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N-GLUCURONIDE FORMATION OF CARCINOGENIC AROMATIC AMINES IN RAT AND HUMAN LIVER MICROSOMES

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Abstract—(1) Sensitive fluorimetric assays were developed for the determination of microsomal UDP-glucuronosyltransferase activities towards 1- and 2-naphthylamine and 4-aminobiphenyl. (2) In rat liver microsomes, enzyme activity towards 1-naphthylamine was orders of magnitude higher than the activities towards 2-naphthylamine, 4-aminobiphenyl or aniline. The differences were less marked with human liver microsomes. (3) Glucuronidation of aniline and 4-aminobiphenyl was not appreciably altered in rat liver microsomes from 3-methylcholanthrene- or phenobarbital-treated rats. UDP-glucuronosyltransferase activities towards 1- and 2-naphthylamine were selectively increased (about 2-fold) by 3-methylcholanthrene-treatment. However the increases were less marked than those observed with representative substrates of the 3-methylcholanthrene-inducible enzyme form. The results suggest that the arylamines investigated are predominantly conjugated by constitutive enzyme forms in rat liver. (4) Arylamine *N*-glucuronides were found to be susceptible to hydrolysis by *E. coli* β-glucuronidase suggesting the release of carcinogenic arylamines in the gut and their enterohepatic circulation.

Many aromatic amines are known to induce tumours predominantly in the urinary bladder but also at other sites such as the liver or the intestinal tract [1, 2]. Although many of these compounds are structurally very similar they differ considerably in their carcinogenic potential probably due to their differing metabolic fate. For example, various species including man are known to be susceptible to tumour development of the urinary bladder by 2-naphthylamine but apparently not by 1-naphthylamine [1]. *N*-Hydroxylation has been shown to play a key role in the toxicity of arylamines [1–3]. However a number of inactivating pathways compete with *N*-hydroxylation [4–6]. *N*-Glucuronidation of the arylamines may be a major factor determining their metabolic fate and their disposition [5, 7–13].

Enzymatic formation of *N*-glucuronides by UDP-glucuronosyltransferase (GT)* was studied first using aniline as substrate [14]. Evidence was obtained that aniline is conjugated by a GT enzyme form different from those conjugating phenols or bilirubin [9, 15]. However, in addition to their enzymatic formation arylamine *N*-glucuronides are also generated spontaneously from the arylamine and D-glucuronate [5, 16–18]. On the other hand arylamine *N*-glucuronides are easily hydrolysed in weakly acidic media [16, 18]. Therefore it is not possible to obtain information about the extent of enzymatic *N*-glucuronidation *in vivo* from the amount of arylamine *N*-glucuronide excreted in urine [19, 20].

It is not known which enzyme forms of GT are responsible for *N*-glucuronidation. Recently it was possible to physically separate multiple enzyme forms of GT with differing but overlapping substrate specificities [21–24]. It was also possible to classify GT activities in various models such as perinatal development [25, 26] or differential induction of rat liver microsomal GT activities [27–29]. GT activities which are chiefly inducible by 3-methylcholanthrene or phenobarbital have been termed group 1 and group 2 activities, respectively [30]. The similarity of 3-methylcholanthrene-inducible GT activities with GT activities of the late-foetal cluster together with results of enzyme purification suggest that the conjugation of these substrates is catalysed by a distinct enzyme form [21, 25, 27–29]. However a third group was also recognized in this model which is not markedly inducible (induction factor <2-fold) by the above inducing agents [29–31]. These group 3 activities may be catalysed by constitutive enzyme forms. In the present study a sensitive and convenient fluorimetric assay is described to investigate glucuronidation of carcinogenic aromatic amines. Classification of these activities in the model of differential induction suggests that the conjugation of aniline and 4-aminobiphenyl is catalysed by constitutive enzyme forms of GT.

MATERIALS AND METHODS

Chemicals. 2-Naphthylamine (m.p. 111°) was purified by sublimation. 4-Aminobiphenyl (m.p. 53°) was recrystallized from aqueous ethanol. 1-Naphthylamine (purity > 99%) was from Serva, Heidelberg, F.R.G. *E. coli* β-glucuronidase type VII was obtained from Sigma, München, F.R.G. β-Glucuronidase from rat preputial glands was purified as described [32]. Chemical synthesis of arylamine *N*-

* Abbreviations: GT, UDP-glucuronosyltransferase (E.C. 2.4.1.17); HPLC, high-performance liquid chromatography.

glucuronides was performed as described [5] with the following minor modification to obtain homogeneous reaction mixtures: The arylamine (10 mM) and D-glucuronic acid, sodium salt (20 mM), were dissolved in a mixture of ethanol and 0.1 M sodium phosphate buffer, pH 7.1 (1:1, v/v). The solutions were kept at 0–4° in the dark overnight. Isolation of *N*-glucuronides was achieved by HPLC.

Isolation of N-*glucuronides by HPLC.* Mixtures containing arylamine *N*-glucuronides were injected into a Waters ALC 202 HPLC system fitted with a 300 × 4 mm μ-Bondapak-C_{18} reversed phase column. Chromatography was followed by u.v. detection at 254 nm. The flow rate was 1 ml/min. Eluents were prepared by addition of methanol to 50 mM sodium phosphate buffer containing 5 mM tetrabutylammonium, pH 7.0. Methanol/buffer ratios of 35:65 and 45:55 (v/v) were used to isolate naphthylamine glucuronides and 4-aminobiphenyl glucuronide, respectively. Retention volumes of *N*-glucuronides of the naphthylamines and of 4-aminobiphenyl were 9 and 7 ml, respectively. Uridine nucleotides and the arylamines themselves were eluted at 3–4 and 20–22 ml, respectively.

N-Glucuronides were identified by their fluorescence spectra (Fig. 1) and by their products after complete hydrolysis by 0.5 N HCl. The arylamines liberated were fluorimetrically determined in 1 M Tris–HCl, pH 7.4, and could be distinguished from the *N*-glucuronides by their different emission spectra (not shown). D-Glucuronic acid in hydrolysed samples was determined by the naphthoresorcinol method [33].

Treatment of animals and preparation of microsomes. Male Wistar rats (180–240 g) were used. 3-Methylcholanthrene (40 mg/kg) dissolved in olive oil

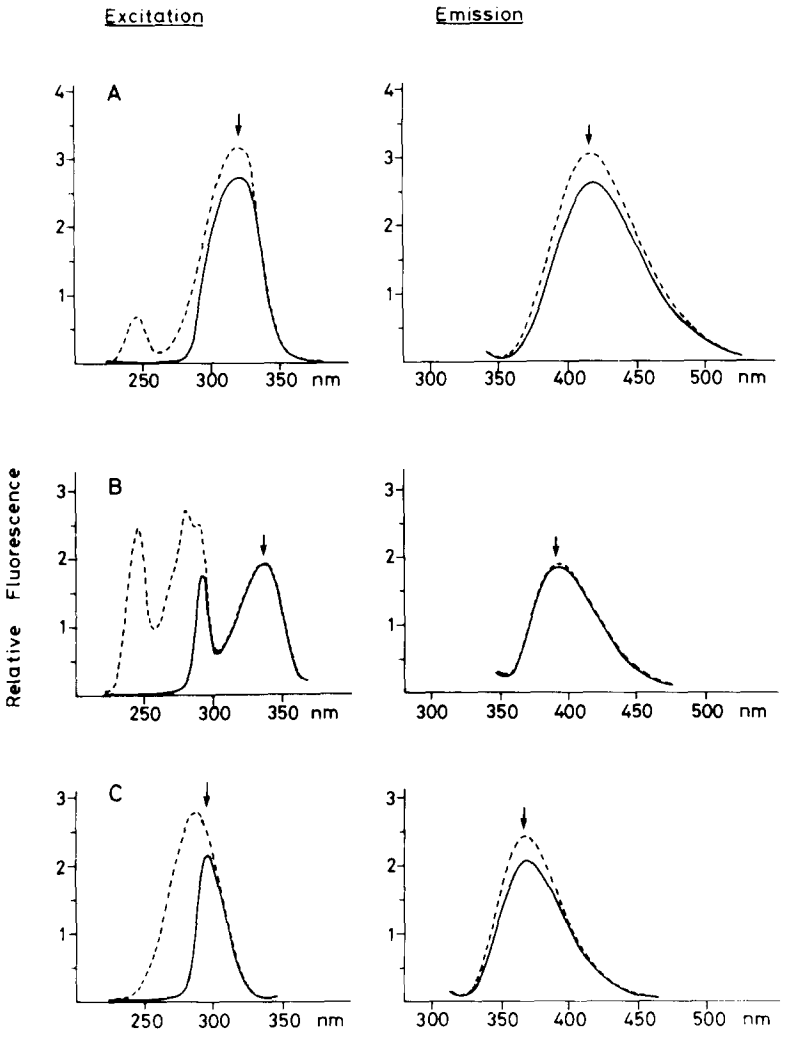

Fig. 1. Fluorescence spectra of the *N*-glucuronides of 1-naphthylamine (A), 2-naphthylamine (B) and 4-aminobiphenyl (C). *N*-Glucuronides obtained by enzymatic synthesis and isolated by HPLC were added to chloroform-extracted incubation mixtures as described in Methods. Fluorescence spectra were recorded in presence (solid lines) or absence (dashed lines) of 3 mM UDP-glucuronic acid. Arrows indicate the wavelengths of excitation and emission selected for the enzyme assays, and for the recording of the spectra. The *N*-glucuronides of 1-naphthylamine, 2-naphthylamine and 4-aminobiphenyl were present at a concentration of 4, 7 and 1 μM, respectively.

was given once i.p. and the animals were killed after 4 days. Phenobarbital, sodium salt, (100 mg/kg) dissolved in saline was given once i.p. followed by 0.1% (w/v) of the drug in the drinking water for 4 days. Microsomes were prepared as described [27]. Samples from human livers were kindly provided from a 'liver bank' and have been characterized elswhere [34–36]. Microsomal protein was determined according to Lowry *et al.* [37].

Enzyme assays. For the determination of GT activities assay mixtures contained 0.1 M Tris–HCl (pH 7.4), 5 mM $MgCl_2$, microsomal protein and 0.5 mM substrate in a total volume of 1.0 ml. Reactions were started after 2 min preincubation by addition of UDP-glucuronic acid (final concentration 3 mM). With freshly prepared microsomes GT activity was studied in the latent state or after partial activation by addition of 3 mM UDP-*N*-acetylglucosamine which is possibly a physiological activator of the enzyme. The enzyme was fully activated by addition of the non-ionic detergent Brij 58 (0.5 mg/mg protein). With 1-naphthylamine, 2-naphthylamine or 4-aminobiphenyl, glucuronidation rates were maximal at 0.5 mM substrate concentration and were linear for up to 30 min or up to 1.0 mg microsomal protein/ml provided that the consumption of substrate was less than 20%.

Reactions were stopped by the addition of 2 ml chloroform. Excess substrate was extracted twice with 2 ml chloroform. Fluorescence of *N*-glucuronides was determined in an aliquot of the aqueous phase (0.5 ml) diluted with the same volume of 0.1 M Tris–HCl, pH 7.4, at the following wavelengths of excitation and emission: for 1-naphthylamine, 318 and 415 nm; for 2-naphthylamine, 335 and 390 nm; for 4-aminobiphenyl, 295 and 363 nm, respectively. Glucuronide fluorescence in the assay mixture was calibrated by the fluorimetric determination of the amount of the arylamine which was liberated by complete hydrolysis of the glucuronide in presence of 0.5 N HCl. In control experiments the *N*-glucuronides isolated by HPLC were used for the calibration of glucuronide fluorescence. As shown in Fig. 1, fluorescence emission of the *N*-glucuronides is moderately quenched by the addition of 3 mM UDP-glucuronid acid at the excitation wavelengths chosen for the enzyme assay. However, fluorescence is markedly quenched at excitation wavelengths below 280 nm. Therefore, fluorescence of the *N*-glucuronides of 1-naphthylamine and 4-aminobiphenyl was calibrated in the presence of 3 mM UDP-glucuronic acid. Fluorescence was quenched to the same extent when UDP-glucuronic acid was replaced by 3 mM UDP or UMP. Accordingly changes in the composition of uridine nucleotides occurring during the incubation did not alter the fluorescence of the *N*-glucuronides. In blanks, UDP-glucuronic acid was added after chloroform extraction.

The sensitivity of the method allows the quantitation of 100–200 pmoles *N*-glucuronide per assay.

N-Glucuronide of aniline was determined as described [14, 38] with the following modifications: After azo dye formation from aniline the reaction mixture was acidified with HCl (1%, w/v) and the colour was read at 550 nm. The extinction coefficient based on aniline in the reaction mixture was 43 $mM^{-1}\,cm^{-1}$.

GT activity towards 1-naphthol was determined as described [39]. GT activity towards 2-naphthol was fluorimetrically determined as described for 4-methylumbelliferone [29]. The fluorescence of 2-naphthol glucuronide was determined at wavelengths of 322 and 345 nm for excitation and emission, respectively.

Hydrolysis of N-*glucuronides by* β-*glucuronidase.* Incubation of the arylamine *N*-glucuronides with β-glucuronidase was carried out at 37° in a final volume of 1 ml containing 0.1 M phosphate buffer, pH 7.0, and 500 'Sigma units' of *E. coli* β-glucuronidase or 10,000 Fishman units of β-glucuronidase from rat preputial glands. Glucuronide hydrolysis was followed by measuring the decrease of fluorescence of the *N*-glucuronide as described above.

RESULTS

Formation of N-*glucuronides in rat and human liver microsomes.* Fluorescence assays to determine the formation of arylamine *N*-glucuronides have not been described. Therefore the identity of the *N*-glucuronides was ascertained as follows:

(1) *N*-Glucuronides synthesized enzymatically were isolated by HPLC. When hydrolysed by HCl the corresponding arylamine and D-glucuronic acid were liberated at a molar ratio of 1:1.

(2) The *N*-glucuronides could also be hydrolysed by bacterial β-glucuronidase (see below).

(3) In the absence of UDP-glucuronic acid or in

Table 1. Properties of arylamine *N*-glucuronide formation in rat liver microsomes

Assay conditions	Glucuronide formation (nmole/min)*			
	1-Naphthylamine	2-Naphthylamine	4-Aminobiphenyl	Aniline
Complete (native microsomes)	2.4	0.25	0.07	0.04
Complete + UDP-*N*-acetylglucosamine	8.6	0.75	0.22	0.10
Complete + Brij 58	53	5.0	2.2	0.45
Complete, UDP-glucuronic acid replaced by D-glucuronic acid (3 mM)	0.002	0.004	0.005	<0.01

* Incubation mixtures were incubated up to 30 min and contained 1 mg microsomal protein. Data are the means of 2 or 3 experiments.

Table 2. Glucuronidation of aromatic amines in human liver microsomes

Sample	UDP-glucuronosyltransferase activity (nmole/min/mg protein)* 1-Naphthylamine	2-Naphthylamine	4-Aminobiphenyl
HL 17	11.9	2.3	3.9
HL 19	8.8	3.6	3.5
HL 20	17.0	6.5	5.8

* The Brij 58-activated enzyme activity is listed.
Data represent the means of 2 determinations differing by less than 10%.

the presence of boiled microsomes no increase of fluorescence was observed in the aqueous phase after chloroform extraction.

Similar to other glucuronidation reactions the formation of *N*-glucuronides could be activated by UDP-*N*-acetylglucosamine or by detergent (Table 1). Nonenzymatic formation of *N*-glucuronides from arylamines and D-glucuronate was orders of magnitude lower than enzymatic formation. Rat liver GT activities towards the arylamines investigated differed up to 120-fold. For example 1-naphthylamine was conjugated about 10 times faster than 2-naphthylamine. These differences were also observed at a lower (50 μM) substrate concentration (not shown).

Arylamine *N*-glucuronidation was also studied in human liver microsomes (Table 2). As in rat liver, 1-naphthylamine was conjugated faster than 2-naphthylamine but the difference was less marked than in rat liver. As expected large inter-individual differences in arylamine glucuronidation were observed in liver tissue from the 3 patients investigated.

Influence of inducing agents on arylamine glucuronidation in rat liver microsomes. GT activities towards the arylamines were classified using the model of differential induction of GT activities [21, 27–31]. Glucuronidation of aniline and 4-aminobiphenyl was not appreciably altered in rat liver microsomes from 3-methylcholanthrene- or phenobarbital-treated rats (Table 3). GT activities towards 1- and 2-naphthylamine were not markedly affected by phenobarbital-treatment (<1.5-fold) but were moderately increased (about 2-fold) by 3-methylcholanthrene-treatment. However, the increases were less marked than those observed using representative substrates of the 3-methylcholanthrene-inducible enzyme form such as 1-naphthol and 2-naphthol. With the phenolic substrates induction factors of about 4-fold were obtained using the same microsomal suspensions (Table 3).

Hydrolysis of N*-glucuronides.* The instability of arylamine *N*-glucuronides below pH 6 is an important factor determining their toxicokinetics. When incubated with β-glucuronidase from *E. coli* the *N*-glucuronides were rapidly hydrolysed. In contrast, they were not hydrolysed by β-glucuronidase from rat preputial glands (Fig. 2). The small loss of *N*-glucuronide in presence of saccharo-1,4-lactone is probably due to nonenzymatic hydrolysis because a similar loss was found in the absence of β-glucuronidase (not shown). Direct synthesis of *N*-glucuronide from the arylamine and D-glucuronic acid may lead to 2 diastereomeric isomers, i.e. α- and β-glucuronides [5, 18]. However, hydrolysis of chemically and enzymatically formed *N*-glucuronides occurred at very similar rates using *E. coli* β-glucuronidase. In addition chemically synthesized arylamine *N*-glucuronides were eluted as one peak from the HPLC column with a retention time and fluorescence spectrum identical to the enzymatically formed glucuronides. The data suggest that under the above conditions the chemical synthesis leads predominantly to the formation of β-glucuronides.

DISCUSSION

A sensitive fluorimetric method is described to determine arylamine *N*-glucuronide formation in rat and human liver tissue. GT activities towards various arylamines were classified in the model of differential

Table 3. Induction of UDP-glucuronosyltransferase activities towards various arylamines, 1-naphthol and 2-naphthol in rat liver microsomes

Substrate	UDP-glucuronosyltransferase activity (nmole/min/mg protein)* Untreated controls	Phenobarbital treatment		3-Methylcholanthrene treatment	
1-Naphthylamine	60 ± 10	77 ± 5	(1.3)	112 ± 15	(1.9)
2-Naphthylamine	5.8 ± 0.4	7.1 ± 0.5	(1.2)	13 ± 2	(2.2)
4-Aminobiphenyl	2.8 ± 0.3	3.6 ± 0.3	(1.3)	2.5 ± 0.3	(0.9)
Aniline	0.45 ± 0.05	0.51 ± 0.03	(1.1)	0.60 ± 0.04	(1.3)
1-Naphthol	67 ± 6	83 ± 6	(1.2)	252 ± 31	(3.8)
2-Naphthol	64 ± 7	85 ± 8	(1.3)	260 ± 40	(4.1)

* The Brij 58-activated enzyme activity is listed. Data are the means ± S.D. of 4 experiments. The induction factor, i.e. the ratio of enzyme activities obtained from treated versus untreated animals, is given in parenthesis.

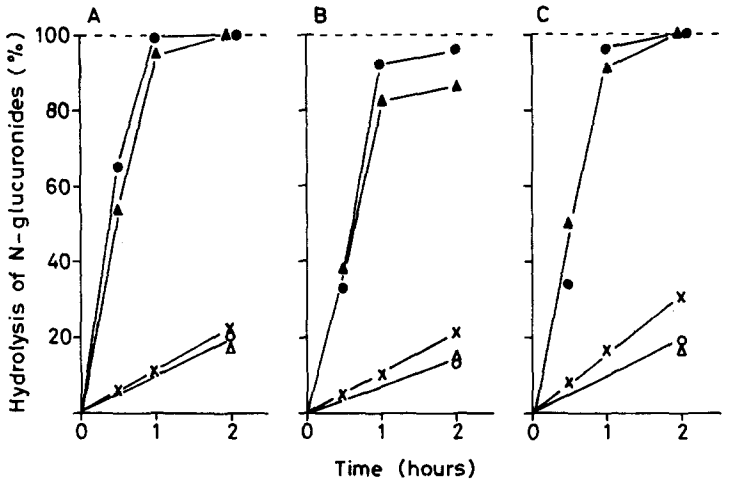

Fig. 2. Hydrolysis of the *N*-glucuronides of 1-naphthylamine (A), 2-naphthylamine (B) and 4-aminobiphenyl (C) by β-glucuronidases. *N*-Glucuronides obtained enzymatically (circles) or non-enzymatically (triangles) were incubated with *E. coli* β-glucuronidase in the absence (closed symbols) or presence (open symbols) of 10 mM saccharo-1,4-lactone. Data indicated by crosses represent experiments with β-glucuronidase from rat preputial glands.

induction [21, 27]. Since glucuronidation of aniline and 4-aminobiphenyl was not appreciably altered by treatment with phenobarbital or 3-methylcholanthrene the two arylamines may be classified as group 3 substrates. GT activities towards 1- and 2-naphthylamine were clearly increased by 3-methylcholanthrene-treatment. However the increases were less marked than those observed with representative substrates of the 3-methylcholanthrene-inducible enzyme form. The purified 3-methylcholanthrene-inducible enzyme form was able to conjugate 1-naphthylamine (not shown). These data taken together suggest that 1- and 2-naphthylamine are overlapping substrates of the 3-methylcholanthrene-inducible enzyme form and of constitutive enzyme forms.

N-Glucuronidation *in vivo* may be limited by competing reactions such as *N*-hydroxylation, *N*-acetylation and sulfation. However *N*-glucuronidation of at least 1-naphthylamine may successfully compete with these reactions thus contributing to the inactivation of 1-naphthylamine in liver. Arylamine *N*-glucuronides are excreted to a considerable extent into the intestine via the bile [10–12, 40]. Since they are susceptible to hydrolysis by bacterial β-glucuronidase the arylamines are released into the gut lumen. The arylamines may then initiate carcinogenesis after metabolic activation by the intestinal mucosa or may undergo enterohepatic circulation [11]. The intestinal tract is known as a target for arylamine tumourigenicity after parenteral application [41, 42].

The sensitive and convenient methods to determine the formation of arylamine *N*-glucuronides described in this paper may greatly facilitate the isolation of those enzyme forms catalyzing *N*-glucuronidation. Moreover they may be useful in studies on the significance of arylamine *N*-glucuronidation in intact cell systems.

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