Demonstration of Two Functionally Heterogeneous Groups within the Activities of UDP-Glucuronosyltransferase towards a Series of 4-Alkyl-Substituted Phenols

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1. A simple colorimetric assay for UDP-glucuronosyltransferase activities towards phenolic substrates, using Folin & Ciocalteu's phenol reagent, is described. The assay is used to measure rat liver transferase activities towards substrates from a series of 4-alkyl-substituted phenols. 2. Activities towards phenol, 4-methylphenol and 4-ethylphenol develop near-adult values before birth, are precociously stimulated by dexamethasone *in utero* and are stimulated 3–4-fold by 3-methylcholanthrene in adult liver. These are assigned to a 'late-foetal' group of transferase activities. 3. Activities towards 4-n-propylphenol, 4-s-butylphenol and 4-t-butylphenol are negligible in late-foetal liver, developing to near-adult values in the first 4 postnatal days, and are not affected by dexamethasone or 3-methylcholanthrene. They are assigned to a 'neonatal' group of transferase activities. 4. Although 4-ethylphenol and 4-n-propylphenol differ only by a single $-\text{CH}_2-$ moiety, this is sufficient to change the acceptability of these substrates respectively from the late-foetal to the neonatal group of transferase activities. The change is distinct, with no overlapping of substrate acceptability between the two groups of transferase activities. 5. From consideration of the above and other substrates, the two groups of transferase activities do not distinguish substrates on the basis of their molecular weights or lipophilicity. The distinguishing feature appears to be the specific molecular configurations of the substrates.

We have recently demonstrated that hepatic activities of the microsomal conjugating enzyme UDP-glucuronosyltransferase (EC 2.4.1.17) exist in the rat as two functionally heterogeneous groups (Wishart, 1978a). Activities of the 'late-foetal' group (toward 2-aminophenol, 2-aminobenzoate, 4-nitrophenol, 1-naphthol, 4-methylumbelliferone and 5-hydroxytryptamine) develop adult or near-adult values prenatally, whereas those of the 'neonatal' group (towards bilirubin, testosterone, oestradiol, phenolphthalein, morphine and chloramphenicol) develop from low to near-adult values during the first 2 postnatal days. The late-foetal group of activities are precociously stimulated by glucocorticoids in foetal liver both in culture and *in utero*, whereas the neonatal activities are not affected (Wishart, 1978a). Furthermore, in adult rats, phenobarbital treatment stimulates neonatal but not late-foetal activities, whereas treatment with 3-methylcholanthrene stimulates late-foetal but not neonatal activities (Wishart, 1978b).

Because the 12 substrates used to identify the two groups of transferase activities were of diverse molecular structures, having been chosen because of an established assay procedure, it was difficult to recognize common characteristics between substrates in a

particular group. Nevertheless, molecular models indicated that neonatal-group substrates were larger and more bulky than the small, planar late-foetal-group substrates. To pursue this correlation further, we have investigated transferase activities towards a series of 4-alkyl-substituted phenols, ranging from the simple planar molecule of phenol to the more bulky 4-t-butylphenol.

For the present investigation we developed a simple colorimetric assay for these phenolic substrates, and found that transferase activities towards the lower-molecular-weight compounds show typical late-foetal-group characteristics and those towards higher-molecular-weight compounds show neonatal-group characteristics. The change in group specificity occurring between 4-ethyl- and 4-n-propylphenol, is distinct, with no overlap. Characteristics by which substrates from each of the two groups may be recognized are discussed. A preliminary note of parts of this work has appeared (Wishart *et al.*, 1978).

Materials and Methods

Rats were Wistar strain, time-mated and (where appropriate) injected with dexamethasone (9-fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-

3,20-dione) (Wishart & Dutton, 1977) or 3-methylcholanthrene (Wishart, 1978b).

Chemicals were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., or BDH Chemicals, Poole, Dorset, U.K. Phenolic substrates were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Glycine/HCl buffer was prepared by adjusting 0.4M-HCl with solid glycine to pH 2.7.

As a source of β -glucuronidase, male or female rat preputial glands were homogenized in 4 vol. of 0.1M-sodium acetate buffer, pH 4.5, and centrifuged at 8000g for 5 min. The supernatant was stored frozen until use.

Phenol-UDP-glucuronosyltransferase assay

Enzyme activity was measured colorimetrically by the disappearance of the unconjugated phenolic substrate, after incubation in the presence of UDP-glucuronic acid. The phenolic group was detected by a modification of the method of Illing & Benford (1976), in which the unconjugated substrate is extracted into an organic solvent, re-extracted into alkali and measured with Folin & Ciocalteu's (1927) reagent. The incubation medium contained, in a final volume of 250 μ l: 75mM-Tris, pH 7.4; 75mM maleic acid; 10mM-MgCl₂; 4mM-UDP-glucuronic acid; 1mM-phenolic substrate, added in 12.5 μ l of ethanol; 50 μ l of a 5–20% (w/v) rat liver homogenate (prepared in 4 vol. of 0.15M-KCl, pH 7.4, and diluted as required); and 0.05–0.2% digitonin, the concentration of detergent varying with homogenate concentration. These conditions allowed maximum activation of the transferase *in vitro*, and enzyme activities quoted in the present work are those of the fully activated rather than the latent enzyme, for reasons discussed previously (Wishart *et al.*, 1977).

After incubation with shaking for 5–20 min at 37.5°C, the reaction was stopped by the addition of 750 μ l of ice-cold glycine/HCl buffer. Control assays had UDP-glucuronic acid added after the glycine/HCl buffer. Activity towards phenol itself was assayed with a 20% homogenate for 20 min. Other phenols were commonly assayed with a 5–10% homogenate for 5–10 min. Enzyme activity developed linearly during incubation for up to 40% disappearance of substrate and was directly variable with homogenate concentration.

After termination of the reaction, samples were shaken by hand with 2 ml of chloroform to extract the unconjugated phenol. After centrifugation at 2000g for 2 min, 1 ml of the organic layer was removed and the phenol re-extracted into 1 ml of 2% (w/v) Na₂CO₃ in 0.1M-NaOH. To 0.75 ml of the alkaline phase was added 75 μ l of Folin & Ciocalteu's (1927) phenol reagent. The colour was developed over 45 min at room temperature and read at 750 nm.

Assay duplicates were, for 50 samples, within

$\pm 6\%$. The absorption coefficients for all substrates used were within the range 70×10^3 – 90×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. Recovery of each authentic phenol when added to the system was 80–100%.

Product-identification procedure

Identification of the product as a glucuronide was performed by taking 1 ml of the post-incubation chloroform-extracted aqueous fraction and extracting this with 1 ml of pentan-2-one. A 0.75 ml portion of the organic phase was removed, evaporated in a stream of air and the residue was taken up in 0.4 ml of 0.1M-acetate buffer, pH 4.5, and incubated at 37°C for 2 h with 0.1 ml of β -glucuronidase preparation. Control incubations contained 10mM-saccharo-1,4-lactone. The reaction was stopped with 0.5 ml of glycine/HCl buffer and the released phenol extracted as for the transferase assay.

Chromatographic separation of phenols was performed by using a standard chromatography tank, cellulose layers (MN 300 grade; Macherey, Nagel and Co., Düren, W. Germany) impregnated with 2.0M-formamide in the slurring solvent, and hexane as the mobile phase (Graham & Daly, 1970).

Determination of partition coefficients

These were determined as described by Illing & Benford (1976) by partitioning substrates between buffer-saturated octanol and octanol-saturated buffer. The buffer was 66mM-Tris/HCl, pH 7.4. Partition coefficients were determined at three concentrations of each test substrate, the amounts of each substrate in the aqueous phase being assayed by the methods described for measuring UDP-glucuronosyltransferase activity (the present paper and Wishart, 1978a).

Results

Identification of reaction products

The UDP-glucuronic acid-dependent disappearance of phenolic substrates was attributed to the formation of β -D-glucuronides by release of phenols on incubation of the aqueous fraction of the transferase assay with crude β -glucuronidase from preputial-gland extracts (see the Materials and Methods section). This release of phenol accounted for the UDP-glucuronic acid-dependent disappearance of the phenols to the following extents: 4-methylphenol, 83%; 4-ethylphenol, 97%; 4-n-propylphenol, 100%; 4-s-butylphenol, 100%; and 4-t-butylphenol, 71%; and was inhibited with 10mM-saccharo-1,4-lactone by, respectively, 100, 76, 61, 77 and 100%. The transferase reaction products were thus identified as β -D-glucuronides.

The phenols released by β -glucuronidase hydrolysis were compared with the original transferase substrates by subjecting them to the chromatographic

procedures described in the Materials and Methods section. Glucuronidation and subsequent deglucuronidation of 4-methylphenol, 4-ethylphenol, 4-n-propylphenol and 4-s-butylphenol yielded phenols with R_F values of respectively 0.10, 0.18, 0.35 and 0.48. These compared favourably with the R_F values of the standard compounds, i.e. 0.12, 0.19, 0.34 and 0.49. The aglycone moieties of the glucuronides formed were thus identifiable as the phenolic substrates originally added to the transferase assay.

Development of transferase activities towards 4-alkyl-substituted phenols

We have previously shown that, at 20 days of gestation, rat liver transferase activities of the late-foetal group are at adult or near-adult values, whereas those of the neonatal group are negligible. During the first postnatal week, both groups of activities are present at near-adult values (Wishart, 1978a).

We have determined the developmental groupings of transferase activities towards the homologous series of 4-alkyl-substituted phenols by assaying these activities in 20-day-foetal liver, 4-day-neonatal liver and adult male liver (see Table 1). Activities towards

phenol, 4-methylphenol and 4-ethylphenol all developed to near-adult activities by day 20 of foetal life, whereas activities towards 4-n-propylphenol, 4-s-butylphenol and 4-t-butylphenol were very low at this time, developing to near-adult activities by the 20th postnatal day.

This apparent near-absence of transferase activity towards 4-n-propylphenol and 4-s-butylphenol on day 20 of foetal life was not attributable to a markedly lower affinity for these substrates. Assayed over the range 0.1–2.0 mM of substrate concentrations, apparent K_m values for 4-methylphenol and 4-ethylphenol in foetal preparations (0.23 and 0.60 mM respectively) were similar to those obtained with adult preparations (0.63 and 0.50 mM respectively). For 4-n-propylphenol and 4-s-butylphenol, apparent K_m values for adult liver (0.33 and 0.29 mM respectively) were of a similar magnitude to the above. In foetal preparations these activities were not detectable at any substrate concentration used. Transferase activities towards phenol, 4-methylphenol and 4-ethylphenol are therefore assigned to the 'late-foetal' group (Wishart, 1978a), whereas activities towards 4-n-propylphenol, 4-s-butylphenol and 4-t-butylphenol are assigned to the 'neonatal' group.

Table 1. *Perinatal development of UDP-glucuronosyltransferase activities towards 4-alkyl-substituted phenols in rat liver*
Foetal samples were pooled foetal livers from different dams; neonatal samples were pooled livers from two or three animals, and adult samples were from separate animals. Each result is the mean $\pm$ S.E.M. for the indicated number of samples. Abbreviation: ND, not determined.

Substrate	UDP-glucuronosyltransferase activity (nmol of phenol conjugated/h per mg of protein)			Percentage of adult-male activity in 20-day foetal liver
	20-day foetal liver	4-day neonatal liver	Adult male liver	
Phenol	96, 75	130, 125	85, 102	91
4-Methylphenol	443 $\pm$ 84 (3)	529 $\pm$ 15 (3)	371 $\pm$ 59 (6)	116
4-Ethylphenol	362 $\pm$ 33 (3)	630 $\pm$ 83 (3)	447 $\pm$ 23 (6)	81
4-n-Propylphenol	29 $\pm$ 18 (3)	549 $\pm$ 35 (3)	399 $\pm$ 59 (6)	7
4-s-Butylphenol	30 $\pm$ 16 (3)	537 $\pm$ 45 (3)	394 $\pm$ 33 (6)	8
4-t-Butylphenol	0, 37	ND	400, 465	4

Table 2. *Effect of dexamethasone on UDP-glucuronosyltransferase activity towards 4-alkylated phenols in 17-day rat foetal liver in utero*

Animals were time-mated. Dams were injected intraperitoneally with 300 μ g of dexamethasone in 0.2 ml of arachis oil on days 14, 15 and 16 of gestation. Control animals were injected with arachis oil only. Each result represents the mean $\pm$ S.E.M. of three samples. Each sample comprises pooled foetal livers from a separate litter.

Substrate	UDP-glucuronosyltransferase activity (nmol of phenol conjugated/h per mg of protein)		Percentage of adult-male activity in dexamethasone-treated foetal livers
	Control	+Dexamethasone	
4-Methylphenol	0	276 $\pm$ 10	74
4-Ethylphenol	47 $\pm$ 5	217 $\pm$ 30	49
4-n-Propylphenol	39 $\pm$ 12	14 $\pm$ 8	4
4-s-Butylphenol	13 $\pm$ 7	22 $\pm$ 12	6

Table 3. *Effect of 3-methylcholanthrene on UDP-glucuronosyltransferase activity towards 4-alkylated phenols in adult male rat liver*

Adult male rats (350–400 g) were injected intraperitoneally with 15 mg of 3-methylcholanthrene in 0.5 ml of arachis oil. Control animals were killed 4 days later. Each sample represents activity from one rat liver. Results are means $\pm$ S.E.M. for three samples.

Substrate	UDP-glucuronosyltransferase activity (nmol of phenol conjugated/h per mg of protein)		Stimulation (%)
	Control	+3-Methylcholanthrene	
4-Methylphenol	360 $\pm$ 51	1705 $\pm$ 251	374
4-Ethylphenol	434 $\pm$ 83	1984 $\pm$ 123	357
4-n-Propylphenol	429 $\pm$ 77	431 $\pm$ 55	1
4-s-Butylphenol	494 $\pm$ 37	423 $\pm$ 104	0

Table 4. *Octanol/buffer partition coefficients and molecular weights of some substrates of UDP-glucuronosyltransferase*

Substrates were partitioned between octanol-saturated 66 mM-Tris/HCl buffer, pH 7.4, and buffer-saturated octanol. The amount of each substrate in the buffered phase was assayed as in the Materials and Methods section.

Substrate	Mol. wt.	Partition coefficient octanol/buffer
Late-foetal group		
4-Methylphenol	108	100
4-Ethylphenol	122	250
4-Methylumbelliferone	176	80
1-Naphthol	144	900
Neonatal group		
4-n-Propylphenol	136	750
4-s-Butylphenol	150	1200
Morphine	285	0.4
Chloramphenicol	323	10.0

Effect of dexamethasone on transferase activities towards 4-alkyl-substituted phenols in utero

As previously shown (Wishart, 1978a), development of the late-foetal group of transferase activities occurs between days 17 and 20 of gestation under the influence of glucocorticoids. This development can be precociously induced in foetal liver *in utero* after maternal injection of dexamethasone, whereas neonatal-group activities are unaffected by this treatment (Wishart & Dutton, 1977; Wishart, 1978a).

To confirm the developmental groupings of transferase activities towards the series of 4-alkylated phenols we therefore examined the effect of dexamethasone on these activities *in utero* (see Table 2). Dexamethasone precociously stimulated activities towards 4-methylphenol and 4-ethylphenol (late-foetal group), whereas activities towards 4-n-propylphenol and 4-s-butylphenol (neonatal group) remained unchanged. Activities towards the former two phenolic substrates would therefore appear to develop under the influence of glucocorticoids.

Effect of 3-methylcholanthrene on transferase activities towards 4-alkylated phenols in adult rat liver

A further distinguishing characteristic between the late-foetal and neonatal groups of transferase activities is the selective stimulation of the former group in adult rats after treatment with 3-methylcholanthrene (Wishart, 1978b). We have therefore investigated this effect on transferase activities towards the 4-alkylated phenols (see Table 3).

We found that activities towards 4-methylphenol and 4-ethylphenol (late-foetal group) are stimulated 4.7- and 4.6-fold respectively, whereas activities towards 4-n-propylphenol and 4-s-butylphenol remain unchanged. This provides evidence of a non-developmental nature for the suggested groupings of the transferase activities towards these substrates (see Tables 1 and 2).

Molecular weight and octanol/water partitions of some transferase substrates

It appeared possible that the features that determine whether a substrate is conjugated by the late-foetal or neonatal group of transferase activities might be its lipophilicity and/or molecular weight.

Such a correlation was found for the 4-alkylated phenols (see Table 4): 4-n-propylphenol and 4-s-butylphenol (neonatal-group substrates) have higher molecular weights and octanol/buffer partition coefficients than have 4-methylphenol and 4-ethylphenol (late-foetal group). However, this relationship was not apparent when other substrates were taken into consideration (see Table 4).

Discussion

The above results demonstrate that as substrates for UDP-glucuronosyltransferase, phenol, 4-methylphenol and 4-ethylphenol are conjugated by a 'late-foetal' activity of the enzyme, whereas 4-n-propylphenol, 4-s-butylphenol and 4-t-butylphenol are conjugated by a 'neonatal' group of activities. The two groups with transferase activities towards these sub-

strates are distinguishable by their differential onset of development and their differential response to stimulation by dexamethasone *in utero* and by 3-methylcholanthrene in adult rat liver. By use of these phenols we have shown that, simply by the addition of $-CH_2-$, a substrate may change from being acceptable to the late-foetal group of transferase activities to become a neonatal-group substrate. This change occurs between 4-ethylphenol and 4-n-propylphenol, and is absolute; 4-ethylphenol shows no neonatal-group substrate characteristics and 4-n-propylphenol shows no late-foetal-group substrate characteristics.

The two groups of transferase activities described in our present and previous (Wishart, 1978a,b) work therefore appear to have distinct and separate substrate specificities.

On what basis might these two groups of transferase activities distinguish their respective substrates?

From the series of phenols described above, the neonatal-group substrates appear to have a higher molecular weight and greater lipophilicity than late-foetal-group substrates. However, when these parameters in other late-foetal- and neonatal-group substrates are considered this relationship breaks down (Table 4). We may conclude that the two groups of

transferase activities distinguish substrates on the basis of specific molecular configuration, as demonstrated by the specific distinction between 4-ethylphenol and 4-n-propylphenol.

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