Pesticide and Xenobiotic Metabolism in Aquatic Organisms

In Pesticide and Xenobiotic Metabolism in Aquatic Organisms; Khan, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

Pesticide and Xenobiotic Metabolism in Aquatic Organisms

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable since symposia may embrace both types of presentation.

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PREFACE

During the 1960's considerable research effort was directed towards elucidating the metabolism and fate of foreign compounds in mammals, notably therapeutic agents and pesticides. However, little attention was paid to the processes involved in the biotransformation and elimination of xenobiotic chemicals in aquatic species. During this period some studies in the literature indicated that a number of species of fish were able to metabolize foreign compounds. Despite this finding, the consensus was that fish do not have nor do they need the ability to biotransform xenobiotic substances. Several pivotal studies in the late Sixties and early Seventies indicated conclusively that fish have the capacity to both oxidize and conjugate xenobiotic substances. These observations, along with a growth in concern for the aquatic environment from both the ecological and human health points of view, led to interest in the metabolism of xenobiotic chemicals in aquatic species. The expansion of research on biotransformation and disposition of xenobiotic chemicals in fish, along with studies of the effects of these chemicals on aquatic organisms, has led to the rapid development of aquatic toxicology research in the past five years.

Monitoring for the presence of xenobiotic chemicals in aquatic organisms may pertain to both ecological and human health interests. Since many xenobiotic chemicals ultimately find their way into aquatic species, knowledge of the location and concentration of these chemicals is germane to both protection of these species and assurance of a safe food supply for humans. In addition, monitoring the state of chemical contamination of the aquatic environment itself is of great importance. Since it is now known that fish and other aquatic organisms can carry out a variety of biotransformation reactions, any surveillance program, regardless of its ultimate purpose, is incomplete unless procedures are designed to take into account the chemical in question and its metabolic products. This is important for two reasons: first, chemical residues may be present in the form of biotransformation products and the identification of these xenobiotic substances in a given aquatic species will not be valid unless the nature and quantity of metabolic product is known; secondly, because it is now known that several biotransformation processes lead to the

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creation of chemicals which may be more toxic than the parent compounds, it is necessary to know the nature and number of metabolites which are formed when this situation arises.

The formation of polar metabolites from nonpolar materials may actually facilitate monitoring programs—in many cases the polar chemicals are highly concentrated in certain body fluids such as bile and urine. On the other hand, materials such as certain cyclodienes and polychlorinated biphenyls, which are very lipid soluble and resistant to metabolism, may accumulate and these chemicals may persist in the environment and may be transferred via the food chain to man. There is also interest in these biotransformation processes in lower organisms since the simplicity of these systems may lead to a better understanding of the phylogenetic development of xenobiotic metabolism.

If in the future unforeseen constraints force us to decide between determining the precise toxicity of all the chemicals which may enter into the aquatic environment, as opposed to knowing which chemicals are formed and where and how long they persist, the latter may be more pertinent. While the former is important, a knowledge of precise toxicity of chemicals to many organisms in all situations may not be feasible, either economically or within a specified time frame. However, knowledge of the toxicokinetic properties of chemicals and their biotransformation pathways in aquatic organisms will lead to surveillance programs reflecting the state of contamination of the aquatic environment. It is possible that this knowledge will aid in averting an ecological or human health crisis in the future.

This international symposium presents research from laboratories concerned with the metabolism and disposition of pesticides and other xenobiotic substances in aquatic organisms. The studies were not restricted to pesticides since current interest concerns a wide variety of chemicals and their disposition in aquatic organisms both in vivo and in vitro. This book is in two sections, one dealing with the in vivo metabolism of chemicals in aquatic organisms and the other describing primarily mechanisms of metabolism and disposition of xenobiotic chemicals in these species. Some of the papers overlap these two sections but were placed in one or the other as a matter of convenience. The authors wish to acknowledge the cooperation and efforts of the contributors both in oral presentations at the 176th ACS Meeting in Miami, Florida in September 1978, and in the preparation of the manuscripts comprising this book.

The authors wish to thank Ms. Cheryl Beyer, Ginger Marlow, and Pat Kelley for their typing and clerical assistance in planning for the symposium and book. Activities of the editors related to the symposium

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Metabolism of Organophosphorus Insecticides in Aquatic Organisms, with Special Emphasis on Fenitrothion

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The organophosphorus compounds constitute one major group of insecticides, and a certain portion thereof may be transported to the aquatic environment resulting either from the actual use on paddy fields or from unavoidable transmittance to waterways. However, possibly because of its relatively shorter persistence, the translocation and transformation of organophosphorus compounds in the aquatic environment has not been extensively investigated as compared with more persistent organochlorine compounds.

Fenitrothion, O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate, is widely used for the control of paddy field insects and forest protection in several countries, and since it is rather highly toxic to some aquatic organisms (LC 50 after 48hr exposure, 1.28 ppm for rainbow trout, 2.72 ppm for bluegill, 4.4 ppm for carp, LC 50 after 3 hr exposure, 0.0092 ppm for daphnia and no-effect dosage after 4 week exposure, 0.02 ppm for carp) (1, 2), the knowledge on degradation and metabolism of the compound in the aquatic environment is important for assessing short-term and long-term impacts on the non-target aquatic organisms.

In this article metabolism and bioaccumulation of fenitrothion in several aquatic species are dealt with under laboratory conditions.

Metabolism in vitro

To acquire information on the intrinsic metabolic activity of aquatic organisms, liver of carp (Cyprinus carpio Linnaeus), rainbow trout (Salmo gairdneri) and freshwater snail (Cipangopaludina japonica Martens) was dissected out, homogenized in 0.1M phosphate buffer, pH 7.5, and centrifuged at 105,000 g for 60 min to obtain the microsome-equivalent (described as the microsomal fraction hereafter) fraction. The protein content of microsomal and submicrosomal (supernatant fractions by Lowry's method, microsomal P-450 content (3), activity of aniline hydroxylase (4) and aminopyrine N-demethylase (5) were determined.

Table I shows the results which reveal that the drug-

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Table I. Drug-metabolizing enzyme activities in liver of animals.

Animal	Cytochrome P-450 content (nmole/g liver)	Activity of aniline hydroxylase (nmole/min/g liver)	Activity of aminopyrine *N*-demethylase (nmole/min/g liver)	Protein content in microsomal fraction (mg protein/g liver)
Mouse	40.1	37.1	89.0	13.4
Rat	31.1	16.5	62.6	18.8
Rainbow trout	21.5	3.7	5.9	75.4
Carp	5.5	2.3	13.9	27.0
Snail	<0.1	1.8	18.9	18.7

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metabolizing enzyme activities of the aquatic organisms are generally much lower than in mammals; especially with respect to P-450 content in snail and aniline hydroxylase in all 3 aquatic organisms, regardless of fairly large amounts of protein in the fraction.

The *in vitro* metabolism of ring-carbon-14 fenitrothion (5.42 mCi/mmole, >99%) was examined by using the microsomal fraction and determining oxidative desulfuration to fenitrooxon, O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphate, oxidation of the m-mehtyl group of fenitrooxon in the presence of NADPH plus EDTA, and hydrolytic cleavage of fenitrooxon to 3-methyl-4-nitrophenol, with calcium ion as a cofactor (6). Also in the presence of reduced glutathione, O-demethylation of fenitrothion and fenitrooxon was tested in the supernatant fraction. During incubation for 5-60 min at 24°C (for aquatic animals) (7) or 37°C (for mammals), an aliquot of the incubation mixture was sampled periodically, and analyzed for substrate disappearance and the respective product formed by two-dimensional thin layer chromatography, followed by determination of radioactivity of the separated spots.

Table II summarizes the results together with the detailed experimental conditions. As is evident, metabolic activities were detectable in these 3 aquatic species, but the rate was far lower as compared with mammalian hepatic enzume preparations, and the oxidative activities in snail were particularly low although the possibility was not ruled out of the presence of inhibitors of mixed-function oxidases in the fractions. The O-demethylation reaction proceeds extremely slowly in the enzyme preparation of aquatic animals, at less than one hundredth that of mammals.

Thus, although organophosphorus compounds like fenitrothion may be metabolized in aquatic organisms through oxidative desulfuration, side chain oxidation, hydrolytic cleavage of P-O-arryl linkage, as well as O-demethylation, the turn-over rate apparently is much lower than in mammals.

Metabolism and bioaccumulation in vivo

In order to obtain metabolic profiles of fenitrothion in fish *in vivo*, 2 yearling rainbow trout weighing on an average 26.6 g were maintained in 10 liters of aerated water at 18±0.5°C containing 0.1 ppm of radioactive fenitrothion labeled at the m-methyl position (3.16 mCi/mmole, >99%) (8). At 6 and 24 hr, and also at 24 hr after transfer to fresh water of fish exposed for the preceding 24 hr to the fenitrothion-containing water (24+24 in Figure 1), the fish were sampled and subjected to autoradiography.

The pattern of distribution of the absorbed radioactivity is shown in Figure 1. After 6 hrs of exposure the concentration of radioactivity was highest in gall bladder and intestine, and the radioactivity was distributed in most tissues except brain and heart after 24 hr. Twenty-four hrs after transfer to fresh water (24+24), most of the radioactivity in tissues had disappeared

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Table II. Metabolic activities in liver of animals for fenitrothion and fenitrooxon.

Animal	Metabolic activity (nmole/min/g liver)				
	oxidation[a] of		hydrolysis[b]	*O*-demethylation[c] of	
	fenitrothion	fenitrooxon	of fenitrooxon	fenitrothion	fenitrooxon
Mouse	11.1	13.8	13.3	652.4	996.8
Rat	7.1	20.5	16.1	513.8	676.2
Rainbow trout	2.7	0.6	1.2	2.8	19.6
Carp	0.9	0.6	1.2	0.4	4.2
Snail	<0.1	<0.1	<0.1	0.3	1.0

a) Ring-carbon-14 fenitrothion or fenitrooxon (70 μM) was incubated for 5-60 min at 37°C (mammals) or 24°C (fish and snail) with microsomal fraction equivalent to 0.15 g liver in the presence of 3 mM NADPH and 0.5 mM EDTA in 0.05 M tris-HCl buffer, pH 7.5.

b) Ring-carbon-14 fenitrooxon (70 μM) was incubated for 5-60 min at 37°C (mammals) or 24°C (fish and snail) with microsomal fraction equivalent to 0.15 g liver in the presence of 0.5 mM $CaCl_2$ in 0.05 M tris-HCl buffer, pH 7.5.

c) Ring-carbon-14 fenitrothion or fenitrooxon (70 μM) was incubated for 5-60 min at 37°C (mammals) or 24°C (fish and snail) with supernatant fraction equivalent to 0.05 g liver in the presence of 5 mM GSH in 0.1 M phosphate buffer, pH 7.5.

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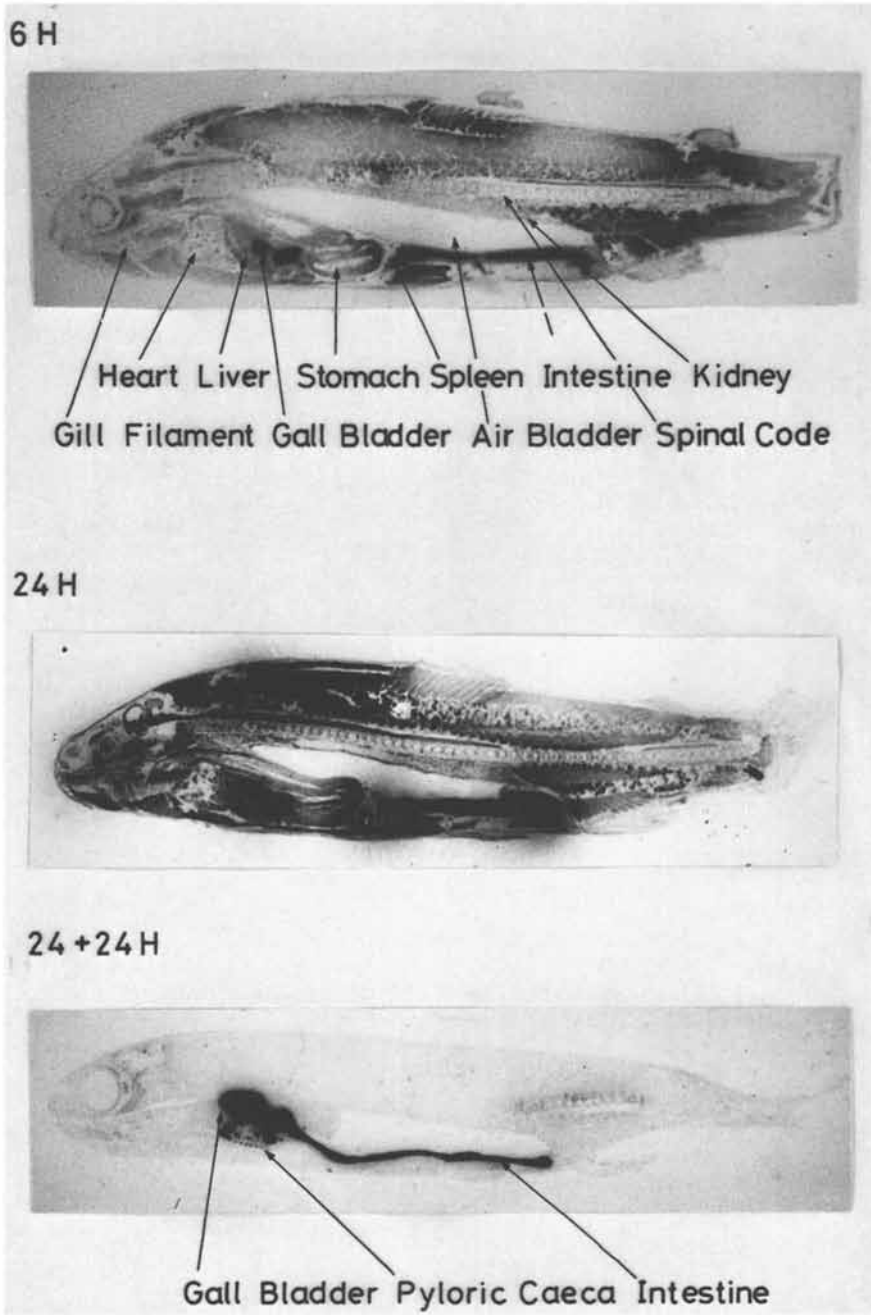

Figure 1. Whole-body autoradiograms of rainbow trout exposed to fenitrothion

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and only gall bladder, intestine and pyloric caeca contained an appreciable amount of radiocarbon.

At intervals, two of the exposed fish as well as an aliquot of aquarium water were analyzed for the radioactive metabolites by extracting them with acidic ether, followed by thin layer chromatographic separation and identification. As shown in Table III, fenitrothion is readily taken up by fish from the surrounding water and approximately one third and one half of the applied radioactivity was present in the fish after 6 and 24 hr, respectively. Transfer of the trout exposed to fenitrothion for 24 hr to fresh water resulted in elimination of the radioactivity from the body, and after 48 hr in fresh water the radioactivity decreased to 20%. The decrease was most likely to be due to excretion since brief washing of the fish removed no radioactivity from fish. Intact fenitrothion accounted for 90% of the total radiocarbon in fish during 24 hr, which is approximately 8 ppm on body weight basis, and the remaining 10% consisted of decomposition products such as 3-methyl-4-nitrophenol, 3-methyl-4-nitrophenyl-β-glucuronide, demethyl-fenitrothion and demethylfenitrooxon. A trace amount of fenitrooxon was present.

In water, the percentage of these degradation products increased with time and amounted to one quarter of the remaining radiocarbon at 24 hr; the quantity of 3-methyl-4-nitrophenol and both demethylated products is *ca.* 7% and 5.5%, respectively. Fenitrooxon was also detected. Because fenitrothion was stable in water under the present experimental conditions, these degradation products are presumably produced by fish metabolism.

Thus, fenitrothion is metabolized in rainbow trout through oxidation to phosphate, cleavage at the P-O-aryl linkage, O-demethylation and conjugation with glucuronic acid. These pathways for fenitrothion in fish are found in other biological systems *in vivo* (9); the former 3 pathways are similar to those found with methylparathion in sunfish *in vitro* (10), and glucuronic acid conjugation was reported to occur in metabolism *in vivo* of 3-trifluoromethyl-4-nitrophenol in rainbow trout (11).

When rainbow trout were transferred to fresh water (24+24 and 24+48 in Table III), both fenitrothion and its radioactive metabolites were eliminated from the fish. Thus after 48 hr in fresh water, 60% of the radioactivity originally contained in the fish had been excreted into the water and the degradation products accounted for one half of the total radioactivity in water. Fenitrothion in the fish biomass steadily decreased, although the rate was lower than in running water as will be described below.

In a similar static condition where approximately 1 ppm of malathion, diazinon or fenitrothion was added, Kanazawa (12) examined uptake and excretion of the compounds in minnows (*Psedorasbora parva* Temminck et Schlegel) at 23±2°C. The concentration of the compounds decreased with the lapse of time, after 4 weeks to 0.27 ppm for diazinon and 0.02 ppm for fenitrothion. Malathion disappeared much more promptly, to less than 0.01 ppm

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Table III. Distribution of fenitrothion and its metabolites in water and rainbow trout.

^{14}C	% Distribution of radioactivity[a]									
	6 hr[b]		12 hr[b]		24 hr[b]		24+24 hr[c]		24+48 hr[c]	
	Water	Fish	Water	Fish	Water	Fish	Water	Fish	Water	Fish
Fenitrothion	57.0	32.6	48.6	37.8	36.5	42.5	10.3	25.2	5.5	17.4
Fenitrooxon	1.4	0.2	2.8	0.2	2.1	0.2	1.1	0.2	1.0	0.1
Demethyl-fenitrothion	0.5	0.1	0.7	<0.1	2.4	<0.1	1.4	<0.1	<0.1	0.2
Demethyl-fenitrooxon	0.8	0.4	1.3	0.2	3.0	0.3	1.0	0.3	0.9	0.3
3-Methyl-4-nitrophenol	1.9	2.1	2.9	1.3	6.4	1.8	2.7	2.3	3.3	1.7
3-Methyl-4-nitrophenyl-G[d]	0.2	0.2	0.7	0.3	0.4	0.2	0.2	0.3	<0.1	0.2
Unextractable	—	1.4	—	2.2	—	3.9	—	3.8	—	1.6
Subtotal	61.8	37.0	55.2	42.0	50.8	48.9	16.7	32.2	10.7	21.5
Recovery	98.8		97.2		99.7					

a) Total radioactivity applied to water is referred to as 100%.
b) Exposure time.
c) After 24 hr exposure to fenitrothion, trout were transferred to fresh water and kept for 24 or 48 hr.
d) 3-Methyl-4-nitrophenyl-β-glucuronide.

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after 1 week. The concentration of the organophosphorus compounds in fish was maximum shortly after initiation of exposure; 211 ppm for diazinon after 3 days, 162 ppm for fenitrothion in 4 days and 2.4 ppm for malathion after 1 day. Thereafter the concentrations decreased gradually due to metabolism and excretion. After 30 days the fish contained 17 ppm or 4.9 ppm of diazinon or fenitrothion, respectively. Malathion was more unstable in fish, being 0.01 ppm after 1 week.

Two different sizes of rainbow trout (underyearling weighing ca. 3.3 g, and yearling fish weighing an average of 26.6 g) and southern top-mouthed minnows about 1.5 g in weight, were kept for a certain period of time in running water containing 0.1 ppm or 0.02 ppm fenitrothion. Fish and water were sampled at intervals and analyzed for fenitrothion adn fenitrooxon. The actual concentration of fenitrothion in water was kept fairly constant, although it was a little lower than the pre-set values.

The concentration of fenitrothion in minnows and in trout increased rapidly after exposure to the compound; it reached a maximum after 1-3 days of exposure (Figure 2). Thereafter, the fenitrothion concentration remained substantially constant. The concentration in underyearling trout exposed to a constant concentration of 0.02 ppm fenitrothion appeared to decrease.

The bioaccumulation ratio of fenitrothion (concentration in fish on test day/average concentration in water during test day) in rainbow trout and minnows was calculated. This revealed that bioaccumulation ratio did not increase on longer exposure and that the ratio was more or less independent of the fenitrothion concentration in water. The ratio was not so different between the two fish species, being approximately 250, 230 and 200 (at its maximum) in underyearling trout, yearling trout and minnow, respectively.

Once the fish were transferred from fenitrothion-containing water to fresh water, the levels of fenitrothion in fish decreased rapidly to around 0.01 ppm in 5 days (that is, by a factor of 1000). This tendency is apparently in good accord with field studies (13).

In contrast, 0.01 ppm DDT in water was rapidly absorbed and accumulated by yearling rainbow trout, the bioaccumulation ratio of total DDT as sum of DDT, DDE and TDE being 2700 after 14 days. The highest content was encountered in intestine (76.8 ppm), followed by stomach (46.0 ppm) and pyloric caeca (29.8 ppm). DDT and its metabolites, DDE and TDE, were hardly eliminated from the fish body during a 30-day clearing period (8).

Translocation and transformation in a freshwater model ecosystem

As one procedure of simulating natural aquatic environment, a freshwater static model ecosystem was established (Figure 3) (14, 15, 16), composed of 7 kg of Katano sandy loam soil (clay content 8%, organic matter content 1.8%, C.E.C. 9.3 me1/100 g dry

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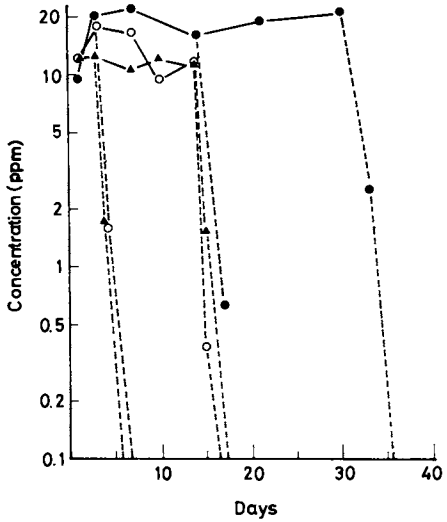

Figure 2. Concentration of fenitrothion in southern top-mouthed minnows and underyearling and yearling rainbow trouts kept in running water containing 0.1 ppm fenitrothion: (——), fenitrothion concentration in fish; (– – –), fenitrothion concentration in fish after transfer to fenitrothion free water. (– ▲ –), (southern top-mouthed minnow; (– ○ –), underyearling rainbow trout; (– ● –), yearling rainbow trout).

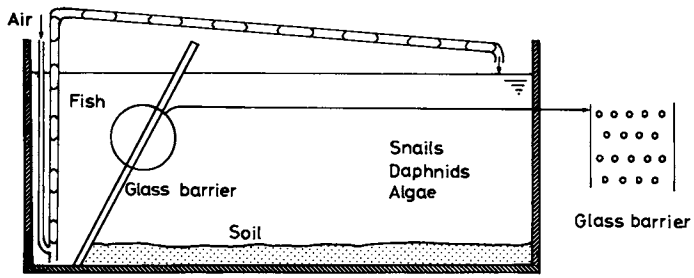

Figure 3. Aquatic model ecosystem. Aquarium (90 × 35 × 45 cm) divided by a glass barrier with holes. Components: 50 L of Freeman's standard reference water and 7 kg Katano sandy loam soil.

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soil, pH 5.8), 50 liters of Freeman's water (17) containing ca. 3 g of Daphnia pulex, ca. 1 g of algae (Oedogonium sp., Filamentous alga sp., Oscillatoria formosa, Scenedesmus bijuga var. alternans, Chlorella vulgaris, Monoraphidium irregulare, Paraphysomonas vestita), 5 freshwater snails (Cipangopaludina japonica Martens) weighing each ca. 20 g and 2 carp (Cyprinus carpio) with body weight of ca. 30 g in another compartment.

Ring-labeled ^{14}C DDT incorporated into bottom soil at the rate of 0.51 ppm was slowly eluted into the water and stabilized after 1 week with the concentration of 0.48 ppb, when 4 organisms were introduced into the aquarium. After 1 week, the total radiocarbon concentration in fish, snails, daphnids and algae was 86, 320, 250 and 190 ppb DDT-equivalents, respectively, while carbon-14 concentration in water was 0.49 ppb. Resolution and quantitation of the radioactive metabolites revealed that, as shown in Table IV, the bioaccumulation ratio of DDT and its metabolites is in the order of several thousand (16).

Table IV. Bioaccumulation of DDT and its metabolites in aquatic model ecosystem

Organism	Bioaccumulation ratio			
	DDT	DDE	TDE	DDT-R*
Alga	4720	2720	6210	4900
Daphnid	2560	—	—	1270
Snail	3660	13700	4460	5820
Carp	2390	8450	2710	3600

* Sum of DDT, DDE and TDE

When, in a preliminary study, ring-carbon-14 fenitrothion (18.4 mCi/mmole, >99%) at 1.0 ppm had been incorporated into the soil, and water poured onto the soil surface, the radiocarbon was eluted into water gradually until the equilibrium (28 ppb) was attained after 28 days at 25°C. The concentration of fenitrothion in water reached maximum, 4 ppb or ca. 3% of the total incorporated radioactivity, on the 1st day, decreasing rapidly thereafter and concurrently the radioactive degradation products such as demethylfenitrothion, 3-methyl-4-nitrophenol and N-acetylaminofenitrothion increased. The findings reflect rapid transformation of fenitrothion in the submerged soil as has been reported elsewhere (18), implying that little fenitrothion would be taken up into the component organisms under the given conditions.

In another trial, at 7 days after building-up the ecosystem, 10 ppb equivalent of the radioactive fenitrothion was added to water. Ten ppb of carbon-14 fenitrothion was added 3 times at the interval of one week. The system was kept at 25°C in a green house. An aliquot of water was sampled periodically, and the content of total radioactivity as well as fenitrothion was monitored.

As indicated in Figure 4 irrespective of single or multiple applications, fenitrothion in water disappeared with similar

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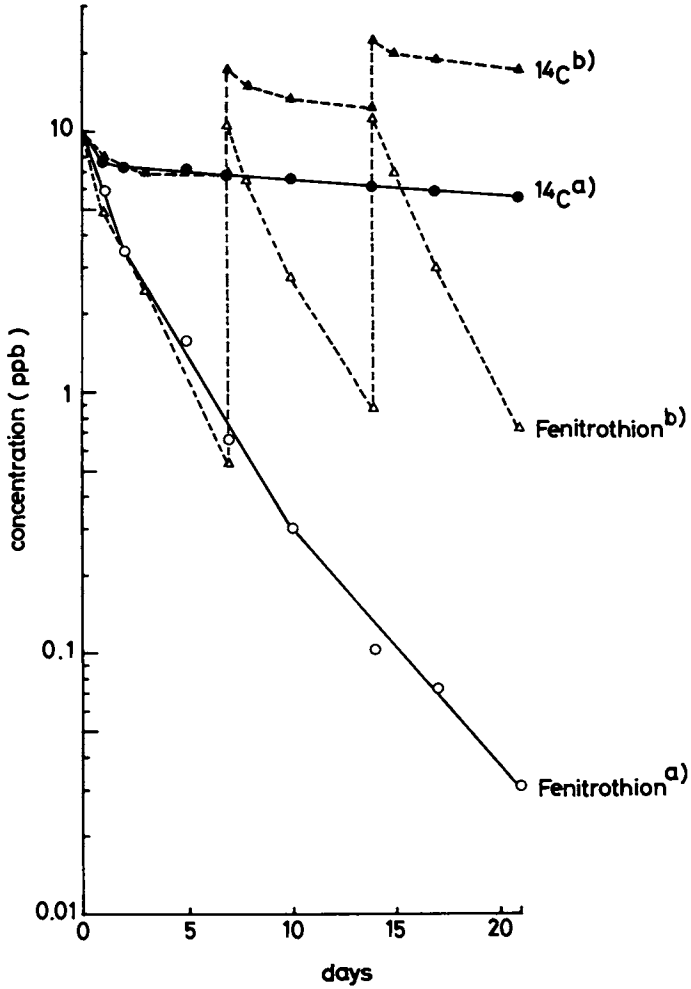

Figure 4. Concentration of ^{14}C and fenitrothion in water of aquatic model ecosystem: (a) fenitrothion added once to the model ecosystem; (b) fenitrothion added to the model ecosystem 2 more times at 7th and 14th day.

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rapidity with a half-life of ca. 2 days, while total radioactivity decreased relatively slowly. At 3, 7 and 21 days after single application or 7 days after 3 applications of fenitrothion, 4 component organisms as well as soil samples were extracted with methanol. Water was passed through an XAD-2 column and the radiocarbon trapped was eluted with acetone. The extracted radiocarbon was separated by thin layer chromatography with the available authentic reference compounds. The radioactivity in the unextractable residue was determined after combustion.

Table V, VI and VII summarize the results of identification of the degradation products. The total radioactivity as well as intact fenitrothion decreased in snail and fish, and 3 consecutive applications of fenitrothion did not affect these concentrations very much. In contrast, in daphnids and in algae total radioactivity tended to increase with the lapse of time, mostly due to an increase of unextractable radioactivity and unidentified products, although the fenitrothion content was constantly decreasing.

Bioaccumulation ratios of total radiocarbon and fenitrothion relative to the water concentrations are shown in Table VIII. They reveal that the ratio is not so high, at the maximum 180, and in fish it tends to decrease with longer incubation of the system, whereas in daphnids and algae the bioaccumulation ratio of fenitrothion was increasing under the present static conditions, due to quite rapid disappearance of fenitrothion in water. In any event, the bioaccumulation ratio was obviously far less than that of DDT and its degradation products.

The number of the degradation products identified was largest in water; they include fenitrooxon, aminofenitrothion, and its N-formyl and N-acetyl derivatives as well as 3 demethylated products. Several phenolic products are found in water, together with a sulfate conjugate of 3-methyl-4-nitrophenol. In soil, no oxygen analogs were demonstrated, but several amino derivatives were present, consistent with previous findings (18). Fish contained amino compounds that have not been identified in metabolism studies described above. They probably result from absorption from the surrounding water. Snails contained a fewer number of degradation products, among which amino derivatives of fenitrothion, demethylfenitrothion, demethylfenitrooxon as well as 3-methyl-4-nitrophenol and its sulfate conjugate are included. Most of the radiocarbon in daphnids and snails is yet to be identified.

Thus, in the static ecosystem the translocation and metabolism of fenitrothion is very complicated, apparently due to decomposition by soil organisms. Nevertheless, the data presented here may imply that rapid bioaccumulation of the radioactive compounds derived from fenitrothion in snail or in fish is unlikely to occur in the natural environment.

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Table V. Distribution of ^{14}C, fenitrothion and its degradation products in individual components of aquatic model ecosystem : Water and soil.

^{14}C-compound	Concentration (ppb of fenitrothion equivalent)							
	Water				Soil			
	3 d	7 d	21 d	21 d[a)]	3 d	7 d	21 d	21 d[a)]
Total ^{14}C	7.00	7.19	5.67	17.1	4.08	7.10	14.9	35.4
Extractable ^{14}C	5.45	5.24	2.73	10.8	2.47	2.63	2.97	6.44
Fenitrothion	3.02	0.97	0.03	0.74	1.18	0.95	0.19	0.65
Carboxyfenitrothion	<0.01	0.01	0.01	0.02	— [b)]	—	—	—
Demethylfenitrothion	0.24	0.23	0.63	0.18	0.36	0.43	0.08	0.53
Aminofenitrothion	0.03	0.02	0.01	0.04	—	0.03	—	0.23
Demethylaminofenitrothion	0.05	0.11	0.19	0.24	—	0.03	0.13	0.34
N-Acetylaminofenitrothion	0.05	0.06	0.06	0.47	0.05	0.02	—	0.20
N-Formylaminofenitrothion	0.03	0.03	0.01	0.05	—	—	—	0.07
Fenitrooxon	0.05	0.05	0.01	0.09	—	—	—	—
N-Acetylaminofenitrooxon	0.01	0.01	0.03	0.11	—	—	—	—
Demethylfenitrooxon	0.25	0.48	0.22	1.38	—	—	—	—
3-Methyl-4-nitrophenol	1.58	2.37	0.46	4.31	0.61	0.87	0.56	1.52
3-Methyl-4-aminophenol	0.01	0.01	0.01	0.02	—	—	—	—
3-Methyl-4-acetylaminophenol	0.01	0.03	0.17	0.24	—	—	0.05	0.08
3-Methyl-4-nitrophenyl sulfate	0.07	0.17	0.12	0.61	—	—	—	—
Unidentified ^{14}C	1.63	0.71	0.79	2.30	0.28	0.30	1.96	2.83
Unextractable ^{14}C	1.55	1.95	2.94	6.30	1.61	4.47	11.9	29.0

a) ^{14}C-fenitrothion added to the model ecosystem 2 more times at 7 and 14th day.
b) Not detected.

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Table VI. Distribution of ^{14}C, fenitrothion and its degradation products in individual components of aquatic model ecosystem : Alga and snail.

^{14}C-compound	Concentration (ppb of fenitrothion equivalent)							
	Alga				Snail			
	3 d	7 d	21 d	21 d[a]	3 d	7 d	21 d	21 d[a]
Total ^{14}C	299	442	525	647	174	157	92.5	219
Extractable ^{14}C	134	139	103	170	168	150	77.9	198
Fenitrothion	71.5	46.9	5.58	11.6	24.8	8.99	1.01	4.16
Demethylfenitrothion	—[b]	—	—	—	71.0	3.74	6.85	34.9
Aminofenitrothion	—	—	—	—	8.55	—	—	2.58
Demethylaminofenitrothion	—	—	—	—	7.21	9.74	8.80	13.3
Demethylfenitrooxon	—	—	—	—	5.03	4.64	3.58	15.7
3-Methyl-4-nitrophenol	5.76	12.5	6.72	9.53	22.0	21.6	8.41	49.2
3-Methyl-4-aminophenol	—	—	0.72	—	—	—	—	—
3-Methyl-4-nitrophenyl sulfate	—	—	—	—	9.90	3.12	22.1	36.1
Unidentified ^{14}C	56.8	79.7	90.0	154	19.5	98.2	27.2	42.1
Unextractable ^{14}C	165	303	422	477	6.00	7.00	14.6	21.0

a) ^{14}C-fenitrothion added to the model ecosystem 2 more times at 7 and 14th day.
b) Not detected.

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Table VII. Distribution of ^{14}C, fenitrothion and its degradation products in individual components of aquatic model ecosystem : Daphnid and carp.

^{14}C-compound	Concentration (ppb of fenitrothion equivalent)							
	Daphnid				Carp			
	3 d	7 d	21 d	21 d[a)]	3 d	7 d	21 d	21 d[a)]
Total ^{14}C	124	333	336	984	1260	1000	236	994
Extractable ^{14}C	93.1	134	85.0	340	1230	972	215	938
Fenitrothion	46.1	49.7	2.13	9.19	547	154	3.01	14.1
Demethylfenitrothion	— b)	—	—	—	82.3	30.1	0.65	36.6
Demethylaminofenitrothion	—	—	—	—	13.5	25.3	18.1	51.6
N-Acetylaminofenitrothion	—	—	—	—	1.23	—	7.09	0.94
Fenitrooxon	—	—	—	—	2.46	—	—	—
N-Acetylaminofenitrooxon	—	—	—	—	—	—	—	4.69
Demethylfenitrooxon	—	—	—	—	6.14	5.83	1.07	12.2
3-Methyl-4-nitrophenol	3.45	8.72	0.85	15.6	55.3	52.5	20.2	44.1
3-Methyl-4-acetylaminophenol	—	—	—	—	—	1.94	7.31	13.1
3-Methyl-4-nitrophenyl-β-glucuronide	—	—	—	—	480	675	129	679
Unidentified ^{14}C	46.3	75.6	82.0	315	42.1	29.3	28.6	81.7
Unextractable ^{14}C	30.9	199	251	644	30.0	28.0	21.0	56.0

a) ^{14}C-fenitrothion added to the model ecosystem 2 more times at 7 and 14th day.
b) Not detected.

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Table VIII. Bioaccumulation ratio of total ^{14}C and fenitrothion in aquatic model ecosystem.

Period (days)	Bioaccumulation ratio							
	Daphnid		Alga		Snail		Carp	
	Total ^{14}C	Fenitro-thion	Total ^{14}C	Fenitro-thion	Total ^{14}C	Fenitro-thion	Total ^{14}C	Fenitro-thion
3	17.7	15.3	42.7	23.7	24.9	8.2	180	181
7	46.3	51.2	61.5	48.4	21.8	9.3	139	159
21	59.3	69.2	92.6	181	16.3	32.8	41.6	97.7
21[a)]	57.5	12.5	37.8	15.8	12.8	5.7	58.1	19.2

a) ^{14}C-fenitrothion added to the model ecosystem 2 more times at 7 and 14th day.

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Abstract

Although metabolism of fenitrothion, O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate in subcellular fractions of rainbow trout and carp is intrinsically not high, as compared with mammalian hepatic enzyme preparations, rainbow trout can metabolize, in vivo, this organophosphorus compound through oxidative desulfuration, cleavage of the P-O-aryl linkage and O-demethylation. The degradation products as well as the parent compound are excreted into the surrounding water. As a result, bioaccumulation of fenitrothion in the fish species proved not to be high. Southern top-mouthed minnows found not to contain much fenitrothion in tissues.

In a static model ecosystem, several amino derivatives of fenitrothion, probably derived from the soil metabolism, were demonstrated in carp tissues, together with the nitro-containing compounds. The concentration of fenitrothion in carp, snails, daphnids and algae decreased with time, although its bioaccumulation ratio relative to the concentration in water tended to increase gradually in snails, daphnids and algae, presumably due to lower metabolic activity and/or slow excretion.

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2

Disposition of Polychlorinated Biphenyls in Fish

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Polychlorinated biphenyls (PCBs) have been used in various industrial processes during the past 40 years but were not recognized as major environmental contaminants until 1966 (1). Fish as a major food source have attained the dubious honor of being the most frequently cited PCB contamination problem (2). In the following presentation disposition of PCBs in fish will be discussed from four points of view: accumulation, metabolism, distribution and elimination. No attempt will be made to cover PCB residue levels found in fish in nature (3) or acute or chronic toxicity of PCBs in fish (4-20).

In the United States PCB preparations are marketed under the trade name Aroclor and consist of complex mixtures of chlorobiphenyls. Table I gives the chlorobiphenyl composition of some

TABLE I

Percent Chlorobiphenyl Composition of Selected Aroclors[a]

Chlorobiphenyl[b]	Aroclor			
	1242	1248	1254	1260
1-CB	3			
2-CB	13	2		
3-CB	28	18		
4-CB	30	40	11	
5-CB	22	36	49	12
6-CB	4	4	34	38
7-CB			6	41
8-CB				8
9-CB				1

[a]Adapted from (22).
[b]1-CB = monochlorobiphenyl, 2-CB = dichlorobiphenyl, etc.

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common Aroclor prepa-ations (21). All Aroclors are assigned a four-digit number. The first two digits, 12, represent chlorinated biphenyl and the last two digits give weight percent of chlorine. In going from Aroclor 1242 to 1260 one is going from a mixture of primarily trichloro- and tetrachlorobiphenyls to hexachloro- and heptachlorobiphenyls.

Accumulation. One of the most important properties of PCBs is their tendency to bioaccumulate in fish to levels higher than ambient water. This property results from high lipid solubility of PCBs and the slow rate with which fish metabolize and eliminate them. Bioaccumulation has been assessed by exposing fish to a constant concentration of PCB in water. Initially PCB uptake is rapid then it levels off until a steady state is reached. At steady state, concentration of PCB in water and fish change but very slowly. Bioaccumulation is determined by calculating a bioaccumulation factor (PCB concentration in whole fish/PCB concentration in water). This factor is a characteristic of the fish and duration of PCB exposure and is independent of PCB water concentration (20). The effect of duration of water exposure on accumulation of Aroclor 1248 and 1254 in channel catfish is shown in Figure 1 (19). For both Aroclors the bioaccumulation factor increases as duration of exposure increases. At the end of exposure, 77 days, a steady state PCB concentration in the fish has not been attained and the bioaccumulation factor at this time is about 60,000 for both Aroclor preparations. These findings demonstrate that to achieve a steady state concentration of PCBs in fish, when exposed to the compounds in water, a long duration of exposure is needed. Table II shows the range of bioconcentration factors for fathead minnows that were exposed to different Aro-

TABLE II

Bioaccumulation of Aroclor 1242, 1248, 1254 and 1260 by Fathead Minnows From Water[a]

Aroclor	Bioaccumulation Factor (range of Individual fish)
1242	32,000 - 274,000
1248	60,000 - 120,000
1254	46,000 - 307,000
1260	160,000 - 270,000

[a]Adapted from (23).

clors in water for 8 months (23). PCB concentration in fish and water had reached a steady state at this time and bioaccumulation

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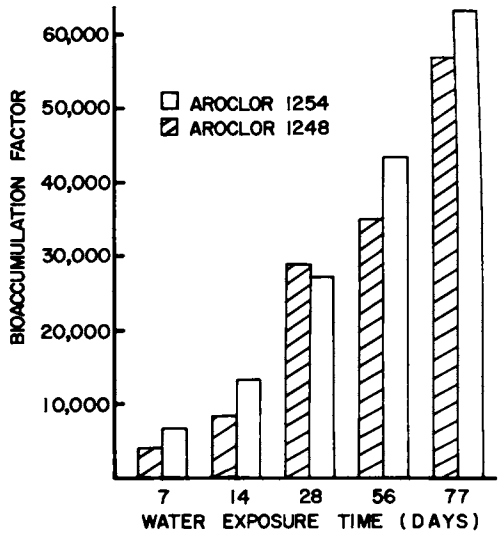

Figure 1. Bioaccumulation of ^{36}Cl-Aroclor 1248 and 1254 by channel catfish from water. Concentrations of Aroclor 1248 and 1254 in water were 5.8 and 2.4 μg/L, respectively (19).

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factors for individual minnows ranged from 32,000 for Aroclor 1242 to 307,000 for 1254. Accumulation factors within this range have been reported for other fish in laboratory experiments (5,19).

Along with water exposure another way that fish accumulate PCB is via the diet. Table III shows accumulation of chlorobiphenyls by coho salmon fed diets containing increasing concentrations of Aroclor 1254 (19). The main finding was that the higher

TABLE III

Concentration of Aroclor 1254 Residues in Coho Salmon After Dietary Exposure [a]

Dietary Concentration (ppm)	Exposure Time (days)						
	14	28	56	112	142	200	260
0.048	0.19	0.23	0.18	0.47	0.35	0.36	0.40
0.48	0.28	0.42	0.69	0.50	0.61	0.68	0.59
4.8	0.96	1.30	2.90	3.80	3.50	2.50	3.90
48.0	8.90	11.00	22.00	22.00	34.00	57.00	54.00
480.0	82.00	114.00	300.00	384.00	361.00	659.00	645.00

[a]Adapted from (19).

the concentration of Aroclor 1254 in the diet the longer it took for whole body PCB concentration to reach a steady state. For salmon fed 0.048 to 4.8 ppm of PCB whole body concentration reached a steady state after 112 days; for fish fed 48 and 480 ppm it took 200 days. An additional observation was that the higher the concentration of PCB in the diet, the higher the steady state concentration in the fish.

The question now is: What happens to the absolute amount of PCB accumulated by fish if they continue to be fed contaminated food after whole body PCB concentration has reached a steady state? The answer is that they continue to accumulate PCB at app-oximately the same rate that they grow (2,24). This is shown in Figure 2 for juvenile rainbow trout fed 15 ppm of Aroclor 1254 (24). Panel A reveals that PCB concentration in whole fish increased until a steady state was reached between 24 and 32 weeks. Panel B demonstrates that the absolute amount of PCB accumulated by the fish increased in an exponential fashion throughout the study and panel C shows that wet weight of the fish also increased exponentially. Taken together these findings demonstrate that whole body PCB concentration reaches a steady state between 24 and 32 weeks od dietary exposure (A) because the exponential increase in total amount of PCB accumulated (B) is offset by the exponential increase in bo-y weight (C).

A factor that determines extent to which PCBs are accumulated

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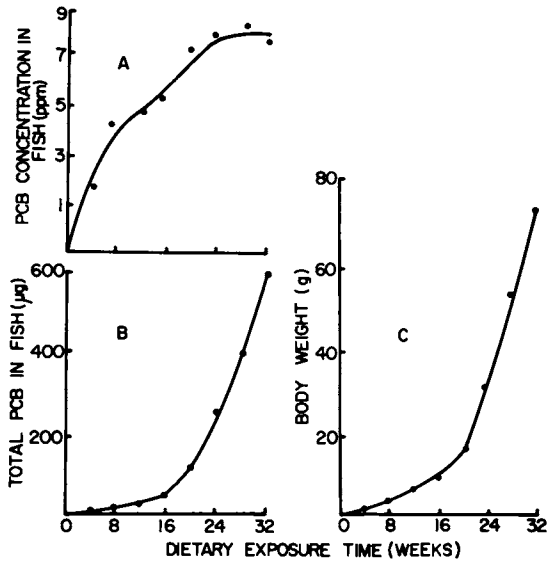

Figure 2. Bioaccumulation of Aroclor 1254 in rainbow trout. The fish were fed 15 ppm of Aroclor 1254 for 32 weeks. All points are mean values (24).

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by fish is degree of chlorination of the biphenyl molecule. Generally lower chlorinated PCB mixtures are accumulated to a lesser extent than higher chlorinated ones. This is illustrated in Figure 3 by channel catfish accumulating Aroclor 1232 and 1248 to a lesser extent than 1254 and 1260 (19). The lower accumulation is due to more rapid metabolism and elimination of lower chlorinated chlorobiphenyls. This has also been demonstrated in fathead minnows exposed to Aroclor 1248 or 1260 for 66 days in water and transferred to PCB-free water for 34 days (20). Figure 4 shows a chromatogram of Aroclor 1248 residues in minnows after the 34 day wash-out period compared to an Aroclor 1248 standard. The composition of PCB residues in minnows (B) is different than the standard (A). In minnows peaks 1, 2 and 4 are reduced and there is a change in doublet peaks 6-7 and 8-9 so they look like single, unresolved components. Also there is an increase in peaks 11, 12 and 13 relative to the standard. The opposite result was obtained for Aroclor 1260. Here PCB residues in minnows were essentially identical on a component basis to Aroclor 1260 (20).

Along with metabolism and elimination determining extent to which PCBs are accumulated by fish, their lipid content is also important. Female fathead minnows accumulated about twice as much Aroclor 1248 and 1260 from water as males and this was due to greater lipid in females (20).

Metabolism. There is a paucity of information on PCB metabolism in fish. With the exception of one study of one study (25) metabolites of PCBs in fish have not been identified other than to say they were more polar than the parent compound (26,27,28,29). Also while effect of degree of chlorination on PCB metabolism in fish has been studied (26) effect of chlorine position has not. What is known is that fish in general metabolize PCBs at a slow rate in comparison to mammalian species (29,30,31) and that rate of metabolism appears to be inversely related to degree of chlorination (20,26). Table IV shows percentage of radioactivity

TABLE IV

Effect of Degree of Chlorination on PCB Metabolism in Green Sunfish[a]

Chlorobiphenyl	% Parent Compound	% Polar Metabolites
2,2',5 - Trichlorobiphenyl	18	82
2,2',5 5' - Tetrachlorobiphenyl	99	1
2,2',4,5,5' - Pentachlorobiphenyl	99	1

[a]Adapted from (26). Green sunfish were exposed to a single ^{14}C-labeled chlorobiphenyl in water on day 0 and day 9. On day 16 fish were killed and percent total radioactivity in fish as parent compound and polar metabolites determined.

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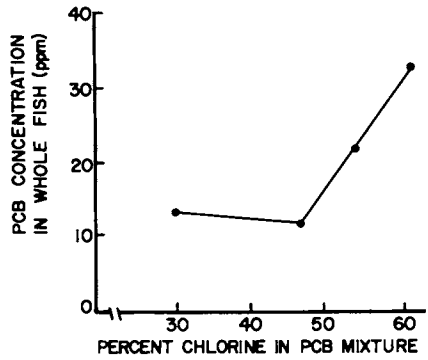

Figure 3. Relation between whole-body PCB residues and percentage chlorine in PCB molecules in channel catfish. The fish were fed Aroclor 1232, 1248, 1254, and 1260 at dietary concentrations of 24 μg/g for 193 days (19).

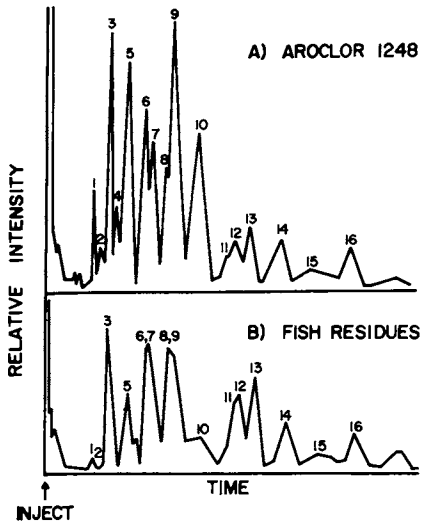

Figure 4. GLC chromatograms of (A), Aroclor 1248 reference standard and (B), Aroclor 1248 residues in fathead minnows (20).

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recovered from green sunfish due to parent chlorobiphenyl and polar metabolites (26). For 2,2',5-trichlorobiphenyl the majority of radioactivity was polar metabolites but for 2,2',5,5'-tetrachlorobiphenyl or 2,2',4,5,5'-pentachlorobiphenyl only 1% of total radioactivity recovered was polar metabolites. Thus, availability of more than two adjacent unsubstituted carbon atoms as occurs in 2,2',5-trichlorobiphenyl appears necessary for an appreciable rate of PCB metabolism.

Along with degree of chlorination another determinant of PCB metabolism in fish is species. Green sunfish and goldfish rapidly metabolize 2,2',5-trichlorobiphenyl whereas bullheads and rainbow trout metabolize it slowly (26,29). There is little evidence for PCB metabolism in brook trout fed 4-chloro-, 4,4'-dichloro-, 2,2',5,5'-tetrachloro- or 2,2',4,4',5,5'-hexachlorobiphenyl (31) and this was confirmed in rainbow trout by less than 1% of 2,2',5,5'-tetrachlorobiphenyl accumulated being recovered as polar metabolites (25).

In fish where metabolism of PCBs has been demonstrated the principal mechanism appears to be hydroxylation. Trout, skate, red crab and lobster metabolize the simplest PCB component, biphenyl, to the 4-hydroxy derivative and to a lesser extent, 2-hydroxy (30,32). In rainbow trout exposed to ^{14}C labeled 2,2',5,5'-tetrachlorobiphenyl polar metabolites were found in gallbladder bile. One of these metabolites was cleaved by β-glucuronidase and identified as 4-hydroxy-2,2',5,5'-tetrachlorobiphenyl (25). Metabolism of 2,2',4,5,5'-pentachlorobiphenyl in dogfish sharks is extremely slow but a small amount of polar metabolites were found in gallbladder bile (28). The metabolites were not converted to organic soluble material by β-glucuronidase hydrolysis. Thus, in dogfish shark glucuronide conjugates of phenols are not major metabolites of PCBs as in rainbow trout (25,28).

Distribution. Generally the highest concentration of PCB residues in fish are in tissues of high lipid content. In Table V juvenile coho salmon were fed equal amounts of three chlorobiphenyls for 117 days. Fish were then killed and lipid content and PCB concentration of various tissues determined (33). Tissues are arranged from top to bottom in order of increasing PCB concentration. For most tissues, but not all, as lipid content increases so does PCB concentration. Lipid content and PCB concentration are low in liver and white muscle, intermediate in spinal column and lateral line muscle, and high in adipose tissue. Lipid content cannot be the sole determinant of PCB concentration in fish tissues because a discrepancy exists between lipid content of brain, heart and spleen and PCB concentration.

To completely describe distribution of PCBs in whole fish data for concentration and total amount of residues in each tissue should be given. Failure to provide both results causes confusion. Figure 5 shows concentration of ^{14}C-labeled

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TABLE V

Lipid Content and Concentration of PCB Residues in Tissues of Juvenile Coho Salmon[a]

	Lipid Content (wt %)	2,2',4,4',6,6'-Hexachloro- (μg/g wet tissue)	2,2',4,5,5'-Pentachloro- (μg/g wet tissue)	3,3',4,4'-Tetrachloro- (μg/g wet tissue)
Brain	7.1	0.38	0.31	0.15
Liver	2.9	0.50	0.35	0.25
White muscle	2.6	0.64	0.63	0.29
Intestines	4.3	0.82	0.79	0.30
Stomach and pyloric caeca	3.3	0.95	0.76	0.43
Spinal column	6.6	1.4	1.4	0.92
Heart	---	1.5	1.2	0.98
Lateral line muscle	6.1	1.9	1.8	0.77
Spleen	---	2.1	2.0	0.85
Adipose tissue	73.0	18.8	19.3	10.6

[a]Adapted from (33).

2,2',5,5'-tetrachlorobiphenyl residues in selected tissues of rainbow trout for up to 56 days after a 36 hour water exposure (34). The highest concentration was in visceral fat, gallbladder bile and eyes plus associated periorbital fat. Skin, carcass and skeletal muscle had lower concentrations. Now, when PCB residues in these same fish are expressed as a percentage of total amount of PCB in the whole fish a different picture of PCB distribution emerges as is shown in Figure 6. Carcass, skeletal muscle and skin contain the highest percentage of tetrachlorobiphenyl residues while visceral fat, eyes and periorbital fat and gallbladder bile contain the lowest. The reason for the different distribution picture is tissue mass. Carcass, skeletal muscle and skin have greater mass than visceral fat, eyes, periorbital fat and gallbladder bile.

There is a large variation in tissue distribution of PCBs among different fish species. In coho salmon and rainbow trout liver contains a low concentration of PCB and a low percentage of the total amount of PCB in the fish (33,34). On the other hand, in spot and dogfish shark liver contains the highest concentration and percentage (5,28).

Elimination. Rate that PCBs are eliminated by fish is strongly influenced by their rate of metabolism. Figure 7 shows concentration of PCB in green sunfish after being exposed to a high or low dose of 2,2',5-trichlorobiphenyl or 2,2',4,5,5'-pentachlorobiphenyl on day 0 and again on day 9 (26). Of these

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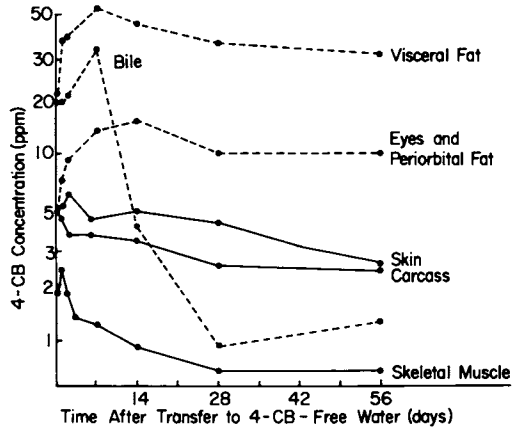

Figure 5. Concentration of 4-CB residues in tissues of rainbow trout exposed to ^{14}C-labeled 4-CB in water and transferred to 4-CB-free water (day 0). Each point is mean of five fish (34).

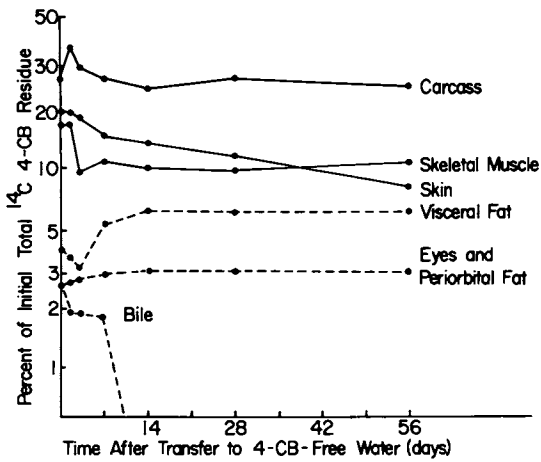

Figure 6. Residues of 4-CB in tissues of rainbow trout expressed as percentage of total amount of 4-CB in whole fish at time of transfer to 4-CB-free water (day 0). Each point is mean of five fish (34).

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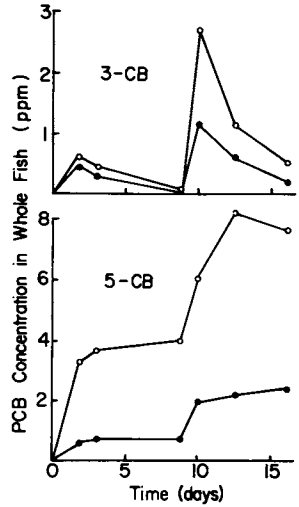

Figure 7. Concentration of PCB residues in green sunfish exposed to 3-CB or 5-CB in water on day 0 and again on day 9: (○ – ○) high dose; (● – ●), low dose (26).

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two PCBs trichlorobiphenyl is metabolized at a more rapid rate (26). Each time fish are exposed to trichlorobiphenyl, concentration of PCB in the fish increases and then decreases during the wash-out period. On the other hand, when fish are exposed to pentachlorobiphenyl PCB concentration increases on the day of exposure and plateaus during the wash-out period with little elimination occurring.

The main organ involved in PCB metabolism and excretion in fish is the liver. Metabolism of PCBs in fish liver homogenates has been demonstrated (29,30,32) and PCB metabolites are excreted into bile (25,28,34). What is not known is extent to which PCB metabolites excreted in bile are eliminated in feces. Also the role of kidneys, gills, intestine and skin in PCB elimination in fish has not been fully elucidated. The only study on urinary excretion of PCBs was in dogfish sharks and revealed that urine was not a major route of elimination (28).

Spawning was recently shown to increase whole body elimination of PCBs in rainbow trout. Figure 8 shows rate of PCB elimination from female trout prior to and after spawning (35). The study lasted for one year and abbreviations for months are given across the top. Fish were exposed to 2,2',5,5'-tetrachlorobiphenyl in water for 36 hours in December, then transferred to an outdoor raceway with flowing, PCB-free water. Bottom panel shows that weight of eggs began to increase in July, reached a peak in October, and decreased in November and December. Top panel demonstrates that during the first two weeks after exposure about 30% of PCB taken up by the fish (531 μg, mean) was eliminated. This initial, rapid rate of elimination is not shown in Figure 8 but is discussed elsewhere (34). What is of interest here is the change in rate of whole body elimination during the spawning season. From January through August PCB elimination was slow ($t_{1/2}$ = 1.76 years) but from September to December it increased ($t_{1/2}$ = 0.52 year). The more rapid elimination was associated with a decrease in weight of eggs contained in the fish. Figure 9, middle panel, shows percentage of PCB in fish that was contained in the eggs. It can be seen that percentage of PCB in eggs increased as weight of eggs increased and when eggs were voided from the fish percentage of PCB in the few eggs that remained was reduced. Thus, whole body elimination of PCBs was more rapid during spawning because the large mass of eggs voided contained PCB.

Top and middle panels of Figure 9 reveal that there was a redistribution of PCB in fish prior to spawning. More explicitly, accumulation of PCBs in eggs was associated with a reduction of PCB in visceral fat and eyes and periorbital fat. Since a sharp reduction of PCB in other tissues did not occur (35) it is possible that PCBs accumulating in eggs were derived in part from body fat. Table VI gives concentration of tetrachlorobiphenyl residues in eggs, visceral fat, eyes and periorbital fat; wet weight is also included. Note that as eggs mature and increase

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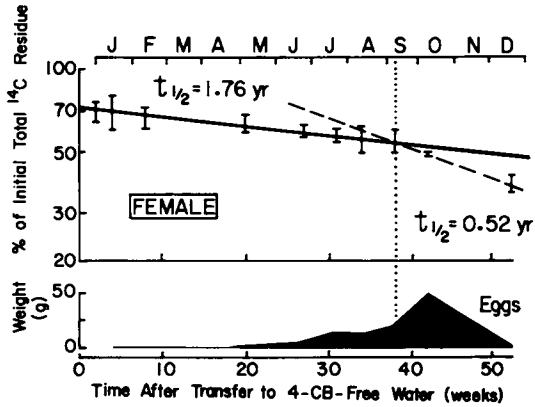

Figure 8. Effect of egg maturation and spawning on whole-body elimination of ^{14}C-4-CB residues in female rainbow trout. Top panel is for 4-CB elimination from whole fish. Each point and associated vertical bar (mean ± SE) represents percentage of whole-body residue of ^{14}C-4-CB at time of transfer to 4-CB-free water (time zero). Vertical dotted line designates last group of fish sampled before whole-body elimination of 4-CB appeared to increase (35).

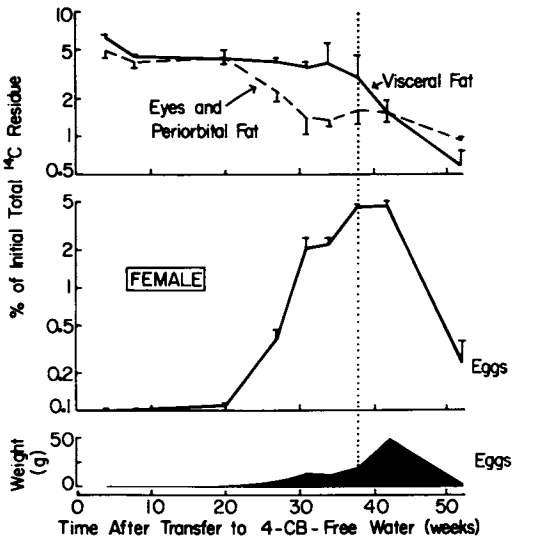

Figure 9. Redistribution of ^{14}C-4-CB residues in body fat depots and eggs of female rainbow trout. In top and middle panel points and associated vertical bars (mean ± SE) represent percent of whole-body residue of ^{14}C-4-CB in indicated tissues at time of transfer to 4-CB-free water (time zero) (35).

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TABLE VI

Concentration of 2,2',5,5'-Tetrachlorobiphenyl (4-CB) in Female Rainbow Trout[a]

Time[b] (weeks)	4-CB Concentration (ppm)			Wet Weight (g)		
	Eggs	Visceral Fat	Eyes and Periorb. Fat	Eggs	Visceral Fat	Eyes and Periorb. Fat
4	0.8	37.9	13.1	0.1	0.6	2.0
8	0.6	26.2	8.6	0.1	0.9	1.8
20	0.7	15.9	6.3	0.7	1.7	2.8
27	0.6	11.2	3.4	5.3	2.4	2.9
31	0.7	6.1	1.9	13.6	2.8	3.3
34	0.7	4.2	1.5	12.2	3.9	3.7
38	0.7	4.6	1.9	19.9	3.2	4.3
42	0.7	2.7	1.0	48.8	2.0	4.6
52	0.5	2.4	0.9	4.2	2.1	4.1

[a]Adapted from (35). Values are mean of 2-5 fish.
[b]Time after transfer to 4-CB-free water.

in weight that PCB concentration in eggs remains constant. On the other hand, concentration of PCB in visceral fat, eyes and periorbital fat decreases. The mechanism for maintenance of a constant concentration of PCB in eggs is not known, but the same phenomenon has been observed in maturing sperm of male rainbow trout and the enhancing effect of spawning on whole body PCB elimination also occurs in males (35).

A final point is that in laboratory studies reducing lipid content of rainbow trout by starvation did not increase whole body PCB elimination (24). Instead PCB concentration in body fat increased as the absolute amount of fat in the fish decreased (24). The latter has also been shown in coho salmon (33). Thus, reducing body fat by starvation does not appear to be a very effective way of increasing PCB elimination in fatty fish.

Concluding Remarks. Results described in preceeding sections have been presented to provide background information on the present status of our understanding of PCB disposition in fish. In comparison to what is known about disposition of chlorobiphenyls in mammalian species such as the rat (36-41) we have only begun to understand how these compounds are handled by fish. Virtually nothing is known about identity of PCB metabolites in fish. Only recently have we started to assess effects of degree of chlorination on fate of chlorinated biphenyls in fish. Impact of position of chlorination on the biphenyl ring system on accumulation, tissue distribution, metabolism and

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elimination of PCBs by fish has not been studied. At present, there is not enough information available to develop pharmacokinetic models that will accurately predict PCB disposition in prototype freshwater and marine fish species. Undoubtedly these gaps in our knowledge will have to be closed if in the future we are to have a better understanding of the disposition of these global contaminants in fish than we do today.

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3

Metabolism of Cyclodiene Insecticides by Fish

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The extensive use of chlorinated cyclodiene insecticides during the last 35 years has resulted in wide-spread contamination of environment with these chemicals as well as with their photoalteration and biotransformation products (1,2). Although their residues are common in water, fish, food and humans (3,4), their fate has been studied only in some mammalian species (5,6,7). Corresponding information about the metabolic fate of these chemicals in fish which are extremely sensitive to cyclodienes (8,9) is almost completely lacking. In vivo metabolism of cyclodienes in model ecosystems (10,11,12,13) does not clearly explain whether the metabolites present in fish are produced by the fish themselves, by other aquatic organisms or by physiochemical factors and then absorbed by fish. For example, a model ecosystem (containing algae, snails, mosquito larvae, and fish) contaminated with heptachlor showed the presence of 9 extractable products including heptachlor along with unextractable(s) in water, snails, and mosquito larvae. The fish (Gambusia) showed the presence of heptachlor, heptachlor-2,3-epoxide, 1-hydroxychlordene-2,3-epoxide, 1-hydroxychlordene, and the unidentified material(s) along with the unextractables (10,11,12,13). Similarly, using a mixture of cis- and trans-chlordane (3:1) at least 21 products, including chlordanes and unextractable(s), were found in various components of the model ecosystem with fish showing only 9 of these including chlordanes along with the unextractable(s) (12,13). Fish hepatic enzymes have been shown to be capable of metabolizing cyclodienes in vitro. These reactions include epoxidation and epoxide hydration (14,15). Metabolism of cyclodiene insecticides and their terminal environmental products i.e., photoisomers (Fig. 1) by fish has been studied in this laboratory. A summary of the work carried out on the absorption, elimination, and metabolism of these chlorinated hydrocarbons is presented.

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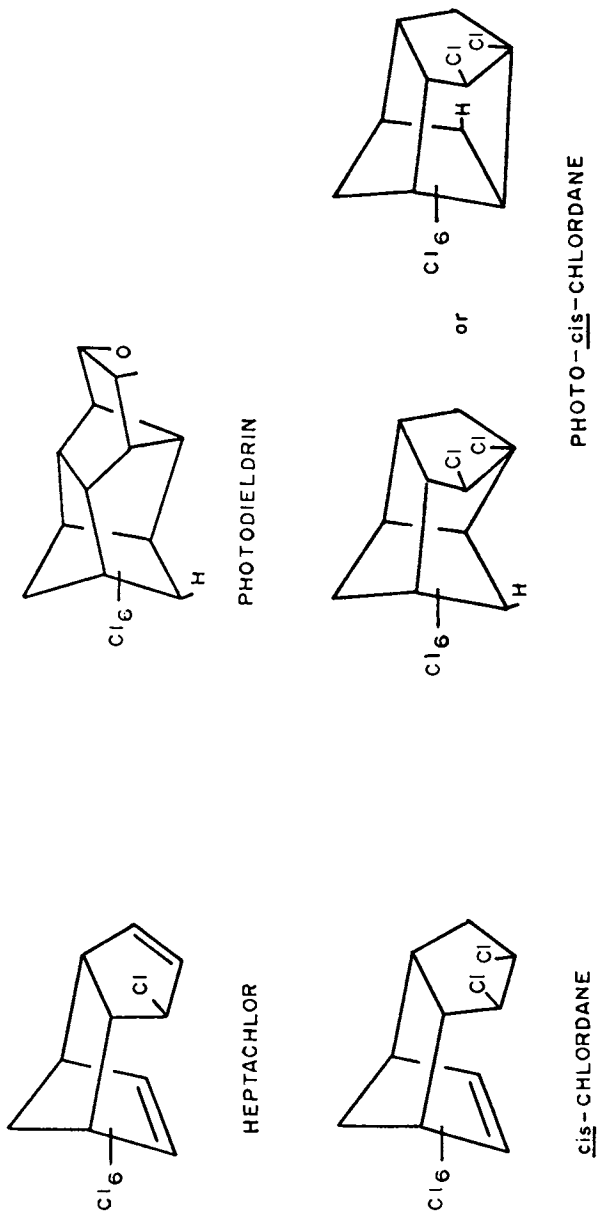

Figure 1. Structural formulae of the compounds used in the studies

Materials and Methods

Goldfish, (Carassius auratus), were purchased from Midwest Aquarium, Inc., Bensenville, Illinois. Bluegill, (Lepomis macrochirus), were a generous gift of McGraw Wildlife Foundation, East Dundee, Illinois. Frogs, (Xenopus laevis), were purchased from NASCO, Fort Atkins, Wisconsin. The tropical fish chichlid (Cichlasoma) were a gift of John G. Shedd Aquarium, Chicago. All these animals were kept in running lake water at 21°C (cichlids were maintained at 26°C) at John G. Shedd Aquarium. They were acclimated in tap water (lake water) at 21°C for at least 10 days before using. The radioactive chemicals were uniformly labeled at all carbons in the hexachloro ring and were 99.9% pure as checked by thin-layer and gas chromatography.

(i) Absorption

To study the absorption of cis-chlordane by bluegill and Xenopus, the exposure was made to 5 ppb of insecticide. The biomass and volume was one 5-g fish/6L and three 27-g Xenopus/8L in 10-liter glass cylinders. The aeration of the water was kept to the minimum and the containers were covered with aluminum foil to reduce loss due to volatilization. The radioactivity in control water without animals and in water with fish was monitored by counting 5 ml of water in 15 ml of Instagel (Packard Instruments) (16).

Whether the isomers of chlordane, namely cis-chlordane and photo-cis-chlordane, were absorbed, retained, metabolized and excreted at similar or different rates was investigated by exposing goldfish or bluegill to 5 ppb concentration of each insecticide. In the case of the goldfish, three 1-g fish were exposed (in duplicates) in 3.8L of water in 4L pickel jars, with no aeration and with lids tightly closed to prevent loss due to evaporation. Because of the oxygen depletion this experiment was carried out for only 16 hours. At each time interval the fish were removed and individually analyzed for radioactivity by digesting in Soluene-350 (15 ml per gm of fish) and then counting 1.5 ml of the digest in 15 ml of Dimilume-30 (Packard Instruments). One ml of the water was counted in 10 ml of Instagel (17). In the case of the bluegill one 50-g fish was exposed in 6L of water (3 replicates) containing 5 ppb of cis-chlordane or photo-cis-chlordane. The 10L jars were covered with aluminum foil and aerated minimally. During the exposure the radioactivity was monitored in water without fish (control) and water with fish. The difference of the two was used to estimate the loss due to absorption by fish (16).

(ii) Elimination

For these studies, the animals were exposed to absorb maximum amounts of the insecticide as mentioned above. They were then

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transferred to insecticide-free water. In the case of the goldfish, radioactivity in fish was monitored, while in the case of the bluegill that released in the water was monitored at regular time intervals. In addition to this, goldfish were injected with ^{14}C-heptachlor in their body cavity (38 µg/44g fish) and the fish were kept in water (19). The radioactivity released in water was monitored with time. In the case of photodieldrin, three bluegill weighing 101-126 g each were exposed to 20 ppb of ^{14}C-photodieldrin in 6L of water for 48 hr and then transferred to clean water (16).

(iii) Metabolism

For metabolism studies larger fish were used. Three cichlids (Cichlasoma sp.) weighing about 300g each were exposed individually in 16L of a 5 ppb solution of ^{14}C-cis-chlordane. After exposure for 72 hours they were analyzed for radioactivity (18). In another similar experiment the treated fish were transferred to insecticide-free water to study the elimination of the absorbed cis-chlordane. Bluegill, as mentioned in the section on elimination, were also analyzed for radioactivity. Goldfish injected with ^{14}C-heptachlor (38 µg/44g fish) (19) as well as those exposed to 26 ppb ^{14}C-cis-chlordane for 24 hr (9 x 16-g fish)(20) were held in insecticide-free water for 10 days and then analyzed for radioactivity.

Fish were individually analyzed for metabolites. They were cut into small pieces and ground with anhydrous sodium sulfate in a mortar with a pestle. The dry powder was vigorously shaken with diethyl ether followed by acetone and then methanol. The pooled extracts were freed of fat either by repeated thin-layer chromatography on silica gel F-254 0.25 or 0.5 mm plates or by column chromatography on Florisil using ether and then methanol as eluting solvents (16,18,19,20). In the case of cichlids, the aqueous phase of fish as well as the exposure water (3L from each jar, after solvent extraction) was lyophilized and then hydrolyzed with HCl and the released product(s) analyzed (19). The identification of metabolites involved co-chromatography (thin-layer and gas chromatography) using authentic reference standards. In the case of polar metabolites derivatization was carried out, wherever mentioned, using Tri-Sil 'Z' (Pierce Chemical Co., Rockford, IL.) (18). Hydrolysis of polar conjugates was generally accomplished with 2N HCl (18). Radioactivity was measured using a Packard Model-3390 spectrometer equipped with a Model-544 Absolute Activity Analyzer. Thin-layer chromatograms were X-rayed (18,19) and radioactive areas scraped and counted. Internal standards of equivalent radioactivity were run in all cases and the counting efficiency ranged from 85-95%. For gas chromatography a Packard Model-7300 gas chromatograph outfitted with electron capture detectors and SE-30 and OV-101 columns (20) was used.

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Results and Discussion

(i) Absorption

Absorption of cis-chlordane and photo-cis-chlordane by fish and Xenopus: Exposure of aquatic vertebrates to 5 ppb of these insecticides shows that under similar conditions the rate of absorption of the same chemical i.e., cis-chlordane can vary in different species. Higher rate of absorption of cis-chlordane by bluegill as compared with Xenopus (Fig. 2) may be due to the greater surface area to body wt ratio in the former because of the gills. Increased absorption through gill surface may also be responsible for this. Similarly, smaller goldfish, because of greater surface area can absorb cis-chlordane at a higher rate than bluegill (Fig. 2). Even the isomers of the same chemical, i.e., cis-chlordane and photo-cis-chlordane, because of differences in their physicochemical properties (water solubility, lipid partitioning, volatility) can be absorbed at different rates by the same species (Fig. 2).

At these concentrations, smaller fish (goldfish) show maximum absorption in only 16 hours as compared with larger ones (bluegill) which take about 24 hours to show the maximum levels, while Xenopus take about 96 hours to absorb maximum amounts. The species differences as well as the properties of the cyclodiene seem to affect the level of its accumulation by the animal. The bioaccumulation ratio: (concentration in animal at the time of maximum absorption/concentration in water at that time) for cis-chlordane which is extremely refractive to degradation by these species (see the following section) is highest for the small fish, medium for bluegill and lowest for Xenopus (Table 1). Bioaccumulation of photo-cis-chlordane by the same fish results in higher values as compared with cis-chlordane. However, since this compound is biodegradable and species differ in their ability to metabolize it, small and large fish do not show any significant difference in its accumulation.

Table 1. Bioaccumulation of cis-chlordane and photo-cis-chlordane by goldfish, bluegill and the amphibian Xenopus following their exposure to 5 ppb concentration in a static system.

Organism	Biomass	Time for maximum absorption hr	Bioaccumulation Ratio	
			cis chlordane	photo-cis-chlordane
Xenopus	80g/8L	96	108	-
bluegill	50g/6L	24	322	1180
goldfish	3g/3.8L	16	990	1143

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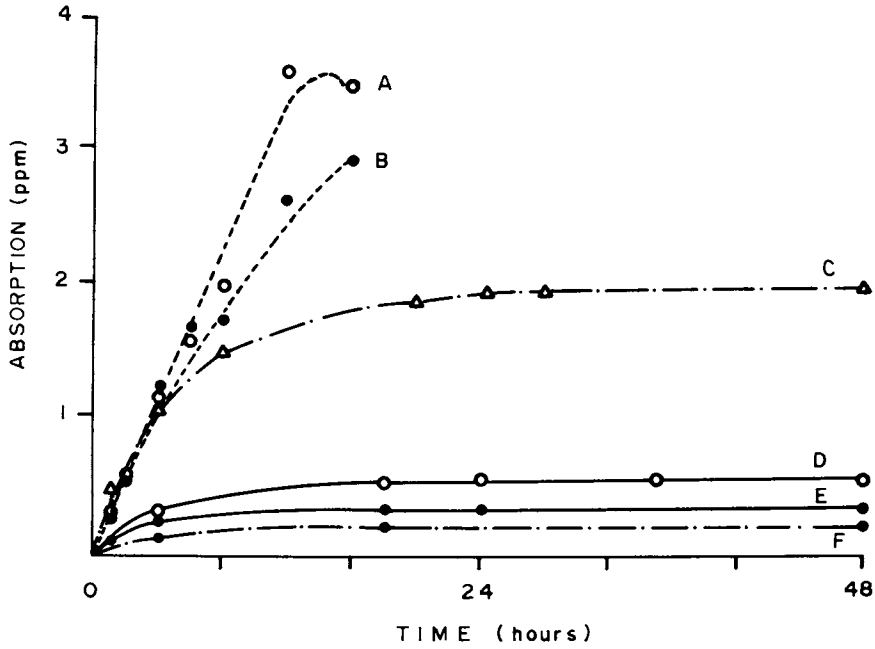

Figure 2. Absorption of cyclodienes by fish and Xenopus *in a static system: (A), goldfish treated with photo-*cis*-chlordane; (B), goldfish with* cis*-chlordane; (C), bluegills with photodieldrin; (D), bluegills with photo-*cis*-chlordane; (E), bluegills with* cis*-chlorane; and (F),* Xenopus *with* cis*-chlordane. All exposures were made at 5 ppb. (see Table 1 for biomass and volume of water).*

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(ii) Depuration

Transfer of the aquatic animals, after absorption of the cyclodiene, to insecticide-free water showed variations in elimination pattern related with the chemical as well as its concentration in the body. It follows a somewhat biphasic response, there is initial rapid elimination of high concentration followed by a slow elimination at lower body concentrations (Fig. 3)(21,22, 23). Xenopus, even at low body levels of cis-chlordane, eliminate it at slightly faster rate than fish. Photo-cis-chlordane seems to be eliminated at a faster rate than cis-chlordane by both goldfish and bluegill (Table 2). The elimination of photo-cis-chlordane by goldfish and bluegill and of photodieldrin by bluegill shows a biphasic response (Fig. 3).

(iii) Metabolism

Since elimination of these apolar cyclodienes does take place in fish, we examined the nature of radioactivity in both fish and water.

(a) cis-chlordane. Cichlids (about 300g/fish) were exposed for 72 hr in 16L of water containing 5 ppb ^{14}C-cis-chlordane. They were then analyzed for metabolites. Of the total radioactivity counted in cichlids, about 99.2% was extractable with organic solvents and the remaining was water soluble. Only about 4.5% of the radioactivity in the organic phase was in the form of about 7 different metabolic products (Fig. 4). Co-chromatography using authentic standards revealed the presence of dichlorochlordene, traces of oxychlordane, chlordene chlorohydrin, and heptachlor diol, plus three unidentified metabolites (19). Of the 0.8% radioactivity in water about 22% was in the form of two different metabolites and about 55% in the form of conjugates (remaining at the origin), and the remaining was unchanged cis-chlordane.

The radioactivity recovered from water showed the presence of unchanged cis-chlordane in the organic phase along with several hydrophilic metabolites (Fig. 4). Most of the radioactivity in both organic and aqueous phases was in the form of conjugates.

A similar exposure of goldfish to cis-chlordane for 24 hours followed by their transfer to and holding in insecticide-free water for 10 and 25 days showed the presence of polyhydroxy and/or conjugated metabolites (unextractable with organic solvents) in fish ranging from 0.24 to 1.2% of the total radioactivity. Organic extracts of the fish showed 99.86% of the radioactivity in the form of cis-chlordane and the ramining as hydroxylated products (Fig. 5a). Based on TLC and GC analyses, these products are tentatively identified as chlordene chlorohydrin (B), monohydroxy derivatives (C and D) and polyhydroxy/ conjugated products (at origin) (20). Similar exposure of

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Table 2. Elimination of cyclodienes by aquatic vertebrates following their transfer, after maximum absorption in a static system, to insecticide-free water.

	fish wt: gm	no. of fish	maximum conc. accumulated: ppm	half-life: weeks	
cis-chlordane					
goldfish	0.75-1.40	6	2.647	4.4	
cichlid	292-324	3	0.155	20	extrapolated
bluegill	72- 75	3	0.32	15.9	extrapolated
Xenopus	20- 35	3	0.207	3.3	
photo-cis-chlordane					
goldfish	.75-1.40	6	3.594	1.1	
bluegill	40-60	3	0.556	9.1	extrapolated
photodieldrin					
bluegill	101-126	3	1.75	3.2	
heptachlor					
goldfish	41- 45	3	2.002	4.1	

Because heptachlor is dechlorinated in water, it was directly injected intraperitoneally (32 µg/44g fish).

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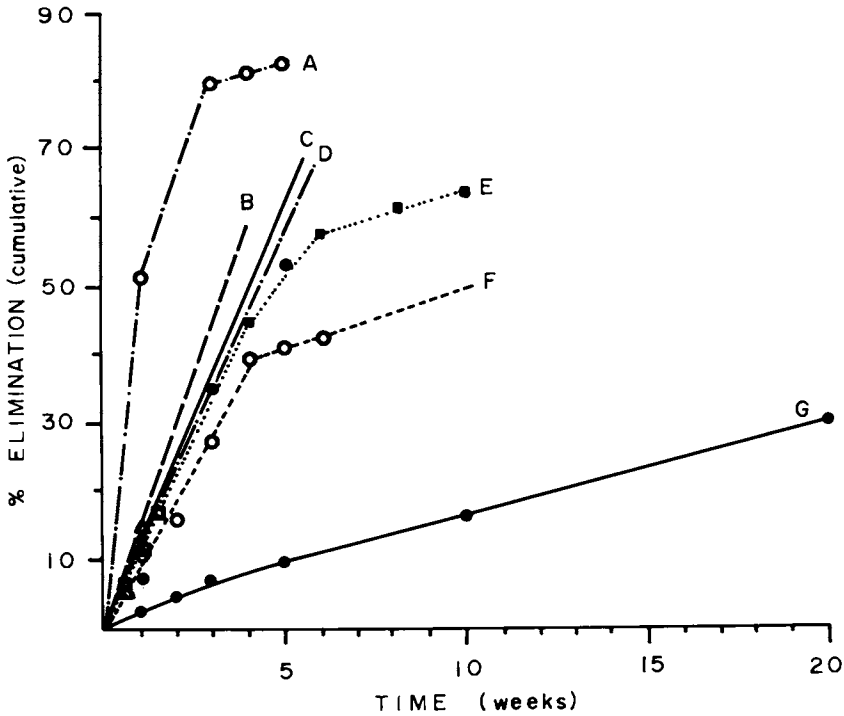

*Figure 3. Rates of elimination of cyclodienes by aquatic vertebrates. Animals were transferred to clean water after maximum absorption in static systems: (A), goldfish treated with photo-*cis*-chlordane; (B),* Xenopus *with* cis*-chlordane; (C), goldfish injected with heptachlor; (D), goldfish with* cis*-chlordane; (E), bluegills with photo-*cis*-chlordane; (F), bluegills with photodieldrin; and (G), cichlids with* cis*-chlordane.*

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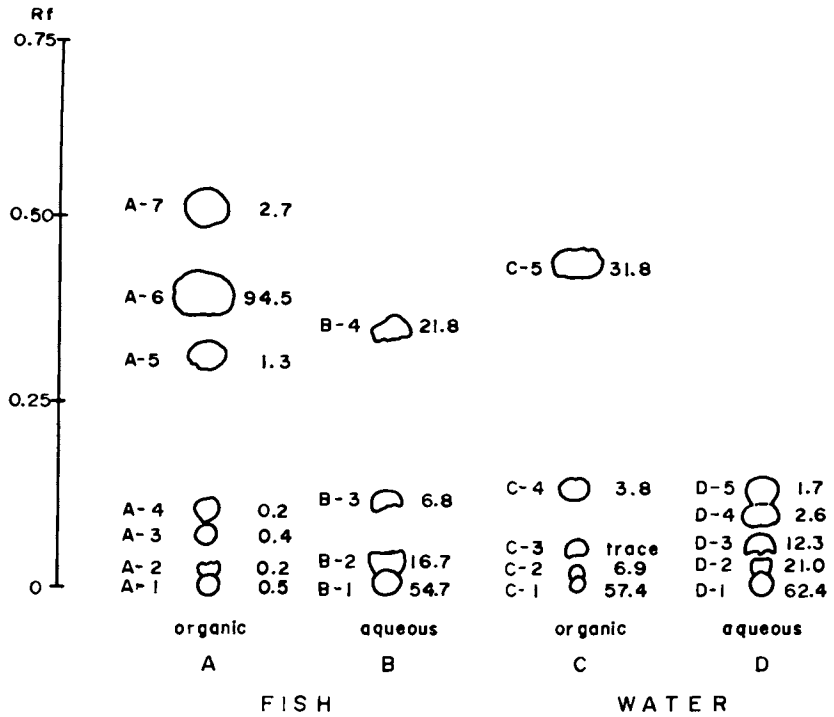

Figure 4. TLC behavior (0.25 mm, Silica gel GF-254; hexane–ethyl acetate, 9:1) of organic extracts of ^{14}C-cis-*chlordane treated cichlids (A) and exposure water (C). Fractions B and D show the compounds released by acid hydrolysis of aqueous phase of fish homogenate and exposure water, respectively.*

The analyses involved the exposure of developed chromatograms to x-ray films followed by scraping of radioactive spots and quantifying them. Relative amounts of various compounds in the fractions are indicated on the righthand side against numbered spots. Spots A-6, B-4, and C-5 represent unchanged cis-*chlordane; A-7 was a mixture of dichlorochlordene and oxychlordane. A-3, B-3, C-4, and D-4 represent chlordene chlorohydrin. A-2, B-2, C-2, and D-2 were complex spots with the heptachlor diol as the major compound. A-1, B-1, C-1, and D-1 were polyhydroxy derivatives or conjugates. Identities of other spots are not known. Recoveries in fractions A, B, C, and D were, respectively, 60.1%, 0.6%, 2.2%, and 2.9% of the applied radiocarbon.*

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bluegill showed the presence of two polar metabolites amounting to 7.3% of the recovered radiocarbon (conjugates) (Fig. 5b) (16).

The pathways of metabolism of cis-chlordane appear to differ in these fish. Cichlids produce oxychlordane not produced by other fish. This may be due to the lack of formation of the intermediate, 1,2-dichlorochlordene in goldfish and bluegill (Fig. 6).

(b) Heptachlor. Goldfish injected intraperitoneally (38μg/44g fish) with ^{14}C-heptachlor eliminated 18% of the dose in 10 days in the form of polar metabolites (Table 3). The extracts of the fish showed, as percent of the recovered radioactivity, heptachlor 91.6, heptachlor-2,3-epoxide 5.4, 1-hydroxychlordene 1.0, 1-hydroxy-2,3-epoxychlordene 1.1, and a conjugate 1.2%. The conjugate on acid hydrolysis yielded a trihydroxy product (18). Formation of a trihydroxy derivative may involve hydration of heptachlor epoxide to diol followed by oxidative dechlorination, or it may involve hydration of 1-hydroxy-2,3-epoxychlordene (Fig. 7). The nature of this conjugate is not known, at least it is not a glucuronide since β-glucuronidase cannot hydrolyze it (18).

(c) Photo-cis-chlordane. Both goldfish and bluegill excrete photo-cis-chlordane at higher rates than cis-chlordane (Fig. 3, Table 2). Analyses showed that 89% of the extractable radioactivity in the fish (about 86% of the total) could be extracted with ether and petroleum ether, the remaining being extractable with methanol. Fourteen percent of the radioactivity in the former extract (the remaining being photo-cis-chlordane) was in the form of 9 different metabolites (Fig. 8). The methanol extracts did not show more than 6 metabolites. Out of the total extractable radioactivity from fish the metabolites amount to at least 20% (Table 4). These metabolites are being characterized (16). It appears that photo-cis-chlordane, a major product of photolysis of cis-chlordane, is biodegradable in fish. Some of the biotransformation products of photo-cis-chlordane in fish appear to be similar to those produced in rats (Fig. 8) (24); however, the fish are less efficient than rats in this respect.

(d) Photodieldrin. Elimination of the absorbed photodieldrin by bluegill appears to be faster than that of cis-chlordane and photo-cis-chlordane (Table 2). About 60% of the absorbed photodieldrin was eliminated in 5 weeks. Extracts of the fish showed most of the radioactivity in the form of photodieldrin (Fig. 9) with about 10% of a less polar metabolite which resembled photodieldrin ketone as checked by thin-layer and gas chromatography. About 0.1% of the extractable radioactivity in fish was in the form of polar metabolite(s) (16) which may be the hydroxylated product(s) as observed in mammals (25). Photodieldrin ketone along with photodieldrin in fish may have chronic toxic effects of the parent cyclodiene, dieldrin, on bluegills and other organisms at higher trophic level.

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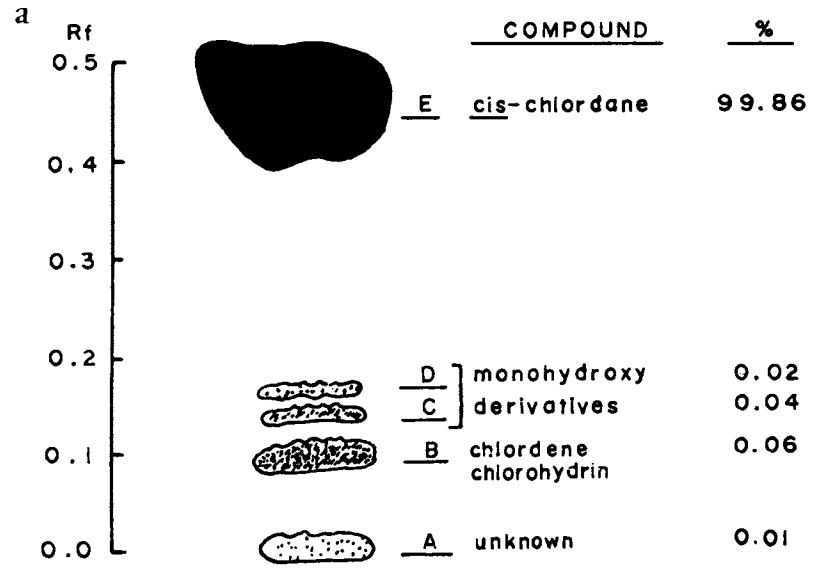

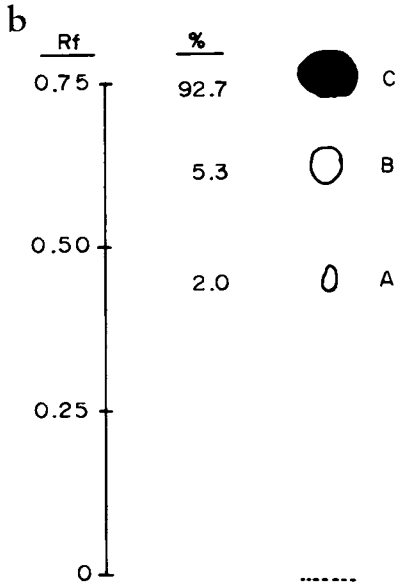

Figure 5. (a) Tracing of an autoradiogram (0.25 mm Silica gel GF-254; hexane–ethyl acetate, 9:1) of organic extract of goldfish treated with 26 ppb ^{14}C-cis-chlordane for 24 hr and then held in clean water for 10 days. Recovery of the radiocarbon from the fish was 85.7% of the dose. Figure also shows possible nature and relative amounts of metabolites. (b) Tracing of an x-ray autoradiogram (0.25 mm Silica gel FG-254, butanol–acetic acid–water, 6:1:1) of organic extract of ^{14}C-cis-chlordane-treated bluegills (25 ppb for 48 hr). Spots A and B represent conjugates and C, unchanged cis-chlordane. Recovery of the dose was 89.5%.

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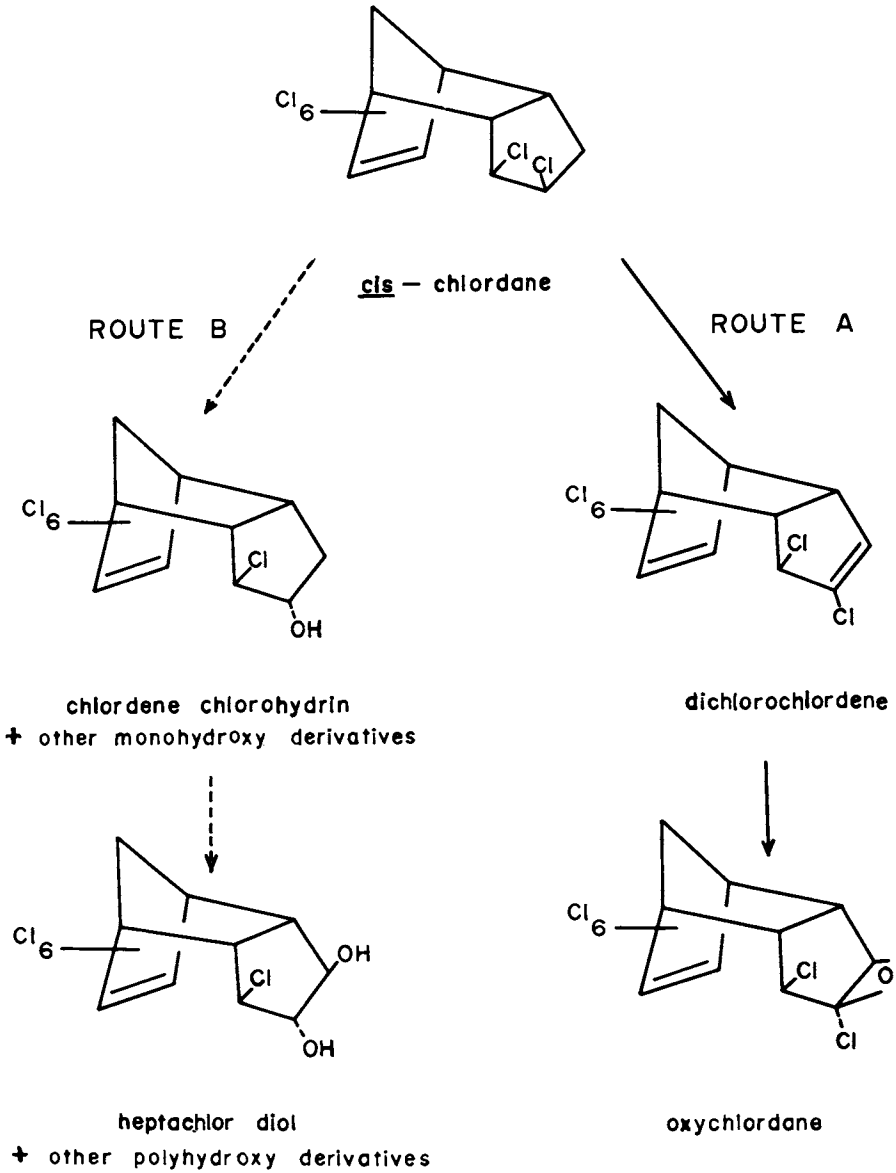

Figure 6. Simplified scheme of metabolic pathways of cis*-chlordane in fish: (Route A), desaturation and epoxidation and (Route B), hydroxylations. Both routes are operative in cichlids (as in mammals). Goldfish and bluegills seem to lack Route A.*

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Table 3. Relative amounts of ^{14}C-heptachlor and its metabolites in fish, water and excreta, 10 days after a single injection (38.2 µg/fish).

Metabolite*	Identity	% of radioactivity recovered**		
		Fish	Water	Feces
A	conjugate	1.21 ± 0.13	72.36 ± 10.68	48.56 ± 7.47
B	1-hydroxy-2,3-epoxy chlordene	1.11 ± 0.06	27.63 ± 10.68	38.55 ± 5.04
C	1-hydroxychlordene***	1.07 ± 0.50	-	12.98 ± 2.48
D	heptachlor-2,3-exo-epoxide	5.40 ± 2.12	-	-
E	heptachlor	91.23 ± 2.56	-	-

* The metabolites are tentatively identified on the basis of co-chromatography by TLC and GLC.
** Recoveries (% of dose) were fish 80.6, water 12.2, and feces 1.0. Sample size 3 in all cases. Values are mean ± S.E.
*** GLC analysis indicated another minor component along with 1-hydroxychlordene.

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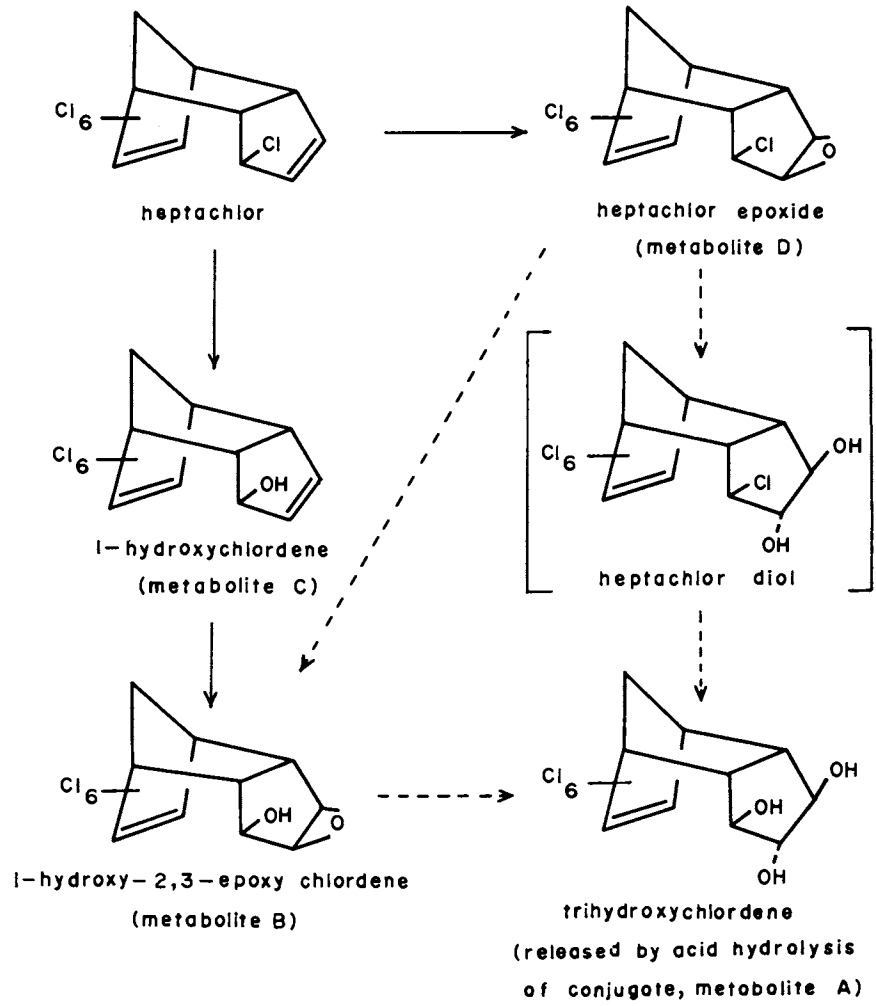

Figure 7. Metabolic pathways of heptachlor in goldfish

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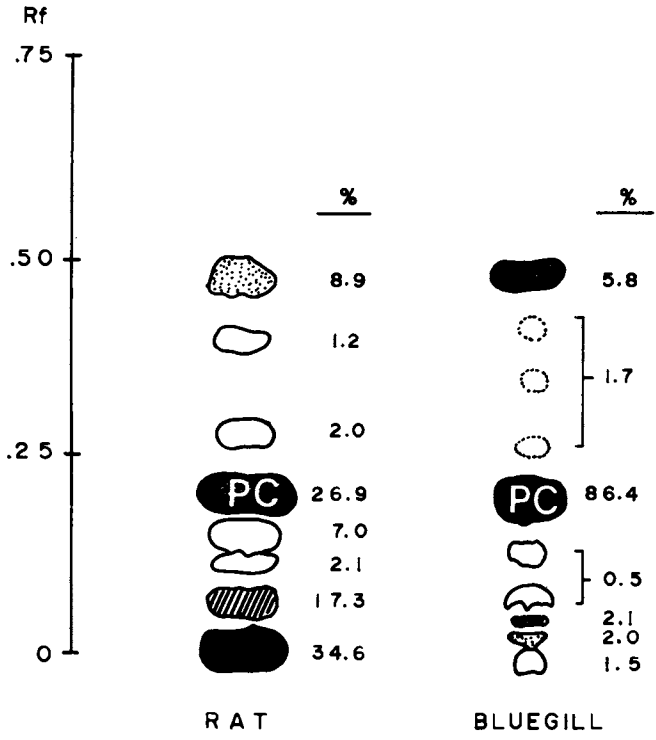

*Figure 8. Comparison of metabilic activity of rat and bluegills toward photo-*cis*-chlordane.*

*Feces from injected (7.9 mg/kg) male rats were collected for one week and extracted with acetone yielding about 50% of the dose. Fish were treated with 5 ppb of the compound in water for 48 hr and then held in clean water for 11 days at the end of which they were extracted whole with organic solvents. Recovery of the compound applied to fish was 74.3%. Chromatographs (0.25 mm Silica gel GF-254) were developed with heptane twice. The figure shows relative abundance of metabolites in the two species. PC is photo-*cis*-chlordane.*

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Table 4. Analysis of metabolites of photo-cis-chlordane in bluegill as analyzed by TLC (silica gel GF-254, 0.25 mm) followed by X-ray autoradiography and scintillation counting (16).

Metabolite*	Rf Values**				% of extracts***		
	I	II	III	IV	ether	pet. ether	methanol
M1	.01	.86	.62	.80	1.47	-	-
M2	.04	.88	.64	.83	2.01	.79	-
M3	.06	.90	.70	.85	2.10	-	-
M4	.10	.91	-	-	0.53	-	-
M5	.15	.91	-	-		-	-
M6	.22	.92	.78	.91	86.85	93.54	30.2
M7	.29	.92	-	-		-	-
M8	.37	.92	-	-	1.68	-	-
M9	.43	.92	-	-		.35	-
M10	.50	.93	-	-		5.10	-
M11	0	0	.25	0	-	-	5.6
M12	0	0	.40	.006	-	-	5.6
M13	0	0	.48	.01	-	-	23.8
M14	0	0	.54	.05	-	-	28.2
M15	0	0	.57	.07	-	-	-

* M6 represents photo-cis-chlordane.

** Solvent systems are: I=heptane - 2 runs, II=benzene-ethyl acetate (3:1) - 2 runs, III=butanol-acetic acid-water (6:1:1), IV=chloroform-methanol (9:1).

*** Of the total radioactivity in fish, ether extracted 78.26, petroleum ether 10.79, and methanol 10.95%. Percent composition of each of these fractions is shown in vertical column.

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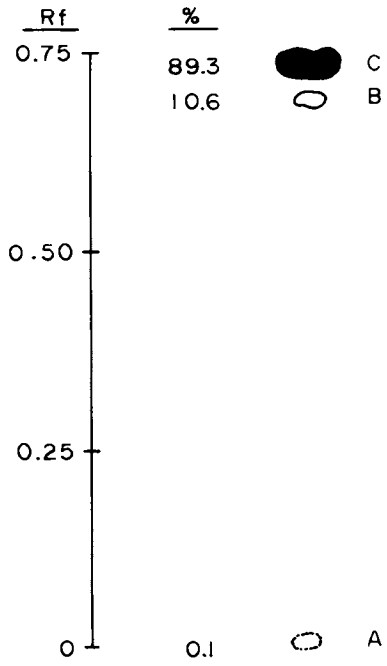

Figure 9. X-ray autoradiogram (Silica gel GF-254, benzene–ethyl acetate 3:1) of ^{14}C-photodieldrin-treated bluegills (20 ppb for 48 hr and held in clean water for 10 days). Recovery of the dose applied, 74.6%. Probable nature of A and B is hydroxy and ketone derivatives of photodieldrin, respectively. C represents unchanged photodieldrin.

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Summary and Conclusions

1. Under similar conditions bluegill absorb cis-chlordane at a higher rate than the amphibian, Xenopus laevis. Photo-cis-chlordane, an isomer of cis-chlordane is absorbed at a higher rate than cis-chlordane by bluegill and goldfish. However, photo-cis-chlordane is eliminated faster than cis-chlordane by the fish.

2. cis-chlordane is metabolized very slowly by fish (cichlid, bluegill, and goldfish) while photo-cis-chlordane and heptachlor are metabolized more efficiently by bluegill and goldfish, respectively. Cichlids resemble rats in the metabolism of cis-chlordane by forming dichlorochlordene and oxychlordane - the products not detected in bluegill and goldfish. Common metabolites in fish include chlordene chlorohydrin and hydroxylated product(s). Goldfish metabolize heptachlor to heptachlor epoxide, 1-hydroxy-2,3-epoxychlordene, 1-hydroxychlrodene, heptachlor diol and trihydroxyheptachlor-conjugate. Photodieldrin is metabolized very slowly by bluegill and the products indicate the presence of photodieldrin ketone, and hydroxylated product(s).

Acknowledgements

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4

Disposition and Metabolism of Aromatic Hydrocarbons in Marine Organisms

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In studies of the fate of hydrocarbons in terrestrial animals, considerable attention is directed toward relations between aromatic hydrocarbon metabolism, interactions of metabolites with macromolecules (e.g., DNA), and the formation of neoplastic lesions (1). A broad perspective exists in studies with marine organisms. In the aquatic forms, exposure to pollutants that are rich in aromatic hydrocarbons, such as petroleum, leads to a wide variety of acute and chronic effects (2). Attempts to delineate these effects require an understanding of the accumulation of the xenobiotics in tissues and an assessment of metabolite formation and retention. The important additional problem of the interaction of metabolites with genetic materials has not been studied to an appreciable degree in marine life.

Reviews on the fate of aromatic hydrocarbons in marine organisms have been published (2,3,4). They indicated that a substantial amount of information exists on the accumulation of these compounds in a variety of phylogenetically diverse organisms. Recently, emphasis has shifted toward studies of bioconversions of these hydrocarbons. Work has been conducted on enzymes mediating the degradation of aromatic hydrocarbons and on the formation and retention of metabolites. Identifications of individual metabolites in tissues and body fluids of several marine organisms exposed to radiolabeled aromatic hydrocarbons have been made; however, insufficient information is available to determine the extent of differences in metabolite profiles as evinced from chromatographic data.

In the present paper, emphasis will be placed on findings obtained in the last three years. Attention will focus on aromatic hydrocarbon accumulations and on the formation, retention, and structure of metabolites. Material appearing in recent reviews will not be emphasized (2,3,4).

INCORPORATION OF HYDROCARBONS INTO TISSUES AND BODY FLUIDS

Marine organisms readily accumulate aromatic hydrocarbons in

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tissues when exposed through the diet, water column, or sediment (2,5,6). The extent of accumulation varies in relation to such factors as species, hydrocarbon structure, route of administration, and environmental conditions. Differences in the tendency of organisms to accumulate aromatic hydrocarbons from the water column are illustrated in Table I (7). The data compare the uptake of substituted and nonsubstituted benzenes and naphthalenes into muscle of coho salmon (Oncorhynchus kisutch) and starry flounder (Platichthys stellatus) exposed under identical conditions to the water-soluble fraction of Prudhoe Bay crude oil. Starry flounder muscle accumulated very high concentrations of the following aromatic hydrocarbons in comparison to coho salmon after two weeks of exposure: C_3-substituted benzenes, C_4/C_5-substituted benzenes, 2-methylnaphthalene, 1-methylnaphthalene, C_2-substituted naphthalenes and C_3-substituted naphthalenes. Starry flounder tended to retain aromatic hydrocarbons for longer periods than coho salmon when both organisms were transferred to clean water. After two weeks in clean water, the muscle of starry flounder contained 26 ppm of C_4/C_5-substituted benzene

Table I Hydrocarbons in muscle tissue of coho salmon *(O. kisutch)* and starry flounder *(P. stellatus)* exposed to the water-soluble fraction of Prudhoe Bay crude oil using flow-through exposure[a]

	Coho salmon								
	Weeks of exposure								
	2		3		5		6		1 Week depuration (6 weeks exposure)
Hydrocarbons	Bioconcentration[b]	ppm dry tissue[c]	Bioconcentration	ppm dry tissue	Bioconcentration	ppm dry tissue	Bioconcentration	ppm dry tissue	
C_2-Substituted benzenes	1.1	0.31 SS[e]	2.4	0.66 SS	2	0.55 SS	1	0.27 SS	NF[f]
C_3-Substituted benzenes	10	0.30 ± 0.12	30	0.90 ± 0.12	50	1.5 ± 0.09	10	0.40 ± 0.18	NF
C_4-C_5-Substituted benzenes	150	1.5 ± 0.61	170	1.7 ± 0.50	550	5.5 ± 1.0	200	2.0 ± 1.5	NF
Naphthalene	20	0.07 ± 0.03	50	0.14 ± 0.07	80	0.24 ± 0.06	40	0.12 ± 0.06	NF
2-Methylnaphthalene	30	0.10 ± 0.05	100	0.31 ± 0.01	190	0.56 ± 0.14	70	0.20 ± 0.10	NF
1-Methylnaphthalene	30	0.10 ± 0.03	70	0.22 ± 0.00	130	0.40 ± 0.08	50	0.16 ± 0.08	NF
C_2-Substituted naphthalenes	30	0.31 ± 0.30	40	0.36 ± 0.00	85	0.90 ± 0.24	40	0.44 ± 0.30	NF
C_3-Substituted naphthalenes	50	0.23 ± 0.09	30	0.15 ± 0.02	140	0.70 ± 0.22	80	0.40 ± 0.40	NF

	Starry flounder							
	Weeks of exposure				Weeks of depuration			
	1		2		1		2	
C_2-Substituted benzenes	20	5.5 ± 2.0	4	1.0 ± 0.30	1	0.27 ± 0.06		NF
C_3-Substituted benzenes	500	15 ± 5.7	70	2.2 ± 1.2	6	0.18 ± 0.03	10	0.30 ± 0.02
C_4-C_5-Substituted benzenes	9300	93 ± 34	1700	17 ± 6.2	980	9.8 ± 0.40	2600	26 ± 1.6
Naphthalene	700	2.1 ± 1.5	240	0.72 ± 0.30	100	0.30 ± 0.02	270	0.80 ± 0.04
2-Methylnaphthalene	2800	8.3 ± 3.6	470	1.4 ± 0.60	110	0.33 ± 0.03	200	0.60 ± 0.02
1-Methylnaphthalene	2000	6.1 ± 4.3	330	1.1 ± 0.50	113	0.34 ± 0.01	270	0.82 ± 0.04
C_2-Substituted naphthalenes	2400	24 ± 9.7	540	5.4 ± 2.3	270	2.7 ± 0.80	700	7.0 ± 1.6
C_3-Substituted naphthalenes	3400	17 SS	1000	5.0 ± 2.0	420	2.1 ± 0.00	1600	8.0 ± 0.10

[a] 0.9 ± 0.1 ppm (total hydrocarbons) in flow-through water.
[b] Bioconcentration = ppm hydrocarbon in dry weight tissue/ppm hydrocarbon in water
[c] Mean ± Standard error of mean for two 10- to 15-g composite samples each prepared from separate groups of coho salmon (2 fish/group)
[d] Mean ± Standard error of mean for two 10- to 15-g composite samples each prepared from separate groups of starry flounder (5 fish/group)
[e] SS = Single sample value
[f] NF = Not found; below limits of detection (≤0.05 ppm)

From Roubal et al. (7).

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fraction, and 7.0 and 8.0 ppm of the C_2- and C_3-substituted naphthalenes, respectively. Levels of petroleum hydrocarbons in the gills and liver of starry flounder were generally below limits of detection. Hydrocarbons were not detected in exposed salmon under comparable conditions of depuration.

The strong tendency for coho salmon and starry flounder to accumulate alkyl-substituted aromatic hydrocarbons through the water column in preference to unsubstituted structures (Table I) suggests that hydrocarbon retention increases in proportion to the extent of ring substitution in these animals. Roubal et al. (8) found that benzene, naphthalene, and anthracene administered in the diet of coho salmon accumulate in tissues, such as liver and muscle, in proportion to the number of benzenoid rings in the molecules. Thus, two structural properties--degree of alkylation and number of fused rings--have been related to the disposition of aromatic hydrocarbons in fish exposed through the diet and water column.

These conclusions were not applicable when sediment was the source of hydrocarbons. McCain et al. (5) studied the bioavailability of petroleum in sediment to English sole (*Parophrys vetulus*). Sediments rich in alkylated and non-alkylated benzenes and naphthalenes, together with fluorene and phenanthrene, were employed. After 11 days of exposure, samples of skin, muscle, and liver were examined. Fluorene and phenanthrene were not accumulated in the test fish; however, significant concentrations of 1-methylnaphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene and 1,2,3,4-tetramethylbenzene, were found in skin and liver (Table II); 1-methylnaphthalene and 2-methylnaphthalene were the major components of muscle. In each tissue examined, 1-methylnaphthalene was the major component; 1,2,3,4-tetramethylbenzene occurred in relatively low concentrations in skin and muscle in comparison to naphthalenes containing one and two alkyl groups.

TABLE I. Petroleum hydrocarbons in the tissues of English sole exposed to oil-contaminated sediment (test, T) and to nonoiled sediment (control, C) for 2 mo continuously.

Polycyclic aromatic hydrocarbons[a]	Concn (ng/gm dry wt)[b]															
	11 d						27 d						51 d			
	Skin		Muscle		Liver		Skin		muscle		Liver		Skin		Muscle		Liver	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1,2,3,4-tetramethylbenzene	—	33	—	44	—	922	—	—	—	—	—	863	—	—	—	—	—	124
Biphenyl	—	156	—	—	—	307	—	—	—	—	—	278	—	—	—	—	—	—
Naphthalene	—	82	—	20	—	100	—	—	—	—	—	—	—	—	—	—	—	—
1-methylnaphthalene	—	1189	—	369	—	1940	—	—	—	—	—	1325	—	—	—	—	—	—
2-methylnaphthalene	—	888	—	279	—	3070	—	—	—	—	—	1500	—	—	—	—	—	60
2,6-dimethylnaphthalene	—	130	—	—	—	69	—	—	—	—	—	—	—	—	—	—	—	—

[a]Arenes with aromatic rings ranging from one to six, i.e. *o*-xylene to benz[*a*]anthracene, were determined and only the compounds listed here were found in significantly higher concentrations in test fish.
[b]Skin and liver samples were pooled from three fish at each analysis; muscle samples were analyzed individually.

From McCain et al. (5).

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ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

These data imply that aromatic hydrocarbons incorporated into sediments are not preferentially accumulated in relation to increased alkyl substitution, as shown with dietary and seawater exposures. Moreover, the apparent lack of accumulation of the fluorene and phenanthrene suggests that unsubstituted aromatic hydrocarbons having more than two benzenoid rings may not be readily sequestered by fish exposed to petroleum-impregnated sediment. These differences are presumably related, at least in part, to physico-chemical interactions of aromatic hydrocarbons with sediment matrices that regulate their bioavailability.

Melancon and Lech (9) (Fig. 1) found with rainbow trout (Salmo gairdneri) that differences existed in the accumulation and excretion of radiolabeled naphthalene and 2-methylnaphthalene, including possible bioconversion products, in exposures through the water column. The alkylated naphthalene was taken up more rapidly than the unsubstituted naphthalene. Moreover, it was initially accumulated to higher levels in all tissues studied and eliminated more rapidly from muscle, liver, and blood. These workers suggest that exposure of trout to naphthalene over extended periods may lead to higher concentrations in tissues of parent compounds and metabolites than would occur with 2-methylnaphthalene. Accordingly, exposure time is important in assessing the retention of aromatic hydrocarbons and their bioconversion products in marine organisms.

Evidence pertaining to the influence of compounds such as PCBs and heavy metals on aromatic hydrocarbon accumulations in marine organisms were discussed in recent review papers (3,4). The influence of such compounds on metabolizing enzyme systems and excretion processes appears to be significant in some cases so that the disposition of hydrocarbons is likely to be affected.

Environmental temperature influences the degree of hydrocarbon accumulation in marine fish. Collier et al. (10) found that depressed temperature (4° vs. 10°C) resulted in significantly ($P<0.05$) increased retention of dietary ^{14}C-naphthalene in brain, liver, kidney, and blood of coho salmon (O. kisutch). Thus, environmental conditions can have a pronounced effect on the disposition of hydrocarbons in exposed marine species.

In exposed marine vertebrates, aromatic hydrocarbons were identified in liver (5,7-13), dark (10) and light muscle (10), brain (8,10,12,13), heart (8,13), gut (8,10,13), kidney (8,10,12), skin (11), eyes (14), gills (7,9,13), blood (8,9,10,12), bile 8,10,13), and mucus (11). Urine of exposed fish did not contain significant amounts of aromatic hydrocarbons (15). Collier et al. (10) found that substantial differences existed in the accumulation of naphthalene between light and dark muscle of coho salmon which were force-fed the hydrocarbon. Compared to light muscle, dark muscle accumulated much higher concentrations of the naphthalene, which is of interest because dark muscle is thought to perform some metabolic functions analogous to liver (16). Collier and Malins (12) found that brain accumulated substantial

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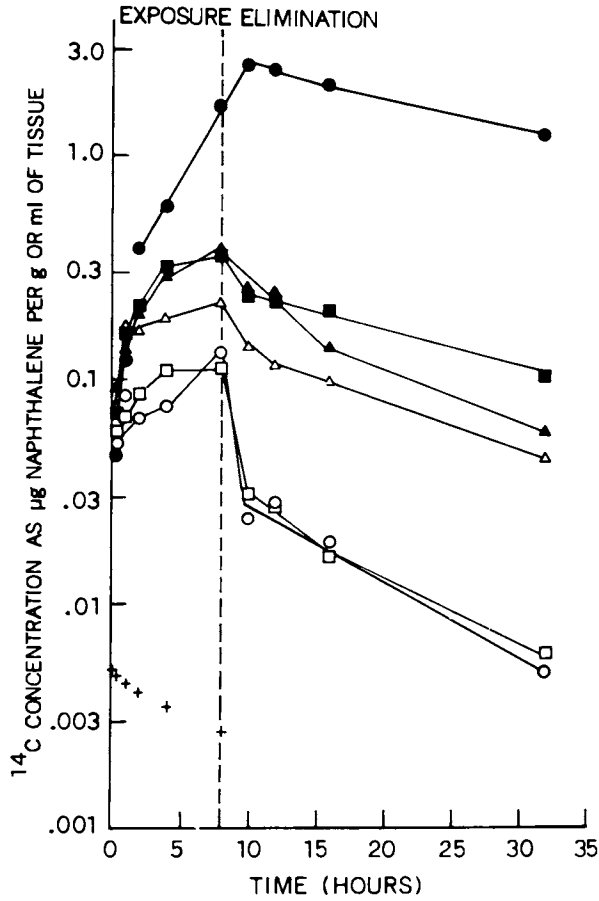

Figure 1. Tissue level of ^{14}C in rainbow trout during exposure to ^{14}C-naphthalene (0.005 mg/L in water) and subsequent elimination. Data are the averages of values from five trout except for the 8-hr values which represent 10 trout: (●), fat; (▲), whole fish; (■), liver; (△), gill; (○), muscle; (□), blood; and (+), exposure water (9).

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levels of naphthalene but, unlike other organs, contained relatively low levels of bioconversion products. Dixit and Anderson (13) and Roubal et al. (8) have also reported accumulations of naphthalene in the brain of exposed fish. Anderson (17) pointed out that hydrocarbon accumulations in brain may be associated with behavioral or physiological changes.

Skin is also a major site of hydrocarbon accumulation in fish. Varanasi et al. (11) (Table III) found that after a few hours of naphthalene exposure, significant concentrations of naphthalene appeared in the skin of rainbow trout (S. gairdneri), regardless of whether the hydrocarbon was administered via force-feeding, intraperitoneal injection, or in flowing seawater. Highest concentrations occurred in the water-immersion study. Smaller concentrations were accumulated by the fish exposed to a single dose of naphthalene via force-feeding or via intraperitoneal injection. Naphthalene in skin was readily discharged after the animals were placed in clean water. Varanasi et al. (11) also showed that epidermal mucus of fish in injection and force-feeding experiments contains naphthalene. They concluded that mucus exudate is involved in the excretion of hydrocarbons because epidermal mucus exists in a state of continual flux (that is, mucus is continually sloughed off and renewed).

Table III. Accumulation and release of naphthalene and its metabolites in skin of rainbow trout

Mode of exposure	Time (hr)	Total radioactivity in skin (μCi)	Relative percentage	
			Naphthalene	Metabolites
Force-feeding	4	0.06	89.4	10.6
	16	0.31	92.6	7.4
	24	0.65	95.6	4.4
	48	0.16	93.2	6.8
	168	0.03	82.4	17.6
Injection	4	0.20	95.4	4.6
	16	0.43	97.2	2.8
	24	0.38	95.4	4.6
	48	0.22	90.0	10.0
	168	0.04	84.9	15.1
Flowing water				
Exposure	24	0.53	95.3	4.7
	72	0.99	84.2	15.8
Depuration	72	0.20	48.0	52.0

Adapted from Varanasi et al. (11).

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Rainbow trout exposed to naphthalene in surrounding water accumulate this hydrocarbon in eyes (14). The retention (including bioconversion products) may be related to observed morphological changes, such as cataract formation, which occurs in marine fish exposed to individual hydrocarbons (18) and petroleum (19). Roubal et al. (20) have demonstrated that gills of salmonids are major sites for discharging naphthalene, which implies that the more water-soluble hydrocarbons in general are cleared via this route.

Invertebrates (crustacea and molluscs) readily accumulate hydrocarbons when exposed for more than a few hours through surrounding water. In crustacea, thoracic and abdominal sections (21,22), gills (22,23), stomach (22, 23), hepatopancreas (23), muscle (23), gonad (23), and blood (23) are sites of hydrocarbon accumulation. In molluscs, gills (24,25), adductor muscle (24, 25), viscera (25), mantle (24,25), and foot (25) are tissues in which hydrocarbons were identified in challenged organisms.

As with marine fish, hydrocarbon structure, physico-chemical interactions with substrates, exposure concentrations and individual differences in physiology and biochemistry are intimately related to hydrocarbon uptake, retention, and release by marine invertebrates. Boehm and Quinn (26) studied the uptake of hydrocarbons into the hard shell clam (Mercenaria mercenaria) from chronically polluted waters of the Providence River. When organisms were transferred to clean water, slight depuration of tissues occurred after 120 days. Only 30% or less of the hydrocarbons accumulated by the clams were lost. The findings indicated that the duration of exposure and the chemical structures of the hydrocarbons are instrumental in determining their retention times in filter-feeding bivalves. Chronically accumulated hydrocarbons were strongly retained by the hard shell clams and tissues were only very slowly depurated. Although other biochemical factors may be responsible, it was proposed (27) that exchange equilibria exist between lipid compartments of organisms and surrounding seawater. According to Boehm and Quinn (26), the findings obtained with the hard shell clam may be accounted for by such equilibrium processes. Reports (28,29) on the relationship between temperature, uptake, and retention of naphthalene in clams (Rangia cuneata, Prototheca staminea) and copepods (Calanus helgolandicus) suggest that retention of this hydrocarbon in test organisms is inversely proportional to exposure temperature. The data indicate that the concentration of naphthalene retained in C. helgolandicus challenged at 6°C would be 44% higher than in copepods challenged at 10°C (29). Earlier work of Corner et al. (30) showed that C. helgolandicus accumulated substantial concentrations of naphthalene in 24 hr at 10°C. These workers (30) also found that there was a linear relation between the concentration of accumulated hydrocarbons in copepods and the concentrations in water. Gills and guts of mussels were shown to accumulate high concentrations of naphthalene from the

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water column (24). Moreover, Sanborn (21) found that the thoracic sections of spot shrimp (Pandalus platyceros) exposed to the water-soluble fraction of Prudhoe Bay crude oil, but not the abdominal sections, accumulated substantial concentrations of alkyl-substituted and non-substituted benzenes and naphthalenes. Thus it is evident that the degree of accumulation of aromatic hydrocarbons in invertebrates varies substantially in relation to different tissue sites.

In mammalian systems, the naphthalenic hydrocarbons are generally non-carcinogenic (31); however, evidence indicates that some alkylnaphthalenes may have co-carcinogenic activity in, for example, the tumor promoting effects of benzo[a]pyrene in lung tissue (31) or skin (32). Thus the tendency for marine organisms to accumulate naphthalenes should be considered in relation to the uptake of known mutagenic/carcinogenic molecules associated with pollutant mixtures.

BIOTRANSFORMATIONS

Mueller and Miller (33) and Brodie et al. (34) were the first to show that enzymes in the microsomal fraction of rat liver could effectively oxidize xenobiotics. Comparable enzymes (aryl hydrocarbon monooxygenases) were later reported in the hepatic tissues of fresh water and marine fish by Creaven et al. (35) and Buhler and Rasmusson (36). Reconstituted hepatic microsomal systems require cytochrome P-450 for monooxygenase activity in both mammals (37) and fish (38,39). Bend et al. (40) have shown that cytochrome P-450 occurs in hepatic microsomes of marine elasmobranch and teleost fish, although the concentrations of this hemoprotein are appreciably lower than those of mammals (e.g., rat and rabbit). Concentrations of P-450 were found to be generally similar in hepatic tissues of fresh water and marine fish. Moreover, P-450 was found in the microsomal fraction of the hepatopancreas from three crustacean species (40). Substantial variations occurred in the amount of P-450 in the hepatopancreas of individual lobsters (Homarus americanus) and blue crab (Callinectes sapidus). Bend et al. (40) observed that a notable difference exists between P-450-dependent mixed-function oxidases in mammals and aquatic forms in that the temperature optima for the conversions *in vitro* are usually lower in the poikilothermic than in homeothermic vertebrates.

Enzymes of the hepatic microsomes of most marine organisms, with the notable exception of certain molluscs, metabolize xenobiotic substrates; however, as much as 600-fold variations in enzyme activities have been noted between different species of marine teleosts (40). The hepatic enzyme activities of aquatic species are generally lower, with most substrates tested, than the hepatic enzymes of mammals (40). The mixed function oxidase enzymes in marine organisms are inducible by hydrocarbons, such as 3-methylcholanthrene or benzo[a]pyrene. Moreover, it is known

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that a number of different other chemicals alter the activities of the mixed function oxidase enzymes in a variety of aquatic organisms (3,4,40).

The microsomal epoxide hydrase activities in organs of marine species were reviewed by Bend et al. (40). Resulting aromatic structures containing alcohol groups undergo conjugating reactions through enzymes, such as the glutathione-S-transferases (40).

Evidence showing that marine organisms are able to extensively metabolize aromatic hydrocarbons to form both conjugated and non-conjugated derivatives leads to a concern about the nature of the metabolic products. Lee et al. (41) studied the metabolism of radiolabeled naphthalene and 3,4-benzo[a]pyrene in mudsucker (Gillichthys mirabilis), sculpin (Oligocottus maculosus), and sanddab (Citharichthys stigmaeus). These workers tentatively identified a dihydrodiol as the major metabolite in both the benzo[a]pyrene and naphthalene exposed fish.

In recent years, using both thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC), it has been possible to separate and identify a variety of conjugated and non-conjugated metabolites of aromatic hydrocarbons in marine organisms (8,10,12,15,42,43,44).

Roubal et al. (8) applied TLC to the separation and identification of metabolites of ^{14}C-labeled naphthalene administered to coho salmon fingerlings via intraperitoneal injection. 1-Naphthol, a dihydrodiol, mercapturic acid, 1-naphthyl glucuronic acid and a glycoside/sulfate fraction were identified in brain, liver, gall bladder, and muscle; 1-naphthol, a dihydrodiol, and 1-naphthyl glucuronic acid were the only metabolites found in heart.

In another study (42) English sole (P. vetulus), rock sole (Lepidopsetta bilineata), and starry flounder (P. stellatus) received ^{3}H-naphthalene through the diet. The hydrocarbons were extensively metabolized; more than 80% of the radioactivity in the blood of each species was associated with metabolic products after 168 hr. Highest concentrations of metabolites occurred in the gall bladder; after 24 hr, TLC showed that the principal metabolite was the 1-naphthyl glucuronide; however, lesser amounts of 1-naphthol, 1,2-dihydro-1,2-dihydroxynaphthalene, 1-naphthyl sulfate, and 1-naphthyl glucoside were also found. The relative proportions of individual metabolites in the bile did not change significantly with time.

Malins et al. (15) showed by TLC that rainbow trout exposed to ^{14}C-labeled naphthalene excrete 1-naphthyl glucuronic acid in the urine almost exclusively. Only 1% of the total radioactivity of the urine was in the form of non-conjugated derivatives. Melancon and Lech (9) demonstrated that rainbow trout exposed to both radiolabeled naphthalene and 2-methylnaphthalene contained significant portions of polar compounds (unspecified) after about four weeks of exposure (Table IV). Malins et al. (15) showed that a variety of metabolic products accumulate in tissues and

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Table IV. Fraction of muscle ^{14}C from rainbow trout exposed to ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene present as polar compounds

	Percent of ^{14}C as polar compounds[a]
Naphthalene	
1 Day exposure	21
27 Days exposure	12
27 Days exposure + 9 days elimination	34
2-Methylnaphthalene	
1 Day exposure	1.5
26 Days exposure	1.1
26 Days exposure + 7 days elimination	24

[a] Values shown are derived from muscle samples analyzed at the end of the indicated experimental periods.
From Melancon and Lech (9).

biological fluids of coho salmon exposed to 2,6-dimethylnaphthalene. Using known standards and colorimetric identifications, glucuronic acid, mercapturic acid, sulphate, monohydroxy, and dihydroxy derivatives were provisionally identified by TLC.

Collier et al. (10) demonstrated that HPLC was an effective technique for the separation of aromatic hydrocarbon metabolites in exposed marine organisms. Radioactive bioconversion products were studied in liver and gall bladder of coho salmon dosed with ^{3}H-naphthalene. Quantitative identifications of glucuronide, sulphate, dihydrodiol, glycoside, and 1-naphthol derivatives were obtained. Three additional polar compounds of unknown structure were found. A typical HPLC profile is shown in Figure 2.

HPLC studies on the brain of mature rainbow trout revealed essentially four metabolites, including the dihydrodiol and 1-naphthol (12). No evidence was found for the presence of conjugated derivatives. It was concluded that the conjugated derivatives of naphthalene were excluded by blood-brain barrier systems that develop in mature organisms.

Thomas et al. (43) showed that phenanthrene is actively metabolized in salmonids and Lee et al. (41) have shown that benzo[a]pyrene is biodegraded in three species of marine fish. Varanasi et al. (11) demonstrated for the first time that the skin of fish exposed to aromatic hydrocarbons via either force-feeding, intraperitoneal injection, or in flowing seawater accumulate substantial concentrations of metabolic products. This is of particular interest since studies of mammalian systems have shown that some alkylnaphthalenes can be accelerators of skin carcinogenesis (32). Varanasi et al. (11) also demonstrated that the mucus of rainbow trout exposed to radiolabeled naphthalene

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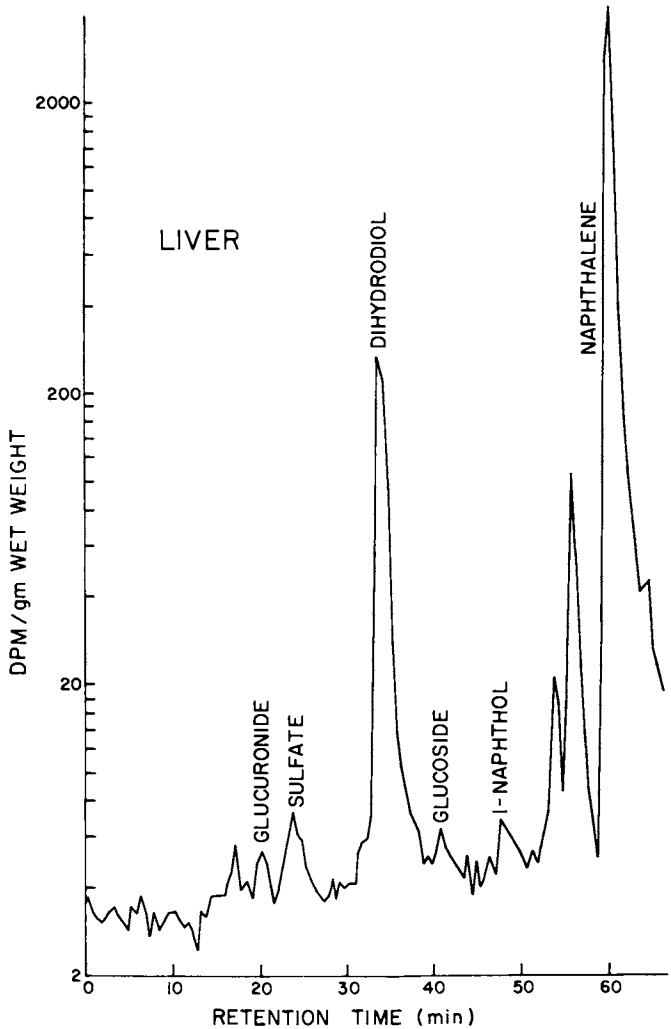

Figure 2. HPLC profile of extracts of liver of ^{3}H-naphthalene-exposed coho salmon. Data were obtained by single analysis of pooled tissue from four fish, each fed 74.6 μCi ^{3}H-naphthalene 16 hr earlier (10).

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by injection or force-feeding contained significant concentrations of metabolites several days after initial exposure. It was concluded that mucus plays an important role in the excretion of metabolites in salmonid fish.

Recent studies in our laboratories have shown that newly hatched herring (Clupea harengus pallasi) larvae exposed to purified ^{3}H-naphthalene in seawater at concentrations of 10 ppb for 9 hr accumulated a variety of conjugated and non-conjugated metabolites. HPLC analysis indicates the presence of the parent compound as well as three additional compounds whose retention times are consistent with a sulfate, a dihydrodiol, and 1-naphthol (Fig. 3).

Reichert et al. (44) investigated the effect of metal exposure on metabolism of naphthalene in starry flounder (P. stellatus): One group of fish was exposed to 150 ppb of cadmium (as $CdCl_2$) in seawater for four weeks. Another group was exposed to lead [as $Pb(NO_3)_2$] under the same conditions. An additional group was maintained in "control" seawater for four weeks. Fish in each group received intraperitoneal injections with radiolabeled naphthalene 24 hr before the animals were sampled. In the groups of metal-exposed fish, concentrations of 1,2-dihydro-1,2-dihydroxynaphthalene (the principle metabolite) in liver were 40 to 60% lower than concentrations in livers of control animals. These findings suggest that metabolite formation and retention in fish may be influenced significantly by metals in the exposure medium.

A limited amount of information is available on the formation of metabolites of aromatic hydrocarbons in aquatic invertebrates; however, some studies have elucidated the structures of conjugated and non-conjugated derivatives produced in these organisms. The bioconversion of naphthalene by the spider crab (Maia squinado) has been studied by Corner et al. (45). These workers identified 1,2-dihydro-1,2-dihydroxynaphthalene, a glucoside of this compound, 1-naphthyl sulfate, and 1-naphthyl glucoside in the urine; 1-naphthyl mercapturic acid was also detected in the urine after acidification. Additionally, Corner et al. (45) found 1-naphthol, 1-naphthyl glucoside, and 1-naphthyl sulfate in the urine of M. squinado dosed with 1-naphthol. Lee (46) demonstrated that marine zooplankton convert benzo[a]pyrene and naphthalene into a number of hydroxy derivatives. Sanborn and Malins (47) demonstrated that Stage V larvae of spot shrimp (P. platyceros) are able to metabolize radiolabeled naphthalene when exposed to the hydrocarbon in seawater (Fig. 4). Metabolic products in larval tissues reached a maximum value of 9% of total accumulated radioactive compounds (based on the molecular weight of naphthol). When larvae were exposed to an equivalent concentration of radiolabeled naphthalene complexed with bovine serum albumin (BSA), the maximum value for accumulated metabolites was 21%. Radiolabeled naphthalene was almost entirely depurated in 24-36 hr, whereas metabolic products were strongly resistant to depuration

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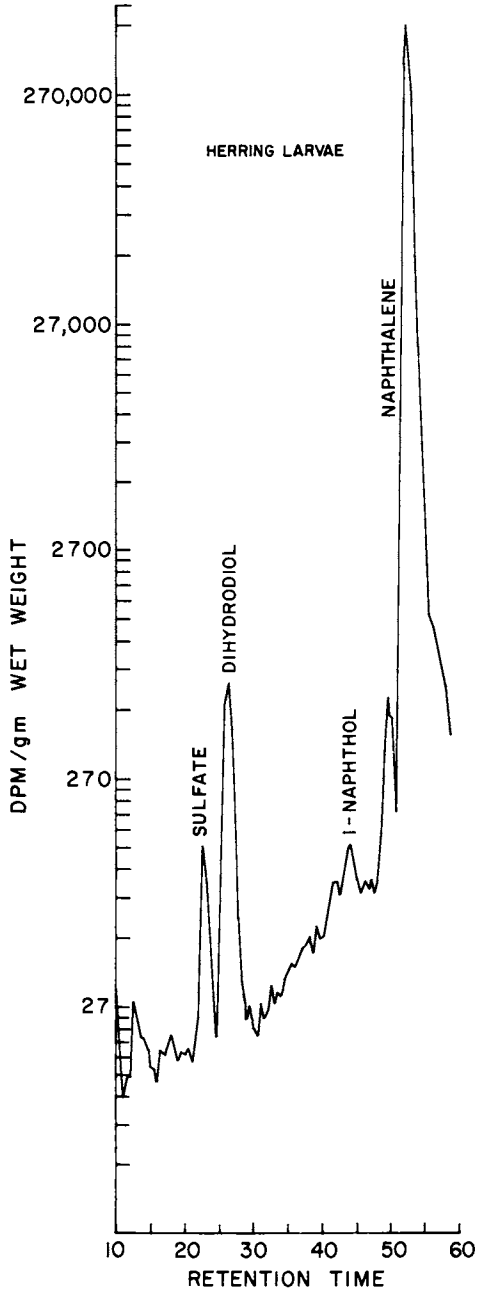

Figure 3. HPLC profile of extracts of ^{3}H-naphthalene-exposed herring larvae

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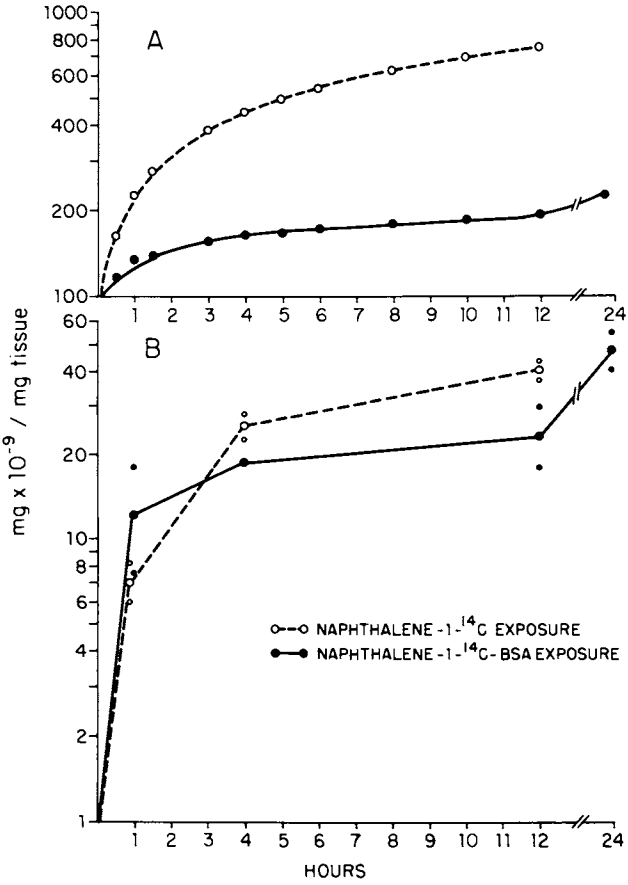

Figure 4. Accumulation of naphthalene and metabolic products (expressed as naphthol) after exposure of stage V spot shrimp to 8–12 ppb of waterborne [1-¹⁴C]-naphthalene and [1-¹⁴C]naphthalene complexed with BSA: (A), regression lines of concentrations of [1-¹⁴C]naphthalene with sampling points indicated; (B), median values of metabolic products with data ranges (47).

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Table V. Excretion of water-soluble metabolites of ^{14}C-anthracene by Daphnia pulex

Time elapsed after ^{14}C-anthracene addition, hr	Fraction of total ^{14}C in form of water-soluble metabolites
0.0	<0.002
0.17	<0.002
0.5	<0.002
1.0	<0.002
2.0	<0.002
4.0	0.008
24	0.045
48	0.040
96	0.22
168	0.21

Data points fitted by least-squares regression to the equation: $C\ (metabolite)_t = 1\text{-}e^{-k(t-2)}$. Computed value of k is 0.00160/hr ($r = 0.91$).

From Herbes and Risi (48).

during this time period. In contrast, studies of Daphnia pulex exposed to water-borne anthracene (48) indicate that metabolized anthracene was readily excreted after a 2 hr latent period (Table V). Recent findings from our laboratories indicate that Stage I spot shrimp larvae convert radiolabeled naphthalene into a variety of conjugated and non-conjugated derivatives. When unfed larvae were exposed to purified, tritiated naphthalene at a concentration of 15 ppb for 9 hr, HPLC separations showed the presence of naphthalene as well as three compounds whose retention times were consistent with those of naphthol, a glucuronide, and a dihydrodiol (Fig. 5).

CONCLUSIONS

Ample evidence exists to show that aquatic vertebrates are able to metabolize aromatic hydrocarbons to a variety of conjugated and non-conjugated derivatives. It was shown with fish that the metabolite:aromatic hydrocarbon ratio tends to increase after hydrocarbon exposure. Under conditions of depuration (clean water environments) either hydrocarbons or metabolites are discharged through gills, bile, urine, skin, and mucus of marine fish. Further work is necessary with phylogenetically diverse species because the above conclusions are based on only a few studies of selected organisms.

Although a number of studies have been conducted on the accumulation of aromatic hydrocarbons in aquatic invertebrates, only a

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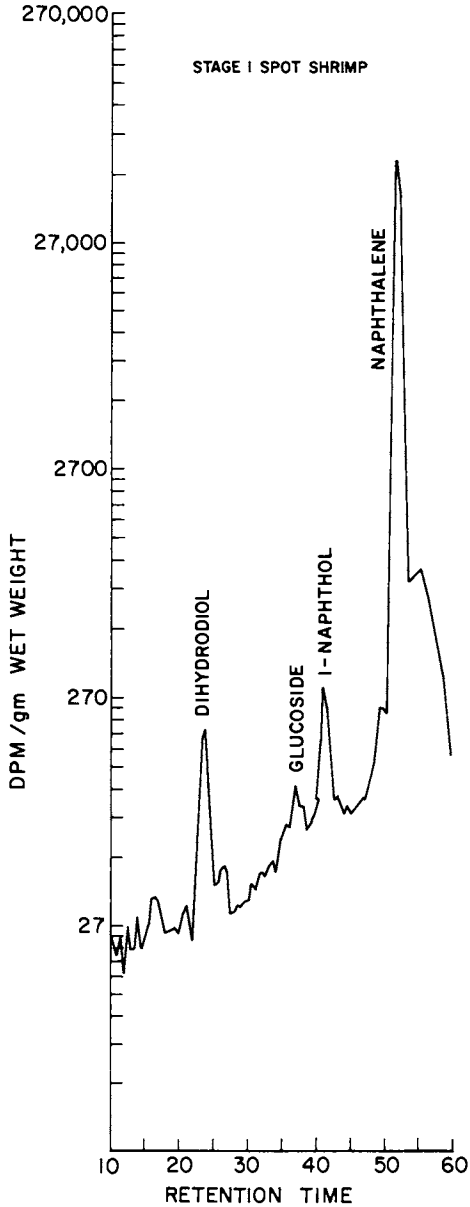

Figure 5. HPLC profile of extracts of ^{3}H-naphthalene-exposed spot shrimp larvae

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limited amount of information is available on the formation and retention of metabolites in these organisms. Accordingly, few conclusions can be drawn at present; however, as discussed, spot shrimp larvae form and retain metabolites, whereas the freshwater unicellular organism D. pulex appears to readily discharge these compounds after exposure (48), as does the spider crab (M. squinado) (45). On the basis of evidence on select species, considerable variations appear to exist in the tendencies of aquatic invertebrates to excrete metabolites of aromatic hydrocarbons.

Identifications of individual aromatic hydrocarbon metabolites in marine organisms have indicated that considerable variations exist in the proportions of individual derivatives, depending upon the species and sites of accumulation. We have pointed out that relatively high concentrations of 1,2-dihydro-1,2-dihydroxynaphthalene appear in liver and other tissues of exposed organisms. The dihydrodiol is obtained from the corresponding epoxide, thus questions arise about the potentially toxic interactions of arene oxides with DNA, particularly because certain epoxides of the higher molecular weight aromatic hydrocarbons are mutagens and carcinogens (1). Unfortunately, no information is presently available to determine whether or not a link exists between metabolite formation and known incidences of cellular damage in marine organisms.

No detailed studies have been reported on the transfer of metabolites through aquatic food webs. Moreover, no specific information is available on the effects of metabolites on aquatic ecosystems. Both of these deficiencies leave important unanswered questions.

The potential threat to aquatic organisms of small amounts of known carcinogens in petroleum products should be viewed in terms of the possible co-carcinogenic role of other hydrocarbons, such as the alkylated structures, that preferentially accumulate in exposed aquatic organisms. It is suggested that a profitable area for future research would be an evaluation of the extent of potentially damaging metabolite interactions with DNA, coupled with a complimentary assessment of the mutagenic potential of these metabolites using procedures such as the Ames test.

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5

Metabolism of Phthalate Esters in Aquatic Species

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Phthalate esters, particularly, di-2-ethylhexyl phthalate (DEHP), are widely used as plasticizers. The total annual U.S. production of phthalate esters was over 400,000 tons in 1970 (1). Many of the applications of phthalate esters such as in construction, home furnishings, the automobile industry, etc. make it likely that they reach the aquatic environment. Since the initial report of the presence of phthalate esters in natural waters and fish in the United States by Mayer and coworkers (2), there have been reports of these chemicals in fish in Canada (3,4) and in Japan (5). The presence of phthalate esters in the ocean waters off the United States and in Gulf of Mexico biota has also been noted (6).

Concern over the fate of phthalate esters in the aquatic environment has resulted in studies of the fate of phthalate esters in a number of aquatic species. Among the aquatic organisms which have been shown to metabolize phthalate esters, are microorganisms present in water, hydrosoil and sewage sludge, several invertebrates and several species of fish.

Metabolism of Phthalate Esters by Microorganisms

The various studies with aquatic microorganisms have shown that a variety of phthalate esters are degraded by river water, hydrosoil and sewage sludge. The data in Table I is derived from work at several different laboratories (7,8,9). While most of these phthalate esters are degraded in the systems studied, those esters containing the longer chain alcohols are broken down more slowly than those containing short chain alcohols. Monobutyl phthalate and phthalic acid were also found to be degraded by activated sludge by Saeger and Tucker (9).

Johnson and Lulves (8) found that di-butyl phthalate was degraded much more rapidly than DEHP and that anaerobic degradation of DEHP and dibutyl phthalate by hydrosoil was much slower

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TABLE I

Degradation of Phthalate Esters by Aquatic Microorganisms

Phthalate Ester	Activated Sludge[a] 48 Hr	Activated Sludge[b] continuous % Degraded	River Water[b] 1 week	Hydrosoil[c] 2 weeks
Di-2-ethylhexyl	91	74	10	47
Butyl benzyl	99	96	80	
Butylglycol butyl		99	95	
Diundecyl		37	10	
Santicizer 711[d]		51	0	
Dibutyl				92

[a]data from Graham (7)
[b]data from Saeger and Tucker (9)
[c]data from Johnson and Lulves (8)
[d]mixture of diheptyl, dinonyl and diundecyl phthalates

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than aerobic (Table II). DEHP, in fact, was completely unaffected by hydrosoil organisms under anaerobic conditions. A comparison of the amounts of dibutyl phthalate and metabolites present under aerobic and anaerobic conditions is shown in Table III. The most striking difference in metabolites is the much larger amount of monoester present under anaerobic conditions. The amount of phthalic acid is also higher, but this represents a much smaller proportion of total ^{14}C. These results suggest that hydrolysis products of dibutyl phthalate are accumulating in the absence of oxygen. This could be due to a shift to another route of metabolism due to the lack of oxygen or to greatly slowed oxidation of the monoester. Because the ^{14}C was in the carbonyl, it was not possible from these studies to determine whether the benzene ring remained intact.

Studies by Saeger and Tucker, however, showed that CO_2 evolution from degradation of dibutyl phthalate, DEHP and Santicizer 711 was 85-95% of the theoretical (9). Johnson and coworkers (10) later showed the evolution of $^{14}CO_2$ from ring labeled DEHP. It seems clear, then, that aquatic microorganisms are able to completely degrade phthalate esters.

Metabolism of Phthalate Esters by Fish in vivo

Although the metabolism of several phthalate esters has been studied *in vitro*, essentially all of the *in vivo* studies have involved DEHP. A summary of these experiments which involved exposure of fish to aqueous ^{14}C-DEHP is presented in Table IV (11,12). Tissue ^{14}C was isolated and separated into parent and the various metabolites by preparative thin layer chromatography on silica gel. Metabolites were hydrolyzed where appropriate and identified by gas chromatography-mass spectroscopy. In whole catfish, whole fathead minnow and trout muscle, the major metabolite was the monoester while in trout bile the major metabolite was the monoester glucuronide. The fact that in all cases the major metabolite was monoester or monoester glucuronide despite the differences in species, exposure level and duration, etc. represented by these data, suggests that hydrolysis of DEHP to monoester is important in the biotransformation of DEHP by fish.

Metabolism of Phthalate Esters by Fish in vitro

Stalling and coworkers (11) studied the metabolism of ^{14}C-DEHP and dibutyl phthalate by channel catfish liver microsomes. They found that the respective monoesters and more polar metabolites were produced, but that DEHP was metabolized to a much lesser extent than dibutyl phthalate. Additional studies (Table V) showed that the production of the respective monoesters was unaffected by the presence of NADPH, but that the production of more polar metabolites is very dependent upon NADPH. The reasons for the apparent lack of a requirement for oxygen are unclear

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TABLE II

Biodegradation of ^{14}C-di-n-butyl Phthalate and ^{14}C-di-2-ethylhexyl Phthalate in Freshwater Hydrosoil[a]

Incubation Duration (days)	di-n-butyl phthalate		di-2-ethylhexyl phthalate	
	aerobic	anaerobic	aerobic	anaerobic
	% recovery of ^{14}C from hydrosoil[b]			
1	95	100		
5	3	69		
7	5	59	100	100
14	8	39	53	100
30	3	2	41	100

[a]data from Johnson and Lulves (8)
[b]recovery from heat killed controls=100%

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TABLE III

Characterization of ^{14}C Recovered from Hydrosoil after Incubation with ^{14}C-di-n-butyl phthalate[a]

		Incubation duration (days)				
		1	5	7	14	30
Aerobic incubation	di-n-butyl phthalate	46.3	85.3	70.0	74.0	76.0
	mono-n-butyl phthalate	46.3	7.0	26.0	22.0	18.6
	Unknown I	2.3	trace	0	0	0
	Unknown II	0.1	0	0	0	0
	Phthalic acid	0.9	2.3	3.0	2.3	3.6
	Other	2.3	3.3	1.0	1.0	1.0
Anaerobic incubation	di-n-butyl phthalate	68.3	29.6	29.0	5.0	37.6
	mono-n-butyl phthalate	30.7	45.3	63.0	85.6	16.9
	Unknown I	trace	4.3	1.3	1.0	0
	Unknown II	0.5	16.3	4.6	6.3	35.0
	Other	trace	4.6	trace	trace	9.0

[a]data from Johnson and Lulves (8);
for % of initial ^{14}C recovered see Table II

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TABLE IV

Biotransformation of ^{14}C-di-2-ethylhexyl Phthalate by Fish *In Vivo*

	Catfish[a] Whole fish 24 hr[c]	Fathead Minnow[a] Whole fish 28d	56d	Rainbow Trout[b] 24 hr Bile	Muscle
	% ^{14}C as di-2-ethylhexyl phthalate & metabolites				
Di-2-ethylhexyl phthalate	14	50	60	1	46
Mono-2-ethylhexyl phthalate					
free	66	37	25	0.5	39
conjugated	14	0.7	1.4	72	
Phthalic acid					
free	4	5.2	4.9	0	
conjugated	0.3	3.0	3.7	2	
Other	2	4.4	1.3	21	

[a]data from Stalling, *et al.* (11)
[b]data from Melancon and Lech (12) and Melancon, *et al.* (15).
[c]exposure duration

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TABLE V

Metabolism of ^{14}C-Phthalate Esters by Catfish Hepatic Microsomes[a]

	Additions		Metabolites formed (DPM)	
	NADPH	O_2	Monoester	Polar Metabolites 0, 1 & 2
Di-n-butyl phthalate	0	0	27,662	2,955
	0	+	31,376	1,992
	+	0	34,569	26,980
	+	+	36,234	21,125
Di-2-ethylhexyl phthalate	0	0	5,498	478
	0	+	4,776	864
	+	0	4,259	2,768
	+	+	5,676	6,130

[a]data from Stalling, et al. (11)

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Sanborn, et al. (13) reported that mosquito fish liver microsomes gave rise to the monoester as a metabolite from dioctyl phthalate and that this metabolism was blocked by paraoxon.

The metabolism of ^{14}C-DEHP by rainbow trout liver subcellular fractions and serum was studied by Melancon and Lech (14). The data in Table VI show that without added NADPH, the major metabolite produced was mono-2-ethylhexyl phthalate. When NADPH was added to liver homogenates or the mitochondrial or microsomal fractions, two unidentified metabolites more polar than the monoester were produced. Additional studies showed that the metabolism of DEHP by the mitochondrial and the microsomal fractions were very similar (Figure 1). Both fractions show an increased production of metabolites of DEHP resulting from addition of NADPH and the shift from production of monoester to that of more polar metabolites. The reduced accumulation of monoester which accompanied this NADPH mediated production of more polar metabolites may help in interpreting the pathway of DEHP metabolism in trout liver. This decreased accumulation of monoester could be explained either by metabolism of the monoester to more polar metabolites or the shift of DEHP from the hydrolytic route to a different, oxidative pathway. The latter explanation is unlikely because in these experiments less than 20% of the DEHP was metabolized.

Because the metabolism of DEHP was catalyzed by so many fractions of the trout liver homogenate, these fractions were characterized by measurement of marker enzymes to determine which organelles actually were responsible for the observed DEHP metabolism. Succinic dehydrogenase activity was used as a marker for mitochondria, whereas glucose-6-phosphatase was used as a marker for microsomes. The distribution of DEHP oxidase activity (production of polar metabolites 1 and 2 with added NADPH) and of DEHP esterase activity (production of monoester without added NADPH) were also determined. It was found (Figure 2) that the distribution of DEHP oxidase activity parallels the distribution of microsomal activity and the distribution of DEHP esterase activity parallels the distribution of microsomal activity, but is also present in the cytosol fraction.

In an effort to characterize further the metabolism of DEHP by trout, the effect of the mixed function oxidase inhibitor, piperonyl butoxide, upon the metabolism of DEHP by these trout liver fractions and serum was examined. Because of the use of piperonyl butoxide as an insecticide synergist, it is possible that fish might be exposed to this chemical in the environment. The data in Table VII show that piperonyl butoxide inhibited overall metabolism of DEHP by liver homogenates and microsomes whether NADPH was added or not. The hydrolysis of DEHP by serum was also blocked by piperonyl butoxide and although not shown, this was also the case with liver cytosol. These latter results were surprising because piperonyl butoxide has been known as a mixed function oxidase inhibitor only, and would not be expected

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TABLE VI

Metabolism of ^{14}C-di-2-ethylhexyl Phthalate by Rainbow Trout Liver Preparations and Blood Serum[a]

		Metabolites Formed		
		Mono-2-ethylhexyl phthalate	Polar Metabolites 1	2
	NADPH	nmol/hr/g liver		
2000xg supernatant	0	3.91	0.53	0.20
	+	3.11	3.38	1.66
Mitochondria (10,000xg pellet)	0	1.99	0.07	0.07
	+	2.19	0.73	0.33
Microsomes (100,000xg pellet)	0	4.51	0.20	0.13
	+	2.23	2.93	1.41
100,000xg supernatnat	0	4.13	0.08	0.10
		nmol/hr/0.08ml serum		
Serum	0	0.61	0.01	0.04

[a]data from Melancon and Lech (14)

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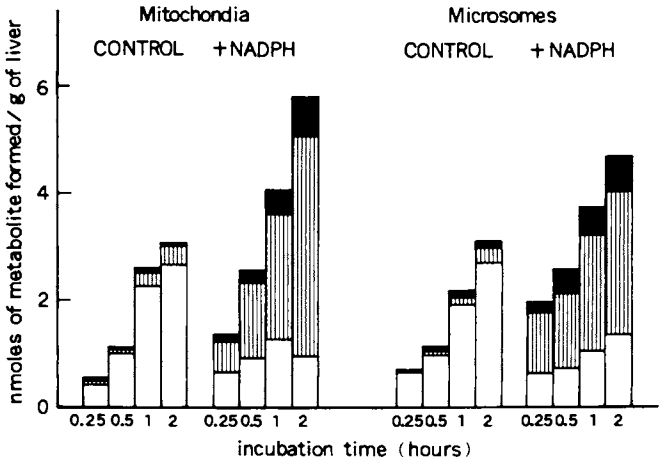

Figure 1. Influence of time on metabolism of ^{14}C-DEHP by trout liver mitochondrial and microsomal fractions. Incubation contained 0.010 μmol of ^{14}C-DEPH in a total volume of 2 mL. Mitochondria equivalent to 0.254 g of liver or microsomes equivalent to 0.361 g of liver were used in each incubation. Open bars represent monoester, striped bars Polar Metabolite I and solid bars Polar Metabolite 2. Each column represents an individual incubation (14).

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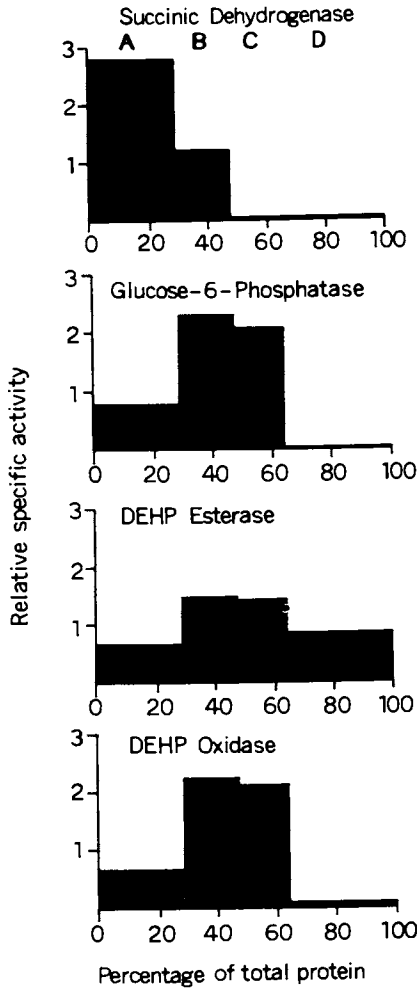

Figure 2. Distribution of marker enzymes and DEHP-metabolizing enzymes in trout liver homogenate fractions. DEHP esterase and DEHP oxidase were each measured by 1-hr incubations of 0.010 μmol of ^{14}C-DEHP in a total volume of 2 mL. Fraction (A), 2,000 g pellet; (B), 10,000 g pellet; (C), 100,000 g pellet; and (D), 100,000 g supernatant. Relative Specific Activity = percent of total activity/ percent of total protein (14).

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TABLE VII

Effect of Piperonyl Butoxide on Metabolism of ^{14}C-di-2-ethylhexyl Phthalate by Rainbow Trout Liver Preparations and Blood Serum[a]

	NADPH (2mM)	Piperonyl butoxide	Metabolites formed: Mono-2-ethylhexyl Phthalate	Polar Metabolites 1	Polar Metabolites 2
			nmol/hr/g liver		
2000xg supernatant	0	0	7.51 ± 0.82	0.22 ± 0.13	0.14 ± 0.02
	0	90μM	1.05 ± 0.18[b]	0.29 ± 0.18	0.06 ± 0.01[b]
	+	0	1.14 ± 0.54	11.29 ± 0.93	1.41 ± 0.46
	+	90μM	1.39 ± 0.37	0.64 ± 0.33[b]	0.08 ± 0.01[b]
Microsomes	0	0	3.93 ± 0.62	0.08 ± 0.05	0.11 ± 0.01
	0	2mM	0.20 ± 0.08[b]	0.04 ± 0.02	0.03 ± 0.02[b]
	+	0	3.10 ± 0.47	4.07 ± 0.64	1.03 ± 0.20
	+	2mM	0.12 ± 0.01[b]	0.11 ± 0.08[b]	0.04 ± 0.02[b]
			nmol/hr/ml serum		
Blood serum	0	0	15.96 ± 0.96		
	0	90μM	1.08 ± 0.12[b]		

[a]data from Melancon, et al. (15).
[b]significantly different from control (no piperonyl butoxide added), $P<0.05$.

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to inhibit hydrolytic activity in liver cytosol and in serum.

Because the metabolism of DEHP was relatively slow, the more readily hydrolyzed 2,4-dichlorophenoxyacetic acid-n-butyl ester was sometimes used for comparison. The hydrolysis of this compound, both by liver preparations and by serum also was inhibited by piperonyl butoxide. Liver homogenates from trout, which had been exposed to piperonyl butoxide *in vivo*, showed decreased capacity to metabolize DEHP (Table VIII).

In Vivo exposure of trout to piperonyl butoxide also affected the disposition and metabolism of ^{14}C-DEHP. The results in Table IX show that piperonyl butoxide reduced biliary ^{14}C but increased ^{14}C in muscle and blood. Because the bile contains mostly DEHP metabolites, this represents decreased metabolism. When the ^{14}C from muscle was examined by TLC, it was found that control muscle contained about equal amounts of DEHP and monoester. Muscle from piperonyl butoxide-treated trout contained about the same amount of MEHP as controls, but 3 times the amount of DEHP.

In additional *in vitro* studies with a number of methylenedioxyphenyl compounds, only tropital, in addition to piperonyl butoxide, had similar inhibitory effects on the metabolism of DEHP by trout liver homogenates and serum (16). Of the methylenedioxyphenyl compounds studied, only tropital had a long side chain like that of piperonyl butoxide. This suggests that similarities in the side chains of these two compounds and the side chains of DEHP and 2,4-dichlorophenoxyacetic acid-n-butyl ester may be responsible for this inhibition.

It was also found that paraoxon, an esterase inhibitor, substantially reduced formation of polar metabolite 1 from DEHP by trout liver microsomes with added NADPH. This suggests that polar metabolite 1 is formed via further metabolism of the monoester, the production of which was reduced by paraoxon.

Results of DEHP metabolism studies *in vivo* and *in vitro*, when considered together, suggest that the major route of metabolism for DEHP in fish consists of hydrolysis to the monoester. The monoester may then be conjugated with glucuronic acid, or further hydrolyzed to phthalic acid, or the remaining sidechain may be oxidized. This is similar to the reported pathway for DEHP metabolism in rats. In rats, dietary DEHP is to a large extent hydrolyzed to the monoester before absorption from the gut. When the monoester is administered orally, the urinary metabolites found are the same as those found after administration of DEHP (17).

Other Studies of the Metabolism of Phthalate Esters by Aquatic Species

Little has been done to study phthalate ester metabolism in other individual species. Mosquito larvae and salt marsh caterpillar larvae, however, were each shown to transform dioctyl

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TABLE VIII

Metabolism of ^{14}C-di-2-ethylhexyl phthalate by Liver Homogenates from Control Trout and Trout Exposed to Piperonyl Butoxide (1mg/1)[a]

	NADPH (2mM)	Metabolites formed: Mono-2-ethylhexyl phthalate	Polar Metabolites 1	Polar Metabolites 2
		nmol/hr/g liver		
Control	0	9.16 ± 1.58	0.72 ± 0.50	0.11 ± 0.05
	+	3.15 ± 0.63	7.68 ± 0.57	1.57 ± 0.61
Piperonyl butoxide	0	3.95 ± 0.21[b]	0.15 ± 0.09	0.06 ± 0.01
	+	2.35 ± 0.31	2.63 ± 1.28[b]	0.15 ± 0.04

[a]data from Melancon, et al. (15).
[b]significantly different from control, P<0.02.

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TABLE IX

Effect of Piperonyl Butoxide (1mg/l) on Tissue Levels on ^{14}C from ^{14}C-di-2-ethylhexyl Phthalate in Rainbow Trout *in vivo*

	Muscle	Blood	Liver	Bile
	Total Tissue ^{14}C expressed as µg DEHP/g or ml			
Control	0.021 ± 0.003	0.142 ± 0.017	0.86 ± 0.08	51.4 ± 5.5
Piperonyl butoxide	0.041[b] ± 0.006	0.234[b] ± 0.019	1.08 ± 0.15	26.2[b] ± 2.8

[a]data from Melancon, *et al.* (15).
[b]significantly different from control, $P<0.01$.

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phthalate to the monoester and some more polar metabolites (13). Piperonyl butoxide and tri-o-cresyl phosphate each affected the pattern of metabolites produced by these species. In the case of caterpillar larvae, only piperonyl butoxide appeared to reduce the metabolism of dioctyl phthalate, while in mosquito larvae, both inhibitors led to increased levels of dioctyl phthalate.

Metcalf and coworkers have studied the fate of DEHP and dioctyl phthalate in model ecosystems (13,18). These small ecosystems contained a number of food chain organisms up to small tropical fish. In the study with DEHP, substantial amounts of metabolites were found in guppy, but little, if any, were found in the lower organisms. With dioctyl phthalate, however, degradation products were found in algae, daphnia, mosquito larvae and snails, in addition to mosquito fish. While ecosystem studies such as this can indicate whether or not a chemical is likely to be degraded in the environment, it is not possible to conclude that the metabolites of a particular chemical which are found in the various organisms, were produced by those organisms.

Summary

1. Phthalate esters are metabolized by aquatic microorganisms, several aquatic invertebrates and several species of fish.
2. Short chain phthalate esters such as bibutyl phthalate are more rapidly metabolized than long chain phthalate esters such as DEHP, both in aquatic microorganisms and fish.
3. Hydrolysis of phthalate diesters to the respective monoesters appears to be the first and the major biotransformation reaction in all of these species, but subsequent oxidative metabolism also may occur.
4. Fish liver microsomes are capable of both hydrolytic and oxidative metabolism of phthalate esters. In addition, trout liver cytosol and blood serum exhibited esterase activity against DEHP.
5. A number of metabolic inhibitors have been shown to modify the metabolism and disposition of phthalate esters in several aquatic species.

Acknowledgements

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6

Metabolism of the Thiocarbamate Herbicide Molinate (Ordram) in Japanese Carp

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Molinate (S-ethyl hexahydro-azepine-1-carbothioate) has been widely used for broadleaf and grass weed control in rice culture. The 96-hour LC_{50} of technical molinate in the common goldfish is 30 ppm (1) and the medium tolerance limits (LTm) for molinate in mosquito fish (Gambusia affinis) are 16.4 ppm for 96 hours (2). Kawatsu (3) estimated the 20-day lethal concentration of molinate in Japanese carp (Cyprinus carpio) var. Yamato koi at 0.18 ppm. However, there was no toxic effect of molinate on American carp at concentrations of 10 ppm during a two-week observation (4).

Molinate has a low toxicity to rats, oral LD_{50}=720 mg/kg, and is rapidly metabolized by plants to CO_2 (1) (5) and naturally occurring plant constituents (1). Molinate is also readily metabolized by soil microorganisms (6). After incubation of molinate with Bacillus sp. 24, Nocardia sp. 119, and Micrococcus sp. 22r which were isolated from Russian garden soils and rice field drains (7,8), it was found that molinate was completely degraded into various hydroxy and oxidized products in the medium. Molinate can be metabolized to its corresponding sulfoxide in the mouse in vivo and by the microsome-NADPH system of mouse liver (9, 10). Hubbell et al. (11) and DeBaun et al. (12) also found molinate sulfoxide along with other polar and nonpolar metabolites in rat urine.

The purpose of this study was to investigate the extent of accumulation and the fate of molinate in the Japanese carp.

Material and Methods

1. Chemicals. [Ring-^{14}C]molinate, S-ethyl hexahydro-[2-^{14}C]azepine-1-carbothioate (8.2 mCi/mM); 3-hydroxy, 4-hydroxy, 4-keto, and 2-keto molinate; and S-carboxymethyl hexahydro-azepine-1-carbothioate (carboxy molinate) were provided by Stauffer Chemical

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Co., Richmond, Calif. Hexamethyleneimine (HMI) was purchased from Matheson Coleman & Bell Co. S-(Hexahydro-[2-^{14}C]azepine-1-carbonyl)-N-acetyl-L-cysteine (molinate mercapturic acid) was provided by DeBaun et al. (12). Caprolactam was purchased from J.T. Baker Chemical Co. Molinate sulfoxide was prepared by reacting [ring-^{14}C]molinate with equimolar m-chloroperbenzoic acid in chloroform (10). The product was purified by preparative thin-layer chromatography (TLC) using acetone:hexane (1:1) as the developing solvent. The final radiopurity was 98%.

2. Treatment of Fish. Japanese carp (Cyprinus carpio) variety Yamato koi imported from Nagano, Japan, were fed carp food No. 3 (Yashima Chemical Co., Japan) containing 30% crude protein, 10% fat and 12% ash. The fish were acclimated for 30 days in the laboratory at 21±2°C.

Fish with an average weight of 17.6 g and a length of 24 cm were used for the studies. Four fish in a 76-liter glass tank were treated with [ring-^{14}C]molinate in 5 ml ethanol at a concentration of 0.2 ppm, and one fish was removed for analysis after 1, 4, 7, and 14 days. The [^{14}C]molinate at 0.2 ppm was also added to fish water (water which no longer contained fish but which did contain fish 14 days previously) and fresh tap water. To reduce the loss of molinate by evaporation, the fish tanks were partially covered with glass plates. Temperature was maintained at 21±2°C throughout the experimental period. Air was supplied to all tanks by diffusers immersed in the water.

3. Isolation of Organosoluble (Nonpolar) Metabolites. Water was sampled at given intervals and thoroughly extracted with equal volumes of chloroform. The chloroform extract was concentrated under nitrogen at 25°C and then developed by TLC. Several metabolites were extracted from the gel with acetone for gas chromatographic-mass spectral characterization.

4. Isolation of Water-Soluble (Polar) Metabolites.

a. Water. The aqueous portion after chloroform extraction was lyophilized, and the dry residue was resuspended in 5 ml methanol. The methanol fraction was diluted with 3 volumes of distilled water for gel filtration through a column of BioGel P-2, 100-200 mesh (Bio-Rad Lab., Richmond, California) 76 cm x 2.5 cm. Metabolites were eluted with distilled water and the broad radioactive peak was lyophilized and resuspended in methanol. The methanol fraction was diluted as before and the pH was adjusted to 1.5 with HCl after which it was extracted with equal volumes of methylene chloride. The remaining aqueous fraction was then adjusted to pH 10 with NaOH for basic extraction as before. Finally, the pH of the aqueous residue was readjusted to pH 7 for lyophilization and the lyophilized sample was reconstituted in methanol for TLC characterization.

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b. Bile. Bile was collected from the gallbladder and diluted with four volumes of water. Following lyophilization, the metabolites were recovered in methanol. In order to remove the bile salts, the methanol-soluble portion was diluted with water and passed through the BioGel P-2 column for gel filtration as described above.

5. Chromatographic Analysis. Unless otherwise stated, thin-layer chromatography (TLC) utilized pre-coated silica gel GF chromatoplates 0.25 cm (Analtech Inc.) which were developed with the following solvent systems:

a. 2,2,4-Trimethylpentane:p-dioxane (2:1)
b. Benzene:ether (2:3)
c. Toluene:ether (2:3)
d. Hexane:ether (1:1)
e. Benzene:ether (7:3)
f. Hexane:ether (9:1)
g. Toluene:ether (7:3)
h. Ethylacetate:formic acid (30:1)
i. 2-Butanone:water:pyridine:acetic acid (55:30:15:2)
j. 1-Butanol:ethanol:water (4:1:1)
k. 1-Butanol:acetic acid:water (15:8:3)

6. Mass-spectral (MS) Analysis. Mass spectral analyses were obtained using a computerized Finnigan Model 1015 gas chromatograph-mass spectrometer (GC-MS) operated at 70 eV. Samples were introduced via direct insertion probe or by utilizing the GC-MS combination, both operated over a programmed temperature range.

7. Radioanalysis. Radiolabeled regions on TLC plates were visualized by autoradiography, while unlabeled standards were detected by uv fluorescent quenching. Percent distribution of labeled metabolites was determined by scraping silica gel regions from the glass support and direct liquid scintillation counting (LSC). Flesh (muscle and skin) samples were homogenized, and an aliquot of the homogenate was then combusted directly for determination of ^{14}C residues in a Packard Sample Oxidizer, Model 306. Blood and all other organs (sectioned or whole) were similarly analyzed by combustion radioanalysis. Following combustion, samples were then analyzed by LSC. Combustion and counting efficiencies were calibrated prior to radioanalysis using [^{14}C]4-chlorobenzenesulfonic acid and [^{14}C]toluene as internal standards.

8. Fish Liver Microsomal Preparation and Assay. Fresh fish liver was homogenized with a teflon-glass homogenizer in sodium phosphate buffer pH 7.4 at 4°C. The liver homogenate was centrifuged at 10,000 x G for 15 minutes and the supernatant was passed through glass wool to remove lipids. For microsomal isolation, the supernatant was centrifuged at 130,000 x G for five minutes using an Airfuge centrifuge (Beckman). The pellets were resuspended in buffer solution. Microsomal protein concentration was

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determined according to Lowry et al. (13). Unless otherwise stated, the standard incubation medium contained: microsomal protein 6 mg/ml, 3 mg NADPH (3.6 μmoles), and molinate (1 μmole), in a 20-ml flask. The flasks were incubated for at least 15 minutes at 15-30°C depending on specific assay conditions. After incubation, samples were extracted with equal volumes of chloroform (3X) for TLC analysis and metabolite characterization. Water-soluble metabolites formed under these conditions were not characterized.

Results and Discussion

1. Molinate Bioaccumulation and Biotransformation in Carp. Table I shows the change of radiocarbon concentration in the water during the 14 days following addition of [ring-^{14}C]molinate. Radiocarbon gradually decreased over the 14-day holding period. The accountability of radiocarbon was 40%, 50%, and 61.5% for tap water, fish water (previously contained fish), and water containing fish, respectively. The loss of radiocarbon was attributed primarily to volatilization (14). Water quality appears to influence the volatilization losses from the water surface. It is likely that the adsorption of molinate on particulate organic matter retarded its rate of volatilization from water previously exposed to fish and from that containing fish. Biodegradation of molinate to less volatile metabolites is probably also a factor involved in the persistence of ^{14}C.

The proportion of molinate in the organosoluble ^{14}C after 14 days, was in decreasing order: 95.5% for tap water, 80.8% for water previously containing fish, and 3.8% for water containing fish. The oxidized products from tap water and water previously containing fish constituted an average of 12.0% of the extractable radiocarbon. The remaining 88% was molinate. However, the proportion of oxidation products formed in water containing fish from 1 to 14 days ranged from 35.6% to 96.5% with an average of 60.3% of the extractable radiocarbon.

The five major organosoluble metabolites in the water were molinate sulfoxide, 3- and 4-hydroxy molinate, 4-keto molinate, and keto hexamethyleneimine (keto HMI). The amount of each metabolite in tap water remained fairly constant (below 2%) throughout the 14 days. In water previously containing fish, molinate sulfoxide was 2.1% 4 days after treatment, while other metabolites remained below 2% for the first 7 days. By the 14th day, however, molinate sulfoxide totaled 12.8% and 4-hydroxy and 3-hydroxy molinate were 2.2 and 3.7%, respectively.

In the presence of carp, molinate was rapidly transformed into more polar metabolites. Twenty-four hours after treatment, conversion of molinate to organosoluble metabolites was extensive (Table II). The proportion of molinate sulfoxide increased from 1.2% on day 1 to 10.2% by the 14th day. Molinate, hydroxylated at the 4 position, decreased from 10.8% to 4.6% in 14 days. However, 4-keto and 3-hydroxy molinate together gradually increased over the 14 days from 6.9% to 77.7%. Another metabolite, a keto HMI,

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Table I. The Accountability of Total Radiocarbon in Water at Various Intervals after the Addition of [Ring-^{14}C]-Molinate

Days After Treatment	Tap Water		% Organosoluble	Fish Water[a]		% Organosoluble	Water Containing Fish		% Organosoluble
	%	PPM		%	PPM		%	PPM	
0	100	0.2	-	100	0.2	-	100	0.2	-
1	80.5	0.161	95.6	86.5	0.173	94.8	94.7	0.189	92.5
4	57.5	0.115	97.4	69.5	0.139	94.2	85.9	0.172	81.5
7	45.5	0.09	95.6	54.5	0.109	96.3	74.8	0.150	72.9
14	40.0	0.08	87.5	50.0	0.1	81.0	61.5	0.123	69.8

a Water which no longer contained fish but which had contained fish for 14 days prior to the addition of the [ring-^{14}C]molinate.

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Table II. Distribution of ^{14}C in Chloroform Extracts of Water Treated with [Ring-^{14}C]-Molinate and Containing Carp

TLC Rf	Identity[a]	Exposure Intervals			
		1 Day	4 Days	7 Days	14 Days
0.26	Molinate sulfoxide[b]	1.2%	2.5%	3.7%	10.2%
0.43	4-Hydroxy molinate[c]	10.8	8.8	0.7	4.6
0.54	4-Keto molinate plus 3-hydroxy molinate[d]	6.9	21.0	58.9	77.7
0.67	Keto hexamethyleneimine[f]	16.8	9.6	4.0	3.6
0.84	Molinate[g]	64.4	58.1	32.7	3.8

a Five major radioactive areas were revealed by TLC with system (a).

b, c, g All were confirmed by TLC with authentic standards with systems (f) and (c).

d After two-dimensional development with solvent systems (a) and (b), it was confirmed that both 3-hydroxy molinate and 4-keto molinate were major metabolites in this area.

f Structure determined from GC-MS, exact position of keto function was not established.

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decreased from 16.8% to 3.6% by the 14th day, and molinate gradually decreased from 64.4% on the first day to 3.8% on the final day.

Figure 1 shows the rapid disappearance of molinate in water containing carp. The data indicate that the fish are largely responsible for molinate biotransformation and/or biodegradation. The relatively small amounts of oxidation products formed in tap water are thought to be formed by direct air oxidation. Metabolism by microorganisms may also contribute to the conversion in the water which previously contained fish.

Figure 2 shows the amount of water-soluble metabolites formed under the three treatment conditions. It is evident that molinate was readily metabolized by carp, and that the amount of conjugated or water-soluble metabolites increased with length of treatment. Water-soluble metabolites from both tap water and water previously containing fish remained constant (below 6%) for the first 7 days. By the 14th day, they rose to 12.5% and 19.0%, respectively. However, water-soluble metabolites increased to 30.2% by the end of the 14th day in the water which contained fish. The data suggest that molinate degrades in water even without carp, but that molinate degradation and/or biotransformation is facilitated by living fish.

Table III summarizes the results of tissue residue analysis. It is evident that the amount of radioactivity in tissues was not directly related to the length of chemical exposure. The average accumulation in fish exposed from 1 to 14 days was 1.35%. In general, liver, kidney, intestine, and bile contained the most ^{14}C. ^{14}C-labeled materials accumulated in the liver at levels 3 to 5 times greater than [^{14}C]molinate concentration in the water. The maximum radiocarbon level in the bile was 14.5 ppm and was reached by the 7th day. On the 14th day, the radiocarbon decreased to 6.09 ppm which was 30-fold higher than the [^{14}C]molinate water concentration. Blood contained negligible amounts of radioactivity, and little of that was associated with the plasma. Twenty percent of total blood radioactivity was detected in the erythrocytes within 4 days after treatment; and by the 14th day, 69% of the radiocarbon in whole blood was present in the erythrocytes. The flesh remaining after removal of the organs listed in Table III also contained very low ^{14}C residues. In general, accumulation of molinate in carp was extremely low as compared with DDT which has been reported to have a fish bioaccumulation factor of 10^6 (15). The level of methoprene in bluegill is also about 10^3 times that in water (16). TLC of liver extracts showed some organosoluble metabolites regardless of the length of exposure to molinate. TLC analysis of bile revealed several minor polar metabolites but no parent compound.

2. Molinate Metabolism in Carp Hepatic Mixed-function Oxidase System. Incubation of molinate with carp liver microsomes produced four major organosoluble metabolites (molinate sulfoxide, 3- and 4-hydroxy molinate, and keto HMI). Parameters affecting

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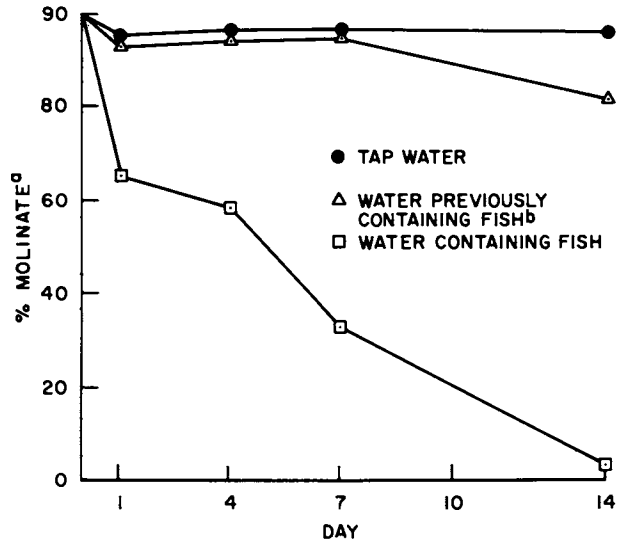

Figure 1. Molinate concentration in the chloroform extract of water with and without carp: (A) expressed as percent ^{14}C molinate in the chloroform extract at each interval; (B) water contained fish 14 days prior to addition of molinate. The fish were removed at the time of addition of the molinate.

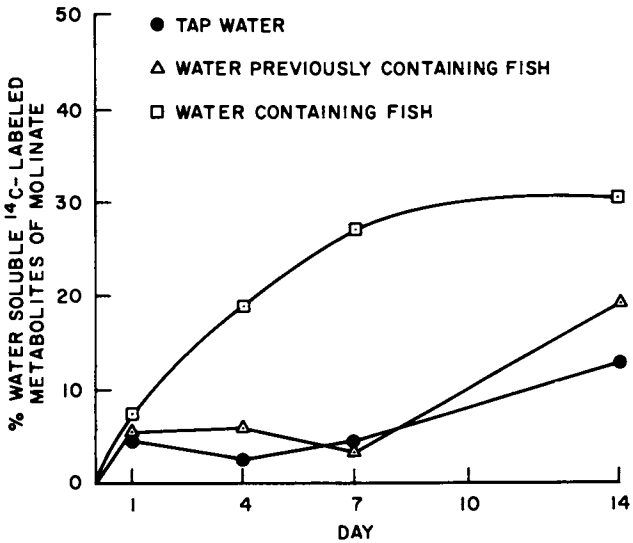

Figure 2. Percent water-soluble ^{14}C-labeled metabolites of molinate in the water with and without carp (water-soluble metabolites are defined as those which did not extract with chloroform under the conditions described in Materials and Methods*)*

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Table III. Residues of Radiocarbon in Tissue of Japanese Carp Exposed to Water[a] Containing 0.2 ppm [Ring-^{14}C]-Molinate

	Exposure Interval							
	1 Day		4 Days		7 Days		14 Days	
Tissue	%[b]	ppm[c]	%[b]	ppm[c]	%[b]	ppm[c]	%[b]	ppm[c]
Liver	0.11	0.79	0.08	0.97	0.05	0.68	0.11	0.72
Kidney	0.03	0.51	0.02	0.50	0.008	0.20	0.009	0.24
Intestine	0.25	0.63	0.24	0.70	0.13	0.42	0.13	0.35
Swimming Bladder	0.02	0.18	0.01	0.09	0.008	0.05	0.004	0.038
Gill	0.06	0.10	0.09	0.15	0.02	0.05	0.04	0.08
Spleen	0.01	0.33	0.008	0.37	0.007	0.30	0.007	0.27
Brain	0.02	0.36	0.009	0.19	0.007	0.17	0.005	0.14
Heart	0.004	0.24	0.003	0.14	0.004	0.20	0.001	0.08
Testes	0.14	0.13	-	-	-	-	-	-
Ovary	-	-	0.006	0.11	0.03	0.12	0.03	0.18
Bile (0.3-0.6 ml)	0.63	7.07	0.63	7.08	1.29	14.58	0.54	6.09
Whole Blood[d]	0.05	0.14	0.02	0.05	0.05	0.13	0.04	0.12
Scales	0.09	0.18	0.09	0.18	0.08	0.18	0.09	0.13
Flesh	0.04	0.23	0.017	0.09	0.023	0.16	0.021	0.11
Total Accumulation:	1.454		1.223		1.707		1.027	

a 21°C ± 2°C water temperature.

b Expressed as total ^{14}C in whole tissue ÷ total ^{14}C added to the water x 100.

c Expressed as molinate equivalents µg/g tissue.

d Computed from 4-ml sample.

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the microsomal mixed-function oxidase (mfo) activity of carp liver were evaluated by determining the amount of sulfoxide formed in the assay.

a. Effect of Temperature of mfo Activity. Figure 3 shows that formation of molinate sulfoxide was optimal at temperatures between 25-30°C, indicating that microsomal mfo enzymes were more active at these higher temperatures. The four major metabolites were found at each temperature, but the amount of total metabolites at higher temperatures was 2- to 3-fold higher than that at low temperatures.

b. Effect of Liver Microsomal Protein Concentration on mfo Activity. The amount of molinate sulfoxide produced was proportional to the microsomal protein concentration when liver microsomes were incubated at optimum NADPH concentration, temperature and pH (Figure 4). The total metabolites were also found to be proportional to the concentration of microsomal protein in the incubation mixture. There were no detectable metabolites produced when microsomes were absent. This indicates that metabolite formation during the incubation period was due mainly to the presence of liver microsomal enzymes.

c. Time-course Evaluation of Sulfoxidation of Molinate by Carp Liver Microsomes. Molinate sulfoxide was detected one minute after the start of incubation and the amount of the sulfoxide increased for up to 30 minutes incubation (Figure 5). In order to generate metabolites, the assay was carried out up to one hour in most experiments. Three major metabolites (molinate sulfoxide, 3- and 4-hydroxy molinate) were formed after incubation for 3 minutes.

3. Quantitation and Identification of ^{14}C-labeled Metabolites. In the *in vitro* microsomal mixed-function oxidase system, 4 major metabolites (72%) and 9 other minor metabolites were detected by TLC of the organosoluble portion. Figure 6 shows a TLC chromatographic pattern and metabolite distribution for carp liver microsomal ^{14}C-labeled metabolites of [ring-^{14}C]molinate. The 4 major metabolites, which included molinate sulfoxide, 3-hydroxy molinate (m-3), 4-hydroxy molinate (m-2) and keto HMI (m-4) were resolved by the two-dimensional solvent systems (a) and (b). Molinate sulfoxide was cochromatographed with authentic standards in three solvent systems, (a), (d), and (e). Molinate, hydroxylated at the 3- and 4-positions, was confirmed by cochromatography with authentic compounds in addition to GC-MS (Figures 7 and 8). The structure of the keto group can be either at the 3- or the 4-position since it did not cochromatograph with authentic caprolactam. The chromatographic pattern of the microsomal generated metabolites is similar to those metabolites generated by the living carp (Figure 10).

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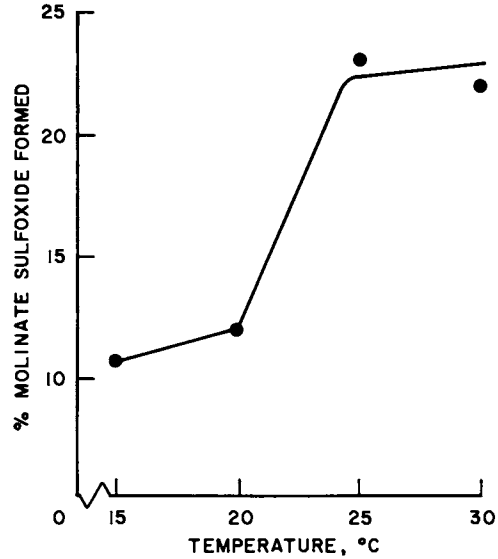

Figure 3. Effect of temperature on conversion of molinate to its sulfoxide by carp liver microsomal mfo (liver microsomal protein 6 mg/mL, pH 7.4, NADPH 3 mg incubated for 15 min)

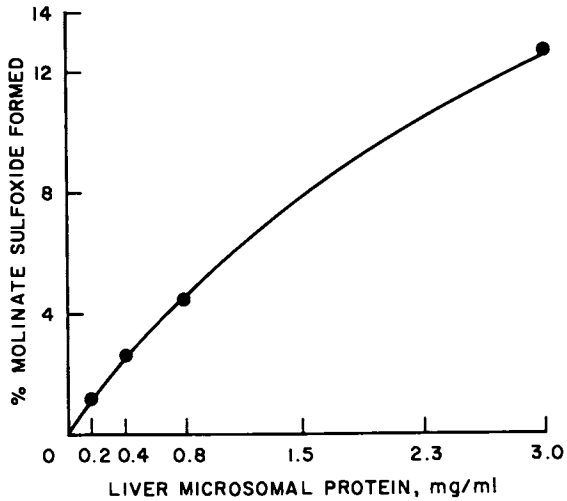

Figure 4. Effect of carp liver microsomal protein concentration on its mfo activity (NADPH 3 mg/mL, pH 7.4 incubated for 15 min at 25°C)

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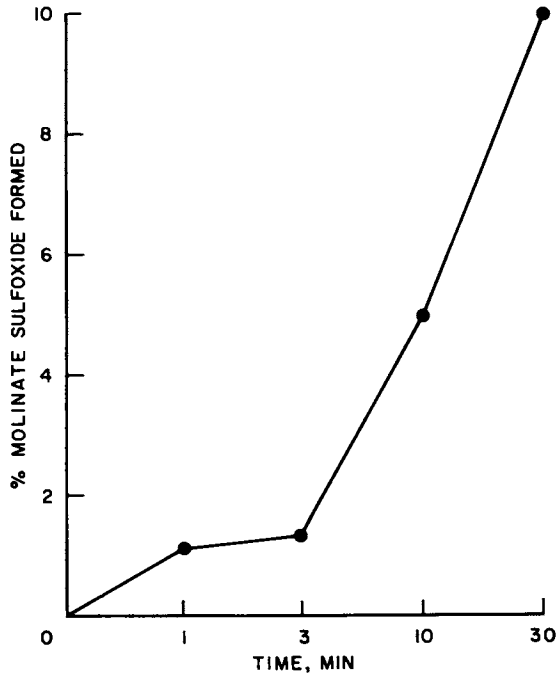

Figure 5. Effect of incubation time on the carp liver microsomal mfo activity (NADPH 2 mg/mL, pH 7.4 incubated at 25°C. Microsomal protein concentration was 1.5 mg/mL.)

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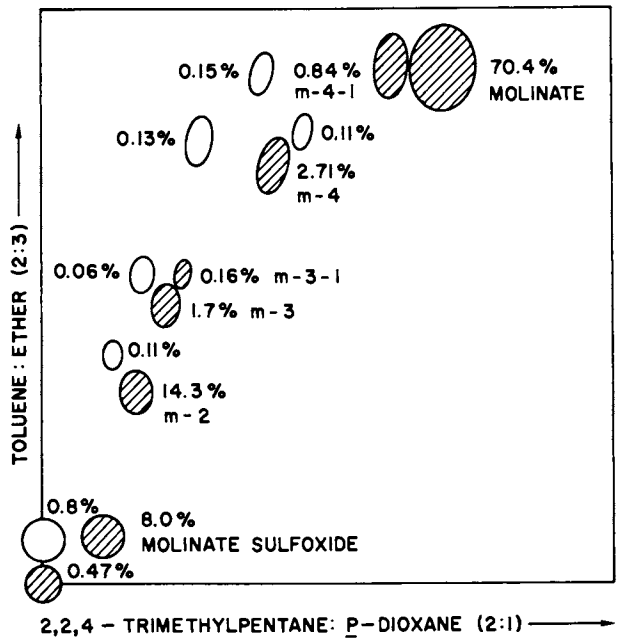

Figure 6. Chromatographic pattern for carp liver microsomal ^{14}C-labeled organosoluble metabolites of [ring-^{14}C]-molinate. Incubation was conducted under standard conditions for one hr; m number represents metabolite code.

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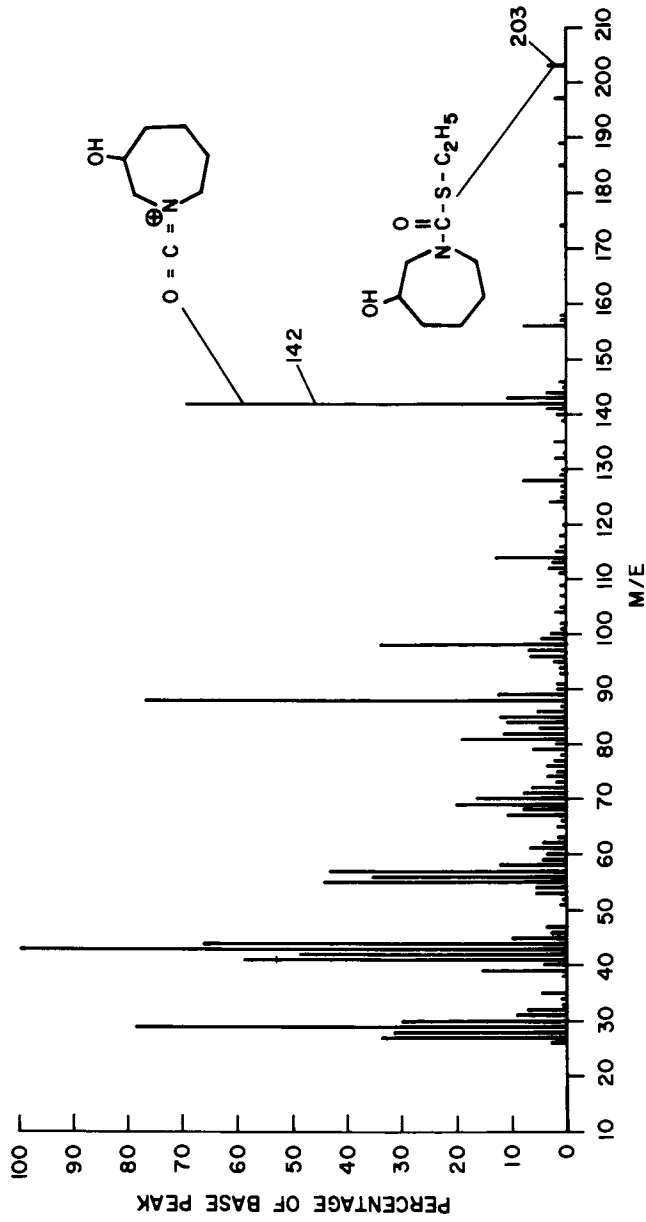

Figure 7. MS of the metabolite 3-hydroxy molinate (GC Conditions: OV 17, 10% on Gas Chrom Q (4'); helium flow rate, 30 mL/min; temperature, 150°C; retention time, 10.0 min)

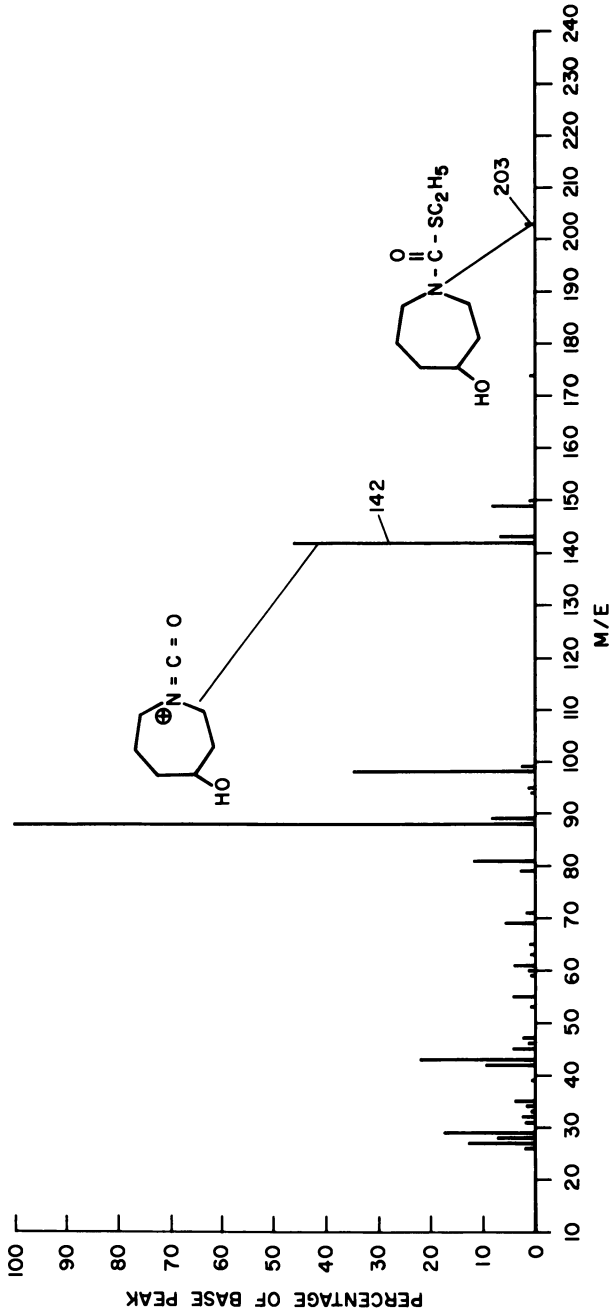

Figure 8. MS of the metabolite 4-hydroxy molinate (GC Conditions: OV 17, 10% on Gas Chrom Q (4'); helium flow rate, 30 mL/min; temperature, 150°C; retention time, 10.0 min)

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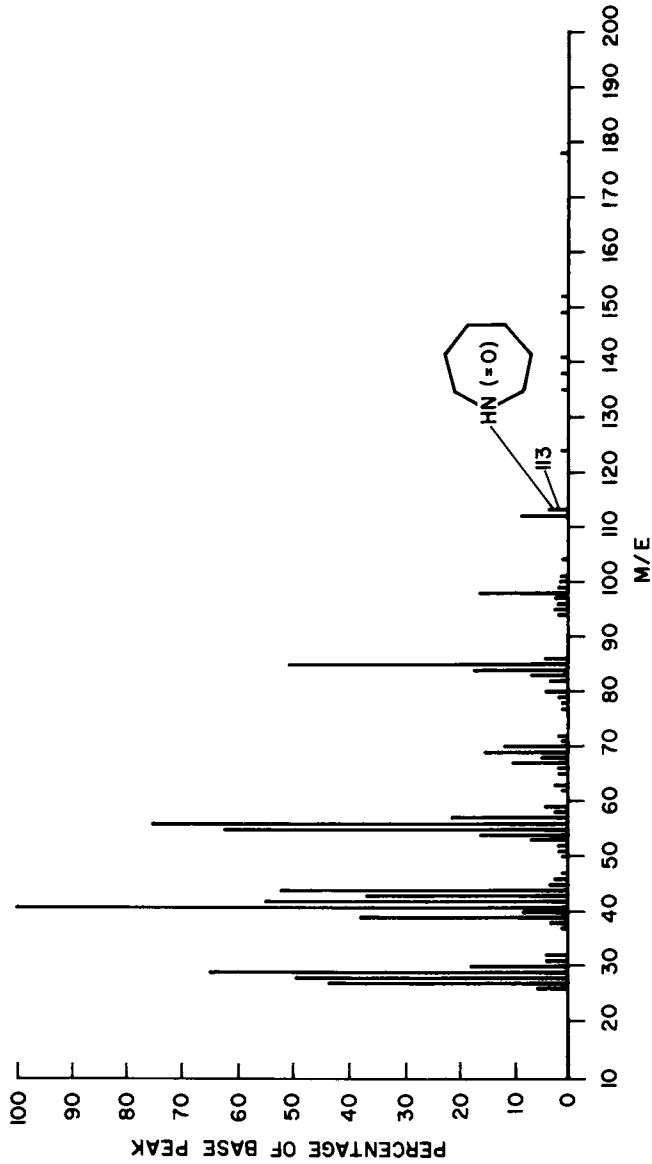

Figure 9. MS of the metabolite keto hexamethyleneimine (GC Conditions: OV 17, 10% on Gas Chrom Q (4'); helium flow rate, 30 mL/min; temperature, 150°C; retention time, 5.1 min)

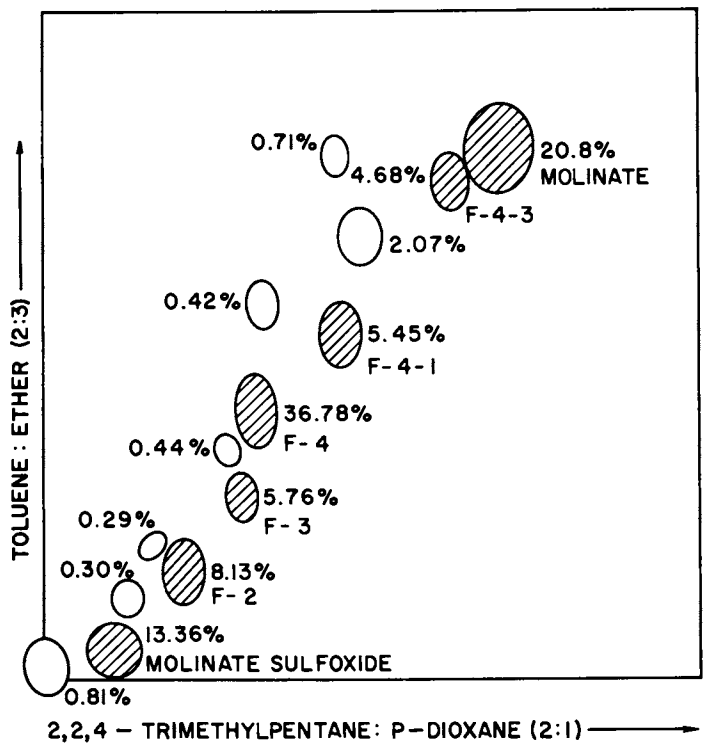

Figure 10. Chromatographic pattern for organosoluble ^{14}C-labeled metabolites from [ring-^{14}C]-molinate-treated water which contained carp for 4 days. F number represents metabolite code.

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In the in vivo system, 13 metabolites were detected, 7 of which were major metabolites. Two-hydroxy molinate (F-4-3) was separated from molinate by programmed multiple development using solvent system (f). The structure was confirmed by GC-MS (Figure 11). This metabolite was not stable and was readily transformed into the stable S-ethyl-5-formylpentylthiocarbamate (12). The sulfoxide and 3- and 4-hydroxy molinate (F-3 and F-2) were readily recovered from the water. Four-keto molinate (F-4) was also confirmed by cochromatography with the authentic compound and by GC-MS (Figure 12). It was found in substantial amounts in the water 4 days after treatment. The amount of 4-hydroxy molinate gradually decreased as the length of exposure increased. In contrast, 4-keto molinate in the water increased over a period of 1 to 14 days, indicating that 4-hydroxy molinate was readily converted to 4-keto molinate.

Organoextraction of the intestine and liver also revealed several metabolites using system (b). However, no unchanged molinate was detected.

In water containing fish, 4-keto molinate constituted 36.8% of all organosoluble metabolites 4 days after molinate addition. However, in the in vitro microsomal mixed-function oxidase system 4-hydroxy molinate was a principal metabolite (14.3%) and 4-keto molinate represented only 0.16% of the total metabolites. Keto-HMI (2.71%) also constituted a substantial proportion of the identified metabolites.

The bile contained the greatest amount of ^{14}C-labeled metabolites (Table III), indicating that bile is an important reservoir and route of excretion for metabolites in fish (17). No [^{14}C]-molinate was detected in bile; however, several polar metabolites were present. Six metabolites were revealed by TLC using solvent systems (g), (h), and (i). HMI constituted 3.16% of the total bile radiocarbon based on TLC cochromatography with an authentic standard in the same solvent system.

The methylene chloride extract of the acidified 14-day aqueous phase, which had previously been extracted at neutrality, accounted for 41% of the total aqueous ^{14}C and contained 11 products as shown by TLC using solvent systems (a) and (b). One of the major metabolites in this extract was identified as carboxy molinate. It comprised 78.5% of the ^{14}C extracted from the acidified aqueous phase. This product was confirmed both by GC-MS (Figure 13) and TLC cochromatography with an authentic standard using solvents (g), Rf=0.14 and (h), Rf=0.67. Incubation of molinate with Micrococcus sp. 22r and Nocardia sp. 119 (7, 8) and treated soil (18) also produced carboxy molinate indicating that both fish and soil microorganisms are capable of oxidizing the molinate to form this polar product via β-oxidation of the S-alkyl moiety (19). The other metabolite in this extract was probably a mercapturic acid derivative. This metabolite, which accounted for 3.8% of the extract, cochromatographs with an authentic standard in solvent systems (g) and (h). However, insufficient material was available for absolute structural confirmation by means of

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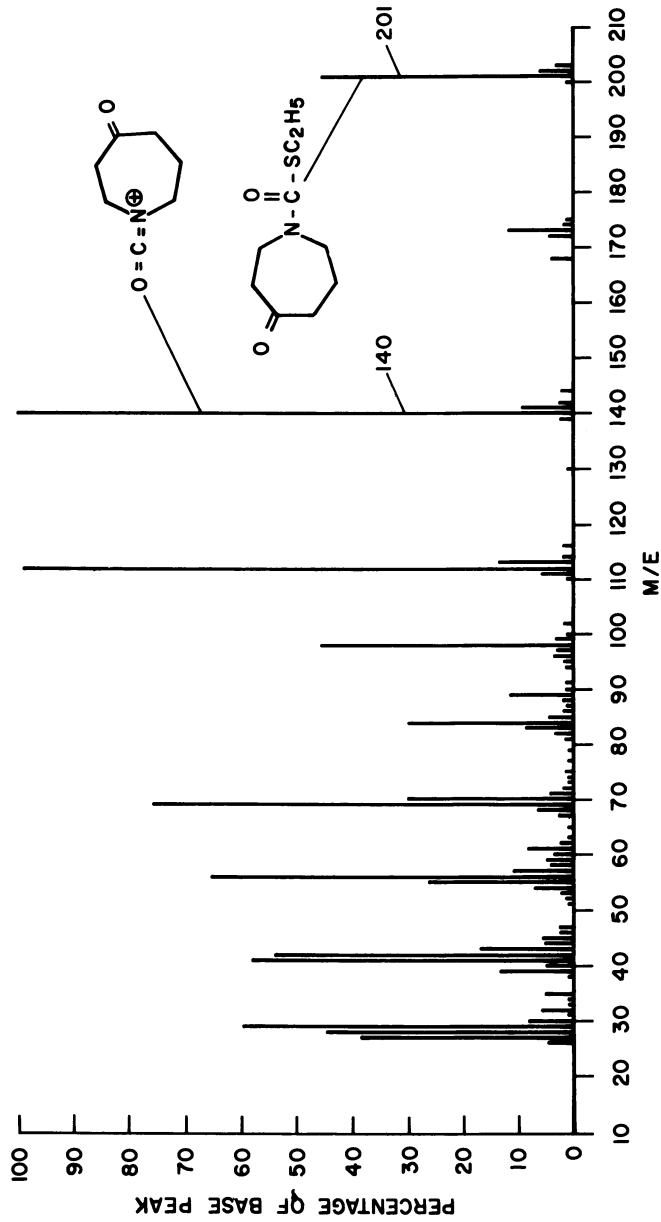

Figure 11. MS of the metabolite 2-hydroxy molinate (GC Conditions: OV 17, 10% on Gas Chrom Q (4'); helium flow rate, 30 mL/min; temperature, 150°C; retention time, 4 min)

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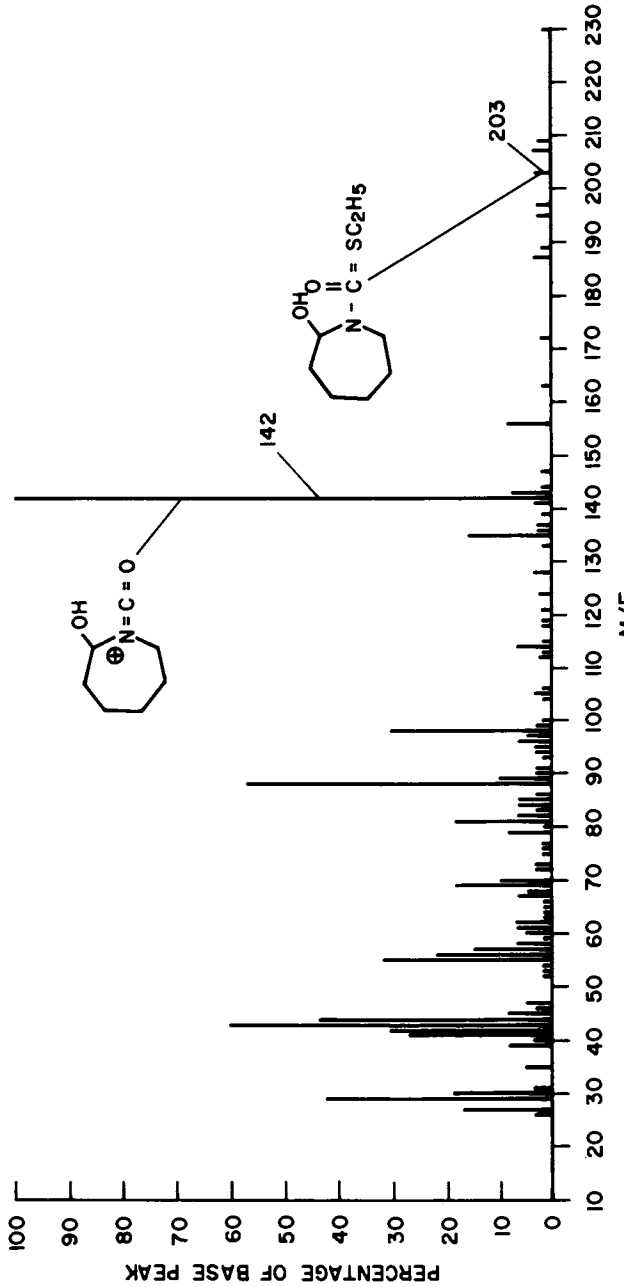

Figure 12. MS of the metabolite 4-keto molinate (GC Conditions: OV 17, 10% on Gas Chrom Q (4'); helium flow rate, 30 mL/min; temperature, 150°C; retention time, 4.0 min)

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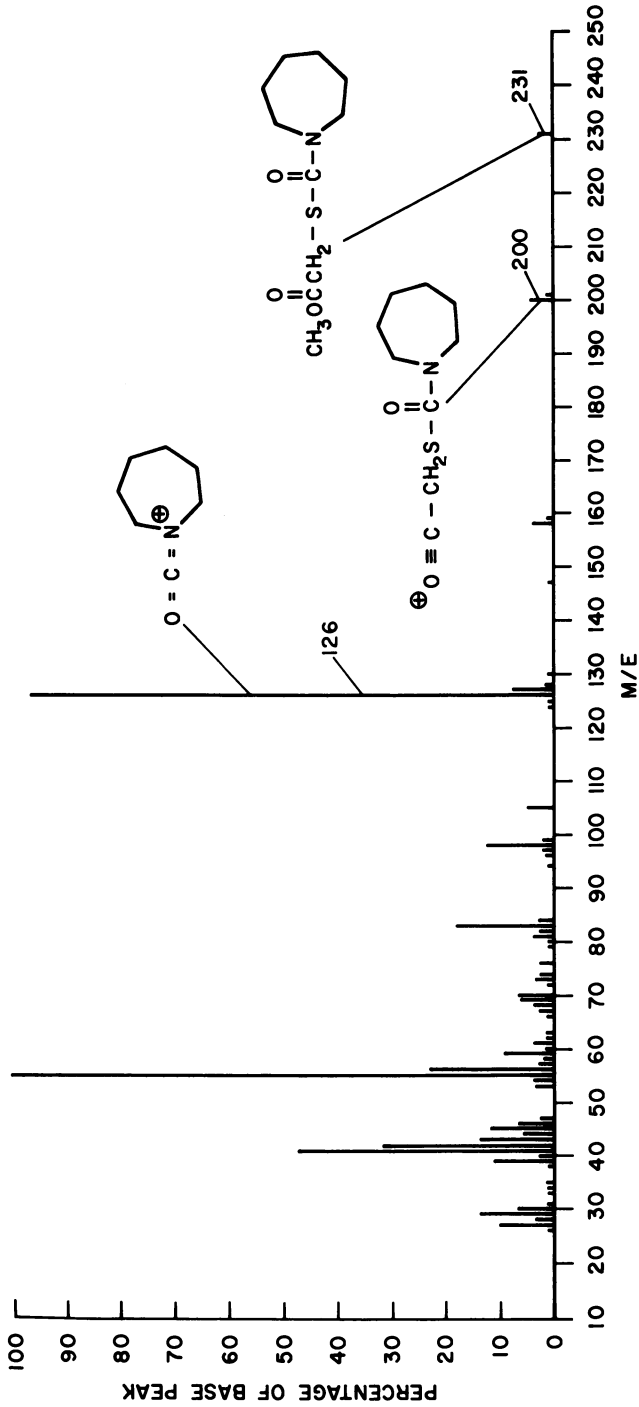

Figure 13. MS of methylated metabolite carboxy molinate (GC Conditions: OV 1, 3% on Gas Chrom Q (4'); helium flow rate, 40 mL/min; temperature, 150°C; retention time, 6.3 min)

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GC-MS spectral analysis. Subsequent extraction of the basified aqueous phase removed another 14% of the aqueous ^{14}C which contained 10 products as determined by the TLC analysis using solvents (j) and (k). HMI, which accounted for 58.8% of the extract, was the major component of this extract as determined by TLC cochromatography in solvents (j) and (k). Five other unidentified products were still present in the aqueous phase (TLC solvent (h)) after neutral, acidic and basic extraction.

Figure 14 shows the proposed metabolic pathway of [ring-^{14}C]-molinate in the Japanese carp based on the identification of ^{14}C-labeled metabolites present in the water and liver microsomal monooxidase system. Apparently, molinate was absorbed rapidly by carp and converted to various more polar metabolites prior to excretion into the water. Structural characterization of various metabolites showed that sulfoxidation of the S-alkyl moiety and ring hydroxylation at various positions were the major biotransformation steps *in vitro* and *in vivo*. In these studies, a substantial amount of 4-keto molinate was found in the fish water. However, it was not detected in rice field water (14) nor in mammalian species (10, 11, 12). Two-keto molinate was present in rice field water (14) but was not found in these studies. HMI may be derived from hydrolysis of the molinate sulfoxide or the sulfone, the latter not being detected in this study. The presence of various keto isomers in the fish water suggests that further oxidation of hydroxylated molinate readily occurs. These studies have shown that the major metabolic pathways of molinate in Japanese carp involve sulfoxidation, ring hydroxylation, cleavage of the carbonyl-N junction, and possible conjugation with GSH. It is further apparent from the foregoing study that, as in the living rat, molinate undergoes extensive metabolic attack in the carp. Oxidative and possible conjugative reactions produced metabolites similar to those detected in the rat (12).

The rapid degradation of molinate and conversion to more polar metabolites which are excreted into water serves to explain the extremely low bioaccumulation potential of this herbicide in fish.

Acknowledgement

Thanks are due to Yashima Chemical Company, Japan and Stauffer Japan Ltd. for arranging carp importation from Japan. The authors are also grateful to the following colleagues from Stauffer Chemical Company's Western Research Center, Richmond and Mtn. View, Calif.: J. Kalbfeld for radiosynthesis, H. Tilles for metabolite synthesis, B. Giang for analytical support, and V.M. Thomas and C.L. Holt for assistance in identifying the carboxy molinate.

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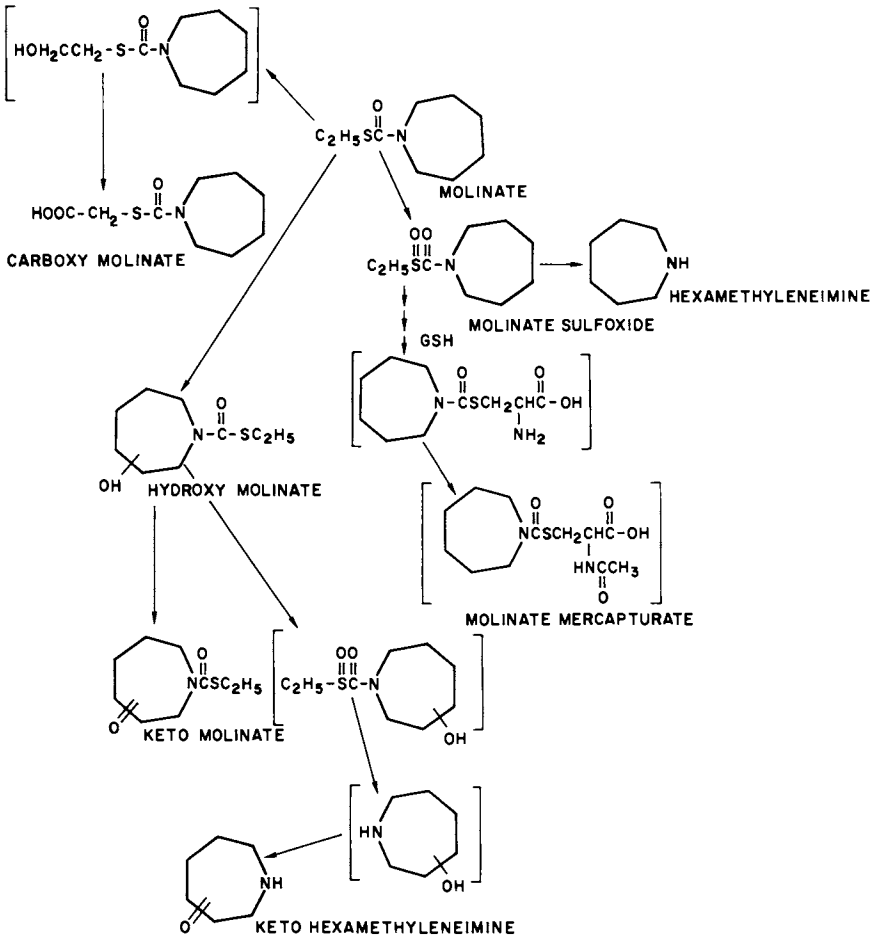

Figure 14. Proposed metabolism of [ring-^{14}C]-molinate by carp in vitro and in vivo

Abstract

The fate of the herbicide, molinate, in Japanese carp, (Cyprinus carpio) var. Yamato koi, was investigated. [Ring-^{14}C]molinate was applied to the water at 0.2 ppm. The overall ^{14}C residues in tissues of fish were low with an average bioaccumulation value of 1.35% during 1- to 14-days exposure. Molinate disappeared rapidly from water containing the carp. It accounted for only 3.8% of the extracted radiocarbon present in the water 14 days after treatment. Molinate was readily converted into various organosoluble and water-soluble degradation products shortly after addition to the water. Molinate sulfoxide, ring-hydroxylated molinate, isomers of keto molinate, keto hexamethyleneimine, hexamethyleneimine, and other metabolites were detected both in the water and/or fish bile. Polar metabolites, but no unchanged molinate, were found in the bile. In vitro, carp liver microsomal mixed-function oxidase systems also produced organosoluble metabolites identical to those found in the water containing living fish. The data suggest that major metabolic pathways for molinate in carp involve sulfoxidation, oxidation to both hydroxy and keto derivatives, and possible conjugation involving carbamoylation of endogenous SH-groups and O-conjugation of HMI.

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7

Biotransformation of Selected Chemicals by Fish

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Fish have been used as experimental animals for almost 200 years. However, their status in this role has not always been held in high regard. Huxley (*1*), speaking through Dr. Obispo, characterized fish as follows:

"The worst experimental animals in the world, he said . . . Nobody has a right to talk about technical difficulties who hadn't tried to work with fish. Take the simplest operation; it was a nightmare. Had you ever tried to keep its gills properly wet while it was anesthetized on the operating table? Or, alternately, to do your surgery under water? Had you ever set out to determine a fish's basal metabolism, or take an electro-cardiogram of its heart action, or measure its blood pressure? Had you ever wanted to analyze its excreta? And, if so, did you know how hard it was even to collect them? Had you ever attempted to study the chemistry of a fish's digestion and assimilation? To measure its speed of its nervous reactions?

"No, you had not . . . And until you had, you have no right to complain about anything."

Despite all the problems attendant on studies of aquatic animals, however, great strides have been made in the past 10 years in defining biochemical pathways used by fishes to biotransform and eliminate xenobiotics (*2*, *3*, *4*, *5*). Many of the earlier studies, especially the extensive work of DeWaide (*6*), defined various biochemical transformations which xenobiotics may undergo in vitro. Only in the past 10 years have in vivo studies been undertaken to define the routes and rates of elimination of xenobiotics by fishes (*7*, *8*, *9*, *10*, *11*).

The studies on which we report here were conducted as part of an ongoing program to evaluate the safety of various chemicals (anesthetics, collecting aids, selective toxicants, and a herbicide) that are used on fish, or that are used in the aquatic environment, or are possible contaminants of that environment. The various biotransformation reactions characterized here represent only a small fraction of the biotransformations that may occur in fish.

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Glucuronide Conjugation

The selective lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), is used to control the sea lamprey (*Petromyzon marinus*) in the Great Lakes (*12*, *13*). Recent studies have shown that in rats TFM is primarily biotransformed to reduced TFM (*14*). The major metabolite (Figure 1) found in rainbow trout (*Salmo gairdneri*), however, was the glucuronide conjugate of TFM (*15*, *16*).

The dynamics of TFM and its glucuronic acid conjugate in rainbow trout were reported by Hunn and Allen (*17*). The major increase in the accumulation of TFM conjugate in the bile occurred at the same time (between 0.75 and 1.0 h of exposure) that the concentration of conjugate in the plasma dropped.

Hunn and Allen (*18*) found that elimination of free and conjugated TFM occurred by way of the kidney in coho salmon (*Oncorhynchus kisutch*) and that conjugated TFM made up the bulk of that excreted. The lampricide rapidly cleared from blood (*18*, *19*) and muscle (*20*) during recovery in fresh water, but the TFM conjugate accumulated in gallbladder bile (*17*, *19*).

Biotransformation of xenobiotics by fish to water-soluble conjugates of glucuronic acid facilitates biliary and urinary excretion and probably decreases toxicity (*16*). Lech and Statham (*21*) reported that sea lampreys demonstrated a lower rate of glucuronide formation than did rainbow trout, on the basis of in vitro glucuronyl transferase assays. They also showed that in vivo sea lampreys had higher circulating levels of free than of conjugated TFM. Pretreatment of sea lampreys and rainbow trout with salicylamide, an inhibitor of glucuronyl transferase, shifted the LC_{50} for trout from 9.7 mg/l to 3.6 mg/l, but did not alter the LC_{50} for sea lampreys. This shift suggests that glucuronide formation may be the mechanism that provides TFM's selective toxicity.

Another lampricide, 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73), appears to be eliminated by rainbow trout in the same manner as TFM (*22*). Statham and Lech (*22*) observed the presence of a polar metabolite in bile; analysis of the metabolite by thin layer chromatography, β-glucuronidase hydrolysis, acid hydrolysis, infrared spectroscopy, and mass spectrometry indicated that the material was the glucuronide conjugate of Bayer 73 (Figure 1). Schultz and Harman (*23*) noted that bile of largemouth bass (*Micropterus salmoides*) exposed to the lampricide contained the glucuronide conjugate of Bayer 73. Allen et al. (*11*) found that Bayer 73 was excreted renally by rainbow trout. They recovered 51 μg of the lampricide in the urine after an intraperitoneal injection of 200 μg of Bayer 73. The analysis of urine before and after β-glucuronidase incubation and thin layer chromatography indicated that most of the material excreted renally was the glucuronide conjugate.

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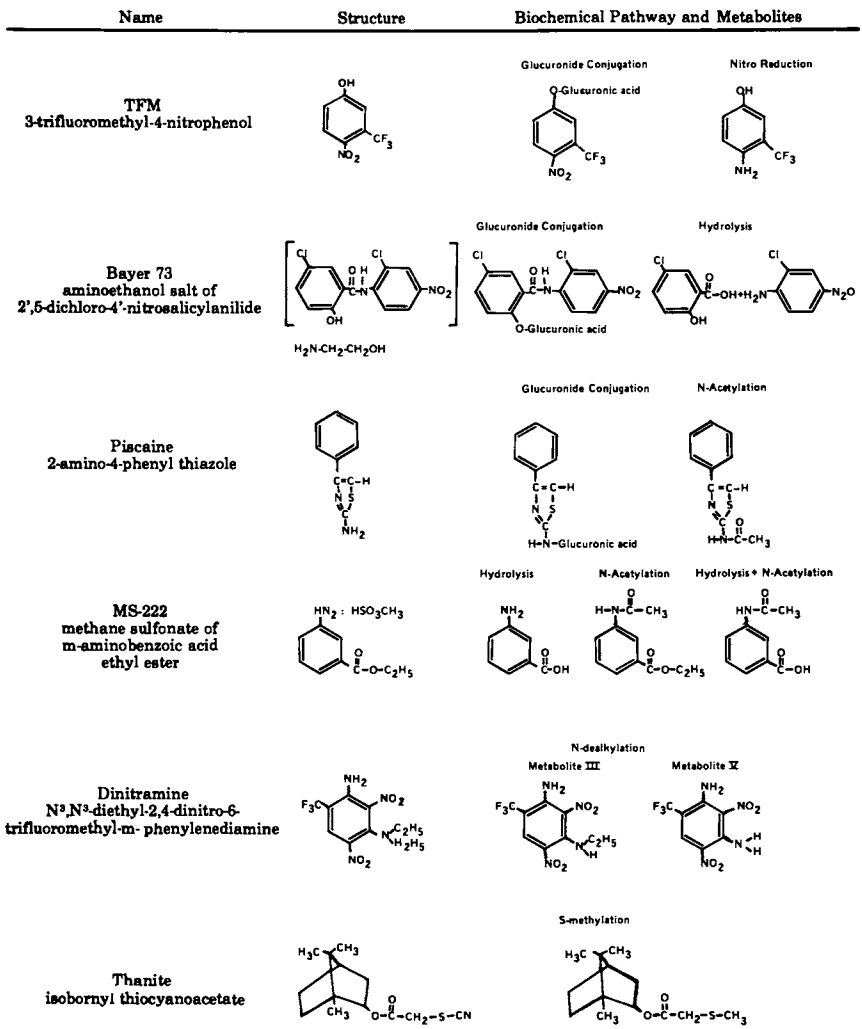

Figure 1. Common name, chemical name, structure, biochemical pathway, and structure of metabolites

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In a series of studies on 2-amino-4-phenylthiazole (Piscaine), a fish anesthetic, Suzuki and co-workers (*24*) isolated and identified an N-glucuronide metabolite (2-amino-4-phenylthiazole-2-N-β-mono-D-glucopyranosiduronic acid) from medaka (*Oryzias latipes*), rainbow trout, and carp (*Cyprinus carpio*) (Figure 1).

Hydrolysis

On the basis of studies on the metabolism of the fish anesthetic, methane sulfonate of *m*-aminobenzoic acid ethyl ester (MS-222), by a shark (the spiny dogfish, *Squalus acanthias*), Maren et al. (*25*) reported the formation of a metabolite, *m*-aminobenzoic acid, by cleavage of the ester bond (Figure 1). This ester-hydrolyzed product was not partitioned across the gill, but was slowly excreted by the kidney.

Luhning (*26*) measured residues of MS-222 and its metabolites in the muscle tissue of striped bass (*Morone saxatilis*), bluegills (*Lepomis macrochirus*), and largemouth bass anesthetized with a 100-mg/l solution of the drug. Striped bass rapidly hydrolyzed MS-222 to *m*-aminobenzoic acid, but bluegills and largemouth bass contained only a small amount of the acid residue. The concentration of *m*-aminobenzoic acid residues in striped bass muscle tissue continued to increase during a 50-min exposure to MS-222, whereas residues of free MS-222 peaked and declined after 30 min of exposure. Hydrolysis of MS-222 also occurred during storage of striped bass muscle samples, but almost none occurred during storage of bluegill or largemouth bass samples. Striped bass apparently possess an esterase not prevalent in bluegills or largemouth bass that can hydrolyze the ester linkage of MS-222.

Hydrolysis of Bayer 73 was also observed in carp, (D. P. Schultz personal communication). He identified the hydrolysis product, 2-chloro-4-nitroaniline, in the bile of carp exposed to 0.05 mg/l of Bayer 73 (Figure 1).

Acetylation

Fish also metabolize xenobiotics by N-acetylation. This process is well documented for the fish anesthetic MS-222 (*25-30*).

Hunn (*28*) showed that rainbow trout which were anesthetized with MS-222 excreted free and acetylated forms of the drug renally (Figure 1). MS-222 injected intraperitoneally was also excreted in both forms. In both experiments, 77 to 96% of the MS-222 excreted renally was acetylated. In the blood, the major form present was free MS-222. Although MS-222 was excreted renally, 79 to 85% of the injected dose was excreted extrarenally, presumably across the gills. In a similar study with the spiny dogfish shark, Maren et al. (*25*) reported that 95% of MS-222 was eliminated essentially intact across the gills within 2 h after injection, but a small percentage (<10%) was the N-acetyl derivative. The remaining 5% was excreted by the kidney, after cleavage of the ester

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bond to yield *m*-aminobenzoic acid and its N-acetyl derivative. These acids were excreted at about the rate of glomerular filtration.

Fish rapidly eliminate residues of MS-222 after exposure to the anesthetic (*27*). Blood, kidney, liver, and muscle show different rates of elimination, which probably reflect the form of the drug present and fluid turnover times in the tissues. The concentration of the anesthetic in these tissues decreased to the detection limit of their method within 5 h.

Acetylated MS-222 was found in much higher concentrations in the urine than in the blood of rainbow trout. This suggests that the kidney concentrated the drug metabolite, or that MS-222 was acetylated in the kidney and excreted in the urine (*28*). Weber (*31*) stated that acetylation of *p*-aminobenzoic acid and sulfamethazine is catalyzed by most of the tissues in the body. He showed that in rabbits the acetylation of these amines by the kidney of rabbit is a small percentage of the total acetylation capability, but that the kidney is involved in this biotransformation.

In vitro studies in our laboratory involving 1-h incubations of 0.5-g liver slices of rainbow trout with 10 ml of 1-, 2.5-, and 5-mg/100 ml concentrations of MS-222, resulted in 8.5, 6.9, and 4.2% (respectively) of the drug being acetylated. Similar incubations of kidney tissue resulted in 0, 0, and 3.2% acetylation. These incubation studies indicate that the liver is the prime site of acetylation of MS-222, but suggest that some may occur in the kidney as well. However, in vitro evaluation of the acetylating capability of rainbow trout kidney is complicated by the diffuse structure and heavy pigmentation of the organ.

In metabolism studies, Lech and Costrini (*15*) observed in vitro acetylation of TFM in liver and kidney extracts of rainbow trout. The nitro group of TFM is apparently first reduced to an amine (reduced TFM), which is then acetylated (Figure 1). In vivo studies of rainbow trout and other species have failed to reveal any significant production of reduced TFM.

Suzuki et al. (*24*) identified a minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout and carp as 2-acetamido-4-(4'-hydroxyphenyl)-thiazole (Figure 1). They were unable, however, to demonstrate the presence of this metabolite in the medaka. These authors also suggested that the effectiveness of the 2-amino-4-phenylthiazole as a fish anesthetic is related to the rate at which the drug is activated by biotransformation.

N-Dealkylation

In vitro demethylation of aminopyrine by fish liver preparations was reported by DeWaide (*6*). Olson et al. (*32*) showed the in vivo stepwise N-dealkylation of the herbicide, N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine (dinitramine), by carp (Figure 1). Biotransformation products were found in

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gallbladder bile but not in blood or muscle, indicating that dinitramine was dealkylated by the liver. Gallbladder bile from carp exposed to 1 mg/l of dinitramine for 12 h at 12 C contained 0.60 μg/ml of N^3-ethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine, but only a trace of 2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine (metabolites III and V) respectively in Figure 1. Concentration of metabolite III in gallbladder bile 1 day after termination of the exposure was approximately equal to that in fish sampled at the end of the exposure. It was not detectable in samples taken 7 days after exposure. Only a trace of metabolite V was found in gallbladder bile at the end of the exposure, but the concentration increased to 4.35 μg/ml 1 day after exposure and slowly decreased to 2.11 μg/ml 7 days after exposure. There was stepwise dealkylation of dinitramine, in which metabolite III was the intermediate product. The presence of dinitramine and metabolite V was confirmed by GC/MS. Metabolite III was not present in sufficient quantities for mass spectral analysis; however, it was identified by using two columns of different polarity in the GC. Retention time on each column was identical with that of standard N^3-ethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine (metabolite III).

S-Methylation

Isobornyl thiocyanoacetate (Thanite) is an insecticide that has been investigated as a potential fish collecting tool. Gas chromatographic analysis with flame photometric detection in the sulfur mode showed that fish exposed to Thanite contained large amounts of a metabolite (Figure 1) but only a trace of the parent compound (*33*). The metabolite had a *p*-value of 0.34 in acetonitrile:hexane, compared with 0.08 for Thanite. Electron impact GC/MS of fish muscle extracts yielded no parent ion. However, a fragment with M/e 61 indicated the presence of a thio-ether on the metabolite. Later synthesis of isobornyl-α(methylthio)acetate showed that it had the same gas chromatographic characteristics as the metabolite of Thanite found in fish. The presence of the metabolite is consistent with the findings of Hunn (*34*) and Lewis (*35*). They suggested that the thiocyanate bond of Thanite is split by the fish, thereby releasing cyanide which produces the incapacitating and toxic effect. Ohkawa et al. (*36*) reported the liberation of hydrogen cyanide from Thanite by the reaction of the chemical with glutathione even in the absence of glutathione-S-transferase.

Allen et al. (*33*) found no accumulation of the Thanite metabolite in gallbladder bile of carp exposed to 1 mg/l of Thanite for 8 h at 12 C, nor could they detect the parent compound in any of the samples. The highest metabolite concentration (0.734 μg/g) was found in muscle samples taken immediately after exposure. After 48 h of withdrawal in fresh water, muscle residue concentration had declined to less than 0.05 μg/g. Plasma and bile, which contained 0.185 and 0.210 μg/ml, respectively, at 0 h had no

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detectable residues (<0.05 μg/g) of the Thanite metabolite after 48 h of withdrawal.

Abstract

Biotransformation of selected chemicals by freshwater fish is accomplished through a diversity of biochemical pathways. Biliary and renal excretion of glucuronide conjugates of two lampricides, 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73), have been demonstrated. Glucuronide conjugation has also been demonstrated with the fish anesthetic, 2-amino-4-phenylthiazole (Piscaine). Preliminary studies have indicated that fish are capable of hydrolyzing Bayer 73 to two fragments, 5-chloro-salicylic acid and 2-chloro-4-nitroaniline. Hydrolysis of the ester linkage of methane sulfonate of *m*-aminobenzoic acid ethyl ester (MS-222) to form *m*-aminobenzoic acid has been shown in freshwater and saltwater fish. Amino groups in MS-222 and Piscaine are subject to N-acetylation. Most of the acid metabolites of the fish anesthetics are excreted renally. Dealkylation of a substituted amine was shown by the stepwise deethylation of dinitramine (N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine) in carp (*Cyprinus carpio*). Fish are also capable of biotransformation involving substitution; fish exposed to Thanite (isobornyl thiocyanoacetate) apparently release cyanide by substituting a methyl group to form isobornyl-α-(methylthio)acetate.

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8

Metabolism of Pentachlorophenol in Fish

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Pentachlorophenol(PCP) is a versatile pesticide widely used as an insecticide, herbicide or fungicide. In Japan, PCP was produced approximately 15,000 ton/year from 1962 to 1970 and used mostly in the summer-time as a herbicide in paddyfields which were filled with water at a depth of few inches. The PCP applied to paddyfields easily flowed out of the fields to rivers and coastal area due to unexpected heavy rainfalls, resulting in a high mortality of fish and shellfish. The use of PCP in Japan was restricted in 1971 by the Government because of its high toxicity to fish.

However, PCP is the second heaviest used pesticide in the United States, although it has been mostly used for the purpose of wood preservation(1). Under such circumstances, an international symposium on "Pentachlorophenol" convened by K. Ranga Rao(University of West Florida) was held in Pensacola, Florida, June 27-29, 1977, concerning the chemistry, pharmacology, and environmental toxicology of PCP. At the symposium, I presented a paper(2) on the metabolism of PCP in fishes, mostly reviewing the works on the absorption, excretion and detoxification of PCP in fish and shellfish, which were done in our laboratory.

The present paper deals with a relation between toxicity and accumulation of chlorophenols in goldfish, Carassius auratus, PCP metabolites and their amounts excreted by the three major routes (branchial, renal and biliary) in the fish, and also with effects of pre-exposure to PCP on PCP-tolerance and on sulfate conjugation with phenol by the liver soluble fraction of the fish.

Toxicity of PCP and Other Chlorophenols

A study has been done regarding the acute toxicity of various chlorophenols to goldfish(av. 2 g), and their accumulation by the fish. Table I (3) shows the 24-h LC_{50} values of tested chlorophenols and the amounts of chlorophenols found in the dead fish in the media containing the chlorophenols at the concentration most close to each 24-h LC50 value, among the test

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Table I. The toxicity and accumulation of chlorophenols in goldfish

Chlorophenols	24-h LC_{50} (ppm)	Amount found in dead fish (μg/g)	Concentration factor*
Penta(2,3,4,5,6)	0.27	95	475 (0.2 ppm)
Tetra(2,3,4,6)	0.75	75	93 (0.8 ppm)
Tri(2,4,5)	1.7	112	62 (1.8 ppm)
Tri(2,4,6)	10.0	200	20 (10 ppm)
Di(2,4)	7.8	268	34 (8 ppm)
Mono(p)	9.0	101	10.1 (10 ppm)
Mono(o)	16	128	6.4 (20 ppm)
Phenol	60	114	1.9 (60 ppm)

* Values were obtained in fish which died in the concentration of each chlorophenol shown in parentheses most closed to 24-h LC_{50} among the tested media.

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media.

An increase of the Cl-atom number in the chlorophenols caused an abrupt increase in toxicity, resulting in the lowest 24-h LC_{50} value of 0.27 ppm for PCP, while that for phenol was 60 ppm. However, many small differences were observed among the concentrations of chlorophenols found in the dead fish during 24 h exposure, as compared with the differences among their LC_{50} values, i.e., the values obtained were within a small range from 75 to 268 μg/g body weight. The concentration factors of chlorophenols found in dead fish increased with an increase of the Cl-atom number in the reverse of the LC_{50} values, e.g., the concentration factors for phenol and PCP were 1.9 and 475, respectively.

From these results, it seems that an increase of the Cl-atom number in chlorophenols promotes an accumulation of the chlorophenols by fish and leads their concentrations in the fish to a lethal level even when the fish were exposed to rather low concentration media, and consequently increases the fish-toxicity of chlorophenols.

Absorption of PCP

When goldfish were exposed to PCP-media, PCP was rapidly absorbed by the fish at a highest concentration factor among the tested chlorophenols(Table I), until a lethal level of approximately 100 μg/g body weight was reached(4).

PCP absorbed by the fish(av. 40 g) from PCP-medium(0.2 ppm) was accumulated in various organs, especially the gall bladder. Although the PCP concentration in the gall bladder was the lowest among the tissues assayed at 1-h exposure, it rapidly increased with time and reached a value of 539 μg/g corresponding to a concentration factor of 2,700 at 24-h exposure. The concentration of PCP in the gall bladder increased linearly even after fish had been transferred to PCP-free running water and reached a level of 1,077 μg/g, corresponding to a concentration factor of 5,400 after 24-h culture in running water, whereas a decrease was observed in all other organs examined(5). Most of the PCP found in the gall bladder must have been transferred from other organs through the liver after conjugation.

Biliary Excretion of PCP-glucuronide

The abrupt increase in biliary concentration of PCP, which was observed with time after 5 h during exposure of goldfish(av. 90 g) to 0.1 ppm PCP, was due to accumulation of a conjugated-PCP (4.46 μmol/g bile at 48-h exposure), whereas the amount of free-PCP in the bile was negligibly small(0.09 μmol/g bile) (6).

The conjugated-PCP in the bile(5.7 g) collected from 30 goldfish(av. 110 g) exposed to 0.1 ppm PCP for 48 h was isolated by treating the bile with activated charcoal columns, followed by elution with an acetone-ammonia mixture and finally by passing

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the concentrated eluate through a Sephadex G-10 column with water. The isolated PCP-conjugate(ca. 13 μmoles) was identified as pentachlorophenyl-β-glucuronide by hydrolysis on incubation with β-glucuronidase, by thin-layer and gas-liquid chromatography and by determination of the molar ratio of PCP to glucuronic acid. No other conjugates including the sulfate-conjugate were detected in the bile(6).

Glickman et al. have also reported that PCP-glucuronide is excreted in the bile of rainbow trout exposed to PCP and pentachloroanisole media(7). The biliary excretion after glucuronide conjugation must be one of general detoxification mechanisms for PCP in fish.

Excretion of PCP-sulfate into Surrounding Water

When goldfish were transferred from PCP-media to PCP-free water, the PCP absorbed by the fish was quickly excreted into surrounding water with a half-life of ca. 10 h(4), mostly in a conjugated-form accompanied with a small amount of free-form(8).

After 15-h exposure to 560 liters of 0.5 ppm PCP, 240 goldfish(av. 35 g) were transferred to 560 liters of PCP-free water and cultured for 24 h. A conjugated-PCP amounting to 0.36 mmoles was excreted in the water during the 24 h culture period. The conjugate was isolated by a procedure similar to that used for the isolation of the PCP-conjugate from bile, as mentioned above.

The isolated conjugate was identified as pentachlorophenyl-sulfate by precipitation with $BaCl_2$, by column and thin-layer chromatography, by UV-absorption spectra, and by determination of the molar ratio of PCP to SO_4. PCP-glucuronide which is excreted in bile was not detected(8).

A PCP-conjugate excreted by short-necked clam, Tapes philippinarum, into surrounding water has been also identified as the sulfate-conjugate(9).

Renal Excretion of PCP-sulfate

The direct excretion of PCP by fish into surrounding water must be mostly from both routes, i.e. gills and kidney. An experiment has been done to confirm the participation of renal route in PCP excretion by goldfish, using a urine collecting apparatus shown in Figure 1.

The apparatus was separated into two compartments(A,B) with a silicone rubber sponge sheet which had a hole in the middle to hold fish at just the back of pectoral fin. Both the head and rear parts of fish were covered with two small boxes which had many holes and were fixed to a cover plate of the apparatus. PCP-free water was supplied into the head compartment(A) at a flow rate of 1 liter/h and the overflow from (A) was reserved in an ice-cooled tank for the analysis of PCP excreted from gills. Fish urine was led by a cannula to an ice-cooled flask through

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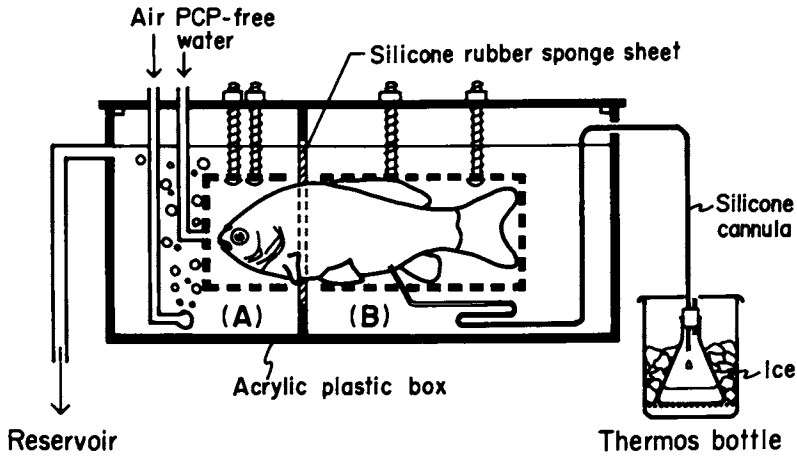

Figure 1. Apparatus for determination of excretion routes of PCP in fish

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a hole in the compartment (B).

After 24-h exposure to 0.1 ppm PCP, goldfish(av. 70 g) were transferred into the urine collecting apparatus and a catheter inserted into the urinary bladder from the urogenital cavity. Approximately 13 ml of urine was collected from each fish for 24 h. The urine collected from 14 fish was pooled and used for the determination of PCP.

More than 95 % of the PCP excreted in the urine was found in a conjugate-form. The isolation and identification of the conjugated-PCP were carried out according to the procedure previously used for the PCP-conjugates excreted in bile and in surrounding water. The PCP-conjugate excreted in the urine was identified as pentachlorophenylsulfate. PCP-glucuronide was not detected in the urine(10).

It is very interesting from the comparative biochemical view point that goldfish excrete PCP in the urine as the sulfate and in the bile as the glucuronide, while rabbits which were orally administered with PCP-Na excreted PCP-glucuronide accompanied by a large amount of free-PCP in the urine, Tashiro et al.(11).

Whole View of Major Detoxification Pathways for PCP

After 24-h exposure to 0.1 ppm PCP, two male goldfish(66 and 70 g) were transferred each into a urine collecting apparatus and a catheter inserted into the urinary bladder, as described above. After holding for 24 h in the apparatus, the fish were removed and dissected after collecting blood by cutting the tail off. Aliquots of the dissected organs were assayed for PCP content. The amounts of free- and conjugated-PCP contained in the overflow from the compartment(A), the water in the compartment(B), the urine and the bile collected from the fish were also determined.

Table II (12) shows a characteristic accumulation of PCP in the gall bladder among the organs, as previously observed in an experiment on the turnover of absorbed PCP in the fish(5).

Table III shows the amounts of free- and conjugated-PCP excreted from each of the biliary, renal and branchial routes. As mentioned above, the amount of PCP accumulated in gall bladder increased linearly for 24 h even after fish had been transferred from PCP-medium to PCP-free water(5). Therefore, the amount of PCP excreted in the bile must be reduced to half the value obtained, as compared with those in other excretion routes.

The amounts of PCP excreted from the branchial, renal and biliary routes corresponded to approximately 52, 24 and 22 % of the whole amount of PCP excreted by the fish, respectively. On the other hand, the amount of PCP in the water of compartment(B), which may be due to excretion from the body surface and leak of urine from the urogenital cavity, was negligibly small compared with those excreted from the above three routes.

Approximately 30 % of the PCP excreted from the gills was in a free-form, whereas almost all the PCP excreted in both the bile

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Table II. Amounts of PCP retained in various tissues of goldfish which were held in the apparatus shown in Figure 1 for 24 h, after 24 h exposure to 0.1 ppm PCP

Tissue	Fish-1			Fish-2		
	Weight	PCP		Weight	PCP	
		Total	Conc.		Total	Conc.
	(g)	(μg)	(μg/g)	(g)	(μg)	(μg/g)
Gills	1.972	52	26	1.996	60	30
Blood	1.450	80	55	1.256	90	72
Digestive tract	1.281	83	65	2.058	95	46
Gall bladder	0.378	633	1675	0.413	520	1260
Testis	2.452	27	11	2.952	48	16
Liver	1.504	50	33	2.877	113	39
Kidney	0.333	12	36	0.439	20	45
Spleen	0.168	3	19	0.265	8	28
Other viscera	1.053	40	38	1.150	47	40
Muscle	12.849	86	7	12.910	115	9
Remainder	42.475	1092	26	43.498	968	22
Whole body	65.913	2158	33	69.813	2082	30

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Table III. Amounts of free- and conjugated-PCP excreted from each route of goldfish, during holding for 24 h in the apparatus shown in Figure 1

Excretion route	Excretion time (h)	Fish-1 (66 g)		Fish-2 (70 g)	
		Free-PCP (μmole)	Conjugated-PCP (μmole)	Free-PCP (μmole)	Conjugated-PCP (μmole)
Biliary	48	0.03	2.35	0.03	1.93
	24*	0.015*	1.175*	0.015*	0.965*
Renal	24	0.07	1.22	0.01	1.06
Branchial	24	0.88	2.22	0.66	1.41
Body surface (including leaked urine)	24	0.00	0.06	0.03	0.09

* Values were calculated as half the amount of PCP found in gall bladder, because the amount of PCP accumulated in gall bladder increased linearly for 24 h, after fish had been transferred from PCP-medium to PCP-free water.

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and urine was in a conjugated-form.

As mentioned above, the conjugated-PCP excreted from both the branchial and renal routes is PCP-sulfate, while that excreted in the bile is PCP-glucuronide. Therefore, the total amount of the sulfate excreted by the fish for 24 h was 2.47-3.44 μmoles, while that of the glucuronide was 0.97-1.17 μmoles, namely the excretion ratio of the sulfate to the glucuronide in the fish was approximately 2.7. Approximately 60 % of the sulfate conjugate excreted by the fish was due to diffusion into the surrounding water through the gills and the rest was excreted in the urine.

The bile containing PCP-glucuronide, however, must be secreted from gall bladder into intestine when fish had fed. For this reason, a further experiment was performed on the hydrolysis of PCP-glucuronide contained in bile on incubation with intestinal mucus of goldfish.

As shown in Table IV (12), a considerable amount of the PCP-glucuronide in bile was hydrolyzed by the intestinal mucus. This indicates that the glucuronide conjugation plays an important role in reduction of the concentration of free-PCP in the fish body, but not in elimination of PCP from the fish body as compared with the sulfate conjugation, because the PCP released from the glucuronide in the intestine must be reabsorbed there.

Effect of Pre-exposure to PCP on PCP-tolerance and Sulfate Conjugation Activity

One hundred goldfish(av. 1.6 g) were divided into two groups of 50 fish each; one group was exposed to 0.1 ppm PCP every other day for 4 days and another was placed in PCP-free water as control, before a toxicity test. On the third day after the end of pre-exposure, each ten fish of both groups were transferred to 0.1, 0.2, 0.3, 0.4 and 0.5 ppm PCP-media, respectively.

Figure 2 (13) shows an obvious increase in PCP-tolerance of the fish pre-exposed to PCP when compared with the control group.

In our previous studies on the sulfate conjugation of phenols by fish livers, all the liver slices of the test fish and shellfish exhibited sulfate conjugation activities with phenol(14), and among various liver cell fractions separated by ultracentrifugation, only the soluble fraction displayed the sulfate conjugation activity for phenol and various phenolic compounds(15).

A further study has been made of an effect of PCP-exposure on the sulfate conjugation activity for phenol by fish liver soluble fraction. Twenty goldfish(av. 66 g) were divided into two groups of 10 fish each; one group was exposed to 0.1 ppm PCP every other day for 4 days and another was cultured in PCP-free running water. On the third day after the exposure, the livers were taken out from the fish, homogenized by a Potter's homogenizer and fractionated using an ultracentrifuge. All the soluble fractions prepared from the individual livers were subjected to

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Table IV. Hydrolysis of PCP-glucuronide in bile of goldfish by its intestinal mucus

Sample	Free-PCP (nmole)	Conjugated-PCP (nmole)	Total PCP (nmole)
No. 1	4	492	496
No. 2	218	282	500

No. 1: 10 ml of bile-0.9% NaCl(1:100).

No. 2: 10 ml of bile-0.9% NaCl(1:100) and 0.69 g of intest. mucus of fish.

Samples were incubated for 5 h at 20 °C with continuous shaking.

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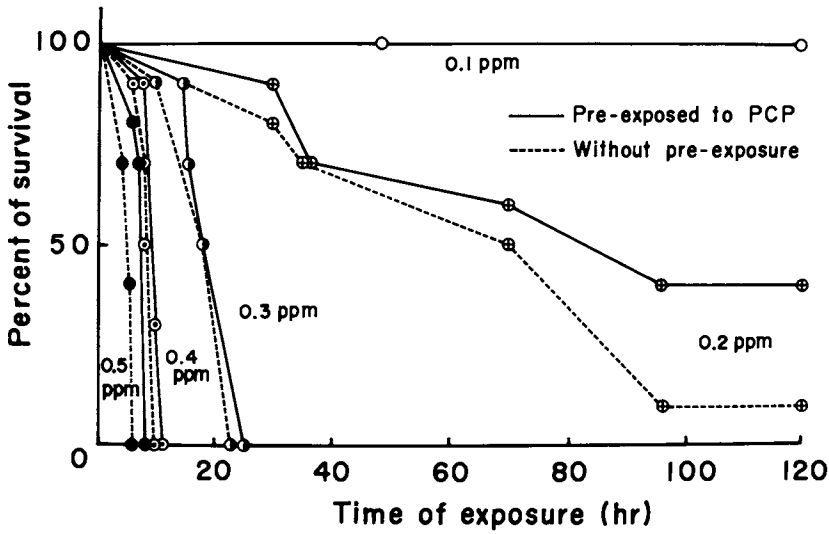

Figure 2. Increase in PCP-tolerance of goldfish pre-exposed to PCP: (——), survival patterns of goldfish which were pre-exposed to 0.1 ppm PCP every other day for 4 days, before the toxicity test; (– – –), survival patterns of goldfish which were not pre-exposed to PCP before the test. The temperature was kept at 20°C.

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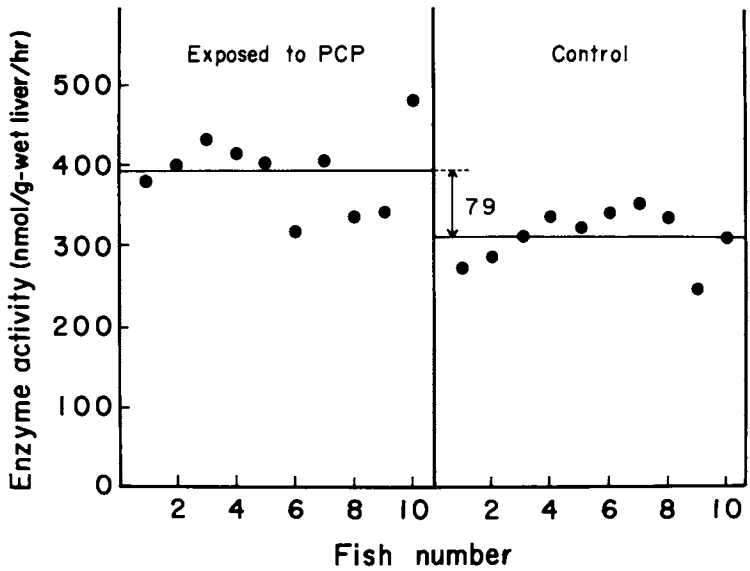

Figure 3. Increase in sulfate conjugation activity of liver-soluble fractions of goldfish exposed to PCP. The fish were exposed to 0.1 ppm PCP every other day for 4 days at 20°C.

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the assay of sulfate conjugation activity for phenol.

Figure 3 (13) shows approximately 26 % augmentation in the sulfate conjugation activity of liver soluble fractions of the fish exposed to PCP for 4 days, when compared with the control group.

The pre-exposure to PCP increased both the PCP-tolerance and the sulfate conjugation activity in goldfish. This suggests that fish have some ability to increase their PCP-tolerance when the fish had been exposed to sublethal PCP-media, and also that the sulfate conjugation activity is an important factor determining the PCP-tolerance of fish.

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9

The Disposition and Biotransformation of Organochlorine Insecticides in Insecticide-Resistant and -Susceptible Mosquitofish

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Selective pressures from agricultural contamination have resulted in the development of a resistant (R) population of mosquitofish (Gambusia affinis) which demonstrates up to a 500 fold greater tolerance of insecticides than does a corresponding susceptible (S) population (Table I). This resistance was caused by agricultural runoff of pesticides used on cotton and soybean fields into drainage ditches subjecting the fish to chronic exposures to insecticides. The resistance is genetically based, and is not merely an expression of environmentally-induced tolerances. The major selective pressure was organochlorine insecticides and the highest levels of resistance are to the chlorinated alicyclic insecticides (40 - 500 fold difference between the 48 hr LC_{50} values of S and R populations). The S population is not abnormally sensitive to organochlorine insecticides, as indicated by aldrin acute toxicities in other fish species (1).

Table I - Comparative 48-hr LC_{50} Values (µg/l) for Insecticide-Susceptible (S) and Resistant (R) Mosquitofish[a]

Insecticide	S	R	Fold difference
DDT	18.9	96.2	5.0
Aldrin	36.2	2735.0	93.5
Dieldrin	8.0	433.6	54.0
Endrin	0.6	314.1	499.0
Toxaphene	11.6	458.7	388.5

[a] From Culley and Ferguson (2).

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Work in our laboratory on various parameters in R and S fish has investigated the factor(s) responsible for resistance. The results have indicated that resistance is multifactorial, involving a barrier to insecticide penetration, insecticide storage, insecticide metabolism, and an apparent "insensitivity" at the target site to the toxic effects of the insecticide. The present report concentrates on two of these factors: insecticide disposition and metabolism.

Disposition

In general, aquatic vertebrates absorb insecticides through the gills (3). As such, the insecticide enters the efferent limb of circulation and may potentially enter the brain directly as one of the first major organs receiving blood from the gills. The site of action of some organochlorine insecticides is believed to be the central nervous system and a barrier to insecticide penetration into the CNS would protect the target organ. A comparison of the ratio of insecticide accumulated in the liver to that in the brain (L/B) of R and S animals following *in vivo* exposure would be an indication of the effectiveness of the brain barrier (Table II). In every case, there is considerably more insecticide in the livers than in the brains of resistant fish. In only one case is this pattern seen in the S fish and in the case of endrin there is 1.7 times as much in the brains as in the livers.

Table II - Ratios of Insecticide Accumulated in Livers to That in Brains in Susceptible (S) and Resistant (R) Fish Exposed to the ^{14}C-labelled Insecticide for 6 hr

	Exposure Concentration, µg/l	L/B		
		R	S	Reference
DDT	20	5.6	1.1	4
Aldrin	80	3.1	2.1	5
Dieldrin	30	2.9	1.2	5
Endrin	10	1.8	0.6	6

This barrier can be further illustrated by comparing tissue insecticide ratios between the S and R populations. Radioactivity accumulated in major organs following exposure to 10 µg/l ^{14}C-endrin is greater in S fish than in R fish for all organs studied except kidney (Table III). Similarly, S fish accumulate more aldrin, dieldrin and DDT in their brains than do R fish.

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With the exception of DDT, there was at least as much or more radioactivity accumulated in livers from S fish than in livers from R fish (Table IV).

Table III - Radioactivity Accumulated in Tissues of Susceptible (S) and Resistant (R) Fish Exposed to 10 µg/l ^{14}C-endrin for 6 hr

	ng endrin equivalents/mg wet weight[a]		
Tissue	S	R	S/R
Brain	192.2 ± 19.2	6.9 ± 0.5	27.8**
Liver	119.9 ± 23.6	12.5 ± 0.6	9.6**
Gill	78.0 ± 23.4	6.5 ± 0.8	12.0**
Gall Bladder	140.4 ± 7.1	20.8 ± 0.7	6.8**
Intestine	30.1 ± 7.3	12.8 ± 0.3	2.4**
Spleen	288.1 ± 13.0	24.1 ± 0.8	12.0**
Kidney	20.2 ± 0.5	66.8 ± 1.8	0.3**

[a] Values are expressed as mean ± SEM, 4-6 replications, 3 fish per replication. From Hunsinger (6).
** Significant differences between the means of the two populations ($P < 0.01$) as determined by the *t*-test.

Table IV - Ratios (Susceptible/Resistant; S/R) of Insecticide Accumulated in Brains and Livers Following 6 hr of ^{14}C-labelled Insecticide Exposure

	Exposure Concentration, µg/l	S/R		
		Brain	Liver	Reference
DDT	20	2.2	0.5	4
Aldrin	80	5.2	3.6	5
Dieldrin	30	2.1	0.9	5

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From the data presented, there is obviously a more effective barrier to insecticide penetration in R fish than in S fish. Further, this barrier apparently operates over a wide range of exposure levels. For example, when the tissue concentration in brains from R fish exposed to 10 μg/l are compared to tissue concentrations in brains of R fish exposed to 314 μg/l endrin, there is a 10-fold increase in endrin concentration in brain tissue (from 6.91 to 73.8 ng endrin equivalents/mg wet weight of tissue), although this represents a 30-fold increase in the insecticide exposure level. However, in S fish, the level of insecticide in brain tissue increased 355-fold (from 0.5 to 192.2 ng endrin equivalents/mg wet weight of tissue) when the insecticide exposure level was raised 17-fold (from 0.6 to 10 μg/l) (submitted for publication). It should be pointed out that the 314 μg/l exposure levels in the R fish represents the 48-hr LC_{50} value while the 0.6 μg/l exposure level is the 48-hr LC_{50} value for S fish. Therefore these data represent comparisons of the S and R populations at both equitoxic and equal exposure levels of endrin.

In most studies insecticide uptake is measured at a specific time of exposure and does not take into account the differences in tolerance to the insecticide within a population. In such studies, the more tolerant individuals of a population are actually selected. If comparisons are made within and between the R and S populations based on toxic effects as expressed by the appearance of symptoms of poisoning, a clearer understanding of the relationship between uptake/disposition and toxicity is possible. The insecticide concentration in the brain expressed as a ratio of symptomatic (s) to asymptomatic (a) would give a measure of the effectiveness of the membrane barrier and some indication of other factors which might be implicated in toxicity. A symptomatic/asymptomatic ratio greater than 1 would be expected if insecticide concentration in the target organ was the only determining factor in toxicity. Likewise, if varying target site sensitivity is a factor, a ratio of 1 or less would be expected. Such comparison of endrin accumulation within the resistant population (R_S/R_a) demonstrated a ratio significantly greater than 1 for the various brain segments (Table V) (7). This is indicative of more effective membrane barriers to insecticide penetration in the more tolerant R fish, which leads to varying degrees of insecticide tolerance within the R population. When a similar comparison is made within the S population between symptomatic and asymptomatic fish (S_S/S_a) the ratios are less than 1 (Table V). Since these comparisons are between less tolerant individuals to more tolerant individuals within the S population, the S_S/S_a ratios indicate a range in target site sensitivity. Such a range shows a basic flexibility within the unselected population upon which selective pressures could have acted to produce the R population.

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Table V - Ratios of Radioactivity Accumulated in Brains of Susceptible (S) and Resistant (R) Fish, Some of Which Were Exhibiting Symptoms (s) and Others not Exhibiting Symptoms (a) of Insecticide Poisoning. Treatment Consisted of a 6 hr Exposure to ^{14}C-endrin[a].

	10 µg/l Endrin			1500 µg/l Endrin		
	Ss/Sa	Ss/Ra[b]	Sa/Ra[b]	Ss/Rs[b]	Rs/Ra[b]	Ss/Ra[b]
Forebrain	0.6	3.1**	5.1**	0.5**	3.7**	1.7*
Midbrain	0.7	2.9**	4.4**	0.5**	4.8**	2.2*
Hindbrain	0.6	3.1**	4.9**	0.4**	4.4**	2.0*

[a] From Scales (7).
[b] All statistical comparisons are between components of each ratio. Significant differences were determined by the t-test, $P < 0.05$ (*), $P < 0.01$ (**).

When comparisons are made between populations, both the effectiveness of the membrane barrier in R fish and the sensitivity of the target site can be demonstrated (Table V). When endrin S_S/R_S ratios are compared, the ratio is less than 1; this suggests that more insecticide is required to elicit symptoms in the R than in the S fish.

This implicated target site insensitivity is more effectively demonstrated when actual amounts of endrin present in brain tissue of S_S fish are compared to R_a fish (7). In S_S fish the endrin concentration in the forebrain is 24 ng endrin equivalents/mg protein while in the same brain fraction of R_a fish there was 1150 ng endrin equivalents/mg protein. Further, when the amount of endrin in various brain fractions from R fish exposed to 1500 µg/l ^{14}C-endrin is monitored with time, up to 4560 ng endrin equivalents/mg protein appears in the brain fractions of R_a fish after 24 hr endrin exposure (Table VI).

This varying sensitivity of the target site might explain why uptake data does not seem to relate directly to toxicity (LC_{50} values). From our data it is possible to suggest that more tolerant individuals within the R population would probably possess a high insensitivity to insecticides at the target site and an effective barrier to insecticide penetration. The less tolerant might possess only one of these factors, or varying degrees of functional effectiveness of one or both factors.

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Individuals in the S population would probably not contain both factors and the factor(s), if present, would not be as functionally effective as in individuals in the R population.

Table VI - Radioactivity in Brain Fractions of Resistant Fish Exposed to 1500 µg/l ^{14}C-endrin not Exhibiting Symptoms of Poisoning[a]

Exposure Time	Brain Fraction[b]		
	Forebrain	Midbrain	Hindbrain
3	970 ± 110	640 ± 40	950 ± 90
6	1150 ± 140	940 ± 80	1100 ± 130
9	1280 ± 170	890 ± 80	1200 ± 80
12	2370 ± 370	1910 ± 170	2240 ± 140
24	4220 ± 230	4350 ± 210	4560 ± 270

[a] From Scales (7).
[b] Each value represents a mean of 5 treatments of 3 fish each expressed as ng endrin equivalents/mg protein ± SEM.

Biotransformation

The biotransformation systems involved in insecticide metabolism have been studied in the R and S populations to determine any differences which might be potential contributory factors to or results of insecticide resistance. In addition, the possibility of mixed-function oxidase induction has been investigated. Specifically, the studies have encompassed a seasonal study of microsomal mixed-function oxidase (mfo) components, and studies of aldrin, dieldrin and DDT metabolism.

Seasonal Study of Mixed Function Oxidases.-- A seasonal study of hepatic microsomal mfo components has been conducted in female R and S fish (submitted for publication). Components studied were cytochromes P-450 and b_5, NADPH-cytochrome c reductase, NADPH-dichlorophenolindophenol reductase, NADH-cytochrome c reductase and NADH-cytochrome b_5 reductase. All were monitored at 30°C by standard spectrophotometric methods following optimization procedures (8, 9, 10, 11, 12). Microsomal and total hepatic protein (13) and liver weight to body weight ratios were also monitored.

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The results indicated that microsomal mfo activities followed a definite seasonal pattern, with highest activities and levels occurring in the cold weather months. All parameters measured, except protein concentration, followed the same trends. Microsomal protein concentration was relatively constant throughout the study. Cytochrome P-450 and NADPH-cytochrome c reductase are presented as representative of the parameters investigated (Figs. 1 and 2). Both the seasonal patterns and the overall range of activities were similar in both populations. The cyclic nature of the parameters investigated may be the result of the relative magnitude of microsomal hydroxylations during the year in relationship to other microsomal processes such as biosynthesis. Although the ranges of enzyme specific activities and cytochrome levels were about the same in both populations, the consistently greater relative liver size in the R than the S fish suggests a greater potential for xenobiotic oxidation (Fig. 3). Lower relative liver size in the summer in both populations reflects the greater proportion of eggs in the total body mass. There has also been a report of seasonal trends in mfo activities in the feral roach (Leuciscus rutilus) with highest activities in the summer and of induction of mfo activity by environmental contaminants (14).

Aldrin and Dieldrin Metabolism.-- The in vivo metabolism of the chlorinated alicyclic insecticides, aldrin and dieldrin, has been measured. Fish were exposed to ^{14}C-labelled aldrin or dieldrin for 6 hours. The metabolism of each compound was monitored by thin layer chromatography of hexane and chloroform-methanol extracts of liver homogenates, followed by liquid scintillation counting of the spots (5,15,16).

When fish of both populations were exposed to 80 µg/l ^{14}C-aldrin, dieldrin was the only detectable metabolite in the organic extracts of the liver. The percentage of radioactivity in the water-soluble fraction in both populations was small. Although previous work had indicated a greater production of water-soluble metabolite(s) in the R population (15), more recent work indicated that the relative proportion of radioactivity in the water-soluble fraction was similar in both populations (Table VII).

The relative conversion of aldrin to dieldrin also varied with the season, with the greatest conversion occurring in the winter (Table VIII). This seasonal phenomenon correlates with the above mentioned seasonal data indicating greater mixed-function oxidase component activity in winter in both populations (Fig. 1 and 2).

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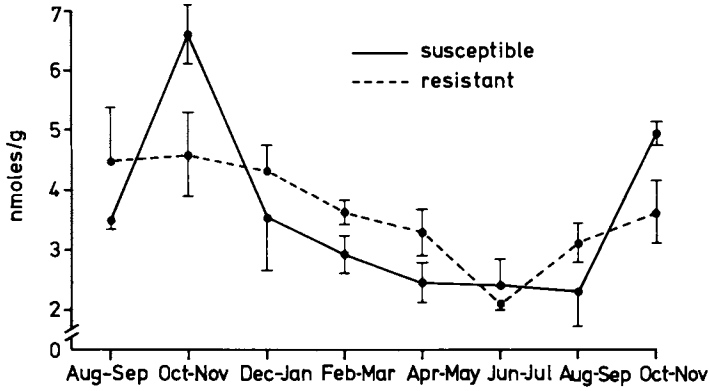

Figure 1. Cytochrome P-450 contents in liver microsomes from insecticide-resistant and -susceptible mosquitofish

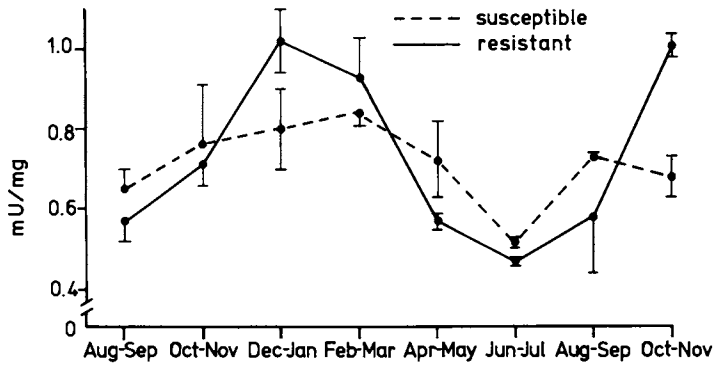

Figure 2. Specific activity of NADPH-Cytochrome c reductase in liver microsomes from insecticide-resistant and -susceptible mosquitofish. A unit (U) of enzyme activity is defined as 1 μmol product formed/min.

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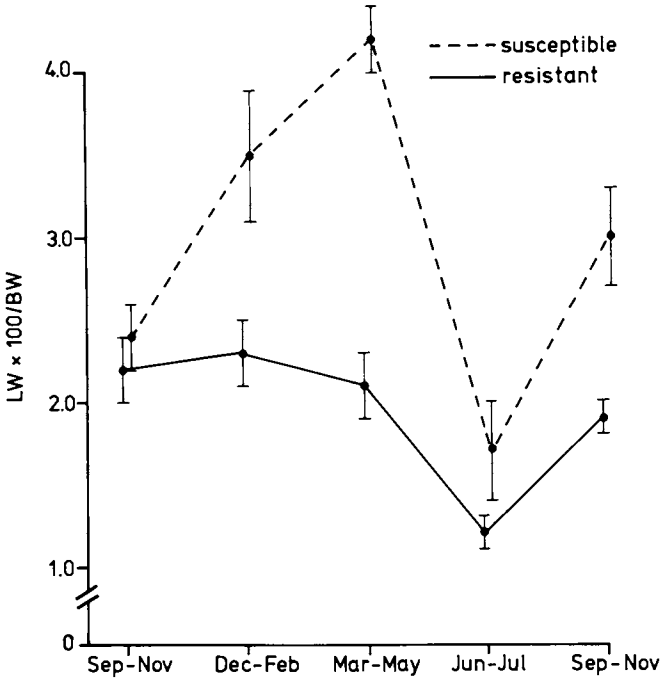

Figure 3. Liver weight to body weight ratios (LW × 100/BW) in insecticide-resistant and -susceptible mosquitofish

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Table VII - Radioactivity in Water-soluble Fractions of Livers of Susceptible (S) and Resistant (R) Fish Exposed to 80 µg/l ^{14}C-aldrin for 6 hr[a]

Population	ng aldrin equivalents/ mg protein	Water soluble radioactivity x 100/ total hepatic radioactivity
S	9 ± 3(11)	1.7 ± 0.3(11)
R	10 ± 2(24)	1.8 ± 0.2(20)

[a] Values are expressed as mean ± SEM (N).

Table VIII - Proportion of Dieldrin Formed (percent of total hepatic radioactivity) in Livers of Susceptible (S) and Resistant (R) Fish Exposed to 80 µg/l ^{14}C-aldrin for 6 hr[a]

Season	S	R
Dec-Feb	47.5 ± 7.2(4)	52.8 ± 2.6(10)
Mar-May	29.1 ± 4.3(2)	13.7 ± 0.0(2)
Sep-Nov	25.0 ± 3.9(4)	28.1 ± 8.7(4)

[a] Values are expressed as mean ± SEM (N).

No metabolites of dieldrin were observed in organic extracts of livers of either S or R fish exposed to 30 µg/l ^{14}C-dieldrin for 6 hr (5) (Table IX). A small percentage of the radioactivity was found in the water-soluble fraction. Therefore, it appears that little metabolism of dieldrin occurs in mosquitofish livers in either population. In addition, the degree of intoxication in S fish did not appear to affect the metabolism either qualitatively or quantitatively.

DDT Metabolism.-- The metabolism of DDT has been studied in R and S fish, following similar protocols to chlorinated cyclodiene metabolism: organic extraction (acetonitrile), thin layer chromatography of organic extracts, and liquid scintillation counting of the resultant spots (4). When S and R fish were exposed to 60 µg/l of ^{14}C-labelled p,p'-DDT for 4 hr, radioactivity was found in the spots which co-chromatographed with

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DDT, DDD and DDE (Table X). There were no qualitative or quantitative differences between the two populations.

Table IX - Radioactivity in Livers of Susceptible (S) Fish Exhibiting Symptoms of Poisoning (s) and S and Resistant (R) Fish not Exhibiting Symptoms of Poisoning (a). Fish were Exposed to 30 µg/l ^{14}C-dieldrin for 6 hr[a]

	Dieldrin		Water-soluble	
Population	ng dieldrin equivalents/ mg protein	%	ng dieldrin equivalents/mg protein	%
S_a	128.6 ± 13.0(4)	97	3.9 ± 0.4(4)	3
S_s	162.7 ± 14.5(4)	95	7.8 ± 0.9(4)	5
R_a	136.3 ± 8.8(5)	98	3.2 ± 0.3(5)	2

[a] Values are expressed as mean ± SEM (N). From Watkins (5).

Table X - DDT and Metabolite Concentrations in Livers of Susceptible (S) and Resistant (R) Fish Exposed to 60 µg/l ^{14}C-*p,p'*-DDT for 4 hr[a]

	S		R	
	ng DDT equivalents/ mg protein	%	ng DDT equivalents/ mg protein	%
DDT	624 ± 36(6)	67.0	483 ± 36(7)	74.1
DDD	75 ± 5(5)	8.0	42 ± 4(5)	6.5
DDE	109 ± 7(6)	11.7	51 ± 6(5)	7.9
Water Soluble	68 ± 10(6)	7.3	48 ± 8(6)	7.4

[a] Data are expressed as mean ± SEM (N). From Hamilton (4).

Mixed-function Oxidase Induction.-- Indirect evidence of environmental induction of detoxifying enzymes in the R fish has been observed as an increase in the acute toxicity of parathion and a simultaneous decrease in NADPH-dependent parathion

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dearylation in R fish with time held in the laboratory (17,18). However, direct attempts to induce mixed-function oxidase activity in mosquitofish have been difficult (19,20). Using DDT as a possible inducer resulted in an inconsistent degree of enzyme induction, with some experiments yielding no induction at all (19).

Discussion

The uptake and distribution of organochlorine insecticides has been studied under a variety of conditions. Although the results indicate that further study is needed on a characterization of extraneous factors that affect disposition, the studies clearly demonstrate the presence of a membrane barrier to insecticide penetration in the R population. This membrane barrier would aid in the protection of target sites in the R fish from the insecticide. This barrier is felt to be an important factor in resistance to organochlorine insecticides in mosquitofish.

Further, by virtue of their larger livers, the R fish have a greater xenobiotic biotransformation potential. However, the *in vivo* studies show few consistent differences in metabolism between the two populations. Biotransformation may be a major contributory factor in mosquitofish resistance to other pesticides, for example, organophosphorus and botanical insecticides, since the level of resistance to these chemicals is very low (4 fold or less) (18,20,21). However, biotransformation does not appear to play a major role in organochlorine insecticide resistance.

The levels of resistance which the mosquitofish demonstrate toward the chlorinated alicyclic insecticides (40 - 500 fold) are the most intriguing of those studied, yet they are impossible to explain in terms of disposition and biotransformation alone. Although insecticide metabolism cannot be completely discounted, it contributes little to chlorinated alicyclic resistance. Barriers to insecticide penetration undoubtedly contribute to chlorinated alicyclic resistance. However, we are led to conclude that these extremely high levels of resistance are the result of a postulated insensitivity of the target site which allows these fish to tolerate elevated internal levels of these toxicants.

We have, therefore, been able to indirectly assess the importance of three factors involved in chlorinated alicyclic insecticide resistance in mosquitofish: disposition, metabolism and target site sensitivity. In a highly polluted environment in which mosquitofish have been placed under severe selective pressures by chronic exposure to insecticides, the system of metabolism appears to be of little significance in resistance; the

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system of disposition with the development of internal and external barriers to insecticide penetration is the next most important; and the implicated target site insensitivity is potentially the most significant factor in the survival of the population.

Abstract

An insecticide-resistant (R) population of Gambusia affinis demonstrates a 5 to 500 fold resistance to organochlorine insecticides when the 48-hr LC_{50} values between the R population and a corresponding susceptible (S) population are compared. Uptake and disposition studies indicate that there is greater insecticide accumulation in tissues of S fish than in those of R fish. However, the difference in uptake between the two populations is not proportional to the degree of resistance for individual insecticides. Resistant fish can accumulate far greater body loads of insecticide without demonstrating symptoms of poisoning than can S fish.

Hepatic mixed-function oxidase activities demonstrated seasonal trends, with higher specific activities in the cold weather months in both populations with few differences in enzyme activities or cytochrome levels between the two populations. Metabolism of aldrin, dieldrin and DDT was similar between the two populations. R fish have larger relative liver size and, therefore, a greater potential for xenobiotic metabolism. However, biotransformation appears to be of minor importance in chlorinated alicyclic insecticide resistance in mosquitofish; barriers to penetration appear to be of greater importance; and an implied target site insensitivity appears to be the most important factor in resistance.

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Acknowledgments

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Metabolism of Insect Growth Regulators in Aquatic Organisms

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Insect growth regulators (IGRs), a new class of indirect insecticides which act by subtly misdirecting normal developmental processes, include compounds which mimic the juvenile hormone of insects as well as compounds which interfere with chitin biosynthesis. Chemicals with either mode of action have much greater specificity than earlier insecticides and have substantial advantages in certain applications. Since representatives of both classes of IGR are useful as mosquito larvicides, their fate in the aquatic environment has been investigated in some detail. This paper will review the degradation of several IGRs in water and aquatic organisms.

Diflubenzuron

Diflubenzuron (Dimilin®, TH-6040) is an IGR which inhibits the normal deposition of chitin. The metabolic fate of diflubenzuron has been studied in sheep (1, 2), cattle (1), rats (1, 3), house flies (4, 5), stable flies (5), chickens (6), swine (6), boll weevils (7), plants (8, 9), and soil (2, 8, 10). Since good reviews of diflubenzuron metabolism have been given by Ivie (11) and Verloop and Ferrell (9), we will present only a tabular summary of the degradation of diflubenzuron in nonaquatic systems for comparative purposes (Table I). The remaining discussion will focus on diflubenzuron degradation in the aquatic environment.

Hydrolysis. Schaefer and Dupras (12) investigated the hydrolytic stability of diflubenzuron as a 0.1 ppm aqueous solution. At pH 7.7 diflubenzuron is stable at 10-24°, but gradually decomposes at 38°. At pH 10 it is stable at 10°, but degrades slowly at temperatures greater than 24°.

Photodegradation. The photochemical degradation products of diflubenzuron in strictly aqueous solution are unreported, perhaps because of the compound's refractory solubility. Exposure of thin films on glass or a 0.1 ppm aqueous solution to sunlight

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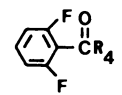
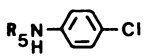
Table I. Nonaquatic Degradation of Diflubenzuron

	Sheep	Cow	Rat	Soil	Plants (5 species)	Insects (5 species)
R_1=R_2=R_3=H	***	***	***	***	***	***
R_1=OH, R_2=R_3=H	*	***	***			
R_1=R_2=H, R_3=OH	*	*	***			
R_1=R_3=H, R_2=OH	*	*	***			***
						
R_4=OH	***	**	**	***		*
R_4=$NHCH_2CO_2H$	**	**				
R_4=NH_2				**		*
						
R_5=$CONH_2$		*	**	***		*
R_5=$COCH_3$						*
R_5=H				*		

* = detectable, but <1% applied dose; ** = 1-10%; *** = >10%

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resulted in minimal photodecomposition (12). Metcalf *et al.* (2) and Ruzo *et al.* (13) irradiated methanolic solutions of diflubenzuron with artificial light as predictive models for environmental photodegradation (Table II). Metcalf *et al.* (2) also detected trace amounts of 4-chloroaniline and aniline from diflubenzuron after irradiation for 4 hr (254 nm) in aqueous dioxane while Ruzo *et al.* (13) found traces of 4-chlorophenyl isocyanate in methanol upon illumination at 300 nm.

Table II. Photoproducts of Diflubenzuron in Methanolic Solution

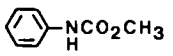
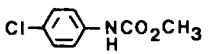
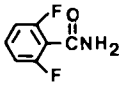
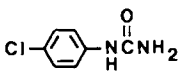
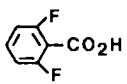
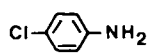
	% Yield	
	Metcalf *et al.* (2)	Ruzo *et al.* (13)
	18	45
	trace	4
	68	49

Table III. Metabolites of Diflubenzuron from Bacteria and Blue-green Algae, Booth and Ferrell (14).

	% Extractable ^{14}C	
	Bacteria (*Pseudomonas*) (10 days)	Blue-green Algae (*Plectonema*) (4 days)
	11	53
	5	trace
	5	36
unmetabolized diflubenzuron	77	5

Microorganisms. Diflubenzuron was stable to degradation by uncharacterized microorganisms from a sewage lagoon (12). *Pseudomonas putida* (soil microbe) also was unable to metabolize diflubenzuron upon incubation of pure cultures (2). Using *Pseudomonas* sp. from aquatic Utah soil Booth and Ferrell (14) followed the metabolic fate of diflubenzuron for twelve days

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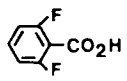
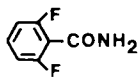
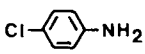
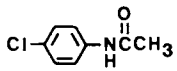
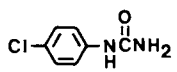
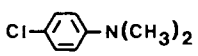
(Table III). *Pseudomonas* was capable of using diflubenzuron as a sole carbon source, but bacterial growth was greatly accelerated when the medium was supplemented with acetate.

Booth and Ferrell (14) found that blue-green algae, *Plectonema boryanum*, were voracious degraders of diflubenzuron (Table III). Just 5 mg of algae cells could metabolize almost 80% of the applied diflubenzuron in just 1 hr. Curiously, this pace was not sustained since 45 mg of algae could degrade only 95% of the applied dose after four days.

Aquatic Ecosystem and Fish. Metcalf *et al.* (2) studied the fate of diflubenzuron (radiolabeled separately in three different positions) in their model ecosystem. Diflubenzuron was dubbed "moderately persistent" in algae, snails, salt marsh caterpillars, and mosquito larvae as evidenced by limited biodegradability (Table IV). However, diflubenzuron and its nonpolar metabolites were not prone to ecological magnification in *Gambusia* fish. The lack of bioaccumulation of diflubenzuron residues in fish was substantiated by Booth and Ferrell (14) who used the channel catfish, *Ictalurus*, in a simulated lake ecosystem. They treated separate soil samples at 0.007 and 0.55 ppm, respectively.

Table IV. Degradation of Diflubenzuron in the Metcalf *et al.* (2) Model Ecosystem.

	% Extractable Radiolabel in				
	Water	Alga *Oedogonium*	Snail *Physa*	Mosquito *Culex*	Fish *Gambusia*
diflubenzuron	24-31	46-74	73-96	84-98	5-17
	6-8	-	-	4	3-10
	1	-	-	-	2
	11	6	-	-	5
	1	-	-	-	1
	10	3	-	2	2
	5	12	-	-	-

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The soil was aged aerobically for two weeks, then submerged under water for two weeks. Finally, additional water and catfish were added with monitoring of residues for twenty-eight days. For soil treated at 0.55 ppm, 64% of the initial dose was released from the soil, but only 2.3% of the applied radiolabel was found in the water upon termination of the study. Water residues consisted mostly (>93%) of 4-chlorophenylurea, accompanied by difluorobenzoic acid (3-5%), 4-chloroaniline (0-1%), and diflubenzuron (0.4-1.5%). An average of 66% of the soil residues were extractable with methanol, consisting predominantly of unmetabolized diflubenzuron (74-84%) and 4-chloroaniline (11-17%). For soil treated at 0.55 ppm, fish residues quickly reached a plateau after three days at about 4 and 10 ppb for muscle and viscera, respectively. Hence, Booth and Ferrell (14) concluded that bioaccumulation of diflubenzuron residues from marsh applications should be minimal.

R-20458

An impressive list of degradative studies has been performed with Stauffer's R-20458 including metabolism by eight insect species (15, 16), rats (15, 17), steers (18, 19), mice (20) and mammalian enzymes (15, 20, 21). It is evident that most published investigations have concentrated on metabolism by mammals and insects. Insect metabolism of R-20458 has been reviewed (22) and a summary of nonaquatic metabolism is given in Table V. Several additional hydroxylated metabolites were identified by Hoffman *et al.* (17) from rats.

Photodegradation. Casida's group (15, 20) has studied the photodecomposition of R-20458 on silica gel and in water. The major aqueous photoproducts are summarized in Figure 1. The predominant photoproduct in aqueous solution resulted from epoxide hydration to the corresponding diol. The photoproducts on silica were quite similar to aqueous products with an enhanced yield of diepoxide and diminished yield of diol. Photosensitizer dyes had little effect on R-20458 photodegradation.

Algae. Gill *et al.* (20) studied the metabolic fate of R-20458 in the algae *Chlorella* and *Chlamydomonas*. Both algae efficiently metabolized R-20458 with *Chlorella* demonstrating a higher metabolic capacity. In *Chlamydomonas*, the main metabolite resulted from hydration of the parent epoxide to the diol. After 48 hr, 78% of the R-20458 was degraded by *Chlorella*, the metabolites consisting mainly of diols (Figure 2).

Epifenonane

Detailed reports of the degradation of this Hoffman-LaRoche compound are limited. Compared to several other IGRs, epifenonane (Ro 10-3108) was relatively stable at pH 4 in the dark (23).

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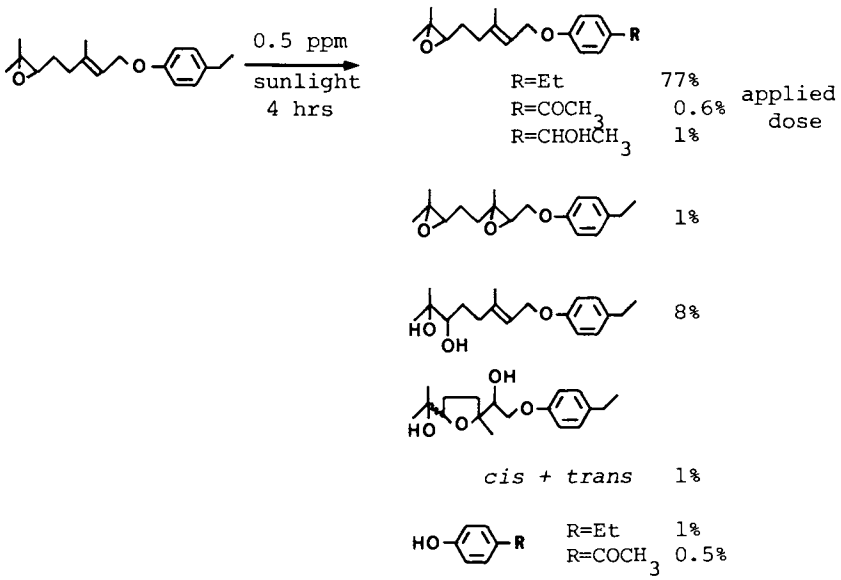

Figure 1. Photoproducts of R-20458 exposed to sunlight in water (20)

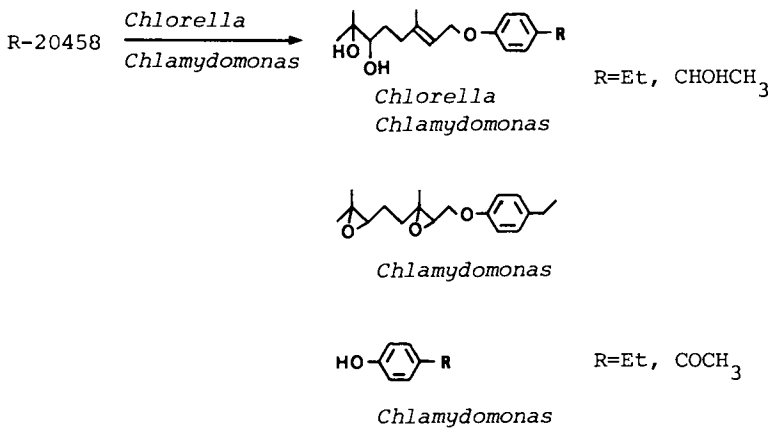

Figure 2. Algae metabolites of R-20458 (20)

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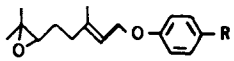
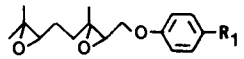
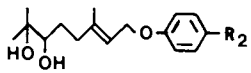
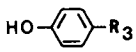
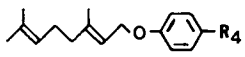
Table V. Nonaquatic Metabolism of R-20458

	mice rats	mammalian microsomes	steer	insects (8 species)
				
R=Et	***	***		***
R=$CHOHCH_3$		**		***
R=CH_2CH_2OH		*		**
R=$COCH_3$		**		***
				
R_1=Et		**		**
R_1=$CHOHCH_3$		*		
R_1=$COCH_3$		*		
				
R_2=Et	**	***	**	***
R_2=$CHOHCH_3$		***		**
R_2=$COCH_3$	**	**	**	**
R_2=OH	**			
Cyclic ethers		**		**
				
R_3=Et	**		***	
R_3=CO_2H	***			
R_3=CH_2CO_2H	**			
R_3=$COCH_3$				
				
R_4=OH	**			
R_4=Et	*		**	
R_4=$CHOHCH_3$	*			

* = detectable, but <1% applied dose; ** = 1-10%; *** = >10%

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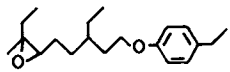
Illumination (290-370 nm) of a thin film on silica with a Xenon lamp (ca. 10x light intensity of sun) gave a half-life of 80 min. This was substantially more stable than methoprene ($t_{\frac{1}{2}}$ = 13 min) or R-20458 ($t_{\frac{1}{2}}$ = 7 min). The Hoffman-LaRoche group attributes the higher stability of their compound, when compared to the structurally similar R-20458, to the absence of an olefinic bond at C-2,3 in epifenonane. Differences in stability of aqueous emulsions of these three IGRs were studied outdoors in WHO standard synthetic field water. The amounts of epifenonane, R-20458, and methoprene recovered after one week were 85, 15, and 0%, respectively. After one week on bean leaves, 65% of the applied epifenonane was recovered relative to DDT as an internal standard (23).

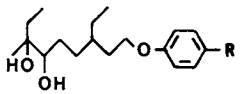
Dorn *et al.* have studied the fate of epifenonane in polluted water (24). When Glatt River water was fortified with epifenonane at 10 ppm and exposed to open air for four weeks, 61% of the applied dose was recovered as intact epifenonane. Volatility losses amounted to 18% of the applied ^{3}H while metabolites contributed a mere 21%. The characterized metabolites are listed in Table VI. The two major degradation routes involved epoxide modification and benzylic oxidation.

Table VI. Environmental Degradation Products of Epifenonane in Polluted Water, Dorn *et al.* (24).

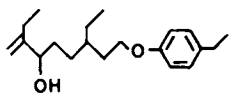


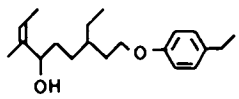
Epifenonane (Ro 10-3108)

major metabolites

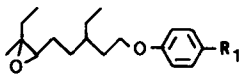


R=Et, $CHOHCH_3$, and $COCH_3$

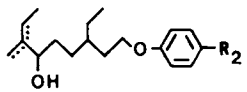




minor metabolites



R_1=$CHOHCH_3$, $COCH_3$



R_2=$CHOHCH_3$, $COCH_3$

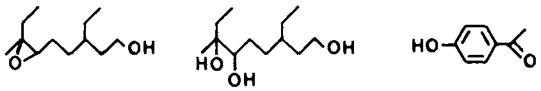


Table VII. Nonmetabolite Residues Formed on Catabolism of [5-^{14}C]methoprene.

Compound	Reference
Cholesterol	30, 31, 32, 33
" esters	30, 31, 32
Cholic acid	30
Deoxycholic acid	30
Acetic Acid	31, 38
Triglycerides	30, 31, 32, 33
(saturated fatty acids)	31
(monenoic " ")	31
(dienoic " ")	31
Diglycerides	32, 33
Chlorophyll	25
Carotenoids	25
Lactalbumin	31
Lactose	31
Casein	31
Structural Proteins	30, 33
Uric Acid	32

Methoprene

Methoprene has been fully registered since 1975 for commercial usage as a mosquito larvicide and for control of horn flies *via* feed-through application to cattle. In addition, methoprene is registered in Japan for administration to silkworms to enhance silk production. As the only IGR currently (July, 1978) registered, it follows that the environmental fate of methoprene has been investigated in detail.

Reports are published on the metabolism of methoprene by plants (25), aquatic microorganisms (26), soil microbes (27), house flies and mosquitoes *in vivo* (28), resistant house flies *in vivo* and *in vitro* (29), a steer (30), a lactating cow (31), chickens (32), and bluegill fish (33). In addition, radioactive material balance studies have been published for a guinea pig, steer, and cow (34), chickens (35), and rats (36, 37), including whole-body autoradiography in rats (37).

Metabolism studies of methoprene in nonaquatic organisms have provided background data which must be considered prior to discussing the fate of methoprene in aquatic systems. All nonaquatic metabolic studies reported to date have utilized [5-^{14}C]methoprene. The location of radiocarbon was selected both for ease of synthesis and for anticipated metabolic stability. However, studies in plants and bovines (25, 30, 31) revealed many presumed "metabolites" to be radiolabeled natural products, or "nonmetabolite residues". Primary metabolites of methoprene resulting from ester cleavage and/or O-demethylation have been

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observed in many organisms, albeit usually in very small yield. Apparently once the ester linkage of methoprene is metabolically cleaved, degradation of the carbon skeleton occurs as though the metabolites were branched-chain fatty acids or terpenoids. Indeed degradation of a [5-^{14}C]methoprene metabolite isolated from a steer, [^{14}C]deoxycholic acid, revealed that this "metabolite" was biosynthesized from [2-^{14}C]acetate (38). The latter finding suggests that degradation of the carbon chain of methoprene proceeds by initial α-oxidation, followed by successive β-oxidations. Such a pathway is well known for phytanic acid, and led us to conclude that methoprene is metabolized in part as a xenobiotic, and in part as a "food" (38). Also by analogy with phytanic acid metabolism, the 2,3- and 4,5-double bonds of methoprene may require saturation before the requisite α- and β-oxidations can occur. In fact, such hydrogenated metabolites have been observed in chicken metabolism, specifically as glyceride conjugates of the saturated acids (32).

Hydrolysis. Aqueous solutions of methoprene (0.5 ppm) were found to be totally stable to hydrolysis for four weeks at pH 5, 7, and 9 at 20° (26).

Photodegradation. Schaefer and Dupras (39) reported that emulsifiable formulations of methoprene at 0.1 ppm in water showed a rapid photodissipation in sunlight, whereas the commercial, microencapsulated formulation remained biologically active in water for several days under similar conditions. Aqueous solutions of methoprene undergo very rapid ($t_{\frac{1}{2}}$ <1 hr) photoequilibration to a mixture (~1:1) of 2E,4E:2Z,4E isomers (26, 39). The 2Z,4E isomer is not biologically active.

The identity of methoprene photoproducts has been studied from aqueous emulsions, thin films on glass or silica gel, and in methanolic solution (Figures 3 and 4, 40). As a thin film (0.1 μm) on glass, the half-life of methoprene was about 6 hr. After 93% degradation of parent, more than 50 photoproducts were observed, only five of these present in 3% or higher yield: 7-methoxycitronellic acid (4%), 7-methoxycitronellal (4%), the 4,5-epoxide of methoprene (6%), a C_{12} methyl ketone (3%), and $^{14}CO_2$ (6%). Similar products were encountered on photolysis of a 100 ppm aqueous emulsion of methoprene, except that methoxycitronellal was isolated only as its dimethyl acetal (9% yield), a presumed artifact of work-up. In addition to the same products identified from thin film studies, at least forty-six other discrete products were detected, but not identified (40).

In contrast, photolysis of methoprene in true aqueous solution gave a simpler distribution of different products (40). Five major products (25, 11, 13, 13, and 8% yield) were separated, but could not be positively identified due to lack of sufficient quantity (methoprene water solubility = 1.4 mg/l) and the singularly uninformative mass spectral fragmentations of the products.

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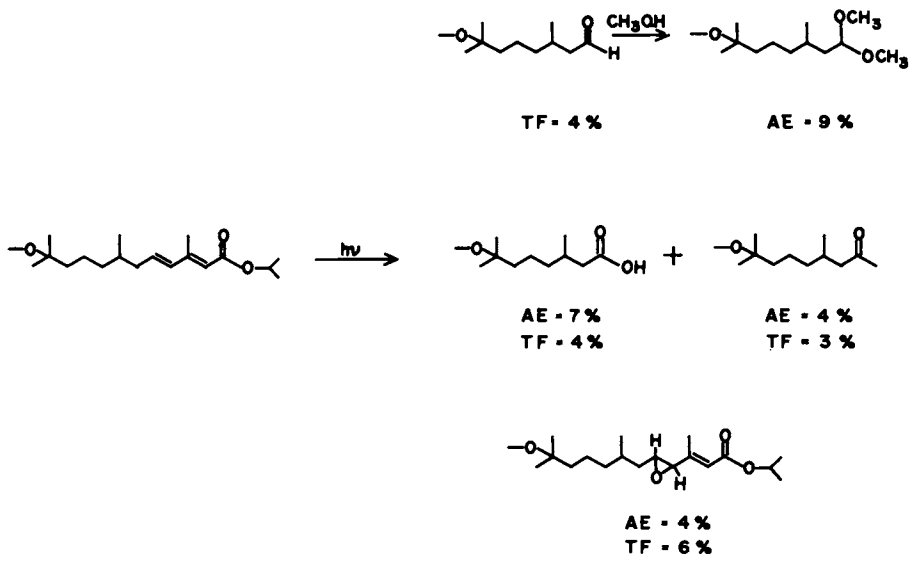

Figure 3. Photoproducts of methoprene from irradiation of an aqueous emulsion (AE) and thin film (TF) on glass (40)

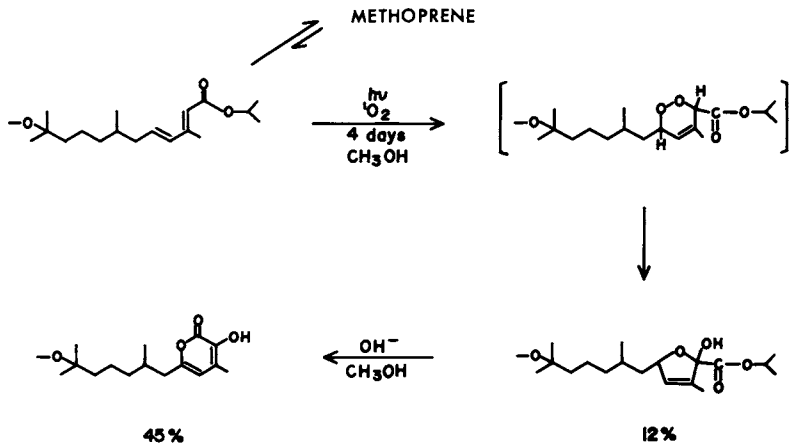

Figure 4. Methanolic photooxidation of methoprene (reaction with singlet oxygen (40)

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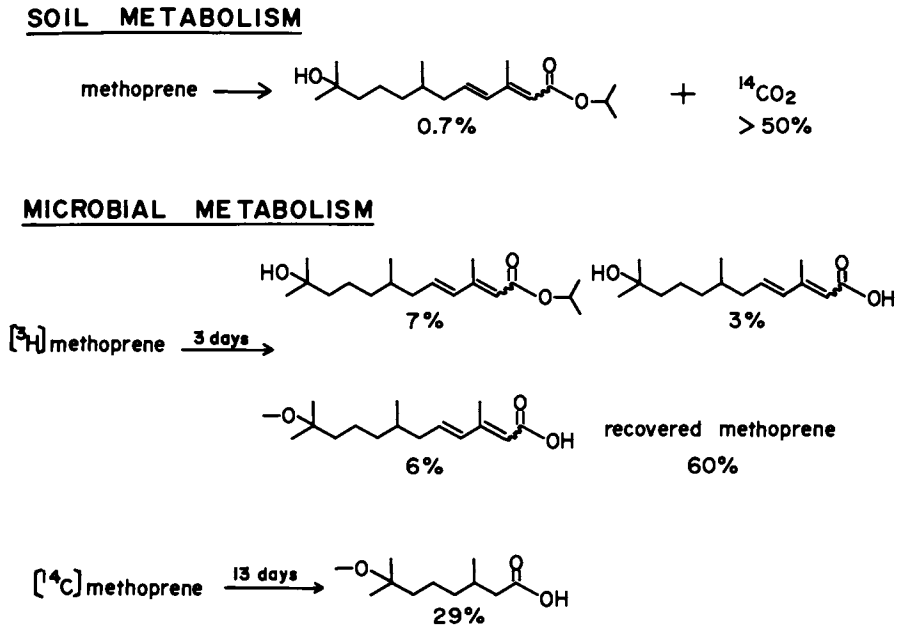

Figure 5. Metabolism of methoprene by soil and aquatic microorganism (26, 27)

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In summary, photodecomposition of methoprene is facile and leads to a multiplicity of products. The lower photostability in sunlight of methoprene compared to epifenonane has been mentioned previously. Because of its photochemical lability and its ready microbial degradation (see below), methoprene is microencapsulated for aquatic use as a mosquito larvicide.

Microorganisms. Outdoor incubation of 0.42 ppm of [10-^{3}H]methoprene with unknown aquatic microorganisms for three days led to production of the hydroxy ester (7.0%), the methoxy acid (5.7%), and the hydroxy acid (2.6%). Recovered methoprene (60%) contained about 7% of the analogous ethyl ester, while the hydroxy isopropyl ester metabolite contained about 15% of the analogous ethyl ester (Figure 5). The genesis of these ethyl esters is uncertain, but believed to arise from ethanol used to dose the incubations (final concentration only 0.08% ethanol in water), with possible enzymic catalysis. Outdoor incubation of [5-^{14}C]methoprene at 0.65 ppm for thirteen days with water from the same source, but collected during August instead of February, led to isolation of a single major metabolite in 29% yield. The metabolite, 7-methoxycitronellic acid, is also known as a photoproduct. However, a simultaneous autoclaved control showed a lower yield of this product. Also, 98% of the radioactivity was recovered from the autoclaved control *vs.* 48% from the microbially-active water. Since C-1 of 7-methoxycitronellic acid is the labeled position, further microbial degradation of 7-methoxycitronellic acid to [^{14}C]acetate and $^{14}CO_2$ can be inferred.

Fall has studied the capability of fourteen species of microorganisms to grow on methoprene as sole carbon source. One of these organisms, *Cladosporium resinae*, was able to utilize methoprene as sole carbon source, while another, *Pseudomonas citronellolis*, was similarly able to utilize 7-methoxycitronellic acid. (41).

Soil microorganisms degrade methoprene rapidly and extensively (27). The hydroxy ester was isolated as a minor metabolite; over 50% of the applied dose was evolved as $^{14}CO_2$. Radioactivity from [5-^{14}C]methoprene incorporated into the humic acid, fulvic acid, and humin fractions of soil.

Fish and Ecosystem Studies. When bluegill sunfish are exposed to a constant level of methoprene in a dynamic flow-through system, they accumulate radiocarbon until a plateau is reached after 7-14 days (33). While levels of methoprene in fish were about 1000x that in water at the plateau, treated bluegill placed into uncontaminated water showed a 93-95% radiocarbon reduction in 14-21 days. Analysis of fish tissues at plateau levels revealed that ∿90% of the radiocarbon was unmetabolized parent, 1% was the hydroxy ester, while the remainder was polar conjugates.

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The fate of methoprene was also studied in an aquatic ecosystem designed to simulate environmental exposure of fish to a mosquito larvicide. Bluegill fish were maintained in two meter diameter pools containing microbially-active water, soil, and bear rush plants. The pool was treated three times at weekly intervals with [5-^{14}C]methoprene sufficient to give 0.011 ppm solution. Radioassay of samples revealed a rather general distribution of radioactivity throughout the system. We analyzed fish tissues two and four weeks after the last treatment. While radioassay revealed an apparent concentration of 2-3 ppm equivalents of methoprene in bluegill, extraction and detailed analysis revealed that less than 0.1% of the radiocarbon was methoprene and its known primary metabolites. Radioactivity in nonpolar extractable fractions was characterized as triglycerides, diglycerides, cholesterol, and free fatty acids (14, 0.2, 1, and 3% of the total residue, respectively) at two weeks posttreatment. Over 50% of the radiocarbon was unextractable, but enzymatically solubilized radioactivity was presumed to be amino acids based on solubility (33). This study provides a telling criticism of ecosystem studies, when performed on readily degraded materials, and/or when not accompanied by cautious analysis of samples.

The fate of methoprene was investigated in the Metcalf ecosystem (42) before the environmental lability of [5-^{14}C]methoprene was documented. It seems likely that the limited data presented in that work gave spuriously high residue levels due to formation of nonmetabolite residues which interfere with the simple tlc analyses performed.

Summary

Insect growth regulators consist of diverse chemical structural types, but appear to share a common feature: quick degradation in the aquatic environment.

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11

The Fate of Highly Brominated Aromatic Hydrocarbons in Fish

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Previous studies of the accumulation of halogenated hydrocarbons by juvenile Atlantic salmon (*Salmo salar*) have indicated that bromobiphenyls with up to 4 bromine atoms in their molecule accumulate similarly to the corresponding chlorobiphenyls (1).

In contrast, penta- and higher bromobiphenyls accumulated in the fish to a lesser extent than similar chlorobiphenyls. The accumulation decreased with increasing bromine substitution and a hexabromobiphenyl was the last bromobiphenyl still accumulated from water (2).

This apparent effect of molecular weight on the accumulation was also confirmed by the lack of accumulation of hexabromobenzene (1), and by a relatively low accumulation of tetrabromo-2-chlorotoluene and pentabromotoluene (3).

The last two compounds were very slowly excreted by the fish. This indicated that their uptake may be very slow as well, which may result in an underestimation of the accumulation coefficient (4).

Octa- and higher brominated biphenyls were accumulated by the fish to a small extent only when administered in food. The main compound found in this case was a hexabromobiphenyl, not present in the originally administered mixture of bromobiphenyls (2).

These observations indicate three possible reasons for the low accumulation of highly brominated biphenyls and benzenes in juvenile Atlantic salmon:

(i) The compounds are likely to have an extremely low solubility in water, preventing their transport to biological membranes. Accumulation generally increases with decreasing water solubility (5), but the relationship has been established only for compounds with water solubilities larger than approximately 5×10^{-2} μmoles/L. Water solubility of highly brominated biphenyls and benzenes has not been measured. Calculations (6) indicate that it may be at least an order of magnitude below the above value. The accumulation/solubility relationship may not be valid in this range.

(ii) The high molecular weight of these compounds may be

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preventing or considerably diminishing their permeation through biological membranes. The permeability of biological membranes is proportional to M_{rel}^{s} (M_{rel} = molecular weight of an non-electrolyte, relative to molecular weight of methanol, s = empirical exponent, ranging from -2.9 to -6) (7).

(iii) The compounds may form bound or polar metabolites, not extractable under the conditions used for the parent molecules. Covalent binding of bromobenzene to tissues has been observed previously (8), and the partial debromination of highly brominated biphenyls indicates the possibility of the formation of reactive metabolites.

This paper describes experiments attempting to simulate the accumulation of halogenated aromatic compounds by measuring their transport rate through a layer of water into hexane, and shows that hexabromobenzene is not transported in this system.

In addition, data on the accumulation of several brominated benzenes by juvenile Atlantic salmon are presented, and the accumulation of brominated benzenes is correlated with the measured transport rates.

Experimental

Measurement of Transport Rates. Halogenated compounds, dissolved in hexane or hexane containing a small amount of toluene in the case of hexabromobenzene (0.5-2 mL), were applied to the bottom of a 25-mL REACTI-VIAL^{R} flask (Pierce Chemical Co.) and the solvent was evaporated gently under a stream of nitrogen. Tap water (25 mL) was added carefully, followed by pesticide-grade hexane (3 mL), and the flask was closed by a Teflon-lined septum. The flask was kept in subdued light at room temperature (20±1°C). Samples of hexane were withdrawn by a 10-μL Hamilton syringe and injected into a gas chromatograph.

The compounds were used in mixtures, prepared in a manner convenient for gas chromatography. Each compound was tested at 2-4 flask loadings, ranging from 0.5-56 μg/flask. At each loading, transport rates (μg/h) were calculated and plotted against the loading (μg/flask), yielding a straight line R(μg/h) = aC(μg/flask) + b, where a, b = empirical coefficients. Experiments were usually carried out over a period of about 60 h.

Accumulation in Fish. Experiments were carried out as described previously (1, 2, 3) for accumulation from water. Average weight of the fish was 2.1 g, and water temperature was 12°C. Fish were exposed for 48 h and placed in clean running water for up to 384 h. Each sample consisted of 2 whole fish, analyzed individually.

Chemical Analyses. Water samples were analyzed as described previously (1). Whole fish were homogenized with tap water (20 mL) in a Sorvall Omni-Mixer, using a 100-mL stainless steel

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container. The homogenate was transferred into a 500-mL round-bottom flask, using an additional 250 mL of tap water, and distilled as described (9) for 1 h, using pesticide-grade 2,2,4-trimethylpentane as the extraction solvent. For halogenated benzenes analyzed by this procedure, the analysis of spiked fish resulted in >85% recovery of the compounds.

Gas chromatography was performed as described previously (10), except that a column temperature of 170°C was used when analyzing for di-tetrabromobenzene, dibromo-toluene and xylene.

Results and Discussion

Transport Rates. The amount of halogenated compounds in the hexane layer increased linearly with time for up to 12 h for all compounds transported. For many compounds the relationship remained linear for at least 24 h and then it started to level off. This increase, expressed as a transport rate in µg/h was a linear function of the amount added to the flask (Table I).

Table I
Transport rate R (µg/h) as a function of the amount in the flask C (µg); $R = aC + b$

Compound	$a \times 10^3$	$b \times 10^3$
p,p'-DDT	-1.0	38
dieldrin	8.0	25
2,4',5-tribromobiphenyl	9.0	21
1,2,4,5-tetrabromobenzene	6.2	13
-nonachlor	3.5	7
hexachlorobenzene	1.4	3
1,3,5-tribromobenzene	26.2	-10
1,2,4- "	54.9	-30
2,5-dibromoxylene	22.9	-32
2,5-dibromotoluene	14.7	-103

Hexabromobenzene at 14 and 56 µg per flask was not detectable in the hexane layer. At 29 µg per flask, 0.57 µg of hexabromobenzene was found at 2.5 h. This might have been caused by a particle of hexabromobenzene released accidentally from the bottom of the flask. The amount of hexabromobenzene in the hexane layer was decreasing steadily throughout this experiment at a rate of about 5.6 ng/h.

These data indicate that hexabromobenzene is not transported as readily as halogenated hydrocarbons listed in Table I.

The coefficient *a* is related to the water solubility of the compound. A low transport rate can be expected when the flask loading is less than the amount of the compound that would dissolve in the volume of water in the flask. The transport rate

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should increase once this amount is exceeded. The data in Table 1 confirm this assumption. The transport rate is practically independent of the amount added to the flask for compounds of low water solubility, but increases considerably with the amount added in the case of compounds appreciably soluble in water.

The coefficient *b*, an extrapolated transport rate, may be interpreted as a relative "driving force" of the transport and related to the accumulation of the compound in aquatic biota. This is indicated by the order of the compounds in Table 1, arranged according to decreasing *b* values. The highly accumulative p,p'-DDT leads the list, and the *b* values of compounds, not expected to accumulate extensively, are much lower.

Accumulation in Fish. The accumulation coefficient and excretion half-life of tri- and tetrabromo-benzenes, dibromotoluene and xylene, are within a range expected on the basis of similar experiments (1), and are summarized in Table II.

Table II
Accumulation and excretion in fish

Compound	Concn. at 48 h water (µg/L)*	fish (µg/g)	Accumulation coefficient	Excretion half-life, h
1,2,4,5-tetrabromo-benzene	15.5	21.6	1390	103
1,3,5-tribromo-benzene	2.29	2.58	1130	95
1,2,4-tribromo-benzene	4.75	5.20	1095	104
2,5-dibromoxylene	30.3	43.3	1430	86
2,5-dibromotoluene	14.0	6.60	470	90

*Mean concentration, 0-48 h

Preliminary experiments indicate that the accumulation coefficient of o- and m-dibromobenzene is about 200, and the excretion half-life is approximately 18 h.

Consequently, with the possible exception of pentabromobenzene, hexabromobenzene may be the only brominated benzene which does not accumulate in fish.

Correlation of Accumulation in Fish with Transport Rates. Equation [1] expresses the accumulation coefficients (Table II) as

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a function of b (Table I):

$$\text{Accumulation coefficient} = 7736b + 1354 \quad (n=5,\ r=0.875) \qquad [1]$$

The amount of data is very limited at this stage, but it appears that the measurement of transport rates, as described above, has a potential to become a tool for predictions of accumulation of chemicals in fish.

From equation [1], the accumulation coefficients of 2,4',5-tribromobiphenyl and of hexachlorobenzene are 1512 and 1378, respectively. Previously reported (4) accumulation coefficients for these compounds are 400-2000, and 753.

In addition, the measurement of transport rates predicted correctly that hexabromobenzene will not accumulate in fish. This prediction would not be possible on the basis of the octanol/water partition coefficient (see for example 8, 11).

Conclusions

Low water solubility, possibly acting in conjunction with low membrane permeability, appears to be the main reason for the lack of accumulation of highly brominated biphenyls and hexabromobenzene from water by juvenile Atlantic salmon.

A method for the measurement of transport rates, described in this paper, is potentially useful for predicting the accumulation of chemicals in fish.

Acknowledgments

I thank Mr. W. G. Carson and Mrs. Sandra Cervantes for valuable technical assistance in a part of the study, Messrs. A. Sreedharan and G. Fawkes for assistance with calculations, and Mrs. Brenda McCullough for typing the manuscript.

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A Terrestrial-Aquatic Model Ecosystem for Evaluating the Environmental Fate of Drugs and Related Residues in Animal Excreta

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When farm animals are treated with drugs both as a prophylactic or curative measure, majority of the drug or drug related residues are eliminated in the excreta. Poultry as well as farm animal excreta is allowed to compost into manure and the manure is used on the farm land. The objective of the present study was to design a terrestrial-aquatic model ecosystem for evaluating the environmental fate of drugs and related residues in the animal excreta used as manure.

Metcalf et al. (1) developed a model ecosystem consisting of a terrestrial/aquatic interface and a seven-element food chain for obtaining valuable information on the biodegradability and ecological fate of numerous pesticides. This study, using a modified system, was initiated to determine the ecological fate of robenidine hydrochloride and related residues present in turkey excreta. ROBENZ® Robenidine hydrochloride, (Figure 1), has been found to be an effective and safe feed additive product for the prevention of coccidiosis in broiler chickens (2). For comparison, a parallel experiment using turkey excreta fortified with carbon-14 DDT as a positive control was conducted since the biodegradability and ecological fate of DDT and its analogs have already been extensively studied (1, 3).

Materials and Methods

Robenidine hydrochloride labeled with carbon-14 in the amino guanidine carbon atom had a specific activity of 18.61 μCi/mg.

Carbon-14 DDT, labeled in the ring, had a specific activity of 83.4 μCi/mg.

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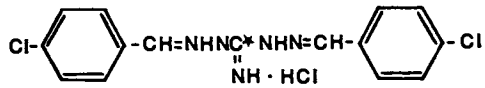
ROBENZ® ROBENIDINE HYDROCHLORIDE



*DENOTES CARBON-14

Figure 1

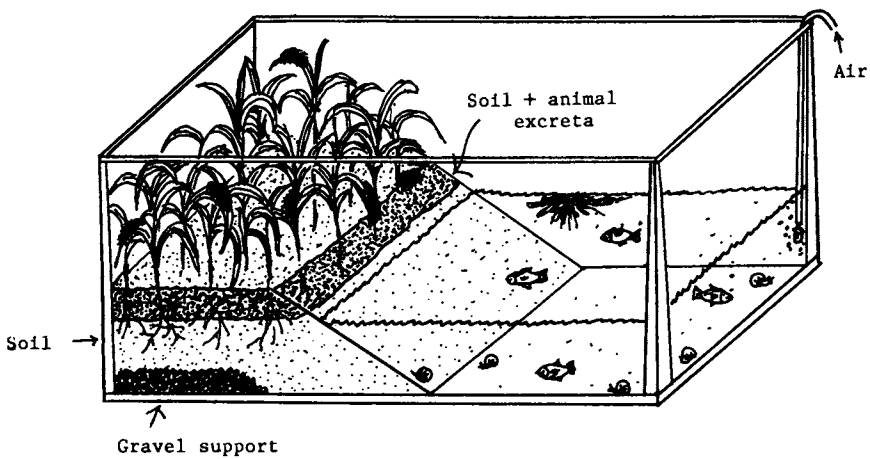


Figure 2. Schematic of the modified model ecosystem detailing a complete terrestrial/aquatic environment for the study of drug biodegradability and ecological magnification

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In this study, three experimental tanks were employed: Tank A containing excreta from a turkey treated with carbon-14 robenidine; Tank B containing excreta fortified with carbon-14 DDT which was used as a positive control and Tank C containing untreated excreta which was used as a control.

To obtain excreta for this study, a turkey was fed 250 grams of a basal diet containing 66 ppm robenidine hydrochloride for ten days. On the eleventh day, the turkey was dosed with 17 mg or 316.37 μCi of carbon-14 robenidine in a capsule. Excreta was collected for two days after treatment. Excreta collected prior to the ten-day conditioning period was also collected and used for the control and DDT experimental tanks.

The model ecosystem was essentially the same as that previously described by Metcalf et al. (1) except that the terrestrial portion consisted of unsterilized sandy loam soil instead of white quartz sand. In practice, 4.5 kg of aquarium gravel was washed thoroughly to remove fines and molded into a support shelf measuring 2 x 6 x 12 inches at the bottom of a 20-gallon glass aquarium. A soil shelf was then molded on top of the gravel consisting of 7 kg of Princeton sandy loam soil. The top 2 inches of the soil shelf was molded from 2.5 kg of soil mixed thoroughly in a ball mill with the dried turkey excreta. The amount of excreta added corresponded to an application rate of 5 tons/acre. The total area of the plateau was 78 square inches (6.5 x 12 inches) with a total height of 6 inches (Figure 2).

The total radioactivity applied to the soil as carbon-14 robenidine residues in excreta was 167.54 microcuries. For Tank B, 100 microcuries of carbon-14 DDT and 9 mg of unlabeled DDT were mixed in a ball mill with 2.5 kg of soil and 52g of control turkey excreta. The amount of DDT added to the soil corresponded to an application rate of approximately 1.5 lbs/acre. For Tank C, control turkey excreta was mixed with the top two inches of soil as previously described.

After the terrestrial portion was formed, 8 liters of reference water (4) were added to the system. The excreta was allowed to age for 4 weeks. During the aging period, distilled water was added whenever needed to keep the level of the aquatic portion constant. At the end of 4 weeks, fifty sorghum (sorghum halpense) seeds were sowed in 5 rows along the flattened terrestrial end. After 3 to 4 days when the seeds had germinated, 3 liters more of reference water were added and the level of water was kept constant throughout the remainder of the study. At this point, the following were added to the aquatic portion: 100 Daphnia magna, 10 Gyraulis snails, a strand of algae (Rhizoclonium and Lyngbia) and 10 milliliters of pond water which provided the plankton culture. When the seedlings were 3 weeks old, ten early fifth instar salt marsh caterpillar (Estigmene acrea) larvae were placed on the sorghum plants. Two to three seedlings were removed prior to addition of the larvae in order to determine the gross uptake of radioactivity by the plants.

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A fine mesh wire screen was then fitted over the tanks to confine the larvae on the plants. The caterpillars consumed the plants within 3 to 4 days and contaminated the water with their excreta and leaf frass. In their search for more food, the caterpillars also ended up contaminating the water themselves. Approximately 300 mosquito larvae (Anopheles quadrimaculatus) were added to the ecosystem after 26 days and 4 days later 50 were removed for determination of gross radioactivity. At this point, three mosquito fish (Gambusia affinis) were introduced and allowed to eat the remaining mosquito larvae and the Daphnia. After three days (Day 33), the experiment was terminated and the different components of the system were analyzed for carbon-14 residues.

Results and Discussion

Approximately 60% of the carbon-14 robenidine administered to the turkey was recovered in the excreta within 48 hours. About 84% of the radioactivity in the excreta was extracted with methanol, another 9% was extracted with a 1% hydrochloric acid/methanol mixture leaving about 7% unextracted. It was found by TLC that 75% of the methanol-soluble radioactivity was due to the presence of unchanged robenidine (Figure 3). Metabolites 1, 2, and 3 accounted for 7, 2 and 0.7% of the extractable radioactivity, respectively.

Sorghum seedlings radioassayed for carbon-14 residues at the time of larval feeding showed low levels of 0.004 and 0.013 ppm for carbon-14 DDT and carbon-14 robenidine, respectively.

Residue levels of carbon-14 in water were low, especially in the case of carbon-14 DDT, indicating that DDT-related residues remain bound to the soil (Figure 4). After an initial concentration of 0.009 ppb at Day 1, the concentration of carbon-14 DDT residues reached an equilibrium of about 0.02 ppb by the third day and then dropped off slightly to 0.012 to 0.013 ppb at the time the mosquito larvae and fish were introduced. Carbon-14 residues in water derived from carbon-14 robenidine showed an initial concentration of 0.344 ppb and then remained fairly constant at about 1 ppb throughout the study, indicating that robenidine-related residues are polar in nature and readily migrate into the water phase and reach equilibrium very rapidly.

The nature of the radioactivity in the water, soil and fish from the carbon-14 DDT experiment was examined by thin-layer chromatography as shown in Figure 5. The radioactivity in the water was very polar in nature and did not migrate appreciably from the origin. About 78% of the radioactivity in the soil was extracted with methanol. The major metabolite in the extractable fraction was DDD which represented 33% of the total radioactivity. The reductive dechlorination of DDT to DDD is a known pathway under anaerobic conditions and has been shown to be due to microbial metabolism (5). Since carbon-14 DDT was incor-

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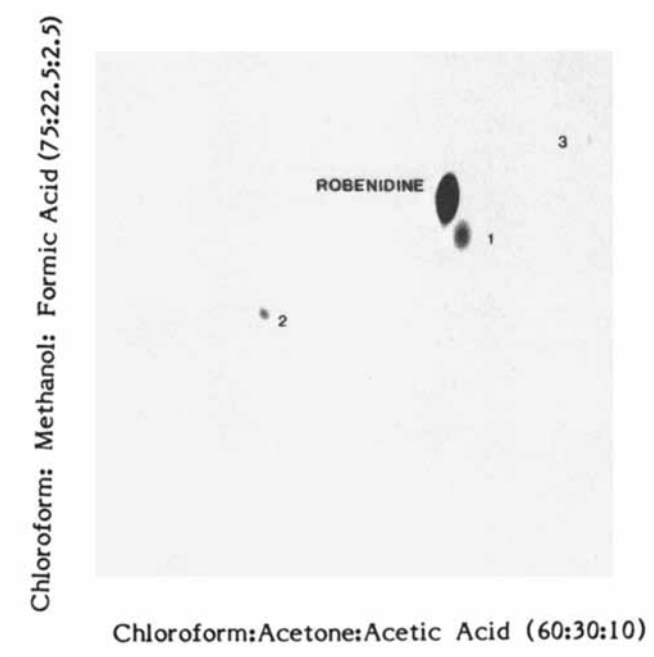

Figure 3. TLC of the extractable radioactivity from the excreta of turkeys

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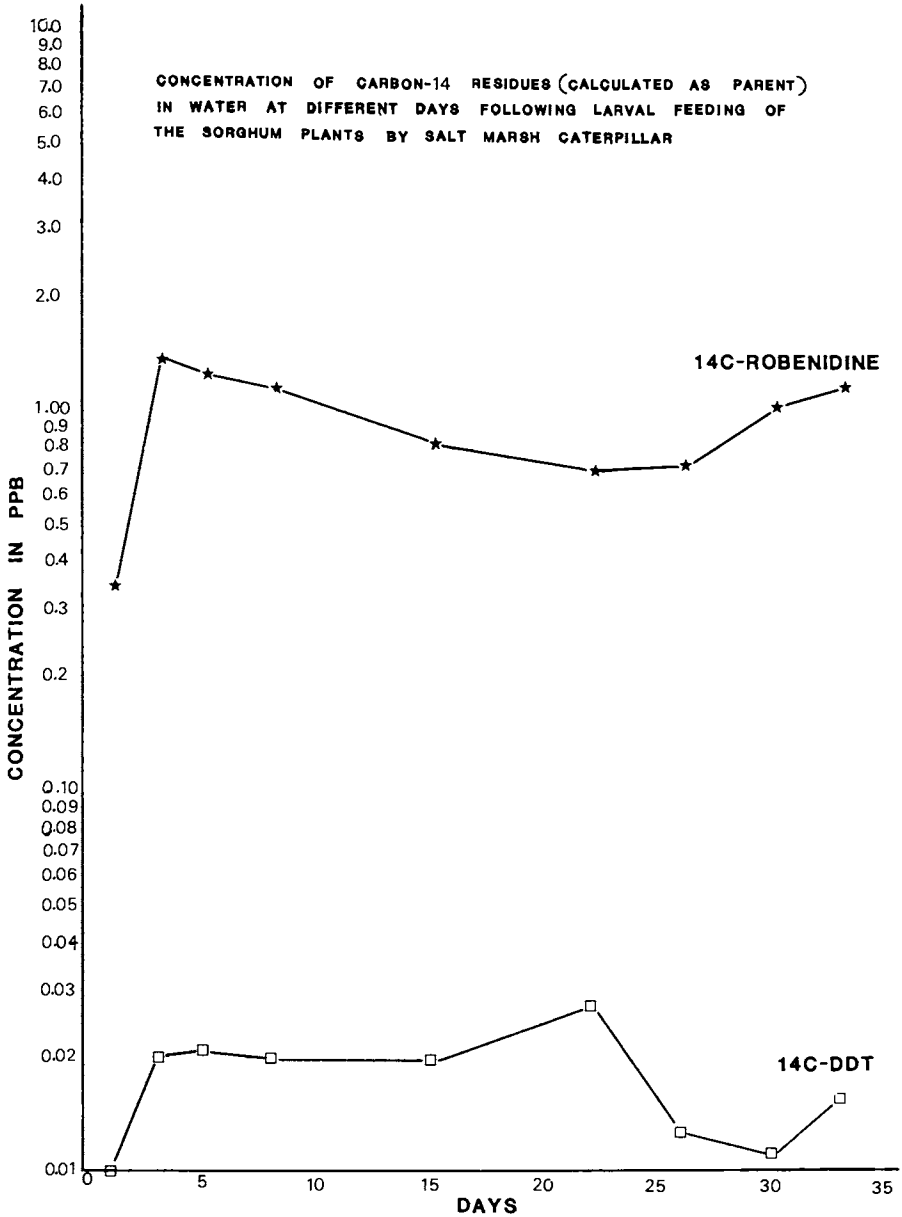

Figure 4

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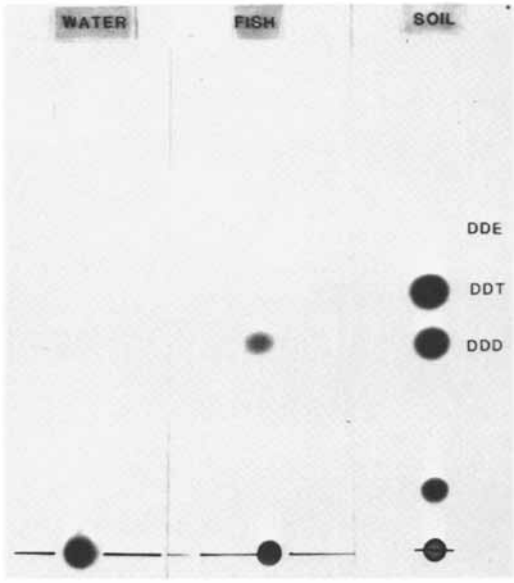

Figure 5. TLC of the fish and soil extractable radioactivity and water from the ^{14}C-DDT experiment. Solvent system used was petroleum ether:diethyl ether (9:1).

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porated into the soil with the excreta it is likely that anaerobic conditions prevailed. Parent DDT accounted for 44% of the extractable radioactivity with traces (1.1%) of DDE. About 13% of the extractable radioactivity was a component more polar than DDT and the remaining radioactivity stayed at or near the origin.

Extraction of the fish showed that 81% of the carbon-14 residues were organosoluble, 13% were polar water-soluble products and 6% unextractable. Chromatography of the organo-soluble radioactivity showed that a large proportion (87%) was still very polar in nature with DDT accounting for 8%, DDE 3% and DDD 2%.

Thin-layer chromatography of the water from the carbon-14 robenidine study showed about 12% (0.138 ppb) parent compound, 27% (0.319 ppb) of Metabolite No. 2 and 61% (0.734 ppb) polar radioactivity which did not migrate far from the origin (Figure 6). The results indicate that even though robenidine was the predominant component of the turkey excreta, it was readily and extensively degraded into polar compounds which end up in the aquatic phase.

About 20% of the radioactivity in the soil from the carbon-14 robenidine tank was extractable at the end of the experiment with 80% remaining unextractable. Chromatography of the extract-able radioactivity showed extensive degradation of the compound as shown in Figure 7.

Robenidine, which was the major component in the excreta, represented about 10% of the extractable radioactivity in the soil. In terms of total carbon-14 residues in the soil, parent compound represented 2.0%. Metabolite 2, which was present only in trace quantities in the excreta, accounted for 21% of the extractable radioactivity or 4.2% of the total carbon-14 residues in the soil. This metabolite was also the only significant compound found in the water. Three other metabolites accounted for about 18% of the extractable radioactivity in the soil, namely, Metabolite 3, 5.3%, Metabolite 6, 7.6% and Metabolite 10, 4.9%. Polar material which was not resolved from the origin represented 25% of the extractable radioactivity in the soil. The remaining radioactivity was distributed among ten minor components.

In the fish, the last element in the food chain web, methanol extracted about 58% of the radioactivity resulting from the carbon-14 robenidine treatment leaving 42% unextracted, indicating that robenidine was being extensively degraded by fish into very polar nonextractable products.

Thin-layer chromatography of the extractable radioactivity did not show any parent compound even though robenidine was one of the components in the environment (water) (Figure 8).

The concentration of carbon-14 residues in the different components of the model ecosystem is compared in Table I. The bioconcentration factor (BCF), defined as the ratio of the

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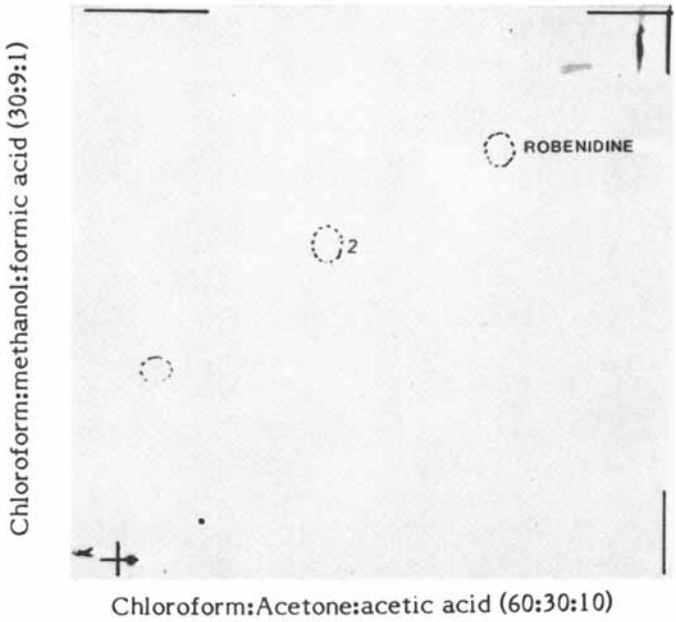

Figure 6. TLC of the water from the ^{14}C-robenidine model ecosystem

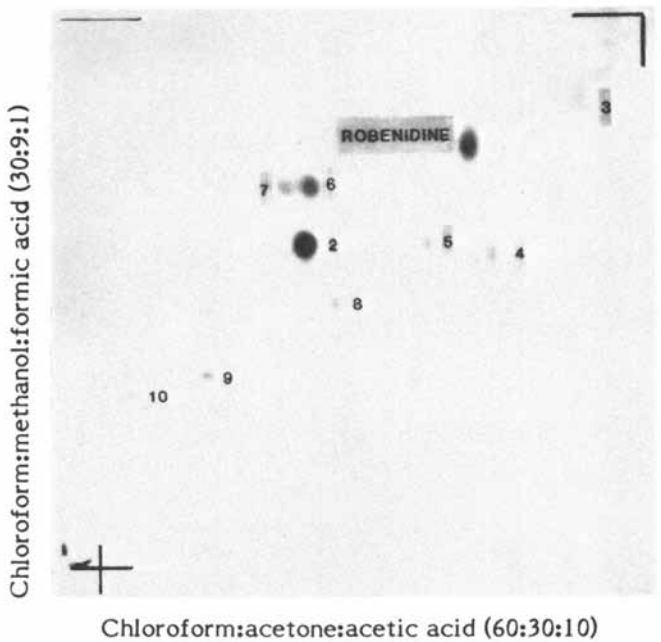

Figure 7. TLC of the methanol-soluble radiactivity in the aged soil/excreta mixture from the ^{14}C-robenidine model ecosystem

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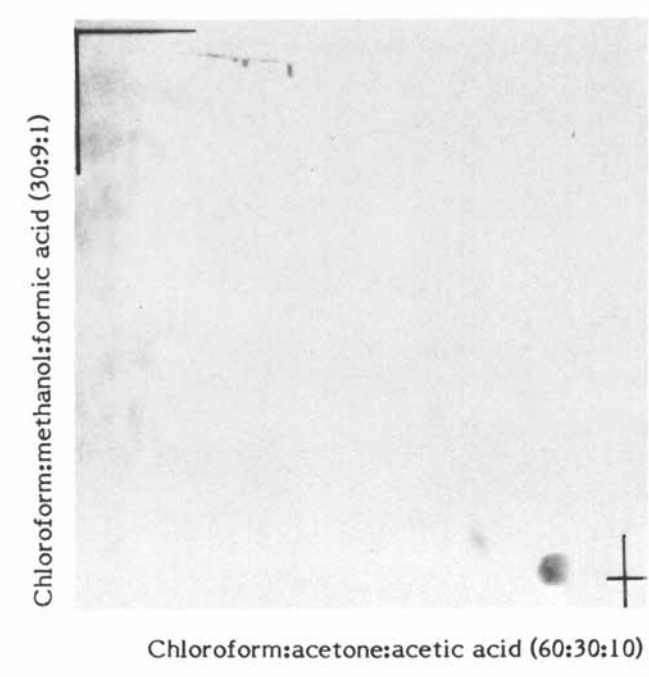

Figure 8. TLC of the methanol-soluble radioactivity in the fish from the ^{14}C-robenidine model ecosystem

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Table I.

COMPARISON OF THE CONCENTRATIONS[1/] OF CARBON-14 RESIDUES AND BIOCONCENTRATION FACTOR (BCF)[2/] IN THE CARBON-14 DDT AND CARBON-14 ROBENIDINE MODEL ECOSYSTEMS

	14C-ROBENIDINE		14C-DDT	
COMPONENT	PPM	BCF	PPM	BCF
WATER	0.001	-	0.000014	-
ALGAE	0.039	33	0.0022	139
SNAILS	0.0124	10	0.0034	214
MOSQUITOLARVAE	0.181	176	0.0036	314
FISH	0.081	66	0.0180	1129

[1/] CALCULATED AS PARENT

[2/] $BCF = \frac{\text{CONCENTRATION OF CARBON-14 RESIDUES IN THE ORGANISM}}{\text{CONCENTRATION OF CARBON-14 RESIDUES IN THE WATER}}$

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concentration of carbon-14 residues in the organism to the concentration of carbon-14 residues in the water, is also shown. The data clearly indicates that the BCF values obtained in all the elements of the food chain were higher for the carbon-14 DDT experiment compared with those for the carbon-14 robenidine experiment. This difference is more prominent in the case of snails and fish, that is, 214 vs 10 and 1129 vs 68, respectively. All these results suggest that the use of excreta as manure from birds kept on a diet containing robenidine will not result in any biological magnification of robenidine-related residues in the elements of the environment.

Conclusion

The modified terrestrial-aquatic model ecosystem described here has been found to be a useful tool in studying the environmental fate of drugs and related residues present in animal excreta used as manure. The operation of the ecosystem is relatively simple and yet it allows one to study the complex metabolic transformations of a drug or related residues in its various components. Especially interesting is the study of the degradation of a compound in the soil in the presence of microorganisms found in the animal excreta. This information is important since it eventually determines whether a compound and/or its metabolites will bioaccumulate in the various elements of the environment.

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Modeling Aquatic Ecosystems for Metabolic Studies

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Model ecosystems have been used for about 8 years to measure the distribution and fate of pesticides in the aquatic environment. Over that period of time numerous design changes have evolved that have increased the versatility of the ecosystem and improved simulation of environmental conditions. In our laboratory, we have used the static model ecosystem primarily to model the pond or small lake environment, and to simulate the likely rates and modes of pesticide entry (1). More recently, we have developed larger systems capable of providing sufficient biomass for accumulation and dissipation rate determinations (2) and for metabolic studies (3).

The primary purpose of this project was to demonstrate that aquatic model ecosystems could be further scaled up in size to provide greater amounts of the components (biomass, soil and water) to satisfactorily study metabolism kinetics. We used trifluralin, a dinitroaniline herbicide, since its metabolic pathways are well known and the metabolites were readily available.

Methods and Materials

Chemicals and Experimental Chambers

The chemical name and structure of trifluralin and the eight metabolites used in this study are given in Table I. All nine compounds had a chemical purity greater than 98.7% and the [^{14}C-ring]trifluralin (specific activity 45.25 μCi/mg) had a radio purity greater than 97%.

Fourteen kg of Matapeake silt loam (pH 5.3, 1.5% O.M.; sand, silt, and clay contents of 38.4, 49.4, and 12.7% respectively) were treated with [^{14}C]trifluralin at the rate of 10 ppm and introduced into the ecosystem tanks as described below. The control tank contained fourteen kg of untreated soil.

Glass aquaria, measuring 75 x 29 x 45 cm, were used as the ecosystem chambers (Figures 1 and 2). Twenty-four soil sampling

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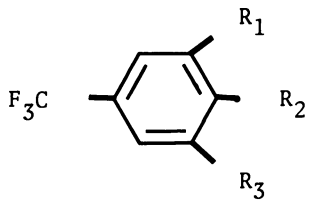
Table I. Trifluralin and its Metabolites.



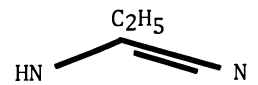
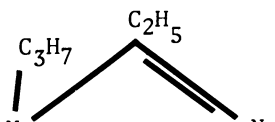
Compound No.	Name	R1	R2	R3
1	2-Ethyl-4-nitro-6-(trifluoromethyl benzimidazole	NO_2		
2	2-Ethyl-7-nitro-1-peopyl-5-(trifluoromethyl)-benzimidazole	NO_2		
3	α,α,α-Trifluoro-2,6-dinitro-N(2-hydroxypropyl)-p-toluidine	NO_2	$HN-CH_2CHOHCH_3$	NO_2
4	α,α,α-Trifluoro-2,6-dinitro-N(3-hydroxypropyl)-p-toluidine	NO_2	$HN-CH_2CH_2CH_2OH$	NO_2
5	α,α,α-Trifluoro-N^4,N^4-dipropyltoluene 3,4,5-triamine	NH_2	$N-(C_3H_7)_2$	NH_2

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Table I (continued)

6	α,α,α-Trifluoro-5-nitro-N^4-propyltoluene-3,4-diamine	NO_2	$HN-C_3H_7$	NH_2
7	α,α,α-Trifluoro-2,6-dinitro-N-propyl-p-toluidine	NO_2	$HN-C_3H_7$	NO_2
8	α,α,α-Trifluoro-5-nitro-N^4,N^4-dipropyltoluene-3,4-diamine	NO_2	$N(C_3H_7)_2$	NH_2
9	α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine	NO_2	$N(C_3H_7)_2$	NO_2

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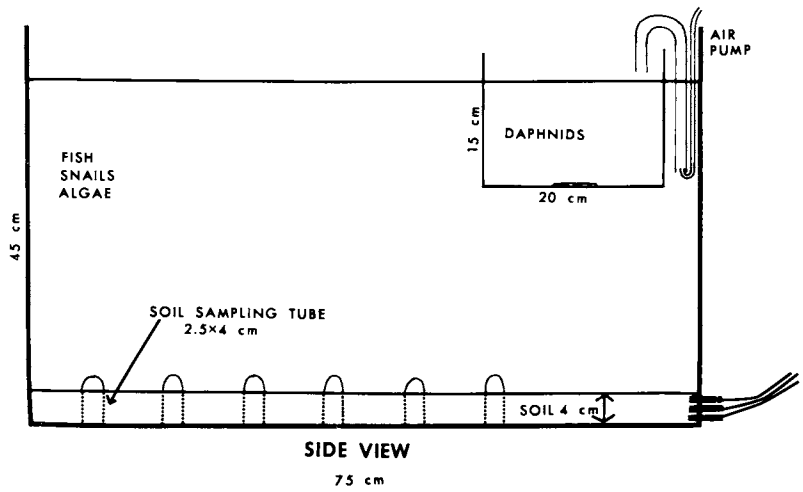

Figure 1. Side view of ecosystem chamber detailing relative proportion and distribution of ecosystem components

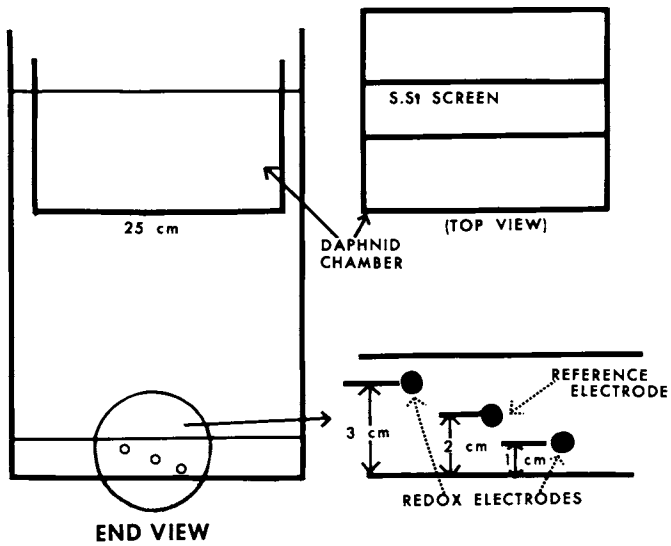

Figure 2. End view of ecosystem chamber showing detail of daphnid chamber and positioning of redox electrodes

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tubes (2.5 cm. dia x 4 cm high; Figure 1) were randomly located on the bottom of the tanks. An aluminum wire hoop was cemented (silicon sealant) to each tube to aid sample retrieval. At one end of the control tank (Figure 2) three holes were drilled, 1, 2, and 3 cm from the bottom and 2 cm apart (horizontally). Two platinum disc (top and bottom holes) and one calomel reference (center hole) electrodes were cemented in place (silicon rubber sealant). These electrodes were used to measure the Eh of the soil. The soil (treated or untreated) was uniformly distributed over the bottom of the tanks and in the sampling tubes. Only the hoop on the sampling tubes protruded from the distributed soil. The three tanks were then filled with 84 liters of water.

One day after flooding, 75 bluegill fish (Lepomis macrochinus), 60 snails (Helisoma sp.), 2 grams algae (Oedogonium cardiacum) and several hundred daphnids (Daphnia magna) were added to the chambers. The daphnids were placed in a special glass tank (25 x 20 x 15 cm) suspended near the water surface in the large tank (Figure 2). An opening in the tank bottom was covered with a stainless steel screen with a mesh sufficiently small (0.38 mm) to restrict the passage of daphnia. A percolator water pump continuously pumped water into the daphnid tank, ensuring uniform mixing of the water and transport of food to daphnids. The experiment was conducted in the greenhouse using natural light at an average temperature of 27 ± 3 C.

Sampling and Analysis. Water samples (triplicate 1 ml) were taken at 2-day intervals and analyzed by standard liquid scintillation (LS) methods for total ^{14}C; 100 ml samples were taken 2, 5, 9, 15, 22, 30, 43, 48, and 58 days after the start of the experiment,; these were extracted twice with 50 ml of ethyl acetate:hexane (7:3 v/v) and the extracts were reduced to 20 ml and analyzed by LS and TLC.

Two soil sampling tubes were removed from each tank 2, 5, 9, 15, 27, 30, 43, and 58, and 72 days after the start of the experiment, then frozen and stored for later analyses. For analyses, the frozen soil cores were removed from the glass tubes (by brief immersion in hot water), then sectioned into four 1-cm cylinders representing 0-1, 1-2, 2-3, and 3-4 cm soil depths. Samples from each depth were shake-extracted with 100 ml ethyl acetate:hexane overnight, and again with 100 ml methanol overnight. Extracts were filtered, concentrated to 20 ml and analyzed by LS and TLC as described below.

Samples of organisms (7 fish, 6 snails, 0.5 g algae, and 0.5 to 1.5 g daphnids) were taken after 2, 5, 9, 15, 22, and 30 days. All remaining organisms were removed after 42 days and additional organisms were added (27 fish, 30 snails, 2 g algae, and several hundred daphnids) the same day. Samples of organisms were again taken on Days 44, 48, 58 and 72. All samples were weighed, then frozen for later processing. Algae samples were oxidized to determine total ^{14}C. Fish, snails, and daphnids were homogenized in methanol and analyzed by LS and TLC.

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The identity of trifluralin and compounds 1-8 were determined by co-chromotography as follows. Extracts from soil, water, and organisms were reduced (under N_2) to 0.1 ml, combined with 10 to 20 µl each of compounds 1-9; then spotted on silica gel TLC plates (20 x 20 cm FG-254, E. Merck, Darmstadt). The plates were developed two dimensionally, first in benzene for 15 cm, then in benzene:ethyl acetate:acetic acid (60:40:1). Spots corresponding to the nine compounds were located visibly and by UV light, then scraped and analyzed by LS. In addition, the origin and a diffuse zone between the origin and compound 1 were scraped and are termed "polar" and "nonpolar" metabolites, respectively (Figure 3).

Results and Discussion

All organisms thrived in the systems with no loss of fish or snails. Numerous egg clusters and small snails were evident in all tanks by Day 42 (when the remaining organisms were harvested). These egg clusters and small snails were not removed since the second set of snails was sufficiently large that identity at harvest was not a problem. The algae weight increased from 2 g (initial) to an average of 9.4 g in the treatment tanks and 26.3 g in the control. However, for the second set of algae, there was no growth difference during the exposure period (Days 42 - 72). The concentration of trifluralin in water decreased rapidly with time (which will be discussed in detail later in this paper). Thus any initial effect of reducing algae growth was lost with time. Daphnids reproduced (in both treatment and control tanks) quickly increasing their mass at least 10 times in 15 days and then maintained this density for up to 42 days. The second set of daphnids behaved similarily.

Degradation of Trifluralin in Submerged Soil. The redox potential of the soil became negative after only 3 days and reached a low of -450 mv after 32 days (Table II). There was little difference in the measurements between the 1 and 3 cm soil depths. We therefore assume that all soil samples (except possibly at Day 2) were anaerobic.

Only about 6% of the original ^{14}C applied to soil as ^{14}C-trifluralin was lost after 72 days (Figure 4). Also, the acetate: hexane and methanol extracts) steadily decreased with time to a low of about 58% after 72 days. (Values shown in Figure 4 are the average of the four depths since there was no significant difference in the ^{14}C content between them). These results show that the diffusion of trifluralin and/or its metabolites from a submerged soil is a slow process, while at the same time, conversion to the "bound" or nonextractable form (35 to 40% at 72 days) occurs. Probst et al. (4) found more rapid conversion to bound residues: nearly 60% of the total ^{14}C from a submerged soil was unextractable after only 14 days.

The analysis of the soil extracts by TLC is shown in Table III. (The percentage values are based on the distribution of ^{14}C

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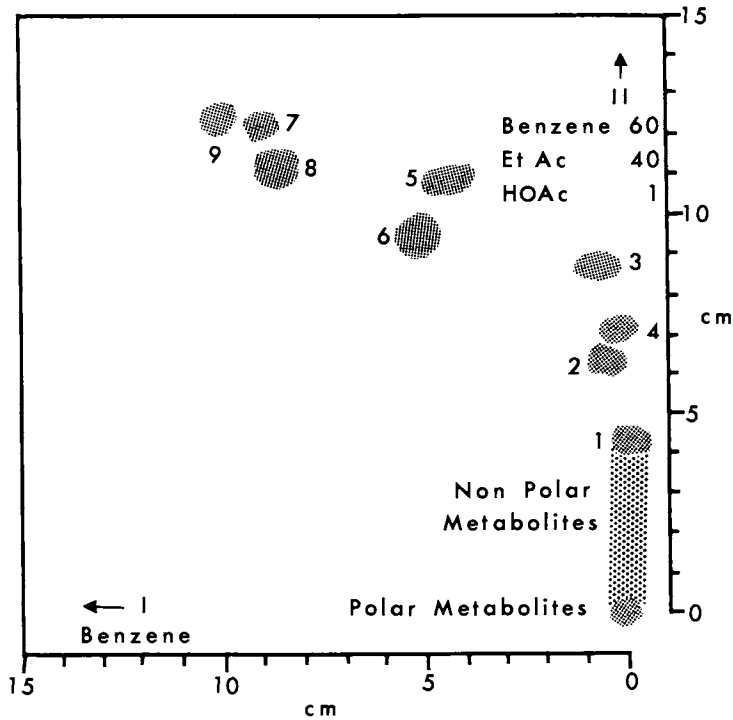

Figure 3. TLC system used to separate trifluralin and metabolites extracted from ecosystem components

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Table II. Redox potentials (expressed in millivolts) at two depths in a flooded soil.

Days	Soil depth[a] 1 cm	3 cm
0	+300	+300
1	+205	+220
2	+15	+40
5	-210	-80
6	-260	-140
7	-290	-250
8	-320	-270
9	-340	-300
12	-360	-320
16	-380	-350
19	-405	-370
22	-430	-395
25	-450	-420
30	-460	-440

[a] Measurements taken 1 and 3 cm below soil surface.

[b] Days after start of experiment.

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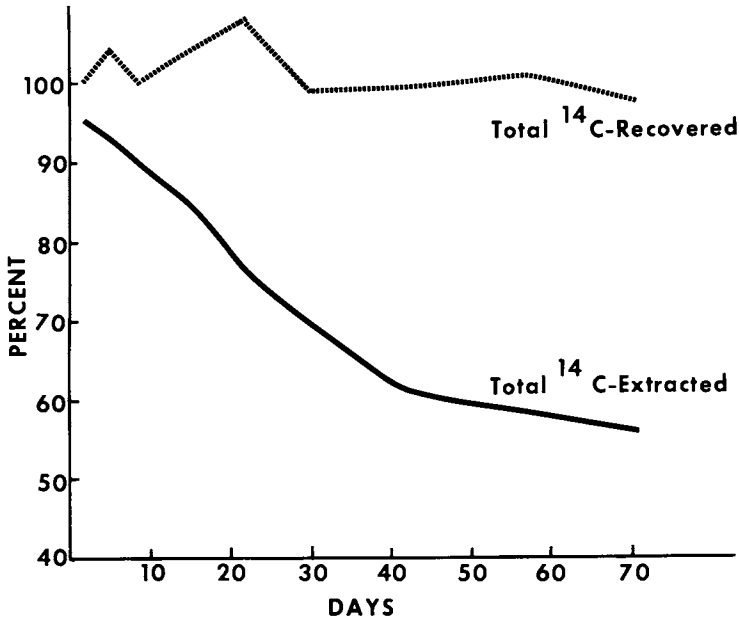

Figure 4. Residual and extractable ^{14}C from ^{14}C-trifluralin-treated anaerobic soil

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Table III. Degradation of [^{14}C]trifluralin in a flooded soil (expressed as percent of extracted radioactivity).

Compounds	Days after start of experiment					
	2	9	22	30	42	71
Polar metabolites[b]	1.2	2.1	5.6	6.8	8.0	17.8
Nonpolar metabolites[c]	0.4	3.0	15.0	18.0	11.7	10.6
1	0.3	.9	2.1	4.2	16.4	14.3
Unknown[d]	3.4	5.0	2.5	1.6	1.6	1.2
2	0.3	0.5	0.9	1.0	1.0	1.0
3	1.1	0.6	0.9	1.0	1.2	1.3
4	1.1	3.6	2.2	2.0	2.2	2.5
5	0.5	0.6	2.0	4.6	6.2	5.9
6	0.1	0.2	0.2	0.3	0.4	0.3
7	2.1	1.8	7.4	2.7	3.0	4.5
8	0.1	2.8	13.3	13.0	7.9	1.3
Trifluralin	89.4	78.9	47.9	44.8	40.4	39.3

[a] Values represent percent of total ^{14}C recovered from soil extracts (ethyl acetate:hexane and methanol) by TLC methods.
[b] ^{14}C recovered from the orgin.
[c] ^{14}C recovered in a zone between orgin and compound 1.
[d] ^{14}C recovered between compounds 1 and 2.

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recovered from the individual TLC plates, representing the ^{14}C in the extracts, not the total soil). Trifluralin appeared to degrade both by sequential reduction of the nitro groups (Compounds 8 and 5) and by dealkylation (Compound 7), Table III, Figure 3. These are all recognized decomposition products in the anaerobic degradation pathway as proposed by Probst et al (4). The intermediates in the formation of Compound 1 in our system are unknown, since it could form either from trifluralin directly (via several intermediators) or from Compound 7 (Figure 5). However, our recovery of Compound 1 supports a previous observation (5) that it forms under anaerobic conditions, but probably is a minor pathway.

The accumulation of polar and nonpolar metabolites also agreed with previous studies. Compounds 2, 3, 4, and 6 were probably not present in our system as indicated by the low recovery of ^{14}C (1 to 3.6%). The level of ^{14}C was too low to confirm the presence of these compounds by TLC.

In general, the degradation products we obtained from the submerged soil of our ecosystem were very similar to those reported by previous investigators (4, 6). However, the rate of trifluralin degradation was much slower. For example, about 8% of the total ^{14}C in the soil was trifluralin after 58 days (confirmed by electron capture gas chromatography) as compared to about 6% after 14 days by Probst et al. (4). Soil organic matter may have been responsible for the degradation rates. Parr and Smith (6) measured the extent of trifluralin degradation in a silt loam, amended and unamended with 1% alfalfa meal, under anaerobic conditions. After 20 days, the amount of trifluralin recovered was 1% and 68% in the amended and unamended soils, respectively. The organic matter in our soil may not have promoted rapid microbial degradation and therefore resulted in a slower rate of degradation.

Degradation of Trifluralin in Water. The amount of ^{14}C recovered from water with time is shown in Table IV. About half of the total ^{14}C (direct count analysis) in water was recovered by extracting twice with ethyl acetate:hexane (7:3), indicating that polar metabolites rapidly formed. Also, the concentration of ^{14}C in water increased most rapidly during the first 22 days, after which the rate decreased. Analysis of the water extracts by TLC is shown in Table V. Trifluralin disappeared very rapidly, decreasing to nondetectable levels between 9 and 22 days. Even as early as 2 days only 46% of the recovered ^{14}C was trifluralin. A sequencial reduction of the nitro group (decrease in the concentration of Compound 8 with time followed by an accumulation of Compound 5) is indicated by the results (Figure 6). Compound 1 also increased rapidly in concentration early in the experiment then maintained a lower but constant concentration between Days 22 and 58. Polar metabolites rapidly increased to 52% of the total recovered ^{14}C by Day 9, then gradually decreased while the concentration of nonpolar metabolites continuously increased with time.

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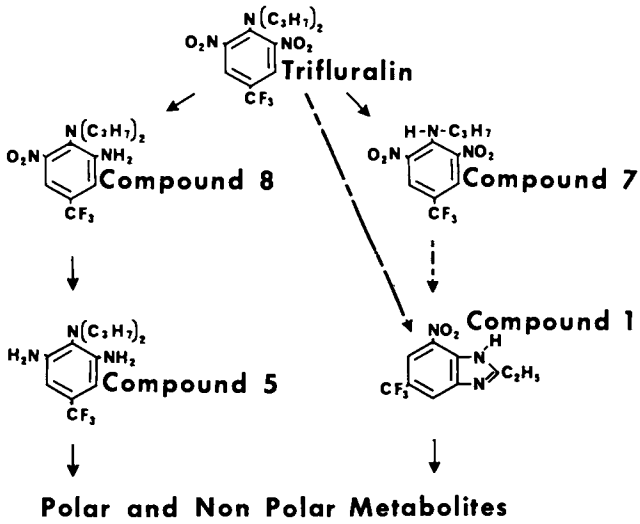

Figure 5. Postulated pathway of trifluralin degradation in an anaerobic soil from a model ecosystem

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Table IV. Concentration of ^{14}C in ecosystem water (expressed on ppb parent compound).

Days [a]	Direct count [b]	Extraction [c]
2	4.7	2.8
5	7.5	3.5
9	11.0	5.4
15	19.1	11.0
22	27.5	9.9
30	29.9	13.6
43	34.8	15.4
48	36.2	14.7
58	38.1	-
72	37.3	-

[a] Days after start of experiment

[b] ^{14}C recovered from 1 ml water samples.

[c] ^{14}C recovered from water extracted 2 times with ethyl acetate:hexane (7:3).

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Table V. Degradation of [^{14}C]trifluralin in ecosystem water (expressed as percent of extracted radioactivity) [a]

Compounds	Days after start of experiment							
	2	5	9	22	30	43	48	58
Polar metabolites [b]	10.7	18.9	52.0	15.5	9.5	13.5	11.2	8.0
Non-polar metabolites [c]	0	8.2	9.1	58.4	72.9	71.2	73.1	62.4
Unknown [d]	2.5	4.8	5.9	8.6	4.8	1.6	3.3	19.4
1	11.2	18.7	16.3	5.8	5.9	5.5	5.6	5.4
2	0.5	4.7	2.0	1.0	.4	0	0	0
3	1.3	2.6	.6	0	0	0	0	0
4	0	0	.9	.3	0	0	0	0
5	0.7	0.6	0.2	8.8	6.5	8.2	6.8	4.8
6	0.6	2.3	2.1	1.2	0	0	0	0
7	0	.5	1.6	0	0	0	0	0
8	26.2	9.3	2.2	0.4	0	0	0	0
Trifluralin	46.3	29.3	7.1	0	0	0	0	0

[a] Values represent percent of total ^{14}C recovered from water extract (ethyl acetate:hexane) by TLC methods.
[b] ^{14}C recovered from the orgin.
[c] ^{14}C recovered in a zone between the orgin and compound 1.
[d] ^{14}C recovered between compounds 1 and 2.

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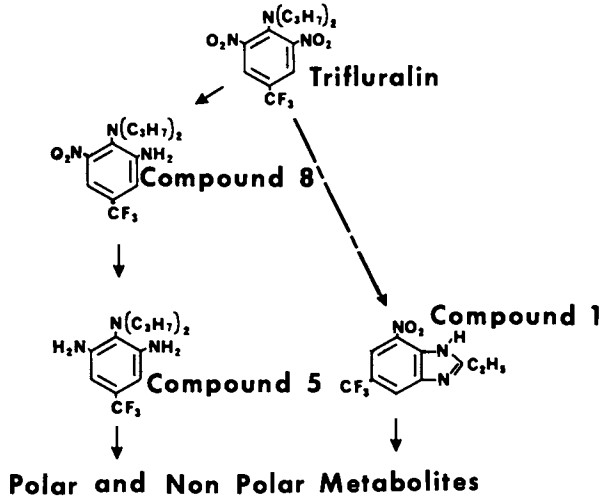

Figure 6. Postulated pathway of trifluralin degradation in water from a model ecosystem

Trifluralin is known to undergo rapid photodecomposition on physical surfaces (7) and degrades rapidly in shallow water exposed to sunlight (8). The recovery of only 46% of the total ^{14}C as trifluralin after 2 days suggests that photodegradation significantly contributed to trifluralin degradation in our ecosystem. The data supports this conclusion since Compound 1, a known photoproduct of trifluralin (9), represents a substantial amount of the total ^{14}C during the first 9 days of the experiment (corresponding to the highest concentration of trifluralin).

Degradation of Trifluralin in Aquatic Organisms. The rapid degradation of trifluralin in water significantly affected the type and amount of metabolites recovered from fish, snails, daphnids and algae (Tables VI - IX). In general, the amount of trifluralin recovered was highest for the initial sampling periods, then decreased to nondetectable levels between 22 and 42 days. This trend closely follows the trifluralin concentration in water, where trifluralin accounted for about 25% of the total ^{14}C (1.3 ppb) on Day 2. This may also explain why polar metabolites accounted for the highest proportion of the ^{14}C recovered, even for the first sampling periods. Compound 1 was the next most prevalent product recovered from organisms. The ^{14}C distribution was more complex in daphnids (Table VIII) than for fish, snails, or algae, where compounds 8, 2 and nonpolar metabolites were also detected. (No data was shown in Table VIII before Day 15 since ^{14}C for TLC analysis was insufficient). Interpretation of the aquatic organism data is difficult since we could not determine whether specific compounds (primarily the polar metabolites) were formed in the organism or were absorbed from water. However, water was probably the major source of the recovered compounds since little difference was noted between organisms analyzed on Day 42 and 44, representing exposure times of 42 vs. 2 days, respectively. (All organisms were removed from the tanks on Day 42 and replaced with a new set the same day).

Far more trifluralin was initially recovered (Days 2 and 5) from algae than any of the other organisms (Table IX). However, after 30 days the polar and nonpolar metabolites accounted for 75% or more of the recovered ^{14}C indicating that the algae were also responding to the rapid loss of trifluralin from water. The initial relatively high concentration of trifluralin may account for the lower accumulation of algae biomass in the treated tanks as compared to the control.

Only one other study has been conducted to evaluate the fate of trifluralin in an aquatic model ecosystem (10). In their system, trifluralin persisted much longer in water than in our study (probably due to less photodegradation through the use of artificial light). As a result, they reported much higher concentrations of trifluralin in snails and fish than we found, but no residues in daphinds. They also reported the presence of Compound 7 plus several other known and unknown metabolites.

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Table VI. Degradation of [^{14}C]trifluralin in bluegill fish (expressed as percent of extracted radioactivity)[a]

Compound	Days after start of experiment							
	2	5	9	15	22	30	42	44
Polar metabolites[b]	58.6	67.5	54.6	60.9	62.9	53.3	60.2	63.4
Nonpolar metabolites[c]	3.3	5.2	5.6	4.1	3.9	5.6	1.5	1.8
1	3.8	4.9	7.0	13.8	20.6	29.8	28.8	25.6
2	3.9	4.4	3.3	3.4	2.1	4.0	4.6	6.0
3	1.2	0.2	1.4	3.2	1.4	1.3	0.9	0.5
4	1.5	1.2	0.6	2.2	1.8	2.0	1.2	0.7
5	1.7	0.8	2.7	0.8	1.7	1.1	0.4	0.9
6	1.8	1.1	4.3	1.0	1.1	0.4	0.6	0.4
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
Trifluralin	24.2	14.7	20.5	10.6	4.5	2.5	1.8	0

[a] Values represent percent of total ^{14}C recovered from fish extracts (methanol) by TLC methods.
[b] ^{14}C recovered from the orgin.
[c] ^{14}C recovered in a zone between the orgin and compound 1.

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Table VII. Degradation of [^{14}C]trifluralin in snails (expressed as percent of extracted radioactivity)[a]

Compounds	Days after start of experiment										
	2	5	9	15	22	30	42	44	48	58	72
Polar metabolites[b]	56.9	60.4	62.7	57.5	79.0	71.6	76.5	75.8	73.6	75.4	79.4
1	22.2	12.6	12.0	15.5	7.7	8.8	10.9	18.7	21.9	16.6	10.3
2	3.5	10.1	7.8	6.6	1.0	5.6	5.3	4.6	2.1	2.7	2.1
3	6.9	3.9	0	3.5	1.4	2.5	2.4	0	0.6	1.4	4.6
4	3.5	4.4	4.9	6.7	0	1.3	3.0	1.4	0	2.0	2.7
5	0	0	0	0	6.2	10.2	1.1	0	2.0	1.6	0.9
6	0	2.6	2.3	0	0	0	0.8	0	0	0	0
7	0	0	1.8	0	1.0	0	0	0	0	0	0
8	2.5	4.3	3.6	3.3	2.8	0	0	0	0	0	0
Trifluralin	4.7	1.9	2.6	6.0	1.1	0	0	0	0	0	0

[a] Values represent percent of total ^{14}C recovered from snail extracts (methanol) by TLC methods.

[b] ^{14}C recovered from the orgin.

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Table VIII. Degradation of [^{14}C]trifluralin in daphnids (expressed as percent of extracted radioactivity)

Compounds	Days after start of experiment							
	15	22	30	42	44	48	58	72
Polar metabolites[b]	14.1	59.7	54.6	73.9	64.6	62.3	65.9	65.9
Nonpolar metabolites[c]	5.2	3.6	8.1	7.8	7.9	8.3	10.0	15.5
1	7.8	3.9	9.8	7.0	16.0	9.4	11.2	8.9
2	16.6	12.1	15.7	6.7	5.5	15.8	11.0	7.0
3	4.0	0.3	0.3	0	0	0.4	0	0.4
4	0	1.5	2.8	1.4	0	1.6	1.2	2.3
5	1.0	0.9	1.7	1.0	6.0	1.2	0	0
6	1.0	0.8	0	0	0	1.0	0.7	0
7	2.3	0	0	0	0	0	0	0
8	12.0	7.5	2.3	0.7	0	0	0	0
Trifluralin	36.0	9.7	4.7	1.5	0	0	0	0

[a] Values represent percent of total ^{14}C recovered from daphnid extracts (methanol) by TLC methods.
[b] ^{14}C recovered from the orgin.
[c] ^{14}C recovered in a zone between the orgin and Compound 1.

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Table IX. Degradation of [^{14}C]trifluralin in algae (expressed as percent of extracted radioactivity)[a]

Compounds	Days after start of experiment									
	2	5	15	22	30	42	44	48	58	72
Polar metabolites[b]	1.8	3.4	34.2	47.7	54.8	54.2	64.3	75.8	61.5	57.2
Nonpolar metabolites[c]	0	0.6	8.6	9.1	22.6	20.4	28.6	21.2	28.7	27.7
1	5.4	0.6	14.3	11.4	2.3	6.1	7.1	3.0	5.8	13.1
2	1.8	1.7	0	2.3	0	0	0	0	2.0	2.0
3	0	0	8.6	8.9	0	8.2	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	1.7	5.7	0	0	0	0	0	0	0
6	0	1.2	0	0	0	0	0	0	0	0
7	11.0	7.6	0	6.8	0	0	0	0	0	0
8	5.4	1.2	0	2.3	0	0	0	0	0	0
Trifluralin	74.5	82.0	28.6	11.4	19.3	6.1	0	0	0	0

[a] Values represent percent of total ^{14}C recovered from algae extracts (methanol) by TLC methods.
[b] ^{14}C recovered from the orgin.
[c] ^{14}C recovered in a zone between the orgin and Compound 1.

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The objective of this investigation, to demonstrate that aquatic model ecosystems can be scaled up in size to perform time-related, metabolic studies, was only partly achieved. We successfully demonstrated that the degradative pathways and rates of trifluralin metabolism in an anaerobic soil can be determined in an ecosystem just as well as they can in laboratory studies (4). Also, the recovery of Compound 1 from water substantiated the significance of photodegradation, which had previously been measured under laboratory conditions (9). However, metabolic studies in aquatic organisms were not very successful because (i) the trifluralin in water decreased very rapidly, and (ii) the origin of the few metabolites that were recovered was not clear. Accumulation of the ^{14}C-labeled compounds in water by the organisms was apparently the predominant source. (The major problem in studying the metabolism of pesticides in aquatic organisms retrieved from model ecosystems is identifying the source of the metabolites, i.e., were they formed in the organisms through some metabolic process or were they absorbed from water?).

These results indicate that our scaled-up model ecosystems are more useful for studying system processes than processes that function in individual components of the environment. In this regard, a preliminary large scale ecosystem study could be very useful to indicate parameter limits such as overall degradation rates and likely concentrations of parent compounds plus metabolites over time. Such information would be useful in the design of metabolic studies in various components of the ecosystem. In addition, the large scale ecosystem study could also be used to determine if processes derived under laboratory conditions continue to function and/or predominate when combined in a complex system.

Abstract

This project was designed to demonstrate that the static water model ecosystems can be scaled up in size to provide sufficient amounts of biomass, soil, and water to study metabolism kinetics of pesticides. Fourteen kg of soil, treated with [^{14}C]-trifluralin at 10 ppm, was flooded with 84 liters water. Bluegill fish, snails, daphnids, and algae were exposed to this system for 72 days. Samples of soil, water, and organisms were periodically analyzed for trifluralin and eight metabolites. The recovered metabolites and their rate of formation closely followed previously reported values for anaerobic soil and water, substantiating the utility of the model ecosystem for metabolic studies. However, residues in the biomass were apparently derived primarily through absorption from water rather than metabolism within the organisms themselves. It was concluded that the scaled-up model ecosystem is more useful for studying system process than processes that function in individual components of the environment.

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Investigation of Xenobiotic Metabolism in Intact Aquatic Animals

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More than 130 years ago, Keller (1) reported the isolation of hippuric acid (benzoylglycine) from the urine of horses fed pure benzoic acid and so ushered in our modern era of metabolism investigations on xenobiotics (foreign substances in the environment). In addition to the valuable basic knowledge of the biological processes of terrestrial animals provided by such studies, the advent of regulations controlling the use of pesticides stimulated research on the disposition of these chemicals by both mammals and insects (2).

Among the requirements for registration of pesticides in the United States, the 1978 guidelines proposed by the U.S. Environmental Protection Agency (3) list general metabolism studies "in at least one mammalian species, preferably the laboratory rat ..." Although similar tests have been conducted on other terrestrial species with increasing frequency, the small rodents have remained the principal source of metabolism data from intact animals.

Standardized techniques and equipment for such investigations are in widespread use. Unfortunately, the same cannot be said for metabolism investigations in aquatic animals. Most of the world's animals exclusive of the insects -- over 200,000 known species -- live at least a part of their lives in water; over 100 species have major economic importance; and they form the populations most often at risk of exposure to a growing number of chemical pollutants, but science remains largely ignorant of the disposition of xenobiotics by intact, living specimens of even the most common of the aquatic animals.

In this article, we propose to discuss some reasons for this lack of information, compare characteristics of the principal experimental systems, and describe our current research on a scientific protocol and technique which would provide the functional equivalent of the standard terrestrial metabolism study but applied to aquatic species.

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Laboratory Metabolism Chambers

According to the EPA guidelines mentioned above, metabolism studies (in rodents) have the following purposes:

(1) To identify and, to the extent possible, quantify significant metabolites;
(2) To determine any possible bioaccumulation and/or bioretention of the test substance and/or metabolites;
(3) To determine [pesticide] absorption as a function of dose;
(4) To characterize routes and rates of [pesticide] excretion;
(5) To relate [pesticide] absorption to the duration of exposure of the animal; and
(6) To obtain an estimate of binding of the test substance and/or its metabolites by target macromolecules in potential target organs.

The required data generally are obtained by administering a measured dose of the candidate compound -- often isotopically labelled -- to the rat or mouse either by injection or *per os*. The animal is housed in a glass metabolism "cage" where it receives food, water, and clean air, and its urine, feces, and respired gases are collected and examined for the parent chemical and its metabolites. Eventual postmortem tissue analysis and calculation of material balance complete the measurements necessary to satisfy the above purposes of metabolism and pharmacokinetic experiments. While *in vitro* biochemical studies are important adjuncts, it is also apparent that only experiments with intact, healthy, living animals will suffice to meet EPA criteria.

Why is it that so little of this information exists for important aquatic species? The following represent some of the reasons:

(1) Lack of experimental animals. Highly standardized rats and mice are readily available in large numbers commercially, but few aquatics are similarly available and, when they are, suitable transportation is difficult.

(2) Maintenance and environmental control. While diet, breathing, temperature, and waste removal are virtually taken for granted in most rodent work, they form serious problems with aquatic animals. Knowledge of dietary requirements and prepared diets generally are nonexistent for most species; oxygen must be supplied and toxic gases removed; temperature maintenance and water composition are very important; and the decay of food waste and excreta must be avoided.

(3) Dosing. Rodents commonly are dosed by intubation, intraperitoneal injection, or in measured amounts of diet. Each of these routes can become extremely difficult to use with most aquatic species -- especially very small forms -- and absorption from the surrounding water becomes the primary

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route of exposure (largely analogous to inhalation exposure in mammals).

(4) Control of remetabolism. Contamination of bedding and coprophagy undoubtedly were drawbacks in earlier rodent work, but modern metabolism chambers remove excretory products rapidly. The excretory products of aquatics are released into the medium and so may be available for repeated reabsorption and remetabolism.

(5) Metabolite collection. Whereas the separation and collection of urine, feces, and respired gases is a simple matter with rodents, the quantitative isolation of microgram amounts of complex metabolites from large volumes of aqueous medium -- especially seawater -- has posed a major hurdle to studies in aquatic organisms.

Experimental aquatic metabolism systems have taken one of four forms -- static water in ordinary aquaria, jars, or beakers; static "model ecosystems"; static outdoor ponds; and continuously-flowing systems (Table 1). A very rough comparison of their advantages is shown in Table II. For example, while the static aquaria doubtless are by far the

Table II. Evaluation of aquatic metabolism chambers.

	SA[a]	ME	P	F
Test simplicity	+++	+	++	++
Environment control	++	++	+	+++
Species flexibility	++	+	++	+++
Dosing	++	+	+	+++
Remetabolism control	+	+	+	+++
Metabolite collection	++	+	+	+++

[a]SA = static aquarium, ME = model ecosystem P = pond, and F = flow-through system

simplest to set up and maintain, they provide less control of oxygenation, chemical loss, and other environmental factors than do flow-through systems, as well as less flexibility in the species used (e.g., some aquatic species are very sensitive to accumulated waste products).

So-called "model ecosystems" (12) offer environmental control similar to that in static aquaria but are much more complex and so far have been limited to a few carefully selected and compatible species -- typically, microcrustaceans, small snails, and minnows -- which are hardy and tolerate each other in reasonable balance. Remetabolism and collection of

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Table I. Examples of Metabolites Formed by Free-Living Aquatic Animals

Chemical	Animal	Major Metabolites	Identified in	Reference
Aldrin	Fresh-water Crustaceans, mussel, snail	Dieldrin	Tissues	4
	Green Sunfish	Dieldrin and hydroxy analogs	Water Tissues	5
2,4-D Butoxy-ethyl Ester	Trout, bluegill, channel catfish	2,4-D	Water Tissues	6
2',5-Dichloro-4'-nitrosalicylanilide	Midge	Chlorosalicyclic acid	Tissues	7
4,4'-Dichloro-biphenyl	Frog	3-Hydroxy analog and other phenols	Water Tissues	8
Dioctyl phthalate	Four fresh water species	Phthalic acid	Tissue	9
Methoprene	Bluegill	Metabolite pool	Water Muscle	10
Methoxychlor	Green Sunfish	Demethylated and dehydrochlorinated analogs	Water	5
3-Trifluoromethyl-4-nitrophenol(TMF)	Sea lamprey Trout	Glucuronide	Bile	11
Trifluralin	Green Sunfish	Dealkylated and reduced analogs	Water Tissues	5

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identifiable quantities of metabolites are still a serious problem, and while the initial input of chemical to the system may be known, the actual extent of exposure of each species presently is unmeasurable. Ponds can be fairly simple and offer the advantage of considerable species flexibility and a more natural environment -- there seems to be no reason why size of the test animal should be limiting in such a system -- although problems of dosing and metabolite collection can be severe. However, ponds add environmental degradation and evaporation processes which may obscure whether product formation and loss is real or an artifact.

Closed flow-through laboratory-scale systems appear to have the greatest potential for analogy to current terrestrial metabolism chambers. While obviously more complex than static aquaria, they allow closer control of environmental variables such as oxygenation and volatilization, adaptability to a wide range of species, maximum freedom from remetabolism, and improved collection of waste products. Dosing can be accomplished through immersion or, beforehand, by injection or intubation where appropriate. On the other hand, none of the four types of metabolism system is ideal, and the most complete data probably will be derived from a combination of methods.

Aquatic Metabolism Protocol

We have developed and tested a metabolism system and regimen which allows collection of data comparable to those from terrestrial animals. The key to our experiments is a metabolism chamber, described previously (13, 14) (Fig. 1), which can be operated in either the static or flow-through mode. Briefly, individuals or groups of animals are held at constant temperature in the jacketed glass chamber (A), on a stainless steel screen (B), while pure water or test solution is passed over them (or held under static conditions). Solid wastes are separated in a jacketed container (C) held near 0°C to minimize microbial action, and the effluent containing dissolved metabolites is passed onto a column of nonionic macroreticular adsoprtion resin where organic solutes are adsorbed from solution (D).

The general experimental protocol is summarized in Table III.

Table III. Proposed test protocol for aquatic metabolism

Step 1.	Toxicity rangefinding (static)
Step 2.	Metabolism/pharmacodynamics rangefinding (static)
Step 3.	Steady-state metabolism and disposition
Step 4.	Metabolite identification and measurement

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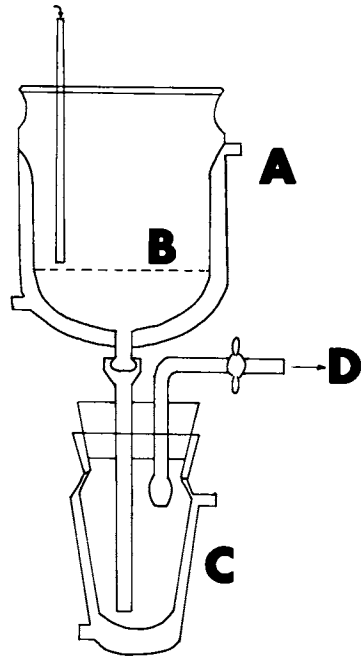

Figure 1. Aquatic metabolism chamber

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After determining a concentration of test compound which elicits no visually detectable response or effect in the aquatic species over a period of 48 hours (Step 1), fresh animals are placed in the chamber, exposed to known concentrations of test chemical (usually ^{14}C-labelled), and the uptake rate and major metabolites determined (Step 2). Depuration rate from the dosed animals also can be estimated at this point by transfer to untreated water. Fresh animals also can be exposed to a constant flow of test solution until an absorption-excretion equilibrium (steady state) has been established, dosed briefly with labelled compound, and release (turnover) rate determined (Step 3). Excreted metabolites are collected on the resin column as a result of both static and steady-state exposures, and their separation is accomplished by thin-layer, gas-liquid, and/or high-pressure liquid chromatography of the eluted residue (Step 4).

Separated metabolites (and parent compound) are quantitated by first scanning developed thin-layer chromatographic plates to locate radioactive spots by means of a radiochromatogram scanner and then accurately measuring radioactivity by scintillation counting. Unknowns are identified by the usual chemical and spectrometric methods.

Although previous applications of this technique in our laboratory had been concerned with aquatic animal metabolism of pesticides such as DDT, parathion, carbaryl, and trifluralin (14, 15), we also became interested in comparing metabolic routes by means of a "metabolic probe". Such a compound ideally should be stable to nonbiological degradation, of low toxicity to maximize the dose, and subject to as many major routes of metabolism as possible without undue analytical complexity.

p-Nitroanisole, whose metabolism in the mouse recently was investigated by Trautman (16), provided a satisfactory probe in which O-demethylation, ring-oxidation, nitro reduction, and O- and N-conjugation might logically be observed. Consequently, the metabolism of p-nitroanisole in marine invertebrates was chosen for comparison with that of the mammal.

p-Nitroanisole Pharmacodynamics in the Sea Urchin

The aquatic species chosen was *Strongylocentrotus purpuratus*, the purple sea urchin. This animal is a common resident of the California coast, a frequent pest in commercial kelp culture, and a specialty food item of growing interest. Phylogenetically, these echinoderms are considered to be in the invertebrate class most directly linked to the vertebrates.

p-Nitroanisole (PNA), uniformly ring-labelled with ^{14}C, was prepared from commercial ^{14}C-p-nitrophenol by the method of Ross (17) and purified by thin-layer chromatography in benzene-methanol (9:1 v/v). Sea urchins were collected at Salt Point, California, and transported in natural sea water

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to our Davis laboratory where they were transferred to artificial sea water (Instant Ocean^R) at the original ocean temperature of 10°C.

The acute toxicity of PNA to the sea urchin was measured by immersion of the animals at 12°C in Instant Ocean^R containing known levels of test compound (Table IV). After 24 hours, the animals were observed and transferred to untreated water,

Table IV. Acute toxicity of p-nitroanisole (PNA) in S. purpuratus[a]

Concentration (mg/l)	24 hr.	48 hr.[b]
10	No effect	No effect
50	2/3 Not attached,	No effect
100[c]	None attached, Spines in disarray	All attached weakly, Spines in rows
500[c]	None attached, Spines in disarray	All dead, Sloughing spines

[a] N = 3

[b] 24 Hrs. in untreated Instant Ocean^R

[c] Solubility of PNA is 71.8 mg/l at 15°C

and observed again after 24 hours. PNA showed no effect on sea urchins at concentrations of 10 mg/l (65 μM), and our further experiments were conducted below this level.

For the metabolism rangefinding experiments, sea urchins were dosed by injection at a level of 2.2 mg/kg of body weight with ^{14}C-PNA into the central cavity, held in untreated water at 12°C, and both the water and coelomic fluid sampled periodically over the course of 8 hours. Most of the PNA was eliminated rapidly into the water (Fig. 2), with a pseudo-first order half-life of appearance of 12 minutes; half of the test compound was lost from the coelomic fluid in about 24 minutes.

To determine absorption rate, another group of animals was immersed in a 5 mg/l solution of PNA in Instant Ocean^R (closed chamber) and the water sampled at intervals to measure the loss of radioactivity compared to controls (Fig. 3). The data were normalized for tissue weight, and equilibrium was reached in about 8 hours at a body burden of 10 μg/g (10 ppm) equivalent to a bioconcentration factor of 2.0 (winter animals). In another experiment, animals were collected at intervals, sacrificed, and their tissue analyzed for ^{14}C

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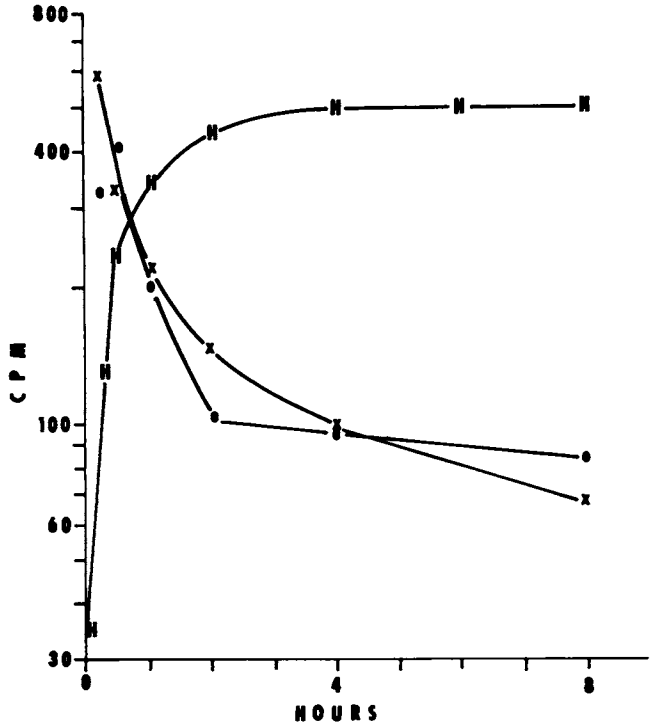

Figure 2. Excretion rate of PNA from S. purpuratus: *(H), radioactivity in the water (×, ○), radioactivity in coelomic fluid (2 replicates).*

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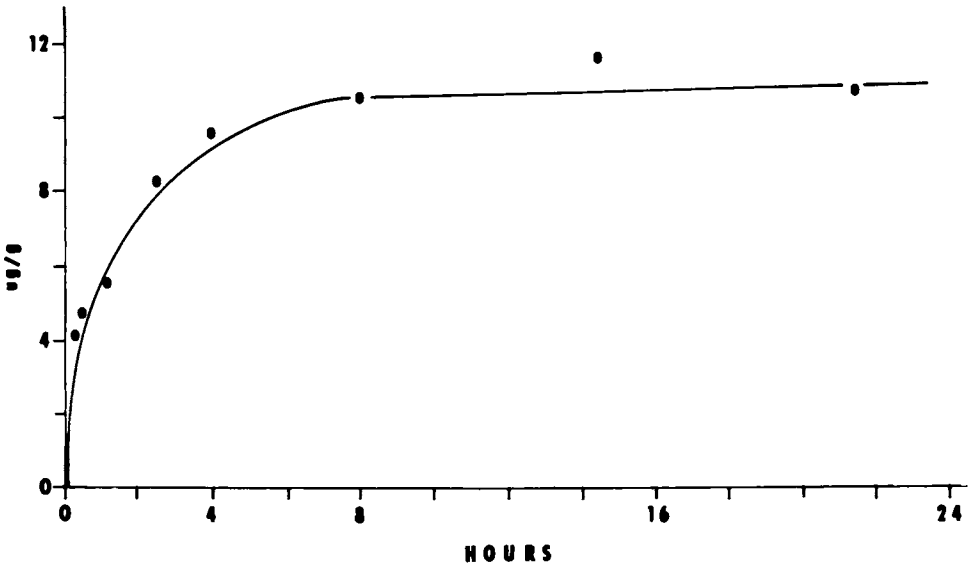

Figure 3. Absorption of PNA by S. purpuratus *from a 5-mg/L solution.*

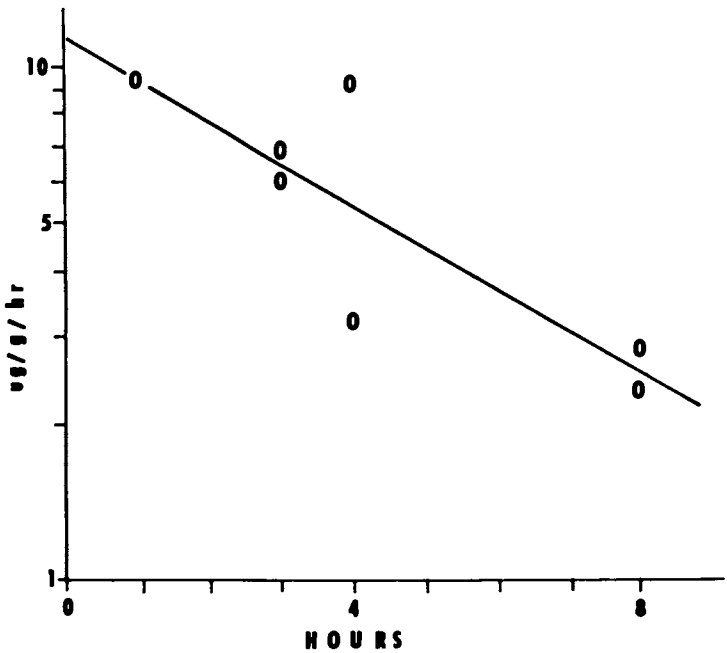

Figure 4. Absorption rate of PNA from a 5-mg/L solution

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(Fig. 4). The initial uptake rate was similar for both types of samples, but the bioconcentration factor in the second instance was 4.0 (summer animals).

When placed in pure water, the animals containing steady-state concentrations of PNA lost it at a rate of 9.4 μg/g/hr with a half-life (appearance in water) of 22 minutes; when they were placed in a 5 mg/l solution of unlabelled PNA, the rate of loss (turnover) of ^{14}C was similar to the depuration rate (Fig. 5).

p-Nitroanisole Metabolism

The soluble metabolites excreted from animals dosed by injection were collected on Amberlite^{R} XAD-4 resin, the resin eluted sequentially with diethyl ether, acetone, and methanol, and the solutes separated by thin-layer chromatography on silica gel and quantitated by liquid scintillation counting.

The products were identified by their chromatographic characteristics in comparison with authentic standards (Fig. 6) and represented 72% of the original dose; acid digestion of the sea-urchin tissue released another 21.5% of ^{14}C bound as unknown products, for a total accountability of 93.5%.

According to Trautman (16), PNA was converted almost exclusively to p-nitrophenol in the mouse with 24 hours, nearly all of which was excreted in the urine as glucuronide, sulfate, glucoside, and unextractable products; the mouse tissue retained only about 1% of the original dose. By comparison, the sea urchin metabolized PNA slowly; p-nitrophenol and its conjugates accounted for only about 6% of the metabolites and most of the remainder (90%) was p-anisidine (p-methoxyaniline) and its *N*-acetyl derivative.

Conclusions

It is apparent that the aquatic echinoderm and terrestrial mammal deal with a chemical probe by very different metabolic pathways. The exclusive formation of oxidized (*O*-demethylated) product in the mouse may partly reflect the animal's highly oxidizing environment, while the relatively anoxic marine environment is represented in the observed reduced metabolites of the sea urchin.

One purpose of metabolic transformations has been assumed to be conversion of a xenobiotic into more water-soluble form (18); as shown by partition coefficients (19), the hydrophilicity of p-nitrophenol (Kp 81) is much greater than that of PNA (Kp 107), and that of the corresponding nitrophenyl sulfate and glycosides must be greater still. On the other hand, while p-anisidine (Kp 9) is relatively soluble compared to PNA, the advantage would seem to be lost in conversion to "insoluble" p-methoxyacetanilide (Kp 14).

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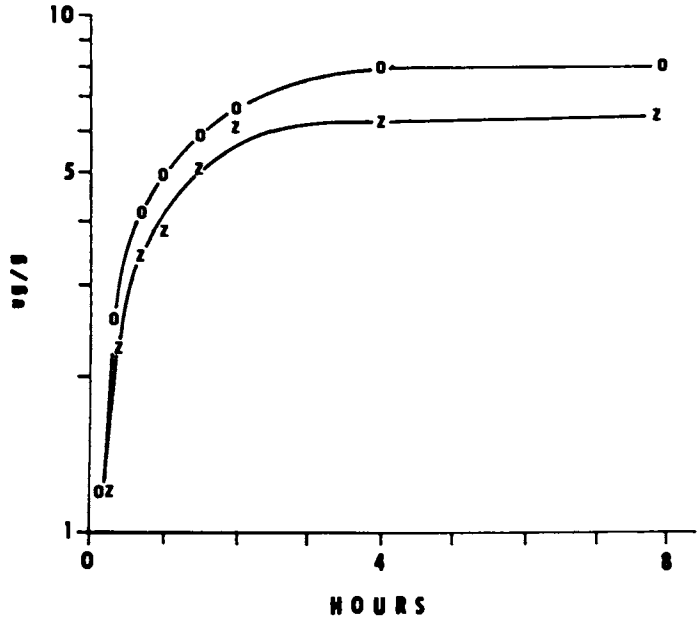

Figure 5. Turnover (O) and depuration (Z) of PNA at steady state

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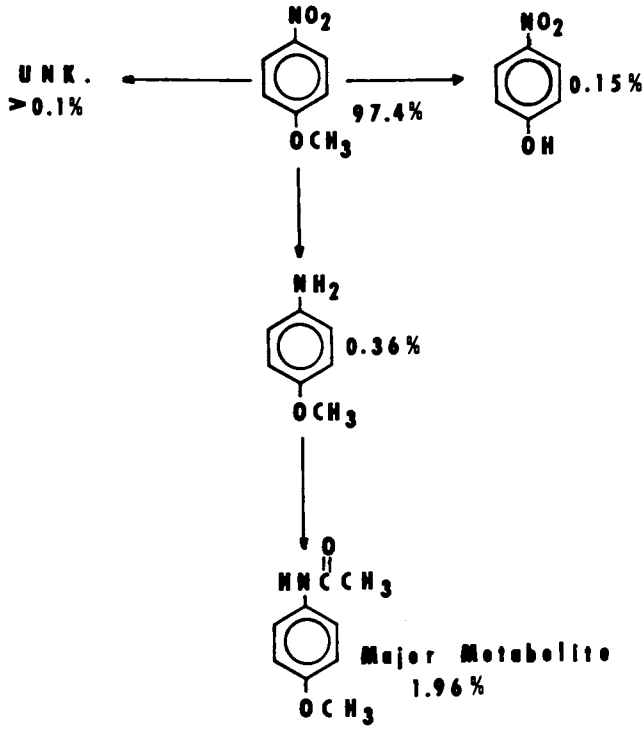

Figure 6. PNA metabolites excreted into water by S. purpuratus *during 8 hr*

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Our research shows that the described system allows the measurement of basic pharmacodynamic properties (not explicitly included in the EPA guidelines) and identification of xenobiotic metabolites from excreta in much the same way as they are obtained with terrestrial mammals. The original purposes of general metabolism studies, outlined at the beginning of this article, can be satisfied -- metabolite identification and quantitation, bioaccumulation, absorption and excretion, and binding to tissue -- as shown with a chemical probe such as p-nitroanisole. While *in vitro* measurements also give important insight and have received the major emphasis in aquatic metabolism studies (20, 21), it is obvious that their results presently are not so directly applicable.

The system is not without major needs and difficulties:

(1) More uniform animals will be required. The wild animals (such as *S. purpuratus*) are quite variable, and the stress of collection and transportation remains a problem;

(2) The system and protocol require extensive standardization and the specification of optimum (or at least generally suitable) conditions of temperature, oxygenation, water composition, flow rates, etc.;

(3) Comparison of results between these and the other major experimental chambers will be important, both because the others are in current use and because each can offer unique benefits;

(4) The model systems eventually must be compared against natural habitats. Although the results of the controlled, standardized laboratory tests give important basic and practical information (as do those from terrestrial metabolism chambers), there is no reason to believe that they represent quantitatively the behavior of the animals in Nature;

(5) Model systems eventually must be extended to accommodate and investigate populations of a species and communities composed of a number of species. Even more than with terrestrial animals, the aquatic communities provide opportunities for a wide range of modified exchange, uptake, excretion, and metabolism which represent more than the sum of the activities of the component species alone.

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Xenobiotic Transport Mechanisms and Pharmacokinetics in the Dogfish Shark

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Our interest in studying the disposition of xenobiotics in aquatic species stems from not only our desire to understand what these substances might do to fish, but also because such studies might forecast problems which might arise in man. Of course, we would also like to know the ultimate sites and mechanisms of action of these compounds, but before such lofty goals are achieved, there is much painstaking work which must be done to study the absorption, distribution (including binding and storage), excretion and metabolism of compounds foreign to living organisms. Having such information enables one to answer a series of questions such as: 1) What kinds of chemicals might cause fish kills? 2) Where in the fish body do they accumulate and 3) Does the xenobiotic itself or some biotransformed product exert the major action?

We were introduced to this fascinating area of research by Dr. David P. Rall, the current Director of National Institute of Environmental Health Sciences. We took what could be described as a pharmacokinetic approach to toxicology. In other words we considered that knowing where the body accumulates a xenobiotic and how long it takes an animal to rid itself of a compound, are important biologic properties to identify. For example, just as one can never ignore a toxicologist's observation that a drug caused a lesion to a particular organ, similar evidence that a xenobiotic localizes in one kind of tissue or organ must be heeded. Thus, in introducing us to such concepts Dr. Rall had us investigate the fate and distribution of the two relevant pollutants, DDT and methyl mercury (MeHg) in a highly relevant species, the lobster. We reported (1, 2) that most of the DDT accumulated in the lobster hepatopancreas and MeHg localized in the tail muscle. These findings are of obvious and profound significance, especially since these animals have been exposed to such pollutants.

Our research moved away from these crustaceans for two primary reasons. First, they are not typical of the more abundant aquatic species. Second, in one of the earliest xenobiotic

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disposition studies done in fish, Rall and Zubrod discovered what appeared to be an appropriate species since it yielded data similar to that reported in mammalian terrestrial species. The species used was the dogfish shark (spiny dogfish, Squalus acanthias) and the xenobiotic was the antimalarial quinine (3). Thus these workers found that the major distribution features which are somewhat unique for this drug, i.e., higher concentrations of the drug in red blood cells, brain and muscle than in plasma, were the same in the dogfish and mammals. This should not be surprising since the dogfish shark is regarded as anatomically and physiologically typical enough to be widely employed in comparative anatomy and physiology textbooks and laboratory manuals (4, 5). More recently, the organ distribution of metallic ions in fish was shown (6) to follow that seen generally in mammals.

Handling Advantages of the Dogfish Shark

Fuller exploitation of the shark as a model for xenobiotic distribution studies had to await the development of methods in two areas: 1) practical techniques for sampling two important physiologic compartments, biliary and urinary; and, 2) validation of pharmacokinetic parameters in this aquatic species. The former was recently accomplished (7, 8) with two substances with well-known predilections for the hepatic (bromsulphalein, BSP) and renal (phenol red) compartments. Thus, for these types of substances, fluid collecting techniques as shown in Figs. 1 and 2 demonstrated that 80% of the dose of BSP was excreted into shark bile in 48 hrs. (7) while in this same time period, 41% of the dose of phenol red appeared in urine and 49% was excreted via the bile in the dogfish (8). In other words most of these drugs were cleared from the shark via the bile and urine. Therefore, these studies demonstrated that the proposed technique greatly improved the "bookkeeping" for substances which would have otherwise been lost if these excretion routes were not adequately sampled. Finally, this fish model proved to be hardy enough to survive the simplest experimental techniques, such as confinement in an aquarium, minor surgery, and exposure to trace quantities of pollutants. With these general features of how the shark handles such xenobiotics Bungay et. al, (9) were able to build on the earlier research of others (10, 11), to establish the applicability of scaling processes in the direction opposite of that usually taken; in this case, pharmacokinetic models developed in intermediary species such as the mouse, were adapted to the lower fish species. Thus, they showed (Fig. 3) that the pharmacokinetic model gave predicted (dotted lines) values for phenol red in kidney, liver, plasma and (Fig. 4), bile and urine. It can be readily seen that most of the experimental points fell very close to the values predicted by the pharmacokinetic model.

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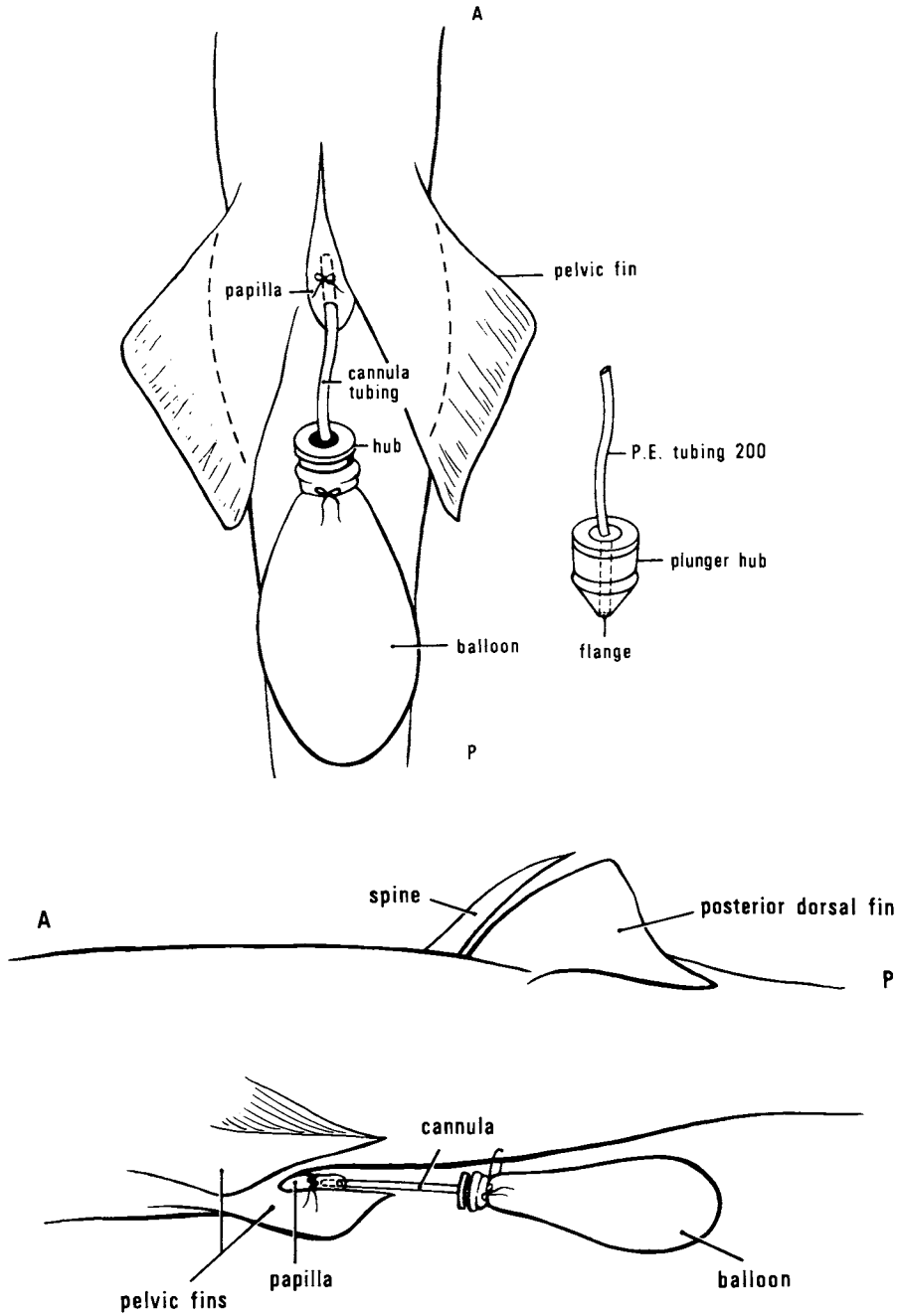

Figure 1. Technique for collecting urine from the dogfish shark

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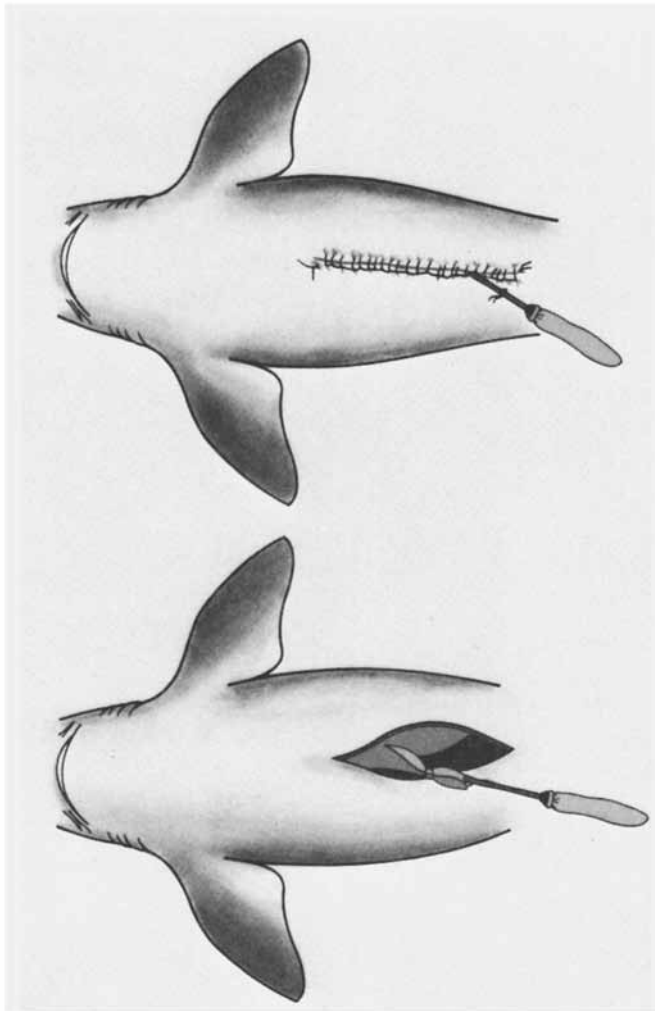

Figure 2. Technique for collection of bile via fistula in the dogfish shark

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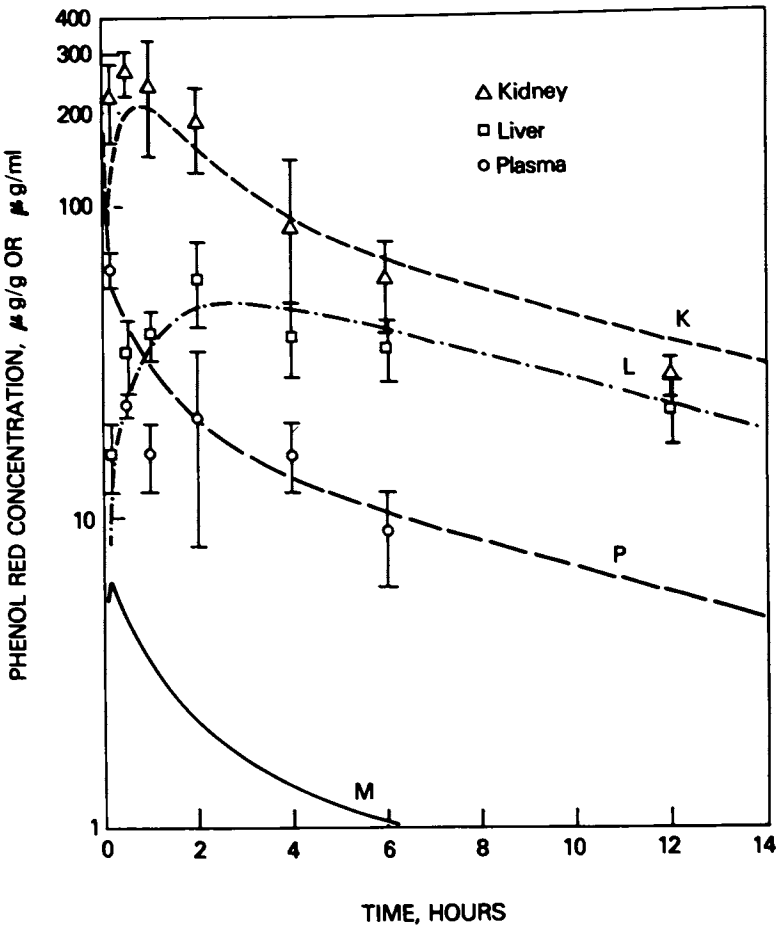

Figure 3. Time course of tissue and plasma concentrations of phenol red: model predictions vs. experimental results. The lines are model predictions; the symbols are experimental data for iv injection of 10 mg/kg into the caudal vein of dogfish sharks. Each symbol represents the average of five to eight female sharks/time point with SD indicated by vertical bars. The limit of sensitivity of the assay was 25, 15, and 5 μg/g or mL for (△), kidney (K); (□), Liver (L); and (○), plasma (P), respectively (9).

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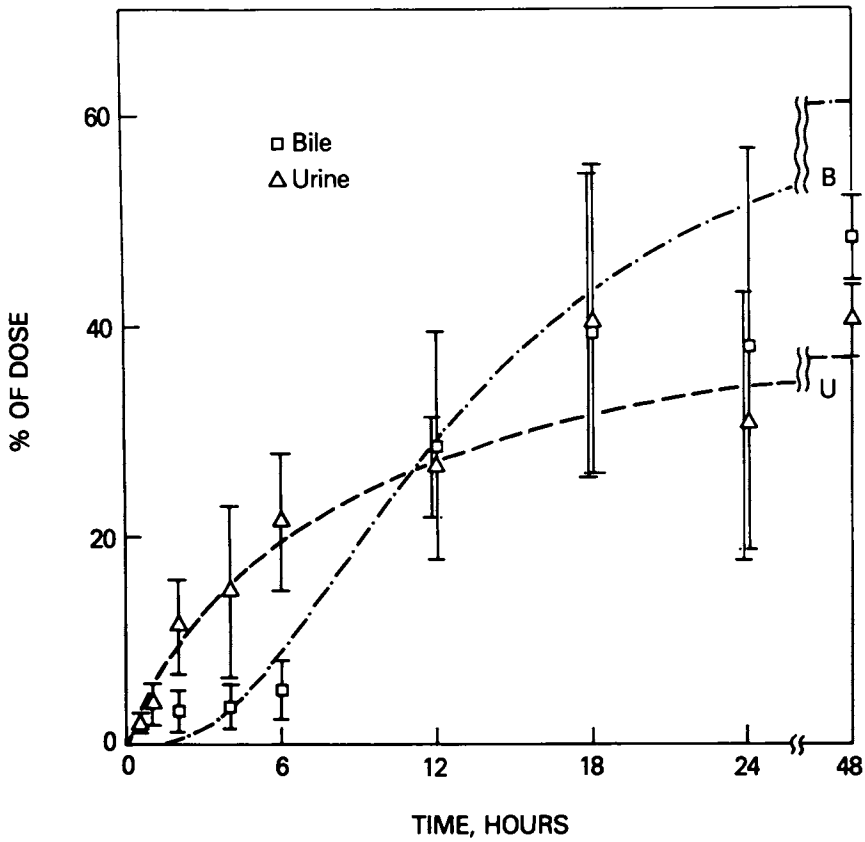

Figure 4. Time course of accumulation of phenol red and glucuronide in the bile and urine. The lines are model predictions and the symbols are experimental data: (□), bile (B); (△), urine (U) (9).

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Our questions broadened to consider how the transport and metabolic capabilities of these aquatic species compare with those of mammalian species. One reason for asking such a question is to assess whether the presence or absence of these capabilities alters the ability of fish to survive in toxic environments. Survival mechanisms fall into two catagories - behavioral and physiologic. An example of a behavioral mechanism could be as simple as a fish avoiding that area of a stream which contains toxic quantitites of phenol. When external perturbations caused by pollutants are small, homeostatic mechanisms such as those of the liver and kidney, allow fish to adapt to the body of water in which they exist. The problem then is related to defining the limits to which homeostatic phenomena can be stressed in aquatic species. An important reason to establish such information in fish is that bodies of water are the "ultimate sink" for a number of pollutants (12). Thus, while a behavioral response such as removing itself from a toxic environment is invariably available to a mammalian species, this type of response is impossible for a fish if a toxic xenobiotic occurs uniformly throughout an entire body of water.

What are some of the general modes by which terrestrial animals can diminish toxic effects of xenobiotics and how do these compare in fish?

1. Routes of entry - fish and mammals share two potential routes of entry, namely oral and percutaneous. Mammals also absorb xenobiotics via the lungs while gill absorption is possible in fish.

2. Plasma binding - both the quantity and types of proteins in plasma differ in these classes of animals. Mammals tend to have large amounts of protein compared with fish (ca. 8 vs 3 g/100 ml) and albumin, the protein which binds most xenobiotics, is negligible in a number of fish species.

3. Storage in fat depots - since the lipid solubility of a xenobiotic is important in its absorption, once in the body it is not unusual that these agents would accumulate in body fat. Mammals tend to distribute their fat somewhat uniformly in the subcutaneous tissues. While some aquatic species also do this, others have distribution patterns where the major site for storing fat is in one organ. For example, the shark and the lobster extensively utilize the liver and hepatopancreas, respectively, for fat storage; furthermore the fat content in these organs constitutes greater than 50% the total tissue wet weight. A further point of comparison is that when fish cast eggs, they often transfer large quantities of fat and therefore lipid soluble xenobiotics, to the yolk (13).

4. Organ blood flow and organ concentration - organ blood flow is an important factor which determines the distribution of a xenobiotic once it has been absorbed. This flow is related to blood pressure and while mammalian pressures are around 100 mm Hg, fish species usually are about one-fifth or less of this value.

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Obviously, then, cardiac output and blood flow through the liver and kidney differ considerably between these two classes of animals. As has been discussed (9) if the distribution of a compound is flow-limited, this factor can be readily considered in pharmacokinetic modeling. While the perfusion of an organ may explain the initially high levels of a xenobiotic in highly vascular tissues, the mammalian liver and kidney have an additional factor favoring concentration of compounds in them, namely a high capacity to bind chemicals more extensively than most other organs. Comparative binding information is desirable therefore, in fish liver, kidney and other organs.

5. Metabolism - a final factor in need of comparative studies is the metabolism of xenobiotics. One obvious difference between mammalian and fish species is that their bodies usually function at temperatures at least 10°C different. This fact undoubtedly explains some differences in metabolic rate but even when *in vitro* incubations are run at optimal temperatures there is a 10 - 100 fold higher rate of mammalian *vs.* fish metabolism (14, 15). In other words, the level of the xenobiotic-metabolizing capacity, especially for oxidative pathways, of the poikilothermic animals is considerably lower than that of the homeothermic species. Elsewhere in this volume Dr. Bend has focused on this aspect of the handling of xenobiotics by fish (16).

In summary then, fish as with most organisms, function competitively in their natural ecosystem. With only a mild additional stress, as might be imposed by an increased pollutant burden, certain species could be placed at a competitive disadvantage due to some degree of selectivity of disposition and/or metabolism. An excellent example of making a "pollutant" work in such manner to advantage is by the use of 3-trifluoromethyl-4-nitrophenol in the upper Great Lakes as a sea lamprey larvicide. This compound has considerably less toxicity to a favored species, the trout (17). A major reason for this selectivity is metabolic, i.e., the trout more readily converts this compound to the easily excreted glucuronide. But compared with the information available on this compound, we are relatively ignorant of how fish handle thousands of other foreign chemicals.

Initial Studies with the Model Compound Phenol Red

Our initial interest was in studying the disposition of phenol red in the shark. This turned out to be a good choice because nearly equal amounts of the model compound appeared in the urine and bile. After solving the body fluid collecting problems, we studied in greater depth, the transport properties of phenol red in both the renal and hepatic systems.
The urine collecting techniques (Fig. 1), were revised from those used by other investigators at Mt. Desert Island Biological Laboratory, Salsbury Cove, ME. Female fish weighing from 3-7 kg were prepared by ligating one end of a 10 cm length of P.E. 190

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tubing to the urinary papilla and the free end to a sturdy rubber balloon. A ligature was passed around the neck of the balloon so that the syringe plunger hub and balloon could be sutured to the skin and muscle. This procedure relieved the tension placed on the papilla which would otherwise cause tearing when the fish swam. For collection periods of less than 4 hrs., balloons with a 20-30 ml capacity were used and for periods of 4-24 hrs., 200-300 ml balloons were used. For more than 24-hour collections, balloons were changed daily. The average urinary output of these sharks is about 1 ml/hr/kg. Usually, bile was collected terminally by perforating the gall bladder with a 19 gauge needle attached to a 6 ml syringe.

To compare the excretion of phenol red into hepatic and gall bladder bile, unanesthetized fish were prepared surgically with biliary fistulae as described in Fig. 2 (7). The cephalad portion of restrained fish was submerged in running sea water while the common bile duct was ligated through an abdominal incision. The gall bladder was drained of bile by aspiration. Tubing (P.E. 200), fitted with a balloon of 5-10 ml capacity, was inserted into the distal tip of the gall bladder so that the cannula occupied most of the lumen of the collapsed gall bladder. The cannula was externalized, sutured in place, and the wound closed by suturing. The two bile collecting techniques were relatively comparable since the sharks were not fed; thus the gall bladder was not emptied during studies conducted for up to one week. Injections of different doses of phenol red were given via the caudal vein and blood samples were drawn from this same location at specified time intervals. After the animals were sacrificed, urine, bile, kidney, liver, muscle and blood (heparinized) samples were collected and, where appropriate, total volumes and total weights were recorded. The colorimetric procedures have been described previously (8) and consisted of homogenization of plasma or tissues with methanol followed by measurement of the optical density of alkalinized solutions. Phenol red glucuronide was determined after either HCl or β-glucuronidase hydrolysis. The presence of the glucuronide was confirmed by thin layer chromatography in three systems (8).

Disposition of Phenol Red

Phenol red is rapidly cleared biphasically from the plasma compartment with an initial $t_{0.5}$ of about 46 min. (Fig. 3) and a second phase with a $t_{0.5}$ of 8.3 hrs. As early as 10 min. there are detectable levels of phenol red in the kidney. The concentration of drug in kidney peaked at 30 min. and decayed with a half-time of about 9 hrs. There also were detectable levels of phenol red within 10 min. in liver but the values were considerably below those of plasma and kidney. Hepatic levels took longer (ca. 2 hrs.) to peak than did those in kidney, and then decayed with a half-time of about 10 hrs. The glucuronide

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conjugate (not shown in Fig. 3 - only total drug appears here) was not detected in plasma, kidney or liver. Phenol red and its glucuronide were apparent in urine within 30 min., increased in concentration for the first 2 hrs. after injection, and then declined. The amount of glucuronide appearing in urine (Fig. 4) was variable, ranging from 7.5% to 32.6% of total material, with higher percentages appearing toward the later times. Unlike urine, the bile had no detectable levels until 2 hrs. and there was no peak in the biliary concentration of the phenol red, since samples from the longest observation period contained the greatest concentration of both free and conjugated drug. The amount of conjugated drug appearing in the bile ranged from 15 to 30% of total material.

Consideration of these data in terms of multi-compartmental analysis is found in Fig. 5. The renal compartment never achieved more than 6.3% of the administered dose and declined rapidly after 1 hr. As early as 10 min. the hepatic compartment contained about 18% of the administered compound where a peak value occurred at 2 hrs. and continued to contain large amounts of phenol red for up to 12 hrs. There is only a slight difference between the amount of phenol red handled by the urinary and biliary compartments in 48 hrs., 40% and 48% respectively. In each compartment most of the material is free drug.

Effects of Interrupted Enterohepatic Circulation on Biliary and Urinary Handling of Phenol Red

The values in Table I compare biliary and urinary excretion of phenol red. The intact animal excretes 49% of the administered dose in 48 hrs. into gall bladder bile and of this 20% is excreted

Table I. Comparison of Urinary and Biliary Excretion of Phenol Red in Intact and in Fistulized Dogfish Shark[a]

	Phenol Red	
	% Total Dose	% as Glucuronide
Intact		
Urine	41	33
Bile	49	20
Fistulized		
Urine	23	30
Bile	47	44

[a]Data from (8). Animals were treated intravenously with 10 mg/kg of phenol red. Values are means for 4-6 animals 48 hr. after treatment.

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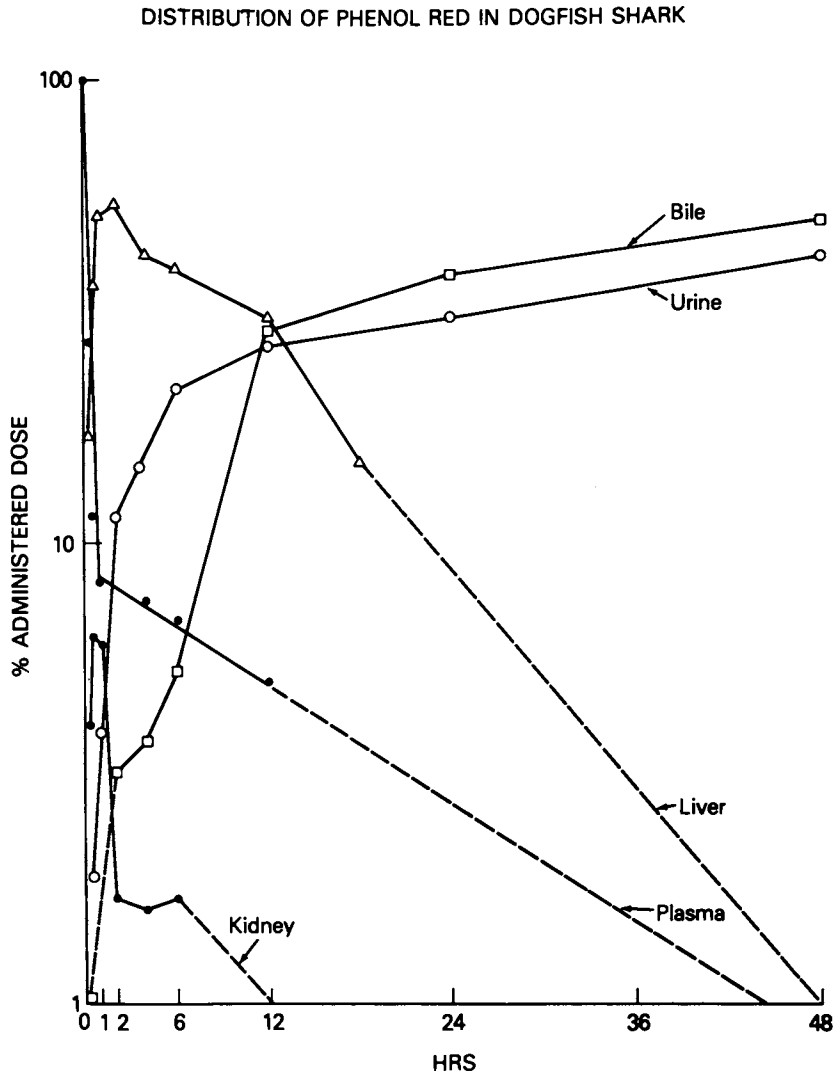

Figure 5. Time course of distribution of phenol red in terms of multicompartment analysis (8)

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as the glucuronide conjugate. Forty-one percent of the administered dose appears in 48 hrs. in the urinary compartment of intact animals and one-third of this is glucuronide. When bile is collected via fistula 47% of the administered dose appears in the bile but about twice as much of this total occurs as the glucuronide compared with the intact animal (44% vs 20%). These data suggest that the liver synthesizes the glucuronide and excretes it into the bile, so that in the animals with fistulae a large proportion of the administered dose is diverted, preventing the reabsorption of the glucuronide (presumably after hydrolysis) via the gastrointestinal tract. The urinary excretion in surgically treated animals confirms this latter point, since in animals with fistulae only 23% of the dose is excreted in the urine in 48 hrs. compared with 41% in the intact animals. On the other hand, the range of values seen in these wild animals (8) often was 30-50% of the mean values, and hence there often were not significant differences between intact and fistulized fish.

Concentrated Transfer of Phenol Red into Renal and Hepatic Compartments

By comparing the tissue and fluid concentrations of free phenol red, one can obtain the degree of concentration occurring in an indicated compartment (Table II). Relative to plasma, the

Table II. Concentration Gradients for Free Phenol Red in Renal and Hepatic Compartments in Dogfish Shark[a]

Ratio of:	Value
Kidney/plasma	6.4
Urine/kidney	4.7
Urine/plasma	30.4
Liver/plasma	3.9
Bile/liver	16.5
Bile/plasma	64.3

[a]Data from (8). Animals were treated intravenously with 10 mg/kg phenol red. Values are mean of 5-6 animals 6 hr. after treatment. This time was chosen because it was the latest time when plasma levels were detectable.

concentration in kidney was twice that of liver. The gradient between bile and liver was about four-fold larger than was the

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urine/kidney ratio indicating a dominance of the biliary transport mechanisms, which became more obvious at later times (Fig 5). Finally, the overall fluid/plasma ratios again favored the biliary route, 64.3 vs 30.4.

Saturation of Excretory Processes

Attempts to gather evidence that both phenol red and its glucuronide are excreted by saturable transfer processes were made by measuring drug disposition at four different doses of phenol red. Of the four doses studied (8) only the two extreme doses are shown in Table III. Over this range of doses, there was no evidence of saturation in terms of concentration, of either the plasma or kidney. There was no proportionality between

Table III. Effects of Different Doses of Phenol Red on its Distribution in the Dogfish Shark[a]

	Dose			
(form of phenol red)	g/g or ml	% Dose	g/g or ml	% Dose
Plasma (free)	16	7.6	204	10.2
Kidney (free)	83	1.6	948	2.2
Liver (free)	38	41.5	155	14.3
Urine (free)	711	13.3	1777	3.9
Urine (glucuronide)	63	1.4	148	0.3
Bile (free)	262	2.6	936	1.0
Bile (glucuronide)	120	1.1	241	0.3

[a]Data from (8). Animals were treated intravenously with 10 or 100 mg/kg phenol red. Values are mean of 5-6 animals per dose 4 hrs. after treatment.

dose and the percentage of administered dose in either the renal or plasma compartment. This provides evidence that both these compartments are not limited in terms of the portion of the administered dose which they can bind and/or contain. In liver, doses of 100 mg/kg of phenol red were significantly different from the 10 mg/kg dose in terms of concentration and percent of administered dose. The percentage of administered dose in liver demonstrates that with increasing doses liver is less able to store phenol red than is kidney. Regarding urinary excretion,

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increased doses of phenol red lead to less than proportional increases in free drug and glucuronide concentrations and a decreased percent of the dose in the urine suggesting saturation. Saturation of biliary transport for free and conjugated phenol red apparently did occur at the 100 mg/kg dose. Glucuronide transport proved to be the most readily saturated in both bile and urine.

Inhibition of Phenol Red Execretion by Probenecid

Since probenecid is used extensively as an inhibitor of the urinary and biliary excretion of carboxylic, phenolic and sulphonic acids in many other animals, it was of interest to determine if probenecid would inhibit the urinary and/or biliary transport of phenol red in the shark (Table IV). The plasma levels determined at 4 hrs. after administration of phenol red alone or in combination

Table IV. Effects of Probenecid Pre-treatment on the Distribution of Phenol Red in the Dogfish Shark[a]

Tissue or fluid (form of phenol red)	Phenol Red Alone µg/g	% Dose	Phenol Red + Probenecid µg/g	% Dose
Plasma (free)	16	7.6	14	7.3
Kidney (free)	85	1.6	25[b]	0.5[b]
Liver (free)	38	42.0	27[b]	28.0[b]
Urine (free)	711	13.3	85[b]	3.6[b]
Urine (glucuronide)	63	1.4	3[b]	0.1[b]
Bile (free)	262	2.6	191	1.7
Bile (glucuronide)	112	1.1	52[b]	0.3[b]

[a]Data from (8). Animals were treated intravenously with phenol red (10 mg/kg) alone, or with probenecid (40 mg/kg) 30 min. prior to phenol red (10 mg/kg) treatment. Values are mean of 5-6 animals 4 hrs. after receiving phenol red.

[b]Significantly different ($P < 0.05$) from phenol red alone.

with probenecid did not significantly differ (8). At this time and at this dose of probenecid, both the concentration and percentage of dose occurring in all other compartments decreased compared with treatment with phenol red alone. The decreases

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were significantly different for all values except free phenol red in bile. Probenecid pretreatment decreased the concentration of free phenol red excreted in the urine by a factor of about 10 and also diminished the percentage of the dose occurring in this compartment by about 70%. More dramatic decreases were seen for the glucuronide in urine, since there was about a twenty-fold reduction in its concentration. Although probenecid decreased biliary values of free phenol red, the changes were not statistically significant in terms of either concentration or percentage of dose. On the other hand, there was a significant reduction of about 50% in the concentration of glucuronide and a 73% decrease in terms of administered dose.

Plasma Binding of Phenol Red

At the dose most commonly employed in the present work (10 mg/kg), 66.6% of phenol red occurred bound as determined by ultrafiltration techniques (8). There were no significant differences in percentage bound at 20 and 40 mg/kg doses whereas there was a slight but significant decrease in the amount of binding by phenol red in the presence of probenecid.

In summary, through the use of the model compound phenol red, by means of pharmacokinetic modeling and disposition studies we have validated that the following features traditionally studied in mammalian systems, are qualitatively similar in the dogfish shark: overall disposition, effects of biliary fistula, transport effects observed from consideration of tissue/plasma or body fluid/plasma ratios, saturability of urinary and biliary excretion mechanism, inhibition of renal and hepatic transport by probenecid, and the presence of significant plasma protein binding. Having developed these experimental and pharmacokinetic techniques for the dogfish, let us now focus on the application of these methods to a number of model compounds and pollutants.

For the purpose of the following discussion, the xenobiotics studied in the dogfish shark were divided into three classes: 1) those relatively hydrophilic (Table V); those relatively lipophilic (i.e., solubility in water less than 1 mg/ml, Table VI); and, 3) metal-containing pollutants (Table VII). Most of these data have been previously reported (18-23) using ^{14}C compounds for assay, with the exception of sodium lauryl sulfate (SLS) (^{35}S), cis-Pt (atomic absorption spectroscopy) and phenol red (spectrophotometry). Unless otherwise stated these data are presented as total radioactivity and the hazards of doing so are recognized (24).

In addition to the standard information regarding the fate and body distribution of these pollutants, we have added the extra dimension of pharmacokinetics. Without going into considerable detail regarding this aspect of our studies, a few terms and their implications should be covered.

The term plasma half-time is perhaps the most familiar since it provides an indication of the rates of xenobiotic metabolism

Table V. Disposition and Pharmacokinetics of Hydrophilic Substances in the Dogfish Shark[a]

	Compound							
	p-Aminohippurate (PAH)		Phenol Red		Phenol		Sodium Lauryl Sulfate (SLS)	
Compartment	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
Plasma	14.6 (7.3)	0.9 (0.4)	16 (7.6)	<5 (<2.5)	3.0 (1.5)	1.5 (0.7)	1.8 (9.0)	0.5 (2.7)
Liver	26.0 (32.2)	31.3 (42.7)	38 (41.5)	<15 (<15)	59.8 (58.5)	21.3 (24.5)	5.4 (61.0)	1.2 (16.9)
Bile	7.1 (0.1)	229.0 (2.2)	374.0 (3.7)	3524.0 (38.1)	2.9 (<1.0)	103.8 (<1.0)	0.4 (<1.0)	27.4 (2.1)
Kidney	310.9 (6.7)	6.6 (<1.0)	85 (1.6)	<25 (<0.5)	12.9 (0.4)	4.0 (0.1)	7.5 (1.5)	1.7 (0.4)
Urine	2850.0 (28.0)	309.2 (56.4)	774 (14.7)	297 (30.6)	83.0 (1.7)	42.3 (4.2)	195.9 (28.4)	39.3 (65.1)
Muscle	2.2 (9.8)	0.5 (2.2)	<5.0 (<1.0)	<5.0 (<1.0)	2.5 (11.4)	0.7 (3.2)	0.4 (19.3)	0.2 (7.0)
Brain	1.9 (<0.1)	0.7 (<0.1)	(<5.0) (<1.0)	(<5.0) (<1.0)	0.9 (<0.1)	0.4 (<0.1)	0.3 (<0.1)	0.2 (<0.1)
CSF	3.1 (<0.1)	2.1 0.1	<5.0 (<1.0)	<5.0 (<1.0)	0.4 (<0.1)	0.3 (<0.1)	0.1 (<0.1)	0.1 (<0.1)
Pharmacokinetic Parameters[b]								
$\alpha t_{0.5}$ (min)	34.4		6.2		11.4		18.6	
$\beta t_{0.5}$ (hr)	4.8		6.6		17.2		10.5	
V_D (% Body weight)	25.1		42.2		239		30	
Cl (ml/min/kg)	0.72		0.81		1.6		0.39	

[a]Values are mean concentration (μg/ml or g) for 4-6 fish at each time point. Values in parentheses are mean percent of administered dose in the given compartment at the time indicated after intravenous administration of the compound at the following doses (in mg/kg): PAH, 10; phenol red, 10: phenol, 10; SLS, 1.

[b]Determined as described in text.

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and excretion. Determination of half-time requires an assay for the parent material in plasma and sampling from a blood vessel at several early time points (less than 2 hrs.) and less frequent sampling at later time points. The availability of radiolabeled pollutants greatly facilitates this area of research, but separation of the parent compound from its metabolites yields the most valid data. It is recognized that the actual plasma levels of a compound represent a group of events where tissue uptake, organ redistribution and excretion are occurring often simultaneously. Thus, the initial plasma half-time (α $t_{0.5}$) is defined as the time in which the concentration of a substance in plasma is decreased by 50% of its initial value. For all the substances reported here there was usually a second (β-phase) half-time and this provides a good index of the persistence of a given pollutant.

Determination of the volume of distribution provides an estimate of rates of uptake and storage of a xenobiotic. This is an abstract parameter (V_D) which is calculated by dividing the dose of administered compound by its plasma concentration. This can be determined either from the initial $t_{0.5}$ curve or from the plasma values after equilibration has been reached. While it might sound naive, particularly for pollutants, the concept of V_D assumes the compound is not bound, metabolized or excreted during the period of observation. Nonetheless, these assumptions actually strengthen the concept in that extremely large values of V_D indicate that at least one of these processes assumed not to be occurring must be contributing to increases in the value of V_D. At the time you may not know which ones are dominating, but you have very important information which helps to define the next step upon which to focus future research. In this summary the steady state V_D will be emphasized. It is the sum of both compartments in a two compartment model and is reported in terms of percent of the body weight. The approximate volumes of a number of body compartments are quite similar for different species and are as follows: body water, 58% of body weight; intracellular space, 41%; extracellular fluid, 13%; plasma volume, 5%.

The term clearance is used here in the sense of total body clearance and is analogous to the term renal clearance. The body as a whole is regarded as acting as a xenobiotic-eliminating system, where the rate of elimination divided by the average plasma concentration of the compound is the total body clearance. Here clearance is calculated (25) by dividing the administered dose of the substance by the area under the plasma concentration-time curve produced by that dose. This pharmacokinetic parameter, as well as others presented in this publication, was calculated by the use of the MLAB on-line computer system established at the National Institutes of Health by Knott and Reece (26). Similar to $t_{0.5}$, the total clearance is a composite of the individual clearances of the material by the various tissues of the body. Thus, for example, if a chemical is eliminated only by renal excretion and hepatic metabolism, total clearance is a composite

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of the individual clearances of the material by the various tissues of the body. The highest possible clearance for a given organ is equal to its blood flow. The units of clearance are given as ml/min/kg.

Distribution and Pharmacokinetics of Water Soluble Compounds

Table V contains data for two model substances, p-aminohippurate (PAH) and phenol red. Consideration of the highest values in this table tells you where the major portions of the substances appear. For example, urine and bile show the largest concentrations of PAH and phenol red. Both compounds appear in significant concentrations in the kidney while the values in muscle, brain and cerebrospinal fluid (CSF) are invariably lower than the values seen in plasma. The values in parentheses (Table V) are percent of the administered dose in a given tissue or fluid compartment. They add to the previous information by revealing the overall importance of a particular compartment in the disposition of a substance. For example, while the hepatic concentrations of PAH and phenol red at 4 hrs. are only about 2-fold those of plasma, the large size of the shark liver relative to its body weight, typically about 10%, leads to the appearance of 30-40% of these substances in the liver. The relative handling of these compounds by the urinary and biliary system is obvious from considering the percentage figures. Thus in 24 hours phenol red is about equally distributed in the bile and urine (38 vs 31%); the urinary route is the dominant route of excretion of PAH, i.e., 56 vs 2%.

In addition to the two model substances, Table V includes similar data on two water soluble pollutants. For phenol, the hepatic compartment at 4 hrs. contained both a large concentration and a high percentage of the administered dose. As is indicated by the urine and kidney levels, the importance of the renal route is evident. While there are no other unique distributional factors noted for phenol from these data, the fact that after 24 hrs. only about 30% of the administered radioactive compound is accounted for, as well as other known properties of phenol, suggests that it may be excreted via the gills (28) or through the skin.

Information regarding the distribution of the very commonly used detergent sodium lauryl sulfate (SLS) also appears in Table V. Twenty-four hrs. after injection of the ^{35}S form of SLS, most of it (65%) has been excreted in the urine of the shark. At the earlier time point, 4 hrs., the hepatic tissue has a higher concentration and quantity of the detergent than any other tissue. Muscle retained the isotope longer than did other tissues in this table and may represent sulfur exchange with endogenous substances.

The pharmacokinetic parameters for these 4 substances are grouped on the bottom of Table V. In general the initial plasma half-times were rapid and usually were less than 30 min. The

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second phase $t_{0.5}$ was most rapid for PAH and phenol red (5-7 hrs.), intermediate for SLS (11 hrs.) and longest for phenol (17 hrs.). For PAH, phenol red and SLS the steady state V_D ranged between that of the intracellular space and that of body water. The extremely high value of the volume of distribution of phenol (239%) is consistent with rapid metabolism and/or rapid gill excretion and/or loss through skin. One reason to have included PAH in this study was to have data on a well-known compound used for renal clearance studies. The clearance of phenol red and PAH were quite similar, while SLS was removed from the body at one-half the rate of these two compounds. Consistent with other information on phenol, it is cleared quite rapidly, i.e., two times faster than PAH.

Distribution and Pharmacokinetics of Lipophilic Pollutants

As complex as is Table VI, consideration of the comments above regarding the fact that the shark liver is quite large and more than 50% fat, and the poor water solubility of these pollutants coupled with their high affinity for lipids greatly simplifies the major facets of this table. First, while comprising 10% of the body weight, considerably greater percentages of these pollutants invariably occurred in the liver. Thus, for DDT, dieldrin and octane at 24 hrs. the liver contained 40-90% of the dose; this is consistent with their approximate water solubilities (in ppm in brackets near each compound name in table) of 0.001 (29), 0.1 (29) and 0.7 (30) respectively. 2,4-D (2,4-dichlorophenoxyacetic acid) is more water soluble, 650 ppm (29) than is 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 238 ppm (29); the solubilities of these pollutants are considerably greater than the other pollutants in this table. DEHP (diethylhexyl phthalate) is listed as "insoluble" in water (31) but it appeared from other values listed in this reference that the limits of the methods were 100 ppm. In any event, we would expect that the hepatic levels of DEHP would fall somewhere between those of the higher lipophilic chlorinated hydrocarbon pollutants and the less fat soluble phenoxyacetic acid herbicides. Note (Table VI) that this is the case.

Some further generalities can be made from data in this table. Except for octane with its known CNS depressant actions, no other compound appeared in significant quantities in the brain. The herbicides were the only compounds which occurred in significant concentrations in the bile and this has been shown (32) to be mainly metabolites, i.e., the taurine conjugates of 2,4-D and 2,4,5-T. Also the kidney and urine samples contained large concentrations of these same herbicides as did those from fish treated with octane or DEHP. Presumably these latter values are at least in part metabolities. Due to its large mass (ca. 50%) relative to the body weight of the shark, the muscle compartment contained relatively large percentages of the dose, 10-30% and these tended to decrease relatively rapidly. For example,

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Table VI. Disposition and Pharmacokinetics of

Compartment	Compound DDT 4 hr	DDT 24 hr	Dieldrin 4 hr	Dieldrin 24 hr	n-Octane 4 hr	n-Octane 24 hr
Plasma	<.1 (5.4)	<.1 (2.3)	0.4 (2.2)	0.2 (0.9)	8.5 (4.2)	1.1 (0.6)
Liver	0.1 (34.6)	0.2 (38.4)	3.6 (48.7)	5.9 (63.6)	71.0 (81.7)	74.5 (78.6)
Bile	0.1 (0.2)	0.1 (0.2)	0.2 (<0.1)	0.4 (<0.1)	2.8 (<0.1)	1.9 (0.1)
Kidney	0.3 (1.6)	0.1 (0.3)	1.2 (0.3)	0.5 (0.1)	19.6 (0.5)	4.1 (0.6)
Urine	0.3 (1.0)	0.1 (3.2)	0.2 (<0.1)	0.1 (<0.1)	1.4 (<0.1)	0.2 (<0.1)
Muscle	0.4 (16.0)	0.3 (15.0)	0.5 (19.8)	0.7 (33.4)	8.2 (37.0)	8.3 (37.0)
Brain	<0.1 (<0.1)	0.8 (<0.1)	3.0 (<0.1)	0.2 (<0.1)	24.1 (<0.1)	2.0 (<0.1)
CSF	<0.1 (<0.1)	<0.1 (<0.1)	<0.1 (<0.1)	<0.1 (<0.1)	0.4 (<0.1)	0.5 (<0.1)
Pharmacokinetic Parameters [b]						
$\alpha t_{0.5}$(min)	3.4		9.8		7.1	
$\beta t_{0.5}$(hr)	1.2		16.3		8.9	
V_D (% Body Weight)	11.1		125.0		101.0	
Cl (ml/min/kg)	1.3		1.1		1.5	

[a] Values are mean concentration (μg/ml or g) for 4-6 animals at each time point. Values in parentheses are mean percent of administered dose in a given compartment at the time indicated after intravenous administration at the following doses (mg/kg) in alcohol - Emulphor-saline (27): DDT, 0.1; dieldrin, 1.0; octane, 10; DEHP, 1.1; 2,4-D, 1.0; 2,4,5-T, 1.0.

[b] Determined as described in text.

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Lipophilic Pollutants in the Dogfish Shark[a]

Di(2-ethylhexyl) phthalate (DEHP)		2,4-D		2,4,5-T	
4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
3.2	<0.1	1.0	0.7	1.2	0.1
(15.9)	(0.2)	(5.0)	(3.3)	(5.8)	(0.4)
3.5	1.5	1.7	1.2	1.9	0.5
(38.2)	(12.3)	(20.8)	(7.4)	(21.5)	(5.2)
0.1)	0.9	17.8	19.4	0.6	12.2
(<0.1)	(0.1)	(1.1)	(1.8)	(<0.1)	(1.1)
3.1	0.3	14.5	1.4	48.9	1.6
(0.8)	(0.1)	(4.0)	(0.4)	(12.1)	(2.4)
1.0	0.7	197.0	59.4	27.3	41.5
(0.1)	(0.8)	(53.2)	(67.5)	(5.5)	(34.8)
-	-	0.5	0.3	0.3	0.3
-	-	(24.4)	(15.4)	(11.0)	(11.4)
0.1	<0.1	0.4	0.4	0.7	0.3
(<0.1)	(<0.1)	(<0.1)	(<0.1)	(<0.1)	(<0.1)
<0.1	<0.1	0.7	0.9	<0.1	0.1
(<0.1)	(<0.1)	(<0.1)	(<0.1)	(<0.1)	(<0.1)

36.1	11.3	32.1
3.0	9.4	38.6
6.5	56.1	83.0
0.33	0.75	0.26

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Table VII. Disposition and Pharmacokinetics of Mercury and Platinum Compounds in the Dogfish Shark[a]

	Compound			
	Methyl Mercury		Cis Diamminodichloro Platinium II	
Compartment	4 hr	24 hr	4 hr	24 hr
Plasma	0.4 (9.4)	0.2 3.7	0.99 (7.6)	0.47 (3.6)
RBC's	1.6 (21.9)	0.8 (0.9)	---	---
Liver	0.7 (23.3)	0.7 (28.1)	0.20 (3.8)	0.21 (2.7)
Bile	0.04 (0.02)	0.10 (<0.01)	<0.1 (<0.1)	1.2 (<0.1)
Kidney	6.6 (6.9)	3.2 (2.9)	3.1 (1.2)	2.0 (0.9)
Urine	0.7 (0.1)	0.7 (0.3)	1.6 (1.3)	0.7 (2.8)
Muscle	0.08 (17.1)	0.10 (30.1)	0.5 (23.4)	0.4 (31.7)
Brain	0.1 (0.02)	0.1 (0.02)	0.40 (<0.1)	0.32 (<0.1)
CSF	<0.1 (<0.01)	<0.1 (<0.01)	0.21 (<0.1)	<0.1 (<0.1)
Pharmacokinetic Parameters[b]				
$\alpha t_{0.5}$(min)	15.4		71.1	
$\beta t_{0.5}$(days)	1.2		17.5	
V_D (% Body Weight)	86.0		103.0	
Cl (ml/min/kg)	0.36		.03	

[a]Values are mean concentration (μg/ml or g) for 4-6 fish at each time point. Values in parentheses are mean percent of administered dose in the given compartment at the time indicated after intravenous administration of the compound at the following doses (in mg/kg): MeHg, 0.1; cis-Pt, 1.0.

[b]Determined as described in text actually employing data collected for up to 6 days.

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while the 4-hour value for 2,4-D was 24%, after 6 days, there was only 0.6% of the administered dose remaining (32).

The pharmacokinetic parameters of octane are more like those of dieldrin than DDT while the clearance of all three of these compounds is somewhat greater than that of PAH. While the initial half life of DEHP is longer (36 min.) than any other compound in Table VI, its second phase $t_{0.5}$ is rapid (3 hrs.) while the V_D is equal to that of the plasma volume. The clearance of DEHP is about one-half that of PAH.

The closely related herbicides have some differences in distribution and pharmacokinetics which are largely resolved by returning to the observation above that the water solubility of 2,4-D is about 3-fold greater than that of 2,4,5-T. Thus, 2,4-D has initial and final $t_{0.5}$ values as well as clearance value, about 3 times those found for 2,4,5-T. These data all fit with the major distribution difference of these 2 compounds, i.e., that considerably more of the dose of 2,4-D is excreted in the urine in 24 hrs.

Distribution and Pharmacokinetics of Metallic Compounds

While our interest in the environmental form of mercury as the methylated form is obvious, the studies with this platinum compound should be explained. First, the presence of Pt catalytic converters in most currently produced automotive exhaust systems as antipollution devices, presents a serious environmental problem of the future. Because of the potential of this heavy metal becoming a pollutant we developed sensitive assays for it in biologic fluids and tissues (33). An interesting convergence of research problems and need in two different areas of toxicology, (environmental and drug) has therefore developed. The National Cancer Institute was developing a Pt-containing drug (cis-Pt), cis-diamminodichloroplatinum (II) which has successfully entered clinical trials (34). Since this compound had an aqueous solubility of 1,000 ppm, it was regarded as a convenient form of Pt to study in fish.

Since MeHg is quite lipid soluble (2), the expected localization in liver occurred (Table VII). Also as expected, due to the presence of abundant -SH groups and their known interaction with Hg, red blood cells (RBC's), muscle and renal levels were high both in terms of concentration and percent of administered dose. For most time points studied for up to 6 days, the RCB/Plasma ratio was about 20. There was some CNS penetrance of MeHg but no behavioral changes were observed in the fish.

The cis-Pt compound showed some selective uptake into -SH abundant tissues such as kidney, muscle and rectal gland (data not shown) but not RBC's. Most other tissues showed no remarkable concentration and/or increased percentage of dose retained over those expected from uniform distribution. There was moderate CNS penetrance of cis-Pt. Neither the Hg nor Pt compound appeared in

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very significant quantities in bile while considerably more Pt than MeHg was excreted via urine.

The initial $t_{0.5}$ for MeHg in plasma is brief (15 min.) while the second half-time is quite long (1.2 days) relative to all other pollutants in this report. The V_D is greater than that of body water and this is consistent with localization in RBC's, muscle and kidney. The clearance is one-half that of PAH. If other forms of Pt are as difficult to model pharmacokinetically and have the same implications as the results of this model, these heavy metal compounds might be troublesome. The slow removal of cis-Pt from the plasma (β-phase) has been noted previously in dogs and rats (22); it is 17.5 days in the shark. The clearance of the Pt compound was less than one-tenth that of MeHg. These cis-Pt data in the shark could be a forecast of problems other species will have in ridding themselves of the soon-to-be-ubiquitous pollutant.

Summary

In the present compilation of the distribution and pharmacokinetic data of a dozen xenobiotics studied in the dogfish shark, this species yielded excellent data consistent with what we know from similar studies on terrestrial mammals. The data from the shark occasionaly provided information not available in other animals. Major transport parameters in this fish were shown to be similar to those found in mammals. This aquatic organism handles lipid-soluble pollutants by sequestering them in its fatty liver. Together with a previous summary (23) we have now studied about three dozen xenobiotics in this species. Because of its ease of handling, low cost, abundance, predictive value of transport mechanisms, and well-developed pharmacokinetics, the dogfish shark is an ideal fish species to use as a model to study aquatic pollutants.

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Disposition of Toxic Substances in Mussels *(Mytilus californianus)*: Preliminary Metabolic and Histologic Studies

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Deaths of target organisms associated with intentional pesticide applications to insect-infested crops, weed-choked roadsides, and nematode-laced fields are predictable, desirable, and relatively easy to measure. Likewise, catastropic releases of chlorine from ruptured tank cars or of crude oil from scuttled supertankers may produce a spectrum of biological effects including toxicity. These events are easily associated with exposures to toxic substances and particular environmental circumstances. In both the intentional and the catastrophic cases, when death is the end-point, the establishment of cause-and-effect relationships is straightforward.

Altered growth and development, reduced vigor, body burdens of persistent chemicals, genetic defects, and impaired reproduction are harmful effects which are more difficult to detect and evaluate. One means to detect such harmful effects is use of monitoring organisms. The concept was explored by Haldane who advocated in the late 19th century (1) that small animals such as mice or birds be used to warn miners of toxic levels of carbon monoxide. The small animals' rapid respiration being very sensitive to carbon monoxide inhibition, symptoms of poisoning gave timely warning of harmful proportions of CO in the workers environment.

More recently, environmental monitoring has been accomplished using bacteria, molluscs, fish, and birds (2,3). Bioaccumulation of mercurials (4), polychlorinated biphenyls (5), and organochlorine pesticides (6,7) has been used to facilitate environmental sampling. An EPA-sponsored "Mussel Watch" program has utilized bivalves from both coasts and the Gulf of Mexico as sentinel organisms in monitoring for halogenated hydrocarbons, artificial radionuclides, petroleum components and heavy metals (8). Results of such studies have been used by regulatory agencies, environmental advocates, and others concerned both with

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microconstituents of the food supply and changes in environmental quality.

Biological consequences of low level body burdens of persistent pollutants are more poorly defined than is the certainty of exposure. Whether or not exposure to persistent chemicals is associated with toxicity will be determined by complex interaction of a number of processes which can be conveniently designated disposition processes. These include uptake (absorption), distribution, receptor interaction, deposition (storage), biotransformation (metabolism), and elimination (excretion, depuration) which determine the biological fate and effects of toxic substances. Histological study of stressed organisms may reveal corresponding changes in tissue form and function. These considerations lend themselves to experimental study, and results of such studies may be valuable in predictive toxicology.

Histological studies and investigations of disposition processes are being coupled in order to assess the condition of populations of California mussels Mytilus californianus. Whether results of future studies of this sort can be used diagnostically to reveal the presence of chemical stressors in the environment and contribute to evaluation of their impact is a concern which motivates much of our experimental work.

Methods and Materials

Mussels. Mytilus californianus were usually collected at low tide in the rocky, intertidal zone of Schoolhouse Beach near Bodega Bay (Sonoma Co.) on the central California coast. Other sites have included Salt Point, Bodega Head, and Fort Bragg. Animals were used on the coast or transported to Davis where they were maintained in aerated aquaria containing Instant Ocean^{R} Synthetic Sea Salt (1.025 specific gravity, 11°C, 12/12 L/D). Except as noted, mussels were used within 2 weeks.

Condition Index. Shell volume, determined volumetrically, and tissue mass were measured for determination of the condition index (9). Tissue (g) divided by shell volume (ml) yielded the index which ranged between 0.45 and 0.60.

Histology. Whole mussels were fixed for 1-2 days in Helly's fluid (10) and stored in 70% ethanol. Tissue was blocked at 2 mm thickness, embedded in paraffin, sectioned at about 7 μm, stained with hematoxylin and eosin, and mounted on glass slides using standard procedures.

In Vivo Exposures. Aldrin (200 μg/200 μl DMSO or methanol) was administered by injection into the posterior adductor muscle or slowly released from a syringe (27 gauge, 1/2 inch) directly onto viscera. Mussels in situ in their natural habitat and others contained in small cheesecloth bags were also treated. After

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designated intervals, mussels were quick-frozen using dry ice and later stored at -15°C prior to analysis.

Uptake and Elimination. Six mussels were placed into 600 ml beakers containing 300 ml aerated Instant Ocean^R (10-12°C) for a 1/2 hr acclimation period before addition of antipyrine (3 μCi/beaker) (or other test compound). Aliquots (1.0 ml) were taken at intervals and placed directly into scintillation fluid (10 ml, 3a70B, Research Products International Corp., Elk Grove Village, IL). Initial experiments showed that uptake was complete within 2 hours. At the end of 2 hours (uptake) the mussels were rinsed, antipyrine solution was replaced with fresh Instant Ocean^R, and sampling at intervals continued. Portions of the water were also analyzed for antipyrine and metabolites. Data expressed in counts per minute per 1.0 ml were used to represent uptake and elimination of antipyrine. Experiments were routinely done in triplicate.

Graphs relating antipyrine concentrations and time were used to calculate clearance rates. A relationship between apparent antipyrine steady state concentrations at 120 and 240 minutes (ap_{120}, ap_{240}) and mussel body water and mantle cavity water was also determined (k). Mantle cavity water is that volume held between the valves when the mussels are closed, e.g., when transferred from the uptake solution (300 ml) to the elimination solution (300 ml). The initial antipyrine concentration (ap_0) was determined at the beginning of the experiment. Assuming no loss of antipyrine, complete mixing of the solutions, and its distribution into total mussel body water, when an apparent steady state is achieved, the following results:

$$300\ ap_0 = (300 + k_{120})\ (ap_{120})$$

$$k_{120} = 300\ \left(\frac{ap_0}{ap_{120}} - 1\right)$$

Similarly, at achievement of the second apparent steady state following transfer of the antipyrine treated mussel to 300 ml water, the following equation can be written:

$$k_{240}\ ap_{120} = (300 + k_{240})\ ap_{240}$$

$$k_{240} = \frac{300\ ap_{240}}{ap_{120} - ap_{240}}$$

where k_{240} is the body and residual water constant derived from steady state antipyrine concentrations.

In identical systems, uptake of aldrin (30, 300, 3000 μg/300 ml) has been demonstrated. Similarly, both uptake and elimination

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of p-nitroanisole (initial concentration 1.5 mg/300 ml) have been measured.

Tissue Homogenates and Microsomes. Preparations were made from gill, mantle, green gland, and viscera (all tissue except adductor muscles) in either ice-cold 1.15% KCl (w/v) or 0.05M phosphate buffer, pH 7.4. Homogenates (10 or 20% w/v) were prepared using either all-glass, hand operated tissue grinders or a Waring Blendor (10 sec at high speed). Microsomal fractions were prepared by centrifugation of the 10,000 g max x 30 min supernatant at 100,000 g max x 60 min. Pellets were resuspended in buffer using tissue grinder with a teflon pestle and final protein (11) concentration ranged between 3 and 10 mg/ml.

Cytochrome Determinations. Microsomal suspensions (1-5 mg protein/ ml) were assayed for cytochromes b_5 and P-450 (12) using a Cary 15 spectrophotometer operated at room temperature (20-23°C). Suspensions in 0.05M phosphate buffer, pH 7.4, were contained in 3 ml cuvettes with a 1 cm path length. Sodium dithionite was the reductant. The extinction coefficient of 171 mM^{-1} cm^{-1} was applied to the 428-490 nm absorbance increment.

Monooxygenase Assays. Incubation media contained the following (final concentrations): 0.05M phosphate buffer, pH 7.4, glucose-6-phosphate (G-6-P, 2.3 mM), G-6-P dehydrogenase (3 units), NADP (0.23 mM), and KCl (2.8 mM), and various tissue preparations. Substrates were added in small volumes (25 µl or less) of MeOH. Samples (1.1 ml) were shaken in a thermostated (usually at 22°C) water bath and reactions terminated by enzyme denaturation. Specific analytical procedures for aldrin epoxidation (13), $^{14}CH_3O$-p-nitroanisole O-demethylation (14), and ^{3}H-benzo(a)pyrene oxidation (15) have been described.

Chemicals. Antipyrine, carbon monoxide (Matheson, Coleman and Bell, Los Angeles, CA), and $^{14}CH_3$-N-antipyrine (11.1 mCi/mM, ICN, Irvine, CA) were purchased. Aldrin (1,8,9,10,11,11-hexachloro-2,3-7,6-endo-2,1-7,8-exo-tetracyclo ($6.2.1.1^{3,6}.0^{2,7}$) dodeca-4,9-diene) and its epoxide, dieldrin were gifts of Shell Development Co. (Modesto, CA). Each was recrystallized from methanol-water solutions and was greater than 99% pure as determined by gas chromatography. $^{14}CH_3O$-p-Nitroanisole (1.9 mCi/mmole) was synthesized (14) and ^{3}H-benzo(a)pyrene (8.3 Ci/mmole) was purchased (Amersham-Searle Co., Arlington Heights, IL). Solvents were nanograde (Mallinckrodt, St. Louis, MO). Other chemicals were at least reagent grade.

Results

Uptake and Elimination. Magnitudes of exposure of lamellibranchs to toxic substances will be determined by rates of flow of water through the gills, concentrations of the toxic substance,

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and the extent of uptake. Rates of ventilation, i.e. volume of water flowing through gills (liters water/hr/mussel) have been measured directly and indirectly (17). Rates of solute removal per unit time are termed clearance. General reviews include those by Owen (18, 19), Jorgensen (20) and Ali (21). Most indirect measurements are made using particulate suspensions of material such as colloidal graphite, silt, and algae. Bayne tabulated ventilation rate data for four species including M. californianus (17).

Quantitative estimation of ventilation by indirect methods in mussels requires four assumptions (16): a) reduction of concentration results from uptake, b) constant ventilation (pumping) rate, c) uptake of a constant percentage of concentration (first order process), d) homogeneity of the test solution at all times. Our transport studies have utilized antipyrine (22, 23) a water soluble, stable chemical of low acute toxicity to mussels. It is readily dissolved in ocean water or Instant Ocean® and is neither adsorbed nor volatilized from the 300 ml test system. Mussels pump throughout the 4 hour test period and this action is apparently sufficient to insure homogeneity of the solution. Inspection of early uptake and elimination curves (antipyrine concentration as a function of time) prompted use of Coughlan's equation (16) for water transport.

Antipyrine uptake rates obtained by measuring declining concentrations of the solute may be obtained from the following equation:

$$\frac{-dC}{dt} = C\left(\frac{mn}{M}\right)$$

where the uptake rate, $\frac{-dC}{dt}$, was the rate of decrease of C; M was the volume of the test system; n was the number of animals; m was the clearance rate of a single animal. This may be solved as follows:

$$m = \frac{M}{n} \times \frac{\ln C_0 - \ln C_t}{t}$$

Indirect measurements of clearance have the advantage of requiring little manipulation of the mussels, but suffer from the fact that rates may be affected by concentrations of suspended particulates and solutes.

Antipyrine from 300 ml solutions containing 0.6, 6, or 60μM was readily taken up by mussels. Within 40-80 minutes an apparent steady state was achieved. Uptake experiments were routinely conducted for 120 minutes (Figure 1). An analogous antipyrine elimination curve is shown in the lower portion of the figure. At the highest dose (60 μM) mussels sometimes closed, but others ventilated with no apparent ill effects. The intermediate concentration (6 μM) was, therefore, used in all subsequent experiments.

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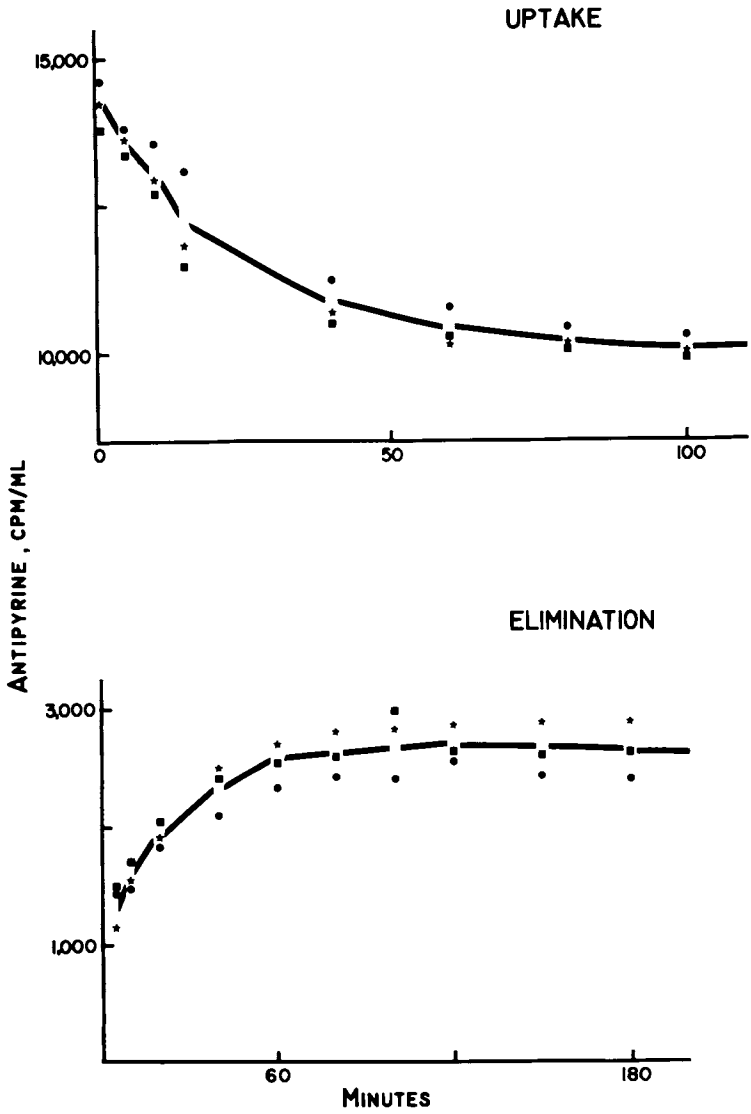

Figure 1. Antipyrine uptake and elimination data taken from 3 experiments. Six mussels in 300 mL Instant Ocean® were used in each. The 1 mL aliquots were taken at the end of each interval.

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From curves including the one shown in Figure 1, antipyrine clearance rates were calculated using Coughlan's formula (16). Rates were calculated for 3 intervals: 0-5 min, 0-20 min, and 5-20 min (Figure 2). Each particular interval yielded apparent rates which were remarkably precise (Table I). Data are presented for two experiments in which 3 sets of mussels were used after different intervals in the laboratory. This series of mussels was the source of most of the animals used in disposition studies to date. The largest changes in antipyrine concentration were observed during the first 5 min interval. The change resulted both from dilution of the antipyrine solution by water within the mantle cavity and from antipyrine uptake. During the 5-20 min interval, mussel pumping provides continuous exposure and uptake is first order. The higher rates for the first two time intervals (0-5, 0-20 min) are influenced by the mantle cavity dilution factor. Antipyrine clearance rates, e.g. 110-150 ml/hr between 5 and 20 min, are calculated rates (Table I) are less than published ventilation rates (17), indicating that antipyrine is not 100% absorbed during a single pass of water through the mussels. Neutral red clearance, an indicator of ventilation (19), yielded rates of 136 to 1181 ml/hr in unpublished preliminary studies. Clearly, antipyrine clearance is not limited by ventilation *per se*.

From the same uptake and elimination data the mantle cavity and body water volume (k) were calculated for mussels at each storage interval (Table II). Since all measurements of uptake and elimination are made with the same test organisms, it is hypothesized that k is a constant that can be calculated from the initial antipyrine (ap_0) and the steady state values (ap_{120} and ap_{240}). The mean ($\pm$S.D.) calculated constants (k_{120} and k_{240}) were 114$\pm$24 ml and 109$\pm$17. The values were not statistically different (Students' t-test, $p<0.05$). At both the high antipyrine concentration of uptake and the low concentration during elimination, the apparent volume of the mussels (mantle cavity and body water) was constant. Whether environmental, e.g. salinity, temperature, position in the water column, season, lattitude, biological and chemical stressors, and biological e.g. age, sex, reproductive status, can effect k will be determined in future experiments.

Determination of k by the means described above requires that antipyrine be distributed in body water. Subsequent to the pioneering studies by Soberman *et al.* (22) in humans, many other experimental biologists have used antipyrine for estimation of body water (23 and references therein). Using a ratio of fresh weight:dry weight of 7.5:1 (17), the concentration of antipyrine in body water is calculated following digestion of whole mussel homogenates (Table III). Table 3 also includes measurements of radioactivity in mantle cavity water. Each of the three compartments (solution, mantle cavity, and body water) had similar antipyrine concentrations indicating complete distribution. These

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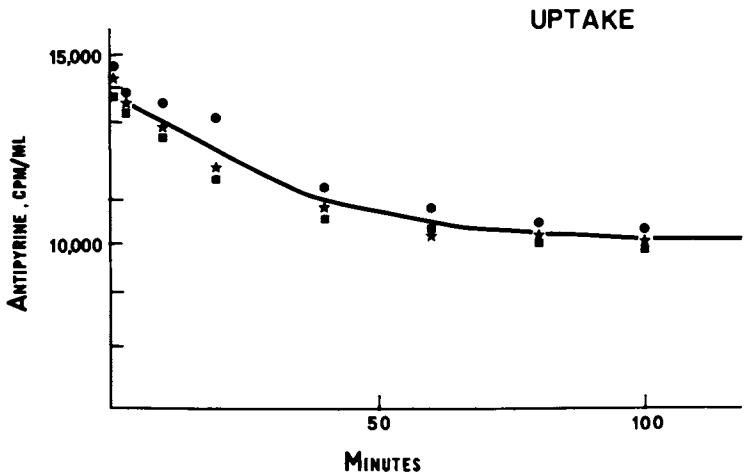

Figure 2. Semilog plot of antipyrine uptake data of Figure 1. Each set of points is from a separate experiment using 6 mussels in 300 mL Instant Ocean®

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TABLE I

Antipyrine Clearance Rates In Static Systems

Days in Laboratory[2]	Interval[3]	Estimated Clearance Rates[1] (liters/hr) 0-5 min	0-20 min	5-20 min
Experiment I				
1 d		0.68	0.26	0.11
2 d		0.46	0.20	0.11
4 d		0.79	0.28	0.11
8 d		0.79	0.31	0.15
Experiment II				
4 d		0.79	0.28	0.11
8 d		0.79	0.26	0.15
16 d		0.68	0.28	0.15
31 d		0.68	0.26	0.11

[1] Liters of water cleared of antipyrine per hour.

[2] Mussels cultured in Instant Ocean[R] (11°C, 12/12 L/D)

[3] Rates determined from antipyrine measurements like those of Figure 2.

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TABLE II
Apparent Steady State Antipyrine Concentrations and Calculated Mussel Mantle Cavity and Body Water

Days in Laboratory	Antipyrine[1] (cpm/ml)			Mussel Water[2] (ml)	
	ap_0	ap_{120}	ap_{240}	k_{120}	k_{240}
Experiment 1					
1 d	21,258	14,586	3,918	139	111
2 d	20,049	15,430	4,174	91	112
4 d	21,846	15,848	3,867	114	97
8 d	21,264	15,213	4,425	120	123
Experiment 2					
4 d	22,096	16,226	4,222	109	106
8 d	20,807	15,112	4,157	113	114
16 d	21,182	15,338	3,972	116	108
31 d	21,377	15,605	3,914	111	102
Mean	21,235	15,420	4,081	114	109

[1] Mussels held at 11°C in aerated Instant Ocean[R]. Antipyrine (6 μM; 1.65 mCi/mmole) in 300 ml. One ml aliquots taken at zero, 120 (uptake), and 240 (elimination) min. Three beakers per set.

[2] Constant "k" calculated from ap_0, ap_{120}, and ap_{240} and includes mantle cavity and body water of the 6 test animals.

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measurements support use of the mussel constant k (above) as an indicator which can be obtained by non-invasive means.

TABLE III
Body Water Determination Using ^{14}C-Antipyrine

Experiment	Antipyrine[1] (dpm/ml) Solution	Mantle Cavity	Body Water[2]
1	11,265	11,060	11,786
2	10,989	10,644	8,879
3	10,834	10,515	10,920

[1]Four measurements per set per experiment. Radioactivity determined using ^{14}C-toluene internal standard.

[2]Body water calculated from fresh weights assuming fresh:dry weight ratio of 7.5:1. Mussels digested.

Three sets of experiments have been done to determine the effect of environmental variables on antipyrine disposition. The conditions included 1) animals transferred to either Instant Ocean® or ocean water antipyrine solutions, 2) animals maintained in Instant Ocean® in the laboratory for periods up to 31 days, 3) mussels placed into antipyrine solutions which contained low levels of other foreign compounds (aldrin, p-nitroanisole, SKF 525-A). Results are summarized in Tables IV and V.

Antipyrine uptake and elimination half-lives were measured in both Pacific Ocean water and Instant Ocean®. Measurements were made immediately after collection of the mussels (0 day). The uptake and elimination half-lives of 176 and 169 min and 27 and 29 min were similar to each other and to half-lives obtained using mussels maintained in the laboratory. Half-lives in the longer term laboratory culture experiments (Table IV) were similar to each other. Similarly, the mantle cavity and body water constants gave no indication of stress (Table II). Mussels used in these experiments were selected by size (ca. 6 g viscera fresh weight) and variability could be reduced by adoption of more objective criteria. Instant Ocean® culture does not directly effect antipyrine disposition and laboratory conditions are suitable for maintenance of animals for at least short times.

Antipyrine uptake and elimination was also assessed in the presence of aldrin and p-nitroanisole, among substrates used in biotransformation studies (Table V). Altered antipyrine half-lives might indicate that the levels of the substrates were stressful to the mussels. The levels used did not overtly effect the mussels pumping activity, and half-lives were within the range of control values. Antipyrine disposition measurements will be included in studies on the biological fate of chemicals.

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TABLE IV
Antipyrine Uptake and Elimination by Mussels
Directly Removed or Laboratory Cultured.

	Experiment Variable	Apparent Half-Time (min) [1] Uptake	Elimination
I	Ocean 0d	176	27
	Instant Ocean 0d	169	29
II	Laboratory 1 d	144	41
	2 d	135	33
	4 d	121	23
	8 d	107	28
III	Laboratory 4 d	166	36
	8 d	163	55
	16 d	124	37
	31 d	235	28

[1] Half-time estimated from the linear portion of semilog uptake (5-20 min) and elimination (5-20 min) curves.

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TABLE V
Effect of Aldrin, p-Nitroanisole, and SKF 525A on Uptake and Elimination of Antipyrine by Mussels

Chemical[1]	Conc.[2] (ppm)	Apparent Half-Time[3] (min) Uptake	Elimination
Aldrin	0	100	55
	0.1	107	20
	1.0	97	43
p-Nitroanisole	0	120	35
	0.5	122	29
	5	107	32
SKF 525A	0	121	26
	1.6	118	38
	16	91	35

[1] Estimated from linear portion of uptake and elimination curves (semilog). Six mussels per 300 ml containing 1.8 μmole antipyrine (0.6 μM).

[2] Calculated concentrations.

[3] Each value duplicate including controls (range ca. 15%).

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Using antipyrine as a diagnostic tool, impacts of other chemicals or chemically uncharacterized solutions/suspensions may be assessable.

Also included in these in vivo studies were trials with the microsomal monooxygenase inhibitor, SKF 525-A. As in the cases of the susbtrates, no remarkable effects were observed. The fate of the inhibitor is unknown. To date, SKF 525-A exposures have not been coupled with measurements of oxidative metabolism.

Biotransformation. Biotransformation is the most frequently investigated disposition process and we have begun studies to define metabolic capabilities of mussels. In general, mussels have been regarded as lacking metabolic capability toward foreign compounds such as oil hydrocarbons (24, 25) and 3,4-benzo(a)pyrene (26). This apparent metabolic deficiency is frequently cited in justification of the usefulness of mussels in environmental monitoring programs.

Oxidative metabolic capability, specifically the ability to use molecular oxygen for the metabolism of foreign compounds, is perhaps common to aerobic organisms. Comparative studies in our laboratory have routinely utilized lipophilic substrates such as aldrin, p-nitroanisole, antipyrine, and benzo(a)pyrene to demonstrate monooxygenase activity. In the present case most work has been done with aldrin and antipyrine. Each is relatively stable, available in pure form, of known toxicity, and transformed to a relatively simple spectrum of metabolites. With the objective being definition and assessment of metabolic capability, such substrates are favored over substances representing particular use classes, i.e. water-soluble petroleum fractions, pesticides, etc. Knowledge about the oxidative metabolic capability of animals is frequently essential for predicting the disposition of foreign compounds.

Aldrin has been administered in vivo by injection into the posterior adductor muscle and by topical application to tissues (primarily gill). During the test period, animals have been left undisturbed in the ocean surf, confined in retrievable traps, or held in beakers at the study site and in the laboratory. Study periods have varied from 2 to 24 hours. Animals frozen and later homogenized and extracted yielded homogenates which contained aldrin and dieldrin and no other electron-capturing metabolites compared to controls.

Aldrin and dieldrin have similar physical and chemical properties, and hence exhibit similar behavior in animals and during analysis. The following relationship is used as an indicator of oxidative metabolic capability, and it is termed the aldrin metabolic index (AMI):

$$\text{AMI} = \frac{\text{Dieldrin (nanomoles)}}{\text{Aldrin + Dieldrin}}$$

Total chlorohydrocarbon recoveries in in vivo work are low in these experiments but variability of the AMI is less than 20%.

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It seems likely that most of the losses following topical aldrin application are due to poor absorption rather than to elimination. The intramuscular route is being used as a means to obtain long-term, low level exposures. Since these procedures permit *in situ* administration of test compounds, an operation difficult or impossible with the more common immersion strategy for dosing, intramuscular injections will receive increased attention and evaluation.

Several factors effecting the metabolic index have been examined. Dose-time relationships have been investigated in mussels dosed by either immersion or topical aldrin applications. *In vivo* metabolism of aldrin has been demonstrated using immersion exposures. The 3 levels of aldrin (30, 300, 3000 µg) added to the 300 ml test systems are several times the water solubility of aldrin (27 ppb; 27). These levels produced no detectable effect on the well-being of the mussels. Mussels removed 58, 46 and 18% of the aldrin added during the 6 hour exposure and from whole mussel extracts AMIs of 0.064, 0.024, and 0.017 were measured. These results are consistent with the suggestion that aldrin metabolism is saturable in living mussels. Time was the independent variable in experiments utilizing topical aldrin applications (release of aldrin solution into mantle cavity). After 8 hour exposures mussels treated *in situ* in their natural habitat, the AMI was 0.008 (n = 5). Mussels which were held 24 hours had an AMI of 0.015 (n = 5). Mussels anchored to their natural habitat will be useful for studying possible influences of environmental variables such as water quality and position of the mussel in the water column. AMIs of similar magnitude have been observed in mussels held in small wire cages during the test period. These experiments demonstrated time and dose dependent dieldrin production under natural and laboratory conditions. Although low levels of activity were obtained, dieldrin was specifically and sensitively detected in mussel extracts. Its formation provides a useful index of oxidative metabolic capability.

Antipyrine metabolism *in vivo* has also been demonstrated. Following extraction of media from uptake and elimination studies, TLC analysis revealed the parent compound and 4-hydroxyantipyrine. Based upon the amount of radioactivity recovered, the metabolite may account for up to 4% of the total. This hydroxylated metabolite is the primary oxidation product in animals studied to date (23). Further characterization of the extracts using high-pressure liquid chromatography will be done in the future.

Similarly, *in vivo* metabolism of p-nitroanisole has been demonstrated by formation of a more polar, unidentified metabolite revealed following extraction and TLC (Benzene: MeOH, 95:5, Silica gel G, 0.25 mm). Indication of *in vitro* anisole metabolism has also been obtained, but the nature of metabolites is uncertain. For the present, these observations are con-

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sistent with the notion that mussels possess oxidative metabolic capability.

These studies with aldrin and antipyrine are sufficient to document the *in vivo* oxidative metabolic capability of mussels. Limits of activity have not been established, but they will be explored by further varying dose and the duration of the test period. With these limits established, the influence of environmental stressors such as salinity, and dissolved and/or suspended particulate matter in water on biotransformation will be assessed. If biotransformation processes are affected by these conditions, their measurement may provide results which can be diagnostically used as indicators of environmental quality.

In vitro studies provide opportunity for more direct control over the experimental system than do investigations with living animals. As a corollary to the *in vivo* work, biotransformation has been studied in cell-free mussel preparations. Results of this aspect of the investigation have been plagued by lack of reproducibility and, therefore, these preliminary results must be considered more tentative than usual.

Biochemical studies with mussel tissue homogenates and microsomal fractions have been conducted using viscera (whole mussel minus adductor muscles), green gland, gill, and mantle. Using aerobic conditions and an NADPH-generating system, the metabolism of aldrin and p-nitroanisole have been observed. During a 30 minute incubation period, low levels of aldrin epoxidation (30-150 picomoles dieldrin/mg protein) were measured compared to those observed using enzyme sources such as aquatic Trichoptera *Limnephilus* sp. gut homogenates (1 pmole/mg protein; 28) or rat liver homogenates (3000 pmoles/mg protein; unpublished) under similar incubation conditions. Anisole metabolism based upon substrate disappearance was detectable but less than 5 picomoles/mg protein were transformed during the incubation period. Characteristics of the enzyme system are incompletely described owing to the low and variable levels of activity which have been obtained.

Several modifications of incubation conditions have neither stabilized the system nor enhanced activity. Acetone and methanol have been used as substrate carriers without affecting activity. Similarly, addition of NADH to the incubation media did not effect epoxidation. The enzymatic nature of the system has been confirmed by use of heat treated homogenates (100°C, 1 min). Incubation temperatures of 8°, 20°, and 30° resulted in progressively greater epoxidation rates and provided no evidence of heat lability. Thus, at this time it is not possible to identify a superior enzyme source for comparative studies in spite of the fact that *in vivo* measurements indicate oxidative metabolic activity in living mussels.

Interferences in the mussel homogenates may be responsible, in part at least, for the low measured epoxidation activity. Properties of endogenous inhibitors in several insect preparations have been reviewed by Wilkinson and Brattsten (29).

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Additions of mussel viscera from animals taken directly from the ocean and from others held several days in Instant Ocean® did not alter rat liver microsomal aldrin epoxidation. In other experiments, inhibition by tissue homogenates has been observed. Homogenates will continue to be screened against rat liver microsomes until this uncertainty is resolved. Characterization of the inhibitory activity in the homogenates may provide leads for stabilization of the mussel preparations.

In vivo oxidation activity may not be expressed in vitro due to inclusion of excessive amounts of "inactive" tissue in the various cell-free preparations. The resulting tissue dilution artifact renders activity unmeasurable due to the sensitivity of the analytical procedures. This consideration warrants further experimental evaluation.

In another approach to indentify tissue(s) with high monooxygenase activity, microsomal fractions of green gland, gill, and viscera have been prepared and analyzed using difference spectroscopy for cytochromes b_5 and P-450. The rationale has been that although endogenous inhibitory factors or preparative artifacts associated with dissection, tissue disruption and centrifugation might destroy or reduce monooxygenase activity, those factors would unlikely destroy cytochromes b_5 and P-450/P-420. Using microsomal suspensions containing 1-3 mg protein/ml and standard spectrophotometric techniques only cytochrome b_5 has been detectable. Visceral preparations contained $0.040 \pm .006$ nmoles cytochrome b_5/mg protein. Use of these chemical constituents will be continued in future efforts to localize monooxygenase activity.

Histology. An important aspect of this study is documentation of histologic evidence of tissue injury or transformation that may be associated with altered disposition. Previous reports of neoplasia, infestations, and non-specific tissue injury have been largely anecdotal; they have not been well correlated with measurements of tissue function or exposure to toxic substances. Assessment of effects is extremely difficult, lacking precise knowledge of concentration-time relationships.

It is anticipated that mussels, as in any other well-studied organism, would develop either specific diseases, such as neoplasms, or demonstrate non-specific acute or chronic changes at the tissue level in response to environmental stress. Histologic evidence of tissue injury may eventually prove to be an important indicator of mussel viability. Our initial pathologic studies will increase knowledge of histologic characteristics of mussel tissues including gill, green gland, and mantle. The latter tissues have received special attention since they may be useful enzyme sources for biotransformation studies.

Tissues are being examined by conventional histologic and electron microscopic techniques. An atlas of normal gross and microscopic mussel structure is being prepared using animals col-

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lected from our relatively uncontaminated sites near Bodega Head (8). Subsequent collections in California at sites characterized by high natural petroleum hydrocarbon levels or chlorinated hydrocarbons will provide material for comparative studies.

Conclusion

If disposition processes in mussels can be measured using indicator chemicals such as antipyrine and aldrin, it may be possible to assess certain biological consequences of some environmental stressors including chemicals. Such information would complement the wealth of environmental monitoring data obtained in programs in which mussels have served to expedite sampling. In calling for more widespread use of organisms such as mussels in the determination of environmental quality Bayne (31) observed, "Chemical analysis, either of water, sediment or of organisms only provides a limited understanding of environmental wellbeing because the ecological consequences of pollution are biological, not chemical." It seems that studies of disposition processes represent useful tools for assessment of biological aspects of environmental quality.

Inclusion of histopathology in experimental protocols enhances the possibility of detecting changes in tissue and cellular form and function that might be associated with stress. Results of histopathological studies might also guide selection of future physiological test parameters. For example, proliferation of endoplasmic reticulum might signal altered oxidative metabolic capability before it can be detected *in vivo* or *in vitro*. The usefulness of tissue studies in predictive toxicology is firmly established, and combination of disposition and histopathological studies enhances the likelihood of detecting biological effects of chemical exposures in mussels.

Acknowledgements

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Cytochrome P-450 in Fish Liver Microsomes and Carcinogen Activation

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Random somatic mutation is thought to be a major event in carcinogenesis which may result from exposure to radiation, viruses, or chemicals. In recent years the role of environmental chemicals in the initiation of cancer has become more evident and the mechanisms involved have been greatly clarified (1).

In mammals the cytochrome P-450 mediated monooxygenase or mixed function oxidase system involved in the elimination of lipophilic environmental contaminants and other foreign compounds, has been implicated in the carcinogen activation process. There are several distinct variants of cytochrome P-450 in mammalian tissues and there may be more than one form of this ubiquitous cytochrome also in fish. The significance of this lies in the fact that different forms of cytochrome P-450 result in different metabolite patterns, which in turn may reflect on the carcinogenicity or toxicity of compounds being metabolized.

Cytochrome P-450 mediated monooxygenase

About twenty years ago a liver microsomal pigment was discovered which in its reduced form was found to bind carbon monoxide, resulting in a complex absorbing light at 450 nm (2, 3). It was established that the pigment is a hemoprotein and was named cytochrome P-450 (4, 5). It was soon realized that cytochrome P-450 plays a central role in the metabolism of a wide variety of drugs, endogenous and synthetic steroids, pesticides, polycyclic aromatic hydrocarbons (PAH) *e.g.* carcinogenic benzo(α)pyrene (BP) and a large number of other compounds which come intentionally or unintentionally into contact with living organisms. Existing information on cytochrome P-450 and its functions has been extensively reviewed by a number of authors (6-13).

Cytochrome P-450 in fish. Cytochrome P-450 with its characteristic spectral properties has since its discovery been detected in a wide range of organisms including several species of fish (13)

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(Table I). The levels of both, cytochrome P-450 (Table I) and its NADPH (reduced nicotinamide adenine dinucleotide phosphate) requiring reducing component (Figure 1)(which can be measured as NADPH dependent cytochrome c reductase) are substantial in fish liver microsomes, although lower than in mammals. NADPH cytochrome c reductase level in trout (*Salmo trutta lacustris*) is 20 nmol cytochrome c reduced/mg microsomal protein/min; the corresponding activity in male Sprague Dawley rat liver microsomes is 96 nmol cytochrome c reduced/mg microsomal protein/min (14).

Table I. Comparison of the liver microsomal levels of cytochrome P-450 in various species of fish with those of some mammals.

Source	Cytochrome P-450 concentration*	Reference
Carp	present	(15, 16)
Female carp	0.38	(17)
Female gibel	0.15	(17)
Trout (*Salmo gairdneri*)	0.22	(18)
Trout (*S. gairdneri*)	present	(19, 20)
Trout (*S. trutta lacustris*)	0.20-0.40	(14, 21)
Trout (*Salvalinus fontinalis*)	0.56 (0.19 starved)	(22)
Kissing gourami	1.03	(22)
Bluegill	0.81	(22)
Winter flounder	0.17	(23)
Little skate	0.22-0.32	(23-26)
Large skate	0.36, 0.41	(21)
Dogfish shark	0.23, 0.29	(23, 26)
Fundulus heteroclitus	0.009	(27)
Stenotomus versicolor	0.35	(28)
Atlantic stingray	0.43	(26)
Bluntnose stingray	0.32	(26)
Sheephead	0.29	(26)
Mangrove snapper	0.25	(26)
Black drum	0.14	(26)
Large mouth bass	0.0215**	(29)
Mammals		
Rat	0.72	(14)
Mouse	0.99	(30)
Rabbit	1.55	(4, 5)

*nanomoles of cytochrome P-450/mg microsomal protein.
**Absorbance/mg microsomal protein; comparative values for rat and mouse are 0.0860 and 0.0970, respectively (29); these authors justifiably avoid calculating molar quantities as the extinction constant available (91 $mM^{-1}cm^{-1}$) is for mammalian cytochrome P-450 (4, 5).

Spectral properties of cytochrome P-450. The spectra of reduced cytochrome P-450.CO complex in fish liver microsomes display consistently a peak at 420 nm when the Omura and Sato method (4, 5) is used (14, 19, 22, 27). It would appear that the fish

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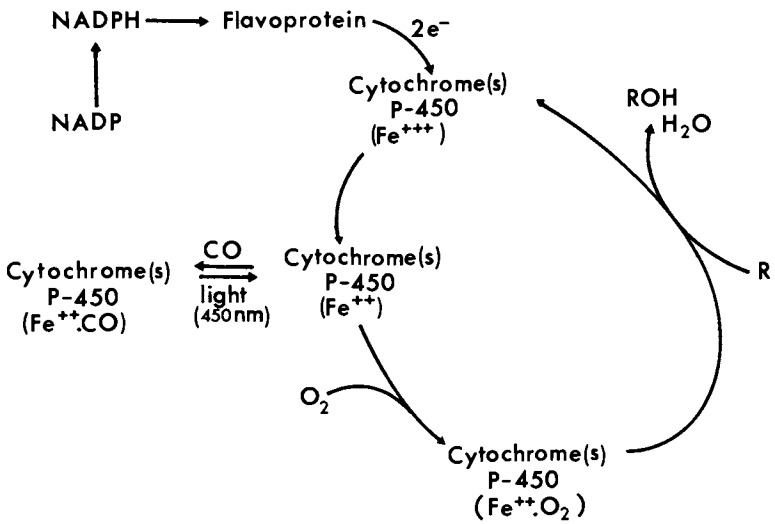

Figure 1. Simplified scheme for the electron transfer in the Cytochrome P-450 mediated monooxygenase activity. In the liver, the flavoprotein is Cystochrome c reductase. R is the compound being metabolized. NAD and Cytochrome b_5 have not been included (36).

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liver microsomal cytochrome P-450 is more sensitive and is converted into inactive form of cytochrome P-450 upon normal handling. If this is the case many of the estimates of cytochrome P-450 in fish liver may be erroneously low. However, according to Stanton and Khan (22) the 420 nm peak is not due to cytochrome P-420, as it increases with time without concomitant decrease in the cytochrome P-450 level. Also, it has been noted by us that if the method of Greim *et al.* (31) is used the 420 nm peak does not appear (21). Fukami *et al.* (16) reported an absorbance peak at 430 nm for reduced carp microsomal preparation + CO, however it must be noted that they bubbled the preparations unusually long (10 min) with carbon monoxide.

Recently Bend *et al.* have succeeded to solubilize and partially purify little skate liver microsomal cytochrome P-450 and have thus been able to record the absolute spectra of fish cytochrome P-450 (25). The spectra are very similar to those of rat liver microsomal cytochrome P-450 and purified rat liver cytochrome P-450 and P-448 (Table II).

Table II. Comparison of the spectral properties of partially purified little skate liver cytochrome P-450 with partially purified rat liver cytochrome P-450 and highly purified rat liver cytochrome P-450 and P-448.

	Little Skate	Rat liver cytochrome		
	P-450*	P-450**	P-450***	P-448***
Specific content (nmol/mg protein)	~3	~3	~17	~20
Molecular weight	?	?	48,000	53,000
Absorption maxima (nm):				
oxidized	420, 482, 571	360, 418, 537, 568	418, 535, 568	417, 535, 568
reduced	421, 543	418, 545	414, 546	411, 545
reduced + CO	450, 553	423, 450, 548	450, 552	447, 552

*Partially purified Little Skate liver cytochrome P-450. The data is from (25) and is as accurate as can be read from published spectra. **Partially purified rat liver cytochrome P-450; the data is from (6). ***Highly purified cytochrome P-450 and P-448 from rat liver; the data is from (32).

Criteria for monooxygenase activity. Metabolism of foreign compounds by microsomes was first described by Mueller and Miller (33, 34). They noted that NADP, NAD (nicotinamide adenine dinucleotide) and molecular oxygen are required in the oxidative N-demethylation of aminoazo dyes. In 1955 Brodie *et al.* (35) showed that liver microsomes metabolized many drugs and for full activity a NADPH generating system, consisting of glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase or NADPH itself is required. The electron transfer system involved in the metabolism of foreign com-

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pounds has been extensively studied and several schemes have been suggested. Figure 1 shows a simplified scheme for the electron transfer (modified after Coon *et al.* (36)) showing also the characteristic light reversible inhibition of the cytochrome P-450 mediated system by carbon monoxide.

The electron transfer system has not been studied in detail in fish, but the metabolism of compounds such as biphenyl (37), benzo(α)pyrene (21) and 2,5-diphenyloxazole (38) by fish liver microsomes has been shown to require oxygen and NADPH generating system. The metabolism of BP (21), 2,5-diphenyloxazole (Ahokas, unpublished observation) and aldrin (27) by fish liver microsomal enzyme system is inhibited strongly by carbon monoxide. This information and the fact that cytochrome P-450, as well as NADPH cytochrome c reductase system are present in fish, suggest strongly that fish have a cytochrome P-450 mediated monooxygenase system which is very similar to that described in mammals.

Multiple forms of cytochrome P-450. It is now clear that there are more than one form of cytochrome P-450. Thomas *et al.* have recently shown by immunochemical means that there are at least six forms of mammalian cytochrome P-450 (39). In 1960's it was noted that there are at least two catalytically and spectrally distinct cytochrome P-450's, *viz.* cytochrome P-450 and cytochrome P-448 or P_1-450 (40, 41). Cytochrome P-448 is inducible by PAH's such as 3-methylcholanthrene (MC) and BP. It metabolizes preferentially PAH's (such as the above carcinogenic inducers). Cytochrome P-448 derives its name from the fact that when reduced and complexed with carbon monoxide it has an absorbance maximum at 448 nm. Cytochrome P-450 induced by compounds such as phenobarbital (PB) appears similar to the control cytochrome P-450 both spectrally and catalytically.

In addition to the 2 nm shift in the absorption maximum, the two cytochromes can be distinguished by the use of ethyl isocyanide interaction spectra (6, 7) and various inhibitors of the monooxygenase activity (Figure 2 and Table III). The relative magnitude of the ethyl isocyanide-cytochrome P-450 interaction spectral peaks at ~430 and ~455 nm is pH dependent (6) and if the absorbance differences are plotted as functions of pH, there is a cross-over point at a certain pH which is characteristic for a particular form of cytochrome P-450; pH ~6.9 for cytochrome P-448 and pH ~7.5-7.6 for PB induced or control cytochrome P-450 (6, 21). The cytochrome P-450 of apparently uninduced trout species (*Salmo trutta lacustris*) has been shown by us to have the pH cross-over point for ethyl isocyanide interaction spectrum at pH 7.8 (21) and the absorption maximum of the reduced trout liver cytochrome P-450 .CO complex is 450 nm, nevertheless its catalytic and inhibitory properties (21)(Table III) are similar to those of cytochrome P-448.

Curiously this parallels the discovery of MC inducible cytochrome P-450 in fetal and neonatal rabbits (43) which, a) fails to

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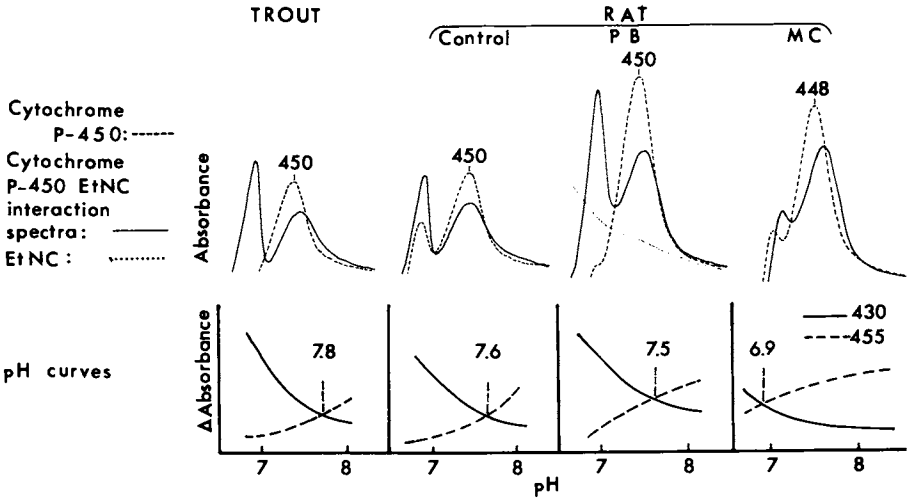

Figure 2. Spectral properties of Cytochrome P-450s from various sources related to the BP hydroxylase activity, the inhibition of BP hydroxylase, and the extent of covalent binding of BP to DNA.

Cytochrome P-450 spectra (reduced + CO) show the 2-nm shift to the blue as a result of 3-MC induction. No such shift is observed in the trout, control rat, and PB-induced rat liver microsomes. Cytochrome P-450 EtNC spectra were recorded at pH 7.4, 2 min after the samples were reduced with dithionite. The absorption peaks are at 430 and 455 nm. For pH curves the Δ Absorbance represents ΔA (430–490 nm) and ΔA (455–490 nm). The number of (+) signifies only the relative activity or inhibition; with respect to BP hydroxylation and covalent binding of BP to DNA (+++) signifies 2–4 times and (++++) signifies 10–36 times the control rat microsomal activity; (n.d.), not determined. Figure 2 is based on data from (6, 21, 69, 70).

Table III. Inhibition of monooxygenase (aryl hydrocarbon hydroxylase) activity in fish and mammalian hepatic microsomes (based on Goujon *et al.* (45) and Ahokas *et al.* (21)).

Inhibitor	Trout cytochrome P-450	Rat	
		Cytochrome P-448	Cytochrome P-450
SKF 525A	-	-	++
Metyrapone	-	-	++
DDT	±	±	++
Pyridine	-	-	++
n-Octylamine	-	-	++
α-Naphthoflavone	++	++	-
Lindane	++	++	-
Testosterone[1]	++	++	-

++, strong inhibition; ±, intermediate inhibition; -, poor inhibition. [1]Applies to mouse; no inhibitory differentiation between rat cytochromes P-448 and P-450 was observed (21).

show a spectral blue shift in the Soret maximum of the reduced cytochrome P-450.CO complex, b) is readily inhibited by α-naphthoflavone (no ethyl isocyanide interaction spectral data was presented).

Induction of aryl hydrocarbon hydroxylase activity in teleost fish (28, 44) and elasmobranch (25) has also been observed without a hypsochromic shift in the spectrum of the cytochrome P-450.CO complex.

Electron paramagnetic resonance (EPR) examination of hepatic microsomes from differently pretreated animals has lead to the conclusion that MC pretreatment leads to the formation of cytochrome P-450 (P-448) with high spin iron (6, 46). Chevion *et al.* (28) failed to demonstrate the presence of high spin cytochrome P-450 in a teleost fish even after induction with MC. The work of Chevion *et al.* (28) indicates further that the fish cytochrome P-450, which is inducible with PAH's and metabolizes readily PAH's, is not identical with the mammalian cytochrome P-448.

Certain compounds (other than ethyl isocyanide) are known to cause spectral changes when added to microsomal suspensions (6, 10). The spectral changes, interaction spectra, have been classified as follows: Type I (an absorption peak at about 390 nm and a trough at about 420 nm), Type II (a trough near 390 nm and a peak between 425 and 435 nm) and modified Type II or reverse Type I spectral change (a trough near 390 nm and a peak near 420 nm). All these spectral changes have been observed to occur with fish liver microsomal cytochrome P-450 (14, 21, 22, 47). Stanton and Khan observed Type I spectral changes in trout liver microsomal preparations (with isodrin, aldrin and hexobarbital as ligands) and in two other species of fish both Type I and Type II spectral changes were observed (22). On the other hand, Ahokas *et al.* (14, 21, 47) could not demonstrate Type I spectral change in microsomal preparations of another species of trout using hexobarbital, SKF 525A

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or 17α-hydroxyprogesterone as ligands. It was possible to demonstrate Type I spectral change in these preparations by using BP (21) and tetrachloroethene (Pelkonen and Ahokas, unpublished observation) as ligands. These results suggest that there are differences in the cytochrome P-450's of even two relatively closely related fish.

Active metabolites and carcinogenicity

Salmonid fish have become a classical example of organisms highly susceptible to chemical carcinogens (48). The incidence of tumors in salmonid and other species of fish has been extensively documented (48, 49, 50, 51) with Wood and Larson reporting the most alarming 50% occurrence of gross tumors among 250,000 adult rainbow trout (52).

A widespread outbreak of hepatoma in cultivated rainbow trout in 1960 marked the beginning of extensive search for a causative agent. This was determined to be almost certainly aflatoxin (53). The carcinogenicity of several other compounds has been investigated in fish and at least the following have increased the incidence of trout hepatoma by more than 20% compared with controls: dimethylnitrosamine, aminoazotoluene, DDT and 2-acetylaminofluorene (2-AAF) (54). The carcinogenicity of aflatoxin B_1 (ATB_1) and 2-AAF to trout has been found to be promoted by cyclopropenoid fatty acids (55). Hepatic tumours have been induced also in the guppy (*Lebistes reticulatus*) by ATB_1, dimethylnitrosamine and 2-AAF (56). The effects of four classes of carcinogens on aquarium fish were investigated by Pliss and Khudoley (57) who studied the carcinogenicity of PAH (MC and 7,12-dimethylbenz(α)anthracene), aromatic amino compounds (benzidine and 2-AAF), azo compounds (o-aminoazotoluene and 4-dimethylaminoazobenzene) and nitroso compounds (diethylnitrosamine, dimethylnitrosamine and nitrosomorpholine). All except benzidine and the two PAH compounds caused hepatic tumors. However, Matsushima and Sugimura (58) demonstrated the production of epitheliomas in aquarium fish by PAH's (MC and BP).

There have also been reports of neoplasms in native bottom-feeding fish with a suggestion that carcinogenic hydrocarbons from motor boat exhausts, rotenone and insecticides such as DDT may be involved as causative agents (59). Similarly (an) unidentified carcinogen(s) are suspected in the case of adematous polyps of gastric mucosa of fish, reported recently (60). The diet of these fish was free of aflatoxins.

It has been proposed that in as many as 70-80% of the cases of human cancer environmental chemicals are the causative factors (1). There is no reason why similar estimates would not be valid for animals. It is noteworthy that the occurrence of chemical carcinogens is widespread in the aquatic environment. For example, of the PAH's, BP is found in the concentration of 50 to 100 $\mu g/m^3$ in what is considered moderately polluted surface water; in waste water as much as 100,000 $\mu g/m^3$ has been measured (61). Several

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other proven carcinogens (benzidine, vinyl chloride) and suspected carcinogens (polychlorinated biphenyls, DDT, chlordane, lindane) are causing concern as widespread water pollutants or potentially as such (61).

Cytochrome P-450 in carcinogen metabolism. In spite of the bewildering number of carcinogens involved the important and unifying fact is, that most of the organic carcinogens are not carcinogenic *per se*, but require metabolic activation *in situ* by cytochrome P-450 mediated aryl hydrocarbon hydroxylase (AHH, also known as BP hydroxylase (EC 1.14.14.2)).

Aryl hydrocarbon hydroxylase has attracted considerable interest in the recent years as it was noted that the hydroxylation of aromatic ring structures occurs via an epoxide intermediate (62). These arene oxide intermediates have since been incriminated as active intermediates responsible for the covalent binding of PAH's to tissue macromolecules, as it was known from the work of Grover and Sims (63) and Gelboin (64), that such binding is dependent on metabolic activation. It is well known that high AHH activity is associated with cytochrome P-448. However, high AHH activity in itself may not result in covalent binding, mutagenicity or carcinogenicity. The predominance of certain metabolites and saturation of competitive elimination processes contribute towards events leading to carcinogenicity (Figures 3 and 4). It is known that cytochrome P-450 and P-448 metabolize certain compounds quite differently (Figure 3). Most of the information has been obtained using rat and other common laboratory mammals and relatively little is known how various carcinogens and other aromatic compounds are metabolized by fish liver microsomes. In 1966 Lotlikar *et al.* (65) reported the metabolism of a potent carcinogen 2-AAF which in rainbow trout caused a low incidence of liver tumors. They found that 2-AAF was metabolized by trout liver preparations, but it was not N-hydroxylated to an intermediate known to be highly carcinogenic. This would suggest that the nature of the cytochrome P-450 involved resembles enzymatically control cytochrome P-450 (cf. Figure 3). Similarly, biphenyl metabolism (non-carcinogen) was catalyzed by trout liver microsomes as if the cytochrome involved was control rat liver cytochrome P-450; the metabolite formed was 4-hydroxybiphenyl (37).

It has been noted that cell cultures derived from trout (66, 67) and trout liver microsomes (42, 47, 68) may have relatively high AHH activity (BP hydroxylase) which sometimes exceeds the activity observed in control rat liver microsomes. The metabolite pattern obtained using trout liver microsomes resembled that produced by MC treated rat liver microsomes (P-448)(69 and Ahokas, Saarni, Nebert and Pelkonen, manuscript in preparation; Table IV). Associated with this pattern of BP metabolites was covalent binding to DNA which was three times as high as obtained by using rat liver microsomes. Another species of fish (roach), on the other hand was found to be almost inactive in catalyzing *in vitro* bind-

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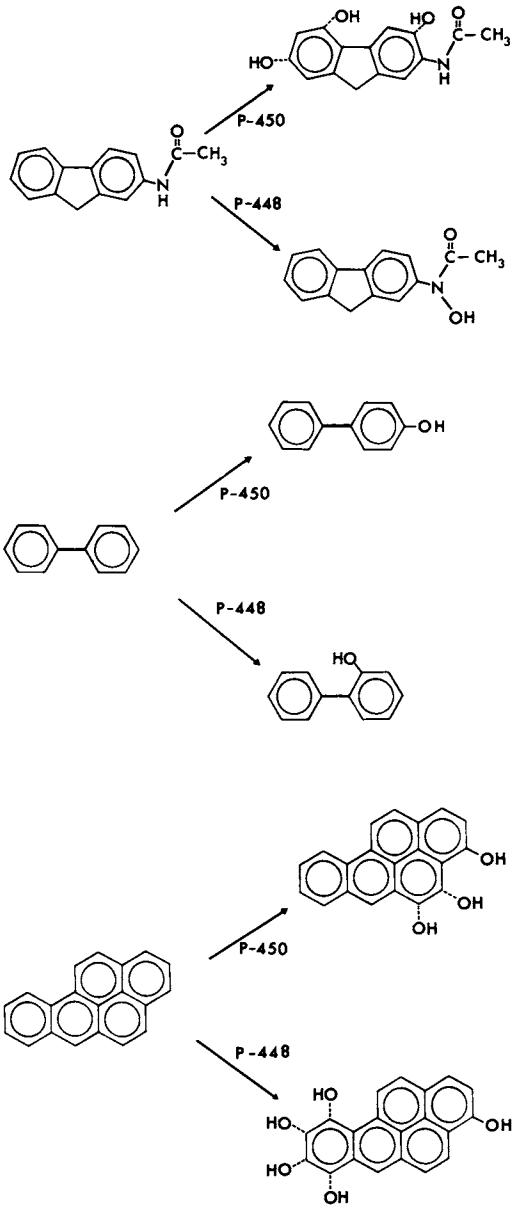

Figure 3. Preferential routes of metabolism of 2-AAF, biphenyl, and BP (from top to bottom) by different forms of rat liver microsomal Cytochrome P-450. Trout has been reported to metabolize 2-AAF (65) and biphenyl (37) like rat liver Cytochrome P-450 and BP like rat liver Cytochrome P-448 (69, 70).

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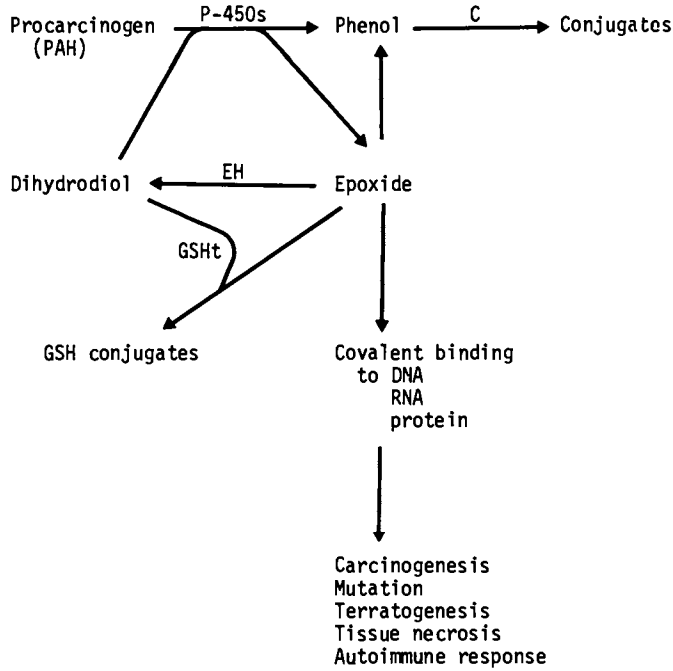

Figure 4. Proposed scheme for Cytochrome P-450 mediated metabolism (inactivation and activation) of procarcinogens (e.g. PAHs).

Conversion of epoxides (arene oxides) into phenols is spontaneous. The conversion of epoxides into dihydrodiols is catalyzed by EH (EC 4.2.1.63). Hydroxyl containing PAHs can act as substrates for conjugases (C) (UDP glucuronsyl transferase (EC 2.4.1.17) and phenol sulphotransferase (EC 2.8.2.1)). This pathway usually leads to inactive excretable products. Epoxides are scavenged by GSH and the reaction is catalyzed by GSHt (EC 2.5.1.18). When GSH is depleted and/or the other pathways are saturated, epoxides of dihydrodiols (particularly 7,8-diol-9,10-epoxides in the case of BP) and phenol metabolites react with cellular macromolecules such as DNA, RNA, and protein. If repair mechanisms are exceeded the detrimental effects of PAH may result.

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Table IV. Comparison of the relative amounts of BP metabolites formed by trout, control rat and MC treated rat liver microsomes.

Metabolite	Trout %	Control rat %	MC-rat %
Total	137	100*	624
9,10-dihydrodiol	22	19	20
7,8-dihydrodiol	28	10	16
4,5-dihydrodiol	1	6	4
3-phenol	33	45	44
9-phenol	2	1	4
quinones	14	20	13

*Total amount of metabolites formed by control rat liver microsomal fraction is arbitarily taken as the reference for the other two totals (data from (70)).

ing of BP to DNA.

The fact that trout liver microsomal preparation can produce significant amounts of nucleoside adducts of BP 7,8-diol-9,10 epoxide and activated BP phenols (70) indicates that fish have enzymatic capability to activate carcinogens to reactive intermediates. The *in vivo* significance of these findings cannot be predicted at the moment. Although the critical cellular targets of chemical mutagens and carcinogens are not fully known, DNA is a prime suspect. Whether or not there is any correlation between *in vitro* activation and covalent binding of a carcinogen and its *in vivo* effects, remains to be seen. However, at least in one instance with respect to mutagenesis in *Salmonella typhimurium* mutagenesis assay (71), we have shown that the reactive intermediates formed by trout liver microsomes can produce biological effects (72). This finding has been recently confirmed (73, 74).

Conclusions

It appears that various fish possess different types of cytochrome P-450, with resultant differences in the metabolism of carcinogens and related compounds.

There are marked species differences in the production of DNA binding metabolites of BP. Several reports indicate that fish liver microsomes can activate promutagens into mutagenic intermediates. However, there is a lack of unified picture, mainly because the characterization of cytochrome P-450, cytochrome P-450 mediated carcinogen metabolism and induction of cancer have been performed in most cases by different laboratories, using different sources of fish and often different species of fish. Not until a consorted effort is made to characterize cytochrome P-450 mediated AHH and carcinogenesis in one population of fish, can we expect conclusive results on inducibility, carcinogen activation and resulting somatic mutation and carcinogenesis.

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Microsomal Mixed-Function Oxidation in Untreated and Polycyclic Aromatic Hydrocarbon-Treated Marine Fish

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The aquatic environment is a repository for numerous foreign organic chemicals (xenobiotics) that occur as environmental pollutants. Dumping of industrial and urban wastes, leaching from landfill sites, atmospheric fallout of airborne particles, runoff from cultivated land that has been treated with insecticides or herbicides, accidental or intentional spillage during shipping, and seepage of hydrocarbons from underwater oil deposits all contribute to this pollution. As certain xenobiotics, such as the polycyclic aromatic hydrocarbon, benzo(a)pyrene, are metabolically activated to products that are toxic (mutagenic, carcinogenic, cytotoxic) to mammals, including man, the ability of aquatic species to biotransform and excrete xenobiotics is of considerable importance, especially in those species that are used for food. Based on this rationale, we have been investigating the *in vitro* and *in vivo* metabolism of xenobiotics in marine vertebrate and invertebrate species. Attention has focused upon the cytochrome P-450-dependent microsomal mixed-function oxidase (MFO) system (1-6), which is very important in the oxidative metabolism of most lipophilic pollutants including hydrocarbons, and those enzymes which are responsible for the further biotransformation of toxic alkene or arene oxides (4) as well as conjugation pathways for chlorinated phenoxyacetic acid herbicides (7, 8).

In general, our studies with cytochrome P-450-dependent metabolism have emphasized the similarity of the hepatic MFO system in marine fish to that found in mammals. Thus, in the little skate (*Raja erinacea*), a marine elasmobranch, enzyme activity is localized in the microsomal fraction, requires NADPH and molecular oxygen for maximum activity, and can be inhibited with CO (1, 2). Moreover, when hepatic microsomes from the little skate were solubilized and separated into cytochrome P-450, NADPH-cytochrome P-450 reductase, and lipid fractions, all three fractions were required for maximal MFO activity in the reconstituted system (3). We have also found, as have others, that the administration of polycyclic hydrocarbons (3-methylcholanthrene, 1,2,3,4-dibenzanthracene [DBA]), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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(TCDD), polychlorinated biphenyls (Arochlor 1254), or polybrominated biphenyls (Firemaster FF1) to marine vertebrates such as little skate, winter flounder (*Pseudopleuronectes americanus*), southern flounder (*Paralichthyes lethostigma*), or sheepshead (*Archosargus probatocephalus*) results in dramatic increases (up to 35-fold) in hepatic microsomal benzo(a)pyrene hydroxylase (aryl hydrocarbon hydroxylase [AHH]) activity (2, 3, 4, 6, 9, 10). This is to be expected from similar observations in several mammalian species. However, in fish treated with DBA or TCDD, under conditions where induction of AHH activity was observed, there was no apparent wavelength shift in the absorption maximum of the CO-bound form of reduced cytochrome P-450 in hepatic microsomes (i.e., no indication of cytochrome P-448 formation) nor was there a significant increase in microsomal cytochrome P-450 content (3). The formation of cytochrome P-448 was anticipated in livers of these DBA- or TCDD-treated fish because increases in AHH activity resulting from administration of polycyclic hydrocarbon-like inducing agents to mammals are normally associated with cytochrome P-448 (11, 12). Consequently, we have investigated the polycyclic hydrocarbon-induced hepatic microsomal system of a few marine fish in some detail.

In this report we compare several properties of hepatic microsomal AHH activity in control and DBA-treated little skates (including metabolic profiles obtained from ^{14}C-benzo(a)pyrene as elucidated by high pressure liquid chromatography [HPLC]), we describe the partial purification of two different forms of cytochrome P-450 (cytochrome P-448 and cytochrome P-451) from hepatic microsomes of DBA-pretreated little skates and we report polycyclic hydrocarbon-like induction in large numbers of winter flounder assayed in Maine during June, July, and August, which was quite different than data obtained with sheepshead examined in Florida during the same period.

Materials and Methods

Fish Collection, Maintenance, and Treatment. Adult fish were collected near Mount Desert Island, Maine, or Marineland, Florida, and were acclimated in aquaria equipped with continuously flowing seawater or in live cars immersed in salt water for at least 24 hr before use. For induction studies little skates were injected IP with 10 mg/kg 1,2,3,4-dibenzanthracene in corn oil on days 1, 2, and 3 and were sacrificed on day 10. Control fish were injected with corn oil only.

Whole Homogenate and Microsome Preparation, Enzyme Assays. All fish were sacrificed between 5:00 and 9:00 a.m. Washed microsomes were prepared from liver homogenates as described previously (3). When whole liver homogenates were used as the enzyme source, 10% w/v homogenates were prepared in 0.15 M KCl, 0.02 M HEPES (N-2-hydroxypiperazine-N'-2-ethane sulfonic acid; Sigma) buffer (pH

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7.4). AHH activities were determined as previously described (3), except that additional incubation mixtures containing 10^{-3} M and 10^{-4} M α-naphthoflavone (microsomes) or 5 x 10^{-3} M and 10^{-3} M α-naphthoflavone (whole homogenate) were assayed simultaneously. One AHH fluorescence unit equals the fluorescence intensity of a 3 μg/ml quinine sulfate · $2H_2O$ in 0.1 N sulfuric acid solution (excitation λ 425 nm, emission λ 555 nm). 7-Ethoxyresorufin deethylase activity was assayed essentially as described by Burke and Mayer (13) at a 7-ethoxyresorufin concentration of 2 μM, a final pH of 7.8, and an incubation temperature of 30° (14). Epoxide hydratase activity, with ^{3}H-benzo(a)pyrene 4,5-oxide as substrate, was assayed by the thin-layer chromatographic procedure of Jerina *et al.* (15). The protein content of microsomal and whole homogenate preparations was determined according to Lowry *et al.* (16), using bovine serum albumin as the standard, and microsomal cytochrome P-450 content was assayed by the method of Omura and Sato (17) on an Aminco DW-2A spectrophotometer.

Metabolic Profile of ^{14}C-Benzo(a)pyrene in Skate Hepatic Microsomes. Microsomal suspensions (1 mg/ml) in 0.5 M HEPES buffer (pH 7.4) were preincubated for 1 min with 100 nmol 7,10-^{14}C-benzo(a)pyrene (4.1 mCi/mmol, radiochemical purity > 97.5%, purchased from California Bionuclear Corporation, Sun Valley, CA) dissolved in acetone (10 μl). The reaction was initiated by the addition of NADPH and was incubated at 31° for up to 30 min. The reaction was terminated by the addition of 2 ml ethyl acetate/acetone, 2:1, v/v, saturated with water, and vortexing. After centrifugation and removal of the upper layer, the incubation mixtures were extracted twice more with 2 ml of the ethyl acetate/acetone solution. The upper phases were combined, stored overnight at -20° to remove residual water, then evaporated to dryness in the dark at room temperature under a stream of nitrogen. The residues were taken up in 40 μl of methanol (glass distilled, Burdick and Jackson Laboratories, Muskegon, MI) and, after addition of a methanolic solution (5 μl) of unlabeled authentic benzo(a)pyrene metabolites (kindly supplied by the National Cancer Institute-NIH Carcinogenesis Research Program) analyzed by HPLC on a 1 m ODS Permaphase column in a DuPont Model 830 instrument, essentially as described by Holder *et al.* (18). Radioactivity was quantitated by liquid scintillation counting (Beckman LS 9000 instrument) using 10 ml Instagel (Packard Instrument Co., Inc., Downers Grove, IL) and appropriately quenched standards.

Solubilization and Partial Purification of Cytochrome P-450 from Hepatic Microsomes of Male, DBA-Pretreated Little Skates. Washed hepatic microsomes (3) from the livers of 10 skates were suspended in 0.25 M sucrose and frozen under nitrogen (-5 to -10°) at the Maine laboratory. They were then packed in dry ice and transported to NIEHS, Research Triangle Park, NC, within 14 days of preparation and were stored at -62°C until use. Microsomes

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from male and female skates (both control and DBA-pretreated) were pooled separately.

Thawed microsomal preparations (500-700 mg protein) from little skates were digested with sodium cholate (1 mg/mg protein) in 10 mM potassium phosphate buffer (pH 7.7) to make a final concentration of 10 mg protein/ml, in the presence of 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol. Digestion was carried out at room temperature for 20 min with constant stirring.

The digested microsomal preparation was centrifuged at 176,000x*g* for 60 min. The precipitate was discarded and the clear supernatant was applied to a DEAE-cellulose column (2.5 x 38 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1% sodium cholate, and 20% glycerol (Buffer I). The column was eluted with 1000 ml buffer I containing 0.5% Emulgen 913 (obtained from Kao-Atlas, Tokyo, Japan; Buffer II). The column was subsequently eluted with Buffer II containing a linear KCl gradient (0-0.5 M) at a flow rate of 60 ml/hr as described by Philpot *et al.* (19). Fractions (250 drops, 8-15 ml) obtained from the column were monitored at 418 nm, and for their cytochrome P-450 content, NADPH-cytochrome *c* reductase activity (determined according to Williams and Kamin [20]), and epoxide hydratase activity.

Cytochrome P-450 fractions were pooled and the free Emulgen 913 removed from the enzyme preparation by stirring with Amberlite XAD-2 beads followed by filtration. The filtrate was concentrated in an Amicon ultrafiltration cell using a YM 10 Diaflo membrane. Dialysis was carried out in 2 liters of Buffer I for 24 hr when required. The fractions containing cytochrome P-450 were stored under nitrogen in 0.5 ml aliquots at -62°.

Reconstitution of Benzo(a)pyrene Hydroxylase Activity in Systems Containing Cytochrome P-448 Obtained from Hepatic Microsomes of DBA-Treated Little Skates. Rabbit hepatic microsomal phospholipids (125 μg, only where required) and sodium cholate (125 μg) in acetone:methanol, 3:1, v/v, were mixed and evaporated to dryness. The cytochrome P-450 preparation (0.02 nmol) and NADPH-cytochrome *c* reductase (115 units, or as specified in 2-10 μl of 10 mM phosphate buffer, pH 7.7; purified from rabbit liver and generously supplied by C. Serabjit-Singh, Laboratory of Pharmacology, NIEHS) were added and preincubated for 40 min at 31° prior to initiating the reaction. HEPES buffer (0.5 M, pH 7.4, 1.0 ml) was added as well as benzo(a)pyrene (100 nmol in 10 μl acetone). NADPH (0.6 mg in 50 μl HEPES buffer) was added to start the reaction, which was incubated at 31° for up to 30 min. The reaction was terminated and analyzed for AHH activity by fluorescence according to Nebert and Gelboin (21), using 3-hydroxybenzo(a)-pyrene as the reference standard. For incubation mixtures containing 1,1,1-trichloropropene oxide or α-naphthoflavone (both from Aldrich Chemical Co., Milwaukee, WI), the addition was made just after the substrate and 1 min before starting the reaction with NADPH.

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When ^{14}C-benzo(a)pyrene (100 nmol) was incubated with the reconstituted MFO system, the reaction components were increased 10-fold (maintaining the original incubation volume, and substrate and NADPH concentrations). Metabolites were extracted and analyzed by HPLC as described for the microsomal incubations.

Results and Discussion

Several properties of hepatic microsomal AHH activity were compared in control and DBA-pretreated male little skates as shown in Table I. Following treatment there was an approximately 35-fold increase in specific enzyme activity, as quantitated by fluorescence of the phenolic metabolites formed (3, 21). The pH optimum, which was fairly broad, and the concentration of benzo(a)-pyrene (0.06 mM) that had to be added to the incubation mixture to achieve maximum enzyme activity were the same for both control and induced skate hepatic microsomes. The shorter periods observed for linearity of product formation with microsomes from the induced skates is thought to be related to the much higher AHH activity present, and may be due to substrate depletion or the formation of products which are inhibitory (i.e., compete with the MFO system as they are substrates themselves). A similar explanation may be relevant for the loss of linear product formation at lower microsomal protein concentrations in the induced animals. The apparent kinetic constants were obtained from Lineweaver-Burk plots of AHH activities recorded in the presence of increasing concentrations of benzo(a)pyrene (0.001-1.0 mM). The plots were linear for both untreated and DBA-induced animals. The apparent V_{max} was 20- to 30-fold higher in hepatic microsomes from the induced skates whereas the apparent K_m values were of the same magnitude in control and treated fish.

An obvious difference was also noted between control and induced skate hepatic microsomal AHH activity in the presence of α-naphthoflavone (10^{-4} M). This compound, when added *in vitro* at this or higher concentrations, caused significant stimulation of AHH activity in control animals (about 3-fold) but inhibition (80%) was found in DBA-pretreated skates. Similar results were earlier reported for control and 3-methylcholanthrene-treated rats (23), where it appears that the response is due to differential effects of α-naphthoflavone on hepatic microsomal cytochrome P-450 (stimulated) and cytochrome P-448 (inhibited) (24). Our data suggests that there may be a novel form of cytochrome P-450 synthesized in skate liver in response to polycyclic hydrocarbon administration, even though there was no hypsochromic shift in the carbon monoxide difference spectrum of dithionite reduced hepatic microsomes from DBA-treated skates (relative to hepatic microsomes from control fish).

Benzo(a)pyrene is converted by the microsomal MFO system of mammals (18) and trout (25) to a number of oxidized products.

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TABLE I

SOME PROPERTIES OF HEPATIC MICROSOMAL BENZO(a)PYRENE HYDROXYLASE (AHH) ACTIVITY IN CONTROL AND DBA-PRETREATED MALE LITTLE SKATES (*Raja erinacea*)[a]

PARAMETER	CONTROL SKATES	DBA-PRETREATED SKATES[b]
AHH Activity (FU/min/mg protein)	0.23 ± 0.06 (4)[c]	8.08 ± 4.32 (4)
pH-Activity Relationship (at 25°)	optimum, pH 7.4-8.0	optimum, pH 7.4-8.0
Temp.-Activity Relationship (at 25°)	linear for 20 min	linear for 15 min
Protein Conc.-Activity Relationship (at 25°)	linear to 1.6 mg/ml	linear to 1.2 mg/ml
Substrate (BP) Conc.-Activity Relationships (at 25°)		
Enzyme-Saturating Concentration (mM)	0.06	0.06
Apparent K_m (mM)	0.005 - 0.006[d]	0.008 - 0.010[d]
Apparent V_{max} (FU/min/mg protein)	0.20 - 0.21[d]	4.2 - 6.9[d]
AHH Activity (no α-naphthoflavone)	0.23[e]	5.23[e]
AHH Activity (+10^{-4} M α-naphthoflavone)[f]	0.73[e]	1.16[e]

[a]Data from reference 22.

[b]Skates were treated (IP) with DBA (10 mg/kg) on days 1, 2, and 3 and sacrificed on day 10.

[c]Mean ± SD (N).

[d]Range of 2 experiments.

[e]Data from a single experiment. The experiment was repeated twice and similar data obtained each time.

[f]α-Naphthoflavone was added to the reaction mixture *in vitro*.

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Since the fluorescence assay only quantitates phenolic metabolites (primarily 3- and 9-hydroxybenzo(a)pyrene in rats (26) and does not accurately measure total metabolism, we have also compared ^{14}C-benzo(a)pyrene metabolism in hepatic microsomes from control and DBA-treated male skates using HPLC analysis. The metabolism of benzo(a)pyrene (per mg microsomal protein) was also much faster (about 15-fold after incubation for 5, 15, or 30 min) in microsomes from DBA-pretreated fish than in those from untreated skates (Fig. 1). As shown in Fig. 2, the metabolites formed from ^{14}C-benzo(a)-pyrene by hepatic microsomes from untreated and DBA-pretreated male skates are qualitatively very similar, if not identical, and the combined radioactivity eluting with the 9-hydroxybenzo(a)-pyrene (9-OH) and 3-hydroxybenzo(a)pyrene (3-OH) standards (i.e., the phenolic metabolites) accounted for greater than 50% of the total biotransformation products in each case. The major quantitative difference in these metabolic profiles was the greater amount of radioactivity chromatographing with the standards in the Q + E region of the chromatogram (benzo(a)pyrene-1,6-, -3,6-, and -6,12 quinones and benzo(a)pyrene 4,5-oxide). It is likely that this is in part due to the accumulation of benzo(a)pyrene 4,5-oxide in the incubation mixtures containing microsomes from DBA-pretreated skates since epoxide hydratase activities in little skate hepatic microsomes, with benzo(a)pyrene 4,5-oxide as substrate, are low (0.19 ± 0.05 nmol/min/mg microsomal protein, mean ± SD, N = 3) and are unaffected by pretreatment with DBA (4).

It was also interesting that significant amounts of benzo(a)-pyrene 7,8-dihydrodiol were formed by hepatic microsomes of both control and DBA-pretreated skates. This dihydrodiol is the metabolic precursor for the isomeric 9,10-epoxide-7,8-dihydro-7,8-dihydroxybenzo(a)pyrenes, which are at least one ultimate carcinogenic and mutagenic form of benzo(a)pyrene (27, 28). Should the formation of benzo(a)pyrene 7,8-dihydrodiol be an important metabolic pathway for benzo(a)pyrene in aquatic species, there is a distinct possibility that potentially dangerous levels of this compound might build up in liver and muscle of fish used for human food. Lee *et al.* (29) previously suggested that benzo(a)pyrene 7,8-dihydrodiol was the predominant benzo(a)pyrene metabolite found in liver, gut, gill, flesh, and heart of mudsucker (*Gillichthys mirabilis*), sculpin (*Oligocottus maculosus*) and sand dabs (*Citharichthys stigmaeus*) although the metabolites were not rigorously identified.

The elution profile of cytochrome P-448 (absorption at 418 nm) and epoxide hydratase activity from a sodium cholate-solubilized hepatic microsomal preparation (from DBA-treated male skates) applied to a DEAE-cellulose column and eluted with Buffer II is shown in Fig. 3. The void volume of the column contained significant amounts of epoxide hydratase activity. Fractions 40-70 (Fig. 3) were combined, and concentrated. The carbon monoxide difference spectrum, which had an absorption maximum at 448 nm in the induced state, is shown in Fig. 4. This form of the cytochrome (i.e.,

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TABLE II

BENZO(a)PYRENE HYDROXYLASE (AHH) ACTIVITY OF A RECONSTITUTED MIXED-FUNCTION OXIDASE SYSTEM CONTAINING HEPATIC CYTOCHROME P-448 FROM DBA-TREATED MALE SKATES

Incubation Conditions	pmoles 3-hydroxybenzo(a)pyrene formed/min/nmol cytochrome P-448	% Maximum Activity
Complete system[a]	775[b]	100
Minus NADPH	0	0
Minus NADPH-cytochrome *c* reductase	225	28
Minus sodium cholate	625	80
Plus rabbit hepatic microsomal phospholipids (125 μg)[c]	275	35
Plus 1,1,1-trichloropropene oxide (50 μM)	500	63
Plus α-naphthoflavone (5×10^{-7} M)	450	57
Plus α-naphthoflavone (5×10^{-4} M)	125	15

[a]Cytochrome P-448 (0.02 nmol), NADPH-cytochrome *c* reductase (115 units; purified from rabbit hepatic microsomes) and sodium cholate (125 μg) preincubated at 31° for 30 min.

[b]Assayed by fluorescence after incubation with benzo(a)pyrene (100 nmol) and NADPH (0.16 mg) in 0.5 M HEPES buffer, pH 7.6 (1 ml) at 31° for 20 min. Product formation was linear for at least 20 min.

[c]See reference 30 for more details.

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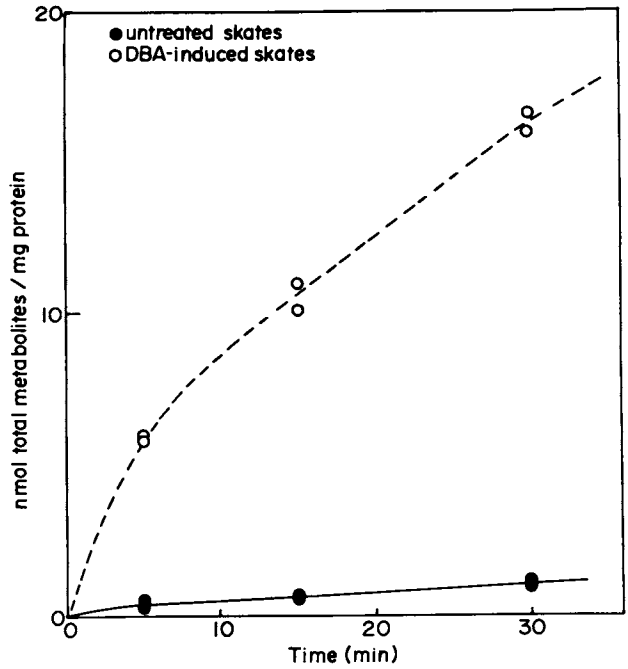

Figure 1. Production of total BP metabolites by hepatic microsomes from control and DBA-pretreated male little skates. Each point is the result of a single incubation and HPLC determination: (○ – – – ○), control skates; (● — ●), DBA-pretreated skates. The DBA-dosing schedule and incubation conditions are described in Materials and Methods.

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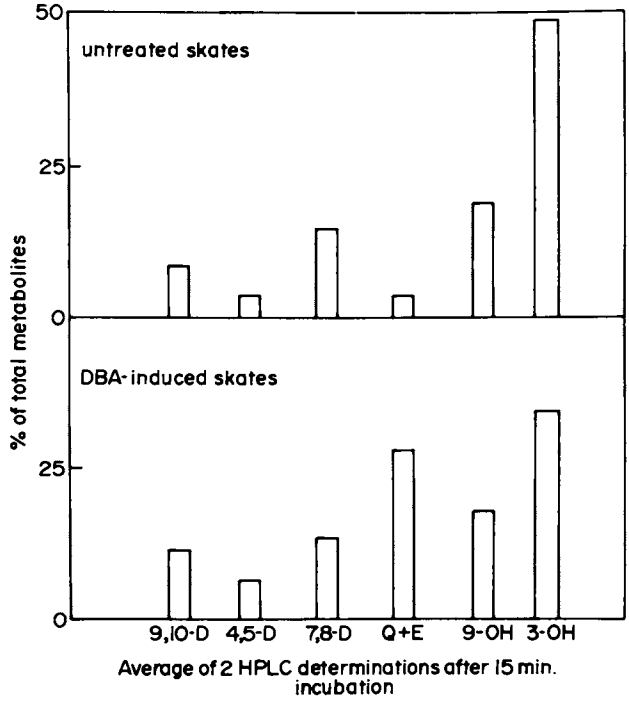

Figure 2. Profile of radioactive metabolites obtained upon incubation of ^{14}C-BP with hepatic microsomes from control or DBA-pretreated male little skates.

The hepatic microsomes were aliquots from pools of 10 control or pretreated fish that were also used for the partial purification of various forms of Cytochrome P-450: 9,10-D, BP-9,10-dihydrodiol; 4,5-D, BP-4,5-dihydrodiol; 7,8-D, BP-7,8-dihydrodiol; Q + E, BP-1,6-, -3,6-, and -6,12-quinones and BP-4,5-oxide; 9-OH, 9-hydroxy-BP; 3-OH, 3-hydroxy-BP. The DBA-dosing schedule and incubation conditions are described in Materials and Methods.

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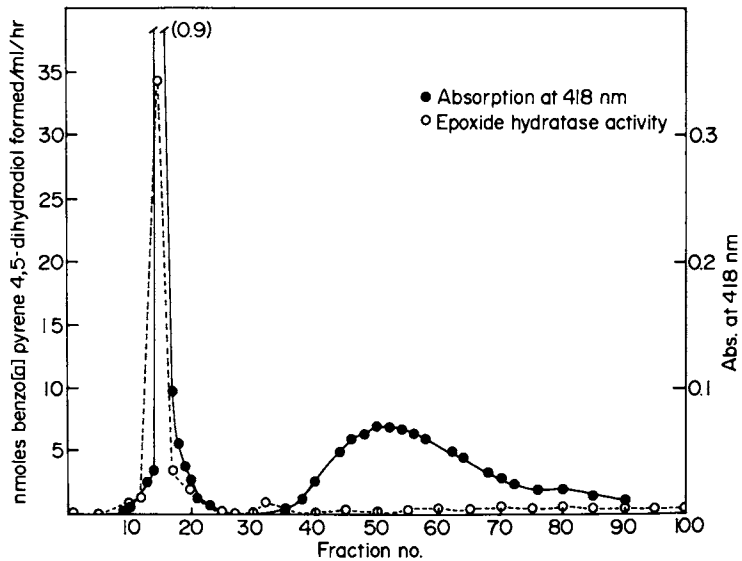

Figure 3. Elution profile of partially purified Cytochrome P-448 and epoxide hydratase activity of solubilized hepatic microsomes from DBA-treated male skates from a DEAE-cellulose column with Buffer II. Epoxide hydratase activity was determined with BP-4,5-oxide as the substrate (see *Materials and Methods*).

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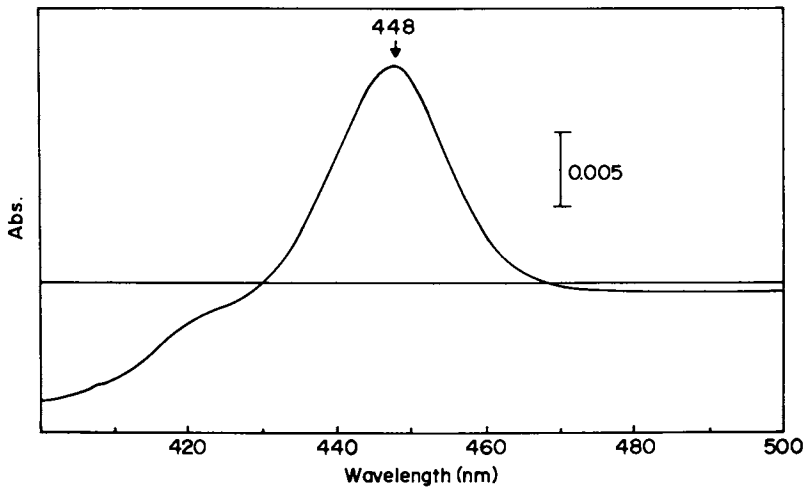

Figure 4. Carbon monoxide difference spectrum of partially purified hepatic microsomal Cytochrome P-448 from DBA-treated little skates. The cuvettes contained dithionite-reduced cytochrome (0.10 mg protein/mL) in 10mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1mM EDTA and 0.1mM dithiothreitol.

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cytochrome P-448) was purified 4- to 5-fold during elution from the DEAE-cellulose column, was free of cytochrome b_5 and contained negligible epoxide hydratase activity. Consequently, it has been further used to study the metabolism of ^{14}C-benzo(a)pyrene in a reconstituted mixed-function oxidase system.

When all of the material absorbing at 418 nm (associated with the cytochrome P-448 fractions) was eluted from the DEAE-cellulose column (which in some experiments required more than 1 liter of Buffer II), elution was continued with a linear KCl gradient (0-0.5 M) in Buffer II, as shown in Fig. 5. A different form(s) of cytochrome P-450 (fractions 130-155), having maximal absorption near 451 nm in the carbon monoxide ligated and reduced form (Fig. 6), was obtained although only 2- to 3-fold purification, relative to microsomes, was achieved. This form of cytochrome P-450 was extensively contaminated with epoxide hydratase activity. However, by combining fractions 130-150 (Fig. 5), it was possible to obtain cytochrome P-451 essentially free of cytochrome b_5. The relative content of cytochrome P-448 and cytochrome P-451 in the DEAE-column eluates ranged from 1:1.1 to 1:1.6 in several different experiments.

The absorbance at 418 nm of fractions 155-168 (Fig. 5) was primarily associated with cytochrome b_5 whereas that in fractions 170-180 was primarily due to NADPH-cytochrome *c* reductase.

Very similar results to those described in Fig. 3-6 were obtained when sodium cholate solubilized hepatic microsomes from DBA-treated female little skates were subjected to chromatography on DEAE-cellulose as described above (data not shown). Also not shown are the results obtained with hepatic microsomes from untreated male and female little skates. With untreated animals, 80-90% of the cytochrome P-450 eluted from the DEAE-cellulose column only at higher ionic strength (i.e., with the KCl gradient). However, in all preparations studied, an appreciable amount of cytochrome P-450 (10-20%), having its absorption maximum in the carbon monoxide-ligated and reduced state at 450 nm, was eluted from the column with buffer II, as was observed with cytochrome P-448 of hepatic microsomes from DBA-treated skates. The further purification of the various forms of cytochrome P-450 from control and DBA-pretreated little skate livers is currently in progress in our laboratory.

Collectively, these experiments have demonstrated that substantial amounts of cytochrome P-448 are indeed present in liver of DBA-pretreated male and female skates but not in untreated skates. Sufficient cytochrome P-448 is present to account for the increased AHH activities observed in hepatic microsomes of DBA-treated skates.

After removal of free Emulgen 913 from partially purified hepatic cytochrome P-448 of DBA-treated male skates an active mixed-function oxidase system was reconstituted by preincubating the cytochrome with purified rabbit hepatic NADPH-cytochrome *c*

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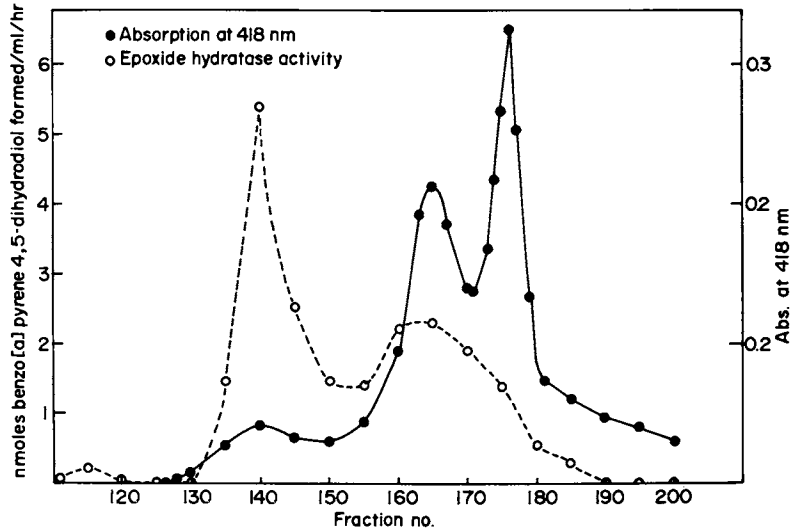

Figure 5. Elution profile of partially purified Cytochrome P-451 and epoxide hydratase activity of solubilized hepatic microsomes from DBA-treated male skates from a DEAE-cellulose column with a linear KCl (0–0.5M) gradient in Buffer II. The KCl gradient was initiated only after all of the Cytochrome P-448 (Figure 3) had been eluted from the DEAE-cellulose column with Buffer II (see Materials and Methods).

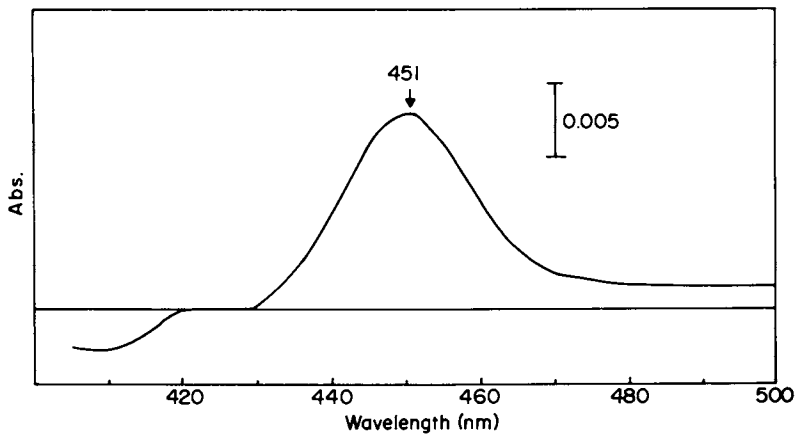

Figure 6. Carbon monoxide difference spectrum of partially purified hepatic microsomal Cytochrome P-451 from DBA-treated male little skates. The cuvettes contained dithionite-reduced cytochrome (0.15 mg protein/mL) in 10mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1mM EDTA and 0.1mM dithiothreitol.

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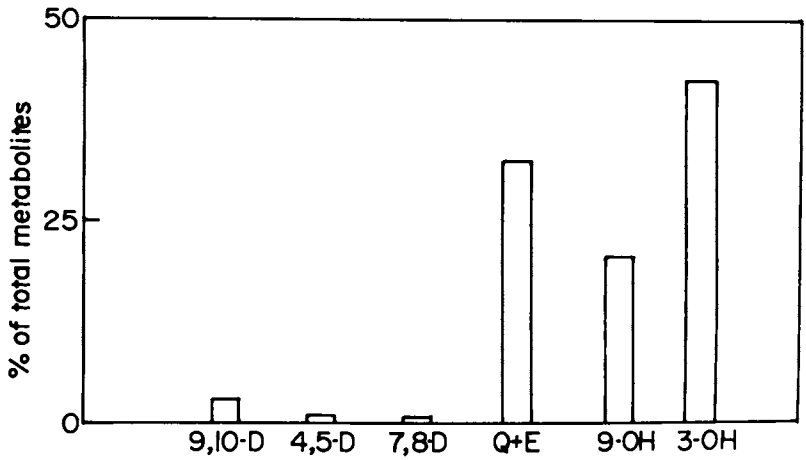

Figure 7. Profile of radioactive metabolites obtained upon incubation of ^{14}C-BP with a reconstituted system containing partially purified Cytochrome P-448 from DBA-pretreated male little skates.

The reconstituted system consisted of Cytochrome P-488 (0.2 nmol), NADPH-Cytochrome c reductase (1500 units) and sodium cholate (1.25 mg). It was preincubated for 30 min at 31°. The final reaction mixture (which was incubated at 31° for 20 min) contained the preincubated system described above, excess NADPH and ^{14}C-BP (100 nmol; 4.1 mCi/mmol) in a final volume of 1 mL 0.5M HEPES buffer, pH 7.6. Rate of BP metabolism was 665 pmol/min/nmol Cytochrome P-488. Abbreviations used for metabolites are described in legend to Figure 2.

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ties in hepatic microsomes from fish captured there may actually be lower than in fish captured from relatively unpolluted waters (32). Finally, there is the potential problem of migration of marine fish from polluted to less polluted areas, and vice versa.

In spite of these limitations there seems little doubt that induction of the hepatic MFO system in certain fish can indicate the presence of selected toxic chemicals (to fish and humans) in both freshwater and marine environments. For this reason we have been studying various aspects of this question for the last few years in freshly captured fish (winter flounder and little skate in Maine and the sheepshead in Florida) and in fish induced by polycyclic hydrocarbon administration.

One of our more interesting observations is illustrated in Table III. The administration of DBA to winter flounder increased hepatic microsomal AHH and 7-ethoxyresorufin deethylase activities as expected, and AHH activity was strongly inhibited in the DBA-treated flounder by 10^{-4} M α-naphthoflavone as we have previously reported for both little skate (4) and sheepshead (9). However, the presence of high AHH and 7-ethoxyresorufin deethylase activities in one control flounder, and the inhibition of AHH activity by α-naphthoflavone in this animal suggested that the hepatic microsomal MFO system of this fish was already induced.

Subsequently, we assayed hepatic microsomal AHH activity (in the presence and absence of 10^{-4} and 10^{-3}M α-naphthoflavone) and 7-ethoxyresorufin deethylase activity in thirteen winter flounder during June-August, 1977 (33). There was a marked variation in microsomal AHH activity (over 60-fold) and eleven of the fish had AHH activity that was inhibited *in vitro* by α-naphthoflavone as well as higher 7-ethoxyresorufin deethylase activities. This again suggested induction of the MFO system in many of the fish similar in nature to that which occurs following treatment with polycyclic hydrocarbon-like inducing agents.

During June-August, 1978 we assayed these same three parameters (i.e., AHH activity in the presence and absence of α-naphthoflavone and 7-ethoxyresorufin deethylase activity) in liver homogenates from more than two hundred winter flounder in Maine, after first demonstrating that the α-naphthoflavone effects (i.e., stimulation or inhibition) were identical in microsomes and whole homogenates of the same fish (Foureman, G. L., Ben-Zvi, Z., Dostal, L., and Bend, J. R., unpublished results). The data obtained with female winter flounder are shown in Table IV. Once again there was a very large range (over 60-fold) between the lowest AHH activity (0.11 FU/min/mg protein) and the highest AHH activity (7.32) observed. Moreover, there was a good correlation between increases in AHH activity in the various arbitrary groups of flounder and 7-ethoxyresorufin deethylase activity (which at least in rats is associated with the induction of cytochrome P-448 (13). Collectively, the three parameters tested indicate that polycyclic hydrocarbon-type induction of the hepatic microsomal MFO system of winter flounder is common in the waters near Mt. Desert Island,

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TABLE III

EFFECT OF 1,2,3,4-DIBENZANTHRACENE (DBA)-PRETREATMENT ON HEPATIC MICROSOMAL BENZO(a)PYRENE HYDROXYLASE (AHH) AND 7-ETHOXYRESORUFIN DEETHYLASE (7-ERF) ACTIVITIES IN WINTER FLOUNDER (*Pseudopleuronectes americanus*)

	AHH Activity (FU/min/mg protein)		7-ERF Activity (pmol/min/mg protein)
	Without α-Naphthoflavone	With α-Naphthoflavone (10^{-4}M)	
Control fish	0.05	0.19	0
	0.40	0.19	23
	1.76	0.83	146
DBA-Treated[a] Fish	1.11	0.63	157
	1.67	1.01	187
	1.84	0.78	261

[a]Injected I.P. with 10 mg/kg DBA in corn oil on days 1, 2, and 3 and sacrificed on day 10.

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TABLE IV

BENZO(a)PYRENE HYDROXYLASE (AHH) AND 7-ETHOXYRESORUFIN DEETHYLASE (7-ERF) ACTIVITIES IN LIVER HOMOGENATE OF FEMALE WINTER FLOUNDER SEPARATED INTO ARBITRARY GROUPS BASED ON AHH ACTIVITY

AHH Activity (FU/min/mg protein)	No. of Fish in Group	AHH Activity (FU/min/mg protein)	7-ERF Activity (pmol/min/mg protein)
0.11 - 0.50[a]	19	0.28 ± 0.10[b]	12.7 ± 18.3
0.51 - 1.00[c]	27	0.78 ± 0.15	71.0 ± 34.0
1.01 - 2.00[c]	29	1.33 ± 0.27	111.5 ± 57.6
2.01 - 4.00[d]	31	2.85 ± 0.47	190.9 ± 85.7
4.00 - 7.36[d]	11	5.71 ± 1.10	358.5 ± 197

[a] α-Naphthoflavone (10^{-3} and 5×10^{-3} M) stimulated AHH activity in all of these fish.

[b] Mean ± SD.

[c] α-Naphthoflavone stimulated AHH activity in some of these fish and inhibited it in others.

[d] α-Naphthoflavone inhibited AHH activity in all of these fish.

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Maine, during the summer months. A very similar response was noted in the male flounder studied. There was also no apparent change in the relative number of induced flounder over the three month period monitored. Since these studies were not conducted during the spawning season it seems unlikely that spawning is a factor. Further studies are in progress to elucidate whether or not this induction is due to exposure to environmental pollutants. However, a further complicating factor is that little skates captured from the same waters during June-August do not have induced hepatic MFO systems, even though they are susceptible to induction by polycyclic hydrocarbon-like compounds (Table I).

Similar studies conducted at our Florida laboratory with the sheepshead have produced quantitatively different results (James, M. O., unpublished data). Of twenty-nine sheepshead assayed in June-August over two years, only four have had partially induced hepatic MFO systems.

Obviously we must obtain considerably more information concerning the causative nature of this interesting enzyme response before any of the species we are currently investigating can be routinely used to monitor the marine environment for pollutants.

Acknowledgments

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Induction of Hepatic Microsomal Enzymes in Rainbow Trout

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Much work has demonstrated the presence of complex multi-enzyme monooxygenase systems within the endoplasmic reticulum of several mammalian species (for Reviews: 1,2,3). These monooxygenase systems are responsible for the oxidative metabolism of many exogenous and endogenous substances, and the unusual non-specificity of these monooxygenase enzymes allows the metabolism of compounds with diverse chemical structures. Early work demonstrated that the terminal microsomal oxidase involved in xenobiotic biotransformation was a hemoprotein, which has been subsequently named cytochrome P-450.

A plethora of investigations has demonstrated that the hemoprotein P-450-mediated monooxygenase activity of mammals may be stimulated by a variety of chemical agents, and it appears that such stimulation may be accounted for by de novo synthesis of enzymes (Reviews: 4,5). The observed induction of microsomal monooxygenation is often too great to be explained merely in terms of increased hemoprotein P-450 levels and it was early recognized that multiple forms of hemoprotein P-450 exist. The relative proportions of these isoenzymes can be altered by various inducing agents (6,7,8,9,10). Classically, the hepatic microsomal hemoproteins may be induced by two major groups of inducing agents, i.e. barbituates and polycyclic aromatic hydrocarbons. These groups are represented by phenobarbital and 3-methylcholanthrene, and induce the synthesis of cytochrome P-450 and cytochrome P_1-450 (P-448) respectively. The initial division of the induction phenomenon into two types is now recognized as an oversimplification, since other types of inducing agents have been discovered, e.g. isosafrole and pregnenolone-16α-carbonitrile (8,11,12). These agents seem to induce hemoprotein(s) P-450 which possess properties in common with both the phenobarbital-and 3-methylcholanthrene-type inducers. Hence, it would appear that multiple forms of cytochrome P-450 and cytochrome P_1-450 exist.

Many studies have examined the effects of inducing agents

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on the microsomal hemoprotein P-450 population of rats, mice and rabbits; however, little information exists regarding the comparative biochemistry of hemoprotein P-450 of lower animals (13).

Early studies on xenobiotic metabolism in fish suggested that fish had little or no capacity to oxidatively metabolize foreign chemicals (14). However, in the last decade, literature has slowly accumulated to indicate that fish possess a relatively well developed drug metabolizing system (15,16,17,18,19). Studies have demonstrated that the livers of several fish species contain hemoprotein(s) P-450 (20,21); however, little information is available as to the properties of the constitutive hemoprotein(s) P-450 or whether multiple forms of the monooxygenase exist. Studies have indicated that certain chemical agents may stimulate monooxygenase reactions in fish. Bend and coworkers (22) have indicated that polycyclic aromatic hydrocarbons and tetrachlorodibenzodioxin can increase the activity of certain monooxygenase systems in the winter flounder (Pseudopleuronectes americanus) and the little skate (Raja erinacea). Similar studies have demonstrated the inducibility of several monooxygenase activities in the rainbow trout (Salmo gairdneri), brown trout (Salmo trutta), scup (Stenotomus versicolor) and the capelin (Mallotus villosus) by polycyclic aromatic hydrocarbons (23,24,25). Despite large increases in mixed function oxidase activity, only small increases in total hemoprotein(s) P-450 were observed.

Studies utilizing barbituate-type inducing agents have generally indicated that fish may be refractive to cytochrome P-450 type of inducers (16,22,26).

In many mammals induction of monooxygenation by polycyclic aromatic hydrocarbons is accompanied by the formation of a hemoprotein not seen to any appreciable extent in non-induced animals. This leads to an alteration in the microsomal hemoprotein populations, a change in the metabolic activity of the microsomes and, hence, possible alterations in the toxicity of other chemicals (27,28).

Whether induction of monooxygenation in fish involves alterations in the relative proportions of multiple form(s) of P-450 remains to be elucidated.

In an attempt to resolve these questions, we have directed studies toward examining the interaction of chemicals with the hepatic microsomal monooxygenase system of fish and to determine whether multiple forms of hemoprotein(s) P-450 exist, and, if so, how the relative microsomal hemoprotein subpopulations are altered by xenobiotics.

Materials and Methods

Rainbow trout (Salmo gairdneri), weighing 50-100g obtained from Kettle Moraine Springs Trout Hatchery (Adell, WI.) were held in flowing, charcoal-filtered, dechlorinated water at 12°C for a

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minimum of 2 weeks before use. A 12h (0600-1800) light cycle was used.

Pretreatment of fish and preparation of microsomes

Polycyclic aromatic hydrocarbons and polyhalogenated biphenyls were administered to fish by intraperitoneal injections dissolved in corn oil (1ml/kg). Actual doses are described in individual figure legends and tables. Fish were sacrificed by a blow to the head, gall bladders carefully removed, and the livers excized into ice-cold 0.154 M KCL. The livers were scissor-chopped and rinsed 3 times in ice-cold 0.154 M KCL. The minced liver was homogenized in 4 volumes of 0.25 M sucrose and washed microsomes obtained by ultracentrifugation as previously described (29,30). The washed microsomal pellets were resuspended in either 0.25 M sucrose or 20 mM Tris-HCL (pH 7.4) containing 0.25 M sucrose and 5.4 mM EDTA, to a final concentration equivalent to 1g wet wt. liver/ml. All microsomes were utilized on day of preparation, except for SDS-PAGE, which was performed on microsomes stored a maximum of 2 weeks at -20°C.

Microsomal enzyme assays

Arylhydrocarbon (benzo[a]pyrene) hydroxylase, benzphetamine-N-demethylation, ethylmorphine-N-demethylation, ethoxycoumarin-O-deethylation and ethoxyresorufin-O-deethylation were performed by published procedures (31,32,33,34), but optimized for use with trout microsomes as described previously (30,35). Hemoprotein P-450 was determined by the procedure of Estabrook et al. (36) to avoid spectral interference by hemoglobin. Microsomal protein content was estimated either by the method of Ross and Shatz (37) or Lowry et al. (38), using bovine serum albumin standards.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially by the method of Laemmli and Favre (39) using 3% and 7.5% (w/v) total acrylamide concentrations in the stacking and resolving gels respectively. Sodium dodecyl sulphate was at a concentration of 0.1% (w/v). Staining for protein was accomplished using Coomassie Brilliant Blue R-250, while peroxidase activity was localized by the method of Thomas et al. (40).

Densitometric scans of Coomassie Blue stained electrophoretograms were obtained at 550nm, using an ISCO gel scanner accessory in combination with an ISCO Type 6 optical unit and Type UA-5 absorbance monitor.

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Results and Discussion

Initial studies designed to obtain a valid subcellular fractionation scheme for rainbow trout liver illustrated the arylhydrocarbon (benzo[a]pyrene] hydroxylase activity separated with glucose-6-phosphatase (35). This observation indicated that the trout hemoprotein P-450-mediated monooxygenation system was located within the endoplasmic reticulum (microsomal fraction).

Several authors have reported varied responses of fish microsomal enzyme activity to inducing agents (16,18,22,23,26). Hence, we initially used several chemicals as potential inducers in the rainbow trout. Figure 1 illustrates the effects of 3-methylcholanthrene, β-naphthoflavone, 2,3-benzanthracene and phenobarbital on several hepatic microsomal enzyme activities of trout. It can be seen that phenobarbital was without effect on glucose-6-phosphatase, glucuronyl transferase and arylhydrocarbon (benzo[a]pyrene) hydroxylase. The polycyclic aromatic hydrocarbons dramatically increased benzo[a]pyrene hydroxylation, but had little or no effect upon glucuronyl transferase **or** glucose-6-phosphatase. Concomitant with increases in monooxygenase activity, total microsomal hemoprotein P-450 was increased by 2,3-benzanthracene, β-naphthoflavone and 3-methylcholanthrene (Table 1). However, although monooxygenase activity was enhanced between 10- and 28-fold, hemoprotein P-450 content was increased only about 1.5-fold.

This initial study demonstrating induction of monooxygenation and hemoprotein P-450 in the rainbow trout by polycyclic aromatic hydrocarbons, but not by phenobarbital, was extended further. The polychlorinated and polybrominated biphenyls are potent inducers of monooxygenation in several mammalian species (41-47). Commercial preparations of the polychlorinated (Aroclors) and polybrominated (Firemaster BP6) biphenyls are mixtures of many congeners (48,49) and appear capable of inducing both cytochrome P-450 and cytochrome P_1-450-like activities. Hence, these compounds were utilized to investigate the inducibility of the rainbow trout monooxygenase system.

Table II demonstrates the effect of two polychlorinated biphenyl mixtures (Aroclors 1254 and 1242), a polybrominated biphenyl mixture (Firemaster BP6), phenobarbital and β-naphthoflavone on various hemoprotein P-450-mediated monooxygenase activities of rainbow trout hepatic microsomes.

Ethylmorphine and benzphetamine are N-demethylated specifically by rodent cytochrome(s) P-450 (as opposed to cytochrome(s) P_1-450); in the rat phenobarbital and the polyhalogenated biphenyls induce these demethylation reactions. However, it is clear that no such stimulation occurred in the rainbow trout (Table II).

On the other hand, the metabolism of benzo[a]pyrene, ethoxycoumarin and ethoxyresorufin, which are preferentially oxidized by rodent cytochrome P_1-450, was greatly induced by the polyhalogenated biphenyls and β-naphthoflavone. Despite large changes in

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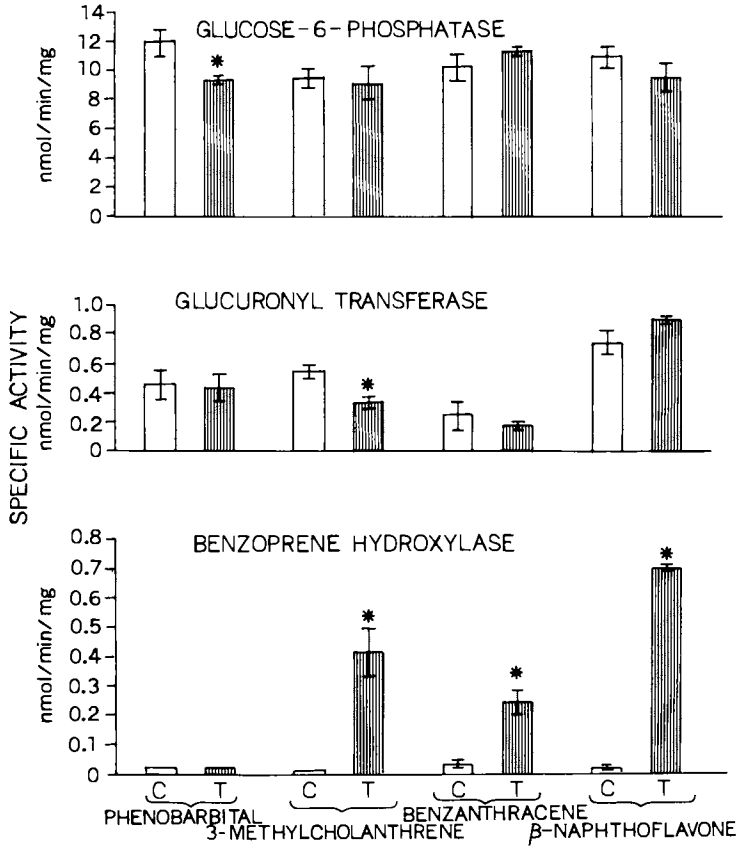

Figure 1. Effect of potential inducing agents on certain hepatic microsomal enzymes of the rainbow trout. Animals were injected intraperitoneally with either phenobarbital (65 mg/kg); 3-methylcholanthrene (20 mg/kg); 2,3-benzanthracene (10 mg/kg); or β-naphthoflavone (100 mg/kg). The animals were sacrified and hepatic microsome prepared 48 hr after injection. Each bar is the mean ± SE (n = 3–5); (*), induced activity (T) significantly different from control (C) activity ($P < 0.05$).

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TABLE I

Hemoprotein P-450 Content of Trout Hepatic Microsomes

Treatment of fish[a]	nmol P-450/mg microsomal protein[b]
Corn oil	0.216 ± 0.003
3-methylcholanthrene	0.337 ± 0.015[c]
2,3-benzanthracene	0.313 ± 0.017[c]
β-naphthoflavone	0.348 ± 0.010[c]

[a] 3-methylcholanthrene, 2,3-benzanthracene and β-naphthoflavone were administered to fish by i.p. injection dissolved in corn oil (1ml/kg) at doses of 20, 10 and 100 mg/kg, respectively. The fish were sacrificed and microsomes prepared 48 hr. after injection.
[b] Assuming extinction coefficient of 100 $mM^{-1}cm^{-1}$. Values are Mean ± SE (n=4).
[c] Significantly different from control value. $p<0.05$.

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TABLE II

Induction of Hepatic Microsomal Monooxygenation in Rainbow Trout

Treatment of fish	Dose (mg/kg)	EMD[a]	BeND[b]	AHH[c]	ECOD[d]	EROD[e]
Corn oil		100[f]	100	100	100	100
Phenobarbital	65	81	ND[g]	104	64	65
Aroclor 1242	150	98	133	1059[h]	808[h]	1367[h]
Aroclor 1254	150	105	49	1300[h]	509[h]	1460[h]
Firemaster BP6	150	89	110	700[h]	547[h]	1564[h]
β-naphthoflavone	100	88	ND	4081[h]	1178[h]	4455[h]

[a]Ethylmorphine-N-demethylation, typical control value: 0.80 nmol/min/mg.
[b]Benzphetamine-N-demethylation, typical control value: 1.10 nmol/min/mg.
[c]Arylhydrocarbon (benzo[a]pyrene) hydroxylation, typical control value: 0.025 nmol/min/mg.
[d]Ethoxycoumarin-O-deethylation, typical control value: 0.025 nmol/min/mg.
[e]Ethoxyresorufin-O-deethylation, typical control value: 0.030 nmol/min/mg.
[f]Values are expressed as % of corn oil control value.
[g]ND, not determined.
[h]Significantly different from corn oil control, $p<0.05$.
Data accumulated from references 29 and 30 and unpublished results.

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monooxygenase activities (500-4000%), only small (10-50%) increases in total hemoprotein P-450 content were observed (Table I and 29,30). This discrepancy between hemoprotein levels and monooxygenase activity may be explained by changes in the relative proportions of hemoprotein P-450 isoenzymes. Such explanations have been validated in the rat and mouse where novel forms of hemoprotein(s) P-450 are induced.

The alteration of hemoprotein(s) P-450 subpopulations in the rat may be observed spectrally, because after treatment of rats with polycyclic aromatic hydrocarbons, the Soret maximum of the carbonmonoxyferrocytochrome complex undergoes a hypsochromic shift from 450 to 448nm (50). This blue shift was not seen with rainbow trout hepatic microsomes (29,30). However, this does not preclude the induction of novel hemoproteins P-450 since (a) the induced hemoprotein(s) may not differ spectrally from the constitutive enzymes and (b) the induced-hemoprotein may account for only a small proportion of total hemoprotein P-450, and hence its contribution to the position of the Soret maximum of carbon monoxide-treated reduced microsomes may be negligible. The latter suggestion is supported by the work of Bend et al. with the little skate. These workers have shown that hepatic microsomes from 1,2,3,4-dibenzanthracene treated skates did not exhibit a hypsochromic shift when compared to control microsomes, however, partially purified hemoprotein exhibited an absorbance maxima at 448 nm (51).

Ethylisocyanide combines with reduced hemoprotein(s) P-450 to form a complex with absorbance maxima at 455 and 430 nm (52). The relative magnitudes of the 455 and 430 nm absorbance maxima may be changed by pretreatment of rats with various inducing agents (53). Figure 2 illustrates that the 455/430 ratio of the ethylisocyanide complex increased from about 0.25 to 1.0 after treatment of female rats with a polycyclic aromatic hydrocarbon (3-methylcholanthrene). However, utilizing rainbow trout, we have observed no significant change in the 455/430 ratio after induction of monooxygenation (29). Figure 3 illustrates that the ratio increased only slightly from 0.35 to 0.53 after induction of monooxygenation by β-naphthoflavone. Again, this lack of alteration in the spectral properties of microsomes from induced-rainbow trout may reflect the small contribution of the induced hemoprotein to the total microsomal hemoprotein spectral properties.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful tool for examining gross changes in microsomal hemoprotein profiles. Utilizing this technique with rainbow trout hepatic microsomes, we have demonstrated several Coomassie Blue staining bands in the 45,000 to 60,000 dalton region (Figure 4; track A). The bands at 59,500, 51,000, 48,000 and 45,000 daltons appeared to be hemoproteins as witnessed by peroxidase staining activity. Hence, the rainbow trout would appear to possess at least 4 constitutive hemoproteins. (Although one

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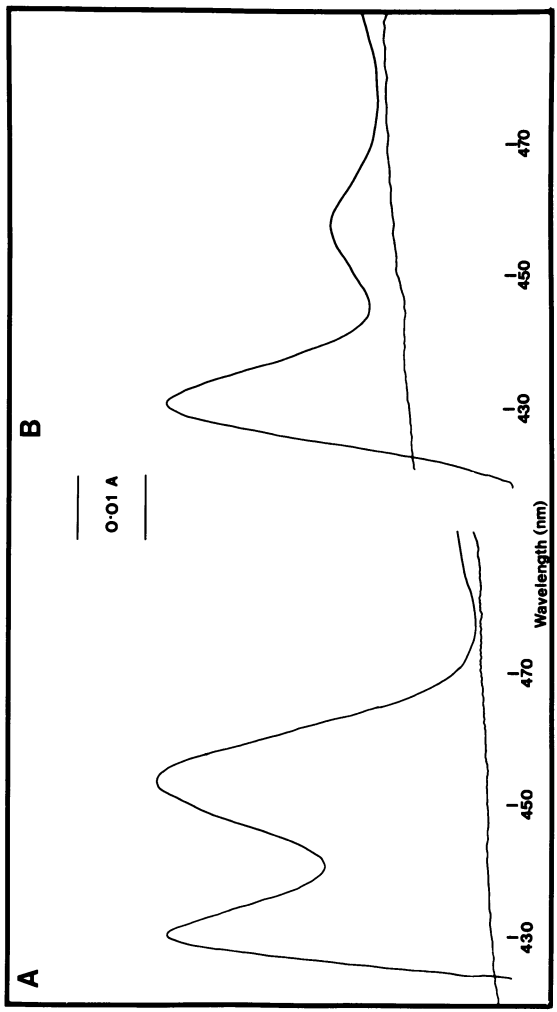

Figure 2. Spectra of ethyl isocyanide–ferrocytochrome P-450 complexes. Female rat.

(A) Hepatic microsomes from female rats pretreated with 3-methylcholanthrene (20 mg/kg, for 4 days) were divided between 2 cuvettes and a baseline of equal light absorbance obtained. A few mg of $Na_2S_2O_4$ were added to the reference and sample cuvettes and ethylisocyanide (final concentration about 10mM) was added to the sample cuvette. The difference spectrum between 390 and 500 nm was then recorded (protein concentration, 0.75 mg/mL; total P-450 concentration; 0.85 μM). (B) Conditions as in (A), but control hepatic microsomes were used. (Protein concentration, 0.72 mg/mL; total P-450 concentration, 0.41 μM).

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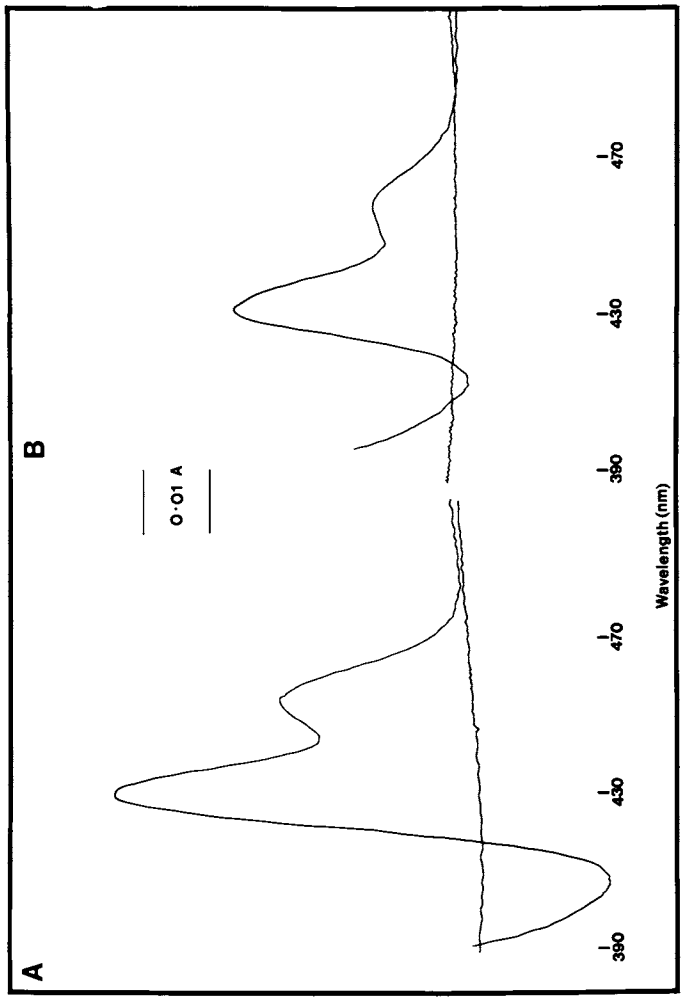

Figure 3. Spectra of ethylisocyanide–ferrocytochrome P-450 complexes, rainbow trout. Conditions were as in Figure 2, except rainbow trout hepatic microsomes were used. (A) Microsomes from rainbow trout treated with β-naphthoflavone (100 mg/kg; sacrificed 4 days later). Protein concentration, 1.4 mg/mL; total P-450 concentration, 0.48 μM. (B) Microsomes from control fish. Protein concentration, 0.93 mg/mL, total P-450 concentration, 0.22 μM.

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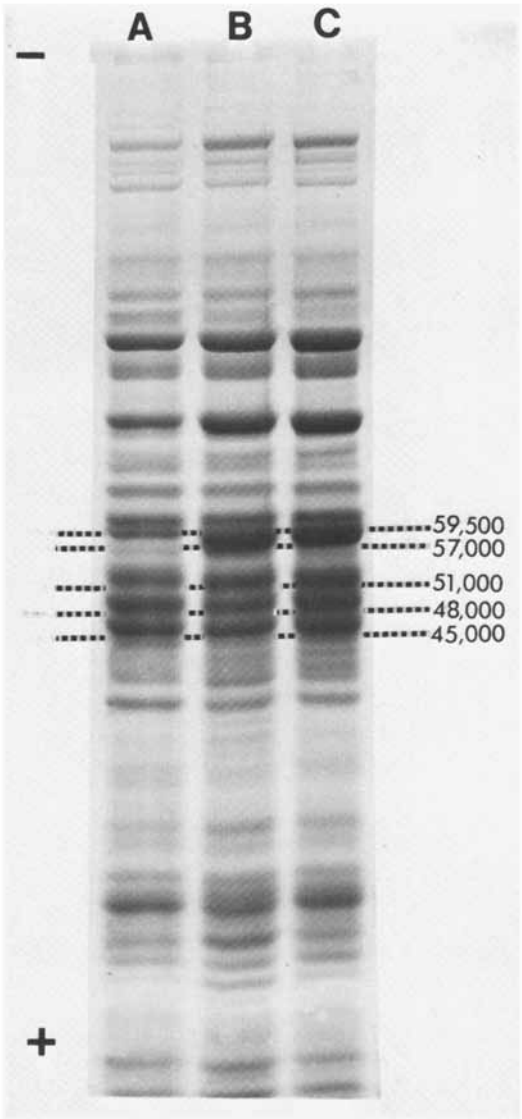

Figure 4. SDS–polyacrylamide gel electrophoresis of microsomes from variously pretreated rainbow trout: (A), control microsomes, 90 μg protein/gel; (B), β-naphthoflavone-induced microsomes, 90 μg protein/gel; (C), Aroclor 1242-induced microsomes, 90 μg protein/gel.

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cannot exclude the non-specific aquisition of heme). Examination of microsomes from β-naphthoflavone-or Arolcor 1242-pretreated trout demonstrated the presence of a new hemoprotein of molecular weight 57,000 (Figure 4). This band is barely visible in control microsomes. It is probable that the protein band at 57,000 daltons seen in induced-fish microsomes is responsible for the large increases in monooxygenase activity after treatment with polychlorinated biphenyls and β-naphthoflavone. The hemoprotein characteristically induced by these agents in the rat has a molecular weight of 53,000 in our system and hence, physically differs from the induced-rainbow trout hemoprotein.

The data so far presented indicates that monooxygenation may be induced in the rainbow trout by polyhalogenated biphenyls and polycyclic aromatic hydrocarbons, but not by phenobarbital-type inducing agents.

To further investigate the refractive nature of trout to phenobarbital-type induction, we have used selected polychlorinated biphenyl isomers. Studies by Goldstein et al. (54) and Poland and Grover (55) have indicated that the "mixed" inducing nature (i.e. inducing cytochromes P-450 and P_1-450) of Aroclors is due to specific polychlorinated biphenyl congeners inducing specific enzymes. The non-coplanar congeners (those with substitutents in the 2,2',6 or 6' positions of the biphenyl nucleus) appear to be inducers of cytochrome(s) P-450 (phenobarbital type), while the planar isomers (lacking the aforementioned substituents) are cytochrome(s) P_1-450 inducers (3-methylcholanthrene-type).

In this study, we have utilized 2,2',4,4'-tetrachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl as representative non-coplanar and coplanar isomers respectively. The 3,3',4,4'-tetrachlorobiphenyl isomer (0.3mmole/kg) induced ethoxycoumarin-and ethoxyresorufin-O-deethylations in the rainbow trout to a similar extent as did Aroclor 1242 (Table III). However, the non-coplanar 2,2'-4,4'-tetrachlorobiphenyl was without effect upon any of the monooxygenase activities examined. Cytochrome P_1-450-like activity as determined by ethoxyresorufin-O-deethylation was increased by the planar 3,3',4,4'-tetrachlorobiphenyl while cytochrome P-450-like activity (benzphetamine-N-demethylation) was unaffected.

SDS-PAGE of microsomal preparations from the livers of rainbow trout treated with either 2,2',4,4'-or 3,3',4,4'-tetrachlorobiphenyl was performed. Figure 5 illustrates densitiometric scans of Coomassie Blue stained electrophoretograms of various microsomal preparations. The vertical broken line indicates the 57,000 molecular weight area. As can be seen, no distinct band was apparent at 57,000 daltons in microsomes obtained from control or 2,2',4,4'-tetrachlorobiphenyl-treated rainbow trout; however, a large absorbance peak was seen at 57,000 daltons in microsomes from Aroclor 1242-or 3,3',4,4'-tetrachlorobiphenyl-treated rainbow trout (Figure 5). This peroxidase staining band corresponds to the β-napthoflavone-induced protein of 57,000 molecular weight seen in Figure 4.

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TABLE III

Induction of Hepatic Microsomal Monooxygenation in the Rainbow Trout by Selected Polychlorinated Biphenyls

Treatment of fish[a]	BeND[b]	ECOD[c]	EROD[d]
Corn oil	100[e]	100	100
Aroclor 1242	113	808[f]	2432[f]
2,2',4,4'-tetrachlorobiphenyl	102	195	340
3,3',4,4'-tetrachlorobiphenyl	111	643[f]	2076[f]

[a]All chemicals given i.p. in corn oil (1ml/kg) at a dose of 0.3mmol/kg.
[b]Benzphetamine-N-demethylation.
[c]Ethoxycoumarin-O-deethylation.
[d]Ethoxyresorufin-O-deethylation.
[e]Values are expressed as % of corn oil control. Typical control specific activities are indicated in Table II.
[f]Significantly different to corn oil control, $p<0.05$.

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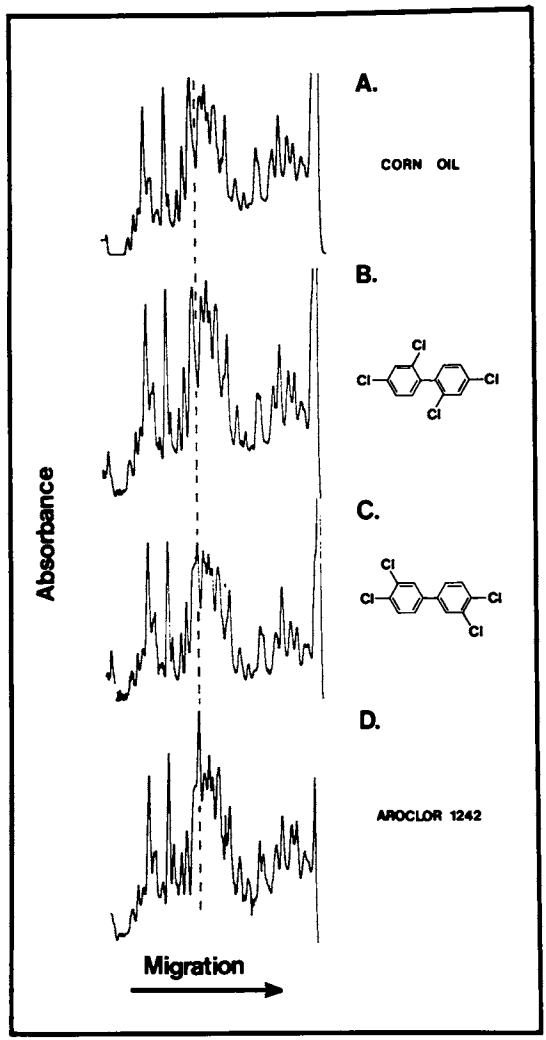

Figure 5. Densitiometric scans of electrophoretograms of hepatic microsomes from rainbow trout pretreated with polychlorinated biphenyl congeners: (A), control microsomes, 90 μg protein/gel; (B), 2,2′,4,4′-tetrachlorobiphenyl-induced microsomes, 90 μg protein/gel; (C), 3,3′,4,4′-tetrachlorobiphenyl-induced microsomes, 90 μg protein/gel; (D), Aroclor 1242-induced microsomes, 90 μg protein/gel. The slab gels were stained with Coomassie Blue-250 and individual sample tracts were cut out and scanned at 550 nm. The vertical broken line is at 57,000 daltons.

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In summary, it would appear that rainbow trout are responsive to cytochrome P_1-450-type inducers (e.g. planar polychlorinated biphenyls and polycyclic aromatic hydrocarbons), but are not responsive to cytochrome P-450-type inducers (e.g. phenobarbital and non-planar polychlorinated biphenyls). Although induced rainbow trout hemoprotein(s) P-450 show high activity with cytochrome P_1-450 substrates, the induced hemoprotein at 57,000 daltons appears, in electrophoretic and spectral properties, to differ from rodent cytochrome P_1-450.

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Further Studies on the Effect of Petroleum Hydrocarbons on Mixed-Function Oxidases in Marine Organisms

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The marine environment acts as a sink for a large proportion of polyaromatic hydrocarbons (PAH) and these compounds have become a major area of interest in aquatic toxicology. Mixed function oxidases (MFO) are a class of microsomal enzymes involved in oxidative transformation, the primary biochemical process in hydrocarbon detoxification as well as mutagen-carcinogen activation (1,2). The reactions carried out by these enzymes are mediated by multiple forms of cytochrome P-450 which controls the substrate specificity of the system (3). One class of MFO, the aromatic hydrocarbon hydroxylases (AHH), has received considerable attention in relation to their role in hydrocarbon hydroxylation. AHH are found in various species of fish (4) and although limited data is available it appears that these enzymes may be present in a variety of aquatic animals (5,6,7,8).

Overwhelming evidence has demonstrated that MFO are subject to modification by a vast number of foreign compounds and drugs. Enzyme induction in fish is effected by exposure to various agents including environmental levels of petroleum (9,10,11), by feeding high concentrations of PCB (12,13) and by feeding or injection of hydrocarbons known to be powerful inducers in mammals (12,14,15). DDT type compounds can induce birds and mammals but were found to be ineffective in fish (13). There are few studies on attempts to stimulate MFO in marine invertebrates by foreign compounds. We previously reported that various species of invertebrates either exposed to emulsified Venezuelan crude in the laboratory or collected from a refinery outfall did not display elevated AHH levels compared to controls (7), but Lee has recently induced crabs with injections of aromatic hydrocarbons (16).

The work reported here principally involves attempts to induce AHH in invertebrates under "extreme" oil exposure conditions. We also investigated induction in fish with various pure hydrocarbons, hydrocarbon analogs, as well as oil and oil spill dispersant mixtures.

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Attempts to Induce AHH in Invertebrates under Extreme Oil Exposure Conditions

Gastropods (snails), echinoderms (sea urchins and sea stars) and annelids (lugworms) were exposed to oil saturated sediments and assayed for AHH activity (17). Sediment was mixed with Venezuelan crude at a concentration of 0.2-0.5% and exposures were for one week (4°C) in a static system with water renewal at 2-3 day intervals. The aim was to determine if oil soaked sediments could induce AHH activity in some representative intertidal benthic organisms common to the subarctic waters of the North West Atlantic. Digestive gland homogenates from snails, sea urchins, and sea stars and a combination of intestinal and gill tissues from annelids were used in the enzyme assays.

Crustaceans (lobsters, Homarus americanus) were exposed to a surface slick of Venezuelan crude for five months in a flow through sea water system. Weathered oil was removed weekly from the surface of the exposure tank and a fresh batch (150 ml) of crude added. The design was to closely simulate a chronic "water soluble" exposure. Control and experimental lobsters were fed an equivalent amount of herring or capelin. Singer (18) recently reported 'high' AHH levels in green gland tissues of female crabs. We assayed male and female lobster hepatopancreatic and green gland tissues with diphenyloxazole (PPO), instead of benzo(a)pyrene, as substrate (19). (Due to the hazards involved with benzo(a)pyrene, we now use PPO with species which display equivalent AHH activity levels with both substrates. Activity with PPO refers to relative fluorescence units. Mass spectral studies of the specific hydroxylated metabolites produced by fish tissues have recently been carried out in this Laboratory (Penrose, unpublished) but standards are not yet available.) In our earlier AHH studies (7,9,17) a Turner-110 Fluorimeter was used; sensitivity has now been increased with the aid of a model (Perkin-Elmer-204) which permits specific wave length selection.

AHH activity was not induced in digestive gland tissues of snails, sea urchins, sea stars, or lugworms (Table I) or in hepatopancreatic or green gland tissues of either male or female lobsters (Table II).

Table I. Effect of Exposing Benthic Invertebrates to Oil-Saturated Sediment on AHH Activity

Organism	No.	Specific Activity (F Units/mg protein/10 min)[a] Control	Experimental
Mollusca			
Snails, Littorina sp	6 + 6	0.10 ± 0.07[b]	0.09 ± 0.05

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Table I. Continued

Organism	No.	Specific Activity (F Units/mg protein/10 min)[a] Control	Experimental
Echinodermata			
Sea urchin, *Strongylocentrotus* sp	6 + 6	0.08 ± 0.04	0.05 ± 0.03
Starfish, *Asterias* sp	6 + 6	0.10 ± 0.08	0.10 ± 0.04
Annelida			
Lugworms, *Arenicola* sp	7 + 7	0[c]	0[c]

[a]Fluorescence (F) units with benzo(a)pyrene as substrate

[b]Mean and standard deviation

[c]< Sensitivity of the assay

Table II. Effect of Exposing Lobsters to a Surface Slick of Oil for Five Months on AHH Activity

Sex	No.	Tissue	Specific Activity (F Units/mg protein/10 min)[a] Control	Experimental
Male	10 + 10	Hepatopancreas	0.25 ± 0.16[b]	0.23 ± 0.13
		Green gland	1.30 ± 1.34	0.70 ± 0.38
Female	10 + 10	Hepatopancreas	0.65 ± 0.39	0.45 ± 0.38
		Green gland	1.08 ± 0.46	1.20 ± 0.52

[a]Fluorescence (F) units with diphenyloxazole as substrate

[b]Mean and standard deviation

This supports our earlier work with invertebrates which were exposed to lower concentrations of oil in the laboratory or selected at a refinery outfall site (7). We recently noted that annelid polychaetes (sandworms) collected near this refinery had slightly higher AHH activity levels than worms from a control site, but attempts to stimulate activity in "control" worms with oiled sediments have been unsuccessful.

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Induction of Bivalve MFO

Previous studies both *in vivo* and *in vitro* have suggested that MFO are not present in marine bivalves, but related animal orders, e.g. gastropods and pelecypods do have the enzyme system (7). Anderson (20) has recently reported AHH in oyster hepatopancreas and slight induction in animals exposed to benzo(a)pyrene or PCB.

We employed various substrates to check for MFO in two bivalve species, a salt water mussel (*Mytilus edulis*) and a fresh water clam (*Anodonta* sp). Cytochrome P-450 was also studied. Organisms were exposed to 100 PPM Venezuelan crude in a stagnant system for up to one month. Enzyme assays were carried out with digestive gland 9000 g homogenates (17) and cytochrome P-450 analysis with microsomes (21). The hydrocarbon substrates investigated included ^{14}C-labelled benzo(a)pyrene, fluorene, anthracene, and naphthalene. The method used for separation of BP metabolites by thin layer radiochromatography has been described (7). The metabolite detection method for the other aromatic hydrocarbons was essentially the same except methylene chloride was used as metabolite extractant as well as TLC developer. Besides the hydrocarbon substrates, we also checked for other MFO reactions, N-dealkylase with ^{14}C-imipramine (22) and O-dealkylase with ethoxycoumarin (15).

There was no indication that either the fresh or salt water bivalves could metabolize any of the aromatic hydrocarbons or the N- and O-heterocyclic compounds. All the compounds were transformed in positive controls with cunner liver extracts, however (Table III).

Table III. Aromatic Compounds Biotransformed by Fish or Bivalve Homogenates

Compound	Metabolites Detected	No Metabolites Detected
	Fish liver	Bivalve hepatopancreas
Benzo(a)pyrene	3	0
Anthracene	2	0
Fluorene	2	0
Naphthalene	2	0
Imipramine	2	0
Ethoxycoumarin*	1	0

*Metabolites detected by fluorimetry instead of radiochromatography

Cytochrome P-450 was also readily detectable in cunner tissue but repeated trials produced no evidence for this cytochrome system in the bivalves.

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Induction of Fish AHH with Pure Hydrocarbons

A variety of aromatic hydrocarbons (often in large doses) have been used to induce MFO in mammalian systems and more recently in fish. 3-methylcholanthrene and benzo(a)pyrene are potent inducers in mammals and are also effective in fish (8, 14,15). Other agents as PCB (12,13) in contrast to DDT type compounds (13) have also been used to induce fish. We are not aware, however, of any studies where fish have been induced by pure hydrocarbons (or other compounds) dissolved or accommodated in water.

Cunners (Tautogolabrus adspersus) were exposed to a variety of compounds from the relative (e.g. benzene) to sparingly (e.g. pyrene) soluble. Although not generally considered to be as potent as such inducers as 3-methylcholanthrene or benzo(a)pyrene, at least five of the seven compounds tested are known to induce MFO in mammalian systems. This experiment is the first of a series planned to assess the inducing potential of various hydrocarbons and hydrocarbon analogs dissolved or accommodated in water. Exposures were carried out in a stagnant water system at -1.0 - 2.0°C when this species is normally torpid (23) and activity was assayed in liver homogenates after a five-day exposure. Preliminary work with this species indicated that PPO was a suitable substrate for monitoring AHH induction with petroleum (24). AHH was not induced in cunner livers with any of the compounds (Table IV).

Table IV. Effect of Exposing Cunners to Various Aromatic Compounds on AHH Activity

Compound	No. Fish	Exposure PPM	Specific Activity (F Units/mg protein/10 min)[a] Control	Experimental
Benzene	6 + 6	5 x 3	2.17 ± 0.74[b]	2.25 ± 0.95
Xylene	4 + 4	2 x 3	2.88 ± 0.68	2.69 ± 1.00
Naphthalene	6 + 6	2 x 2	3.53 ± 1.99	2.17 ± 0.61
Phenanthrene	6 + 6	5	2.17 ± 0.74	1.81 ± 0.59
Pyrene	6 + 6	5	3.53 ± 1.99	2.30 ± 0.47
Dibenzothiophene	4 + 4	5	2.21 ± 0.64	2.49 ± 0.76
Iso-octane	6 + 6	50	2.27 ± 1.14	2.88 ± 0.66

[a]Fluorescence (F) units with diphenyloxazole as substrate

[b]Mean and standard deviation

By comparison, Venezuelan crude (exposed to 1-3 mls as a surface slick in a 20 l aquarium for 3 days) was effective in inducing liver as well as heart, kidney, and spleen tissues (Table V).

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Table V. AHH Induction in Cunners Exposed to Venezuelan Crude Oil

Tissue	No. Fish 4 + 4	Specific Activity (F Units/mg protein/10 min)[a] Control	Experimental
Liver		9.9 ± 4.4[b]	61.59 ± 10.26
Heart		0.48 ± 0.35	4.98 ± 1.83
Kidney		1.29 ± 1.22	8.47 ± 2.31
Gills		0.54 ± 0.19	2.10 ± 0.20
Spleen		0[c]	0.52 ± 0.36
Brain		0[c]	0[c]

[a]Fluorescence (F) units with diphenyloxazole as substrate

[b]Mean and standard deviation

[c]< Sensitivity of the assay

Basal enzyme levels for control fish in the Venezuelan crude experiment (Table V) were higher than the control values for experiments with pure hydrocarbons (Table IV). Increase in basal activity was correlated with an increase in water temperature and the initiation of feeding. Enzyme activity was consistently low in cunners during the winter, and appears to rise in late spring to a summer peak (24).

Induction of AHH in Fish with Oil and Oil Spill Dispersant Mixtures

Dispersants are being increasingly used to combat oil spills in the marine environment. The new generation of dispersants are commonly fatty acid-polyethoxylate esters (25) and are relatively non toxic. The specific compounds in petroleum responsible for MFO induction in fish have not been defined. Dispersed oil could increase the availability of inducing components, either the particulates or solubles, but alternatively, soluble compounds may be rapidly lost from dispersed oil (26). Preliminary experiments have been carried out to assess the effectiveness of dispersed oil in AHH induction. Venezuelan crude and bunker (distillation cut above 300-400°C) and two polyethoxylate oil spill dispersants, BP 1100X (BP Trading Ltd.) and Oilsperse 43 (Diachem of B.C. Ltd.) were studied. Canadian regulations recommend a ratio of 1:10 for the application of dispersant on spilled oil; to effect "maximal dispersion" we used a larger proportion of dispersant.

Preliminary evidence suggests that dispersants have little effect in enhancing the inductive potential of spilled oil. Dispersed crude was no more effective than crude alone in AHH induction (Table VI)

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Table VI. AHH Induction in the Livers of Cunners Exposed to Oil and Oil-Spill Dispersant Mixtures

Treatment (Addition to the surface of 20 l water)	No. Fish	Specific Activity (F Units/mg protein/10 min)[a]
Control	5	7.4 ± 0.91
1 ml Venezuelan crude	4	53.2 ± 28.6*
1 ml Venezuelan crude + 1 ml Oilsperse 43	4	39.6 ± 8.7*
1 ml Venezuelan bunker + 1 ml Oilsperse 43	4	10.89 ± 3.39
3 mls BP 1100X	4	6.58 ± 2.57
3 mls Venezuelan bunker + 3 mls BP 1100X	4	7.01 ± 1.44

[a]Fluorescence units with diphenyloxazole as substrate

*Significantly different from controls ($P < 0.05$, Student's T test)

and neither dispersed nor undispersed bunker stimulated enzyme activity. It appears that the major inducers in oil are in the soluble fraction and these soluble compounds may not necessarily become "more available" for induction as a result of oil dispersion. It should also be noted that the degree of dispersion in these studies (by using a greater proportion of dispersant) would not ordinarily be attained in the environment. Besides the surfactant compounds, the possibility exists for induction by glycols (27) which are potential decomposition products from these dispersants (25,28). There was no evidence, however, for induction in fish by dispersants alone.

Conclusions

Exposure of various invertebrate species to high concentrations of petroleum did not induce mixed function oxidase activity. Enzyme activity was stimulated, however, in a number of fish tissues by petroleum. Different permutations can be addressed as to the significance of basal or induced levels of mixed function oxidases and hydrocarbon toxicity. AHH may have a physiological role in enhancing hydrocarbon clearance but may also increase the mutagenic-carcinogenic potential of hydrocarbons. Both of these concepts have been demonstrated in studies with fish (29,30). Induced AHH levels may permit a more rapid oxidative transformation with concomitant "disappearance" of parent hydrocarbons, but potentially toxic metabolites could be retained in tissues for longer periods (31). It is likely that at the enzymic level the

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balance between the rate of production (viz. mixed function oxidases) and rate of destruction of reactive metabolites (viz. hydratases, conjugases, etc.) could be critical in determining hydrocarbon toxicity for any species.

Induction did not occur under our exposure conditions in fish exposed to water saturated with a variety of pure hydrocarbons. The compounds studied are commonly found in other pollutants besides petroleum. It is reasonable to speculate that enzyme induction may not be a common response of fish in the environment to various pollutant hydrocarbons which may be available in the water column for short periods.

The soluble component may be of prime importance for MFO induction in fish by petroleum. Dispersion should increase the "availability" of this component in the water column, but preliminary evidence suggests that dispersants have little effect in enhancing the inductive potential of petroleum.

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In Vivo and In Vitro Studies of Mixed-Function Oxidase in an Aquatic Insect, *Chironomus riparius*

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Introduction

Few insects have been studied in detail in regard to the metabolism of insecticides by mixed function oxidases (MFO) (1). Most of those studies dealt with terrestrial insects. Information on the metabolism of insecticides by non-target aquatic insects is fragmentary.

High population densities of midge larvae are found in "polluted" waters, i.e. waters having very little dissolved oxygen and/or a high biological oxygen demand. MFO enzymes require oxygen. Hence, investigation of an oxygen-requiring enzyme system is especially important in midges, since they reside in habitats that may be oxygen deficient. Moreover, midges are among the most sensitive aquatic insects, responding to insecticides in the ug/L range. Thus, fundamental studies on midge metabolism of insecticides would be elucidating.

In addition to the above, Chironomus riparius was selected as the test organism for the following reasons: 1) C. riparius is a common aquatic insect having a wide distribution; 2) A recent review of the taxonomic status of C. riparius Meigen and C. thummi Kieffer concluded that the designations are synonymous, the former being the correct name (2). Larvae of both names have been used extensively in physiological, biochemical and genetical research. 3) Chironomids are considered target or non-target species. The adults of certain midge species are pests in several areas of the United States, while midge larvae comprise a major portion of the diet of certain fish species. Information about midge-insecticide interactions may relate to more effective control measures or ecological effects on midges.

Experiments in this study, done exclusively with midge larvae, include: 1) 24-hr toxicity data for representative insecticides, with and without synergists; 2) in vivo absorptive uptake and metabolic studies of aldrin and dieldrin, with and without piperonyl butoxide (PBO); 3) body depuration rate (loss to water) for dieldrin; 4) determination of optimal in vitro

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assay conditions for aldrin epoxidation by MFO's with whole body homogenates; and 5) measurement of aldrin epoxidation with larval microsomes, with and without PBO.

Materials and Methods

Insects. Midge larvae were collected at the Jackson Pike sewage treatment plant in Columbus, Ohio and reared to the adult stage in the laboratory. From them, a colony of Chironomus riparius (Meigen) was established and maintained in aerated tap water (21 ± 2°C) in a bin, 48 X 37 X 23 cm, covered with a screen flight cage, 50 X 35 X 75 cm. The larvae were fed pulverized Hartz Mountain Dog Yummies^{R} and reared according to a described method (3) without the addition of substrate. The larvae were maintained for 3 years as a laboratory culture prior to experimentation.

Chemicals. Insecticides, at least 95% pure, were prepared as acetone solutions: p-p' DDT, lindane, parathion, paraoxon, malathion, malaoxon, propoxur, carbaryl, Landrin^{R}, aminocarb, mexacarbate, allethrin (90%), piperonyl butoxide (PBO) and sesamex. Aldrin was 98.5% and dieldrin was 99+% pure.

All other chemicals were analytical reagent grade. All solvents were redistilled in glass.

Immersion Toxicity: Assay Procedure, Criteria of Response and Data Analysis. The assay procedure was a modification of published methods (4, 5). Toxicity assays were conducted at 21 ± 2°C in narrow-mouth, quart (0.95 L) glass jars containing conditioned Columbus tap water (pH 7.5 to 8.5, aged 24 hr). No food, substrate, or aeration were used during the test. Cannibalism did not occur with well-fed larvae and short term (24 hr) assays.

All aqueous solutions or suspensions were prepared by adding insecticide in 0.5 ml of acetone to 500 ml of conditioned tap water and vigorously shaking the capped container. New acetone solutions of insecticide were prepared for each experiment. Each assay contained untreated controls and solvent controls.

Twenty fourth-instar midge larvae were placed in test containers, 10 larvae/container, 2 containers/insecticide concentration. Mortality was recorded after 24 hr, moribund larvae being recorded as dead. Larvae used in synergism experiments were pretreated for 1 hr in 1 mg/L PBO or sesamex (sub-lethal doses). Pretreated larvae were transferred to jars containing insecticide with synergist, and mortality was recorded after 24 hr. The synergistic ratio (SR) was obtained by dividing the LC_{50} of the insecticide alone by the LC_{50} of the insecticide-synergist mixture. LC_{50} values are from pooled data of 3 experiments performed on different days. Organisms that pupated during the assay period or test concentrations where mortality was 0% or

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100% were excluded from analysis. Toxicity assays were corrected for control mortality (6). The LC_{50} values and their 95% confidence level were determined by computer (7).

Various criteria have been used to define midge mortality including lack of movement when touched with a probe, inability to make undulating movements, and color changes (4, 5, 8, 9). We used mobility changes as a measure of midge mortality. Normal midges exhibit body movements that we define as a swimming cycle which, in composite, resembles a figure eight. Normal larvae generate continuous figure eights as they swim. Any larva which could not respond with 3 swimming cycles when pinched with tweezers in the region of the anal papillae was considered moribund.

Homogenizing Procedure. Larvae in whole body homogenate or subcellular fraction assays were homogenized in Tris-HCl buffer unless otherwise noted. In uptake or depuration experiments, midges were rinsed once with water and homogenized in 5 ml of distilled water. All homogenates were prepared in a glass Potter-Elvehejm tissue grinder at a low speed for approximately 20 sec, 10 passes through the brie.

Extraction Procedure. We modified the extraction procedure of Nelson et al (10). Brie acidified with 2 ml of 5% trichloracetic acid (TCA) was extracted 3 times with 20 ml of petroleum ether. The combined extracts were reduced to 5 ml in a rotating evaporator, returned to the separatory funnel, and combined with 60 ml each of acetonitrile and distilled water. The acetonitrile-water-insecticide mixture was extracted twice with 60 ml of petroleum ether and anhydrous Na_2SO_4 was added to the combined 120 ml extract. The extract was evaporated just to dryness and the residue was dissolved in benzene for analysis by gas-liquid chromatography (GLC). Extraction efficiencies in spiked experiments were 73% (aldrin) and 83% (dieldrin).

Water samples were extracted 3 times with 50 ml of petroleum ether and anhydrous Na_2SO_4 was added to the combined 150 ml extract. The extract was evaporated just to dryness and dissolved in benzene for GLC analysis. The extraction efficiency for dieldrin in water was 95%.

Gas-Liquid Chromatography (GLC). A Varian Aerograph GLC Model 1440 equipped with a ^{3}HSc electron capture detector and a 150 cm X 2 mm (i.d.) glass column packed with 3% SE-30 on Gas Chrom Q was used. The following operating parameters were employed: injector 240°C, column 200°C, detector 260°C, and a N_2 flow of 37.5 ml/min. The chromatogram was recorded on a Varian Model A-25 recorder with a chart speed of 0.1 in/min. Insecticides were quantified by peak height using daily standard curves. Each analysis was determined by averaging the peaks of three injections.

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Insecticide Absorption, Conversion and Depuration. Aqueous suspensions of insecticides were prepared as for toxicity assays. Fourth instar larvae, 20 per container, were exposed for 2 hr to 20 ug/L of aldrin or dieldrin. When a synergist was used, 20 midges were pretreated for 1 hr in 1 mg/L PBO, then exposed for 2 hr to 20 ug/L of aldrin or dieldrin and 1 mg/L PBO. In depuration experiments, 60 midges were exposed for 1 hr to 20 ug/L dieldrin. The water and groups of 20 midges each were analyzed for dieldrin content after being held for 0, 3 hr and 6 hr in clean water after exposure. Extraction and analysis of midges and water were as previously described. Mean values were computed from two separate experiments.

Assay of Homogenate for Aldrin Epoxidation. The following experimental sequence was designed to determine the optimum *in vitro* conditions for aldrin epoxidation in larval whole body homogenates: 1) the effect of component chemicals generally included in an incubation mixture, 2) a pH profile, 3) a temperature profile, 4) a molarity profile, 5) a reaction time profile, 6) a larval concentration (enzyme concentration) profile, 7) a substrate concentration profile, and 8) a restudy of the effects of component chemicals in the initial incubation mixture (Step 1) upon aldrin epoxidation under optimum conditions as defined by steps 2-7 above. The effect of PBO, FMN, and FAD upon enzyme activity was also tested.

In Step 1, an incubation mixture (11) was tested using 20 midge larvae homogenized in 8.3×10^{-2} M Tris-HCl buffer, pH 7.5. Each 5 ml incubation mixture contained 20 homogenized midges, 5.0×10^{-2} M Tris HCl buffer, pH 7.5, 2.4×10^{-3} M glucose 6-phosphate (G-6-P), 1.6 units glucose 6-phosphate dehydrogenase (G-6-P dH), 5.1×10^{-5} M NADP, and 2.7×10^{-3} M KCl. In addition, the following chemicals were included in the final concentration indicated: 5.1×10^{-5} M NADH, 1% (W/V) bovine serum albumin (BSA), and 1.0 mg aldrin in 0.1 ml ethanol. Whole body homogenate experiments included all of the above chemicals unless otherwise noted. Reaction mixtures were incubated with swirling in test tubes at 30 ± 1°C. Reactions in Steps 1-4 of the experimental sequence were stopped after 1 hr and Steps 6-8 after 15 min, by the addition of 2 ml 5% TCA.

Microsome Preparation. Fourth instar midge larvae, 1,000/experiment, were weighed to the nearest 0.1 mg and homogenized in 8.3×10^{-1} M Tris-HCl buffer, pH 7.5.

Preparatory centrifugations were performed in a Sorvall Model RC2-B centrifuge with a SS-34 rotor. The homogenate was sedimented at 2,400g max (4,500 RPM) for 15 min at 1 ± 1°C to remove large cell fragments and debris. The supernatant was centrifuged at 20,000g max (13,000 RPM) for 15 min at 1 ± 1°C to isolate the mitochondrial pellet. The post-mitochondrial supernatant was sedimented in a Beckman Model L centrifuge with a 50

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rotor at 128,000g max (40,000 RPM) to isolate the microsomes. A portion of the microsome pellet was resuspended in 1.5 X 10^{-1} M KCl, pH 7.5 and centrifuged as before (washed microsomes). All equipment, solutions, and glassware were precooled and all processed material was kept in crushed ice until incubated.

Assay of Subcellular Fractions for Aldrin Epoxidation. Mitochondrial and microsomal pellets were resuspended in Tris-HCl buffer. Each 5 ml incubation mixture contained the following: 2.4 X 10^{-3} M G-6-P, 1.6 units G-6-P dH, 5.1 X 10^{-5} M NADP, 1.0 mg aldrin in 0.2 ml ethanol when used alone, or 1.0 mg aldrin and 1.0 mg PBO, each in 0.1 ml ethanol, when used in combination. In synergism experiments, mixtures were pretreated with PBO for 3-5 min prior to the addition of substrate. The reaction mixtures were incubated with agitation in test tubes at 30 ± 1°C in a water bath shaker for 15 min. Reactions were stopped by acidifying with 2 ml 5% TCA. The acidified mixtures were extracted and analyzed by GLC.

Protein Analysis. Protein concentrations were determined with a Spectronic 20 spectophotometer employing BSA as a standard (12). Each 0.1 ml sample was spotted on 3 cm^2 Whatman No. 42 filter paper and air dried. Samples were stained with Xylene Brilliant Cyanin G (K and K Laboratories, Cleveland, Ohio), and the absorbance at 610 nm was recorded against a blank containing distilled water. Samples were corrected using controls containing all components except protein.

Results

Toxicity Assays. The computed LC_{50} and 95% confidence interval and the synergistic ratio (SR) for each insecticide are contained in Table I. Control mortality was 5% or less in all experiments.

In general, organochlorine and organophosphate insecticides had LC_{50} values in the same order of magnitude (LC_{50}'s from 0.5 ug/L to 6.2 ug/L), while carbamate insecticides were generally less toxic (LC_{50}'s from 12.2 ug/L to 376.6 ug/L).

Dieldrin, the oxidative metabolite of aldrin, was the most toxic of all insecticides in this study but was only slightly more toxic than its parent compound. The oxidative metabolites of parathion and malathion, paraoxon and malaoxon, were slightly less toxic than their parent compounds.

The range for the regression coefficients of all insecticides was generally medium to high (3.0-10.9) except for parathion and carbaryl which were 2.1 and 2.6, respectively.

Low synergistic ratios indicating antagonism by PBO (ratios from 0.15 to 0.33) were obtained with aldrin, parathion, malathion, aminocarb, and mexacarbate. PBO strongly synergized allethrin (SR 102). Assays where synergism was weak or absent

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Table I. LC_{50} values (24 hr) for fourth instar larval *Chironomus riparius*

Insecticide	LC_{50} (95% C.I.), ug/L	Synergistic ratio
Organochlorine		
DDT	4.7 (4.2-5.3)	-
DDT+Sesamex	2.9 (2.6-3.2)	1.63
Aldrin	0.8 (0.7-0.8)	-
Aldrin+PBO	2.2 (1.9-2.6)	0.33
Dieldrin	0.5 (0.4-0.6)	-
Dieldrin+PBO	0.3 (0.3-0.4)	1.39
Lindane	3.6 (3.1-4.0)	-
Organophosphate		
Parathion	2.5 (1.7-4.1)	-
Parathion+PBO	17.1 (14.3-21.6)	0.15
Paraoxon	6.2 (5.8-6.7)	-
Paraoxon+PBO	5.5 (4.8-6.5)	1.13
Malathion	1.9 (1.7-2.2)	-
Malathion+PBO	6.8 (6.0-7.8)	0.28
Malaoxon	5.4 (4.9-5.9)	-
Malaoxon+Sesamex	1.9 (1.6-2.3)	2.78
Carbamate		
Carbaryl	104.5 (83.3-151.7)	-
Carbaryl+PBO	62.4 (54.0-72.9)	1.68
Landrin^{R}	51.4 (45.0-59.3)	-
Landrin^{R}+PBO	45.7 (37.5-54.5)	1.12
Aminocarb	376.6 (329.7-428.5)	-
Aminocarb+PBO	1,172.2 (1,072.0-1,308.1)	0.32
Mexacarbate	12.2 (11.0-13.5)	-
Mexacarbate+PBO	59.3 (52.9-66.2)	0.20
Propoxur	64.4 (59.5-69.4)	-
Propoxur+PBO	29.2 (25.2-33.8)	2.21
Synthetic Botanical		
Allethrin	41.9 (38.6-45.3)	-
Allethrin+PBO	0.4 (0.3-0.5)	102.10

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(ratios from 0.81 to 2.78) included: malaoxon or DDT with sesamex; propoxur, carbaryl, dieldrin, paraoxon, or Landrin^{R} with PBO.

Insecticide Absorption, Conversion and Depuration. Midge larvae immersed in 20 ug/L aldrin for 2 hr absorbed 25.4 ng of insecticide per larva, converting 58% of it to dieldrin (Table II). With 1.0 mg/L PBO present, midges absorbed 18.4 ng of aldrin per larva, but no aldrin was converted to dieldrin. With or without PBO, midge larvae absorbed dieldrin at the same rate as aldrin (Table II).

Table II. The absorption of insecticides by C. riparius larvae

	Body Content, ng/larva (ug/g)		
Insecticide	Aldrin	Dieldrin	Total
Aldrin	10.7 (0.8)	14.7 (1.1)	25.4 (1.9)
Dieldrin	-	23.9 (1.9)	23.9 (1.9)
Aldrin+PBO	18.4 (1.5)	N.D.[a]	18.4 (1.5)
Dieldrin+PBO	-	24.9 (2.0)	24.9 (2.0)

[a]Not Detected

All insecticides affected the mobility of C. riparius larvae in a similar manner. A normal swimming motion was generally reduced to 1 cycle at the onset of toxic symptoms. The effect of the toxicant increased until the larva lost all ability to move. Death soon followed. Similar symptoms of insecticide poisoning have been reported for stonefly naiads (13, 14). Changes in larval color were unreliable for determining toxic affect. Toxic symptoms were observed in approximately 50-75% of the midge larvae immersed in 20 ug/L aldrin at the end of the 2 hr exposure but no effects were noted with aldrin plus PBO. All larvae immersed in dieldrin, or dieldrin with PBO were moribund.

The depuration of dieldrin from midge larvae was relatively slow (Table III). Larvae transferred to clean water for 3 hr released 0.011 ng of dieldrin/larva/hr, or 0.2% of the total dieldrin absorbed. Another group of larvae transferred to clean water for a total of 6 hr, released dieldrin to the water at approximately the same rate, 0.014 ng/larva/hr, or 0.5% of the total dieldrin absorbed.

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Table III. Depuration of dieldrin by C. riparius larvae

Time Hours	Dieldrin, ng/midge	Dieldrin in water, ng	Loss, ng/midge/hr
0	19.0	-	-
3	19.5	0.65	0.011
6	17.8	1.65	0.014

Establishing Optima for Aldrin Epoxidation Using Whole Body Homogenates. The addition of all component chemicals increased dieldrin production greater than 3X compared to the unmodified homogenate (Table IV). As the optimum of each factor was established, it was used in all subsequent experiments.

Table IV. Aldrin epoxidase requirements of C. riparius whole body homogenates

Incubation Medium	nmoles dieldrin/min	% Maximum
Crude Homogenate[a]	0.015	28
+ 1% BSA	0.016	30
+ 1% BSA G-6-P (2.4×10^{-3} M) G-6-P dH (1.6 units) NADH (5.1×10^{-5} M) NADP (5.1×10^{-5} M)	0.38	72
+ KCl (2.7×10^{-3} M) and all of above	0.053	100

[a]20 midges/5 ml, 0.05 M Tris buffer, pH 7.5, 1 mg aldrin. All incubations at 30°C for 1 hr. Not optimum conditions (Table V); mean of 2 experiments.

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Table V. Aldrin epoxidase requirements of C. riparius whole body homogenates under optimum conditions[a]

Incubation Medium	nmoles dieldrin/min	% Maximum
Crude Homogenate	0.116	36
+ (electron generator) G-6-P (2.4×10^{-3} M) G-6-P dH (1.6 units) NADP (5.1×10^{-5} M)	0.324	100
+ KCl (2.7×10^{-3} M) and electron generator	0.314	97
+ 0.1% BSA, KCl and electron generator	0.279	86
+ NADH (5.1×10^{-5} M) and all of above	0.275	85

[a]Larvae were homogenized in 8.3×10^{-1} M Tris-HCl, pH 7.5 buffer. Complete incubation medium (final concentrations): 3 ml of homogenate (20 larvae); 5.0×10^{-1} M Tris HCl buffer, pH 7.5; 1.0 mg aldrin in 0.5 ml ethanol; total volume, 5 ml.
Reaction mixtures were incubated at 30°C for 15 min.
Mean values from 2 experiments.

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The effect of pH on *in vitro* aldrin epoxidase activity was established over a pH range 6.5-8.5 (Figure 1). A pH of 7.5 was used as optimum. The effect of temperature on *in vitro* aldrin epoxidase activity was determined over a range of 20°-40°C (Figure 2). An optimum incubation temperature of 30°C was used. The maximum epoxidase activity was attained at a Tris-HCl buffer concentration of 5.0×10^{-1} M (Figure 3).

After establishing optimum reaction conditions, the effect of chemical supplements upon epoxidase activity was re-examined. Several chemicals used in preliminary incubation mixtures and throughout optimizing experiments had no effect upon dieldrin production under optimum conditions. These are BSA, KCl and NADH (Table V). Overall, there was a 22-fold enhancement of *in vitro* epoxidase activity when initial conditions (crude homogenate, Table IV) are compared to optimum conditions (homogenate plus electron generator, Table V). FMN decreased, FAD slightly increased and PBO completely inhibited *in vitro* epoxidase activity (Table VI).

Table VI. Effect of FMN, FAD, and PBO upon *in vitro* *C. riparius* aldrin epoxidase in whole body homogenates[a]

Incubation Medium	nmoles dieldrin/ midge/min	Percent
Homogenate	0.081	100[b]
FMN (10^{-3} M)	0.075	92
FAD (10^{-3} M)	0.089	110
PBO (6.0×10^{-4} M)	N.D.[c]	0

[a]Optimum conditions of Table V. Mean values of 2 experiments

[b]Standard

[c]Not Detected

The cumulative amount of dieldrin increased during incubation times of 15 min and 30 min, then approached a plateau over the following 45 min (Figure 4). The plot of dieldrin/insect/min vs. time of incubation had a negative slope (Figure 4). A 15 min incubation time was used as optimum.

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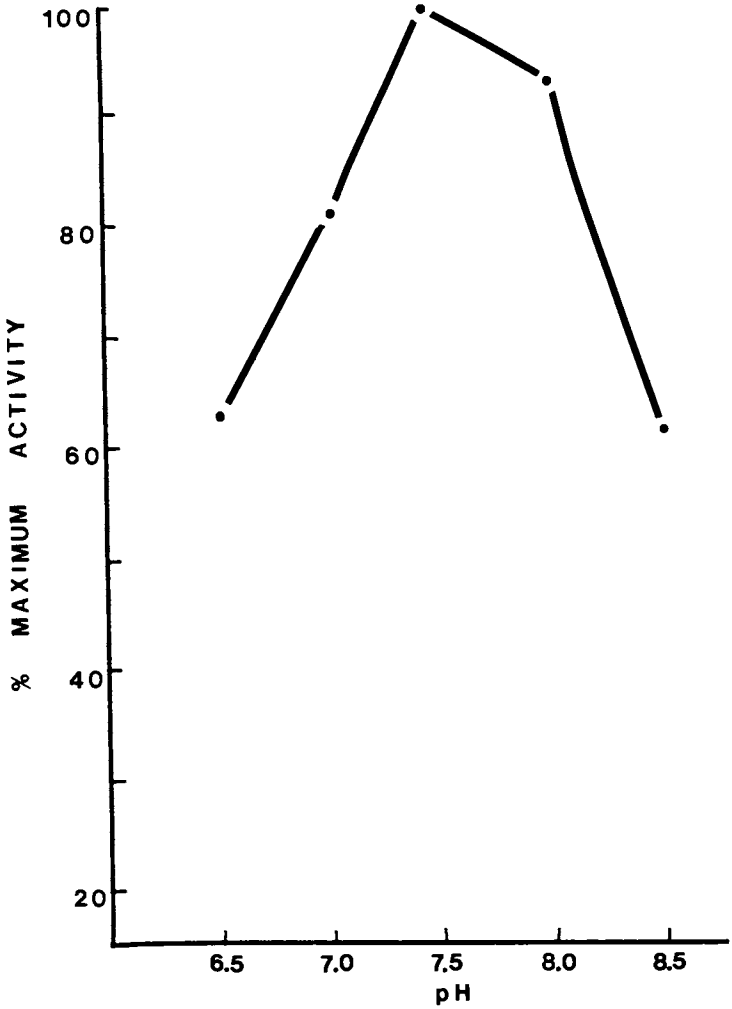

Figure 1. Effect of pH on aldrin epoxidation by midge homogenate (mean of 2 experiments)

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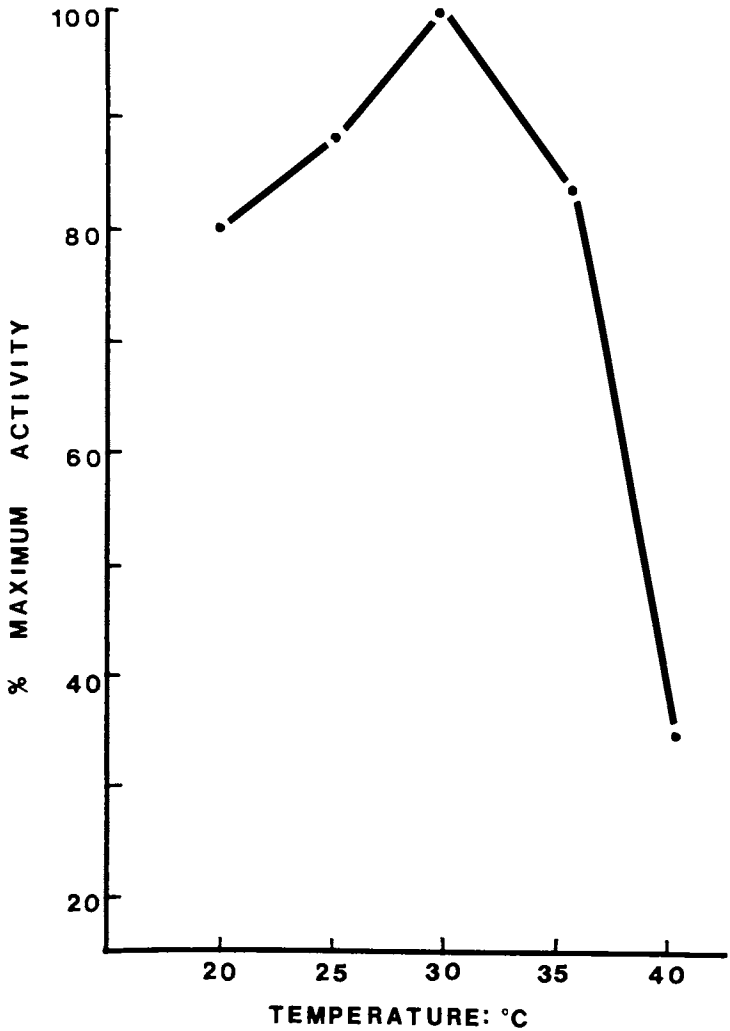

Figure 2. Effect of temperature on aldrin epoxidation by midge homogenate (mean of 2 experiments)

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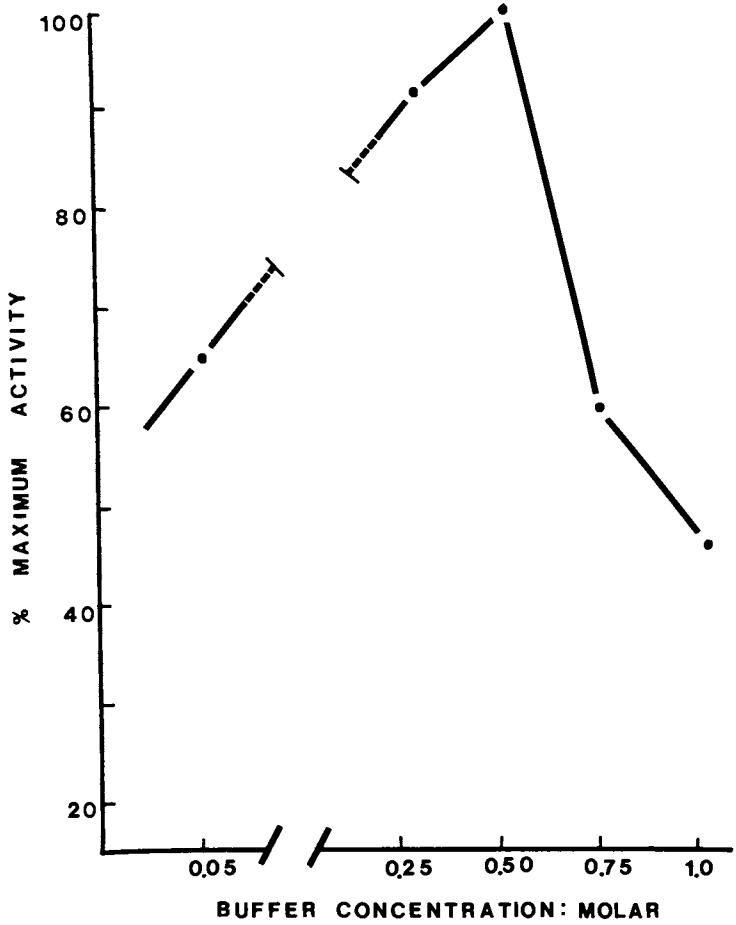

Figure 3. Effect of buffer concentration on aldrin epoxidation by midge homogenate (mean of 2 experiments)

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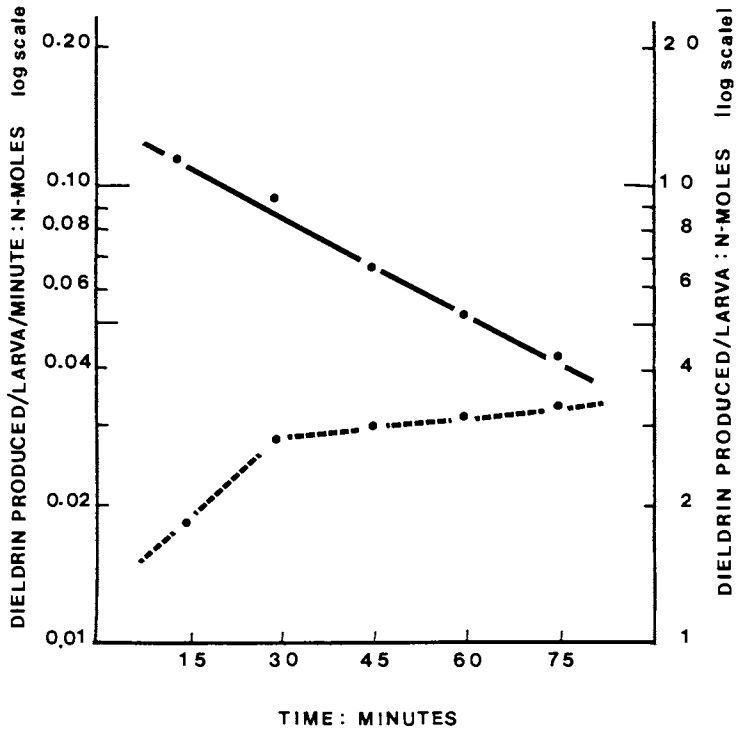

Figure 4. Effect of incubation time on aldrin epoxidation by midge homogenate (mean of 2 experiments). Rate analysis: (—— and left ordinate), nmol/larva/min and (– – – and right ordinate), cumulative aldrin epoxidized, nmol/larva.

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The *in vitro* aldrin epoxidase activity was linearly correlated with homogenate concentration (enzyme concentration) over a range of 1 to 5 larvae/ml (Figure 5).

Dieldrin production increased from 0.39 to 1.91 nmoles/insect when the substrate concentration was increased from 0.01 mg to 1.0 mg in 5 ml of incubation mixture (Table VII).

Table VII. The effect of aldrin concentration and BSA upon aldrin epoxidase of *C. riparius* whole body homogenates[a]

mg aldrin/5 ml	% BSA	nmoles dieldrin/midge
1.0	1.0	1.91
1.0	0.1	2.12
0.1	1.0	1.20
0.01	1.0	0.39

[a]Optimum conditions of Table V. Mean values from 2 experiments.

Assay of Subcellular Fractions for Aldrin Epoxidation. There was considerable epoxidase activity in the mitochondrial fraction but the highest activity was in the washed microsome fraction. Microsomes washed in KCl were more active than unwashed microsomes. No epoxidase activity was detected in the post-microsomal supernatant, or when PBO was added to microsomes (Table VIII).

Discussion

Allowing for differences due to species, assay techniques and the subjective nature of assessing midge mortality, the susceptibility of *C. riparius* to insecticides (Table I) is generally similar to other chironomid species. *C. riparius* and *C. tentans* (5) exhibit similar susceptibility to two organochlorines, dieldrin and DDT. Likewise, the LC_{50}'s of several organophosphates to *C. riparius*, *Tanypus grodhausi* (4) and *C. tentans* (5) are in the same range, although LC_{50} values higher than 10 ug/L have been reported for other organophosphates and *Chironomus sp.* 51, *Goeldichironomus holoprassinus* and *Chironomus*

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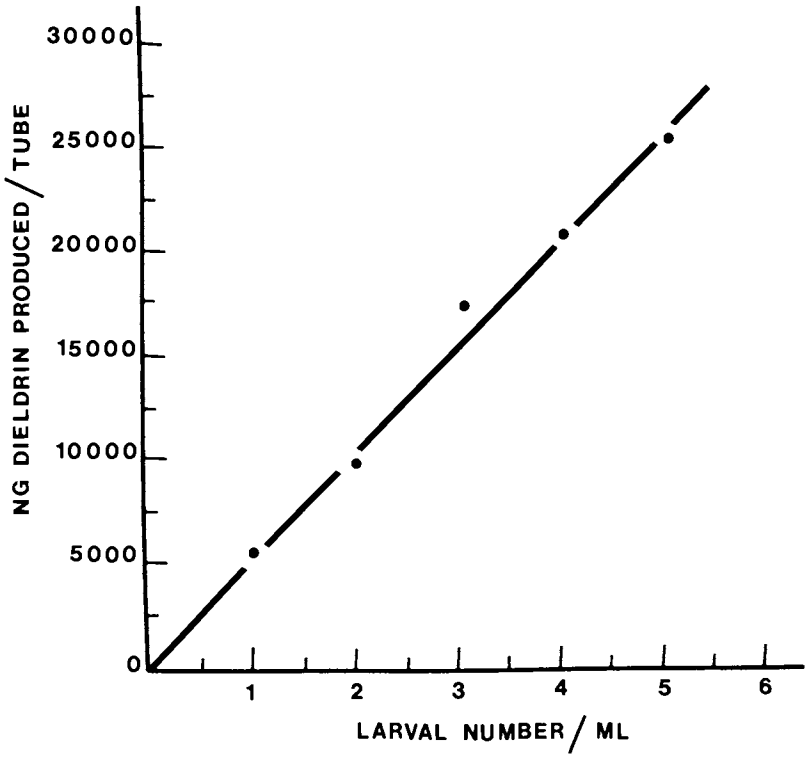

Figure 5. Effect of enzyme concentration (larvae/mL) on aldrin epoxidation by midge homogenate (mean of 2 experiments)

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Table VIII. Subcellular localization of aldrin epoxidase activity in C. riparius larvae[a]

Fraction	pmoles dieldrin/mg protein/min (pmoles dieldrin/larval equiv./min)
Homogenate	236 (86.7)
Mitochondrial	757 (6.6)
Microsomal	798 (19.4)
Microsomal (washed)	1,303 (23.8)
Microsomal+PBO	N.D.[b]
Post-microsomal Supernatant	N.D.[b]

[a]Optimum conditions of Table V. Mean values of 2 experiments.

[b]Not Detected.

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sp. (4, 15). In a comparison of specific insecticides, the LC_{50} values for parathion (4), malathion (4, 5) and allethrin (5) among several midge species closely correspond to the respective LC_{50} values for C. riparius. However, carbamates appear to be approximately 10X more toxic to C. tentans (5) than to C. riparius.

The insecticide-PBO assays provide evidence that C. riparius larvae have an active MFO system: 1) PBO, a selective inhibitor of MFO, antagonized aldrin, two phosphorothioates (parathion and malathion) and two carbamates (mexacarbate and aminocarb) which are metabolized by MFO,to products that are more toxic or more potent acetylcholinesterase inhibitors than the parent compound (16, 17, 18, 19, 20) and 2) allethrin was strongly synergized by PBO, which inhibits allethrin detoxication (21). A high allethrin-PBO ratio with C. riparius contrasts with C. tentans (5) and other insects (21), indicating a significant metabolic difference with respect to allethrin in two related midge species. None of the carbamates were synergized very much by PBO including carbaryl, a carbamate used to assay for MFO in joint action studies (22). C. tentans also exhibited a low PBO-carbaryl ratio (5). Paraoxon is not apparently metabolized very much by MFO, in contrast to malaoxon. Indirect evidence for an active MFO system in midges was provided by finding dieldrin in larvae exposed to aldrin (23).

Aminocarb and mexacarbate, both antagonized by PBO in midges, were demethylated at the phenyl-N-dimethyl group by rat liver MFO, forming more potent acetylcholinesterase inhibitors than the parent compounds (20). Landrin^{R}, with no phenyl-N-dimethyl group but otherwise similar to aminocarb and mexacarbate, was unaffected by PBO in midges. Although no metabolites were identified in the present study, the midge toxicity data and enzyme inhibition studies (20) suggest that demethylation of phenyl-N-dimethyl groups of carbamates occurs in midges as an activation reaction.

Midges exposed to aldrin or dieldrin (20 ug/L,2 hr) contained (body weight basis) 95X the aqueous concentration. Furthermore, midges lost less than 0.1% of absorbed dieldrin per hr in a 6 hr depuration experiment. Therefore, C. riparius rapidly concentrate dieldrin from the water and retain most of the absorbed dose. Midges exposed to 0.02 ug/L aldrin or 0.05 ug/L DDT accumulated them by factors (dry weight basis) of > 12,000 (aldrin) and 7,800 (DDT) in 24 hr (23). In a longer exposure with a different midge, body accumulation of DDE increased for 30 days and had not completely equilibrated at termination (24). Thus, rapid penetration plus bioaccumulation may contribute to the high toxicity of insecticides to midges, at least for the organochlorines.

Midges converted 58% of absorbed aldrin to dieldrin but midge epoxidase was completely inhibited *in vitro* and *in vivo* by PBO, demonstrating that PBO is a potent inhibitor of midge MFO

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and supporting the earlier discussion of MFO in joint action studies. Under our conditions, no *in vivo* conversion of dieldrin was detected and dieldrin was the only detectable metabolite of aldrin *in vivo* and *in vitro*. In another study, midges converted less than 25% of aldrin to dieldrin and dieldrin also was the only product detected (23).

An optimum pH of 7.5-8.0 for midges is similar to the slightly alkaline optima for aldrin epoxidase of other insects (11, 25, 26, 27, 28) including caddisflies, an aquatic species (29). An optimum temperature of 30°C for midge epoxidase is also near that of other insects (1, 11, 26, 30), although considerable midge epoxidase activity, 80-89% of maximum, was obtained at 20-25°C. Caddisfly aldrin epoxidase was near maximum at 20-25°C (29) and mosquito larvae homogenates oxidatively metabolized more propoxur at 25°C than at 30°C (31). Wilkinson and Brattsten (1) speculated that aquatic insects, living at lower ambient temperatures, may have lower optimum temperatures for MFO than terrestrial insects and midge data do not strongly refute that notion. The temperature profile of midge epoxidase is not much different than aldrin epoxidase of caddisfly fat body (29).

Maximum epoxidase activity was obtained with 5 X 10^{-1} M Tris buffer. KCl enhanced activity only at suboptimal buffer concentrations, probably due to ionic strength effects (1). NADH, FMN and FAD had little or no effect on midge epoxidase.

In vitro dieldrin production increased proportionally to larval concentration (enzyme concentration) up to 5 larvae/ml and each 10X increase in substrate (aldrin) concentration tripled and doubled, respectively, dieldrin formation. An approximation of 2 X 10^{-5} M aldrin as the Km value for midge epoxidase was obtained from a double reciprocal plot of data in Table VII, which closely corresponds to values for aldrin epoxidase in the house fly (28) and the southern armyworm (11).

Dieldrin accumulated in proportion to incubation time during the first 30 min and declined thereafter, like the biphasic curves for aldrin epoxidation in other insects (11, 26). The rate curve declined continuously to 50% of maximum after 60 min of incubation at 30°C. BSA did not increase epoxidase activity in 15 min incubations (Table V) or 60 min incubations (Table IV). Consequently, reduced epoxidase activity is probably not due to endogenous proteases in the homogenate (1).

The higher activity of washed microsomes is probably due to the removal of inactive protein and/or endogenous MFO inhibitors. Based on results with whole body homogenates, BSA was not used in subcellular studies. The light pink color of unwashed microsomes is undoubtedly due to midge hemoglobin. Hemoglobin, porphyrins and heme compounds may bind to mammalian microsomes (32, 33, 34) and such binding may inhibit MFO activity (34). Besides the possible inhibition by hemoglobin, we have no evidence of any endogenous inhibitors in midge homogenates.

The specific activity (protein basis) of aldrin epoxidase

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in midge microsomes is high among other insects or insect tissues (11, 25, 26, 27, 28, 29, 35-43) but optimum conditions were not established in all of those studies. The epoxidase activity of midge body homogenates is much greater than tissue preparations of another aquatic insect (29), providing evidence for extremes among aquatic insects in this regard. The relatively high levels of MFO activity in midges may be an evolutionary consequence of the generally polluted conditions of their natural habitat.

Electron microscopic examination of midge microsomes prepared by a slightly different procedure than Table VIII revealed a homogeneous mixture of vesicles derived from rough and smooth endoplasmic reticulum, ribosomes and a few mitochondria. Midge preparations are similar in composition to microsomal fractions of southern armyworm fat body and gut (44).

Summary

1) Chironomus riparius larvae were susceptible to insecticides in ug/L concentrations.
2) Results of joint action experiments with PBO were typical of an insect with an active MFO system.
3) Midge larvae rapidly accumulate aldrin or dieldrin and readily epoxidize aldrin to dieldrin without further conversion.
4) Within expected variation, the optimum conditions for in vitro epoxidase activity of midges are typical of other insects. Maximum activity was obtained with 1 mg aldrin in 5 ml homogenate, an electron generator system with NADP, pH 7.5 buffer of 5 X 10^{-1} M and incubation for 15 min at 30°C.
5) Midge aldrin epoxidase is highly active and may be completely inhibited in vivo or in vitro by PBO.
6) The ease of preparation, minimal endogenous inhibitor problems plus points 2, 4 and 5 above suggest that midge preparations may be a convenient, effective tool for insect MFO studies.

Acknowledgement

Dr. N. W. Britt and Mr. R. Stoffer identified the midge species in this research.

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22

Degradation of Pesticides by Algae and Aquatic Microorganisms

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Microorganisms are considered to be one of the most significant factors in the degradation and eventual elimination of pesticides and other xenobiotics in the environment. Such a conclusion has been well supported by many documents published during the last two decades. It is also well known that microorganisms metabolize xenobiotics mainly by oxidative, hydrolytic, reductive, and isomerization processes (1).

Despite the wealth of information on the roles of bacteria and fungi in degrading pesticidal chemicals, we don't have any knowledge on the role of aquatic algae for the same purpose. Considering the importance of algae in aquatic systems, such a neglect seems unjust. The most likely reason for the lack of interest is that algae themselves, when they are tested alone, do not show appreciable degradation capabilities. It must be stressed here that the functions of algae cannot be studied without due regards to their association with sunlight, since by definition no algae should live where sunlight does not reach.

The role of aquatic microorganisms in affecting photochemical reactions has not been carefully studied in the past probably because of the lack of knowledge that such reactions do take place. Yet algae constitute the bulk of biomass in many aquatic systems. They are known to collect pesticidal chemicals because of their large surface areas.

Many organic chemicals such as chlorophyll, benzophenone, rotenone, aromatic amines and FMN are known to sensitize and enhance photochemical reactions of xenobiotics (2, 3, 4, 5). For example, diethylamine sensitizes the photodegradation of DDT to yield DDE, TDE, dichlorobenzophenone, and two other unidentified compounds (6). These reactions are thought to involve a charge transfer from the amine to DDT. Similarly, rotenone is highly effective in enhancing the photochemical alteration of dieldrin to photodieldrin (7). Rotenone also catalyzes the photochemical alteration of aldrin, isodrin, endrin, heptachlor, and heptachlor epoxide but it is less effective or ineffective with DDT, DDE, lindane, and endosulfan (7, 8).

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The purpose of this paper is to study the role of algae in altering pesticidal chemicals in aquatic environments with special reference to their influence on photochemical degradation activities.

Experimental

Microorganisms. A strain of Pseudomonas putida (ATCC, 17484 Med.-3 colony type) was cultured in 250-ml flasks containing 100 ml of the modified Fred-Waksman (9) medium. Flasks were inoculated from the stock culture and kept at room temperature for 3 days with continuous shaking. The bacterial cells were harvested by centrifugation at 6,000 g for 10 minutes.

Two algal species, Anacystis nidulans (TX-20 strain) and Microcystis aeruginosa (IND 1036) were maintained in the laboratory. The bacteria free culture of the TX-20 was supplied by Dr. John Myers (Lab. of Algal Physiology, University of Texas, Austin, Texas 78712) and was cultured under an aseptic conditions by the method described by Batterton et al. (10). The IND 1036 was provided by Dr. G. C. Gerloff (Dept. Botany - Mineral Nutrition Laboratory, University of Wisconsin, Madison, Wisconsin 53706) and cultured on Gorham's medium. Algal cells were harvested after 2-3 weeks of rearing by centrifugation at 6,000 g for 10 minutes.

Cell-Free Suspension. Cells were washed twice in phosphate buffer (pH 7.2, 0.02 M) by repeating the suspension and centrifugation processes at 6,000 g for 10 min. The washed cells were suspended in the standard phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 6.0, 0.02M) at the rate of 65 mg fresh weight per milliliter buffer. To break cell walls, a lysozyme preparation (Sigma Chem., St. Louis, MO) was added to the cell suspension at the rate of 2 mg enzyme per ml suspension. The mixture was incubated for 3 hrs. at 37°C with continuous shaking. The lysozyme-treated suspension was sonicated at 250W for 3 minutes using Braunsonic 1510 (B. Braun, Melsungen AG). The resulting cell-free suspension was subjected to protease treatment. This was accomplished by incubating the suspension with protease (2 mg/ml) for 4 hrs at 37°C with continuous shaking.

Partial Purification. The cell-free suspension, before and after protease treatment, was subjected to gel-filtration chromatography on Sephadex G-75 according to the procedure described in our earlier report (11). Peak II of the sephadex column eluate will be referred to as flavoprotein preparation (Fig. 1).

Incubation Conditions. The incubation mixtures consisted of the cell preparation, with or without a flavin cofactor (FMN, Flavin mononucleotide; 100 µg) and 10 µl 95% ethyl alcohol containing the insecticide substrate in 2.5 ml (or 5 ml in the experiments shown in Tables II and III, Figs. 3, 4 and 5) standard

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phosphate buffer. Anaerobic incubation was carried out in Thunberg tubes while the aerobic ones in the regular 20 ml culture tubes. These tubes were incubated in metabolic shaker for 2 hrs at 37°C under normal laboratory light (80-120 foot candles) or in dark. In certain experiments (Tables II and III; Figs. 3, 4 and 5) tubes were placed immediately in front of a fluorescent lamp (Westinghouse, F20T12/CW, 20W approx. 3 to 5 cm from the light source) for seven to nine days at room temperature in order to study the photodegradation of certain insecticides by algal cell-free preparation.

Insecticidal substrates used in these studies were: ^{14}C-mexacarbate [(4-dimethyl-3,5-^{14}C-3,5-xylyl)N-methylcarbamate] with specific activity of 8.62 mCi/mM (lot no. 5169-52-47, Dow Chemical Co.); (ethyl-1-^{14}C-)parathion with specific activity 19 mCi/mM (Amersham); ^{36}Cl-toxaphene (0.5005 gm/0.026 mCi, Lot no. X1790l-80-1, Hercules, Inc.; DDT [(2,2-bis(p-chlorophenyl)1,1,1-trichloroethane]; dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-1,4-exo-5,7-dimethanonaphthalene); lindane (1a,2a,3β,4a,5a,6β)-1,2,3,4,5,6-hexachlorocyclohexane).

Analytical Procedures. Incubation mixtures were extracted with diethyl ether except in the case of toxaphene where a mixture of chloroform-methanol (5:1, v/v) was used instead. Ether extracts of DDT, dieldrin, and lindane were dried over anhydrous sodium sulfate, evaporated using a gentle stream of nitrogen, and the residues were redissolved in n-hexane. Aliquots of the hexane solutions were directly injected into a gas liquid chromatograph (GLC, Varian, model 2400) equipped with an electron capture (EC) detector (Aerograph Sc ^{3}H detector) and a 1.5% OV-101 on chrom GHP 100/120, 5' x 1/8" stainless steel column. The detector temperature was 245°C, injection port 235°C, and the oven temperature was 125°C for lindane, 180°C for DDT and 200°C for dieldrin. Carrier gas was nitrogen at the flow rate of 40 ml/min.

Both the organic and water layers from the radioactive insecticides were counted in a liquid scintillation counter (Isocap/300, Searle). The results are expressed as nanomoles or micrograms equivalent of the substrate per mg protein.

Protein was estimated by the method of Lowry et al. (12), using fresh bovine serum albumin as a standard.

Excitation and emission spectra for the flavoprotein preparation was obtained in 100 mM acetic acid using an Aminco Bowman Spectrofluorometer.

Results

Flavoprotein Preparations. During the studies on algal degradation of mexacarbate it was accidentally discovered that the degradation activities were actually higher when algal cells, denatured by boiling, were substituted for alive algal cells, in

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the presence of light. Since no degradation activities were observed in the dark, this finding suggests that the degradation activities of the algae, TX-20 in this case, are largely due to photochemical reactions, and that the factor(s) promoting such reactions must be heat stable.

A purification scheme was devised to isolate and identify the factor for promoting photochemical reactions, by using ^{14}C-mexacarbate as the substrate (Table I). It must be noted that the factor obtained here is only partially purified. Also in some cases it requires the presence of FMN to fully express its stimulatory potency for this substrate.

To study the effect of the protease treatment cell-free suspension, with or without protease treatment, was subjected to gel-filtration chromatography on Sephadex G-75 and the elution patterns were compared (Fig. 1). In each case, two major peaks were detected by monitoring column fractions with absorbance at 280 nm. Degradation activities on mexacarbate, in the presence of FMN and light under anaerobic condition, were measured for each fraction. It was found that the highest activity was associated with peak II. It is interesting to note that protein(s) associated with peak II were detected with or without protease treatment; these will be referred to as natural flavoprotein (B, Fig. 1) and protease-liberated flavoprotein (A, Fig. 1). The molecular weight of the protein(s) associated with peak II is about 6,000 to 10,000 using standard proteins and Andraws' procedure (13).

Table I. Degradation activity on ^{14}C-mexacarbate using live or dead algal cells and partially purified fractions of TX-20[a/]

Biological Material Used		Amount of water-soluble products nanomoles per mg protein per two hrs, formed in the following incubation mixtures:	
		Control	Control + FMN
(A)	Living algal cells	0.3	0.7
(B)	Boiled algal cells	0.4	1.6
(C)	B + Lysozyme	0.8	1.6
(D)	C + Sonication	0.7	1.6
(E)	D + Protease	0.5	3.6
(F)	Peak II, Sephadex Column	1.3	24.0

[a/] Incubation was carried out aerobically in the presence of light

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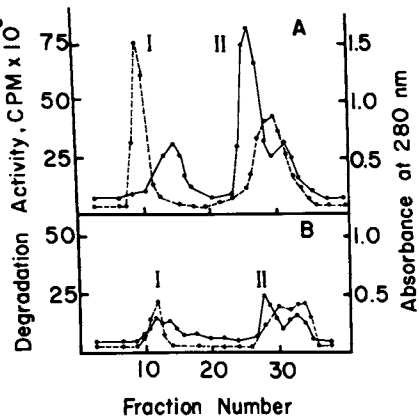

Figure 1. Column gel filtration chromatography using Sephadex G-75 for the TX-20 cell-free preparation (A), after and (B), before protease treatment. (– – –), degradation activities on mexacarbate; (——), protein readings as measured at 280 nm.

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Emission and excitation spectra were obtained for both natural and protease-liberated flavoprotein materials in 100 mM acetic acid. The λmax for excitation was 330 for the natural and 410 nm for the protease-liberated flavoprotein while that for emission were 420 and 480 nm, respectively. These spectroscopic data show that these are flavoproteins and that the protein composition of natural and protease-liberated flavoprotein are somehow different.

Substrate Spectra. Photodecomposition of five insecticidal chemicals stimulated by protease-liberated flavoprotein was studied and results are shown in Tables II and III and Figures 3, 4 and 5. Generally the flavoprotein(s) was significantly more active in stimulating the photodegradation process in the absence than in the presence of the flavin cofactor (FMN).

With respect to ^{14}C-parathion and ^{36}Cl-toxaphene, protease-liberated flavoprotein was significantly more active than phosphate buffer in photodegrading these chemicals to water-soluble products (Tables II and III). The amount of ^{14}C-water-soluble products formed from parathion was 5-7 times greater in the presence than in the absence of flavoprotein. It should be noted that the presence of FMN in the mixture caused a slight change in amount of water-soluble products formed (Table II). ^{36}Cl-toxaphene was photodegraded by TX-20 in a similar fashion as in the case of ^{14}C-parathion (Table III).

Table II. Photodegradation[a] of ^{14}C-parathion[b] by protease-liberated peak II from TX-20.

Incubation Mixtures	Distribution of radioactivity, nanomoles per mg protein, in: Water Layer	Ether Layer	% Recovery
TX-20 Peak II			
Control	3.6	0.4	102
Control + FMN	2.4	0.9	82
Phosphate Buffer			
Control	0.5	2.7	82
Control + FMN	0.5	2.8	84

[a] Exposure to a fluorescence light source; Westinghouse, 20W F20T12/CW, 800 foot candles for 9 days.

[b] 10 nanomoles ^{14}C-parathion were added to each tube.

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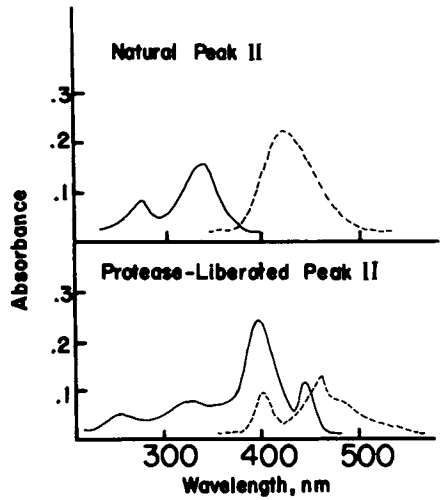

Figure 2. Emission (– – –) and excitation (——) spectra for TX-20 natural and protease-treated flavoprotein (peak II).

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Table III. Photodegradation[a] of ^{36}Cl-toxaphene[b] by protease-liberated flavoprotein (peak II) from TX-20.

Incubation Mixtures	Distribution of Radioactivity microgram per mg protein in Water Layer	Ether Layer	% Recovery
TX-20 Peak II			
Control	3.4	33.4	92
Control + FMN	3.9	30.5	86
Phosphate Buffer			
Control	1.4	37.5	97
Control + FMN	2.1	35.5	94

[a] Exposure to a fluorescence light source; Westinghouse 20W F20T12/CW, 800 foot candles for 7 days.

[b] 100 micrograms ^{36}Cl-toxaphene were added to each tube.

Organochlorine insecticides were degraded more efficiently with the flavoprotein alone than in the presence of added FMN. For example, DDT was degraded by light to three products; peaks 1, 2 and 3 in Fig. 3. Peaks 2 and 3 are DDE and TDE,respectively. It is clear from Figure 3 that TX-20 was by far the most active in stimulating DDT photolysis to yield TDE as a major product. Addition of FMN to TX-20 results in inhibition of the photodegradation processes judging by the amount of original DDT recovered in ether extract and detected by GLC (B, Fig. 3). DDT in the phosphate buffer alone with or without FMN, (i.e. in the absence of added flavoproteins) seems to be rather stable. However, small amounts of peaks 1, 2 and 3 were detected under these conditions (C and D, Fig. 3).

Protease-liberated flavoprotein from TX-20 algae was relatively less active in stimulating lindane as compared to the case with DDT. As illustrated in Figure 4, one major and two minor degradation products with short retention times were formed by the flavoprotein, whereas one major product was formed when FMN was added to the incubation mixture. When lindane was incubated with buffer or buffer + FMN no degradation products were detected by GLC analysis. The major degradation product formed by TX-20 had the same retention time as γ-BTC in two GLC systems (2.3 min, A in Fig. 4 and 3 min on QF-1 at 130°C).

Photodegradation of dieldrin by the flavoprotein of TX-20 alone resulted in the formation of photodieldrin as the major

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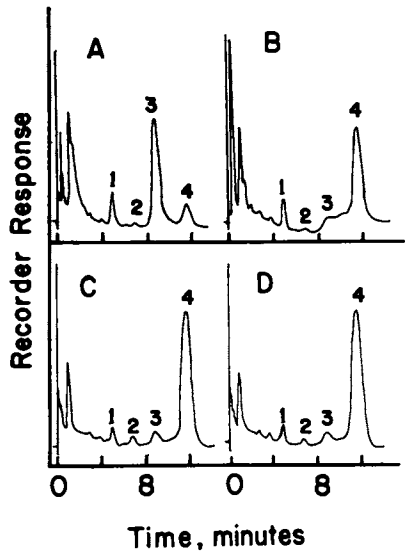

Figure 3. Typical GLC of DDT photodegradation by protease-liberated flavoprotein from TX-20 algae: (A), TX-20; (B), TX-20 + FMN; (C), phosphate buffer; (D), phosphate buffer + FMN; (1), unknown; (2), DDE; (3), TDE; (4), DDT.

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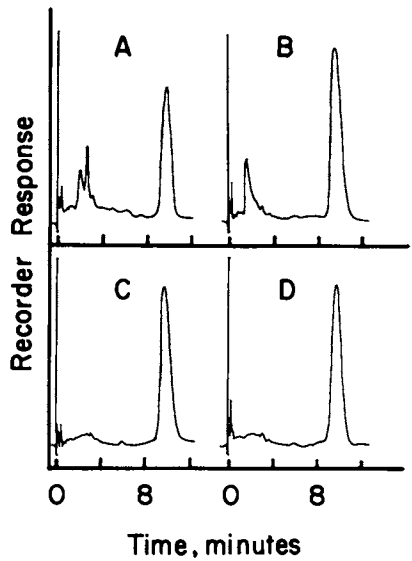

Figure 4. Typical GLC of lindane photodegradation by protease-liberated flavoprotein from TX-20 algae: (A), TX-20; (B), TX-20 + FMN; (C), phosphate buffer; (D), phosphate buffer + FMN; R_t for γ-BTC = 2.3 min and γ-BHC = 9.7 min.

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product (peak 2, Fig. 5). Addition of FMN to the mixture did not affect the photolysis of dieldrin with respect to the relative amount of photodieldrin produced (A and B, Fig. 5). When dieldrin was illuminated in the standard phosphate buffer, a relatively small amount of photodieldrin was detected by GLC. Also, addition of FMN to the incubation mixture resulted in a slight increase in the dieldring photolysis to photodieldrin (C and D, Fig. 5). At least two to three minor peaks were detected in all incubation mixtures tested; the identity of these minor products are unknown.

Comparative Studies on Reductive Metabolism by Flavoproteins from Various Biological Sources. To study the factors affecting degradation by flavoprotein (peak II) of TX-20 ^{14}C-mexacarbate was incubated under various conditions. ^{14}C-mexacarbate was found to be degraded to water-soluble products more efficiently under anaerobic than aerobic conditions. As shown before, the presence of a flavin cofactor, such as FMN, was required to elicit the aerobic or anaerobic stimulation of mexacarbate degradation. In the absence of FMN, mexacarbate was practically stable under the incubation conditions. Regardless of the presence or absence of oxygen, mexacarbate degradation was stimulated by the presence of light (ca. 20 foot candles). Using the standard phosphate buffer and in the presence of FMN, mexacarbate degradation was much less extensive than in the presence of peak II flavoprotein(s) (Table IV).

Table IV. Effects of various incubation conditions on the degradation activity of flavoprotein preparations from TX-20 on mexacarbate[a/b/].

Flavoprotein Preparation	Amount of water-soluble products, nanomoles per mg protein per two hours, formed under:			
	Light		Dark	
	Anaerobic	Aerobic	Anaerobic	Aerobic
Natural				
Control	0.07	0.02	0.13	0.04
Control + FMN	6.33	2.58	4.40	1.94
Protease-Liberated				
Control	0.08	0.03	0.02	0.01
Control + FMN	2.68	1.48	2.06	1.38
Blank (Buffer + FMN)	0.56	0.22	0.25	0.19

a/ Ten nanomoles of ^{14}C-mexacarbate were added to each incubation.

b/ Average of two experiments carried out in duplicate.

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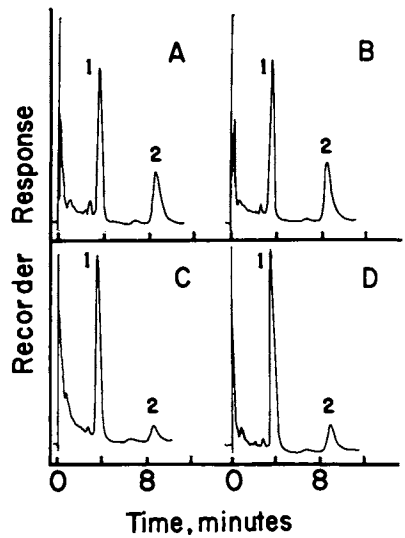

Figure 5. Typical GLC of dieldrin photodegradation by protease-liberated flavoprotein from TX-20 algae: (A), TX-20; (B), TX-20 + FMN; (C), phosphate buffer; (D), phosphate buffer + FMN; (1), dieldrin; (2), photodieldrin.

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A comparison of the degradation activities of flavoproteins (Sephadex peak II's) from various sources were then made (Table V). The flavoproteins from three microorganisms, two algal species and a bacterium, and the rat intestine and liver showed varying degrees of stimulating activities on mexacarbate degradation. The highest specific activity was associated with the natural flavoprotein of TX-20, while the lowest was that of Pseudomonas. Natural flavoprotein from the two algal species and that from the rat intestine showed comparable activities to each other. When mexacarbate degradation was calculated per fluorescence unit (Table V), natural and protease-liberated flavoproteins from TX-20 were found to be the most active protein(s). This was followed by those from the alga, Microcystis and the rat intestine; while that from Pseudomonas again showed the lowest activity.

Table V. Anaerobic degradation[a/] of ^{14}C-mexacarbate by flavoprotein (peak II) from rat and microorganisms.

Flavoprotein Source	Specific Activity nanomoles degradation products per mg protein per two hours	Specific activity per fluorescence unit[b/]
Rat		
Intestine	5.4	0.46
Liver	1.9	----
Anacystis nidulans (TX-20)		
Natural	6.33	1.51
Protease-liberated	2.68	1.27
Microcystis aeroginosa (IND 1036)		
Natural	5.0	0.68
Protease-liberated	1.3	0.23
Pseudomonas putida		
Protease-liberated	0.8	0.08

a/ Incubation was carried out in Thunberg tubes anaerobically in the presence of FMN and in dark.

b/ An arbitrary unit where the height of λmax of the emission spectra (Fig. 2) was used as the fluorescence unit.

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Discussion

Photodegradation of DDT by the protease-liberated flavoprotein from TX-20 resulted in the formation of TDE as the major product in addition to three other minor compounds. It has been well established that DDT conversion to TDE, anaerobically, is a reductive process involving replacement of a chlorine atom by hydrogen. On the other hand, it has been suggested that photolytic reactions involve a charge transfer from an amine to DDT and a subsequent pickup of a proton. Thus there is a possibility that the photochemical reaction involving flavoproteins undergoes a similar reaction scheme. Much more data are, however, needed to confirm this point.

With respect to dieldrin, photodieldrin was the major product formed. This photolytic conversion is the result of intramolecular rearrangement and has been reported by several researchers (3, 5, 14). Photodieldrin has also been found in nature. For instance, Robinson et al. (5) and Korte (15) found photodieldrin on the surface of plant leaves. Lichtenstein et al. (16) found photodieldrin in soil samples which had been treated previously with a large amount of aldrin in the field. In addition, dieldrin is converted to photodieldrin by microorganisms (17). Algal cultures, Dunaliella sp. and Agmenellum quadraplicatum, have been shown to convert dieldrin to photodieldrin (14). In view of the potency of the flavoprotein in promoting the photochemical reactions, it would not be surprising if such reactions commonly occur throughout these biological materials in addition to the one promoted by chlorophyll.

Among the organochlorine insecticides studied, lindane was the least affected chemical. This somewhat surprising, since it has been known that lindane is susceptible to dechlorination as well as dehydrochlorination just like DDT. Such differences in substrate susceptibility to this flavoprotein-stimulated photodegradation process indicate some degree of specificity and point to the need for future studies.

Degradation of DDT, dieldrin and lindane by the flavoprotein preparation was almost more efficient in the absence than in the presence of FMN (e.g. Figure 3). On the contrary, photodegradation of mexacarbate was greatly enhanced by FMN and other flavin cofactors. It is well known that flavin cofactors, such as FMN, are active photosensitizers. Hence it is possible that the mechanisms or pathways involved for the photodegradation of DDT, dieldrin and lindane and that for mexacarbate are different.

As was the case with dieldrin, photodecomposition of ^{14}C-parathion and ^{36}Cl-toxaphene was relatively extensive, and the addition of FMN has almost no effect on the enhancing or decreasing the rate of degradation to water-soluble products. Since the organic-soluble products were not analyzed in the case of parathion and toxaphene, it is difficult to speculate on the type of degradation products formed and the pathways involved on

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these chemicals. However, parathion was labeled at the 1-C of the ethyl group, and hence some of the water-soluble products could be the result of de-ethylation reactions. As for the ^{36}Cl-toxaphene, the radioactivity in the water layer could be largely due to dechlorination and dehydrochlorination.

As we reported recently (18), ^{14}C-mexacarbate was used in the processes involved in characterization, isolation, and partial purification of a flavoprotein system from the rat intestine and liver. Indeed an identical procedure was used in the present study where it was found that the flavoprotein preparation (peak II) was present in algae before and after digestion with protease. These natural and protease-liberated peak II flavoproteins were active in the reductive degradation of mexacarbate to N-desmethyl-mexacarbate and water-soluble products under anaerobic conditions. We were also able to isolate the flavoprotein system from a bacterium, Pseudomonas putida, which shows the same degradation activity on mexacarbate. It should be emphasized that this algal flavoprotein system was active in degrading mexacarbate under all conditions tested: i.e. aerobic and anaerobic conditions and in the presence or absence of light. However, the light did greatly enhance mexacarbate degradation. Thus one must conclude that the flavoproteins act as photosensitizers and agents to promote reductive degradation which occurs only under anaerobic conditions. Such reductive degradation also have been observed to occur with DDT (11) and to a lesser extent with toxaphene, but not with parathion, lindane and dieldrin (Esaac and Matsumura, unpublished data). The overall results show that flavoproteins have roles in degrading xenobiotics in two distinct ways: 1) they act as photosensitizers and/or promoting agents for photochemical reactions, and 2) they act as a reductive agent in the presence of a flavin cofactor under anaerobic conditions. Since dead algae can be precipitated, it is possible to find anaerobic niches in which flavoproteins act as the reducing agent in aquatic sediment in nature. Judging by the rate of degradation and the number of susceptible compounds found so far, we conclude that the role of flavoproteins as agents to aid photochemical reactions is the more prevalent one as compared to the one involving reductive reactions in nature.

In conclusion, the flavoprotein systems isolated from the algae and the bacteria, have a good potential to play significant roles in pesticide degradation in aquatic environments. Such flavoprotein systems are active in degradation of xenobiotics both under aerobic and anaerobic conditions by promoting photochemical and reductive reactions.

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Abstract.--The nature of the factor which promotes photodecomposition of pesticidal chemicals in blue-green algae was studied. As a result of partial purification and spectroscopic studies it was identified to be small weight flavoproteins. In their presence the photodegradation of DDT, lindane, dieldrin, toxaphene, parathion and mexacarbate was greatly enhanced. The same flavoproteins were found to play a role in reductive degradation of mexacarbate, DDT and to a lesser extent toxaphene under anaerobic conditions. In the case of mexacarbate degradation, this reaction was promoted by exogenously added flavin cofactors, Thus flavoproteins were found to play two distinct roles in degrading xenobiotics: as agents promoting photochemical and reductive degradation activities. Because of the biomass, the large surface area, and the relative abundance of algae in many aquatic systems, such algae derived flavoproteins may be considered to play important roles in pesticide degradation in the aquatic environment.

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Dietary Casein Levels and Aflatoxin B_1 Metabolism in Rainbow Trout *(Salmo gairdneri)*

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The influence of nutrition on chemical carcinogenesis has been reviewed by Clayson (1). Dietary protein reportedly affects the toxicity and carcinogenicity of a variety of chemical carcinogens in animals, presumably by altering the activities of enzymes involved in their activation and/or detoxification (2, 3, 4).

Aflatoxin B_1 (AFB) is a mold metabolite which has been observed to be acutely toxic and carcinogenic to a wide variety of animals (5, 6) and has been implicated in human primary hepatic carcinoma (7, 8). Diets deficient in protein have been reported to increase the susceptibility of mammals to acute AFB toxicity and the induction of cancer (2, 9, 10, 11, 12, 13). Increased dietary proteins have increased the carcinogenic activity of AFB fed to rats (14) and trout (15). Supportive of this latter finding has been the reported direct relationship between dietary protein content and AFB-DNA adduct formation *in vivo* in rats (16, 17).

AFB has been shown to require metabolic activation to its ultimate carcinogenic species (18, 19, 20, 21, 22) which is believed to be a 2,3-epoxide form of AFB (OAFB) (19, 23-29). This epoxidation of AFB has been associated with aldrin epoxidase (AE) activity in trout (30). As with other epoxide carcinogens, OAFB may be a substrate for epoxide metabolizing enzyme systems such as epoxide hydrase (EH) (EC4.2.1.63) and glutathione-S-epoxide transferase (GTr) (EC4.4.1.7) found in mammals and fish (31, 32, 33, 34). AFB also undergoes a variety of other reactions, generally to less toxic metabolites depending on the species of animal involved (35, 36). The primary AFB metabolite in rainbow trout has been shown to be a reduced form of AFB, aflatoxicol (AFL) (24).

The present study was undertaken to determine the influence of several levels of dietary casein upon the activities of trout hepatic enzyme systems which may be involved in the *in vitro* activation and detoxification of AFB. In addition, the effect of

[1]Technical Paper No. 4883, Oregon Agricultural Experiment Station.

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dietary casein upon the conversion of AFB to an active mutagen by trout hepatic enzymes is described.

Experimental Procedures

Diets. Semipurified diets, as shown in Table I, were prepared as described by Sinnhuber et al. (37). Casein at levels of 32, 42, 52 and 62% (dry weight) provided the protein source along with an 8% gelatin binder. All diets were isocaloric and each test group received the same amount of feed each day.

Fish. One year old Mt. Shasta strain rainbow trout (Salmo gairdneri) weighing an average of 140g were used for the study and housed in circular tanks supplied with 15.2 l of well water/min. at 11-12°C. Fish were fed twice daily for seven months prior to sacrifice at which time they weighed an average of 800g.

Hepatic Enzyme Preparations. Fish were killed by a cranial blow between 6 and 8 A.M. Livers were immediately removed, weighed and perfused with ice cold saline (0.9%) and then homogenized with four volumes of 0.25M sucrose solution in a Potter-Elvejhem apparatus by four complete passes of the pestle. Homogenates were centrifuged for 15 minutes at 12,000xg, the supernatant recovered (PMF) and recentrifuged at 105,000xg for one hour. Fatty layers were discarded and the 105,000xg supernatant recovered. Microsomal pellets were resuspended in an original weight of either 0.25M sucrose or 0.073M potassium phosphate buffer (pH 7.6). All steps were carried out at 1°C and isolated fractions were frozen in dry ice and stored at -45°C until used (<2 days). Three livers were pooled from each diet and sampling was repeated in one week. For bacterial mutagen assays and AFL production assays six livers/diet were aseptically removed from fish and sterile solutions utilized. Protein content of each fraction was determined by the method of Lowry et al. (38).

Cytochrome and Enzyme Assays. Liver fractions were thawed at R.T. and kept on ice until used. AE activity was determined using a slightly modified method of Chan et al. (39). Basically, this involved the incubation at 25°C for 30 min. of 4mg microsomal protein in 0.25M sucrose, an NADPH generating system (40) with 2U glucose-6-phosphate dehydrogenase, 260μmoles Tris buffer (pH8.2) and 25nm aldrin (gift of Shell Oil Company) in 10μl methyl cellusolve in a total volume of 6ml. Mixtures were extracted three times with 4ml n-hexane and extracts passed through a glass column containing 20g alumina (10% deactivated), concentrated and their dieldrin content determined by electron capture GC (39).

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Table I. Diets

Ingredient	Percent			
Casein	30.8[a]	42	52	62
Gelatin	7.7	8	8	8
Dextrin	20	15	10	8
α-Cellulose	17	12	7	2
Mineral mix[b]	4	4	4	4
Carboxymethyl cellulose[c]	1	1	1	1
Choline-chloride (70%)	1	1	1	1
Vitamin mix[d]	2	2	2	2
Fish oil (salmon or herring)	15	15	15	15

[a]Plus 0.6% arginine, 0.2% L-cysteine, 0.4% methionine and 0.3% DL-tryptophan.

[b]Calcium carbonate ($CaCO_3$, 2.100%), calcium phosphate ($CaHPO_4 \cdot 2H_2O$, 73.500%), potassium phosphate (K_2HPO_4, 8.100%), potassium sulfate (K_2SO_4, 6.800%), sodium chloride (NaCl, 3.060%), sodium phosphate ($Na_2HPO_4 \cdot 6H_2O$, 2.140%), magnesium oxide (MgO, 2.500%), ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$, 0.558%), manganese carbonate ($MnCo_3$, 0.418%), cupric carbonate [$2CuCO_3Cu(OH)_2$, 0.034%], zinc carbonate ($ZnCO_3$, 0.081%), potassium iodide (KI, 0.001%), sodium fluoride (NaF, 0.002%) cobalt chloride ($CoCl_2$, 0.020%), and citric acid ($C_6H_8O_7 \cdot H_2O$, 0.686%).

[c]Hercules Powder Company, San Francisco, Calif.

[d]Thiamine hydrochloride (0.3200%), riboflavin (0.7200%), niacinamide (2.5600%), biotin (0.0080%), D calcium pantothenate (1.4400%), pyridoxine hydrochloride (0.2400%), folic acid (0.0960%), menadione (0.0800%), vitamin B_{12} (cobalamine, 3000 μg/g, 0.2667%), i-inositol (meso, 12.5000%), ascorbic acid (6.0000%), p-aminobenzoic acid (2.0000%), vitamin D_2 (500,000 USP/g, 0.0400%), vitamin A (250,000 units/g, 0.5000%), dl-a-tocopherol (250 IU/g, 13.2%), and α-cellulose (60.0293%).

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EH and GTr activities were assayed after the method of James et al. (33), which in the former case was a modification of Oesch et al. (34) method. Incubation mixtures contained 1μmole styrene-^{14}C-oxide (specific activity 47.7μCi/mmole) in 2μl tetrahydrofuran. Styrene-^{14}C-oxide was prepared from ^{14}C-styrene (purchased from California Bionuclear Corp.) after the method of Oesch et al. (34). Incubations were carried out at 25°C for 15 minutes.

Cytochrome c reductase activity and cytochrome P-450 were determined by methods outlined by Mazel (41) and the method of AFB conversion to AFL was carried out by the method of Loveland et al. (42).

Microbial Mutagen Assay. The conversion of AFB to an active mutagen for Salmonella typhimurium TA 98, a plasmid containing frameshift mutant, was accomplished using the Stott and Sinnhuber (43) modification of the Ames mutagen assay (44). Several levels of AFB standard (0.05μg to 0.15μg AFB/assay) were utilized to ensure a linear relationship between mutagenic response and AFB concentration.

Results and Discussion

As noted in Table II, the protein content of the hepatic microsomal fractions showed an increase with diets 32% through 52% casein. These observations are consistent with the findings of other workers with rats (45, 46, 47, 48, 49). The decrease noted in the protein content of the liver fractions of fish fed the 62% casein diet has also been observed by Sachan (49) in rats fed a high protein diet.

Unlike hepatic proteins, cytochrome P-450 content in isolated trout microsomes was observed to decrease 21% with increasing casein in the diet (Table II). The large standard deviations noted were a result of the averaging of two samplings data, but in each case the trend was identical. These findings are at variance with reported increases in cytochrome P-450 content of rats fed increasing levels of casein (45, 46, 47, 49, 50). Only slight differences were observed in cytochrome c reductase activities between the diets, with the highest activity of 20nmoles cytochrome c reduced/min/mg protein occurring in fish fed the 32% casein diet (Table III).

The decrease in cytochrome P-450 content correlated with a lowering of trout AE activity observed in hepatic microsomes recovered from fish fed high levels of casein, versus those from fish fed low casein diets. As shown in Table III, up to a 32% decrease in the production of the epoxide dieldrin was noted. Similar results have been observed in 10 month old rainbow trout with a nearly identical maximum decrease (unpublished data). Since AFB activation has been shown to involve a cytochrome P-450 dependent enzyme system (19, 20, 22) and trout

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Table II. Liver Weights, Protein and Cytochrome P-450 Content of Hepatic Fractions in Rainbow Trout Fed 32, 42, 52 and 62% Casein Diets[a]

Diet (% Casein)	Liver Weight[b] (% Body Weight)	Liver Fraction	Protein Content (mg/g wet wt. liver)	Cytochrome P-450(nmoles /mg protein)
32	0.89	Microsomes	34.0(±1.7)[c]	0.160(±0.000)
		Cytosol	54.8(±1.7)	
42	1.03	Microsomes	36.8(±1.1)	0.154(±0.017)
		Cytosol	53.6(±6.2)	
52	1.00	Microsomes	39.9(±3.5)	0.146(±0.025)
		Cytosol	60.2(±3.1)	
62	1.07	Microsomes	32.9(±1.3)	0.124(±0.018)
		Cytosol	59.2(±9.6)	

[a]Values with different superscript numbers are significantly different ($P<.05$, Analysis of Variance).
[b]Average of 6 livers.
[c]±Standard deviation.

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Table III. Effect Dietary Casein Levels on the *in vitro* Activities of Trout Hepatic Cytochrome c Reductase, Aldrin Epoxidase, and the Conversion of Aflatoxin B_1 to Aflatoxicol[a]

Diet (% Casein)	Cytochrome c Reductase (nmoles Cytochrome c Reduced/ min/mg Protein)	Aldrin Epoxidase[c] (pmoles Dieldrin/ mg Protein)	Aflatoxin B_1 Conversion to Aflatoxicol[c] (nmoles Aflatoxicol/nmoles Total Aflatoxin/mg Protein)
32	20.0 (± 0.0)[b]	99.6[1] (±13.2)	5.77[1] (±1.63)
42	17.9 (± 3.0)	72.4[1,2] (±13.9)	7.99[1,2] (±2.05)
52	16.8 (± 7.1)	69.8[2] (± 5.3)	9.66[2] (±1.57)
62	18.4 (± 1.4)	67.2[2] (± 9.2)	9.02[1,2] (±1.25)

[a]Values with different superscript numbers are significantly different ($P<.05$, Students t Test on n assays).
[b]±standard deviation.
[c]n=3.

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aldrin epoxidase related directly to AFB activation (30), these findings may reflect a decrease in the AFB activation potential of trout fed high casein diets. Similar effects of dietary protein on pesticide activation have been reviewed by Campbell and Hayes (45). However, total cytochrome P-450 measurements may be deceiving as a poor correlation between total cytochrome P-450 contents and conversion of AFB to a mutagen has been noted (51).

There was a significant ($p<.05$) decrease in GTr activity in fish fed increasing levels of casein, while EH activity remained unchanged except for a decrease in activity observed in fish fed a 42% casein diet (Figure 1). Trout hepatic GTr and EH activities were observed to be linear for up to 15 minutes incubation at 25°C with the use of up to 3mg cytosol and microsomal protein. The decrease in GTr activity from 171.8nmoles conjugate to 126.8nmoles conjugate/mg protein in fish fed 32% and 52% casein diets respectively may have represented a loss in the ability of fish fed the higher levels of protein to detoxify OAFB. Indeed, GTr has been implicated in the detoxification of activated AFB. Mgbodile et al. (52) have reported that AFB induced hepatic necrosis was increased over the controls in rats depleted of glutathione (GSH) by treatment with diethyl maleate, while pretreatment with cysteine, a precursor of GSH, prevented necrosis. Similarly, Allen-Hoffmann and Campbell (16) have noted that in rats fed a high casein diet and in those treated with diethyl maleate, hepatic GSH levels were depleted and *in vivo* DNA binding of administered AFB increased. Since GSH is required for GTr activity, decreased hepatic GSH may result in lowered GTr activity with the resultant drop in OAFB detoxification via this mechanism. The significance of free GSH interaction with activated AFB has been questioned as its addition to incubation mixtures failed to decrease toxicity of AFB to mutant bacteria (20, 30). The apparent lack of dietary casein effects upon *in vitro* hepatic EH activity may not be significant in terms of potential detoxification of OAFB. The addition of an EH inhibitor, cyclohexene oxide, to *in vitro* incubation mixtures reportedly failed to increase AFB binding to DNA (21) and the mortality of *Salmonella typhimurium* exposed to AFB (20).

Unlike EH and GTr activities, the production of AFL from AFB by trout hepatic enzymes was observed to increase in fish fed the higher casein diets (Table III). AFL has been shown to be carcinogenic (30) and mutagenic (53) and may be oxidized back to AFB by trout hepatic enzymes (54). It has been suggested that AFL represents a reserve pool of toxin *in vivo* (35, 55) and that its production is indicative of a sensitive animal species (56, 57). If AFL does indeed represent a reserve pool of AFB in trout, then its increased production by fish fed high levels of casein may predispose these particular animals to the induction of cancer.

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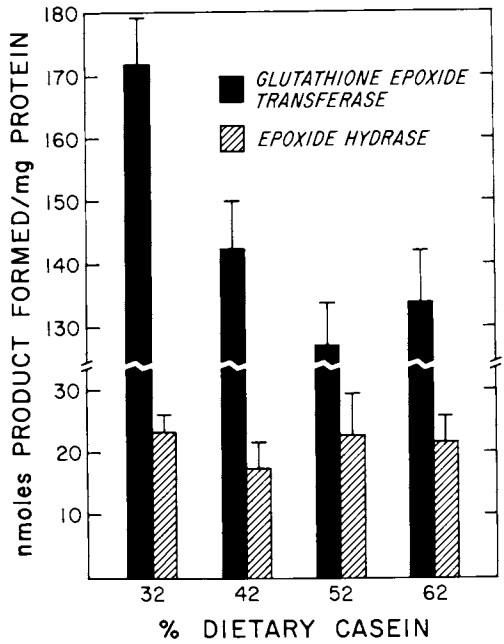

Figure 1. Epoxide hydrase and GSHt activities of trout hepatic microsomes and cytosol respectively

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Ames mutagen assay responses of S. typhimurium TA 98 to AFB followed a pattern similar to that of AFL production (Table IV). A 62% increase in the mutagenic response of these bacteria to AFB was noted in assays using PMF from trout fed high casein diets as compared to those using PMF from trout fed low casein diets. It has been suggested that the mutagenic character of compounds in this assay system is related to their carcinogenic activity (44). Evidence of this has been observed in rainbow trout treated with polychlorinated biphenyls and AFB (43, 58). Thus, these findings indicate that trout fed higher casein diets produce a greater amount of an active form of AFB which may result in an enhanced carcinogenic activity of AFB.

Table IV. Effect of Casein Intake Upon Conversion of Aflatoxin B_1 by Trout PMF to a Mutagen for Salmonella typhimurium TA 98[a]

Diet (% casein)	Response[b] (Number Revertants/ μg Aflatoxin B_1/mg Protein)
32	502(±47)[c]
42	482(±80)
52	673(±96)[1]
62	814(±17)[d,2]

[a]Values with different superscript numbers are significantly different ($P<.05$, Students t Test on n assays).
[b]n=8.
[c]± standard deviation.
[d]n=4.

The observed mutagenic responses to AFB reflect the overall effects of activation and detoxification systems on the in vitro metabolism of AFB. It appeared that the effect of high casein levels fed to trout was that a greater amount of activated AFB was produced and/or that less could be detoxified by these fish than by those fed lower casein diets. If lowered cytochrome P-450 content and AE activities in fish fed the high casein diets represented a decrease in the activation of AFB, then these effects were overcome by the observed decreases in GTr activity and/or increases in AFB conversion to AFL relative to those of trout fed lower casein diets. Alternately, the results could be explained by dietary effects upon some unknown OAFB metabolizing enzyme system in trout, upon free GSH levels in hepatic tissue, or that the levels of the cytochrome P-450 involved in AFB activation were not reflected by the observed total cytochrome P-450 levels.

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Abstract

The effect of dietary protein (casein) levels (32%, 42%, 52%, 62%) upon the activities of several hepatic activating and detoxifying enzyme systems of aflatoxin B_1 (AFB) in rainbow trout (Salmo gairdneri) was investigated. It was observed that in fish fed increased casein, cytochrome P-450 content, in vitro epoxidase and glutathione epoxide transferase activity dropped, up to 21%, 32% and 25% respectively. Fish fed the higher levels of protein converted a greater amount of AFB to aflatoxicol and showed a 62% increase in AFB mutagen assay responses. In vitro cytochrome c reductase and epoxide hydrase activities were not observed to be affected by the diets. The enzyme data provided a possible explanation for the mutagen assay results.

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Alterations in Rainbow Trout Liver Function and Body Fluids Following Treatment with Carbon Tetrachloride or Monochlorobenzene

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It is becoming increasingly evident that drug or chemical induced tissue damage is produced by some foreign compounds through metabolic conversion to a more highly reactive intermediate product. Recent evidence suggests that the mode of hepatic and renal necrosis found following intoxication by certain of the aromatic organic compounds (1, 2); or carbon tetrachloride (CCl_4)(3) results from activation of the parent compound to a more reactive species. This process of activation is thought to be mediated primarily through a group of mixed function oxidase enzymes which are found in the smooth endoplasmic reticulum.

Considerable interest has developed concerning the nature of the mixed function oxidase system in fish and the role that this system may play in the development of toxic responses in these animals. Studies have shown that components of the mixed function oxidase system are present in relatively high concentrations in fish liver (4, 5, 6); and that the enzyme systems in this organ are capable of many of the biotransformation reactions already described for the mammalian liver (7, 8, 9). The presence of this complement of enzymes in the livers of many fishes suggests that this organ too may be particularly sensitive to insult from sublethal concentrations of many waterborne toxicants. For this reason, methods to evaluate liver function in fish may be particularly useful in identifying the sublethal effects of certain classes of toxicants.

The use of clinical diagnostic procedures by which to identify and evaluate specific organ dysfunction is used widely in human and mammalian toxicology. Despite the apparent usefulness of such techniques little has been done to develop similar methods for use in studying toxic responses in aquatic organisms. Recently we have evaluated several exogenous and endogenous tests of liver function in rainbow trout following intoxication by the model hepatotoxicant CCl_4. The results of these studies indicate that elevated plasma activity of the enzyme glutamate-pyruvate transaminase (GPT) is the most sensitive endogenous index of

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liver damage in trout of the several enzyme systems tested (10). Similarly, we have presented data indicating that liver function studies based on plasma clearance of the organic anion sulfobromophthalein (BSP) also are useful in evaluating liver damage in rainbow trout (11, 12). The intent of the present study was to compare and contrast two model mammalian hepatotoxic agents for their potential to produce hepatotoxicity in the rainbow trout.

Materials and Methods

Rainbow trout (100-250 g) were purchased from Roaring River fish hatchery, Scio, Oregon and held under conditions previously described (11). Chlorobenzene (MCB) and carbon tetrachloride (CCl_4) were obtained from commercial sources and used without further purification. Chlorobenzene was dissolved in an equal volume of corn oil and administered as a single intraperitoneal (i.p.) injection (either 0.5 or 1.0 ml/kg). Control animals received a similar volume of corn oil. Carbon tetrachloride was given undiluted by i.p. injection (0.2, 1.0, or 2.0 ml/kg) and control animals received a similar volume of physiological saline.

Acute Lethality of MCB or CCl_4. Four groups of 3 trout were given MCB using a dose range of from 1.0-3.0 ml/kg. The number of dead animals per treatment group was recorded daily for 3 days and the median lethal dose was calculated for 24, 48 and 72 h by the method of Weil (13). The median lethal dose of CCl_4 was determined in two groups of 5 fish by the Up and Down Method of Brownlee et al. (14) using a dose range of from 1.6 to 5.0 ml/kg.

Effect of Intoxication by MCB or CCl_4 on Plasma Clearance of BSP and Plasma Activity of GPT. Twenty four, 48, 72 or 96 h after administration of the toxicants BSP (5.0 mg/kg) was injected into the caudal vein of animals in each treatment group. After 45 min. fish were killed by a sharp blow on the head and a single blood sample was drawn by cardiac puncture. Each animal was weighed, the liver removed and weighed and soft organs examined for gross pathological changes. For plasma enzyme activity studies a single blood sample was drawn into a heparinized tuberculin syringe from the caudal vein and placed into a test tube rinsed with a 2.5% (W/V) solution of potassium oxalate.

Effect of CCl_4 Intoxication on Water Balance in Rainbow Trout. In studies of water balance the urinary bladder of treated and control animals was catheterized (PE 50 tubing) and individual fish were placed in plexiglass restraining chambers for 24 h to allow urine flow rates to stabilize. Urine was collected into individual test tubes at one hour

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intervals. After 24 h fish were injected with CCl_4 (1.0 ml/kg) or physiological saline (1.0 ml/kg) and urine flow rates determined at hourly intervals for 24 h. In a separate group of fish total plasma protein concentration and plasma osmolality were determined from a single blood sample drawn from the caudal vein.

Analytical Methods. The concentration of BSP in the plasma was determined by the method of Richterich (15). The activity of GPT in the plasma was determined on a Gilford Model 2400 recording spectophotometer fitted with a recirculating constant temperature water bath. The assay conditions were the same as those previously described (10).

Total plasma protein concentration was determined by the buiret method (16) after correction of plasma hemoglobin which was determined by the cyanmethemoglobin method (15). Plasma osmolality was measured by a Wescor model 5100 vapor pressure osmometer.

Statistical Methods. Means of treatment groups for plasma retention of BSP, plasma osmolality, total plasma protein concentration and urine flow rates were compared by students t test for independent sample means (17). Plasma enzyme activity data were converted to a quantal form and analyzed by the Fischer Exact Probability Test (18). Values greater than 2 standard deviations ($P < 0.05$) from the control value were chosen to indicate a positive response in treated fish.

Results

Acute Lethality. The median lethal dose (LD_{50}) of MCB to rainbow trout was estimated to be 1.8 ml/kg at 24 h. No mortalities were observed in any of the treated animals after this time. The 24 h LD_{50} value for CCl_4 was estimated to be 4.75 ml/kg but, unlike fish treated with MCB, fish intoxicated with CCl_4 continued to die throughout the entire 96 h observation period.

Effect of Intoxication by MCB or CCl_4 on Plasma Clearance of BSP and Plasma Activity of GPT. Sublethal doses of MCB and CCl_4 were chosen as simple multiples of those used in mammalian studies. For MCB the doses 0.5 ml/kg and 1.0 ml/kg represented 0.28 and 0.56 respectively of the estimated LD_{50} value for the trout. The doses 1.0 ml/kg and 2.0 ml/kg used for CCl_4 represent .21 and .42 of its estimated LD_{50} value. Fish treated with MCB thus tolerated a relatively greater sublethal dose of toxicant than did those fish treated with CCl_4. Our previous experience has indicated that this volume range is suitable for these types of studies with trout.

A higher concentration of BSP ($P < 0.05$) was evident in

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the plasma of fish treated with 1.0 ml/kg of MCB only at 24 h (Fig. 1). No significant elevation of BSP was found in fish treated with 0.5 ml/kg MCB. Elevated plasma BSP concentrations ($P < 0.05$) were evident in fish treated with 2.0 ml/kg CCl_4 after 24, 48 and 96 h and in one group of fish treated with 0.2 ml/ kg CCl_4 after 24 h.

Plasma GPT activity varied greatly in animals receiving MCB at 1 ml/kg and was significantly increased in test animals only 72 h post-treatment (Fig. 2). Conversely, plasma GPT activity was elevated ($P < 0.01$) in groups of fish receiving either 1.0 ml/kg or 2.0 ml/kg of CCl_4 at both 24 and 48 h.

Gross pathological responses of the trout to the toxicant were comparable in fish treated with either MCB or CCl_4. Slight to moderate hemorrhaging, which persisted throughout the study, was noted at the base of both single and paired fins after 12 h in fish treated with either dose of MCB or CCl_4, however hemorrhagic areas were observed more consistently in fish treated with CCl_4. Inspection of the peritoneal cavities of animals treated with both chemicals revealed areas of mild to moderate inflammation in sections of both the peritoneal linings and the small and large intestines. The surface of both the liver and spleen were mottled with blanched areas.

The liver to body weight ratio was never significantly different from controls in any treatment group. The spleens of animals treated with both doses of CCl_4 and with the high dose of MCB were enlarged in the majority of fish examined, however wet weights of this organ were never taken. Unlike control fish and those treated with CCl_4, the surface of the spleens from fish treated with MCB had a bumpy, coarse texture.

Hemoglobinemia was pronounced in fish treated with both doses of CCl_4 and with the highest dose of MCB. After 24 h the mean plasma hemoglobin concentration in animals treated with 2.0 ml/kg CCl_4 was greater than 200 mg/100 ml while in fish receiving MCB (1.0 ml/kg) the plasma hemoglobin concentration was not more than 150 mg/100 ml. Hemoglobinuria was evident in fish treated with CCl_4 but was never apparent in MCB treated animals.

Effect of CCl_4 Intoxication on Water Balance in Rainbow Trout. Because our earlier work had indicated that CCl_4 intoxication resulted in significant weight gain in spinal transected rainbow trout (12) weight change was determined in intact fish treated with MCB or CCl_4. The whole body wet weight of trout treated with MCB did not change appreciably from control animals at any time during the course of the intoxication (Fig. 3). Significant differences ($P < .05$) were observed in whole body wet weight change in fish treated with CCl_4 at both 1.0 ml/kg and 2.0 ml/kg. Fish treated with CCl_4 either lost less weight than control

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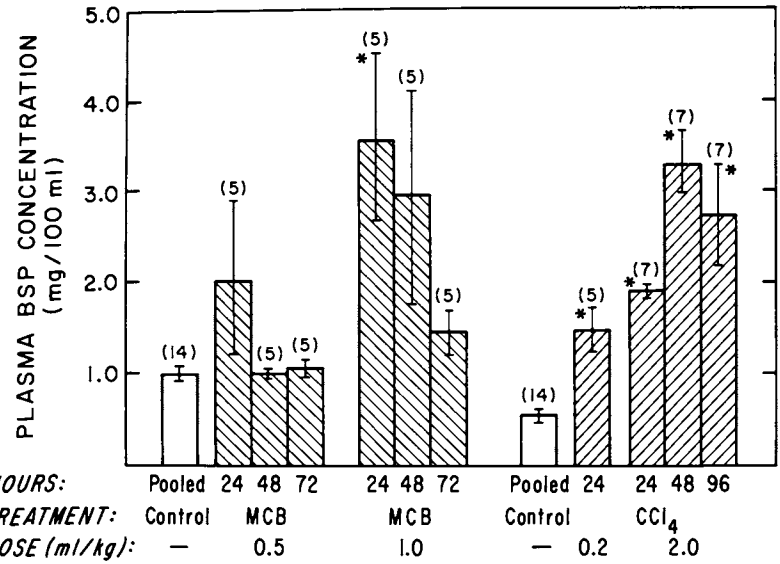

Figure 1. Plasma BSP concentrations in rainbow trout following single ip injections of either CCl_4 (0.2 mL/kg or 2.0 mL/kg) or MCB (0.5 mL/kg or 1.0 mL/kg). Plasma dye concentrations were determined 45 min after a single dose of BSP (5.0 mg/kg) was given. Values represent the mean ± SE of the number of animals indicated in parentheses; (*), values which are significantly different from controls.

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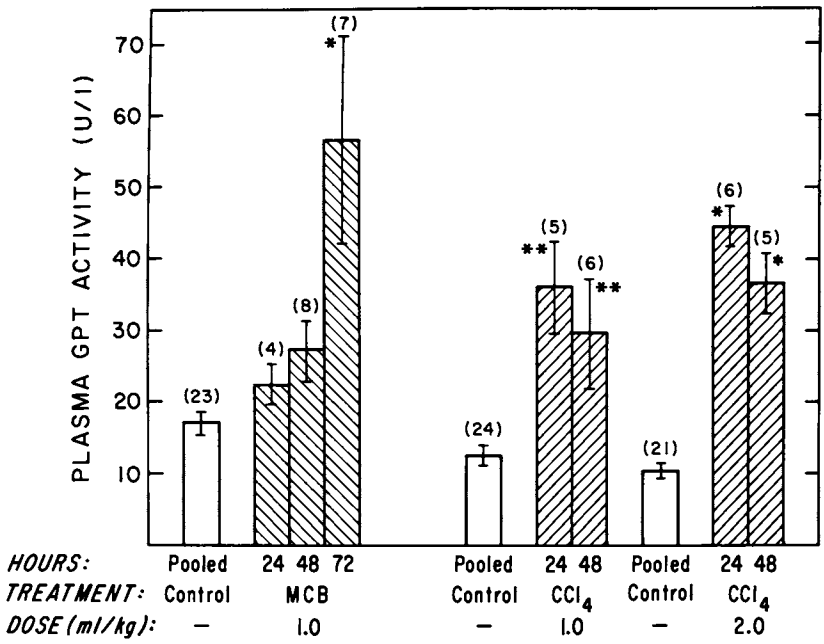

Figure 2. Plasma GPT activity in rainbow trout following treatment with either CCl_4 (1.0 mL/kg or 2.0 mL/kg) or MCB (0.5 mL/kg or 1.0 mL/kg). Values represent the mean ± SE of the number of animals indicated in parentheses; (*), values that are significantly different from controls (, $P < 0.05$; **, $P < 0.01$).*

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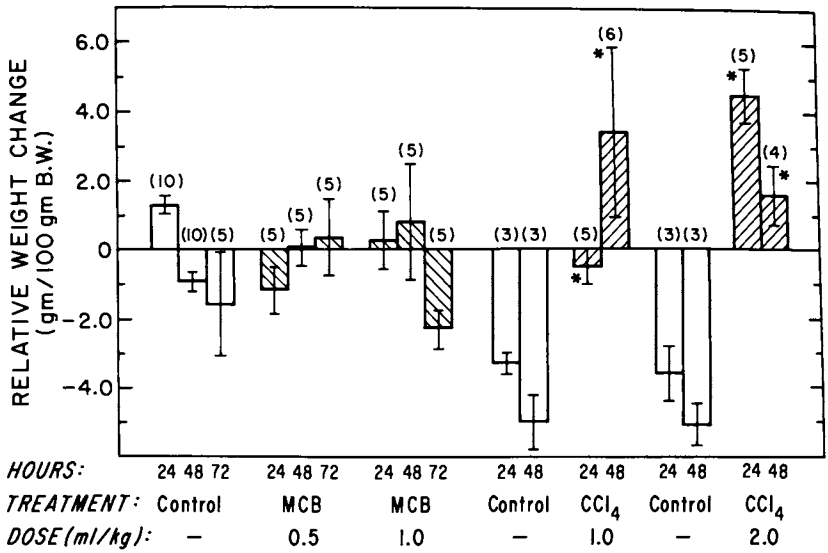

Figure 3. Relative weight change in rainbow trout following treatment with either MCB (1.0 mL/kg) or CCl_4 (2.0 mL/kg). Values represent the mean ± SE of the number of animals indicated in parentheses; (*), values that are significantly different from controls ($P < 0.01$).

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fish or tended to gain weight.

To investigate whether the difference in weight changes in CCl_4 treated fish was associated with impaired water clearance a study of water balance was conducted. Within 24 h differences ($P < 0.05$) were observed in whole wet body weight change, total plasma protein concentration and rate of urine flow in fish treated with CCl (Table 1). Plasma protein concentration in treated fish was reduced to 46.7% of that of control fish while no significant difference was observed in plasma osmolality. Slight proteinuria was observed in the majority of fish treated with CCl_4. The urine flow rates of all fish treated with CCl_4 dropped precipitously during the first hour after treatment and were depressed for the remainder of the 24 h experimental period. The mean urine flow rate after 24 h was reduced to 29.8% of that of the control fish and the mean 24 h urine output in treated fish (23 ml/kg) was only 22% of control animals (104 ml/kg).

Discussion

Elevated plasma GPT activity (10, 19) and attenuated clearance of BSP from plasma (11, 12) both have been used as diagnostic criteria by which to assess liver damage in rainbow trout. In the present study significant changes in both of these tests of liver function were apparent following treatment of trout with CCl_4 at both doses of toxicant and at all sample periods. In addition, we previously have reported that CCl_4 intoxication produces histological alterations in trout liver including necrosis of hepatocytes surrounding central veins (12). In contrast, the results of studies with MCB were variable and inconclusive. Significant alterations of these indicators of liver dysfunction were evident only at the highest dose of MCB used and only at specific times either early or late in the course of the intoxication. These results suggest that the liver of rainbow trout is somewhat more sensitive to intoxication by CCl_4 than by MCB.

The seemingly greater effect of CCl_4 in producing liver damage in trout may be attributed to several factors. Because CCl_4 was not administered in corn oil vehicle it may have been absorbed more readily and had greater access to body compartments than did MCB. In addition, the highest dose of CCl_4 administered to the trout (19 mM/kg) was 1.4 times greater than the highest dose of MCB (13.55 mM/kg). However, when these doses are compared relative to their respective LD_{50} values it is apparent that trout treated with MCB received a proportionately greater toxic dose than did those treated with CCl_4. The likelihood of effects other than specific organ toxicity influencing these diagnostic indexes therefore is increased.

The attenuated clearance of BSP observed in MCB or CCl_4 treated fish may be due to factors unrelated to liver dysfunction. For example, anesthetic effects associated with CCl_4 or MCB intoxication may have influenced plasma clearance of BSP

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Table I.

Alterations in Selected Biochemical and Physiological Parameters of the Rainbow Trout 24 Hours after Treatment with CCl_4 (2.0 ml/kg, i.p.)

Parameters	N	Control	N	Treated
GPT Plasma Activity				
(U/l)[b]	3	8.4 ± 1.2[a]	5	44.7 ± 2.8
(U/g protein)	3	0.27± 0.03	5	3.24± 0.42
Weight Change				
(g/100 g B.W.)	3	-3.6 ± 0.8	5	4.5 ± 0.8*
Plasma Osmolality				
(mOs/kg)	3	290 ± 8	5	273 ± 2
Plasma Protein				
(mg/ml)	3	30.8 ± 1.2	5	14.4 ± 1.2*
Urine Flow Rate				
(ml/kg/hr)	8	4.7 ± 0.4[c]	10	1.4 ± 0.5**

[a] Values are mean ± S.E. for N number of animals.

[b] U = One international unit of activity at 25°C and pH 7.5.

[c] Values are hourly mean for the 6 hr interval (19-24 hours) post-treatment

* $P < .05$

** $P < .01$

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by altering total hepatic blood flow. It has been demonstrated that the depressant, chlorpromazine, can attenuate BSP clearance in mice and rats through the production of hepatic ischemia (20). If reduced plasma clearance of BSP were related to anesthetic induced alterations in hepatic blood flow it should be evident early in the course of the intoxication when these types of effects would be most pronounced. We have reported that significant plasma retention of BSP in rainbow trout occurred only within the first 24 h after treatment with MCB (21) whereas maximum plasma BSP retention in CCl_4 treated trout occurred 48 h after the intoxication and persisted for as long as 96 h. Additionally, plasma BSP retention was evident in trout treated with 1.9 mM/kg, a dose that produced no observable anesthetic responses in the trout. It thus appears that extrahepatic effects of both toxicants may have contributed at least partially to the impaired clearance of BSP however these responses probably are more prominent with MCB intoxication.

Rainbow trout appear to be less susceptible to liver damage by both of the chemicals used in this study than are laboratory mammals. The highest dose of each toxicant used in the present study was twice that used in similar studies with mice and rats and greater than that used in a previous study with rainbow trout (Table 2). Despite this fact, histological evidence of liver damage is inconsistent among groups of fish treated with either toxicant. In preliminary histological studies we have reported an incidence of pericentral necrosis of 25 percent in trout treated with 2.0 ml/kg CCl_4 (12) and less than 15 percent occurrence of this lesion in trout treated with 1.0 ml/kg MCB. The incidence of centrilobular necrosis in rats treated with half this dose is nearly 100 percent 24 h after treatment (1).

The increase in relative whole wet body weight in CCl_4 treated trout appears to be related in part to impaired water clearance. The decrease in total 24 h urine output, proteinuria and hemoglobinemia suggests that renal function may have been impaired in treated animals. Similar symptoms of renal damage have been reported in humans (23) and laboratory mammals following acute exposure to CCl (24, 25) however these symptoms are evident only after 1-4 days. Previously we have reported that severe hemoglobinemia results from CCl_4 intoxication in rainbow trout (12). Jaenike and Schneeburger (26) have demonstrated that intravascular and intratubular hemoglobin aggregation resulting from hemoglobinemia act to produce renal ischemia and reduced glomerular filtration in rats. Similarly, Smith et al. (27) have demonstrated severe congestion of glomeruli in chinook salmon (Oncorhynchus tshawytscha) rendered anemic by the hemolytic agent phenylhydrazine. It is possible that the hemoglobinemia observed in trout treated with CCl_4 may be responsible in part for the reduced urine flow rates observed in treated fish in this study.

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Table II

Toxicant	Dose Administered (mM/kg)			
	Rainbow Trout	Reference	Mammals	Reference
CCl_4	19mM/kg	Present Study	9.5mM/kg	22
	12.6mM/kg	(19)		
	9.5mM/kg	Present Study		
Monochlorobenzene	13.55mM/kg)	Present Study	7.4mM/kg	1

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It is not likely that the drastic drop in urine flow rate observed with the first hour after CCl_4 treatment was the result of hemoglobinemia or the direct effects of this toxicant on the kidney. Hemoglobinemia was not apparent in treated fish for at least 3 h after treatment and yet urine flow rate was reduced to only 30 percent of that of controls after one hour. Carbon tetrachloride treatment has been shown to produce an immediate decrease in systemic blood pressure in laboratory dogs (24), presumably by sensitizing cardiac muscle. A similar decrease in systemic blood pressure in the trout could lead to redistribution of blood through the splanchnic drainage resulting in reduced renal blood flow, reduced glomerular filtration and abnormal back diffusion in the renal tubules.

The use of simple clinical diagnostic tests to screen for liver dysfunction appears to be practical in trout. Cutler (28) has shown that an elevation in plasma GPT activity is closely associated with the development of histopathological lesions in rats treated with CCl_4. Our work (10) and that of others (19) suggests that this also is the case in rainbow trout. However this criteria alone is not adequate to detect functional impairment of the liver in the absence of morphological changes. Therefore a test of organ function used in combination with enzyme tests and histological screening appear to be most useful in the overall assessment of organ dysfunction provided that the limitations of each test are recognized.

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