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COMMENTARY

THE GLUCURONOSYLTRANSFERASES: WHAT PROGRESS CAN PHARMACOLOGISTS EXPECT FROM MOLECULAR BIOLOGY AND CELLULAR ENZYMOLOGY?

GÉRARD SIEST,* BÉNÉDICTE ANTOINE, SYLVIE FOURNEL, JACQUES MAGDALOU and JACQUES THOMASSIN

Centre du Médicament, UA CNRS 597, 30 rue Lionnois, 54000 Nancy, France

The UDP-glucuronosyltransferases (UDPGT, EC 2.4.1.17) are a family of membrane-bound enzymes involved in the conjugation of various exogenous (such as drugs) and endogenous (such as bilirubin or steroid hormones) aglycones with UDP-glucuronic acid (UDPGA). They act either on hydroxylated compounds or directly on chemical structures that have hydroxyl, carboxyl, or sulfhydryl groups, permitting them to be eliminated in a water-soluble form. Although UDPGTs operate mainly in the liver, their activity is detectable in practically all organs and tissues. In the liver, UDPGT activity is associated with various types of subcellular membranes, particularly the endoplasmic reticulum (ER), where it accounts for up to 1% of total membrane proteins. Its presence in nuclear membranes has suggested that it may protect against the activity of carcinogenic substances.

Study of the physicochemical and molecular properties of UDPGTs has not hitherto enjoyed the same development and success as has the characterization of the cytochrome P-450-dependent monooxygenase complex. In the past 10 years, progress in purification techniques and increasing familiarity with the methods of molecular biology have led to the unequivocal description and identification of several forms of cytochrome P-450 and glutathione *S*-transferases. During that period, attempts to characterize and define the properties of UDPGTs have run into considerable difficulties and have not yielded results as clear-cut as those on cytochrome P-450. Furthermore, the marked phospholipid dependence of these membrane enzymes has prompted much study. In particular, the development of two models of regulation—one involving compartmentation, the other constraint—to explain how UDPGTs function in biological membranes has fuelled many arguments and often contradictory theories which have not really explained the main enzymatic characteristics of the reaction.

Why this discrepancy in the progress of knowledge between the monooxygenase and the UDPGT system? The following are some of the most likely reasons: (1) the weak inducibility of transferases (generally 1- to 5-fold) compared with cytochromes P-450 (1- to 100-fold); (2) a lack of specific inhibitors, which became available for monooxygenase very early; (3) the relative insensitivity of UDPGTs, aside from their "activation", to phenomena of binding and rapid regulation, and the absence of receptors; (4) the difficulty of purifying the enzyme, mainly due to its phospholipid-dependent character and also the weak immunogenicity of the purified protein; and finally (5) the perception that their toxicological or pharmacological importance is low.

In this commentary, following the first workshop devoted to this subject [1], we evaluate the relative chances of success of different approaches (molecular biology, cell biology, and enzymology) applied to UDPGT. One question is whether these disciplines offer answers to various questions of pharmacological or toxicological interest:

(a) in view of the early results obtained by molecular biology, confirming the heterogeneity of this family of proteins, are there actually some enzymes more specifically responsible for transforming endogenous substrates, and others for xenobiotic substrates?

(b) What is the pharmacological significance of the presence of UDPGT in plasma membranes or nuclear membranes? How can the specificity of these enzymes come into play at these subcellular levels? How do these enzymes get from the endoplasmic reticulum to these destinations?

(c) How can one approach the structure of the active site of the isoenzymes and their topology? Does the use of purified proteins (enclosed in liposomes) really reflect the functioning of the enzyme *in vivo*? What information can the use of inhibitors provide?

To discover the metabolic fate of a drug or the risk of toxicity of a xenobiotic, it is essential to know which UDPGT isoenzyme conjugates it, and how and where this isoenzyme is specifically regulated.

Are UDPGTs products of a single gene family?

Functional heterogeneity of UDP-glucuronidation is suggested by the existence of at least seven or eight molecular forms of UDPGT in various mammals. Up to now, these have been distinguished mainly on the basis of physiological criteria (such as perinatal development, or induction) or physicochemical properties (such as the structure of their substrates)

* Address all correspondence to: Dr. Gérard Siest, Laboratoire de Biochimie Pharmacologique, Centre du Médicament, University de Nancy-1, 30, rue Lionnois-B.P. 403, F 54001 Nancy Cedex, France.

Table 1. Summary of the criteria used to separate UDPGT forms

Substrate specificity	Specific induction [10, 25–27, 54–57]	Ontogenic development [20, 58–60]	UDPGT form [10, 55, 61–63]	Subcellular localization* [9, 11–18]	Inhibition [49–53, 64, 65]	Purification: Chromatographic separation [61, 63, 66–70]	Purification: Chromatofocusing pI [71]	Characterized mRNAs [3, 5]
1. Aromatic flat < 0.4 nm group 1 substrates	TCDD, 3-MC β-naphthoflavone	Late fetal	GT_1/pnpGT	ER Nuclear envelope (mitochondria)	Arginyl reagents† Ethanol‡ Organophosphates§ Piperine‡ MOAI‖	GTA	GT I 8.9	3
2. Bulky molecules > 0.4 nm group 2 substrates	PB¶ TSO	Neonatal	GT_2	ER Golgi apparatus Plasma membranes		GTD	—	—
3. Monoterpenoid alcohols**	PB††	—	GT_2A	ER Plasma membranes		—	—	—
4. Bilirubin	Clofibrate and related hypolipidemic agents	Neonatal	Bilirubin GT/ GT_3	ER Golgi apparatus	Triphenylacetic acid‖ and related compounds MOAI‖ Novobiocin‡	GTB	GT V 7.5	—
5. Digitoxigenin monodigitoxoside**	Spironolactone	Weaning	—	ER		GTF	—	—
6. Estrone (3 hydroxysteroids)	Arochlor 1254	Neonatal	—	ER			GT VI 6.5	—
7. Testosterone (17 hydroxysteroids)	—	Neonatal	—	ER Golgi apparatus Plasma membrane		GTE	GT II/III 8.4/8.0	3?
8. Androsterone (3 hydroxysteroids)	—	Weaning	—				GT IV 7.8	1

* Other subcellular locations are mentioned when specific activity in percent of microsomal activity is higher than 50%.
† Inactivation towards the UDPGA binding site (J. Thomassin, unpublished observations).
‡ Aspecific inhibition by action on various steps of the reaction (action on the cofactor, chelation of lipids, action on the lipid surrounding).
§ Specificity towards group 1 substrates. No inhibition of testosterone, phenolphthalein activities.
‖ Higher specificity towards the bilirubin conjugating form (S. Fournel, unpublished observations).
¶ Considered as specific inducer of group 2 substrate conjugation, but also inducer of other forms of UDPGT.
** Considered to be a separate form on the basis of selective induction.
†† Specific inducer in the guinea pig [55].

(Table 1). Purification made possible a partial biochemical characterization of the various protein entities. The estimated molecular weight of UDPGT, which varies with the species of animal and also with the purification methods used, is of the same order of magnitude as that of other drug-metabolizing membrane enzymes, such as cytochrome P-450 or epoxide hydrolase. The UDPGTs probably have an oligomeric structure that comprises one to eight subunits of apparent molecular weights 50,000 to 60,000 when analyzed on sodium dodecyl sulfate/polyacrylamide gels. Isoelectrofocusing experiments have suggested that there are at least seven isozymes. Their substrate specificities generally exhibit partial overlapping, though there is a strict specificity with regard to endogenous substrates, particularly bilirubin and steroids.

Although direct evidence, i.e. primary structure, has until now been difficult to obtain by purification, it is likely that the great strides in molecular biology techniques will soon make it possible to isolate the corresponding DNA sequences, and that these will confirm the real existence of one or more gene families. Studies of how the expression of these genes is regulated will contribute to an understanding of what "induction" of certain drug-metabolizing enzymes by xenobiotics means.

The earliest finding obtained by means of molecular biology, in Bethesda [2, 3] and Dundee [4, 5], suggested that part of UDPGT polymorphism may be due to differential processing of pre-mRNA and, also, to differential maturation of the translation products of the different, but very homologous mature mRNAs.*

Indeed, in contrast with cytochrome P-450 (of which five different gene families have already been described) [6], a single UDPGT pre-polypeptide (52 kD) was obtained when differently induced mRNAs were translated *in vitro* [2, 4]. Mackenzie *et al.* [3] found evidence for three rat UDPGT cDNAs—two structural cDNAs in the same subfamily and one phenobarbital-inducible UDPGT cDNA in a second subfamily.

Similarly, Burchell's team determined the sequence of four classes of rat cDNAs, which were found to be 85–95% homologous, and thus to belong to the same subfamily [5]. However, Mackenzie and Burchell seemed to have isolated cDNAs corresponding to at least distinct gene subfamilies, perhaps coding for distinct isoenzymes. Indeed, in this regard, while the cDNAs obtained by Mackenzie were isolated using an antibody which showed a specialization for immunoadsorbing GT_1† (or xenobiotic) activities, Burchell characterized a different gene subfamily involving the coding sequence for androsterone UDPGT, using an antibody which was affinity-purified against testosterone and bilirubin UDPGTs, i.e. endogenous aglycones. These findings suggest that there may be, at least partially, distinct coding sequences for the UDPGTs involved in the conjugation of either endogenous or exogenous compounds. The notion of two distinct populations of UDPGT, one for endogenous and the other for exogenous compounds, had already been suggested by biochemical data.

First, purified isoforms towards endogenous substrates have more restricted specificities (see Table 1). Moreover, the endogenous compounds and "natural" xenobiotic UDPGTs generally show low capacities (low V_{max}) but higher affinities (low K_m) in conjugating their substrates [7–9], their high specificity providing for reliable elimination of physiological molecules. Conversely, xenobiotic UDPGTs seem to compensate for their low affinity (and thus low specificity) towards a diversity of substrate structures by a high rate of handling to detoxify these various drugs efficiently [7–10], as well as greater inducibility.

Thus, it can be speculated that the initial DNA coding sequences might have been for UDPGTs conjugating endogenous substrates and then have been adapted to the transformation of new xenobiotics.

Is the subcellular location of an isozyme a clue to its function?

In this respect, we would like to emphasize the less studied notion of subcellular localization according to function of the different molecular forms of UDPGT. The ER is considered to be the main site of hepatic drug metabolism. This simple terminology already suggests an adaptive function of the hepatic ER in detoxifying xenobiotics. But it seems possible that some physiological molecules may be conjugated, and thus eliminated, in a more appropriate and specialized sublocation. Very few studies of the subcellular distribution of the different forms of UDPGT have been carried out until now [9, 11–18].

The existence of carcinogen-induced UDPGTs (GT_1), whose distribution seems to be restricted to the ER and the nuclear envelope, i.e. close to genetic material, could be the best example of the evolutionary concept of "UDPGT sublocation according to function". There are many arguments in favor of the existence of GT_1 enzymes which conjugate and detoxify polycyclic hydrocarbons and phenols. It has even been shown that only the molecules that have carcinogenic potential are able to induce these forms [19]. Though GT_1 have broad substrate specificity, numerous observations confirm a functional specialization in protecting genetic material against carcinogenic compounds, which often bind to DNA:

—GT_1 are increased in chemically induced preneoplastic nodules, together with strictly specialized cytochrome P-450s and epoxide hydrolase, in a phenotypic biochemical response of the hepatocyte to toxic compounds;

—They are expressed before birth [20], whereas the

* According to Dayhoff's definition, two proteins belong to different gene families if they have similar biological functions but less than 50% similarity in amino acid sequence [6].

† The abbreviation GT_1 is operationally used to denote UDPGTs conjugating group-one substrates or planar phenolic aglycones (very often 4-nitrophenol) and sharing in common various properties such as inducibility or structure of the substrates accepted. Nevertheless, recent findings in molecular biology indicate that GT_1 comprises at least two forms of UDPGT. Similarly, GT_2, classically used in the literature, means UDPGTs conjugating group-two substrates or bulky molecules.

forms involved in endogenous metabolism develop postnatally;
—They are ubiquitous. GT_1 are not only present in liver and intestine (as are GT_2) but in a great number of other tissues [21]. For example, only GT-naphthol seems to be localized in the brain, perhaps to protect neurones against certain phenols [22];
—They are quickly restored after partial hepatectomy [23]. This characteristic shows, first, that GT_1 are regulated independently and also suggests the high priority the organism assigns to protect DNA;
—The GT_1 forms seem to be mainly localized in the centrolobular part of the liver [24], i.e. the part most richly irrigated by blood carrying (possible) toxic molecules;
—They have a good inducibility and, particularly, substrate induction in response to polycyclic hydrocarbons [25–27], in contrast to endogenous substrate conjugating forms.

All these individual characteristics strongly support the existence of some UDPGTs which specialize in protection against toxic compounds, which are differently regulated, and which seem to appear in the context of the organism's adaptation to the environment and its pollutants. Thus, it is logical to think that this specialization of GT_1 could be associated with a specialized "address" of the proteins in the hepatocyte endomembranes. They are the only enzymes which are so situated in both the rough ER and the nuclear envelope [9, 11, 16] with even similar substrate specificities between both these membranes [11].

UDPGTs that act on endogenous substrates seem to be distributed more widely inside the hepatocyte. Although very few studies have been done, our own findings suggest a less restricted "address" for the UDPGT forms that conjugate bulky substrates, including most endogenous molecules. We have found, for example, that Golgi and plasma membranes may contribute significantly in glucuronoconjugating testosterone in the hepatocyte [16]. The same observations were made with regard to morphine, which could be, by structural analogy, specially conjugated by an "endorphin" UDPGT. In each type of membrane, this bulky substrate has a high affinity for the enzyme [9].

The questions that come to mind in a discussion of plurilocalization are, first, what is the mechanism by which membrane proteins migrate, and, second, what is the control (the signal sequence for a specific "address"?) of such intracellular traffic.

The peptide precursors to diverse membranes and organelles are known to follow a common pathway from their site of synthesis in the ER to the Golgi apparatus, where they separate to their final destination (see Rothman and Lenard [28] and Sabatini *et al.* [29] for reviews). Thus, in a preliminary experiment, we attempted to trace the possible migration of different newly synthesized UDPGT forms after a single phenobarbital injection. These preliminary results suggested that, in contrast to 4-nitrophenol GT, which seems to be sequestered in its site of synthesis (ER), morphine UDPGT may migrate to Golgi membranes, where an increase of activity was observed, following its increase in the ER [30]. Similarly, the bilirubin transferase was shown to be effective in the Golgi membranes [17], where a single dose of phenobarbital was able to increase its activity by 200% 24 hr after the injection (B. Antoine, unpublished observation). These findings should be compared with those of Mackenzie *et al.* [31], who showed, first, the glycosylation maturation of some UDPGTs with specificity towards testosterone and morphine (this raised the question of an eventual passage in the Golgi) and, second, the proteolytic cleavage of at least one other UDPGT form, synthesized *in vitro* [2] (that exhibits a similarity with the secretory protein pathway).

This observation of Mackenzie naturally led us to wonder about the significance of proteolytic cleavage in the ER, mailing newly synthesized polypeptides. Signal sequences (commonly at the NH_2 terminal) were recognized as distinguishing between secreted proteins, which are to be translocated into the lumen of the ER, and cytoplasmic and membrane proteins, which are not. At present, we do not know how a protein signals its route, nor how the information is encoded in the many different kinds of transport vesicles that are needed to carry membrane traffic. But the remarkable discovery about UDPGTs is that they are the first drug-metabolizing membrane proteins [32] which have been found to be cleaved proteolytically in the ER. Moreover, this phenomenon does not seem to affect all UDPGT forms [2]. Though this information is not evidence that some UDPGT forms migrate, it is surprising enough to deserve mention.

Many endogenous substrates for UDPGTs are bulky molecules, which are generally ready to be conjugated (i.e. already hydroxylated). The presence of some capacity to glucuronidate such endogenous compounds, outside the ER, could contribute to a more balanced metabolic capacity in the cell.

In contrast to GT_1, few arguments point to a specialized sublocation of the UDPGTs that act on endogenous substrates in the ER. These forms, whose activities are commonly lower than the others (especially than GT_1), could be more difficult to detect, even with immunocytochemical techniques using nonspecific antibodies [33]. They are perhaps present in smaller proportions in the different membrane preparations, and it could be easy to miss them until specific probes are found.

To conclude this section, we would like to emphasize that very little is known about "endobiotic" UDPGTs and the regulation of their expression. Since differential peptide maturation seems to be a critical point when considering UDPGT polymorphism, it might be sensible to first study the "ancestral" forms of the enzyme and their pharmacologically specialized sublocations to understand better their evolutionary adaptation (differentiation and localization according to function) in response to the changing environment.

A kinetic approach to the study of the active sites of UDPGTs

Thorough kinetic analysis is an essential part of characterizing an enzyme system and the working out of how it functions *in vivo*. The dependence of

UDPGTs on surrounding lipid membranes probably explains in part why, in this respect also, they are the least-well-understood of the drug-metabolizing enzymes. Surprisingly, the catalytic functioning of this enzyme is not known; UDPGT investigators cannot even agree on a definition of its kinetic type. It is accepted that glucuronoconjugation of 4-nitrophenol works by a "random rapid equilibrium bi-bi" mechanism [34]. While it does indeed seem that the enzyme kinetics described have always been of the sequential type, nevertheless other types have also been postulated [35, 36]. We should also mention that the kinetic studies have been mainly of the conjugation of planar phenolic substrates. Virtually nothing is known about the conjugation of other substrates, phenolic or not, which are probably acted on by distinct forms.

UDPGT really does represent a puzzle in enzymological terms. It is usually described as a latent enzyme. Its activity in hepatic microsomes is expressed completely only after treatment with an activator, generally a detergent. Furthermore, in native microsomes glucuronoconjugation does not follow the Michaelis–Menten equation until after activation [37, 38]. Questions have always been asked about the physiological significance of this "state of latency" *in vitro*:

(a) Does this catalytic characteristic really correspond to a regulatory process of the organism itself, or is this latency mostly of an artefactual nature as suggested for other membrane bound enzymes [39]?
(b) Should the non-Michaelis–Menten enzyme kinetics be interpreted in terms of cooperativity of enzyme subunits [37] or, rather, as evidence of the existence of two populations of protein molecules localized differently in the membrane of the endoplasmic reticulum [38]?
(c) What is the real role of UDP-*N*-acetylglucosamine, which is considered to be a physiological activator mainly because its activator effect operates at physiological concentrations?

This characteristic latency of UDPGT fits the notion of an enzyme situated deep in the membrane phospholipid bilayer. The most recent study of the amino-acid composition of the enzyme even suggests that 95% of one phenobarbital-inducible UDPGT form would be on the luminal side of ER, with the protein anchored to the membrane by the carboxyl-terminal hydrophobic segment [40]. But no one knows as of now what the real factors are that regulate the accessibility of the substrates at their active sites. In particular, it may be asked how the UDPGA nucleotide sugar, a large ionic molecule, can penetrate into the membrane. The existence of a permease [41] responsible for this transport remains to be established.

A vigorous debate, engaged in by two schools, British and American, proposed models called, respectively, "compartmented" and "constraint", which attempted to explain the kinetics and catalytic functioning of the enzyme, conditioned by its lipid environment. The confrontation between these two schools has been fuelled by a series of publications providing partial answers based more on speculative interpretations than firm evidence (see, for example, Refs. 38, 42, and 43). The problem of its catalytic behavior seems so complex that it has somewhat discouraged UDPGT investigators outside these two schools, and knowledge about the matter is stagnating.

The most thorough of recent investigations has been of the glucuronoconjugation of bilirubin, which for a long time was left to one side because of technical difficulties inherent in its measurement and because of the additional problem represented by the formation of several types of glucuronides. For example Cuypers *et al.* [44] analyzed the kinetics of the formation of bilirubin diglucuronide, and Blanckaert and Vanstapel [45] tried to define the substrate specificity of this reaction using structural analogues of bilirubin.

This difficulty in characterizing the kinetic functioning of UDPGTs is mainly related to the problems posed by purified preparations. The use of purified material, essential for kinetic studies, has serious disadvantages in the case of UDPGTs. Although purification techniques have improved considerably as regards solubilization (for example using Chaps as detergent) and affinity chromatography (using UDP-hexanolamine), it is not really possible to separate the different isoforms clearly. Also, reconstitution of the enzyme activity of the delipidated enzyme with phospholipids does not restore an enzyme system with catalytic properties identical to those of UDPGTs *in situ*. In particular, the substrate specificity may be altered profoundly.

Therefore, the results obtained with purified preparations should be constantly compared with those obtained on systems closer to physiological, such as isolated perfused liver or hepatic microsomes. UDPGT, more than any other enzyme, must not be regarded as an isolated protein entity.

The architecture and amino-acid composition of the active sites are also part of the puzzle of the catalytic functioning of UDPGTs. In our laboratory we use a dual approach to the active sites. From structure/activity relationships, one can characterize the physicochemical properties of the substrates conjugated by one or the other of the isoforms and, consequently, their interaction with the enzyme protein. Our early studies were limited to gaining some idea of the size of the molecules and established a limiting thickness of the substrates acted on by the isozymes called GT_1 [46]. Using a more sophisticated approach, by means of calculated or experimental steric parameters (such as Van der Waals volume) or energy parameters (such as orbital energy, delocalization energy, and electron affinity), we succeeded in defining the physicochemical criteria of the substrates that affect their glucuronoconjugation [10, 47]. Among these criteria were the following:

(a) Steric hindrance. The more hindered the region near the active group, the more difficult the conjugation will be. It even seems that the more fully the para-position of a phenol is occupied by a hindering substituent, the less the aglycone reacts as a planar aglycone (GT_1).
(b) The reactivity of the hydroxyl group. The lower the pK_a and the more easily dissociated the OH

proton, the more easily the hydroxyl group will be conjugated.
(c) The orientation of the aglycone in the active site. We have shown that the dipolar moment of coumarins directs them more or less advantageously to the site, and that this orientation greatly affects their rate of glucuronoconjugation.

A complementary approach is the classic study of the inhibition of enzyme function, and inhibitors are valuable molecular tools in attempts to approach the active site of these proteins. Unlike the long-recognized powerful inhibitors of cytochrome P-450 and epoxide hydrolases—such as carbon monoxide, SKF-525A, or 1,1,1-trichloropropene 2,3-oxide—such compounds that act on UDPGTs remain largely undiscovered. Various compounds that reduce the potential of glucuronoconjugation have been described. However, these products act more on general cell metabolism, in particular on the biosynthesis of UDP glucuronic acid [48] or specifically on the enzyme reaction, as in the case of novobiocin complexing Mg^{2+} [49], or of monoamine oxidase inhibitors disturbing the membrane lipid environment [50], or by nonselectively inhibiting all drug-metabolizing enzymes, as piperine does [51]. Furthermore, many studies of competitive inhibition between various substrates of UDPGTs, which theoretically should provide information about which isoforms metabolize which aglycones, turn out to be generally disappointing and do not provide definitive answers as to their specificity, probably because of overlap between the forms, and their topological and structural similarities.

Research on specific inhibitors is thus a very promising area in the field of glucuronoconjugation. The use of reactive molecules (2,3-butanedione) targeted on the active sites and covalently binding there has made it possible to identify an arginine in the protein structure that plays a preponderant role in the binding to UDP-glucuronic acid by interaction with its carboxyl group [52]. On the other hand, the discovery in our laboratory of a potent competitive inhibitor of glucuronoconjugation of bilirubin, with a triphenylcarboxylic structure, gave us a way of approaching the molecular events in the binding of this substrate to its active site and the spatial organization of this site [53]. Thanks to its specific binding, it can be predicted that such an inhibitor can also be used as an affinity ligand for purposes of purification.

In conclusion, the reality of UDPGT molecular forms will soon be clarified by molecular biology. The regulation of these enzymes during development, induction or carcinogenesis mechanisms and the statement of pharmacogenetic variations will then be easier to study with molecular probes. The relationships between UDPGTs and the other drug-metabolizing enzymes (cytochromes P-450, glutathione-*S*-transferases, epoxide hydrolases, gamma-glutamyltransferases) could also be defined more precisely, facilitating the prediction of risk of toxicity. But the completion of such investigations will need time. During this period, and to complement the genetic information, it is necessary to understand how the enzymes work, namely by characterizing the active sites and their exact topology in the membrane and the real molecular basis of their lipid-dependence and latency. The use of better fitting models, the detailed study of protein conformation in membranes, and the development of specific inhibitors should be priorities in bringing precious information on the "working enzyme" to pharmacologists and toxicologists.

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