The Comparative Metabolism and Toxicity of the Pyrethroid Insecticide Cypermethrin in Vertebrates.

by

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The author visits the grand academy of Lagado during a voyage to Balnibarbi

The first man I saw, was of a meagre aspect, with sooty hands and face; his hair and beard long, ragged and singed in several places, His clothes, shirt, and skin, were all of the same colour: he had been eight years upon a project for extracting sun-beams out of cucumbers; which were to be put into vials, hermetically sealed, and let out to warm the air, in raw inclement summers. He told me, he did not doubt, in eight years more, that he should be able to supply the Governor's garden with sun-shine at a reasonable rate; but, he complained that his stock was low, and intreated me to give him something as an encouragement to ingenuity, especially since this had been a very dear season for cucumbers.

Johnathan Swift
Gulliver's Travels
1735

Contents

Abstract

The target organ sensitivity and metabolism of the synthetic pyrethroid insecticide cypermethrin has been studied in rainbow trout, frog, mouse and quail in order to help explain observed species differences in the acute toxicity of this compound (fish > amphibians > mammals > birds).

Toxic oral doses of cis-cypermethrin were most readily absorbed by quail, but were then rapidly metabolised and excreted. Trout, though very sensitive to both cis- and trans-cypermethrin when dissolved in the water, failed to take up the majority of the pyrethroid from the intestine and eliminated the unchanged insecticide dispersed in secreted bile. Concentrations of cypermethrin in the brains of animals exhibiting toxic signs showed a neuro-sensitivity in the order of frogs > trout >> mouse > quail. In addition, potentially toxic metabolites of cypermethrin were identified in the brains of frogs and trout.

Biotransformation studies with cypermethrin isomers in vivo and in vitro have also revealed interesting species differences. Quail and mice are considerably more active in detoxifying cypermethrin than frogs or trout, with the bird metabolising oral doses more rapidly and more extensively than the mammal. Unlike the other species, quail form large amounts of the products of ester cleavage from cis-

cypermethrin. Studies with liver microsomes suggest that this may be due to oxidative de-esterification, the other vertebrates largely forming hydroxylated derivatives of intact cis-cypermethrin, with species variations in the preferred sites of oxidation. Trans-cypermethrin was largely hydrolysed by esterases in mouse, quail and frog, with mouse tissues being the most active. In the trout, esterases in the intestine and plasma were particularly important, as their overall biotransformation exceeded that of the liver. Important phase II reactions were glucuronidation (all species), sulphation (quail) and amino acid conjugation (mouse).

The results suggest that both differences in biotransformation and target organ sensitivity can largely account for the species-selective toxicity of pyrethroid insecticides.

Acknowledgements

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I should especially like to thank my supervisors, Dr. Peter Millburn and Dr. David Hutson, for their advice, interest and encouragement throughout my training. I am also indebted to the many individual staff at St. Mary's and Shell Research who have given up their time to help me, over the last three years. In particular, I should like to acknowledge Dr. Christopher Logan for his advice on the *in vitro* studies, Dr. Richard Stephenson for his assistance with the fish studies and George Stoydin and the staff of the Animal House (St. Mary's) for their general technical expertise. Finally, my thanks go to Mrs. Joy Dexter for all the typing, my wife for all the proof reading, and my family for their continued support.

Chapter 1. Introduction

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1. Introduction

Man's wish to control pest insect species dates back to classical times, with Homer and Pliny the elder describing the uses of sulphur and arsenicals as insecticides (Hassal, 1982). In addition to these compounds, fluorine-containing toxicants and natural plant extracts such as nicotine, rotenone, and pyrethrum have also been developed as insecticides over the centuries. Such preparations have been described as first generation insecticides and due to their high toxicity to non-target species, or poor stability under field conditions, have now been largely superseded by synthetic organic compounds (see Table 1:1). These second generation insecticides are more potent and stable than many of their predecessors and may be divided into four major groups, namely; the organophosphates, the methyl-carbamates, the organochlorines and the pyrethroids. The third generation insecticides, those compounds specifically influencing insect behaviour and biochemistry (e.g. hormones) are still under development and seem unlikely to supplant the more traditional insecticides (Elliott, 1979). In addition to the synthetic pesticides, systems of biological insect control such as viruses, bacteria and fungi are also in limited use today.

The large scale use of some second generation insecticides has given rise to a number of environmental problems. The organochlorines, such as

DDT and dieldrin, have been shown to be unfavourably persistent and toxic to non-target species especially birds, both through application and by accumulation in food chains. The carbamates and organophosphates can be hazardous to man and wildlife during manufacture and field use, due to their high acute toxicity. By contrast, the recently developed pyrethroid insecticides are readily broken down by biological systems and show a remarkable selective toxicity towards insects (see: Casida, 1980; Elliott, 1976; Miyamoto, 1976). The bioactivity of the pyrethroid insecticides compared with their competitors is illustrated in Table 1:1. As the pyrethrins have been the natural precursors of the pyrethroids, a brief account of their history and development has been included.

Table 1:1

The relative toxicity of insecticides to pest and non-pest species

[a] Approximate LD_{50} Values ($mg\ kg^{-1}$)	Insects (topical)	Rats (oral)	Selective Toxicity Factor [b]
First Generation Insecticides			
Arsenic compounds	165	100	0.6
Fluorinated toxicants (Cryolite)	300	200	0.7
Nicotine	650	55	0.1
Pyrethrin (I)	15	420	28
Second Generation Insecticides			
Organochlorines (DDT)	18	120	7
Organophosphates (Malathion)	31	1400	45
Carbamates (Carbaryl)	31	700	23
Pyrethroids (Permethrin)	0.7	>2000	>2,800

[a] LD_{50} values obtained from a number of studies with male and female rats, and a variety of insect species. Median values of ranges reported by Elliott, 1976; 1979.

[b] Calculated as ratio of rat LD_{50}:insect LD_{50}.

2. Pyrethrins

2.1. History

Extracts from the secretory ducts of the achenes contained in the flowers of the daisy Tanacetum cineraiifolium have long been known to possess insecticidal activity; their use being widespread in Persia in the early 19th century for controlling body lice (Casida, 1980). Having originated in the mountainous regions of the Middle East, the plants were first commerically grown in Yugoslavia from 1840 up until the First World War. The centre of world production then moved to Japan, finally residing in Kenya and Tanzania where some 15,000 tonnes of dried flowers are picked and processed annually. An active preparation is obtained by solvent extraction with hexane or kerosene. The concentrated extract being normally cleaned up by re-extraction with methanol and charcoal treatment to remove allergenic impurities. Though superseded as an agricultural insecticide in the 1940's by the organochlorines and organophosphates, pyrethrum extracts continue to be useful in the domestic environment and for use with stored food products. Common methods of application are in the form of a dust, an aerosol or as a smoke generated by mosquito coils.

2.2. Structure and activity

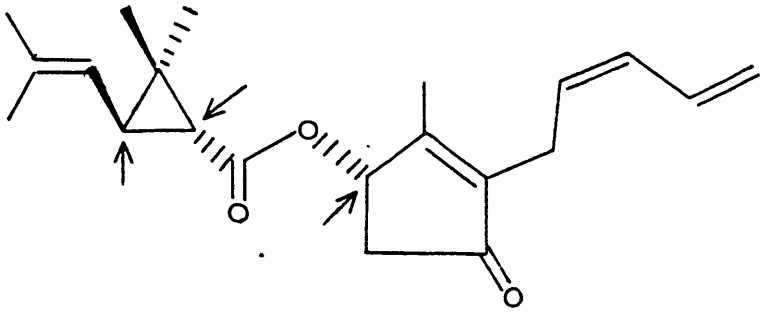
Due to the insecticidal properties of pyrethrum

extracts, a considerable amount of research has gone into the structural elucidation of the active components (see: Elliott, 1979; Casida, 1980). The pyrethrins are a group of six esters differing in the terminal substituents in the side chains of the cyclopropanecarboxylic acid and the cyclopentenolone alcohol . The acid moiety is formed in the plant from mevalonic acid, but the biosynthesis of the cyclopentenolone derivatives has not been elucidated (Casida, 1980). Following condensation, the various chrysanthemates and pyrethrates are formed by oxidation of the methyl group of the isobutenyl side chain and by methylation. These substitutions yield six active compounds, which are classified as pyrethrins, cinerins or jasmolins, depending on the plant species they were first characterised in.

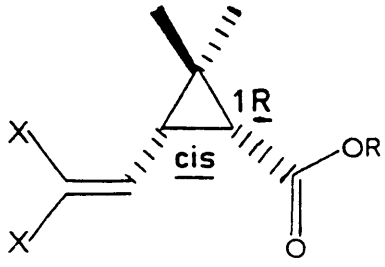
Pyrethrum extracts paralyse, or "knock down" flying insects and at sufficiently high doses will subsequently kill them. Early experiments with the isolated components showed pyrethrin I to be the most active killing agent, and pyrethrin II to have maximum knockdown activity (Sawicki and Thains, 1962). In addition to structural differences, the stereochemical configuration of these molecules has also been shown to greatly influence their insecticidal activity (Elliott, 1976). In particular, the juxtapositions of the methyl groups at the C-2 position of the cyclopropane ring, of the unsaturated side chain at C-3, and the ester

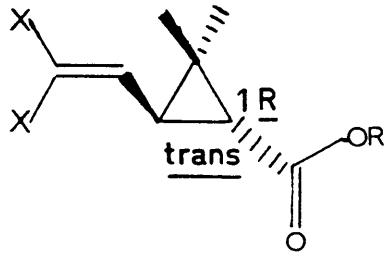
linkage at C-1 define biological activity. With three optical centres (see Figure 1:1), pyrethrin I has a total of 8 stereo-isomers which are best described using a hybrid Cahn-Prelog-Ingold system of nomenclature as described by Elliott and co-workers. The conventional R (rectus:right) and S (sinister:left) terminology is used to describe the orientation of three of the bonds on a carbon atom when viewed from the bond of lowest priority (lowest atomic weight of attached group). However, in addition to the way the orientation at C-1 may be defined as R or S, the configuration at C-3 in relation to C-1 is described as cis (on the same plane of the cyclopropane ring) or trans (on the opposing plane) as shown in Figure 1:1. The orientation of the α-carbon of the ester linkage can then be referred to as being either αR or αS.

Despite the excellent insecticidal activity of the natural pyrethrins, their development as agricultural insecticides has been inhibited by their photo-instability. Susceptible sites of photo-oxidation are the unsaturated side chains and the cyclopentenolone substituent (Figure 1:1). Typical light mediated reactions are epoxidation, peroxidation and methyl-oxidation (Casida, 1980). Commercial exploitation of these natural insecticides has therefore largely depended on the development of synthetic analogues which are more stable and in some cases, more effective

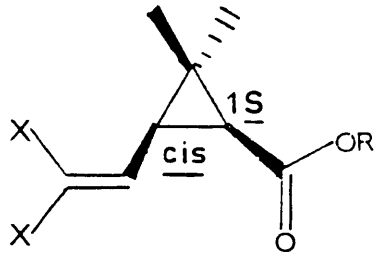


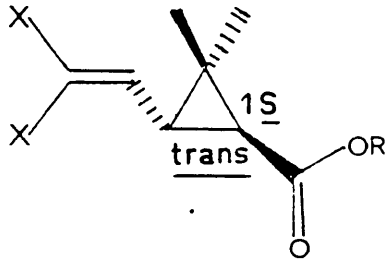
Pyrethrin I (↑ chiral centres)



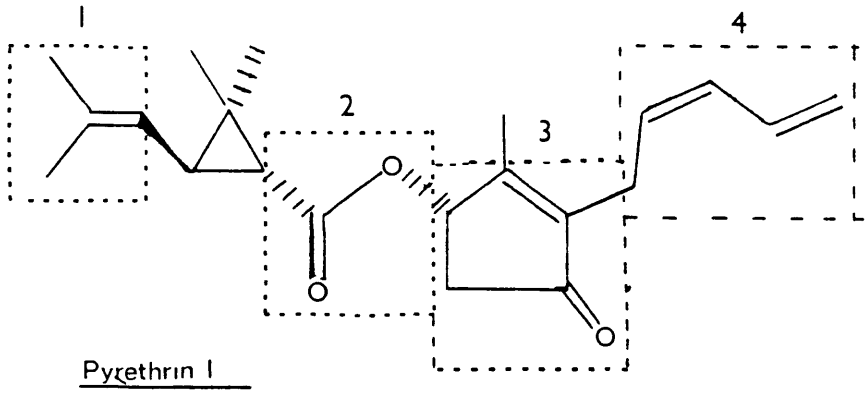


1R isomers insecticidal





1S isomers non-insecticidal



Pyrethrin I

Photolabile centres

Figure 11 Chemistry of the pyrethrins

as insecticides.

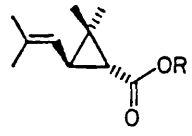
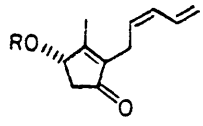
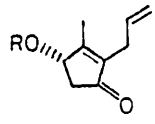
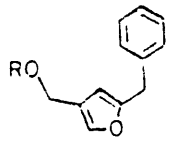
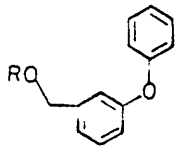
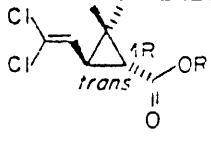
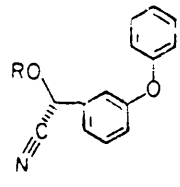
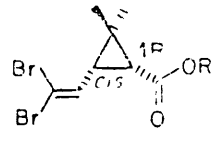
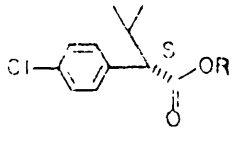
3. Synthetic pyrethroids

3.1. Development

In the last 20 years a number of synthetic pyrethroid insecticides have been developed, principally at the Rothamstead Experimental Station (England) and the Sumitomo Chemical Company (Japan). Elliott (1977, 1979) has reviewed this subject in considerable detail and described some of the parameters which define biological activity in the synthetic analogues of the pyrethrins. Figure 1:2 summarises the important structural modifications of the acid and alcohol moieties, which have increased the stability and insecticidal activity of the pyrethroids. In particular, the isoteric replacement of the labile alcohol groups with the phenoxybenzyl moiety, the substitution at the C-2 position of the cyclopropane ring with the dihalovinyl group and the inclusion of the nitrile group at the α-carbon of the ester linkage have greatly increased photo-stability and insecticidal activity. The presence of the chlorophenyl-methyl-butyrate substituent as the acid moiety in fenvalerate, demonstrates that pyrethric acid is not essential for insecticidal activity. Similarly, in fluvalinate (structure not shown), the acid moiety is replaced by the 2-chloro-4-trifluoromethyl-anilino group. Pyrethroid development is still continuing, with many

Figure 1:2

The development of the synthetic pyrethroids

Acid moiety.	Alcohol moiety.	Name. (date)
		Pyrethrin
		Allethrin (1949)
		Resmethrin (1967)
		Phenothrin (1971)
		Permethrin (1973)
		Cypermethrin (1975)
		Deltamethrin (1974)
		Fenvalerate (1976)

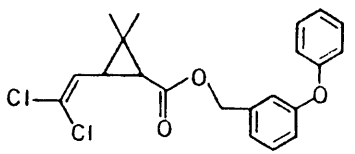
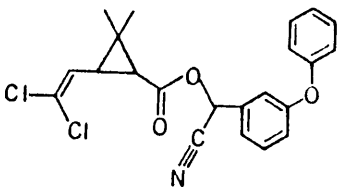
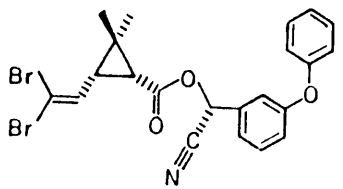
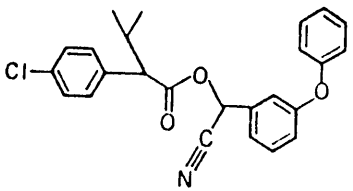
hundreds of potential compounds tested annually. Elliott (1984) has reported that aryl-substitution with fluorine atoms at various positions, and replacement of the pyrethroid alcohols with α-isopropyl aryl-acetates may improve existing pyrethroids. Some success has also been obtained in replacing the ester linkage with oxime derivatives (Nanjyo et al., 1980).

In common with the pyrethrins, the pyrethroids are dependent on their three-dimensional conformation for their insecticidal activity. Most of the pyrethroids are generally available as a racemic mixture of the constituent isomers. The exceptions to this are deltamethrin and FASTAC which consist of one and two enantiomers respectively. The structures and names of the major pyrethroids used in crop protection, permethrin, cypermethrin, deltamethrin and fenvalerate are shown in Figure 1:3. The remaining discussion will apply largely to these four compounds unless otherwise stated.

3.2. Physical and chemical properties

The vapour pressure and lipophilicity of a compound determine its environmental distribution between air, soil and water (Briggs, 1984). The synthetic pyrethroids have high boiling points (>550^{o}C) and show low volatility from soil and water surfaces. Being non-polar these compounds are also virtually insoluble in water (Schimmel et al., 1983; Briggs,

Figure 1:3 Major pyrethroids used in crop protection.

Structure	Systematic Name	Trivial Name	Trade Name	Isomers
	3-Phenoxybenzyl (1R,1S, cis, trans)-2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylate.	Permethrin	Ambush	4
	(S,R)-α-cyano-3-phenoxybenzyl (1R,1S, cis,trans)-2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylate.	Cypermethrin	Ripcord Fastac	8 2 (1R,cis,αS 1S,cis,αR)
	(S)-α-cyano-3-phenoxybenzyl (1R,cis)-2,2-dimethyl-3-(2,2-dibromovinyl) cyclopropane carboxylate.	Deltamethrin	Decis	1
	(S,R)-α-cyano-3-phenoxybenzyl (S,R)-2-(4-chlorophenyl)-3-methylbutyrate.	Fenvalerate	Sumicidin	4

1984) and on spraying over ponds tend to reside in surface water only (Crossland, 1982). The bio-concentration potential of xenobiotics in aquatic systems has been shown to be directly proportional to their partition coefficient (P-value) between n-octanol and water (Neely et al., 1974). The pyrethroid insecticides have very high P values (log P approximately 6, Schimmel et al., 1983; Briggs, 1984) and therefore have considerable potential for accumulation in the lipid and protein fractions of aquatic life forms. However, lipophilic interactions with soil and plant surfaces ensure that aquatic contamination is minimised, both by the low incidence of leaching (Briggs, 1984) and by extensive binding to aquatic flora and sediment (Sharom and Solomon, 1981; Schimmel et al., 1983).

Though the pyrethroids are far more resistant to photochemical degradation than the natural pyrethrins, several degradative reactions have been observed both on surfaces and in solutions exposed to sunlight (Ruzo et al., 1977). Photo-decomposition may occur by cis - trans isomerisation, dehalogenation, breakage of the diphenyl-ether linkage, or most importantly, by homolysis of the ester linkage and some subsequent decarboxylation. Rates of abiotic degradation of pyrethroids are so low however, as to make them potentially more persistent in the environment than other synthetic insecticides (Casida, 1980).

Fortunately, they are readily metabolised by living organisms to non-toxic products (see metabolism section) and have short biological half-lives. For example, the half-life of permethrin in soil has been calculated as 9 days, compared with 1800 days for DDT (Briggs, 1984). In aquatic systems, pyrethroids are almost entirely bound to sediment (95%), and this enhances their stability (Sharom and Solomon, 1981). Once adsorbed, the cis-isomer is more stable than the trans-isomer and under some conditions (eg: as found in estuaries) pyrethroids may be more persistent than other insecticides (Schimmel et al., 1983).

3.3. Commerical use

The pyrethroids are now an increasingly important class of insecticide. Their uses include crop protection, public health, and domestic pest control. They are formulated as dust, powder, or an emulsifiable concentrate which can be diluted with water. Common methods of spray application are from knapsacks, tractors, aircraft or mist-blowers. The pesticide may therefore be presented to insect pests and non-target organisms in forms which may be inhaled, absorbed through the cuticle or skin, or ingested. Extensive environmental screening has examined these factors and satisfied the regulatory authorities as to the long term safety of pyrethroids.

Though more expensive than the other synthetic

insecticides (pyrethroids >carbamates> organophosphates>organochlorines: Hassal, 1982), application rates in the field are so low as to make the pyrethroids viable alternatives to their competitors. Typical application rates of cypermethrin (RIPCORD*) are 25-75 grams of active ingredient per hectare (Review Tox. 82-002). The use of only the active isomers of pyrethroids, such as the pair of cypermethrin enantiomers comprising FASTAC*, (Figure 1:3), further reduces environmental contamination.

Crop protection applications of pyrethroid insecticides are now widespread, varying from rice to cotton, oilseed rape, and topfruit. Effects on terrestrial non-target arthropods following field applications are far less severe and of shorter duration than those produced by treatment with organophosphates or organochlorines (Inglesfield, 1984). A particularly interesting observation is the repellency effect shown by treated flowers to foraging bees (Shires, 1984). This feature of the pyrethroids protects a beneficial insect species, which is normally extremely sensitive to these insecticides. Effects on aquatic insects are more severe however. Kingsbury and Kreutzweiser (1984) have reported massive increases in the stream drift of dead and paralysed surface-insects

* Registered trademark, Shell Limited.

(e.g. water-boatmen) following aerial applications of permethrin along forest streams. In contrast, more modest 'spray' drift contamination arising from ground-based applications of cypermethrin have been shown to have negligible effects on aquatic organisms (Crossland et al., 1982). Deleterious effects on vertebrates have generally not been observed in the field, with the exception of dead and poisoned fish obtained after direct overspraying of Canadian lakes (Kingsbury and Kreutzweiser, 1984). In addition, when residues of fenvalerate in aquatic organisms, mammals and birds were measured following treatment of cotton fields, concentrations in avian and mammalian species were negligible (<0.02ppm), though higher levels of the insecticide were observed in reptiles and fish present in adjacent draining ditches (Bennett et al., 1983). Environmental problems associated with pyrethroid applications to date have therefore been negligible, compared with the usage of other synthetic insecticides. However, careful consideration should be given to large scale use near some aquatic ecosystems (Schimmell et al., 1983).

3.4. Insecticidal activity

The pyrethroids, in common with the other synthetic insecticides, are potent neurotoxicants, and disrupt the function of the central and peripheral nervous system (see mode of action). Toxic effects of

hyperactivity, ataxia, convulsions and paralysis are observed in insects following oral or topical treatment (Miller and Adams, 1982). Aquatic insects in particular are unusually sensitive to pyrethroids dissolved in the water (Stephenson, 1982). Insecticidal efficacy has been shown to be determined by rapid penetration of the compound to its active site as well as its subsequent activity. The lipophilicity of the pyrethroids is an important feature in controlling cuticle penetration and rate of knockdown (Briggs et al., 1974). Bioavailability in insects has also been shown to be influenced by pharmacokinetic considerations and rates of detoxificiation, which are in turn dependent on structural and conformational factors (Soderlund et al., 1983b; 1983c). Similar considerations influence neuro-activity. Gammon et al., (1981) have demonstrated that only those esters of cyclopropane-carboxylates in the 1R conformation, and isoteric 2S isomers of non-cyclopropane acids are active neurotoxicants and insecticides (see Table 1:2). The substitution of phenoxybenzyl pyrethroids with an α-cyano group in the S conformation futher enhances insecticidal activity, but does not cause a corresponding increase in cercal nerve response(Gammon et al., 1981; see Table 1:2). The most potent pyrethroid insecticides are thus the 1R cis αS isomers of cypermethrin and its dibromo-derivative, deltamethrin.

Despite their insecticidal potency and favourable environmental properties, the pyrethroids may be ultimately restricted in application by the development of insect resistance. This has arisen most notably in Egypt and Denmark, both through the indiscriminate use of these insecticides in pest control, and through insect cross-resistance conferred from previous DDT applications (Miller and Adams, 1982). Such a loss in insecticidal potency (often many hundred-fold), renders the pyrethroids uneconomic in use (Farnham, 1984). A series of elegant genetic experiments have revealed a number of acquired characteristics which confer pyrethroid resistance in insects (Casida, 1980). Identified phenotypes are impaired insecticide penetration, nerve insensitivity or knock down resistance (Kdr), and improved detoxification mediated by the mixed function oxidase system. The first two characteristics are recessive genotypes carried on chromosome three of house flies, while the latter is carried as a dominant gene on chromosome two. Nerve insensitivity seems particularly important and this is also found with DDT resistant insects. Chang and Plapp (1983) have shown impaired binding of pyrethroids and DDT to membrane receptors in brain preparations from resistant flies, and proposed that this demonstrated target organ insensitivity. Other genes have also been implicated in modifying the characteristics

Table 1:2

Pyrethroid structure-activity relationships in the cockroach Periplaneta americana and the mouse

Pyrethroid	Isomeric form	Cockroach [a] Topical LD_{50} $\mu g\ g^{-1}$	Cercal nerve sensitivity (M)	Mouse [b] i.c. LD_{50} $\mu g\ g^{-1}$ brain
Non α-cyano substituted				
Permethrin				
	1R cis	0.09	7×10^{-9}	11
	1R trans	0.6	8×10^{-9}	>860
	1S cis	>500	$>10^{-6}$	
	1S trans	>500	$>10^{-6}$	
α-cyano substituted				
Cypermethrin				
	1R cis αS	0.02	$>10^{-6}$	0.6
	1R trans αS	0.01	$>10^{-6}$	1.6
Deltamethrin				
	1R cis αS	0.01	$>10^{-6}$	1.2
Fenvalerate				
	2S αS	0.01	$>10^{-6}$	1.0

i.c. intracerebral administration

a Gammon et al., 1981

b Lawrence and Casida, 1982

contained on chromosome two and three. Additionally, resistant strains of cotton leafworms have been shown to possess elevated levels of pyrethroid-hydrolysing esterases (Riskallah, 1983).

Although insect tolerance to these compounds is a serious development, an integrated approach to insecticide management should overcome the problems associated with resistance and greatly prolong the commercial life of pyrethroids (Farnham, 1984).

4. Toxicology of pyrethroids in vertebrates

The modes of action of pyrethroids in vertebrates have been comprehensively reviewed by Miller and Adams (1982) and Vijverberg and Van den Bercken (1982). Currently the mammalian toxicology of these compounds is under review by Gray and Soderlund (1985). This account will therefore confine itself to the current ideas on mechanisms of pyrethroid action and summarise the available toxicological data in a number of vertebrate species.

4.1. Structure-activity relationships

In common with insects, the conformation and structure of pyrethroids influences their toxicity in vertebrates; both by controlling routes and rates of detoxification (see metabolism section) and by regulating pharmacokinetics and activities at the target organ (Gray and Soderlund, 1985). As shown in

Table 1:2, the most active pyrethroids in mice are phenoxybenzyl esters containing an α-carbon substituent in the S configuration (Lawrence and Casida, 1982; Staatz et al., 1982a). The most active enantiomers are those in the 1R conformation (cyclopropanecarboxylates) or 2S conformation (non-cyclopropanecarboxylates). In mammals, unlike insects, the trans-isomers of 1R non α-cyano substituted pyrethroids are largely inactive (e.g. permethrin; Table 1:2), indicating a greater target organ stereospecificity in mammals (Gray and Soderlund, 1985). However both the cis- and trans-isomers of α-cyano containing 1R, αS phenoxybenzyl esters have similar neuro-activities (Lawrence and Casida, 1982).

In addition to differences in potency, pyrethroid structure also dictates the type of toxic response observed. When applied to isolated insect nerves, non-cyano pyrethroids induce repetitive nerve firing, while α-cyano phenoxybenzyl esters cause an increased rate in miniature excitatory post-synaptic potentials (Gammon et al., 1981; Saldago et al., 1983). Similarly, toxic signs may be differentiated on this structural basis in insects (Gammon et al., 1981), rats (Verschoyle and Aldridge, 1980), and mice (Lawrence and Casida, 1982; Staatz et al., 1982a). Initial toxic effects in mammals induced by non-cyano containing pyrethroids are increased sensitivity to external stimuli, followed by progressively worsening tremors

(Verschoyle and Aldridge, 1980). On the other hand, α-cyano phenoxybenzyl esters cause hyperactivity, followed by salivation, inco-ordination, whole body writhing (choreoathetosis) and finally clonic and tonic seizures (Barnes and Verschoyle 1974; Ray and Cremer, 1979).

Verschoyle and Aldridge (1980) have termed these two types of poisoning syndromes T (tremor) and CS (choreoathetosis-salivation), respectively. However, to describe toxic effects in non-mammalian species, as well as distinguishing the two types of electrophysiological responses, Lawrence and Casida (1982) have suggested that the type I and type II nomenclature of Gammon et al., (1981) should be adopted. Thus the non-cyano substituted phenoxybenzyl ester permethrin is a type I pyrethroid, while its α-cyano analogue cypermethrin is a type II pyrethroid. Pyrethroids without an α-cyano phenoxybenzyl moiety are almost invariably type I compounds, though some synthetic derivatives such as fenpropathrin, fluororesmethrin, and cyphenothrin are more difficult to classify by this system (Gray and Soderlund, 1985).

4.2. Acute toxicity

The available data on the acute toxicity of the major crop protection pyrethroids in crustacea, fish, mammals and birds are summarised in Table 1:3. Additional toxicity information on the natural

pyrethrins, and the other classes of synthetic pyrethroids is also available in the references listed. These non-insect species are considered in turn (with reference to Table 1:3).

4.2.1. Invertebrates

Crustacea are very susceptible to pyrethroids present in the surrounding water, though the effects of other routes of exposure (eg: oral) have not been examined. By contrast mollusca species such as snails and oysters are unusually tolerant to dissolved pyrethroids (Cole and Casida, 1983) despite very extensive bioaccumulation (Ohkawa et al., 1980a; Schimmel et al., 1983). Acute toxicity data on other non-insect invertebrates are unavailable, but no potential problems have been defined during field studies.

4.2.2. Fish

A wide range of xenobiotics are toxic to fish, with most classes of compound showing a direct correlation between bioconcentration potential and toxicity (Birge and Cassidy, 1983). However the pyrethroids are even more toxic to fish than predicted from such structure-activity relationships (Zitko et al., 1977; 1979) and are extremely poisonous at µg per litre concentrations (Table 1:3). Toxic signs of hyperactivity and loss of equilibrium have been

Table 1:3 Acute toxicity of synthetic pyrethroids (types I & II) to non-insect species..

Species	Ref.	Pyrethrin (I)	Permethrin (I) cis-	Permethrin (I) trans-	Cypermethrin (II) cis-	Cypermethrin (II) trans-	Deltamethrin (II)	Fenvalerate (II)
		Aqueous exposure LC_{50} 24hr ($\mu g\ l^{-1}$)						
Crustacea.								
shrimp	a		0.2		0.01			0.8
lobster	b		0.4	7.0	0.0003	0.04	0.0014	
Fish.								
atlantic salmon.	b,c	32	1.3	8.8	0.7		2.0	
rainbow trout	(d)		25	(135)		(55)		
	e,f,g		12.5	12.5 14	1.1	0.5	1.0	
		LD_{50} 24hr (mg kg^{-1})						
rainbow trout (i.p)	f		22	7				
Amphibia.[a]								
grassfrog (i.p)	h		0.14	7.5	0.16	0.65	0.35	0.13
Mammals.								
mouse (i.p.)	f,i	240	108	> 800	28	> 500	10	500
mouse (p.o.)	i,j		85	3200	76			
rat (p.o.)	j,k	340	> 270	> 270	215	500	20	450
Birds. (p.o.)								
quail/chicken(p.o.)	l,m		> 13500		> 2000			> 1600
duck	m,n		> 4640		> 10000		> 4000	

Footnotes to Table 1:3

LC_{50} - Concentration in the water giving 50% lethality.

LD_{50} - Dose causing 50% lethality.

Figures represent mean values of reported ranges using analytical grade material. Underlined figures refer to use of only those cis- and trans-isomers in the 1R conformation.

i.p. intraperitoneal administration in hydroxylated vegetable oil (emulphor) or methoxytriglycol.

p.o. oral administration in corn oil, dimethyl-sulphoxide or glycerol formal.

References

a. Schimmel et al., 1983

b. Zitko et al., 1979

c. Zitko et al., 1977

d. Coats and O'Donnell-Jeffrey, 1979

e. Mulla et al., 1978

f. Glickman et al., 1981a

g. Stephenson, 1982

h. Cole and Casida, 1983

i. Soderlund and Casida, 1977

j. Hutson, personal communication

k. Verschoyle and Barnes, 1972

l. Bradbury and Coats, 1982

m. Review Series Tox. 82-002, 1982

n. Elliott et al., 1978.

a In a study with bullfrog tadpoles, Jolly et al., (1978) reported a 96h LC_{50} of permethrin = 7,0300 $\mu g l^{-1}$

reported by Glickman et al., (1981) in rainbow trout following exposure to permethrin. Such toxic effects are freely reversible if fish are only briefly exposed to contaminated water (Francois et al., 1982). This exceptional sensitivity may be partly explained by the uptake of pyrethroids across the gills, thus gaining direct access to the blood supply and hence to the sites of toxic action. However, Glickman et al., (1981a) have demonstrated that cis- and trans— permethrin are five times and over one hundred times respectively, more toxic to trout than mice when dosed intraperitoneally (i.p.). Unlike mammals, fish are sensitive to the trans— isomers of type I pyrethroids (Miyamoto, 1976). Furthermore, Glickman et al., (1981a) have reported that trans— permethrin is more toxic to trout than the cis-isomer.

Apart from pyrethroid structure, several factors have also been shown to influence pyrethroid acute toxicity in fish. Coats and O'Donnell-Jeffrey (1979) have shown that formulated pyrethroids are more toxic to trout than the analytical material. Different fish species show varying susceptibilities to dissolved pyrethroids, the Salmonids being particularly sensitive (Mulla et al., 1978; Stephenson, 1982). Toxicity is also increased by reducing the water temperature (Kumaraguru and Beamish, 1981), but is decreased by increasing the water particulate content (Review TOX. 82-002) and by using larger and older fish (Mauck et

al., 1976; Kumaraguru and Beamish, 1981).

4.2.3. Amphibians

In a comparative study, Jolly et al., (1978) reported that bullfrog tadpoles were far less sensitive to dissolved permethrin than crayfish, catfish, bass and mosquitofish. Similarly, Cole and Casida (1983) have observed that grassfrogs are less sensitive to a range of dissolved pyrethroids than goldfish. Tadpoles and frogs are equally susceptible to pyrethroids, the toxicity increasing at lower temperatures. With all the compounds tested the cis-isomers are more toxic than the trans-isomers. Type I pyrethroids induce toxic signs of hyperactivity and tremors, while type II derivatives cause tonic seizures and choreoathetosis.

4.2.4. Mammals

The low toxicity of pyrethroids to mammals, illustrated in Table 1:3 for mice and rats, is also seen in rabbits (Hutson, 1979) and dogs, sheep and cattle (James, 1980). Acute toxicity is dependent on species, the sex of the animal, and the vehicle used for dosing (Gray and Soderlund, 1985). White et al., (1976) have also shown that lowering the ambient temperature increased the oral toxicity of cismethrin to rats, though this was not due to increased target organ sensitivity. When pyrethroids are administered by routes which give direct access to the animal's

blood supply, such as intravenous injection or inhalation, they are far more toxic than by oral or intraperitoneal exposure (Gray and Soderlund, 1985). Dermal absorption however, does not present an acute toxicological hazard (James, 1980; Kavlock et al., 1979).

Comparable toxic signs to those previously described for rats (see structure-activity section), are seen in other mammalian species following exposure to type I and type II pyrethroids. In man, transient facial effects of paresthesia have been reported after exposure to synthetic α-cyano substituted pyrethroids (Le Quesne et al., 1980) though no long term effects have been noted. However, accidental oral ingestion of a large quantity of cypermethrin has resulted in a human fatality (Poulos et al., 1982).

4.2.5. Birds

Natural pyrethrum extracts have very low toxicities to sparrows and pigeons (>50mg kg^{-1}), though toxic signs of hyperactivity were observed (Saxena and Saxena, 1973; Saxena and Bakre, 1976). Similarly, the oral toxicities of the major synthetic pyrethroids are very low in mature birds (Table 1:3), though young quail have been shown to be more suceptible to fenvalerate than adults (Bradbury and Coats, 1982). Miyamoto (1976) has also reported that synthetic pyrethroids are essentially non-toxic to lovebirds

(LD_{50} 1R *trans*-allethrim 1600mg kg^{-1} (male), 840mg kg^{-1} (female)). Toxic signs of hyperactivity, irregular locomotion, ataxia, seizures and paralysis have been observed in bobwhite quail treated with fenvalerate (Bradbury and Coats, 1982) or deltamethrin (David, 1982). Also a significant loss in body weight was observed during acute studies.

4.3. Toxicokinetics

A limited amount of data are now available on the mammalian pharmacokinetics of pyrethroids dosed orally and intravenously (i.v.) (see Gray and Soderlund, 1985). The *cis*- and *trans*-isomers of resmethrin, (i.e. cismethrin and bioresmethrin respectively), have slow rates of intestinal uptake when administered at toxic oral doses to rats (White *et al.*, 1976). This slow absorption accounts for the reduced toxicity of pyrethroids when dosed orally compared to i.v., though the rates of intestinal uptake are dependent on the vehicle used (Gray and Soderlund, 1985). When resmethrin was dosed to rats i.v., it was rapidly cleared from the circulation, with a corresponding increase in the concentrations of hydrolysed metabolites being observed in the blood (White *et al.*, 1976; Gray *et al.*, 1980). Products of pyrethroid ester-cleavage derived from the alcohol moiety have been shown to be non-toxic to mice when administered i.p. (Casida *et al.*, 1979), and to fish when dissolved

in the water (Zitko et al., 1977). However, there is some evidence to suggest that the cyclopropane acid in the 1R conformation is toxic to mice (LD_{50} values for [1R,t] Cl_2CA and [1R,c] Cl_2CA are respectively 210 and 370mg kg^{-1}, i.p. (Gaughan et al.; 1977)).

Accompanying the rapid turnover of resmethrin isomers in i.v. dosed rats, there is an accumulation of parent compound and metabolites in all tissues, with peak concentrations in the brain occuring 1-2.5 minutes after administration (Gray et al., 1980). White et al., (1976) demonstrated that the onset of toxic signs would then only occur above a critical concentration of the parent cis-isomer, cismethrin in the brain. The trans-isomer, bioresmethrin, had negligible neurotoxic activity, the observed toxic effects being due to 5% contamination with the cis-isomer (Gray et al., 1980). Ruzo et al., (1979) have similarly shown that a threshold concentration of deltamethrin in the brain is required before toxic signs are shown in the mouse.

Toxicokinetic studies in rats dosed i.v. with deltamethrin have shown that the pyrethroid and its metabolites are rapidly distributed throughout the body (Gray and Rickard, 1981; 1982a). Half-lives of the parent deltamethrin were greater in the blood and liver than was the case with cismethrin. In these studies, pyrethroid concentrations in the brain were lower than those found with the resmethrin isomers and did not correlate well with toxic signs. Instead, an

unidentified,non-pentane extractable component was suggested as being the active neurotoxicant (Gray and Rickard, 1982a). The low levels of deltamethrin in the CNS may be partly due to the impaired penetration of cyano substituted pyrethroids into the brains of rats (Marei et al., 1982). Such blood-brain barrier considerations may help explain the poor correlation between concentrations of deltamethrin in the blood, and the severity of observed toxic signs (Gray and Rickard, 1981; 1982a). In some cases, delayed toxicity has been observed with i.v. injected pyrethroids due to precipitation of the compound in the lungs of treated animals (Gray and Connors, 1980).

Toxicokinetic studies have also been carried out in rainbow trout with the cis- and trans— isomers of permethrin dissolved in water (Glickman et al., 1981a) and with trans-permethrin dosed i.v. and i.p. (Glickman and Lech, 1982). Exposure to [^{14}C] permethrin dissolved in aquarium water over a 24 hour period, resulted in radioactive residues steadily increasing in blood, muscle, liver and fat with bioaccumulation factors of 30, 30, 300 and 400 respectively. The tissue potential for bioconcentration of pyrethroids in fish has been shown to be directly proportional to lipid content (Bennett, 1984). When trans-permethrin was administered i.v. to trout and mice, the fish cleared the pyrethroid and its metabolites from the blood at a slower rate than the mammal. Similarly, the

accumulation of intact trans-permethrin in the brains of trout was slower than in mice, as was the development of acute toxic signs. Glickman and Lech (1982) also demonstrated that the severity of toxic signs was dependent on the concentrations of parent pyrethroid in the brains of both species, the trout having a greater target organ sensitivity.

No other toxicokinetic studies with pyrethroids have been reported in other vertebrate species, though such investigations have been carried out in insects (Soderlund et al., 1983b). However, Bradbury and Coats, (1982) have observed that specific levels of fenvalerate were required in the brains of immature quail before any fatalities were recorded. Concentrations of the pyrethroid in the CNS associated with mortality were lower than those determined in similar acute toxicity studies with DDT (Hill et al., 1971) and endrin (Ludke, 1976); even though these organochlorine insecticides are more toxic to birds than pyrethroids (Hassal, 1982).

4.4. Sites of action

The pyrethroids have a complex mode of action in insects and mammals, and affect both the central and peripheral nervous systems (Vijverberg and Van den Bercken, 1982; Miller and Adams, 1982). Injections of very small quantities of pyrethroids (µg amounts) directly into the CNS of rats (Gray and Rickard, 1982b)

and mice (Lawrence and Casida, 1982; Staatz et al., 1982a), induce similar toxic effects to those observed after peripheral administration. This suggests a largely central site of action in mammals. Additionally, the quantities of i.c. administered pyrethroids required to produce toxic signs are similar to those observed in the brain following oral or i.p. dosing. When administered i.v., deltamethrin was uniformly distributed throughout the brain (Gray and Rickard, 1981). However, in an i.c. dosing study, Gray and Rickard (1982b) have suggested that the brain stem and cerebellum are the critical sites of action in the CNS. Electro-physiological experiments in rats undergoing deltamethrin induced choreoathetosis further suggests that the primary action of type II pyrethoids is on the extra-pyramidal motor system (Ray, 1980).

An activity at peripheral target organs in mammals should not be discounted however, as i.c. administration of type II pyrethroids failed to induce the salivation response induced by i.v or oral dosing (Gray and Rickard, 1982b; Staatz et al., 1982a). Gray and Soderlund, (1985) have also suggested that an action on the cardiovascular and respiratory systems may contribute to the actue toxicity of type II pyrethroids. Cardiac output, blood pressure and respiration have all been shown to alter following i.v administration of deltamethrin (Ray and Cremer, 1979; Forshaw and Bradbury, 1983). Some of these effects are

due to elevated catecholamine levels in the blood (Cremer and Seville, 1982), though deltamethrin has been shown to have a direct action on isolated hearts (Forshaw and Bradbury, 1983). Type I pyrethroids have no activity on the cardiovascular system (Forshaw and Bradbury, 1983), though they do invoke physiological responses associated with increased exercise e.g. increased body temperature and respiration rate (White et al., 1976; Cremer and Seville, 1982).

4.5. Mechanisms of action

The precise mode of action of pyrethroids in the nervous systems of insects and mammals remains unclear, though it now appears that this may be due to their multiple activities. Two schools of thought currently exist as to the mechanisms of pyrethroid action, namely; those who propose that there is an action solely on the excitable membranes of neurones (i.e. pre-synaptically; Vijverberg and Van den Bercken, 1982) and those who suggest that interactions occur principally with receptor-ionophores on the post-synaptic membrane (see: Lawrence and Casida, 1983; Staatz et al., 1982a; 1982b). The available evidence for these two proposals is considered in turn.

4.5.1. Effects on ion channels

In common with DDT, pyrethroids inhibit the closing of sodium gating channels that open during

depolarisation, such that the sodium tail current persists after excitable membranes have been repolarised. Voltage-clamp experiments with Xenopus nodal membranes have shown this to be due to stereospecific interactions between the pyrethroid and the activation gate of the sodium channel (Vijverberg et al., 1982). Similarly, Jacques et al., (1980) demonstrated that the action of pyrethroids is antagonized by tetrodotoxin, a compound known to selectively "plug" open sodium channels. Such a membrane-mediated mode of action adaquately explains the inverse temperature dependence observed with pyrethroid-receptor binding and neurotoxicant activity in insects (Chang and Plapp, 1983) and poikilothermic vertebrates (Cole and Casida, 1983). Similar reversible, temperature dependent electro-physiological responses are observed with DDT (Vijverberg and Van den Bercken, 1982). Kinetic considerations of pyrethroid-sodium channel interactions can also explain the observed differences in nerve electro-physiological response to type I and type II pyrethroids (Vijverberg et al., 1983; Lund and Narahashi, 1983). Type I pyrethroids retain sodium channels in the open conformation only transiently (msec) and induce large depolarising after-potentials with minimal variation in resting-potential. This causes the nerve to fire repetitively, an activity not invoked by type II compounds, which cause small but long-lasting depolarising after-potentials in isolated

frog nerves (Vijverberg and Van den Bercken, 1979). This difference is caused by the longer interaction of type II pyrethroids with the sodium channel in the open position (100 msec) (Lund and Narahashi, 1983). Apart from the action on the sodium channels of neurones, pyrethroids have also been found to affect ion transport in the acetylcholine receptor-complex of Torpedo electric organs (Abbassy et al., 1983: see Table 1:4) and sodium uptake by frog skin (Salibián, 1983).

In addition to pyrethroid structure and conformation, the electro-physiological sensitivities of vertebrate nerves are dependent on their neural function. In particular, the ends of afferent and efferent nerves and sensory organs, are very sensitive to pyrethroids in vitro (Vijverberg an Van den Bercken, 1982). Similarly EEG studies with rats (Ray, 1980), rabbits (Carlton, 1977) and rainbow trout (Glickman et al., 1981b), have all shown abnormal electrical activity associated with tactile or auditory stimulation in the early stages of acute toxicity. Since these EEG patterns are associated with hypersensitivity and excitability, a direct correlation between some of the observed toxic signs and sodium channel modification can be established. However in insects, Salgado et al., (1983) have show that although repetitive nerve firing induced by type I pyrethroids is correlated positively with knockdown activity, there

is no such relationship as regards lethality.

4.5.2. Effects on neurotransmitter receptor-complexes

In order to explain some of the aspects of pyrethroid activity in the CNS, a number of drugs and toxins have been used in neuropharmacological and ligand-binding studies. The results summarised in Table 4, clearly demonstrate that many compounds which are active in the vertebrate CNS can modify the toxicology of pyrethroids. Some drugs such as atropine can only suppress some of the peripheral cholinergic responses of pyrethroid toxicity such as salivation (Ray and Cremer, 1979). Others, such as mephenesin and diazepam, can afford total protection against toxic effects and have been proposed as antidotes to pyrethroid poisoning (Bradbury et al., 1981; Gray and Soderlund, 1985). To date, work on receptor interactions has largely concentrated on the L-glutamate (Staatz et al., 1982b) and Y-aminobutyric acid (GABA) (Leeb-Lundberg and Olsen, 1980; Lawrence and Casida, 1983), neurotransmitter systems. This has been due to the synergistic or protective effects observed in vivo with those drugs active on these two systems, or the similarity in observed toxic signs shown by known toxins (see Table 1:4).

Staatz et al., (1982b) have demonstrated that the structural analogue of L-glutamate, kainic acid (KA), is displaced from mouse brain-membrane receptors by

Table 1:4

Pharmacological and receptor-binding studies with pyrethroids in vertebrates

Neurotrasmitter system studied	Drug /toxin	Type of pyrethroid	Effects of drug on pyrethroid toxicity	Effects of pyrethroid on drug-receptor binding	Species used and reference
Peripheral nervous system					
Acetylcholine receptor - channel-histrionicotoxin	Acetylcholine	I/II	n.d.	no effect	Torpedo a
		I	n.d.	reduced (fast)	
		II	n.d.	reduced (slow)	
	Atropine	II	reduced (no salivation	n.d.	Rat, j
Central nervous system					
Acetylcholine, Dopamine, Norephinephrine, Serotonin	) variety of receptor blockers		potentiated or no effect (drug dependent)	n.d.	mouse b
GABA	GABA	II	n.d.	no effect	rat, c
	Picrotoxinin	II	analogous toxic effects	ligand displacement	mouse, d
	butylbicyclophosphorothionate	II	analogousn toxic effects	(1R isomers only)	
	Diazepam	I	no effect	n.d.	mouse, f
		II	reduced	n.d.	frog, g
	Phenobarbital	I/II	slightly reduced	n.d.	mouse, f
L-glutamate	Kainic acid	I		displacement (low affinity)	mouse, h
		II	analogous toxic effects	diplacement (high affinity)	
Mephenesin		II	reduced	n.d.	rat, i
Cycloheximide		I	reduced	n.d.	mouse, b

pyrethroids in a dose-dependent way. Also, the potency of ligand displacement follows the order deltamethrin> cis-permethrin>trans-permethrin, which is identical to the observed i.c. toxicity of these compounds (Staatz et al., 1982a). A difficulty arising from these studies, has been the poorly defined activity of KA in modifying glutamate neurotransmitter systems. Interest in this mechanism of activity has been generated as a chemical model for the disease Huntington's chorea (Ray and Cremer, 1979; Staatz et al., 1982b).

Rather more favoured at present, are the interactions of pyrethroids with the GABA-receptor-ionophore complex. Using the ligands picrotoxinin and t-butyl-bicyclophosphorothionate, Leeb-Lundberg and Olson (1980), and Lawrence and Casida (1983) have demonstrated that ligand displacement by pyrethroids is dependent upon the structure and conformation of the insecticide. Type II pyrethroids are more potent in displacing toxins than type I compounds and only show activity in their insecticidally active 1R, αS conformation. The cis-isomers are more active than the trans-isomers, and in all cases pyrethroid binding activity shows an absolute correlation with i.c. toxicity. Whether such ligand interactions are with the receptor, or with the ion-channel remains unclear, though Abbassy et al., (1983) have shown that these insecticides only bind to the ionophore component of the acetylcholine receptor complex. In addition,

Cremer et al., (1980) did not observe any reduced binding of GABA to rat brain synaptosomes following deltamethrin treatment. In the context of the known interactions of pyrethroids with ion channels, these observations suggest that these compounds bind specifically to the ionophore component of receptor complexes. Recently stereospecific pyrethroid-receptor binding has also been reported in mouse brain membranes (Soderlund et al., 1983a) though its toxicological significance is unclear.

4.5.3. Other neurochemical/biochemical effects

A number of other biochemical changes have also been observed in the CNS, principally in the cerebellum, which appears to be the centre of pyrethroid action (see Table 1:5). This is reflected by the increase in cerebellar blood flow and glucose utilisation, which presumably support increased neuronal activity (Gray and Soderlund, 1985). Changes in the energy utilisation of the brain are accompanied by a general increase in the concentrations of glucose and lactose in the blood during acute toxicity (Ray and Cremer, 1979; Cremer and Seville, 1982).

During pyrethroid-induced acute toxicity, there are variations in the concentrations of intracellular and extracellular neurotransmitters in the cerebellum; namely, a lowering in acetylcholine content, and an increase in cyclic guanosine mono-phosphate (cGMP)

(Aldridge et al., 1978; Lock and Berry, 1981). Brodie and Aldridge (1982) have shown that such increases in cGMP are time dependent and they have suggested that such variations reflect the increased motor activity, several other convulsants producing similar effects. Such biochemical changes appear to be the secondary effects of modified neuronal activity and not primary toxic events (Gray and Soderund, 1985). An exception to this may be the pyrethroid-induced release of hormones in the rat neurohypophysis, which is mediated by a direct action on membrane ion-channels (Dyball, 1982).

4.6. Other toxic effects

Currently there is no evidence to suggest that pyrethrins or pyrethroids are potential mutagens, carcinogens, or teratogens in mammals (Miyamoto, 1976; Gray and Soderlund, 1985). Thus, this brief account will concern itself with those physiological and biochemical effects which have been observed in fish, mammals and birds, even though their toxicological significance is uncertain in some cases.

4.6.1. Fish

Following exposure to dissolved permethrin, rainbow trout undergo histopathological changes to their gills and show an increased basal metabolic rate (Kumaraguru et al., 1982; Kumaraguru and Beamish, 1983). The

Table 1:5

Biochemical changes in nervous tissue associated with pyrethroid acute toxicity

Tissue Studied	Biochemical Parameter	Effect of Pyrethroid Administration	Reference
Rat Cerebellum	Blood flow	regional increases	a, b, d
	Glucuose utilisation	increased	a, b, d
	[Glucose]	transient increase only	e, f
	[Lactate]	increased	e, f
	[GABA]	slight increase	a, e
	[Glutamate]	no effect	a, e
	[Acetylcholine]	reduced	g
	[ATP]	no effect	e, f
	[cAMP]	no effect	e, f, g
	[cGMP]	large increase	e, f, g, h
Squid Nerve	Ca+Mg-ATPase	inhibition	i
Rat Neurohypophysis	Hormone release	inhibition	j

For references see footnote to Table 1:5.

[ ] - figures in parenthesis refer to concentration per tissue weight

Pyrethroids tested Cismethrin, b, g
Cypermethrin, d
Deltamethrin, a, b, c, d, e, f

Footnotes to Table 1:4

n.d.	not determined
G.A.B.A.	Y-aminobutyric acid
References	a Abbassy et al., 1983
	b Staatz et al., 1982a
	c Cremer et al., 1980
	d Leeb-Lundberg and Olsen, 1980
	e Lawrence and Casida, 1983
	f Gammon et al., 1982
	g Cole and Casida, 1983
	h Staatz et al., 1982b
	i Bradbury et al., 1981
	j Ray and Cremer, 1979

Footnotes to Table 1:5

a, b	Cremer et al., 1980, ;1983
c	Cremer and Ray, 1983
d	Lock and Berry, 1981
e	Aldridge et al., 1978
f	Brodie and Aldridge, 1982
g	Marshall-Clark and Matsumura, 1982
h	Dyball, 1982

increase in respiration during the acclimatisation period is due to physiological stress, detoxification and tissue repair, and leads to a reduction in swimming activity. No other sub-acute toxicological studies have been conducted with pyrethroids in fish, due to their very high acute toxicity.

4.6.2. Mammals

Sub-lethal doses of pyrethroids can cause lesions to the sciatic nerves of rats (Gray and Soderlund, 1985), though Le Quesne *et al.*, (1980) have reported no impairment of nerve function in workers occupationally exposed to cypermethrin. Short term feeding studies with large doses of pyrethroids result in liver enlargement, largely through an increase in hepatic lipid content (Springfield *et al.*, 1973). Additionally, such exposure can lead to a transient selective induction of liver mixed function oxidases (mfo's), depending on the pyrethroid (Litchfield, 1983).

Other effects on hepatic biochemistry, such as protein binding, alterations in mitochondrial activity and increases in ATPases and Kynurenine aminotransferases/hydrolases have also been reported (see Gray and Soderlund, 1985). The toxicological hazard of such biochemical changes remains obscure, though long term feeding studies with pyrethroids (Parker *et al.*, 1983) suggest that they are of

negligible pathogenic significance.

4.6.3. Birds

Unlike the organophosphates, no neuropathic effects on peripheral nerves were observed in chickens following exposure to toxic doses of pyrethroids (Gray and Soderlund, 1985). David (1980) has reported that during sub-acute feeding trials, formulated deltamethrin exhibited a repellant effect on quail, which were averse to eating treated food. Ingestion of the technical material led to behavioural abnormalities, though no changes in weight were observed compared to controls. In a later study, David (1982) demonstrated that deltamethrin lowered the embryogenic germ potential of the genital tract of adult and hatchling female quail following oral exposure, or treatment of eggs. Similarly, Lutz-Ostertag and Lutz (1974) have reported that pyrethrins can reduce fertility in birds.

No other toxic responses have been reported in avian species: cypermethrin and deltamethrin having failed to induce oxidases in quail liver or intestine (Riviere et al., 1983).

5. Metabolism of pyrethroids

Synthetic pyrethroids have a complex metabolic fate in living systems, the subject being most recently reviewed in general by Chambers (1980), in mammals by

Hutson (1979), in insects by Soderlund et al., (1983c)and in soil and plants by Roberts (1981). This account will concentrate on the metabolism of the major crop protection pyrethroids, especially in those species which have not been so comprehensively reviewed. Also by using the pyrethroids as a chemical model, many of the features of xenobiotic metabolism may be illustrated.

5.1. Role of oxidation

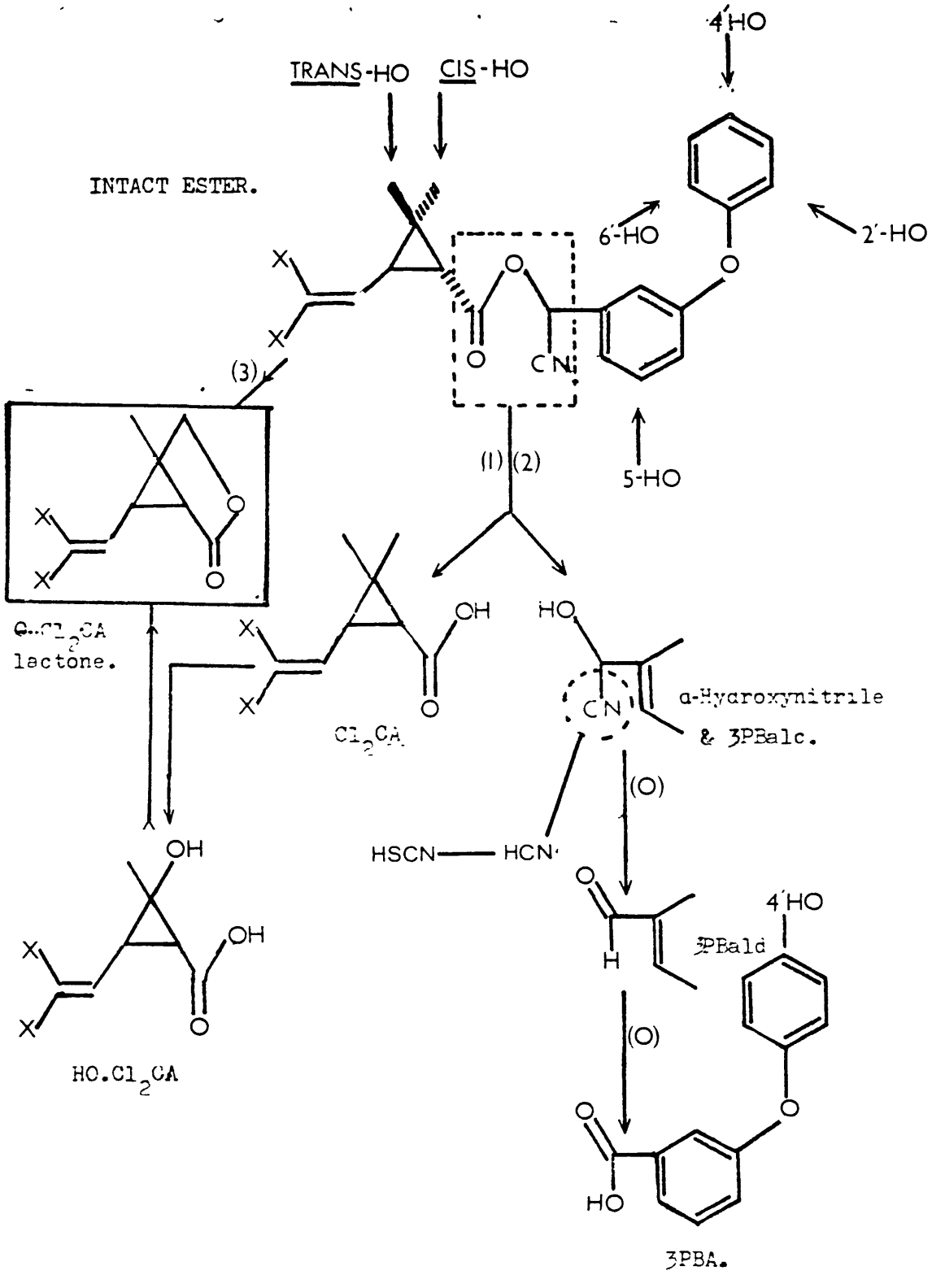
The mechanisms of pyrethroid metabolism have been investigated largely using washed liver microsomes in conjunction with specific cofactors and inhibitors (Soderlund and Casida, 1977). Such systems give qualitatively similar phase I metabolites to those observed in whole animal studies (Casida et al., 1979).

The oxidation of pyrethroids is carried out by microsomal mfo's. The cytochrome P_{450} mediated enzyme system is particularly important, with type I difference spectra being obtained on substrate binding (Kulkarni et al., 1975). The cis-isomers of pyrethroids are more readily acted on by such systems than the trans-isomers, in preparations derived from insects (Shono et al., 1979), mammals (Soderlund and Casida, 1977; Shono et al., 1979), and fish (Glickman et al., 1979). The sites of oxidative attack in the aromatic rings of the phenoxybenzyl substituent (2',4',6', and 5 positions) and the methyl groups of

the cyclopropane ring (cis-(c) or trans-(t)to the carbonyl group) are illustrated in Figure 1:4. In general the 4' position of the phenyl ring, and the cyclopropyl methyl group in the trans-conformation are the preferred sites of hydroxylation. However, the site specificity for oxidation is dependent upon the structure and conformation (cis/trans) of the pyrethroid and the species under study. This is demonstrated with the isomers of permethrin in Table 1:6. Oxidation of α-cyano substituted phenoxybenzoyl pyrethroids is qualitatively very similar to that of the corresponding non-cyano containing isomers, though overall reaction rates are lower (Soderlund and Casida, 1977), and some hydroxylation at the 5 position of the benzyl ring is seen in mice (Shono et al., 1979). Further oxidation of the hydroxy-methyl derivatives may occur to yield the aldehyde and carboxylic acid (Shono et al., 1979), or alternatively lactonisation may occur (Soderlund and Casida, 1977) (Figure 1:4). Hutson (1979) has suggested that the formation of the lactone of the acid substituent of the intact pyrethroid would lead to ester cleavage and this is indicated in Figure 1:4. This may account for the appreciable quantities of the lactone of the dichlorovinyl-dimethyl-cyclopropanecarboxylic acid formed from permethrin in mice (Shono et al., 1979), cows (Gaughan et al., 1978a), and goats (Ivie et al., 1980). Hutson (1979) has also suggested that oxidation of the

Figure 1:4

Phase I reactions of cypermethrin (X= Cl),and deltamethrin (X= Br).



Mechanisms of ester cleavage. (1).Hydrolysis by esterases.
(2).Oxidation of α-carbon.
(3).Lactonization.

Table 1:6

Major phase I reactions of permethrin isomers in insects, plants, fish, mammals and birds

Species	Reference	Ester hydrolysis	Sites of oxidative attack				
			Acid moiety		Alcohol moiety		
			c-me	t-me	2'	4'	6'
cis-permethrin							
Cabbage Looper	a	+++		++		+	
Snapbean	b	++++			+	+	
Rainbow trout	c					++++++	
Carp	c	+		+		++++	
Mouse	a	++	+	++	~	+	~
Rat	a,d	++	~~	++	~	++	
Goat	e	+++		++		+	
Cow	f	+++		++		+	
Hen	g	++++		+		+	
trans-permethrin							
Cabbage looper	a	++++++					
Snapbean	b	++++++					
Rainbow trout	c	++				++++	
Carp	c	+++++	~			+	
Mouse	a	++++	+	+			
Rat	a,d	++++		+		+	
Goat	e	++++++					
Cow	f	+++		+++			
Hen	g	++++++					

\+ - refers to proportions of metabolised material recovered in that form, (total of 6 per line). ~ significant reaction.

a Shono et al., 1979
b Roberts, 1981
c Glickman et al., 1979
d Gaughan et al., 1977
e Ivie et al., 1980
f Gaughan et al., 1978a
g Gaughan et al., 1978b

phenoxybenzyl moiety at the α-carbon of the ester linkage may also catalyse ester cleavage (see Chapter 6). Such a mechanism may be particularly relevant to the metabolism of the cis-isomers of pyrethroids (Ueda et al., 1975; Crawford and Hutson, 1977), and may lead to binding to macromolecules through the formation of acylating intermediates (Hutson et al., 1981).

The importance of oxidative processes in controlling the acute toxicity of pyrethroids has been examined by pre-treatment of animals with inhibitors of mfo activity, eg: piperonyl butoxide (PB). The enhancement, or synergism, of toxic activity using such compounds is very high in insects and is utilised commercially (Casida, 1980). In mice, though PB was found to alter the metabolite profile of deltamethrin in the excreta, synergism was negligible (Ruzo et al., 1979). Rather more unusually, inhibition of the mfo's of rainbow trout potentiated the acute toxicity of trans-permethrin (Glickman et al., 1982). This is due to an unusually high dependence on trout liver oxidases for detoxification of trans-permethrin, which is a consequence of the low levels of hepatic hydrolases present (Table 1:6). Similarly PB also increased the toxicity of trans-permethrin and trans-resmethrin to frogs, though synergism was more pronounced with the respective cis-isomers (Cole and Casida, 1983).

In order to help predict species variations in hepatic oxidase activities, Gregus et al., (1983) have

compared the oxidation of a number of substrates in trout and quail with that of mammals commonly used in toxicity testing. Though possessing lower levels of cytochrome P_{450} than the mammalian species, trout and quail showed considerable variation in the specific activities of a number of oxidase functions. For example, the activities of ethoxy-resorfuin O-de-ethylase in the fish, and benzo-a-pyrene hydroxylase activity in the bird, exceeded that of many of the mammals. However with other substrates, oxidase activity in the non-mammalian species was very low. The possible role of extra-hepatic mfo in pyrethroid detoxification is at present unknown, even though a number of tissues possess such activity in mammals (Burke and Orrenius, 1982), and birds (Riviere and Bach, 1979).

5.2. Role of esterases

Hydrolysis of permethrin yields the dichlorovinyl-dimethyl-cyclopropanecarboxylic acid (Cl_2CA) and 3-phenoxybenzyl alcohol (3PBalc) (see Figure 1:4). Similarly, cypermethrin and deltamethrin yield the acid and the α-hydroxynitrile derivative of the alcohol. The cyanide ion is then liberated and eliminated as thiocyanate, or in the case of deltamethrin dosed rats, as 2-iminothiazolidine-4-carboxylic acid (Hutson, 1979). No adverse toxicological effects have been observed due to this release of cyanide (Ohkawa et al., 1979). In

the case of the α-cyano-phenoxybenzyl esters, the alcohol moiety is more commonly recovered as its oxidised derivatives 3-phenoxybenzyl aldehyde (3-PBald), or 3-phenoxybenzoic acid (3-PBA). Clearly, hydrolysis may also occur after hydroxylation of the intact ester, the combination of these two reactions yielding hydroxylated alcohol and acid derivatives. Alternatively, Cl_2CA and 3PBA produced by hydrolysis alone may subsequently serve as substrates for mfo's as has been shown to be the case with 3PBA in a number of species (Huckle et al., 1981c).

Just as pyrethroids in the cis- conformation are preferentially metabolised by oxidation, then the trans-isomers are better substrates for liver microsomal esterases (Abernathy et al., 1973; Soderlund and Casida, 1977). This is demonstrated in various species, both in vivo and in vitro, by the extensive formation of hydrolysed products of trans-permethrin (Table 1:6). In addition to these conformational restraints on pyrethroid hydrolysis, α-substitution of the alcohol moiety can reduce esterase activity, such that the α-cyano-3-phenoxybenzyl ester cypermethrin is less readily cleaved than its non-cyano analogue permethrin (Soderlund and Casida, 1977; Shono et al., 1979). Such structure-activity relationships may not apply universally to all species however. Thus, rat plasma esterases possess no cis-/trans-isomer specificity with resmethrin (White et al., 1976; Gray

et al.,1980). Similarly, rabbit liver microsomes are highly active at hydrolysing cis—pyrethroids relative to their trans- isomers (Crawford and Hutson, 1977), as are human hepatic esterases (Croucher and Logan, 1982).

Species variations also exist in the total activities of pyrethroid-hydrolysing esterases found in the liver. Thus in a comparative study using post-mitochondrial supernatants, Miyamoto et al., (1974) determined trans—phenothrin hydrolase activities in the ratio:- guinea pig (5):rabbit (3):dog (1.3):rat (1). However, these differences in activity were minor compared to those obtained with rainbow trout and mice using trans-permethrin as a substrate (Glickman and Lech, 1981; 1982). In these studies, mouse liver incubates at 37°C were 166 times more active in hydrolysing trans-permethrin, than similarly prepared trout tissues at 12°C (typical environmental temperature). Even when compensating for temperature differences, the mammalian tissues were far more active than those of the fish, though interestingly frog liver esterases have been found to be equally active to those of mice at 30°C (Cole and Casida, 1983).

Subcellular studies with liver showed that these pyrethroid hydrolases were associated with the microsomal fraction and that they were susceptible to inhibition by organophosphate insecticides such as S,S,S-tributyl phosphorotrithioate (DEF) or tri-o-tolyl phosphate (TOTP), both in vitro (Abernathy et al.,

1973; Glickman and Lech; 1982), and *in vivo* (Ruzo *et al.*, 1979). Similarly, inhibition of pyrethroid esterases has also been observed with a number of carbamate and organophosphate insecticides in insect species (Ishaaya and Casida, 1980; Chang and Jordan, 1983; Riskallah, 1983). Using such inhibitors selectively, Soderlund *et al.*, (1982) demonstrated that several mammalian microsomal esterases were active in hydrolysing permethrin isomers. Paraoxon completely inhibited hydrolysis of the *trans*-isomer, but only partially affected that of *cis*-permethrin. Similarly, separate inhibition curves were obtained for the two isomers when the esterases were treated with α-napthyl N-propylcarbamate. In a previous study, a carboxylesterase had been isolated from rat liver microsomes which showed many of the features of pyrethroid hydrolases (ie: inhibition by carbamates and organophosphates and greater activity towards pyrethroids in the *trans*-conformation rather than the *cis*- Suzuki and Miyamoto, 1978). The isolated enzyme (EC 3.1.1.1.) had a molecular weight of 74,000 and a pH optima 7-9. It also actively hydrolysed malathion and p-nitrophenylacetate, and showed many similar properties to a carboxylesterase recently isolated from cattle ticks (Riddles *et al.*, 1983).

The possible role of extra-hepatic esterases in hydrolysing pyrethroids remains unclear, even though tissues such as plasma, kidney and the intestinal tract

show appreciable hydrolytic activity towards a variety of esters, including several insecticides (Heymann, 1982). In a detailed study with trans-permethrin, Glickman and Lech (1981) compared the hydrolytic biotransformation potential of kidney and plasma preparations in mouse and trout. In all cases, the activity of the mouse tissues exceeded that of the trout, irrespective of temperature. Interestingly, trout plasma was more active in hydrolysing trans-permethrin than the liver. Similarly, the plasma of mice (Ruzo et al., 1979) and rats (White et al., 1976; Gray et al., 1980; Gray and Rickard, 1982a) contain esterases which hydrolyse resmethrin and deltamethrin. No such activity has been detected in the plasma of humans (Abernathy et al., 1973) or frogs (Cole and Casida, 1983).

Apart from plasma, limited deltamethrin hydrolase activity has been determined in mouse stomach, kidney and brain (Ruzo, et al., 1979). In all the tissues except stomach, this hydrolysis was reduced by the esterase inhibitor tetraethyl-pyrophosphate. Pyrethroid hydrolysis by brain fractions has alos been studied, as it may influence target organ sensitivity. No activity was detected in rat brain (Gray and Rickard, 1982a), though Ghiasuddin and Soderlund (1984) have shown that mouse brain esterases readily hydrolysed non-cyclopropane esters such as fenvalerate and fluvalinate.

The significance of pyrethroid hydrolysis in detoxification has been demonstrated in acute toxicity studies using organophosphate esterase-inhibitors as synergists. Following pre-treatment with DEF, TOTP or similar compounds, the toxicities of several trans-pyrethroids were greatly increased in mammals (Gray and Soderlund, 1985), insects (Ishaaya and Casida, 1980; Riskallah, 1983), and amphibians (Cole and Casida, 1983). However, such compounds do not increase the acute toxicity of trans-permethrin in rainbow trout (Glickman and Lech, 1982). In particular, the synergism by organophosphates may represent a toxicological hazard to workers using pyrethroids in conjunction with other insecticides (Hutson, 1979).

5.3. Species differences in metabolism

Due to differences in the activities of enzymes involved in phase I and phase II reactions, the metabolic fate of many xenobiotic compounds is dependent upon the species under study. Excellent examples of such species diversity can be seen with the biotransformation of the aryl-acids (see Millburn, 1978). In general these investigations have been confined to mammals (Caldwell, 1981), though a limited amount of information is also available for birds (Pan and Fouts, 1978), and fish (Chambers and Yarbrough, 1976; Allen et al., 1979).

The pyrethroids which have been developed from

natural products, provide a useful biochemical probe to investigate species variations in xenobiotic biotransformations. In particular, the metabolite 3PBA has been found to undergo remarkably diverse reactions in various mammals and birds (Huckle, 1981). The metabolic fate of the pyrethroids will now be briefly reviewed in the major biotic systems.

5.3.1. Soil and plants

Soils readily metabolise pyrethroids, largely by hydrolysis (Roberts, 1981); this activity being dependent upon microbial activity (Kaufmann et al., 1977). Degradation is optimal under aerobic conditions, with some evolution of CO_2 being brought about by mineralisation of pyrethroid metabolites into the soil organic fraction (Roberts and Standen, 1977).

The pyrethroids are non-systemic and remain tightly bound to cuticular waxes after topical application. Bioavailability for metabolism is therefore reduced, though photo-isomerisation of the adsorbed insecticide may occur, such that formation of the more stable cis-isomer from the trans-isomer is favoured (Roberts, 1981). Plants metabolise pyrethroids principally by ester hydrolysis (Table 1:6), and most actively cleave the trans-isomers. Hydrolysed metabolites may then be conjugated with glucose, or rather more unusually with disaccharides (Wright et al., 1980) or amino acids (Gaughan and

Casida, 1978).

Oxidation at the 4', trans-methyl, and to some extent the 2' positions of permethrin, cypermethrin and deltamethrin have been observed in a number of plant species (Roberts, 1981). Additionally, the α-nitrile group of cypermethrin and fenvalerate may also be oxidised to the amide and carboxylic acid (Ohkawa et al., 1980). None of these metabolites have been shown to be phytotoxic to plants.

5.3.2. Insects

The metabolism of pyrethroids in insects has tended to concentrate on phase I detoxification processes, which ultimately limit the acute toxicity of these compounds. Insects metabolise permethrin and cypermethrin in similar ways to mammals, but show species and sex variations in the preferred sites of metabolism both in vivo (Soderlund et al., 1983c) and in vitro (Shono et al., 1979). In these studies, cypermethrin was generally more resistant to metabolism than permethrin, with the cis-isomers being more persistent than their trans- counterparts. In vivo, oxidation of the intact pyrethroid was more important than that observed in vitro, though the majority of the metabolites were derived from ester hydrolysis (Stuart-Holden, 1979). However, this appeared to be due to oxidative cleavage.

Phase II reactions of pyrethroids and their

metabolites in insects, consist of conjugations with glucose and amino acids (Soderlund et al., 1983c), though overall rates of elimination are frequently slow.

5.3.3. Aquatic vertebrates

It is now recognised that in vivo, fish can metabolise and eliminate xenobiotics, but that their biotransformations are less rapid and less varied than those of similarly treated mammals (Allen et al., 1979). In many cases this poor metabolic capacity is reflected in the high acute toxicity of many pesticides and pollutants to fish (Lech and Bend, 1980). In particular, the bioaccumulation of lipophilic compounds such as pyrethroids in fish (see "toxicokinetics"), can lead to the saturation of detoxification mechanisms even at very low concentrations of dissolved xenobiotic in the water.

Once absorbed, non-polar compounds may only be eliminated following metabolism to water-soluble derivatives, which can be excreted either in the urine, by passive diffusion through the gills, or in secreted bile. Biliary elimination has been shown to be the major excretion route for the metabolites of several lipid-soluble pesticides, and because of the attendant bioconcentration, fish bile has been suggested as an aid for monitoring water contamination (Lech et al.,

1973). When rainbow trout were exposed to [^{14}C] *cis*- and [^{14}C]*trans*-permethrin dissolved in the aquarium water (5 µg l^{-1}), radioactivity rapidly accumulated in the bile, particularly with the *cis*— isomer (Glickman *et al.*, 1981*a*). Analysis of the biliary metabolites showed that they were β-glucuronidase labile, and that the major aglycone co-chromatographed with 4'HO-permethrin. Similarly, the biliary excretion of glucuronide conjugates in fish has been observed with a number of other phenols such as 3-trifluoromethyl-4-nitrophenol (Lech and Costrini, 1972), phenol (Nagel and Urich, 1983), 1-napthol , 1-chlorophenol, and *m*-cresol (Layiwola *et al.*, 1983*a*), pentachlorophenol (Glickman *et al.*, 1977) and phenolphthalein (Curtis, 1983). Glucuronidation in fish is dependent on a number or conditions, such as the pH of the water (Laitinen *et al.*, 1982), and the species of fish used (Nagel, 1983). However, seasonal variations and changes in ambient temperature had no significant effect on the biliary excretion of phenolphthalein glucuronide in trout (Curtis, 1983), though such factors are known to influence other xenobiotic reactions in fish (Chambers and Yarbrough, 1976).

In addition to the glucuronide of 4'HO-permethrin, Glickman *et al.*, (1981) also determined the presence of uncharacterised sulphate conjugates in trout urine. A number of phenols form sulphate conjugates in fish, though their presence has only previously been

confirmed in the bile, or the aquarium water, and not the urine (Layiwola et al., 1983a; Nagel, 1983). Nagel (1983) has demonstrated that such sulphation is related to both the species of fish used and the size of the administered dose, such that increases in the exposure of phenol to goldfish favoured glucuronide formation. Similar relationships between species, dose and sulphation are recognised in mammals (Capel et al., 1974), while trout are reported to have low levels of sulphotransferase in their livers (Gregus et al., 1983). Glickman et al., (1981) did not identify any amino acid conjugates of permethrin metabolites, presumably due to the low levels of ester hydrolysis. This impaired metabolic capacity was reflected in a slow elimination of [^{14}C] permethrin in all tissues (half-lives (t 1/2) = 10-30 hours, except fat t 1/2 > 50 hours). No significant difference in elimination rates between the cis- and the trans-isomers was noted.

No information is available on the in vivo metabolism of pyrethroids in other aquatic vertebrates, though toxicity studies with esterase and oxidase inhibitors would suggest that frogs metabolise cis- and trans—isomers primarily by oxidation and hydrolysis, respectively (Cole and Casida, 1983). Amphibians are known to form glucuronide and glycine conjugates of benzoic acid and its derivatives (Millburn, 1978).

5.3.4. Mammals

Detailed information is now available on the metabolism of the cis- and trans-isomers of permethrin in rats (Gaughan et al., 1977), cows (Gaughan et al., 1978a) and goats (Hunt and Gilbert, 1977; Ivie and Hunt, 1980). Similar investigations have been carried out with the α-cyano-phenoxybenzyl esters. Thus, detailed metabolism studies have been reported with cypermethrin in mice (Hutson and Casida, 1978; Hutson et al., 1981) and rats (Crawford et al., 1981a; 1981b); with deltamethrin in mice (Ruzo et al., 1979); and with fenvalerate in rats (Ohkawa et al., 1979). Most recently, the biotransformation of the anilino acid ester-derivative of the cyano-phenoxybenzyl pyrethroids, fluvalinate, has been investigated in cows (Quistad et al., 1982a).

The major excreted conjugates of the cyclopropanecarboxylate esters, and the routes and rates of elimination of metabolites are summarised for a number of vertebrate species in Table 1:7. In all the mammals studied, the rate of excretion of radioactivity of the trans-isomers of radiolabelled pyrethroids exceeded that of the corresponding cis-isomers, though this difference was less than would have been predicted from in vitro studies. Radioactivity derived from orally dosed [^{14}C]cis-pyrethroids was more extensively eliminated in the faeces than was the case with the trans-isomers. The faecal metabolites of pyrethroids

Table 1:7

The metabolism in vivo of permethrin, cypermethrin and deltamethrin by vertebrates

Species	Pyrethroid	Elimination (major route and isomer preference)		Major conjugated metabolites Alcohol	Acid
Trout	PER	bile	c=t	4'O.PERgluc	
Mouse	CYP	c-faeces t-urine	t>c	PBA tau (PBA gluc)	Cl_2CAgluc
	DEL	urine	-	PBA tau	Cl_2CAgluc
Rat	PER,CYP	c-faeces=urine t-urine	t>c	4'.HO.SO_2O.PBA	Cl_2CAgluc
	DEL	urine		4'.HO.SO_2O.PBA	Cl_2CAgluc
Cow	PER	faeces	t>c	PBA gluc	Cl_2CAgluc
Goat	PER	c-faeces t-urine	t>c	PBAgly	Cl_2CAgluc
Hen	PER	(excreta)	c=t	t'HO.SO_2O.PER 4'HOPBAconj	Cl_2CAgluc

Abbreviations PER. permethrin, CYP. cypermethrin
DEL. deltamethrin
c - cis t - trans
gluc. glucuronide; glu. glutamate; gly. glycine; tau. taurine; conj. unknown amino acid.

Fish exposed to dissolved pyrethroid, the other species dosed orally.

For references see text: fish, mammals birds.

consist largely of unchanged insecticide and non-polar hydroxylated derivatives of the intact ester. Urinary metabolites tend to be derived from the products of ester-hydrolysis.

The slower excretion of cis-permethrin and cis-cypermethrin compared to their trans-enantiomers, is associated with both higher tissue residues and an increased elimination of the cis-isomers and their related metabolites in the milk of lactating animals (Casida, 1980). In depletion studies with permethrin and cypermethrin, the residues of the cis-isomers were more persistent in rats, particularly in adipose or fatty tissue (Crawford et al., 1981a; Marei et al., 1982). Hutson et al., (1981) have suggested that this increased stability in fat reserves may be due to the reduced activity of pyrethroid-hydrolysing lipases towards cis-cypermethrin compared with trans-cypermethrin. Persistent residues in fatty tissues have also been shown to be caused by conjugation of the 3PBA moiety of pyrethroids with glycerides (Crayford and Hutson, 1981).

The species-dependent variations in the phase I metabolism of permethrin isomers shown in Table 1:6, are also seen with cypermethrin and deltamethrin. In particular, the presence of considerable quantities of the hydroxylated derivatives of the free acid and alcohol in mammals, has led to some conjecture as to whether this oxidation occurs before, or after, ester

cleavage. Little t'-hydroxylation of the acid moiety was observed when dibromovinyl-dimethyl-cyclopropanecarboxylic acid was administered on its own to mice, suggesting that such oxidation occured prior to the hydrolysis of deltamethrin (Ruzo et al., 1979). However, when 3PBA was administered on its own to mice (Hutson and Casida, 1977) and rats (Huckle et al., 1981a), both the extent and the pattern of hydroxylation was similar to that observed with the alcohol moiety of intact cypermethrin. This suggests that aryl-hydroxylation occurs after hydrolysis, with rats in particular being extremely active at forming 4'HO-3PBA in vivo (Huckle et al., 1918a).

In all the mammalian species, phase II conjugations are largely restricted to the products of ester hydrolysis (Table 1:7). However, the glucuronide of t'HO-permethrin and the sulphate of 4'HO-cypermethrin, have been reported in cows (Gaughan et al., 1978a) and mice (Hutson et al., 1981), respectively. The majority of the conjugated cyclopropyl acid and its oxidised derivatives are recovered as glucuronides (Table 1:7). However, the glycine, taurine and sulphate conjugates have also been identified in mouse urine as minor metabolites (Ruzo et al., 1979; Hutson et al., 1981). The relatively simple metabolic profile of the acid moiety of pyrethroids has facilitated dose-excretion studies with unlabelled cypermethrin in man (Eadsforth and Baldwin, 1983).

Analysis of conjugated and free Cl_2CA in the urine, showed that trans-cypermethrin was excreted more rapidly over a 24 hour period (78% of the dose recovered) than the cis-isomer (49%). The acid component of fenvalerate, 3-(4-chlorophenyl)-isovalerate and its hydroxylated derivatives are also largely conjugated with glucuronic acid (Ohkawa et al., 1979). However, the anilino acid of fluvalinate gives a more complex metabolic profile, forming conjugates with glycine, taurine, glucuronic acid and bile acids (Quistad et al., 1982a; 1982b).

The alcohol moiety of the phenoxybenzyl esters gives a complex range of phase II metabolites when administered as either the intact pyrethroid, or as free 3PBA. Such biotransformations are species-dependent (Huckle, 1981) and have also been demonstrated with a number of other aromatic acids (see Millburn, 1978). The presence of the ether and ester glucuronides of 3PBA and 4'HO-3PBA in the urine of marmoset, rabbit, guinea pig and hamster (Huckle et al., 1981c), and to a lesser extent mouse (Hutson et al., 1981; Ruzo et al., 1979), indicate that glucuronidation is the major phase II reaction of the alcohol moiety in these species. Huckle et al., (1981a) have also demonstrated that rats excrete these glucuronides in the bile, but that de-conjugation occurs in the intestine due to the action of bacteria. The aglycones are then reabsorbed, sulphated and

excreted in the urine, largely as 4'HO.SO_2.0.3PBA. In other species, 3PBA is eliminated in the urine as amino acid conjugates. Thus, 3PBA is largely conjugated with glycine in sheep, cats, gerbils and goats (Huckle et al., 1981c; Ivie and Hunt, 1980), with taurine in mice (Hutson and Casida, 1977) and with glutamate in the cow (Gaughan et al., 1978a). These species variations in amino acid conjugation have been shown to be due to differences in the activity and substrate specificity of the hepatic and renal biosynthetic enzymes involved in these reactions (Huckle et al., 1981d). The biochemistry of amino acid conjugation has recently been reviewed by Huckle and Millburn (1982).

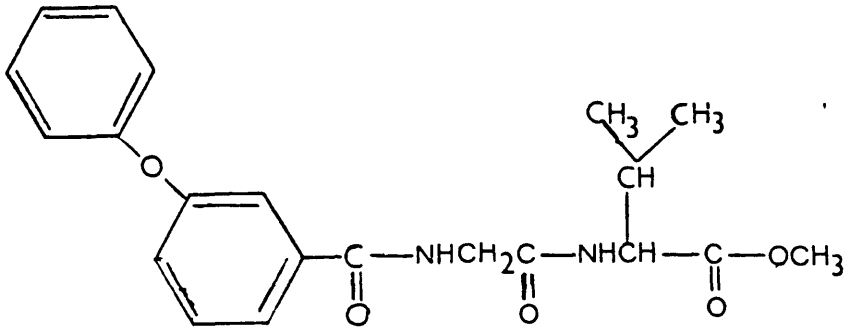
5.3.5. Birds

Although detoxification appears to be a major factor in determining the low acute toxicity of fenvalerate to birds (Bradbury and Coats, 1982), few metabolism studies with pyrethroids have been reported in avian species. Following oral administration of [^{14}C] permethrin (Gaughan et al., 1978b), or [^{14}C] fluvalinate (Staiger et al., 1982), hens rapidly eliminate radioactivity in their excreta irrespective of isomer conformation (Table 1:7). Such rapid metabolism and excretion are indicative of the high metabolic rate, and speed of food passage in chickens (Bell and Freeman, 1971), and have been observed with a number of orally dosed xenobiotics (Pan and Fouts,

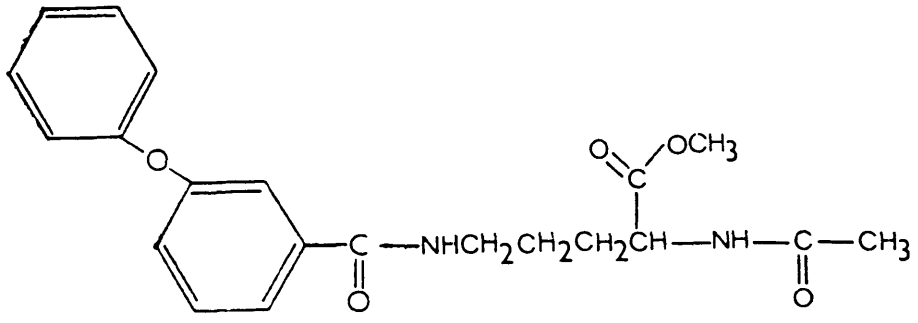
1978). Dermally applied [^{14}C] permethrin was also readily eliminated, though some residual radioactivity was associated with the skin and fatty tissue (Hunt et al., 1979). In all the studies with pyrethroids, low concentrations of radioactivity were associated with the eggs. Residues of cis-permethrin in egg-yolk, skin and fat were higher than those associated with trans-permethrin (Gaughan et al., 1981b). In this respect, birds are similar to mammals (see previous section), though residue concentrations are significantly lower.

In hens, trans-permethrin was metabolised almost exclusively by hydrolysis (Gaughan et al., 1981b). The cis-isomer was also actively hydrolysed, but there was significant hydroxylation of the trans-methyl group of the intact ester, followed by sulphation (Table 1:7). Metabolites resulting from hydrolysis were largely recovered as their hydroxylated derivatives, c, or t-HO.Cl_2CA, 4'HO.3PBalc, and 4'HO.3PBA. The available evidence suggested that this oxidation occured largely after ester cleavage. The acid moiety was largely conjugated with glucuronic acid, though taurine derivatives and HO.SO_2O.Cl_2CA were also identified. The alcohol moiety and its 4'hydroxy derivatives were recovered as glucuronides, sulphates and uncharacterised amino acid conjugates. In studies with 3PBA, Huckle et al., (1981b) demonstrated that in the mallard duck, conjugation occurred mainly with the dipeptide glycylvaline (Figure 1:5). In the hen

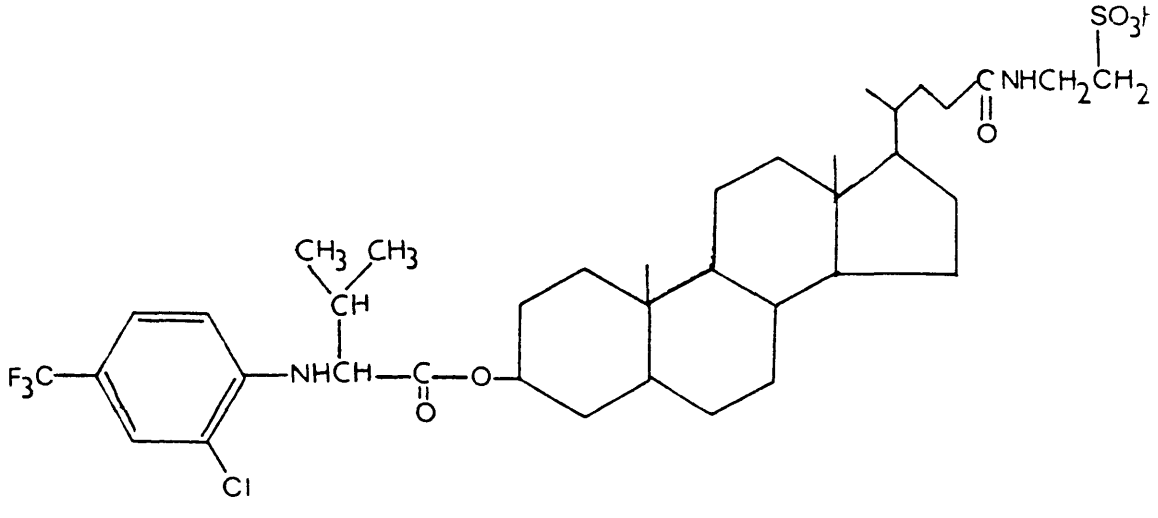
Figure 1:5, Unusual conjugates of pyrethroids in birds.



N-(3-phenoxybenzoyl) glycylvaline



α-N-(3-phenoxybenzoyl) acetylornithine



2-[2-chloro-4-(trifluoromethyl) anilino]-taurochenodeoxycholate

however, 3PBA was excreted conjugated with glucuronic acid, glycine, glycylvaline (minor metabolites) and N-acetylornithine (major metabolite : see Figure 1:5) (Huckle et al., 1982). Significant amounts of 4'HO.SO_2.O.3PBA were also detected. The pyrethroid fluvalinate, was metabolised by 4'—hydroxylation of the intact ester and by hydrolysis to the anilino acid, which was recovered as its taurine, and taurochenodeoxycholic acid conjugates (Staiger et al., 1982, see Figure 1:5).

These metabolic products illustrate the wide range of conjugations carried out on xenobiotics by birds (Pan and Fouts, 1978). Hens, and other Galliforms, are well known to form sulphate and glucuronide conjugates with phenols, and ornithine conjugates with aromatic acids (Millburn, 1978). An interesting feature of the metabolism of the pyrethroids in hens is the possible relative roles of urinary and biliary excretion. Chipman and Walker (1981), have demonstrated that sulphate conjugates of a chloro-cyclodiene were excreted in the urine of colostomised pigeons, while the glucuronides were eliminated in the bile. Such a balance would depend upon the molecular weight threshold for biliary excretion in the bird species under study, as determined with other species (Millburn, 1976). A greater understanding of such mechanisms would help to explain the rapid metabolism and elimination of pyrethroids in birds.

6. Scope and objectives of the project

From a review of the available literature, it is apparent that the acute toxicity of pyrethroids to vertebrates is regulated by metabolic and toxicokinetic parameters, as well as the sensitivity of the CNS to their neurotoxicant action. These factors in turn are controlled by the structural and conformational characteristics of these insecticides, as well as the species under study. Thus the acute toxicity of a given pyrethroid follows the pattern fish>amphibians>mammal>birds.

To explain these species differences, cypermethrin isomers have been used to examine the biotransformation potential, and the target organ sensitivities of rainbow trout, frog, mouse and quail. This has involved a multiple approach to the problem; namely in each species:

i) To determine concentrations of cypermethrin isomers in the brain and in other tissues associated with toxic signs.

ii) To study the *in vivo* metabolism of cypermethrin at sub-toxic and toxic doses.

iii) To compare the rates and mechanisms of biotransformation in various tissues *in vitro*.

Such studies will assist in answering questions which have arisen in other investigations, and may help explain some of the biochemical mechanisms underlying pyrethroid toxicology in vertebrates.

Chapter 2

Materials and general methods

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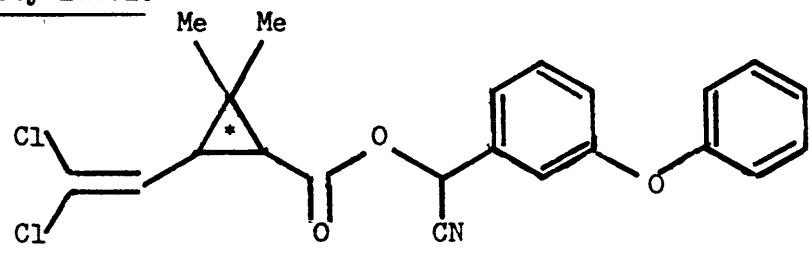
1. Radiochemicals

All radiolabelled compounds were synthesised by the Organic Chemistry Division, Shell Biosciences Laboratory, Sittingbourne, Kent, UK. (see figure 2:1). Preparations obtained in the form of resolved isomers were [^{14}C-cyclopropyl] *cis*-cypermethrin (48 μCi mg^{-1}), [^{14}C-benzyl] *cis*-cypermethrin (30 μCi mg^{-1}) and [^{14}C-phenyl] *trans*-cypermethrin (28.6 μCi mg^{-1}). Racemic mixtures used were [^{14}C-cyclopropyl] *cis*- and *trans*-cypermethrin (48 μCi mg^{-1}) (*cis*:*trans* 1:1), and [^{14}C-benzyl] *cis*- and *trans*-cypermethrin (22.5 μCi mg^{-1}) (*cis*:*trans* 1:1.15). Single isomer preparations, and *cis*/*trans* mixtures which required no further resolution of the enantiomers were purified prior to use by t.l.c. using toluene as the developing solvent (Rf: *cis*=0.49, *trans*=0.45) and eluted from the silica-gel with chloroform. Racemates requiring resolution into their *cis*- and *trans*-isomer components were purified by h.p.l.c. system I. The radiochemical purity of all preparations was confirmed by t.l.c. (solvent E), or h.p.l.c. (system III) and was found to be >99% in all cases, as determined by l.s.c. . 3-Phenoxy [*ring*-U-^{14}C] benzoic acid (58.2 μCi mg^{-1}), and 3-(4-hydroxy-phenoxy) [*ring*-U-^{14}C] benzoic acid (24.7 μCi mg^{-1} were purified by t.l.c. (solvent E) prior to use (radiochemical purity 98.8% and 97% respectively).

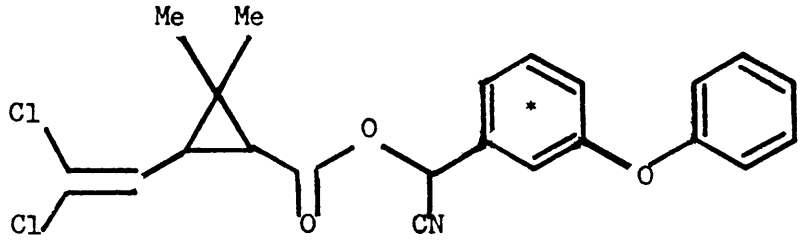
Figure 2:1. Radiolabelled preparations of cypermethrin.

Acid moiety label.



[^{14}C-cyclopropyl] cis-& trans-cypermethrin.

Alcohol moiety label.



[^{14}C-benzyl] cis+trans-cypermethrin.

[^{14}C-phenyl] cis-& trans-cypermethrin labelled in the outer aromatic ring.

* = position of radiolabel.

2. Reference compounds

Unlabelled analytical grade *cis*- and *trans*-cypermethrin and all reference metabolites were obtained from Shell Biosciences Laboratory, Sittingbourne, except 3-phenoxybenzoic acid which was purchased from the Aldrich Chemical Co. Ltd., Dorset, UK. The syntheses of these compounds has been described (Shono *et al.*, 1979; Huckle, 1981). The methylated derivatives of hydroxylated 3-phenoxybenzoic acid were either obtained from Shell Limited, or prepared by reaction with diazomethane (see: identification of conjugates). All standards were stored with dessicant at $4^{o}C$ in the dark. For chromatography, reference solutions were prepared in methanol (5 mg ml^{-1}), or hexane (cypermethrin only, 10 mg ml^{-1}), and stored at $-20^{o}C$.

3. Animals

Animals were obtained and fed as detailed in Table 2:1. Frogs were maintained at approximately $8^{o}C$ in flowing mains water without food for up to 3 months. Fish were fed daily and held in flowing de-chlorinated mains water for at least seven days prior to use. Ambient conditons were:

Temperature	:	$15^{o} \pm 2^{o}C$
Lighting	:	12 hour cycle
Water quality	:	pH:7.5-8.0
Dissolved O_2	:	9.1-9.7 mg l-1

Table 2:1

Data for animals used

Common Name	Strain	Species	Diet	Sex	Weight (g)	Supplier
Trout	Rainbow	*Salmo gairdneri* (Richardson)	A	M,F	13±2	Parkwood Farm, Harrietsham, Kent.
		Salmo gairdneri	B	M,F	208±32	Zeals Fish Farm Wolverton, Wilts.
Frog	Common	*Rana temporaria*	-	M,F	27±3	Frog Farm, Kells, Eire.
Rat	Wistar	*Rattus norvegicus*	C	M	255±11	Lions Laboratory, Ringwood, Hants.
Mouse	CF-1	*Mus musculus*	C	M	37±2	Shell Research Ltd. Sittingbourne, Kent.
Quail	Japanese	*C. Coturnix japonica*	D	F	231±24	Lincolnshire Pheasantries Boston, Lincs.

Diets

A Mainstream salmon fry food (0.3), Special Diets Services Ltd., Witham, Essex.
B Mainstream trout fingerling (2), Special Diets Services Ltd., Witham, Essex.
C Labsure modified 41B, RHM, Poole, Dorset.
D Turkey starter crumbs, BOCM, Silcock.

Water hardness : (as $CaCO_3$): 250-265 mg l^{-1}

Mice, rats and quail were all housed at ambient temperature and lighting, and allowed food ad libitum. Mammals and birds were only used after a three week quarantine period.

4. Necropsy

Following sacrifice (see Chapters 3- 6), all tissues were rapidly removed and frozen; samples for h.p.l.c. analysis being placed directly into liquid N_2 (<1 min). In the case of fish and frogs, acetone rinsed instruments were used for all dissections. Blood samples were collected from the tail vein (trout), or by cardiac puncture, using pre-weighed swabs, or heparinised syringes, and stored at 4°C. Gall bladder (trout and frogs only), liver, kidney, intestinal tract, brain, fat and skin samples were also removed and stored in pre-weighed containers (glass for residue studies, plastic otherwise) at -20°C pending analysis.

5. Measurement of radioactivity

5.1. Liquid scintillation counting (l.s.c.)

Solutions for radioassay were mixed with scintillator, the vials wiped with a damp tissue to remove static charges and the mixture allowed to equilibrate for at least 30 min prior to counting. At St. Mary's Hospital Medical School (SMHMS) l.s.c. was

carried out using a Packard Prias model 240 cl/d scintillation spectrometer, or a Packard scintillation spectrometer model 2450. Scintillant (4 ml) consisted of either (i) 5-phenyloxazole (PPO) 5 g l^{-1} toluene, 1,4-bis (5-phenyloxazol-2-yl) benzene (POPOP) 0.3 g l^{-1} in toluene. Toluene mixture: Triton X-100 2:1 v/v (BDH, Dorset, UK.), (ii) Packard toluene scintillator (Packard Instrument Co.):Triton X-100 (2:1 v/v), or (iii) 3 mls of liquiscint (National Diagnostics, N.J.).

At the Shell Research Centre (SRC) a Packard Tri-Carb 460CD counter and ES299 scintillator (10 ml; Packard Supplies Ltd.) were used. In all cases, counting efficiences were corrected for using the external standard ratio method.

5.2. Determination of tissue radioactivity

Non-aqueous, or highly coloured biological fluids (eg. blood), were radioassayed by oxidation analysis, or tissue solubilisation. Weighed samples for oxidation (50-200 mg depending on fat content) were placed in paper combusta-cones and covered with combust-aid and cellulose powder (United Technologies, Packard). Combustion analysis was carried out in duplicate or triplicate, in a Packard model 306 sample oxidiser using carbosorb (8 ml) and permafluor (13 ml) (United Technologies Packard), as the absorber and scintillator solutions, respectively. Recoveries were corrected for using Amersham CFR 101 ^{14}C standards

(recoveries 98.5±1.0%). Tissue digestion was carried out with NCS solubiliser (Amersham, Il, USA). Weighed samples (50-300 mg) were digested for 24 hr at 50°C with 5 v/w NCS solubiliser. Digestates were then bleached with 2 v/w of benzyl peroxide solution (20% w/v toluene) for 60 min at 50°C prior to adding of scintillant (16 ml). Counting efficiencies were corrected for using an internal standard of [^{14}C]toluene (4850 dpm, Amersham International, Amersham, UK).

No preparation was required for homogeneous samples such as blood and egg-yolk. In other cases, tissue homogenisation was either carried out by maceration with scissors, or treatment with an Ultra-Turrax homogeniser (Janke and Kunkel, W. Germany), with water added if required. Skinned carcasses were homogenised in 0.3 v/w distilled water using a MSE Atomix. Four dorsal skin samples with associated skin/feathers (approx. 100 mg each) were removed from various sites for oxidation analysis. In all cases, untreated control tissues were used to subtract for biological background radioactivity. Radioassay of adipose tissue was carried out by extracting fat (x3) by gentle heating with 5 v/w acetone:hexane (1:1 v/v). The pooled, filtered extract was then taken to dryness at 40°C, and the residue re-dissolved in hexane. After making up to volume in a graduated flask, samples were taken for l.s.c.. This procedure was shown to extract

95.4% of the total radioactivity associated with fat.

5.3. Determination of radioactivity in excreta

Urine (20 µl) and bile (5 µl) samples were counted in triplicate. Rat and mouse faeces were soaked in methanol (10 v/w) for 1 hr at 4°C, then homogenised with the Ultra-Turrax three times at half maximal speed for 2 min. After extracting for 4 hr at 4°C with constant stirring, the mixture was centrifuged (1,000g, 10 min:MSE-Minor) and the decanted supernatant analysed by l.s.c. (3x50 µl) and chromatography. The extracted residue was air dried, weighed and sub-samples taken for oxidation.

Samples of whole quail excreta (QEX) were radioassayed following either homogenisation of the lyophillised material with a pestle and mortar, or by soaking the droppings in distilled water (2 v/w) for 1 hr at 4°C prior to treatment with the Ultra-Turrax homogeniser. Several extraction cocktails were assessed for their efficiency in recovering radioactivity from dessicated QEX and the results and extraction conditions are summarised in Table 2:2. Methanol:acetonitrile:water (2:2:1 v/v) (x2) was subsequently used in all extractions, since this gave recoveries of radioactivity from water soaked QEX of 90%±8.7% with [^{14}C-benzyl] cypermethrin metabolites and 97.2±1.2% in [^{14}C-cyclopropyl] labelled experiments. QEX extracts were centrifuged at 3,000g for 15 min

Table 2:2

Extraction of radioactivity from dessicated quail excreta containing metabolites of [^{14}C-benzyl] cypermethrin.

Solvent (proportions by vol.)	% of ^{14}C extracted	
Methanol	57.1±2.1	
Methanol/water (9/1)	71.8±6.9	
Methanol/water (1/1)	69.9±1.9	
Acetonitrile	11.6± 1.4	
Acetonitrile/water (9/1)	61.5± 0.5	
Acetonitrile/water (5/1)	69.1± 1.8	
Acetonitrile/water (1/1)	80.6± 2.6	
Acetonitrile/water (1/2)	83.5±11.7	
Water	72.4± 4.8	
Methanol/acetonitrile (1/1)	52.1± 2.3	
Methanol/acetonitrile/ water (2/2/1)	76.7± 1.3	(90.0±8.7)a
Methanol/acetonitrile/ water (1/1/1)	77.2± 2.5	

Mean values ± SD (n=3)

Dessicated quail excreta samples were shaken (100 oscillatins min^{-1}) with 20 volumes of solvent at 4°C for 16 hr.

a % of ^{14}C extracted by 2 x 20 volumes of solvent.

(Sorvall GLC-4), and the supernatant decanted and concentrated to its aqueous component by rotary film evaporation (RFE) at 40°C. Sub-samples (5 ml; pH 2) were then loaded onto SEP-PAK C18 columns (Waters Associates Inc., Hartford, Cheshire, UK.) which had been previously washed and equilibrated with methanol (10 ml), followed by distilled water (10 ml). The de-salted, bound material, was then eluted with methanol (10 ml), such that 98% of the column radioactivity was recovered. By repeating this clean-up procedure (x3) 92.2%±3.0% of the extracted ^{14}C was recovered as a methanolic extract.

6. Chromatographic analysis

6.1. Thin-layer chromatography (t.l.c.)

Conventional t.l.c. plates (20x20 cm, glass and plastic backed) and high performance thin-layer chromatography (h.p.t.l.c.) plates (10x10 cm, glass backed), were purchased pre-coated with silica-gel F_{254} from Merck, Darmstadt, W. Germany. Preparative silica-coated plates were similarly obtained. Following clarification by centrifugation, or filtration (Millipore, pore size = 0.5 μm), samples for chromatography were applied to 1-2 cm wide bands, 2 cm from the bottom of the t.l.c. plate at a typical application rate of 5 μl cm^{-1} for (excreta extracts). The plates were placed in Shandon chromatotanks, containing the pre-equilibrated (approx. 1-2 h) solvent

system. After developing and drying, uv absorbing reference compounds were visualised in a CC-20G chromato-view cabinet (Ultra-violet Products Inc., San Gabriel, Ca., USA.) operating in the short wave mode. Radioactive zones were located by autoradiography, or with a Bertholdt LB 2723 radiochromatogram scanner. Autoradiography was carried out with Fuji X-ray film (Fuji Film Co. Ltd., Japan), with up to four weeks exposure at -70°C. Development (4 min) and fixing (2 min) utilised the proprietary Fuji products. Plates were quantified by scraping off the silica-gel either in 5mm wide strips, or just associated with those zones detected by autoradiography, into scintillation vials containing methanol (0.3 ml). After vigorous mixing and extraction (30 min), scintillant was added and samples quantified by l.s.c. Control experiments showed that 98.8% of the applied ^{14}C was recovered by this method.

Identification by co-chromatography was based on the coincidence in location, shape and size of bands visualised under uv light and by autoradiography, following development of radiolabelled metabolites over-spotted with reference standards. The solvent systems used and the Rf values of important metabolites, are shown in Table 2:3.

6.2 Gas liquid chromatography (g.l.c.)

G.l.c. of cypermethrin was carried out with a

Compound. (c= cis isomer) (t= trans isomer)	Rf in t.l.c. system BAW	EFW	CA	TEA	Elution volume (ml) in h.p.l.c. system IV	V
c-/t- cypermethrin	0.85	0.97	0.92	0.83	34.4/34.2	35./35
4'HO.c-cypermethrin	-	-	-	0.55	31.5	34.5
t'HO.c-cypermethrin	-	-	-	0.55	31.5	34.5
t'HO.4'HO.c-cypermethrin	-	-	-	-	28.7	31.8
4'O.cypermethrin.glucuronide	-	0.43	-	0	26.6	26.0
3PBalc	-	-	-	0.45	-	-
3PBA	0.81	0.95	0.64	0.32	-	26.3
3PBA.glucuronide	-	0.48	-	0	-	-
3PBA.glycine	0.69	0.88	0	0.04	-	18.9
3PBA.glycylvaline	0.72	0.85	-	0.13	-	21.8
3PBA.α-ornithine	0.28	0.05	-	-	-	-
3PBA.δ-ornithine	0.42	0.05	-	-	-	-
3PBA.α-N-acetyl-ornithine	0.62	0.55	-	0	-	18.3
3PBA.taurine	0.50	0.08	0	0	-	12.0
2'HO.3PBA	-	0.89	0.55	-	-	19.2
4'HO.3PBA	0.77	0.89	0.33	0.25	-	18.3
4'HO.3PBA.glucuronide	-	0.35	-	0	-	-
4'O.3PBA.glucuronide	-	0.40	-	0	-	-
2'HO.SO_2.0.3PBA	0.55	0.26	0	0	-	11.7
4'HO.SO_2.0.3PBA	0.59	0.25	0	0	-	11.9
c-Cl_2CA	0.72	0.93	-	0.49	26.4	-
t-Cl_2CA	,0.72	0.93	-	0.45	25.8	-
t'HO.c-Cl_2CA	0.70	0.83	-	-	20.4	-
t'O.Cl_2CA	0.80	0.93	-	-	24.8	-
c-/t-Cl_2CA.glucuronide	0.41	0.33	-	-	20.2	-

Table 2:3. T.l.c. and h.p.l.c. data for cypermethrin and it's major metabolites.

T.l.c. solvent systems.
B.A.W. :- Butan-1-ol :acetic acid:water.(6:1:1 v/v.)
E.F.W. :- Ethyl acetate:formic acid:water.(35:2:2 v/v.)
C.A. :- Chloroform::acetic acid.(95:5 v/v.)
T.E.A. :- Toluene :ethyl acetate :acetic acid.(75:25:1.)

Hewlett-Packard 5710 gas chromatograph coupled to a model 7671A automatic sampler and model 3380A integrator. Detection was in the electron capture mode with a ^{63}Ni source. Operating conditions were

Column (glass) : 1mx4mm (i.d.) packed with Gas Chrom Q100/200 mesh supporting methyl silicone oil (OV-1) 3%M.

Carrier Gas : O_2 free N_2 40 ml min^{-1}

Injection port temperature: 250°C

Detector temperature : 350°C

Under these conditions no isomer resolution was achieved. Analytical grade standards were prepared in hexane in the range 0.001-0.020 μg ml^{-1}.

6.3. High pressure liquid chromatography (h.p.l.c.)

All solvents were of h.p.l.c. grade. Hexane and dichloromethane (CH_2Cl_2) were obtained from Fisons, Ltd., Loughborough, Leics, UK. and acetonitrile and water from Rathburn Chemicals Ltd., (Walkerburn Pebbleshire, UK.). At SMHMS a Waters dual-pump solvent delivery system and model 660 programmer was employed (Waters Associates), while at SRC a comparable LDC apparatus (Milton Roy, UK.) was used. In both cases a Waters U6K injection loop was used. Metabolites derived from the phenoxybenzyl moiety absorb at 254 nm and therefore this wavelength was used to detect [^{14}C-benzyl] labelled samples, the eluate being monitored

with a Waters model 440 detector, coupled to a Philips PM 8252 dual-pen recorder. The acid moiety of cypermethrin contains no aromatic groups, and the eluate derived from [^{14}C-cyclopropyl] cypermethrin metabolites was therefore monitored with a Pye Unicam LC3 variable wavelength detector set at Cl_2CA λmax = 240 nm, in conjunction with a Linseis 2000 tri-pen recorder. Fractions (0.3 ml) for radioassay were collected with a LKB 2112 Redirac fraction collector (Croydon, Surrey, UK.) and quantified by l.s.c. Mobile phases were:-

System I : normal-phase 25cm x 8mm (i.d.) column containing 5 µm Hypersil stationary phase (Hichrom, Woodley, Bucks, UK.) eluted with hexane:dichloromethane:dichloromethane (water saturated) (40:7:3 v/v) at 4 ml min^{-1}.

System II : normal-phase, 25cm x 4.9mm (i.d.) column containing 5µm Hypersil stationary phase (Hichrom) eluted with hexane:dichloromethane:dichloromethane (water saturated), 40:7:3 v/v, 1.5 ml min^{-1}.

System III : reversed-phase, 25cm x 4.9mm (i.d.) column containing 5 µm Spherisorb-ODS C_{18} stationary phase (Hichrom) eluted with acetonitrile:water:acetic acid, 85:15:0.5 v/v, at 1.5 ml min^{-1}.

System IV : column as in system III, eluted with a linear gradient containing increasing amounts of acetonitrile; 5-100% water/acetonitrile (+0.5% v/v acetic acid), for 20 min at 1.5 ml min^{-1}.

System V : reversed-phase, 25cm x 4.9mm (i.d.)

containing 5 μm Spherisorb-ODS stationary phase (Hichrom) with a Co:pell ODS pre-column (Whatman, NJ., USA.) eluted as in system IV with a three step linear gradient, 5-40% (4 min), 4-50% (10 min), and 50-100% (6 min) at 1.5 ml min^{-1}.

System VI : Column as in system V, acetonitrile/water, Waters gradient profile M=3, 20-50% (15 min) 1 ml min^{-1}.

System VII : column as in system V, acetonitrile/water (no acetic acid), Waters gradient profile M=5, 60-100% (40 min) 0.5 ml min^{-1}.

The elution volumes of acid and alcohol derived metabolites of cypermethrin, as determined by systems IV and V respectively, are given in Table 2:3.

The above h.p.l.c. systems were utilised as follows: System I was used to preparatively resolve cypermethrin isomers, systems II and III to analyse for parent insecticide in animal tissues, system IV for analysing acid metabolites, system V and VI for analysing alcohol metabolites, and system VII for separating methylated derivatives. Identification of metabolites was based on co-elution of radioactivity with the uv trace of co-injected reference compounds.

7. Identification of conjugates

Conjugated metabolites were identified by co-chromatography and by chemical and enzymic hydrolysis. All enzyme preparations were obtained from Sigma

Chemicals, Dorset, UK. For hydrolysis experiments, radiolabelled metabolites were taken to dryness in the reaction vessel either under a stream of N_2, or by lyophilisation (Edwards-Modulyo freeze-drier), such that on redissolving the sample with the assay-buffer, a specific activity of 1,000 dpm per µl was attained. In all cases, reactions were terminated with 1 vol. of acetonitrile and the samples were then centrifuged (3,000 g, 5 min) prior to chromatographic analysis of the supernatants. Controls were prepared using buffer only.

7.1. Sulphates

Sulphates were identified by their lability to mild acid hydrolysis (1M HCl) and Helix pomatia sulphatase (type H-2 500 units ml^{-1}) following incubation at 37°C for 12 hr. Saccharic acid 1,4-lactone (25 mM; Callbiochem, La Jolla, USA.) was added to sulphatase preparations to inhibit endogenous β-glucuronidase activity. Radioactive metabolites liberated by hydrolysis were analysed directly as described previously, or were first derivatised. Methylation was carried out using a solution of diazomethane in ether, freshly prepared from Diazald (N-methyl-N-nitroso-p-toluenesulphonamide (Aldrich Chemicals). Sulphatase hydrosylates were prepared for derivatisation by extracting the incubations with 2 vol. diethyl ether (x3) and concentrating the dried

solvent extract with anhydrous Na_2SO_4. Methylation was then carried out at 4°C for 12 hr in the presence of excess diazomethane to afford a mixture of ester- and ether—methyl-derivatives.

7.2. Glucuronides

Several commercial preparations of β-glucuronidase were assessed as to their suitability in specifically hydrolysing glucuronic acid conjugates of 3PBA and Cl_2CA. Thus, enzymes derived from bovine liver (type BI), Patella vulgata (type LII), Helix pomatia (type HI), and Escherichia.coli (type VII) were all tested under Sigma recommended buffer conditions, at 1,000 units ml^{-1} for 4 hr at 37°C. E.coli (type VII) was found to be the most active preparation and contained minimal sulphatase activity under the incubation conditions (0.1M phosphate buffer pH 6.8). It was therefore used in all subsequent glucuronide hydrolysis experiments, with samples being withdrawn for chromatographic analysis at timed intervals.

8. Gut contents incubations

Animals were killed by cervical dislocation and the complete intestinal tract removed. This was flushed out using a 10 ml syringe filled with 0.1M phosphate buffer pH 6.9 containing 0.6% w/v tryptone, 0.6% w/v yeast extract and 0.6% w/v D-glucose (Oxoid, Hants., UK). Samples (1 ml) were then incubated with

the substrate (12 hr) at the appropriate body temperature, under an anaerobic atmosphere of H_2 and CO_2 generated in a BBL Gas-Pak (BBL Microbiology Systems, Md., USA.). Reactions were terminated with the addition of ice-cold acetonitrile (1 ml) and the samples were centrifuged at 3,000 g for 5 min prior to h.p.l.c. analysis.

9. Determination of partition coefficients (P-values)- between n-octanol and buffer (pH 7.4)

The partitioning of a compound between n-octanol, an organic solvent possessing many of the characteristics of lipid membranes (Leo et al., 1971) and neutral buffer, can help predict the bioconcentration potential of compounds in aquatic systems (Neely et al., 1974) and the likely routes of elimination of metabolites from animals (Millburn, 1976). Thus, Hirom et al., (1974) have shown that polar metabolites are preferentially excreted in the bile if they are amphipathic in character, ie: possess a certain balance between hydrophobic and hydrophilic properties within their structure. Such properties are determined by the molecular weight of the compound as well as the presence of certain functional groups (eg: OH and COOH). It has been suggested that P-values can be predicted from the chromatographic properties of compounds as measured by reversed-phase h.p.l.c., or alternatively by summing the hydrophobic coefficents of

the chemical constituents using commerically available computer models such as M.A.C.C.S. (Molecular Design Ltd., Hayward, Ca., USA.) as described by Eadsforth and Moser, 1983. In order to predict both the bioaccumulation potential and the possible routes of elimination, the P-values of cypermethrin and of several of its major metabolites were determined.

Pure radiolabelled preparations were obtained as described previously, or isolated from the biosynthetic sources by t.l.c. as detailed in Table 2:4. Samples (x3), containing 50,000 dpm, were taken to dryness under N_2 in a 4 ml glass vial. Octanol (0.25 ml), previously saturated with 0.1M phosphate buffer pH 7.4, was then added and the vial shaken vigorously. Buffer (0.25 ml saturated with n-octanol) was then introduced and the vial inverted 100 times by hand. After resolution of the phases by centrifugation (1,000 g, 5 min), duplicate samples (50 µl) were removed from each phase with a Hamilton syringe, and analysed by l.s.c.

The P-values determined by this method are listed in Table 2:4 and are compared with some of the values obtained by h.p.l.c. and M.A.C.C.S. (Eadsforth, 1984: personal communication). As can be seen, considerable variation exists between the predicted values and those obtained by this procedure, particularly with cypermethrin and 4'HO.cypermethrin. Similar discrepancies have been reported for other pyrethroids (Schimmel et al., 1983), which seem to result from the very small quantities of

Table 2:4

Determination of the partition coefficients (P-values) of cypermethrin and some of it's major metabolites.

Metabolite	Biosynthetic source	T.l.c. purification	P-value obtained	P-value predicted b
cypermethrin a	-	Toluene	1833 ± 33.1	1580000
4'HO.cyper	trout liver in vitro	TEA	9.63 ± 0.2	400000
4'O.cyper.gluc	trout bile in vivo	EFW	10.67 ± 0.23	0.01 160
3.PBA	-	TEA	4.28 ± 0.20	-
4'HO.3PBA	-	TEA	0.88 ± 0.01	-
4'HO.SO_2.O.3PBA	rat urine	BAW, EFW	0.01 ± 0.00	-
Cl_2CA	quail liver in vitro	EFW, TEA	10.05 ± 0.27	0.2 790 3200 c
Cl_2CA.gluc	quail liver in vitro	EFW	0.07 ± 0.00	0.001 3

$$P = \frac{[\text{compound in n-octanol}]}{[\text{compound in 0.1 M buffer pH 7.4}]}$$

Values reported as mean ± S.D.

a. cis- or trans-isomer.

b. Values predicted from M.A.C.C.S. computer model in ionized form and neutral form (underlined).

c. Values obtained by reversed-phase h.p.l.c. method.

(b./c. -Eadsforth, 1984; personal communication .)

equilibrated water-soluble material, compared to the larger amounts of compound present in the aqueous phase as a stable emulsion. Such differences can easily account for several hundred-fold variations in observed n-octanol: buffer ratios, and hence in the P-values. Similar differences are also apparent using h.p.l.c. data in comparison with values obtained by M.A.C.C.S. (eg: P-values for Cl_2CA).

Of greater interest to subsequent discussions are the values for the more polar metabolites ($P<10$), as these are the major excreted products *in vivo*. Thus, the polarity of metabolites is found to sequentially increase following phase I and phase II reactions, such that the compounds are sufficiently non-lipophilic to be eliminated in the urine or bile. The relative proportions of the products excreted by these two routes will largely depend upon the threshold molecular weight for biliary elimination for the species under study (Millburn, 1976).

Chapter 3

The metabolism of cis- and trans-cypermethrin in rainbow trout and frogs

Contents

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1. Materials and Methods

1.1. Animal experiments

Metabolism studies with [^{14}C-cyclopropyl] *cis*-cypermethrin and [^{14}C-benzyl] *cis*- and *trans*-cypermethrin were carried out at 15°C±1°C, under a 12 hour photoperiod between April-June (trout) and November-December (frogs). Experimental conditions were as follows.

1.1.1. Rainbow trout

6 small fish, or 3 large fish, previously starved for 24 hours, were placed in 40 litres of fresh, O_2-saturated water (quality as detailed in Chapter 2). One litre of water was then withdrawn and the dose contained in ethanol (4 ml), added. The radiolabelled solution was returned to the aquarium and mixed in with a glass rod. Final concentrations of cypermethrin isomers in the water were 5 μg l^{-1} for the large fish studies, and 10 μg l^{-1} for the small trout experiments. Full oxygenation of the water was maintained throughout the 24 hour exposure period, any dead fish being removed by netting. The effect of oral exposure on the metabolism of cypermethrin in trout was also investigated. The dose was prepared by adding a concentrated solution of cypermethrin (50 mg ml^{-1} in hexane), to individual pellets (weight 6.6±0.6 mg) with a Hamilton syringe and allowing it to air dry. Small fish were individually held in 10 litres of oxygenated

water and starved for 48 hours. After determining that the animal was feeding with a single pellet, two treated pellets were sequentially added, and it was observed that they were subsequently ingested.

For both routes of exposure, representative samples of water (120 ml) were collected at varying depths, pooled, and analysed immediately using all-glass apparatus. Fish were sampled as required by netting. Pooled water samples were assayed for total radioactivity by l.s.c. (2 ml). Tank water (100 ml) was also extracted (x3) with 15 ml of hexane (pesticide residue grade, Fisons Ltd.). The hexane fractions were pooled, and dried and filtered through a funnel containing pre-washed anhydrous Na_2SO_4. The filtrate was then made up to 50 ml in a volumetric flask, and assayed for cypermethrin isomers by g.l.c. (Chapter 2). Subsequent dilutions were made if required.

At the end of the exposure period, small trout were killed with a blow to the head, and large fish were killed with an overdose of MS-222 SANDOZ (0.1g l^{-1}) (Sandoz Ltd. Basle, Switzerland). Tissue samples were prepared for oxidation analysis (see Chapter 2), and bile was obtained by gall bladder puncture and 5 µl taken for l.s.c.

1.1.2. Frogs

Frogs were housed individually in 1 litre glass beakers, containing distilled water (20 ml). The dose was

introduced as a suspension in 0.1 ml Mulgofen EL 719:ethanol:water (1:1:8 v/v) (Mulgofen:GAF Ltd., Manchester, UK) into the aquarium water, to give a final concentration of 6 mg ml^{-1}. After 24 hours exposure, frogs were killed by pithing, and samples of bile and liver removed for analysis as described previously. Water samples (5 ml) were extracted (x3) with hexane (10 ml), and cypermethrin residues were quantified directly by h.p.l.c. system II (0.2 ml injected).

1.2. Analysis of metabolites

1.2.1. Aquarium water

Samples of fish aquarium water (1 litre), were adjusted to pH 2 with 2M HCL and pumped (Watson-Marlow peristaltic pump) onto a 168mm x 40mm (i.d.) column, containing 100g of pre-washed and equilibrated Lichroprep RP-18 (25-40 µm: Merck), at a flow rate of 5 ml min^{-1}. After washing with 500 ml deionized water (pH 2), the bound radioactivity was recovered by elution with methanol (5 ml min^{-1}), with collection of fractions from 220-400 ml. This extract was then concentrated *in vacuo* at 40°C and analysed by h.p.l.c.. Using this procedure, 91.9% of the radioactivity was recovered from [^{14}C-cyclopropyl] *cis*-cypermethrin studies. For frog experiments, aquarium water was adjusted to pH 2 as above, and 5 ml applied to a pre-equilibrated (C18 SEP-PAK column see Chapter 2). Bound

radioactivity was then eluted with 10 ml of methanol to give 95.7% recovery of the applied ^{14}C, which following concentration, was analysed by t.l.c.

1.2.2. Bile

Bile was analysed directly by t.l.c. and h.p.l.c.. For enzymic analysis and metabolite isolation, pooled bile samples (pH 6.8) were diluted with distilled water (20 vols), adjusted to pH 2 with 0.1M HCl, and extracted (x3) with diethyl ether (2 vols). The pooled ether extracts were then concentrated under a stream of N_2, and the residue taken up in methanol. Mean recoveries of ^{14}C were 92.6% for [^{14}C-cyclopropyl] labelled metabolites and 84.4% for [^{14}C-benzyl] metabolites.

Identification of metabolites was based upon enzyme hydrolysis studies, and co-chromatography with available standards by t.l.c. (solvents EFW, TEA) and h.p.l.c. (system IV and V). Major radiolabelled metabolites were purified by preparative and analytical t.l.c. (solvent EFW), and if necessary by h.p.l.c (system IV), to a single moiety as determined by uv absorbance and l.s.c..

2. Results

2.1. Uptake, elimination, and disposition of cypermethrin isomers

2.1.1. Rainbow trout

Following aqueous exposure to [^{14}C-cyclopropyl] cis-cypermethrin (5 µg l^{-1}) both total soluble ^{14}C and parent compound were found to decrease rapidly over the first hour (Figure 3:1a). By 24 hours, some 40% of radioactivity in the aquarium water was in the form of metabolites. Conversely, following oral administration of cis-cypermethrin (220mg kg^{-1}), radioactivity in the water was found to rise rapidly over the first 4 hours, with a corresponding decrease in intestinal radioactivity (Figure 3:1b). This elimination of ^{14}C from the gut was presumably effected by dispersion in secreted bile. Carcass levels showed a linear increase with time, some 10% of the dose being accumulated in 24 hours, largely from the surrounding water.

Table 3:1 shows the concentration of radioactivity in trout tissues and aquarium water, following aqueous exposure (10 µg l^{-1}), and oral dosing (100 mg kg^{-1}), of cis- and trans-cypermethrin. The concentration of ^{14}C in trout exposed to dissolved cypermethrin shows some differences between the two isomers in all samples eg: the bile (trans>cis 30:7 ppm). In addition, overall metabolite concentrations in the aquarium water were found to be similar at 24 hours (cis= 2.3, trans= 2.1 µg l^{-1}). This indicates that the

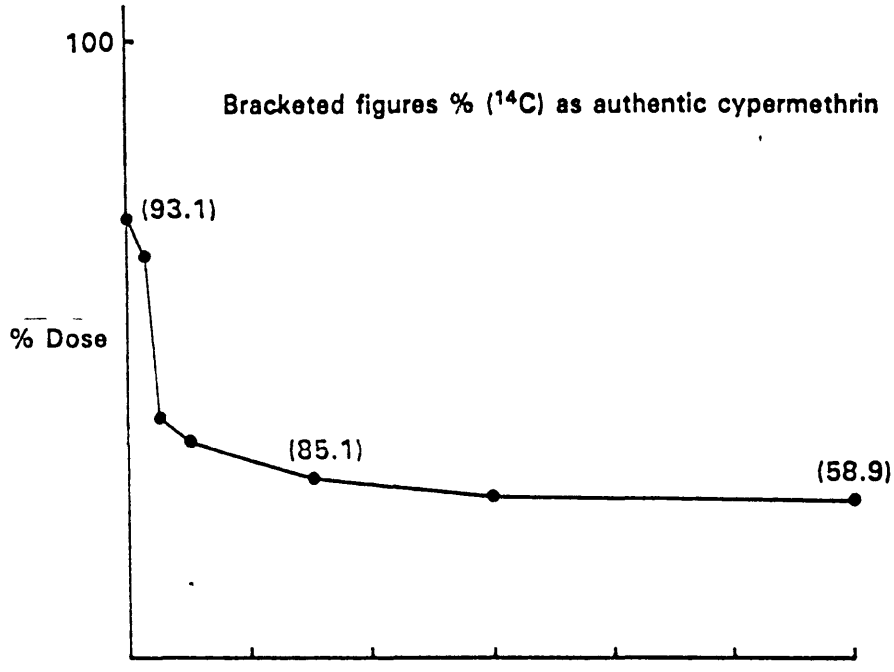

Fig3:1a Disappearance of soluble ^{14}C from aquarium water following static exposure of Rainbow Trout (11.3 g fresh weight per litre water) to 5 µg l^{-1} *cis*-cypermethrin

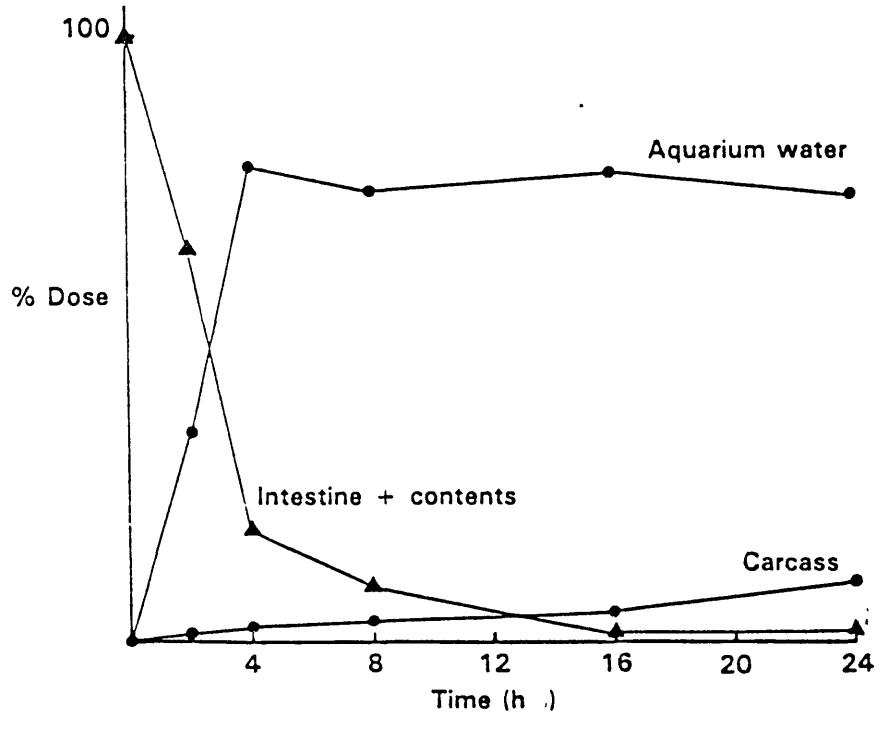

Fig3:1b Disposition of ^{14}C following a single oral exposure of Rainbow Trout to (^{14}C cyclopropyl) *cis*-cypermethrin (188 + 31 mg kg^{-1})

Table 3:1

Concentrations of cypermethrin and it's metabolites in rainbow trout tissues and aquarium water, following aqueous and oral exposure (24h) .

Tissue	Concentration µg [^{14}C]-cypermethrin equivalent per g			
	Cis- aqueous exposure 9µg/l	Trans- aqueous exposure 10µg/l	Cis-Oral exposure 100mg/kg	Trans-Oral exposure 116mg/kg
CARCASS	0.03±0	0.5±0.1	0.8±0.4	4.2±2.1
INTESTINE	0.7±0.3	1.7±1.0	45.2±15.3	145.0±6.0
BLOOD	0.02±0	0.3±0.1	0.8±0.3	6.7±4.3
LIVER	2.6±0.6	2.5±0.8	6.7±3.1	4.7±0.8
BILE	16.7±5.3	29.7±4.3	117.6±42.7	103.9±31.8

FINAL AQUARIUM WATER CONCENTRATION µg l^{-1}

Total ^{14}C equivalents	5.3	5.4	75.9±30.8	35.4±1.9
cypermethrin	3.0	3.3	56.2	19.6

± S.D. Standard deviation. (n= 6)
Values without S.D. values refer to the means of duplicate determinations on pooled samples.

rate of metabolism for the two isomers was similar over a 24 hour period. In oral dosing studies (100 mg kg^{-1}), the concentration of tissue radioactivity was found to be significantly higher in the intestine, carcass and blood of fish treated with trans-cypermethrin, than in their cis—isomer dosed counterparts (Table 3:1). This, in conjunction with the lower concentrations of trans-cypermethrin and its metabolites determined in the 24 hour aquarium water compared with cis-cypermethrin treatments (total ^{14}C cis= 56 μg l^{-1}, trans= 20 μg l^{-1}), indicate a preferential retention of the trans-isomer following oral dosing

The orally administered cis- and trans-cypermethrin represented an equivalent aqueous exposure to the trout, of 130 μg l^{-1}, and 150 μg l^{-1}, respectively. Thus, the higher concentrations of pyrethroid residues associated with trout tissues following oral dosing compared with the 10 μg l^{-1} exposure (Table 3:1), were not unexpected. The time course studies (Figure 3:1b) suggest that the uptake of cypermethrin isomers occurred largely by absorption of the secreted insecticide present in the water. By 24 hours, the aqueous concentrations of insecticide greatly exceeded the true solubility of cypermethrin in water (5-10 μg l^{-1}; Stephenson, 1982), and this implied that the eliminated bile may have a detergent action. Concentrations of cypermethrin in all the trout tissues

except liver, were very much higher after oral treatment than following aqueous dosing. The lower levels of residues in the liver may be due to the rapid removal of metabolites into the bile. The very high concentrations of radioactivity associated with the intestine, were largely associated with the surface intestinal mucosa, and could be readily extracted with methanol washes (3 x 5 vol.). Analysis of the pooled extract by h.p.l.c., showed that 98% of the applied ^{14}C was unchanged pyrethroid. Thus, it appears that orally administered cypermethrin is not effectively absorbed into the intestinal lumen.

Table 3:2 shows the disposition of [^{14}C-cyclopropyl]- and [^{14}C-benzyl]-labelled cis-cypermethrin in three large rainbow trout (contained in a 40 litre aquarium), following a 24 hour exposure to the dissolved pyrethroid (5 $\mu g\ l^{-1}$). A total of 70-80% of the administered radioactivity could be accounted for under these conditions, the remaining ^{14}C being associated with the glass walls of the tank. The recovered radioactivity was divided almost equally between the carcass, bile and surrounding aquarium water. Assuming that each fish received a third of the administered dose, then 19.5%±5.4% of the [^{14}C-cyclopropyl] cis-cypermethrin, and 27.9%±7.0% of the [^{14}C-benzyl] cis-cypermethrin, was recovered as accumulated biliary metabolites.

Table 3:2

The elimination and disposition of cis-cypermethrin in rainbow trout following a 24h aqueous exposure (5 $\mu g\ l^{-1}$).

	% DOSE [^{14}C-cyclopropyl] cis-cypermethrin(n=6)		% DOSE [^{14}C-benzyl] cis-cypermethrin(n=3)	
	Per fish±S.D.	TOTAL[b]	Per Fish±S.D.	TOTAL
TOTAL ^{14}C ADMINISTERED		100		100
^{14}C IN SOLUTION 0hr		69.1		63.0
^{14}C IN SOLUTION 24hr		22.1		27.3
CARCASS	8.5 ±3.7	25.5	7.2 ±1.6	21.6
LIVER	0.4 ±0.1	1.2	0.3 ±0.1	0.9
BILE	6.5 ±1.8	19.5	9.3 ±2.6	27.9
[a] BLOOD	0.6 ±0.2	1.8	0.1 ±0	0.3
TOTAL RECOVERY		70.1		78.0

[a] Figure for % body weight obtained from Conte et al.,(1963)
Brain levels < 0.02% dose

[b] Total refers to the sum of the radioactivity recovered in each treatment for the 3 fish.

2.1.2. Frogs

Since cypermethrin was dosed to the frog in the aquarium water as a suspension, no attempt was made to study rates of uptake or elimination. At 24 hours, 70.7% of the administered [^{14}C-benzyl] trans-cypermethrin was recovered in the aquarium water as radioactivity, with 16% of this ^{14}C in the form of metabolites (11.3% of the dose). Of the absorbed radioactivity, 1.3% of the dose was recovered in the bile, and 1.1% in the liver (8.0±1.1 $\mu g\ g^{-1}$ liver weight), other tissues were not analysed. Detailed metabolism studies with cis-cypermethrin were not carried out, due to the very high acute toxicity of the cis-isomer to frogs under these experimental conditions. However, during studies with 6 $\mu g\ ml^{-1}$ [^{14}C-cyclopropyl] cis-cypermethrin (see Chapter 5), frogs had low concentrations of radioactivity in the bile (1.7 $\mu g\ g^{-1}$) after a four hour exposure, and only 1.6% of the aquarium water ^{14}C was in the form of metabolites.

2.2. Biliary metabolites of cypermethrin isomers

2.2.1. cis-cypermethrin

2.2.1.1. Rainbow trout

After determining that the metabolite profiles of bile from individual trout were qualitatively similar by t.l.c., pooled samples were ether extracted and subjected to chromatographic analysis. When the

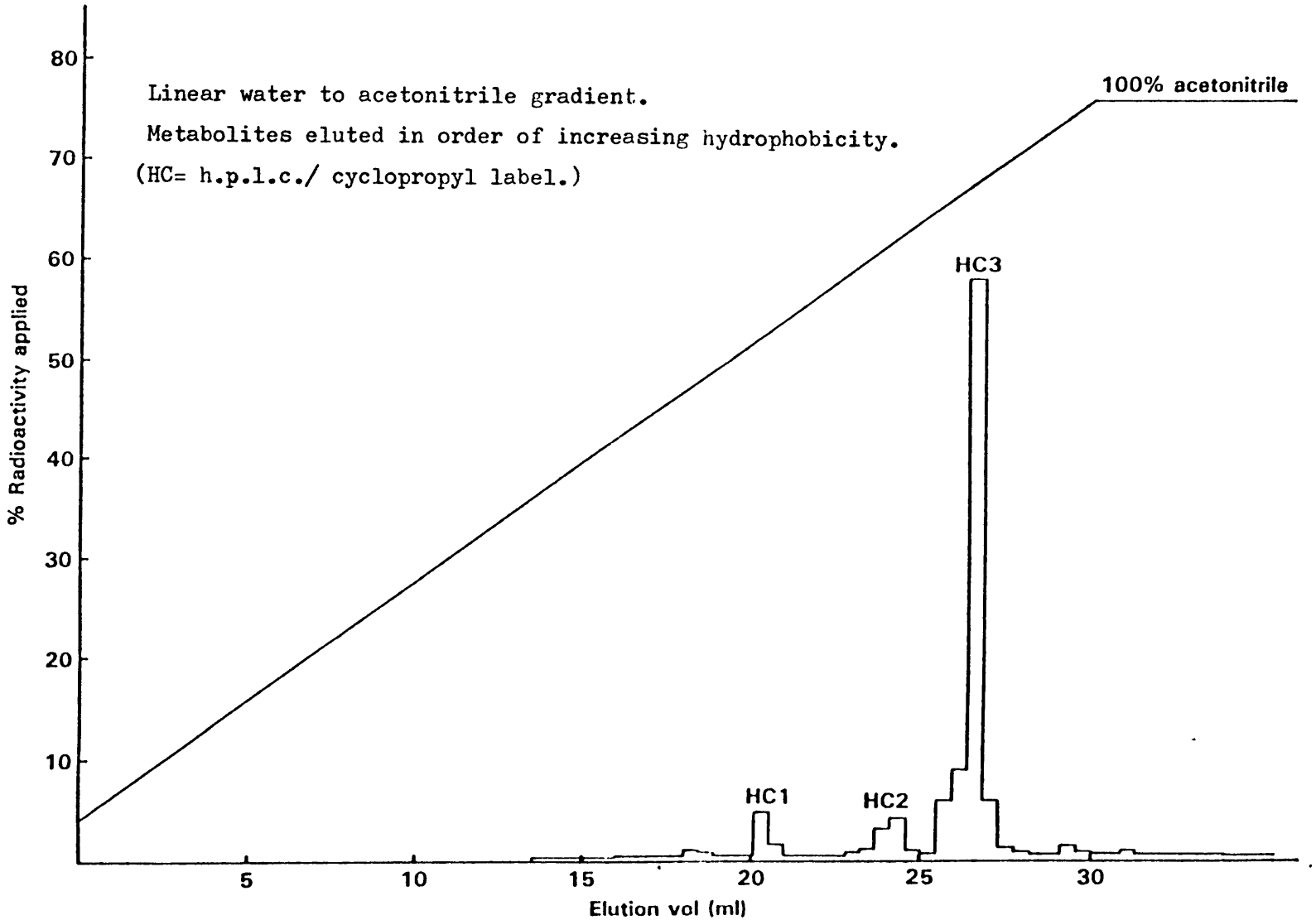

Fig 32 Profile of Rainbow Trout biliary metabolites of (^{14}C-cyclopropyl)-*cis*-cypermethrin by HPLC system IV

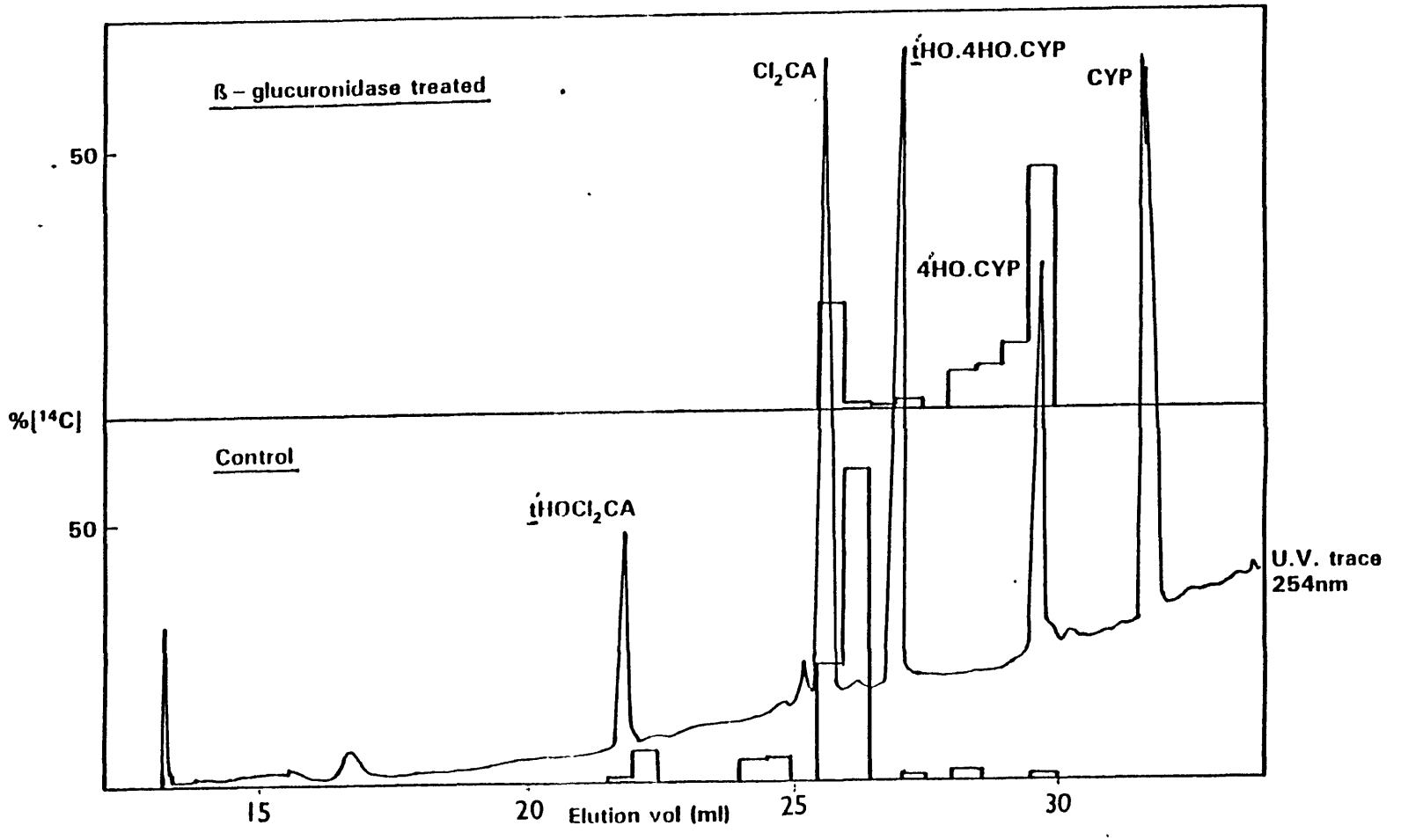

Fig3:3 Effect of ß-glucuronidase on the bi liary metabolite profile of (14C-Cyclopropyl)-*cis*-cypermethrin

biliary metabolites of [^{14}C-cyclopropyl] cis-cypermethrin were analysed by h.p.l.c. (system IV), three major peaks of radioactivity were obtained (see Figure 3:2). These components were termed HC-1, HC-2 and HC-3, and represented 5.0%, 7.1% and 77.1%, respectively, of the applied ^{14}C. All three peaks were readily hydrolysed by ß-glucuronidase (Figure 3:3). With peak HC-3, 72% of the original radioactivity co-chromatographed with 4'HO-cis-cypermethrin following hydrolysis, with 20% yielding an uncharacterised product eluting just before 4'HO-cypermethrin. This minor product could well be a breakdown product of 4'HO-cypermethrin (eg: its quinone).

Similarly, when the biliary metabolites of [^{14}C-benzyl] cis-cypermethrin were analysed by h.p.l.c. and t.l.c., the major radioactive component (79.6% of the bile ^{14}C) co-chromatographed with the material identified as [^{14}C-cyclopropyl] 4'HO-cis-cypermethrin glucuronide, and yielded 4'HO-cypermethrin on treatment with ß-glucuronidase. Following purification (t.l.c. EFW followed by h.pl.c. system IV), the identification of this glucuronide conjugate was further confirmed by mild base hydrolysis (1M NaOH, 37^{o}C, 60 min) of the [^{14}C-cyclopropyl]- and [^{14}C-benzyl]-labelled materials. Oxidation of the hydrolytic products (stream of O_2, 60 mins, 30^{o}C) gave Cl_2CA and the ether glucuronide of 4'HO.3PBA (yield 35% of initial radioactivity). However satisfactory mass

spectra of 4'O.cypermethrin glucuronide were not obtained following either fast atom bombardment, or chemical ionization. The purified metabolite was also used to investigate the possibility of intestinal de-conjugation occuring. Under anaerobic conditions (see Chapter 2), very little β-glucuronidase activity was observed in trout gut contents compared to controls (2.7% of initial ^{14}C released as 4'HO.cypermethrin in 12 hours). However over the same period, the glucuronide conjugate was ester-cleaved (31.0%), to yield Cl_2CA and 4'O.3PBA glucuronide.

T.l.c. analysis revealed eleven other metabolites of [^{14}C-cyclopropyl] cis-cypermethrin in trout bile (Table 3:3). The h.p.l.c. peaks HC-1 and HC-2 gave only a single aglycone species, Cl_2CA, on β-glucuronidase treatment (Figures 3:2 and 3:3). Peak HC-2 corresponded to the radioactive band running in solvent EFW (Rf = 0.33) and was tentatively identified as the glucuronide of Cl_2CA. Peak HC-1 could be resolved into 2 minor metabolites by t.l.c. in EFW (Rf 0 - 0.33), and probably consisted of Cl_2CA conjugated with lactonised glucuronic acid. Similar artefacts of glucuronide conjugates have been observed with the acid moiety of permethrin (Gaughan et al., 1977).

When the bile from trout treated with [^{14}C-benzyl] cis-cypermethrin was analysed in detail by h.p.l.c and t.l.c. (Table 3:4), a total of 13 minor metabolites were detected. These were largely glucuronic acid

Table 3:3

Metabolites of [^{14}C-cyclopropyl]-cis-cypermethrin in rainbow trout and frog bile as determined by t.l.c.

Metabolite identity	T.l.c. solvent & Rf	% of biliary radioactivity.	
		Trout	Frog
	EFW		
Unknown.gluc (HC1)	0- 0.26	7.5 ±0.3	3.5 ±0.2
Unknown	0.27	0.9 ±0.2	2.6 ±0.1
Cl_2CA.gluc (HC2)	0.33	6.9 ±1.0	22.7 ±1.3
4'O.cyper.gluc (HC3)	0.42	79.7 ±0.2	8.3 ±1.1
t'O.cyper.gluc [a]	0.42	n.d.	7.4 ±0.8
	TEA		
Unknown	0.09	1.0 ±0.2	2.5 ±0.2
t'HO.Cl_2CA	0.13	0.9 ±0.0	1.8 ±0.1
O.Cl_2CA.lac	0.16	1.0 ±0.0	9.8 ±0.3
Unknown [b]	0.23,0.25	n.d.	2.7 ±0.2
Unknown	0.26-0.48	0.9 ±0.1	1.4 ±0.0
Cl_2CA	0.46	0.9 ±0.1	32.0 ±0.0
t'HO.cyper	0.49	n.d.	2.7 ±0.2
4'HO.cyper	0.58	0.3 ±0.1	2.6 ±0.4
Total recovery		100%	100%
% of administered dose		19.5 [c]	1.0 [d]

Abbreviations :- cyper, cypermethrin
gluc, glucuronide
lac, lactone
n.d., not detected

Values represent the means of triplicate determinations, ±S.D.

[a] Resolved by analysis of the aglycones.

[b] Metabolite seperated into two components by t.l.c., but elutes as one peak in h.p.l.c. system IV.

[c] As recovered in 24 hours.

[d] As recovered in 4 hours.

conjugates of 3PBA and its hydroxylated derivative, 4'HO.3PBA, Identification was based upon enzymic hydrolysis of metabolites derived from the trans-isomer, as they were present in larger quantities than the corresponding products formed from cis-cypermethrin. In addition, small amounts of 3PBA taurine, 4'HO.3PBA and its sulphate conjugate, and 4'HO.cypermethrin, were also detected. No free 3PBA was identified however.

2.2.1.2 Frogs

Frogs appeared to be more effective than trout at ester cleavage of [^{14}C-cyclopropyl] cis-cypermethrin, and accumulated Cl_2CA and its glucuronide as the major radioactive products in the bile. This only represented a very small proportion of the dose however (Table 3:3). When the radioactive zone from EFW developed plates (Rf 0.42), was excised and treated with ß-glucuronidase, two aglycone species were resolved by t.l.c. (solvent TEA.), which co-chromatographed with 4'HO-cis-cypermethrin and t'HO-cis-cypermethrin, respectively. No t'HO-cypermethrin glucuronide was identified in trout bile however (Table 3:3). An additional interesting species difference was the presence of appreciable amounts of the lactone of $HO.Cl_2CA$ in frog bile (9.8% biliary ^{14}C). Generally, the proportions of unconjugated metabolites were higher in frog than in trout. This may be due to, either the

Table 3:4

Metabolites of [^{14}C-benzyl] cis- and trans-cypermethrin in rainbow trout and frog bile (24 h),as determined by t.l.c.

Metabolite identity.	T.l.c. solvent & Rf	% of biliary radioactivity		
		Trout (cis-)	Trout (trans-)	Frog (trans-)
	EFW			
Unknown	0.0	3.8 ±0.8	1.8 ±0.2	13.7 ±1.2
3PBA taurine	0.08	1.4 ±0.4	1.3 ±0.2	n.d.
Unknown	0.14	1.5 ±0.1	1.2 ±0.1	8.8 ±0.3
Unknown sulphate [a]	0.20	2.7 ±0.1	3.0 ±0.1	9.6 ±0.9
4'HO.SO_2.0.3PBA	0.22	1.0 ±0.1	15.2 ±2.6	23.8 ±1.0
4'HO.3PBA.gluc [b]	0.28	1.0 ±0.1	10.8 ±1.5	11.5 ±0.6
4'O.3PBA.gluc [c]	0.35	1.2 ±0.3	10.7 ±1.0	11.0 ±1.1
4'O.cyper.gluc	0.42	79.6 ±1.6	35.5 ±1.7	7.0 ±0.5
t'O.cyper.gluc [d]	0.42	n.d.	n.d.	6.1 ±0.4
3PBA.gluc	0.48	2.4 ±0.4	3.1 ±0.8	1.0 ±0.1
Unknown	0.60	1.2 ±0.3	2.3 ±0.2	0.8 ±0.1
	TEA			
Unknown	0.05-0.1	2.7 ±0.4	5.2 ±0.1	1.1 ±0.2
4'HO.3PBA	0.18	1.0 ±0.2	7.4 ±0.5	3.0 ±0.4
4'HO.3PBA.art [e]	0.24	n.d.	n.d.	0.4 ±0.0
3PBA	0.28	n.d.	n.d.	0.2 ±0.0
3PBalc	0.43	n.d.	n.d.	0.1 ±0.0
t'HO.cyper	0.48	n.d.	n.d.	0.3 ±0.0
4'HO.cyper	0.60	0.3 ±0.1	0.2 ±0.0	0.3 ±0.0
cyper	0.6-1.0	n.d.	n.d.	1.3 ±0.1
Total recovery		99.8%	99.7%	100%
% of administered dose.		27.9	24.6	1.6

For abbreviations see Table 3:3

a Unknown sulphate characterised more fully in chapter 4.

b Ester glucuronide of 4'HO.3PBA.

c Ether glucuronide of 4'HO.3PBA.

d Metabolite resolved from 4'O.cyper.gluc by treatment with β-glucuronidase and analysis of aglycones in solvent TEA.

e Artefact of 4'HO.3PBA observed during t.l.c.

greater exposure of the amphibian to cis-cypermethrin, resulting in the saturation of glucuronide conjugation, or alternatively, a lower UDP-glucuronyltransferase activity towards these substrates in frogs, compared with trout.

2.2.2. Trans-cypermethrin

2.2.2.1. Rainbow trout

Experiments with the trans-isomer were restricted to benzyl-labelled cypermethrin. Combined h.p.t.l.c. and h.p.l.c. analysis showed that a total of 13 metabolites were present in trout bile (Table 3:4). The glucuronide of 4'HO. cypermethrin was the major radioactive product, representing 35.5% of biliary ^{14}C. As determined by hydrolysis with sulphatase or ß-glucuronidase and analysis of the released aglycones, the other major conjugated metabolites were 4'HO.SO_2.0.3PBA, and the ether and ester glucuronides of 4'HO.3PBA. No free 3PBA of 3PBalc were identified in the bile, though some 3PBA was conjugated with glucuronic acid, and to a lesser extent with taurine. The only other important biliary metabolite was 4'HO.3PBA.

2.2.2.2. Frogs

As determined with cyclopropyl-labelled cypermethrin, only a very small proportion of the administered dose (<2%), was recovered in frog bile.

The t.l.c. metabolite profile of [^{14}C-benzyl] trans-cypermethrin in the bile was more complex in frogs than in trout, largely due to the presence of a number of minor unconjugated metabolites (Table 3:4). The principal metabolite, representing 23.8% of the biliary ^{14}C, was labile to sulphatase treatment (85% hydrolysed in 1 hr), and was identified as 4'HO.SO_2.O.3PBA.. 4'HO.3PBA was also extensively conjugated with glucuronic acid, though the glucuronides of 4'HO-, and t'HO-trans-cypermethrin, were only minor biliary metabolites. In common with trout, 3PBA and 3PBalc were very minor components in the amphibian. However, less of the biliary radioactivity was recovered as 3PBA.glucuronide in the frog, than in the fish.

2.3. Metabolites of cypermethrin in the aquarium water

2.3.1. Rainbow trout

Following a 24 h exposure to 5 $\mu g\ l^{-1}$ [^{14}C-cyclopropyl] cis-cypermethrin, a concentrated extract of the tank water representing 22.1% of the administered radioactivity, was analysed by g.l.c., h.p.l.c. and t.l.c.. As expressed as a percentage of the extracted ^{14}C, the identified constituents were cypermethrin (58.9%), 4'O.cypermethrin glucuronide (5.7%), c-Cl_2CA (6.0%), the lactone of HO-c-Cl_2CA (7.5%), and Cl_2CA glucuronide (12.2)% (total 90.3%). The remaining radioactivity consisted of uncharacterised polar material, probably arising from

photo-oxidation and bacterial degradation of cypermethrin metabolites. In control experiments with [^{14}C-cyclopropyl] cis-cypermethrin dissolved in distilled water (10 µg l^{-1}), only 1.5% of the starting material was degraded in 24 hours at 15°C, largely by ester hydrolysis. The persistence of glucuronide conjugates in the water indicates the lack of any appreciable de-conjugating activity, either mediated by fish enzymes, or by intestinal micro-organisms. Lactonisation of t'HO-c-Cl_2CA was probably brought about by the acid conditions used during extraction of the tank water.

Analysis of [^{14}C-benzyl] cis-cypermethrin aquarium water metabolites, showed similar proportions of recovered radioactivity associated with cypermethrin (61%) and 4'O.cypermethrin glucuronide (4.6%), as found with the cyclopropyl labelled material. The remaining ^{14}C consisted of metabolites whcih were hydrolysed by sulphatase and ß-glucuronidase (6.2% and 8.9% of aqueous radioactivity, respectively), with 4'HO.3PBA being released on enzyme treatment. In addition, unconjugated 4'HO.3PBA (8.5% of recovered ^{14}C), 3PBA (2.0%) and polar breakdown products (8.8%) were also observed.

2.3.2. Frogs

Following a 24 h exposure to 6 µg ml^{-1} [^{14}C-benzyl] trans-cypermethrin, 11.3% of the dose was

present as metabolites in the frog aquarium water. Analysis of the concentrated extract by t.l.c. in solvents EFW and TEA, showed that 84% of the aqueous ^{14}C was unchanged trans-cypermethrin. Eleven metabolites were also identified. As expressed as proportions of the recovered radioactivity, these were largely 4'HO.cypermethrin (1.0%), and 4'HO,3PBA (3.0%) and its glucuronide (3.5%) and sulphate (5.3%) conjugates. Small amounts of t'HO.cypermethrin, 3PBA, 3PBalc and 2'HO.3PBA were also determined.

3. Discussion

In metabolism studies in mammals, the trans-isomers of pyrethroids are invariably eliminated at faster rates than those observed with the cis-isomers (Hutson, 1979). In this study, following a 24 hour exposure to cis- and trans-cypermethrin administered either orally, or dissolved in the water, tissue residues in trout associated with the trans-isomer treatment were appreciably higher than those observed with cis-isomer. The preferential retention of trans-cypermethrin by trout has been observed previously during long term depletion studies with a cis/trans- mixture of cypermethrin (Bennett, 1984). In a detailed study with cis- and trans-permethrin, Glickman et al., (1981a) have shown that over a 24 hour period, there are no significant isomer-dependent differences in either uptake, or tissue elimination of the pyrethroid.

However, over an initial 4 h period, biliary metabolites of cis-permethrin accumulated more rapidly than those derived from the trans-isomer. Differences in the relative rates of elimination of cypermethrin and permethrin isomers, may be explained by the lower activity of pyrethroid hydrolysing esterases towards α-cyano substituted compounds, such as cypermethrin (Soderlund and Casida, 1977). Thus, the limited, largely extra-hepatic esteratic activity in rainbow trout (Glickman and Lech, 1981), may be more readily inhibited by the presence of the α-cyano group, than those oxidative processes of the liver which favour cis-isomer metabolism (Glickman et al., 1979). From these experiments, it is not possible to determine the relative rates of metabolism of cis- and trans-cypermethrin in frogs.

The importance of urinary excretion of cypermethrin metabolites was not investigated, though it seems likely that the majority of the polar radioactivity in the frog aquarium water was due to this route of elimination, as no faeces were observed in the tanks after a 24 h exposure. In the case of trout, no distinction could be made as to the origins of the metabolites in the water. However, Glickman et al., (1981a) have shown that urinary elimination of permethrin metabolites was very limited in catheterised fish. Using permethrin radiolabelled in its alcohol moiety, the major urinary metabolite was tentatively

identified as the sulphate of 4'HO.3PBA. Similarly in this study, 4'HO.SO_2.O.3PBA was identified as an important metabolite of [^{14}C-benzyl] trans-cypermethrin in the trout aquarium water. Ethereal sulphates are known to be formed in amphibians (Williams, 1967), and recently a number of freshwater fish species have been shown to form these conjugates with phenol (Nagel, 1983), quinol (Nagel and Urich, 1983), and cresol, naphthol, and chlorophenol (Layiwola et al., 1983a). In some cases, (eg: phenyl sulphate), excretion seem to be restricted to the urine (Kobayashi et al., 1976). However, the sulphate conjugates of naphthol and chlorophenol have also been observed in the bile (Layiwola et al 1983a). Similarly in these studies, 4'HO.SO_2.O.3PBA was observed as a biliary metabolite of [^{14}C-benzyl] trans-cypermethrin in trout and frogs.

In both amphibians and fish, the accumulated metabolic products in the bile were assumed to be representative of the routes of biotransformation used in vivo, even though only very small percentages of the administered dose were recovered in this form in the frog. Subsequent experiments with liver subcellular fractions (see Chapter 6), have shown this assumption to be valid for hepatic metabolism, though this may not be a model for whole-body detoxification. In the fish, the bile was a major route of elimination of cis- and trans-cypermethrin, and 80% and 36% respectively, of the recovered biliary radioactivity was in the form of

a single metabolite, the glucuronide of 4'HO. cypermethrin. Similarly, Glickman et al., (1981a) have reported that the glucuronide of 4'HO.permethrin was the major metabolite of cis- and trans-permethrin in rainbow trout bile. The elimination of glucuronide conjugates in the bile of fish has been shown to be an important mechanism of excretion for a number of phenolic compounds (see Chapter 1). In addition, the glucuronide conjugates of Cl_2CA,3PBA, and 4'HO.3PBA (ether and ester derivatives), were all eliminated in trout and frog bile. Glucuronide conjugates of the hydroxylated alcohol moiety are the principal biliary metabolites of 3PBA in the rat (Huckle et al., 1981b).

Several species-dependent differences were seen in the biliary metabolites of cypermethrin in frog and trout. Unlike the fish, frogs formed the glucuronide of t'HO. cypermethrin, a conjugation previously reported in cows treated with permethrin (Gaughan et al., 1978a). Also, proportionally more of the products of ester cleavage of both cis- and trans-cypermethrin are formed in frogs, than trout. This probably reflects the more active pyrethroid-hydrolysing hepatic esterases in grassfrogs (Cole and Casida, 1983). The possiblity of de-conjugation of 4'O. cypermethrin glucuronide by the intestinal contents was investigated, since such a reaction could result in the entero-hepatic circulation of a potentially toxic metabolite (4'HO.cypermethrin). The intestinal re-

absorbtion of hydrolysed aglycones has previously been observed with 3PBA and 4'HO.3PBA in rats (Huckle et al 1981b), and with phenol in goldfish (Layiwola et al., 1983b). However, no de-conjugation of 4'O.cypermethrin glucuronide, or of 4'O.3PBA glucuronide which was released following ester hydrolysis of the pyrethroid, was observed. However, it is of interest to note that at neutral pH the glucuronide of 4'HO.cypermethrin remains sufficiently lipophilic (see Chapter 2) to be re-absorbed by the fish through the gills, and thus possibly undergo an alternative form of re-circulation.

Oral exposure of trout to very large doses of cis- and trans-cypermethrin (100 mg kg^{-1}) resulted in higher concentrations of the pyrethroid and its metabolites in tissues, than those obtained following aqueous exposure at 10 $\mu g\ l^{-1}$. These residues appear to be largely derived from the dissolved pyrethroid, following its elimination in secreted bile, and not from intestinal uptake (see Chapter 5).

Chapter 4

Comparative metabolism of cypermethrin in quail, rat and mouse

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1. Introduction

This chapter describes comparative metabolic studies carried out with [^{14}C-benzyl] *cis*- + *trans*-cypermethrin (2mg kg^{-1}) in quail, rat and mouse, following i.p. administration. In addition, the biotransformation of orally dosed [^{14}C-benzyl] *cis*- + *trans*-cypermethrin (2mg kg^{-1}) and [^{14}C-cyclopropyl] *cis*-cypermethrin (20, 37, 300 and 750mg kg^{-1}) has been investigated in Japanese quail, to establish the rates and routes of metabolism at sub-toxic and toxic doses. These investigations complement the studies of Hutson *et al.*, (1981) and Crawford *et al.*, (1981a; 1981b) carried out with orally dosed cypermethrin (2mg kg^{-1}) in mice and rats, respectively.

2. Materials and methods

2.1. Quail

Young, laying female Japanese quail were dosed i.p. with 0.5 ml of Mulgofen (EL-719):ethanol:water (1:1:8 v/v) containing [^{14}C-benzyl] *cis*- + *trans*-cypermethrin (2mg kg^{-1}, 13 µCi per bird). Alternatively, oral doses of [^{14}C-benzyl] *cis*- + *trans*-cypermethrin (2mg kg^{-1}, 13 µCi per bird), or [^{14}C-cyclopropyl] *cis*-cypermethrin (20, 37, 300 and 750mg kg^{-1}; each at 20 µCi per bird) were administered by gavage in corn oil (0.5 ml). The specific activity of these preparations was determined by h.p.l.c. (see Chapter 5). For the duration of the study, birds were

housed individually in metal cages, and excreta and any eggs collected 4 and 24h after dosing, and then daily for up to 7 days at room temperature on trays covered with aluminium foil. Excreta samples were stored at -20°C prior to analysis. Food and water were available ad libitum throughout the study. At the end of the experiment, birds were killed by cervical dislocation and tissue samples assayed for radioactivity as described in Chapter 2 (section 5.2.).

2.2 Rat and mouse

Young male Wistar rats and CF-1 mice, were dosed i.p. with [^{14}C-benzyl] cis- + trans-cypermethrin (2mg kg^{-1}), 10 µCi and 1 µCi being administered respectively per animal, using the same vehicle as used in the quail studies (0.5 ml per rat, 0.1 ml per mouse). The rodents were housed in appropriately sized all-glass Metabowls (Jencons Ltd., Hemel Hempstead, Herts., UK.), designed for separate collection of urine and faeces. Excreta samples were removed at 4 and 24h after dosing, and thereafter at daily intervals. Food and water were available ad libitum throughout the experiment. At the end of the study animals were killed and samples taken for analysis as described previously.

2.3. Analysis of metabolites

Urine and extracts of faeces and bird excreta, were analysed directly by t.l.c. and h.p.l.c.. Identification of metabolites was based on enzyme hydrolysis studies, and co-chromatography with reference compounds, as described in Chapter 2. No attempt was made to characterise ^{14}C in the tissues.

3. Results

3.1. Elimination of [^{14}C] cypermethrin in quail

When orally dosed with 2mg kg^{-1} [^{14}C-benzyl] *cis*- + *trans*-cypermethrin, quail eliminated the radioactivity extremely rapidly in the excreta, with 65% of the administered ^{14}C recovered in 4 hours (Figure 4:1). Analysis of the excreted metabolites showed that this rapid detoxification was not due to voiding of unabsorbed cypermethrin, as only 4.4% of the dose was recovered as unchanged pyrethroid (see Table 4:2). Similarly, Gaughan *et al.*, (1978b) have reported that orally dosed *cis*- and *trans*-permethrin were very readily absorbed, metabolised, and excreted in laying hens. By contrast, the radioactivity derived from [^{14}C-benzyl] *cis*+*trans*-cypermethrin was eliminated more slowly in quail dosed i.p. than *per os* (p.o.) (Figure 4:1), with almost 20% of the dose remaining in the tissues after 7 days (Table 4:1). This residual ^{14}C was largely associated with fatty tissues (adipose reserves, liver and skin) and this suggests that

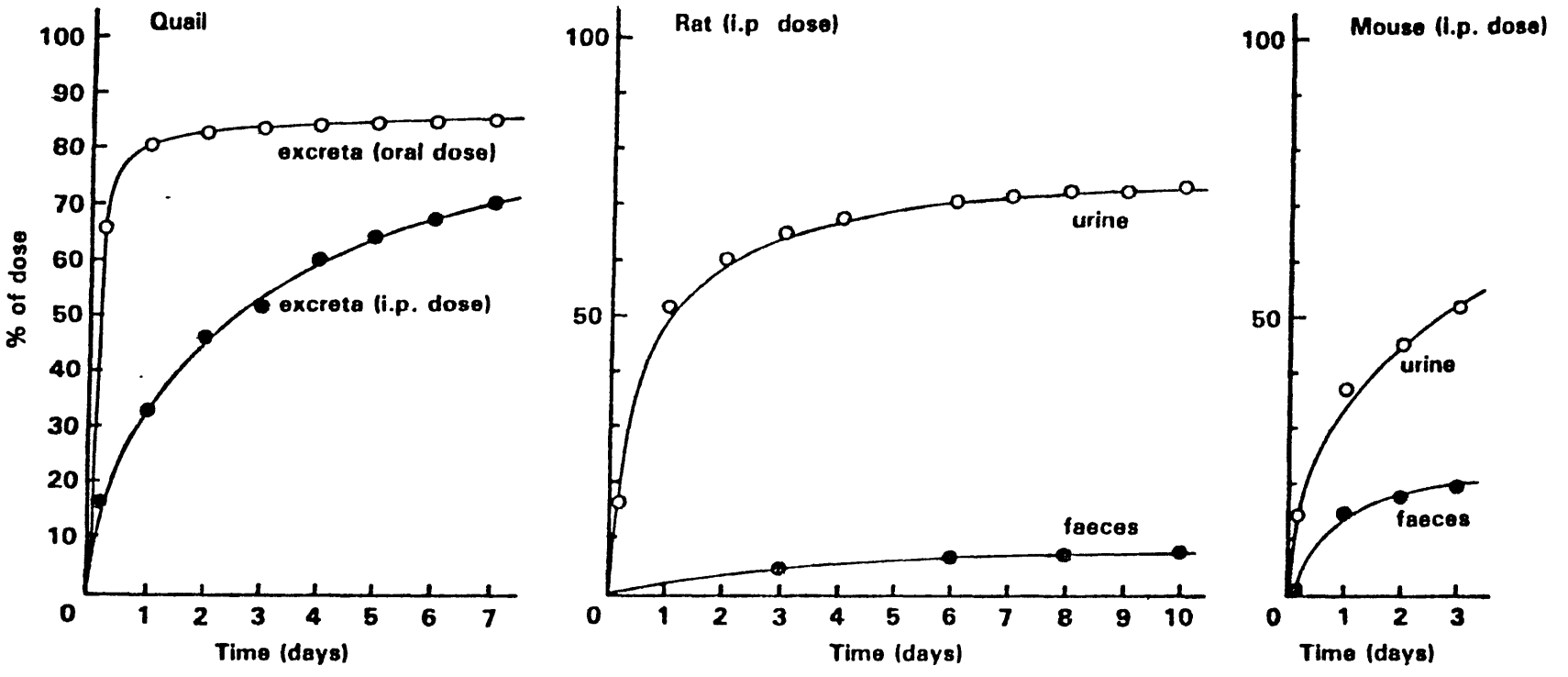


Fig4:1 Elimination of radioactivity from quail, rats and mice administered [^{14}C benzyl]-labelled cypermethrin

Table 4:1 Elimination and disposition of radioactivity following the administration of [^{14}C —benzyl] labelled cypermethrin to quail, rats and mice.

Parameter	Quail		Rat	Mouse
Route of administration	oral	i.p.	i.p.	i.p.
Dose of cypermethrin (mg kg^{-1} body wt)	2	2	2	2
Dose of radioactivity (μCi per animal)	13	13	10	1
Number of animals per dose	3	4	3	6
Days until sacrifice	7	7	10	3
Radioactivity found as % of that administered [a]				
Excreta	84.7±3.6	70.6±5.0	-	-
Urine	-	-	73.9±3.3	52.3±6.9
Faeces	-	-	7.2±3.0	19.4±1.6
Cage washings	2.2±0.7	4.7±3.0	0.2±0	8.7±2.5
Total excreted	86.9	75.3	81.3	80.4
Tissue residues at day of sacrifice as % of dose of radioactivity [a]				
Adipose tissue	0.1±0	8.1±5.0	0.95±0.5	-
Intestine and contents	)	)	0.59±0.5	-
Liver	) 0.1±0	) 5.8±5.0	0.07±0	-
Kidney	)	)	0.01±0	
Skin	0.6± [b]	2.2±0.7 [c]	0.24±0.1	0.3±0.1
Brain	0	0.01	-	-
Carcass	0.1±0.1	1.9±0.5	0.45±0.15	5.2±0.8
Total tissue radioactivity	0.9	18.0	2.3	5.5
Total recovery of radioactivity	88.1±3.6[d]	93.3±5.1	83.6±4.2	85.9±7.1

[a] Data are expressed as means ± SD.

[b] Skin, 0.3±0%; feathers, 0.3±0.2%.

[c] Skin and feathers together.

[d] Eggs collected over the 7-day period of the experiment contained 0.3±0.1% of the dose of ^{14}C.

following injection, the lipophilic pyrethroid becomes sequestered in fat deposits, and that this lack of availability for metabolism limits the rate of excretion. The retention of pyrethroid residues in fatty tissues has been similarly observed in hens, following dermal exposure to permethrin (Hunt et al., 1979). Fat depletion studies with cypermethrin in rats, show that the cis-isomers are particularly persistent (Crawford et al., 1981a; Marei et al., 1982), though the individual isomers were not investigated in this study.

Residues in orally dosed quail were very low (0.9% of dose at 7 days), and only 0.3% of the administered ^{14}C was recovered in the eggs, largely associated with the yolk (Table 4:1). Studies with orally dosed permethrin (Gaughan et al., 1978b), and fluvalinate, (Staiger et al., 1982) in chickens,have also shown negligible residues in the eggs and tissues.

When [^{14}C-cyclopropyl] cis-cypermethrin was given orally to quails at toxic doses (n= 2-5 birds per treatment), the elimination of radioactivity was monitored, so as to assess the protective role of metabolism in determining the low acute toxicity of pyrethroids in birds. When administered at 20, 37, 300 and 750mg kg^{-1}, 44.3%, 19.4%, 11.0% and 12.3% respectively, of the dose was recovered in the excreta in 2 hours. In a longer term study, 50.1% of the radioactivity was recovered in 4 hours and 60.0% in 5

hours, following oral exposure to 300mg kg^{-1} [^{14}C] *cis*-cypermethrin. Analysis of the excreted metabolites (Table 4:3), showed that at these toxic doses more unabsorbed and unchanged cypermethrin was eliminated than at 2mg kg^{-1} p.o.. However, the recovery of half of the 300 mg kg^{-1} dose in 4 hours compared favourably with the 65% elimination achieved at the sub-toxic dose of 2mg kg^{-1}. Furthermore, the majority of the radioactivity in the excreta (63%) was in the form of metabolites, and was not unabsorbed pyrethroid.

3.2. Elimination of [^{14}C] cypermethrin in rat and mouse

Following i.p. administration of [^{14}C-benzyl] *cis*- + *trans*-cypermethrin, the total rate of elimination of radioactivity in the urine and faeces, was greater in the rodent species than in similarly dosed quail, with no appreciable difference between the rat and mouse. In the rat, the majority of excreted ^{14}C was found in the urine, with only 10% of the dose being recovered in the faeces over a 10 day period (Figure 4:1, Table 4:1). This contrasts with the distribution of excreted radioactivity following oral exposure to cypermethrin, where a greater proportion of the dose (20-50%) was found in the faeces, with a corresponding reduction in urinary elimination (Crawford *et al.*, 1981a). The decreased faecal elimination of radioactivity following i.p. dosing compared with p.o. administration, can be accounted for by the extensive elimination of the

unabsorbed, unchanged pyrethroid in oral studies (Crawford et al., 1981b).

In mice, the major route of excretion of radioactivity was in the urine following i.p. injection of the pyrethroid (Figure 4:1). However over a three day period, more of the eliminated ^{14}C was recovered in the faeces, than was the case with similarly dosed rats. Hutson et al., (1981) reported that whereas the metabolites of orally administered trans-cypermethrin were largely excreted in the urine, those of cis-cypermethrin were more extensively recovered in the faeces (40% of the dose). Thus, the faecal elimination of radioactivity observed in this study has presumably arisen mainly from the metabolism of the cis-isomers of the racemate, and not from trans-cypermethrin.

3.3. Metabolism of [^{14}C-benzyl] cis- + trans- cypermethrin in quail.

Solvent extracts of the excreta were prepared for individual quail as described in Chapter 2 (section 5:3), and analysed by t.l.c. in solvents EFW and TEA. The 0-4 h , 4-24 h , and 24-48 h extracts from each of these birds showed negligible variation in their metabolite profiles, such that 0-48 h pooled samples were prepared for detailed analysis by t.l.c. (solvents BAW, EFW, CA and TEA) and h.p.l.c. (systems V and VI). The metabolites of orally administered [^{14}C-benzyl] cypermethrin were not characterised in the period from

3 to 7 days after dosing, due to the very low levels of excreted radioactivity. However, the 48-72 h excreta extracts from i.p. dosed birds were shown to be qualitatively similar in metabolic profile to the 0-48 h preparations. Analysis by h.p.l.c. (system V), showed the metabolism of both i.p. and p.o. administered [^{14}C-benzyl] cypermethrin to be extremely complicated, with 35 metabolites being resolved by a combination of reversed-phase h.p.l.c. (Figure 4:2) and t.l.c. (Table 4:2). In order to simplify the analysis of the components, the h.p.l.c. water to acetonitrile gradient (ie: compounds eluted in order of increasing hydrophobicity), was sub divided into fractions as indicated in Figure 4:2 and Table 4:2. The eluates from several runs were then pooled and concentrated in vacuo (40^{o}C), prior to further analysis. These fractions are considered in turn.

Fraction 1. (eluted with 5-40% acetonitrile): This fraction represented 21-22% of the administered i.p. and oral doses, and was resolved into five radioactive zones by t.l.c. in solvent BAW (Table 4:2), none of which co-chromatographed with any available standards. On treatment of these metabolites with H-1 sulphatase, some 50% of the total radioactivity was released as two moieties, which had Rf values in solvent EFW of 0.66 and 0.80, respectively. However, Fraction 1 was stable to a range of β-glucuronidase preparations. It was

Figure 4:2 H.p.l.c. analysis of excreted metabolites of [^{14}C-benzyl]-cypermethrin in orally dosed quail.

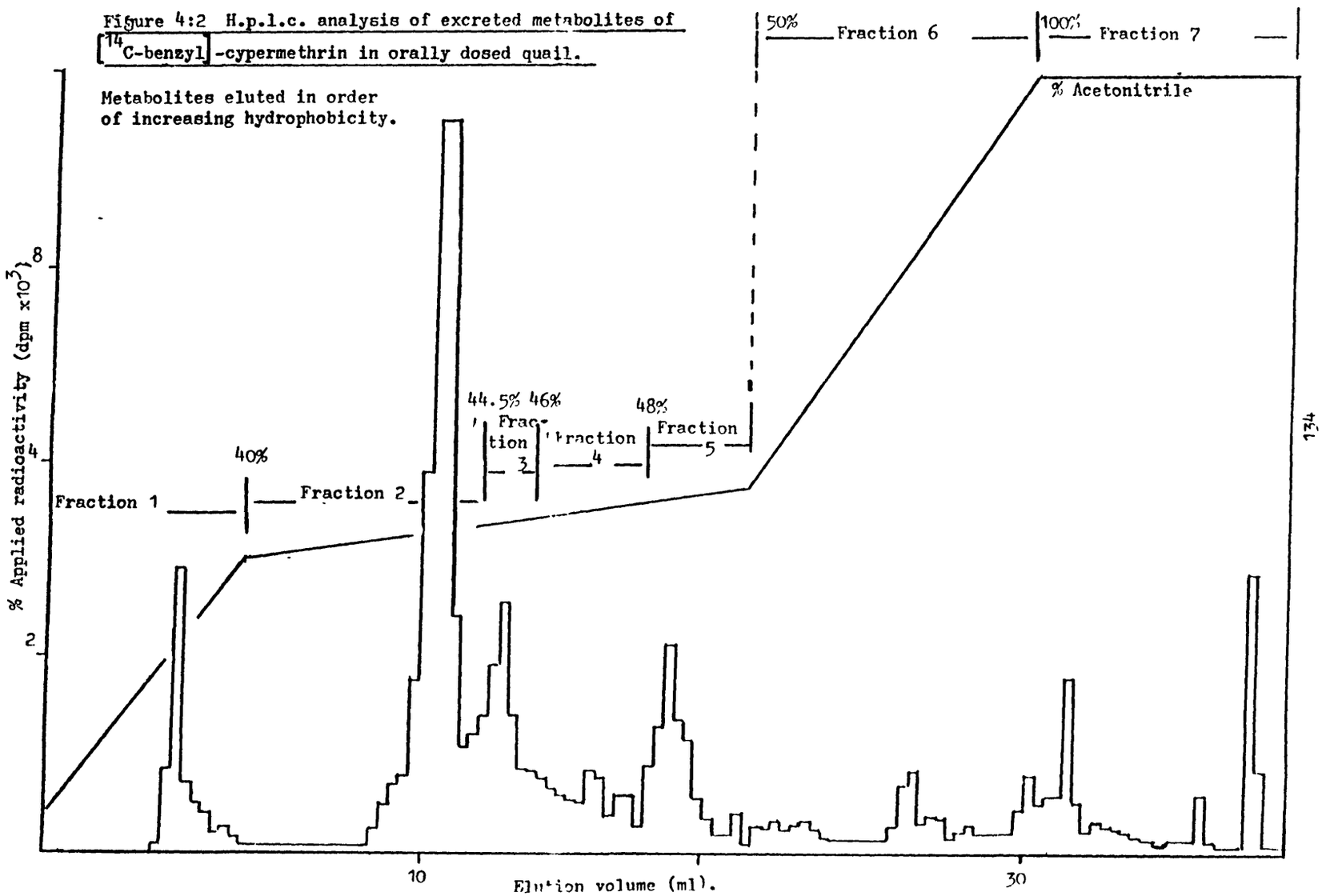


Table 4:2 Metabolites of [^{14}C-benzyl] cypermethrin in quail excreta (0- 7 days).

H.p.l.c.[a] fraction system V	Metabolite characterization Total number	Identity	t.l.c.. solvent/Rf	% of chromatographed ^{14}C (% dose) Quail p.o.	Quail i.p.
1. 5-40%	5 [b]	EFW			
	3	unknowns	0-0.05	24.2 (21.3)	28.9 (21.8)
	2	U/Nsulphates			
2. 40-44.5%	6	3PBA taurine	0.10	2.6 (2.3)	- (-)
		unknown	0.12	4.6 (4.0)	2.0 (1.5)
	I.	U/N.sulphate I	0.15	5.2 (4.6)	2.3 (1.7)
	II.	4'HO.SO_2.0.3PBA	0.20	11.6 (10.2)	10.0 (7.5)
	III.	U/N.sulphate	0.24	6.7 (5.9)	11.8 (8.9)
	IV.	U/N.sulphate	0.27	2.8 (2.5)	- (-)
3. 44.5-46.0%	3	unknown	0.30	1.7 (1.5)	- (-)
	V.	4'O.3PBA.gluc	0.33	1.6 (1.4)	9.0 (6.8)
		unknown	0.43	1.2 (1.1)	2.3 (1.7)
4. 46.0-48.0%	4	α/δ.3PBA orn	0.08	4.3 (3.8)	3.6 (2.7)
	2	unknowns [c]	0.48	4.9 (4.3)	1.1 (0.8)
		3PBA glutamate	0.79	3.5 (3.1)	1.4 (1.0)
5. 48.0-50.0%	7	3PBA N-Ac-orn	CA 0.05	3.0 (2.6)	2.2 (1.7)
		3PBA glycine	0.08	2.9 (2.6)	3.7 (2.8)
		unknown	0.11	0.5 (0.4)	- (-)
		unknown	0.17	1.3 (1.1)	- (-)
		4'HO.3PBA	0.22	3.4 (3.0)	3.0 (2.3)
		4'HO.3PBA.art	0.28	0.4 (0.4)	2.4 (1.8)
		2'HO.3PBA	0.41	0.5 (0.4)	- (-)
6. 50.0-100%	7	EFW			
	VI.	4'O.cyper.gluc	0.37	2.6 (2.3)	1.6 (1.2)
	3	unknowns [d]	0.58	0.9 (0.8)	6.1 (4.6)
		3PBA serine	0.66	0.6 (0.5)	1.3 (1.0)
		3PBA glyval	0.79	1.7 (1.5)	2.1 (1.6)
		3PBA	0.95	- (-)	1.4 (1.0)
7. 100%	4	TEA			
		t'HO.cyper	0.44	0.6 (0.5)	0.8 (0.6)
		4'HO.cyper	0.50	0.9 (0.8)	0.5 (0.4)
		unknown	0.80	0.6 (0.5)	0.3 (0.2)
		cyper	0.85	5.0 (4.4)	1.4 (1.1)
Totals	36 possible metabolites			99.8 (87.8)	99.2 (74.7)

Abbreviations used: cyper, cypermethrin (cis- & trans-)
gluc, glucuronide; glyval, glycylvaline
orn, ornithine; art, artefact.

Values represent the means of duplicate (h.p.l.c.) or triplicate (t.l.c.) determinations

a Water to acetonitrile gradient, metabolites eluted in order of decreasing polarity.

b 5 components resolved by t.l.c. in solvent BAW Rf's 0,0.06,0.10, 0.16,0.19.

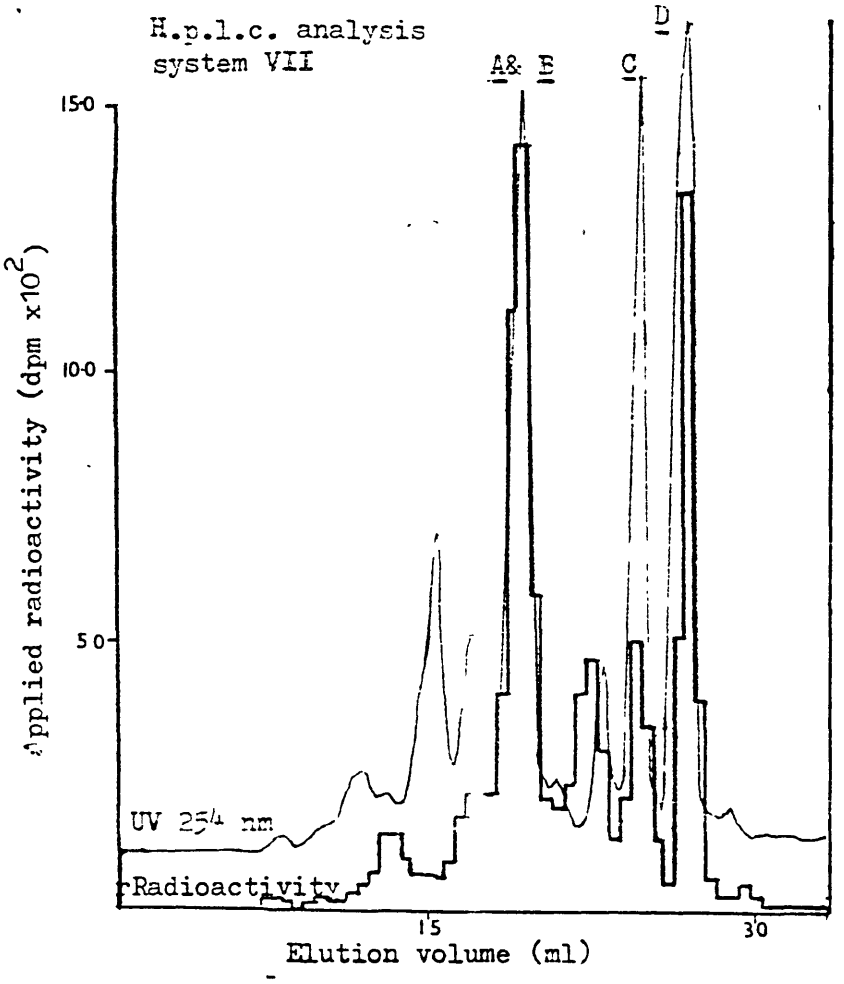
c 2 components as resolved by h.p.l.c. system VI

d 3 components resolved by t.l.c. in solvent TEA

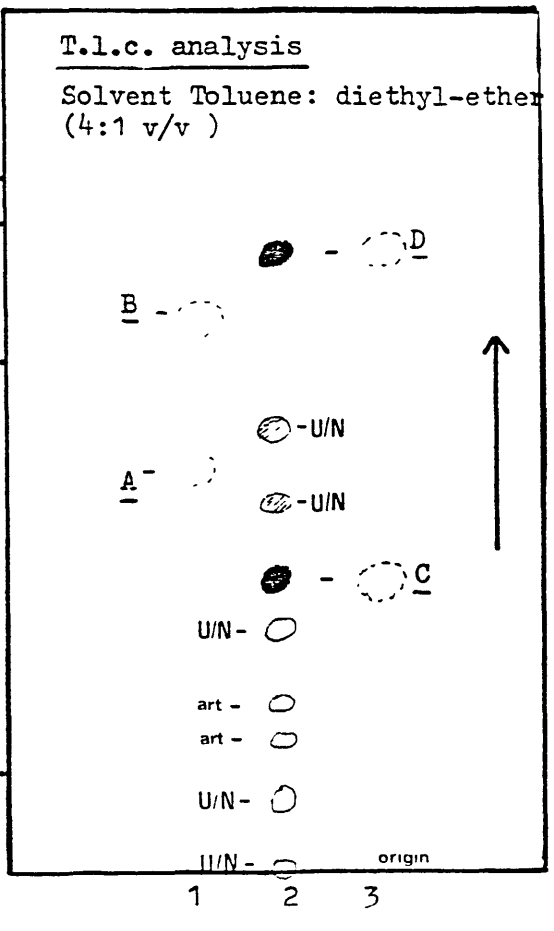
therefore concluded, that at least two of these components were polar sulphate conjugates of hydroxylated 3PBA. Gaughan et al., (1978b) reported the presence of an uncharacterised amino acid conjugate of 4'HO.3PBA in chicken excreta following oral administration of permethrin. Sulphation of such a derivative would produce metabolites possessing the polar characteristics of some of the Fraction 1 components. Extensive contamination of these compounds with bile salts, and the difficulty in analysing such hydrophilic metabolites (ie: resolution of metabolites only possible in very polar t.l.c. systems, eg: BAW), prevented any further characterisation.

Fraction 2 (eluted with 40-44.5% acetonitrile): When derived from orally dosed birds, this fraction was eluted as a single peak in h.p.l.c. system V (Figure 4:2), but could be resolved into 3 peaks by h.p.l.c. sytem VI, and six distinct bands by t.l.c. in solvent EFW as determined by autoradiography (Table 4:2). When treated with sulphatase, four of these six metabolites were quantitatively hydrolysed, and the released radioactive moieties were methylated (see Chapter 2) and analysed by h.p.l.c., and t.l.c. (Figure 4:3). Analysis by h.p.l.c. (system VII) indicated that there were four major peaks present, containing 36.6%, 15.9%, 11.5% and 21.2% of the applied radioactivity, respectively. Co-chromatography with similarly

Figure 4:3 Identification of methylated derivatives of hydroxylated 3PBA released following treatment of quail metabolites with sulphatase.



Derivitive	Identity
A	mono-methyl-2'HO.3PBA
B	di-methyl-2'HO.3PBA
C	mono-methyl-4'HO.3PBA
D	di-methyl-4'HO.3PBA



U/N unknown derivative

art artefact of methylation

Key for thin-layer chromatogram.

1. Methylated standards A& B.
2. Methylated metabolites relesed on sulphatase action.
3. Methylated standards C& D.

methylated standards suggested that the derivatives of 4'HO.3PBA, 2'HO.3PBA and an unknown were present in the ratio 2:1:1. However, when analysed by t.l.c. in toluene:diethyl ether (4:1 v/v), though the presence of methylated 4'HO.3PBA was confirmed, no methylated 2'HO.3PBA was detected; the remainder of the radioactivity being largely associated with two unknown derivatives (Figure 4:3). In addition, there was no co-incidence of u/v absorbance and radioactivity following t.l.c. of Fraction 2 in solvent EFW, when reference 2'HO.SO_2.O.3PBA was added as a carrier.

On the basis of these studies, and the stability of Fraction 2 to β-glucuronidase, metabolites I, II, III and IV (Table 4:2) were identified as sulphates, though only II was positively characterised (4'HO.SO_2.O.3PBA). Metabolite I possesses some of the chromatographic properties of 2'HO.SO_2.O.3PBA, and may be the sulphate conjugate of 6'HO.3PBA or 5HO.3PBA. These phenol derivatives have been observed in mice *in vivo* (Hutson *et al.*, 1981). Metabolite III and IV appear to be related to 4'HO.SO_2.O.3PBA. Similar unidentified sulphates have been obtained in previous studies with cypermethrin in mice (Hutson *et al.*,1981) and with 3PBA in rats (Huckle *et al.*, 1981a) and chickens (Huckle *et al.*, 1982). In all of these investigations, a sulphate conjugate which was distinguishable from 4'HO.SO_2.O.3PBA in solvent EFW (Rf = 0.29) consistently broke down to yield 4'HO.3PBA

derivatives following treatment with sulphatase. Hutson et al., (1981) suggested that the metabolite may be the sulphated conjugate of dihydroxylated 3PBA. Chromatographic evidence suggests that this corresponds to band III, as obtained in this investigation. Metabolite I may be the sulphate of 4'HO.cypermethrin, this conjugate having been previously reported in mice (Hutson et al., 1981). The corresponding derivative of permethrin is a major metabolite in orally dosed chickens (Gaughan et al., 1978b). Any 4'HO. cypermethrin released on sulphatase treatment would be degraded following methylation (Hutson, personal communication), and thus would not be identified by this procedure. Metabolite IV was not found in i.p. dosed birds.

In addition to sulphate conjugates, two minor peaks were resolved by t.l.c. in solvent EFW, which were stable to hydrolysis by β-glucuronidase, sulphatase, and 1M NaOH. Co-chromatography (h.p.l.c. system VI and t.l.c. (BAW)) identified one of these metabolites as 3PBA taurine, though it was only present in orally dosed quails (Table 4:2). Taurine has been shown to be an important conjugating amino acid for several aryl-acetic acids in some bird species, notably the Passeriforms such as pigeons (Idle et al., 1976).

Fraction 3. (Eluted with 44.5-46.0% acetonitrile): This fraction contained three components as resolved by

t.l.c. in solvent EFW. Metabolite V (Rf 0.33) was resistant to hydrolysis by sulphatase and mild base treatment (1M NaOH), but labile to β-glucuronidase. From these characteristics, and the mobility of the compound in the t.l.c. systems BAW and EFW (c.f. Huckle et al., 1981a), it was concluded that this compound was the ether glucuronide of 4'HO.3PBA. Comparison of the proportions of this metabolite formed by the two different routes of administration, showed that the formation of this product was greater in birds dosed i.p., than in quail given cypermethrin orally (Table 4:2). Similarly Capel et al., (1974) have shown that the conjugation of phenol with glucuronic acid was more pronounced in hens dosed i.p., than in birds exposed p.o.. In the case of phenol, this can be explained by the more rapid uptake of the material into the liver following direct injection into the peritoneum, rather than the slower absorption through the intestinal lumen. This results in the higher hepatic substrate concentrations which promote glucuronic acid conjugation rather than sulphation. However, such an explanation may not be relevant with a xenobiotic such as cypermethrin, which undergoes a slow release from peritoneal fat following administration by i.p. injection.

The other two metabolites present in this fraction were stable to chemical and enzymic hydrolysis, and could not be identified by co-chromatography with

available standards. It was concluded that they were probably unknown amino acid conjugates, or uncharacterised products of partial pyrethroid degradation.

Fraction 4. (Eluted with 46.0-48.0% acetonitrile): This eluate contained four metabolites as determined by t.l.c. in solvents EFW and BAW (Table 4:2). Both 3PBA glutamate, and an uncharacterised (α- or δ-) ornithine conjugate of 3PBA were tentatively identified using reference compounds. Ornithine is a major conjugating agent of benzoic acid in several Galliforms , but not apparently in other avian orders (Huckle and Millburn, 1982). In studies with 3PBA in the chicken, Huckle et al., (1982) identified an ornithine conjugate of 3PBA (α - or δ- substituted) as a minor excreted metabolite (6.3% of dose), though none were reported in the excreta of mallard ducks (an Anseriform: Huckle et al., 1981b).

Fractions 5 (Eluted with 48.0-50.0% acetonitrile): This fraction contained a total of seven components, with the glycine and N-acetyl-ornithine conjugates of 3PBA being identified by two-dimensional t.l.c. (solvents EFW followed by CA). The presence of 3PBA N-acetyl-ornithine is particularly significant, as this is the principal metabolite of hens dosed orally with 3PBA (Huckle et al., 1982). The presence of this conjugate

in quail dosed with [^{14}C-benzyl] cypermethrin confirms this metabolic pathway in another Galliform species.

In addition to these metabolites, unconjugated 2'HO.3PBA and 4'HO.3PBA were identified by t.l.c. in solvent CA, and by t.l.c. and h.p.l.c. following methylation. Using solvent system CA,several unknown metabolites which appear to be hydroxylated forms of 3PBA be resolved (Rf 0.11, 0.17, 0.28). In particular, the moiety described in Table 4:2 as an artefact of 4'HO.3PBA consistently appeared in chromatograms of 4'HO.3PBA developed in solvent CA and may be the quinone derivative of the parent compound. The two other related metabolites (Rf 0.11 and 0.17), which remain uncharacterised, were also observed in the studies with rats and mice.

Fraction 6. (Eluted with 50-100% acetonitrile): By a combination of t.l.c. (solvents BAW, EFW) and h.p.l.c. (system VI), this fraction was found to contain 3PBA, and its serine and glycylvaline conjugates as minor metabolites. The formation of 3PBA glycylvaline by the quail is interesting, as this conjugate has been found to be a metabolite of 3PBA in chickens (Huckle et al., 1982) and mallard ducks (Huckle et al., 1981b). In addition to these components, metabolite VI (Table 4:2) was identified by its lability to β-glucuronidase, its stability to sulphatase, and its chromatographic properties as being the glucuronide of 4'HO.

cypermethrin. Unlike trout (see Chapter 3), this was a very minor metabolic product of cypermethrin in both i.p. and p.o. dosed birds. However, the presence of this metabolite in quail is of interest as it confirms the presence of pathways which conjugate derivatives of the intact ester. Similarly, sulphation of 4'HO-permethrin has been reported in chickens (Gaughan et al., 1977b). Three other components present in this eluate could not be characterised by hydrolysis or co-chromatography. Their identity therefore, remains unknown.

Fraction 7. (Eluted with 100% acetonitrile): This fraction contained four metabolites, whose hydrophobicity and co-chromatography with available standards confirmed them as being metabolites of the intact ester. When analysed by t.l.c. in solvent TEA, cypermethrin and its 4'-, and t'- hydroxylated derivatives were all identified as minor metabolites. As expected, rather more of the administered radioactivity was recovered as unchanged cypermethrin following oral dosing than was the case after i.p. exposure. The unknown metabolite, with an Rf in solvent TEA of 0.80 has been observed as a minor metabolite in a number of species following in vitro studies with cypermethrin isomers (see Chapter 6), but has not yet been characterised.

The metabolism of [^{14}C-benzyl] cypermethrin in quail dosed orally and i.p.is therefore extremely complex, with 35 metabolites of cypermethrin being resolved by a combination of reversed-phase h.p.l.c. and normal-phase t.l.c. (Table 4:2). When excreta extracts from p.o. dosed quail were analysed by two-dimensional t.l.c. in solvent EFW, followed by BAW (ie: two polar systems), 40 metabolites could be resolved by autoradiography of the developed chromatogram. This discrepancy in the total numbers of metabolites present, may have arisen due to partial degradation of compounds (eg: sulphates) in the highly acidic BAW solvent system.

The metabolite profiles obtained following i.p.and oral exposure to [^{14}C-benzyl] cypermethrin were very similar, with some additional minor metabolites being present in the excreta of p.o. dosed birds. Whether these differences were a function of the route of cypermethrin administration, or due to the greater maturity of the quail in the oral study (ie: laying birds), remains unclear. The overall metabolic scheme for the detoxification of [^{14}C-benzyl] cypermethrin is summarised in Table 4:3.

3.4. Metabolism of [^{14}C-cyclopropyl] cis-cypermethrin in quail

These studies with [^{14}C-cyclopropyl] cis-cypermethrin were primarily carried out to establish

Table 4:3. Summary of metabolism of [^{14}C-benzyl] cypermethrin in quail.

Biotransformation Pathway [a]	% of chromatographed radioactivity (% dose)	
	Quail p.o.	Quail i.p.
Hydroxylation of intact ester	4.7 (4.1)	3.2 (2.4)
Ester cleavage [b]	77.0 (67.8)	76.1 (57.3)
4'hydroxylation	20.5 (18.1)	26.5 (20.0)
Alternative hydroxylation	15.9 (13.9)	16.9 (12.7)
Sulphation [c]	38.4 (33.8)	38.5 (29.0)
Glucuronidation	4.2 (3.7)	10.6 (8.0)
Amino acid conjugation	18.6 (16.4)	14.3 (10.8)
Unknown (polar)	27.8 (24.5)	40.7 (30.6)

a The proportions of radioactivity representing each pathway were determined from the positively identified phase I and phase II metabolites added together (eg: both 4'HO.3PBA and it's sulphate ester are products of ester cleavage.)

b Includes hydrophilic components of fraction 1.

c Includes sulphatase labile fraction of fraction 1.

the extent of detoxification of the pyrethroid at acute toxic doses in the quail, and are not directly comparable to the 2mg kg^{-1} experiments with the [^{14}C-benzyl]-labelled pyrethroid. However, when pooled excreta samples (0-2 h) from birds dosed orally at 20mg kg^{-1} were extracted (overall recovery of radioactivity = 83.2%) and analysed by h.p.l.c. (Figure 4:4) and h.p.t.l.c. (Table 4:4), the majority of the recovered radioactivity (78.6%) was in the form of metabolites of cypermethrin. It was therefore assumed, that although subsequent reductions in the administered dose would have increased the proportion of the recovered ^{14}C in the form of metabolites, there would not have been a corresponding increase in the number of radioactive products in the excreta. Thus the data presented in Table 4:4 and Figure 4:4, represent a similar range of metabolic options for the acid moiety in quail as would be obtained at sub-toxic doses (eg: 2mg kg^{-1}).

The results show that [^{14}C-cyclopropyl] cypermethrin has a simpler metabolic profile than [^{14}C-benzyl] cypermethrin, with the excreta samples from 20mg kg^{-1} treatments being resolved into only 15 peaks of radioactivity by h.p.l.c. system IV, and 14 zones by h.p.t.l.c. (solvent EFW). When cis-cypermethrin was orally administered at 750mg kg^{-1}, only seven metabolites of [^{14}C-cyclopropyl] cis-cypermethrin, representing 26.5% of the recovered ^{14}C, could be

identified by h.p.t.l.c.. Analysis of the metabolites showed that this was largely due to a saturation in biotransformation capacity, such that the formation of non-toxic polar conjugates was reduced. Presumably this was brought about by a combination of exceeding the maximal turnover rate of the detoxification enzyme systems, depletion of the available supply of conjugating cofactors, and saturation of the intestinal uptake mechanisms.

The identification of metabolites was based upon h.p.t.l.c. (solvents EFW and TEA) and h.p.l.c. analysis of pooled excreta extracts obtained from quail dosed with 20mg kg^{-1} [^{14}C-cyclopropyl] cis-cypermethrin. Co-chromatography with available standards identified both the glycine and taurine conjugates of Cl_2CA as being present (Table 4:4); Cl_2CA glycine being resolved from t'HO-c-Cl_2CA by h.p.l.c.. The presence of four other unidentified metabolites, which were resistant to hydrolysis (enzymic and 1M NaOH), and which had intermediate polarity in solvent EFW (Rfs 0.44, 0.53, 0.57 and 0.63; see Table 4:4) also suggested that other amino acid conjugates of the acid moiety may be present. Dihalovinyl-dimethyl-cyclopropanecarboxylic acids have previously been reported as conjugating with glycine in mice (Ruzo et al., 1979; Hutson et al., 1981) and rats (Crawford et al., 1981b), with glutamate in cows (Gaughan et al., 1978a) and with taurine in

Figure 4:4 H.p.l.c. analysis of excreted metabolites of [^{14}C-cyclopropyl]-cis-cypermethrin in orally dosed quail

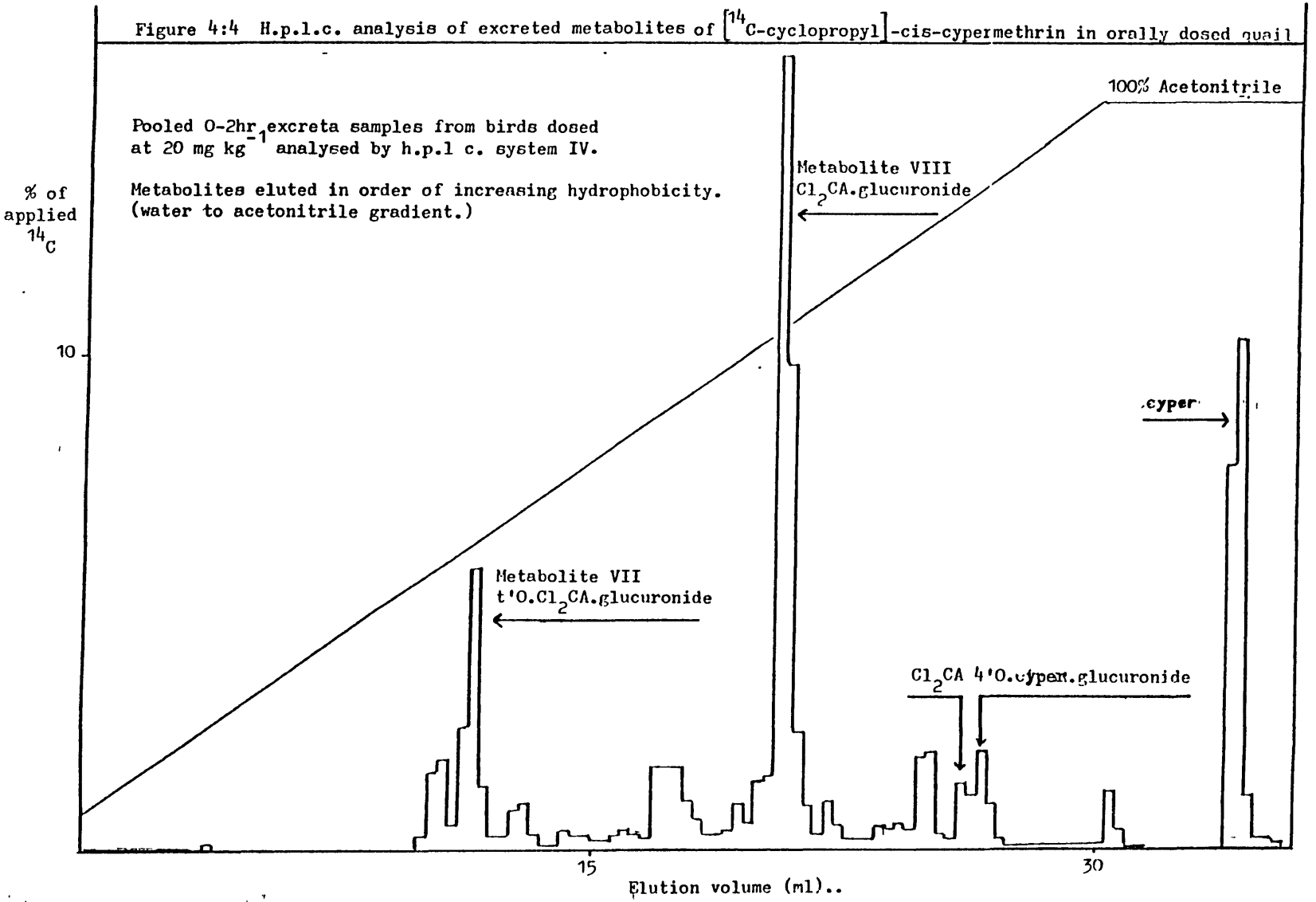


Table 4:4 Metabolites of [^{14}C-cyclopropyl]-cis-cypermethrin in quail excreta (0-2 h) following oral exposure to toxic doses.

Metabolite identity.	Rf (EFW)	[a] % of chromatographed ^{14}C			
		20mgkg^{-1}	37mgkg^{-1}	300mgkg^{-1}	750mgkg^{-1}
U/N	0	2.4	5.2	1.7	3.3
Cl_2CA.tau	0.10	7.2	2.5	2.6	0.8
tHOcCl_2CA.gluc VII	0.17	14.5	18.0	15.5	4.0
Cl_2CA gluc VIII	0.30	25.4	33.5	20.5	8.0
4'O.c.cyper.gluc IX	0.37	6.4	7.2	2.5	1.4
U/N	0.44	1.0	1.0	0.7	-
U/N	0.53	1.4	1.0	1.9	-
U/N	0.57	1.2	1.0	-	-
U/N	0.63	3.7	2.2	2.6	-
tHOcCl_2CA) cCl$_2$CA.gly)	0.73	4.2	5.0	2.8	1.4
cHOcCl_2CA.lac	0.83	6.6	4.0	2.8	-
cCl_2CA	0.87	2.1	3.3	5.0	7.6
c-cyper	0.93	20.0	10.9	37.3	70.0
& unknown [b]		2.0	1.5	-	-
TOTAL [c]		98.6	95.4	95.9	96.5

Values represent the means of duplicate determinations on pooled samples.

Abbreviations :- cyper, cypermethrin; gluc, glucuronide; gly, glycine; lac, lactone; tau, taurine.

[a] As determined by h.p.t.l.c.

[b] Unknown hydrophobic metabolite Rf= 0.8 in solvent TEA.

[c] Applied ^{14}C not accounted for represents radioactivity not associated with zones detected by autoradiography.

mice (Hutson et al., 1981) and hens (Gaughan et al., 1978b). The serine and glutamine conjugates have also been determined in a number of insect species (Soderlund et al., 1983c).

The major metabolites of [^{14}C-cyclopropyl] cis-cypermethrin, bands VII and VIII, were identified by their stability to sulphatase treatment and lability to β-glucuronidase and 1M NaOH, as glucuronic acid conjugates. Analysis of the released aglycones by h.p.l.c. subsequently identified VII and VIII as the ester glucuronides of t'HO-c-Cl_2CA and c-Cl_2CA, respectively. In mammalian species, these are also the major conjugated metabolites of the cyclopropane acid moiety (Hutson, 1979). However, in studies with cis- and trans-permethrin in chickens (10mg kg^{-1}), Cl_2CA glucuronide was a minor metabolite and no tHO.Cl_2CA conjugates were reported (Gaughan et al., 1978b). Instead, the majority of the excreted radioactivity was in the form of unconjugated Cl_2CA and HO.Cl_2CA. In these studies with quail, even at doses as high as 300mg kg^{-1}, the amount of cyclopropane acid and its hydroxylated derivatives conjugated with glucuronic acid greatly exceeded that of the free material. From this it may be concluded that the quail has more active UDP-glucuronyltransferases towards Cl_2CA, than that found in the chicken.

In these studies, no unconjugated, hydroxylated derivatives of cypermethrin were identified, though the

presence of the glucuronide of 4'HO.-cypermethrin (metabolite IX) demonstrated that the aryl-oxidation of the intact ester was occuring. The presence of this conjugate also confirms the existence of this glucuronidation reaction previously determined with benzyl-labelled cypermethrin. The other identified metabolites were, Cl_2CA, t'HO-c-Cl_2CA, and the lactone of HO.Cl_2CA. It was interesting to note, that as the oral exposure to cis-cypermethrin increased, so did the proportion of radioactivity excreted as the free acid, even though the relative amounts of its hydroxylated derivatives (tHO.Cl_2CA and lactone) decreased. This suggests that ester cleavage is not the rate limiting step in the formation of the trans-hydroxy derivatives of Cl_2CA.

3.5 Metabolism of [^{14}C-benzyl] cis- + trans-cypermethrin in rat and mouse

After determining that the 0-24 h urinary metabolite profiles of individual animals were similar following t.l.c. in solvent EFW, 0-48 h samples of urine were pooled and analysed directly. Faeces (0-72 h) were also combined and extracted with methanol as described in Chapter 2. The recovered radioactivity represented 56% and 81% of the total faecal ^{14}C of rat and mouse, respectively. Extracts were also prepared from 72-144 h rat samples, so as to establish whether any variation in biotransformation was occuring with

Table 4:5 . Metabolites of [^{14}C-benzyl]-*cis*- + -*trans*-cypermethrin in rat (0-7 days) and mouse (0-3 days).

Metabolite identity	T.l.c. solvent & Rf	% of chromatographed ^{14}C (% of dose)					
		Rat n=3			Mouse n=6		
		Urine	Faeces	Total	Urine	Faeces	Total
	EFW						
Unknown	0-0.03	3.9(2.9)	5.4(0.4)	(3.3)	6.5(3.4)	4.0(0.8)	(4.2)
3PBA tau	0.10	3.1(2.3)	1.7(0.1)	(2.4)	18.8(9.8)	6.5(1.3)	(11.1)
Unknown sulphate.I	0.19	- (-)	1.8(0.1)	(0.1)	- (-)	- (-)	(-)
4'HO.SO_2.O. 3PBA. II	0.25	74.1(54.8)	12.5(0.9)	(55.7)	6.5(3.4)	1.2(0.2)	(3.6)
4'O.3PBA gluc. V	0.32	2.5(1.8)	2.0(0.1)	(1.9)	12.0(6.3)	0.7(0.1)	(6.4)
4'O.cyper gluc VI	0.40	0.5(0.4)	- (-)	(0.4)	4.4(2.3)	1.5(0.3)	(2.6)
3PBA gluc X	0.44	- (-)	- (-)	(-)	3.1(1.6)	0.8(0.2)	(1.8)
3PBA gly	0.80	0.7(0.5)	- (-)	(0.5)	0.9(0.5)	3.7(0.7)	(1.2)
	TEA						
Unknown	0.10-0.13	- (-)	3.2(0.2)	(0.2)	- (-)	- (-)	(-)
4'HO.3PBA	0.20	3.1(2.3)	18.4(1.3)	(3.6)	16.5(8.9)	3.8(0.7)	(9.6)
4'HO.3PBA-artefact	0.24	- (-)	3.5(0.3)	(0.3)	- (-)	21.3(3.9)	(3.9)
Unknown	0.26	- (-)	1.2(0.1)	(0.1)	- (-)	15.4(2.8)	(2.8)
Unknown	0.29	- (-)	- (-)	(-)	- (-)	1.7(0.3)	(0.3)
3PBA	0.31	8.6(6.4)	5.4(0.4)	(6.8)	27.8(14.5)	4.1(0.8)	(15.3)
t'HO.cyper	0.45	- (-)	4.7(0.3)	(0.3)	1.3(0.7)	30.0(5.5)	(6.2)
4'HO.cyper	0.59	- (-)	11.4(0.8)	(0.8)	- (-)	1.0(0.2)	(0.2)
cyper	0.84	0.7(0.5)	24.3(1.7)	(2.2)	- (-)	- (-)	(-)
Total % accounted for[a]		97.2(71.9)	95.5(6.7)	(78.6)	97.8(51.4)	95.7(17.8)	(69.2)
No of metabolites		15			14		

Values represent the means from triplicate determinations (standard deviations not shown).

Abbreviations used; cyper- cypermethrin (*cis* & *trans*)
gluc- glucuronic acid conjugate
gly- glycine conjugate
tau- taurine conjugate

[a] Applied ^{14}C unaccounted for represents radioactivity not associated with zones detected by autoradiography.

time. In all cases, metabolites were identified by co-chromatography with reference compounds using t.l.c. (solvents BAW, EFW, CA, TEA), and by hydrolysis studies. The metabolite profiles in systems EFW and TEA shown in Table 4:5 represent the summarised results of these investigations. The identification of metabolites was confirmed using at least two developing solvents. When rat (72-144 h) urine and faeces samples were compared with these earlier profiles (0-48 h and 0-72 h respectively), the only apparent difference was a slight increase in the proportions of radioactivity excreted as metabolite II. For comparison with the avian species, the total proportion of the dose excreted by as a given metabolite by the rodents, has been calculated as the sum of the urinary and faecal elimination.

In the rat, the major metabolite (II) was isolated by preparative t.l.c. in solvent EFW (Rf = 0.25), and identified as being the sulphate conjugate of 4' HO.3PBA by co-chromatography and its lability to sulphatase. This has been shown to be the major urinary product of the 3-phenoxybenzoyl moiety in rats dosed orally with permethrin (Gaughan et al., 1977), cypermethrin (Crawford et al., 1981b), and fenvalerate (Ohkawa et al., 1979). Unlike permethrin however, no 2'HO.SO_2.O.3PBA was identified, though an unknown sulphate conjugate (metabolite I), which was also identified in the quail studies, was present. Similar

uncharacterised sulphates have been made by rats dosed orally with cypermethrin (Crawford et al., 1981b). Two minor metabolites, V and VI, were identified as the ether glucuronides of 4'HO.3PBA, and 4'HO.cypermethrin, respectively. Bile-duct-cannulation experiments have shown that rats readily form glucuronides of 4'HO.3PBA in the liver, but that following biliary excretion, de-conjugation by the intestinal microflora occurs, thus greatly reducing the amounts of these metabolites recovered in the final excreta (Huckle et al., 1981a).

The other minor conjugated metabolites were 3PBA glycine and 3PBA taurine. The glycine derivative has prevously been identified in rats dosed orally with phenoxybenzyl-pyrethroids (Gaughan et al., 1977; Ohkawa et al., 1979; Crawford et al., 1981b), though there have been no previous reports of 3PBA taurine being present in either the urine, or the faeces, of this rodent species. The principal unconjugated metabolites recovered in this study were 3PBA and is 4'hydroxylated derivative, which were eliminated in the urine, and to a lesser extent in the faeces. Presumably such compounds may be partially derived from aglycones released by the action of β-glucuronidases in the intestine. As expected, a smaller proportion of the dose was recovered as unchanged cypermethrin, or as hydroxylated metabolites of the intact ester, than was the case following oral exposure (Crawford et al., 1981b).

In the mouse, following i.p. administration of [^{14}C-benzyl] cypermethrin, proportionally more of the radioactivity was recovered as unconjugated phase I metabolites (notably as 3PBA and 4'HO.3PBA (Table 4:5)) than was the case in similarly dosed rats or quail. In addition, appreciable amounts of t'HO.cypermethrin, an unknown metabolite (Rf 0.26 in solvent TEA), and the artefact of 4'HO.3PBA observed previously in quail excreta, were all present in the faeces. When cypermethrin was administered orally to mice, similar faecal metabolites of cypermethrin were identified, though they represented a smaller proportion of the dose (Hutson et al., 1981). However, unlike the case following oral exposure, no unchanged pyrethroid was recovered in the faeces of i.p. dosed animals.

The majority of the polar metabolites were eliminated in the urine, and the major component was identified by t.l.c. in solvents BAW and EFW as the taurine conjugate of 3PBA. This is also the major metabolite of 3PBA, cypermethrin and deltamethrin following oral administration to mice (Hutson and Casida, 1978; Hutson et al., 1981; Ruzo et al., 1979). A small amount of 3PBA glycine was also found in both the urine and the faeces. In the mouse, sulphation was a less important conjugation reaction of the alcohol moiety of cypermethrin than in quails or rats, though mice readily formed glucuronides. These were identified by their chromatographic properties and lability to β-

glucuronidase, as being the glucuronic acid conjugates of 3PBA and 4'HO.3PBA (ether and ester linked). Glucuronides have been recovered in the urine of mice orally dosed with [^{14}C-benzyl] cypermethrin (Hutson et al., 1981), though the presence of the glucuronides of 5HO.3PBA and 3PBalc could not be confirmed in this experiment. Huckle et al., (1981c) have reported that when 3PBA was administered i.p. at 10mg kg^{-1}, mice glucuronidated 3PBA more readily than 4'HO.3PBA. In this study, the principal conjugation was to form the ether glucuronic acid derivative of 4'HO.3PBA. In conjunction with the observation that 4'O.cypermethrin glucuronide is present, this suggests that 4'O.3PBA glucuronide may be partially bio-synthesised by the ester cleavage of the glucuronic acid conjugate of 4'HO, cypermethrin. This was also observed in vitro (see Chapter 6).

4. Discussion

The routes of biotransformation of [^{14}C-benzyl] cypermethrin in quail, rat and mouse are not appreciably affected by the method of exposure (i.p. or p.o.). This is presumably due to both the complex pharmacokinetic properties of such lipophilic compounds in mammals and birds (see Gray and Soderlund, 1985), and the multiplicty of metabolic options available for pyrethroid degradation (Hutson, 1979). In all three species, the primary phase I reaction is ester cleavage

combined with aryl-hydroxylation of the alcohol moiety at the 4' position. As determined by the proportions of excreted radioactivity present as related derivatives, these vertebrates showed a specificity for 4'hydroxylation of 3PBA in the order rat (61.5%)>quail (27%)>mouse (23%). Additional positions for aryl-oxidation were also observed in the quail, and have previously been reported in the mouse *in vivo* (at the 2' and 5 positions: Hutson *et al.*, 1981). Other studies with 3PBA in a number of species suggest that this hydroxylation occurs after ester cleavage in mammals (Huckle *et al.*, 1981c). There is also some evidence that this may be the case in chickens (Huckle *et al.*, 1982), though possibly not in quail (see Chapter 6).

Sulphation of hydroxylated 3PBA derivatives was the major conjugation in rats, with 71% of the excreted radioactivity being recovered as 4'HO.SO_2.O.3PBA. This reaction was also important in the quail (38% of eliminated ^{14}C), though a larger number of other sulphated derivatives were formed. However, mice did not synthesise large amounts of 4'HO.SO_2.O.3PBA, but were the most active species in forming glucuronides. Presumably as demonstrated in the rat, the proportions of metabolites eliminated as sulphate, or glucuronic acid, conjugates are dependent upon the substrate specificities of the two competing phase II reaction systems (Mulder *et al.*, 1984), as well as the relative

activities of the de-conjugating enzymes present in the gut microflora (Huckle et al., 1981a). Gregus et al., (1983) have shown that the activities of sulphotransferases and UDP-glucuronyl-transferases in mouse, rat and quail, are largely determined by the chemical and physical properties of the substrate. It is therefore interesting to speculate, that in the case of the ether derivatives of the phenoxybenzoyl moiety, the major route of conjugation may be dependent on whether sulphation and glucuronidation occur before or after, ester cleavage of the pyrethroid. For example, 4'HO. cypermethrin may be a better substrate for UDP-glucuronyltransferases than 4'HO.3PBA, which in turn may be more readily sulphated than tne intact ester. Inter-species variations in the amino acid conjugation of 3PBA were also observed in these studies. Thus the ornithine, N-acetylornithine and glycylvaline, conjugates were restricted to the quail, and appear to be exclusively formed by avian species (Huckle et al., 1981d; 1982). However, the formation of 3PBA glycine was observed in all three species, and this conjugation appears to be an important phase II reaction in a number of mammals and birds (Huckle et al., 1981b; Ivie and Hunt, 1980).

The most striking difference between the metabolism of [^{14}C-benzyl] cypermethrin in quail, rat and mouse, was the number and diversity of conjugated metabolites formed by the bird, compared to the

mammals. This may be of some significance, as these conjugates are very polar (eg: P- value of 4'HO.SO_2.O.3PBA = 0.01) and would be readily eliminated, particularly in the urine. By contrast, mice form fewer conjugates of the alcohol moiety, and the free 3PBA remains sufficiently lipophilic at physiological pH (P value = 4.3), to be retained in some tissues. In addition, the multiple phase II reaction pathways available to quail, ensure that conjugate formation is not as potentially rate-limiting, as it may be in the elimination of detoxification products in the mammalian species.

This metabolic diversity was also demonstrated with cyclopropyl-labelled cypermethrin, which though largely metabolised in the avian species by similar mechanisms to those observed in rats, (Crawford et al., 1981b) and mice (Hutson et al., 1981)(ie: principally by glucuronidation of Cl_2CA), also formed a larger number of minor products, which represented 66% of the metabolised radioactivity in the bird, compared to only 21% in the rat. This multiplicity in metabolic options for both the alcohol and acid moiety of cypermethrin, allied to a large capacity for both ester cleavage and for some phase II reactions (eg: glucuronidation of Cl_2CA), ensure that in the quail even very large oral doses of the pyrethroid are rapidly broken down to non-toxic products.

Similarly, when radiolabelled permethrin was administered orally to chickens at 10mg kg^{-1} (Gaughan et al., 1978b), the dose was rapidly recovered in the excreta, though the proportions of unchanged, unabsorbed pyrethroid were higher than those determined with quail following treatment with 2mg kg^{-1} cypermethrin, and the range of metabolites was more restricted (13 from alcohol label). When hens were orally dosed with [^{14}C] fluvalinate at 1mg kg^{-1}, and 100mg kg^{-1}, it was reported that 93% and 62%, respectively, of the administered radioactivity was eliminated in 24 h (Staiger et al., 1982). Analysis of the excreta subsequently showed that 9% of the lower dose, and 44% of the higher dose, were unchanged pyrethroid. Preliminary analysis of a sample of excreta recovered from chickens dosed orally with [^{14}C-benzyl] cis+trans-cypermethrin (sample obtained from D.H. Hutson, Shell Research Centre, UK.), failed to demonstrate the presence of any parent pyrethroid. The metabolite profile was simpler than that of the quail, with more unconjugated 3PBA (3% of applied radioactivity) and 4'HO.3PBA (16%) present. The major conjugation reaction was sulphation (26% of applied radioactivity), though a number of highly polar uncharacterised metabolites were also present.
The biotransformation of cypermethrin in the quail compared with other species is examined in more detail using in vitro methods in Chapter 6.

Chapter 5 The target organ sensitivities of trout, frog, mouse and quail to cis-cypermethrin

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1. Introduction

In Chapter 1 (section 4), the available toxicological data on the mechanisms of pyrethroid action in vertebrates were reviewed, and this suggested that these insecticides exert their toxicant effects principally on the CNS. Studies with cismethrin and bioresmethrin in rats (White *et al.*, 1976; Gray *et al.*, 1980), and with deltamethrin in mice (Ruzo *et al.*, 1979), have shown that the development of toxic signs is dependent on the concentration of parent insecticide in the brain. This relationship between target organ levels of pyrethroid and acute toxicity, also seems to be the case for permethrin in rainbow trout (Glickman and Lech, 1982), and for fenvalerate in bobwhite quail (Bradbury and Coats, 1982). Therefore, in order to establish whether there are species-dependent differences in the neuro-sensitivites to *cis*-cypermethrin in trout, frog, mouse and quail, the concentrations of the pyrethroid in the brain associated with acute toxicity have been determined in these species. Also the uptake, distribution and elimination of cypermethrin has been investigated, in order to assess the other parameters which influence toxicity.

2. Methods

2.1. Chemicals and animals

[^{14}C-cyclopropyl] cis-cypermethrin and [^{14}C-phenyl] trans-cypermethrin were obtained and purified as detailed in Chapter 2. Dose solutions were prepared by diluting radiolabelled material with analytical grade cis- or trans-cypermethrin. Specific activities for oral studies were 0.2mCi mmol^{-1} for quail and mouse, and 1mCi mmol^{-1} for trout. When fish were exposed to cypermethrin in aquarium water, the radiolabelled preparation was used undiluted. During experiments, trout and frogs were individually maintained in 10 litres of dechlorinated, oxygenated mains water and 20 ml of distilled water, respectively. Mouse and quail were both housed in groups of 3 or more animals and were dosed after a 24 hour acclimatisation period. Trout and frogs were maintained at 15^{o}C under a 12 hour photoperiod. Mouse and quail were held at ambient light and temperature conditions (18-21^{o}C).

2.2. Toxicity studies

Dose solutions of [^{14}C] cis-cypermethrin were prepared in corn oil and administered by gavage to mice (0.1 ml) and quail (0.5 ml). Trout were exposed to cypermethrin absorbed onto food pellets, and dissolved in the aquarium water (added in 1 ml ethanol). In the case of frogs, cypermethrin was dispersed in the water with 0.1 ml Mulgofen:ethanol:H_2O (1:1:8 v/v). At

defined toxic signs as compared to controls, animals were killed by cervical dislocation and tissues rapidly removed and frozen in dry ice or liquid nitrogen. Samples were analysed within one week of storage (at -20°C).

2.3. Analytical procedure

Tissues were assayed for total radioactivity as described in Chapter 2. Weighed brain samples were extracted (x3) by gentle heating with 10 v/w hexane:acetone (2:1 v/v). The residual brain was then assayed for total radioactivity. The pooled extract was concentrated to dryness *in vacuo* at 40°C and after redissolving this in hexane (10 ml), removal of lipids was achieved by partitioning (x 3) against acetonitrile (10 ml). The combined acetonitrile phases containing cypermethrin were pooled and then concentrated to 1 ml *in vacuo* at 40°C. A similar procedure was employed for liver samples, with an additional stage of partitioning the initial extract against water (1 vol., pre-hexane extracted), to remove polar impurities. The hexane phase was then dried by filtering through anhydrous Na_2SO_4. Blood samples were extracted directly with hexane (2 vol.) and then cleaned up as for liver. In all cases, recoveries of *cis*-cypermethrin were monitored using 1 µg of [^{14}C] *trans*-cypermethrin (10,000 dpm) added prior to extraction. This internal standard was then resolved from the *cis*-

cypermethrin by h.p.l.c. system II. Mean recoveries were 95% for brain, 88% for liver and 60% for blood. All solvents used were of h.p.l.c. or pesticide residue grade. H.p.l.c. was carried out using system II (normal-phase) and system III (reversed-phase), as detailed in Chapter 2.

3. Results

After administration of the pyrethroid, animals were observed at regular intervals and their behaviour compared to similarly housed controls, which had been treated with the dose vehicle only. The time taken for half of the dosed group to exhibit a defined toxic effect (TE_{50}) is shown for each treatment in Table 5:1. The results refer to studies carried out with both labelled and unlabelled cypermethrin. Following oral (100mg kg^{-1}) or aqueous (10 $\mu g\ l^{-1}$) exposure to *cis*-cypermethrin, trout exhibited toxic signs of gill-flailing and hyperactivity, followed by loss of buoyancy and trim control. This latter stage was normally followed by death. Similar toxic effects have been observed in trout exposed to the type I pyrethroid, permethrin (Glickman *et al.*, 1981a). Under identical experimental conditions, *trans*-cypermethrin caused similar toxic effects, though over a 24 hour period fewer fish developed severe toxic signs and died than was the case following *cis*-isomer treatment (see

Table 51 The acute toxicity of cypermethrin to trout, frogs, mice and quail.

Species	n	Isomer	Dose [a] mg kg	Median time (hr) TE_{50} [b] & (range)	% fatality [c] (24 h)	Progression of observed toxic signs [d]
Rainbow trout			Aqueous			sensitivity to external stimuli
	12	cis	10 µg l^{-1}	2.5 (2.0-3.0)	10	aggressive and excitable behaviour
	10	trans	10 µg l^{-1}	2.0 (1.5-2.5)	10	loss of bouyancy and trim control
			Oral			
	10	cis	100	3.5 (3.0-4.0)	50	
	8	trans	116	3.5 (3.0-4.0)	10	
Frog			Aqueous			
	8	cis	300 µg l^{-1}	0.8 (0.7-0.9)	50	facial rubbing and salivation loss of leg co-ordination choreoathetosis and seizures
Mouse	6	cis	20	2.0 (1.9-2.1)	30	facial licking and salivation
	6	cis	40	1.5 (1.3-1.7)	100	unco-ordinated movements
	6	cis	50	1.5 (1.4-1.6)	70	choreoathetosis and seizures
	6	cis	100	0.5 (0.4-0.6)	100	
	6	cis	150	0.5 (0.4-0.6)	100	
Japanese quail	4	cis	20	no toxic effect	0	aggressive behaviour, head flicking
	3	cis	37	2.0 (1.0-3.0)	0	sudden un-controlled movements
	3	cis	300	1.0 (0.9-1.1)	30	loss of balance and recumbancy
	9	cis	750	1.0 (0.7-1.3)	60	

[a] Dose orally administered unless indicated otherwise.

[b] TE_{50} refers to the time at which 50% of the treated individuals were showing initial toxic signs.

[c] % of individuals killed by a single treatment over a 24 h period.

[d] As compared with controls dosed with the vehicle only.

Table 5:1). The development of initial toxic signs was delayed in orally dosed trout by approximately an hour, compared with fish which had been exposed to the cypermethrin isomers initially dissolved in the aquarium water.

Frogs showed toxic signs, ie: seizures and choreoathetosis, within two hours of exposure to a 6000$\mu g\ l^{-1}$ suspension of cis-cypermethrin. Similar effects have been described for several type II pyrethroids in grassfrogs (Cole and Casida, 1983), and rodents (see Chapter 1). For the determination of pyrethroid concentrations in the brain associated with toxic signs, mice were orally dosed with 32mg kg^{-1} and 50mg kg^{-1} [^{14}C] cis-cypermethrin (Table 5:1). Quail treated with cypermethrin (20, 37, 300 and 750mg kg^{-1} p.o.) showed initial toxic signs of compulsive pecking and head flicking for 1.0 - 2.0 hours following administration, at all doses except 20mg kg^{-1}. At 750mg kg^{-1}, this was followed by ataxia and finally sternal recumbancy. Similar toxic effects have been observed with fenvalerate in bobwhite quail (Bradbury and Coats, 1982).

The analogous toxic responses in all four species, namely hyperactivity followed by loss of balance, indicate a similar mode of action on the CNS. On this basis, an arbitrary classification of comparable toxic effects was defined in three stages as: (i) no toxic effects, ie: no behavioural differences from controls,

(ii) initial effects or onset of toxic signs (hyperactivity, tremors), and (iii) severe effects (choreoathetosis, loss of equilibrium). Using this system of classification, concentrations of cis-cypermethrin in the brain associated with acute toxicity are shown in Figures 5:1 and 5:2, irrespective of route of administration or size of dose.

As has been shown in rats (White et al., 1976; Gray et al., 1980), and in mice and trout (Glickman and Lech, 1982), a threshold concentration of the pyrethroid in the brain was required before toxic effects were observed in the bird, or the fish. The CNS of both trout and frog were very sensitive to cypermethrin, with an onset of toxic signs occurring at brain concentrations above 0.02$\mu g\ g^{-1}$ (Figure 5:1). In trout brain, the cis- and trans-isomers of cypermethrin are equally active. Target organ sensitivities to cis-cypermethrin were lower in mouse and quail, than trout or frog, with a threshold concentration of 1 $\mu g\ g^{-1}$ being required in the brain to initiate toxic signs in both species (Figure 5:2). The concentrations of cis-cypermethrin in the CNS associated with severe toxic signs in the quail (2.5 - 8.5$\mu g\ g^{-1}$), were far higher than those found in similarly affected mice (0.8 - 2.3$\mu g\ g^{-1}$). This indicates that the avian species has a lower brain sensitivity to the lethal effects of cypermethrin, than the mammal. These results are summarised in Table 5:2, with the concentrations

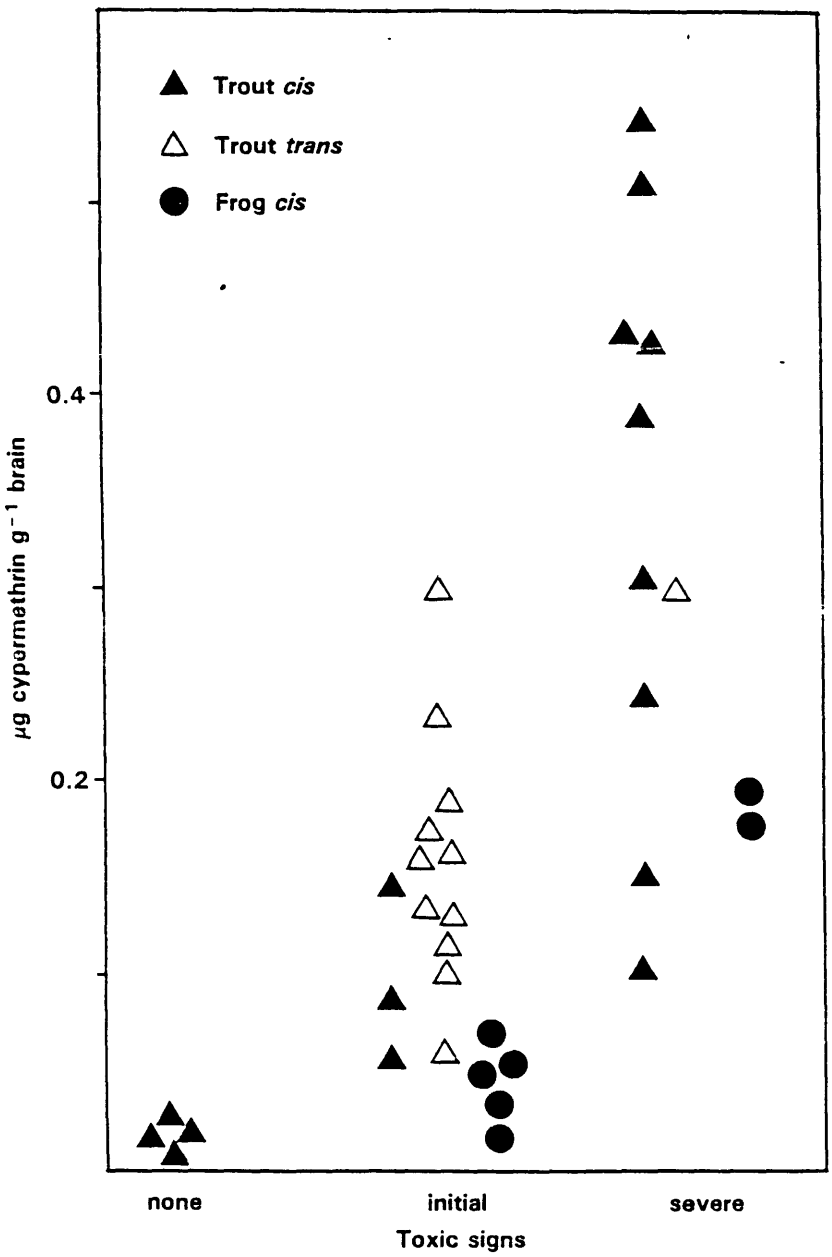

Figure 5:1 Concentrations of cypermethrin in the brains of frogs and trout in relation to toxic signs.

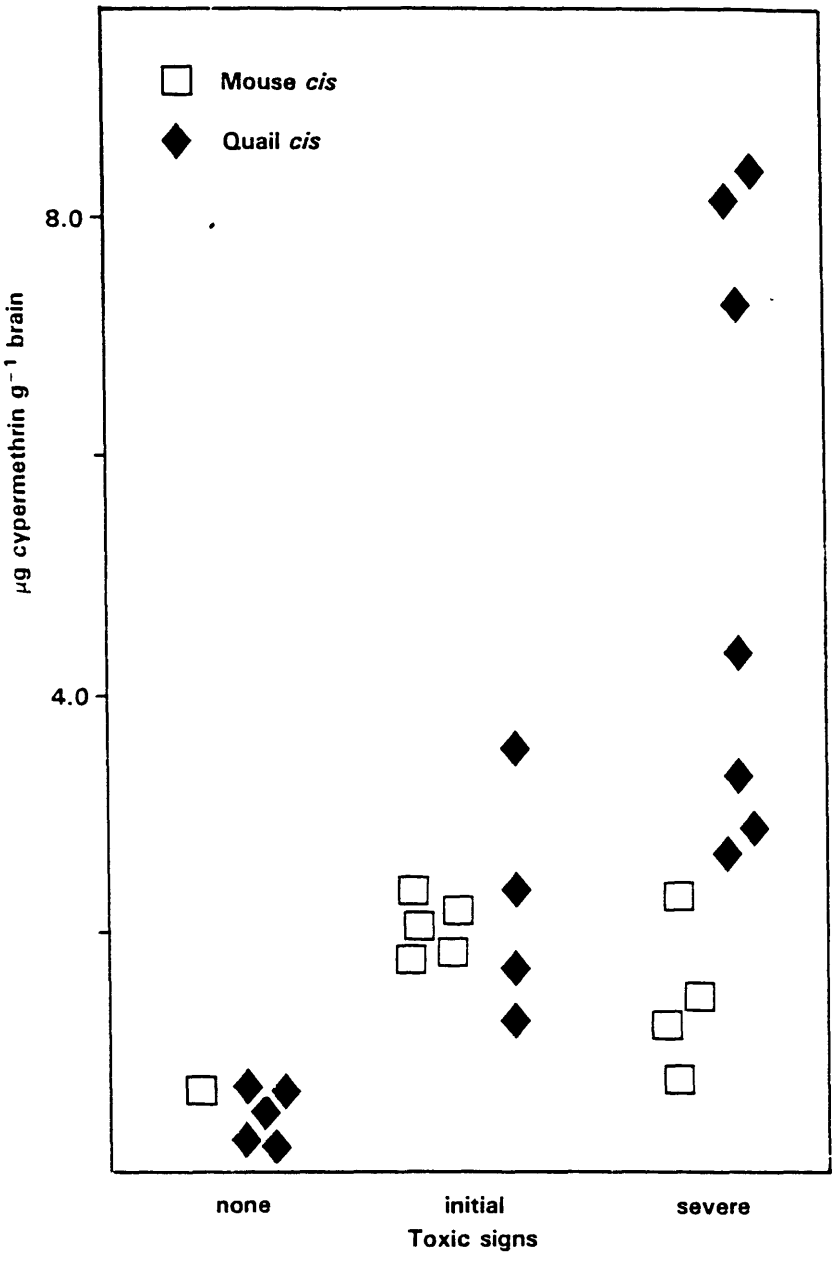

Figure 5:2 Concentrations of cypermethrin in the brains of mice and quail in relation to toxic signs.

of cypermethrin in the brain associated with both initial and severe toxic effects being combined to give a single value representing the target organ sensitivity of each species to this pyrethroid.

H.p.l.c. analysis showed that some 90% of the total radioactivity present in the brains of both mouse and quail showing acute toxic signs was in the form of unchanged [^{14}C] cis-cypermethrin (Table 5:3). A typical separation obtained by h.p.l.c. system II (normal-phase) is shown in Figure 5:3. However, in trout and frog, only 56% and 32% respectively, of the total brain radioactivity was identified as parent insecticide, following analysis by h.p.l.c. system III (reversed-phase). A typical elution profile is shown in Figure 5:4. Detailed analysis of the radioactivity extracted from trout and frog brains was carried out using h.p.l.c. system IV. The results (Table 5:3) indicate that the major metabolites in trout brain are hydroxylated derivatives of cypermethrin, and in frog brain, the products of ester cleavage. It seems likely that the metabolites detected in brain have accumulated from the circulating blood, as they are still readily lipid soluble (see Chapter 2, section 9).

Figure 5:5 shows the relationship between severity of toxic signs and concentrations of cis-cypermethrin and its metabolites in blood and liver associated with acute toxicity. Concentrations of cis-cypermethrin equivalents in trout were much lower than those

Table 5:2

The concentration of cis-cypermethrin in the brain associated with acute toxic signs

Species	Concentration of cypermethrin $\mu g/g^{-1}$ (brain fresh weight)
Frog	0.08±0.03
Trout	0.23±0.05 *
Mouse	1.71±0.33
Quail	3.94±0.83

* Concentration of trans-cypermethrin in the brain of trout associated with acute toxicity was 0.05±0.02 µg/g.

Values represent the mean of determinations carried out on animals exhibiting toxic signs ± S.E.M. (n>7).

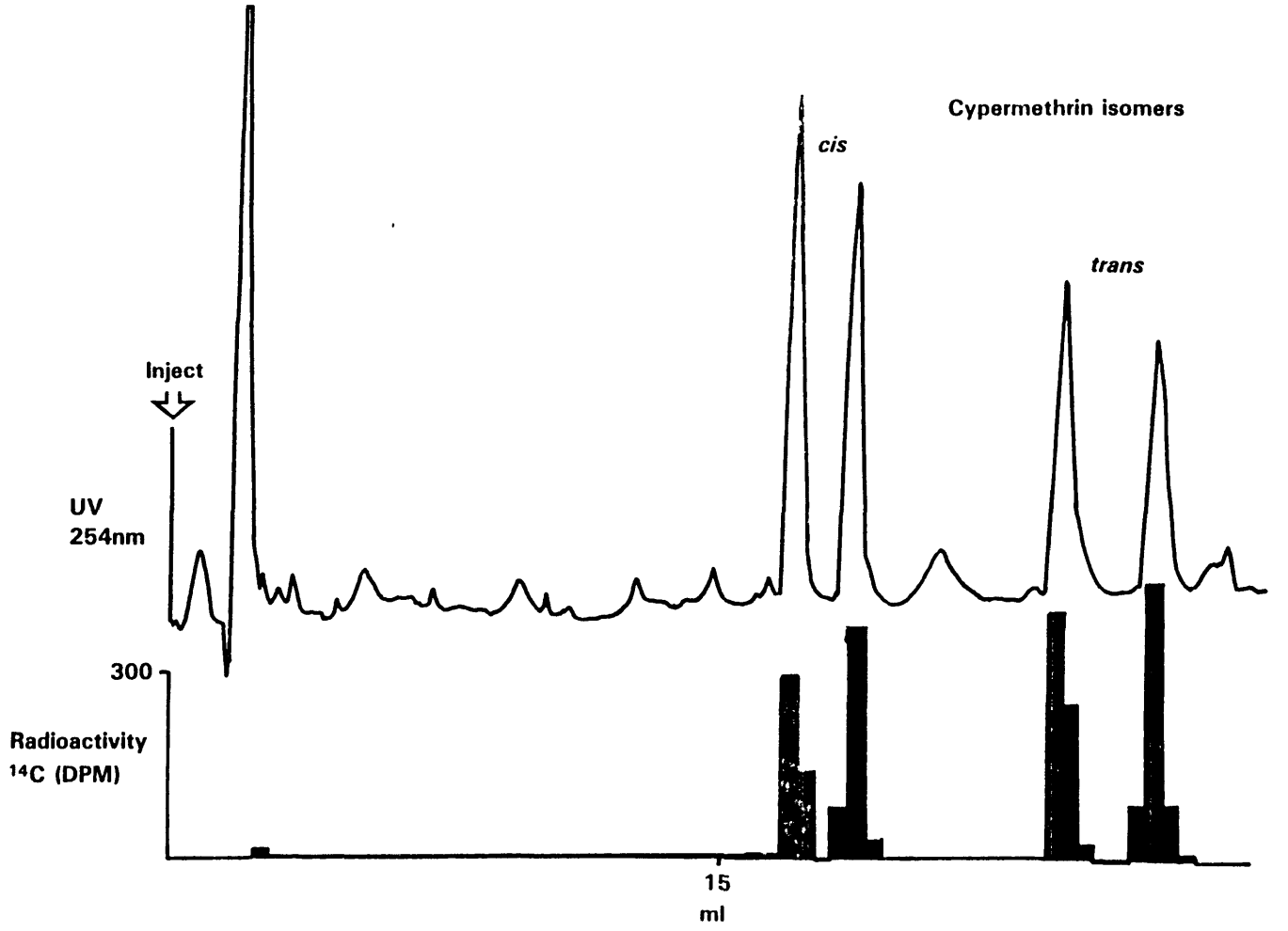

Figure 5:3 Analysis of quail brain extract for cis-cypermethrin by normal-phase radio-h.p.l.c.

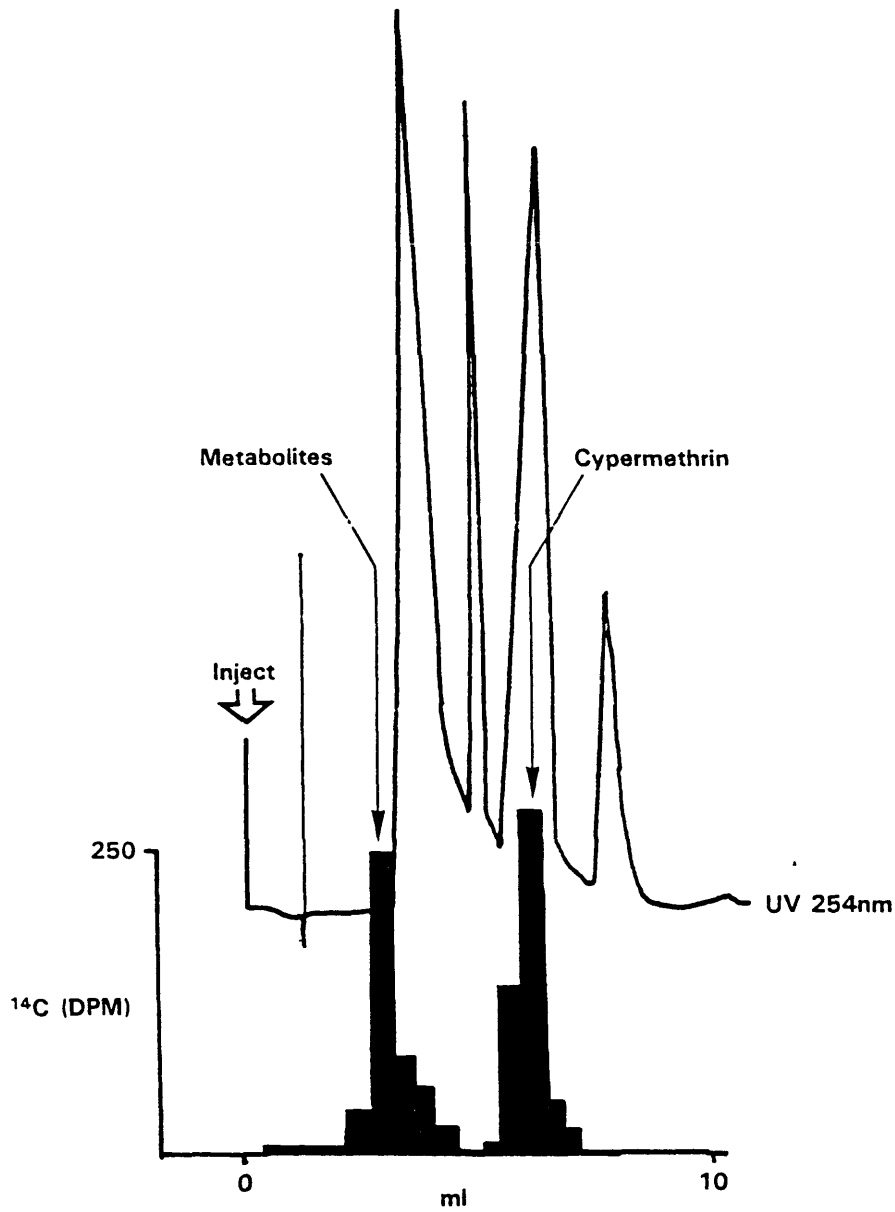

Figure 5:4 Analysis of trout brain extract for cypermethrin and metabolites by reversed-phase radio-h.p.l.c.

TABLE 5:3

Identification of metabolites of [^{14}C] cis-cypermethrin present in the brains of animals showing acute toxic signs.

	% total radioactivity				
	Unextracted	cis-cypermethrin	[a] c.4'HO cyper	[b] c.Cl_2CA	Other
Trout	25.9±1.7	56.4±2.6	14.4	0.3	3.0
Frog	6.3±0.6	31.8±9.2	3.8	48.7	10.1
Mouse	3.4±0.4	92.7±3.1	-	-	3.9
Quail	5.4±0.5	90.6±4.2	-	-	4.0

Mean figures ± S.E.M. (Standard error of the mean: n= 6)
h.p.l.c. system II 5-100% linear gradient, 20 mins ,1.5 ml min^{-1}
[a] 4'-hydroxy-cis-cypermethrin
[b] cis-3-(2,2-Dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylic acid.

1. Trout (aqueous exposure); 2. Trout (oral exposures); 3. Mouse, oral; 4. Quail, oral.

Where shown, the solid areas represent parent cypermethrin. The bars indicate the standard errors of the means.

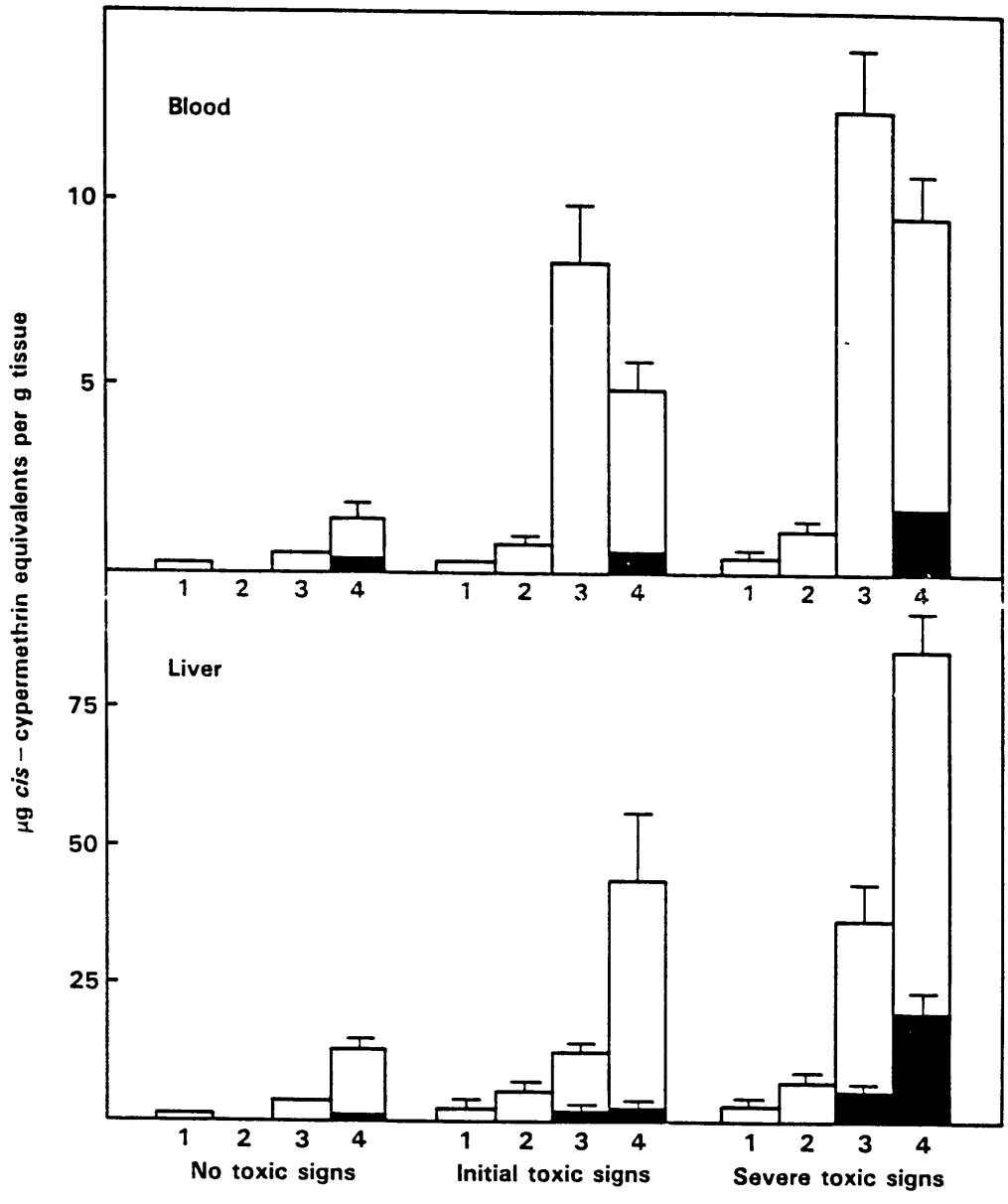

Figure 5:5 Concentrations of cypermethrin equivalents (^{14}C, expressed as µg per g of tissue) in blood and liver during intoxication with cis-cypermethrin

determined in mouse and quail tissues, with very similar values being obtained with trans-cypermethrin. During acute toxicity studies with frogs, mean concentrations of cis-cypermethrin equivalents were 1.6 $\mu g\ g^{-1}$ and 6.2 $\mu g\ g^{-1}$ respectively, were recorded in the liver during initial and severe toxic signs. These values are similar to those associated with acute toxicity in trout liver. In mouse and quail, analysis of blood and liver samples for unchanged cis-cypermethrin, showed the majority of the radioactivity present in these tissues to be in the form of polar metabolites, which were eluted prior to the pyrethroid following h.p.l.c. in system III. Similarly rapid appearance of metabolites of the pyrethroids deltamethrin (Gray and Rickard,1982a) and resmethrin (Gray et al., 1980) have been reported in the blood of rats following iv administration of toxic doses. The proportions of total radioactivity in the liver in the form of parent pyrethroid, and the progression of toxic signs was also studied (Figure 5:5). At severe toxic signs, in addition to having higher concentrations of radioactivity in the liver, quail had a larger proportion of ^{14}C in the form of cis-cypermethrin (24% of total ^{14}C) than the mouse (14%). This indicates that lethality is associated with a saturation of the hepatic biotransformation capacity in the quail, since at initial toxic signs, the parent pyrethroid accounted for only 6% of total radioactivity compared with 15% in

the mouse.

The overall tissue distribution of radioactivity two hours after oral exposure to toxic doses of pyrethroid, showed an interesting species difference (Table 5:4). Quail absorbed more cis—cypermethrin from their intestines, than trout, at all the doses tested. Following uptake, the insecticide was then readily metabolised to non-toxic products even at doses as high as 300mg kg^{-1} (see Chapter 4). At higher doses, both biotransformation and excretion of cis-cypermethrin appeared to be impaired. Following oral exposure to cypermethrin (100mg kg^{-1}), carcass residues in trout were very low, indicating poor intestinal absorption of the cis- and trans-isomers. Instead, the unchanged pyrethroid was largely elimininated from the intestines of fish dispersed in secreted bile. This was reflected in the delayed toxicity of oral doses in trout (see Table 5:1), which appeared to be due to gill-absorption of the dispersed cypermethrin from the aquarium water, which by 24h had reached a toxic concentration of 76µg l^{-1} and 35µg l^{-1} for the cis- and trans-isomers respectively.

4. Discussion

The results show a clear gradation of species as regards brain sensitivity to cypermethrin, namely; frogs>trout>>mammals>birds. Additional evidence of this species-dependent target organ sensitivity has

TABLE 5:4

Distribution of radioactivity in trout, mouse and quail 2 hours after oral exposure to [^{14}C] cis-cypermethrin

Species	Dose mg kg^{-1}	n	% Administered Radioactivity			
			Carcass	Intestine	Eliminated	Eliminated as cypermethrin[a]
Trout	100	3	2.0±0.3	27.1±1.8	59.5±5.8	56.8
Mouse	32	5	23.6±1.6	60.3±4.4	n.d.	n.d.
Quail	37	3	23.1±4.2	17.0±3.5	44.3[b]	5.1
Quail	750	3	61.9±6.4	9.9±1.9	12.3[b]	8.6

Mean ± S.E.M.

n.d. not determined

a - as determined by h.p.l.c. II on extracts from pooled excreta samples.

recently emerged for other pyrethroids, and the values obtained in this study are compared with those of other workers in Table 5:5. The data also show that trout brain is far more sensitive than mouse brain to the effects of the type I pyrethroid trans-permethrin (Glickman and Lech, 1982). As predicted from structure-toxicity relationships in mice (Lawrence and Casida, 1982), the type II pyrethroid cypermethrin was more active in trout brain than permethrin. However, similarities in the toxic effects produced by permethrin and cypermethrin in trout (eg: loss of equilibrium), in addition to the greater acute toxicity of the trans-isomer of permethrin to trout with respect to the cis-isomer (Glickman et al., 1981a), suggest that the type I/ type II system of pyrethroid classification may not be relevant for all classes of vertebrates. This presumably arises from the multiple sites of action of pyrethroids in the CNS and peripheral nerves of vertebrates (see Chapter 1),with these pesticides exerting differential effects on the nervous systems of different species. Frogs are significantly more neuro-sensitive to cis-cypermethrin than trout, though the 1R cis αS and 1Rtrans αS isomers have been shown to be more toxic to fish than to grassfrogs (Cole and Casida, 1983). The amphibian therefore presumably derives its protection against the toxic effects of pyrethroids from species differences in toxicokinetics and detoxification.

The cis- and trans-isomers of cypermethrin were equally active in trout brain. This lack of isomer specificity has been observed with several type II pyrethroids in mouse brain, but not with type I pyrethroids, where the cis enantiomers are more active than their trans counterparts (Lawrence and Casida, 1982; Staatz et al., 1982a). The presence of appreciable concentrations of 4'HO-cis-cypermethrin in the brains of trout, and to a lesser extent in frog, may be important, as hydroxylated forms of intact pyrethroids may still be active neurotoxicants (Ruzo, personal communication), and thus contribute to the toxicity. By contrast, the major biotransformation reaction of cypermethrin in mouse and quail is ester cleavage (see Chapters 4 and 6), and these products have negligible toxicity in mammals (White et al., 1976; Gaughan et al., 1977). Low concentrations of pyrethroid metabolites in the brains of animals showing toxic signs have been reported with deltamethrin in rats (Gray and Richard, 1982a), and with permethrin in mice and trout (Glickman and Lech, 1982). However, a study with cismethrin and bioresmethrin in rats (Gray et al., 1980), showed an extensive accumulation of both extractable and non-extractable brain metabolites with time. It is possible that some of these products of cypermethrin metabolism have accumulated in the brains of frog and trout due to the extended period of the toxicity study (4-24 h) when compared with 2-4 h in

TABLE 5.5

Concentrations of pyrethroid in the brains of animals associated with acute toxic signs, in this (*) and other studies.

Species	Pyrethroid (type)	Dose route	Brain concentration nmol g^{-1} cis-	trans-
Quail	Fenvalerate[a] (II)	p.o.	0.3-3.6	
	Cypermethrin* (II)	p.o.	9.5±2.1	
Mouse	Resmethrin[b] (II)	i.c.	132.3(77.4-206.5)	27740
	Permethrin[c] (II)	iv/ip	15.6±0.5	77.2±0.3
	Fenvalerate[b] (II)	i.c.	2.8(1.1-6.3)	
	Deltamethrin[b] (II)	i.c.	2.4(1.6-3.4)	
	Deltamethrin[d] (II)	p.o.	1.0	
	Cypermethrin[b] (II)	i.c.	1.4(0.7-3.1)	3.8(2.6-10.3)
	Cypermethrin* (II)	p.o.	4.1±0.8	
Rat	Resmethrin[e] (I)	p.o.	1.6-16.5	12.9-112.9
	Resmethrin[f] (I)	i.v.	73.5	
Frog	Cypermethrin* (II)	aq.	0.2±0.1	
Rainbow Trout	Permethrin[c] (I)	iv/ip	5.1±1.3	5.1±0.3
	Cypermethrin* (II)	aq/po	0.6±0.1	0.4±0.0

i.c. intracerebral LD_{50} values

aq. administered in aquarium water

mean values with 90% confidence limits (bracketed figures) or ± S.E.M. where applicable.

Underlined figures refer to the use of 1R, cis isomers only.

a Bradbury and Coats, 1982
b Lawrence and Casida, 1982
c Glickman and Lech, 1982
d Ruzo et al., 1979
e White et al., 1976: Value for trans-resmethrin (bioresmethrin) obtained with 1-5 % contamination with the cis- isomer (cismethrin), Gray and Connors, 1980
f Gray et al., 1980

b, e, f : All studies with resmethrin carried out with 1R- isomers only.

the case of mouse and quail. However, the relationship between concentrations of cis-cypermethrin and its metabolites in blood and brain suggest that differences may exist in the "blood/brain barrier" for pyrethroids in the different species.

The extent of intestinal absorption of orally dosed cis-cypermethrin lies in the order: quail>mouse>trout. The effect of using different vehicles for dose delivery was not studied however, and this has been shown to strongly influence the oral toxicity of pyrethroids in rats (Verschoyle and Barnes, 1972). The rapid elimination by trout of unabsorbed cis-cypermethrin dispersed in secreted bile is particularly significant, as ingestion of dead insects or vegetation, contaminated with pyrethroids can be an important route of exposure in the field (Kingsbury and Kreutzweiser, 1984). In the environment where fish are swimming in a large volume of water, the risk of pyrethroid reabsorption should be greatly reduced, and the overall toxic hazard of orally ingested pyrethroid lessened. Orally administered cypermethrin was also poorly absorbed by mice, though during the two hour test period little of the dose radioactivity was recovered in the faeces. White et al., (1976) have similarly reported a slow intestinal uptake of toxic doses of cismethrin and bioresmethrin in rats, and proposed this as an explanation of the greatly reduced toxicity of the pyrethroid when administered orally,

than when given i.v. In the quail however, *in vivo* studies have shown cypermethrin to be readily absorbed, then following extensive metabolism to a large number of products, rapidly eliminated in the excreta (see Chapter 4). Similarly, Bradbury and Coates (1982) have suggested that the rapid detoxification of fenvalerate in bobwhite quail can explain the low oral toxicity (LD_{50}>4000mg kg^{-1}).

Chapter 6. Biotransformation of cypermethrin isomers in liver, plasma, brain and intestine in trout, frog, mouse and quail

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1. Introduction

The observation that metabolic inhibitors increase the toxicity of pyrethroids to several classes of vertebrates (see Chapter 1, section 5) implicates biotransformation as partially determining species-dependent variations in the toxicology of these insecticides. Direct *in vivo* comparisons of the metabolic capabilities of aquatic and terrestrial chordates to detoxify insecticides is technically difficult (Allen *et al.*, 1979). Therefore, the majority of comparative metabolic studies have utilised *in vitro* methods, with an emphasis on liver preparations (see Chapter 7). Glickman and Lech (1981) and Glickman *et al.*, (1982) examined the hydrolysis of permethrin isomers in the liver, plasma, and serum, of trout and mouse in order to determine the relative importance of detoxification in defining the susceptibility of these species to pyrethroids. This approach has been extended in this study to include a bird and amphibian. In addition, the potential importance of oxidation and phase II conjugation (glucuronidation) in the biotransformation of pyrethroids has also been investigated in trout, frog, mouse and quail.

2. Methods

2.1. Chemicals and animals

[^{14}C-cyclopropyl] cypermethrin and [^{14}C-benzyl]

cypermethrin were resolved into their cis- and trans-isomers by preparative h.p.l.c. (system I). The radiolabelled preparations were then added to the respective unlabelled analytical grade isomers, to give a stock solution in hexane of specific activity 6.8mCi $mmol^{-1}$, which was stored at -20°C prior to use. Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6P dehydrogenase), β-nicotinamide adenine dinucleotide NADP, uridine diphosphate glucuronic acid, UDPGA and Bovine serum albumin (BSA), were all obtained from Sigma Chemicals (Dorset, UK.). Triphenyl phosphate (98% purity) was purchased from the Aldrich Chemical Co. Ltd. (Dorset, UK.), and β-diethylaminoethyl-diphenyl-n-propylacetate-HCl (SKF-525A) from Smith Kline and French Limited (Welwyn, Herts., UK.).

2.2. Preparation of tissue fractions

Animals were killed with a blow to the head (trout), by pithing (frog), or by cervical dislocation (mouse and quail). Heparinised blood samples were collected from the tail vein (trout), or by cardiac puncture, and plasma prepared by centrifugation (3000g, 10 min, 4°C). Intestinal contents were collected and assayed immediately as described in Chapter 2, at a substrate concentration of 10^{-5}M. Brain, intestine and liver samples were rinsed in ice-cold 0.15M KCl, and after cutting into sections, homogenised in 0.1M

phosphate buffer pH 7.4 (5 vol.) in a Potter-Elvjhem glass-Teflon homogenizer. After straining through muslin, homogenates were centrifuged at 10,000g (20 min, 4°C) to afford a post-mitochondrial supernatant which was used directly. In addition, the supernatant was centrifuged at 195,000g (40 min, 4°C) to give microsomal and cytosolic fractions. The microsomes were then washed in phosphate buffer, recentrifuged, and finally resuspended in buffer to the same volume as the post-mitochondrial supernatant from which they were prepared. All subcellular fractions were used within 4 hours of preparation.

2.3. Incubation conditions

Duplicate incubations (3 ml) were prepared as detailed in Table 6:1, and cis- or trans-cypermethrin (final assay concentration 10^{-5}M) added in acetone (50 µl). Following pre-incubation, the reaction was initiated by the addition of the tissue fraction (1 ml) to the substrate and cofactors, with the boiled equivalent serving as a control. The reactions were carried out in 25 ml Erlenmeyer flasks in a reciprocating water bath (100 osc min^{-1}) at 15°C for trout and frogs, 37°C for mice, and 42°C for quail. Total metabolism of cypermethrin isomers was monitored by removing samples (25 µl) at timed intervals and applying them as a 2cm wide band to a t.l.c. plate.

Table 6:1

Use of cofactors in characterising mechanisms of biotransformation in microsomal fractions

Type of activity	Incubation conditions
A. Esterases only	0.1M Phosphate buffer (pH 7.4) $^{+}$0.005M $MgCl_2$
B. Esterases and Oxidation	A + 0.0015M NADP + 0.016M G6P/G6P-Dehydrogenase (4 units)
C. Esterases, Oxidation and Glucuronidation	B + 0.0016M UDPGA

Following development in toluene, the radioactivity in the region Rf 0- 0.1 was then quantified by l.s.c.. This chromatography system clearly separates cypermethrin isomers (Rf: cis- 0.49, trans- 0.45) from all its metabolites (Rf: 0- 0.1).

Reactions were terminated with ice-cold ethanol (2 ml) and the resulting precipitate removed by centrifugation (3000g, 10 min, 4°C). After decanting the supernatant, the pellet was re-extracted with ethanol (2 ml) at 4°C for 30 min and then centrifuged. Pooled supernatant fractions were analysed directly by h.p.l.c. (system IV). If additional identification was required, extracts were adjusted to pH 2 with 0.1M HCL and extracted (x3) with diethyl ether (2 vol.). Samples of this combined ether extract were then concentrated to dryness under N_2, redissolved in methanol (0.2 ml) and applied to h.p.t.l.c. plates and developed in EFW or TEA.

2.4. Protein determination

The method of Lowry et al., (1951), as modified by Hartree (1972) was employed. Duplicate samples (1 ml) of the tissue preparation were treated with 1 ml 20% w/v trichloroacetic acid (TCA) and the precipitate collected by centrifugation (3000g, 10 mins). After washing the pellet with 10% w/v TCA (2 ml) and recentrifuging, the precipitate was solubilised in 0.1M NaOH (2ml) at 50°C. This extract (0.1 ml) was then

heated at 50°C for 10 min with distilled water (1 ml) and 0.9 ml Na_2CO_3 (10% w/v) K.Na.tartrate (0.2% w/v) contained in 0.5M NaOH. After cooling, 0.1 ml $CuSO_4$ (1% w/v), K.Na.tartrate (2% w/v) in 0.1M NaOH was added, and the mixture allowed to stand at room temperature for 15 min. Finally, 6.6% v/v Folin Ciocalteau's reagent was added (3 ml) and after development at 50°C for 10 mins, the absorbance at 650nm was determined with a Cecil CE 373 spectrophotometer. Protein concentrations were then calculated from a standard curve prepared from BSA.

3. Results

3.1. Tissue distribution of cypermethrin biotransformation activity

The weights of brain, liver, intestine (less stomach) and plasma were determined on sacrifice, and from published sources (Table 6:2). Biotransformation activities of tissues were then determined by incubating subcellular fractions under standard assay conditions and in the presence of all cofactors, and calculating the nanomoles of cypermethrin isomers metabolised by the tissue per kilogram of body weight, as described by Glickman and Lech (1981). The total activities of the various tissues towards cypermethrin isomers, were determined from initial rates (i.e: product formation linear with respect to time), and are shown in Table 6:3. No attempt was made to study the

Table 6:2

The weights of liver, brain, intestine and plasma expressed as a percentage of total body weight

Species	Liver	Brain	Intestine	Plasma
Trout	1.1	0.1	7.4	2.1[a]
Frog	2.1	0.4	-	-
Mouse	4.7	1.2	12.6	3.0[b]
Quail	1.8	0.4	6.4	6.3[c]

a Value obtained from Conte et al., (1963)

b Value obtained from Sassen et al., (1968)

c Value obtained from Mitruka and Rawnsley (1981) (for chicken).

effect of temperature on these incubations.The concentration of cypermethrin in these standard assays (10^{-5}M) corresponds to a tissue concentration of 63 μg g^{-1}. This exposure level is well in excess of concentrations observed in tissues during acute toxicity experiments (Chapter 5). The mouse metabolised cis- and trans-cypermethrin more readily than trout, frog or quail in all the tissues studied. In both mouse and quail, the liver was the major site of detoxification,and in the presence of all cofactors metabolised the cis- and trans-isomers at very similar rates. Overall biotransformation of the pyrethroid by the liver was higher in mouse than quail due to the relatively larger liver (Table 6:2). In trout liver, cis-cypermethrin was metabolised three times more readily than trans-cypermethrin.

Detoxification of the trans-isomer by plasma and intestinal fractions appeared to be particularly important in the trout, with the activity of both tissues exceeding that of the liver (Table 6:3). Metabolism of cypermethrin isomers by brain 10,000g homogenates was very poor in all the species studied. Identification of the metabolites of [^{14}C-cyclopropyl] cis- and trans-cypermethrin in intestine, brain and plasma by h.p.l.c., showed them to be largely derived from ester hydrolysis (Table 6:4). This accounts for the faster biotransformation of trans-cypermethrin compared to the cis-isomer. Hydroxylated derivatives

Table 6:3

Biotransformation rates of cypermethrin [^{14}C-cyclopropyl]-labelled isomers (10^{-5}M) by 5v/w tissue post-10,000g supernatants and plasma

Species	nmols cypermethrin metabolised min^{-1} Kg body $weight^{-1}$				
	liver	brain	intestine	gut-contents	plasma
			cis-		
Trout	10.1	0.03	4.6	<0.01	1.2
Frog	24.3	0.16	n.d.	<0.01	n.d.
Mouse	263.5	0.46	22.0	n.d.	21.8
Quail	170.2	0.08	13.7	0.14	1.5
			trans-		
Trout	3.4	0.08	4.4	<0.01	7.0
Frog	19.7	0.31	n.d.	<0.01	n.d.
Mouse	297.3	1.60	63.8	n.d.	77.2
Quail	186.6	0.39	38.7	0.14	2.7

Values represent the means of separate determinations carried out on tissues pooled from n> 3 animals.

n.d. not determined.

of 3-(2,2-dichlorovinyl)-2,3-dimethyl-cyclopropane-carboxylic acid (Cl_2CA) and the respective lactones were also identified as major metabolites of cis-cypermethrin in trout and mouse intestinal fractions. An unknown non-polar metabolite, U/N 1, was also observed in many of the tissues studied, particularly in trout. It was associated with ester hydrolysis and was most readily formed from trans-cypermethrin. The chromatographic properties of this metabolite are described in Table 6:4, but no further characterisation was attempted. Biotransformation of cypermethrin isomers by the gut contents was very low in all the species studied, with quail being the most active. H.p.l.c. analysis showed that Cl_2CA was the major metabolic product.

3.2. Metabolism of cypermethrin isomers by liver subcellular fractions

3.2.1. [^{14}C-cyclopropyl] cypermethrin

Studies with liver subcellular fractions showed that the majority of the pyrethroid biotransformation activity was locatedin the microsomes in all four species (Table 6:5). Active ester hydrolysis in the cytosol was also observed in quail, and to a lesser extent in the mouse. In all the species except mouse, a loss in total activity was observed on preparation of microsomes and cytosol, such that 10,000g supernatant was found to be the most satisfactory fraction for

Table 6.4

Metabolism of [^{14}C-cyclopropyl] cypermethrin isomers by 5v/w intestine and brain 10,000g supernatants and plasma.

cis-cypermethrin

% of metabolised cypermethrin

Metabolite (identified by h.p.l.c.)	Intestine			Brain			Plasma		
	Trout	Mouse	Quail	Trout	Mouse	Quail	Trout	Mouse	Quail
HO-cyper [a]	n.d.	12.9	14.0	n.d.	21.0	n.d.	n.d.	n.d.	n.d.
U/N 1 [b]	14.1	n.d.	n.d.	51.4	n.d.	26.7	34.5	4.9	26.7
Cl_2CA	5.4	25.8	64.0	8.6	26.3	66.7	25.9	84.4	23.3
CH_2OH-Cl_2CA	60.9	41.9	n.d.	n.d.	13.1	n.d.	n.d.	n.d.	n.d.
CH_2OH-Cl_2CA-lac	n.d.	4.0	22.0	5.7	n.d.	6.7	n.d.	n.d.	n.d.
U/N [c]	19.6	15.3	n.d.	34.3	39.5	n.d.	39.6	10.7	50.0
Total	100.0	99.9	100.0	100.0	99.9	100.1	100.0	100.0	100.0

trans-cypermethrin

HO-cyper [a]	n.d.	3.1	n.d.	n.d.	3.4	n.d.	n.d.	n.d.	n.d.
U/N 1 [b]	45.1	0.9	10.3	55.9	16.9	16.9	23.4	1.5	48.8
Cl_2CA	17.6	88.4	80.1	15.5	66.3	61.5	68.5	96.9	27.9
CH_2OH-Cl_2CA	15.4	2.8	n.d.	n.d.	3.4	n.d.	1.1	n.d.	n.d.
CH_2OH-Cl_2CA-lac	n.d.	n.d.	n.d.	5.9	n.d.	n.d.	n.d.	n.d.	n.d.
U/N [c]	22.0	4.7	9.6	22.7	10.1	21.5	6.9	1.5	23.3
Total	100.1	99.9	100.0	100.0	100.1	99.9	99.9	99.9	100.0

Metabolite abbreviations as detailed in Table 1.

Results represent the means of duplicate determinations.

n.d. not detected

[a] t'HO and 4' hydroxylated derivatives of cypermethrin not resolved by h.p.l.c.

[b] U/N 1 unknown metabolite. Associated with ester hydrolysis, elution volume by h.p.l.c. cis- 30.8 ml trans- 30.0 ml.

hptlc. Rf in solvent TEA cis- and trans- = 0.74

[c] Uncharacterised polar radioactivity (eluted in void volume)

metabolite analysis. Activities per mg of protein were much higher in the microsomal fraction than in the post-mitochondrial fraction, however (Table 6:5).

The metabolites of [^{14}C] cypermethrin isomers were identified and quantified by h.p.t.l.c. and h.p.l.c., and the results represented as the proportions of radioactivity recovered as metabolised products (Table 6:6). Trout liver biotransformed cis-cypermethrin almost entirely by hydroxylation at the 4' position in the phenyl ring. The 4'HO. cypermethrin formed was then readily glucuronidated. In comparison with the other species, trout liver had low activities of pyrethroid-hydrolysing esterases and this was reflected in both the higher proportions of the trans-isomer recovered as 4'HO. cypermethrin, and the overall preferential biotransformation of the cis-isomer (Table 6:3). In frog and mouse, hydroxylation of cis-cypermetnrin was observed at the 4' position of the phenyl ring and also in one of the methyl groups of the acid moiety. Both derivatives were then glucuronidated, with 4'HO. cypermethrin being the preferred substrate. The lactone of CH_2OH-Cl_2CA was also readily formed from cis-cypermethrin in the frog, but not in the mouse. In contrast, hydroxylated derivatives of cis-cypermethrin were minor metabolites in the quail, with cis-Cl_2CA and its glucuronide being the major products. In all four species, trans-cypermethrin was largely metabolised to trans-Cl_2CA but proportionally less of this was

Table 6.5

Total biotransformation of cypermethrin isomers by liver subcellular fractions.

Species	Incubation temp °C	Total Product Formation nmols min^{-1} $mg\ protein^{-1}$ [a]					
		10,000g supernatant		cytosolic fraction		microsomal fraction	
		cis-	trans-	cis-	trans-	cis-	trans-
Rainbow Trout	15	0.030	0.008	0.003	0.003	0.029	0.011
Frog	15	0.020	0.016	0.007	0.008	0.071	0.101
Mouse	37	0.066	0.077	0.005	0.073	0.100	0.337
Quail	42	0.128	0.117	0.031	0.043	0.315	0.347

Values refer to the mean initial rates of duplicate determinations carried out on pooled samples ($n \geqslant 3$) on two separate occasions.

Incubations carried out in presence of NADPH and UDPGA.

a Specific activities only. Total biotransformation by a subcellular fraction = specific activity x total protein content.

Table 6:6

Metabolites of [^{14}C-cyclopropyl] cypermethrin isomers formed by liver 10,000g supernatant supplemented with NADPH and UDGPA

% of metabolised cypermethrin

Metabolites (identified by h.p.l.c. and hptlc)	cis-cypermethrin				trans-cypermethrin			
	Trout	Frog	Mouse	Quail	Trout	Frog	Mouse	Quail
Hydroxylated derivatives of intact cypermethrin.								
4'HO-cyper	44.9	13.0	12.3	4.6	25.6	8.1	1.5	1.6
CH_2OH-cyper	n.d.	10.0	25.8	n.d.	n.d.	1.9	2.4	n.d.
CH_2OH-4'HO-cyper	0.7	n.d.	6.6	n.d.	1.1	n.d.	4.3	n.d.
4'O-cyper-gluc	40.7	6.6	9.5	6.1	12.9	1.1	3.7	1.3
CH_2O-cyper-gluc	n.d.	3.0	4.0	n.d.	n.d.	0.8	1.0	n.d.
Total	86.3	32.6	58.2	10.7	39.6	11.9	12.9	2.9
Derivatives of ester cleavage								
U/N 1[a]	4.5	1.4	n.d.	4.2	20.7	11.4	n.d.	2.3
Cl_2CA	4.4	6.9	15.7	34.7	32.6	56.9	83.4	84.5
CH_2OH-Cl_2CA	0.9	4.1	3.9	5.5	1.8	2.8	0.3	4.2
CH_2OH-Cl_2CA-lac & U/N [b]	4.0	37.5	7.0	9.3	5.3	14.2	0.4	0.2
Cl_2CA-gluc	n.d.	14.8	9.3	28.0	n.d.	1.4	2.3	5.3
U/Ngluc[c]	n.d.	2.7	6.2	7.6	n.d.	1.4	0.8	1.0
Total	13.8	67.4	42.1	89.3	60.4	88.1	87.2	97.5
Total accounted for	100.1	100.0	100.3	100.0	100.0	100.0	100.1	100.4

n.d. not detected

gluc glucuronide

Trout and Frog incubations analysed at 45 mins. mouse and quail at 15 mins.

a U/N 1 Unknown product of ester hydrolysis (see Table 5).

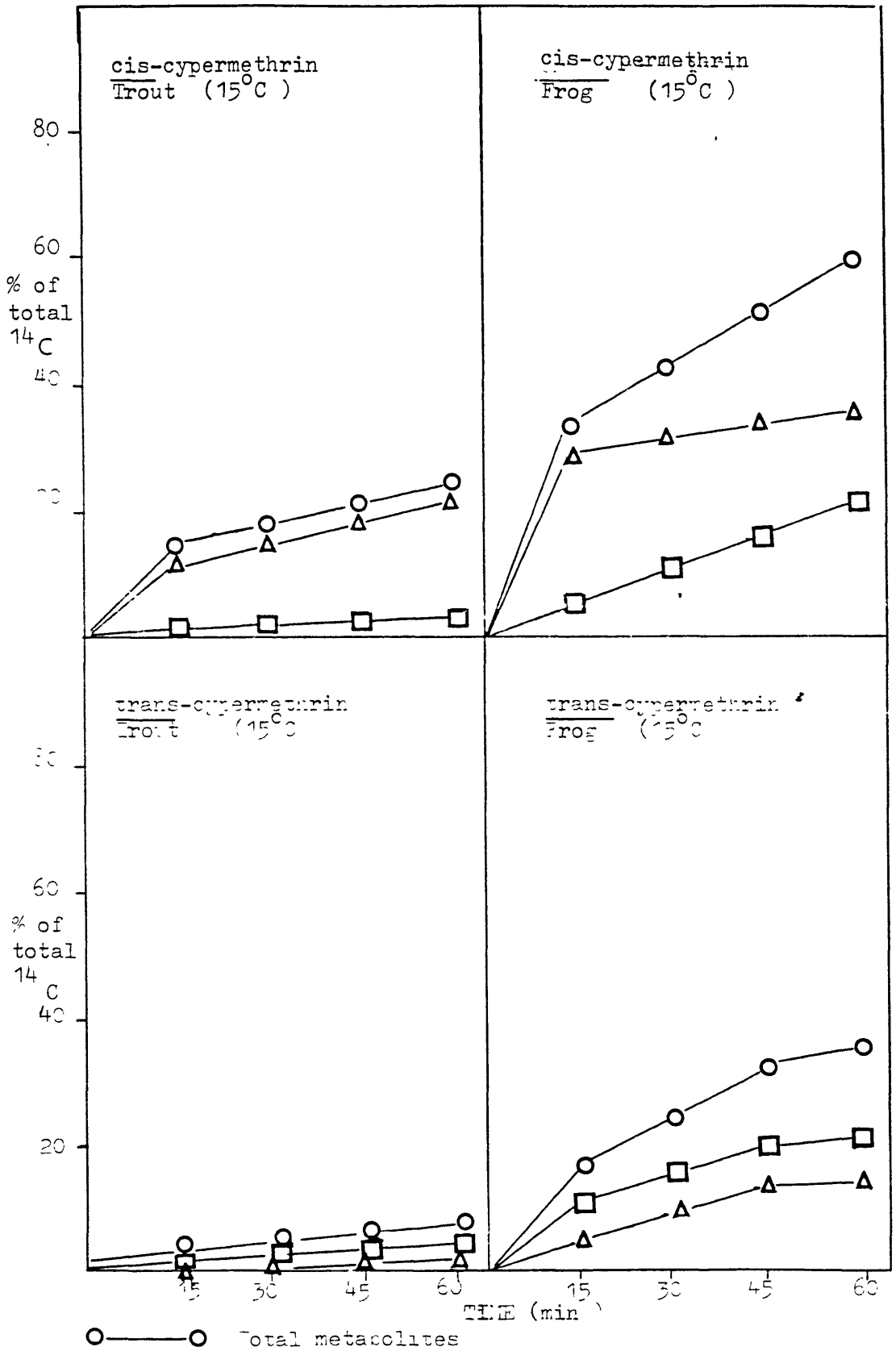
b Peak resolved into three bands by hptlc in solvent TEA; Rf 0.21, 0.23, 0.25 major band Rf 0.25 = lactone.

c Mixture of polar products : β-glucuronidase labile (Rf 0, 0.24; solvent EFW)

glucuronidated than with the cis-isomer. The unknown metabolite U/N 1 was detected in the liver 10,000g supernatant of all the species except mouse. Time course studies in the four species (Figures 6:1 and 6:2) confirmed these species variations in routes of metabolism, with the combined rates of hydroxylation and ester cleavage of cypermethrin being determined as separate components of overall biotransformation. Similar profiles were obtained with microsomal preparations as with 10,000g supernatants, when incubated under identical conditions, though overall conversion rates tended to be low.

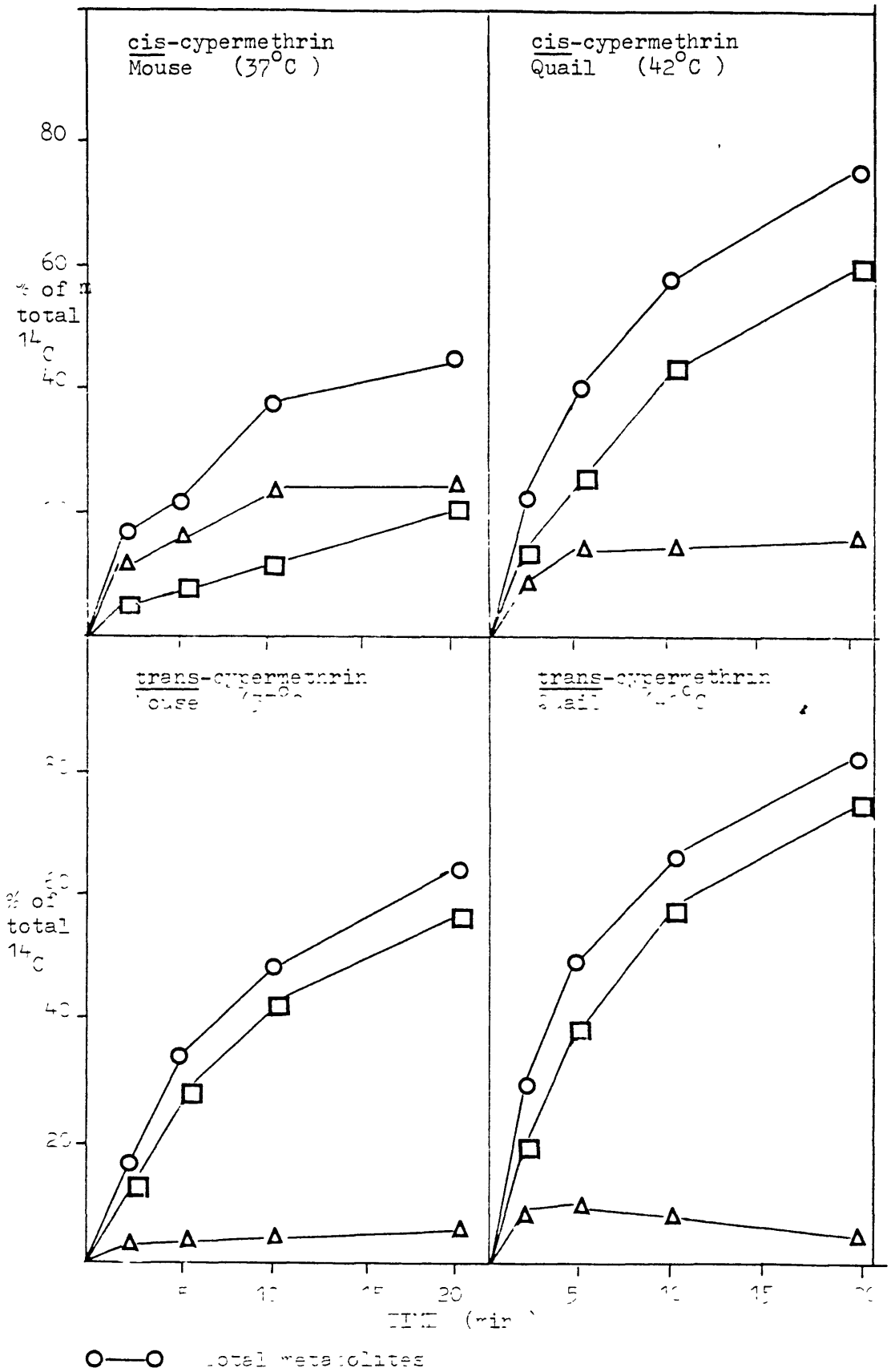
To investigate the differences in metabolism in trout, mouse and quail in more detail, washed microsomes were incubated under varying conditions as detailed in Table 6:1, and the metabolites identified by h.p.l.c.. The effect of cofactors on total biotransformation rates are shown in Figure 6:3. Addition of the oxidative cofactor NADPH increased the metabolism of cis-cypermethrin in all three species, though only in the quail was this due to the greater rate of formation of cis-Cl_2CA. The addition of UDPGA further increased biotransformation in trout and quail, due to the formation of the glucuronides of 4'HO-cis-cypermethrin and cis-Cl_2CA respectively. By contrast trans-cypermethrin was largely hydrolysed by esterases,with mouse microsomes being particularly active.

Figure 6:1 Time-course studies of the biotransformation of cis- and trans-cypermethrin in trout and frog liver post-10,000g supernatant.



Assay conditions: 10^{-5} M cypermethrin isomers
1 ml 5 % w/w 10,000g supernatant
+NADPH +UDPGA
total volume 3 ml

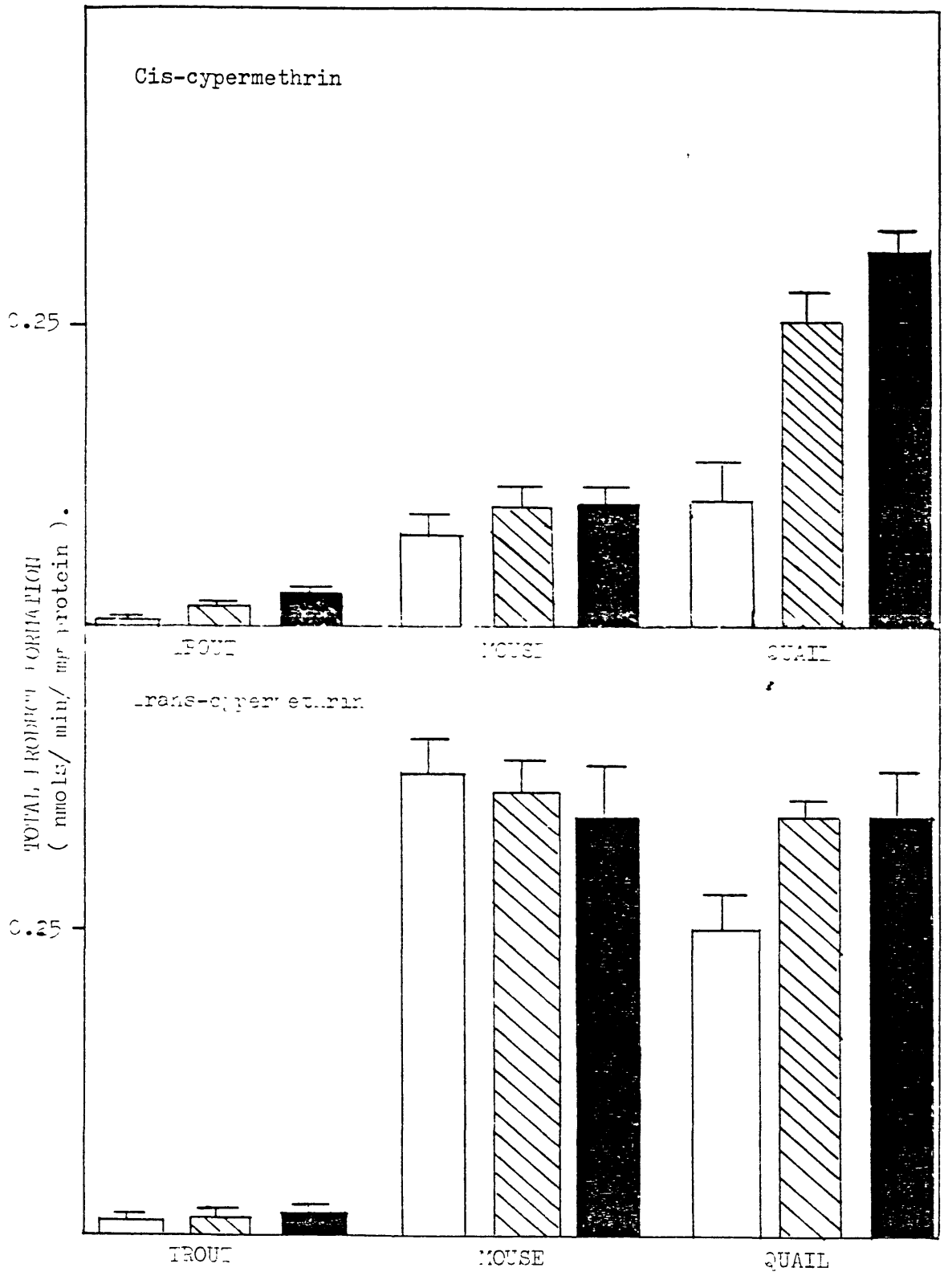
Figure 6:2. Time-course studies of the biotransformation of cis- and trans-cypermethrin in mouse and quail liver post-10,000g supernatant.



Assay conditions as detailed in Figure 6:1

Figure 6:3.

The effect of cofactors on the total biotransformation of cypermethrin isomers by washed liver microsomes in trout, mouse and quail.



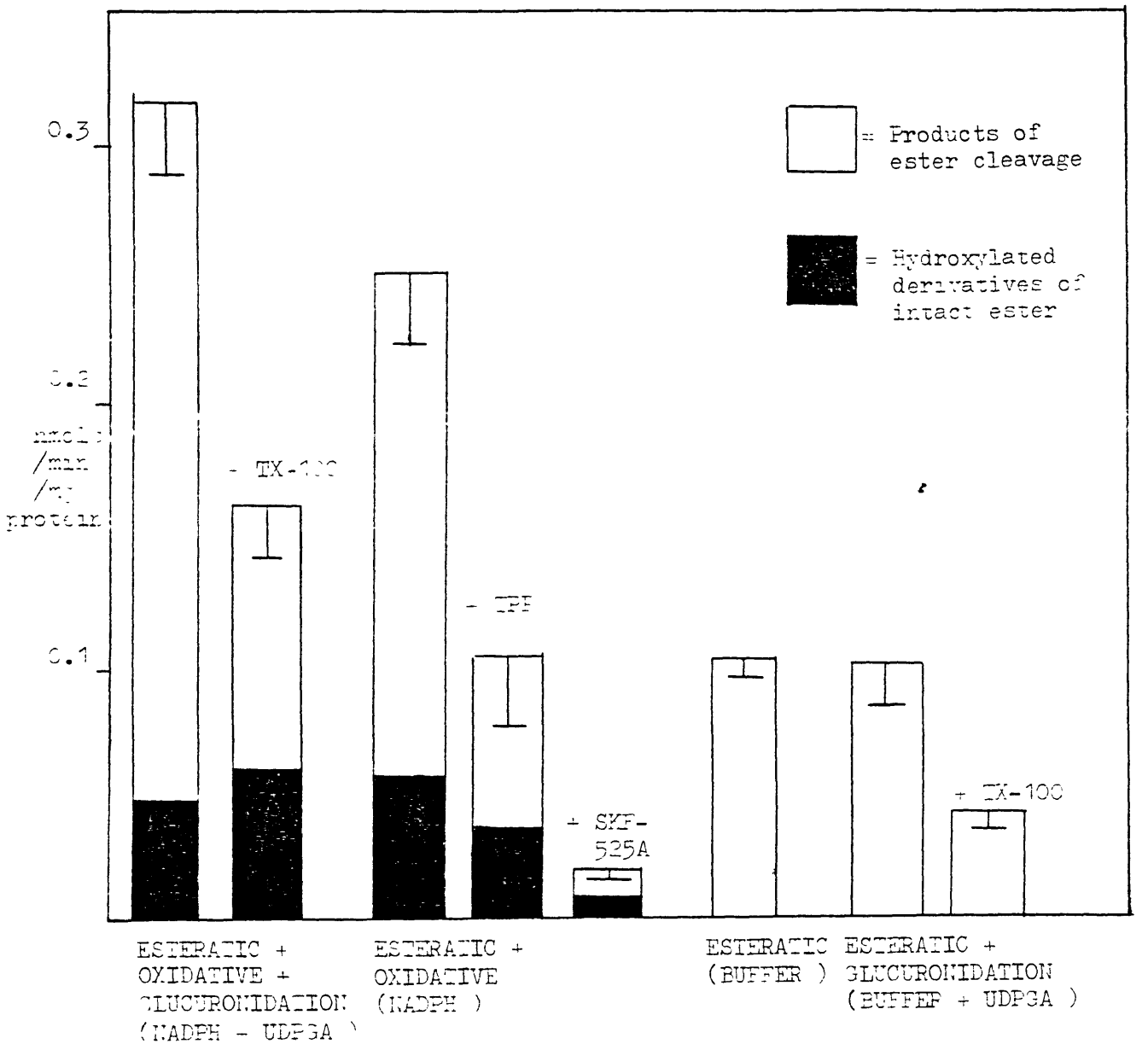
The metabolism of cis-cypermethrin by liver quail microsomes was also investigated using the esterase inhibitor triphenyl phosphate (TPP; 10^{-5}M), the mixed function oxidase inhibitor SKF 525A, (10^{-4}M), and the surfactant triton X-100 (0.25%). The results are summarised in Figure 6:4. In the presence of NADPH, the addition of TPP to microsomes caused a 60% loss in biotransformation activity due to a reduction in ester hydrolysis. However, SKF 525A caused a 92% reduction in microsomal activity, indicating that both esterases and the oxidative enzymes involved in ester cleavage were being inhibited. Triton X-100 failed to increase the rate of glucuronidation of released cis-Cl_2CA formed by the action of esterases, and caused a marked reduction in total biotransformation, presumably due to membrane disruption of the microsomes.

3.2.2. [^{14}C-benzyl] cypermethrin

Liver 10,000g fractions were incubated with [^{14}C-benzyl] cypermethrin under identical conditions to those described previously, and the products of biotransformation are shown in Table 6:7. The metabolite profiles for the hydroxylated derivatives of [^{14}C-benzyl]-labelled cypermethrin isomers were very similar to those obtained with [^{14}C-cyclopropyl] cypermethrin. The primary products of ester cleavage of cis-cypermethrin in frog, mouse and quail were 3-phenoxybenzoic acid (3PBA) and its hydroxylated

Figure 6:4

The effect of cofactors and inhibitors on the hydrolysis and oxidation of cis-cypermethrin by quail liver microsomes.



TPP 10^{-5}M triphenyl phosphate : Inhibitor of carboxylesterases

SKF-525A 10^{-4}M β-diethylaminoethyl-diphenyl-n-propylacetate-HCl : Inhibitor of microsomal oxidation.

TX-100 Triton X-100 : Surfactant

Bars represent standard deviations of triplicate determinations.

derivatives (largely 4'HO.3PBA). In addition, the ether glucuronide of 4'HO.3PBA was identified in these three species, though it could not be determined as to whether this had arisen from ester cleavage of the glucuronide of 4'HO.cypermethrin or by conjugation with 4'HO.3PBA. The ester glucuronide of 3PBA was only observed in quail liver. Trans-cypermethrin was metabolised to similar products, though higher yields of the products of ester hydrolysis were obtained. The unknown metabolite U/N 1 seen with [^{14}C-cyclopropyl] cypermethrin was not found with the benzyl label. 3-phenoxybenzyl alcohol and 3-phenoxybenzyl aldehyde were not major metabolites in any of the incubations.

4. Discussion

4.1. Routes of biotransformation

Trout, frogs, mice and quail showed considerable variation in the routes used in the metabolism of cypermethrin isomers in the liver. Trout hepatic esterases showed a low activity towards cis- and trans-cypermethrin, with hydroxylation at the 4' position of the phenoxybenzyl moiety being the principal phase I reaction (Tables 6:6 and 6:7). Similar metabolic profiles have been obtained with permethrin isomers in trout liver microsomes, but not in carp, which actively hydrolyse trans-permethrin to Cl_2CA and 3PBalc (Glickman et al., 1979). The in vitro formation of the glucuronide of 4'HO.cypermethrin is particularly

Table 6:7

Metabolites of [^{14}C-benzyl] cypermethrin isomers formed by liver 10,000g supernatant supplemented with NADPH and UDPGA

Metabolites (identified by hptlc.)	% of metabolised cypermethrin. cis-cypermethrin				trans-cypermethrin			
	Trout	Frog	Mouse	Quail	Trout	Frog	Mouse	Quail
Hydroxylated derivatives of intact cypermethrin								
4'HO-cyper	51.9	17.2	3.7	3.5	35.9	21.4	1.1	2.4
CH_2OH-cyper	n.d.	18.0	20.6	n.d.	n.d.	5.2	1.1	n.d.
CH_2OH-4'HO-cyper	n.d.	n.d.	1.3	n.d.	n.d.	n.d.	1.6	n.d.
4'O-cyper-gluc	44.0	8.6	5.8	1.0	11.1	6.0	2.7	1.0
CH_2O-cyper-gluc	n.d.	4.0	2.9	n.d.	n.d.	2.1	0.5	n.d.
Total	95.9	47.8	34.3	4.5	47.0	34.7	7.0	3.4
Derivatives of ester cleavage								
3PBA	n.d.	10.5	21.9	7.2	37.8	27.2	73.6	9.8
[a] HO-3PBA	3.4	14.7	10.0	37.4	n.d.	20.2	6.2	28.9
3PBA-gluc	n.d.	n.d.	n.d.	15.8	n.d.	n.d.	n.d.	15.5
4'O-3PBA-gluc	n.d.	15.1	21.1	10.0	n.d.	1.1	4.3	11.3
[b] U/N gluc	0.3	5.3	8.4	20.4	13.3	5.7	4.4	25.8
[c] U/N	-	6.2	2.9	4.7	-	11.0	2.4	5.2
Total	3.7	51.8	64.3	95.5	51.1	65.2	90.9	96.5
Total accounted for	99.6	99.6	98.6	100.0	98.1	99.9	97.9	99.9

Trout and Frog incubations analysed at 45 mins, Mouse and Quail at 15 mins.

n.d. not detected

3PBA 3-phenoxybenzoic acid gluc. glucuronide

a resolved into two bands by hptlc. Rf in solvent TEA: 0.21, 0.24. Major band (0.24 co-chromatographed with 4'HO.3PBA.

b Polar metabolites, β-glucuronidase labile, Rf EFW 0, 0.18.

c Uncharacterised radioactivity.

interesting, as this is a major metabolite of cis-cypermethrin observed in trout in vivo (Chapter 3). Recently Gregus et al., (1983) have reported that when compared with other chordates, trout liver UDP-glucuronyltransferase is surprisingly active towards some substrates. Interestingly they also detected appreciable quantities of endogenous hepatic UDPGA in this fish species. This was confirmed in these studies by the rapid formation of the glucuronide of 4'HO-cypermethrin in trout liver post-10,000g supernatant supplemented only with oxidative cofactors. This was not observed in the other species studied.

In both frogs and mice, the methyl group of the acid moiety as the major site of oxidative attack on cis-cypermethrin, with 4' hydroxylation of the phenyl ring also being important. This specificity of oxidation has also been reported by Shono et al., (1979) in mouse microsomes incubated with permethrin and cypermethrin. Alternative sites of hydroxylation (2', 6'and 5) were not investigated, though they have been reported in mice in vitro (Shono et al., 1979). Frog liver was more active in hydrolysing trans-cypermethrin than trout, though this activity was far lower than that of quail and mouse. Interestingly, Cole and Casida (1983) have reported that activities of hepatic esterases towards trans-permethrin in grassfrogs were similar to those found in mice at 30°C.

Quail were unique amongst the species tested in

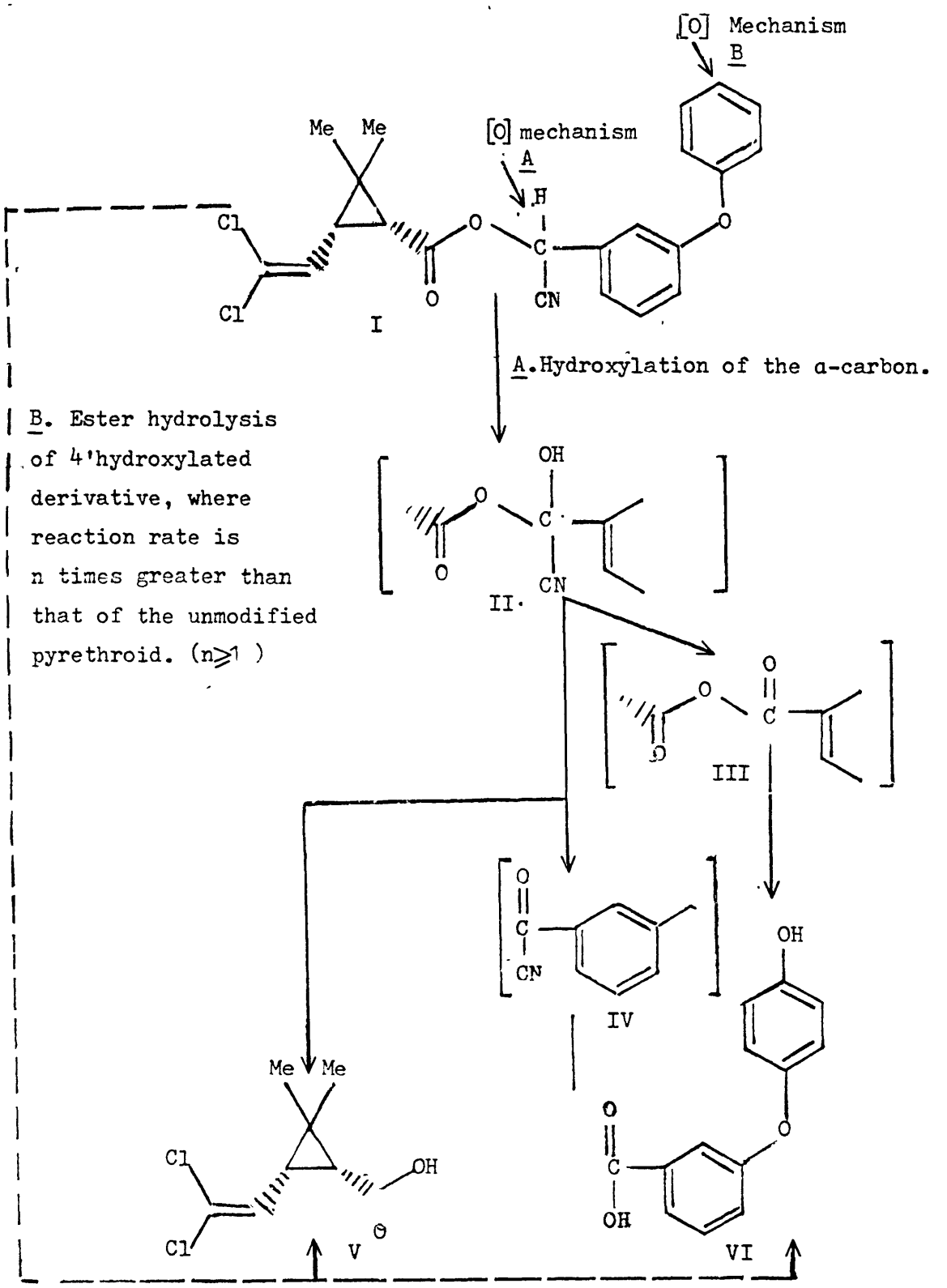


Figure 6·5 The role of oxidation in ester cleavage of cis-cypermethrin: Two possible mechanisms in the quail.

Mechanism A as proposed by Hutson, 1979. By this route of metabolism aryl hydroxylation may occur before or after ester cleavage.

I. cis-cypermethrin.
II. α-hydroxy derivative of I.
III. anhydride derivative of I.
IV. 3-phenoxybenzoyl cyanide.
V. cis-dichlorovinyl-dimethyl-cyclopropanecarboxylic acid.
VI. 3-phenoxybenzoic acid.(3PBA).

III & IV are acylating agents and would react with water to yield 3PBA.

metabolising cis-cypermethrin largely by ester cleavage, forming Cl_2CA, 3PBA and 4'HO.3PBA. The experiments with cofactors show that much of this de-esterification is dependent on oxidation. Using rat liver microsomes, NADPH-dependent ester cleavage of pyrethroids in the cis-conformation has been demonstrated with resmethrin (Ueda et al., 1975) and fenpropathrin (Crawford and Hutson, 1977), while Bedford et al., (1978) have characterised the oxidative de-esterification of flamprop-isopropyl. The involvement of oxidation in the ester cleavage of cis-cypermethrin may be explained (Figure 6:5) by the formation of an unstable α-hydroxy intermediate of cypermethrin (II), which is oxidised to give the anhydride derivative of the pyrethroid (III), or 3-phenoxybenzoyl cyanide (IV), and Cl_2CA. Both derivatives (III and IV) would then break down to give 3PBA in the presence of water. Alternatively, a less direct role for oxidation in the ester cleavage of cis-cypermethrin in quail may be proposed, whereby aryl-hydroxylation of the intact pyrethroid gives rise to a better substrate for esterases than the original insecticide (Figure 6:5). The evidence for such this latter mechanism is partly circumstantial, since quail readily form 4'HO.3PBA following hydrolysis of cis-cypermethrin. In addition, the pyrethroid allethrin will only undergo ester hydrolysis in rats, following it's oxidation to the acid (see Hutson, 1979). However

in vitro, the oxidative cleavage of fenpropathrin in the rat was not associated with 4'HO.3PBA formation (Crawford and Hutson, 1977), so mechanism B would seen unlikely to apply in rodent species. Quail also metabolised trans-cypermethrin by ester cleavage, though this was largely due to the action of hydrolases, with microsomal specific activities (nmols hydrolysed mg protein^{-1}) being rather lower than the mouse.

The relationship between oxidation and glucuronidation is demonstrated by the increase in biotransformation of cis-cypermethrin on the addition of UDPGA to quail liver microsomes (+NADPH). Similar observations have been made following the 12' hydroxylation of dieldrin (Hutson, 1976). This increase in reaction rate is presumably due to removal of phase I products from the vicinity of the active site of the oxidase and was not observed with the products of esterase action, even in the presence of Triton X-100. In all the species studied, the total rates of biotransformation of both the cis- and trans-isomers of cypermethrin were not affected by the position of the radiolabel (data not shown). The range of products formed from [^{14}C-cyclopropyl] and [^{14}C-benzyl] cypermethrin isomers in vitro in the liver were qualitatively similar to the metabolite profiles obtained in vivo (see Chapters 3 and 4).

The metabolism of [^{14}C-cyclopropyl] cypermethrin

isomers by fractions prepared from brain and plasma was largely esteratic in trout, mouse and quail (Table 6:4). No studies were carried out in the extra-hepatic tissues of the amphibian, but Cole and Casida (1983) have reported that there was no detectable pyrethroid-hydrolysing activity in the plasma of grassfrogs. Pyrethroid-hydrolases have previously been reported in the blood of rats (White et al., 1976), mice (Abernathy et al., 1973; Ruzo et al., 1979) and trout (Glickman and Lech, 1981). However in this study, the extent of hydrolysis of cis- and trans-cypermethrin in quail plasma was very low. Low levels of A-esterases have previously been reported in bird plasma (Brealey et al., 1980). By the classification system of Aldridge and Reiner (1972) the pyrethroid-hydrolases of the liver are more similar to B-esterases, as they are inhibited by several organophosphates (Abernathy et al., 1973). However, there is now some evidence to suggest that A-esterases in sheep serum can hydrolyse cypermethrin isomers (Mackness et al., 1984). Recently Westlake et al., (1983) have compared the activities of nitrophenylacetate-esterases (NPAE) in the brains and plasma of several wild birds and animals. Phasianidae species such as quail, had lower levels of NPAE's in their plasma than rodents, but similar activities in the brain. This is particularly interesting, as NPAE's are closely related to the pyrethroid-hydrolases of rat liver microsomes (Suzuki

and Miyamoto, 1976). On the basis of enzyme activities per gram of tissue, NPAE activities appear to correlate well with the relative hydrolysis of cypermethrin isomers in the brain and plasma of quail and mice. The hydrolysis of pyrethroids in mouse brain has previously been reported with deltamethrin (Ruzo et al., 1979), and recently Ghiasuddin and Soderlund (1984) have partially characterised an active brain esterase in the soluble fraction. This hydrolase has a high affinity for pyrethroids, particularly in the cis- conformation, though overall activities were low compared to the hepatic esterases. The toxicological significance of hydrolysis of pyrethroids in the brain may be relevant, since the activity is present at the site of toxic action. However in all the species studied, overall biotransformation rates of both cis- and trans-cypermethrin in the brain were very low (Table 6:3), and studies in mice with intracerebrally administered pyrethroids suggest that detoxification in the CNS during acute toxicity is negligible (Lawrence and Casida, 1982).

Metabolism of cis- and trans-cypermethrin in the intestinal post-10,000g supernatant was largely hydrolytic, though hydroxylation of one of the methyl groups of Cl_2CA (cis- or trans-) was a significant reaction in mice and trout. Unusually, little oxidative biotransformation was detected in quail gut, even though the intestine of this species is known to

have a very active mixed function oxidase activity towards some insecticides (Riviere and Bach, 1979). Glucuronidation of cypermethrin metabolites by gut preparations was not detected in any of the species studied, though UDP-glucuronyltransferases are known to be present in the intestine of mammals (Crawford and Hutson, 1979) and fish (Hänninen et al., 1984).

4.2. Rates of biotransformation

On the basis of nmoles of cypermethrin metabolised/ kg body weight/ min by the liver, mouse, quail and frog were respectively 27, 17 and 2 times more active in metabolising cis-cypermethrin than rainbow trout, and 87, 55 and 6 times more active towards trans-cypermethrin. The rates of biotransformation of trans-cypermethrin by mouse and trout liver and plasma preparations were lower than those obtained in similar studies with trans-permethrin (Glickman and Lech 1981; Glickman et al., 1982). This is probably due to the reduced susceptibility of α-cyano substituted pyrethroids to hydrolysis (Soderlund and Casida, 1977). Trout plasma and intestine were more active in the biotransformation of trans-cypermethrin than the liver. Similarly, Glickman and Lech (1981) and Glickman et al., (1982) have reported that trout plasma esterases are more active in hydrolysing trans-permethrin than liver hydrolases. In the other species, the liver was the major organ of

pyrethroid degradation, though the biotransformation of trans-cypermethrin by intestine and plasma (not quail) may be toxicologically important and account for the more rapid metabolism of trans-isomers observed in a number of species in vivo (Hutson, 1979). Certainly in the frog, mouse and quail, the preferential metabolism of the trans-isomer of pyrethroids in the liver would only arise if inadequately supplied with oxidative and conjugative cofactors; since cis- and trans-cypermethrin were metabolised at similar rates in the presence of NADPH and UDPGA. The possible contribution made by other tissues in pyrethroid detoxification was not investigated. However kidney preparations have also been shown to actively hydrolyse deltamethrin (Ruzo et al., 1979) and permethrin (Glickman and Lech, 1981), in mice and trout, respectively.

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Note: Abbreviations used in Figures 7:1 and 7:2

gluc. glucuronic acid

tau. taurine

1. Introduction

In this concluding chapter, the combined role of detoxification and target organ sensitivity in determining the species-dependent toxicity of cypermethrin will be assessed; the preceeding chapters having contained individual detailed discussions of their respective results. In addition, several other comparative metabolism/toxicity studies which have been carried out with other insecticides in fish, mammals and birds, will be briefly reviewed.

2. Species differences in the detoxification of cypermethrin

The metabolism of cypermethrin in fish, mammals and birds is summarised in Figure 7:1. The studies with cypermethrin *in vivo* (Chapters 3 and 4) showed that the number of biotransformation pathways used in detoxification followed the order quail>mouse>trout. In particular, this was clearly shown when the benzyl-labelled pyrethroid was used, and the metabolic profiles of [^{14}C-benzyl] cypermethrin in each species, as determined by reversed-phase h.p.l.c., are shown in Figure 7:2. Quail formed a large number of polar metabolites which were derived from ester cleavage. At the other extreme, trout were largely confined to forming the glucuronide of 4'HO.cypermethrin. Such differences in metabolic capability were also accompanied by species variations in the rates of

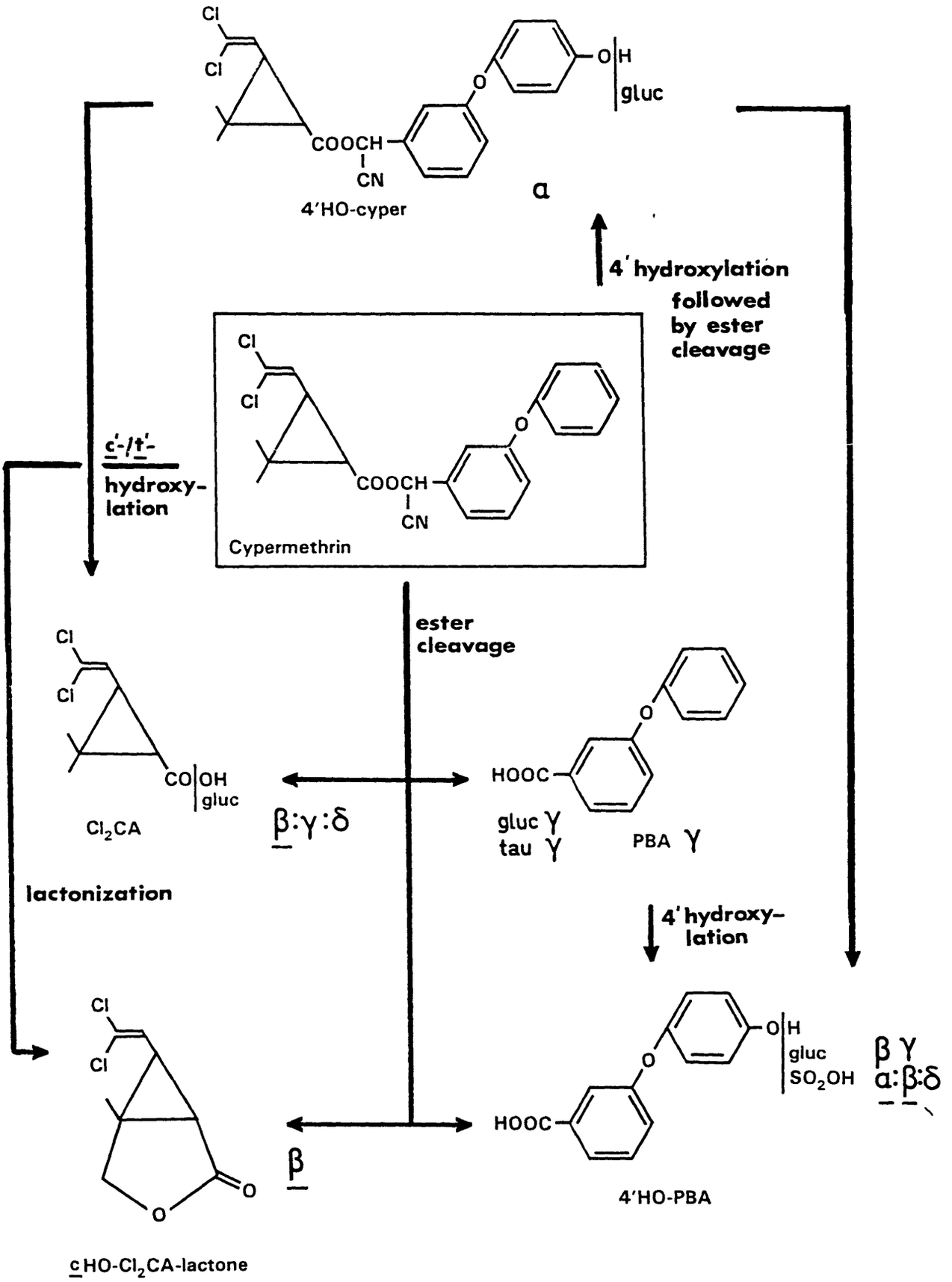
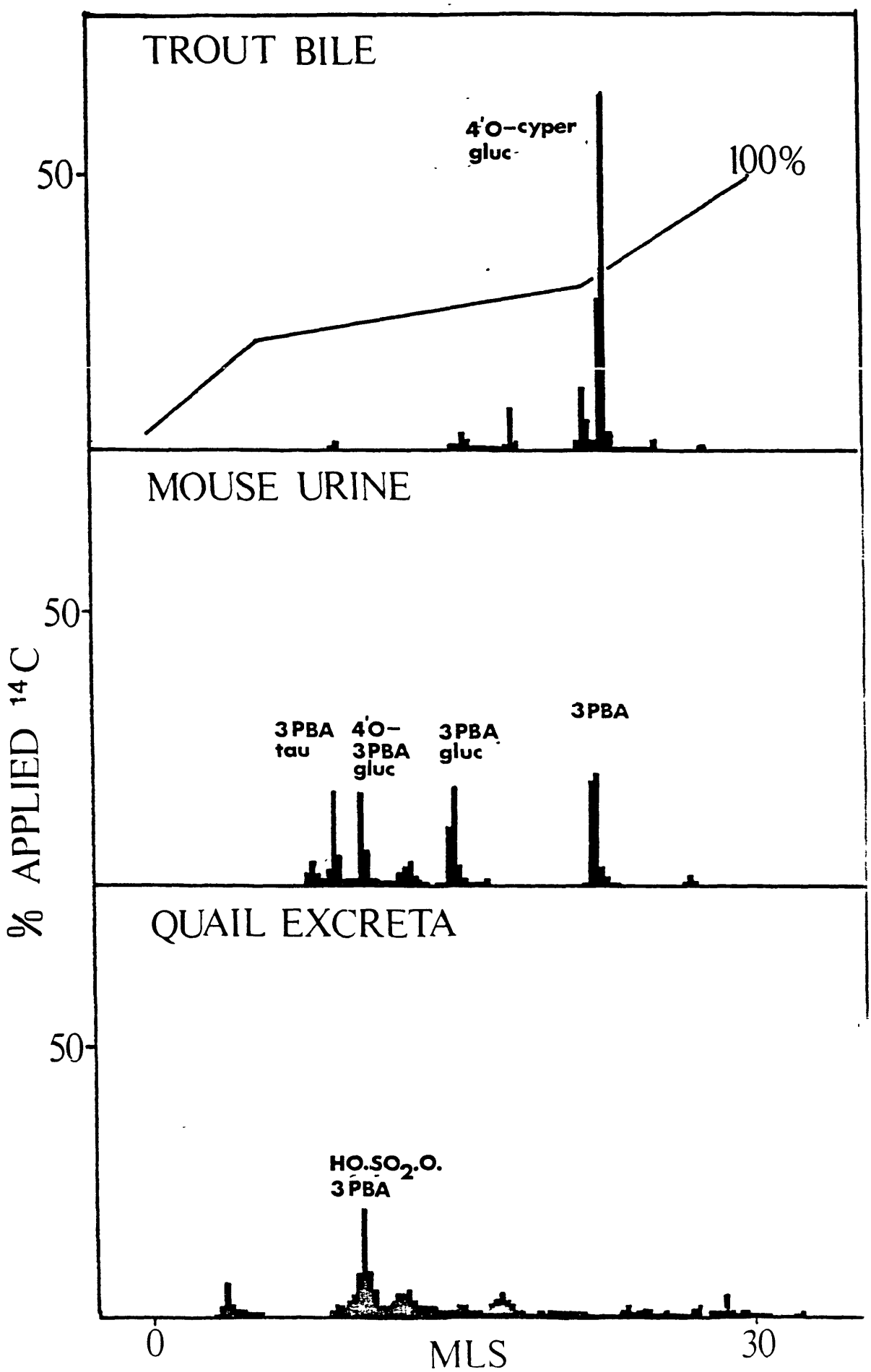


Figure 7:1 The metabolism of cypermethrin in trout, frog, mouse and quail.

Figure 7:2 The metabolite profile of [^{14}C-benzyl] cypermethrin in trout, mouse and quail.



cypermethrin detoxification. Thus, quail formed a larger range of metabolites than mice, and also eliminated the orally administered pyrethroid more rapidly than mammals. Unfortunately, direct inter-species comparisons between the *in vivo* biotransformation rates of cypermethrin in fish and birds were not possible,because of differences in the routes of dose administration. However, the *in vitro* studies with a number of tissues also indicate that quail are far more efficient at metabolising cypermethrin than trout or frogs.

Thus in the avian species, rapid pyrethroid-detoxification was associated with the use of a large number of biotransformation pathways, even at very high doses. This may indicate that through the use of a diverse range of metabolic pathways, no single detoxification reaction becomes rate-limiting; either through substrate inhibition, or cofactor depletion. By contrast, trout have a strictly limited ability to metabolise cypermethrin, and this seems to largely stem from the low levels of pyrethroid-hydrolases present in the liver. This confirms the observations of Glickman and Lech, (1981), who suggested that the slow hydrolysis of *trans*-permethrin in rainbow trout compared with mice could partially account for the sensitivity of this species to pyrethroids. Their subsequent toxicity studies with esterase inhibitors appeared to substantiate this claim (Glickman *et al.*,

1982).

However, not all fish species are deficient in pyrethroid-hydrolysing enzymes. There is some evidence to suggest that Cyprinidae species can actively ester-cleave the trans-isomers of pyrethroids, and this has been demonstrated with trans-permethrin in vitro using carp-liver microsomes (Glickman et al., 1979). Similarly, when carp were contained in a model aquatic ecosystem and exposed to [^{14}C-cyano]-S-fenvalerate, the amide and carboxylate derivatives of the intact ester, and 4'HO-S-fenvalerate were only detected as minor metabolites in fish tissues (Ohkawa et al., 1980). Instead, the concentrations of radiolabelled polar metabolites (presumabley HCN or thiocyanate) greatly exceeded that of the derivatives of the parent fenvalerate. Such a release of water-soluble ^{14}C would be dependent upon ester-cleavage, and this suggestd that carp are less dependent on 4'hydroxylation of the intact ester than trout. In addition, Bahadir and Freitag (1984) have reported that 3PBA and 4'HO.3PBA were the major extracted metabolites of cypermethrin in golden orfe following an aqueous exposure to the pyrethroid. Such a hydrolytic capability in carp may account for the lower sensitivity of this species to dissolved cypermethrin (96hr LC_{50} = 1.0µg l^{-1}) compared with trout (96hr LC_{50} = 0.5µg l^{-1}) (Stephenson, 1982).

There are also two other species differences in metabolism which are of toxicological interest. Firstly, the

polar metabolites formed by the quail (eg: P values: Cl_2CA glucuronide; 0.7;4'HO.SO_2,0.3PBA; 0.1) will be more readily eliminated in the urine than the more lipophilic glucuronide of 4'HO cypermethrin (P = 10.7). In the trout, the elimination of biliary detoxification products requires that food should be ingested, so as to allow gall-bladder empyting (Balk et al., 1984). In addition, the non-polar nature of 4'O cypermethrin glucuronide resulted in it's association with lipids and proteins in vitro, and this was confirmed in vivo by the presence of this metabolite in brain samples (Chapter 5). Thus, the formation of this product would be more likely to lead to a greater persistence of residues, than would be the case with the polar metabolites formed by mice and quail. Secondly, and from a toxicological point of view more importantly, are the primary detoxification steps used by each species. The products of ester cleavage of pyrethroids are essentially non-toxic (Gray and Soderlund, 1985), but some oxidative derivatives of pyrethroids, such as epoxy-chrysanthemates retain activity as neurotoxicants (Casida et al., 1983). This is particularly relevant with respect to the metabolism of the cis-isomers of pyrethroids, which in most mammalian species are acted on principally by oxidases (Hutson,1979). This was clearly demonstrated in vitro by mouse liver microsomes, which formed 4' and t' hydroxy derivatives of cypermethrin with the cis-isomer, but largely

products of ester cleavage with the trans—isomer (Chapter 6). By contrast, quail metabolised cis-cypermethrin to Cl_2CA, 3PBA and 4'HO.3PBA, and at a similar rate to the hydrolysis of the trans-isomer. Thus, unlike the mouse and the trout, the initial biotransformation of cis-cypermethrin in quail rendered the pyrethroid non-toxic. Therefore,the oxidative de-esterification of cis-cypermethrin in the quail may be of primary significance by achieving a more rapid and complete detoxification than in the other species. It may also explain the very similar rates of elimination of the cis- and trans-isomers of permethrin in chickens (Gaughan et al., 1978b).

Oxidatively mediated cleavage of cypermethrin may confer an additional advantage to the quail, in that the Cl_2CA so formed is readily glucuronidated (Chapter 6). This phase II reaction actually increased the overall biotransformation rate. No similar effect was observed with the acid released by the action of esterases, presumably due to poor substrate availability. Trout also showed a significant increase in the overall metabolism of cis-cypermethrin in the presence of UDPGA. This may be even more important than in the quail, since as 4'HO.cypermethrin is a potential neurotoxicant, the rate-limiting detoxification would be its subsequent glucuronidation. This assumes that 4'O.cypermethrin glucuronide would be non-toxic, which would appear to be reasonable, on the

basis of its increased polarity. Glucuronidation has previously been implicated in determining the species-dependent toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol to the sea lamprey, compared with rainbow trout (Lech and Statham, 1975).

A feature of the *in vitro* studies (Chapter 6), was the difficulty in extrapolating from the results obtained with subcellular fractions, to the likely detoxification capabilities of the respective vertebrate *in vivo*. For example, the studies with mouse tissue fractions suggested that the mammal would metabolise both *cis*- and *trans*-cypermethrin more readily than the quail, but this was not observed *in vivo*. Another difficulty in the use of hepatic microsomes, was the complex cofactor requirements for optimal biotransformation. Much of the current literature on pyrethroid detoxification has concentrated on the activities of plasma and liver hydrolases (see reviews by Casida *et al*., 1975/6; 1983). In the case of mammals, such as the mouse, measurement of the hydrolysis rates of *trans*-cypermethrin using an esterase-only microsomal assay, would give an accurate estimation of the true biotransformation potential of the liver towards this isomer. However in the quail, such a determination would underestimate detoxification rates by up to 25% (ie: in the presence of NADPH), and in trout would predict a five fold lower metabolic activity with the

cis-isomer, than observed in the presence of NADPH and UDPGA. Recent studies have shown that considerable inter-species variation exists in the proportional contribution made by hydrolases in the hepatic metabolism of cypermethrin isomers (Croucher and Logan, 1983). Thus, rabbit and human liver have very active pyrethroid-hydrolysing esterases, which in the case of man, show an unusually high activity towards cis-cypermethrin. However, in view of the reduced importance of hepatic hydrolases in pyrethroid detoxification in the non-mammalian species as determined in these studies, the use of esterase-only assays to predict likely in vivo biotransformation rates should be treated with caution.

Another feature of the in vivo studies was the contribution made by extra-hepatic tissues in cypermethrin metabolism, particularly in the trout. Similarly, Gray and Soderlund (1985) have stressed the importance of pyrethroid degradation in plasma and kidney. However, these investigations have also shown that the intestine may be an important organ of insecticide detoxification, as has previously been shown with dieldrin in the Japanese quail (Riviere and Bach, 1979). Several intriguing questions remain unanswered by these metabolism studies. In particular, the oxidative de-esterification of cis-cypermethrin seems worthy of further investigation. The exact role of oxidation in the ester cleavage could be determined

by ^{18}O studies. For example, labelling resulting from the use of $^{18}O_2$ should demonstrate whether α-carbon hydroxylation is occurring, since mixed-function oxidases (mfo's) require molecular oxygen. If ^{18}O was solely incorporated from labelled water however, then this mechanism could be discounted in favour of the proposed increased hydrolysis following aryl-hydroxylation of the intact ester. This latter suggestion could also be tested by Km determinations of the microsomal hydrolases in quail using cypermethrin and 4'HO.cypermethrin as substrates. Another interesting series of experiments could be carried out to investigate the effects of temperature, and water acidification, on pyrethroid metabolism in trout. Glickman et al., (1981) demonstrated that the biotransformation of permethrin by rainbow trout tissues in vitro, was increased by raising the incubation temperature over a moderate range (12-25°°C). Similarly, in vivo, trout convert DDT to DDE more rapidly at 18°C, than at 2°C or 10°C (Zinck and Addison, 1975). However in the field, such considerations may be less important in fish which are acclimatised to the temperature, since trout are capable of partially compensating their mfo and UDP-glucuronyltransferase activities during seasonal cooling and warming of the water (Koivusaari and Andersson, 1984). This is particularly relevant in view of the known increase in pyrethroid toxicity to

fish, which decreases with the water temperature (Kumaraguru and Beamish, 1981). Under laboratory conditions, this is likely to be a function of both reduced biotransformation, and increased neuro-sensitivity.

The role of water acidification in the metabolism of pyrethroids in fish is also of interest, since these insecticides are now under field-evaluation in Canadian pine forests (Kingsbury and Kreutzweister, 1984), where the aquatic eco-system is known to be under threat from acid pollution (Haines, 1981). Laitinen et al., (1982) have reported that following exposure to water at pH 3, the Salmonidae species, lavaret and splake, had significantly reduced hepatic mfo activity, though the UDP-glucuronyl-transerases were apparently unaffected. Since trout are dependent upon oxidation to metabolise pyrethroids, this warrants further investigation.

3. Species differences in the toxicology of pyrethroids

The neuro-sensitivities of trout, mouse and quail to cypermethrin, as determined by the concentrations of the pyrethroid in the brain during the manifestation of toxic signs, showed a positive correlation with the known susceptibilities of these species to pyrethroids ie: fish>mammals>birds (Miyamoto, 1976). An exception to this pattern was the frog, which though known to be less sensitive to dissolved pyrethroids than several

fish species (Jolly et al., 1978; Cole and Casida, 1983), had lower brain threshold to cis-cypermethrin than rainbow trout (Chapter 5). A partial explanation to this observation can be found in the hepatic biotransformation potentials of the two species, which showed the amphibian to be more active in the detoxification of both cis- and trans-cypermethrin, than the fish.

In all the species studied, concentrations of cis-cypermethrin in the brain and the severity of observed toxic signs showed a positive relationship, and indicated a largely central site of neuro-activity. This confirms the observations of other workers with pyrethroids in mammals (Gray and Soderlund, 1985), and fish (Glickman and Lech, 1982), but is the first time such a relationship has been demonstrated in birds. Fortunately, specific activities (micrograms of pyrethroid per gram of whole brain) appear to give an accurate estimate of neuro-sensitivity, since the insecticide appears to be distributed uniformly in the brain during toxic signs (Gray and Rickard, 1981). This is particularly relevant in inter-species comparative studies, where the neurotoxicant may be exerting differential effects on various components of the nervous system.

In addition to the species differences in brain sensitivities to cypermethrin, it was also apparent that various other factors such as intestinal

absorption and blood-brain barrier considerations, as well as detoxification, were also regulating the toxicity of the pyrethroid. The rate of penetration of a centrally-acting insecticide into the blood stream may be considered to be the organisms first line of defence. In the case of cypermethrin, the least susceptible species, quail, absorbed the pyrethroid from the intestine more readily than orally dosed trout and mice. However, the bird was then able to complement this with rapid detoxification. In mammals, poor intestinal uptake of pyrethroids appears to be a protective mechanism (see Chapter 5). This also seems to be the case in trout, which use the bile secreted into the intestine after ingesting cypermethrin-dosed pellets as an effective dispersant of the lipophilic insecticide.

The uptake of dissolved pyrethroids by fish, is complicated by the three possible modes of absorption; namely, through the gills, the skin, or the intestine. Balk et al., (1984) have studied the uptake of dissolved benzo-(a)-pyrene in pike and found that the majority of the xenobiotic was absorbed through the gills, though intestinal absorption occurred when the compound was administered orally. This is of particular interest, as benzo-(a)-pyrene has many of the physical properties of cypermethrin which influence bioaccumulation in fish, ie: low water solubility (5 $\mu g\ l^{-1}$) and high lipophilicity (Lech and Bend, 1980). A

study of the gill absorption of pyrethroids could provide some valuable toxicokinetic data on the uptake and elimination of these insecticides in fish. McKim and Goeden (1982) have recently described such a technique for determining the absorption efficiency of endrin across the blood-water interphase of brook trout.

Another factor which appears to be influencing species-dependent pyrethroid toxicity is the equilibration of the insecticide between brain and blood. Clearly, the exchange of the insecticide from the blood into the brain would be dependent on both plasma-protein binding, as well as the compounds affinity for brain phospholipids. Interestingly, Wells and Yarbrough (1972a and b) have reported that mosquitofish which were partially resistant to the effects of DDT and endrin, appeared to have a more effective blood-brain barrier to the insecticides, than susceptible individuals of the same species. This appeared to be due to the increased binding of DDT to brain plasma membranes, thus reducing the penetration of the neurotoxicant into the CNS. This may be pertinent to the aquatic toxicology of pyrethroids, since DDT and type I compounds appear to have similar modes of action (Miller and Adams, 1982), and also have similar physical properties, such as lipophilicity (Briggs, 1984). In fact, there have been reports of the resistance of some fish species to organochlorine

insecticides, being conferred to the pyrethroids (Chambers and Yarbrough, 1976). Similarly, the differential, non-covalent binding of cypermethrin to non-target membrane lipids and proteins, may partially explain species differences in target organ sensitivities. An alternative proposal accounting for the differences in vertebrate brain sensitivities to cis-cypermethrin may be variations in ligand binding affinity at the active site, as demonstrated with pyrethroid-resistant and sensitive housefly brain preparations (Chang and Plapp, 1983). Similarly, binding studies with the GABA receptor-ionophore complex in chordate brains may help determine whether species differences exist in the susceptibility to disruption of the active site, (Lawrence and Casida, 1983). In addition, examination of the biochemical changes in the CNS known to be brought about by pyrethroids, such as increased cerebellar cGMP (Aldridge et al., 1978; Brodie and Aldridge, 1982), may help explain species variations in target organ sensitivities. Ultimately, a true understanding of the factors influencing the neuro-sensitivity of vertebrates to pyrethroids must await a more definitive understanding of their mode of action at the biochemical level.

4. Implications of these studies

4.1. Comparison with other insecticides

The organophosphate and organochlorine classes of pesticides are generally more toxic to fish and birds than they are to mammals (O'Brien, 1967). In the case of some insecticides, this species-dependent toxicity has been found to be due to differences in the biotransformation capabilities of the different classes of vertebrates, as has been shown with cypermethrin in this study.

Thus, the natural insecticide rotenone is more toxic to roach and carp than rodents because the fish species have a reduced oxidative metabolic capacity in the liver, kidney, brain and intestine (Fukami et al., 1969). In the case of the organophosphates, a reduced oxidative capacity may have toxicological advantages, since the conversion of P=S to its oxygen analogue P=O (eg: parathion to paraoxon) is an activation step (O'Brien, 1967). When parathion was incubated with liver slices, trout were less active in forming paraoxon than rats, rabbits, pigeons and frogs (Potter and O'Brien, 1964). However, subsequent studies which examined the detoxification of thiophosphate insecticides as determined by anticholinesterase assays, showed that the reduced activation of organophosphates by piscine species was more than matched by a correspondingly reduced ability to detoxify these compounds by oxidation (Murphy, 1966)

and by nitro-reduction (Hitchcock and Murphy, 1966). In a later study with parathion and methyl-parathion, sunfish were found to be less sensitive to the i.p. administered insecticides (LD_{50}:110 and >2,500 mg kg^{-1} respectively) than mice (LD_{50}:14 and 11 mg kg^{-1} respectively) (Benke et al., 1974). This selective toxicity was shown to be due to a reduced inhibition of the piscine acetylcholinesterase, and a subsequent delay in toxicity in the fish species which enabled detoxification to occur by A-esterases and glutathione-dependent demethylation.

The metabolism of the organochlorine insecticides has been studied in fish, mammals and birds. However, no comparative studies which have examined the relationship between biotransformation and toxicity in these vertebrates have been reported. In a series of papers, the metabolism of the cyclodiene insecticides has been examined in fish (Sudershan and Khan, 1980; 1981) and found to be largely similar to mammals, though a more limited range of metabolites were formed, and at a slower rate. A relationship between cyclodiene metabolism and aquatic toxicology has been shown by the increased oxidation of these insecticides in mosquitofish which were resistant to aldrin (Wells et al., 1973). This appeared to be partially due to mfo induction by environmental contaminants. This development of fish resistance to insecticides through detoxification, may be relevant with pyrethroids, since

organochlorine-resistant mosquitofish also show an increased tolerance to pyrethrum extracts and allethrin, which was associated with increased mfo activity (Chambers and Yarbrough, 1976).

The studies with cypermethrin in vertebrates would further confirm the role of biotransformation in partially regulating toxicity. However, more importantly, they illustrate the need for a multiple approach in determining the toxicology of insecticides in aquatic, terrestrial and avian species.

4.2. Environmental toxicology

These studies confirm the environmental safety of pyrethroids in crop protection. Birds and mammals are extremely unlikely to ingest sufficient quantities of cypermethrin, either in the form of dead insects, or treated vegetation, to be at toxicological risk from acute poisoning. This has also been deduced by calculation for fenvalerate in bobwhite quail (Bradbury and Coats, 1982). Furthermore, the rapid metabolism of cypermethrin in birds ensures negligible teratological risk, by ensuring low body-residues of the insecticide. This is of current interest, due to the previous deleterious effects of organochlorines on wild life. In aquatic ecosystems, the use of the resolved, active 1R *cis* αS isomers, as contained in FASTAC, will reduce the potential bio-hazard of cypermethrin to fish and amphibians, since compared with RIPCORD, application

rates are reduced. This is particularly relevant, as these studies indicate that the trans-isomers contained in RIPCORD are as toxic as the cis-isomers to Salmonids. These investigations also indicate that pyrethroids which are adsorbed into vegetation or dead insects, are less toxic to some species of fish than possibly anticipated, since cypermethrin was slowly absorbed from the intestines of trout.

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