

Chapter 2: Literature Review

2.1 A historical perspective on the study of glycine conjugation

The urinary excretion of hippurate after ingestion of benzoate was first observed by Alexander Ure in 1841 (Ure, 1841). This credits Ure with the first discovery of a biotransformation reaction, a finding that started the whole field of drug metabolism research. However, interest in glycine conjugation faded significantly after this great discovery, probably because very few pharmaceuticals are metabolised by conjugation to glycine (Badenhorst et al., 2013, Knights et al., 2007). This explains why, now more than 170 years later, the significance of glycine conjugation in metabolism is still not clearly understood. As mentioned in Chapter 1, GLYAT conjugates several endogenous and xenobiotic organic acids to glycine. Acylglycines from endogenous sources include butyrylglycine, hexanoylglycine, and isovalerylglycine. The xenobiotic acylglycines include hippurate, salicylurate, and methylhippurate (Bartlett and Gompertz, 1974, Nandi et al., 1979, Schachter and Taggart, 1954, Mawal and Qureshi, 1994). It seems as though this unusual range of metabolites formed by GLYAT has contributed to the lack of understanding of the glycine conjugation pathway.

Some historical perspective sheds light on this situation. Initial studies of glycine conjugation were similar to Ure's original experiments. Benzoic acid was ingested by human or animal test subjects, followed by detection and quantification of hippurate in the urine. This led to several interesting observations such as the decreased synthesis of hippurate in individuals with schizophrenia and in hepatitis patients (Quastel and Wales, 1938, Probst and Londe, 1940, Wong, 1945, Saltzman and Caraway, 1953). In 1953 Schachter and Taggart showed that the synthesis of hippurate from benzoate and glycine is dependent on benzoyl-CoA, a high-energy form of benzoate (Schachter and Taggart, 1953). Then, in 1954, they purified GLYAT from bovine liver mitochondria and tested its ability to use several acyl-CoAs, including isovaleryl-CoA, as substrates

(Schachter and Taggart, 1954). In 1966 Tanaka and co-workers discovered isovaleric acidemia, a defect of leucine catabolism that results in accumulation of isovaleric acid in the bodily fluids (Tanaka et al., 1966). The following year they discovered isovaleryl-glycine in the urine of isovaleric acidemia patients (Tanaka and Isselbacher, 1967). This confirmed their hypothesis that isovaleric acidemia is caused by a defect of isovaleryl-CoA dehydrogenase, resulting in the accumulation of isovaleryl-CoA, which was shown by Schachter and Taggart to be a substrate for glycine conjugation (Schachter and Taggart, 1954, Tanaka and Isselbacher, 1967). These findings seem to have led Bartlett and Gompertz in 1974 to investigate the relationship between the substrate selectivity of bovine GLYAT and the acylglycines excreted in the urines of organic acidemia patients (Bartlett and Gompertz, 1974). Studies by Kølvrå and Gregersen further demonstrated the affinity of GLYAT, isolated from rat and human liver, to the straight- and branched-chain acyl-CoAs known to accumulate in patients with organic acidemias (Kolvraa and Gregersen, 1986). Then, in 1978, Batshaw and co-workers suggested that sodium benzoate could be used to treat the hyperammonemia resulting from urea cycle disorders. This therapeutic strategy is based on the conversion of excess ammonia to glycine, which is conjugated to benzoate and excreted in the urine (Batshaw et al., 1988). As a result of these discoveries, glycine conjugation became a subject of great interest to those studying and treating inborn errors of metabolism (Bartlett and Gompertz, 1974, Gregersen et al., 1986, Tanaka and Isselbacher, 1967, Barshop et al., 1989, Batshaw and Brusilow, 1981). While very important, this focus on glycine conjugation as an alternative pathway for the treatment of metabolic disorders seems to have drawn attention away from the normal role of glycine conjugation in metabolism. The rest of this chapter consists of two review articles that discuss the role and importance of glycine conjugation in metabolism.

2.2 The role of glycine conjugation in normal metabolism

In the first review (Paper I) it was argued that the primary role of glycine conjugation is the detoxification of benzoate and related aromatic acids, which are encountered in the diet. The focus was on the importance of glycine conjugation in preventing the CoASH sequestration that would result from the accumulation of acyl-CoA metabolites such as benzoyl-CoA. It was further argued that, because of its dependence on glycine, ATP, and CoASH, the glycine conjugation pathway can influence metabolism on several levels (Figure 2 and Figure 4 of Paper I).

The second review (Paper II, manuscript submitted to *Drug Metabolism Reviews*) was written in response to a recent publication in which it was argued that glycine conjugation should be viewed as a neuroregulatory process, important for the regulation of CSF glycine levels, rather than as a detoxification mechanism (D Beyoglu and JR Idle. The glycine deportation system and its pharmacological consequences. *Pharmacology & Therapeutics* 2012; 135: 151-167). According to the glycine deportation hypothesis, accumulation of the neurotransmitter glycine to toxic levels is prevented by the irreversible urinary excretion of glycine as a conjugate to benzoate (Beyoglu and Idle, 2012, Beyoglu et al., 2012). In Paper II these two perspectives were carefully analysed and compared. It was concluded that, while providing a valuable new perspective, the glycine deportation system does not provide a suitable alternative to the view of glycine conjugation as a detoxification mechanism.

∞ Paper I ∞

Glycine conjugation: Importance in metabolism, the role of glycine N-acyltransferase, and the factors that influence interindividual variation

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EXPERT OPINION

1. Introduction
2. Acyl-CoA metabolism and toxicity
3. Glycine conjugation and interindividual variation
4. GLYAT, liver cancer, hepatitis, and musculoskeletal development
5. Glycine *N*-acyltransferase
6. Summary
7. Expert opinion

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Glycine conjugation: importance in metabolism, the role of glycine *N*-acyltransferase, and factors that influence interindividual variation

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Introduction: Glycine conjugation of mitochondrial acyl-CoAs, catalyzed by glycine *N*-acyltransferase (GLYAT, E.C. 2.3.1.13), is an important metabolic pathway responsible for maintaining adequate levels of free coenzyme A (CoASH). However, because of the small number of pharmaceutical drugs that are conjugated to glycine, the pathway has not yet been characterized in detail. Here, we review the causes and possible consequences of interindividual variation in the glycine conjugation pathway.

Areas covered: The authors review the importance of CoASH in metabolism, formation and toxicity of xenobiotic acyl-CoAs, and mechanisms for restoring levels of CoASH. They focus on GLYAT, glycine conjugation, how genetic variation in the GLYAT gene could influence glycine conjugation, and the emerging roles of glycine metabolism in cancer and musculoskeletal development.

Expert opinion: The substrate selectivity of GLYAT and its variants needs to be further characterized, as organic acids can be toxic if the corresponding acyl-CoA is not a substrate for glycine conjugation. GLYAT activity affects mitochondrial ATP production, glycine availability, CoASH availability, and the toxicity of various organic acids. Therefore, variation in the glycine conjugation pathway could influence liver cancer, musculoskeletal development, and mitochondrial energy metabolism.

Keywords: acyl-coenzyme A, benzoate, CASTOR disorder, coenzyme A, coenzyme A sequestration, GLYAT, glycine conjugation, glycine *N*-acyltransferase, hepatocellular carcinoma, xenobiotics

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1. Introduction

The study of drug metabolism started with the discovery of glycine conjugation. The excretion of hippuric acid after ingestion of benzoic acid was discovered in 1841 by Alexander Ure [1]. This was later confirmed by Wilhelm Keller in 1842 who ingested 32 grains of benzoic acid and isolated hippuric acid from his urine the next morning [2]. Later, in 1845, it was demonstrated by Dessaignes that hippuric acid was in fact an amide conjugate between glycine and benzoic acid, making this the first conjugation reaction to be discovered [3]. Since this epic discovery, interest in glycine conjugation has faded significantly, and only sporadically has anything on the subject been published in the last 168 years. In this review, we wish to re-emphasize the importance of glycine conjugation and clarify its influence on the metabolism of CoASH and glycine. We also point out some

Article highlights.

- GLYAT is the enzyme responsible for glycine conjugation of the acyl-CoA esters of several xenobiotic organic acids.
- Glycine conjugation is important for the detoxification of benzoate and hydroxybenzoates that are conjugated to coenzyme A in the liver and kidneys.
- Interindividual variation in glycine conjugate excretion has been observed but the mechanisms underlying this variation are not understood.
- SNPs in human GLYAT have been shown to influence the enzymatic activity, but it is not clear how this influences variation in the glycine conjugation pathway.
- The high exposure to xenobiotics in modern times may exacerbate dietary glycine deficiency.
- Because of its influence on glycine availability, GLYAT may play a role in the development of hepatocellular carcinoma and may be involved in musculoskeletal development.

This box summarizes key points contained in the article.

serious deficiencies, of paramount importance, in our understanding of the glycine conjugation pathway.

Humans have several biotransformation systems, including conjugation to sulfate, glucuronate, and glycine, that convert various endogenous and xenobiotic metabolites to more hydrophilic conjugates that can be excreted in the urine [3–8]. The resulting conjugates are often less toxic than the parent compound, with some exceptions such as reactive acyl-glucuronides [9]. Glycine *N*-acyltransferase (GLYAT) is responsible for the glycine conjugation of xenobiotics such as benzoic acid (Figure 1). Although the small range of substrates for glycine conjugation, when compared with glucuronidation, may have contributed to the relatively little research that has been done on GLYAT [3], we will argue that the enzyme plays a central role in maintaining CoASH homeostasis in the liver. We briefly review and discuss acyl-CoA metabolism, glycine conjugation, interindividual variations, and some factors that may influence glycine conjugation. Finally, we review the literature on the GLYAT gene and enzyme, and what is known about genetic variation in the GLYAT gene and its consequences.

2. Acyl-CoA metabolism and toxicity

2.1 The importance of coenzyme A in metabolism

Coenzyme A is an extremely important molecule that can be seen as a central hub around which much of metabolism revolves [10–13]. Acyl-CoA esters are important intermediates in many anabolic and catabolic reactions. Almost all catabolic reactions result in the formation of acetyl-CoA, the fuel for both oxidative phosphorylation and lipogenesis (Figure 2) [10,14]. It is thus clear that disturbances of coenzyme A metabolism, and changes in the relationships between

CoASH and acyl-CoAs, can have severe and far-reaching consequences for metabolism as a whole [12,13]. Therefore, coenzyme A metabolism is tightly regulated, and even under ischemic conditions, levels of free and acylated CoASH in the liver stay the same, despite a doubling in acetyl-CoA levels [12].

An interesting study in which rats were fed with the pantothenate analog hopantenate demonstrates the tight regulation of hepatic coenzyme A metabolism. Hopantenate inhibits CoASH biosynthesis, and the rats died of hypoglycemia within 2 weeks with fatty liver and mitochondrial dysmorphology [13]. It was shown that hopantenate initiates a transcriptional reprogramming of the liver, which leads to an increase in expression of acyl-CoA thioesterases, and pyruvate dehydrogenase kinase isoform 1, which decreases pyruvate dehydrogenase activity. The result is increased liberation and decreased consumption of CoASH [11–13]. These observations emphasize the importance of tight regulation of hepatic CoASH metabolism and the consequences of disruption of CoASH homeostasis.

2.2 Formation of xenobiotic acyl-CoAs

Several fatty acids and xenobiotic carboxylic acids that are conjugated to amino acids must first be activated to acyl-CoAs by ATP-dependent acid:CoA ligases [3,4,15,16]. These ligase enzymes exhibit selectivity for short-, medium-, long-, or very long-chain fatty acids [15]. Several long-chain forms have been identified, which have different activities and tissue localization, and enable site-specific activation of fatty acids for specific metabolic requirements [12,17]. Most xenobiotics that undergo glycine conjugation are activated by the mitochondrial medium-chain ligases, which also activate C4–C12 acids for β -oxidation [15,18–20]. This dual role of the medium-chain ligases for fatty acid oxidation and xenobiotic activation is one of the reasons why mitochondrial accumulation of xenobiotic acyl-CoA esters may interfere with β -oxidation and disturb mitochondrial metabolism [3,21–23].

Four distinct medium-chain ligases, XL-I, XL-II, XL-III, and XL-J, have been identified in bovine liver, and have overlapping substrate specificities [18,19,22,24,25]. XL-I, XL-II, and XL-III all activate C3–C10 fatty acids and a range of arylacetic and aromatic carboxylic acids, including benzoate, 4-amino-benzoate, 4-chlorobenzoate, 4-nitrobenzoate, naphylacetate, and salicylate [15,18,20]. In humans, there are five medium-chain xenobiotic-activating enzymes. These are ACSM1, ACSM2A and ACSM2B, ACSM3, and ACSM5 [26]. Vessey *et al.* characterized two human liver medium-chain ligases, HXM-A and HXM-B, with activity toward a range of xenobiotics [3,18,27,28]. HXM-A is encoded by the ACSM2A gene [26,28]. These enzymes are less well characterized than the corresponding bovine enzymes, but have been shown to activate benzoate, hexanoate, octanoate, and decanoate. There is evidence of activation of valproate by HXM-A and salicylate by HXM-B [3,27,28]. Xenobiotics that involve activation to an acyl-CoA ester include pivalate,

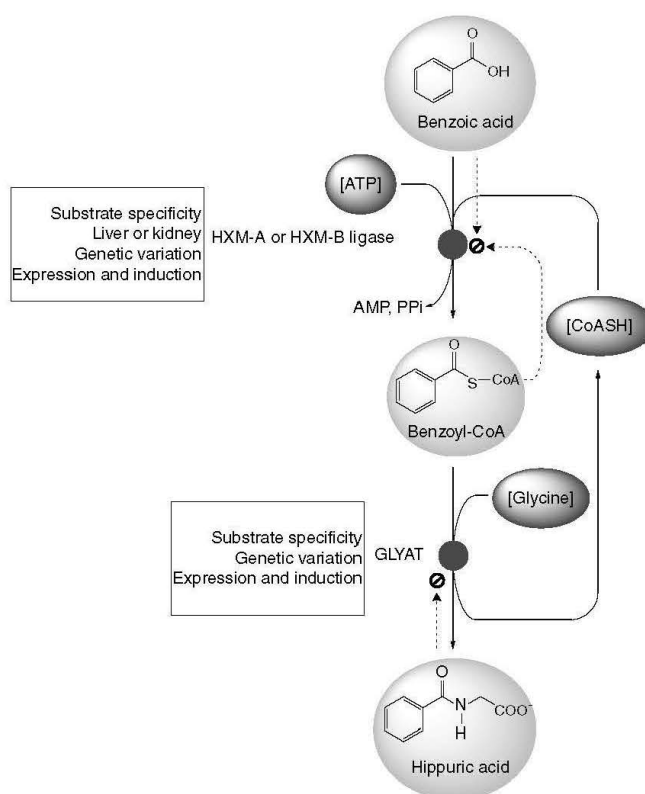


Figure 1. Glycine conjugation of benzoic acid. The glycine conjugation pathway consists of two steps. First benzoate is ligated to CoASH to form the high-energy benzoyl-CoA thioester. This reaction is catalyzed by the HXM-A and HXM-B medium-chain acid:CoA ligases and requires energy in the form of ATP. Some acyl-CoA esters can competitively inhibit the ligase enzymes. The benzoyl-CoA is then conjugated to glycine by GLYAT to form hippuric acid, releasing CoASH. In addition to the factors listed in the boxes, the levels of ATP, CoASH, and glycine may influence the overall rate of the glycine conjugation pathway. The black circles indicate the ligase and GLYAT enzymes.

AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PPI: Pyrophosphate.

valproate, benzoate, salicylate, phenylbutyrate, and several others, summarized in Table 1. Depending on the xenobiotic, glycine conjugation may occur primarily in either the liver or kidney, reflecting differences between hepatic and renal acyl-CoA formation, but this is not discussed here [29]. If a xenobiotic acyl-CoA is formed that cannot be metabolized further, it will accumulate, resulting in toxicity [4,23,30]. The mechanisms of acyl-CoA toxicity are briefly described in the following section, before looking at pathways that can restore CoASH levels and homeostasis.

2.3 Mechanisms of acyl-CoA toxicity and pathogenesis

All disorders, acquired or inherited, that involve coenzyme A sequestration, toxicity, or redistribution were conceptually

united into a group called CASTOR disorders by Mitchell *et al.* [12]. In CASTOR disorders, the degradation of acyl-CoA esters is impaired [10,14]. Grouping of the CASTOR disorders enables a clearer grasp of the underlying pathophysiology and enables better understanding of potential therapeutic strategies. The mechanisms of pathogenesis can be divided broadly into effects caused by depletion of CoASH, and effects caused by the accumulated acyl-CoA itself [4,12,30].

2.3.1 Depletion of CoASH

Depletion of CoASH is often one of the most severe consequences of acyl-CoA accumulation [4,12,13,31,32]. As described in Section 2.1, coenzyme A is a central metabolic hub and depletion can, indirectly, have far-reaching implications for both intermediary and energy metabolism. When CoASH

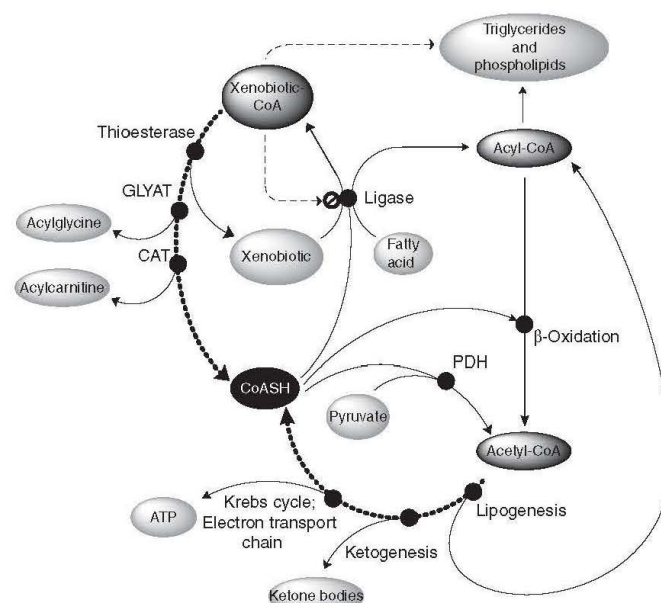


Figure 2. An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is extremely important that CoASH always be available in the cell because of its role in β -oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting the ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. The black circles indicate important processes involved in the formation and degradation of acyl-CoAs.

CAT: Carnitine acyltransferase; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PDH: Pyruvate dehydrogenase.

becomes limiting, energy metabolism is impacted on several levels [13,14,33]. The consumption of glucose, a primary metabolic fuel, results in the formation of pyruvate, which requires CoASH in order to be converted to acetyl-CoA by pyruvate dehydrogenase [13]. CoASH is also needed for β -oxidation of fatty acids, which are broken down to two-carbon units in the form of acetyl-CoA [10,13,14,31,33]. Thus, if CoASH is depleted, glucose and lipids cannot be efficiently utilized for the production of energy by oxidative phosphorylation. The result is diminished capacity for mitochondrial ATP production, increased dependence on glycolysis, and altered ratios of cellular NAD^+ and NADH [12,13,31,32]. NAD^+ is required for activity of the sirtuins, a family of NAD^+ -dependent deacetylases and ADP-ribosyltransferases [34]. These proteins play important roles in energy metabolism by regulating the activities of enzymes involved in gluconeogenesis, β -oxidation, and the electron transport chain [34]. Disturbances of NAD^+ levels can thus negatively impact the regulation of energy metabolism, but this falls outside the scope of this review.

The effects of CoASH sequestration are demonstrated by the metabolism of valproate, an anti-epileptic drug that is metabolized to valproyl-CoA. As valproyl-CoA is not a substrate for glycine conjugation, it can accumulate in the liver, deplete CoASH, and may eventually cause hepatic steatosis [3,30,31,33]. This effect is not caused by an α -fluorinated derivative of valproate, which is either not a good substrate for ligation to CoASH, or because of its increased acidity forms a less stable thioester that spontaneously hydrolyses. As a result, this α -fluorinated derivative does not cause CoASH sequestration and hepatic steatosis [3].

2.3.2 Toxic effects of accumulating acyl-CoAs

Accumulation of acyl-CoAs can also negatively influence energy metabolism by causing a depletion of carnitine, which is the transporter of fatty acids over mitochondrial membranes [10,14]. When an acyl-CoA accumulates to high enough amounts, it may become a substrate for carnitine acyltransferases, resulting in the formation of an acyl-carnitine that can be

Table 1. Xenobiotics that are metabolized to acyl-CoA and glycine conjugates.

Xenobiotic	Glycine conjugate formed in humans	CoA sequestration or toxicity	Notes
2,4,5-Trichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
2,4-Dichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
3-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
4-Aminobenzoate (PABA)	Yes	No	Seems to be well tolerated; slow glycine conjugation of PABA has been correlated to liver failure and hepatitis probably because of decreased formation of aminobenzoyl-CoA [3,47,65,66]
4-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
Astemizole	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Benzoate	Yes	No	Good substrate for glycine conjugation; large doses of benzoate are tolerated; we believe that benzoate would cause severe CoASH sequestration in the absence of GLYAT activity [2,27,45,59,78]
Brompheniramine	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Ferulic acid	Yes	No	From metabolism of ferulate-containing plant material by gut microorganisms [100]
Hypoglycine	Yes	Sequestration and toxicity	Glycine conjugation is not fast enough to detoxify the acyl-CoA metabolite, which is an irreversible inhibitor of dehydrogenase enzymes [3,4]
Ibuprofen	No	Sequestration	Taurine conjugate is formed; interaction between salicylate and ibuprofen was observed for the bovine medium-chain ligase enzymes; causes CoASH sequestration in rat liver [3,4,22]
Indoleacetic acid	Yes	No	Usually conjugated to glutamine; associated with gut microbe dysbiosis; not activated to acyl-CoA by HXM-A or HXM-B [27,81,100]
Naphthylacetic acid	No	Unknown	Weak activation by HXM-A and HXM-B; unlikely to cause CoASH sequestration [27]
Nicotinic acid	Unknown	No	Very weak activation by HXM-B [27]
Permethrin	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Phenylacetic acid	Yes	Unknown	Usually conjugated to glutamine; associated with gut microbe dysbiosis [81,100]
Pivalic acid	No	Sequestration	Not a substrate for human GLYAT [3,32]
Salicylate	Yes	Uncertain, toxic	Activation of salicylate to salicylyl-CoA is slow, making CoASH sequestration unlikely; toxicity and associated Rye-like syndrome are possibly caused by inhibition of carnitine acyltransferases by salicylyl-CoA; salicylic acid at therapeutic doses can also inhibit bovine ligase enzymes, suggesting another mechanism of toxicity [3,22,27,39,78]
Toluene	Yes	No	Metabolized to hippuric acid; no interaction was observed between toluene and the xylenes at the doses used in the investigation [83]
Triflusal	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Valproate	No	Sequestration and toxicity	Can cause hepatic steatosis because of CoASH sequestration [3,18,67]
Xylenes	Yes	No	These solvents are metabolized to methylhippurates; <i>m</i> -xylene has been shown to interact with salicylate conjugation [63,83]

PABA: *Para*-aminobenzoic acid.

excreted in the urine [21,32,35,36]. For example, benzoic acid administration results in benzoyl-carnitine excretion and a decrease in plasma-free carnitine levels [35].

Xenobiotic acyl-CoAs can substitute for acetyl-CoA in lipogenesis, resulting in odd-chain, branched-chain, aromatic, and other unnatural fatty acids, which cannot be properly catabolized and may be incorporated into cell membranes [3,30,31]. For example, propionyl-CoA, which accumulates in propionic acidemia, can be the substrate for synthesis of odd- and branched-chain fatty acids [37]. It has also been shown that 2-arylpropionyl-CoA esters, metabolites of the nonsteroidal anti-inflammatory drugs (NSAIDs), can be incorporated into adipocyte triglycerides [15,31]. Enzymes may be competitively or allosterically inhibited by acyl-CoAs, with effects that are difficult to predict [12,21–23,30,38,39]. For example, protein kinase C activity, important in signal transduction, is perturbed by ciprofibril-CoA, a metabolite of the hypolipidaemic drug ciprofibrate [15]. Propionyl-CoA, at high concentrations, inhibits formation of N-acetylglutamate by N-acetylglutamate synthetase, resulting in urea cycle dysfunction and hyperammonemia [37].

2.4 Restoration of CoASH levels

There are a few basic mechanisms that can restore depleted CoASH reserves, including conjugation to amino acids or to carnitine, and hydrolysis of acyl-CoAs by thioesterases (Figure 2) [3,12,40]. Acyl-CoA thioesterases hydrolyze acyl-CoA esters to free organic acids and CoASH. This is an indispensable metabolic necessity, because CoASH must always be available to maintain a proper metabolic milieu [10,14]. Thioesterases may have selectivity for short-, medium-, long-, and very long-chain acyl-CoAs, and are found in almost every compartment of the cell, including the cytoplasm, peroxisomes, microsomes, and mitochondria [3,12,41]. There is a direct relationship between cellular levels of CoASH, long-chain acyl-CoAs, peroxisomal β -oxidation, and cellular thioesterase activity, with thioesterases playing a role in regulation of peroxisomal and intracellular lipid metabolism [10,41]. It has been suggested that the accumulation of a particular xenobiotic acyl-CoA will, in part, reflect the relative activity and substrate selectivity of the various thioesterases [15]. In the following section, the main focus is on glycine conjugation, a primary mechanism for the restoration of CoASH levels.

3. Glycine conjugation and interindividual variation

3.1 The metabolic role of glycine conjugation

Although GLYAT can conjugate a variety of endogenous and xenobiotic acyl-CoAs to glycine, the normal metabolic role of GLYAT seems to be the detoxification of dietary benzoates [4,42–45]. On a daily basis, humans consume varying quantities of benzoate, a metabolite found in plant material [45]. In addition, plant material contains complex

polyphenols, which are fermented by the colonic flora to benzoate, 3- and 4-hydroxybenzoates, and the corresponding hydroxyphenyl-propionates [44]. After intestinal absorption, these compounds are transported to the liver, where they are conjugated to CoASH by the medium-chain xenobiotic acid:CoA ligases [3,15,18,45]. Because these benzoates are ubiquitous in plant-containing diets, it is clear that hepatic CoASH would be rapidly sequestered if the xenobiotic acyl-CoAs could not be further metabolized. GLYAT plays a major role in restoring CoASH levels by conjugating these xenobiotic acyl-CoAs to glycine. Therefore, excretion as the corresponding glycine conjugates is the major metabolic fate of ingested polyphenols [44,45]. Other natural substrates for conjugation to glycine include salicylate, a common plant metabolite, and 4-aminobenzoate [39,46–48]. About 83 – 90% of ingested benzoate and about 75 – 84% of ingested salicylate are excreted as glycine conjugates [49]. Decreased benzoate production by the gut microorganisms in patients with Crohn's disease is correlated with decreased hippurate excretion in the urine [45].

3.2 Glycine conjugation in metabolic diseases

In several organic acidemias, an acyl-CoA accumulates to toxic levels because of a defect of the enzyme acting on it [14,43,50]. This results not only in CoASH sequestration, but because of thioesterase activity, free organic acids are released, causing potentially deadly acidoses [51–53]. Because some of the acyl-CoAs that accumulate in organic acidemias are substrates for GLYAT, glycine conjugation impacts on the biochemical profiles and clinical outcomes of some of these metabolic defects [42,43,53–55]. Glycine conjugation under these abnormal conditions sheds light on the important role GLYAT plays in maintaining CoASH levels.

In some cases, the accumulating acyl-CoA can be conjugated to glycine by GLYAT, decreasing the severity of CoASH sequestration and avoiding acidosis, as a less toxic acylglycine is formed and excreted [42,43,50,56]. It was demonstrated that a relationship exists between the kinetics and substrate selectivity of a bovine liver GLYAT, and the acylglycines excreted in the urine of patients with organic acidemias [42]. For example, in isovaleric acidemia, where isovaleryl-CoA accumulates, large amounts of isovalerylglycine are excreted in the urine because isovaleryl-CoA is a good substrate for GLYAT [50,55,57]. However, in propionic acidemia, only relatively small amounts of propionate are excreted as propionylglycine [37]. This is because, despite having similar K_M values for isovaleryl-CoA and propionyl-CoA, bovine GLYAT conjugates propionyl-CoA at a much lower rate [42,58]. Unfortunately, a similar comparison cannot yet be made for the human enzyme, as its kinetic parameters are not as well characterized. Glycine conjugates are also excreted in several other organic acidemias, and include 3-methylcrotonylglycine, hexanoylglycine, butyrylglycine, and tiglylglycine [14,43,52,53]. There is no simple relationship between GLYAT substrate selectivity, in terms of K_M and V_{max} parameters, and acyl

group structure. This makes it difficult to predict the extent to which glycine conjugation will influence the outcome of any particular organic acidemia [4,42].

3.3 Glycine availability can be a limiting factor in glycine conjugation

The presence of isovaleryl-glycine in the urine of patients with isovaleric acidemia suggested that glycine supplementation may help to detoxify the accumulating isovaleryl-CoA [50,55]. In fact, glycine supplementation has been shown to increase the levels of isovaleryl-glycine excreted, and usually decreases the severity of the disease to allow normal physical and mental development [50,55,57]. This suggests that availability of glycine may be a limiting factor in glycine conjugation under some conditions [50,59,60]. When glycine conjugation is maximal, glycine may be depleted, and other amino acids may be used, as suggested by the detection of isovaleryl-conjugates of 19 other amino acids in the urine of isovaleric acidemia patients [61]. It was proposed, although not experimentally verified, that these conjugates are also formed by GLYAT. Glycine availability also influences benzoate conjugation, as demonstrated by the dose-dependent increase in hippurate formation after administration of glycine [59]. Cysteamine, which decreases the activity of the glycine cleavage system and doubles hepatic glycine content, increases the benzoate clearance by 50% in rats [59].

3.4 Interindividual variation in glycine conjugation

Glycine conjugation is also manipulated for the treatment of hyperammonemia in urea cycle disorders, by administration of benzoate, which is conjugated to form hippurate, allowing excess nitrogen to be excreted from the body [36,60]. Interindividual variation in responsiveness to administration of glycine and benzoate, respectively, was observed in both isovaleric acidemia and hyperammonemia [56,57,62]. For example, variation in clinical outcome and responsiveness to glycine supplementation was observed in a group of South African isovaleric acidemia patients, all homozygous for the same isovaleryl-CoA dehydrogenase mutation [56]. It was suggested that interindividual variation in GLYAT activity may partly account for this, but further investigation is needed.

It has also been shown that there is significant interindividual variation in the rate of glycine conjugation of xenobiotics [35,48,49,62,63]. Greater similarity between identical twins than between fraternal twins in the glycine conjugation of salicylate suggested that there is a genetic component to this variation [48]. Using human liver samples, it was demonstrated that there is interindividual variation in the capacity for hippurate synthesis from benzoate, and that the elderly seem to have a slightly decreased capacity [64]. In a large group of subjects, a coefficient of variation of approximately 15–24% was found for the formation of hippurate and salicylurate from benzoate and salicylate, respectively [49]. However, no significant difference was found between the mean values for

children, adults, the elderly, or patients with liver disease. There was, however, higher variation between the individuals with liver disease, suggesting that the rate of glycine conjugation is influenced by liver disease (Section 4.1) [47,49,65,66]. Glycine conjugation is a saturable process, and this has consequences for co-administration of different substrates for this pathway [3,4,39,45,48,49,64]. For example, although there is inter-individual variation in the total amount of glycine conjugates excreted by healthy adults, the total amount of glycine conjugates excreted is the same for each individual whether aspirin and *m*-xylene (excreted as 3-methylhippurate) are administered separately or simultaneously [63].

It is important to note that all these studies on glycine conjugation of xenobiotics report variation for the whole pathway, including ligation to CoASH and conjugation to glycine [3,35,48,49,62–64]. Several factors can influence the overall rate of glycine conjugation, including the availability of ATP, CoASH, and glycine, variation in acid:CoA ligase activity, and GLYAT enzyme activity (Figure 1) [3,4,55,59,67]. The limiting step is substrate dependent and can be either ligation to CoASH, as with salicylate, or conjugation to glycine, as with benzoate [3,29,64].

4. GLYAT, liver cancer, hepatitis, and musculoskeletal development

4.1 GLYAT, liver cancer, and hepatitis

Recently, a complete downregulation of transcription of the GLYAT gene was observed in 32 of 41 hepatocellular carcinoma specimens investigated, with significant downregulation in the other nine specimens [68]. This was confirmed by immunohistochemistry using a GLYAT-specific antibody, which revealed that GLYAT is not expressed in cancerous cells, but is expressed in neighboring healthy hepatocytes. Interestingly, GLYAT expression was found to be significant and similar in all noncancerous liver specimens studied, including 60 samples from patients with chronic hepatitis of various etiologies [68]. This observation may be explained by the expression of GLYAT in differentiated hepatocytes, but not in dedifferentiated cancerous cells. On the basis of these findings, Matsuo *et al.* proposed that suppression of GLYAT transcription may be a novel marker of hepatocellular carcinoma and is a key event in the development of liver cancer. There could also be a relationship between GLYAT activity, glycine availability, and cancer cell proliferation [69,70]. This is further elaborated on in Section 7.

It has been reported that the fraction of 4-aminobenzoate excreted as glycine conjugates correlates well to functional hepatic reserves in patients with hepatitis. Therefore, the measurement of glycine conjugation of 4-aminobenzoate has been proposed as a liver function test [47,65,66]. GLYAT expression is normal in hepatitis specimens and it has been suggested that the lower glycine conjugation observed for hepatitis patients could be explained by impaired hepatic β -oxidation and lower availability of ATP for ligation of benzoate to CoASH [3,68].

4.2 GLYAT, glycine, and musculoskeletal development

It was recently proposed that the GLYAT gene may be involved in determining lean muscle mass and bone size in humans [71]. About 690 000 single nucleotide polymorphisms (SNPs) were analyzed in large groups of unrelated Han Chinese (1627) and American Caucasian (2286) individuals to search for variations in the genome that correlate to variation in lean muscle mass and bone size. Fourteen SNPs with significant correlation were identified, three of which are located in or near the GLYAT gene (rs2507838, rs7116722, and rs11826261). Guo *et al.* explained this correlation by stating that GLYAT is important in the metabolism of glucose, but we are not aware of a direct relationship between GLYAT and glucose metabolism. The correlation they report is significant, however, suggesting that GLYAT may play an as yet unknown role in musculoskeletal development [71]. The significance of this observation is further elaborated on in Section 7.

5. Glycine *N*-acyltransferase

5.1 Biochemical and enzymatic characteristics of GLYAT

GLYAT is a monomeric detoxification enzyme found in the mitochondrial matrix of mammalian liver and kidney [58,60,68,72-80]. GLYAT was first identified in bovine liver mitochondria in 1953 and subsequently isolated and characterized from human liver mitochondria in 1976 [16,81]. GLYAT catalyses the transfer of an acyl group from an acyl-CoA to the amino group of glycine, forming an acylglycine and CoASH. Both products of the reaction are powerful inhibitors, and product inhibition is readily observed in enzyme assays [73,82]. Human GLYAT can use several endogenous and xenobiotic acyl-CoAs as substrates, as is evidenced by excretion of corresponding acylglycines in urine (Table 1) [4,35,42,43,50,54,63,83]. However, very little information is available on the kinetic parameters of human GLYAT [60,72,74,78,81]. The apparent K_M (benzoyl-CoA) value is reported to range from 13 μ M to 57.9 mM, and the V_{max} value using benzoyl-CoA and glycine is reported as 700 nmol/min/mg and 17.1 μ mol/min/mg (Table 2). This large variation in reported values is difficult to explain, but differences in the method of kinetic analysis, substrate quality, enzyme quality, experimental technique, and perhaps genetic heterogeneity of the GLYAT gene may be responsible [81,84]. The molecular mass of human GLYAT has been reported as 24, 27, 30, and 30.5 kDa [60,74,78,81]. This variation in reported values may be partly explained by the different techniques used in the different studies. For example, Kelley and Vessey [73] found that bovine GLYAT bound to their gel filtration matrix, resulting in erroneous molecular mass estimates.

No structure has been reported for GLYAT; thus, little is known about structure-function relationships. However, GLYAT is a member of the GNAT (Gcn5-related *N*-acetyltransferase) superfamily. Because of the remarkable structural conservation in the GNAT superfamily, a molecular model of

bovine GLYAT could be generated by homology modeling [85]. The model was used to propose that Glu²²⁶, a highly conserved residue, is catalytically important. Kinetic characterization and pH profiling of an E226Q mutant demonstrated that Glu²²⁶ acts as a general base that deprotonates glycine before nucleophilic attack on the carbonyl of the acyl-CoA thioester (Figure 3) [85].

5.2 The GLYAT gene and genetic variation

The human GLYAT gene is located on chromosome 11 at position 11q12, spans over 23 000 base pairs, and contains six exons [40]. Two splice variants of human GLYAT mRNA exist, coding for isoforms a (296 residues) and b (162 residues). The transcript for isoform b does not contain exon 6, and there is no protein level evidence for the existence of isoform b [86]. Within the GLYAT gene, there are approximately 668 known SNPs (www.ensembl.org, February 2013), of which 12 are synonymous and 39 are nonsynonymous. Only two studies on relatively small groups of Japanese and French Caucasian individuals have reported novel genetic polymorphisms and allele frequencies of SNPs in human GLYAT [86,87]. The N156S variant had allele frequencies of 97 and 85% in the French Caucasian and Japanese populations, respectively. Because of this high frequency, it was suggested that the N156S allele, rather than the reference sequence (NM_201648.2), should be considered as the wild-type allele [86,87].

In a recent study, the relative enzyme activities of six known polymorphisms (K16N, S17T, R131H, N156S, F168L, and R199C) of a recombinant human GLYAT were compared to the enzyme encoded by the reference sequence (NM_201648.2) [84]. The N156S variant had a greater relative activity than the reference sequence, and this might further support the suggestion that the N156S allele represents the wild-type enzyme. It is interesting to note that the variants with low allelic frequencies (R131H, F168L and R199C) had higher apparent K_M (benzoyl-CoA) values or lower relative enzyme activity when compared to the reference sequence [84,86,87]. The V_{max} values of the variants investigated range from approximately 500 to 1200 nmol/min/mg, and the apparent K_M (benzoyl-CoA) values range from approximately 20 to 70 μ M (Table 3). Compared to the reference sequence, the K16N, S17T, and R131H variants had similar activities, the F168L variant had decreased activity and an increased K_M (benzoyl-CoA) value, while the R199C variant had < 5% activity. These results indicate that SNP variations found in the human GLYAT gene may result in altered properties of the enzyme, and could perhaps explain some of the differences in kinetic parameters reported in the literature [84]. A molecular model of human GLYAT was used to help explain the altered kinetic properties of the R131H, F168L, and R199C variants of human GLYAT [84].

5.3 Paralogs of the human GLYAT gene

GLYAT is one of four putative glycine-conjugating enzymes. Two GLYAT-like genes, GLYAT-L1 and GLYAT-L2, are

Table 2. Kinetic parameters of human GLYAT.

Parameters	Values
K_M (benzoyl-CoA) (μM)	13 [60] 67 ± 5 [74] 57900 [78]
$V_{\max}$ (nmol/min/mg)	700 [60] 17100 [78]

located with the GLYAT gene on chromosome 11q12.1, while the GLYAT-L3 gene is located on chromosome 6p12.3 [40,68,88]. In addition to GLYAT, primates have another transferase that conjugates arylacetyl-CoAs to glutamine, forming phenylacetylglutamine and indoleacetylglutamine [74,81]. The GLYAT-L1 gene codes for the glutamine-conjugating enzyme in humans [68]. Both mitochondrial and cytoplasmic localizations of GLYAT-L1 have been reported, and this could be explained by the two alternative splice variants of GLYAT-L1 mRNA, which code for two isoforms (333 and 302 residues) with distinct N-termini and possibly different subcellular localization [40,81]. The 302-residue isoform of GLYAT-L1 is located in the cytoplasm and transcriptionally activates the heat shock factor pathway in HEK293T cells [40]. The two isoforms might thus have different functions in the mitochondria and cytoplasm [40,68,81]. It has not been investigated why primates, unlike other mammals, conjugate arylacetates to glutamine instead of glycine.

GLYAT-L2 mRNA is expressed in salivary gland, trachea, spinal cord, and skin fibroblasts. The enzyme is localized to the endoplasmic reticulum, and a recombinant GLYAT-L2 catalyses the formation of long-chain acylglycines such as N-arachidonoylglycine and N-oleoylglycine [88,89]. These are members of a class of cannabinoid-like signaling hormones that activate G-protein-coupled receptors and have antinociceptive, anti-inflammatory, and antiproliferative effects [90]. GLYAT-L2 activity is regulated by acetylation on Lys¹⁹ and mutation of Lys¹⁹ of a recombinant GLYAT-L2 to arginine or glutamine resulted in a 70–80% decrease in enzyme activity [89]. Mutation of the equivalent Lys²⁰ residue of a recombinant human GLYAT to arginine or glutamine did not cause a similar reduction in enzyme activity, suggesting that acetylation of this lysine residue is not important in regulation of GLYAT activity [84,89]. No enzyme activity has been reported for GLYAT-L3, which does not seem to have the catalytic glutamate residue proposed for the GLYAT reaction mechanism, but the significance of this is unclear [85,88].

6. Summary

Compared to the cytochrome P450 and UDP-glucuronosyltransferase superfamilies of biotransformation enzymes, GLYAT is not very well characterized [3,4]. This may be because of the small number of pharmaceutical drugs that are metabolized to glycine conjugates and the difficulty in

obtaining human material and xenobiotic acyl-CoA substrates for research [3]. In this review, we have demonstrated that glycine conjugation is an important metabolic pathway that plays a role in the metabolism of CoASH and glycine and can influence mitochondrial energy production (Figures 2 and 4) [4,84]. Recent studies suggest that GLYAT may also be an important factor in the development of hepatocellular carcinoma and could influence musculoskeletal development and growth [68,71].

A range of xenobiotic acylglycines are excreted in urine, indicating that either the parent xenobiotic or a carboxylate metabolite is a substrate for ligation to CoASH, and that the acyl-CoA is a substrate for glycine conjugation (Table 1) [4,29,35,44,50,63,65,83]. The toxicity of xenobiotic carboxylates is partially determined by the extent to which an acyl-CoA, that cannot be conjugated to glycine or some other acceptor, is formed [3,4,31,33]. This leads to accumulation of the acyl-CoA, which can have several toxic effects in addition to disrupting mitochondrial energy production [23,33]. In severe cases, this can lead to hepatic steatosis and death [3,12]. As the glycine conjugation pathway is saturable, variation in the rate of glycine conjugation influences the clearance of xenobiotics and thus toxicity [3,64]. If the rate of conjugation by GLYAT is low, glycine conjugation may not prevent the toxicity of an acyl-CoA, even if it is a substrate for the enzyme (Table 1) [3,37,42,57].

It is often unclear whether variation in the rate of glycine conjugation results from differences in acid:CoA ligase activity or GLYAT activity (Figure 1) [4,55,59,67]. This is complicated by the observation that the limiting step depends on the xenobiotic, making it difficult to compare the results of different studies [29,64]. The recent expression and characterization of recombinant human GLYAT enzymes made an important contribution to our understanding of variation in glycine conjugation by demonstrating that genetic variation in the GLYAT gene can influence GLYAT enzyme activity [68,84,88,89].

7. Expert opinion

7.1 GLYAT and its relationship to liver cancer and musculoskeletal development

It was recently proposed that the GLYAT gene may play important roles in development of both hepatocellular carcinoma and the musculoskeletal system [68,71]. We suggest that these relationships may be explained by the role of GLYAT in glycine metabolism. Glycine is commonly considered as a nonessential amino acid because it can be synthesized from serine (Figure 4) [91,92]. However, studies over the past two decades have shown that glycine is in fact a semi-essential amino acid, that humans may have a daily shortage of about 10 g of glycine and that this may impact on collagen turnover and the synthesis of bile acids, creatine, glutathione, and heme [8,91–93].

Under certain conditions, GLYAT can conjugate sufficient amounts of glycine to limit its availability for other metabolic

C. P. S. Badenhorst *et al.*

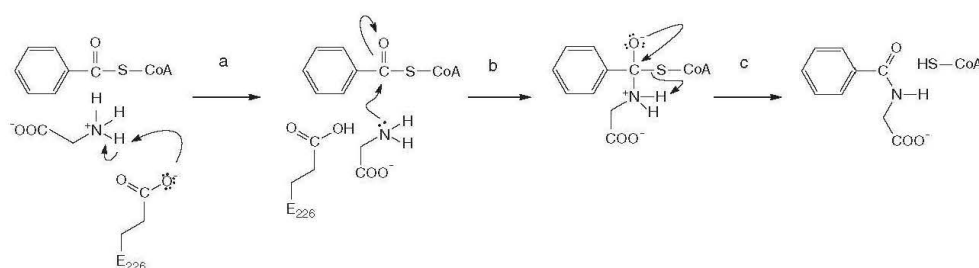


Figure 3. The catalytic mechanism proposed for bovine GLYAT. Bovine GLYAT employs a ternary complex mechanism, where Glu²²⁶ serves as a general base catalyst. (a) For nucleophilic attack to take place, the glycine amino group must be deprotonated by Glu²²⁶; (b) A tetrahedral intermediate is formed, following the nucleophilic attack by the amino group of glycine on the thioester carbonyl group. (c) Finally, the tetrahedral intermediate collapses, forming benzoylglycine and CoASH. ChemDraw 10.0 (CambridgeSoft, Cambridge, MA) was used to produce this schematic.

Reproduced with permission [85] from the American Society for Pharmacology and Experimental Therapeutics.

Table 3. Kinetic parameters of recombinant human GLYAT enzymes.

Recombinant GLYAT	K_M (benzoyl-CoA) (μM)	V_{max} (nmol/min/mg)
Flag-His ₆ -hGLYAT (N156S)	209	807 [68]
Trx-His ₆ -hGLYAT (NM_201648.2)	24 $\pm$ 3	730 $\pm$ 30 [84]
Trx-His ₆ -hGLYAT (K16N)	21 $\pm$ 1	1030 $\pm$ 20 [84]
Trx-His ₆ -hGLYAT (S17T)	28 $\pm$ 5	665 $\pm$ 40 [84]
Trx-His ₆ -hGLYAT (R131H)	71 $\pm$ 11	1040 $\pm$ 85 [84]
Trx-His ₆ -hGLYAT (N156S)	38 $\pm$ 4	1230 $\pm$ 60 [84]
Trx-His ₆ -hGLYAT (F168L)	53 $\pm$ 6	500 $\pm$ 30 [84]
Trx-His ₆ -hGLYAT (R199C)	Not determined	Not determined [84]

processes (Figure 4) [61,94,95]. For example, it has been shown that administration of benzoate to rats can reverse chemically induced porphyria by diverting glycine away from heme biosynthesis. This results in normalization of urinary δ -aminolevulinat, porphobilinogen, and porphyrin levels, an effect cancelled out by co-administration of glycine [8,94]. We suggest that the recently reported correlation of SNPs in and near the GLYAT gene to variation in lean muscle mass and bone size could be explained, in part, by the impact of GLYAT on the availability of glycine for the synthesis of creatine, collagen, and elastin [71,91,92]. It is interesting that apart from the normal expression of GLYAT in liver and kidney, low levels of GLYAT expression have also been observed in skeletal muscle, but the significance of this observation is unclear [68].

It was recently demonstrated that glycine is a metabolite crucial for rapid division of cancer cells and that inhibition of glycine uptake or biosynthesis impaired the cancer cell growth, probably by slowing the synthesis of nucleic acids (Figure 4) [69]. We suggest that this helps to explain why GLYAT is not expressed in hepatocellular carcinoma, as depletion of hepatic glycine by GLYAT would inhibit rapid proliferation of cancer cells [68-70,96,97]. This could have

significant implications for both the diagnosis and treatment of liver cancer.

7.2 The increased demand for glycine conjugation in modern life

In modern times increasing exposure to benzoate, salicylate, solvents, and drugs that are metabolized to acyl-CoA intermediates places more pressure on the glycine conjugation pathway, possibly exacerbating metabolic glycine shortage discussed previously [3,4,63,83,84,91,93]. Therefore, the consequences of interindividual variation in the glycine conjugation pathway may become more significant as more xenobiotic organic acids are encountered in the future [3].

In addition to xenobiotics, SCFAs produced by intestinal microbes are another potential source of substrates for glycine conjugation and may contribute to glycine depletion under some conditions [98]. Gut microbes produce large amounts of SCFAs that account for 5 – 10% of the total dietary energy intake in humans [98]. Indeed, the gut contains an active SCFA ligase for metabolizing these organic acids [99]. Gut dysbiosis, caused by antibiotic use, for example, can result in increased SCFA production and this has been associated with obesity and diabetes [100,101]. SCFAs are not usually

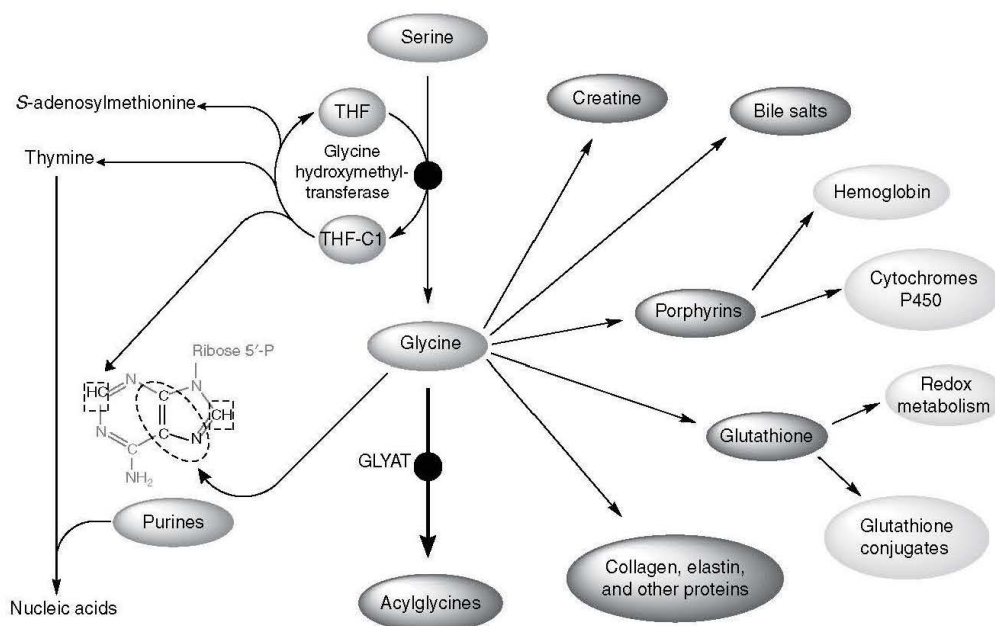


Figure 4. Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesized from serine by glycine hydroxymethyltransferase. The reaction converts tetrahydrofolate (THF) to tetrahydrofolate-C1 (THF-C1) for each molecule of glycine produced. The total amount of glycine synthesized can thus not exceed the amount of THF-C1 consumed through the production of S-adenosylmethionine, thymine, and purines. Glycine is used in the production of glutathione, creatinine, bile salts, porphyrins, collagen, elastin, and other proteins. The bold arrow indicates the formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively. The black circles indicate the glycine hydroxymethyltransferase and GLYAT enzymes.

conjugated to glycine, as this would be energetically wasteful [14,42,43]. However, it is our opinion that if sufficiently large amounts of SCFAs are produced, hepatic metabolism of CoASH and glycine will be affected. A recent observation in our laboratory seems to support this idea. A patient with unusually high levels of urinary butyrate complained of bad body odor and was referred to our laboratory by a physician. The increased butyrate excretion was not the result of a short-chain acyl-CoA dehydrogenase defect, and gut dysbiosis was suspected. Glycine supplementation was recommended and this resulted in significantly increased butyrylglycine excretion, decreased butyrate excretion, and disappearance of the body odor (unpublished results).

7.3 Future investigations of interindividual variation in glycine conjugation

In conclusion, we believe that it is important to study the relationships between genetic variation in the GLYAT gene, GLYAT enzyme activity, the *in vivo* rate of glycine conjugation, and physiological consequences of variation in the glycine conjugation pathway.

Existing publications on interindividual variation in the glycine conjugation pathway do not discriminate between variation in acyl-CoA formation and variation in glycine conjugation. It is important to remember that glycine conjugation is a two-step process and that the overall rate of glycine conjugation can be influenced by several factors (Figure 1) [3,4,55,59,67]. Most importantly, the limiting step in the glycine conjugation pathway depends on the xenobiotic used [29,64]. We suggest that future studies employ at least salicylate and benzoate as probe compounds, on separate occasions, to enable differentiation between variation in acid:CoA ligase and GLYAT activities, respectively.

The use of benzoate as a probe compound is, however, complicated by the metabolism of gut microorganisms. It was mentioned in Section 3.1 that gut dysbiosis in Crohn's disease results in decreased microbial benzoate production and lower levels of hippurate in urine [45]. Gut metabolism will thus influence relative increases in urinary hippurate levels after benzoate ingestion without necessarily affecting the rate of hepatic glycine conjugation [44,45]. Therefore, it is important to determine increases in hippurate excretion rather

than ratios to baseline levels [45]. An alternative probe compound is 4-aminobenzoate [65]. However, substituted benzoates are generally activated to acyl-CoAs more slowly than benzoate [24,27,29]. This suggests that glycine conjugation of 4-aminobenzoate, as with salicylate, is limited at the acid: CoA ligase step. This is consistent with the suggestion that the decreased glycine conjugation of 4-aminobenzoate by hepatitis patients is because of decreased mitochondrial ATP production [3]. Determination of hippurate formed from an oral dose of stable isotope labelled benzoate is one suggestion to simplify the interpretation of benzoate conjugation data, which we believe cannot be substituted for by salicylate or 4-aminobenzoate.

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A new perspective on the importance of glycine conjugation in the detoxification of benzoic acid

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A new perspective on the importance of glycine conjugation for the detoxification of benzoic acid

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Abstract

Glycine N-acyltransferase is responsible for conjugating a number of endogenous and xenobiotic organic acids to glycine. Glycine conjugation has generally been assumed to be a detoxification mechanism, increasing the water solubility of organic acids in order to facilitate urinary excretion. However, the recently proposed glycine deportation hypothesis states that the role of glycine conjugation is to prevent accumulation of the neurotransmitter glycine to toxic levels in the brain. This hypothesis is based on the observation that, compared to glucuronidation, glycine conjugation does not significantly increase the water solubility of aromatic acids. In this review it will be demonstrated that the primary function of the glycine conjugation pathway is in fact the detoxification of dietary benzoates. It will be argued that glucuronidation, which occurs in the endoplasmic reticulum, would not be ideal for the detoxification of benzoate, which accumulates in the mitochondrial matrix. It is further argued that the mitochondrial accumulation of benzoate is prevented by glycine conjugation, which forms a more hydrophilic conjugate that is more readily transported out of the mitochondrial matrix. Finally, it will be explained that the glycine conjugation of benzoate, a commonly used preservative, exacerbates the dietary deficiency of glycine in humans. Because the resulting shortage of glycine can negatively influence the synthesis of collagen, nucleic acids, porphyrins, and other important metabolites, the risks of using benzoate as a preservative should not be underestimated.

Key words:

Coenzyme A, glycine N-acyltransferase, GLYAT, hippurate, biotransformation, schizophrenia, hepatitis, hepatocellular carcinoma, mitochondria, mitochondrial permeability transition.

1. Introduction

1.1 The history of glycine conjugation

Benzoic acid was the first compound investigated with regard to its fate in the body. As early as the 18th century, benzoate was used “therapeutically” as a tonic to stimulate respiration. In 1801 it was suggested that urine contained a compound similar to benzoate, rather than benzoate itself, and in 1829 Liebig discovered hippuric acid in the urine of horses (reviewed in (Conti and Bickel, 1977)). In 1841 Ure discovered that ingestion of benzoic acid resulted in urinary excretion of hippurate, a finding confirmed in 1842 by Keller (Keller, 1842, Ure, 1841). In 1845, Dessaignes demonstrated that hippurate is a peptide conjugate of benzoic acid and glycine (reviewed in (Conti and Bickel, 1977)). These discoveries marked the start of the new era of biotransformation research, and by the end of the 19th century most of the major pathways of drug metabolism had been discovered. These include oxidation and reduction, and conjugation to glucuronic acid, sulfate, and acetate (Conti and Bickel, 1977). Subsequently, very large numbers of enzymes and substrates were characterised for the oxidation, reduction, hydrolysis, glucuronidation, and sulfonation pathways (reviewed in (Conti and Bickel, 1977, Coon, 2005, Gamage et al., 2006, Guillemette, 2003, Oates and West, 2006, Ritter, 2000, Rodriguez-Antona et al., 2010, Weinshilboum et al., 1997)). These biotransformation reactions are still the subject of much research in medicine and pharmacology because of their impact on metabolism and predisposition to adverse drug responses (Gamage et al., 2006, Guillemette, 2003, Ritter, 2000, Rodriguez-Antona et al., 2010). Interest in glycine conjugation, however, significantly faded shortly after its discovery. It has been argued that this may be because of the small number of pharmaceutical drugs that are conjugated to glycine (Badenhorst et al., 2013, Knights and Miners, 2012, Knights et al., 2007). More recently, interest in the importance of glycine conjugation has increased again, and a recent review concluded that glycine conjugation, the “poor cousin” of the drug metabolism family, may now have “inherited a fortune” (Beyoglu and Idle, 2012, Beyoglu et al., 2012). This is because it is becoming clear that glycine conjugation is a very important metabolic pathway that can influence the

metabolism of glycine, ATP, and CoASH, versatile metabolites that participate in a large number of metabolic pathways (Section 2 and Section 6).

1.2 Conjugation reactions are commonly thought to be detoxification mechanisms

Collectively, conjugation reactions have traditionally been referred to as phase II biotransformation processes, indicating that the conjugates formed are less toxic or more readily excreted than the parent metabolites (Caldwell, 1982, Caldwell, 1984). Although the more recent discovery of reactive conjugates such as some acylglucuronides has led some researchers to abandon the concept of phase II biotransformation, it is still generally useful to think of conjugation reactions as mechanisms to accelerate excretion of a vast number of bioactive compounds (Caldwell, 1982, Ritter, 2000). Recently the “detoxification theory” of glycine conjugation was directly challenged by the introduction of the “glycine deportation hypothesis”. This hypothesis states that the true purpose of amino acid conjugation reactions is to regulate the levels of amino acids that are also utilised as neurotransmitters (Beyoglu and Idle, 2012, Beyoglu et al., 2012). In this paper we intend to show that glycine conjugation should be viewed as a detoxification mechanism, by arguing that benzoate is toxic, and by offering an explanation for the lower toxicity of glycine conjugates (Section 5). We also discuss the glycine deportation hypothesis in detail, and argue that it does not seem to contradict the detoxification theory of glycine conjugation, and may in fact support it.

2. A brief introduction to glycine conjugation

2.1 The glycine conjugation pathway

Several xenobiotic organic acids are detoxified by conjugation to glycine in the mitochondrial matrix in mammalian liver and kidney, followed by urinary excretion (Knights and Miners, 2012, Knights et al., 2007). The most abundant amino acid conjugate excreted in the urine of almost all mammals is hippuric acid, the glycine conjugate of benzoate (Lees et al., 2013). As demonstrated in Figure 1, hippurate is formed from benzoate in two steps. First, benzoate is activated to benzoyl-CoA by the action of a mitochondrial ATP dependent acid:CoA ligase, which in humans has been identified as HXM-A, encoded by the ACSM2 gene (Knights, 1998, Knights and Drogemiller, 2000, Schachter and Taggart, 1953, Vessey et al., 1999, Vessey et al., 2003). Second, the enzyme glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) binds benzoyl-CoA and catalyses the acylation of glycine to form hippurate and CoASH (Schachter and Taggart, 1953, Schachter and Taggart, 1954). Benzoyl-CoA is the preferred substrate for the orthologs of GLYAT that have been isolated from the livers and kidneys of several mammals, including rats, rabbits, sheep, cows, rhesus monkeys, and humans (Gregersen et al., 1986, Kelley and Vessey, 1990, Kelley and Vessey, 1994, Kelley and Vessey, 1993, Kolvraa and Gregersen, 1986, Mawal and Qureshi, 1994, Nandi et al., 1979, Schachter and Taggart, 1954, Webster et al., 1976). Several other acyl-CoAs, including salicylyl-CoA, 4-aminobenzoyl-CoA, hexanoyl-CoA, and isovaleryl-CoA, can act as acyl donor substrates, but much less efficiently (Badenhorst et al., 2012, Bartlett and Gompertz, 1974, Kolvraa and Gregersen, 1986, Mawal and Qureshi, 1994, Nandi et al., 1979). While glycine is always the preferred acyl acceptor substrate, the amino acids asparagine, glutamine, glutamate, alanine, serine, and threonine can also be used, but at low rates that are physiologically irrelevant (van der Westhuizen et al., 2000, Webster et al., 1976). A second N-acyltransferase, glycine phenylacetyltransferase (E.C. 2.3.1.192), is responsible for the glycine conjugation of phenylacetate (Kelley and Vessey, 1990, Nandi et al., 1979, Vessey and Lau, 1998). In primates, including rhesus monkeys and humans, phenylacetate is conjugated to glutamine, instead of glycine (Knights and Miners, 2012, Webster et

al., 1976). In humans the glutamine phenylacetyltransferase (E.C. 2.3.1.14) is encoded by the GLYAT-L1 gene (Matsuo et al., 2012). It is currently not understood why separate acyltransferases exist for benzoyl-CoA and phenylacetyl-CoA, or why the primate enzymes use glutamine instead of glycine for the conjugation of phenylacetyl-CoA (Badenhorst et al., 2013, Knights and Miners, 2012).

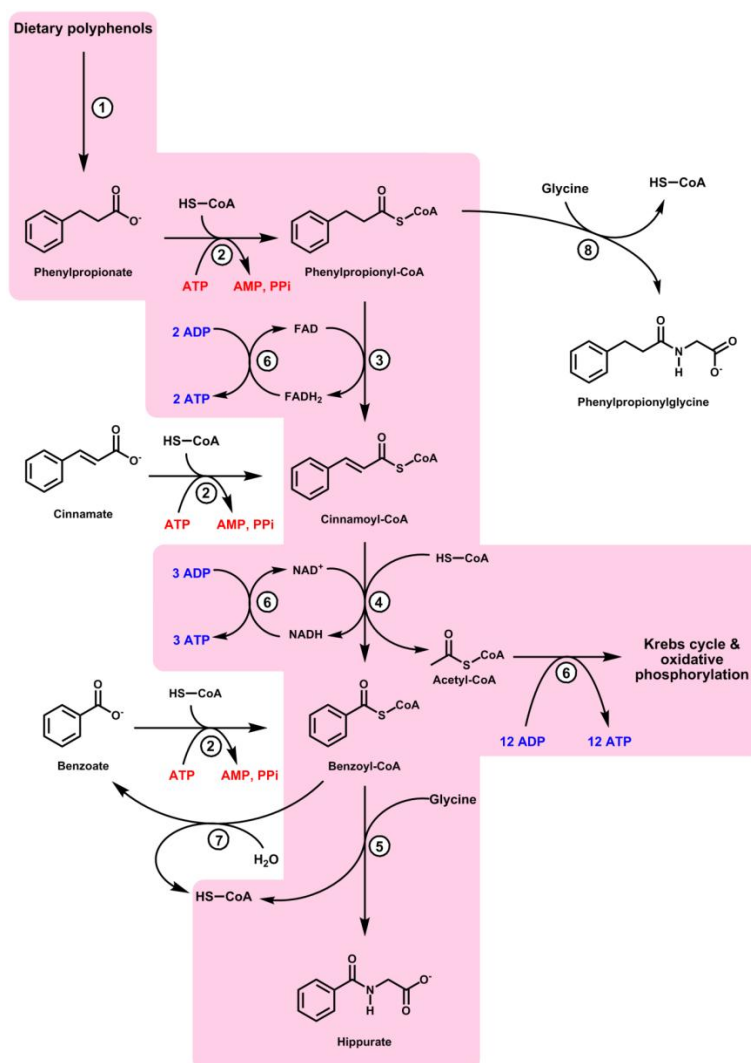


Figure 1: A schematic representation of the glycine conjugation pathway. The part of the pathway on the pink background depicts the natural glycine conjugation pathway, as it is described in this paper. **1)** Dietary polyphenols are converted by the gut microbiota to simple aromatic acids such as phenylpropionate, cinnamate, and benzoate. **2)** After absorption and transport to the liver, the aromatic acids are activated to acyl-CoA thioesters by ATP dependent acid:CoA ligases. The consumption of ATP is demonstrated by red. **3)** Phenylpropionyl-CoA is converted to cinnamoyl-CoA by medium-chain acyl-CoA dehydrogenase, the first step of the β -oxidation cycle. **4)** Cinnamoyl-CoA is further oxidised to benzoyl-CoA and acetyl-CoA. **5)** Benzoyl-CoA is converted to hippurate by glycine N-acyltransferase. **6)** The β -oxidation of phenylpropionate generates FADH₂, NADH, and acetyl-CoA, which are used by the Krebs cycle and the electron transport chain to produce ATP. The production of ATP is demonstrated by blue. **7)** When the capacity of the glycine conjugation pathway is exceeded benzoyl-CoA accumulates, resulting in CoASH sequestration. Hydrolysis of benzoyl-CoA, by thioesterases with high affinity for benzoyl-CoA, releases benzoate and CoASH. **8)** In individuals with a deficiency of medium-chain acyl-CoA dehydrogenase, phenylpropionyl-CoA accumulates and is converted by glycine N-acyltransferase to phenylpropionylglycine.

2.2 Variation in urinary hippurate excretion

The natural substrates for glycine conjugation include benzoate, salicylate, 4-hydroxybenzoate, 3-hydroxybenzoate, 4-aminobenzoate, 2-furoate, and microbial metabolites of polyphenols (Knights and Miners, 2012). The major families of polyphenols in food are the flavan-3-ols, flavonols, flavanones, anthocyanins, and hydroxycinnamates. These are metabolised by the gut microbiota to simpler aromatic compounds such as phenylpropionate, 3-hydroxyphenylpropionate, and 4-hydroxyphenylpropionate (Bravo, 1998, Jenner et al., 2005, Rechner et al., 2002, Tsao, 2010). These aromatic compounds are transported to the liver where they are first activated to acyl-CoA esters and then converted to the corresponding glycine conjugates (Fedotcheva et al., 2008, Rechner et al., 2002, Smith and Macfarlane, 1996). It is now well known that the amount of glycine conjugates excreted in the urine largely depends on the dietary intake of free aromatic acids and polyphenolic compounds, and the extent to which the polyphenols are fermented in the colon (Lees et al., 2013, Phipps et al., 1998, Rechner et al., 2002). The polyphenol fermentation products produced in the colon depend on the type and amount of food consumed, its transit time through the digestive system, and the composition of the gut microbiome (Bravo, 1998, Fedotcheva et al., 2008, Rechner et al., 2002, Smith and Macfarlane, 1996). Suppression of microbial activity by antibiotic use, for example, results in decreased production of compounds like phenylpropionate and consequently lower urinary hippurate excretion (Lees et al., 2013). This is further supported by the observation that germ free mice, bred under sterile conditions, excrete about 17 times less hippurate than conventional mice fed the same diet (Wikoff et al., 2009). Exposure of germ free mice to a normal environment results in microbial colonisation of the colon and normalisation of urinary hippurate levels after about three weeks (Wikoff et al., 2009). For humans, significant interindividual variation in urinary hippurate excretion has been documented. One study determined the daily hippurate excretion as 6285 ± 4008 μmol for men and 4793 ± 3239 μmol for women. The Human Metabolome Database reports urinary hippurate concentrations ranging from 27.92 to 932.66 $\mu\text{mol}/\text{mmol}$ creatinine (Lees et al., 2013, Wijeyesekera et al., 2012).

2.3 Variation in the rate of glycine conjugation

Because the glycine conjugation system is readily saturable, the metabolism of benzoate is dose dependent (Amsel and Levy, 1969, Knights and Miners, 2012, Knights et al., 2007, Kubota and Ishizaki, 1991, Poon and Pang, 1995). The major metabolite of benzoate is hippurate, but when very large doses are administered benzoylcarnitine and benzoylglucuronide may also be formed (Bray et al., 1951, Sakuma, 1991). The formation of these secondary metabolites depends on both the dose of benzoate and the capacity of the individual glycine conjugation system in question (Bray et al., 1951, Saltzman and Caraway, 1953). It is important to distinguish between variation in the amount of hippurate excreted, and variation in the rate of glycine conjugation. These two concepts are usually not clearly distinguished in the literature, which can lead to confusion. For example, patients with Crohn's disease are characterised by significantly lower urinary hippurate levels. This is associated with significant alterations of the gut microbiome, which result in decreased fermentation of dietary polyphenols and lower production of phenylpropionate. However, these individuals do not have a decreased rate of hepatic glycine conjugation, as demonstrated by normal conversion of an oral dose of benzoate to hippurate (Williams et al., 2010). In the rest of this section the factors that influence the rate and capacity of the glycine conjugation pathway are discussed.

2.3.1 Availability of ATP, CoASH, and glycine

The first step in the glycine conjugation pathway requires CoASH for the synthesis of benzoyl-CoA (Figure 1). The availability of unacylated CoASH can therefore directly limit the maximum rate of glycine conjugation (Gregus et al., 1996a, Gregus et al., 1992). In one study pantothenate, a precursor for CoASH biosynthesis, was administered to a hyperglycinemia patient on benzoate therapy. This resulted in increased urinary hippurate excretion and attenuation of seizures, but there was no way to determine whether hepatic CoASH levels were actually increased. However, it was demonstrated that addition of pantothenate to the culture

medium of HepG2 cells resulted in significant increases in cellular CoASH content and increased conversion of benzoate to hippurate (Palekar, 2000). The synthesis of benzoyl-CoA is also dependent on ATP (Figure 1). Since ATP is hydrolysed to AMP, two molecules of ATP are consumed per molecule of benzoate (Schachter and Taggart, 1953, Schachter and Taggart, 1954). A shortage of ATP will, therefore, limit the rate of glycine conjugation by limiting the formation of benzoyl-CoA (Gregus et al., 1996a). The rates of glycine conjugation of cinnamate, benzoate, and 4-aminobenzoate have been measured extensively as a test of liver function, and correlates well with hepatic reserve (Duffy et al., 1995, Furuya et al., 1995, Krahenbuhl et al., 1997, Probst and Londe, 1940, Saltzman and Caraway, 1953). It is thought that the mitochondrial dysfunction associated with liver disease, which leads to decreased ATP production, explains the lower rate of glycine conjugation in hepatitis patients (Badenhorst et al., 2013, Gregus et al., 1996a, Knights et al., 2007). The availability of glycine is another factor known to strongly influence the rate of hippurate synthesis (Figure 1) (de Vries et al., 1948, Gregus et al., 1992, Gregus et al., 1993, Levy, 1979). Therefore, although hippurate synthesis in humans is saturable with large doses of benzoate, the co-administration of glycine can significantly increase the rates of benzoate clearance and hippurate formation (Knights and Miners, 2012, Levy, 1979). The availability of glycine also limits hippurate synthesis in rat liver. In one study the hepatic glycine content of rats was increased 2-3 times by the administration of cysteamine, a potent inhibitor of the mitochondrial glycine cleavage system, without affecting the levels of ATP and CoASH. The result was a 50% increase in the rate of benzoate conjugation (Gregus et al., 1993). Because the increase in the rate of hippurate formation was smaller than the increase in glycine concentration, other factors such as CoASH availability seem to limit hippurate synthesis when excess glycine is available (Gregus et al., 1996b, Gregus et al., 1996a, Gregus et al., 1992, Gregus et al., 1993). Although glycine supplementation stimulates hippurate synthesis, the glycine conjugation of salicylic acid is not enhanced by co-administration of glycine (Amsel and Levy, 1969). This is because the limiting step in the pathway depends on the substrate; hippurate formation for benzoate, and salicylyl-CoA formation for salicylate. This is consistent with the observation that the rate of salicylyl-CoA formation by human liver HXM-A is less than 1% of that for benzoyl-CoA formation (Knights et al., 2007, Vessey et al., 1999).

Further complexity arises because of the existence of multiple precursors for hippurate synthesis, including benzoate, phenylpropionate, and cinnamate (Figure 1). The use of benzoate as the typical substrate in many studies on glycine conjugation that have been published may be misleading (reviewed in (Lees et al., 2013)). This is because the major natural precursor for hippurate synthesis appears to be phenylpropionate, a product of microbial fermentation in the gut. Phenylpropionate is absorbed from the colon and transported to the liver, where it is activated to phenylpropionyl-CoA in the mitochondrial matrix (Fedotcheva et al., 2008, Jenner et al., 2005, Lees et al., 2013, Rechner et al., 2002). Phenylpropionyl-CoA is a good substrate for medium-chain acyl-CoA dehydrogenase (MCAD) and is rapidly converted to cinnamoyl-CoA (Mao et al., 1994). This is why phenylpropionyl-CoA is used in enzyme assays for the confirmation of MCAD deficiency (Rinaldo et al., 1990). MCAD patients excrete phenylpropionylglycine, indicating accumulation of phenylpropionyl-CoA in the livers of these individuals (Bennett et al., 1990). Cinnamoyl-CoA continues through the β -oxidation cycle to yield acetyl-CoA and benzoyl-CoA (Saltzman and Caraway, 1953). There are three important differences when phenylpropionate, rather than benzoate, is the substrate for conjugation (Figure 1). First, the conjugation of benzoate consumes two molecules of ATP, while the β -oxidation of phenylpropionate can theoretically yield 15 molecules of ATP. Second, an extra molecule of CoASH is needed for the oxidation of phenylpropionate (Houten and Wanders, 2010). The availability of ATP and CoASH for hippurate synthesis may thus depend on whether the substrate is phenylpropionate, cinnamate, or benzoate. Finally, the rates of mitochondrial uptake and activation to acyl-CoAs may be different for these substrates, because of differences in structure and lipophilicity (Masuda et al., 1996, Hansch et al., 1968, Kasuya et al., 1990, Kasuya et al., 1991).

2.3.2 Genetic variation in HXM-A and GLYAT

Early investigations revealed a greater similarity in the metabolism of aspirin between identical twins than between fraternal twins, suggesting that genetic variation is partly responsible for interindividual variation in

the glycine conjugation of salicylate (Furst et al., 1977). However, very little is known about the influence of genetic variation on the glycine conjugation pathway. One study described an association between aspirin intolerance and an SNP (rs1133607) in the ACSM2 gene, which encodes HXM-A, the salicylate activating enzyme (Agundez et al., 2009, Vessey et al., 1999). This suggests that genetic variation may influence the rate of activation of aromatic acids by HXM-A and HXM-B, although very little is known about the actual influence of genetic variation on the enzyme activities of these ligases (Boomgaarden et al., 2009). Genetic variation in the human GLYAT gene can influence the enzyme activity of a bacterially expressed recombinant human GLYAT (van der Sluis et al., 2013). Although the effects of only six SNPs on enzyme activity were determined, it is interesting to note that the variant with the highest enzyme activity had the highest allele frequency, and that those with negative effects on enzyme activity occur very rarely (van der Sluis et al., 2013, McVean, 2012). For example, it was suggested that the N156S variant should be considered the “wild-type” allele because it has a much higher allele frequency and higher enzyme activity than that encoded by the reference sequence (NM_201648.2). In contrast, only a single case of each of the R131H, F168L, and R199C variants, which negatively impact GLYAT enzyme activity, have been reported (Lino Cardenas et al., 2010, van der Sluis et al., 2013, Yamamoto et al., 2009, McVean, 2012). It is not yet known if genetic variation in the GLYAT gene influences the overall rate of glycine conjugation *in vivo* (Badenhorst et al., 2013, van der Sluis et al., 2013).

2.3.3 Expression of the ligase and transferase genes

Both the ligase and transferase enzymes are inducible, although the mechanisms involved are not well understood. Pre-treatment of rats with salicylate for six days increases the capacity of liver and kidney homogenates for synthesis of salicylylglycine, hippurate, and 4-aminohippurate (Irrjala, 1972). Salicylate pre-treatment also increases the rate of salicylylglycine formation in humans (Furst et al., 1977). Recently it was demonstrated that dietary restriction results in increased urinary acylglycine excretion, which correlates

with increased expression of GLYAT in the liver of rats (Wen et al., 2013). Also, investigation of hepatocellular carcinoma specimens further demonstrated that GLYAT expression is transcriptionally regulated (Matsuo et al., 2012).

3. The glycine deportation hypothesis

3.1 A brief overview of the glycine deportation hypothesis

The recently proposed glycine deportation hypothesis states that amino acid conjugation is best viewed as a neuroregulatory mechanism, rather than a detoxification process (Beyoglu et al., 2012). This is because benzoate is usually conjugated to glycine, glutamine, glutamate, arginine, ornithine, or taurine, amino acids that are also utilised as neurotransmitters in the CNS of animals (Beyoglu and Idle, 2012, Beyoglu et al., 2012). Accumulation of these neurotransmitters in the CNS, which would be neurotoxic, is avoided by the irreversible urinary excretion of these amino acids as conjugates to benzoate. The basis of this argument is that amino acid conjugation does not significantly increase the water solubility of aromatic acids, as has generally been assumed (Beyoglu and Idle, 2012, Beyoglu et al., 2012). For example, the solubility of benzoic acid in water is 3.4 g/l, and that of hippuric acid is only 3.75 g/l. In the case of phenylacetic acid, water solubility decreases from 16.6 g/l to 7.3 g/l or 2.12 g/l, when conjugated to glycine or glutamine, respectively. In contrast, the glucuronidation of benzoate increases water solubility to 263 g/l. According to the glycine deportation hypothesis, glucuronidation would thus be a better choice of conjugation reaction if the goal was to increase water solubility and facilitate urinary excretion (Beyoglu and Idle, 2012, Beyoglu et al., 2012). The glycine deportation hypothesis is summarised in Table 1, with brief comments and references to relevant parts of this review. The apparent conflict between the views of glycine conjugation as a system for disposing of glycine, or as a system for the detoxification of benzoate, will be carefully analysed in Section 5. In the rest of this section it will briefly be argued that glycine conjugation does not seem to be primarily a neuroregulatory process.

Table 1: A summary of the glycine deportation hypothesis with some comments

	The glycine deportation hypothesis[†]	Comments	Sections of interest in this review	Figures of interest in this review
1	“Compared with glucuronic acid conjugation, for example, amino acid conjugation does not render aromatic acids significantly more water soluble. In many cases, the converse is true. Thus, the detoxication value of amino acid conjugation is low.”	1.1) While the solubility of benzoic acid is only 3.4 g/l, that of sodium benzoate is 623 g/l, which is much higher than physiological concentrations. Therefore, water solubility is probably not the factor determining the detoxification value of glycine conjugation.	Sections 3.1 and 5.3	Figure 3 (numbers 2, 3, and 7)
		1.2) Most phase II conjugation systems, including sulfonation, acetylation, glutathione conjugation, and glucuronidation, are located in the cytoplasm.	Sections 3.1 and 5.3	Figure 3 (number 8)
		1.3) Because of the pH gradient over the inner mitochondrial membrane, benzoate accumulates in the mitochondrial matrix to approximately 50 times the external concentration. Glucuronidation, which occurs in the endoplasmic reticulum, would therefore not efficiently detoxify benzoate.	Sections 3.1 and 5.3	Figure 3 (numbers 1, 2, and 8)
		1.4) Glycine conjugation significantly decreases the lipophilicity of benzoic acid. The lower lipophilicity of hippuric acid can explain why it is more readily excreted, and therefore less toxic, than benzoate.	Sections 3.1 and 5.3	Figure 3 (numbers 2, 3, and 7)
2	“The principal amino acid conjugations in vertebrates utilize glycine, glutamine, and taurine and glycine, glutamate, and taurine are CNS neurotransmitters.”	2.1) Glycine conjugation is by far the most common amino acid conjugation reaction, for both benzoate and phenylacetate. The other amino acid conjugations are much less common.	Section 2.1	Figure 1
		2.2) The observation that the same amino acids are sometimes used as both neurotransmitters and conjugation cofactors can be explained by the fact that these amino acids are readily synthesized, as needed, from mitochondrial metabolites.	Section 3.1	
		2.3) Uricotelic animals such as birds, reptiles, and some insects, have a high demand for glycine, which is used for uric acid synthesis. Benzoate is therefore conjugated to ornithine instead of glycine.	Section 6.4	Figure 4
3	“The efflux of glycine and glutamate from the brain is a neuroprotective mechanism against elevated and neurotoxic concentrations of these amino acids.”	3.1) While this statement is true, it is unlikely that the disposal of amino acids, which are often limiting factors in animal growth and development, is the primary purpose of the amino acid conjugation reactions.	Sections 3.1, 3.3, and 6.1	Figure 4
4	“Trafficked amino acids are scavenged by metabolic processes that lead to irreversible urinary excretion of amino acid conjugates of aromatic acids.”	4.1) There is strong evidence to suggest that humans have a moderate deficiency of glycine, despite the commonly held view that glycine is a nonessential amino acid. This further supports the idea that it is unreasonable to think of glycine conjugation only as a means of disposing of excess glycine.	Sections 3.1 and 6.1	Figure 4
5	“Experiments in rats demonstrate that scavenging of plasma glutamate by activation of blood transaminases also removes glutamate from the	5.1) This statement is related to the conjugation of phenylacetate to glutamine. However, since this occurs almost exclusively in primates, it is of little relevance to understanding the metabolic significance of amino acid conjugation in general.	Sections 2.1 and 3.3	

	brain.”	5.2) In humans CSF glutamine concentrations (284-680 μM) are much higher than glycine concentrations (0.7-14.7 μM). However, the levels of phenylacetylglutamine excreted in urine are usually about tenfold lower than that of hippurate. It is therefore unlikely that plasma or CSF glutamine levels are significantly influenced by the conjugation of phenylacetate to glutamine, at least under normal conditions.	Sections 2.1 and 3.3	
6	“Both benzoic acid and phenylacetic acid are used clinically to scavenge glycine and glutamine for the purpose of excess nitrogen excretion in urea cycle defects.”	6.1) This therapeutic strategy is known as “alternative pathway therapy”. Ammonia is first incorporated into glycine or glutamine, which are then conjugated to either benzoate or phenylacetate, respectively. Excess ammonia is thus removed from the body by the irreversible urinary excretion of the amino acid conjugates.	Section 4.1	Figure 1 and Figure 4
7	“Clinical use of phenylacetic acid and its metabolic precursors, which result in considerable glutamine scavenging, are commonly associated with CNS side-effects, a possible reflection of brain glutamate depletion.”	7.1) Very large doses of these aromatic acids are used in this kind of therapy, suggesting that this phenomenon is not relevant to normal physiological conditions.	Sections 3.3 and 4.1	
8	“Psychiatric conditions with reduced psychomotor activity display reduced glycine scavenging by benzoic acid, which can be reversed by glycine loading.”	8.1) The availability of glycine is one of the major factors determining the rate of hippurate synthesis from benzoate. Furthermore, plasma glycine levels are lower than normal in individuals with schizophrenia, an example of a psychiatric condition with reduced psychomotor activity. The lower rate of hippurate synthesis in schizophrenia is thus a consequence of reduced glycine availability, and not of an abnormal glycine conjugation pathway.	Sections 2.3.1, 3.2, and 3.3	Figure 1
9	“Psychiatric conditions with enhanced psychomotor activity display enhanced glycine scavenging by benzoic acid.”	9.1) Individuals with free anxiety have higher than normal plasma glycine levels. The increased rate of hippurate synthesis in these individuals is a reflection of the increased glycine availability, and not of an abnormal glycine conjugation pathway.	Sections 2.3.1, 3.2, and 3.3	Figure 1
10	“Glycine scavenging by various aromatic acids is enhanced in autism.”	10.1) Individuals with autism have higher than normal plasma glycine levels. The increased rate of hippurate synthesis in these individuals is a reflection of the increased glycine availability, and not of an abnormal glycine conjugation pathway.	Sections 2.3.1, 3.1, and 3.2	Figure 1
11	“Glycine, glutamate, and arginine are scavenged by benzoic acid and its derivatives in certain species of insects and spiders that use these same amino acids as neurotransmitters in the CNS.”	11.1) These amino acids are rapidly synthesized in mitochondria from readily available metabolites. This explains the use of these amino acids as both conjugation cofactors and neurotransmitters, which have to be rapidly synthesized and degraded.	Sections 3.1 and 6.4	

[†]Taken from: Beyoglu, D. and Ilde, JR. The glycine deportation system and its pharmacological consequences. *Pharmacology & Therapeutics* 2012;135:151-167)

3.2 Abnormal hippurate synthesis in psychiatric disorders reflects differences in substrate availability

Much of the glycine deportation hypothesis is based on the relationship between psychiatric disorders and altered urinary hippurate excretion (Beyoglu and Idle, 2012, Beyoglu et al., 2012). For example, it is well known that patients with schizophrenia excrete less hippurate than healthy individuals (Fabisch and Fellner, 1957, Quastel and Wales, 1938, Wong, 1945). In contrast, hippurate synthesis is enhanced in individuals with free anxiety (Persky et al., 1952, Persky et al., 1950). These relationships between hippurate synthesis and psychiatric disorders are, however, more readily explained by differences in the availability of glycine and ATP than by abnormal function of the glycine conjugation pathway itself (Ben-Shachar, 2002, Beyoglu et al., 2012, Sumiyoshi et al., 2004). The increased synthesis of hippurate in patients with anxiety can be explained by increased availability of glycine, whereas the decreased synthesis of hippurate in schizophrenia can be explained by decreased availability of glycine and ATP (Ben-Shachar, 2002, Heresco-Levy et al., 1999, Javitt et al., 2001, Koersner, 1947, Ohnuma et al., 2008, Persky et al., 1952, Persky et al., 1950, Sumiyoshi et al., 2004). Interestingly, administration of glycine to schizophrenic individuals significantly increases the rate of hippurate synthesis, supporting the view that decreased glycine availability, rather than a defective glycine conjugation system, is responsible for the low hippurate excretion in schizophrenia (Koersner, 1947). Therefore, the altered excretion of hippurate in psychiatric disorders does not seem to support the suggestion, made by the glycine deportation hypothesis, that the glycine conjugation of benzoate is a neuroregulatory mechanism.

3.3 The glycine conjugation pathway does not seem to be important for glycine homeostasis

The major system involved in glycine homeostasis is the glycine cleavage system, which has a high metabolic capacity and is expressed in several tissues, including liver and brain (Kikuchi et al., 2008). Defects of the glycine cleavage system result in nonketotic hyperglycinemia (NKH), characterised by significant elevations

of glycine concentrations in plasma and CSF (Conter et al., 2006, Hiraga et al., 1981, Kure et al., 2004, Perry et al., 1975, Hamosh and Johnston, 2013). The most useful diagnostic parameter is the CSF/plasma glycine ratio, which is normally below 0.02, with values of 0.09 or greater being diagnostic for NKH (Krieger et al., 1977, Perry et al., 1975, Hamosh and Johnston, 2013). Although there are no completely satisfactory treatments for this disorder, administration of sodium benzoate is used to decrease plasma and CSF glycine levels, as the excess glycine is excreted in the urine as hippurate. The effectiveness of this therapy is variable and glycine levels or CSF/plasma glycine ratios are rarely decreased to satisfactory levels (Applegarth and Toone, 2001, Barshop et al., 1989, Krieger et al., 1977). The failure of this therapy to completely normalise glycine levels and CSF/plasma glycine ratios, despite the large doses of benzoate used, suggests that the contribution of the glycine deportation system to glycine homeostasis is small, compared to that of the glycine cleavage system (Krieger et al., 1977, Hamosh and Johnston, 2013).

After phenylketonuria, NKH is the second most prevalent defect of amino acid catabolism, affecting up to one in 64 000 individuals in some populations (Hamosh and Johnston, 2013). Therefore, being a carrier of NKH is not lethal or extremely deleterious for human health, despite the higher plasma and CSF glycine concentrations observed in carriers, compared to controls (Kure et al., 2004, Von Wendt and Similä, 1982). If the purpose of the glycine conjugation pathway was the regulation of glycine levels, one might then expect defects of this system to be at least as common as NKH, since glycine conjugation makes the smaller contribution to glycine homeostasis. However, no defect of glycine conjugation has ever been reported, suggesting that glycine conjugation is a vital metabolic process, and that its primary purpose is not the regulation of glycine levels. The rest of this review will focus on the toxicity of benzoate (Section 4), its detoxification by conjugation to glycine (Section 5), and the consequences of this detoxification pathway for metabolism as a whole (Section 6).

4. The toxicity of benzoate and other aromatic acids

4.1 The toxicity of benzoate and phenylacetate to animals and cultured cells

Benzoate and phenylacetate are toxic to normal metabolism on several levels. It seems to be generally thought that benzoate and phenylacetate are not seriously toxic compounds (Beyoglu and Idle, 2012, Beyoglu et al., 2012). However, this is probably only because of the efficient detoxification of these compounds by conjugation to glycine, as is discussed in Section 5. Conjugation of large doses of benzoate or phenylacetate results in significant decreases in plasma glycine, which is associated with neurological symptoms in rats, dogs, and humans (Abdo et al., 1998, Beyoglu and Idle, 2012, Beyoglu et al., 2012, de Vries et al., 1948). High doses of benzyl acetate result in severe brain damage in rats as a result of glycine depletion, an effect cancelled out by the administration of glycine but not alanine (Abdo et al., 1998). When glycine is depleted, the detoxification of benzoate is significantly impaired, and significant damage occurs to the livers and kidneys of rats and dogs (Abdo et al., 1998, Griffith et al., 1989, Sherwin and Kennard, 1919, White, 1941). Plasma concentrations of aspartate amino transferase and alkaline phosphatase, markers of liver damage, are increased in rats following benzoate administration. There is also evidence that benzoate and phenylacetate are neurotoxic (Batshaw and Brusilow, 1981, Praphanphoj et al., 2000). In addition to gut abnormalities, defects of pronephric tubes, cardiac oedema, and misalignment of muscle fibres, sodium benzoate causes motor neuron innervations and abnormal expression of the acetylcholine receptor, tyrosine hydroxylase, and a dopamine transporter in zebrafish embryos and larvae (Bichara et al., 2013, Chen et al., 2009, Tsay et al., 2007). In human and mouse neurons and astrocytes, benzoate increases the expression of DJ-1, a protein with neuroprotective properties in Parkinson's disease (Khasnavis and Pahan, 2012). Benzoate can directly inhibit brain enzymes and can also increase the turnover of serotonin in the brains of mice and humans by competing with tryptophan for binding to albumin (Batshaw et al., 1988). Phenylacetate causes permanent disruption of neurological development if administered to new born rats (Wen et al., 1980). Similarly, abnormal neurological development of the foetus has been reported in cases of

untreated maternal phenylketonuria, which is characterised by increased phenylacetate levels (Fulton et al., 1980). A potential mechanism of pathogenesis is the formation of phenylacetyl-CoA in the brain, where it strongly inhibits choline acetyltransferase and disrupts neuronal utilisation of acetyl-CoA (Loo et al., 1980, Loo et al., 1979, Loo et al., 1985).

Incubation of cultured primary rat hepatocytes with benzoate results in dose dependent depression of protein and DNA synthesis and decreased activities of ornithine transcarbamylase and tyrosine aminotransferase (Oyanagi et al., 1987). Incubation of human leukocytes with benzyl acetate, benzyl alcohol, benzaldehyde, or benzoate results in significant DNA damage, reflecting the genotoxicity of these compounds (Abe and Sasaki, 1977, Demir et al., 2010, Yilmaz et al., 2009, Zengin et al., 2011). Benzoate influences the mitochondrial membrane potential and matrix calcium levels in cultured rat cortical neurons and human clonal epithelial cells, and induces cell death in a dose dependent manner (Park et al., 2011, Park et al., 2013). The mechanisms of toxicity of aromatic acids like benzoate and cinnamate have not been clearly defined, but involves sequestration of CoASH, formation of toxic acyl-CoA thioesters, and disruption of mitochondrial integrity by interaction of the aromatic acids with the inner mitochondrial membrane. These mechanisms are briefly reviewed in Sections 4.2 and 4.3. The mechanisms of detoxification by means of glycine conjugation are discussed in Section 5.

4.2 Toxicity mediated by acyl-CoA formation

Comprehensive understanding of coenzyme A metabolism was advanced by the recent introduction of the concept of CASTOR disorders (Coenzyme A Sequestration, Toxicity, and Redistribution). In CASTOR disorders the synthesis of CoASH or the degradation of acyl-CoA thioesters is impaired, resulting in a combination of CoASH sequestration and accumulation of toxic acyl-CoAs (Mitchell et al., 2008). Therefore, it is very useful to keep in mind that the toxicity of an organic acid is largely determined by the extent to which an acyl-CoA

is formed that cannot be further metabolised (Figure 2) (Badenhorst et al., 2013, Knights and Miners, 2012, Knights et al., 2007, Mitchell et al., 2008).

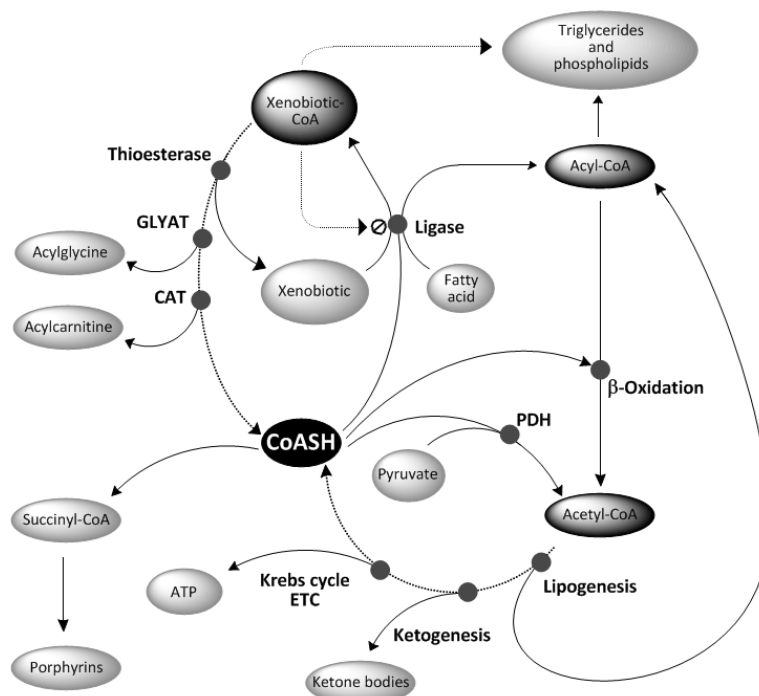


Figure 2: An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is very important that CoASH always be available in the cell because of its role in β -oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. Black circles represent processes or enzymes involved in the metabolism of CoASH. Abbreviations: GLYAT, glycine N-acyltransferase; CAT, carnitine acyltransferase; PDH, pyruvate dehydrogenase; ETC, electron transport chain; CoASH, coenzyme A. Reproduced with minor modifications (Badenhorst et al., 2013) with permission from Informa Healthcare.

4.2.1 The metabolic consequences of CoASH sequestration

The first step in the metabolism of several organic acid xenobiotics is the ATP dependent activation to an acyl-CoA thioester by hepatic acid:CoA ligases (Badenhorst et al., 2013, Caldwell, 1982, Knights et al., 2007, Schachter and Taggart, 1953). In addition to the toxicity of the thioesters, acyl-CoA formation can cause sequestration of CoASH, which negatively impacts metabolism on many levels (Knights and Drogemiller,

2000, Knights et al., 2007, Mitchell et al., 2008, Zhang et al., 2007). Coenzyme A is perhaps one of the most important molecules in biochemistry, participating in β -oxidation, ketogenesis, lipogenesis, glucose catabolism, gluconeogenesis, and several other metabolic processes such as amino acid metabolism and the Krebs cycle (Figure 2). Sequestration of CoASH can therefore inhibit these vital metabolic processes (Mitchell et al., 2008, Zhang et al., 2007). Oxidation of propionate by bovine liver tissue is inhibited by cinnamate, phenylpropionate, and benzoate (Cremin Jr et al., 1994). Sequestration of CoASH by benzoate also inhibits β -oxidation, lipogenesis, ureagenesis, gluconeogenesis, ketogenesis, and the utilisation of ketone bodies in cultured rat hepatocytes or isolated mitochondria (Beynen and Geelen, 1982, Cyr et al., 1991, Griffith et al., 1989, Kalbag and Palekar, 1988, Palekar and Kalbag, 1991, Yao et al., 1994). The levels of ATP and acetyl-CoA in the brains of hyperammonemic rats treated with benzoate also decrease as a result of CoASH sequestration (Ratnakumari et al., 1993). The main routes for regeneration of free CoASH from xenobiotic acyl-CoAs are carnitine conjugation, hydrolysis, and glycine conjugation (Knights and Drogemiller, 2000, Knights et al., 2007, Mitchell et al., 2008). The importance of glycine conjugation in the maintenance of hepatic CoASH levels and normal metabolic function is discussed in Section 5.

4.2.2 The toxic effects of accumulated acyl-CoA thioesters

It is well known that formation of a more reactive acyl-CoA is an important mechanism mediating the toxicity of organic acids such as benzoate, phenylacetate, salicylate, valproate, and clofibrate. A major reason for the increased reactivity of acyl-CoA metabolites is the electrophilic nature of the thioester bond (Caldwell, 1984, Grillo and Benet, 2002, Grillo and Lohr, 2009, Knights et al., 2007, Li et al., 2002, Li et al., 2003, Olsen et al., 2007, Sidenius et al., 2004). This makes acyl-CoAs reactive to nucleophiles such as glutathione and the cysteine, serine, lysine, and histidine residues of proteins. For example, covalent modification of glutathione and cellular proteins by the acyl-CoA metabolites of phenylacetate, 2-phenylpropionate, and 2,4-dichlorophenoxyacetate has been demonstrated (Caldwell, 1984, Grillo and

Benet, 2002, Grillo and Lohr, 2009, Knights et al., 2007, Li et al., 2002, Li et al., 2003, Olsen et al., 2007, Sidenius et al., 2004).

Acyl-CoA metabolites can also inhibit several enzymes, causing complex effects that are difficult to predict (Knights et al., 2007, Mitchell et al., 2008). Examples include the inhibition of choline acetyltransferase by phenylacetyl-CoA, inhibition of pyruvate carboxylase by propionyl-CoA and isovaleryl-CoA, inhibition of acid:CoA ligase enzymes by benzoyl-CoA and salicylyl-CoA, inhibition of the glycine cleavage system by valproyl-CoA and propionyl-CoA, and perturbation of protein kinase C function by clofibroyl-CoA (Erflé and Sauer, 1969, Knights et al., 2007, Kolvraa, 1979, Loo et al., 1980, Mortensen et al., 1980). In addition to acting as inhibitors, acyl-CoAs can also behave as alternative substrates for enzymes, resulting in the biosynthesis of abnormal metabolites. For example, when propionyl-CoA accumulates it can substitute for acetyl-CoA, resulting in the formation of methylcitrate by citrate synthase and N-propionylglutamate by N-acetylglutamate synthetase. The resulting shortage of N-acetylglutamate results in disruption of the urea cycle (Fenton et al., 2013). Accumulated propionyl-CoA and isovaleryl-CoA can substitute for acetyl-CoA in lipogenesis to produce odd-chain and branched-chain fatty acids (Fenton et al., 2013, Sweetman and Williams, 2013). Similarly, 2-arylpropionyl-CoAs, metabolites of nonsteroidal anti-inflammatory drugs, can be incorporated into adipocyte triglycerides (Knights et al., 2007). Very important cases involve the reaction of isovaleryl-CoA and benzoyl-CoA with carnitine, catalysed by carnitine acyltransferase enzymes (Itoh et al., 1996, Sakuma, 1991). This results in the excretion of acylcarnitines in the urine and can cause systemic carnitine depletion, which negatively impacts fatty acid oxidation and energy metabolism (Itoh et al., 1996, Lheureux and Hantson, 2009, Sakuma, 1991).

4.3 Benzoate and other aromatic acids stimulate ROS production and inhibit complex I

The aromatic acids phenylpropionate, cinnamate, and phenylacetate accumulate in the plasma of septic patients as a result of microbial metabolism in the blood. Sepsis is characterised by extremely high ROS production, oxidative stress, and mitochondrial damage (Beloborodova et al., 2012). The accumulating aromatic acids stimulate the production of ROS in isolated liver mitochondria and inhibits the activity of complex I of the electron transport chain (Beloborodova et al., 2012, Fedotcheva et al., 2008, Fedotcheva et al., 2012). Although the mechanisms of mitochondrial toxicity of the aromatic acids are not well understood, several recent advances in our understanding of this process will be briefly reviewed here.

Benzoate, salicylate, phenylpropionate, cinnamate, valproate, and isovalerate are all known to induce the mitochondrial permeability transition, an important mechanism in the pathogenesis of disorders related to Reye's syndrome (Battaglia et al., 2005, Beloborodova et al., 2012, Fedotcheva et al., 2008, Trost and Lemasters, 1996). Mitochondrial permeability transition is the common final pathway leading to acute cell death as a result of mitochondrial dysfunction, is induced by several mild oxidants such as t-butylhydroperoxide and diamide, and is inhibited by cyclosporin A (Fedotcheva et al., 2008, Kushnareva and Sokolove, 2000, Lemasters et al., 2002, Nakagawa and Moldeus, 1998, Nakagawa and Moore, 1999, Trost and Lemasters, 1996, Trost and Lemasters, 1997). It is characterised by the opening of a large non-selective pore which leads to uncoupling of oxidative phosphorylation, generation of ROS, mitochondrial swelling, release of cytochrome c, and apoptosis (Beloborodova et al., 2012, Bradshaw and Pfeiffer, 2006, Kushnareva and Sokolove, 2000, Trost and Lemasters, 1997). An important first step leading to the permeability transition seems to be the formation of a small non-selective pore, distinct from the pore involved in mitochondrial permeability transition. The opening of this pore allows the passage of cations such as calcium, but not protons, and is thought to be stimulated by pro-oxidants and by salicylate in the inner mitochondrial membrane (Battaglia et al., 2005). Calcium efflux from this pore, coupled to calcium import by the calcium uniporter, results in rapid collapse of the membrane potential, which is stimulated by external

calcium and inhibited by EGTA (Battaglia et al., 2005, Fedotcheva et al., 2008, Kushnareva and Sokolove, 2000, Trost and Lemasters, 1997). Collapse of the membrane potential precedes opening of the permeability transition pore and is not inhibited by cyclosporin A (Kushnareva and Sokolove, 2000).

Although it is not clear how aromatic acids induce ROS production and the mitochondrial permeability transition, it has been suggested that diffusion of these compounds into the inner mitochondrial membrane alters the properties of the membrane and stimulates opening of the initial small pore (Bernardi et al., 1994, Broekemeier and Pfeiffer, 1995, Trost and Lemasters, 1997). N-ethylmaleimide can promote opening of the pore, which is inhibited by dithiothreitol, suggesting that oxidation of critical sulfhydryl groups on the inner mitochondrial membrane is important for opening the pore or for keeping it in an open state (Fedotcheva et al., 2008, Trost and Lemasters, 1997). In the mitochondria of cultured tobacco cells, benzoate and salicylate directly inhibit the transfer of electrons from complex I to ubiquinone, stimulating ROS generation (Norman et al., 2004). This could cause oxidation of sulfhydryl groups in the membrane, leading to inhibition of complex I and opening of the initial pore. This is supported by the observation that reducing agents like dithiothreitol can prevent the inhibition of complex I caused by N-ethylmaleimide or aromatic acids (Fedotcheva et al., 2008). The inhibition of complex I results in ROS production upon oxidation of complex I and complex II substrates (Fedotcheva et al., 2008, Murphy, 2009). Oxidation of complex II substrates is not inhibited, since this complex is less sensitive to oxidative stress than complex I (Beloborodova et al., 2012, Fedotcheva et al., 2008, Fedotcheva et al., 2012, Murphy, 2009).

A related explanation for the damaging effects of aromatic acids on mitochondria is the ability of lipophilic compounds to disrupt the structural integrity of biological membranes by partitioning into the phospholipid bilayer, thereby increasing its permeability to small molecules and ions in a nonspecific manner (Politycka, 1997, Sikkema et al., 1995, Ye et al., 2006). For example, benzoate can increase the conductance of biological membranes, and causes leakage of lactate from intact mitochondria (Cremin Jr et al., 1994, Gutknecht, 1992). Increased membrane permeability can result in dissipation of membrane potential and

uncoupling of oxidative phosphorylation, as demonstrated by the effects of cyclohexane on yeast mitochondria (Sikkema et al., 1995). This effect on membrane integrity also explains the observation that intracellular membrane trafficking in yeast is disturbed by benzoic acid, which also seems to negatively influence the fission and fusion of mitochondria (Hazan et al., 2004, Park et al., 2011, Park et al., 2013). The ability of a compound to diffuse into and accumulate in biological membranes is directly related to its lipophilicity (Lakeram et al., 2007, Sikkema et al., 1995). For example, the ability of the parabens, alkyl esters of 4-hydroxybenzoic acid, to induce mitochondrial damage depends on the alkyl-chain length and is linearly related to the octanol/water partition coefficient, a reflection of lipophilicity (Lakeram et al., 2007, Nakagawa and Moldeus, 1998, Nakagawa and Moore, 1999). The ROS production and complex I inhibition in isolated mitochondria induced by aromatic acids also seems to be influenced by the lipophilicity of the compound (cinnamate > benzoate > phenylpropionate > phenylacetate) (Beloborodova et al., 2012, Fedotcheva et al., 2008, Fedotcheva et al., 2012, Politycka, 1997, Ye et al., 2006). The hydroxylated aromatic acids such as phenyllactate, 4-hydroxyphenylacetate, and 4-hydroxyphenyllactate are less lipophilic, do not stimulate ROS generation, and because of their hydroxyl groups, can act as free radical scavenging antioxidants (Fedotcheva et al., 2008).

5. Glycine conjugation as a detoxification mechanism

5.1 Glycine conjugation restores CoASH levels

As discussed in Section 4.2, several metabolic processes are dependent on CoASH and are inhibited by CoASH sequestration (Figure 2). Glycine conjugation is important for the maintenance of CoASH levels, by preventing its sequestration as benzoyl-CoA (Knights and Miners, 2012, Knights et al., 2007). For example, the inhibition of gluconeogenesis in bovine liver mitochondria by benzoate-induced CoASH sequestration is reversed by the addition of glycine, which stimulates hippurate synthesis, decreases benzoyl-CoA levels, and increases CoASH levels. As a result acetyl-CoA levels are restored and the rate of gluconeogenesis normalises (Griffith et al., 1989). The accumulation of benzoyl-CoA and sequestration of CoASH caused by benzoate is similar to the situation in isovaleric acidemia, suggesting that isovaleric acidemia can shed some light on the importance of glycine conjugation (Badenhorst et al., 2013, Trost and Lemasters, 1996). Isovaleric acidemia is a defect of leucine catabolism caused by mutations in the isovaleryl-CoA dehydrogenase gene, resulting in accumulation of isovaleryl-CoA in mitochondria (Tanaka et al., 1966). This accumulation results in sequestration of CoASH and formation of large amounts of isovaleric acid, which is toxic to mitochondria (Trost and Lemasters, 1996). Because isovaleryl-CoA is a good substrate for GLYAT, glycine conjugation restores CoASH levels and produces isovalerylglycine, a less toxic metabolite that is more readily excreted by the kidneys (Bartlett and Gompertz, 1974, Tanaka and Isselbacher, 1967). As a result of the excessive glycine conjugation, depletion of glycine is often observed in these patients. Glycine supplementation restores glycine levels, increases isovalerylglycine excretion, and generally results in normal mental and physical development of these patients (Dercksen et al., 2012, Itoh et al., 1996, Tanaka and Isselbacher, 1967, Sweetman and Williams, 2013). Alternatively, supplementation with carnitine leads to increased isovalerylcarnitine excretion, which also restores CoASH levels, detoxifies isovaleric acid, and results in effective treatment of the disease. Interestingly, carnitine supplementation does not necessarily decrease isovalerylglycine excretion, and may even stimulate it (Itoh et al., 1996, Sweetman and Williams, 2013).

5.2 Glycine conjugates are less toxic than the unconjugated acids and acyl-CoA thioesters

An important mechanism by which glycine conjugation reduces the acyl-CoA mediated toxicity of organic acids is by converting an electrophilic thioester to the inert peptide bond of the glycine conjugate (Caldwell, 1982, Caldwell, 1984). Unlike thioesters, and even esters such as acylglucuronides, peptide conjugates are chemically stable and do not react with nucleophiles such as proteins and glutathione (Caldwell, 1982, Caldwell, 1984, Grillo and Benet, 2002, Grillo and Lohr, 2009, Li et al., 2002, Li et al., 2003, Olsen et al., 2007, Sidenius et al., 2004). Since covalent modification by acyl-CoAs is dependent on both time and the concentration of the acyl-CoA thioester, it is reasonable to assume that the toxicity of these electrophiles are significantly decreased by conjugation to glycine, although this is yet to be experimentally verified (Caldwell, 1984, Grillo and Lohr, 2009).

Glycine conjugates are also usually less toxic than the original carboxylic acids. For example, hippurate is about fifteen times less toxic to cultured mosquito larvae than benzoate. Addition of glycine stimulated hippurate synthesis and reduced the toxicity of benzoate in a dose dependent manner. Interestingly, the toxicity of benzoate was enhanced by 4-hydroxybenzoate at concentrations where the 4-hydroxybenzoate itself was not toxic. This suggested competition with 4-hydroxybenzoate for glycine conjugation and detoxification, although the formation of 4-hydroxyhippurate was not confirmed (Casida, 1955). The enzyme D-amino acid oxidase is inhibited by benzoate *in vitro*, an effect also observed *in vivo* in rat kidneys. Lack of D-amino acid oxidase activity in rats is associated with abnormal kidney function, altered methionine metabolism, and amino aciduria, suggesting that benzoate may interfere with nephrocyte metabolism (Williams and Lock, 2005). Activity of D-amino acid oxidase is also important in the brain, where it is responsible for degradation of the neurotransmitter D-serine. Interestingly, altered levels of D-serine in the brain are associated with neurological abnormalities such as schizophrenia, where increased D-amino acid oxidase activity results in decreased brain D-serine levels (Madeira et al., 2008). It is possible that benzoate may interfere with brain D-serine metabolism, but this has not been investigated. However, it has been

shown that inhibition of D-amino acid oxidase by benzoate influences the conversion of D-3,4-dihydroxyphenylalanine to L-3,4-dihydroxyphenylalanine in rat brain and influences locomotor behaviour (Moses et al., 1996). Hippurate, however, is not an inhibitor of D-amino acid oxidase. In one study it was demonstrated that the inhibition of succinate oxidation, anaerobic glycolysis, and glutamate decarboxylation, caused by some substituted benzoates in rat brain homogenates, is decreased by glycine conjugation (Ross and Wootton, 1964). Benzoate also inhibits human tyrosinase and the aspartate amino transferase, alanine aminotransferase, and alkaline phosphatase of human erythrocytes (Chang, 2009). However, a search of the literature for enzymes inhibited by hippurate yielded only the glutaminase enzymes, which are discussed in Section 6.2.

5.3 Amino acid conjugation decreases lipophilicity to decrease toxicity and facilitate urinary excretion

As mentioned in Section 3.1, the commonly held view that glycine conjugation increases the water solubility of aromatic acids is incorrect (Beyoglu and Idle, 2012, Beyoglu et al., 2012). However, the water solubility of sodium benzoate is 629 g/l, which is much higher than physiologically relevant concentrations, suggesting that solubility is not the factor mediating its toxicity. We suggest that increased hydrophilicity, rather than increased water solubility, explains the detoxification of aromatic acids by glycine conjugation. In Section 4.3 the linear relationship between lipophilicity and the membrane accumulation and mitochondrial toxicity of the parabens was discussed (Lakeram et al., 2007, Nakagawa and Moldeus, 1998, Nakagawa and Moore, 1999). In one study it was demonstrated that diazinon, a carboxylesterase inhibitor, significantly increased the toxicity of propyl-paraben by inhibiting its hydrolysis to the more hydrophilic and less toxic compound 4-hydroxybenzoate (Lakeram et al., 2007). This suggests that the conversion of toxic organic acids to more hydrophilic metabolites, by biotransformation processes such as conjugation, is an important mechanism of decreasing toxicity to biological membranes. Interestingly, plants also seem to make use of conjugation reactions to decrease the toxicity of aromatic acids. For example, cucumber roots take up benzoates and

cinnamates in a dose-dependent manner, with the more lipophilic cinnamates being absorbed more rapidly. Diffusion of the aromatic acids across the plasma membrane causes membrane damage, leakage of ions, and membrane depolarisation. The plant responds by converting the acids to acylglucuronides, which are less toxic and do not cause membrane disruption (Politycka, 1997, Ye et al., 2006).

Hippurate is transported out of the kidneys into urine by rat organic anion transporter 1 (Deguchi et al., 2005). Benzoate competes with hippurate for the hepatic monocarboxylate transporters, indicating that both benzoate and hippurate can be exported by these transport proteins (Schwab et al., 2001, Yoshimura et al., 1998). Therefore, it is important to ask why benzoate has to be converted to the more hydrophilic hippurate in order to facilitate excretion. This seems to be because benzoate rapidly accumulates in the mitochondrial matrix to concentrations about 50 times higher than the external concentration (Gatley and Sherratt, 1977, Gatley and Sherratt, 1976). This indicates that the rate of benzoate export from the mitochondrial matrix is significantly lower than the rate at which it can enter. The explanation for this is that the pH in the intermembrane space (as low as pH 6.0) is about two units lower than in the matrix (pH 7.8) (Davies et al., 2011, Strauss et al., 2008, Porcelli et al., 2005). This high pH gradient over the inner mitochondrial membrane favours formation of undissociated benzoic acid in the intermembrane space and diffusion into the matrix, where the higher pH favours dissociation and trapping of the benzoate ion (Gatley and Sherratt, 1977, Gatley and Sherratt, 1976, Porcelli et al., 2005). Efflux of benzoate by monocarboxylate transporters would therefore be futile, since the anion can simply reform benzoic acid and re-enter the matrix space. In fact, yeasts gain resistance to benzoic acid not only by actively transporting benzoate out of the cell, but also by decreasing the permeability of the plasma membrane to decrease the ability of benzoic acid to diffuse over the membrane (Piper et al., 2001, Piper, 1999). We think that conversion of benzoate to hippurate facilitates excretion, since hippurate is less lipophilic and therefore less capable of partitioning into biological membranes. This concept is demonstrated in Figure 3. Despite being only slightly more water soluble, hippuric acid is significantly more hydrophilic than benzoic acid, as demonstrated by their LogP values of 0.31 and 1.87, respectively (Hansch and Leo, 1995). The lower pKa of hippurate, compared to

benzoate, may further contribute to its lower toxicity. The pKa value of hippuric acid is about 0.6 pH units lower than that of benzoic acid, indicating that the amount of undissociated acid (toxic form) present in the mitochondrial intermembrane space will be about four times lower for hippuric acid than for benzoic acid.

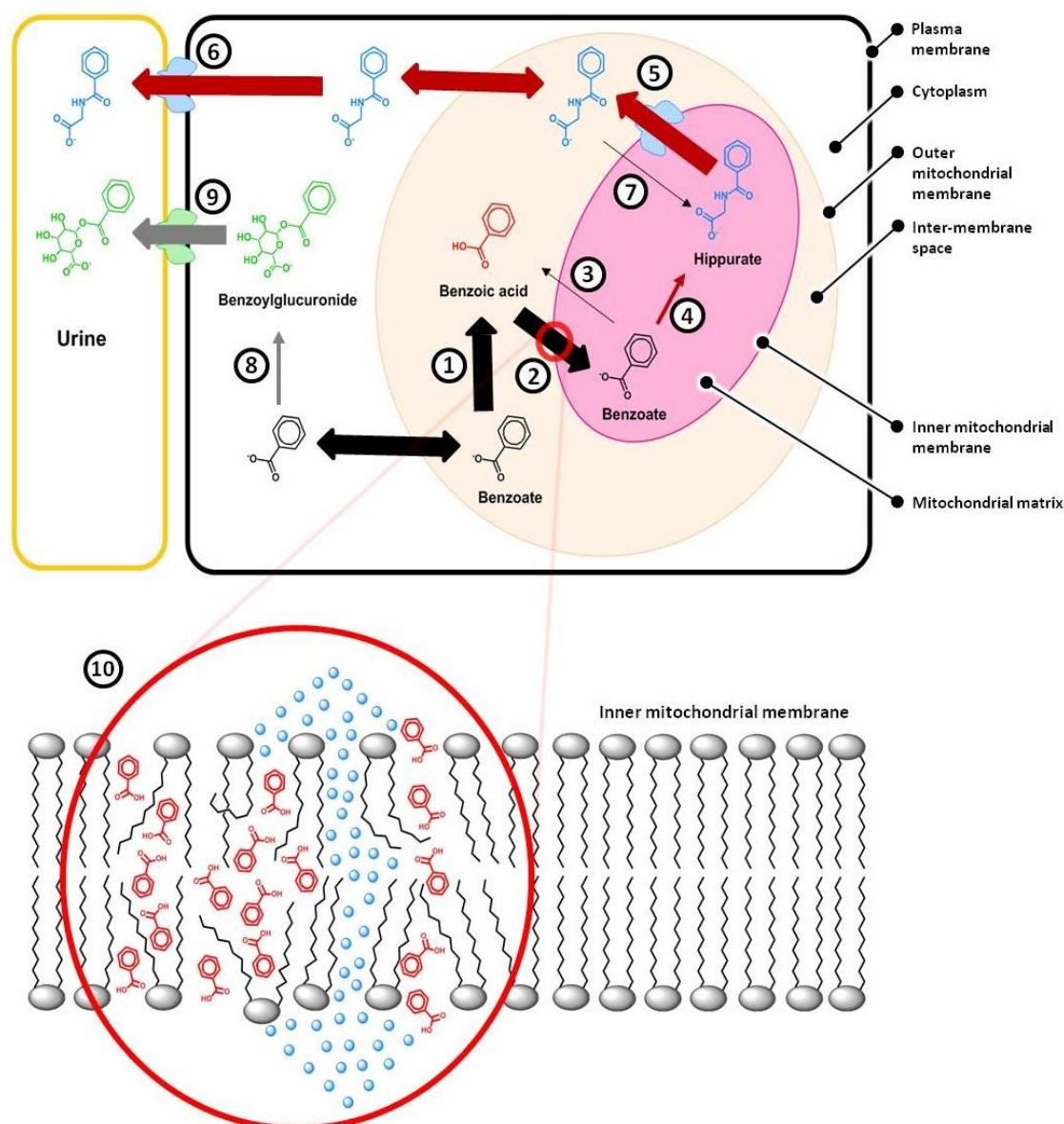


Figure 3: The detoxification of benzoate by means of glycine conjugation in hepatocytes. 1) Because the pH in the mitochondrial inter-membrane space is lower than that of the cytoplasm and the mitochondrial matrix, the conversion of benzoate (black) to benzoic acid (red) is favoured. 2) Benzoic acid can freely diffuse across the mitochondrial inner membrane into the mitochondrial matrix. In the matrix the higher pH favours the dissociation of benzoic acid to form benzoate. 3) Benzoate accumulates in the mitochondrial matrix to concentrations about 50 times higher than that in the surrounding solution. This is demonstrated by the width of the black arrows. Therefore, the thick black arrow 2, pointing to the matrix, is 50 times wider than arrow 3, pointing out of the matrix space. 4) In the mitochondrial matrix benzoate is converted to hippurate (blue). 5) Hippurate is transported out of the matrix by organic anion transporters. The rate at which hippurate can be excreted is about five times higher than that at which it can be synthesised. This is demonstrated by the red arrow 5 being five times wider than red arrow 4, which represents hippurate synthesis. 6) Finally, hippurate is irreversibly excreted into urine (yellow block) by organic anion transporters. 7) Hippuric acid is less lipophilic than benzoic acid and therefore diffuses into the matrix space at a significantly lower rate. This is indicated by black arrow 7 being 40 times thinner than black arrow 2, which represents the diffusion of benzoic acid into the matrix. 8) In the cytoplasm, benzoate can also be converted to benzoyl-glucuronide by UDP-glucuronosyltransferases, as represented by the grey arrows. Because benzoate accumulates in the mitochondrial matrix, benzoylglucuronide synthesis, which is localised to the endoplasmic reticulum, only occurs when the capacity of the mitochondrial glycine conjugation system is exceeded. 9) Benzoylglucuronide is then also excreted in the urine. 10) This section zooms in on a part of the inner mitochondrial membrane. It represents the diffusion of the lipophilic benzoic acid into the inner mitochondrial membrane, which causes disruption of the phospholipid bilayer and results in permeabilisation of the membrane. This is represented by the small blue spheres leaking across the membrane.

6. The impact of glycine conjugation on metabolism and health

6.1 Benzoate and human glycine deficiency

Glycine is commonly thought to be a nonessential amino acid since it can be synthesised from serine by serine hydroxymethyltransferase (Wu et al., 2013). Serine itself is synthesised from 3-phosphoglycerate, an intermediate of glycolysis (Figure 4). Serine hydroxymethyltransferase converts serine to glycine, 5,10-methylenetetrahydrofolate (THF-C1), and NADH. Additional THF-C1 and NADH are produced from glycine by the glycine cleavage system. In recent years it has become apparent that glycine is a conditionally essential amino acid, as the metabolic demand for glycine often exceeds the capacity for glycine synthesis (Melendez-Hevia et al., 2009, Wu, 2013, Wu et al., 2013). For example, glycine availability has been shown to be limiting in the developing human foetus, in young men, birds, and in milk-fed pigs (Jackson et al., 1997, Jackson et al., 1996, Wang et al., 2013, Wu, 2010, Wu, 2013, Wu et al., 2013). In fact, it has been estimated that human adults have a shortage of about 10 grams of glycine per day. This glycine deficiency, and the resulting deficiency of THF-C1 units, can influence the metabolism of collagen, glutathione, creatine, nucleic acids, and porphyrins (Brosnan et al., 2011, Melendez-Hevia et al., 2009, Wang et al., 2013). It has been argued that glycine shortage can be exacerbated by benzoate and other substrates for glycine conjugation (Au et al., 2013, Badenhorst et al., 2013). In addition to natural sources of benzoate (Section 2), humans are nowadays exposed to ever increasing amounts of this compound, since it is used as a preservative in food and pharmaceuticals (Piper, 1999). In some populations where a lot of preserved food is consumed, benzoate intake can be as high as 280% of the recommended daily allowance, which is reflected by unusually high levels of urinary hippurate (Lees et al., 2013, Tfouni and Toledo, 2002).

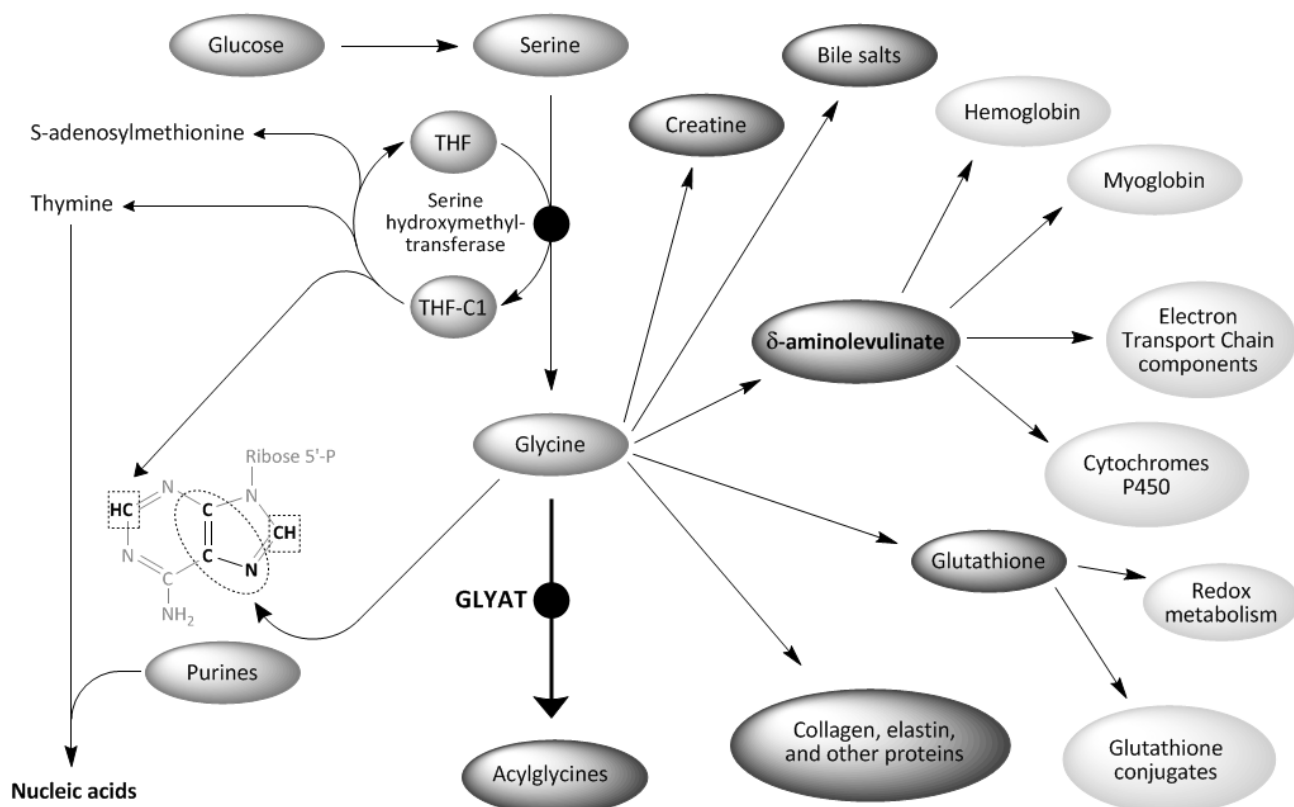


Figure 4: Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesised from serine by serine hydroxymethyltransferase. The reaction converts THF (tetrahydrofolate) to THF-C1 (tetrahydrofolate-C1) for each molecule of glycine produced. The total amount of glycine synthesised can thus not exceed the amount of THF-C1 consumed through production of compounds such as S-adenosylmethionine, thymine, and purines. Glycine is consumed in the production of creatine, bile salts, porphyrins, collagen, elastin, other proteins, and glutathione. Black circles represent the serine hydroxymethyltransferase and GLYAT enzymes. The bold arrow indicates formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively. Reproduced with minor modifications (Badenhorst et al., 2013) with permission from Informa Healthcare.

6.2 Glycine conjugation, glutathione, and diabetes

A useful marker of glycine depletion is the urinary excretion of pyroglutamate, which is spontaneously formed when insufficient glycine is available for glutathione synthesis (Jackson et al., 2004, Jackson et al., 1997, Jackson et al., 1996). Vegetarians, who consume large amounts of substrates for glycine conjugation, and often insufficient protein, excrete larger amounts of pyroglutamate than omnivorous humans (Metges et al., 2000, Persaud et al., 1996). Benzoate administration to humans significantly increases pyroglutamate excretion, indicating glycine shortage and impaired glutathione synthesis (Jackson et al., 1987). Glutathione

is crucial in all cells of the body for maintaining a reducing environment and for neutralising the harmful ROS constantly produced by mitochondria (Wu et al., 2004). A deficiency of glutathione is associated with inhibition of mitochondrial complex I, increased levels of ROS, and oxidative damage such as lipid peroxidation (Jha et al., 2000, Sekhar et al., 2011a, Sekhar et al., 2011b).

Metabolites associated with glycine and glutathione metabolism are the most important metabolites differentiating diabetics from controls in metabolomics studies. However, this is not clearly understood at present, and warrants further investigation (Xie et al., 2013). Diabetes is characterised by a depletion of glutathione and very high levels of ROS (Bandeira et al., 2013, Pitocco et al., 2010, Sekhar et al., 2011a). The shortage of glutathione is due to impaired synthesis, resulting from a shortage of glycine and cysteine. Dietary supplementation with glycine and cysteine results in increased glutathione synthesis and decreases the levels of ROS and lipid peroxidation to normal (Sekhar et al., 2011a). It is interesting to note that diabetes is associated with an abnormal gut microbiome and significantly elevated urinary hippurate, indicating increased glycine conjugation of microbial metabolites (Lees et al., 2013, Zuppi et al., 2002). Although it is not known whether the increased synthesis of hippurate is responsible for the glycine shortage in these individuals, they may be more sensitive to the negative consequences of consuming benzoate as a preservative, which can exacerbate the glycine deficiency (Badenhorst et al., 2013, Lees et al., 2013, Sekhar et al., 2011a, Zuppi et al., 2002). Glycine shortage could also decrease the rate of benzoate clearance, and therefore increase the levels of benzoate circulating in plasma. This can stimulate ROS production and damage mitochondria, important factors in the development of insulin insensitivity (Beloborodova et al., 2012, Park et al., 2013, Trost and Lemasters, 1996, Trost and Lemasters, 1997, Wang et al., 2010). Interestingly, administration of benzoate to sheep rapidly increases plasma levels of glucose, insulin, and glucagon, but the significance of this observation is unclear at present (Mineo et al., 1995).

6.3 Glycine N-acyltransferase and the musculoskeletal system

A well-known consequence of glycine shortage is decreased turnover of collagen, an important structural protein. Although decreased collagen turnover is not lethal, it leads to accumulation of damage to connective tissues such as the skin, thereby accelerating the ageing process, especially in long-lived animals such as humans (Melendez-Hevia et al., 2009, Wu et al., 2013). In addition to influencing collagen turnover, glycine shortage seems to be related to the growth and development of the musculoskeletal system in several complicated ways. A recent study revealed a statistically significant correlation between lean muscle mass and bone size, and three SNPs in the human GLYAT gene. Unfortunately, the effect of these genetic variations are not known, but it has been suggested that the influence of GLYAT on glycine metabolism could explain this observation (Badenhorst et al., 2013, Guo et al., 2013). Depletion of glycine by conjugation to benzoate, for example, has been shown to result in reduced weight gain and weight loss in rats, an effect cancelled out by co-administration of glycine (White, 1941). The amount of hippurate synthesised, however, depends mostly on the extent of exposure to hippurate precursors like benzoate (Section 2.2). Genetic variation in GLYAT is therefore not likely to significantly influence the amount of glycine consumed in the synthesis of hippuric acid, and other factors must explain the relationship between genetic variation in GLYAT and development of the musculoskeletal system. The effects of increased levels of plasma benzoate on muscle function is not yet clearly understood, but its negative effect on mitochondrial energy production may be a contributing factor (Section 4). Other factors that could influence muscle growth and function are plasma levels of glucose, insulin, and glucagon, which may be influenced by benzoate (Mineo et al., 1995). It is known that hippurate can inhibit the utilisation of glucose by human muscle and kidney cells, but this effect is only observed at the high concentrations of hippurate resulting from chronic renal failure (Spustova et al., 1989, Spustova and Dzurik, 1991, Spustova et al., 1987, Spustova and Oravec, 1989). Interestingly, GLYAT is expressed weakly in skeletal muscle and the pancreas, but glycine N-acyltransferase activity has not been reported in these tissues and the significance of this observation remains unclear (Matsuo et al., 2012).

6.4 Glycine conjugation and cancer

In hepatocellular carcinoma specimens the expression of GLYAT is significantly or completely downregulated at the transcriptional level (Matsuo et al., 2012). This may be explained by the dependence of rapidly proliferating cancer cells on the availability of glycine, which is needed for nucleic acid synthesis (Figure 4) (Jain et al., 2012). Two molecules of glycine and one THF-C1 are needed for the synthesis of purines, and *de novo* thymidine synthesis requires THF-C1 produced by the glycine cleavage system (Melendez-Hevia et al., 2009, Wu, 2010, Wu, 2013, Wu et al., 2013). The downregulation of GLYAT expression in hepatocellular carcinoma could therefore be a strategy to conserve glycine, which is needed for DNA synthesis (Badenhorst et al., 2013). Benzoate has been shown to inhibit DNA synthesis in cultured rat hepatocytes, but it is not clear whether this is because of glycine depletion (Oyanagi et al., 1987). The impact of benzoate conjugation on the availability of glycine for purine synthesis is demonstrated by the observation that uricotelic birds and reptiles, which excrete waste nitrogen as uric acid, do not synthesise hippurate (Bridges et al., 1970, Smith, 1958). Two molecules of glycine are needed for uric acid synthesis, perhaps explaining why benzoate is conjugated to ornithine instead (Smith, 1958). Further indirect evidence for decreased purine synthesis, as a result of increased hippurate synthesis, is the reduced urinary excretion of uridine by diabetic patients, who also excrete significantly more hippurate than controls (Lees et al., 2013, van Doorn et al., 2007).

In addition to glycine, glutamine is a crucial nutrient for cancer cells (Wise and Thompson, 2010). Utilisation of glutamine is initiated by its conversion to glutamate by glutaminases, followed by transamination of glutamate to α -ketoglutarate, which enters the Krebs cycle. The rate of growth of cancer cells is proportional to glutaminase activities (Knox et al., 1969, Linder-Horowitz et al., 1969). Phosphate-dependent and phosphate-independent glutaminases, with different metabolic functions, are found in humans and other animals. Phosphate-independent glutaminase activity, thought to be important in the regulation of renal ammoniogenesis and pH balance, is stimulated by hippurate. In contrast, the mitochondrial phosphate-dependent glutaminases are inhibited by hippurate under acidic conditions (Dzurik et al., 2001, Krivosikova

et al., 1998). Hippurate could thus inhibit the utilisation of glutamine by tumours, which prefer acidic environments. This is supported by the observation that hippurate can significantly inhibit tumour growth (Spustova and Oravec, 1989). Furthermore, hippurate has recently been identified in metabolomics studies as important for the prediction of cancer recurrence, with decreased hippurate levels correlating to recurrence of oral cancer (Xie et al., 2012, Ye et al., 2012).

6.5 Benzoate and glycine conjugation may influence porphyrin metabolism

Porphyrins are crucial components of haemoglobin, myoglobin, electron transport chain complexes, and cytochrome P450 enzymes (Figure 2 and Figure 4). Porphyrin synthesis starts with the mitochondrial condensation of glycine and succinyl-CoA to form δ -aminolevulinate (Piper et al., 1973). To date, there is no direct evidence that glycine availability limits porphyrin synthesis. However, benzoate decreases the faecal excretion of porphyrin metabolites by rats, an effect cancelled out by administration of glycine, which by itself increases the excretion of these metabolites (Lucas and Orten, 1954). Benzoate can also be used to treat chemically induced porphyria in rats, by decreasing glycine availability and inhibiting porphyrin synthesis, an effect cancelled out by the co-administration of glycine (Piper et al., 1973). In a recently published hypothesis it was argued that the glycine dependence of cancer cells cannot be completely explained by the increased demand for nucleic acid synthesis. The authors point out that cancer cells are specifically dependent on mitochondrial glycine synthesis, while purine biosynthesis occurs in the cytoplasm (di Salvo et al., 2013). Furthermore, Chinese hamster ovary cell lines with defects of mitochondrial serine hydroxymethyltransferase are glycine auxotrophs and have reduced activities of mitochondrial complex IV, which assembles incorrectly if haem is deficient (Atamna et al., 2001, Ye et al., 2010).

An issue that has not been addressed in the literature is whether succinyl-CoA availability can limit the synthesis of δ -aminolevulinate, and therefore porphyrins. Synthesis of succinyl-CoA from either succinate or

α -ketoglutarate requires CoASH. It is therefore possible that sequestration of CoASH by benzoate would limit the synthesis of succinyl-CoA and therefore decrease porphyrin synthesis. This is indirectly supported by the observation that ketone body oxidation in rat heart causes CoASH sequestration, which results in decreased synthesis of succinyl-CoA by α -ketoglutarate dehydrogenase (Russell and Taegtmeyer, 1992). If benzoate influences the availability of succinyl-CoA and glycine it is possible that the synthesis of the cytochromes P450 may be affected, which could result in abnormal phase I detoxification. This topic must be urgently investigated, as abnormal succinyl-CoA ligase activity has been linked to deleterious effects on mitochondrial DNA synthesis (Elpeleg et al., 2005, Furuyama and Sassa, 2000).

6.6 Benzoate consumption may influence appetite and feeding behaviour

In ecology, the “detoxification limitation hypothesis”, based on observations of animal feeding behaviour over the past four decades, states that animals can sense the toxic load of food and either reduce food intake, or switch diets, in response to a high intake of toxic plant secondary metabolites. This prevents the animal from ingesting an amount of toxins exceeding its detoxification capacity (Marsh et al., 2006). The emetic system is one mechanism mediating an aversion to the diet, explaining why anti-emetic drugs such as metoclopramide and ondansetron can suppress this response. However, the decreased consumption of diets containing benzoate by possums is not suppressed by anti-emetics, but supplementing the benzoate containing diet with glycine restores food intake to normal (Marsh et al., 2005). This suggests that plasma or CSF glycine levels may be important in mediating the response to benzoate. Although it has been argued in this review that regulation of CNS glycine concentration is not the primary function of the glycine conjugation system, short-term fluctuations of plasma and CNS glycine do result from ingestion of benzoate and other substrates for glycine conjugation (Beyoglu and Idle, 2012, Beyoglu et al., 2012, de Vries et al., 1948). Imbalanced diets rapidly influence the levels of amino acids in rat brain, which is sensed in the anterior piriform cortex by a GCN2 kinase mediated signal transduction pathway that results in an aversive

response to the food source (Maurin et al., 2005). We hypothesise that this mechanism could partly explain the decreased appetite induced in humans, monkeys, dogs, rats, possums, and hens by the administration of benzoate or phenylacetate (Batshaw et al., 1988, Marsh et al., 2005, Sherwin and Kennard, 1919). In this view the glycine deportation hypothesis (Section 3) seems to support, rather than contradict, the detoxification theory of glycine conjugation (Badenhorst et al., 2013, Beyoglu and Idle, 2012, Beyoglu et al., 2012).

7. Summary and conclusions

7.1 The primary purpose of the glycine conjugation pathway is the detoxification of benzoate

The main objective of this review has been to develop a deeper understanding of the role of glycine conjugation in metabolism. It was demonstrated that glycine conjugation, far from being an uninteresting and unimportant metabolic process, is one of the fundamentally important homeostatic mechanisms in animal physiology. It was demonstrated that glycine conjugation is crucial to the maintenance of adequate levels of CoASH in the liver and kidney, and to decrease the intrinsic toxicity of organic acids by increasing their hydrophilicity (Figure 3). It was also argued that the recently proposed glycine deportation hypothesis is not a suitable replacement, but rather seems to support, the detoxification theory of glycine conjugation (Beyoglu and Idle, 2012, Beyoglu et al., 2012, Caldwell, 1982, Caldwell, 1984). This is an important perspective, since modern humans are constantly exposed to large amounts of benzoate and other toxic organic acids, and underestimation of the toxicity of these compounds may pose a public health risk (Park et al., 2013).

7.2 The glycine conjugation pathway is still very poorly characterised

The levels of hippurate excreted in urine seem to correlate with a wide range of pathological conditions such as diabetes, obesity, gut dysbiosis, autism, schizophrenia, depression, hepatitis, and cancer (Lees et al., 2013). However, it is currently impossible to accurately interpret the relationships between hippurate excretion and these disease conditions, since the glycine conjugation pathway is still rather poorly understood (Badenhorst et al., 2013, Knights and Miners, 2012, Knights et al., 2007, Lees et al., 2013). Therefore, the influence of genetic variation in the ACSM2A and GLYAT genes on the activities of the HXM-A and GLYAT enzymes, as well as the relationship between these factors and the *in vivo* rate of glycine

conjugation, must be investigated in more detail. Here some studies, that could significantly advance our understanding of the glycine conjugation pathway, are suggested.

7.2.1 Determination of *in vivo* and *in vitro* glycine conjugation rates

Previous *in vivo* studies of glycine conjugation are difficult to compare because of differences in the substrates, substrate dosages, sample collection protocols, metabolite analyses, and test animals used. Most studies have made no attempt to identify the limiting step of the glycine conjugation pathway, making it difficult to estimate the relative importance of variation in HXM-A and GLYAT enzyme activities to this process (Badenhorst et al., 2013, Knights and Miners, 2012). As discussed in Section 2.3.1, the commonly accepted view of benzoate as the primary substrate for glycine conjugation may be misleading. The major precursor for hippurate synthesis is phenylpropionic acid, produced by the gut microbiota from dietary polyphenols (Fedotcheva et al., 2008, Jenner et al., 2005, Rechner et al., 2002). This means that the rates of glycine conjugation measured using benzoate as a probe compound may not always be physiologically relevant. Phenylpropionate, cinnamate, and benzoate differ in their ability to stimulate ROS generation and inhibit respiration (Beloborodova et al., 2012, Fedotcheva et al., 2008, Fedotcheva et al., 2012). It would therefore be valuable to compare the *in vivo* rates of hippurate formation from phenylpropionate, cinnamate, and benzoate, and to compare the stimulatory effect of glycine supplementation in each case. It is also important to compare the *in vitro* rates of activation of these three hippurate precursors to acyl-CoA esters by the human liver HXM-A and HXM-B ligases, as well as the rate of benzoyl-CoA formation through β -oxidation. This information is crucial to developing an understanding of the factors that influence variation in the rate of glycine conjugation *in vivo*. It would also be valuable to compare glycine cleavage system activities to glycine availability, which is a major factor influencing the rate of glycine conjugation *in vivo* (de Vries et al., 1948).

7.2.2 Investigation of the influence of genetic variation in the ligase and transferase enzymes

As discussed in Section 2.3.2, it is now known that genetic variation in the human GLYAT gene influences the enzyme activity of a recombinant human GLYAT. However, of the 55 nonsynonymous SNPs in the human GLYAT gene that are currently known, only six have been investigated (van der Sluis et al., 2013). It would therefore be valuable if more of these variations were characterised in terms of enzyme activity, substrate selectivity, stability, and mitochondrial import. The influence of variation in the non-coding parts of the gene also need to be investigated, since it has been reported that some of these variations may influence development of the musculoskeletal system (Guo et al., 2013). It is possible that these genetic variations influence expression of the gene, but at present this is not understood at all. It is known that hepatic expression of GLYAT is influenced by diet in rats, and that decreased transcription of GLYAT results in decreased GLYAT protein content of hepatocellular carcinoma specimens (Matsuo et al., 2012, Wen et al., 2013). However, it is not yet known how variation in hepatic GLYAT activity influences the *in vivo* rate of glycine conjugation, since we do not yet understand the conditions under which GLYAT activity is the limiting factor in this pathway.

The study of Temellini and co-workers demonstrated significant interindividual variation in the ability of human liver homogenates to synthesise hippurate from benzoate, ATP, CoASH, and glycine. This suggests that the amounts and activities of the ligase and transferase enzymes are responsible for the variation, but these factors were not determined separately (Temellini et al., 1993). As far as we know, the association between aspirin intolerance and an SNP (rs1133607) in the ACSM2 gene, which encodes HXM-A, is the only information currently available to suggest that genetic variation in the acid:CoA ligase enzymes can influence the metabolism of salicylate and other benzoate derivatives (Agundez et al., 2009, Boomgaarden et al., 2009, Vessey et al., 1999). The use of standardised protocols for the characterisation of recombinant enzymes and their variants seems to hold great promise for this field, given the difficulty in obtaining human liver tissues for research (Knights et al., 2007, van der Sluis et al., 2013, Vessey et al., 1999).

References

The references for this manuscript are included in the final reference list at the end of this thesis.