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GLUCURONIDATION AND ITS ROLE IN REGULATION OF BIOLOGICAL ACTIVITY OF DRUGS

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INTRODUCTION

Glucuronidation is a major drug-metabolizing reaction. It involves the transfer of the glucuronic acid group from UDP-glucuronic acid (UDPGlucA) to an acceptor group on a substrate. For a long time, glucuronidation has been considered a detoxification reaction that terminates the biological or pharmacological activity of drugs (or xenobiotics in general) and endobiotics. It was therefore considered to be a "safe," somewhat boring process and so was left alone by those who were looking for more exciting biotransformations. However, that view is no longer tenable, since it has been shown over the past decade that glucuronides may have very potent effects themselves. For instance, glucuronidation of morphine leads to pharmacologically very active conjugates: the glucuronide conjugate formed at the 6-position of morphine is a much more potent agonist of the morphine $\mu 1$ receptor than is morphine itself, whereas the glucuronide at the 3-position is an extremely potent antagonist (1, 2). It has already been suggested that morphine activity in vivo may be due to its 6-glucuronide conjugate rather than to unconjugated morphine. Another example is glucuronidation of steroids at the 17β position, leading to cholestatic conjugates of (endogenous) estradiol and (the oral contraceptive) ethinylestradiol. This may be the reason why jaundice was

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occasionally observed in women on oral contraceptives, especially the former high-dose preparations (3). Of interest in this respect may be that estradiol-17β-glucuronide increases tight junctional permeability in rat liver (4). Even if a glucuronide itself has no biological effects, it may be indirectly responsible for such effects because very active β-glucuronidases are present in all tissues in the body, so that circulating glucuronides can be converted to their biologically active aglycone in target tissues.

An important reason why only few effects of glucuronides have been identified so far seems that investigators have not been looking for them; the reaction was dogmatized to be "just" detoxification. Admittedly, for pharmacological agents that were developed for their effects on a certain receptor, a change in chemical structure would indeed be expected to decrease the particular effect for which this structure had been optimized in, for instance, quantitative structure-activity relationship (QSAR) studies. Addition of the glucuronic acid moiety changes the overall structure of the compound profoundly, in particular its water and lipid solubility. Most probably, therefore, it will also change its receptor specificity and binding. In most cases this will decrease binding, so that glucuronidation will result in a loss of the effect with respect to the parent compound ("detoxication"). For these reasons, glucuronidation indeed is a major factor in the termination of drug action for most medicinal drugs. Similarly, it "detoxifies" endogenous compounds such as bilirubin or thyroid hormones. However (as for morphine), in special cases the glucuronide may fit even better to the receptor or have a greater efficacy. Alternatively, it may bind to a different receptor, with a new biological effect ("toxification"). For this reason, glucuronides may turn out to be responsible for side effects. For instance, the toxicity of drugs that contain a carboxylic group may be due to the formation of acylglucuronides at that group. These conjugates are reactive, so they may bind covalently to, e.g., plasma proteins, thus presenting haptens to the immune system (5).

In this review, properties of glucuronidation in vivo and in vitro will be discussed with some emphasis on areas that show rapid progress and those that need more investigation. For a recent in depth review of glucuronidation, the reader is referred to Mulder et al (6); the older literature is exhaustively covered by Dutton (7).

THE GLUCURONIDATION SYSTEM

Formation of glucuronides requires the cosubstrate UDPGlucA and a transferase, UDPglucuronosyltransferase (UDPGT; EC 2.4.1.17). The latter is a multienzyme family, comprising many forms with different substrate specificities. There are pronounced species differences in glucuronidation. Thus, in pigs phenols are almost exclusively glucuronidated (there is little

sulfation), whereas in cats the opposite is true in that sulfation is predominant (7), although acylglucuronidation, which cannot be replaced by sulfation, shows appreciable activity (8). Since glucuronidation and sulfation are complementary for phenols, this often does not make much difference in elimination of the substrates. Species differences become very important, however, if glucuronidation and sulfation lead to conjugates with very different profiles of biological action. For instance, the sulfate conjugate of *N*-hydroxy-2-acetylaminofluorene is extremely reactive, whereas the glucuronide conjugate is stable (9). In that case, a big species difference in sulfation versus glucuronidation will obviously have major consequences for drug toxicity.

In general, glucuronidation is a low-affinity/high-capacity conjugation, as compared with sulfation, which is a high-affinity/low-capacity conjugation (6). However, in the rat intestine this situation may be reversed (10). Most species, including humans, have a high activity of transferases in the liver, but appreciable activity in other organs as well. In general, therefore, glucuronidation is a very common reaction of a xenobiotic substrate. After oral administration, first-pass conjugation in the gut mucosa and the liver may lead to a low bioavailability. As yet it is not clear whether any blood cell contains UDPGT activity that can be used in a diagnostic sense, to evaluate the glucuronidation capacity of human individuals.

UDPGlucA is a ubiquitous substance in cells. It serves not only as a substrate for glucuronidation of low-molecular-weight compounds, but also for the synthesis of glucuronic acid–containing glycosaminoglycans or glycoproteins. Furthermore, it is an intermediate in the pentose phosphate shunt from UDP-glucose to the glucuronic acid pathway. There are no special dietary requirements for its synthesis other than "energy." However, it can be depleted at high demands, for instance at very high doses of substrates for glucuronidation.

The substrates must enter the cell before they can be conjugated, since this process occurs exclusively intracellularly. The rate of conjugation, therefore, may be limited by cellular uptake of substrates, if these are not sufficiently lipid soluble to enter the cells readily. The glucuronides, once formed, are much less lipid soluble. In certain cases carrier proteins may be needed for their excretion from the cell. In particular, in rats, but also in humans, the glucuronides are excreted at high concentration in bile (11). This may change after certain treatments. For instance, phenobarbital treatment in rats results in an increased glucuronidation of paracetamol, but a decreased biliary excretion (12).

In an evaluation of the conjugation rate in humans (or other animals), the elimination of the model substrate from blood rather than only the rate of excretion of the glucuronide in urine should be measured, since the latter certainly does not directly reflect the rate of conjugation. Transport of the

conjugate from the cell into the blood and the distribution process may be rate determining for the urinary excretion.

Before further details about the glucuronidation system are discussed, one example, the role of glucuronidation in the regulation of morphine action, will be presented.

IS THE PHARMACOLOGICAL ACTIVITY OF MORPHINE IN VIVO DUE TO THE 6-GLUCURONIDE CONJUGATE?

The morphine used clinically is the natural (−) enantiomer (Figure 1). The major metabolite in humans is the glucuronide conjugate at the 3-position: approximately two-thirds of the dose is recovered in urine as morphine-3-glucuronide (M-3-G). The second major metabolite is the 6-glucuronide, M-6-G, which is present in plasma at approximately 10% of the concentration of M-3-G in patients who use morphine orally or parenterally (13, 14). The 10-fold difference in plasma concentrations between the two conjugates is also observed in children and neonates. This suggests that the postnatal development of glucuronidation at the two positions occurs in parallel (15). The conjugate level in plasma is much higher than that of unchanged morphine. A pronounced first-pass conjugation is observed after oral administration, so that the levels of the conjugates in plasma are much higher after oral than after intravenous administration. In the rat, biliary excretion is a major pathway of elimination for the conjugates. In humans they are excreted in urine; therefore, in patients with various kidney diseases, the conjugates may accumulate in the blood.

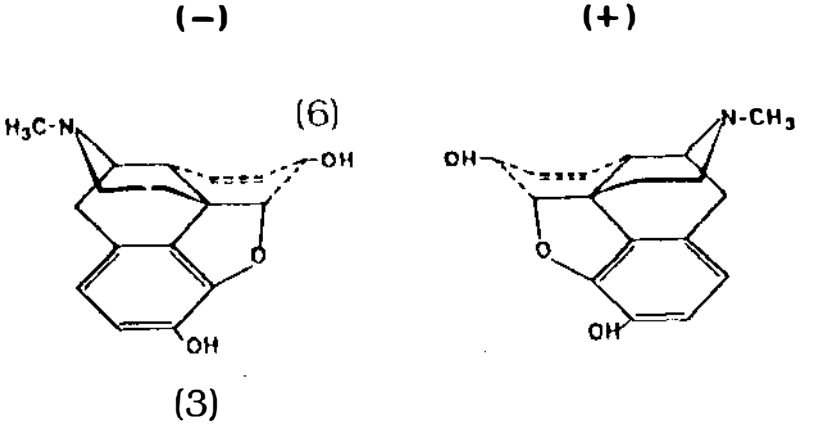

Figure 1 Structure of (+) and (−)-morphine. The 3- and 6-hydroxy groups are indicated.

Such accumulation of the conjugates may have important pharmacological consequences, because it has been discovered that both conjugates have effects on the morphine receptor: M-6-G is an extremely potent agonist, and M-3-G is an antagonist. When M-6-G is administered intrathecally or intracerebroventricularly (i.c.v.), it is ca 800 and 100 times, respectively, more active than morphine itself (1). Even when it is given intraperitoneally (i.p.), it is much more active as an analgesic than morphine. In fact, the analgesic effect of morphine may be due to that of M-6-G (16). Recently M-6-G was infused into patients and shown to be an effective analgesic agent (17). The authors suggest that this is the reason why there was no correlation between morphine levels in plasma and the clinical effect: the contribution of M-6-G was overlooked! Both M-6-G in the blood (which can reach the receptors in the brain) and M-6-G synthesized in the brain may contribute to the effect. Although the glucuronidation of morphine in the brain has not been investigated in detail, UDPGTs are present in various tissues in the brain (18). Thus, the data indicate that M-6-G may play a major role. Its effects after i.p. or i.c.v. injection are mediated by $\mu 1$ receptors (naloxone is a good antagonist), whereas after intrathecal administration $\mu 2$ receptors may be involved (1).

M-3-G is a potent antagonist of morphine (2). Its concentration in blood exceeds that of morphine by a factor of 20 and that of M-6-G by a factor of 10 (13, 14). After i.p. or i.c.v. administration it decreased the effect of both morphine and M-6-G. The authors speculate that accumulation of M-3-G under certain conditions might lead to a decrease in the pharmacological effect of morphine (2), although one would expect that simultaneous accumulation of M-6-G would counteract this. Such accumulation occurred in late-gestation fetal lambs. Formation of M-3-G from morphine in the lambs in utero was quite high. Since it could not be rapidly eliminated across the placenta, M-3-G accumulated significantly (19). The same could happen in pregnant women, but no data are available.

In patients with severe cirrhosis, the clearance of morphine was decreased (13, 14), apparently because glucuronidation was affected. Thus, clearance was 28 ± 2 ml min^{-1} kg^{-1} in controls and 11 ± 1 ml min^{-1} kg^{-1} in cirrhotic patients (14). The hepatic extraction of morphine decreased from 0.52 to 0.39 as measured by catheterization of the hepatic vein in groups of patients (13). In agreement with this, the first-pass conjugation was greatly decreased in cirrhotic patients who took morphine orally (14). In patients with less severe cirrhosis there seemed to be little effect on morphine elimination (20). Part of this may be due to extensive extrahepatic glucuronidation of morphine: the data suggest that in cirrhotic patients some 30% of conjugation may be outside the liver (13). Such extrahepatic glucuronidation (ca 30%) was confirmed in studies in patients with a hepatic insulinoma in whom the various blood vessels to and from the liver were sampled simultaneously (21).

There is evidence suggesting that the glucuronidation of morphine at the 3- and the 6-positions in humans is catalyzed by different forms of UDPGT, which is not altogether surprising since the 3-position represents a phenolic hydroxyl group and the 6-position an alcoholic one (22). The activity of UDPGT for morphine in liver biopsy correlated with in vivo M-3-G formation in patients (23). The UDPGT in rat liver that catalyzes the glucuronidation at the 3-position of (−)-morphine does not conjugate at all at the 6-position (22). Studies with the unnatural (+)-morphine showed that different enzymes must be involved in the conjugation of the various sites: one form (24) converted the (−)-3 and the (+)-6 sites, whereas a distinct enzyme converted the (+)-3 site. The latter may be the bilirubin UDPGT, since only the activity toward the (+)-3 site is absent in the Gunn rat but is increased by clofibrate treatment in normal rats (22). In human liver, both a high- and low-affinity UDPGT seemed to contribute to 3-glucuronidation, based on biphasic kinetics in liver microsomes (25). These findings show the complexity of the various forms of UDPGT when species are compared.

Interestingly, the 6-sulfate conjugate of morphine is also a potent agonist of the morphine receptor, whereas the 3-sulfate is not (26). Clearly, the role of the conjugates in morphine pharmacological activities has been underestimated in the past.

It would be of interest to find whether the deficient glucuronidation of morphine in the cat would have major consequences for the pharmacological effects of morphine in this species.

PROPERTIES OF UDPGTs

Kinetics and Inhibition

The glucuronidation reaction chemically is an S_N2 reaction in which a nucleophilic acceptor group on the substrate attacks the electrophilic C-1 atom of the glucuronic acid group (Figure 2). Therefore, every electrophilic group can serve as acceptor, such as hydroxyl, phenol, carboxylic acid, and thiol groups. Certain nitrogen-containing groups (tertiary amines, aromatic amines) may form *N*-glucuronides. For a high rate of conjugation, appreciable lipid solubility is required (27), possibly because the substrate-binding site is very hydrophobic. Although several group-specific reagents inhibit enzyme activity in vitro, the precise chemical mechanism of catalysis has not yet been worked out (28). Studies with a number of bilirubin structural analogs gave some insight into binding interactions in the bilirubin UDPGT-active site (29). Photoaffinity labeling by [^{3}H]flunitrazepam of a morphine UDPGT may help to locate the acceptor substrate-binding site on the enzyme (30).

Although enzyme kinetic studies with UDPGT are difficult because the activity is dependent on the membrane structure and phospholipid layer

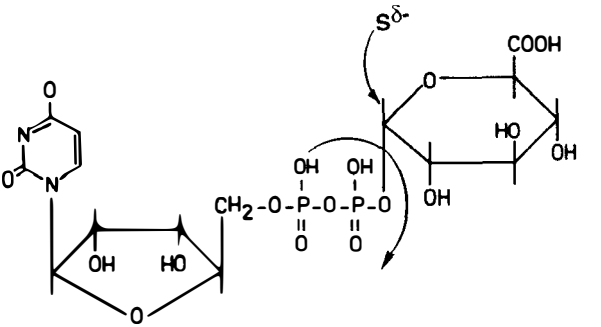

Figure 2 Attack of the C-1 carbon atom of glucuronic acid in UDPGlucA by a nucleophilic substrate ($S^{\delta-}$).

around the enzyme molecule, the results of several studies suggest that the catalysis is of the random-order, sequential (31), or rapid-equilibrium (32) type. Only with a few glucuronides is the reaction reversible in the presence of UDP, such as with 4-nitrophenylglucuronide (7, 33). Phospholipids and membrane fluidity affect the kinetic properties of the enzyme molecule very profoundly (32); however, it seems inappropriate to define different "allosteric" forms of the enzyme on the basis of (small) differences in kinetic properties induced by, for instance, temperature changes, pressure, or alkali treatment (34, 35). One of the reasons for this is that "allosteric" carries the connotion of physiological relevance. It is uncertain whether the various forms of the enzyme as obtained after solubilization have any relevance to the physiological conformation and activity in the endoplasmic reticulum (ER) membrane. An additional problem in kinetic studies is that many substrates are only very slightly water soluble; bilirubin is an extreme example of this. It is likely that physicochemical properties of the membrane phase in which UDPGT is located strongly influence the presentation of substrate to the active site of UDPGT (36).

As yet, there are no inhibitors of glucuronidation that can be used in vivo. These would be very desirable, for instance, to evaluate the role of glucuronidation in the pharmacological activity of morphine. The only means available so far are the use of competitive substrates to inhibit the enzyme, e.g. a carboxylic acid substrate such as probenecide (37, 38), or metyrapone, which is a strong inhibitor in vivo (39). However, these are only short acting and probably are not very specific. A new approach is the synthesis of transition state analogs such as triphenylethyl-UDP (40). They are based on the structure of very strong in vitro inhibitors, ω,ω,ω-triphenyl alkylcarboxyic acids (41). When these are linked to UDP, two binding sites of UDPGT are utilized. Triphenylethyl-UDP inhibits the glucuronidation of a number of

substrates in isolated rat hepatocytes, but, unfortunately, it was not active in the rat in vivo, most probably because it was too rapidly eliminated (41a). The lack of such inhibitors (which, ideally, are selective for only one form of UDGPT) is the major obstacle in studies on the relevance of glucuronidation in detoxification and toxification.

Induction

The mechanisms by which expression of UDPGTs is regulated have not yet been completely unraveled. The UDPGTs can be induced by many typical microsomal enzyme inducers, although it should be mentioned immediately that induction, i.e. increased mRNA production, is usually not proven to be the cause of the observed increase in UDPGT activity. Some UDPGT forms are induced coordinately with cytochrome P-450 species, since their induction is linked with the Ah receptor involved in the induction of those P-450 species by arylhydrocarbons (Ah's). Moreover, it was discovered that there are several groups of coordinately induced UDPGT forms: 3-methylcholanthrene (3-MC) and related compounds induce the glucuronidation of planar phenolic compounds, such as 1-naphthol and *N*-hydroxy-2-naphthylamine, whereas phenobarbital (PB) is an inducer of the glucuronidation of bulky phenols such as chloramphenicol and 4-hydroxybiphenyl (43). Other compounds, such as clofibrate, rather selectively induce bilirubin conjugation. These findings helped define the concept of different UDPGT forms and clearly showed that their expression was under separate control. This could later be confirmed by the use of cDNAs specific for different UDPGT messengers. The induction of UDPGT activity, and the subsequently increased clearance of substrates for glucuronidation, can be easily demonstrated in animals. Thus, PB treatment of rats leads to increased glucuronidation of the thyroid hormones. This is followed by functional adaption of thyroid size and activity, leading, after a few weeks, to a new steady state (44). It is much more difficult to prove induction in humans. Studies on the effect of smoking or of using oral contraceptive steroids report conflicting conclusions (45), which may be due to the use of different test drugs or experimental methods. Since a good model substrate to monitor glucuronidation capacity in humans in vivo is not yet available, studies of this process are difficult.

Localization of the Enzyme: Latency

Without exception, UDPGTs are membrane-bound enzymes. The highest activities are found in the ER membrane, in particular the smooth ER, but appreciable activity is found in the nuclear or even the cellular plasma membrane. Recent data on the overall structure of UDPGTs suggest that they are anchored in the membrane by only one peptide fragment (Figure 3; 46). Most of the enzyme is supposed to be inside the lumen of the ER; only a small

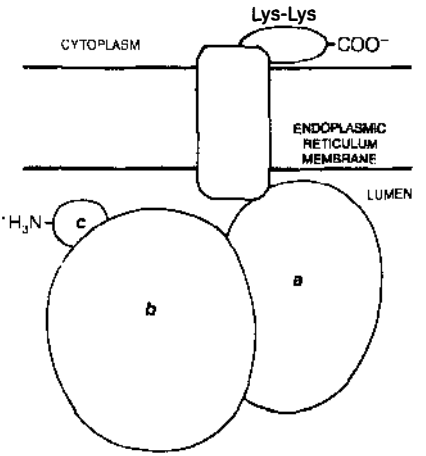

Figure 3 Model of the topology of UDPGT in the endoplasmic reticulum membrane. *a* indicates the (conserved) UDPGlucA-binding domain; *b* indicates the acceptor substrate-binding domain (variable sequence); *c* indicates the (conserved) N-terminal region; Lys-Lys indicates the stop transfer sequence. (Reprinted with permission from Ref. 46.)

fragment of the protein seems to be located on the cytosolic side. The activity is strongly dependent on phospholipids: if the membrane lipids are removed totally, very little activity is left. The type of phospholipid is rather critical: a structure–activity study of 1-palmitoyl-*sn*-glycero-3-phosphocholines (47) suggested that negatively charged phospholipids are inhibitory, whereas neutral or positively charged phospholipids are activating.

A feature of UDPGT activity that has caused a lot of controversy in UDPGT research is the fact that the measurable enzyme activity in the microsomal fraction can be much increased by treatment of this preparation with agents that disrupt the membrane structure: detergents, phospholipases, and physical procedures such as ultrasonic treatment or rehomogenization of the microsomal pellet (7). The microsomal fraction, of course, is an artifact of the ER; therefore, the question of relevance of this activation became important. Investigators have been divided into two camps for a long time: the "compartmentationalists," who believed that activation procedures opened a compartment of restricted accessibility (e.g. the access of water-soluble UDPGlucA to the microsomal lumen), and the "conformationalists," who believed that activation removed constraints that prevented full expression of enzyme activity. Although not all the details are clear, most data suggest (but do not prove as yet!) that the active site of UDPGT is located inside the lumen of the ER (and the microsomal vesicles). There are several arguments for this

(48). First of all limited proteolysis of intact microsomal preparations does not inactivate the UDPGT, whereas the same treatment in detergent-treated vesicles does. Further, UDPGT synthesized in intact microsomes in vitro is not detected by antibodies, unless the vesicles are opened (49). Finally, the extent of activation of UDPGT closely follows that of mannose-6-phosphatase activity, in which the active site is also located in the lumen of the vesicles.

Such a localization would require transport of the poorly lipid-soluble UDPGlucA across the ER or microsomal membrane to reach its binding site on UDPGT. The "activation" would take away this barrier, leading to a higher activity for the transferases. The barrier might be crossed by using a specific UDPGlucA carrier. The evidence for such a carrier is still indirect. A temperature-dependent association of radioactive [*glucuronate*-^{14}C]UDPGlucA with microsomal vesicles has been demonstrated (50). The characteristics of this binding suggested that UDPGlucA became associated with the membrane phase of the vesicles. The interpretation of these results, however, is not clear, since the nature of the accumulated radioactivity was not established. It is possible that the [^{14}C] glucuronate moiety was transferred enzymatically to an acceptor group in the microsomal membrane, so that, instead of uptake of UDPGlucA, covalent binding of [^{14}C]glucuronate was measured. If it were enzyme mediated, such a process would show all the characteristics determined by Hauser et al (50). Therefore, as yet the carrier for UDPGlucA has not been proven to exist. It has been suggested that the increase in glucuronidation rate in intact membrane vesicles by UDP-*N*-acetylglucosamine is due to an activation of the UDPGlucA carrier; again, however, the evidence is very indirect (51). What is needed is the isolation of the carrier protein, its incorporation in an intact membrane vesicle, and proof that it is functional. If such a carrier exists, its biochemistry may in fact determine part of the properties of the overall glucuronidation reaction in the intact ER and microsomal vesicles. Other carriers might be involved in the transport of other reactants, such as in glucose-6-phosphatase action (52).

A related question is that of the activity status of UDGPT in the cell in situ. If the latency in vitro reflects the situation inside the cell, the activity in the intact cell should be much lower than that of the fully activated enzyme. The results so far are inconclusive. In perfused-organ experiments (mainly with the liver) and in isolated hepatocytes, the maximum rate of glucuronidation has been determined and found to be somewhere between the "native" and fully activated microsomal levels (6). One of the problems in these studies is that very high concentrations of the substrates are added to the perfusion medium of the incubation, so that these may have toxic effects in the cells. Such toxicity might in fact result in activation of UDPGT in situ if it results in membrane perturbation. However, this could not be a long-lasting effect, since such damage to the ER would be expected to result in a loss of cell functions so that, for instance, UDPGlucA would be depleted.

Since many procedures may result in the partial activation of "native" UDPGT in the microsomal fraction, and the degree of latency may change with, for instance, age or pretreatment of the animals, it is essential to fully activate the UDPGT before activity assays are done. Only then is a reliable estimate obtained for UDPGT activity in a particular tissue.

Purification

Every purification of the enzyme starts with its solubilization from the membrane, followed by various chromatographic techniques. Purification of the protein, in particular removal of the lipids, will lead to inactivation of the enzyme, so that in activity assays these must be added to reconstitute the active enzyme. A major improvement was the introduction of affinity columns that afforded much higher purification in a single step. The application of chromatofocusing allows the preparation of really pure enzyme forms. Thus, UDPGTs with very small differences in properties can be separated.

With pure enzymes, specific antibodies can be produced; these can be used to isolate polysomes containing nascent UDPGT protein. Then the UDPGT mRNAs can be isolated, which are needed for synthesis of cDNAs.

Along these lines, many forms of UDPGT have been isolated from many species, mostly from the liver but also from other organs such as the kidneys (53) or olfactory epithelium (54). Amino acid sequencing of (parts of) the protein (usually the amino terminus) has been done, as well as some physiochemical characterization. In combination with cDNA data, this allowed rapid analysis of sequences and identity of UDPGT forms (55). However, properties of the enzyme will depend very strongly on the phospholipid environment, which precludes "simple" studies on properties of the "native" enzyme, i.e. the enzyme in its natural membrane localization.

CLASSIFICATION OF THE UDPGT FORMS

Despite the numerous reports on enzyme purification, no valid classification of the various form of UDPGT has been possible so far. Reasons for this are that no unique substrates have been identified that are highly specific for one single UDPGT form and that cross-reactivity of antibodies against pure UDPGT forms occurs. Moreover, since the various forms have very similar molecular weights and physicochemical properties, it is hard to separate them. However, since cDNAs have become available, it will be easier to make a logical classification. At a recent international workshop in Noordwijkerhout, the Netherlands, a proposal to classify UDPGTs according to gene product identity, in analogy to the cytochrome P-450 classification, was accepted. It is expected that such a classification will turn out to be logical with respect to, e.g., substrate specificity, regulatory control, and genetic variation.

Although substrate specificity allows some discrimination, it is not unequivocal, because there is too much overlap between the forms of UDPGT. Since it seems likely that several UDPGT forms play an important role in the elimination of endogenous substrates, such as bilirubin or thyroid hormones, it might have been possible to base a nomenclature on that. However, there is too little insight into the specificity toward endobiotics, and therefore it is uncertain whether they (or some of them) have such an exclusive function toward an endogenous compound. For instance, bilirubin UDPGT still can convert a number of other substances. A recently described 3,4-catechol estrogen UDGPT seems quite specific for such compounds, but it still converts other structures (56). It has been possible to discriminate among different UDPGT groups according to the bulkiness or flatness of the substrate structure, which must be related to be topology of the binding site for the acceptor substrate in the UDPGTs (57). This classification coincides to a certain extent with one that is based on certain regulatory properties.

Regulation of expression is controlled by endogenous factors (steroid hormones, etc) or exogenous compounds. Postnatal development of UDPGT activity toward different substrates showed different characteristics: there are several clusters of substrates, one of which (comprising planar phenols) showed an early rise (late fetal cluster), whereas another developed only after birth (neonatal cluster) (58). This and similar data for still other clusters suggest separate regulation for different forms of UDPGT. Another change in regulation takes place in preneoplastic and neoplastic liver: 2-acetylaminofluorene-induced nodules express higher levels of certain UDPGTs (59). Similarly, a daunorubicin-resistent cell line had increased UDPGT activity (60).

Induction of UDPGT activity toward different groups of substrates by, on the one hand, PB and, on the other, 3-MC also indicates separate control of the UDPGT forms involved. GT1 and GT2 UDPGT forms were identified (43, 61): GT2 accommodates flat, planar substrates (1-naphthol, *N*-hydroxy-2-naphthylamine, or 3-hydroxy-benzo[*a*]pyrene), whereas GT1 accepts bulky ones (4-hydroxybiphenyl, morphine, and chloramphenicol). Further work indicated that within those groups further subdivisions would be required to account for all the facts (62).

Certain strains or species lack some UDPGT activities; therefore, this is evidence that different forms of UDPGT must be involved. For instance, the Gunn rat lacks bilirubin UDPGT and the activity toward some phenols, whereas other substrates are very well conjugated.

These and similar considerations have led to some preliminary classification of different UDPGT forms, but the underlying molecular differences, i.e. structural differences and sequence homology between the different forms, remained obscure. Only since these tools have been provided by molecular biology is a biologically logical classification becoming possible.

MOLECULAR BIOLOGY OF UDPGTs

In the last decade molecular-biological approaches have enabled rapid progress in characterization of UDPGTs in terms of their structure and their genomic expression. cDNAs of UDPGT messengers have been made and sequenced and have provided probes to identify related cDNAs coding for other forms of the enzyme. Subsequently, these cDNAs have been transfected into COS or V79 cells, in which UDPGT activity can be expressed. This allowed studies on the substrate specificity of the UDPGT corresponding to a particular cDNA. Thus, human liver UDPGTs that conjugate a wide range of phenols (63, 64), estriol (55), hyodeoxycholate (64), and 3,4-catecholestrogens (56) and rat liver UDPGTs that conjugate several phenols (65, 66), bilirubin (67), or steroid hormones such as testosterone in a more or less selective way (56, 68–70) have been identified.

The length of the UDPGT cDNAs is usually between 1.8 and 2.3 kb, although longer sequences have been identified. The open reading frame is some 1600 nucleotides, coding for 500–530 amino acids. After completion of the protein, a signal peptide of some 20–25 amino acids is removed from the N terminus. The cDNAs have noncoding regions at both the 5' and 3' termini, with an adenylylation site(s) at the 3' end. A signal peptide sequence at the N terminus of the protein takes care of the translocation of the peptide chain through the membrane of the ER, so that most of the protein is present inside the lumen of the ER. In most of the derived protein sequences, asparagine-linked glycosylation sites were identified; some, like the pUDPGTr-3 protein, have no such site (69). A hydrophobic segment at the C terminus of the protein may serve as a transmembrane fragment; it is followed by a short sequence with basic amino acids, which sticks out in the cytosol. After transfer of the protein into the lumen of the ER during its synthesis, the signal peptide will be cleaved off and glycosylation can occur in the lumen. Interestingly, if the signal peptide is not hydrolyzed, the enzyme of the pUDPGTr-3 is not incorporated in the ER, so that this cleavage may be associated with membrane incorporation of the enzyme (69). As yet it is not clear how the folded enzyme interacts with the phospholipid phase of the membrane.

cDNAs that were induced by PB or 3-MC, as well as noninduced ones, have been isolated by various groups. Whereas the related cDNAs pUDPGTr-1 and pUDPGTr-3, which are not inducible by PB (69), showed high homology, the PB-inducible pUDPGTr-2 (65) was much less homologous with these forms. Most of the clones reported by Mackenzie (65, 68–70) yielded enzyme forms that could glucuronidate testosterone and some other steroids when expressed in COS cells. Even though the cDNAs are very similar, the specificity of the enzymes may be different: the primary sequences of pUDPGTr-3 and pUDPGTr-5 are 93% similar, yet the enzyme formed from

the latter does not conjugate estradiol (70). The physiological substrate of the pUDPGTr-3 form may not yet have been identified, since the activity for the substrates tested so far is very low.

Some cDNAs have been isolated from human tissues. One was rather specific for the bile salt hyodeoxycholate, at the 6α-position (64). Such a narrow substrate specificity may be a typical feature for UDPGT forms that primarily convert an endogenous substrate. Another illustration of that is the cDNA from human liver that codes for a UDPGT that convert 3,4-catecholestrogens very specifically (56). A human phenol UDPGT was very similar to the rat liver cUDPGT in size and showed a 76% sequence identity to the phenol UDPGT from rat liver (63, 66). As for most UDPGTs studied so far, the homology was highest in the C terminus, where the UDPGluA-binding site may be located. The HLUGP1 protein accepted many phenols as substrate, but was devoid of activity toward, for instance, (−)-morphine, phenolphthalein, or 4-hydroxybiphenyl. Furthermore, it did not catalyze the glucuronidation of several steroids (63). Recently, Ritter et al (67) cloned two bilirubin UDPGTs from human liver that were identifical after codon 287 but showed only 60% identity in the amino terminus. They were expressed in COS-1 cells.

Iyanagi et al (71) have characterized the deficiency of Gunn rat UDPGT. This strain of rat is deficient not only in bilirubin UDPGT but also in the 3-MC-inducible phenol UDPGT. They discovered that in the cDNA of the latter enzyme in the Gunn rat, 1 bp was deleted at position 1239/1240, which led to a completely changed sequence for the terminal 115 amino acids and to a nonfunctional enzyme. This deletion was confirmed by the use of nucleotide probes specific for either the mutated sequence or the normal sequence. The cDNA of bilirubin UDPGT (a 1762-bp cDNA) shares a 913-bp sequence with the 3-MC-inducible phenol UDPGT cDNA from rat liver, which codes for the C-terminal 247 amino acids (71). This part includes the position of the deletion mutation in the Gunn rat UDPGT gene, which explains why both the bilirubin and 3-MC-inducible phenol UDPGT activities in the Gunn rat are deficient. Sato et al (72) suggested that both UDPGTs are derived from a common primary transcript, which then is spliced alternatively to yield two different messengers. This may be a more common mechanism for the synthesis of different UDPGT forms.

Recently, a clone of the entire UDPGT gene was isolated that codes for a UDPGT form glucuronidating testosterone and several phenols (73). A cDNA was used to detect the gene. It is ca 12 kbp long and contains six exons. The first exon contains the signal peptide, and the last codes for the hydrophobic sequence. A promotor region and elements that may be involved in its liver-specific expression, as well as in induction by certain agents, were identified on the 5' end of the gene.

GENETIC DEFICIENCIES IN GLUCURONIDATION

So far, few "inborn errors" of glucuronidation have been discovered. Deficiencies in bilirubin glucuronidation have been identified in humans (the Crigler–Najjar syndrome and Gilbert's disease) and in rats (Gunn rat). The deficiency in bilirubin conjugation is easily observed as a result of the appearance of jaundice of the unconjugated type.

The deficiency in the Gunn rat has been most extensively studied. It is due to a mutation in the DNA coding for bilirubin UDPGT, which also affects the 3-MC-inducible 4-nitrophenol UDPGT. The reason is that these two forms of UDPGT seem to derive from one common primary transcript with alternative splicing of the 5'-end region, such that both carry the same inactivating mutation (72). Indeed, the two UDPGTs had already been located on the same chromosome (74). Induction of the mRNAs for 4-nitrophenol UDPGT by 3-MC resulted in increased mRNA levels, whereas the level of enzyme activity was not increased, because a nonfunctional truncated protein was formed (75–77).

The Crigler–Najjar type I syndrome is the more serious deficiency in humans, since patients with Gilbert's disease demonstrate only intermittent jaundice. Patients with the Crigler–Najjar type I syndrome usually do not survive for more than some 15 years, since they are totally unable to conjugate bilirubin (78). These patients are a rather heterogeneous collection, probably with very different mutations leading to the same result, as demonstrated by the use of various antibodies against bilirubin UDPGT in liver microsomes from several patients (79). Patients with the Crigler-Najjar type II syndrome show a milder form of deficiency.

In many rat strains there is a heterogeneity of androsterone (3α-hydroxysteroid) glucuronidation: part of the population has a high activity, and part has a low activity. This is inherited in an autosomal-recessive fashion (74, 80). The cDNA was isolated by Jackson & Burchell (81), and the amino acid sequence was determined from the cDNA. They showed that the rats with low androsterone activity had no mRNA for this UDPGT. Recently it was shown that the glucuronidation of 3,3',5-triiodothyronine (T3) and several aromatic amines followed the heterogeneity of aldosterone; apparently, these forms of UDPGT are regulated similarly, or their glucuronidation is by the same UDPGT form (82, 83).

UDPGlucA AVAILABILITY

A high rate of glucuronidation requires a sufficient supply of UDPGlucA; the cytosolic UDPGlucA concentration may determine the rate of UDPGT activity. Under normal conditions the rate of UDPGlucA synthesis in rat liver is

equal to that of its precursor, UDPglucose: 100 nmol min^{-1} g of $liver^{-1}$ (84). When a substrate for glucuronidation is given, a high turnover of UDPGlucA may occur. This may lead to a reduced "steady-state" concentration of UDPGlucA. Thus, a high dose of salicylamide decreased the UDPGlucA level by 90% and thereby reduced diglucuronidation of bilirubin severely (85). Whether a decrease of UDPGlucA by itself leads to a reduced glucuronidation rate is uncertain, since neither the K_m in situ for UDPGlucA nor the precise concentration of the cosubstrate in the cell cytosol or lumen of the ER is known. Experiments with isolated hepatocytes suggested that the K_m is of the order of the UDPGlucA levels in the cell (86).

The ATP level, and therefore sufficient oxygenation, determines the rate of UDPGlucA synthesis (87, 88). The UDPGlucA concentration can also be influenced by many other compounds, especially by diethyl ether, which results in a rapid decrease of UDPGlucA in the liver: within a few minutes it is decreased from 0.5 to 0.02 μmol g of $tissue^{-1}$. It recovers soon after exposure; rapid effects on nucleotide pyrophosphatase activity may mediate this change (89).

UDPGlucA availability is dependent on glucogen as a spare capacity; when glycogen is depleted by fasting, the availability of UDPGlucA is decreased upon challenge with a high dose of a substrate (90). Administration of D-galactosamine depletes the uridine pool and hence glucuronidation because no new UDPGlucA can be synthesized. It may affect only hepatic glucuronidation (91). However, galactosamine is rather toxic, and so its use is not a good method for inhibition of glucuronidation.

HEPATIC AND EXTRAHEPATIC CONTRIBUTION TO GLUCURONIDATION

In the past, the contribution of an organ to total glucuronidation capacity has usually been assessed from its UDPGT activity. However, this approach does not take into consideration two other important factors: the availability of the cosubstrate UDPGlucA and the "pharmacokinetic localization" of an organ in the body. For instance, if a drug is given orally, a major site of glucuronidation can be the gut mucosa, because that is the organ to which the drug is initially presented. Also, if a substrate is present in the splanchnic blood, intestinal glucuronidation may contribute heavily to glucuronidation: as much as 30% of the concentration of a substrate may be converted prehepatically in this region (92, 93). In the once-through vascularly perfused rat intestine–liver preparation, glucuronidation was the only intestinal biotransformation of salicylamide, whereas sulfation was predominant in the liver (94). The lungs receive the total cardiac output, but the liver and kidneys receive only 20–25%, so that a lower UDPGT activity in the lungs may be offset to some extent by a higher substrate presentation. It is likely, therefore, that the

importance of the liver, which in general certainly has the highest activity of (and often capacity for) UDPGT in the body, has been overestimated. Unfortunately, the data on the quantitative importance of other organs are very limited. Studies involving the specific activity difference ratio (SADR) technique demonstrated that the kidneys contribute considerably to the total body glucuronidation capacity (95).

Within an organ the UDPGT activity may be distributed nonhomogeneously among the different cell types. For instance, in the kidneys the UDPGT is localized mainly in the proximal tubular cells. Such distributions may be revealed either by microdissection studies (96) or by immunohistochemistry, using UDPGT-specific antibodies (97).

Within the liver, the same cell type, the hepatocyte, may deploy different properties depending on its localization relative to the portal venule and hepatic venule. For glucuronidation the activity seems rather homogeneous, with possibly slightly higher activity in zone 3 than in zone 1. These conclusions were based on studies of the perfused rat liver by Pang et al (98, 99) and Conway et al (90). The substrates tested were phenols such as harmol or 4-methylumbelliferone, for which sulfation is a competing reaction. Sulfation can easily be excluded by either omission of sulfate from the perfusion medium or addition of a sulfation inhibitor. On the basis of immunochemical localization, the distribution of UDPGT enzyme forms was homogeneous for some, but showed a higher concentration in zone 3 for others (97). In isolated perfused organs, uptake and transport of drugs or their metabolites can be studied. Thus, in the perfused mouse liver the biliary clearance seemed rate limiting in elimination of oxazepam glucuronide formed from oxazepam in the liver (100).

GLUCURONIDATION OF DRUGS IN HUMANS

Glucuronidation is also a major biotransformation in humans. Many drugs are direct substrates of the various forms of UDPGT, such as paracetamol, (−)-morphine, (±)-oxazepam, (±)-temazepam, amitriptyline, (±)-ritodrine, diflunisal, and the anti-AIDS drug zidovudine (101, 102). Most of these have a phenolic acceptor group, and therefore sulfation is a competing reaction. Carboxylic acids such as (±)-ibuprofen, probenecid, and (endogenous) bilirubin are eliminated almost exclusively by glucuronidation. Some compounds such as diflunisal (103) and 6-hydroxy bile acids (104) have both phenolic and carboxyl acceptor groups. Also, N-glucuronidation in humans has been studied (105).

In addition to these direct substrates, most other drugs can be metabolized by phase I reactions to generate acceptor groups for glucuronic acid. As a result, very many drugs are excreted to at least a minor extent in the form of glucuronides; for some it is the major route of excretion.

In view of the important role of glucuronidation outlined above, it is surprising that so little has been done to characterize this conjugation in humans. The available data suggest a rather wide variation in conjugation capacity: in human liver samples the UDPGT activity showed a 10–20-fold variation (106). Furthermore, in healthy volunteers the excretion of temazepam as glucuronide in urine varied over a 4-fold range (107) and that of paracetamol over a 5-fold range (108). However, these results are difficult to interpret: certainly for paracetamol, but also for temazepam, other metabolic pathways are present, and variation in glucuronidation may be due, in fact, to primary variations in the competing pathways, which are then reflected in increased or decreased availability of the substrate for glucuronidation. No substrate is available for human studies that is virtually exclusively glucuronidated, so that its rate of elimination from the blood reflects only its rate of glucuronidation. The "paracetamol test" as proposed by Kietzmann et al (109) is affected too greatly by small changes in the urinary excretion of free paracetamol to be reliable unless big differences are found. Moreover, paracetamol is mainly sulfated, so that its sulfation strongly influences glucuronidation. Obviously, as for oxidative metabolism, a number of substrates will be required to characterize the complete glucuronidation system because different UDPGTs are involved and each might show separate variation.

Although glucuronidation may be low in neonates, there is already appreciable capacity for certain drugs at birth, even though bilirubin UDPGT has not yet developed. In fetal hepatocytes, sulfation but not glucuronidation of paracetamol was measurable at 22 weeks of gestation (110). A sex difference in the glucuronidation of diflunisal at a phenolic hydroxyl group was observed, men having higher clearance than women. The use of oral contraceptive steroids increased the clearance in women to the level in men (45). These results confirmed earlier findings with, e.g., paracetamol, oxazepam, or temazepam, although the findings of different investigators may seem conflicting. In part this may be due to a rather big interindividual variability, certainly when small groups are used.

Severe liver disease will impair glucuronidation efficiency (13, 14, 111), although the UDPGT activity in liver samples of such patients was hardly affected (106). An extrahepatic contribution to conjugation may ensure that there is a residual capacity in case the liver function is lost. Kidney disease hardly affects glucuronidation of paracetamol in humans, but leads to accumulation of the conjugates in the blood (112).

ROLE OF GLUCURONIDATION IN TOXIFICATION

As discussed above. glucuronidation is a major detoxification reaction for most substrates that have been investigated in this respect. The example of

morphine showed, however, that this is not a rule; it depends on the substrate chosen. There are a few cases in which a substrate becomes more toxic upon glucuronidation. Good examples of this are the glucuronides of harmol and ethinylestradiol, which are cholestatic in the rat (3, 113). The molecular mechanisms have not been precisely established. Low solubility of harmol glucuronide, leading to precipitation of crystals in the bile canaliculi, may be the mechanism for harmol. For the steroid D-ring glucuronides (ethinylestradiol glucuronide), interactions with bile salt carriers in the canaliculus membrane may be involved (3).

Acylglucuronides are rather reactive, so that the acyl group can be transferred to, e.g., glutathione, protein, or other acceptor molecules (5, 114). They react with amine or thiol groups. The resulting adducts, which may be very long lived (115), serve as haptens and generate an immune reaction toward the drug. Idiosyncratic reactions may be the result of these adducts, for instance, those of zomepirac or tolmetin (116). Antibodies against this type of drug could be identified in patients who use the drugs.

CONCLUSION

Over the past 10 years there has been rapid development in certain areas of glucuronidation research, whereas some of the "old" problems have still not been solved. Examples of the latter problems are the physiological relevance of "latency" and the biological significance of glucuronidation in humans in vivo; a few more have been mentioned in the text. Very rapid progress is being made on the molecular biology of UDPGT expression and gene organization, which will solve the old problem of multiplicity of UDPGTs. Also, the role of glucuronidation in the metabolism of endogenous compounds such as thyroid hormones or bilirubin, as well as deficiencies in bilirubin metabolism in humans, is increasingly well understood. However, in the area of steroid glucuronidation, little insight is available, since although the formation of many steroid glucuronides has been demonstrated, their physiological significance has not been systematically investigated. The findings for morphine, however, will revive interest in the biological effects of glucuronides. A recent paper which demonstrates that the glucuronide conjugate of a retinoid derivative was more effective and at the same time less toxic than the aglycone (117) illustrates the potential advantage of glucuronides as drugs. A very interesting development is the role of glucuronidation in odorant signal termination in olfactory tissue (54, 118). The UDPGTs isolated from olfactory tissue have rather special properties and show high activity toward odorants. These UDPGTs may therefore have a physiological function toward these "physiological" xenobiotics.

These recent development suggest that in the years to come we will see a

rapidly growing insight in the physiological role of UDPGT, which obviously includes the metabolism of "physiological" xenobiotics in, for instance, the natural diet.

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