Biochemical Pharmacology, Vol. 29, pp. 495–500.
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0006-2952/80/0215–0495 $02.00/0

FUNCTIONAL HETEROGENEITY OF UDP-GLUCURONYLTRANSFERASE IN RAT TISSUES

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(*Received* 11 *July* 1979; *accepted* 27 *September* 1979)

Abstract—Tissue distribution of UDP-glucuronyltransferase was investigated using two substrate groups which were shown to be conjugated by two different forms of this enzyme in previous studies with rat liver. These enzyme forms were found to be differentially inducible by 3-methylcholanthrene (form 1) and phenobarbital (form 2). Group 1 substrates (conjugated by form 1) include 1-naphthol, *N*-hydroxy-2-naphthylamine and 3-hydroxybenzo[*a*]pyrene; group 2 substrates (conjugated by form 2) comprise 4-hydroxybiphenyl, morphine and chloramphenicol. Group 1 substrates are conjugated in a number of tissues, for example, liver, kidney, small intestinal mucosa, lung, skin, testes and spleen. However, conjugation of group 2 substrates is detectable only in liver and intestine to an appreciable extent. It is concluded that enzyme(s) efficient in the conjugation of group 1 substrates is ubiquitous in the investigated organs, whilst only liver and intestine possess enzyme(s) efficient in the conjugation of group 2 substrates.

In contrast to 3-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 7,8-dihydrodiol cannot be clearly associated with only one of the 2 substrate groups. Glucuronidation of benzo[*a*]pyrene 7,8-dihydrodiol is enhanced by both phenobarbital and 3-methylcholanthrene in liver. Conjugation of the dihydrodiol is detectable in all tissues examined. However, enzyme activity towards the dihydrodiol is much lower than that towards 3-hydroxybenzo[*a*]pyrene. It is disproportionately low with skin microsomes.

Conjugation with glucuronic acid catalysed by microsomal UDP-glucuronyltransferase (GT, EC 2.4.1.17) is quantitatively the most important phase 2 reaction of drug metabolism [1,2]. The enzyme converts a wide variety of lipid soluble drugs, environmental chemicals and endogenous compounds such as bilirubin and steroid hormones into biologically inactive glucuronides which are easily eliminated from the organism. Many substrates of GT are metabolites of phase 1 reactions, some of which are highly cytotoxic, for example *N*-hydroxyarylamines and benzo[*a*]pyrene phenols.

Primary metabolites can be further converted by recycling through a second monooxygenase-catalysed activation step to ultimate toxic metabolites as in the case of aryl hydrocarbons [3–6]. Glucuronidation (together with sulfation) may effectively prevent recycling and thus prevent the formation of ultimate carcinogens. In some instances glucuronidation may lead to chemically stable substances which are actively transported from the site of formation to the urinary tract or the intestine where they may be reactivated to electrophilic species [7, 8]. There are also examples of glucuronides which are reactive compounds themselves, for example *N*-hydroxy-2-acetylaminofluorene-*N*-*O*-glucuronide [9] and *N*-hydroxy phenacetin-*N*-*O*-glucuronide [10].

Tissue distribution studies of drug metabolizing enzymes may help to understand organ specificity of toxic reactions since very often a delicate balance between activating and inactivating enzymes in a given tissue may be decisive for the accumulation of reactive intermediates [11, 12]. Although GT has already been found in many tissues [2, 13, 14], studies on substrate specificity of GTs in extrahepatic tissues are rare.

In this report we demonstrate functional heterogeneity of tissue specific GTs towards two groups of substrates which in rat liver are conjugated by two different forms of GT [16]. These forms of GT have been recently separated and purified to apparent homogeneity [15, 16]. The two enzyme forms are differentially inducible† by 3-methylcholanthrene and phenobarbital and are tentatively denoted GT_1 and GT_2, respectively. The results are discussed with regard to possible roles of glucuronidation in benzo[*a*]pyrene metabolism.

MATERIALS AND METHODS

Chemicals. *N*-Hydroxy-2-naphthylamine was synthesized according to Willstätter and Kubli [18]. (±)-*trans*-7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene was prepared as described by McCaustland and Engel [19] using the synthetic modification suggested by Fu and Harvey [20]. It was purified by preparative reversed phase high performance liquid chromatography.

† The term induction is used in this study to denote an increase in enzyme content with no implications as to the underlying mechanism. The term "enzyme forms" is used in a broad sense as outlined in Nomenclature of multiple forms of enzymes [17]. The substrate groups are derived from the substrate specificity of two purified enzyme fractions separated by DEAE–cellulose chromatography [16]. However, characterization of these enzymes forms is still incomplete.

1-(1-^{14}C)naphthol, (*N*-methyl-^{14}C)morphine hydrochloride, D-threo-(dichloroacetyl-1-^{14}C)chloramphenicol, UDP-D-(U-^{14}C)glucuronic acid ammonium salt were from The Radiochemical Centre, Amersham. Aroclor 1254 was kindly supplied by Monsanto Chemical Co, St. Louis, MO. Brij 58 (a condensate of hexadecyl alcohol with 20 mol ethylene oxide/mol) was a gift of Atlas, Essen. Samples of 3-hydroxybenzo[*a*]pyrene were generously provided by ITT Research Institute, Chicago, on behalf of the NCI Carcinogenesis Research Program.

Preparation of organ homogenates and of microsomal fractions. Male Wistar rats (200 g) were used. Organs were excised, homogenates and microsomal fractions were prepared as described for liver microsomes [21]. The duodenum and small intestine (20 cm from the pylorus) was rinsed extensively with ice-cold 0.9% NaCl. The mucosa was scraped off with a spatula and homogenates (20%, w/v) were prepared in 0.25 M sucrose [22]. After killing, the animals were shaved at the dorsal area (4 × 6 cm) with an electrical clipper. The shaved areas were excised and cooled in 1.15% KCl containing 10 mM potassium phosphate buffer, pH 7.4. Adherent fat tissue was scraped off using a scalpel. The remaining skin preparation was minced and homogenized in 1.15% KCl containing 10 mM potassium phosphate buffer, pH 7.4, using an Ultra-Turrax for 5 times 15 sec at high speed as described previously [23]. Samples were cooled for 5 min between each Ultra-Turrax treatment. Microsomes were prepared as described above for liver tissue.

Treatment of animals with inducing agents. Male Wistar rats (200 g) were used.

Phenobarbital-treatment: An initial dose of 100 mg/kg was given once i.p. and was followed by 0.1% (w/v) in drinking water for 4 days.

3-Methylcholanthrene-treatment: A dose of 40 mg/kg, dissolved in olive oil, was given once i.p.; animals were killed 4 days after treatment.

Treatment with Aroclor 1254: A dose of 500 mg/kg, dissolved in olive oil, was given once i.p.; animals were killed 7 and 14 days after treatment.

Assays of UDP-glucuronyltransferase. Enzyme activity towards various substrates was assayed by the following aglycone concentrations by methods already described: 0.5 mM 1-naphthol [24], 0.05 mM 3-hydroxybenzo[*a*]pyrene [25], 0.5 mM *N*-hydroxy-2-naphthylamine [7], 1.5 mM morphine [26], 1.5 mM chloramphenicol [27], 0.5 mM 4-hydroxybiphenyl and 0.05 mM benzo[*a*]pyrene 7,8-dihydrodiol (see below). For purpose of comparison and standardization assays were performed at 37° in the presence of 0.1 M Tris–HCl, pH 7.4, Brij 58 (0.05%, w/v) and 5 mM $MgCl_2$. Reactions were started by addition of 3 mM glucuronic acid. In blanks, UDP-glucuronic acid was omitted. When GT was assayed in homogenates 10 mM saccharic acid-1,4-lactone was also present to inhibit β-glucuronidase. Assays were performed under conditions leading to linear reaction rates with time and protein concentration. Units of enzyme activity (U) represent nmol product formed per min. Glucuronidation of 3-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene 7,8-dihydrodiol and of 4-hydroxybiphenyl was studied fluorometrically using a Farrand spectrophotofluorometer, Mark I. 3-Hydroxybenzo[*a*]pyrene–GT was assayed as described [25], except that emission was measured at 425 nm with excitation at 378 nm. The previously described method (measurement of emission at 450 nm with excitation at 300 nm) leads to the same reaction rates, but is less sensitive.

UDP-glucuronyltransferase activity with 4-hydroxybiphenyl as substrate. The reaction mixture, specified above, was incubated in 0.5 ml. The reaction was stopped by the addition of 1 M trichloroacetic acid (0.5 ml). More than 95 per cent of the excess substrate could be extracted with 1 ml chloroform. An aliquot of the aqueous phase (0.5 ml) was added to 1.0 ml 1.6 M glycine buffer, pH 10.3, and fluorescence of the glucuronide was determined at 325 nm with excitation at 290 nm. Calibration of glucuronide fluorescence was done by comparing the increase of fluorescence with the disappearance of the phenolic substrate measured with the Folin–Ciocalteu reagent.

UDP-glucuronyltransferase activity with benzo[a]pyrene 7,8-dihydrodiol as substrate. The substrate (50 nmol) dissolved in 10 μl acetone was added to the assay mixture (1.0 ml). At various times 0.25 ml aliquots were extracted with 6 ml dichloromethane to remove the substrate. A 0.1 ml portion of the aqueous phase was added to 0.5 ml methanol and 1.0 ml 1.5 mM sodium citrate, pH 7.5, containing 15 mM NaCl. Fluorescence was determined at 410 nm with excitation at 350 nm. Fluorescence of benzo[*a*]pyrene 7,8-dihydrodiol glucuronide was calibrated as follows. Similar incubations were performed in the presence of UDP-(^{14}C)glucuronic acid. The radioactive nucleotide and benzo[*a*]pyrene 7,8-dihydrodiol-(^{14}C)-glucuronide were separated by thin layer chromatography on silica gel (Merck, Darmstadt) with a solvent mixture of ethyl acetate–methanol–water–formic acid (100 : 25 : 20 : 1, v/v). The glucuronide could be readily identified by both its strong fluorescence and radioactivity. The radioactive glucuronide was scraped off the plate into scintillation vials and counted for radioactivity. The assay was calibrated by comparing the amount of benzo[*a*]pyrene 7,8-dihydrodiol-(^{14}C)glucuronide with fluorescence determined in an aliquot of the same incubation mixture.

Protein was determined according to the method of Lowry *et al.* [28] using bovine serum albumin as standard.

RESULTS

As shown in Table 1, GT activity towards 1-naphthol, *N*-hydroxy-2-naphthylamine and 3-hydroxybenzo[*a*]pyrene (GT_1 substrates) was clearly detectable in all tissues examined. Enzyme activity towards 4-hydroxybiphenyl, morphine and chloramphenicol (GT_2 substrates) was found only in liver and intestine to an appreciable extent. Low enzyme activity towards 4-hydroxybiphenyl in tissues other than liver and intestine (1 per cent or less than the specific activity found in liver) may be due to some overlapping substrate specificity of GT_1 towards 4-hydroxybiphenyl. Chloramphenicol-GT was not

Table 1. Distribution of UDP-glucuronyltransferase activities towards various substrates in microsomal fractions from rat organs

Organ	UDP-glucuronyltransferase activity (U/mg protein)*					
	1-Naphthol	*N*-Hydroxy-2-naphthylamine	3-Hydroxy-benzo[*a*]pyrene	4-Hydroxy-biphenyl	Morphine	Chloramphenicol
Liver	71 ± 12	38 ± 6	1.35 ± 0.02	26 ± 4	8 ± 1	1.0 ± 0.5
Kidney	27 ± 8	6 ± 3	0.14 ± 0.04	0.3 ± 0.1	<0.1	<0.2
Intestine	13 ± 5	5 ± 2	0.20 ± 0.07	4 ± 1	1.5 ± 0.5	<0.2
Lung	10 ± 3	5 ± 1	0.22 ± 0.07	0.10 ± 0.05	<0.1	<0.2
Skin	13 ± 4	n.d.†	0.08 ± 0.01	<0.08	n.d.	n.d.
Testes	23 ± 5	6 ± 2	0.16 ± 0.06	0.12 ± 0.03	<0.1	<0.2
Spleen	4 ± 2	n.d.	0.12 ± 0.04	<0.08	<0.1	<0.2

* Values represent the mean ± S.D. of at least 4 microsomal preparations.
† Not determined.

detectable in microsomes from intestinal mucosa, possibly due to the low sensitivity of the assay.

Low enzyme activities in extrahepatic tissues might become detectable after induction. Therefore 1-naphthol-GT and morphine-GT were studied after the administration of Aroclor 1254, a mixture of polychlorinated biphenyls containing approximately 54% chlorine. Aroclor 1254 has been shown to possess inducing properties of both phenobarbital and 3-methylcholanthrene on the various cytochromes P-450 [29, 30]. It also induces hepatic epoxide hydratase [31], whereas no significant changes of epoxide hydratase activity were noted in 13 investigated extrahepatic tissues [31]. It also appears to be an inducer of both GT_1 and GT_2 in liver [32]. Moreover, due to its excellent penetration within the organism and long half life, Aroclor 1254 is well suited for induction studies of GT with extrahepatic tissues [33]. GT activities increased up to 14 days after a single dose of Aroclor 1254 (500 mg/kg, i.p). In microsomes obtained after 14 days, 1-naphthol-GT was induced 3.5-fold in liver (Table 2). It was also markedly enhanced in kidney, lung and spleen. Morphine-GT was induced 3.4-fold in liver. It was not enhanced in the intestinal mucosa (not shown) and remained undetectable in other tissues.

There are obvious difficulties in comparing enzyme activities in microsomal fractions from various organs due to differing contamination of true endoplasmic reticulum elements with other membrane fragments. Therefore enzyme activity towards 1-naphthol was also determined in homogenates from different organs (Table 3). Enzyme activity per g organ allows a much better comparison of enzyme contents in various organs than enzyme activity per mg microsomal protein. When enzyme activity per g tissue in the homogenate is divided by the enzyme activity per mg protein in microsomes (the ratio being operationally used as "microsomal protein" [34]), these ratios are different for various organs. For a given organ this ratio should be similar for different constituents of endoplasmic reticulum membranes. Similar ratios were found for cytochrome P-450 and glucose-6-phosphatase in rat liver [34]. Moreover, for enzymes with similar distribution, the enzyme content per g organ can be readily obtained by multiplying the specific enzyme activity in microsomes with this factor. In all tissue homogenates, GT activity could be activated by the addition of Brij 58, although to variable extents. This also holds true for homogenates from intestinal mucosa. In this tissue it is difficult to prevent spontaneous activation of GT during the isolation of microsomes [22]. Therefore GT activity in microsomes from intestinal mucosa was assayed both in the presence and absence of detergents since in the fully activated state of the enzyme detergents have inhibitory effects [21].

Glucuronidation of 3-hydroxybenzo[*a*]pyrene in liver is chiefly inducible by 3-methylcholanthrene [25]. In contrast, the glucuronidation of benzo[*a*]-pyrene 7,8-dihydrodiol was enhanced by both phenobarbital and 3-methylcholanthrene, and the degree of enhancement was similar with both inducers (Table 4). Reaction rates were much lower for the dihydrodiol than for the phenol, in agreement with the results of Nemoto and Gelboin [35]. Glucuronidation of the phenol and the dihydrodiol was detectable in two non-target and two target tissues of benzo[*a*]pyrene carcinogenicity. However, conjugation rates of the dihydrodiol were disproportionately low with skin microsomes.

DISCUSSION

The substrate specificity of GT from various rat tissues was investigated using typical GT_1 and GT_2 substrates. It was found that GT activity towards GT_1 substrates was clearly detectable in all tissues examined (liver, kidney, small intestinal mucosa, lung, skin, testes and spleen), whereas enzyme activity towards GT_2 substrates was only found in liver and intestine to an appreciable extent. This implies that the functional heterogeneity of our GT_1 and GT_2 forms extends further than a differential response in adult rat liver to induction by phenobarbital and 3-methylcholanthrene. Such a possibility has already been suggested by the correlation of this response with development of GT and its perinatal induction by glucocorticoids [36], e.g. our GT_1 substrates are included with those substrates displaying "late foetal" characteristics and our GT_2 substrates with those displaying "neonatal" characteristics [36]. Bilirubin and steroids (included in the "neonatal" group) are, however, probably not sub-

Table 2. Effects of Aroclor 1254 on UDP-glucuronyltransferase activities in various rat organs

Organ	Treatment *in vivo**	UDP-glucuronyltransferase activity (U/mg protein)†	
		1-Naphthol	Morphine
Liver	−	69 ± 12	7 ± 2
	+	239 ± 37	24 ± 7
Kidney	−	23 ± 6	<0.1
	+	81 ± 8	<0.1
Lung	−	11 ± 3	<0.1
	+	21 ± 5	<0.1
Spleen	−	3 ± 2	<0.1
	+	6 ± 2	<0.1

* Aroclor 1254 (500 mg/kg) was administered once i.p., dissolved in olive oil. Three rats were killed after 14 days and microsomal fractions from various organs were prepared.

† Values represent the mean ± S.D. of three different induction experiments.

Table 3. UDP-glucuronyltransferase activity towards 1-naphthol in homogenates of rat organs

Organ	UDP-glucuronyltransferase activity (U/g tissue)*		Ratio of enzyme activities: U/g tissue
	(native)	(activated)	U/mg microsomal protein†
Liver	111 ± 30	2397 ± 87	34
Kidney	73 ± 15	798 ± 11	30
Intestine	80 ± 10	340 ± 21	26
Lung	30 ± 3	100 ± 17	10
Testes	35 ± 11	414 ± 22	18
Spleen	41 ± 7	142 ± 6	36

* Values represent the mean ± S.D. of 4 homogenate preparations.

† GT activity in microsomes is taken from Table 1.

Table 4. UDP-glucuronyltransferase activity towards 3-hydroxybenzo[*a*]pyrene and benzo[*a*]pyrene 7,8-dihydrodiol in microsomal fractions from various rat organs

Organ	Treatment *in vivo*	UDP-glucuronyltransferase activity (U/mg protein)*	
		3-Hydroxy-benzo[*a*]pyrene	Benzo[*a*]pyrene 7,8-dihydrodiol
Liver	—	1.6 ± 0.1	0.28 ± 0.05
Liver	Phenobarbital	2.7 ± 0.2	0.41 ± 0.02
Liver	3-Methylcholanthrene	9.9 ± 1.6	0.44 ± 0.05
Kidney	—	0.14 ± 0.04	0.028 ± 0.006
Lung	—	0.22 ± 0.07	0.037 ± 0.007
Skin	—	0.08 ± 0.01	0.003 ± 0.001

* Values represent the mean ± S.D. of 4 experiments.

strates for our GT_2 enzyme, since evidence indicates that bilirubin [37–39] and steroids [40–41] may be glucuronidated by other GT forms. Interestingly, some general differences in molecular configuration between GT_1 and GT_2 substrates are noticeable. GT_1 appears to be specific for planar molecules, whereas GT_2 accepts non-planar structures [16, 42]. Using a series of phenols with alkyl substituents of various chain length at different positions of the phenolic nucleus, Wishart and Campbell were able to define limiting configurations within which a substrate displayed "late foetal" characteristics and outside of which it displayed "neonatal" characteristics [42]. In these studies no overlap was found. However there may well exist a number of overlapping substrates. 4-Hydroxybiphenyl is in line with the general configuration of GT_2 substrates, since the phenyl group in the para-position seems to be preferentially in the non-planar conformation. The dihedral angle between the two aromatic rings of 4-hydroxybiphenyl was estimated to be 33–40° [43, 44]. Probably an equilibrium between planar and nonplanar conformations exists. On this basis some overlapping activity with GT_1 may occur. This overlap is much greater using 2-hydroxybiphenyl as substrate for purified GT_1 and GT_2 preparations from rat liver (Lilienblum and Pfeil, unpublished results). Benzo[*a*]pyrene 7,8-dihydrodiol also appears to be an overlapping substrate between GT_1 and other GTs. Its glucuronidation is induced by both 3-methylcholanthrene and phenobarbital to similar degrees (Table 4). The compound is conjugated by purified GT_1 (unpublished). However, conjugation of the dihydrodiol has not yet been studied with purified GT_2. Compared with 3-hydroxybenzo[*a*]pyrene, the dihydrodiol is a poor substrate of GT_1. Although the conformation of the 7,8-dihydrodiol is such that both hydroxyl groups occupy pseudoequatorial positions [45], the portion of the molecule which serves as substrate for GT is not planar.

If some overlap in substrate specificity is taken as a realistic possibility, enzyme activity towards GT_2 substrates seems to be restricted to liver and small intestine in the rat, although, alternatively, the other extrahepatic tissues may possess GT_2 but only very low amounts. Similarly, testosterone–GT, like other "steroid group" substrates, was only found in rat liver [46], although our own results suggest that GT_1 may have some enzyme activity towards testosterone [16]. Functional heterogeneity of GT activity between various tissues may be explained by the existence of multiple enzyme forms which are differently distributed among tissues. Investigations with antibodies to purified rat liver GT_1 are currently under way to further resolve the molecular similarity of GTs in various tissues. Tissue distribution of GTs is probably different in other species. We observed high GT activity towards 4-hydroxybiphenyl and morphine in human kidney, in contrast to rat kidney (unpublished).

GT_1 substrates include metabolites of toxic compounds such as the carcinogens benzo[*a*]pyrene and 2-naphthylamine. Hence GT may have important implications for the tissue specificity of toxic lesions produced by these compounds. A few conclusions

emerge from studies on the glucuronidation of 3-hydroxybenzo[*a*]pyrene and benzo[*a*]pyrene 7,8-dihydrodiol. Due to rapid conjugation in many tissues the phenol is probably not an ultimate carcinogen in the animal, although it is a mutagen in *in vitro* systems [47, 48]. On the other hand, the dihydrodiol as a poor substrate for GT_1 is readily available for epoxidation to an ultimate carcinogen, benzo[*a*]pyrene 7,8-dihydrodiol 9,10-oxide [3–5]. Similarly benzo[*a*]pyrene 4,5-oxide as a good substrate for epoxide hydratase [23] is only a weak carcinogen *in vivo* [49]. Hence inactivating enzymes may be decisive for the selection of ultimate carcinogens.

Acknowledgements—We thank Ms. M. Blume, U. Heine, S. Zachmann and cand. med. S. Grüne for expert technical assistance and the Deutsche Forschungsgemeinschaft for financial support.

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