Part One
Biochemistry and Molecular Genetics of Drug Metabolism

1
Drug-Metabolizing Enzymes – An Overview

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1.1
Introduction: Fate of a Drug in the Human Body

Drugs and, more generally, all substances foreign to the human body enter the organism in many ways. Intentional administration of a drug implies that the route of administration is selected depending on the clinical status of the patient, on the target tissue or organ, and on the chemical nature of the drug. For example, highly ionized compounds cannot easily penetrate barriers such as that of the gastrointestinal tract and therefore should be administered parenterally. Peptides or proteins are degraded to a great extent in the gastrointestinal tract by the action of hydrolytic enzymes and hence are often given to patients in ways other than the most common oral route (e.g., by intranasal application). Intravenous application implies an immediate interaction of a drug with plasma enzymes (e.g., carboxyesterases).

In many cases, the enzymes performing the biotransformation of a drug are needed to convert a parent drug (a prodrug) to the active molecule. Lovastatin – a hypolipidemic drug – is a good example of this process as it requires metabolic activation by carboxyesterases. Carboxyesterases in the plasma, liver microsomes, and liver cytosol convert 18, 15, and 67%, respectively, of the orally given drug to the active hydroxyacid molecule [1].

In general, after its administration a drug should be absorbed; subsequently, it is distributed in the body, often it is also metabolized, and finally excreted. These processes determine the pharmacokinetics of a drug; in other words, the time course of the drug level in the tissue or organ of interest.

The majority of drugs are administered orally and, hence, the uptake of a drug from the gastrointestinal tract is the most frequent way of drug absorption; consequently, the action of liver (and intestinal) drug-metabolizing enzymes starts already in the process of absorption, even before the drug reaches the systemic circulation. The enzymes of drug biotransformation often lower the amount of drug available in the systemic circulation by converting it into metabolites (active, inactive, or with an altered activity) – this process is known as the "first-pass effect." The enzymes of drug biotransformation often decide the biological availability of

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a drug (i.e., the level of a drug available at the site of its action). This book focuses on drug metabolism and on the respective enzymes responsible for this process.

However, this is not the only role of drug-metabolizing enzymes. Changes in drug metabolism may be responsible for the incidence of adverse reactions to drugs, such as when a drug's metabolism is blocked by another compound (e.g., due to competition with another drug – then the level of the "victim" drug may increase and even exceed the toxic levels) or, on the contrary, by induction of drug-metabolizing enzymes. Then, the metabolism of another drug ("victim"), metabolized by the same enzyme, is quicker and it may fail to reach its therapeutic range. This type of drug–drug interaction has been intensively studied as it is a potential reason for failure of pharmacotherapy [2]. The situation may be further heavily influenced by genetic predisposition of a patient to metabolize the respective drug, such as in many examples of drugs metabolized by cytochromes P450 (CYPs). This is, for example, the case for antidepressants metabolized by CYP form 2D6, where the genetically determined ability of a patient to metabolize the drug may lead to effective dose variations approaching an order of magnitude [3]. For example, when a slowly metabolizing patient on a somewhat lower dose of the "victim" drug takes another drug metabolized by the same CYP2D6 enzyme, both the effects of drug interaction caused by the competition for the enzyme active site and the pharmacogenetic predisposition come into play, and the patient could easily be overdosed. This is why this book deals not only with the respective enzymes and drugs, but also with the pharmacogenetic implications of patients' predispositions to variations in drug metabolism.

1.2 Classification Systems of Drug-Metabolizing Enzymes According to Different Criteria

Drug-metabolizing enzymes have been traditionally grouped into two main classes, reflecting the fact that a drug is often primarily transformed to a more polar metabolite either by insertion of a polar group into the molecule (e.g., by hydroxylation) or by liberation of an already present functional group (e.g., by demethylation of a hydroxymethyl derivative yielding a free hydroxyl). These "first-phase" reactions are in most cases followed by conjugation reactions ("second phase") during which the molecule is typically attached to a more polar molecule to facilitate its excretion. Since 1959 when Williams [4] introduced this terminology, it has been shown (with progress in elucidation of the drug metabolic pathways of many drugs) that there are possibly more exceptions to this general rule than expected; for example, some "phase II" reactions may not be preceded by "phase I" biotransformation – morphine that is glucuronidated directly or paracetamol (acetaminophen) that is also predominantly glucuronidated or conjugated with sulfate (Figure 1.1) [5].

The original concept of the drug or its metabolite being converted to an even more polar molecule in the "phase II" process is also not generally valid. As has

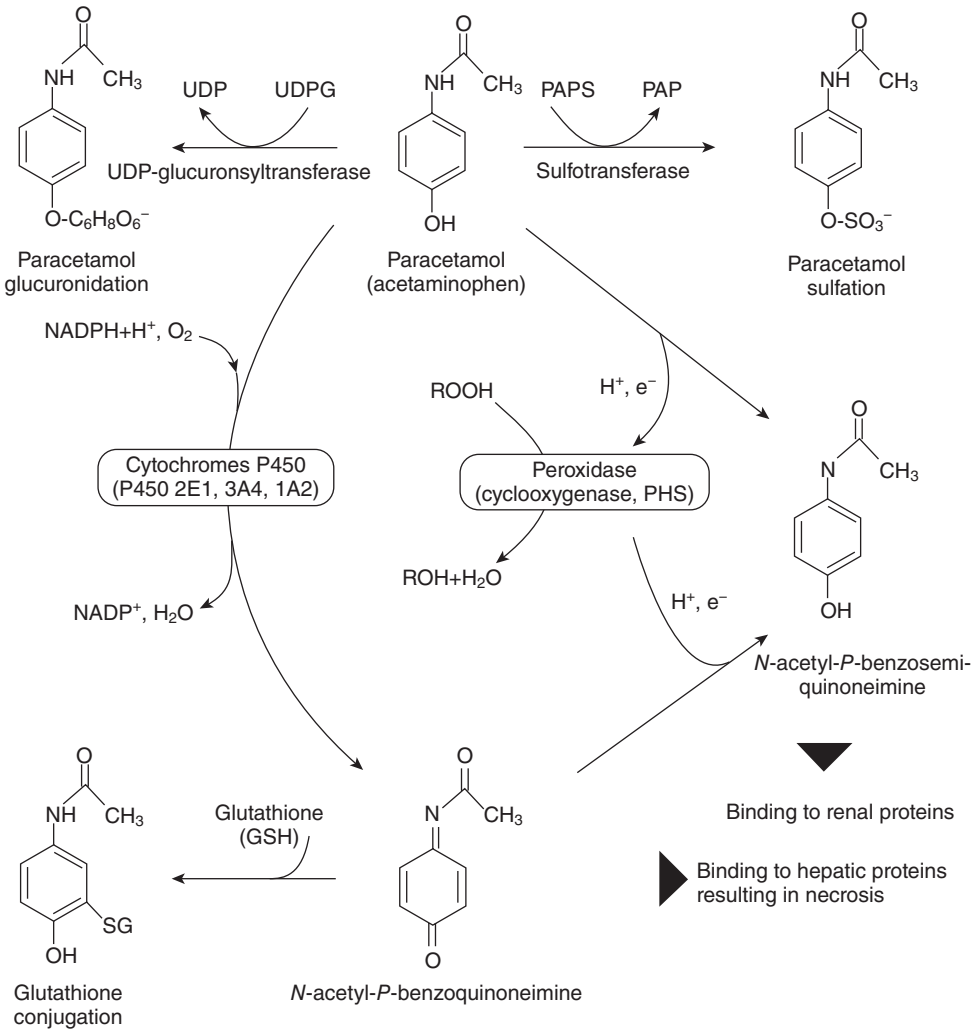

Figure 1.1 Pathways of paracetamol (acetaminophen) metabolism. Paracetamol is primarily converted to a sulfate or glucuronide by SULT (with a PAPS coenzyme) and UGT, UDPG is the UDP-glucuronide; oxidation reactions catalyzed by CYPs or peroxidase lead to the formation of reactive toxic products; for detoxication, conjugation with glutathione (GSH) by GST is available under physiological conditions [5].

been recently pointed out, for example, the *N*-acetylation of aromatic amines or the methylation of catechols usually decreases the water solubility of the resulting compounds [6].

The apparent complexity of this problem is also reflected in a classification of biotransformation reactions suggested in [6] based on the chemical nature of the process:

i) Oxidations, including reactions mediated by CYPs and peroxidases, but also by alcohol hydrogenases and others.

ii) Reductions, performed by, for example, ketoreductases and azoreductases.

iii) Conjugations, limited, however, to reactions in which the electrophilic nucleoside-containing cofactors (such as adenosine triphosphate, activated sulfate 3′-phosphoadenosine-5′-phosphosulfate (PAPS), acetyl-CoA, UDP-glucuronic acid, *S*-adenosylmethionine, etc.) play a crucial role in interactions with nucleophilic sites in a xenobiotic molecule (e.g., the amino or hydroxy group).

iv) Nucleophilic trapping processes, when electrophilic xenobiotics react with cellular nucleophiles – often represented by water or by glutathione (including formation of protein and DNA adducts).

Clearly, the future will show whether the new classification is more viable than the original one.

Figure 1.1 gives an introduction to drug metabolism processes by showing the pathways of paracetamol metabolism. Reactions of the "first phase" involve oxidation of the parent molecule to quinone structures catalyzed by CYPs and peroxidase (cyclooxygenase (COX, also called prostaglandin H synthase); the conjugation processes are both sulfation and glucuronidation (the majority of paracetamol is metabolized by these two reactions) as well as a detoxication reaction by conjugation of glutathione to the toxic and reactive *N*-acetyl-*p*-benzoquinone imine (NAPQI).

In the first part of this book, the focus is on the main classes of biotransformation enzymes without attempting to divide or group them into categories. As in most textbooks, the description will start with CYPs as they represent the most important family of drug-metabolizing enzymes. Formation of adducts of activated xenobiotics with biological macromolecules (proteins, DNA, but also polysaccharides) as well as with other structures is not covered here since the aim of this book is to give the interested professional information on the interaction of drugs with enzymes of metabolism and on the related consequences.

1.3 Overview of the Most Important Drug-Metabolizing Enzymes

1.3.1 CYPs

CYPs are the best known drug-metabolizing enzymes [7]. They deserve this attention – more than three-quarters of all known drug oxidations are catalyzed by CYPs and this is certainly not the final count. The oxidation reactions are started by one-electron reduction of the heme iron central atom, which is followed by binding of molecular oxygen. In other words: (i) CYPs are proteins that possess heme in

their active site (just as with hemoglobin or other cytochromes), (ii) their function needs electrons to be supplied from a suitable source – another protein having the ability to transfer electrons from NADPH to CYPs (for drug-metabolizing microsomal CYPs, in most cases a flavoprotein), and, finally, (iii) the heme iron should be able to bind molecular oxygen (no wonder – as in hemoglobin), but in this particular case it should be endowed with "magical" force to split the dioxygen and activate it. This "extra" force is provided by donation of electron density coming from a sulfur atom serving as the sixth ligand of the heme iron. To refresh the chemistry of hemes, an iron atom can be bound to six partner atoms or ligands; here, in the resting state, four bonds are occupied by nitrogen atoms of the heme, the fifth bond joins the heme iron to a negatively charged sulfur atom ("thiolate" sulfur) of a Cys amino acid residue from the protein chain and the sixth bond is with an oxygen from a water molecule present in the active center (during the catalytic process, a dioxygen is bound here). The result can be described by a relatively simple equation summarizing the reaction in which the molecular oxygen (dioxygen) is activated and split, yielding a water molecule and a monooxygenated (mostly hydroxylated) substrate. Very recently, the crucial intermediate of this process, the Fe(IV) oxo porphyrin radical species (so-called Compound I of the heme enzymes) has been prepared in high yield from microbial CYP119 [8]. A detailed description of the catalytic mechanism is given in Chapter 2. The reaction summarizing most of the processes in which the CYP enzymes take part can be written as:

$$NADPH + H^+ + R{-}H + O_2 \rightarrow NADP^+ + H_2O + R{-}OH$$

The reaction involves the source of electrons (reduced NADPH cofactor with a proton H^+), a substrate (R–H), dioxygen, and oxidized $NADP^+$, water, and a monooxygenated or hydroxylated molecule of substrate (R–OH).

CYP enzymes metabolizing xenobiotics are localized in many tissues, typically in the liver, intestines, lung, and kidney, but also in the brain, heart, and nasal mucosa. Subcellular localization of these enzymes is mainly in microsomes (formed after cell disruption from the endoplasmic reticulum); however, drug-metabolizing CYPs are present also in cellular membranes and mitochondria [9].

In most cases, the products (i.e., metabolites) are welcome as the drugs should be easier to conjugate after hydroxylation (e.g., with a polar residue of glucuronic acid) or excreted as hydroxy derivatives. However, in many cases the metabolites formed by monooxygenation are harmful to the body or, more precisely, the products of the CYPs-mediated reaction are further converted to species that lead to toxic responses of organism or are involved in mechanisms underlying serious diseases (e.g., initiation of chemical carcinogenesis) [5]. Typical cases of such processes are the formation of reactive NAPQI (which binds covalently to proteins of liver and kidney, Figure 1.1) from paracetamol or generation of reactive dihydrodiol epoxide of benzo[*a*]pyrene as well as activation of *N*-nitrosamines leading to the formation of reactive carbenium ions that bind to bases of nucleic acids.

The aim of this introduction is to provide basic information on this highly important class of drug-metabolizing enzymes; a more fundamental discussion

on CYPs is the subject of Chapter 2. Clinical implications will be given, as a rule, in the respective chapters with individual classes of drugs and active substances.

1.3.2 Peroxidases

With the exception of glutathione peroxidases (selenium-containing enzymes), the majority of peroxidases are hemoproteins (like CYPs) that can couple the reduction of hydrogen peroxide (more generally, peroxides) to oxidation of another substrates, such as drugs and other xenobiotics [10]. This cooxidation reaction is typical for prostaglandin H synthase (Figure 1.2), better known by the name COX [11].

The CYP and peroxidase enzyme systems are generally considered to be the most important groups of enzymes involved in the bioactivation of potentially toxic compounds, producing either electrophilic or radical metabolites known to be related to chemical carcinogenesis and toxicity [10].

For example, aflatoxin B_1 is converted by CYP as well as by peroxidases to its 8,9-epoxide, which causes liver tumors or renal papilla neoplasia [5]. Another example of the participation of peroxidase (here, prostaglandin H synthase) as well as of CYP in drug toxicity is the well-known formation of NAPQI (Figure 1.1) from paracetamol, leading to the hepatotoxicity (and to the nephrotoxicity) of this drug [5, 12]. Prostaglandin H synthase itself is an enzyme exhibiting two types of catalytic activities: COX as well as peroxidase activity. This is why this enzyme (or, more precisely, these enzymes as there are at least two known forms – the first constitutively expressed in many tissues and the second inducible, for example, by cytokines such as interleukin-1β or by bacterial endotoxins) is known as COX (COX1, 2, or 3 for the respective forms). Both activities require the activation of the oxygen moiety (Figure 1.2). In the first step, two molecules of oxygen are inserted into the substrate molecule (arachidonic acid) – one forming a cyclic endoperoxide bridge and the second inserted into the molecule of arachidonic acid to create a hydroperoxide group. In the peroxidase reaction, the

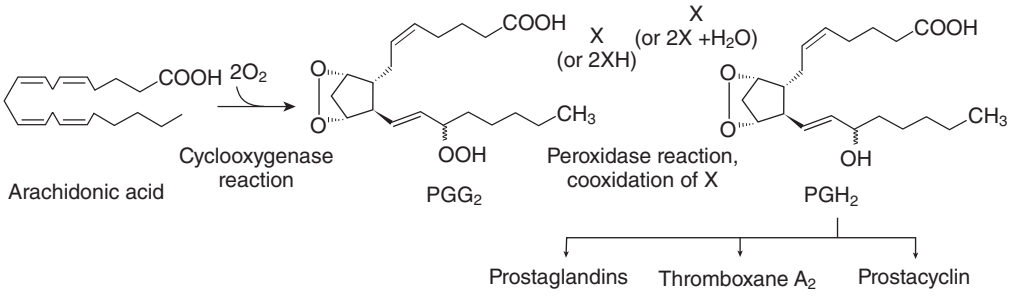

Figure 1.2 Schematic description of reactions catalyzed by prostaglandin H synthase (COX). Prostaglandins PGG_2 and PGH_2 are formed; the reactions involve activation of molecular oxygen and cooxidation of cosubstrate X.

OOH hydroperoxide group is reduced to an OH group; simultaneously, a substrate is cooxidized either by insertion of an oxygen atom or into a radical that may then undergo further reaction (for reviews on peroxidases and COXs, see [13–15]). Cyclophosphamide – an antineoplastic drug – is a typical example of a drug causing lung damage due to its oxidative biotransformation [16]. Activation of cyclophosphamide is known to proceed via CYPs; however, in this study the lung toxicity has been shown to be caused by cooxidation through the action of the COX enzyme because typical inhibitors of the CYP enzymes have not interfered with biomarkers of drug toxicity.

Prostaglandin H synthase or COX is in fact unique among peroxidases due to its mechanism of action. Metabolism of drugs and other xenobiotics is often realized by the action of ubiquitous peroxidases, such as myeloperoxidase of polymorphonuclear leukocytes (neutrophils), eosinophil peroxidase, or lactoperoxidase. Hydroperoxide substrates of these peroxidases are typically formed from hydrogen peroxide, which is a product of cellular respiration; its level is controlled by scavenging mechanisms (e.g., by glutathione peroxidase or catalase). Myeloperoxidase has been implicated in reactions leading to inactivation of the anticancer anthracyclines doxorubicin and daunorubicin [17]. However, another anthracycline-derived drug – mitoxantrone has been shown to exhibit particular effectiveness against tumors expressing high levels of heme peroxidases (myeloid leukemia, and breast and ovarian carcinoma); a recent systematic *in vitro* study has demonstrated the formation of oxidized derivatives of mitoxantrone from covalent complexes with DNA leading to DNA strand breakage and inhibition of topoisomerase II [18]. These studies open the possibility that certain types of solid tumors (lymphoma, and prostate and breast carcinoma) secreting peroxidases may activate mitoxantrone by this mechanism; in other words, that the mechanism of mitoxantrone action may be understood in terms of peroxidase/hydrogen peroxide-mediated reactions.

Airway peroxidases such as myeloperoxidase and lactoperoxidase, which are naturally present in these tissues, can apparently contribute to peroxidative metabolism of phenolic β_2-adrenoreceptor agonists such as salbutamol, fenoterol, and terbutaline [19]. Under inflammatory conditions associated with asthma, these drugs function as substrates for airway peroxidases and the resulting products (of a radical nature) may result in diminishing therapeutic efficacy of the drug [20]. Myeloperoxidase-catalyzed reactions (i.e., oxidations by the myeloperoxidase/hydrogen peroxide/Cl^- system generating HOCl) were also found to be the most probable reason of leukopenia (agranulocytosis) observed as an adverse effect after treatment with many drugs such as ticlopidine, clozapine, aminopyrine, dapsone, sulfonamides, or procainamide (e.g., see [21, 22]). In many countries, patients who are prescribed clozapine must be nationally registered in order to monitor white blood cell counts and absolute neutrophil counts. Idiosyncratic drug reactions including agranulocytosis seem to be generally caused by several factors and have more mechanisms underlying the process; however, formation of reactive metabolites typically by peroxidases and CYPs, which subsequently act as antigens, seems to be one of the most frequent ones [23].

1.3.3
Flavin Monooxygenases

Non-CYP (and nonheme)-mediated oxidative biotransformation plays an important role in the metabolism of xenobiotics. Among enzymes possessing the flavin moiety, the microsomal flavin monooxygenases (FMOs), molybdenum-containing hydroxylases (xanthine oxidase (XO) and aldehyde oxidase (AO) (Section 1.3.4)), and FAD-dependent amine oxidases (Section 1.3.5) should be discussed. As a rule, however, these enzymes have many endogenous substrates, which means that the possibility of interactions of drugs metabolized by these enzymes with natural substrates should be considered. A review of FAD-dependent enzymes participating in the metabolic oxidation of xenobiotics has been published recently [24].

FMOs were considered as "sister enzymes" of CYPs as they also catalyze microsomal NADPH-dependent *N*- or *S*-monooxygenation by activated oxygen. The reactive oxygen species (ROS) originates from a dioxygen molecule bound to reduced flavin, yielding a hydroperoxide moiety (Figure 1.3). Contrary to CYPs, which can insert the oxygen atom into a non-nucleophilic substrate, FMOs catalyze monooxygenation of compounds that have in their structures a highly polarizable nucleophilic N or S heteroatom. Tertiary amines are then converted to *N*-oxides, secondary amines to hydroxylamines or *N*-oxides, and primary amines also to derivatives of hydroxylamine or to oximes. Thiols (possessing a free SH group) are oxidized to disulfides by joining two sulfur atoms by an -S–S- bridge; sulfur atoms in compounds of the R–S–R′ structure are converted by FMOs to

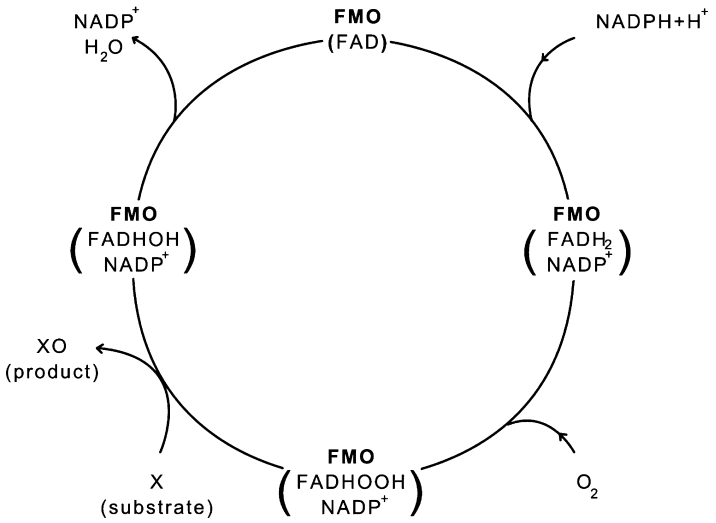

Figure 1.3 Scheme of FMO catalytic cycle; the enzyme possesses the FAD cofactor that undergoes reduction, then binds the molecular oxygen forming a hydroperoxide; subsequently, it is converted to a hydroxy form and finally is regenerated in its resting state.

S-oxides. An analogous reaction takes place at the phosphorus atom; a *P*-oxide is the product [5].

FMO enzymes are in principle inducible [25], at least in rats, by ligands of aryl hydrocarbon receptor (AhR) [26] and are also regulated by different factors such as nitric oxide [27]. Their pharmacogenetics is rather complex. There are five known forms in humans (FMO1–5); their genes are clustered on chromosome 1 [28]. Most humans are homozygous for a nonsense mutation that inactivates FMO2. However, a substantial proportion of sub-Saharan Africans expresses a functional FMO2, and thus are is predicted to respond differently to drugs and other foreign chemicals. FMO3 is a well-known liver enzyme, catalyzing the oxygenation of drug substrates such as clozapine, amphetamine, metamphetamine, sulindac, tamoxifen, and nicotine [28, 29]. Loss-of-function mutations of *FMO3* cause a disorder called trimethylaminuria – also known as fish odor syndrome, because these individuals are not able to metabolize this compound to the odorless *N*-oxide. More common variants that decrease enzyme activity are associated with increased drug efficacy [5, 30].

1.3.4 Other Oxidases: Amine Oxidases, and Molybdenum-Containing XO and AO

Amine oxidases – monoamine oxidase (MAO, typically mitochondrial), diamine oxidase (DAO), and polyamine oxidase (PAO, mostly cytosolic) – mediate oxidative deamination of primary, secondary, and tertiary amines, and their substrates hence are both of natural origin (endobiotics), such as 5-hydroxytryptamine (serotonin, substrate of MAO), putrescine (substrate of DAO), or spermine (substrate of PAO), as well as of foreign origin (xenobiotics, e.g., drugs) [24, 31]. Amine oxidases MAO and PAO typically possess flavin cofactor (FAD); products of these reactions are dehydrogenated and further oxidized. For example, propranolol – a β-blocking agent – forms two metabolites by two different forms of CYPs (CYP2D6 and CYP2C19): a hydroxylated product and a dealkylated primary amine, which is further oxidized by MAO to an aldehyde (in parallel, ammonia is formed with hydrogen peroxide) [32]. Activity of MAO is primarily associated with inactivation of neurotransmitters; its dysfunction (either increased or diminished activity) is associated with neurological disorders (such as depression, schizophrenia, and migraine). Interestingly, its activity seems to be suppressed in the adipose tissue of obese patients [33].

DAO is often employed as a clinical indicator of the integrity of intestinal mucosa. It is not a flavoenzyme; instead, it has a copper atom and trihydroxyphenylalanine quinone as cofactors. Among its natural substrates, histamine is probably the most important [34]. Drug substrates are rare.

Molybdenum-containing flavoenzymes or molybdenum hydroxylases (i.e., XO (more precisely, xanthine oxidase/dehydrogenase) and AO (aldehyde oxidase)), are cytosolic metalloflavoproteins catalyzing among their endogenous functions also oxidation and reduction of many drugs and xenobiotics. The molybdoenzymes possess not only the Mo(VI) ion bound to oxygen and sulfur atoms and the FAD

coenzyme, but also two Fe_2S_2 iron–sulfur centers. Detailed mechanisms of their redox reactions are hence not simple and not fully understood yet; what is known is that the substrate is bound directly to the molybdenum atom by a Mo–C bond and that the oxygen atom for hydroxylations or, in general, for insertion to substrate molecule, is taken from a water molecule present in the active site [5, 35]. Electrons flow either to the NAD^+ (dehydrogenase reaction) or to the oxygen atom with ROS formed (XO reaction). Among reactions catalyzed by the XO enzyme, conversion of purines ultimately to uric acid, and its participation in the metabolism of the immunomodulating drugs azathioprine and 6-mercaptopurine are often listed (see also methyltransferases below and Figure 1.4). In this respect, it should be recalled here that the deposition of uric acid in joints and other places is the cause of inflammatory arthritis known as gout. Allopurinol is the leading antigout drug, which is *de facto* a prodrug metabolized in the first step by XO and AO to oxypurinol that then subsequently inhibits the XO enzyme directly, thus reducing the formation of uric acid [36]. Febuxostat – a novel nonpurine selective inhibitor of XO – has been introduced recently [37]. There are not many drugs known to be metabolized by XO or AO; the acyclovir prodrug 6-deoxyacyclovir is an example of a prodrug activated by XO into its active compound [38]. The AO enzyme has been recently found to metabolize a drug called FK3453 in humans and in experimental animals [39]. Both molybdoenzymes – XO and AO – are able to interact with flavonoids, acting as inhibitors of these enzymes [40]. Interestingly, in a medium with low partial pressure of oxygen and in the presence of easily oxidized substrates (in fact "cosubstrates"), the AO may catalyze the reduction of, for example, azo or nitro groups [5].

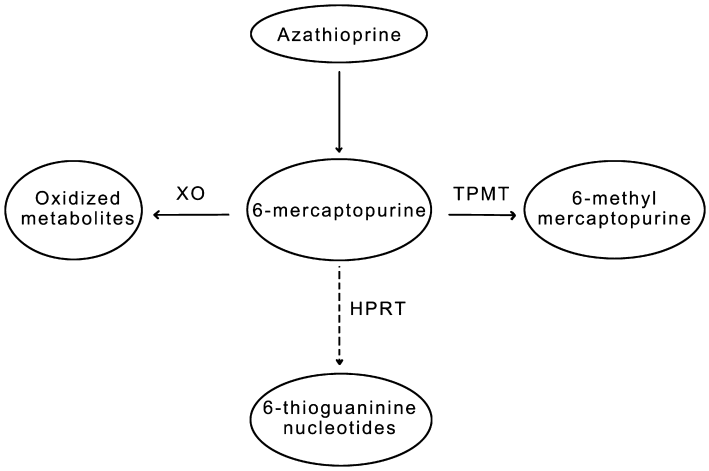

Figure 1.4 Metabolic conversion of azathioprine and 6-mercaptopurine. Levels of 6-thioguanine nucleotides are controlled by xanthine oxidase (XO) and/or thiopurine *S*-methyl transferase (TPMT). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) participates in the formation of 6-thioguanine nucleotides.

1.3.5 NADPH Oxidase, NAD(P)H Oxidoreductase, and Dihydropyridine Dehydrogenase

Two oxidoreductases should be mentioned here as they represent a bridge to reductive processes in xenobiotic metabolism. First, the NADPH oxidase is a cell membrane-bound enzyme catalyzing the production of superoxide from oxygen and NADPH. It is a family of enzymes comprised of membrane proteins with both a heme and flavin moiety of which five isoenzymes were described, and of other cytosolic subunits [41]. This complex enzyme exhibits only one known function – generation of superoxide and ROS in general. Rather than searching for drugs metabolized by this enzyme, the pharmacologic interest is in finding an inhibitor or class of inhibitors of this enzyme as the generation of ROS is connected with many pathologies (hypertension, diabetes, atherosclerosis, and inflammation, just to name the most important) [42].

The second enzyme is the NAD(P)H oxidoreductase, also named DT-diaphorase since it can use both NADH (abbreviated DPNH in earlier literature) and NADPH (abbreviated TPNH earlier) as sources of electrons for reduction of its typical substrates, quinones [43]. The soluble cytosolic enzyme is a dimer of two identical subunits, each containing the FAD moiety, and in humans is coded by four genes in four forms; the most active (and important) is the form labeled NQO1 (NADPH : quinone oxidoreductase-1). Interestingly, it is itself polymorphic, which has important consequences for the metabolism of drugs by this enzyme (i.e., quinone anticancer agents). As the NQO enzyme is more active in cancer cells, activation of anticancer drugs by NQO leading to formation of cytotoxic products is a way that may lead to the development of a novel class of anticancer agents [44]. This is the mechanism of activation of mitomycin C, which is, however, not the best substrate of NQO; the most promising drug of this class seems to be RH1 (possessing a quinone moiety), which has been recently shown to exhibit promising pharmacodynamic and pharmacokinetic properties [45]. In the same study, a patient homozygous for an inactive allele has been shown not to respond as expected. The enzyme is inducible by two mechanisms, one of which involves the AhR (see Chapter 8) which means that its expression is controlled by the same mechanisms as that of, for example, CYP1A1 or glutathione *S*-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) [46].

Dihydropyridine dehydrogenase (DPD) is the last enzyme to be discussed here. It is a cytosolic enzyme that is highly conserved among mammals, being a homodimer with each subunit having one FAD, one FMN, and four 4Fe–4S clusters [47]. The DPD gene exhibits many mutations and polymorphisms, which may lead to defective activities of this enzyme and hence to increased levels of drugs such as 5-fluorouracil (reduced by DPD to fluorodihydrouracil). Unfortunately, high levels of this drug, known for its narrow therapeutic window, result in hematotoxicity and gastrointestinal toxicity [48].

5-Fluorouracil is the standard treatment for stage II–IV colon cancer. As already been mentioned, DPD metabolizes 5-fluorouracil to inactive 5-fluorodihydrouracil, which means a lowering of the level of the parent drug and simultaneously also

a lowering of the formation of fluorouracil derivatives (nucleotide analogs) that enter RNA and DNA of cancer cells causing the desired anticancer effect. As the cancer cells grow rapidly, the cytotoxic effect of fluorouracil derivatives should be primarily observed in them. DPD was also the reason for lethal drug interactions caused by concomitant administration of two drugs – 5-fluorouracil and sorivudine (an antiviral drug used to treat herpes zoster) – in Japan almost 20 years ago. Sorivudine – a uracil derivative – was converted by gut flora to an intermediate that was further reduced by DPD to a compound covalently bound to this enzyme. Sorivudine thus inhibited DPD, which in turn resulted in elevated and lethal levels of 5-fluorouracil [49].

1.3.6 Reductases

Reductive processes are common in cells, and are not limited just to tissues and compartments with a lower partial pressure of oxygen (as in the intestines with gut microflora reducing and splitting azo groups to amines [5]). As has been mentioned earlier, many oxidoreductases are able to catalyze the reduction of substrates such as azo or nitro derivatives (aldehyde oxidase), quinones (NAD(P)H oxidoreductase, DT-diaphorase), uracil derivatives (DPD) or unsaturated aldehydes (CYPs) and halogenated hydrocarbons (e.g., reductive dehalogenation of halothane by CYPs). However, the main task of (mostly cytosolic) NAD(P)H-dependent reductases is to reduce carbonyl groups of ketones and aldehydes. These enzymes belong to two protein superfamilies – aldo-keto reductases (AKRs) and short-chain dehydrogenase/reductases (SDRs) [50, 51].

AKRs are soluble enzymes, acting as NAD(P)H oxidoreductases; 10 enzymes were found in man. AKRs are involved in the reduction of aldehydes and ketones to primary and secondary alcohols, and their substrates are either detoxified or activated. For example, they contribute to tobacco-induced carcinogenesis by activating *trans*-dihydrodiols of polycyclic aromatic compounds to reactive quinones; they also participate in the detoxication of aflatoxin-derived aldehydes and lipid peroxides [50].

To date, the SDRs constitute a superfamily of nearly 50 000 mostly soluble cytosolic enzymes in all domains of life [51, 52]. Three SDRs, NADPH-dependent carbonyl reductases, are known in humans, namely carbonyl reductases 1, 3, and 4 (abbreviated as CBR1, CBR3, and CBR4; according to the nomenclature of SDRs, the systematic names of these enzymes are SDR21C1, SDR21C2, and SDR45C). The CBR2 enzyme was found only in rodents and pigs. CBR1 is known to reduce a variety of aldehydes, ketones, and quinones such as the anthracycline-based anticancer drugs doxorubicin and daunorubicin. It has been shown recently that resistance to these drugs correlates with increased activity of CBR1 [53]. This enzyme is also known for its participation in the reduction of haloperidol and related structures (haloperidol metabolism is rather complicated and includes glucuronidation, reduction, as well as oxidation by CYP enzymes [54]). Interestingly, the expression and activity of CBR1 has been found to be increased in tumor

tissues [52]. CBR3 shares high structural similarity to CBR1; however, its activity towards most of the CBR1 substrates is lower, as is the number of its known substrates. Contrary to most of the CBRs, CBR4 is localized in the mitochondrial matrix and its substrates are predominantly quinones; however, daunorubicin is a poor substrate of this enzyme.

1.3.7 Conjugating Enzymes

Enzymes helping to join two molecules, one being a xenobiotic and the other a helping particle such as glutathione or a UDP-glucuronic acid, with the aim of facilitating the excretion of the xenobiotic part of the conjugate, are usually named phase II enzymes and will be treated here as one group of drug-metabolizing enzymes, regardless of the fact of whether the xenobiotic binds by a nucleophilic (e.g., an amino or hydroxyl group) or by an electrophilic site of its molecule (e.g., as in conjugation to glutathione) (see also Figure 1.1). Only several, more or less classical examples of these reactions will be listed here, as detailed discussions of individual enzymes and their importance for drug metabolism are given in the following chapters.

UDP glycosyltransferases, including UGTs, are a superfamily of enzymes transferring (at the expense of energy supplied by the UDP) a sugar moiety to another particle forming a glycosidic bond [55]. When the sugar moiety is represented by glucuronic acid, then as a result of the glucuronidation reaction, the drug is bound to glucuronic acid by a glycosidic bond (i.e., at the C1 carbon atom of the carbohydrate),whereas the acid, carboxylic group at the C6 position, thanks to which the glucuronic acid exhibits polar properties, is free and thus enables better excretion. There are four families of genes coding the UGTs found in the human genome (UGT1, 2, 3, and 8).

Mammals utilize six sugar nucleotide donors for UGTs: UDP-glucuronic acid, UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, and UDP-xylose. For conjugation reactions with drugs, UDP-glucuronic acid is utilized as a donor; the UGTs of family 1 and 2 that need this donor molecule are the well-known "UGTs." Put another way, the abbreviation "UGT" has a broader meaning than solely "glucuronyltransferase," as it encompasses also the two other UGT families that, however, catalyze mostly reactions involving UDP-*N*-acetylglucosamine (UGT3) or UDP-galactose (UGT8) [56, 57]. The focus in this book is hence on the UGTs of families 1 and 2.

Drugs are glucuronidated by enzymes of both the UGT1A and UGT2B subfamily (the UGT2A enzymes are rare and their role in drug metabolism is marginal). In both these families of microsomal proteins, pharmacogenetic variants of the respective enzymes have been described in detail (see Chapter 3 and the web site http://www.pharmacogenomics.pha.ulaval.ca/sgc/ugt_alleles). Paracetamol (Figure 1.1), oxazepam, or morphine are typical examples of substrates for UGT1A6 and UGT2B7 [57]. As glucuronidation is the most common type of drug conjugation [57, 58], its clinical importance, including the pathways

of drug elimination and evaluation of the possible drug interactions, is relatively high.

Methods of studying drug interactions that are able to provide data on the specificity of drug–enzyme interactions involve, for example, *in vitro* studies with purified or, more often, recombinant enzymes, making it possible to identify the respective form of a particular enzyme (e.g., CYP or UGT) responsible for the drug metabolism, or studies with microsomal preparations examining possible inhibition of a prototypic activity of a particular drug-metabolizing enzyme by another drug acting as an inhibitor. For UGTs, more or less specific substrates and inhibitors for the most important UGTs have been identified [57, 59]. As an example, an analysis of quantitative prediction of *in vivo* drug interaction based on *in vitro* data was presented with fluconazole (a selective UGT2B7 inhibitor) and zidovudine [60]. Complexity and difficulty in interpretation of drug interaction studies with UGTs and CYPs has been recently demonstrated in a study describing the inhibition of CYP enzyme (CYP2C8) by glucuronide of the interacting drug (gemfibrozil, formed by UGT2B7 and 1A3) leading to increased levels of another concomitantly administered drug, repaglinide; leading to an order of magnitude increase of the repaglinide bioavailability due to inhibited CYP2C8 [61].

It is not only the pharmacogenetics that may significantly influence the ability of a human subject to glucuronidate the respective substrates. Recent studies have found another factor affecting the efficacy with which the drugs are glucuronidated and, hence, excreted from the human body. Namely, induction of UGT enzymes via the AhR (see also Chapter 8). For example, a very recent paper has documented that drinking coffee induces expression of glucuronosyltransferases including the already mentioned form UGT1A3 in the liver and stomach via the AhR and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways [62].

Sulfotransferases (SULTs) are members of a superfamily of enzymes catalyzing transfer of a sulfonyl moiety (SO_3H or, more generally, SO_3^- anion) from the universal donor PAPS to an acceptor group possessing an O, N, or S nucleophilic atom. Hence, the name is *de facto* not correct because the transferred group is a sulfonate, not a sulfate (SO_4H); however, it remains in use for practical reasons. SULTs were formerly classified by their subcellular localization as either cytosolic or membrane-bound. Sulfonate conjugation is important also from the ontogenetic point of view: the human fetus does not possess a functional UGT transcript until week 20 of gestation [63]; on the contrary, cytosolic fetal liver fractions exhibit SULT activities towards drug substrates such as paracetamol [64].

SULTs are known to participate in conjugation reactions involving endogenous (hormones, neurotransmitters) and exogenous compounds (xenobiotics, including drugs). They are present in the main organs involved in interaction with xenobiotics [5, 65]. Human SULT1A1, which represents over a half of liver SULTs and is the major form present in the kidney [65], catalyzes sulfate conjugation primarily of phenolic xenobiotics such as paracetamol (Figure 1.1). Thanks to this property, and also thanks to its remarkable thermostability, it was formerly called phenol SULT or thermostable phenol SULT [66]. Human monoamine-preferring SULT (SULT1A3, present in the kidney, small intestine, and lungs [65]) catalyzes

sulfate conjugation of phenolic monoamines, including neurotransmitters such as dopamine, noradrenaline (norepinephrine), and serotonin, as well as conjugation of phenolic drugs [67]. Human SULT1E1 – the major form in the lungs – plays a major role in sulfation of estrogens [68] and human hydroxysteroid SULT (SULT2A1, present in the liver, but also in the lungs, small intestine, and to a lesser extent in the kidneys) is important for regulation of dehydroepiandrosterone and other hydroxysteroid hormones [65, 69].

Inhibition of SULTs may interfere with their ability to detoxify xenobiotics, as well as with other processes in which sulfated (more precisely, sulfonated) compounds play a role. For example, an inhibitory effect of nonsteroidal anti-inflammatory agents such as nimesulide or piroxicam on SULT1A1 was described recently; other drugs of this class (sulindac, ibuprofen) act as inhibitors of the SULT1E1 enzyme [70]. Studies with flavonoids and related polyphenols present in food including beverages have shown that many compounds of this and similar structure such as curcumin or quercetin are inhibitors of SULT enzymes [71]; SULT1A1 and SULT1A3 have been recently shown to be inhibited by grapefruit juice, green tea, black tea, and oolong tea [72]. Interestingly, inhibition of SULT may be beneficial: Some compounds (e.g., safrole) may be bioactivated by sulfation and the subsequent reactions may lead to electrophilic nitrenium or carbenium ions that bind to DNA, hence initiating the process of chemical carcinogenesis; inhibition of the respective SULT by flavonoids from the diet in such cases helps to protect the organism [73].

Induction, regulation of biosynthesis, and effects of gene polymorphism are usual factors complicating the study of drug metabolism efficacy and toxicity. These aspects that should be taken into account also with SULTs are largely covered in Chapter 4.

N-acetyltransferases (NATs) are cytosolic enzymes found typically in the liver, but also in other tissues, catalyzing in two steps the transfer of an acetyl group from acetyl-CoA at first to a Cys residue of the enzyme and, in the next step, the transfer of the acetyl group from the enzyme to the amino group of a substrate. Typical substrates are aromatic amines and hydrazides; aliphatic primary amines are rarely substrates of these enzymes. Interestingly, the products – acetylated amines – are often less hydrophilic than the substrates, which is seemingly in contrast with the general concept of making the xenobiotic more polar to be excreted. However, the elimination process is more complex and the properties of the protein transporters following the conjugation should also be taken into account – these proteins may prefer acetylated substrates.

NATs became well known for their genetic polymorphism already more than 50 years ago (for a review, see, e.g., [74]). The concept of the slow and rapid or fast acetylator phenotype was known in the pharmacology of the antituberculotic isoniazide, and was in fact the beginning of the era of clinical pharmacogenetics. In humans, there are two known genes coding for NATs – for NAT1 and NAT2 – with 85% homology of nucleotide sequences; the respective proteins exhibit a certain overlap, but they have distinctly different substrate specificity. Typical substrates of NAT1 are *p*-aminosalicylic acid, *p*-aminobenzoic acid, and sulfamethoxazole;

NAT2 is known to acetylate isoniazid, hydralazine, dapsone, and sulfonamides; sulfamethazine is a selective substrate of NAT2 [5, 75, 76]. Also, their tissue distribution differs – NAT1 is fairly ubiquitous, whereas NAT2 is present in the liver, intestinal epithelium, and colon [77].

The NAT enzymes, similarly to other drug-metabolizing enzymes, are able to catalyze a variety of reactions that may lead both to inactivation or detoxication as well as to activation. *N*-hydroxylation and *N*-acetylation of aromatic amines may lead to formation of nitrenium-containing structures that may readily bind to DNA [5, 74]. Also, the presence of genetic variants of these enzymes was shown to affect the extent of formation of DNA adducts [74].

GSTs catalyze formation of thioether conjugates joining an essentially hydrophobic, xenobiotic molecule (its electrophilic site) with glutathione via its thiol group (more precisely, through the thiolate anion of glutathione). A typical reaction of this type is the detoxication of NAPQI formed from paracetamol (Figure 1.1) by metabolic activation (oxidation) by CYPs and peroxidases such as prostaglandin H synthase (COX, see also Section 1.3.2) [5]. GSTs are divided into three main classes: cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs (also know as (MAPEGs: membrane-associated proteins in eicosanoid and glutathione metabolism) [78]. GSTs are widely associated with detoxication reactions; however, many other activities were recently found to be associated with GSTs, such as participation in sterol and leukotriene biosynthesis, peroxide degradation, double-bond *cis*/*trans* isomerization, and ligand binding and transport. They may also lead to activation of xenobiotics (e.g., by binding to haloalkanes where they substitute the halogen atom; eventually a reactive particle is formed that is able to bind to DNA) [79]. The best-known GSTs are the soluble GSTs forming dimers (usually homodimers); classes of these enzymes are classically denoted by Greek characters (α, μ, π, κ, θ, σ, ζ, and ω). Numerous reviews on GSTs are available in the literature (e.g., [5, 80]); an overview of GSTs and their properties including regulation and polymorphisms is given in Chapter 5.

Methyltransferases represent a relatively less common pathway of drug conjugation reactions. Methylation, usually at the O, N, and S atom of a xenobiotic, apparently leads to less polar products with lower solubility in water as has been already discussed with *N*-acetylation; in other words, methylation must give the compound another (yet unclear) advantage facilitating its excretion. Only in limited cases does methylation result in the formation of a more polar compound; an example of such a reaction is the *N*-methylation of nicotine where a quaternary N atom of a pyridinium ring of nicotine isomethonium metabolite possesses a positive charge [81]. The methyl group comes from methyl-*S*-adenosyl-L-methionine cofactor. Two methytransferases are often discussed due to their function and pharmacogenetics – catechol-*O*-methyltransferase (COMT) and thiopurine *S*-methyltransferase (TPMT).

COMT substrates are catecholamine neurotransmitters such as adrenaline (epinephrine), noradrenaline (norepinephrine), dopamine, and related structures (e.g., L-DOPA (3,4-dihydroxyphenylalanine – an antiparkinsonic agent, recently suggested to be effective in treatment of the restless leg syndrome [82]) or meth-

yldopa (L-α-methyl-3,4-dihydroxyphenylalanine – an antihypertensive drug used mainly to treat hypertension in pregnancy)). COMT substrates also include melanin, and drugs such as isoprenaline, dobutamine, rimiterol, or flavonoids and tea catechins [83]. In human tissues, the majority of COMT is ubiquitous; however, there are differences between its soluble and membrane-bound form (the latter protein is 50 residues longer and it is the main form present in the brain) [83, 84].

COMT is known to exhibit genetic polymorphism; the Val150/158Met (soluble/membrane bound form) polymorphism has been found to be associated with many disorders such as Parkinson's disease, schizophrenia, alcoholism, and brain cancer [84]. Interestingly, most recent studies revealed an association of this genotype with post-traumatic stress disorder, and a relationship to altruism and empathy [85, 86].

TPMT is a cytosolic enzyme catalyzing *S*-methylation of aromatic and heterocyclic sulfhydryl compounds including anticancer and immunosuppressive drugs, such as 6-mercaptopurine, 6-thioguanine, and azathioprine. These drugs are used to treat childhood acute lymphoblastic leukemia, inflammatory bowel disease, and autoimmune diseases, and are prescribed to organ transplant recipients [84, 87]. Soon after marketing, myelotoxicity of these drugs was discovered and an association with impaired function of TMPT was identified. The mechanism of action of these drugs is that they are all in fact prodrugs that are activated (in an ultimate step, from 6-mercaptopurine either being the parent drug of formed from other drugs such as azathioprine, Figure 1.4) by hypoxanthine-guanine phosphoribosyl transferase (HPRT) to 6-thioguanine nucleotides (decoy nucleotides altering the synthesis and function of primarily DNA and RNA of rapidly dividing cells). Levels of 6-thioguanine nucleotides are controlled by two reactions that reduce their availability: (i) XO-mediated conversion of 6-mercaptopurine to thiouric acid and (ii) TPMT-catalyzed conversion of 6-mercaptopurine to 6-methylmercaptopurine. In hematopoietic tissue, XO is not expressed and hence the genetically determined variations in TPMT activity have a greater impact on the availability of 6-mercaptopurine, and consequently lead to higher levels of 6-thioguanine nucleotides and more pronounced resulting drug-induced myelosuppression. Hence, patients homozygous for alleles known to result in low TPMT activity suffer from elevated levels of 6-thioguanine nucleotides when treated with standard doses of these drugs – these patients should be given 1/10–1/15 of the standard dose. Genetic polymorphism of TPMT has become one of the first documented cases of pharmacogenetics and is routinely investigated in clinical laboratories in developed countries [84, 87]. TPMT pharmacogenetics are also treated in Chapter 9.

Amino acid conjugation was the first reaction in drug metabolism described in the literature, already more than 160 years ago when the formation of hippuric acid was correctly ascribed to conjugation of benzoic acid with glycine. Only later were sulfate and glucuronide conjugations discovered; hydroxylations by CYPs were discovered and started to be studied only in the early 1960s [88]. Glycine conjugation is probably not just the best known, but also the most important amino acid conjugation. The reaction takes place in three steps: (i) activation of a xenobiotic (e.g., salicylate) by binding the AMP (i.e., reaction of a xenobiotic with

ATP, formation of acyl-AMP conjugate); (ii) formation of acyl-CoA (by acyl-CoA synthetase (more correctly, ATP-dependent acid : CoA ligase, which catalyzes both the first steps)), and (iii) ligation of the amino acid (Gly, but also Gln or taurine) with the CoA-activated acyl to get the conjugate by formation of the acyl carbonyl-NH (α-amino group from the amino acid as glycine) peptide-like bond. The third step is catalyzed by acyl-CoA : amino acid *N*-acyltransferase; CoA is liberated. Reactions with xenobiotics including drugs occur in mitochondria where the corresponding enzymes as well as the ATP are available. Conjugated drugs (e.g., hippuric acid, product with benzoic acid conjugated to glycine) are excreted in urine, which is the last step of this detoxication process – there are no known pathways leading to reactive nucleophiles as it is the case with glucuronidation, glutathione conjugation, *N*-acetylation by NAT, or sulfation.

This introduction does not claim to be an exhaustive list of all reactions – only those enzyme systems that are most important have been discussed here, and only the basic characteristics and the principles covered. The following chapters will give more detailed information focusing on selected enzymes of drug metabolism.

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