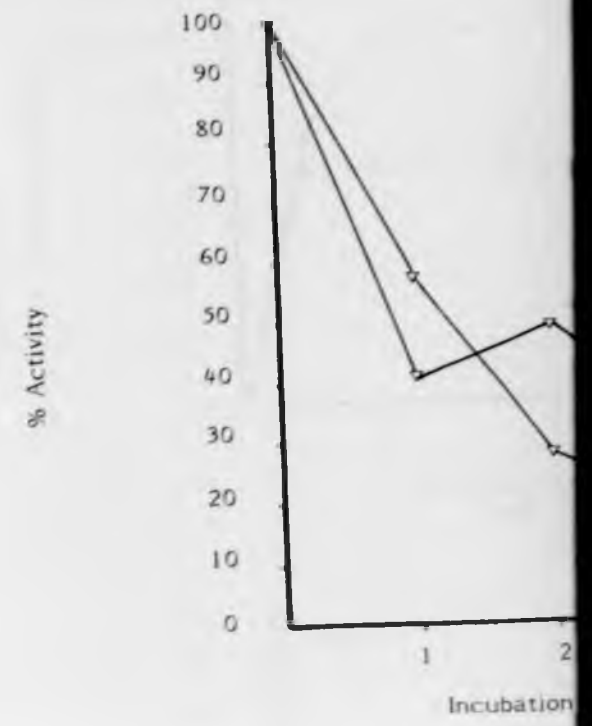
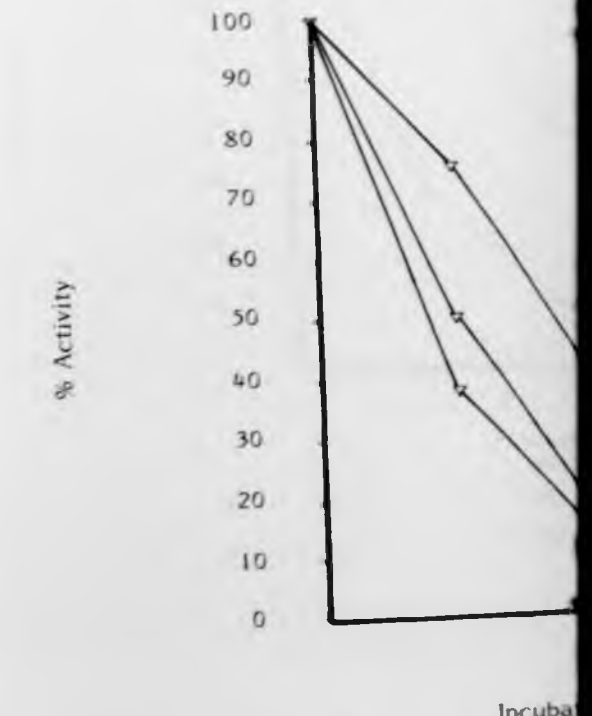
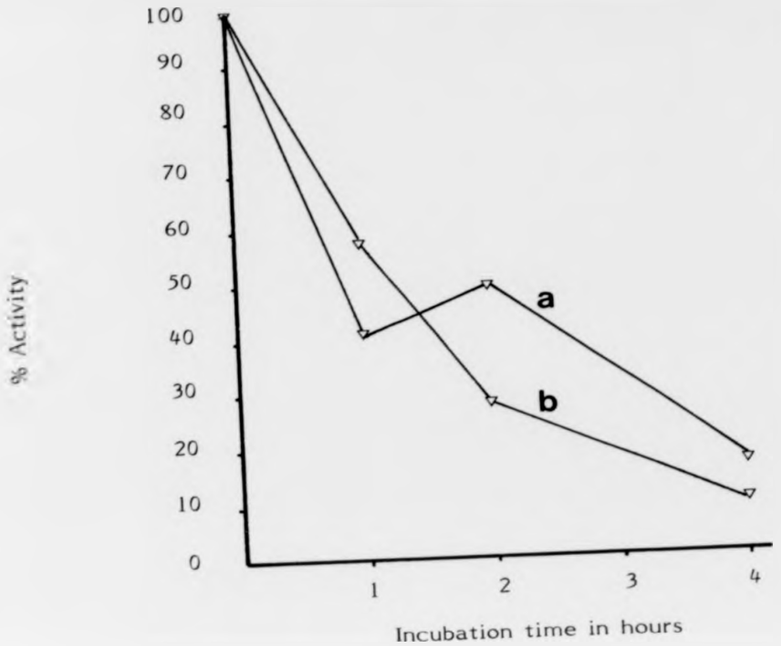
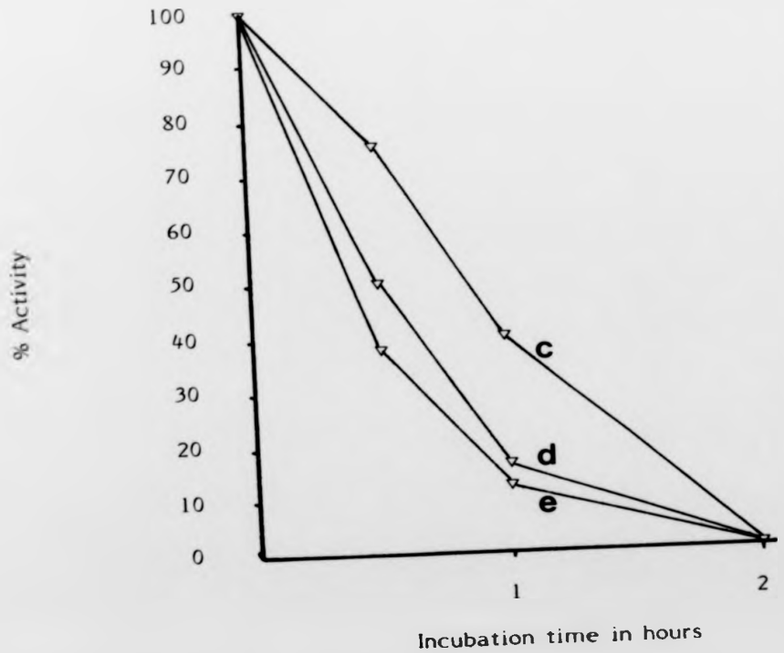


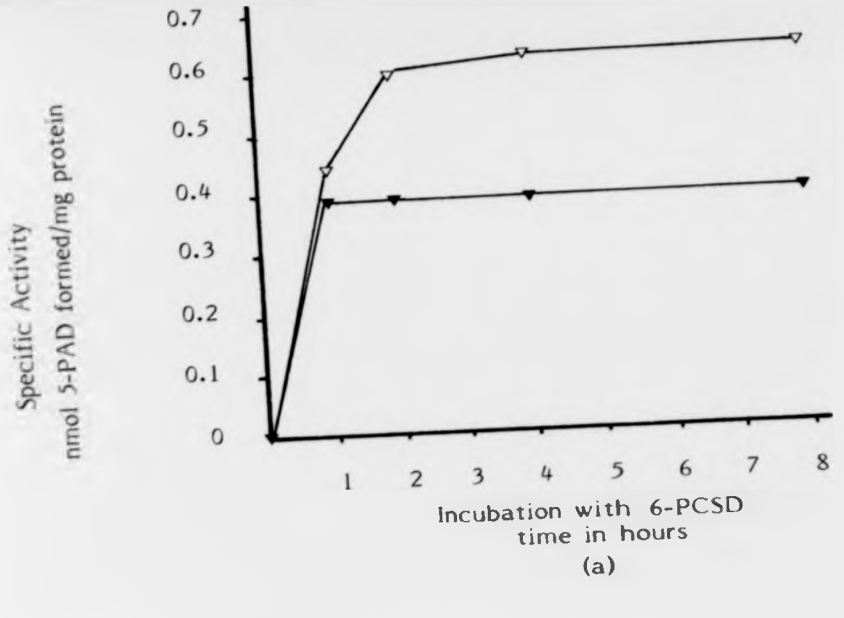
Figure 33

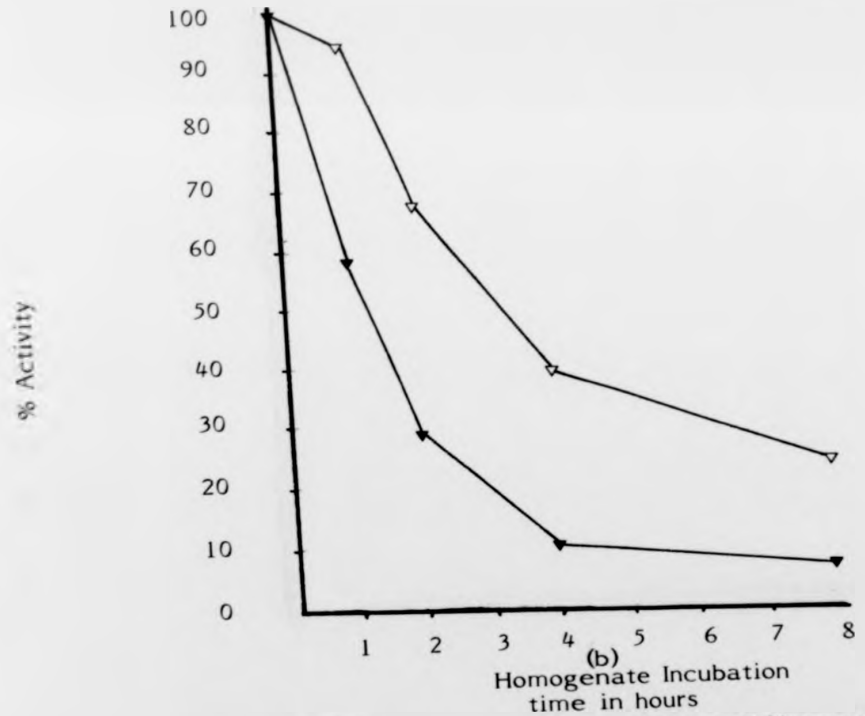
Loss of 5-PAD forming activity in goldfish homogenates upon incubation at 19°C.




100
90
80
70
60
50
40
30
20
10
0
% Activity
a
b
1
2
3
4
Incubation time in hours


100
90
80
70
60
50
40
30
20
10
0
% Activity
c
d
e
1
2
Incubation time in hours





(a) Incubation of Homogenate with 6-PCSD

(b) Incubation of Homogenate (19°C) prior to incubation with 6-PCSD

Fig 34. The effect of DTT on the 5-PAD forming activity of Goldfish liver homogenates

HPLC profiles of incubation mixtures of 6-PCSD goldfish liver homogenate in the presence of:

(a) potassium phosphate buffer pH 7.4, alone

(b) potassium phosphate buffer pH 7.4/5mM DTT

(c) HPLC profile of 5-PAD standard

B - DTT peak, absorbs strongly at 230 nm

P_1 - 6-PCSD

P_2 - 5-PAD

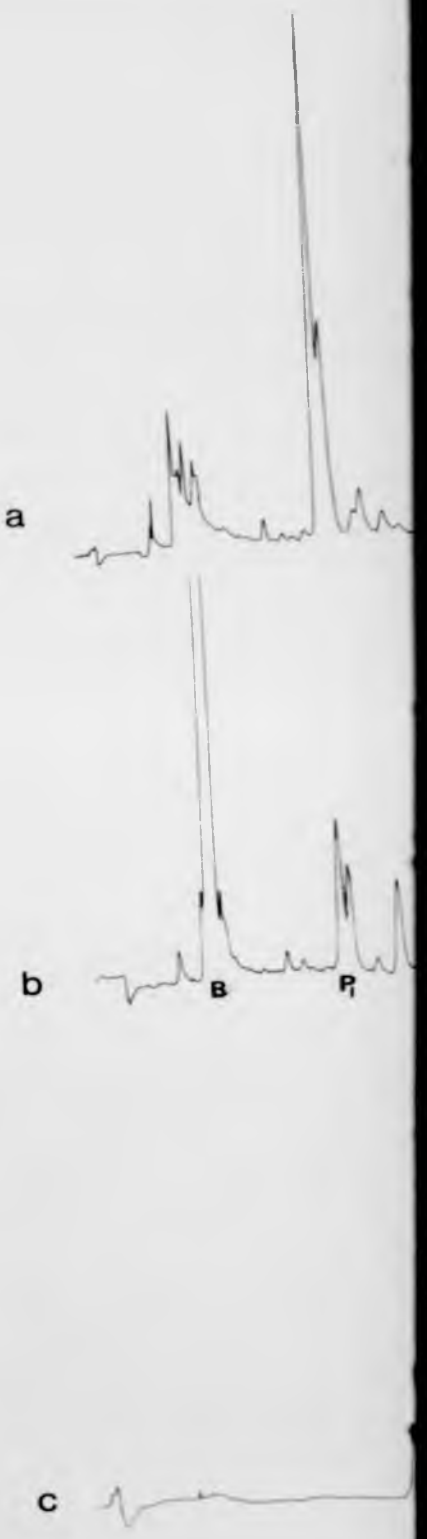


Figure 35

Increased 5-PAD formation by Goldfish liver homogenate upon addition of DTT to incubation mixtures.

s of 6-PCSD goldfish liver

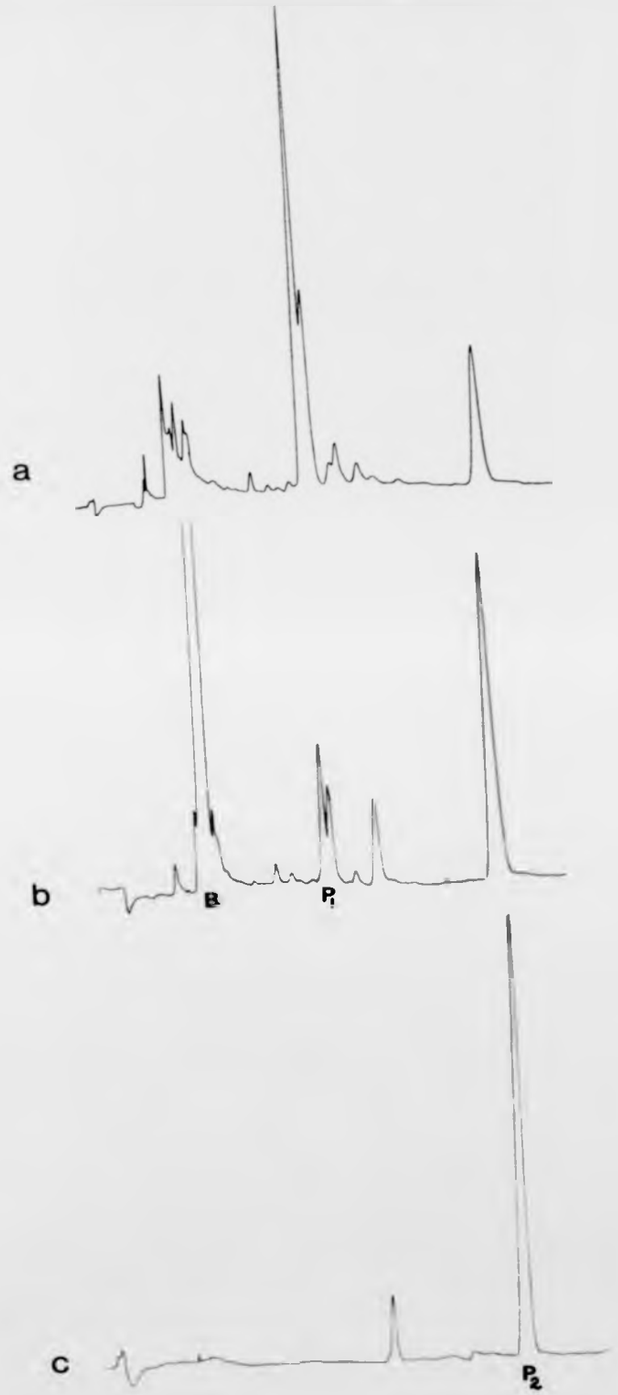
7.4, alone

7.4/5mM DTT

230 nm

ish liver homogenate upon

ures.



HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) pike liver crude homogenate prepared and incubated in potassium phosphate buffer pH 7.4

(b) pike liver crude homogenate prepared and incubated in potassium phosphate buffer pH 7.4/5 mM DTT

(c) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

B - DTT peak

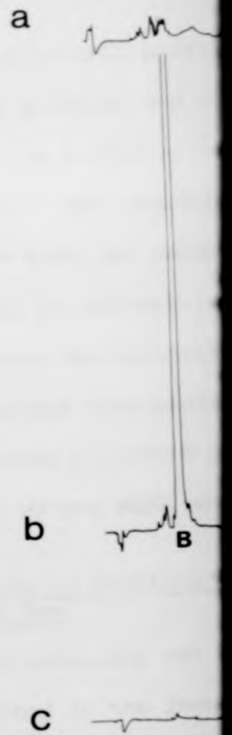
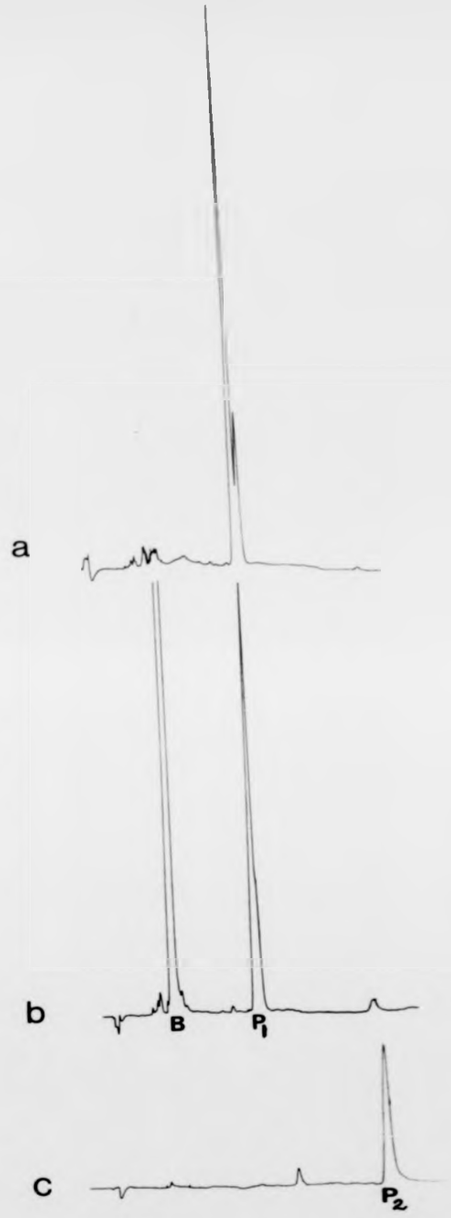


Figure 36

The presence of a detectable 5-PAD forming activity in Pike liver crude homogenate when incubated with DTT (5 mM) and 6-PCSD.


a
b
B
P1
c
P2

3.4.8 Comparison of the *in vitro* metabolism of 6-PCSD and Eulan WA New by liver homogenates of different species of freshwater fish

3.4.8.1 Incubation of liver homogenates with 6-PSCD

Crude liver homogenates from Goldfish, Carp, Pike, Trout, Perch and Eel were incubated with 6-PCSD in the presence of DTT (5 mM). 5-PAD formation was observed in incubation mixtures of Goldfish, Carp and Pike liver crude homogenates. No detectable 5-PAD formation was observed in Perch, Eel and Trout liver homogenates (Fig. 37). The specific activities of 5-PAD formation of the liver homogenates studies are given in Table 9.

The 5-PAD peaks in HPLC profiles of the extracted incubation mixtures containing goldfish and carp homogenates are quite similar. However, the amount of 6-PCSD extracted from these incubation mixtures are quite different. The reduction in the 6-PCSD component does not therefore correlate with the amount of 5-PAD formed. These differences may be accounted for by differing extraction efficiencies of 6-PCSD and 5-PAD components from the incubation mixtures containing different homogenates. A further interpretation would be to propose the formation of either a metabolite of 6-PCSD or 5-PAD by goldfish homogenate, which is not extractable by the MTBE method employed.

3.4.8.2 Incubation of Goldfish and Carp liver homogenates with Eulan WA New

Incubation of both carp and goldfish liver homogenates with Eulan WA New resulted in the formation of PAD products. HPLC profiles of incubation mixtures showed two product peaks resolved from the PCSD components of Eulan WA New (Fig. 38 peaks II and IV). In total three product peaks were proposed and were identified as the 5-PAD products of the parent 6-PCSD (Fig. 38, peaks I and III) [the minor component, which was observed upon incubation with 6-PCSD, coelutes with the 7-PCSD

HPLC profile of extracted incubation mixtures of 6-PCSD with the following liver crude homogenates in the presence of DTT (5 mM):

(a) Trout, Perch and Eel (sample profile)

(b) Carp

(c) Pike

(d) Goldfish

(e) HPLC profile of 5-PAD standard

B - DTT absorbance peak

P_1 - 6-PCSD

P_2 - 5-PAD

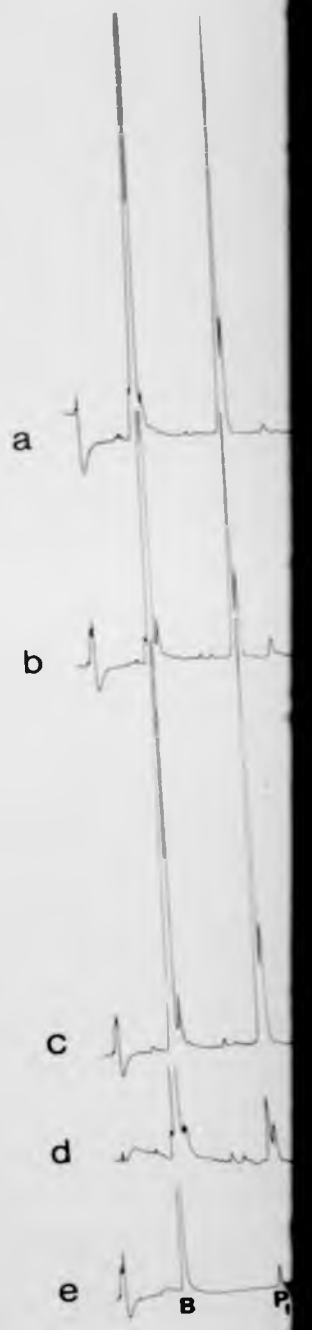


Figure 37

The *in vitro* metabolism of 6-PCSD to 5-PAD by crude liver homogenates of different species of freshwater fish.

n mixtures of 6-PCSD with

es in the presence of DTT

rofile)

to 5-PAD by crude liver

of freshwater fish.

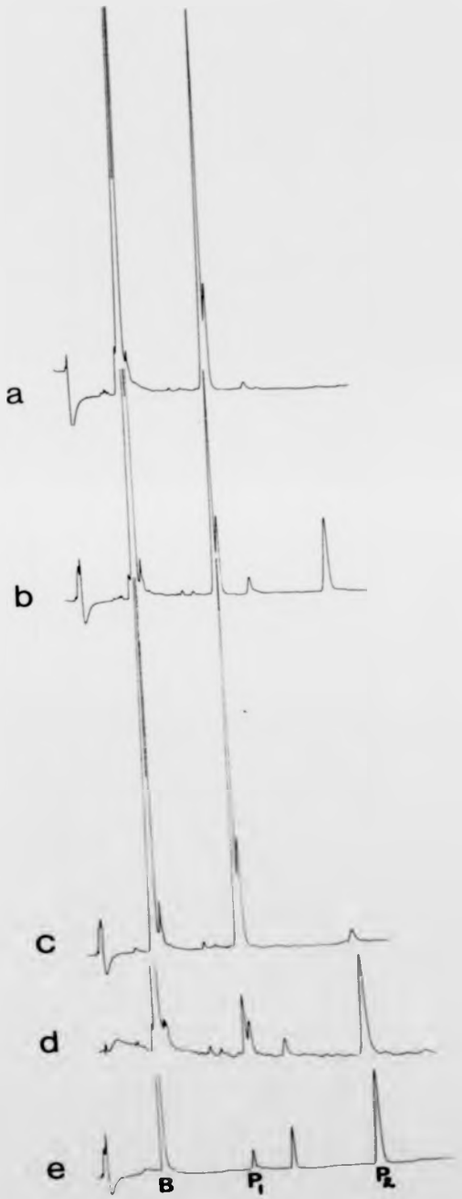


Table 9

Specific activities of 5-PAD formation by liver homogenates prepared from different species of freshwater fish, when incubated with 6-PCSD in the presence of DTT.

Crude liver homogenates prepared from the following fish	Specific activities nmol 5-PAD formed/mg protein/ incubation period
Goldfish	0.83
Carp	0.43
Pike	0.07
Trout	ND
Perch	ND
Eel	ND

ND - no detectable activity (to be detected the specific activity must be >0.01 n mol 5-PAD formed/mg protein/ incubation period)

HPLC profile of the extracted incubation mixture of:

(a) Eulan WA New in the absence of carp liver homogenate

(b) Eulan WA New in the presence of carp liver homogenate with DTT (5 mM)

(c) Carp liver homogenate in the absence of Eulan WA New with DTT (5 mM)

B - DTT peak

P_1 - 6-PCSD

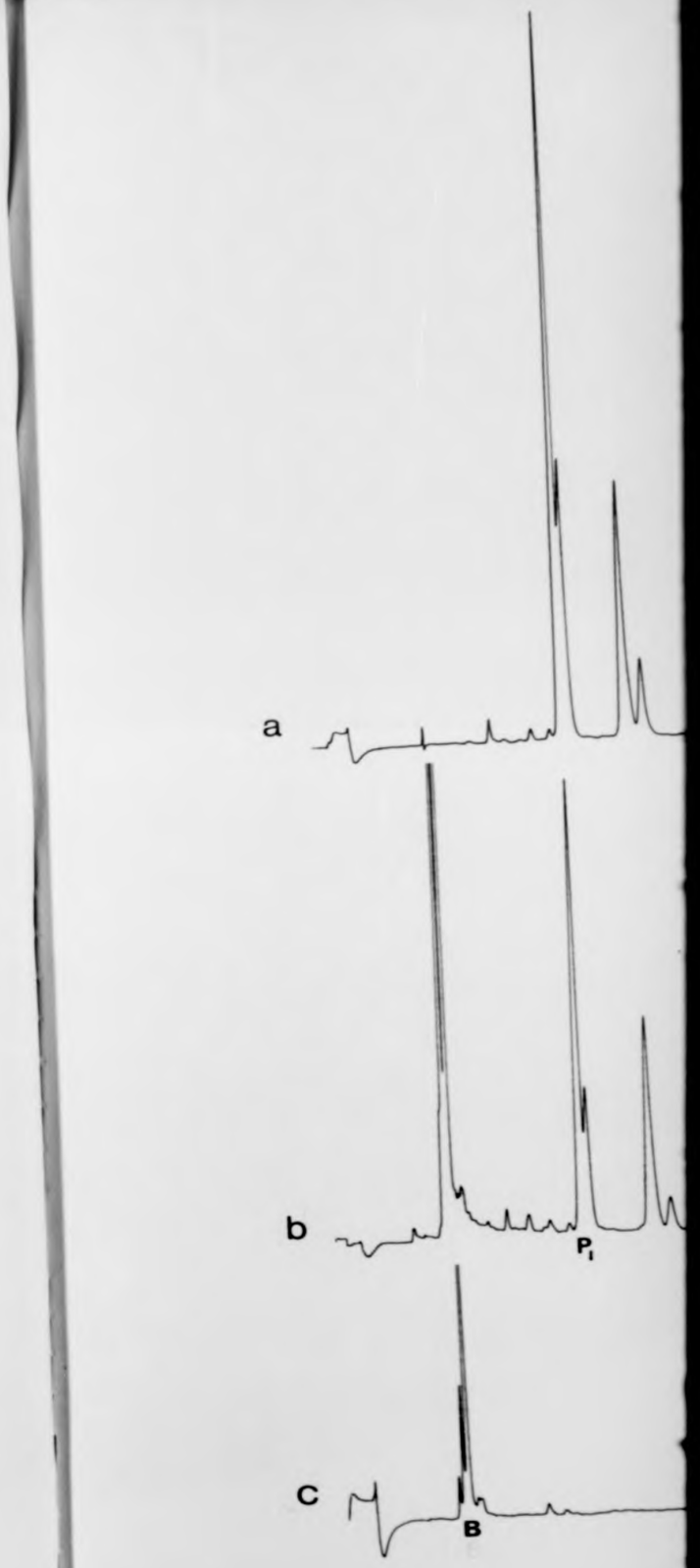


Figure 38

The *in vitro* metabolism of Eulan WA New by carp crude liver homogenate.

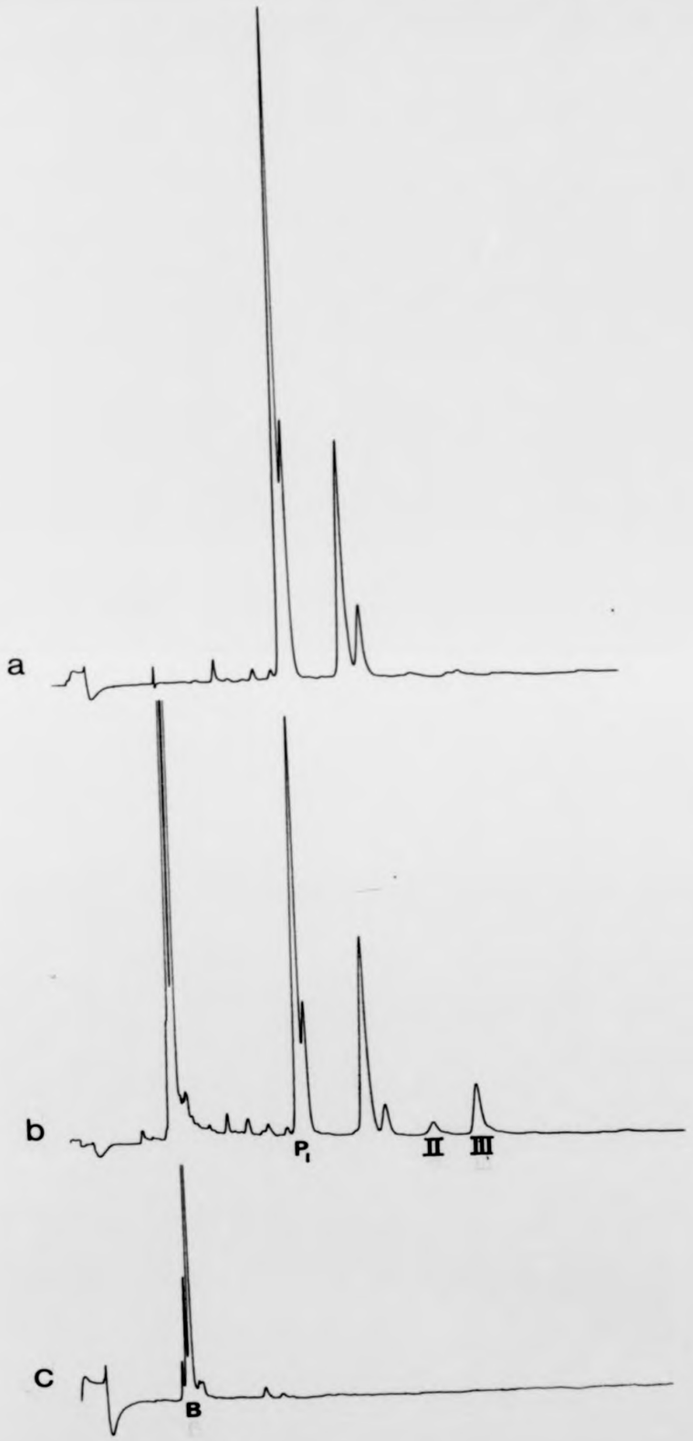
ubation mixture of:

of carp liver homogenate

of carp liver homogenate

absence of Eulan WA New

WA New by carp crude liver



HPLC profiles of:

(a) the extracted incubation mixture of goldfish liver crude homogenate with Eulan WA New

(b) 5-PCSD and its alkaline hydrolysis product

(c) 6-PCSD and its alkaline hydrolysis products

I - Unknown (PAD product of peak coeluting in HPLC profiles with 7-PCSD)

II - 4-PAD

III - 5-PAD

P_1 - 6-PCSD

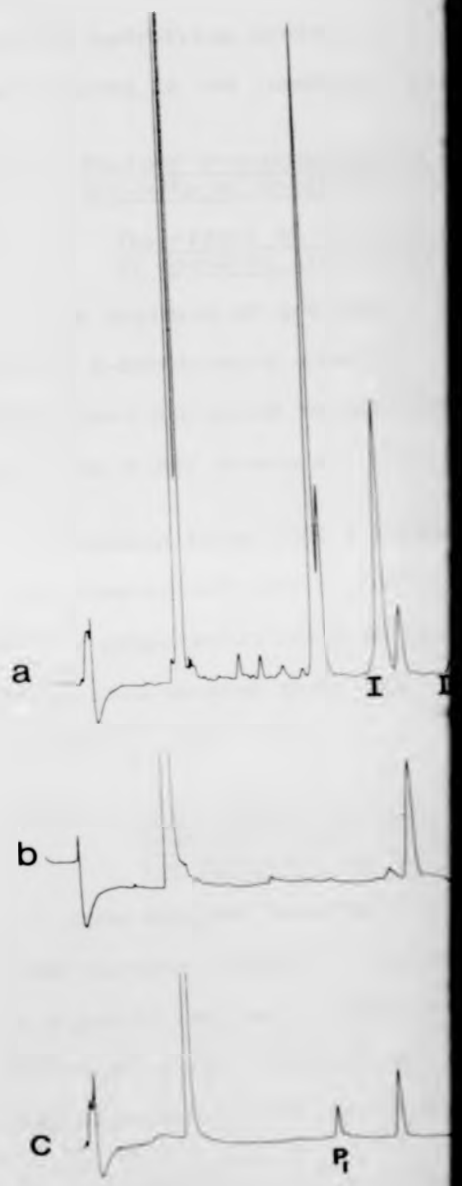
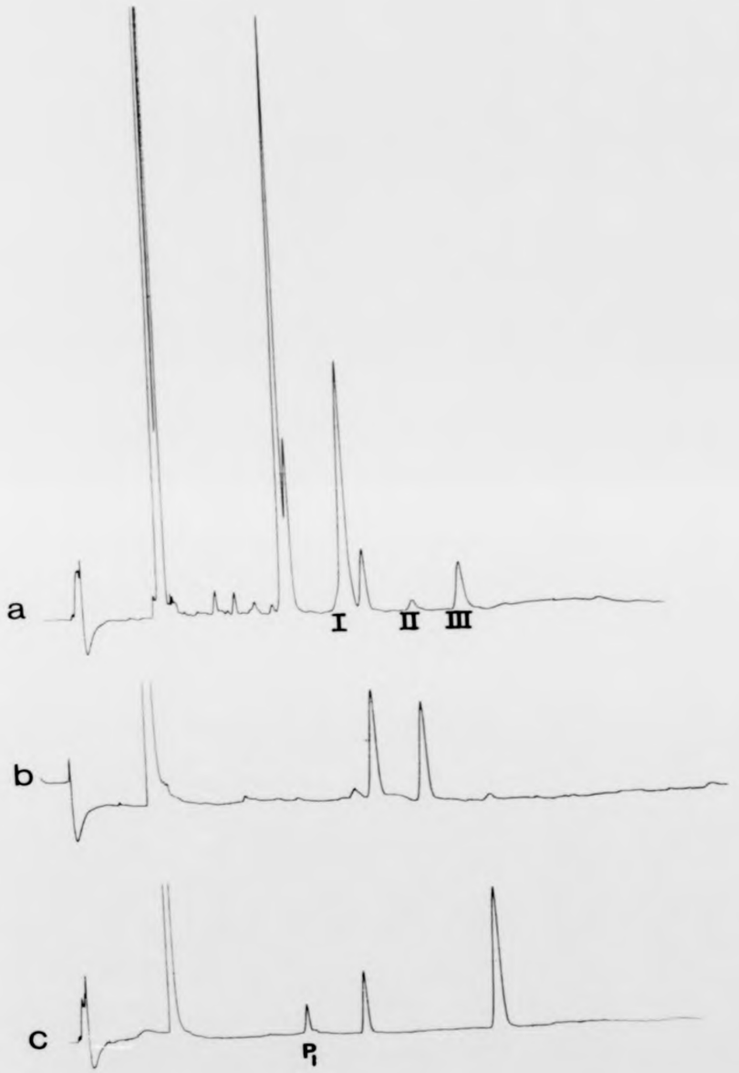


Figure 39

Identification of the *in vitro* metabolic products of Eulan WA New upon incubation with Goldfish liver crude homogenate


a
I
II
III
b
c
P1

component of Eulan WA New, upon HPLC analysis]. Also the PAD product of 5-PCSD (4-PAD) was identified by its coelution with the 5-PCSD alkaline hydrolysis product (Fig. 38, peak II). No detectable 6-PAD was observed in the incubation mixture extracts.

3.4.9 Further characterisation studies of the 5-PAD forming activity of Goldfish liver homogenates

3.4.9.1 The effect of dialysis on the 5-PAD forming activity of Goldfish liver 1000 g supernatant

The dialysis of goldfish liver 1000 g supernatant resulted in the loss of 5-PAD forming activity. Addition of a sample of the lyophilised dialysate to the dialysed 1000 g supernatant, restored in part, the 5-PAD forming activity (Fig. 40).

Goldfish liver 1000 g supernatant was prepared and assayed for 5-PAD forming activity. Addition of a sample of lyophilised dialysate to this preparation resulted in an increased 5-PAD forming activity (three fold greater than that observed in the 1000 g supernatant upon preparation, Fig. 41).

3.4.9.2 The stimulation of 5-PAD formation in dialysed goldfish homogenate and 1000 g supernatant, upon addition of some low molecular weight components to incubation mixtures

Upon dialysis goldfish liver crude homogenate showed no detectable 5-PAD forming activity. However addition of NADPH (2 mM) resulted in a partly restored 5-PAD forming activity (Table 10). A similar magnitude of HPLC response was observed upon the addition of lyophilised dialysate to the dialysed homogenate (Fig. 42).

The addition of particularly NADPH (2 mM), NADH (2 mM) and DTT (5 mM) increased 5-PAD formation in goldfish liver 1000 g supernatants (Table 11 and Fig. 43).

HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) goldfish liver 1000 g supernatant

(b) dialysed goldfish liver 1000 g supernatant

(c) dialysed goldfish liver 1000 g supernatant plus a sample of lyophilised dialysate

P_1 - 6-PCSD

P_2 - 5-PAD

The additional peaks observed in these HPLC profiles are tissue homogenate coextractants, since they were also observed in the HPLC profile of an extracted 1000 g supernatant sample in the absence of 6-PCSD

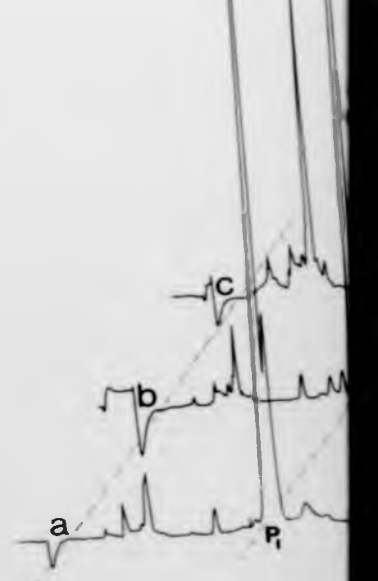
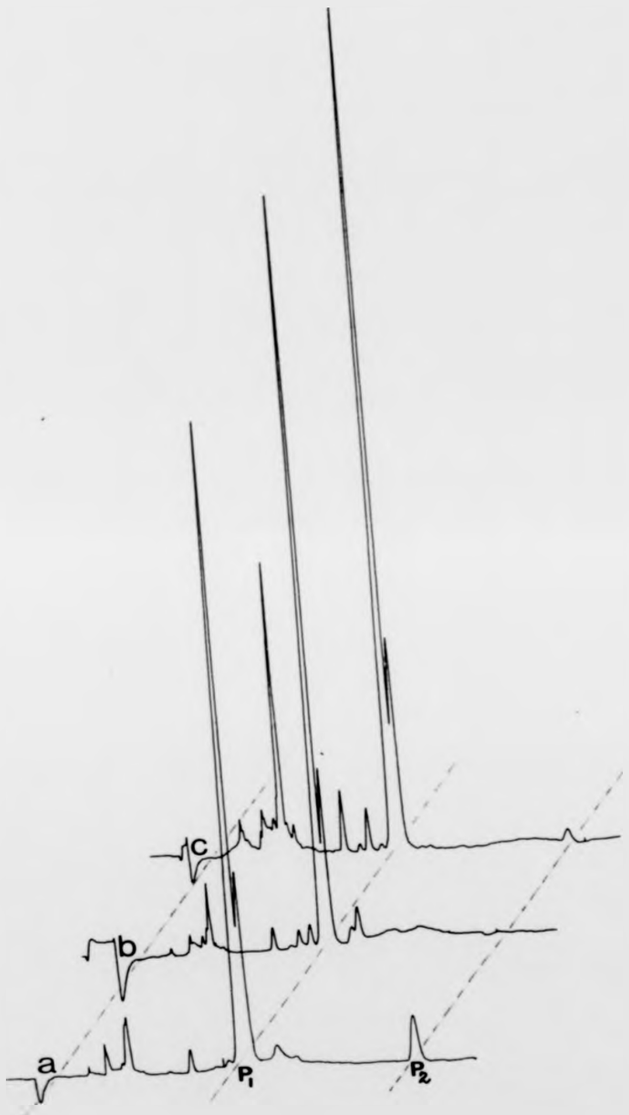


Figure 40

The loss of 5-PAD forming activity in dialysed goldfish 1000 g supernatant and its reappearance upon addition of lyophilised dialysis.


c
b
a
P1
P2

HPLC profile of extracted incubation mixtures of 6-PCSD with:

(a) goldfish liver 1000 g supernatant

(b) goldfish liver 1000 g supernatant plus the lyophilised dialysate from dialysed goldfish liver crude homogenate

(c) lyophilised dialysate from goldfish liver crude homogenate

P_1 - 6-PCSD

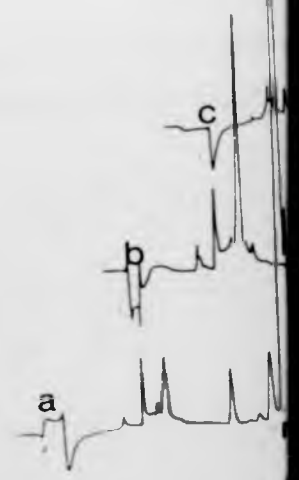
P_2 - 5-PAD

P_3 - ?

The peaks other than 6-PCSD and 5-PAD are tissue homogenate coextractants, since they appeared also in the HPLC profile of an extracted 1000 g supernatant sample in phosphate buffer, in the absence of 6-PCSD. P_3 (a peak which appears in the HPLC profile of the MTBE extract of phosphate buffer) is of interest because during incubation with 6-PCSD, the peak decreases. The correlation between its decrease and PAD formation suggests the possibility of its involvement in the metabolic events leading to the degradation of 6-PCSD to 5-PAD. The nature of the component is at present unknown.

Figure 41

<u>The stimulation of 5-PAD formation in Goldfish liver 1000 g supernatant upon addition of lyophilised dialysate.</u>



tion mixtures of 6-PCSD

rnatant

rnatant plus the lyophilised

ldfish liver crude homogenate

goldfish liver crude

5-PAD are tissue homogenate

red also in the HPLC profile

ant sample in phosphate

D. P_3 (a peak which appears

extract of phosphate buffer)

ncubation with 6-PCSD, the

on between its decrease and

ibility of its involvement in

the degradation of 6-PCSD to

onent is at present unknown.

tion in Goldfish liver 1000 g

yophilised dialysate.

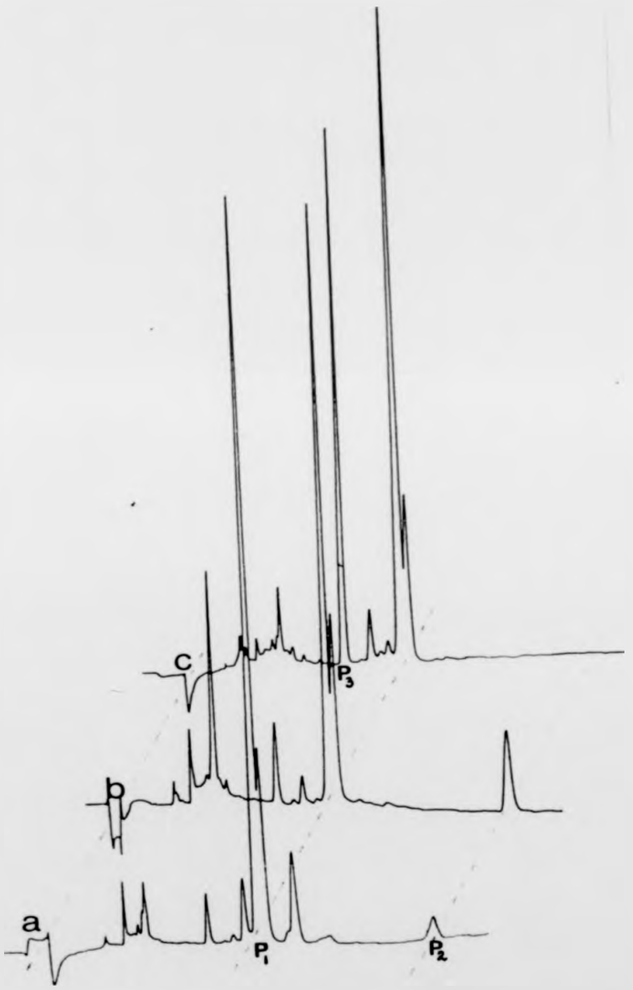


Table 10

The stimulation of 5-PAD forming activity in dialysed Goldfish liver homogenate upon addition of some low molecular weight components.

	µg 5-PAD formed/tube/ 3h incubation period
Dialysed goldfish crude homogenate alone	ND
+ GSH (5 mM)	0.10
+ UDPGA (2 mM)	0.13
+ ATP (1 mM)	0.03
+ ADP (1 mM)	ND
+ NADPH (2 mM)	0.19
+ lyophilised dialysate	0.19

ND Not detected - a value less than 0.03 µg/5-PAD/tube

8h incubation period is not detected by the HPLC system

Table 11

The stimulation of 5-PAD formation in Goldfish liver 1000 g supernatant upon addition of some small molecular weight components to incubation mixtures.

		µg 5-PAD formed/ tube/8h incubation period	Stimulated 5-PAD formation (µg/tube/ incubation period)
Goldfish liver 1000 g supernatant plus:		0.99	-
+ UDPGA	2 mM	0.99	-
+ GSH	2 mM	1.02	0.03
+ NADPH	2 mM	1.44	0.45
+ $NADP^+$	2 mM	1.02	0.03
+ NADH	2 mM	1.29	0.30
+ NAD^+	2 mM	1.02	0.03
+ DTT	5 mM	2.30	1.31
+ UDPGA, GSH NADPH each	2 mM	1.41	0.42

HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) dialysed goldfish liver crude homogenate

(b) dialysed goldfish liver crude homogenate plus lyophilised dialysate

(c) dialysed goldfish liver crude homogenate plus NADPH (2 mM)

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 42

Stimulation of 5-PAD forming activity in dialysed Goldfish liver homogenate upon addition of lyophilised dialysate or NADPH.

ation mixtures of 6-PCSD

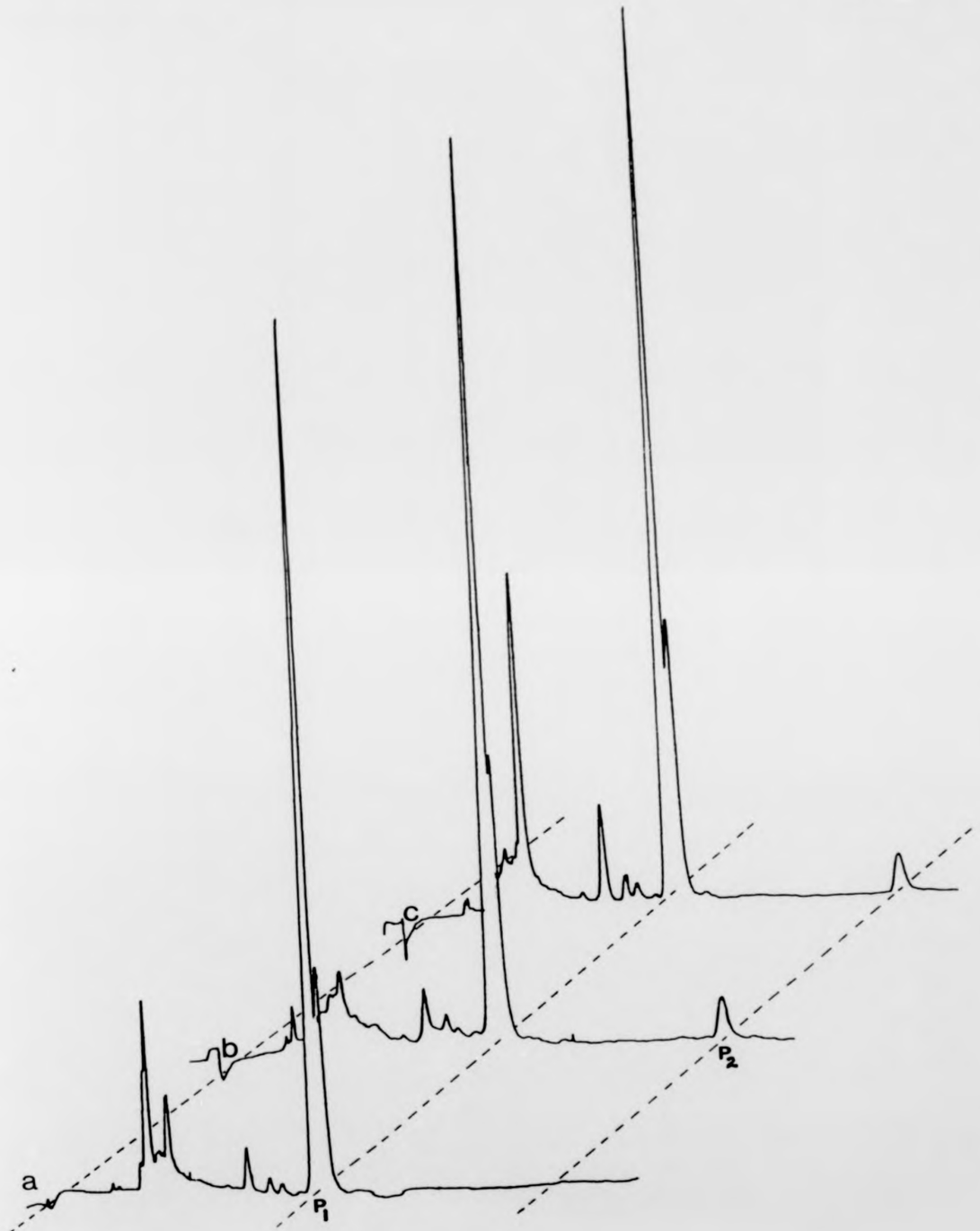
de homogenate

de homogenate plus

de homogenate plus NADPH

ctivity in dialysed Goldfish

of lyophilised dialysate



HPLC profiles of extracted incubation mixtures of:

(a) 6-PCSD in the absence of goldfish liver 1000 g superantant

(b) 6-PCSD with goldfish liver 1000 g supernatant

(c) 6-PCSD with goldfish liver 1000 g supernatant plus NADH (2 mM)

(d) 6-PCSD with goldfish liver 1000 g supernatant plus NADPH (2 mM)

(e) goldfish liver 1000 g supernatant

(f) HPLC profile of 5-PAD standard

P_1 - 6-PCSD

P_2 - 5-PAD

P_3 - ? unknown peak extracted from phosphate buffer

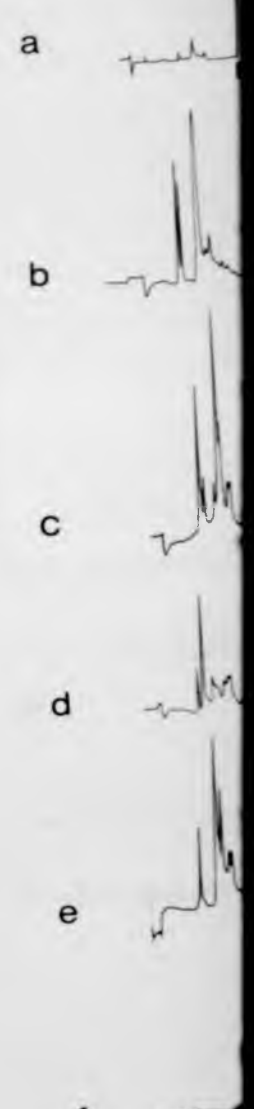
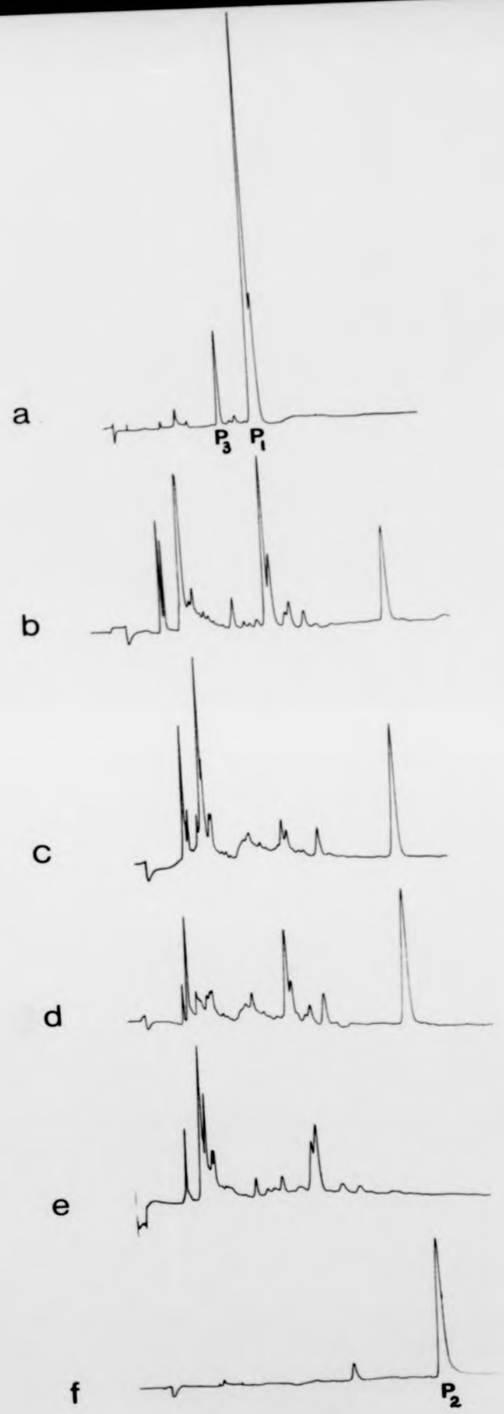
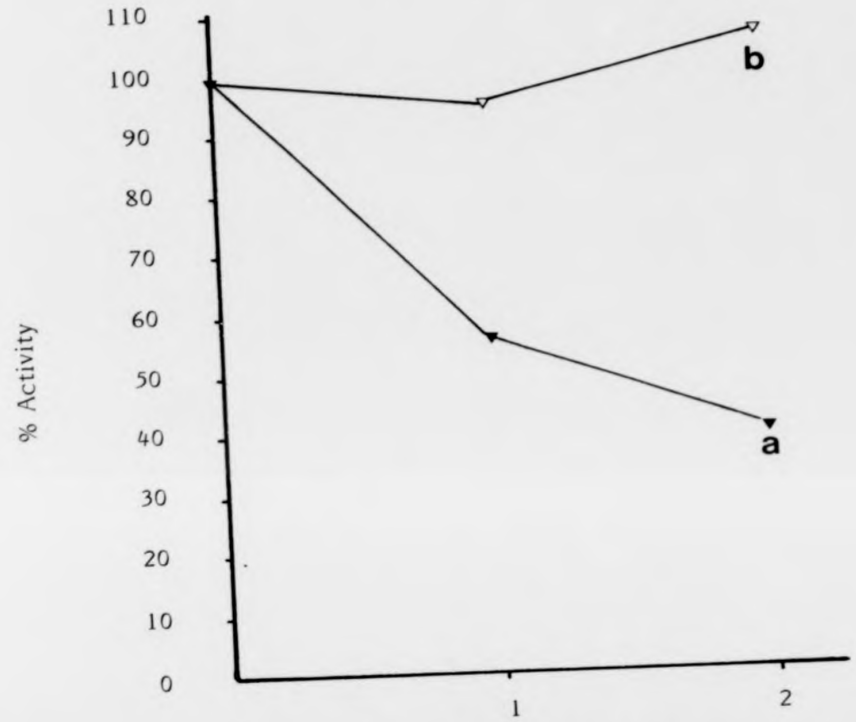


Figure 43

Increased 5-PAD forming activity in Goldfish liver 1000 g supernatant upon addition of NADH (2 mM) and NADPH (2 mM)


a
P3
P1
b
c
d
e
f
P2



a 40mM potassium phosphate buffer / 5mM DTT, pH 7.4

b 40mM potassium phosphate buffer / 5mM DTT / 2mM NADPH, pH 7.4

Fig 44. No loss of 5-PAD forming activity in Goldfish homogenates incubated with DTT and NADPH

3.4.9.3 A comparison of the 5-PAD forming activity of Goldfish liver homogenate in incubation mixtures containing DTT and DTT/NADPH

The addition of DTT to goldfish liver homogenate, increased both the activity and stability (i.e. a decreased loss of activity with time) of 5-PAD formation (section 3.4.6.4). However, a general trend of decreasing 5-PAD formation is still observed when homogenates are incubated at 19°C in the presence of DTT. This trend was not observed when NADPH was added to homogenates before incubation at 19°C. The initial 5-PAD forming activity of the goldfish liver homogenate (with DTT/NADPH) was maintained throughout a 2h homogenate incubation period. By contrast, the homogenate with DTT alone, possessed only 40% of the initial 5-PAD forming activity after an identical 2h incubation period (Fig. 44).

3.4.10 Subcellular location of the 5-PAD forming activity in Goldfish and Carp livers

Subcellular fractions were prepared from goldfish and carp livers as described in section 3.4.10.1.

3.4.10.1 Carp liver subcellular fractionation

A sample of carp liver crude homogenate was stored at 4°C upon preparation, during the subcellular fractionation period. This sample was assayed both upon preparation and after incubation for the period of the subcellular fractionation, giving a measure of the 5-PAD forming activity to be found in the subcellular fractions (Fig. 45a and b).

Each subcellular fraction from carp liver was assayed for 5-PAD forming activity by incubation with 6-PCSD. Activity was observed in the 1000 g and 10,000 g supernatants, but no 5-PAD forming activity in either of the 140,000 g supernatant or 140,000 g pellet

HPLC profiles of extracted incubation mixtures of 6-PCSD

with:

(a) carp liver crude homogenate assayed upon preparation

(b) carp liver crude homogenate incubated for the subcellular fractionation period

(c) carp 140,000 g supernatant

(d) carp 140,000 g pellet

(e) carp 140,000 g supernatant plus 140,000 g pellet

Preparation and incubation buffer:

0.25 M sucrose/40 mM potassium phosphate pH 7.5/ 5 mM DTT

B - DTT peak

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 45

The requirement of components of both the 140,000 g supernatant and pellet fractions for 5-PAD formation by subcellular fractions of carp liver.

ubation mixtures of 6-PCSD

te assayed upon preparation

te incubated for the

period

t

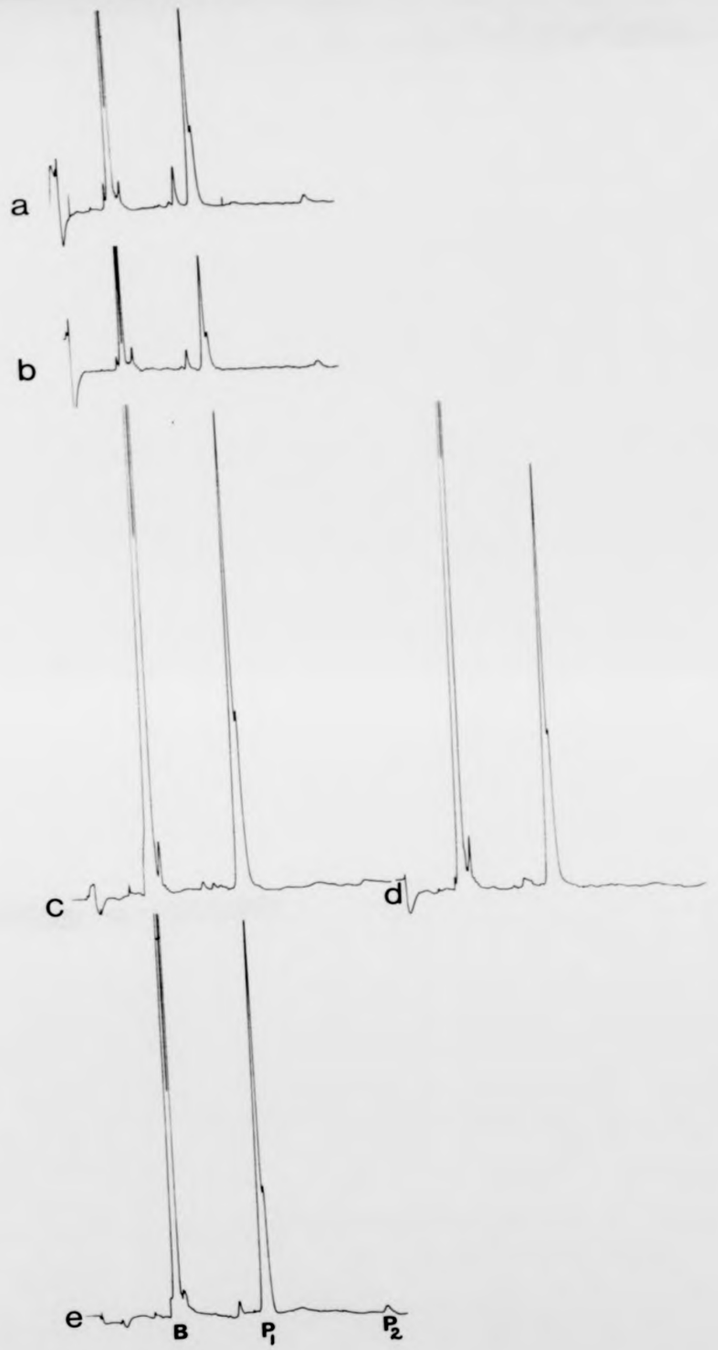
t plus 140,000 g pellet

ffer:

ssium phosphate pH 7.5/ 5 mM DTT

of both the 140,000 g supernatant

D formation by subcellular fractions



(Fig. 45c and d). However the addition of a sample of the 140,000 g supernatant to that of the 140,000 pellet and incubation with 6-PCSD, did result in detectable 5-PAD formation (Fig. 45e). The incubation of aliquots of the 140,000 g supernatant with 6-PCSD and each of the following; UDPGA (2 mM), GSH (5 mM), NADH (2 mM), NADPH (2 mM) or acetyl coA (0.5 mM) did not result in 5-PAD formation in any of the incubation mixtures. No new peaks were observed in the HPLC profiles of the extracted incubation mixtures. Incubation of the latter molecules with 140,000 g pellet and 6-PCSD, resulted in 5-PAD formation in only the incubation mixtures containing NADPH (2 mM) or NADH (2 mM) (Fig. 46). The 5-PAD forming activity therefore resides in the prepared 140,000 g pellet and requires either of the components NADPH or NADH for activity.

3.4.10.2 Goldfish liver rapid subcellular fractionation

A sample of goldfish liver crude homogenate was stored upon preparation at 4°C. This sample was assayed for 5-PAD forming activity upon preparation and also upon incubation for the period of the liver subcellular fractionation (Fig. 47a and b).

Both 140,000 g goldfish liver supernatant and pellet fractions showed no 5-PAD forming activity when incubated individually with 6-PCSD (Fig. 47c and d). However, the addition of a sample of 140,000 g supernatant to 140,000 g pellet and their incubation with 6-PCSD, gave detectable 5-PAD forming activity (Fig. 47e). 5-PAD formation was stimulated in the 140,000 g pellet fraction upon addition of NADPH (Fig. 48a and b). Adding both NADPH and NADH to the 140,000 g pellet fraction resulted in a very similar 5-PAD formation in HPLC profiles to that observed with NADPH alone (Fig. 48c).

HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) carp liver 140,000 g pellet and UDPGA (2 mM) or GSH (5 mM) or Acetyl coA (0.5 mM) sample profile

(b) carp liver 140,000 g pellet plus NADH (2 mM)

(c) carp liver 140,000 g pellet plus NADPH (2 mM)

(d) HPLC profile of 5-PAD standard

Preparation and incubation buffer:

0.25 M sucrose/40 mM potassium phosphate/5 mM DTT, pH 7.5

B - DTT peak

P_1 - 6-PCSD

P_2 - 5-PAD

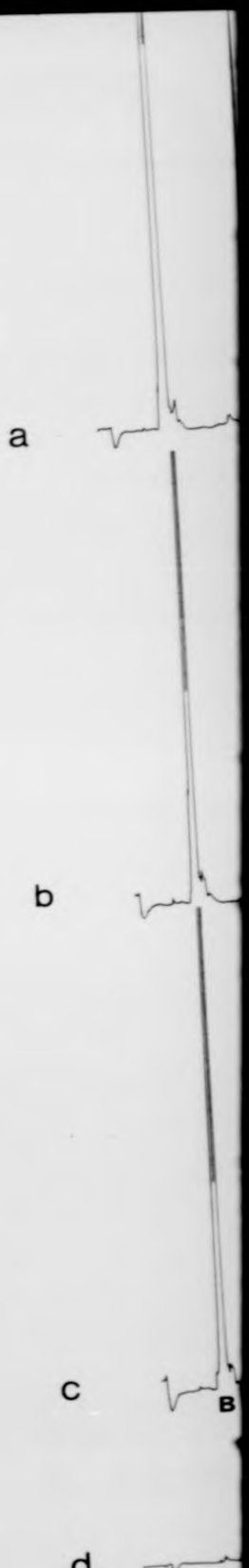


Figure 46

The presence of a NADH or NADPH stimulated 5-PAD forming activity in carp liver 140,000 g pellet fraction.

ubation mixtures of 6-PCSD with:

let and UDPGA (2 mM) or

(0.5 mM) sample profile

let plus NADH (2 mM)

let plus NADPH (2 mM)

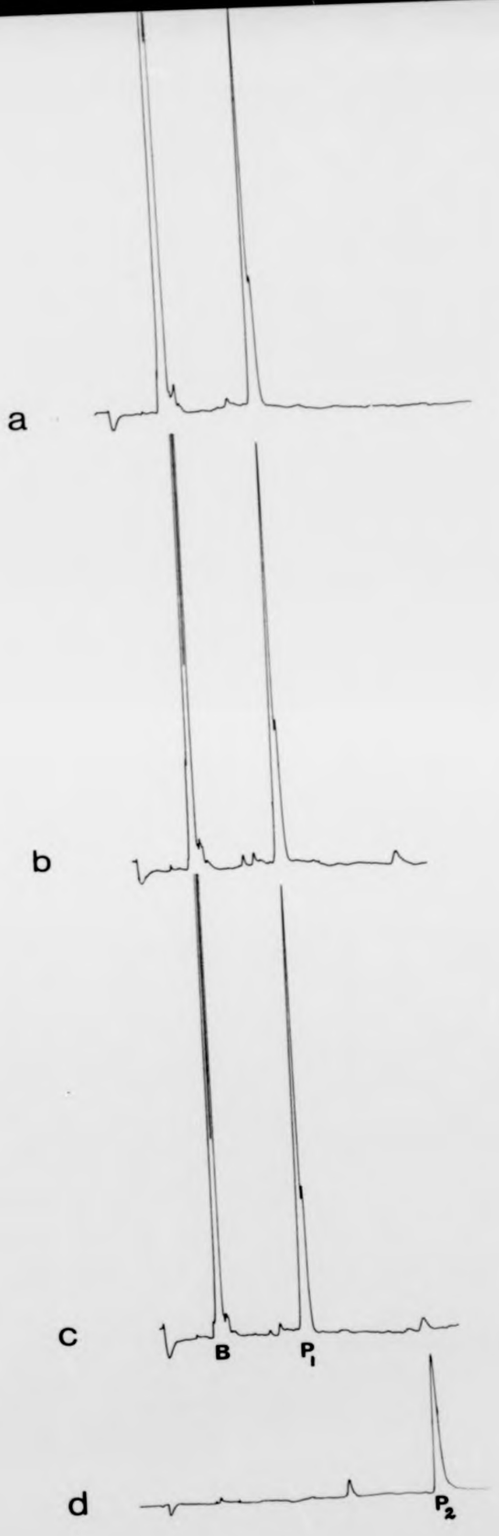
andard

ffer:

assium phosphate/5 mM DTT, pH 7.5

PH stimulated 5-PAD forming

00 g pellet fraction.



HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) goldfish liver crude homogenate assayed upon preparation

(b) goldfish liver crude homogenated assayed after incubation for the period of the subcellular fraction

(c) goldfish liver 140,000 g supernatant

(d) goldfish liver 140,000 g pellet

(e) goldfish liver 140,000 g supernatant plus 140,000 g pellet

Preparation and incubation buffer:

0.25 M sucrose/40 mM potassium phosphate/5 mM DTT, pH 7.5

P_1 - 6-PCSD

P_2 - 5-PAD

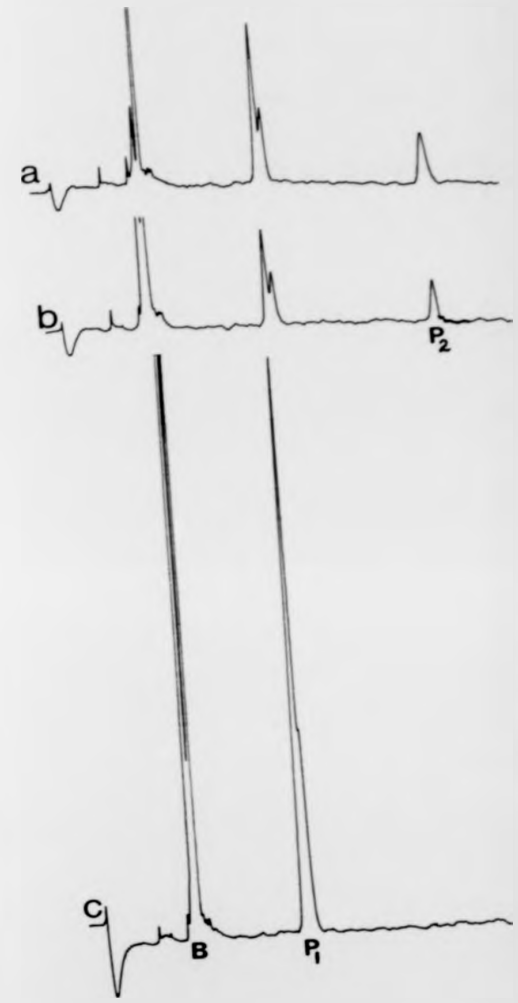


Figure 47

The requirement of components of both the 140,000 g supernatant and pellet for 5-PAD formation by subcellular fractions of goldfish liver.

cubation mixtures of 6-PCSD

ogenate assayed upon preparation

ogenated assayed after incubation

bcellular fraction

g supernatant

g pellet

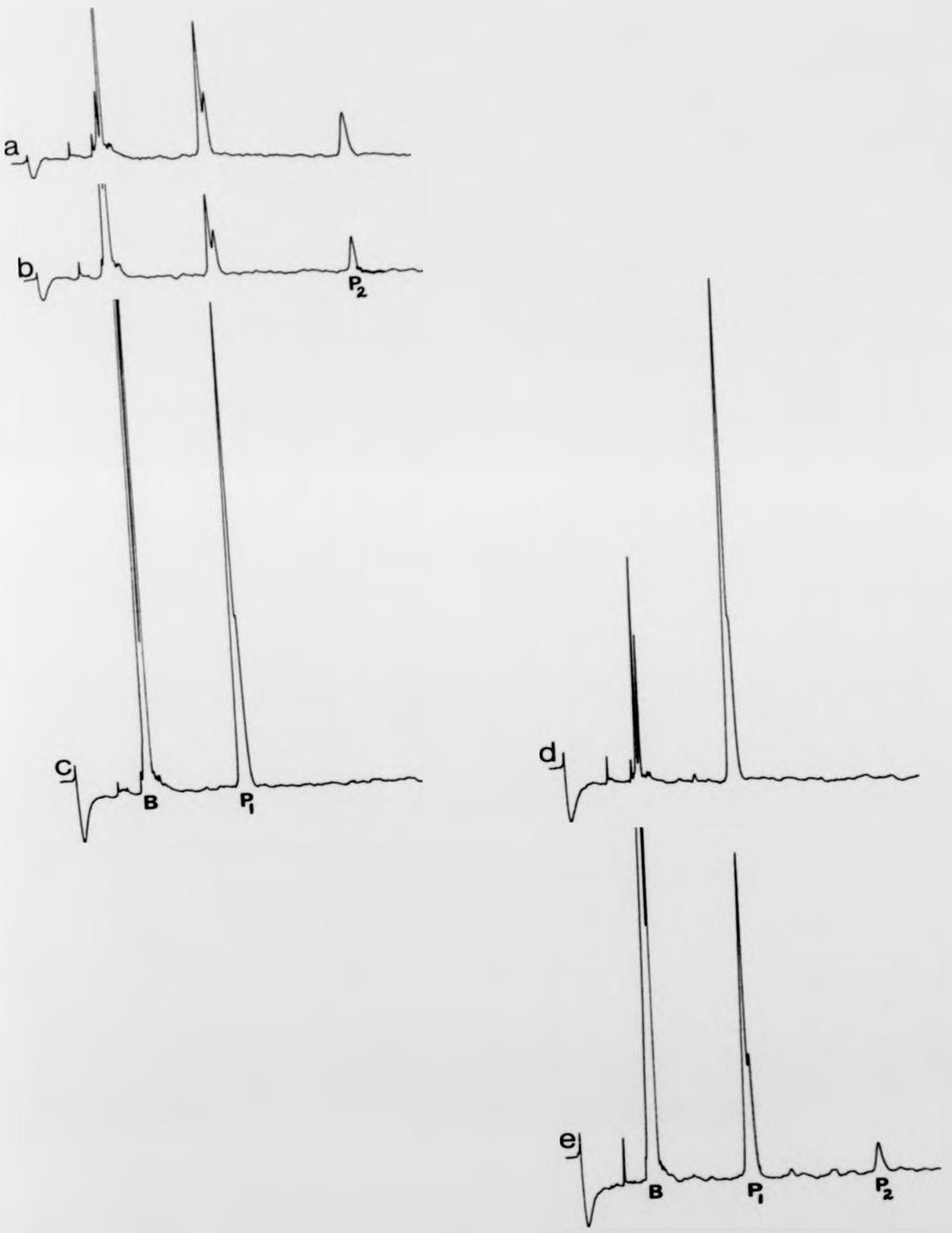
g supernatant plus

uffer:

tassium phosphate/5 mM DTT, pH 7.5

s of both the 140,000 g supernatant

on by subcellular fractions of



HPLC profiles of extracted incubation mixtures of 6-PCSD with:

(a) goldfish liver 140,000 g pellet

(b) goldfish liver 140,000 g pellet plus NADPH (2 mM)

(c) goldfish liver 140,000 g pellet plus NADPH (2 mM) and NADH (2 mM)

(d) HPLC profile of 5-PAD standard

B - DTT peak

P_1 - 6-PCSD

P_2 - 5-PAD

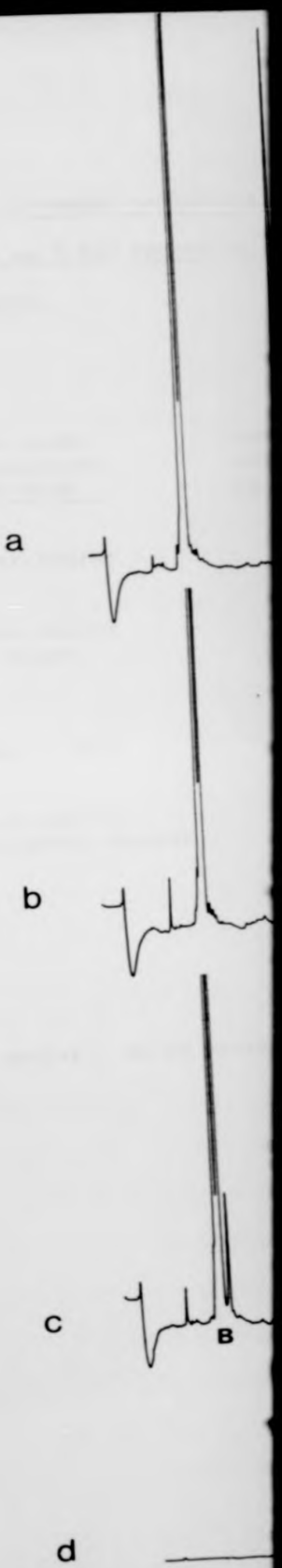


Figure 48

The presence of a NADH and NADPH stimulated 5-PAD forming activity in Goldfish liver 140,000 g pellet fraction.

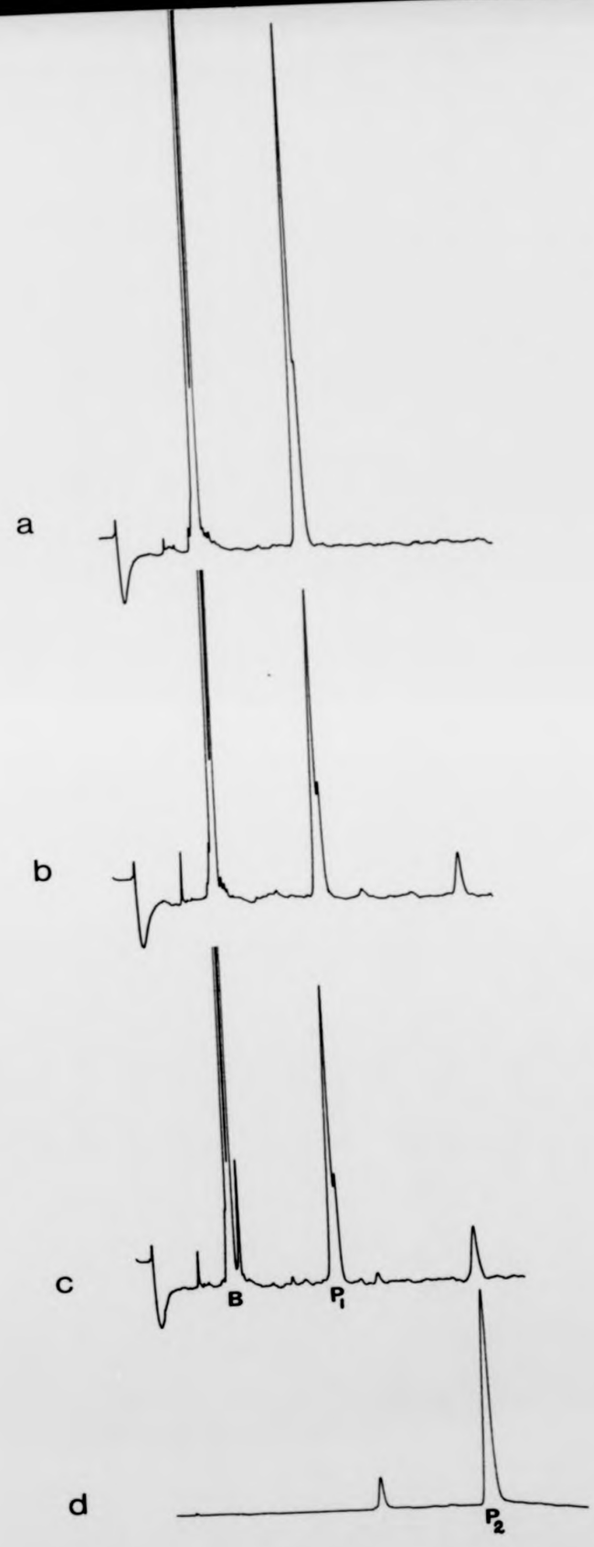
ubation mixtures of 6-PCSD with:

pellet

pellet plus NADPH (2 mM)

pellet plus NADPH (2 mM)

andard



ADPH stimulated 5-PAD forming

40,000 g pellet fraction.

Table 12

The effect of increased concentrations of oxygen and carbon dioxide on 5-PAD formation by Goldfish liver crude homogenates.

Goldfish liver crude homogenate incubation mixture with 6-PCSD	Specific activity nmol 5-PAD formed/ mg protein/8h incubation period
40 mM phosphate buffer	0.39
40 mM phosphate buffer flushed with oxygen (sealed)	0.68
40 mM phosphate buffer	0.18
40 mM phosphate buffer flushed with carbon dioxide (sealed)	0.09

Preparation buffer - 40 mM potassium buffer pH 7.4

3.4.11 The effect of oxygen and carbon dioxide on 5-PAD formation of Goldfish liver crude homogenate

An incubation mixture of goldfish liver homogenate with 6-PCSD, flushed with oxygen and sealed, gave almost double the specific activity of 5-PAD formation observed in an open incubation mixture. By comparison, the 5-PAD forming activity of goldfish liver homogenate was reduced by 50% upon flushing with carbon dioxide (Table 12).

SUMMARY

The first positive identification of *in vitro* 5-PAD formation was observed in goldfish liver homogenate. 5-PAD formation was highest at pH 7.4, lower at pH 9.0 and absent at pH 3.0 and 5.0. No 5-PAD forming activity was detected in spleen or digective tissue homogenates from the goldfish. An increased 5-PAD formation was observed upon preparation and incubation of goldfish liver homogenate in 40 mM potassium phosphate buffer pH 7.4, compared with a range of other buffers of the same concentration and pH.

A time course study of 5-PAD formation by goldfish liver homogenate when incubated with 6-PCSD, showed no further formation of 5-PAD after 1h incubation at 19°C. Incubation of the homogenate at this temperature prior to its assay for 5-PAD formation, showed that 5-PAD forming activity decreased rapidly with incubation time. The addition of DTT to the homogenate in phosphate buffer, increased both the activity of 5-PAD formation and the stability of the 5-PAD forming activity upon incubation. However, in the presence of DTT, a general trend of decreasing 5-PAD formation was still observed upon incubation. The 5-PAD forming activity of goldfish liver homogenate in phosphate buffer was observed to increase substantially when incubation mixtures

were flushed with oxygen, and greatly decrease when flushed with carbon dioxide.

Dialysis of goldfish liver 1000 g supernatant resulted in the loss of 5-PAD forming activity. The activity was restored upon addition of the lyophilised dialysate to the dialysed supernatant. Similarly, addition of particularly NADPH to the dialysed or non-dialysed supernatant stimulated 5-PAD formation. Incubation of goldfish liver homogenate and NADPH at 19°C resulted in no loss of 5-PAD forming activity over a 2h period. Incubation of the same homogenate in the presence of DTT alone for this period resulted in the loss of 60% of the initial 5-PAD forming activity.

Rapid subcellular fractionation of goldfish and carp livers located the 5-PAD forming activity in the 140,000 g pellet (contaminated with mitochondria). 5-PAD forming activity was only observed in this fraction upon addition of either NADPH or NADH to incubation mixtures, or upon addition of a sample of the 140,000 g supernatant to the 140,000 g pellet.

Goldfish, carp, pike, trout, perch and eel liver homogenates were prepared in the presence of DTT, and incubated with 6-PCSD. 5-PAD formation was only observed by goldfish, carp and pike homogenates. The 5-PAD forming activity was an order of magnitude greater in goldfish and carp compared with that of the pike liver homogenate. The presence of DTT was necessary to result in detectable 5-PAD forming activity in pike liver homogenate. Early *in vitro* studies involving pike liver homogenate did not include the addition of DTT to incubation mixtures. This mainly accounts for the reported absence of detectable 5-PAD forming activity during the early stages of this work (sections 3.4.3.2 to 3.4.3.6).

3.5 DISCUSSION

5-PAD forming activity of goldfish liver homogenate was progressively lost upon incubation at 19°C. Factors which may account for the observed lability of the 5-PAD forming activity include:

(a) Digestion by proteolytic enzymes released during homogenate preparation.

(b) Accumulation of an inhibitor.

(c) Enzyme inactivation by processes modifying important structural and/or functional groups of the protein(s).

(d) Loss of a cofactor essential for enzyme activity.

Proteolytic digestion may arise from the release of proteinases upon homogenisation of tissues. The lysosomes represent a major site of compartmentation of hydrolytic enzymes. Lysis of this organelle results in the release of such degradative activities. The addition of sucrose prior to homogenisation affords osmotic protection against organelle disruption and enzyme release. The addition of BSA to incubation mixtures provides large substrate protein concentrations for active proteinases and so effectively reduces the digestion of protein(s) under study. For example the addition of BSA to incubation mixtures containing the southerm army worm gut homogenate, effectively protected microsomal monooxygenase activities from digestion by inhibitory proteinase factors present (Brattsten and Wilkinson, 1973). In addition to this role BSA non-specifically binds a wide range of potential enzyme inhibitors.

Addition of sucrose, BSA or the serine proteinase inhibitor PMSF to samples of goldfish homogenate did not substantially prevent the loss of 5-PAD forming activity upon incubation. Therefore the

observed loss of 5-PAD forming activity is not likely to be in most part due to proteinase digestion or apparently the accumulation of an inhibitor.

The addition of DTT did however partially prevent the progressive loss of 5-PAD forming activity observed upon incubation of goldfish homogenates. Both the initial activity and stability of 5-PAD forming activity was increased, when DTT was added to goldfish liver homogenate in phosphate buffer, prior to incubation. DTT is a sulphydryl protecting agent which because of its low redox potential, maintains monothiols in their reduced state and reduces disulphides quantitatively (Cleland, 1964). Its role in enhancing 5-PAD formation implicates the presence of important structural and/or functional sulphydryl groups as part of the 5-PAD forming activity. Complementary to this hypothesis was the observed inhibition of 5-PAD forming activity in goldfish liver homogenate by the addition of the sulphydryl binding agent pHMB. Although the addition of DTT to homogenates increased 5-PAD formation above that observed in phosphate buffer, the general trend of decreasing 5-PAD formation upon incubation was still observed. This suggests that the observed loss of 5-PAD forming activity involves an interplay of a number of factors in addition to the apparent loss of important sulphydryl groups associated with the activity.

The dialysis of goldfish liver 1000 g supernatant resulted in the loss of 5-PAD forming activity and further, readdition of a fraction of lyophilised dialysate resulted in the stimulation of 5-PAD formation. A low molecular weight dialysable factor is therefore required for 5-PAD formation by the goldfish liver 1000 g supernatant. The addition of NADPH to dialysed, and NADPH or NADH

to non-dialysed goldfish liver supernatant, resulted in stimulation of 5-PAD forming activity. That a similar increase in 5-PAD formation was observed upon addition of lyophilised dialysate or NADPH to the dialysed goldfish liver supernatant suggests that NADPH is an essential cofactor required for 5-PAD formation, and that 5-PAD formation is limited by the loss of this molecule upon dialysis.

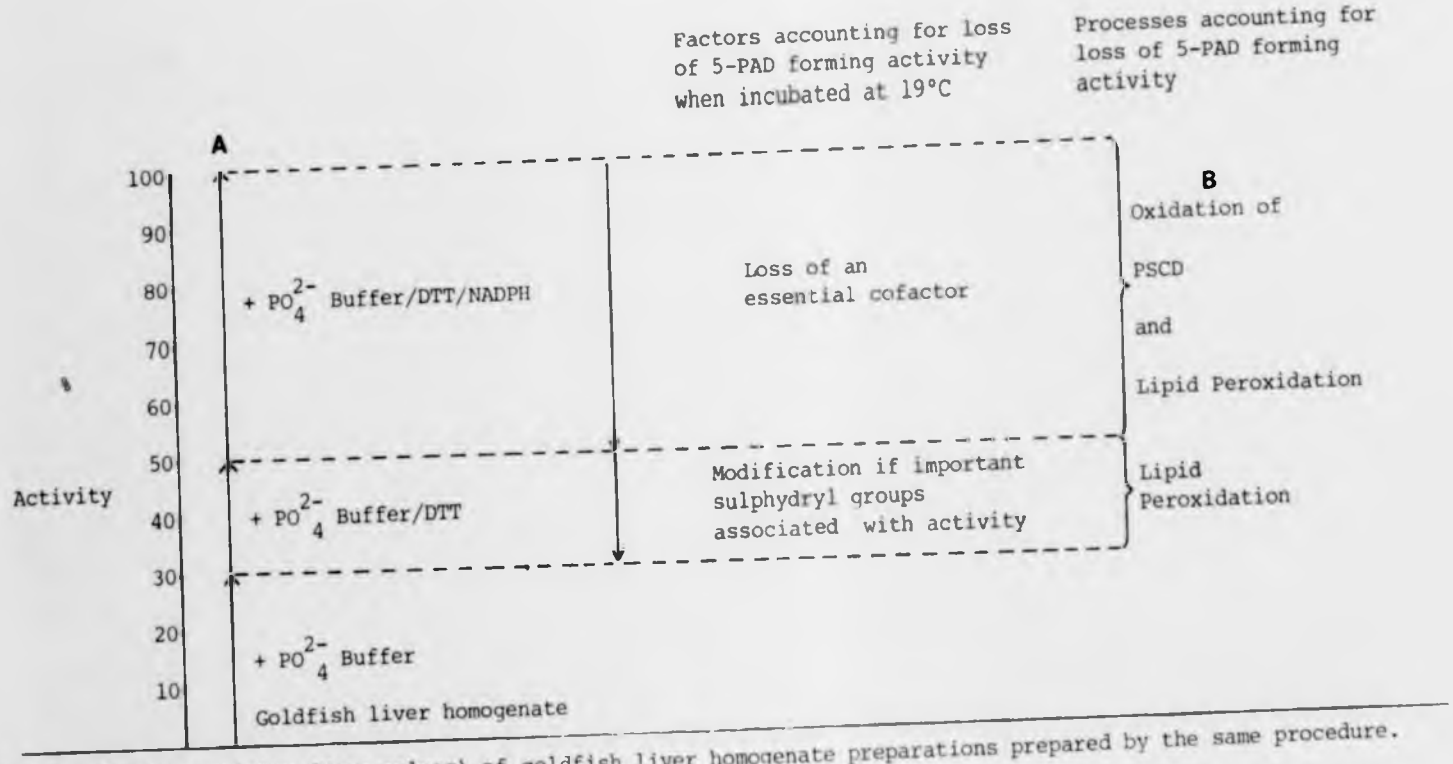
Rapid subcellular fractionation of both goldfish and carp livers (in buffer containing sucrose and DTT) located the 5-PAD forming activity in the 140,000 g pellet (containing both microsomes and mitochondria). 5-PAD formation was observed in an incubation mixture containing samples of both the 140,000 g supernatant and 140,000 g pellets. However, no detectable 5-PAD formation was observed when these fractions were assayed individually. It was found that the 140,000 g supernatant fraction of goldfish and carp liver could effectively be replaced by either NADPH and/or NADH, which similarly stimulated 5-PAD formation by the 140,000 g pellet fractions. NADPH and/or NADH are therefore essential cofactors required for 5-PAD formation by fractions of goldfish and carp livers. The enhanced 5-PAD forming activity observed upon the addition of either NADPH or NADH to goldfish liver 1000 g supernatant, suggests that during the preparation period of this fraction, these cofactors are in part lost, so limiting formation of 5-PAD. From these observations it is reasonable to propose that the substantial loss of 5-PAD forming activity upon incubation of goldfish liver homogenate in the presence or absence of DTT, may be due to the loss of the essential cofactors NADPH and/or NADH required for 5-PAD formation. In agreement with this hypothesis, goldfish liver homogenate in phosphate buffer containing DTT showed 60% of the initial 5-PAD forming activity after

2h incubation at 19°C. In contrast, the further addition of NADPH to the same homogenate sample containing DTT gave a calculated 106% value of the initial activity, after similar incubation for 2h at 19°C. The addition of NADPH therefore prevented the progressive loss of 5-PAD formation observed upon incubation of goldfish homogenate. Two independent factors therefore account for the loss of 5-PAD forming activity of incubated goldfish homogenate:

(a) Modification of important sulphydryl groups of the 5-PAD forming activity.

(b) The loss of the essential cofactors NADPH and/or NADH. The major process most probably accounting for these factors resulting in the loss of 5-PAD forming activity is the peroxidation of cellular components present in the goldfish liver homogenate (Fig. 49).

Oxidative deterioration of mitochondria was observed by Tappel and Zalkin (1959). Oxygen uptake correlated with the concommitant loss of activities associated with mitochondrial oxidative phosphorylation. Two components, unsaturated fatty-acids and/or sulphydryl groups were reported to be of sufficient lability to account for the oxidative deterioriation of the mitochondria. Wills (1961) studied the effect of unsaturated fatty-acids and their peroxides upon sixteen enzyme activities, and observed that enzymes containing functionally important sulphydryl groups were particularly susceptible to peroxide inactivation. It was reported that after short periods of peroxidation enzyme inhibition could be reversed by addition of sulphydryl protecting agents. A low basal rate of non-enzymic lipid peroxidation was observed in microsomal membranes and is proposed to be mediated in part by haemoproteins (O'Brien and Rahimtula, 1975). Both haemoproteins cyt-b_5 (Tappel and Zalkin, 1960) and cyt.p-450 (Levin et al, 1973) are inactivated during lipid



(A) 5-PAD forming activity (Mean values) of goldfish liver homogenate preparations prepared by the same procedure.

(B) The oxidation of PCSD by a monooxygenase will utilise NADPH and so reduce the endogenous concentration of this cofactor.

Figure 49 Possible processes accounting for loss of 5-PAD forming activity upon incubation of goldfish liver homogenate.

peroxidation by a mechanism which involves destruction of the haem group rather than loss of membrane integrity. The basal rate of lipid peroxidation was accelerated in rat liver microsomes upon addition of NADPH in the presence of ferric ions and chelators such as ADP or pyrophosphates (Hochstein and Ernester, 1963). It has been reported that both microsomal membranes and phosphate buffer contain sufficient contaminating iron to facilitate NADPH-dependent microsomal lipid peroxidation (Poyer and McCay, 1971). NADPH-cyt.c reductase was solubilised from rat liver microsomes by proteolytic digestion and was observed to catalyse the peroxidation of microsomal lipids (Penderson and Aust, 1972). The antibody against NADPH-cyt.c reductase inhibits lipid peroxidation by 90% (Penderson et al, 1973). Also a reconstituted system of purified NADPH-cyt.c reductase, iron (Fe^{3+}) chelated by ADP and EDTA and either microsomal lipid (Penderson et al, 1973) or lipoprotein particles (Noguchi and Nakano, 1974) promoted NADPH-dependent lipid peroxidation. The peroxidation process is initiated by induction of transient free radical species which interact with cellular proteins leading to their modification and polymerisation (Roubal and Tappel, 1966a and b). The mechanism of lipid peroxidation was studied in more detail by Poyer and Stanley (1975), and was shown to involve; (i) abstraction of a hydrogen atom from an unsaturated fatty-acid resulting in formation of a radical; (ii) rearrangement of double bonds and formation of conjugated dienes; (ii) oxygen attack resulting in formation of a lipid hydroperoxide or a lipid endoperoxide. The process is complex and leads to a large number of reaction products. For example the peroxidation of linoleic acid alone results in the formation of at least twenty degradation products (Weisleder, 1974). Microsomal membranes are particularly susceptible to lipid peroxidation owing to the presence of high concentrations of

polyunsaturated fatty-acids. Recently the rat microsomal lipid content of saturated/unsaturated fatty-acids has been observed to vary depending on the composition of the fatty-acids of the diet. A correlation between composition of fatty-acids and rate of microsomal lipid peroxidation has also been shown (Lokesh et al, 1981). It is of interest that endogenous sulphydryl protecting agents present in the cell have recently been implicated as representing a cellular defence against lipid peroxidation. Younes and Sieger (1981) reported that when molecules known to conjugate GSH were added to phenobarbital pretreated rat liver the endogenous concentration of GSH was reduced. At a critical concentration (20% of the normal level of GSH) an enhancement of lipid peroxidation was observed. Hepatocytes from rat liver were treated with halogenated acetamides, e.g. iodoacetamide, known to conjugate GSH. Lipid peroxidation was induced and resulted in cell lysis *in vitro* and tissue necrosis *in vivo* (Anundi et al, 1979).

A further feature of the 5-PAD forming activity of goldfish liver homogenate was the increased 5-PAD formation observed with the homogenate in phosphate buffer compared with a range of buffers, of the same pH and concentration. High concentrations of inorganic phosphate have been reported to inhibit lipid peroxide formation by liver mitochondria (Weinstein et al, 1963) and in microsomes (Thiele and Huff, 1964). The mechanism of inhibition was not reported.

The process of peroxidation could therefore account for both the reduction of levels of endogenous NADPH, (a cofactor also essential for 5-PAD formation), and modification of the sulphydryl groups of the 5-PAD forming activity and also possibly the variation of 5-PAD forming activity observed in different buffers. The process of peroxidation results in uptake of oxygen from the surrounding environment

when goldfish liver homogenate in phosphate buffer was bubbled with oxygen the 5-PAD forming activity was increased above that observed with incubation mixtures equilibrated with air. One interpretation of this is to suggest that in phosphate buffer alone, the peroxidative process depletes the oxygen concentration such that it is limiting the rate of 5-PAD formation. Further work is necessary to determine whether oxygen concentration is limiting 5-PAD formation by goldfish homogenate in the presence of DTT and NADPH under the present *in vitro* assay conditions.

The established features of the activity studies in both goldfish and carp livers resulting in the cleavage of the sulphonamido bond of the 6-PCSD molecule and formation of the corresponding amine are:

(i) The activity is located in the microsomal and/or mitochondrial pellet fractions of goldfish and carp livers.

(ii) 5-PAD forming activity in goldfish liver homogenate is enhanced in the presence of DTT and inhibited by the sulphydryl binding agent pHMB.

(iii) NADPH and NADH are essential cofactors for 5-PAD formation by goldfish and carp.

(iv) A preliminary study suggested that oxygen was required for 5-PAD formation by goldfish liver homogenate.

The major group of enzymes showing reported characteristics very similar to those of the 5-PAD forming activity are the microsomal monooxygenases or alternatively named mixed function oxidases. In fish these enzymes have been located in the microsomal fraction of the liver and shown to require both oxygen and NADPH for maximal activity (Buhler and Rasmusson, 1968a; Pohl et al, 1974). The mechanism of oxidation of those microsomal enzymes involves the incorporation of

an oxygen atom derived from molecular oxygen into a substrate. Therefore by definition these enzymes are oxygenases. They are of bifunctional nature, since they catalyse both an oxygen-fixation reaction and an oxidase type reaction. As a result of this, they are referred to as mixed function oxidases (Mason, 1957 and 1965). This group of enzymes catalyse diverse types of hydroxylation reactions, however the primary chemical events of these reactions are basically identical, that is the hydroxylations are initiated by the incorporation of one atom of molecular oxygen into the substrate. Consequently these enzymes are alternatively referred to as the monooxygenases (Hayaishi, 1969). Here recently a further sub-division of this group of enzymes has appeared. Two types of monooxygenase activities have been reported; those mediated through cyt.P-450 and those catalysing monooxygenation reactions independently of cyt.P-450 (e.g. in the presence of cyt.P-450 inhibitors). The latter type of enzyme has been purified from hog hepatic microsomes and has been referred to as a mixed function amine oxidase (Ziegler and Mitchell, 1972). It has been shown to catalyse the monooxygenation of a wide number of sulphur containing substrates as well as the nitrogen containing amine substrates. The purified activity was devoid of cyt.p-450 and NADH or NADPH cyt.c reductase activities. In this work the mono-oxygenases discussed will be divided into two types, referred to as a cyt.P-450 dependent monooxygenases and a cyt.P-450 independent monooxygenases, on the basis of published data of cyt.P-450 inhibitors and their effect on monooxygenase activity. An example of both types of monooxygenase activity in fish are given in Table 13.

Table 13

Evidence for the presence of both cyt.P-450 dependent and cyt.P-450 independent monooxygenation activities in fish livers.

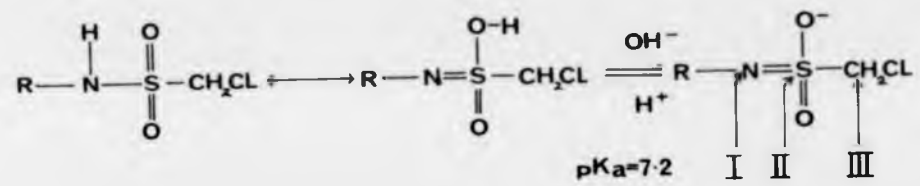
Fish liver studied	Substrate	Reaction Catalysed	Requirement for NADPH and Oxygen	CO Inhibition	Subcellular Location	Optimal pH
Nurse shark[1]	Trimethylamine	Nitrogen Oxidation	+	-	Microsomal	7.8
Trout[2]	Aldrin	Epoxidation	+	+	Microsomal	7.4

[1] Goldstein and Dewitt-Harley (1973)

[2] Burns et al (1976)

(The criteria used to distinguish the activities of Table 13 was the inhibition of the cyt.P-450 dependent monooxygenase by the specific binding [co-ordination] of carbon monoxide with the reduced haemo-protein.)

From observation of published reactions catalysed by the microsomal monooxygenases, three sites of possible oxidative attack have been proposed within the PCSD molecule:



(a further possible site of hydroxylation is that of the aromatic rings, however it is difficult to see how this modification would lead to cleavage of the sulphonamido bond).

The types of monooxygenation reactions which may occur at each of the three sites are given in Table 14.

Table 14 Types of reaction catalysed by cyt.P-450 dependent/independent monooxygenases which may account for the biotransformation of PCSD.

Sites of oxidation in the PCSD Molecule	Type of reaction Catalysed	Examples of Substrates modified by these types of monooxygenase reactions	
		Cyt.P-450 Dependent	Cyt.P-450 Independent
I	Nitrogen oxidation	Dibenzylamine[1]	Trimethylamine[2]
II	N-Dealkylation	Dimethylnitrosoamine[3]	p-chloro-N-methyl-aniline[4] *
	S-oxidation	Chloropromazine[5]	Diaminodiphenylsulphide[6]
III	Aliphatic oxidation	N-Heptane[7]	-
	Oxidative Dehalogenation	Chloramphenicol[8]	-

References:
1 - Beckett and Gibson (1975)
2 - Baker et al (1963)
3 - Lotlikar et al (1975)
4 - Prough and Ziegler (1976)
5 - Coccia and Westerfeld (1967)
6 - Gillette (1969)
7 - Frommer et al (1972)
8 - Pohl et al (1977)

* An alternative pathway of N-Dealkylation by a cyt.P-450 dependent mechanism has also been observed for this substrate.

Enzymic oxidation at the nitrogen atom of the PCSD molecule (site I) would most probably proceed by a two stage oxidation as described for the monooxygenation of the secondary amine N-methyl benzylamine (Poulsen et al, 1974). The resulting nitrone species may hydrolyse rapidly in aqueous solution (Exner, 1951) giving rise to bond cleavage and hydroxylamine formation. The reduction of some hydroxylamines have been reported by a microsomal NADH/NADPH dependent reductase (Kadlubar et al, 1973), giving rise to the corresponding amine of the hydroxylamine substrates. *In vitro*, 5-PAD may be formed by a similar mechanism. (However, the alkaline hydrolysis of PCSD results in the formation of the amine and not the hydroxylamine product?) It would therefore appear more likely that oxidation(s) would occur at the sulphur and/or aliphatic group of PSCD (sites II and III). Sulphur oxidation reactions have been reported to be catalysed by both cyt.P-450 dependent and independent monooxygenases (Table 14). The cyt.P-450 independent monooxygenase from Hog microsomes has recently been shown to catalyse a wide range of sulphur oxidation reactions. The purified monooxygenase is reported to be a better sulphur oxygenase than a nitrogen oxygenase, since compounds bearing both nucleophilic sulphur and nitrogen atoms are preferentially oxygenated at the sulphur atom (Ziegler, 1980). A sulphoxide oxidising activity resulting in the conversion of tetrahydrofurfuryl sulphoxide to the corresponding sulphone was observed in rat liver microsomes. The activity was inhibited by SKF 525A but was only slightly inhibited by CO. The enzyme was also inhibited by pHMB (Fuijita and Suzuoki, 1967). Poulsen et al (1979) observed that the oxygenation of sulphur of 2-mercaptoimidazole and thiourea was exclusively catalysed by the flavin containing cyt.P-450 independent monooxygenase of hog and hamster liver. Some of the highly oxidised

sulphur substrates, e.g. Imidazole-2-sulphinic acid, rapidly hydrolyse in aqueous environments, yielding sulphite and the corresponding imidazole (Balaban and King, 1927). A similar oxidation and hydrolysis may occur with 6-PCSD resulting in the cleavage of the sulphonamido bond. However at present the mechanism by which the sulphonamido bond is cleaved is unknown.

The proposed reactions catalysed by the microsomal monooxygenases which may account for the oxidative metabolism of PCSD have been reported to result in the activation of some substrates, forming metabolites which can bind to cellular components. For example, the microsomal N-oxidation of amphetamine compounds result in the formation of metabolites which can complex with cyt.P-450 (Franklin, 1974a and b). Chloramphenicol is oxidatively dehalogenated by a cyt.P-450 dependent monooxygenase, giving rise to a metabolite which binds to cellular protein (Pohl and Krishna, 1978). Monooxygenase catalysed sulphur oxidation of thioacetamide results in the formation of an hepatotoxic S-oxide metabolite (Porter and Neal, 1978).

The observed characteristics of the 5-PAD forming activity are now discussed with respect to related published features of cyt.P-450 dependent and independent monooxygenases.

Two pathways supplying cyt.P-450 with reducing equivalents have been identified in mammalian microsomes. One from NADPH via a flavoprotein to cyt.P-450 and secondly an alternative route from NADH via cyt.b_5 to cyt.P-450 (details are given in section 3.1).

Mammalian microsomes when incubated with NADH alone could sustain NADPH-dependent monooxygenation of substrates at 1/5 to 1/2 the level of the corresponding NADPH dependent activity. The addition of

NADH and NADPH was observed to enhance monooxygenase activity to levels higher than that observed by NADPH dependent monooxygenation alone (Krisch and Staudinger, 1961; Nilsson and Johnson, 1963) and was later referred to as 'NADH-synergism' of cyt.P-450 dependent monooxygenase reactions (Hildebrandt and Estabrook, 1971).

In comparison the purified cyt.P-450 independent monooxygenase from porcine microsomes was observed to catalyse the oxidation of substrates equally in the presence of either of the cofactors NADH or NADPH. However, NADPH saturated the enzyme at 1/10 the concentration of that of NADH (Ziegler and Poulsen, 1978). Goldstein and Dewitt-Harley (1973) report however that in the cyt.P-450 independent monooxygenation of TMA, an NADH-generating system was only 6% as effective as an NADPH-generating system.

In fish the cyt.P-450 dependent aniline hydroxylation and phenacetin dealkylation occurred at much lower rates in the presence of NADH compared with that of NADPH (Buhler and Rasmusson, 1968a). The cyt.P-450 dependent hydroxylation of benzo(a)pyrene by microsomes from the sheepshead (*Archosargus probatocephalus*) was 10% greater in the presence of both NADPH and NADH than with NADPH alone (James and Bend, unpublished data), providing evidence for the 'NADH-synergism' observed in mammals.

The 5-PAD forming activity of carp microsomes was very similar when incubated with either NADPH or NADH. The amount of 5-PAD formed upon addition of both NADPH and NADH was 10% greater than that observed with NADPH alone.

DTT enhanced 5-PAD formation in goldfish liver homogenate and pHMB inhibited this activity. These observations suggest the

presence of functionally important sulphydryl groups associated with the 5-PAD forming activity.

Microsomal cyt.P-450, upon aerobic incubation is converted to a denatured form referred to by its characteristic absorbance property as cyt.P-420. The conversion of cyt.P-450 to cyt.P-420 results in concomitant loss of monooxygenase activity (Imai and Sato, 1967). This conversion can also be induced by treatment of cyt.P-450 with the sulphydryl binding agent pHMB (Cooper et al, 1965); and under some conditions reversion to the active cyt.P-450 can be achieved by the addition of sulphydryl protecting agents such as DTT (Yu and Gunsalus, 1974). DTT has also been reported to protect cyt.P-450 from inactivation by lipid peroxidation (Lu and Coon, 1968). From structural studies cyt.P-450 of both bacterial and mammalian microsomal sources shows the presence of half cysteine residues (Dus et al, 1974). In bacterial cyt.P-450, six half cysteines are present as free sulphydryl groups which can be tritiated by pHMB. At least two of these appear to be related to the spectrum and activity of cyt.P-450 (Yu and Gunsalus, 1974).

Reconstitution of cyt.P-450 dependent monooxygenase systems in mammals (Lu et al, 1969) and fish (Philpot et al, 1977; Bend et al, 1977) has shown that monooxygenation requires both cyt.P-450 and NADPH cyt.c reductase (cyt.P-450 reductase). In addition to its role in monooxygenation NADPH cyt.c reductase also catalyses lipid peroxidation (Penderson and Aust, 1972). Peroxidation catalysed by NADPH cyt.c reductase exhibits a high sensitivity to the sulphydryl binding agent pHMB (Ernester and Nordenbrand, 1967) suggesting that sulphydryl groups are also important in this component of the monooxygenase system, for its functional role as reductase. The

cyt.P-450 dependent dealkylation of phenacetin by trout liver homogenate was inhibited by pHMB; however only slight inhibition of aniline hydroxylation was observed in another cyt.P-450 dependent monooxygenase activity studies (Buhler and Rasmusson, 1968a). Some indirect evidence for the importance of sulphydryl groups in the monooxygenase system is provided from studies of reduction of oxidised substrates involving cyt.P-450. In addition to the catalysis of oxidation reactions cyt.P-450 has been implicated in reduction reactions. Reduction requires NADPH and occurs only at detectable rates under anaerobic conditions *in vitro*. Consequently it is questionable whether these reactions proceed at a significant rate *in vivo*, in the oxygenated physiological environments of animal tissues. These reactions may therefore be only of academic interest. The reduction reactions studied in mammalian hepatic microsomes involving cyt.P-450 include nitroreduction of nitrobenzoic acid (Fouts and Brodie, 1957; Gillette et al, 1968); azo reduction of neoprontosil (Shargel and Mazel, 1972); tertiary amine N-oxide reduction of N,N-dimethylalanine N-oxide (Sugiura et al, 1976); arene oxide reduction of benz[a]anthralene (Booth et al, 1975) and reductive dehalogenation of carbontetrachloride (Uehleke et al, 1973). In fish the nitro reduction to p-aminobenzoic acid has been studied (Buhler and Rasmusson, 1968b) and proposed as in mammals to involve cyt.P-450. This activity was found to be inhibited by either pHMB or oxygen. The oxidisable components resulting in this loss are thought to be important functional sulphydryl groups associated with the (cyt.P-450 dependent) nitro reductase activity. Further evidence for this was shown by the reversal of nitro reductase inhibition after short periods of oxygenation, by sulphydryl protecting agents (Buhler and Rasmusson, 1968b). The process activated by oxygen resulting in the loss

of the important functional sulphydryl groups is most probably that of lipid peroxidation.

There are very few published studies of the effects of sulphydryl agents on defined cyt.P-450 independent monooxygenase activities. One report, the oxidation of imipramine (which proceeds by a cyt.P-450 independent mechanism) was inhibited by pHMB (Bickel, 1969).

The cyt.P-450 dependent monooxygenases appear in general to possess functionally important sulphydryl groups showing a high sensitivity to sulphydryl binding agents. A general statement of the sensitivity of cyt.P-450 independent monooxygenases to sulphydryl binding agents cannot at present be made.

Increased 5-PAD formation was observed in phosphate buffer compared with other buffers of equal concentration and pH. Also the addition of sucrose or glycerol substantially decreases 5-PAD forming activity in goldfish liver homogenate. This decreased activity was also observed in carp and goldfish subcellular fractionation studies which were carried out in the presence of sucrose. One possible explanation of these observations is that the 5-PAD forming activity is sensitive to variations in ionic strength of the immediate aqueous environment. Such variations in ionic strength have been reported to effect cyt.P-450 dependent monooxygenation reactions with a focus particularly on the NADPH cyt.c reductase activity. NADPH cyt.c reductase activities of rat and rabbit microsomes are easily solubilised by protease treatment. The rabbit enzyme when solubilised remains active in reduction and antibodies raised to this activity effectively inhibited the NADPH cyt.c reductase and the associated cyt.P-450 catalysed monooxygenation reactions of rabbit liver microsomes (Prough and Ziegler, 1977). The activity of the NADPH cyt.c reductase

activity when solubilised, and its microsomal inhibition by the antibody, suggests that its position in the microsomes is one largely exposed to the aqueous environment. In contrast antibodies to purified pig liver amine oxidase (cyt.P-450 independent) elicited in rabbits are not inhibitory of the microsomal activity, suggesting its position to be largely embedded within the hydrophobic membrane (Prough and Ziegler, 1977). The activity of the NADPH cyt.c reductase of rat liver microsomes was observed to vary with the ionic strength of the aqueous environment (Penderson and Aust, 1972). Also it is of interest that the activity of NADPH-cyt.c reductase from pike liver microsomes increased in activity by 25% when the ionic strength of sodium phosphate buffer (pH 7.5) was increased from $\mu = 0.2$ to $\mu = 1.0$ (Balk et al, 1980).

A further characteristic feature of the 5-PAD forming activity from *in vitro* studies was the differing abilities of liver homogenates from a variety of freshwater fish species to catalyse the formation of 5-PAD. Detectable 5-PAD forming activity was observed by goldfish, carp and pike liver homogenates in contrast to an absence of detectable activity in trout, perch and eel homogenates. The specific activities of 5-PAD formation in goldfish and carp was larger than that observed in the pike, with an order of magnitude difference between goldfish and pike activities. (This comparison is made from studies where DTT was included in both preparation and incubation steps of the liver homogenates. Further characterisation of the goldfish 5-PAD forming activity showed that exogenous NADPH and NADH were required for maximal activity. The reported 5-PAD forming activities of the liver homogenate from different fish species will therefore be dependent on the levels of endogenous NADPH and NADH.)

Reports of contrasting species differences in the metabolism of xenobiotic substrates are rare, but it is of interest that the *in vitro* cyt.P-450 monooxygenation of TMA showed qualitative species differences in a variety of fish (Baker et al, 1963). Some quantitative differences between species of fish in cyt.P-450 dependent monooxygenation of some xenobiotic substrates have been reported, from an *in vitro* study of the cyt.P-450 dependent monooxygenation of benzo-(a) pyrene by a variety of fish, showed a thirty-fold difference in activity between the mangrove snapper (*L. griseus*) and the bluntnose stingray (*D sayi*) when both assayed under optimal conditions for monooxygenation (James and Bend, 1979). Such species differences will be discussed together with the species dependent formation of 5-PAD in Chapter 4 of this work.

The 5-PAD forming activity therefore resembles that of a microsomal monooxygenase. Further classification relating to the type of monooxygenase activity is not possible from the data of the preliminary characterisation studies. The following discussion of the characteristic features of the cyt.P-450 dependent and independent monooxygenase activities may provide the basis for future experimental approaches to further define the 5-PAD forming activity. Criteria implicating microsomal cyt.P-450 dependent metabolism will first of all be considered.

(a) Inducers and inhibitors of cyt.P-450

Induction of microsomal monooxygenase activity involving cyt.P-450 was first observed by Brown et al (1954). These workers reported an increase in the *in vitro* N-demethylation of 4-dimethylaminoazobenzene by pretreatment of rats with 3-methylcholanthrene. Since this observation a large number of potential cyt.P-450 monooxygenase inducers have been reported (Table 15).

Table 15

Inducers and inhibitors of cyt.P-450.

In vivo	*In vitro*
Inducers	**Inhibitors**
Phenobarbital	SKF 525-A
3-methylcholanthrene	metyrapone
benzo[a]pyrene	7,8-benzoflavone
5,6-benzoflavone	2,4-Dichloro-6-phenyl phenoxyethylamine (DPEA)
pregnenolone-16α-carbonitrile	carbon monoxide
2,3,7,8-tetrachlorodibenzo-p-dioxin	antibodies to purified cyt.P-450
Inhibitors	
Cobalt chloride	
Carbon tetrachloride	

Many of these mammalian cyt.P-450 inducers have been observed to elevate monooxygenase activity when fed or injected into fish (Payne and Penrose, 1969; Stratham et al, 1978). Induction results in an increase of microsomal cyt.P-450 in liver, with concomitant increased metabolism of a substrate by the microsomal monooxygenase system. Inhibition involves the reverse, a decrease in cyt.P-450 and monooxygenase activity. Consequently, an increase in metabolism of a compound following treatment with an inducer, and a decrease in metabolism after treatment with an inhibitor would suggest the involvement of cyt.P-450. [In contrast, cyt.P-450 independent monooxygenases do not appear to be induced by a reported cyt.P-450 dependent monooxygenase inducers (Burke and Mayer, 1974).]

Inhibitors of cyt.P-450 interacting with the haem or protein moeity, have been reported (Table 15). Carbon monoxide is a commonly used inhibitor which inhibits cyt.p-450 dependent monooxygenases by its ability to co-ordinate with the reduced cyt.P-450. To study the effect of carbon monoxide on the oxidation of different molecules, a series of gas mixtures of carbon monoxide/oxygen must be prepared with different nitrogen/oxygen mixtures as controls. This method allows correction for inhibition of monooxygenase activity due to limiting oxygen (Cooper et al, 1965). The inhibition by carbon monoxide can be reversed by monochromatic light of different wavelengths so relieving monooxygenase inhibition. The inhibition of the cyt.P-450 dependent monooxygenase systems by antibodies raised to cyt.P-450 (Thomas et al, 1977) and NADPH cyt.c reductase (Prough and Ziegler, 1977) have proved to be useful tools in identification of cyt.P-450 dependent monooxygenase activities.

(b) Characteristic spectral changes associated with molecules interacting with cyt.P-450

Many substances interact with cyt.P-450 resulting in three main types of cyt.P-450 dependent spectral changes, e.g. those resulting in the designated Type I spectra, Type II spectra and reverse Type I spectra (Imai and Sato, 1966b; Schenkman et al, 1967; Schenkman et al, 1973) such spectral changes coupled with the observation of monooxygenase activity implicate cyt.P-450 as a component of the monooxygenase system. Some metabolites oxidised by a cyt.P-450 dependent activity have been observed to result in the formation of a cyt.P-450/metabolite complex, when NADPH is added to microsomes. The complex has a characteristic absorbance profile (Franklin, 1977).

(c) Reconstitution of monooxygenase systems containing cyt.P-450

Unequivocal involvement of cyt.P-450 in monooxygenation reactions comes from reconstitution of the monooxygenase system. Cyt.P-450 was an essential component of the reconstituted monooxygenase system isolated from the 1,2,3,4-dibenzoanthracene treated liver of the little skate (*R. erinacea*). The reconstituted system catalysed the cyt.P-450 dependent monooxygenation of benzo(a)pyrene (Bend et al, 1979).

Care must be taken in the interpretation of a negative result from the latter approaches since the broad substrate specificity of the monooxygenases has been attributed to the existence of multiple forms of the haemoprotein, cyt.P-450. These isoenzymes show an individual specificity with respect to some substrates and also an overlapping substrate specificity with respect to others (discussed in section 3.1). Consideration must be given to the substrate used and the preparation and tissue source of cyt.P-450. For example, the forms of cyt.P-450 employed may not catalyse the oxidation of the particular substrate under study.

(d) Characteristic properties of the cyt.P-450 independent monooxygenases

The pig liver microsomal cyt.P-450 independent monooxygenase has at least one modified site which can bind positively charged lipophilic alkylamines, e.g. n-octylamine (Ziegler et al, 1978). These compounds consistently activate the enzyme (Ziegler, 1980). In contrast n-octylamine has been reported as a cyt.P-450 inhibitor (Jefcoate et al, 1969). In *in vitro* studies using pig liver microsomes n-octylamine inhibited (cyt.P-450 dependent) demethylation of tertiary amines and consistently stimulated the cyt.P-450

independent N-demethylation of N-methylamphetamine (Prough and Ziegler, 1977). The unusual thermolability of the cyt.P-450 independent monooxygenases has been used as a characteristic by which to differentiate this type of monooxygenase from cyt.P-450 dependent monooxygenases in microsomes (Uehleke, 1971). A specific inhibitor of the purified hog microsomal cyt.P-450 independent monooxygenase has not been found (Ziegler, 1980).

Gorrod (1978) has proposed that substrates for each type of monooxygenase can be identified by the observation that basic amines are the preferred substrates for the cyt.P-450 independent monooxygenases (microsomal amine oxidases); and less basic amines are the preferred substrates for the cyt.P-450 dependent monooxygenases. There are many exceptions however to this general rule. Both types of monooxygenase show broad substrate specificities catalysing the oxidation of both nucleophilic nitrogen and sulphur atoms of those substrates. To propose that monooxygenation of a particular substrate proceeds by only one type of monooxygenation may be a naive over-simplification.

Hlavica and Kehl (1977) provided unequivocal evidence for the participation of both a cyt.P-450 dependent and a cyt.P-450 independent monooxygenases, catalysing the oxidation of nitrogen in a single substrate, N,N-dimethylaniline. The rabbit liver microsomal cyt.P-450 dependent pathway was estimated to account for 50-60% of the N,N-dimethylaniline N-oxide formed with the remainder of the metabolite being formed by the cyt.P-450 independent monooxygenase. Also 65% of p-chloro-N-methylaniline was oxidatively N-demethylated in pig liver microsomes by a cyt.P-450 dependent monooxygenase and 35% by a cyt.P-450 independent monooxygenase (Prough and Ziegler, 1977).

Differentiation of the two activities in order to assess their separate contributions to the oxidation of the substrates was achieved by some of the approaches described previously in this section, e.g. cyt.P-450 inhibition by use of *in vitro* inhibitors and the antibody against NADPH cyt.c reductase.

The *in vitro* conditions for the cleavage of the sulphonamido bond of 6-PCSD, implicate a monooxygenase activity. Preliminary characterisation of this activity has not provided sufficient evidence to determine the type of monooxygenase activity, either cyt.P-450 dependent and/or cyt.P-450 independent. Further characterisation will enable identification of the type of monooxygenase activity, the mechanism of sulphonamido bond cleavage, and allow a comparison of this with other published sulphamidase type activities (section 3.1). It is of interest that the metabolism of the mothproofing agents Dieldrin (Reddy and Khan, 1978; Khan et al, 1979) and Permethrin (Glickman et al, 1979) involve mainly the microsomal monooxygenases. A detailed understanding of the nature of these enzymes will therefore be of value in the study of the environmental metabolism of the major mothproofing agents.

CHAPTER 4

Apparent Qualitative Differences in the Metabolism of Eulan WA New by Species of Freshwater Fish, Exhibited by Complementary *in vivo* and *in vitro* Studies.

'Oh, East is East, and West is West,
and never the twain shall meet,
Till Earth and Sky stand presently
at God's great judgment seat;
But there is neither East nor West,
Border, nor Breed, nor Birth,
When two strong men stand face to face,
though they come from the ends of earth!'

Rudyard Kipling (1865-1936)

The animal kingdom is represented by seventeen different phyla encompassing an enormous diversity of living organisms. With respect to the study of the metabolism of xenobiotics two of these phyla have in part been explored; the Arthropoda including the insects, and the phylum Chordata including fish, birds and mammals. These phyla contain those organisms mainly of interest to man in terms of his medicine and agriculture. Man's concern to define the impact of xenobiotics on his environment and secondly, to establish model systems in which to study the metabolism of xenobiotics, in relation to Man; nas necessitated an investigation into the metabolic differences that exist between species. Species alone is regarded as a primary factor accounting for differences in metabolism of xenobiotics. However other factors including age, sex, season, diet, route of administration of xenobiotic, exposure to other xenobiotics, genetic differences, disease etc. can also result in differences in xenobiotic metabolism. Some of these factors have been studied in fish. Mammalian hepatic microsomal monooxygenase activity has been observed to vary depending on diet (Campbell and Hayes, 1974). In contrast similar variations of xenobiotic metabolising enzymes were not observed in the few fish dietary studies reported (Buhler and Rasmusson, 1968a; Dewaide, 1971). Environmental factors, including the exposure of organism to xenobiotics can influence xenobiotic metabolising activities. The induction of particular microsomal cyt.P-450 dependent monooxygenase activities has been reported in fish upon exposure to environmental xenobiotics including DDT (Pohl et al, 1974), Arochlor 1254 (Hill et al, 1976) and petroleum compounds (Payne, 1976). The activity of xenobiotic metabolising enzymes has also been reported to vary with season. Dewaide (1971) reported high aniline hydroxylase and aminopyrine demethylase activities in the roach

(*L. rutilus*) during the summer months, and significantly decreased activities during the winter months.

In mammals sex differences in the metabolism of xenobiotics have been reported. Quinn et al (1958) observed a more rapid oxidative metabolism of many xenobiotics in male rats compared with females. These differences were minimised by either castration of the males or alternatively administration of testosterone to the females (Kato et al, 1968). The sex hormones are implicated as factors accounting for these differences in microsomal activities. From studies in fish, a number of sex differences in the metabolism of xenobiotic substrates have been reported. Stegeman (1977) showed that the cyt.P-450 content of hepatic microsomes in the male rainbow trout (*S. gairdneri*) and the brook trout (*S. fontinalis*) was twice that of the females during spawning. This correlated with a three-fold greater specific microsomal aminopyrine demethylase activity in the male rainbow trout compared with the female.

Studies of species differences are mainly carried out *in vivo*, involving the dosing of organisms with xenobiotics and the subsequent monitoring of blood plasma, tissues and excretory routes for the parent compound and metabolites. In the complex living organism the xenobiotic is involved in the processes of absorption, distribution and excretion which may each show species differences. However Gillette (1977) considers the quantity and quality of the xenobiotic metabolising enzymes and their interaction with endogenous and exogenous compounds to be the most important factor determining species differences. In addition to the *in vivo* studies a more simplified, fundamental approach is required to study those particular metabolic events thought to vary between species. An approach amenable to such study is the *in vitro* method. Using this approach quantitative studies present

more problems than quantitative ones, and generally problems of extrapolation increases as the system becomes more defined. Quantitative aspects of xenobiotic metabolism are best studied using *in vitro* methods involving tissue slices of explants in which the *in vivo* cellular integrity is maintained. Sullivan (1972) has defined a tissue maintenance technique for comparative drug metabolism studies, in which pieces of tissue can be incubated with the xenobiotic for up to 18h. Alternative studies with the isolated hepatocyte (Moldeus et al, 1978) removes the problems of artifacts arising from mechanical injury and limited diffusion of substrate and oxygen associated with the latter method. Further homogenisation of tissues and their subcellular fractionation allows better control of conditions for the study of the metabolism of xenobiotics but sacrifices organizational completeness. This most fundamental *in vitro* approach has the inherent problems of the release of normally compartmented degradative enzymes and other potential inhibitors of enzyme activity. Initial studies must therefore define the need for protective agents as well as characterisation of the particular activity under study, in terms os temperature, pH optima, cofactor requirement(s) etc.. This approach allows the study of individual steps of xenobiotic metabolism, and the substrate specificity, and also kinetic parameters of the enzyme(s). Comparative *in vitro* studies in different species generally involves the defining of optimal conditions for the assay of the particular metabolic event(s) *in vitro* in one species and an assessment of this event in other species under identical assay conditions. However, possible errors may arise in assuming that the defined conditions for optimal activity in one species are identical for the assay of the same activity in another species. A particular activity may not always be localised in the same subcellular fraction in different species.

For example nitro reductase activity is localised in the soluble fraction of fish livers (Buhler and Rasmusson, 1968b) and yet is equally distributed between the soluble and microsomal fractions in the rat (Fouts and Brodie, 1957). The stability of a xenobiotic metabolising enzyme may also differ between species. For example, centrifugation and resuspension (with tissue grinder) of trout and of rat liver microsomes results in 31% loss of N-demethylase activity in the trout and a negligible loss in the rat preparation. Further the incubation of rat and roach 9000 g liver supernatants at room temperature for 5h resulted in a 14% loss of N-demethylase activity in the rat and 42% loss in the roach (Dewaide, 1971). A fifteen-fold difference in the activity of the lipid peroxidase system of rat and rabbit liver microsomes has been observed (Kamataki and Kitagawa, 1973), this may account for differential losses of xenobiotic metabolising activities between species. The affinity of a particular enzyme for a substrate may show species differences for example, the K_m of a substrate N-demethylated by a microsomal monooxygenase was observed to vary twenty-four fold in sixteen species studied (from 0.42 mM for the pigeon to 10 mM for the pike). The temperature optimum for the microsomal N-demethylase activity was found to vary widely in eight species studied. Dewaide (1971) agreed with earlier studies of Adamson et al (1965) that the liver demethylase of birds should be incubated at 42°C, in mammals 37°C and in fish 25°C.

A complementation of the *in vivo* and *in vitro* methods should provide a valuable 'reliability test' for the data, since both should agree in the patterns and products of xenobiotic metabolism. However the extrapolation of results obtained *in vitro* to the *in vivo* condition must be qualified. The optimal conditions for enzyme assay *in vitro* are those most suitable for good kinetics (e.g. rate

linear with time and protein concentration). This may not relate directly to the situation *in vivo* where the penetration of the substrate to the enzyme may be restricted or facilitated, or natural modifiers may be present. Also an interaction of metabolic processes may occur affecting reaction rates.

It has been proposed that species differences in the metabolism of xenobiotics may arise from one or more of three origins:

(a) The existence of species which are defective in individual metabolic reactions.

(b) The restriction of certain reactions to an individual species or group of species.

(c) Variations in the relative extents of two or more competing reactions which a compound may undergo.

Examples of species defective in a xenobiotic metabolising activity have been observed in the order mammalia (Table 1). Defects have most commonly been observed in phase II type, conjugation activities, some of these defects have been explored further by coupling *in vitro* studies with those *in vivo*. The guinea pig was found to be defective in the formation of mercapturic acids. *In vitro* studies showed that the guinea pig was unable to catalyse the final step of the sequential metabolic pathway to mercapturic acid formation (Bray et al, 1959). With respect to small water soluble substrates, the cat was reported to be unable to form glucuronic acid conjugates. Studies have suggested that a form of UDP-glucuronosyltransferase responsible for the conjugation of these small molecular weight substrates is absent from the cat liver (Dutton et al, 1976). In fish, the parasitic sea lamprey (*Petromyzon marinus*) has shown a very limited capacity to glucuronidate, the xenobiotic substrate

Table 1 Examples of species defective in a particular metabolic reaction involved in the metabolism of xenobiotic substrates in the order mammalia.

Reaction	Substrates	Defecting species	Reference
Aliphatic amine N-hydroxylation	Chlorophentermine	Rat, marmoset	Caldwell et al (1975)
Arylacetamide N-hydroxylation	2-acetamidofluorene	Guinea pig, Steppe lemming	Weisburger et al (1964)
Arylamine N-acetylation	p-aminobenzoic acid, sulfanilamide (N^4-amino) sulfadimethoxine (N^4-amino) Isonazid, 4-Aminobiphenyl	Dog	Williams (1967)
Glucuronidation	phenol, 1-naphthol, 2-naphthol acetaminophen benzoic acid, 1-naphtholacetic acid clofibric acid	Cat, lion, lynx	Williams (1974) Hirom et al (1976)
Mercapturic acid formation	chlorobenzene, benzyl chloride p-chlorobenzyl chloride 3,4-dichloronitrobenzene 2,3,4,6-tetrachloronitrobenzene 2,3,5,6-tetrachloronitrobenzene	Guinea pig	Bray et al (1959) Corner and Young (1954)

3-trifluoromethyl-4-nitrophenol (TFM) compared for example with the high conjugation activity of the rainbow trout. The toxicity of the non-conjugated TFM has been elegantly shown by *in vivo* inhibition of the conjugation reaction in the trout (Lech and Statham, 1975). Here the complementation of *in vivo* and *in vitro* studies has provided an understanding of both the mechanism of action of TFM and also the molecular basis of the species differences. This defect in the lamprey has allowed the use of TFM as a selective lamprey larvicide in the Great Lakes (Applegate et al, 1961).

Only two defects in mammals have been reported of phase I type xenobiotic metabolising activities and are described in Table 1. Both of these involve a nitrogen hydroxylation reaction. The molecular basis of these defects have not been explored. However the enzymes catalysing the oxidation of 2-acetamidofluorene have been studied in mammals and characterised. This activity was found to be induced by methylcholanthrene (Lotlikar et al, 1973) was inhibited by antibodies raised against cyt.P-450 reductase and was inhibited by treatment with cobalt chloride (Thorgeirsson et al, 1973). Partially purified cyt.P-450 fractions from 3-methylcholanthrene treated rats (Lotlikar and Zaleski, 1975) and hamster (Lotlikar et al, 1974) were reconstituted in systems which catalysed monooxygenation of the substrate. This evidence suggests that N-hydroxylation of 2-acetamido-fluorene is catalysed by a microsomal cyt.P-450 dependent monooxygenase type activity. The guinea pig and steppe lemming may therefore represent species defective in a particular isoenzyme of cyt.P-450 monooxygenase type. Similar characterisation studies have not been reported for the activity catalysing the N-hydroxylation of chloro-phentermine in mammals. In association with these reports it is interesting to note the presence of contrasting species differences

in the ability of fish to N-hydroxylate the substrate trimethylamine, (TMA) from *in vitro* studies involving the oxidation of TMA was observed in preparations of rat, guinea pig, sheep, hog and rabbit liver homogenate. However, the distribution of this activity in both marine and freshwater fish was sporadic, with no clear rationale accounting for the observed distribution. For example, of seven phylogenetically similar species of flat-fish obtained from the same area of the Pacific ocean; two catalysed the oxidation of TMA and five gave no detectable activity (Baker et al, 1963). Further studies by Goldstein and Dewitt-Harley (1973) reported a similar sporadic distribution of this activity in the livers of elasmobranchs. The nurse shark (*G. cirratum*), lemon shark (*N. brevirostris*) and smooth dogfish (*M. canis*) showed detectable TMA oxidase activity. However no detectable activity was observed with spiny dogfish (*S. acanthias*), little skate (*R. erinacea*), large skate (*R. ocellata*) and the American stingray (*D. americana*). From *in vitro* characterisation studies involving cyt.P-450 inhibitors, these workers identified the activity to be a microsomal cyt.P-450 independent monooxygenase type (mixed function amine oxidase). From these studies it would appear that species defects in both cyt.P-450 dependent and cyt.P-450 independent monooxygenase activities do occur resulting in contrasting species differences in xenobiotic metabolism. Studies including alternative substrates would be useful in defining more clearly the nature of the specific defects, particularly those of the phase I type reactions.

Metabolic reactions concerned with the metabolism of xenobiotic substrates, of restricted species occurrence have been reported in mammals. Four reactions have been observed to be restricted to man and non-human primates. These are; glutamine conjugation of arylacetic acids (James et al, 1972); the aromatization of quinic

acid (Adamson et al, 1970a; Bridges et al, 1965); the N'-glucuronidation of sulphadimethoxine (Adamson et al, 1970b) and the O-methylation of 4-hydroxy-3,5-diiodobenzoic acid (Wold et al, 1973).

The metabolism of a xenobiotic rarely involves a single step. In general substrates contain a number of functional groups which are modified sequentially or by competing enzyme catalysed reactions. Common interspecies differences have been observed in the order mammalia, in the relative extents of various reactions which a xenobiotic compound may undergo. An example is shown by the metabolism of amphetamine congeners in different species. In the rat aromatic hydroxylation predominates; side chain cleavage is the major route of metabolism in the guinea pig and mixed patterns occur in other species. How reliable these species patterns of metabolism of xenobiotics are, in predicting the metabolism of a xenobiotic in different species is at present under debate (Smith, 1974).

It is clear therefore that species differences in the metabolism of xenobiotics are present in both the mammalia and fish. However there is an essential need to deepen our understanding of such differences by exploring their molecular basis using available biochemical techniques.

In vitro metabolism of 6-PCSD and Eulan WA New, resulted in the formation of amine metabolites by cleavage of the sulphonamide bond of the PCSD components. No other metabolites of the parent PCSDs or PAD products were detected by HPLC analysis of MTBE incubation mixture extracts. The PCSD components of Eulan WA New are therefore unusual as xenobiotic substrates since metabolism involves apparently a single major event, that of the cleavage of the sulphonamido bond. A

comparative study of the 5-PAD forming activity in liver homogenates of different species of freshwater fish has been described (section 3.4.8). The metabolic cleavage of a sulphonamido bond in a substrate is an extremely rare event with few documented reports of the *in vitro* conditions required for the assay of such sulphamidases. It was therefore necessary to characterise the 5-PAD forming activity *in vitro*. The activity however was only partially characterised when the *in vitro* comparative study was conducted. This study did not include the addition of the exogenous cofactor NADPH, which was later found to be necessary for maximum 5-PAD formation throughout the assay period. (Higher 5-PAD formation *in vitro* may be further obtained following a more specific characterisation of optimal *in vitro* conditions, e.g. pH, temperature optima etc.). Even though optimal conditions for the assay of 5-PAD formation were not employed in the comparative study, contrasting species differences were observed.

Wells and Cowan (1983) analysed samples of different species of freshwater fish taken from Loch Leven, an environment contaminated with Eulan WA New. A comparison of the 5-PAD : 6-PCSD ratio found in the livers of the brown trout, pike and perch taken from Loch Leven are given in Table 2. The high 5-PAD : 6-PCSD ratio in pike

Table 2

The ratio of 5-PAD : 6-PCSD in different species of freshwater fish taken from an environment contaminated with Eulan WA New

Fish livers	5-PAD : 6-PCSD
trout	0.24
pike	119.7
perch	0.06

Table 3 A qualitative assessment of 5-PAD forming activity observed in different species of freshwater fish from both *in vivo* and *in vitro* studies.

	Pike (*C. lucius*)	Goldfish (*C. auratus*)	Carp (*C. carpio*)	Rainbow trout (*S. gairdneri*)	Brown trout (*S. trutta*)	Perch (*P. fluviatilis*)	Eel (*A. anguilla*)
in vivo study							
a) dosing fish with 6-PCSD	+[1]	+[1]	+[2]	-[1]			
b) analysis of fish taken from Loch Leven	+[3]				-[3]	-[3]	
in vitro study							
a) metabolism of 6-PCSD by crude liver homogenates	+[4]	+[4]	+[4]	-[4]		-[4]	-[4]

1 - In this work, sections 2.4.4, 5 and 6
2 - Hamburger et al, (1981)
3 - Wells and Cowan (1983)
4 - In this work, section 3.4.8

liver strikingly contrasts with the much lower ratios found in the livers of the trout and the perch, which are not dissimilar to the ratio of 5-PAD:6-PCSD in Eulan WA New, <0.10. The very small ratios found in perch and trout liver suggest the absence, or the presence of a very low 5-PAD forming activity. In contrast, the particularly high 5-PAD:6-PCSD in pike liver is indicative of an *in vivo* metabolic conversion of 6-PCSD to 5-PAD. Such contrasting species differences were also observed when different species of fish from a 'clean' environment were dosed with either Eulan WA New or 6-PCSD (sections 2.4.4, 5 and 6) and also from *in vitro* studies previously discussed (section 3.4.8). Both *in vivo* and *in vitro* are complementary, showing the same distinctive pattern in the distribution of the 5-PAD forming activity, in species of freshwater fish common to the studies (Table 3). Evidence is provided for the metabolism of 6-PCSD to 5-PAD by goldfish, carp and pike with little evidence for 5-PAD formation in the eel, perch, rainbow and brown trout. The ability to cleave the sulphonamido bond of 6-PCSD by freshwater fish is therefore species dependent. The differences in activity between species of freshwater fish generally appear to be qualitative, with identification of the presence or absence of activity. This most probably accounts for the close agreement of *in vitro* and *in vivo* methods. One interpretation of the species differences is to propose that species showing no PAD formation are defective with respect to this activity.

In *in vitro* studies, both carp and goldfish liver homogenates were observed to metabolise Eulan WA New. PAD products were identified arising from the cleavage of the 6- and 5-PCSD components of Eulan WA New. However, the 7-PCSD component was not metabolised to give detectable 6-PAD (section 3.4.8.2). The relative amounts of

amine product formed during these metabolism studies suggest differential rates of metabolism of the individual PCSD components of Eulan WA New. The alkaline hydrolysis of Eulan WA New shows a similar pattern of product formation. These differential rates of hydrolysis and metabolism of the PCSD components appear to be inversely related to their degree of chlorination; the more chlorinated the component the slower it is cleaved. The rate of cleavage of the sulphonamido bond of the PCSD components appear therefore to be dictated by steric factors related to the degree of chlorination. The commercial mixtures of polychlorinated biphenyls, e.g. Arochlor 1254, consists of structurally related components (congeners) differing in their degree of chlorination. The rate of metabolism of the different congeners was found to be related to their degree of chlorination in the rat (Grant et al, 1971), in mice (Grage and Holm, 1976), in the Japanese quail (Bailey and Bunyan, 1972) and in the sunfish *L. Cyanellus* (Sandborn et al, 1975). As well as showing different rates of metabolism with respect to the degree of chlorination of the substrates, different species oxidise this group of molecules to different extents. For example, Hutzinger et al (1972) compared the metabolism of PCB congeners by the rat, pigeon and brown trout. The pigeon and rat metabolised mono, di and tetrachlorobiphenyls but not the hexachlorobiphenyls. In comparison the trout did not metabolise any of the congeners. The very low metabolic ability of the rainbow trout to metabolise PCBs was also shown by Melancon et al (1976). A limited metabolism of PCB components has been reported in some other fish species including the dogfish, *S. acanthias* (Hart et al, 1973; Bend et al, 1976). A comparative study of the metabolic ability of the trout, bullhead, goldfish and rat to metabolise PCBs showed that not all fish species have a limited capacity to metabolise PCBs. The

rat and goldfish showed similarly high activities in the metabolism of PCBs in comparison with the much lower activities of the bullhead and trout (Hinz and Matsumura, 1977). The green sunfish appears to be similar to the goldfish metabolising 50% of the dose of trichlorobiphenyl to the oxidised metabolite (Sandborn et al, 1975). It is therefore interesting to note the differing abilities of species of fish to metabolise PCBs. Of the fish species studied the goldfish and the trout represent extremes in ability to metabolise PCBs. A similar contrasting pattern of metabolism is shown with PCSD components as xenobiotic substrates, by the goldfish and trout. The molecular basis of the PCB species differences in metabolism has not been reported, although the metabolism is thought to involve a cyt.P-450 dependent monooxygenase (Hinz and Matsumura, 1977).

In the *in vitro* metabolism of Eulan WA New by carp and goldfish the 7-PCSD component was not metabolised. Consequently the tissues of those fish exposed to Eulan WA New would most probably show a selective enrichment with respect to the 7-PCSD component. Trout, perch and eel show no detectable PAD formation consequently the ratio of PCSDs in these fish tissues *in vivo*, will most probably be similar to the ratio observed in Eulan WA New. Therefore, in some of the fish species studies of the PCSD components of Eulan WA New will be metabolically inert. It is of interest that an inhibitory role of fluorinated hydrocarbons as uncouplers of monooxygenation type reactions has been reported (Ullrich and Diehl, 1971; Staudt et al, 1974). These fluorocarbons were not metabolised by cyt.P-450 dependent monooxygenates but were of sufficient lipophilic nature to form the cyt.P-450 substrate complex. Oxygen uptake was

stimulated upon formation of this complex but stereochemical factors of the fluorocarbons prevented incorporation of the activated oxygen into the molecule. These compounds therefore inhibit monooxygenase activity by uncoupling electron transport. In fish species, the metabolically inert highly chlorinated PCSD components may similarly interact with microsomal monooxygenases. A detailed study of the interaction of PCSDs with monooxygenases may provide valuable information of the molecular basis of their possible mechanism of action *in vivo*. It has been proposed earlier in the discussion that the absence of 5-PAD formation may reflect a defective enzyme or isoenzyme. A possible alternative explanation of the data is that the 5-PAD forming activity may show a species dependent differential inhibition of metabolism by PCSDs. Also the substrate specificity of the enzyme may vary with species. Quantitative differences in substrate specificity and kinetic properties between species have been described for a microsomal cyt.P-450 independent monooxygenase activity. For example the stimulation of N-oxygenation by alkylamine was catalysed by the hog, hamster and guinea pig microsomes but could not be demonstrated in rat or rabbit liver microsomes (Poulsen et al, 1974; Hlavica and Kehl, 1977). It is interesting to note that qualitative differences in this type of activity have been observed in species of freshwater and saltwater fish (Baker et al, 1963; Goldstein and Dewitt-Harley, 1973). The multiplicity of the second type of monooxygenase activity (cyt.P-450 dependent) has been discussed previously in this work (section 3.1). It is interesting that differential inhibition by SKF 525A and 7,8-benzoflavone of the molecular forms of cyt.P-450 has been recently reported. The effect of these compounds on monooxygenase inhibition therefore appears to be cyt.P-450 dependent (Kawalek and Lu, 1975;

Johnson et al, 1979). It may therefore be possible that species differences in inhibition of 5-PAD formation by PCSD may be dependent on the molecular forms of the cyt.P-450 present.

In general the molecular forms of cyt.P-450 are characterised by both an overlapping substrate specificity and also an individual specificity with respect to certain xenobiotic and endogenous substrates. In fish, individual cyt.P-450 isoenzymes have not been studied in detail in terms of substrate specificity. However rainbow trout hepatic microsomes have been shown to contain at least four constitutive haemoproteins upon treatment with inducing agents (Elcombe et al, 1979). The existence of a number of molecular species of cyt.P-450 with both differing and overlapping substrate specificities, introduces the possibility of qualitative species differences relating to a defective (absent or inhibited) cyt.P-450 isoenzyme catalysing a specific monooxygenation reaction which in the case of Eulan WA New may be the oxidative cleavage of the sulphonamido bond of 6-PCSD. Studies of this activity suggest that it involves a microsomal monooxogenase (cyt.P-450 dependent and/or independent, section 3.5). Some evidence has been provided for the existence of species defective in monooxygenase activities. However to date, the molecular basis of these apparent species defects in the metabolism of Eulan WA New and other xenobiotics has not been explored.

As a result of the differences between species in the metabolism of Eulan WA New, the tissues of species of freshwater fish will be exposed to differing concentrations of PCSD and PAD in the contaminated environment. An assessment of the environmental impact of Eulan WA New demands consideration of both the toxicity of PCSD components of Eulan WA New and their corresponding PAD metabolites. At present, the

Table 4

The acute lethal toxicity of Eulan WA New formulation to different species of freshwater fish.

References	Fish Studied	Test Conditions	Temp. °C	Water Hardness mg/l $CaCO_3$	pH	Median Lethal Concentration µg/l 48h
1	Rainbow trout fry	Constant flow	13	20	6	20
1	"	"	13	20	8	74
2	"	"	15	250	7.4	62
2	"	"	15	250	7.4	38
1	Brown trout fry	"	13	20	6	32
1	"	"	13	20	8	105
3	Golden orfe	Static	20	267	7-8	3330
4	Carp	"	20	267	7-8	3332

References:

1 - Tooby (communication)
2 - Abram et al (1981)
3 - Caspers and Hamburger (1981)
4 - Hamburger et al (1981)

only toxicity data available is that of the toxicity of Eulan WA New to some species of freshwater fish (Table 4). Of particular interest is the contrasting differences in the tolerance of fish species to Eulan WA New. For example both the carp and its relative the golden orfe can survive for a period of 48h in almost a hundred fold greater concentration of Eulan WA New than can the rainbow trout. With these species as examples, a correlation can be shown between their differing tolerances to Eulan WA New and their similarly contrasting metabolic abilities to metabolise 6-PCSD to 5-PAD. Carp is active in the metabolism of 6-PCSD and trout inactive (Table 3). It is tempting therefore to speculate that such contrasting metabolic species differences may underly the similar trends shown in the toxicity data. One interpretation of the data would be to propose that PCSD is more toxic to fish than its metabolite PAD, the conversion of PCSD to PAD therefore representing a detoxication of the xenobiotic. That Eulan WA New is relatively more toxic to trout than to goldfish was observed in dosing studies (section 2.4.6). When goldfish and trout were dosed with a similar concentration of 6-PCSD, the goldfish survived an eight hour dosing period, whereas the dosed trout died after 45 min. Another salmonid, salmon fry (*Salmo solar*) were dosed with 40 ppb Eulan WA New, prior to being fed to pike (section 2.3.4). After 24-48h exposure to Eulan WA New, substantial losses of fish were recorded. The trout therefore and maybe salmonid relatives, appear to be particularly sensitive to Eulan WA New.

Verification of the relationship between toxicity to Eulan WA New and the ability to metabolise this xenobiotic, would require comparative toxicity studies of both PCSD and PAD, using chosen species of freshwater fish. The exposure of fish to equal concentrations of PCSD or PAD in surrounding water would not be a successful approach

because of the relative insolubility of PAD in aqueous environments. An alternative approach may involve injection of equal doses of PCSD or PAD into a fish species unable to metabolise PCSD to PAD, e.g. trout, with subsequent determination of the relative toxicities of these components. Another approach is to further characterise the 5-PAD forming activity *in vitro* and selectively inhibit PAD formation (using a fish species active in 5-PAD formation, e.g. goldfish) by a specific *in vivo* inhibitor. The relative toxicities in the presence and absence of inhibitor would give a measure of the relative toxicities of PCSD and PAD respectively. (Many such inhibitors have been defined for cyt.P-450 dependent monooxygenases, however a specific inhibitor has not so far been identified for the cyt.P-450 independent type of monooxygenase). Data on the relative toxicity of PCSD and PAD to fish would allow identification of 'target species' i.e. those most at threat in an environment contaminated with Eulan WA New. These sensitive species can then be used as environmental 'indices' to monitor the extent of pollution. This can be approached by measuring tissue levels of xenobiotic and metabolite [e.g. muscle, liver, and a particularly useful monitor of Eulan WA New contamination, the bile (section 2.4.4)]. A histological study may complement the latter approach, relating the extent of environmental contamination with histological changes in target species. A preliminary histological study of rainbow trout dosed for periods up to one week with Eulan WA New (40 ppb), identified some histological changes appearing upon exposure to the xenobiotic. Eosinophilic cell remnants were observed in stomach tissue and also the presence of some necrotic cells in the terminal gut. Further detailed histological studies are however necessary to verify these preliminary observations.

An assessment of the environmental impact of a xenobiotic demands consideration of the toxicity of both parent compounds and metabolites to the future generations of fish, and other aquatic organisms. This necessitates ontogenic study, assessing the ability of different developmental stages of aquatic organisms to metabolise xenobiotics. Only through this approach can one assess the sensitivity of the developing organism to xenobiotics.

The observed contrasting differences also demand care in the choice of suitable model systems from which to assess both environmental impact and in which to study the metabolism of Eulan WA New, representative of that of aquatic organisms. It is clear that the choice of a single test fish would be insufficient and could yield grossly misleading data with respect to residue concentrations of PCSD and PAD in freshwater fish. In the light of this work, an advisable choice of fish species would be trout and carp, since these show the contrasting species differences in the metabolism of Eulan WA New, representative of freshwater fish. Consequently these species provide model systems in which to assess and study the environmental impact of Eulan WA New on freshwater fish. Of the freshwater fish species studied two members of the family Cyprinidae, the goldfish and carp and one member of the family Escoidei showed the ability to metabolise 6-PCSD to 5-PAD. In contrast rainbow and brown trout from the family Salmonidae the perch (family Percidae) and the eel (family Anguillidae) gave no conclusive evidence for 5-PAD formation. The rationale behind such a distribution of the 5-PAD forming activity is not at present clear. It would be interesting to see if these trends in xenobiotic metabolism extend to other members of these families and also to the more primitive classes of fish such as the Elasmobranchs. Some xenobiotic metabolising activities have been identified in this

group (Adamson and Guarino, 1972). The species distribution of the 5-PAD forming activity may show some evolutionary influences. Williams (1976) recognised the possible contributions that the characteristics of xenobiotic metabolism of a species might make to the overall problem of zoological classification and introduced the basis of pharmacotaxonomic study.

Species differences therefore exist in the ability of freshwater fish to metabolise 6-PCSD to 5-PAD with complementary evidence provided from both *in vivo* and *in vitro* approaches. Such a close correlation of *in vitro* and *in vivo* studies allows an assessment of environmental metabolism of Eulan WA New by aquatic organisms to be made from *in vitro* studies. This approach circumvents the tedious and time consuming sampling of contaminated environments, sample dissection, tissue extraction and extract "clean-up" methods, necessary for determination of concentration of xenobiotic and metabolites in tissues. Using the *in vitro* method a rapid comparative assessment of ability of aquatic organisms to metabolise Eulan WA New can be made.

A more complete assessment of environmental impact demands complementary histological and toxicity studies, in addition to the xenobiochemical approach employed in this work. Preliminary characterisation of the 5-PAD forming activity suggests that a microsomal monooxygenase activity accompanies cleavage of the sulphonamido bond of 6-PCSD. The molecular basis of such contrasting species differences has not been explored. Such studies may provide a further insight into the multiplicity of the microsomal monooxygenases and will also serve to further highlight the necessary care which must be taken in the choice of organisms as model systems in which to study xenobiotic metabolism.

APPENDIX

CHAPTER 5

The Metabolism of Eulan WA New by the Fungus *Verticillium lecanii*.

The natural multicellular design of the mammalian system complicates studies of xenobiotic metabolism. Particular tissues as sites of metabolite production may be studied *in vitro*, however the perfused tissue may remain physiologically active for only relatively short periods. The production of metabolites of xenobiotics in sufficient quantity for structure elucidation and for studies of biological activity is not easily achieved from mammalian systems. Such limitations have resulted in the search for simpler model systems and the proposal of the use of microorganisms as models for mammalian metabolism (Rosazza and Smith, 1979). The production of xenobiotic metabolites by microorganisms can be enhanced by varying both growth and incubation conditions, and sizeable (gram) quantities can be obtained. Biological material can be supplied in large quantities necessary for enzyme purification procedures and further biochemical characterisation studies. Microorganisms have provided the source of cyt.P-450 dependent monooxygenases, most extensively studied, a consequence of the ease of study and abundance of pure components. This has led to the detailed characterisation of cyt.P-450 of *Pseudomonas putida*, grown on camphor as a sole source of carbon. The oxidative intermediates, metabolite pathway, and their genetic organisation has been elucidated (Rheinwald et al, 1973); the electronic states of the metallocatalytic iron centres have been studied (Gunsalus et al, 1974) and the reaction dynamics and energetics for both the individual components and the multicomponent enzyme system have been reported (Gunsalus and Sligar, 1978).

The fungi have recently been subject to study as models for mammalian xenobiotic metabolism, mainly concentrating at present on Cunninghamella species. Ferris and co-workers (1973) have shown O-demethylation of 4-nitroanisole; aryl hydroxylation of anisole,

aniline and naphthalene; and reduction of nitro and azo groups of xenobiotic substrates by fungi. These types of microsomal monooxygenase oxidative/reductive activities are typical of the mammalian hepatic microsomal system, showing a similar broad substrate specificity. In addition to the ability to catalyse a similar diversity of types of monooxygenase activity, the fungi also appear to possess a very similar complement of xenobiotic metabolising enzymes to those observed in mammals. For example Cunninghamella *elegans* oxidises naphthalene and also produces a trans 1,2-dihydro-1,2-dihydroxynaphthalene metabolite by an epoxide hydrolase activity (Cerniglia and Gibson, 1978). Glucuronic acid and sulphate conjugates of xenobiotic substrates have been isolated from culture filtrates of this fungus (Cerniglia, 1981), providing evidence for the presence of enzymes active in the conjugation of xenobiotics. Further, the presence of UDP-glucuronosyltransferase and glutathione transferase activities have recently been reported in this fungus (Wackett and Gibson, 1982). Such close similarities in the metabolism of xenobiotics by mammalian and fungal systems may relate to their eukaryotic nature (Gibson et al, 1975). Fungal cyt.P-450 dependent microsomal monooxygenases are also inducible, analogous to those in mammals. Induction of the fungal monooxygenases has been observed by naphthalene, phenobarbitol and 3-methylcholanthrene (Cerniglia and Gibson, 1978). The comparative similarity of features of both fungal and mammalian xenobiotic metabolism highlight the potential of utilising fungal systems to study and possibly predict the metabolic fate of xenobiotics in mammals.

This work reports the *in vivo* cleavage of the sulphonamido bond of the xenobiotic 6-PCSD (the major component of Eulan WA New) by the fungus *Verticillium lecanii*. This 5-PAD forming activity appears to be species dependent in freshwater fish (section 3.4.8.1).

Growth and Morphology of *V. lecanii*

50 mM potassium phosphate buffer was prepared containing Eulan WA New (20 ppm). The solution was left at 20°C overnight (uncovered) and then stored routinely at 4°C, over a period of six weeks this stock Eulan WA New solution showed a decrease in Eulan WA New concentration which at the time could not be explained. The solution was not used further but was still stored at 4°C. After a period of two months, microscopic analysis of samples of the stock Eulan WA New solution showed the presence of fungal mycelia forming an amorphous mass at the base of the glass container. The fungus was identified by the Commonwealth Mycological Institute (Kew, Richmond, Surrey) to be *Verticillium lecanii*. The growth of *V. lecanii* occurred with apparently Eulan WA New as the sole carbon and nitrogen source. (The sample referred to as the 'stock solution' in this section, is the original Eulan WA New/phosphate buffer solution containing *V. lecanii*).

Grams (1971) classified *V. lecanii* in the Prostrate section of the Cephalosporia. Colonies grown in agar and in liquid medium were described:

On agar, colonies were 18-22 mm in diameter, white or pale yellow, cottony/velvety. Phialides were awl-shaped, very variable in size, single or in small groups of verticiliate whorls on aerial mycelia. Conidia were observed in a terminal head of slime, on phialides, which were cylindrical to ellipsoidal with symmetrically rounded ends (Fig. 1A). Chlamydospores were absent. In liquid media the fungus assumed a semi-yeast morphology (probably in response to accumulation of carbon dioxide) forming budding elements, known as blastospores (Fig. 2B).

Figure 1

Slime head of *V. lecanii* containing conidia (A) (× 400)

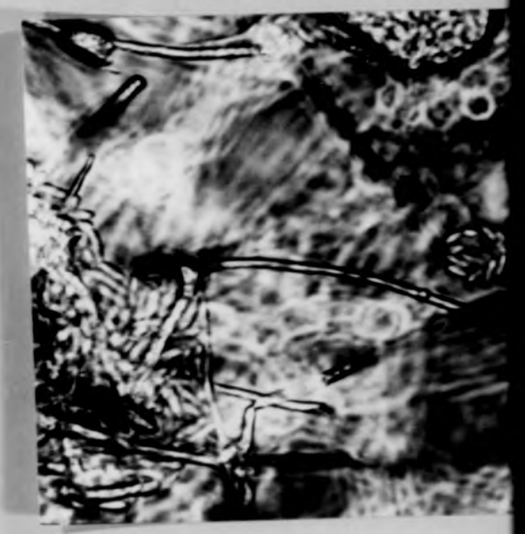


Figure 2

Budding elements (blastospores) of *V. lecanii* in liquid medium (B) (× 400)

g conidia (A) (× 400)

f V. lecanii


A


B

Substrates for growth include wood and soil, and this fungus has also been observed to etch upon plastic contact lenses. The fungus is non-fastidious and will grow on all conventional mycological media so far tested, including a medium containing chitin as a sole source of carbon and nitrogen.

This fungus is also a microbial insecticide (entomopathogen), its major hosts being aphids and scales (Neuzilova, 1957; Samsináková and Kálalová, 1976)

Methods

Samples of fungus from the stock solution were rinsed by immersing in 50 mM phosphate buffer (pH 7.4) free of Eulan WA New. The mycelial mass was divided equally by eye. Samples of the fungus were placed into 5 ml aliquots of 50 mM potassium phosphate buffer, pH 7.4. Eulan WA New (5 µl of a 4 mg/ml Eulan WA New in DMF solution) was added to some of the fungal solutions; and to others purified 6-PCSD (25 µl of a 2 mg/ml 6-PCSD in DMF solution). To the remaining controls was added either 5 µl or 25 µl of DMF. The samples were incubated at room temperature. Upon completion of the dosing periods, samples were spun in a bench centrifuge for 5 min. The buffers were carefully decanted from the fungal pellets, into clean tubes and further extracted with MTBE (section 3.3.1). The fungal pellets were each ground with anhydrous sodium sulphate and extracted with MTBE (section 2.3.7.1). The tissue and buffer MTBE extracts were evaporated to dryness under a stream of dry air and the residues redissolved in DMF. Aliquots of each were injected for HPLC analysis.

HPLC profiles of:

(a) MTBE extracted buffer (50 mM potassium phosphate pH 7.4) of the stock solution containing Eulan WA New (20 ppm) and the fungus *V. lecanii* (time zero)

(b) an MTBE extracted fungus of a sample of *V. lecanii* from the stock solution after 3 months growth.

The new peaks of profile 'b', I and II, coeluted with the PAD products of 6-PCSD
(A = DMF injection solvent)

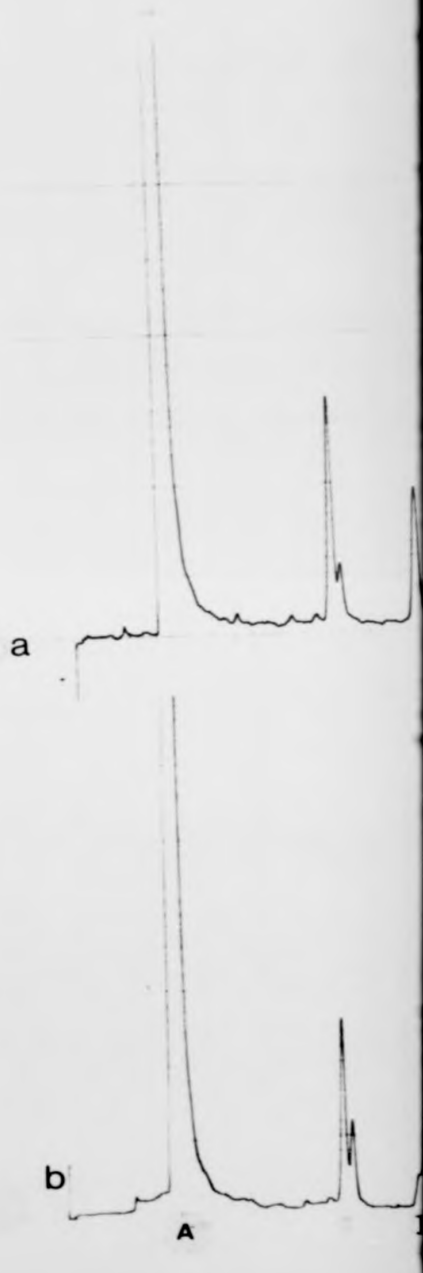


Figure 3

The presence of PAD in the fungus *V. lecanii* after growth in 50 mM phosphate buffer pH 7.4 containing Eulan WA New.

otassium phosphate pH 7.4)

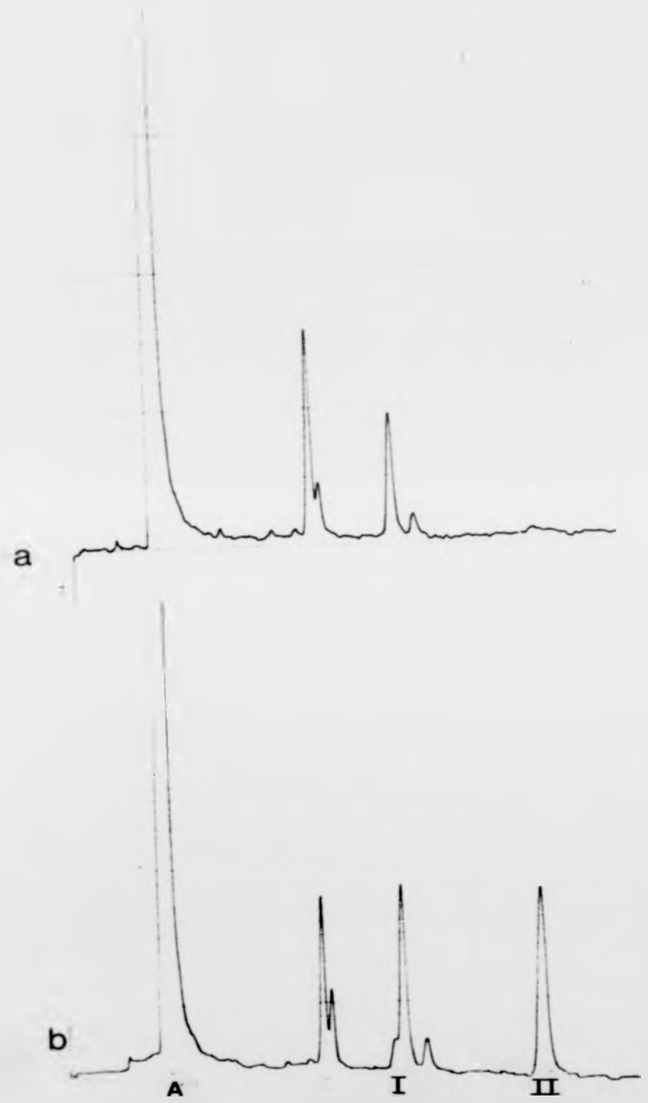
g Eulan WA New (20 ppm)

e zero)

ample of *V. lecanii* from

hs growth.

II, coeluted with the



. lecanii after growth in

lining Eulan WA New.

HPLC profile of MTBE extracted:

(a) surrounding buffer (5 ml) Eulan WA New (4 ppm) at time zero.

(b) samples of *V. lecanii* at time zero

(The presence of Eulan WA New is shown associated with the fungus at the start of the dosing study. The fungus is taken from the stock sample which contains Eulan WA New.)

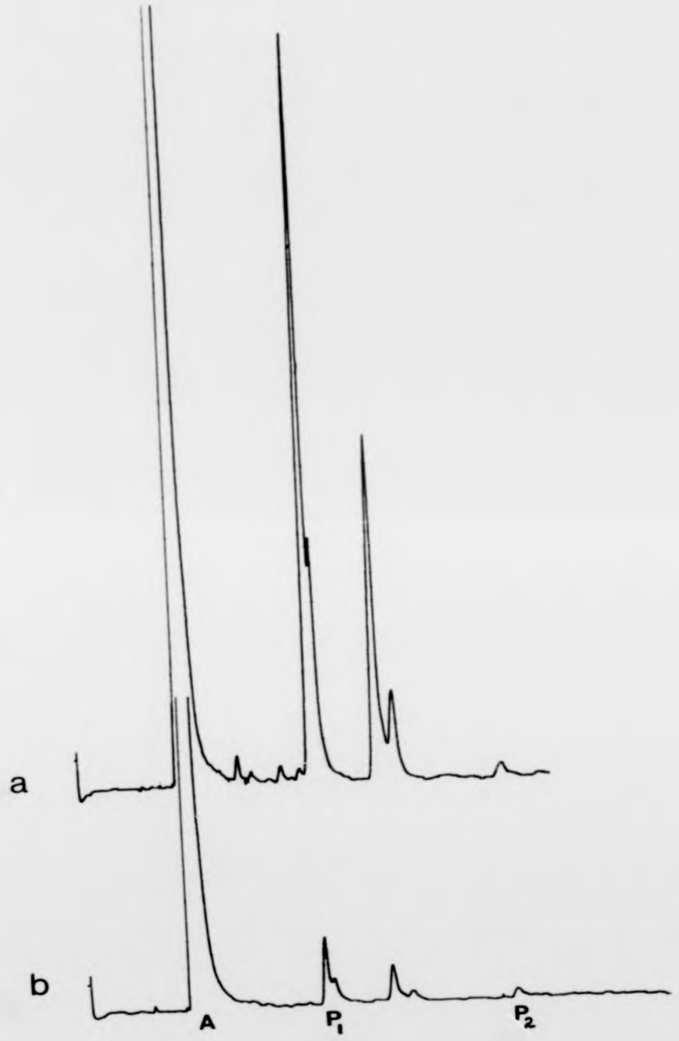
A - DMF injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 4

HPLC profiles of extracted fungus and surrounding buffer at the start of the dosing period with Eulan WA New.



WA New (4 ppm)

zero

own associated with the
tudy. The fungus is
ontains Eulan WA New.)

and surrounding buffer

with Eulan WA New.

HPLC profile of MTBE extracted:

(a) sample of *V. lecanii* after dosing with Eulan WA New in surrounding buffer for 48h.

(b) surrounding buffer after 48h

A - DMF injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

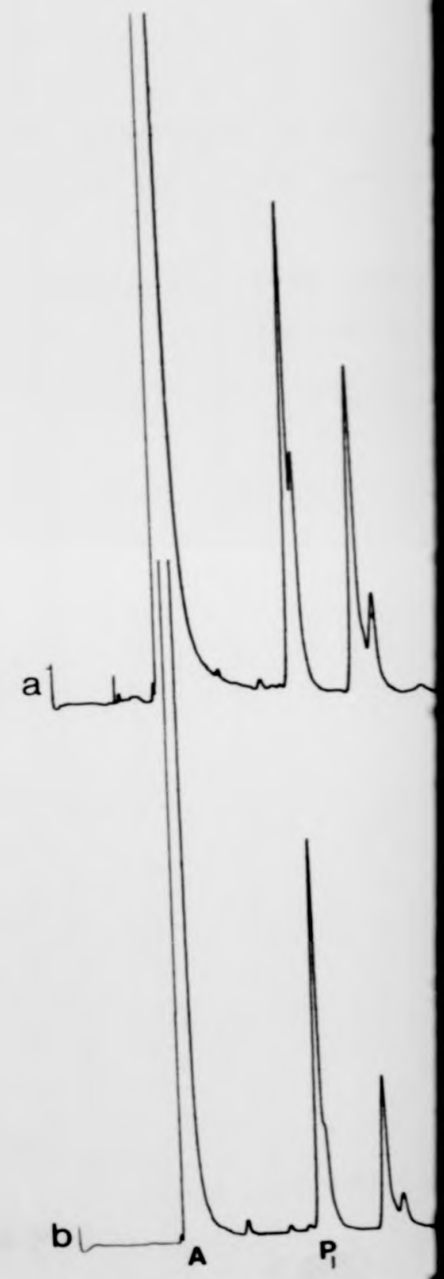
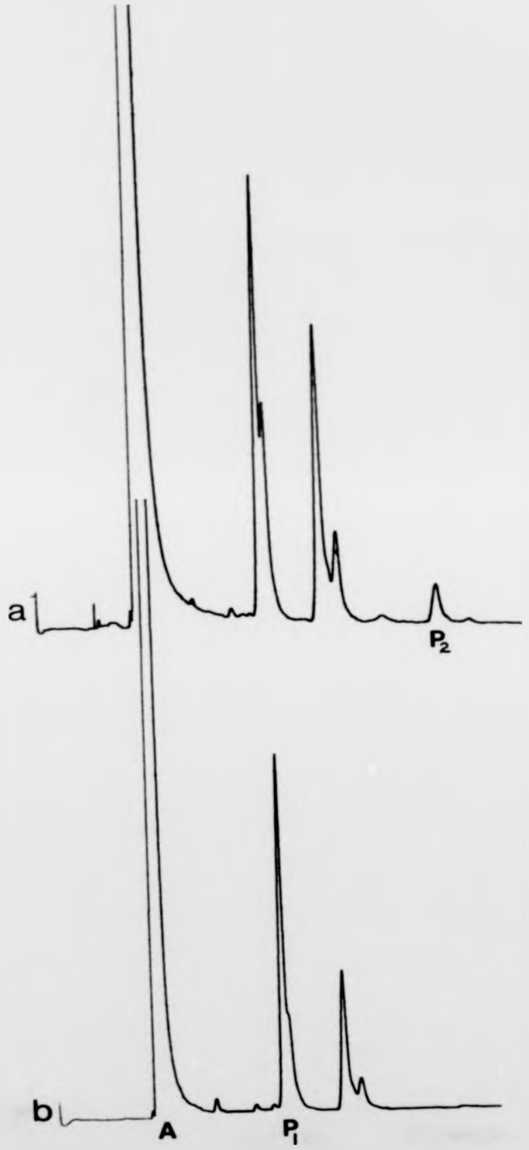


Figure 5

HPLC profiles of extracted fungus and surrounding buffer after dosing with Eulan WA New for 48h.

ling with Eulan WA New



and surrounding

A New for 48h.

HPLC profile of:

(a) 5-PAD standard

(b) MTBE extracted sample of *V. lecanii* after dosing with 6-PCSD (10 ppm) in surrounding buffer (5 ml) for 5-days

(c) 5-PAD standard plus an aliquot of the MTBE extracted *V. lecanii* sample, identifying 5-PAD

A - DMF injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 6

The presence of 5-PAD in fungus tissue after dosing with 6-PCSD.

canii after dosing with

g buffer (5 ml) for 5-days

t of the MTBE extracted

g 5-PAD

issue after dosing

HPLC profile of:

(a) 6-PCSD standard

(b) 6-PCSD plus an aliquot of MTBE extracted *V. lecanii* sample (Fig.6b) identifying the presence of 6-PCSD

A - DMF injection solvent

P_1 - 6-PCSD

P_2 - 5-PAD

Figure 7

Identification of 6-PCSD in fungus tissue.

extracted *V. lecanii*

e presence of 6-PCSD

as tissue.

Results and Discussion

Extraction and HPLC analysis of a mycelial sample of *V. lecanii* after three months growth in the stock solution showed the presence of a high 5-PAD : 6-PCSD ratio of 1.2 (Fig. 3) compared with 0.05 for the Eulan WA New solution used for dosing. This observation suggested that the fungus was active in the metabolic cleavage of the sulphonamido bond of 6-PCSD, resulting in the formation of the amine (5-PAD) product. Dosing of samples of the fungus with Eulan WA New over a period of 48h, showed an increase in 5-PAD associated with fungal tissue at the end of the dosing period (Figs. 4 and 5). However analysis of the surrounding buffer at the end of the 48h period showed no detectable 5-PAD (Fig. 5b - normally 5-PAD constitutes approximately 2-5% of Eulan WA New). The accumulation of 5-PAD in fungal tissue may therefore be accounted for by either a selective or differential absorption of 5-PAD from the surrounding buffer and/or the *in vivo* fungal metabolism of 6-PCSD to 5-PAD. To resolve this question, *V. lecanii* was dosed with 6-PCSD (free of 5-PAD impurity). Upon completion of a five day dosing period, a high concentration of 5-PAD was found in fungal tissue (Figs. 6 and 7) with a 5-PAD : 6-PCSD ratio of 0.33. Therefore part or possibly all of the 5-PAD in the fungal sample dosed with Eulan WA New, arose through *in vivo* metabolism of 6-PCSD to 5-PAD. Attempts to identify the 5-PAD forming activity *in vitro* in the presence of DTT (section 3.4.6.4) have so far been unsuccessful. It was noted however that homogenisation of the fungus by grinding with acid washed sand led to surprisingly large changes in pH of the homogenisation buffer. 1g fungus (mycelia) when homogenised in 4 ml 50 mM potassium phosphate buffer changed the buffer pH from 7.4 to 5.5. Future *in vitro* studies should also include the addition of NADPH which is a cofactor

Algae was collected from the walls of a goldfish bowl containing 6-PCSD (0.3 ppm), after standing for a period of six weeks. The algal sample was ground with anhydrous sodium sulphate and extracted with MTBE. The MTBE extract was further 'cleaned' upon a mixed alumina column. The successive eluates from the column, MTBE, methanol, MTBE (following acidification) were analysed by HPLC. HPLC profiles of the mixed alumina column eluates:

(Procedures described in sections 2.3.7 and 2.4.3).

(a) 25 ml MTBE (5-PAD containing eluate, P_2)

(b) 25 ml Methanol (6-PCSD containing eluate, P_1)

A - DMF injection solvent

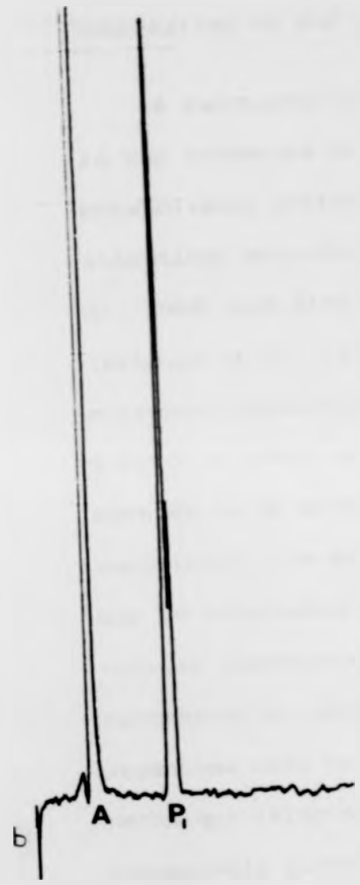


Figure 8

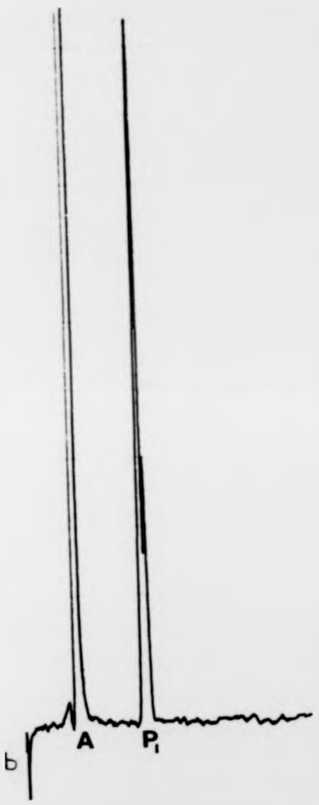
<u>The presence of 5-PAD in algal tissue grown in water containing 6-PCSD.</u>

alls of a goldfish bowl
standing for a period of
ground with anhydrous sodium
The MTBE extract was
nina column. The successive
hanol, MTBE (following
LC. HPLC profiles of the

2.3.7 and 2.4.3).

eluate, P_2)

ining eluate, P_1)



tissue grown in water

required for 5-PAD formation (section 3.4.9.2). *V.lecanii* therefore provides a model system in which to study the *in vivo* metabolism of the xenobiotic 6-PCSD to its amine product 5-PAD. This reaction in freshwater fish is species dependent (section 3.4.8), it would be interesting to see if this dependency also extends to the fungi.

A functionally important site of compartmented microorganisms is the intestine of higher organisms. Specific xenobiotic metabolising activities have been found to be catalysed by the intestinal microflora including a reduction of azo dyes (Gringel et al, 1969) and also the metabolism of the 'N-S' bond of cyclamate (Niimura et al, 1974). Preliminary studies of the ability of the microbial populations of goldfish digestive contents to metabolise 6-PCSD to 5-PAD gave no evidence of 5-PAD formation (section 3.4.4). However in an environment where there is constant exposure to a xenobiotic, the microorganism populations of the digestive tract may be alternated such that particular populations most able to survive (through metabolic adaptations) will predominate. This succession of microbial forms is that observed in the isolation of organisms able to metabolise xenobiotics by the selective enrichment technique (Kluyver and Van Niel, 1956). In the aquatic environment, innumerable microorganisms inhabit both deposits and surface waters constituting the lowest trophic levels of the aquatic ecosystem. Wells and Cowan (1983) have reported that zooplankton and/or phytoplankton metabolise 6-PCSD to 5-PAD. In agreement with this observation, algae taken from goldfish bowls in which solutions of 6-PCSD had been left for six weeks were shown to contain 5-PAD (Fig. 8), most probably arising from *in vivo* metabolism of the 6-PCSD. The role of the diverse range of aquatic microorganisms in the metabolism of Eulan WA New awaits to be further defined. These organisms provide

a major food supply for the lower members of the aquatic food chain. Consequently studies of their ability to metabolise Eulan WA New may be useful in helping define the magnitude of 6-PCSD and/or 5-PAD accumulating in nutritionally inter-related aquatic organisms through biomagnification.

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