Conjugation of Organic Pollutants in Aquatic Species
by Margaret O. James*

Aquatic organisms can take up organic pollutants from their environment and subsequently excrete the pollutant or its biotransformation products (metabolites). Phase II (conjugation) biotransformation products are almost always less toxic than the unmetabolized organic pollutant. For many organic pollutants, the extent to which conjugates are formed is extremely important in determining the rate of excretion of the pollutant. This is because most conjugates (glycosides, sulfates, amino acid conjugates, mercapturic acids) are organic anions which are readily water-soluble and are rapidly excreted by fish (and probably higher invertebrates) by a combination of glomerular filtration and tubular transport. In this paper, each major conjugation pathway is discussed with respect to what is known about its occurrence in fish and aquatic invertebrates, both from in vivo and in vitro data. Although limited data are available, this paper also considers what is known about how each conjugation reaction affects the toxicity and potential for renal and biliary excretion of organic xenobiotic substrates.

Introduction

Many aquatic environments are polluted with organic chemicals as a result of discharge of industrial chemicals, run-off of agricultural chemicals and fall-out of combustion products in rain (1, 2). It is known that aquatic organisms living in chemically polluted environments will absorb lipophilic organic pollutants (3). Organic chemicals usually undergo biotransformation in animals via phase I (functionalization; i.e., oxidation, reduction, etc.) and phase II (conjugation) reactions to more polar derivatives, which are more readily excreted than the parent compound (4, 5). The most important phase II reactions are glycosylation, sulfation, mercapturic acid formation, amino acid conjugation, and acetylation. Metabolites formed by conjugation reactions are usually less toxic than the unconjugated compound, although there are notable exceptions to this rule (6, 7). Thus, conjugation is usually a detoxication reaction, and as such is a desirable process. Most conjugates (glycosides, sulfates, amino acid conjugates, mercapturic acids) are organic anions which are readily water-soluble. In mammals and fish, the organic anions formed by phase II reactions are frequently substrates for facilitated renal tubular transport and are therefore rapidly excreted in urine by a combination of glomerular filtration and tubular transport (5). Higher invertebrates can also excrete organic anions into urine by facilitated transport (8, 9). Thus, depending on structure, and the extent of phase I biotransformation of a particular organic pollutant, the rate of excretion will be influenced by the extent to which the pollutant is conjugated. Pollutants that are rapidly excreted usually show no lasting toxicity. Compared with phase I reactions, there have been few studies of phase II reactions in aquatic animals, and this is especially true of invertebrates (10). In this paper, I will summarize what is known about the most important conjugation reactions in fish and aquatic invertebrates.

Glycosylation

Organic molecules containing phenolic or alcoholic hydroxyl groups, carboxylic acid groups, nitrogen atoms, thiol groups, or other nucleophilic centers can undergo glycosylation, as shown in Equation (1):

\[ RXH + UDPG \rightarrow RXG \]  

where \( X = O, N, S \) (and under exceptional circumstances C), \( R \) = the rest of the xenobiotic, and UDPG = uridine diphospho-\( \beta \)-d-glucuronic acid or uridine diphospho-\( \beta \)-d-glucose. Whether the sugar moiety is glucuronic acid or glucose depends on the species of animal and on the structure of the xenobiotic. Table 1 shows presently available data on the species occurrence and types of substrates that undergo conjugation with glucose or glucuronic acid in marine species. Glucuronides are more water-soluble than glucose conjugates by virtue of the carboxylic acid group, and should therefore be more readily excreted, especially by animals with organic anion transport systems.

Glucuronides

Xenobiotics containing phenolic hydroxy groups either in the parent molecule or as a result of cyto-

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Table 1. Occurrence of glycosylation in aquatic animals.

| Pathway            | Species       | Substrate                      | References       |
|--------------------|---------------|--------------------------------|------------------|
| Glucuronidation    | Teleost fish  | Phenolic group (10–23)         | (22–41)          |
|                    | Elasmobranch  |                                |                  |
|                    | Crustacea     |                                |                  |
|                    | Teleost fish  | Dihydrodiols (24–30)          |                  |
|                    | Crustacea     |                                |                  |
|                    | Teleost fish  | Aliphatic hydroxy group        | (26)             |
|                    |                | Carboxylic acid                | (31)             |
| Glucose conjugation| Molluscs      | Phenolic group (19,25,42) (11,39,43)|                  |
|                    | Crustacea     |                                |                  |
|                    | Teleost fish  |                                |                  |

Chrom P-450 monoxygenase have been found in bile or urine of several teleost fish species as conjugates with glucuronic acid (11–23). Glucuronic conjugates of dihydrodiols metabolites of aromatic hydrocarbons, e.g., naphthalene, benzo(a)pyrene, have also been found in fish bile after administration of the parent hydrocarbon (24–28). Conjugates of dihydrodiols and phenols which could be hydrolyzed by β-glucuronidase have been found in extracts of shrimp (29) and spiny lobster (30) after administration of aromatic hydrocarbons. Glucuronic conjugates of an aliphatic hydroxy group (26) and a carboxylic acid (31) have been found in trout bile after administration of 2-methylnaphthalene (26) or di-2-ethylhexyl phthalate (31). The aliphatic hydroxy group was formed by hydroxylation of the methyl group in 2-methylnaphthalene and the carboxylic acid by ester hydrolysis of di-2-ethylhexyl phthalate to mono-2-ethylhexyl phthalate.

Formation of a glucuronide in liver and excretion in bile does not necessarily mean that all of the glucuronide conjugate will be rapidly excreted in urine, even though the glucuronide conjugates are water-soluble and readily excreted by kidney. This is because glucuronides can be hydrolyzed by β-glucuronidases present in intestine and other organs. For example, in the goldfish, phenol glucuronide was formed in liver and excreted in bile but subsequently hydrolyzed by intestinal β-glucuronidase; the phenol was reabsorbed and finally excreted in urine as a sulfate conjugate (10). Guarino showed that the 48 hr urinary excretion of phenol red and its glucuronide by dogfish shark was reduced by removing bile through a surgically implanted fistula (22). In this case, the phenol red and its glucuronide were artifically removed from the fish with the collected bile, preventing hydrolysis of the conjugate and reabsorption of the parent drug (23). If, in the intact animal, glucuronide conjugates are excreted in bile but then undergo extensive intestinal hydrolysis to parent xenobiotic that is reabsorbed (enterohepatic circulation) the excretion of the xenobiotic glucuronide conjugate could be delayed.

Glucuronidation has also been studied at the level of the enzyme. In vitro studies have shown that UDP-glucuronosyltransferases in fish liver possess many of the same properties as the mammalian enzymes. The activity is microsomal and is enhanced by treatment of the microsomes with detergent, digitonin or other agents which disrupt the vesicle structure (36–40). Trout liver UDP-glucuronosyltransferase is inducible by β-naphthoflavone, and there is preliminary evidence that multiple forms of the enzyme exist with different substrate selectivities (41).

Glucosidation

There have been very few studies in which glucoside conjugates have been identified as metabolites in aquatic species (Table 1). In the teleost fish, glucosides were found as minor metabolites (19,25) in tissue extracts, and it is not known if glucosides are excreted. Glucosidation may be a more important pathway in invertebrates (11,39,42,43), but so far insufficient data are available to draw this conclusion. Glucosides have been found in excreta of invertebrates (42).

It is of interest from a public health standpoint that there is evidence from at least one study that glucoside conjugates ingested by mammals are rapidly hydrolyzed and the aglycone metabolized and excreted exactly as if the aglycone were administered. Thus, Crayford and Hutson (44) showed that the glucoside conjugate of 3-phenoxybenzoic acid (a major plant metabolite) was rapidly hydrolyzed when administered to rat, and the excreted metabolites were the same as those found when 3-phenoxybenzoic acid was administered.

Sulfation

Organic molecules containing aliphatic or aromatic hydroxyl groups can also be sulfated, as shown in equation (2)

$$\text{ROH} + \text{PAPS} \rightarrow \text{ROSO}_3\text{H}$$

where R = the rest of the molecule and PAPS = phosphoadenosyl phosphosulfate. Some reports of sulfate conjugates of organic xenobiotics in aquatic species are summarized in Table 2. Data on sulfation are somewhat incomplete, because in many cases investigators have focused on identifying metabolites formed by monoox-

Table 2. In vivo evidence for sulfate conjugation in aquatic species.

| Substrate                  | Species                  | Reference |
|----------------------------|--------------------------|-----------|
| Phenol                     | Goldfish, guppy, tench, bream, rudd perch, roach | (12, 13)  |
| 4-Nitrophenol              | Gum boot chiton, starfish, lobster | (42, 43)  |
| Hydroxynaphthalene         | Shrimp                   | (29)      |
| 1- and 3-Hydroxy-2,6-dimethylnaphthalenes | Sea urchin | (45)      |
| Phenols and dihydrodiols of benzo(a)pyrene | Southern flounder, English sole | (20, 27)  |
ygenation and have not attempted to distinguish between sulfate and glucuronide conjugates of hydroxyl groups. When finding a metabolite that cannot be extracted into organic solvents, many investigators hydrolyze the polar metabolite with a mixture of β-glucuronidase and arylsulfatase and then attempt to identify the unconjugated molecule. It is noteworthy that the rainbow trout does not appear in the list of species in which sulfate conjugates have been found. At least three groups have looked for evidence of sulfate conjugation in rainbow trout, using a variety of substrates including pentachlorophenol (16), p-nitrophenol (32), 7-ethoxycoumarin (33), and acetaminophen (34). Studies in southern flounder have shown that sulfate conjugates of 7-hydroxybenzo(a)pyrene and 7,8-dihydroxybenz(a)pyrene were excreted more rapidly in urine than the corresponding glucuronide conjugates, and that this was because the sulfate conjugates were better substrates for renal tubular transport than the glucuronides (20).

Another point of interest is that sulfate conjugates have frequently been identified as the major metabolites of hydroxylated xenobiotics in invertebrates (29,42,43,45) (Table 2). In sea urchins exposed to 2,6-dimethylnaphthalene, sulfate conjugates of ring-hydroxylated products were the major excreted metabolites. The sulfate conjugates appeared to be cleared fairly rapidly, through the digestive tract (45). There is no far no evidence that sulfate conjugates of xenobiotics, once formed, are hydrolyzed back to the parent molecule in aquatic species, although this has been shown for some sulfate conjugates in mammals (7).

Mercapturic Acid Biosynthesis

Mercapturic acids (N-acetylcysteine conjugates) are the ultimate excreted metabolites formed by further metabolism of glutathione conjugates of organic molecules, as shown in equations (3)–(6).

\[
\begin{align*}
R-X + GSH & \rightarrow R-SG \\
RSG & \rightarrow R-cys-gly \\
R-cys-gly & \rightarrow R-cys \\
R-cys & \rightarrow R-cys(N-acetyl)
\end{align*}
\]

where R-X is a xenobiotic with an electrophilic center, GSH is the tripeptide glutathione (γ-glutamylcysteinylglycine), R-cys-gly is the cysteinylglycine conjugate formed by cleavage of the glutamyl group of RSG, R-cys is the cysteinyl conjugate formed by cleavage of the peptide bond in R-cys-gly, and R-cys(N-acetyl) is the mercapturic acid, formed by acetylation of the cysteinyl amino group of R-cys. R-cys conjugates are sometimes referred to as premercapturic acids. Details of the enzymology of mercapturic acid biosynthesis may be found in Jakoby (46,47). Electrophilic centers are present in reactive chemical pollutants, such as alkylating agents, and may also be introduced into a previously unreactive molecule by monoxygenation: for example, the epoxide group is introduced into molecules containing double bonds by cytochrome P-450. In the absence of GSH and GSH S-transferases, these reactive chemical groups can bind to tissue macromolecules and initiate a variety of toxic reactions. Studies in mammals have shown that GSH S-transferases have three functions: catalysis, reversible binding of organic molecules, and irreversible binding of electrophiles. The catalytic and irreversible binding functions are important in detoxication of electrophiles, and the reversible finding function may be important in the transport of organic molecules from cells. Therefore, knowledge of the GSH S-transferases and related systems of aquatic animals is important in understanding processes of detoxication in these animals.

Mercapturic acids and premercapturic acids have been found in excreta of sea urchins fed 2,6-dimethylnaphthalene (45), in bile of Japanese carp fed the herbicide molinate (48), and in urine and bile of winter flounder injected with the glutathione conjugates of styrene oxide (49). In the winter flounder, the cysteinyl conjugate was the predominant urinary metabolite, showing that this metabolite can be efficiently excreted (49). Bile from English sole administered benzo(a)pyrene contained predominantly glutathione and cysteinyl-glycine conjugates of benzo(a)pyrene metabolites (28).

The important first step in mercapturic acid biosynthesis has been studied in vitro in many aquatic animals (50). All vertebrate and invertebrate species so far examined seem competent to form glutathione conjugates, although there is wide variation in the rapidity with which the conjugates are formed (10). It has been assumed that these glutathione conjugates are then processed as shown in Equations (4)–(6), prior to elimination from the animal. Glutathione S-transferase enzymes have been purified from the rainbow trout (51), the little skate (52), and the thorny-back shark (53), and their properties investigated. In each of these species multiple forms of the enzyme were found, and each enzyme was shown to consist of two protein subunits with molecular weights in the 25,000 dalton region (51–53).

| Table 3. Acid-soluble thiol concentrations in fish liver. |
|----------------------------------------------------------|
| Species               | Acid-soluble thiol concentration, mM | Method used* | Reference |
|-----------------------|-------------------------------------|--------------|-----------|
| Little skate          | 2.3                                 | OPT (54)     |           |
| Large skate           | 1.4                                 | DTNB (54)    |           |
| Thorny skate          | 2.5                                 | OPT (54)     |           |
| Thorny-back shark     | 1.3                                 | DTNB (53)    |           |
| Sheephead             | 1.9                                 | OPT          | b         |
| Pinfish               | 2.8                                 | OPT          | b         |
| Sea bass              | 1.3                                 | DTNB (55)    |           |
| Rainbow trout         | 1.8                                 | DTNB (51)    |           |
| Mullet                | 2.1*                                | DTNB (56, 58)|           |
| Croaker               | 2.7*                                | DTNB (57)    |           |
| Winter flounder       | 1.3                                 | DTNB (58)    |           |

*OPT: o-phenylaldehyde method (59); DTNB: 5,5'-dithiobis-2-nitrobenzoic acid (56).
*James, M. O. Unpublished results.
*Thomas and Wofford (56, 58) have shown that in mullet and croaker, glutathione accounts for 60–70% of the acid soluble thiols in liver, as measured by reaction with DTNB.
Table 4. Acetylation of xenobiotic by aquatic species.

| Species          | Substrate                           | References |
|------------------|-------------------------------------|------------|
| Dogfish shark    | Ethyl m-amino benzoic acid          | (60)       |
| Rainbow trout    | Ethyl m-amino benzoic acid          | (61) (62)  |
|                  | 2-Amino-4-phénylthiazole            | (62)       |
|                  | 4-Nitroaniline                      | (63)       |
|                  | 3-Trifluoromethyl-4-nitrophenol     | (64)       |
|                  | Sulfanilamide                       | (65)       |
|                  | Sulfadimidine                       | (65)       |
| Carp             | 2-Amino-4-phénylthiazole            | (62)       |
| Snail urchin     | Sulfamethazole                     | (66)       |
| Sea urchin       | p-Toluidine                         | (42)       |
|                  | p-Aminobenzoic acid                | (42)       |
|                  | p-Nitroanisole                     | (67)       |
| Gum boot chiton  | p-Nitroanisole                     | (42)       |

Table 5. Environmental chemical substrates for amino acid conjugation.

| Environmental chemical | Substrate for amino acid conjugation |
|------------------------|--------------------------------------|
| 2,4-Dichlorophenoxyacetic acid (2,4-D) | 2,4-D                                 |
| 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) | 2,4,5-T                               |
| 1,1,1-Trichloro-2,2-bis (p-chlorophenyl)ethane (DDT) | Bis(p-chlorophenyl) acetic acid (DDA) |
| Toluene                | Benzoic acid                         |
| Alkyl substituted benzenes | Substituted benzoic acids           |
| Alkanes, alcohols, glycols | Alkyl or alkoxycarboxylic acids    |
| Pyrethroid insecticides | Substituted cyclopropene carboxylic acid |

Table 6. Occurrence of taurine conjugation of carboxylic acids.

| Substrate                          | Species                | Reference |
|------------------------------------|------------------------|-----------|
| Phenylacetic acid and other substituted acetic acids | Winter flounder       | (71–77)   |
|                                    | Southern flounder      | (73, 77)  |
|                                    | Mullet                 |           |
|                                    | Sheephead              |           |
|                                    | Pinfish                |           |
|                                    | Drum                   |           |
|                                    | Redfish                |           |
|                                    | Dogfish shark          |           |
|                                    | Stingray               |           |
|                                    | Skate                  |           |
| Benzoic acid                       | Southern flounder      | (77–79)   |
|                                    | Red drum               | (77–79)   |
|                                    | Stingray               |           |

This is consistent with what has been found in studies with mammals (46). When kinetic parameters of the fish enzymes with a commonly used substrate, 1-chloro-2,4-dinitrobenzene (CDNB), were investigated, it was found that apparent \( K_m \) values for CDNB ranged from 0.2 to 0.7 mM, and apparent \( K_v \) values for GSH ranged from 0.2 to 4.3 mM (51–53). In studies with hepatic cytosol from the sheepshead, \( K_m \) values for GSH of 0.3 to 0.5 mM were found with styrene oxide or benzo(a)pyrene 4,5-oxide as substrates (50). The \( K_m \) values for GSH are in the same range as the concentration of total acid-soluble thiols of fish liver (Table 3). In studies with mullet and croaker, Thomas and Wofford have shown that the GSH concentration of fish liver is about 60 to 70% of the concentration of total acid-soluble thiols, as measured by the reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNE) (56,58). Total acid-soluble thiols measured by reaction with o-phthalaldehyde (OPT) are reported to be >90% GSH (59). Because the \( K_m \) values for both GSH and CDNB are so high, rates of formation of the CDNB-GSH conjugate in vivo could be very sensitive to changes in concentration of either CDNB (or substrates with similar properties) or GSH. Since conjugation with GSH is a very important route of detoxification of electrophilic xenobiotics, which can also react with cellular macromolecules, this is of toxicological importance.

**Acetylation**

Organic xenobiotics which contain amino groups may be biotransformed to acetylated metabolites as shown in Equation (7).

\[
R-\text{NH}_2 + \text{AcetylCoA} \rightarrow R-\text{NHCOCOCH}_3
\]  

(7)

where \( R \) is the rest of the molecule. The acetylated product is frequently less polar than the parent xenobiotic, and may not be excreted from the animal as readily. The role of acetylation in excretion of amines has been studied in several species as shown in Table 4.

The widely used fish anesthetic, tricaine methane sulfonate, MS-222, contains a free amino group. The drug is excreted rapidly across the gills, largely as unchanged drug, but it was shown many years ago that a small fraction was eliminated across gills as the acetylated derivative (60). Other studies showed that MS-222 was also excreted renally: the urine contained some unchanged MS-222, but >75% was acetylated MS-222 (62,62).

**Amino Acid Conjugation**

Carboxylic acid groups in xenobiotics can be conjugated with amino acids prior to excretion. The enzymatic reaction mechanism is shown in Equations (8) and (9).

\[
\text{RCOOH} + \text{CoASH} \rightarrow \text{RCOSCoA}
\]  

(8)

\[
\text{RCOSCoA} + R^'\text{NH}_2 \rightarrow \text{RCONHR}' + \text{CoASH}
\]  

(9)

where \( R \) is the rest of the xenobiotic molecule, \( R^' \) is the rest of the endogenous amino acid, CoASH is coenzyme A. Metabolic energy to form the coenzyme A intermediate is supplied by ATP. The amino acid used for this reaction varies with species. The most commonly used amino acid in mammalian species is glycine, although conjugates with glutamine and taurine are also
frequently found (68,69). Examples of organic pollutant substrates for this reaction are shown in Table 5. A few pollutants are themselves substrates for conjugation (e.g., 2,4-D and 2,4,5-T), but most are first oxidized to the carboxylic acid. This pathway is somewhat unusual in that the substrate carboxylic acids (organic anions) are themselves good candidates for urinary excretion. Glycine conjugates, however, are even better candidates for urinary excretion: indeed, p-aminobenzoic acid (p-aminohippurate) is perhaps the best known substrate for the renal organic anion transport system, and has been used in marine fish and crustacea as well as in mammals to probe excretory systems (5,8,9).

The only rigorously identified metabolites of carboxylic acids found so far in aquatic animals are the taurine conjugates. Taurine conjugates of several carboxylic acids have been found in marine fish and crustacea, as shown in Table 6. To date there have been no published reports in which amino acid conjugates have been unequivocally indentified as metabolites of carboxylic acids in freshwater fish. One paper states that hippuric acid was found as a metabolite from goldfish exposed to toluene (70), but the “hippuric acid” was not isolated from tank water and chemically identified. In the absence of definitive evidence to the contrary, taurine conjugation may be said to be a major route of conjugation of carboxylic acids in marine animals.

The renal excretion of several $^{14}$C-labeled carboxylic acids and their taurine conjugates has been studied in winter flounder and southern flounder (74,76,79). The carboxylic acids studied were accumulated from medium into isolated flounder renal tubules, apparently by the organic anion transport system, but the $^{14}$C present in tubules was mainly the taurine conjugate. Clearly, the taurine conjugates can be formed in flounder kidney, and it is therefore difficult to compare the excretion properties of the free acid and the taurine conjugate. By studying uptake after a short period of time (5 min), it was possible to show that the taurine conjugate of benzoic acid was accumulated to a greater extent than the unconjugated acid (79).

Other in vitro studies showed that in the presence of $^{14}$C-labeled taurine, kidney and liver mitochondria from several marine fish could catalyze the conversion of phenylacetyl Coenzyme A and benzoyl Coenzyme A to the respective taurine conjugates (see Table 6) (71,77,78).

Several factors influence the rate of urinary excretion of carboxylic acids. Table 7 shows the variation in 24 hr excretion of several carboxylic acids by southern flounder, and lists values for some of the parameters known to affect urinary excretion. It is clear from Table 7 that the amount of taurine conjugate present in urine does not correlate with the amount of acid excreted in 24 hr, but rather that several factors influence excretion.

Studies of the disposition of some environmentally important carboxylic acids have been conducted in a marine crustacean, the spiny lobster (75). The amount excreted by spiny lobster in 24 hr ranged from 89% for 2,4-D to 24% for DDA (Table 8). Each acid was taken up to some extent by hepatopancreas, where it was metabolized to the taurine conjugate, but the rate of excretion was faster if uptake and metabolism by hepatopancreas was less avid than uptake by green gland. Once taken up by green gland, the acids seemed to be excreted unchanged in urine (75). In this instance, conjugation was not needed to facilitate excretion.

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