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14 The Amino Acid Conjugations

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Introduction

In 1829 Liebig isolated a compound from horse urine which he called hippuric acid (Greek: acid from horse urine) and was able to show that the material contained both a benzoyl group and nitrogen. Keller isolated the same compound from his own urine, in 1842, following self-administration of benzoic acid and the structure of hippuric acid was determined by Dessaignes, three years later, when he found both benzoic acid and glycine on treatment of the material with inorganic acid (Conti and Bickel 1977). Thus conjugation of benzoic acid with glycine to yield hippuric acid or benzoylglycine (Figure 14.1) is generally accepted to be the first xenobiotic transformation reaction to be discovered (Smith and Williams 1970; Conti and Bickel 1977). The other major metabolic transformation of xenobiotic carboxylic acids, namely conjugation with glucuronic acid, was also discovered in the nineteenth century (Williams 1959; Conti and Bickel 1977).

The significance of xenobiotic metabolism and particularly that of the xenobiotic carboxylic acids to the initial development of biochemistry cannot be overemphasised. For example, the application of higher homologues of both benzoic acid and the phenylacetic acids ultimately resulted in the elucidation of the β-oxidation pathway of fatty acid metabolism (Dakin 1922), and glycine was shown to be a constituent of hippuric acid before it was found in glycocholic acid, a bile acid (Young 1977).

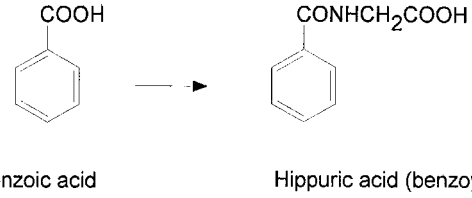

Figure 14.1 Conjugation of benzoic acid to hippuric acid: the first reaction of drug metabolism.

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Since these initial observations, conjugation of xenobiotic carboxylic acids with endogenous amino acids has been shown to be an important pathway in the biotransformation of a number of compounds in a variety of species. The reaction involves the formation of an amide or peptide bond between the carboxyl group of the xenobiotic acid and the amino group of the endogenous compound. The conjugation reaction is generally accepted to be a two-step process involving initial activation of the carboxyl group to yield a reactive acyl-CoA thioester (Figure 14.2, equations 1 and 2), followed by acyl transfer to the amino group of an amino acid (Figure 14.2, equation 3) (Killenberg and Webster 1980; Caldwell 1982). Thus selectivity, or specificity, may be exerted at either the activation and/or acyl-transfer steps.

As pointed out above, the two major metabolic options of carboxylic acids have been known for well over a century and it was only relatively recently that a number of alternative metabolic pathways have been elucidated and their potential toxicological significance appreciated (Figure 14.3) (Hutson 1982; Caldwell 1984, 1985; Fears 1985). Conjugation with glucuronic acid is discussed elsewhere in this book (Chapters 5 and 8) and will not be examined in any detail here. The formation of acyl-coenzyme A (acyl-CoA) thioester intermediates (Figure 14.2, equations 1 and 2) is of significance in both the metabolism of xenobiotic acids and in intermediary biochemistry, and the alternative pathways associated with carboxylic acids are those of the acyl-CoA thioester intermediates. It is therefore appropriate to briefly provide an overview of the fate of these reactive intermediates.

1. $\text{Ar-COOH} + \text{ATP} \rightarrow \text{Ar-CO}\sim\text{AMP} + \text{PPi} + \text{H}_2\text{O}$

2. $\text{Ar-CO}\sim\text{AMP} + \text{CoA-SH} \rightarrow \text{Ar-CO}\sim\text{S-CoA} + \text{AMP}$

3. $\text{Ar-CO}\sim\text{S-CoA} + \text{NH}_2\text{CH}_2\text{COOH} \rightarrow \text{Ar-CO-NHCH}_2\text{COOH} + \text{CoA-SH}$

Figure 14.2. Reaction sequence of amino acid conjugation.

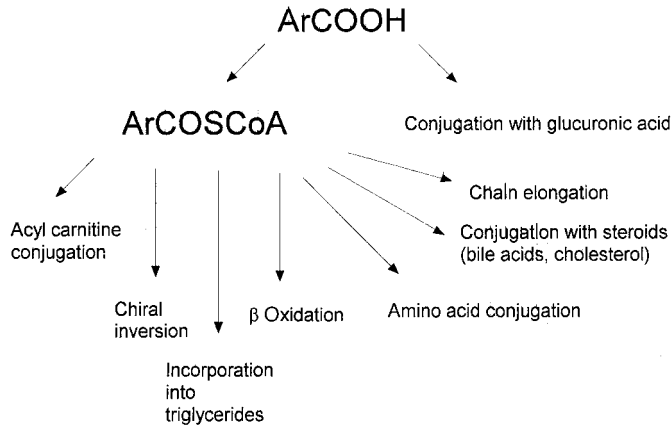

Figure 14.3 Biotransformation of xeno/endobiotic carboxylic acids.

Reactions of Acyl-coenzyme A thioesters

With respect to the topic of this chapter, the transfer of the acyl moiety to the amino group of an amino acid is obviously the most significant of the reactions outlined in Figure 14.3. However, a number of alternative pathways are possible and what may appear as relatively minor changes in the structure of the xenobiotic acid may result in significant alterations in the product ultimately formed.

Carnitine, an essential cofactor required for the transport of long-chain fatty acids into mitochondria, has been shown to yield conjugates with xenobiotic carboxylic acids following acyl transfer onto the secondary alcohol group. Thus cyclopropane carboxylic acid (Quistad *et al*. 1978a–c, 1986), pivalic acid (Vickers *et al*. 1985; Totsuka *et al*. 1992; Mizojiri *et al*. 1995) and valproic acid (Millington *et al*. 1985) are all excreted as carnitine conjugates following administration either as such, or as metabolic precursors, to both animals and man. In the case of cyclopropane carboxylic acid, the corresponding glycine conjugate is also excreted (Quistad *et al*. 1978a) and Quistad *et al*. (1986) were able to detect very small (*ca* 0.04% of the dose) quantities of benzoylcarnitine following administration of benzoic acid to the rat. Kanazu and Yamaguchi (1997) have carried out a comparative *in vitro* study to examine the relative extent of carnitine and glycine conjugation using rat hepatocytes and kidney slices. Cyclopropane and cyclobutane carboxylic acids were found to be the best substrates for carnitine conjugation in both tissues, both compounds also yielding glycine conjugates.

Acyl-transfer to oxygen may also result in the formation of sterol esters. For example, the pyrethroid insecticide fluvalinate undergoes ester hydrolysis to yield an anilino acid derivative which undergoes conjugation with bile acids in rats, chickens and cows (Quistad *et al*. 1982). Similarly, the hypolipidaemic drug, CCD (1-(4-carboxyphenoxy)-10-(4-chlorophenoxy)decane) forms a cholesteryl ester conjugate in the rat (Fears *et al*. 1982).

Acyl-transfer to carbon results in the addition of either single or multiple acetate units and elongation of the carbon chain (Caldwell and Marsh 1983). For example, 5-(4-chlorobut-1-yl)picolinic acid undergoes addition of a two-carbon unit to yield products corresponding to the β-ketoacid, α,β-unsaturated acid and the corresponding saturated analogue (Miyazaki *et al*. 1976). These metabolites were found in the urine of both animals and man following drug administration. Similarly, 3-hydroxy-3-phenylpropionic acid has been identified in horse urine following administration of benzoic acid (Marsh *et al*. 1982).

The active acyl group may become involved with the intermediates of lipid biosynthesis (Caldwell 1984) and undergo acyl transfer to oxygen to yield hybrid triacylglycerols (Hutson 1982; Fears 1985) or alternatively may be incorporated into triglycerides following chain elongation (Hutson 1982; Caldwell 1985). The pharmacological and toxicological significance of these alternative pathways, the majority of which are quantitatively minor, have been discussed elsewhere (Fears 1985; Hutson *et al*. 1985).

In the case of the 2-arylpropionic acid non-steroidal anti-inflammatory drugs (NSAIDs), e.g. ibuprofen and fenoprofen, the formation of the acyl-CoA thioesters is of pharmacological significance. These agents are used as racemic mixtures even though

their main pharmacological activity, inhibition of cyclooxygenase resides in the enantiomers of the *S*-configuration. However, the *R*-enantiomers of a number of these agents form the corresponding CoA thioesters which subsequently undergo inversion of chirality of the propionic acid moiety, followed by hydrolysis to yield the active enantiomer (Hutt and Caldwell 1983; Caldwell *et al.* 1988). Recent evidence, associated with the formation of amino acid conjugates, has indicated that the reaction, in some species, may be highly stereoselective rather than stereospecific (see below).

Amino acids and conjugation

As pointed out above, conjugation of benzoic acid with glycine is generally accepted to be the first reaction of drug metabolism to be discovered but it was not until the 1980s that Marsh *et al.* (1981) demonstrated the formation of hippuric acid following the administration of benzoic acid to the horse. Since the initial observation, a number of alternative amino acids have been shown to be involved and the history of the amino acid conjugations is summarised in Table 14.1.

The amino acid utilised for conjugation is highly dependent on both the structure of the xenobiotic carboxylic acid and the animal species under investigation. The most frequently observed amino acid conjugates are those with glycine, which is utilised by the majority of animal species for the conjugation of a wide variety of carboxylic acids including aliphatic, aromatic, heteroaromatic and phenylacetic acid derivatives.

The first example of an alternative to glycine conjugation was reported by Jaffe, in 1877, who found that benzoic acid underwent conjugation with ornithine in the hen. Ornithine conjugation, unlike the other amino acid conjugations, involves the acylation of both amino groups, thus in the case of benzoic acid the product is N^2, N^5-dibenzoylornithine or ornithuric acid. Conjugation with ornithine has been found to occur in other avian and in some reptile species (Smith 1958), and appears to be associated with uricotelic species (Killenberg and Webster 1980), species which excrete uric acid as the major nitrogenous waste product of amino acid metabolism, a

Table 14.1 Discovery of the amino acid conjugations

Amino acid	Carboxylic acid	Species	References
Glycine	Benzoic acid	Man	Keller (1842)
Ornithine	Benzoic acid	Hen	Jaffe (1877)
Glutamine	Phenylacetic acid	Man	Thierfelder and Sherwin (1914)
Serine	Xanthurenic acid	Rat	Rothstein and Greenberg (1957)
Glutamic acid	4-Nitrobenzoic acid	Spider	Smith (1962)
Arginine	4-Nitrobenzoic acid	Spider	Smith (1962)
Histidine	Benzoic acid	*Peripatus*	Jordan *et al.* (1970)
Taurine	Phenylacetic acid	Pigeon	James *et al.* (1971)
Alanine	4,4′-Dichlorodiphenylacetic acid	Mouse	Wallcave *et al.* (1974)
Aspartic acid	2,4′-Dichlorodiphenylacetic acid	Hamster	Reif and Sinsheimer (1975)
	2,4′-Dichlorodiphenylacetic acid	Rat	Reif and Sinsheimer (1975)

process characteristic of terrestrial species which develop within a shell where the nitrogenous waste products are stored in an insoluble form. Ornithine conjugation is not a general reaction of all avian species. For example, benzoic acid yields ornithine conjugates in domestic fowl (Galliformes), ducks and geese (Anseriformes), and hippuric acid in pigeons and doves (Columbiformes), whereas parrots yield neither amino acid conjugate (Baldwin *et al*. 1960). The formation of an *N*-acetylornithine conjugate of 3-phenoxybenzoic acid has been reported in the chicken (Huckle *et al*. 1982).

L-Glutamine conjugation, first reported to be the route of biotransformation of phenylacetic acid in humans (Thierfelder and Sherwin 1914), appears to be restricted in the main to arylacetic acids, e.g. phenylacetic acid and related compounds in mammals. The conjugation of phenylacetic acid and related compounds (e.g. 4-chlorophenylacetic acid and indol-3-ylacetic acid) with L-glutamine was believed to be confined to the anthropoid apes, Old and New World monkeys and humans (James *et al*. 1972a,b; Bridges *et al*. 1974). However, both phenylacetic acid and 4-chlorophenylacetic acid yield small quantities of the corresponding glutamine conjugates in the ferret (Hirom *et al*. 1977; Idle *et al*. 1978) and 2-naphthylacetic acid undergoes extensive conjugation with L-glutamine in the ferret, rabbit and rat (Emudianughe *et al*. 1977, 1978). Formation of L-glutamine conjugates of benzoic acid derivatives in the house fly and arachnids has also been reported (Smith 1962; Hitchcock and Smith 1964; Esaac and Casida 1968). With respect to drug metabolites, diphenylmethoxyacetic acid, a metabolite of diphenhydramine, yields an L-glutamine conjugate following drug administration to the Rhesus monkey (Drach and Howell 1968; Drach *et al*. 1970). In addition, α-fluorovalproic acid and the corresponding fluorinated 2-propyl-4-pentenoic acid oxidation product undergo glutamine conjugation in rats and mice (Tang and Abbott 1997; Tang *et al*. 1997).

Taurine, 1-aminoethane sulphonic acid, while not strictly an amino acid, yields conjugates with xenobiotic carboxylic acids and taurine conjugation is generally classified as an amino acid conjugation. The reaction appears to be primarily associated with carnivorous species, e.g. dog (Jordan and Rance 1974; Sakai *et al*. 1984) and ferret (Idle *et al*. 1978), and some aquatic species (James and Bend 1976; James 1982) but is known to occur also in the rat (Emudianughe *et al*. 1978; Egger *et al*. 1982; Peffer *et al*. 1987), horse (Marsh *et al*. 1981) and to a minor extent in humans (Shirley *et al*. 1994). In terms of xenobiotic acids, taurine conjugation was thought to be restricted to aryl- and aryloxyacetic acids but some aromatic, e.g. 3-phenoxybenzoic acid (Hutson and Casida 1978; Huckle *et al*. 1981c) and aliphatic acids, e.g. trimoprostil (Kolis *et al*. 1986), have been shown to undergo this conjugation.

The most commonly encountered alternative amino acids to glycine are L-glutamine, L-ornithine and taurine but there are, however, several examples of other amino acids involved in conjugation reactions but these appear to be restricted both in terms of their species occurrence and the substrate utilised (Table 14.2) (Caldwell *et al*. 1980; Quistad 1986). In addition, a small number of dipeptide conjugates have also been reported and these are summarised in Table 14.2.

Polyglutamyl conjugates of methotrexate, a folic acid antagonist, have been reported in several species including humans (Baugh *et al*. 1973; Shin *et al*. 1974;

Table 14.2 Atypical amino acids and dipeptides used in conjugation reactions

Amino acid or dipeptide	Carboxylic acid	Species	References
Alanine	4,4′-Dichlorodiphenylacetic acid	Mouse	Wallcave *et al.* (1974)
	Piperonylic acid	Hamster	Gingell (1976)
		Housefly	Esaac and Casida (1968)
Aspartic acid	2,4-Dichlorodiphenylacetic acid	Rat	Reif and Sinsheimer (1975)
Serine	4,8′-Dihydroxyquinaldic acid	Rat	Rothstein and Greenberg (1957)
	2,4′-Dichlorodiphenylacetic acid	Rat	Feil *et al.* (1973) Reif and Sinsheimer (1975)
	4,4′-Dichlorodiphenylacetic acid	Mouse	Gingell (1976)
	Piperonylic acid	Housefly	Esaac and Casida (1968, 1969)
Histidine	Benzoic acid	*Peripatus*	Jordan *et al.* (1970)
Glutamic acid	Benzoic acid	Indian fruit bat	Idle *et al.* (1975)
		African bat	Collins *et al.* (1977)
	trans 3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid	Cow	Gaughan *et al.* (1977)
	Piperonylic acid	Housefly	Esaac and Casida (1969)
	3-Phenoxybenzoic acid	Cow	Gaughan *et al.* (1977)
Arginine	Benzoic acid	Scorpion	Hitchcock and Smith (1966)
	p-Aminobenzoic acid	House spider	Smith (1962)
		Millipede	Hitchcock and Smith (1964)
		Arachnids	Hitchcock and Smith (1964)
Glycyltaurine	Quinaldic acid	Cat	Kaihara and Price (1961) Morris and Price (1963)
Glycylglycine	Quinaldic acid	Cat	Kaihara and Price (1965)
Aspartic acid and serine[a]	4,4′-Dichlorodiphenylacetic acid	Rat	Pinto *et al.* (1965)
Glycylvaline	3-Phenoxybenzoic acid	Mallard duck	Huckle *et al.* (1981a)

[a]Sequence unknown

Israili *et al.* 1977). The significance of this biotransformation is unclear but these conjugates may contribute to the biological activity of the drug (Montgomery *et al.* 1979).

Structure–metabolism relationships

The biotransformation of xenobiotic carboxylic acids is dependent on the size and the nature of the substituents surrounding the carboxyl group. The presence and nature of other functional groups within the molecule and their possible biotransformation will also influence the ultimate fate of the acid. To date there have been few systematic attempts to examine the structure–metabolism relationships of amino acid conjugation. The majority of the literature resulting from an examination of the metabolic fate of a particular xenobiotic carboxylic acid in a range of animal species, an approach

which is handicapped by both species variation in the amino acid used for the conjugation and alternative, possibly competing, metabolic pathways.

Structure–metabolism relationships for amino acid conjugation need to be carried out with some care as selectivity, or specificity, may be exerted at either of the two steps of the reaction, i.e. formation of the acyl-CoA thioester or in the acyl-transfer. In *in vivo* studies of the two major metabolic options available to carboxylic acids, i.e. conjugation with either an amino acid or glucuronic acid, the ultimate fate of the compound will depend on a number of factors including the administered dose, availability of the conjugating agent, diet and species under examination (Hutt and Caldwell 1990).

Aliphatic and alicyclic carboxylic acids

There are few examples of exogenous aliphatic carboxylic acids, or 3-aryl substituted acids containing three carbon atoms in a straight chain, undergoing amino acid conjugation, presumably due to their facile β-oxidation. However, in addition to the metabolism of xenobiotic carboxylic acids, amino acid conjugation is an important metabolic pathway for the elimination of endobiotic acids that may accumulate in a number of metabolic diseases which result in acidaemia, e.g. isovaleric acid undergoes conjugation with glycine (Tanaka and Isselbacher 1967). In cases of medium-chain acyl-CoA dehydrogenase deficiency, 3-phenylpropionic acid, a compound that normally undergoes β-oxidation to benzoic acid followed by excretion as hippuric acid, is excreted as 3-phenylpropionylglycine (Bennett *et al*. 1992). In such cases, unusual amino acids, not normally associated with amino acid conjugation, may be utilised, e.g. sarcosine (Lehnert 1983). Several investigations using purified enzyme systems for the formation of both CoA thioesters and *N*-acyltransferase activity have indicated that short-chain aliphatic acids may form amino acid conjugates *in vitro* (Mahler *et al*. 1953; Schachter and Taggart 1954; Nandi *et al*. 1979).

Several 3-arylpropionic acid derivatives have been reported to yield amino acid conjugates, the carboxylic acid metabolites of the anti-histamines brompheniramine and chlorpheniramine have been found to yield amino acid conjugates in the urine of both humans and dogs (Bruce *et al*. 1968). Glycine conjugates have similarly been reported for the unsaturated acid, cinnamic acid and the related compounds β-methylcinnamic and 3,4-dimethoxycinnamic acids (Williams 1963; Solheim and Scheline 1976; Hoskins 1984).

The metabolism of the anticonvulsant agent valproic acid involves cytochrome P450-mediated formation of 2-propyl-4-pentenoic acid which is implicated in the hepatotoxicity of the drug via β-oxidation to yield a reactive metabolite. Valproic acid also undergoes glycine conjugation in rats but to a minor extent, less than 1% of the dose. The α-fluorinated derivatives of both valproic acid and 2-propyl-4-pentenoic acid have been synthesised in order to prevent β-oxidation and hence hepatotoxicity. As a result of the structural modification, neither compound undergoes β-oxidation and the metabolic pathway switches to amino acid conjugation. Both yield L-glutamine conjugates in the rat and mouse (Tang and Abbott 1997; Tang *et al*. 1997).

The formation of taurine conjugates of relatively long chain aliphatic acids has been reported. For example the metabolism of prostaglandin E_2(PGE_2) in rat hepatocytes

yields taurine conjugates of the major metabolites arising from β-oxidation dinor and tetranor prostaglandin E_1 (PGE_1) and dinor-PGE_2 (Hankin *et al*. 1997). Whether these metabolites are produced *in vivo* is yet to be investigated. However, taurine conjugates of the PGE_2 analogue trimoprostil and its β-oxidation products have been identified in rat bile following administration of the parent drug (Kolis *et al*. 1986). Similarly both *all-trans* and 9-*cis* retinoic acids have been reported to undergo a variety of biochemical transformations in the rat resulting in the formation of highly polar metabolites identified as taurine conjugates (Skare *et al*. 1982; Shirley *et al*. 1996).

Glycine conjugation of isopropoxyacetic acid has been found in both rat and dog following the administration of isopropyl oxitol (Hutson and Pickering 1971). While the glycine conjugate of cyclopropylcarboxylic acid, a metabolite of the miticide, hexadecyclopane carboxylate, has been reported in the urine of rats, cows and dogs (Quistad *et al*. 1978a–c). The glutamic acid conjugate of *trans*-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropane carboxylic acid has been found in the cow. Both geometrical isomers have been reported to give rise to glycine, serine and glutamic acid conjugates in insects (Unai and Casida 1977). The formation of glycine conjugates of cyclohexanoic acid derivatives, hexahydrohippuric acid and 3,4,5,6-tetrahydrohippuric acid, together with hippuric acid, have been reported in rats and perfused rat liver following the administration of cyclohexanoic acid and shikimic acid (Brewster *et al*. 1977a,b, 1978). Hexahydrohippurate has also been reported to occur in the urine of cattle, horses and elephants (Balba and Evans 1977).

Aromatic carboxylic acids

Benzoic and heterocyclic aromatic acids are mainly conjugated with glycine in mammalian species, other amino acid conjugates are also generated but their occurrence is restricted in terms of species. Between 1944 and 1955, Bray and coworkers examined the fate, in terms of conjugation with either glycine or glucuronic acid, of a variety of substituted benzoic acids in the rabbit (summarised in Williams 1959), and this group of compounds is the most extensively examined in terms of quantitative structure–metabolism relationships. Hansch *et al*. (1968) using Bray's data for the *para*-substituted compounds established a parabolic relationship between the logarithm of the percentage of the dose undergoing conjugation with glycine (Log MR) and log P:

$$\text{Log MR} = -0.665(\text{Log P})^2 + 3.153\text{Log P} - 1.763$$

$$r = 0.916,\ s = 0.187,\ n = 8$$

Subsequently, examination of the data obtained for the *ortho*-substituted compounds indicated that the steric bulk of the substituent, as measured by Taft's steric parameter, E_s, was the determining factor (Caldwell *et al*. 1980), the extent of glycine conjugation expressed as a percentage of the dose decreasing as steric bulk of the *ortho* substituents increased, which Caldwell *et al*. (1980) interpreted as steric hindrance of the *ortho*-substituent for the formation of the CoA thioester.

Kasuya *et al*. (1990, 1991) examined structure–metabolism relationships for the

glycine conjugation of a series of benzoic acids using liver and kidney mitochondria from rat and mouse. Physicochemical parameters found to be of significance for the rate of glycine conjugation were Van der Waals volume and the calculated logarithm of the octanol/water partition coefficient (CLOGP). These workers also re-examined Bray's data for *para*-substituted benzoic acid conjugation in the rabbit, and similar to their *in vitro* observations both Van der Waals volume and CLOGP were found to be significant parameters (Kasuya *et al*. 1990). Overall conjugation with glycine was found to increase with lipophilicity and decrease with steric bulk of substituents in the 3- and 4-positions of the aromatic ring. More recent investigations have examined the overall fate of benzoic acids, i.e. excretion either unchanged or as the glycine or glucuronide conjugates, following administration to rabbits (again using Bray's data) and rats (Ghauri *et al*. 1992; Cupid *et al*. 1996, 1999). Using computational chemistry and multivariate statistical methods, relationships were derived which allow prediction of the urinary excretion of both conjugates together with the unchanged acid. Interestingly, the urinary excretion of benzoylglycines in the rat was found to be dependent on molecular weight and the energy of the highest occupied molecular orbital (HOMO) of the acid (Cupid *et al*. 1999), whereas in the case of the rabbit the most significant parameters were CLOGP, the molar refractivity, the partial charge on C_1 of the aromatic ring and the second principal ellipsoid axis, several of which relate to molecular size and shape (Cupid *et al*. 1996; 1999). Similar to the *in vitro* study by Kasuya *et al*. (1990), the most important physicochemical property was CLOGP (Cupid *et al*. 1996). Such species differences in the structure–metabolism relationships of a relatively simple series of compounds serve to illustrate the complexity of the reaction sequence involved in amino acid conjugation.

Arylacetic acids

The amino acid utilised for conjugation of arylacetic acids varies between species, rodents such as rats producing predominantly glycine conjugates whereas primates, including humans, utilise L-glutamine (Table 14.3). Substitution of these compounds at the α-carbon atom, also has a marked effect on their metabolic fate. For example, 2-phenylpropionic and diphenylacetic acids undergo glucuronidation rather than amino acid conjugation in both animals and humans (Dixon *et al*. 1977a,b).

Table 14.3 Variation in the amino acid conjugation (% dose) of arylacetic acids with structure and species

Species	Rat	Man	Rhesus monkey	Capuchin monkey
Amino acid	Glycine	Glutamine	Glutamine	Glutamine
Phenylacetic acid	99	93	32	64
4-Chlorophenylacetic acid	92	90	40	14
4-Nitrophenylacetic acid	61	–	–	–
1-Naphthylacetic acid	15	0	0	5
2-Naphthylacetic acid	8.6	–	–	–

Data from Caldwell *et al*. (1980), Dixon *et al*. (1977c) and Emudianaghe *et al*. (1978).

The conjugation of the regioisomers 1- and 2-naphthylacetic acid is of interest as the pattern of conjugation both in terms of the pathway, glucuronidation versus amino acid conjugation and the amino acid utilised varies considerably. The major metabolic pathway of the 1-isomer in human, rat and rabbit is glucuronidation, the cat yielding both glycine and taurine conjugates while the glycine conjugation of the compound is relatively low (15% of dose) compared to the substituted phenylacetic acids (61-99%) (Dixon *et al*. 1977c). In contrast the 2-isomer undergoes conjugation with three amino acids simultaneously, namely glycine, L-glutamine and taurine, in addition to glucuronic acid conjugation in the rat, rabbit and ferret (Emudianughe *et al*. 1978), the overall urinary recovery of the 2-isomer being reduced in comparison to 1-naphthylacetic acid but the total undergoing amino acid conjugation being greater. This difference in amino acid conjugation may be due in part to steric hindrance in the case of 1-naphthylacetic acid for the formation of either the CoA thioester or *N*-acyltransferase(s). Thus it would appear that the 2-naphthylacetic acid readily undergoes activation but that selectivity is exerted in the acyl transfer step. These data suggest that 2-naphthylacetic acid may be a useful probe compound for investigating these mechanisms.

The fate of 2-phenylpropionic acid is of interest as this compound undergoes chiral inversion in the rat (Fournel and Caldwell 1986), the initial step of which is formation of a Coenzyme A thioester. As amino acid conjugation does not take place in this species, the selectivity presumably occurs at the level of *N*-acyltransferase. In recent years a number of 2-arylpropionic acid NSAIDs, together with 2-phenylpropionic acid, have been reported to undergo conjugation with taurine, mainly in the dog (Sakai *et al*. 1984; Mori *et al*. 1985; Asami *et al*. 1995; Konishi *et al*. 1999) but also in the rat and mouse (Mohri *et al*. 1998; Egger *et al*. 1982), and to a minor extent in humans (Shirley *et al*. 1994). In addition, 2-phenylpropionic acid has been reported to yield a glycine conjugate following administration to dogs (Tanaka *et al*. 1992). In the case of some compounds, the stereochemistry of the amino acid conjugates has been investigated and there are indications that the *S*-enantiomers of the 2-arylpropionic acids may also form CoA thioesters, but to a much smaller extent than their *R*-antipodes, and that the conjugation reaction may also be stereoselective and vary with species (Tanaka *et al*. 1992; Shirley *et al*. 1994; Mohri *et al*. 1998; Konishi *et al*. 1999).

Aryloxyacetic acids

Aryloxyacetic acid derivatives, e.g. the herbicides 2,4-dichlorophenoxyacetic acid (2, 4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) together with the structurally related hypolipidaemic agent, clofibric acid, are known to undergo taurine conjugation in carnivorous, marine and other species (James and Bend 1976; James 1982; Emundianughe *et al*. 1983). 3,4-Dichlorobenzyloxyacetic acid, an agent with potential for the treatment of sickle cell anaemia, has been shown to undergo extensive taurine conjugation (60% of dose) in the rat (Peffer *et al*. 1987). This compound may be useful as a probe for taurine conjugation in other species. Both 2,4-D and 2,4,5-T have been reported to yield small quantities of amino acid conjugates following administration to rats (Grunow and Bohme 1974). An explanation for this observation,

in terms of the affinity of the corresponding CoA thioesters for the glycine *N*-acyltransferase(s) has been postulated (see below; Kelley and Vessey 1986).

Biochemical and molecular mechanisms of amino acid conjugation

ACYL-CoA SYNTHETASES

As pointed out above, the initial reaction in amino acid conjugation is the formation of an acyl CoA thioester mediated by an acyl CoA synthetase or ATP-dependent acid: CoA ligases. These synthetases are divided into three ATP- and one GTP-dependent systems, thus: short-chain/acetyl-CoA synthetase/acetate: CoA ligase (AMP), EC 6.2.1.1; medium-chain/butyryl-CoA synthetase/medium-chain fatty acid: CoA ligase (AMP), EC 6.2.1.2; long-chain fatty acyl-CoA synthetase/acyl-CoA synthetase/long-chain fatty acid: CoA ligase, EC 6.2.1.3 and medium-chain fatty acid: CoA ligase (GDP), EC 6.2.1.10.

Of these four systems, the medium-chain CoA synthetase (EC 6.2.1.2) is principally associated with the activation of benzoic acids and phenylacetic acids whereas the long-chain CoA synthetases (EC 6.2.1.3) are primarily involved in the activation of the 2-arylpropionic acids (Sevoz *et al*. 2000).

The medium-chain CoA synthetase was initially purified by Mahler *et al*. (1953) from bovine hepatic mitochondria. The enzyme showed broad substrate specificity for straight chain aliphatic acids (C_4-C_{12}) with optimal activity at C_7. In addition, benzoic and phenylacetic acids, together with several branched chain aliphatic carboxylic acids were found to be activated by this enzyme. However, salicylic and *p*-aminosalicylic acids were found not to be substrates (Schachter and Taggart 1954). Killenberg *et al*. (1971), again using the same enzyme source, purified two medium-chain CoA synthetases, one being able to activate salicylic and *p*-aminosalicylic acids while the other could not. Differences in enzyme stability were also reported, the salicylate CoA synthetase being less stable than the non-salicylate CoA synthetase. By the mid-1970s, three soluble CoA synthetases, medium-chain acyl-CoA synthetase, a salicylate CoA synthetase and a propionyl-CoA synthetase, had been purified from guinea pig liver mitochondria (Groot and Scheek 1976).

More recently, Kasuya *et al*. (1996) reported the purification and characterisation of a medium-chain acyl-CoA synthetase from bovine hepatic mitochondria. Enzyme specificity was examined using aliphatic acids (C_3-C_{10}), substituted benzoic acids and 1- and 2-naphthylacetic acid. Optimal activity was found with hexanoic acid, but benzoic acid derivatives with large alkyl and alkoxy groups in the *para*- or *meta*-positions were also highly active whereas *ortho*-substituted derivatives exhibited no activity (Kasuya *et al*. 1996). Such data corresponded to *in vivo* observations where the extent of glycine conjugation decreased with increased steric bulk of the *ortho*-substituent (Caldwell *et al*. 1980). Also of interest is that both regioisomers of naphthylacetic acid had activities similar to that of benzoic acid. The molecular mass of the Kasuya-enzyme was determined to be 65 kDa, whereas the mass of the Mahler-enzyme was estimated to be between 30 and 60 kDa (Mahler *et al*. 1953) and that of a similar enzyme isolated from rat liver mitochondria was 47 kDa (Groot and Scheek

1976). However, stability may be a problem and storage may result in either dissociation and/or degradation (Kasuya *et al*. 1996).

The rat possesses five long-chain acyl-CoA synthetases (ACS1-ACS5) (Sevoz *et al*. 2000). Each ACS appears to have a marked tissue distribution and completely different regulation from those of the others (Suzuki *et al*. 1995). Rat ACS1 is found predominantly in the liver, heart and adipose tissue (Suzuki *et al*. 1991), while ACS2 and ACS3 are the major forms in the CNS (Fujino and Yamamoto 1992, Fujino *et al*. 1996). The remaining enzymes, ACS4 and ACS5, are highly expressed in steroidogenic tissues and the small intestine (Kang *et al*. 1997; Oikawa *et al*. 1998). These regulator and tissue distribution differences may reflect the biological roles of these enzymes with regard to their function in fatty acid metabolism (Oikawa *et al*. 1998). Using rat recombinant ACS1 and ACS2 expressed in *E. coli* and (−)-(*R*)-ibuprofen or (−)-(*R*)-fenoprofen, Sevoz *et al*. (2000) reported that ACS1 appeared to be the major enzyme involved in the first step of the chiral inversion of the 2-arylpropionic acids *in vitro*.

ACYL-CoA: AMINO ACID *N*-ACYLTRANSFERASE

The transfer of the acyl group from CoA thioester to the amino group of the amino acid is catalysed by an *N*-acyltransferase. The first example of this type of enzyme to be partially purified was the glycine *N*-acyltransferase (EC 2.3.1.13) from bovine hepatic mitochondria (Schachter and Taggart 1954). The enzyme was found to show absolute specificity with respect to the amino acid but to catalyse the transfer of a variety of both aliphatic (C_2-C_{10}) and aromatic acyl groups. Moldave and Meister (1957) partially purified a glutamine *N*-phenylacetyltransferase (EC 2.3.1.14) from the cytosolic fractions of human liver and kidney. This enzyme was found to catalyse both phenacylation of glutamine and the benzoylation of glycine, the latter reaction being carried out at a considerably slower rate than the former. The kidney enzyme appeared to have a higher specific activity than the hepatic enzyme (Moldave and Meister 1957). Glycine *N*-acyltransferases have also been purified from human and bovine hepatic mitochondrial preparations (Tishler and Goldman 1970; Forman *et al*. 1971). Both enzyme preparations were found to transfer salicyl and benzoyl acyl groups from their corresponding CoA thioesters to produce salicyluric and hippuric acid respectively.

Webster *et al*. (1976) isolated and purified two acyl-CoA: amino acid *N*-acyltransferases, with a molecular mass approximately 24 kDa, from Rhesus monkey and human hepatic mitochondrial preparations. Both enzymes were shown to exhibit acyl-acceptor specificity with either glycine or L-glutamine. The 'glycine' conjugating enzyme showed acyl-donor specificity for benzoyl and salicyl Co-A while the 'glutamine' conjugating enzyme used either phenylacetyl or indolylacetyl-CoA (Webster *et al*. 1976). The presence of only one glutamine *N*-acyltransferase was indicated by the nearly constant ratio of phenylacetyl and indolylacetyl transferase activities during purification of the enzyme isolated from Rhesus monkey tissues.

Webster *et al*. (1976) also reported that the amino acid *N*-acyltransferase activity of both enzymes was inhibited by the acyl donors for the other enzyme. Nandi *et al*. (1979) found similar results following isolation of two enzymes from bovine hepatic mitochondria. However, in addition they observed that glycine was the preferred acyl

acceptor for both enzymes, with L-glutamine and L-asparagine being weak acyl acceptors. The molecular mass of these two enzymes of approximately 33 kDa was in agreement with those of Lau *et al*. (1977) and Forman *et al*. (1971) of 36 and 32 kDa respectively.

The specificity of the two bovine transferases to the CoA thioesters of 2,4-D, 2,4,5-T and phenoxyacetic acid has been examined using benzoyl- and phenylacetyl-CoAs as reference standards for activity (Kelly and Vessey 1986). Phenoxyacetyl-CoA and 2,4,5-T-CoA were found to be substrates for the phenylacetyl- and benzoyl-transferases respectively, whereas 2,4-D-CoA was a substrate for both enzymes. Both enzymes showed a high affinity for the herbicide-CoA thioesters but the reaction rates were low, which was found to be due to increased K_m values for glycine in comparison to the normal substrates (Kelly and Vessey 1986).

Organ location

Although the liver is a major site of amino acid conjugation, the kidney has been known to be involved in this biotransformation since 1870. Quick (1931) extended the early work of Schmeideberg in the 1870s, in showing that in the dog hippuric acid biosynthesis was effected by the kidney and not the liver. Amino acid conjugation has been reported at very low levels of enzymic activity in rat intestinal slices and everted sections (Strahl and Barr 1971). The formation of *p*-aminohippuric acid from *p*-aminobenzoic acid has been reported for rat and guinea pig duodenum homogenates (Irjala 1972). Rabbit small intestine extracts possessed very low levels of glycine *N*-acyltransferase activity. Lung tissue, however, was found to contain no enzymatic activity at all (James and Bend 1978). A similar pattern of results were reported in that human brain, lung, intestine and heart possessed little or no ability to form hippuric acid from benzoic acid (Caldwell *et al*. 1976).

The relative contributions of the kidney and liver to the formation of amino acid conjugates is both species and substrate dependent. The formation of glycine conjugates from *p*-aminobenzoic acid, benzoic acid and salicylic acid was undertaken in tissue slices, homogenates and mitochondria from hepatic and renal sources in the rat, guinea pig, cat and dog (Irjala 1972). The conjugation of *p*-aminobenzoic acid was greater in renal tissue slices than the hepatic tissue in all four species investigated. Benzoic acid conjugation was greater in renal than hepatic slices from cats and dogs, but no significant differences were seen in the rat and guinea pig. With salicylic acid as substrate, the renal slices showed greater activity than the liver in rat, guinea pig and dog. When homogenates were used instead of the tissue slices, a different picture was seen. Both *p*-aminobenzoic acid and benzoic acid conjugation were greater in the liver than kidney in rat and guinea pig. However, this observation was reversed in the cat and dog. Using salicylic acid as substrate, little activity was found in rat hepatic homogenates whereas using guinea pig tissue, hepatic activity was greater than renal, and in the dog the reverse was observed. Finally, using mitochondrial preparations from both renal and hepatic sources in the rat, hepatic mitochondria showed greater activity in glycine conjugation of *p*-aminobenzoic acid than renal mitochondria.

The conjugation of benzoic acid with glycine has been investigated using human

hepatic and renal cortex homogenates (Temellini *et al*. 1993). The kidney cortex was found to have the higher activity of the two organs but that the activity was normally distributed in both organs. The enzymic activity of both hepatic and renal preparations from rat, mouse, hamster, gerbil and ferret have been investigated using 3-phenoxybenzoic acid as substrate (Huckle *et al*. 1981b). These investigators examined the overall conjugation reaction, CoA thioester formation and glycine *N*-acyltransferase activity. Examination of the overall reaction indicated that in the ferret and mouse, renal activity was the greatest, the activities were similar in hamster and gerbil renal and hepatic tissue, and that the rat had greater activity in the hepatic tissue. When the activation of the substrate with CoA was investigated, similar results were observed. However, when the glycine *N*-acyltransferase activity was investigated the rat, ferret and gerbil showed higher activity in renal tissue than the liver, whereas the opposite was true for the hamster and mouse (Huckle *et al*. 1981b). It was also found that acyl-CoA formation was 10 to 300-fold slower than glycine *N*-acyltransferase activity, thus making the CoA thioester formation the rate-limiting step in glycine amino acid conjugation (Forman *et al*. 1971, Huckle *et al*. 1981c).

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