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STEREOCHEMICAL HETEROGENEITY OF HEPATIC UDP-GLUCURONOSYLTRANSFERASE ACTIVITY IN RAT LIVER MICROSOMES

IRÉNA OKULICZ-KOZARYN*, MICHEL SCHAEFER†, ANNE-MARIE BATT†, GÉRARD SIEST† and VINCENT LOPPINET‡

* Institut Farmakologie, Akademia Medyczna Poznan, Poland, † Laboratoire de Biochimie Pharmacologique, E.R.A. C.N.R.S. 698 Université de Nancy I, France and ‡ Laboratoire de Chimie Organique Pharmaceutique, Université de Nancy I, France

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Abstract—Rat liver UDP glucuronosyltransferase activities may be divided into at least two groups with differential responses towards substrates. This paper deals with an attempt to describe on what chemical basis the two groups may be distinguished. We studied the glucuronidation of 24 substrates in liver-microsomes of Sprague–Dawley rats pretreated with 3-methylcholanthrene or phenobarbital. The conjugation of 11 substrates was stimulated most strongly by 3-methylcholanthrene and that of the others, by phenobarbital. The estimated thickness of the molecules in their most likely conformation was below 4 Å for molecules of the first group and more than 4 Å for the second. The thickness or the bulkiness of the molecules seems to play an important role. However, for phenol substituted in the position 2, a steric effect or intramolecular interactions may change the substrate's classification within these two groups. It was also noticed that phenobarbital stimulated more than 3-methylcholanthrene the glucuronidation of the corresponding hydroxylated metabolite.

'Functional heterogeneity' of rat liver UDP glucuronosyltransferase activities (EC 2.4.1.17) is a concept already developed by Wishart *et al.* [1]. It has now been suggested by four main types of evidence: data of studies of the enzymes activity during perinatal development [2, 3], its induction by phenobarbital and 3-methylcholanthrene [4, 5] and attempts to purify the enzyme or enzymes [6–8] and tissue distribution [9].

Wishart [5] described 'a late foetal' group of UDP glucuronosyltransferase substrates which glucuronidation was preferentially stimulated by 3-methylcholanthrene, and a 'neonatal group' which was stimulated by phenobarbital.

In an effort to distinguish between these two groups Wishart *et al.* [1] finally rejected molecular weight, lipophilicity and route of excretion for their two groups. They detailed ring substitution changes which they demonstrated were criteria for their two groups, and they suggested that planarity or bulkiness of the molecule determined its acceptance or not by either of the two enzyme forms [10].

Dealing with the more general aspect of the spatial structure of the molecules, this present work sought to give a more quantitative aspect to the idea of 'specific molecular configurations' introduced by Wishart from a series of substituted phenols [10].

The present paper deals with the glucuronidation of 24 substrates after stimulation of the enzyme(s) by phenobarbital or 3-methylcholanthrene. In an effort to discriminate among the substrates, we studied alcoholic or phenolic, monohydroxylated or dihydroxylated compounds as well as planar or bulky molecules, excreted in urine or in bile.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Centre des Oncins, Saint-Germain sur l'Arbresle, France) weighing 350–400 g were injected with phenobarbital (sodium salt, Merck, Darmstadt, Germany) (80 mg/kg in 0.9% NaCl, on days 1, 2, and 3) or 3-methylcholanthrene (Sigma Chemical Co. Saint-Louis, U.S.A.) (15 mg/kg in 0.4 ml peanut oil, on day 2). Control animals received no injection. Animals were killed on day 5 after 12 hr fasting.

Liver-microsomal fractions were prepared by the method of Beaufay *et al.* [11] in 0.25 M sucrose, buffered with 1 mM Tris–HCl (final pH 7.4) and were frozen at −30°.

Chemicals. Phenol, 4-bromophenol, 2-bromo-4-chlorophenol, 4-ethylphenol, 4-nitrophenol and 1,3-naphthalenediol were obtained from Merck. 1-naphthol was from Kuhlman (Paris, France); catechol, resorcinol, hydroquinone, eugenol, menthol, *l*-borneol, 4,4'-dihydroxybiphenyl and 4-hydroxybiphenyl were purchased from Fluka (Bucks, Switzerland).

Trans-1,4-decalindiol, *cis*, *trans*-1,2-cyclohexanediol, 5,6,7,8-tetrahydro-1-naphthol and 1,2,3,4-tetrahydro-1-naphthol were supplied by Aldrich (Beerse, Belgium).

Morphine hydrochloride, was supplied by Cooperation Pharmaceutique Française (Melun, France).

Oxazepam and Lorazepam were the kind gift of Prophac (Paris, France).

Chloramphenicol was supplied by Boehringer-

* Present address.

Mannheim (Germany) 5-(4-hydroxyphenyl)-5-phenylhydantoin was the gift of Dr. A. J. Glazko (Parke Davis, Ann Arbor, U.S.A.), 3-hydroxybenzo-(a)-pyrene was generously given by Dr. M. Litwack (National Institute of Health, Bethesda, U.S.A), and 4-hydroxyphenyl, 5-ethyl-5-(p-hydroxyphenyl) barbiturate (4-hydroxyphenobarbital) was synthetized by one of us (V.L.) and identified by mass spectrometry.

Biochemical reagents: UDP glucuronate (disodium salt), phosphoenolpyruvate (potassium salt), NADH (disodium salt, grade II) pyruvate kinase from rabbit muscle and lactate dehydrogenase from beef heart were all obtained from Boehringer-Mannheim (Germany).

Triton X-100 (ICI Trade Mark) was from Sigma Chemical Co.

Determination of enzyme activity. Microsomal protein was measured by the method of Lowry *et al* [12] with bovine serum albumin (Sigma Chemical Co.) as standard.

UDP-glucuronosyltransferase activities were determined by the method of Mulder and Van Doorn [13] except that the temperature was 37° and the decrease in E 365 was followed on an Eppendorf spectrophotometer. Triton X-100 was added to the microsomal suspension, 20 min before assay, for a ratio (w/w) of detergent/microsomal protein of 0.2. The final concentration of UDP glucuronate was 4.5 mM, and of each aglycone, 0.3 mM, in the medium. Appropriate controls were performed without aglycone, without UDP-glucuronic acid and without phosphoenolpyruvate.

Estimation of the molecular thickness. The bulkiness of the substrate molecules were calculated by solid geometry from Dreiding molecular models [14]. The values of valence angles, bond lengths and covalent radii were taken from Gillespie [15]. We have defined 'thickness' as the distance between the two parallel planes to the mean plane of the molecule and tangential to the covalent shalls of the furthest atoms in a perpendicular axis against this mean plane, as it is shown in the case of the *trans*-1,2-cyclohexanediol (See Fig. 1).

The molecules have been taken in their most stable

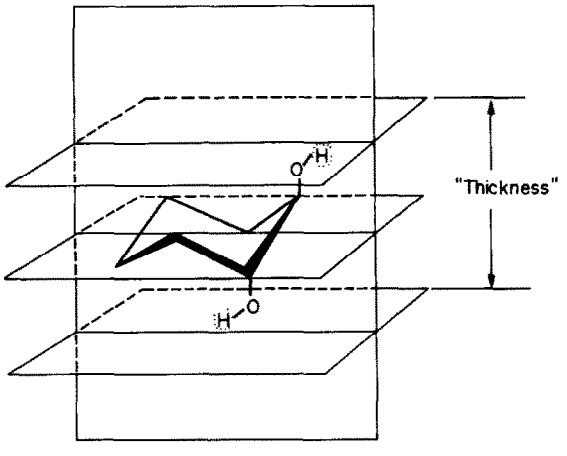

Fig. 1. Thickness evaluation of the molecule of *trans*-1,2-cyclohexanediol.

Table 1. Microsomal rat liver UDP-glucuronosyltransferase activities stimulated by 3-methylcholanthrene

Substrate	Approximate thickness (Angstroms)	UDP-Glucuronosyltransferase activities (nmol min^{-1} mg^{-1} microsomal protein)				
		Control rats	3-Methylcholanthrene-treated rats	Percentage stimulation	Phenobarbital-treated rats	Percentage stimulation
4-Bromophenol	2.3	43.48 ± 1.23	162.17 ± 5.62	370	48.84 ± 2.34	110
2-Bromo-4-chlorophenol	2.3	83.90 ± 2.22	169.00 ± 9.56	200	94.00 ± 3.49	110
1-Naphthol	1.7	51.06 ± 1.75	120.00 ± 7.24	230	54.66 ± 3.07	110
1,3-Naphthalenediol	2.1	32.28 ± 2.21	109.27 ± 1.75	340	33.95 ± 0.60	105
4-Nitrophenol	3.4	12.71 ± 0.39	32.45 ± 0.63	260	12.69 ± 1.84	100
Phenol	1.7	6.13 ± 0.44	12.12 ± 0.14	200	9.10 ± 0.96	150
Hydroquinone	3.4	12.63 ± 1.07	29.04 ± 0.73	230	17.70 ± 0.83	140
Resorcinol	3.4	23.53 ± 1.19	53.67 ± 0.67	230	25.57 ± 0.79	110
5,6,7,8-Tetrahydro-1-naphthol	3.3	21.10 ± 0.44	29.15 ± 0.93	140	23.78 ± 0.07	110

Glucuronidation activities are given as the means ± standard deviation for 5 determinations.

Table 2. Microsomal rat liver UDP-glucuronosyltransferase activities stimulated by phenobarbital

Substrate	Approximate thicknessess (Å)	UDP-Glucuronosyltransferase activities (nmol min^{-1} mg^{-1} microsomal protein)				
		Control rats	3-Methylcholanthrene-treated rats	Percentage stimulation	Phenobarbital-treated rats	Percentage stimulation
Chloramphenicol	>5	3.35 ± 0.51	2.95 ± 0.58	90	5.69 ± 0.46	170
4-Hydroxyphenobarbital	>5	6.86 ± 0.55	5.41 ± 0.06	80	17.40 ± 1.27	250
5-(4-hydroxyphenyl) 5-phenylhydantoin	>7	5.60 ± 0.53	4.45 ± 0.49	80	10.90 ± 0.64	190
4-Hydroxybiphenyl	4.3	18.99 ± 0.19	20.65 ± 2.36	110	48.25 ± 3.56	250
4,4'-Dihydroxybiphenyl	4.3	11.54 ± 0.66	11.39 ± 0.46	100	17.85 ± 0.27	150
Lorazepam	>4	4.21 ± 0.71	3.11 ± 0.40	80	10.26 ± 0.78	240
Oxazepam	>4	5.04 ± 0.69	3.72 ± 0.32	70	11.91 ± 0.84	240
Morphine	5.67	4.89 ± 0.31	4.86 ± 0.17	100	11.57 ± 0.67	130
l-Borneol	>4.5	23.66 ± 0.55	17.67 ± 0.67	80	31.52 ± 0.82	130
Menthol	>6.5	14.86 ± 0.59	14.15 ± 0.65	100	24.04 ± 0.71	160
Eugenol	1.87–4.6*	41.96 ± 1.03	80.51 ± 0.61	190	95.26 ± 0.94	230
Catechol	3.4	8.20 ± 0.86	9.55 ± 0.60	120	12.00 ± 1.23	140
trans-1,4-Decalindiol	≥6	5.27 ± 0.29	3.25 ± 0.51	60	8.49 ± 0.54	160
cis, trans-1,2-Cyclohexanedol	>4	3.29 ± 0.39	2.82 ± 0.25	90	7.97 ± 0.16	240
1,2,3,4-Tetrahydro-1-naphthol	4.1	11.31 ± 0.44	9.38 ± 0.69	80	21.54 ± 2.43	190

Glucuronidation activities are given as the means ± standard deviation for 5 determinations.
* See Fig. 2.

conformations from the thermodynamical point of view, when the Pitzer's strains are minimal (staggered and 'chair' conformers for the acyclic and the cyclohexane molecules respectively); this corresponds to their highest populations. For the aromatic compounds, we have chosen to place the substituents on both sides of the cycle.

In these cases the molecules tend generally to occupy the largest space possible, in accord with the free rotation around the bonds. Some examples will be given later on.

RESULTS

The results are summarized in two tables, depending on the inducer.

The substrates shown in Table 1 had their conjugation stimulated more by 3-methylcholanthrene under the conditions used. They include molecules with one or two hydroxyl-substituents, as example 1-naphthol and 1,3-naphthalenediol. For all of them their approximate molecular bulkiness is less than 4 Å.

The substrates shown in Table 2 had their conjugation stimulated more by phenobarbital pretreatment. They also include molecules with one or two hydroxyl moieties. But their estimated bulkiness are more than 4 Å except for catechol.

In the cases of commercial mixtures of diastereoisomeric forms, we always considered all the possibilities: Both *cis*- and *trans*-cyclohexane-1,2-diols are more than 4 Å thick and form weak intramolecular hydrogen bonds [20]. *trans*-Decalin-1,4-diol can exist in three different forms (a, a), (e, e) and (a, e) = (e, a); except for the (e, e) form, they are more than 4 Å thick. Because of the asymmetric carbon atoms in position 1 and 4, i.e. all three diols form racemates.

The same geometric estimation of bulkiness was applied to substrates taken from published information. 5-Hydroxytryptamine and 4-methylumbelliferone are respectively 3.7 Å and 2.28 Å bulky and belongs to group 1 according to Wishart. Testosterone and estradiol are both more than 5 Å bulky, and phenolphthalein more than 8.5 Å. They are described as belonging to the second group(s) [5].

DISCUSSION

Some of the data obtained in this report have been compared with previously reported results from other workers [4, 5]. 4-Nitrophenol and 1-naphthol have already been described as sharing the properties of so called 'group 1' substrates as their conjugation was preferentially induced in rat liver respectively by 200–300 per cent and 170–300 per cent after 3-methylcholanthrene pretreatment. Chloramphenicol and morphine have been reported as typical substrates preferentially induced by phenobarbital. The glucuronidation of 4-hydroxybiphenyl was also demonstrated to belong to the so called group 2 [9].

In the present work we have concentrated on the basis of the bulkiness of the molecules used as substrates, to separate the two groups of substrates after induction of the enzyme activity by 3-methylcholanthrene or phenobarbital. The approximate limiting bulkiness may be estimated near 4 Å. The case of 1,2,3,4-tetrahydro-1-naphthol compared to 5,6,7,8-tetrahydro-1-naphthol is particularly demonstrative as these molecules do not differ in their molecular weights, they both bear one hydroxyl substituent, phenolic or alcoholic, but they differ in the spatial structure around the hydroxyl group. 5,6,7,8-Tetrahydro-1-naphthol belongs to the 3-methylcholanthrene group, whereas 1,2,3,4-tetrahydro-1-naphthol which is non planar in the neighbourhood of the hydroxyl group belongs to the phenobarbital-group. In control rat liver microsomes the activity was 50 per cent less for the alcoholic than for the phenolic compound.

Bock *et al.* [9] having examined glucuronidation of 3-hydroxybenzo-(a)-pyrene compared to benzo-(a)-pyrene-7,8-dihydrodiol, also observed that the dihydrodiol was a poor substrate of their GT_1, as the portion of the molecule which serves as substrate is not planar. Our data with tetrahydronaphthols lead us to the same conclusion.

These new substrates also confirm that the molecular weight and the lipophilicity themselves were not criteria.

Moreover 'small' molecules like menthol (mol. wt 156.26) and borneol (mol. wt 154.24) belong to the second group, while heavier ones, like 2-bromo-4-chlorophenol, belong to the first one.

For the bile or urine excretion lorazepam and oxazepam are both excreted in rat urine [17] while nevertheless belonging to the phenobarbital group.

In a study of some 2-, 3-, and 4-substituted phenols, Wishart *et al.* [1, 10] found that 4-ethylphenol and 4-*n*-propylphenol, which differ only by a single-CH_2 moiety, did not belong to the same group of UDP-glucuronosyltransferase substrates. The authors attributed this difference to 'limiting molecular configurations' or 'specific molecular configurations' without, however, being more explicit.

5-Hydroxytryptamine and 3-propylphenol are almost as planar, and Wishart *et al.* [1] found 5-hydroxyptamine to belong to group 1 and propylphenol to group 2.

This confirms that thickness is not the only criterion and that steric hindrance near the hydroxyl conjugated may also play a distinguishing role: thickness concerning the whole molecule, and steric hindrance reflecting an effect near the conjugated hydroxyl.

Catechol was another example in this work [1], as were in the series of phenols reported by Wishart and Campbell [10], 2-ethylphenol, 3-methyl-5-ethylphenol, which are planar molecules (less than 4 Å) but belong to the group more stimulated by phenobarbital. A steric effect (with 2-ethylphenol) and/or intramolecular interactions in the aglycone, also plays a role in determining the specificity of the enzyme or enzymatic site for the substrate.

Eugenol glucuronidation (Table 2) was stimulated mostly by phenobarbital, but 3-methylcholanthrene was also a potent inducer. In addition to the possible steric hindrance due to the *O*-methoxy group, eugenol is a molecule whose conformation varies because of a rotation around the C–C bond and whose thickness thus varies from 1.87 Å to 4.6 Å allowing each rotamer to have the optimal thickness

either for 3-methylcholanthrene-group or for phenobarbital-group. The thickness of molecules in their most likely conformation, and taking into account the steric hindrance or molecular interaction near the hydroxyl group seems then to be a useful feature to guide the future investigations concerning the heterogeneity of the UDP-glucuronosyl transferase activities.

We noticed here that the glucuronidation of 4-hydroxyphenobarbital was increased by phenobarbital pretreatment; on the other hand, when studying 3-hydroxybenzo-(a)-pyrene, we noticed a 2.5-fold increase in its glucuronidation by Wistar rat liver microsomes after 3-methylcholanthrene pretreatment [18]. This tends to demonstrate that a parent xenobiotic preferentially induced the glucuronidation of its hydroxylated metabolite and of compounds resembling its metabolite.

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