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HETEROGENEITY OF HEPATIC MICROSOMAL UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES

CONJUGATIONS OF PHENOLIC AND MONOTERPENOID AGLYCONES IN CONTROL AND INDUCED RATS AND GUINEA PIGS*

JEAN A. BOUTIN,†‡ JACQUES THOMASSIN,† GERARD SIEST†§ and ALAIN CARTIER‖

†Centre du Medicament, UA CNRS 597, et Faculté des Sciences Pharmaceutiques et Biologiques, BP 403, 54001 Nancy Cédex, France; and ‖Laboratoire de Chimie Théorique (Pr. Rivail), Faculté des Sciences, BP 239, 54506 Vandoeuvre-Lès-Nancy, France

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Abstract—In this report we present evidence that the heterogeneity of hepatic microsomal UDP-glucuronosyltransferase(s) (UDPGT) activities depends on the chemical structures of the aglycones as well as their biophysical constants. Three animal models were used: Wistar rats, which have active UDPGTs; Gunn rats, in which some of the UDPGT activities are reduced, but which can be induced by phenobarbital; and guinea pigs. In Wistar rats, we found that some coumarins were poor substrates of UDPGT (GT_1) and that twenty monoterpenoid alcohol activities showed typical phenobarbital-inducible behavior. In Gunn rats, we showed that substitution of the phenolic aglycone by bulky (alkyl- or methoxy-) groups in the 2-position of the phenolic ring decreased UDPGT (GT_1) activity, whereas substitution in the 4-position resulted in an increase in this activity. We also showed that, in this particular strain, activities toward terpenes were less affected than activities toward flat (aromatic) aglycones. Induction by phenobarbital in Gunn rats increased the activity and limited the deficiency for monoterpenoid alcohols. In guinea pigs, we confirmed that phenobarbital selectively increased the activities of UDPGT towards twenty monoterpenoid alcohols without affecting other typical phenobarbital-induced activities such as those for conjugation of morphine. Finally, we showed that orientation of the aglycone molecule in the active site was apparently related to its dipole moment and that the distance between "acceptor-oxygen" (hydroxyl) and the carbons out of the general plane of the molecule was an important factor. These studies clearly suggest that rat and guinea pig contain a UDPGT(monoterpenoid alcohols) with restricted specificities and also that UDPGT(GT_1) comprises at least two or three different isoenzymes, each with a slightly different restricted specificity towards flat aromatic aglycones.

An increasing number of foreign molecules (xenobiotics) are present in our environment as drugs or pollutants. In mammals most of these synthetic or natural products are metabolized by conjugation with glucuronic acid which is the main pathway of the Phase II (elimination) of the detoxication process, catalyzed by UDP-glucuronosyltransferase(s) (UDPGT, EC 2.4.1.17). These enzymes are membrane-bound and are located mainly in the endoplasmic reticulum [1] of hepatocytes [2]. UDPGT activities are strongly dependent on their microenvironment, and especially on the phospholipids of the membrane [3].

Knowledge of the number of isomolecular forms of these enzymes, and understanding of their regulation (and especially their response to induction by xenobiotics), their developmental patterns, and their substrate specificities are important for pharmacology and drug design [4].

Since the original demonstration that UDPGT comprised at least two molecular forms of enzymes [5] with probably overlapping specificities, five such forms have been identified [6, 7]. These isofunctional enzymes appear to have restricted substrate specificity, but definitive data on these specificities are lacking [8]. In particular, information on the structural requirements of the aglycones is limited. From previous studies by our laboratory [9] and others [10, 11], it appears that the shape of the aglycones, i.e. whether they are bulky (>4.5Å) or planar, may play a key role in determining whether the molecule shows complementarity to the active site, and whether these compounds will be detoxified. The current studies were designed to investigate the substrate specificities of these isofunctional enzymes, and to determine possible differences in their regulation.

To carry out these studies, three systems were used. First, we examined the activities toward forty-five flat and bulky aglycones in control and induced Wistar rats. Next, we studied conjugation in Gunn rats, which lack UDPGT-bilirubin [12] and are partially deficient for other UDP-glucuronosyltransferases [13–15]. Some of these activities can be induced by phenobarbital, however, which permitted comparison with the same experimental situation in Wistar rats. Third, we examined the conjugation of

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‡ Present address: Laboratory for Molecular Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, U.S.A.

§ From whom reprints should be requested.

monoterpenoid alcohols in both control and phenobarbital-induced guinea pigs.

The use of microsomal systems permits an evaluation of UDPGT specificity under conditions similar to those in the intact animal by taking into account the interrelationships of the different isofunctional enzymes and effects of membrane phospholipids in these interactions, in particular the role of these phospholipids on the spatial conformation of the UDPGT [16, 17].

We have chosen to study three main chemical families: hydroxycoumarins, alkyl or arylphenols, and monoterpenoid alcohols, since these substrates are relatively actively conjugated under our experimental conditions. Furthermore, these compounds are available with a large variety of chemical substituents which allows us to define the substrate requirements. All UDPGT activities were measured by a modification of the procedure of Mulder and van Doorn [18], thus avoiding the problem of differences attributable to different analytical methods. We compared our results with data obtained by other methods, particularly that of Bansal and Gessner [19], and found reasonable agreement between our data and those presented in the literature [20]. We adapted our method to a centrifuge analyser [21].

Based on this approach, we attempted to determine the structural requirements of the aglycones to provide information on the conformation(s) of the active site(s) and also to understand the differences in induction and the overlap between substrate specificities.

We found a clear relationship between the defect of some Gunn rat activities and the structure of the aglycones. We confirmed that the monoterpenoid alcohols are probably conjugated by a rather specific isoenzyme. We provide new data suggesting that hydroxycoumarins are also conjugated by a different isoenzyme, as suggested by other recent data [8, 14, 22].

MATERIALS AND METHODS

Chemicals were obtained from the following sources: from Sigma (St. Louis, MO): Triton X-100 and 3-methylcholanthrene; from Boehringer (Mannheim, West Germany): UDPGT measurement reagents—phosphoenolpyruvate, lactate dehydrogenase, UDP-glucuronic acid (UDPGa), NADH, and pyruvate kinase; from Aldrich (Beerse, Belgium): carveol, dihydrocarveol, terpinen-4-ol, 3-methyl-2-norbornane methanol, borneol, 4,8-dimethyl-7-hydroxycoumarin, 7-hydroxy-4-phenylcoumarin, 4-hydroxy-6-methylcoumarin, eugenol, 4-isopropylphenol, 4-hydroxybiphenyl, 5,6,7,8-tetrahydro-2-naphthol, and 1,2,3,4-tetrahydro-2-naphthol; from Fluka (Buchs, Switzerland): 4-benzylphenol, linalool, citronellol, nerol, geraniol, menthol, terpineol, *trans*-myrtanol, *cis*-myrtanol, nopol, *cis*-verbenol, fenchol, scopoletin, 4-methylumbelliferone, umbelliferone, thymol, cedrol, carvacrol, and phenobarbital; from Merck (Darmstadt, West Germany): 2-naphthol, 4-nitrophenol, 4-nitro-3-cresol, 6-nitro-3-cresol, 2-nitro-4-cresol, 2-nitro-3-cresol, 3-methoxyphenol, and 4-methoxyphenol; from Ega-Chemie (Steinheim, West Germany): 4-hydroxycoumarin, 4-chromanol, guaiacol, isoeugenol, isopulegol, limonen-10-ol, patchenol, isopinocampheol, and myrtenol.

4-Hydroxydibenzyl was donated by Pr. R. R. Scheline (Bergen, Norway).

Male Wistar and Gunn rats (200 g) were obtained from IFFA-CREDO (Saint-Germain sur l'abresle, France). They had free access to food (UAR, Villemoisson/Orge) and water.

Male "tricolores" guinea pigs, weighing 350–400 g, were obtained from the Centre d'Elevage d'Animaux de Laboratoire, Ardenay, France.

Groups of four rats were assigned to each treatment. Each animal was treated by intraperitoneal injection of 0.5 ml of vehicle per 200 g of body weight. One group received phenobarbital (80 mg/kg) dissolved in 0.9% NaCl (w/v) daily for 4 days. The corresponding controls received saline solution only. Two groups of Gunn rats received the same treatment. Another group of Wistar rats received 3-methylcholanthrene in corn oil, as a single injection of 80 mg/kg on the first day and these animals were killed on day 5. Corresponding controls received corn oil only.

A group of four guinea pigs received daily intraperitoneal injections of 20–40 mg of phenobarbital per kg of body weight, for 9 days, (total dose, 320 mg/kg of body weight).

Animals were decapitated 24 hr after the last treatment. Liver microsomes were prepared in 0.25 mM sucrose, 1 mM Tris–HCl buffer, pH 7.4, according to the method of Beaufay *et al.* [23]. Protein was measured by the method of Lowry *et al.* [24].

UDPGT activities were measured by a modification [21] of the method described by Mulder and van Doorn [18]. The microsomal suspensions were activated with Triton X-100 such that the ratio of Triton to protein (w/w) was 0.4. Each cuvette of the centrifuge analyser (Cobas-Bio Roche) contained 120 μl of a medium composed of the following: 5 μl of microsomal suspension (2.5 mg protein/ml); 50 μl of water; 55 μl of Mulder and van Doorn medium [UDP-glucuronic acid (4.5 mM), pyruvate kinase (25 μg/ml, 200 units/mg), phosphoenolpyruvate (0.2 mM), NADH (0.2 mM), lactate dehydrogenase (2.5 μg/ml), in Tris–HCl buffer (75 mM), $MgCl_2$ (5 mM), pH 7.4, at 4°]; and 10 μl of aglycone (0.3 mM final concentration, or as indicated) to start the reaction. Each run (twenty-nine assays) took 10 min, and readings at 340 nm were recorded every 20 sec. The specific activity was calculated by linear regression. Each value is given as a mean of four determinations.

A blank without UDP-glucuronic acid was included with each run to determine possible interaction between aglycones and NADH [21]. In the presence of Triton X-100, no interference was detected with any of the forty-five aglycones. Activities were also measured at Triton to protein ratios of 0.2 and 0.6 at the same time to verify that the maximum activation was reached with a ratio of 0.4.

For kinetic studies, the determinations of apparent V_{max} and K_m have been performed with six different concentrations of aglycones (0.05 to 0.6 mM) at saturating concentration in UDPGa of 4.5 mM. Once the

double-reciprocal plot was obtained, the constants were calculated by linear regression.

RESULTS

Activity of UDPGT in Wistar rats: Effect of induction by phenobarbital and 3-methylcholanthrene

Wistar rats have been studied extensively with respect to the heterogeneity of their UDP-glucuronosyltransferase activities [5, 7, 9]. We first examined the activity of microsomal preparations from control, phenobarbital-, and 3-methylcholanthrene-treated animals towards forty-five substrates which had been shown previously to be conjugated *in vivo* [25]. We found that 3-methylcholanthrene and phenobarbital produced different levels and/or patterns of induction for different groups of compounds (Table 1, Figs. 1–4). Thus, with respect to 3-methylcholanthrene, activity toward 7-hydroxylated coumarins (compounds I to V) was increased from 2.8 to 13.2 times over control values, whereas the activities for 4-hydroxylated coumarins (VI, VII) and 4-chromanol (VIII) were not increased. All the activities toward phenolic compounds (IX to XXIV) were increased by 2 to 4 times, except for thymol (XX), 4-hydroxybiphenyl (XXII), and 4-benzylphenol (XXIII) where only limited induction by 3-methylcholanthrene was observed. None of the activities towards terpenoids (XXV to XLV) was enhanced by 3-methylcholanthrene treatment.

In contrast, phenobarbital had very little effect on compounds I to VII (the hydroxylated coumarins) and compounds IX to XXIV, except for eugenol (XVIII), isoeugenol (XIX), 4-isopropylphenol (XXI) and the arylphenols (XXII to XXIV), the activities for which were induced from 1.6 to 2.0 times. Moreover, all the activities toward terpenoid compounds (XXV to XLV) were enhanced to various extents (from 1.4- to 2.1-fold over the controls) by phenobarbital treatment.

Kinetic studies on control and induced Wistar rats

We selected twenty-one aglycones from the previous experiment based both on high activities and simplest structure. We measured apparent K_m and V_{max} values to determine whether induction altered these enzymatic parameters. A wide range of K_m and V_{max} values for these aglycones was observed; K_m values ranged from 1 μM (VI, VII, XI, XX and XXIX) to 0.1 mM (I) in control rats (Table 2). Except for 4-nitro-3-cresol (XI), these values were similar whether corn oil or saline solution was used.

Furthermore, there was a very little change in K_m values after phenobarbital induction, and 3-methylcholanthrene induction enhanced only the phenol K_m values (XI to XXI), therefore diminished the corresponding affinity.

In controls, with respect to V_{max}, a large range of values was observed from 7-hydroxycoumarin (I to IV) with a $V_{max} = 120$ nmoles $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$ to 4-hydroxycoumarin (V, VI) with 5 nmoles $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$. As previously pointed out for specific activities, we found that V_{max} was altered after induction. However, whereas phenobarbital enhanced all the V_{max} values to 1.5 to 3.5 times over the controls, 3-methylcholanthrene induction resulted in even greater increases (up to 5-fold) in the V_{max} for 7-hydroxycoumarins.

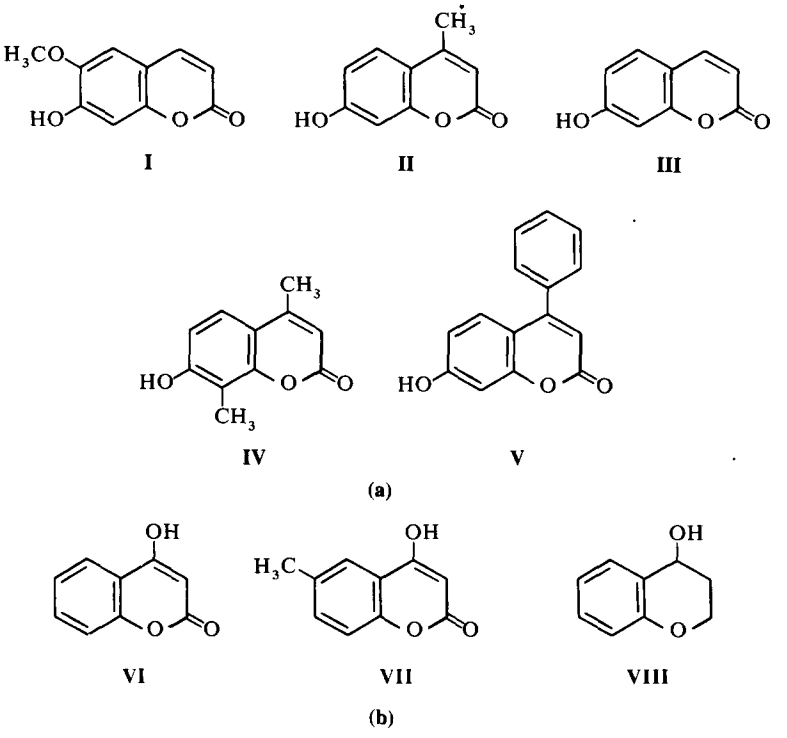


Fig. 1. Structural formulae of (a) 7-hydroxylated coumarins, and (b) 4-hydroxylated coumarins.

Table 1. UDP-glucuronosyltransferase specific activities of forty-five aglycones measured in liver microsomes from Wistar rats induced by either phenobarbital or 3-methylcholanthrene*

Aglycones (concentration in the assay)	UDPGT specific activities (nmoles/min/mg)			
	Controls (NaCl)	PB	Controls (oil)	MC
Coumarins				
I Scopoletin (0.5 mM)	58.1 ± 5.1	70.1 ± 1.1	29.2 ± 1.1	81.5 ± 2.5
II 4-Methylumbelliferone (0.25 mM)	62.7 ± 1.3	80.3 ± 2.0	65.9 ± 2.7	227.9 ± 3.0
III Umbelliferone (0.5 mM)	82.3 ± 2.1	118.2 ± 4.5	58.3 ± 1.1	218.6 ± 10.8
IV 4,8-Dimethyl-7-hydroxy coumarin (0.25 mM)	89.3 ± 7.8	102.5 ± 3.5	55.1 ± 1.4	221.4 ± 8.3
V 7-Hydroxy-4-phenyl coumarin (0.5 mM)	30.7 ± 1.7	75.2 ± 6.9	13.4 ± 2.0	172.4 ± 14.8
VI 4-Hydroxycoumarin (0.5 mM)	3.2 ± 0.1	5.1 ± 0.8	4.4 ± 0.1	3.0 ± 0.3
VII 4-Hydroxy-6-methyl coumarin (0.5 mM)	7.5 ± 0.7	9.1 ± 0.2	4.6 ± 0.1	2.8 ± 0.4
VIII 4-Chromanol (0.25 mM)	4.4 ± 0.1	6.7 ± 0.2	4.3 ± 0.1	4.0 ± 0.1
Naphthols and phenols				
IX 2-Naphthol (0.25 mM)	59.2 ± 8.3	63.3 ± 1.4	42.9 ± 0.8	188.9 ± 4.0
IL 1,2,3,4-Tetrahydro-2-naphthol (0.5 mM)	8.3 ± 0.3	16.2 ± 0.5	5.3 ± 0.1	7.6 ± 0.3
XLVIII 5,6,7,8-Tetrahydro-2-naphthol (0.5 mM)	15.2 ± 1.3	94.9 ± 2.0	32.0 ± 1.5	122.8 ± 2.4
X 4-Nitrophenol (0.5 mM)	45.5 ± 0.4	66.2 ± 5.4	27.2 ± 0.7	161.4 ± 3.1
XI 4-Nitro-3-cresol (0.5 mM)	54.2 ± 1.2	78.0 ± 1.9	35.4 ± 0.5	177.0 ± 3.6
XII 6-Nitro-3-cresol (0.5 mM)	22.5 ± 1.4	25.6 ± 1.8	13.1 ± 0.6	35.6 ± 0.4
XIII 2-Nitro-4-cresol (0.5 mM)	41.8 ± 2.6	67.7 ± 6.3	35.2 ± 0.6	134.4 ± 4.7
XIV 2-Nitro-3-cresol (0.25 mM)			46.9 ± 0.7	193.1 ± 1.3
XV 4-Methoxyphenol (0.25 mM)	10.7 ± 1.3	12.8 ± 1.7	11.7 ± 0.1	34.7 ± 0.5
XVI 3-Methoxyphenol (0.25 mM)	21.2 ± 0.2	22.8 ± 0.3	13.3 ± 0.2	44.1 ± 0.5
XVII Guaiacol (0.25 mM)	8.9 ± 0.4	10.1 ± 0.8	13.0 ± 0.4	44.3 ± 0.8
XVIII Eugenol (0.25 mM)	26.6 ± 1.8	45.7 ± 2.5	28.0 ± 0.6	85.1 ± 1.4
XIX Isoeugenol (0.25 mM)	16.7 ± 0.8	26.3 ± 2.0	27.9 ± 0.6	66.9 ± 1.5
XX Thymol (0.25 mM)	7.2 ± 0.2	11.4 ± 0.6	8.1 ± 0.2	11.4 ± 0.3
XXI 4-Isopropylphenol (0.25 mM)	35.8 ± 0.6	54.8 ± 5.1	24.7 ± 0.3	56.1 ± 0.4
XXII 4-Hydroxybiphenyl (0.25 mM)	14.7 ± 0.8	33.2 ± 0.8	13.8 ± 0.8	18.4 ± 0.7
XXIII 4-Benzylphenol (0.25 mM)	25.4 ± 0.8	39.9 ± 1.6	22.0 ± 0.2	36.3 ± 4.3
XXIV 4-Hydroxydibenzyl (0.25 mM)	10.9 ± 2.4	22.4 ± 1.6	10.8 ± 0.3	21.9 ± 0.4
Monoterpenoid alcohols				
XXV Linalool (0.25 mM)	7.4 ± 0.3	9.7 ± 0.1	7.5 ± 0.1	6.8 ± 0.2
XXVI Citronellol (0.25 mM)	12.9 ± 0.5	30.6 ± 3.7	9.5 ± 0.4	10.0 ± 0.5
XXVII Nerol (0.25 mM)	9.4 ± 0.6	22.1 ± 0.7	7.6 ± 0.3	8.7 ± 0.0
XXVIII Geraniol (0.25 mM)	13.2 ± 0.8	24.7 ± 1.0	13.5 ± 0.3	12.3 ± 0.1
XXIX Menthol (0.25 mM)	8.9 ± 0.4	14.3 ± 0.4	5.9 ± 0.1	6.6 ± 0.1
XXX Terpineol (0.25 mM)	9.0 ± 0.3	13.2 ± 0.4	6.6 ± 0.1	7.8 ± 0.0
XXXI Carveol (0.25 mM)	12.3 ± 2.6	17.3 ± 0.6	6.4 ± 0.2	7.0 ± 0.2
XXXII Dihydrocarveol (0.25 mM)	6.7 ± 0.2	10.1 ± 0.1	4.8 ± 0.3	5.1 ± 0.2
XXXIII Terpinen-4-ol (0.25 mM)	8.3 ± 0.8	10.4 ± 0.4	8.6 ± 0.2	8.7 ± 0.2
XXXIV Isopulegol (0.25 mM)	6.6 ± 0.1	10.5 ± 1.9	5.8 ± 0.2	5.9 ± 0.2
XXXV Limonen-10-ol (0.25 mM)	11.8 ± 0.1	18.7 ± 0.5	10.9 ± 0.3	11.1 ± 0.2
XXXVI *trans*-Myrtanol (0.25 mM)	16.4 ± 3.8	24.8 ± 4.5	12.1 ± 0.3	15.4 ± 0.2
XXXVII *cis*-Myrtanol (0.25 mM)	19.0 ± 2.7	40.6 ± 3.2	11.4 ± 0.5	13.6 ± 0.4
XXXVIII Patchenol (0.25 mM)	10.7 ± 0.4	20.1 ± 1.0	10.0 ± 0.4	11.6 ± 0.2
IXL Isopinocampheol (0.25 mM)	10.6 ± 0.2	18.4 ± 2.8	8.9 ± 0.1	10.2 ± 0.3
XL Nopol (0.25 mM)	15.6 ± 0.3	35.1 ± 0.9	12.7 ± 0.1	10.7 ± 0.4
XLI Myrtenol (0.25 mM)	10.5 ± 2.2	15.8 ± 2.6	9.4 ± 0.4	9.5 ± 0.4
XLII *cis*-Verbenol (0.25 mM)	11.6 ± 0.6	17.6 ± 0.3	9.1 ± 0.4	10.2 ± 0.2
XLIII 3-Methyl-2-norbornane methanol (0.25 mM)	11.3 ± 0.4	17.7 ± 2.0	8.3 ± 0.1	10.1 ± 0.2
XLIV Fenchol (0.25 mM)	8.0 ± 0.1	9.6 ± 0.4	4.6 ± 0.1	4.6 ± 0.1
XLV Borneol (0.25 mM)	15.6 ± 0.4	27.0 ± 0.5	8.2 ± 0.3	10.2 ± 0.2
Aliphatic alcohol				
L1-Butanol (0.25 mM)	6.9 ± 0.1	8.5 ± 0.1	3.0 ± 0.1	3.3 ± 0.1

* Activities were determined on microsomal preparations obtained from liver of rats treated with phenobarbital (PB) or 3-methylcholanthrene (MC). The corresponding controls received the vehicle only. Values represent mean ± S.D. of four determinations on pooled microsomes of four animals. The data are expressed in nmoles·min^{-1}·(mg protein)$^{-1}$. The Roman numeral on the left side refers to structural formulae shown in Figs. 1–4.

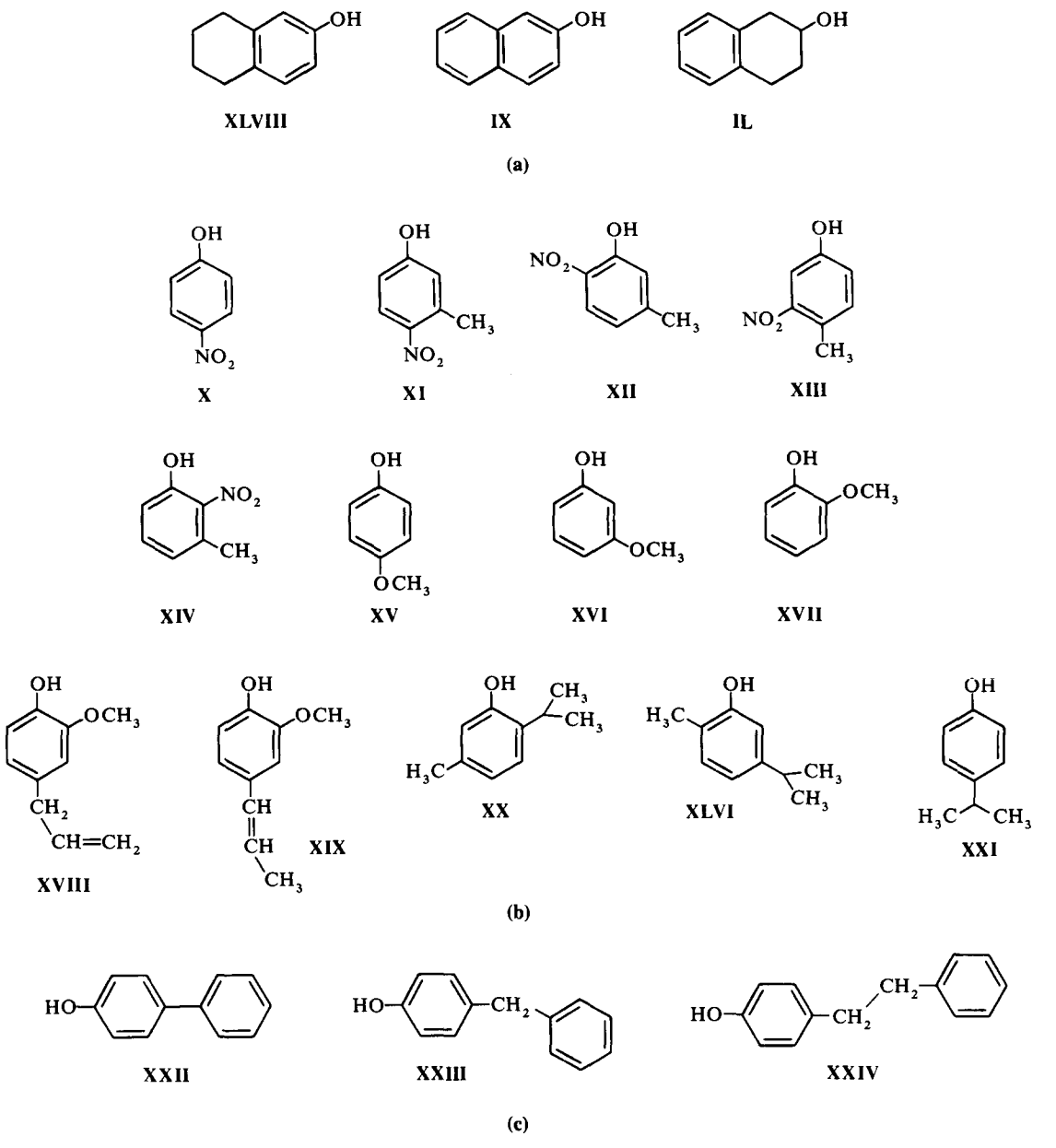


Fig. 2. Structural formulae of (a) naphthols, (b) substituted phenols, and (c) aryl phenols.

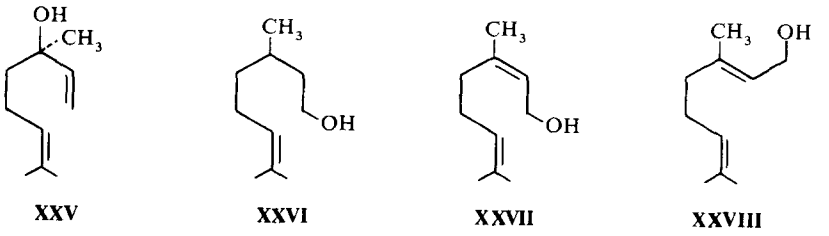
Activity of UDPGT in Gunn rats: Effect of induction by phenobarbital

Gunn rats have been shown to have partially reduced UPDGT activities toward some substrates and to be completely lacking in UDPGT-bilirubin activity [13, 14, 26]. We therefore used this strain to study substrate specificity and induction by phenobarbital to determine how conjugation is affected under these conditions.

We focused our attention on a family of natural compounds (monoterpenoid alcohols), which have not been studied previously *in vitro*.

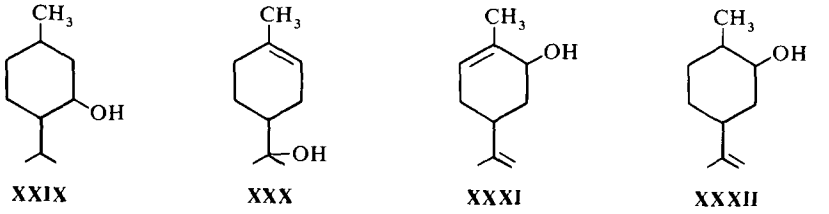
We found that the compounds fell into three classes (Table 3), those with activities similar to Wistar rat control values (XXVI, XXIX, IXL and XLI), those with levels of conjugation about 20% of Wistar rat control values, and those with activities about 60–70% of the control Wistar rat activities.

The activities toward all compounds tested were induced significantly (1.3 to 4.0 times control values) after phenobarbital treatment. The ratio of induced activities in Gunn rats compared with induced activities in Wistar rats for monoterpenoid alcohols (XXXVI to XLV) was about 0.8.

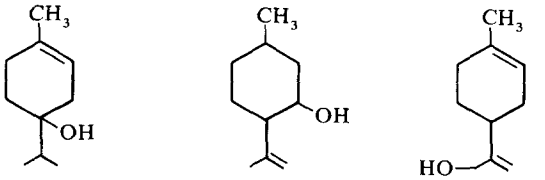


XXV XXVI XXVII XXVIII

(a)

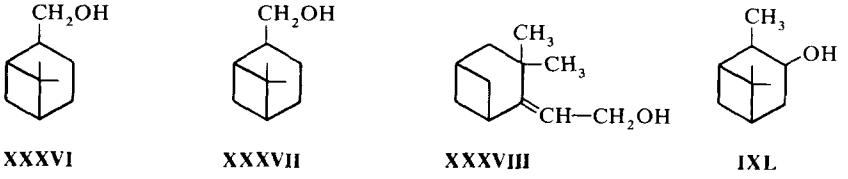


XXIX XXX XXXI XXXII

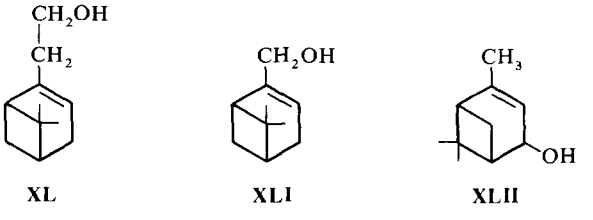


XXXIII XXXIV XXXV

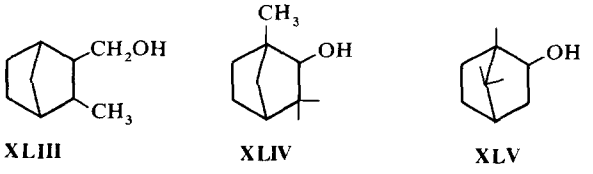
(b)



XXXVI XXXVII XXXVIII IXL



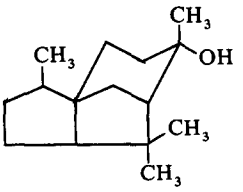
XL XLI XLII



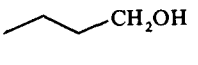
XLIII XLIV XLV

(c)

Fig. 3. Structural formulae of (a) monoterpenoid alcohols—acyclic compounds, (b) monoterpenoid alcohols—monocyclic compounds, and (c) monoterpenoid alcohols—bicyclic compounds [designated according to the nomenclature of terpen hydrocarbons in *Nomenclature for Terpene Hydrocarbons*, American Chemical Society, Washington, DC (1955)].



XLVII



L

Fig. 4. Structural formulae of cedrol and 1-butanol.

Table 2. K_m and V_{max} values for twenty-one aglycones studied with hepatic microsomal UDP-glucuronosyltransferases obtained from controls, phenobarbital-treated, and 3-methylcholanthrene-treated Wistar rats*

Aglycones	K_m ($\times 10^5$)				V_{max}			
	C_1	PB	C_2	MC	C_1	PB	C_2	MC
Coumarins								
I Scopoletin	18	41	10	14	30	106	71	138
II 4-Methylumbelliferone	7	8	9	11	79	120	80	354
III Umbelliferone	8	9	6	8	94	124	59	291
IV 4,8-Dimethyl-7-hydroxycoumarin	5	7	16	19	100	145	108	540
VI 4-Hydroxycoumarin	≈0.5	≈0.5	≈0.2	≈0.2	5	6	6	6
VII 4-Hydroxy-6-methylcoumarin	≈0.1	≈0.2	≈0.1	≈0.1	5	6	5	5
Naphthol and phenols								
IX 2-Naphthol	3	4	5	4	76	105	57	245
XI 4-Nitro-3-cresol	≈0.5	≈0.5	6	9	57	79	26	108
XVI 3-Methoxyphenol	9	11	3	12	15	20	11	48
XVII Guaiacol	5.5	6	5.5	13	10	13	16	65
XVIII Eugenol	7	12	7	11	47	120	22	58
XX Thymol	≈0.6	1	≈0.8	3	8	13	7	11
XXI 4-Isopropylphenol	9	10	6	9	39	59	21	55
XXIII 4-Benzylphenol	2.5	2	1	4	31	57	21	29
Monoterpenoid alcohols								
XXVI Citronellol	1.3	3	4	4	24	57	9	11
XXIX Menthol	≈0.5	1	≈0.5	1	11	18	6	8
XXXI Carveol	2	4	3	3	13	21	8	9
XL Nopol	1	3	2	12	17	38	12	20
XLI Myrtenol	1	3	1	2	17	29	12	15
XLII *cis*-Verbenol	1	7	2	4	16	21	10	12
XLIV Fenchol	2	5	1.5	2.5	8	9	6	6

* Details of induction are given in Materials and Methods. Key: 3-methylcholanthrene-treated rats (MC) and corresponding controls (C_2); phenobarbital-treated rats (PB) and corresponding controls (C_1). The data were obtained using five concentrations of aglycones (from 0.05 to 0.6 mM) and were made in triplicate. K_m values are expressed in moles, V_{max} in nmoles·min^{-1}·(mg protein)$^{-1}$. They were calculated by linear regression using double inverse plots. The symbol (≈) means that, under our experimental conditions, the K_m in the micromolar range could only be indicative.

Activity of UDPGT in guinea pigs: Control and phenobarbital induction

Phenobarbital induction of UDP-glucuronosyltransferases towards a wide range of bulky compounds has been described for several species: pig [27], rat [5, 7, 9, 28] and mouse [29]. In contrast, guinea pig hepatic UDP-glucuronosyltransferase activities towards monoterpenoid alcohols (terpineol, *t*-myrtanol, citronellol) were the only ones induced by phenobarbital.*

In the present study, after having checked that 4-methylumbelliferone (II), 4-hydroxycoumarin (VI), 4-nitrophenol (X), 4-hydroxybiphenyl (XXII), 1-butanol (L) and morphine activities were not induced, we measured the conjugation of twenty-eight other compounds, mostly monoterpenoid alcohols, but also some alkyl substituted phenolic compounds (Table 4).

The extent of the induction varied considerably from one aglycone to another, ranging from: 1.5 to 1.8 for phenolic compounds (XVII to XXI, XLVIII) to 2.5 to 3 for monoterpenoid alcohols (XXIX to XLV), except terpinen-4-ol (XXXIII) and fenchol (XLIV) for which no induction was observed and

* J. A. Boutin *et al.* manuscript in preparation.

Table 3. Activities of hepatic UDP-glucuronosyltransferases from control and phenobarbital-treated Gunn rats*

Aglycones (0.25 mM)	Control	Ratio of activity Gunn : Wistar	PB	Ratio of activity Gunn : Wistar
Coumarin				
VIII 4-Chromanol	2.1 ± 0.2	0.6	3.5 ± 0.3 (170)	0.5
Naphthol and phenols				
XLVIII 5,6,7,8-Tetrahydro-2-naphthol	14.2 ± 0.9	0.2	33.0 ± 0.2 (230)	0.3
XVI 3-Methoxyphenol	3.3 ± 0.2	0.2	4.2 ± 0.2 (130)	0.2
XVII Guaiacol	3.3 ± 0.1	0.4	5.3 ± 0.9 (160)	0.5
XVIII Eugenol	12.7 ± 0.6	0.6	59.8 ± 3.5 (470)	1.2
XIX Isoeugenol	10.0 ± 0.2	0.7	38.4 ± 1.0 (380)	1.5
XX Thymol	4.3 ± 0.4	0.5	8.5 ± 1.0 (200)	0.6
XLVI Carvacrol	3.7 ± 0.1	NDW†	4.7 ± 0.5 (130)	NDW
XXI 4-Isopropylphenol	6.4 ± 0.6	0.2	28.5 ± 0.8 (450)	0.6
XXII 4-Hydroxybiphenyl	1.0 ± 0.1	0.1	4.1 ± 1.3 (400)	0.1
XXIV 4-Hydroxydibenzyl	1.3 ± 0.1	0.1	5.2 ± 0.8 (400)	0.1
Monoterpenoid alcohols				
XXVI Citronellol	11.1 ± 0.3	0.9	25.6 ± 1.0 (230)	0.9
XXVII Nerol	7.2 ± 0.6	0.7	22.8 ± 0.4 (320)	0.9
XXVIII Geraniol	7.8 ± 0.3	0.6	21.3 ± 1.5 (270)	0.7
XXIX Menthol	9.3 ± 0.4	0.9	14.8 ± 0.2 (160)	0.9
XXX Terpineol	6.4 ± 0.3	0.8	8.8 ± 1.0 (140)	0.7
XXXI Carveol	9.0 ± 1.3	0.6	14.2 ± 0.2 (160)	0.7
XXXII Dihydrocarveol	5.6 ± 0.5	0.8	7.9 ± 0.4 (140)	0.7
XXXIII Terpinen-4-ol	4.1 ± 0.1	0.6	6.6 ± 0.6 (160)	0.7
XXXIV Isopulegol	5.2 ± 0.1	0.8	8.7 ± 0.4 (170)	0.8
XXXV Limonen-10-ol	8.6 ± 0.2	0.6	14.9 ± 1.4 (170)	0.6
XXXVI *trans*-Myrtanol	11.2 ± 0.6	0.7	19.1 ± 1.3 (170)	0.8
XXXVII *cis*-Myrtanol	10.7 ± 0.4	0.6	25.7 ± 2.0 (240)	0.7
XXXVIII Patchenol	7.0 ± 0.9	0.7	16.3 ± 0.1 (230)	0.7
XLI Myrtenol	11.1 ± 0.3	1.0	19.6 ± 0.5 (180)	1.0
XLII *cis*-Verbenol	9.2 ± 0.1	0.7	13.6 ± 0.8 (150)	0.6
XLIII 3-Methyl-2-norbornane methanol	8.7 ± 0.4	0.8	15.8 ± 0.8 (180)	0.8
XLIV Fenchol	4.5 ± 0.5	0.7	6.5 ± 0.4 (140)	0.8
XLVII Cedrol	13.2 ± 1.0	NDW	16.5 ± 0.6 (130)	NDW
XLV Borneol	8.0 ± 0.1	0.5	12.7 ± 0.5 (160)	0.5
XL Nopol	10.7 ± 1.3	0.8	27.9 ± 1.3 (260)	0.8
IXL Isopinocampheol	13.2 ± 1.0	1.3	20.3 ± 0.2 (160)	0.9

(*continued*)

Table 3 *continued*

Aglycones (0.25 mM)	Control	Ratio of activity Gunn:Wistar	PB	Ratio of activity Gunn:Wistar
Others				
Aniline	4.9 ± 0.2	0.8	8.4 ± 0.8 (170)	1.4
Paracetamol	2.2 ± 0.4	NDW	5.0 ± 0.3 (230)	NDW

* Values represent the means ± S.D. of four determinations from pooled fractions from four animals, and they are expressed in nmoles·min^{-1}·(mg protein)$^{-1}$. The numbers in parentheses provide the percentage of induction by phenobarbital. The ratios refer to the similar situation in Wistar rats (from Table 1).

† These activities were not determined in Wistar rats, and no ratio could be calculated.

Table 4. Hepatic microsomal UDP-glucuronosyltransferase activities from control and phenobarbital-treated guinea pigs*

Aglycones (concentration in the assay)	Controls (A)	PB (B)	Ratio B:A
Coumarins			
II 4-Methylumbelliferone (0.25 mM)	69.3 ± 0.7	76.0 ± 3.6	1.1
VI 4-Hydroxycoumarin (0.175 mM)	3.8 ± 0.3	4.8 ± 0.2	1.3
Naphthol and Phenols			
XLVIII 5,6,7,8-Tetrahydro-2-naphthol (0.175 mM)	45.4 ± 0.3	67.4 ± 1.2	1.5
X 4-Nitrophenol (0.062 mM)	14.5 ± 0.1	16.8 ± 0.9	1.2
XVII Guaiacol (0.175 mM)	16.1 ± 1.0	30.9 ± 1.6	1.9
XVIII Eugenol (0.175 mM)	51.0 ± 0.5	91.0 ± 4.6	1.8
XIX Isoeugenol (0.175 mM)	29.8 ± 0.3	47.8 ± 0.6	1.6
XX Thymol (0.175 mM)	7.9 ± 0.2	12.8 ± 2.4	1.6
XXI 4-Isopropylphenol (0.175 mM)	25.5 ± 0.5	44.3 ± 2.0	1.7
XLVI Carvacrol (0.175 mM)	11.6 ± 0.2	15.4 ± 0.8	1.3
XXII 4-Hydroxybiphenyl (0.175 mM)	40.5 ± 0.5	46.5 ± 0.8	1.1
IL 1,2,3,4-Tetrahydro-2-naphthol (0.175 mM)	9.1 ± 0.2	20.8 ± 0.3	2.3
Monoterpenoid alcohols			
XXV Linalool (0.175 mM)	4.0 ± 0.3	6.3 ± 0.1	1.6
XXVI Citronellol (0.175 mM)	10.1 ± 1.0	28.3 ± 0.9	2.8
XXVII Nerol (0.175 mM)	8.0 ± 0.3	18.5 ± 0.8	2.3
XXVIII Geraniol (0.175 mM)	10.0 ± 0.4	26.9 ± 1.1	2.7
XXIX Menthol (0.175 mM)	7.5 ± 0.2	16.2 ± 0.7	2.2
XXX Terpineol (0.175 mM)	8.2 ± 0.2	22.2 ± 0.4	2.8
XXI Carveol (0.175 mM)	7.0 ± 0.3	12.9 ± 0.4	1.8
XXXII Dihydrocarveol (0.175 mM)	7.7 ± 0.3	12.1 ± 0.5	1.6
XXXIII Terpinen-4-ol (0.175 mM)	6.1 ± 0.1	7.4 ± 0.3	1.2
XXXIV Isopulegol (0.175 mM)	7.4 ± 0.2	11.7 ± 0.3	1.6
XXXV Limonen-10-ol (0.175 mM)	8.8 ± 0.2	18.3 ± 0.4	2.1

(continued)

Table 4 *continued*

	Aglycones (concentration in the assay)	Controls (A)	PB (B)	Ratio B:A
XXXVI	*trans*-Myrtanol (0.175 mM)	11.8 ± 0.4	35.7 ± 0.9	3.0
XXXVII	*cis*-Myrtanol (0.175 mM)	10.9 ± 0.6	28.8 ± 1.4	2.6
XXXVIII	Patchenol (0.175 mM)	13.6 ± 0.3	28.6 ± 1.3	2.1
IXL	Isopinocampheol (0.175 mM)	8.6 ± 0.7	24.0 ± 0.4	2.8
XL	Nopol (0.175 mM)	9.4 ± 0.1	25.2 ± 0.4	2.7
XLI	Myrtenol (0.175 mM)	10.1 ± 0.3	24.7 ± 0.6	2.4
XLII	*cis*-Verbenol (0.175 mM)	7.0 ± 0.3	12.8 ± 0.4	1.8
XLIII	3-Methyl-2-norbornane methanol (0.175 mM)	8.4 ± 0.1	24.6 ± 1.2	2.9
XLIV	Fenchol (0.175 mM)	6.5 ± 0.1	7.7 ± 0.4	1.2
XLV	Borneol (0.175 mM)	9.6 ± 0.0	23.9 ± 1.5	2.5
Others				
	Morphine (0.25 mM)	6.6 ± 0.2	7.5 ± 0.3	1.1
L	1-Butanol (0.25 mM)	6.6 ± 0.2	7.9 ± 0.3	1.2

* Activities were determined on microsomal fractions from guinea pigs treated with phenobarbital. The corresponding controls received the vehicle only. The values are given as means ± S.D. of four determinations, from pooled fractions from four animals, and are expressed in nmoles·min^{-1}·(mg protein)$^{-1}$. The ratio B/A represents the measured induction by phenobarbital.

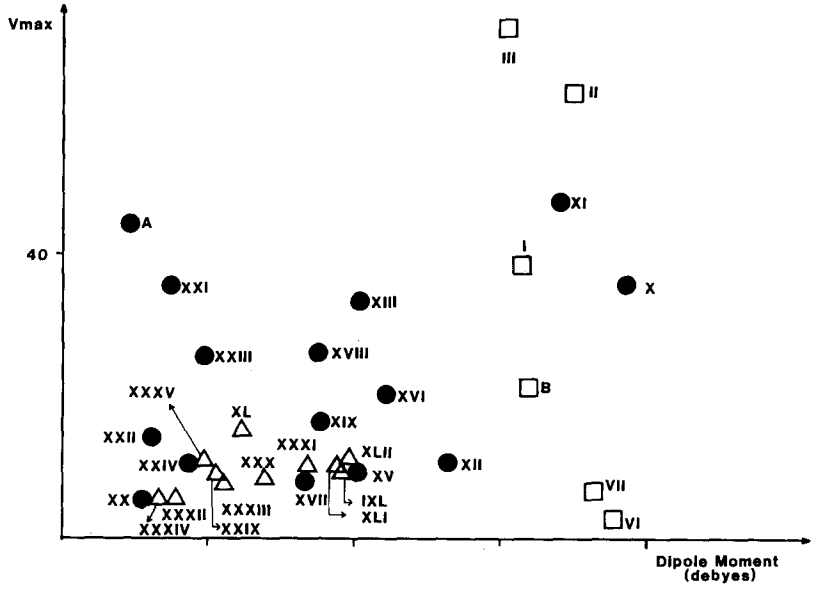


Fig. 5. Relationship of the apparent maximal velocity of untreated rat hepatic microsomal UDP-glucuronosyltransferase(s) measured with various aglycones to the calculated value of the aglycone dipole moment (μ). The apparent V_{max} values were obtained by measuring the activities with six concentrations of aglycone (0.05 to 0.6 mM) in the presence of 4.5 mM UDP-glucuronic acid with use of a microsomal preparation maximally activated with Triton X-100. The dipole moments were obtained from SCF calculations by using the experimental geometry of the molecules obtained by the CNDO/2 method [30]. 4,8-Dimethyl-7-hydroxycoumarin (IV) with $\mu = 7.4$ debyes was out of the range and is not plotted. A is 1-naphthol; B is aesculatin.

1,2,3,4-tetrahydro-2-naphthol (IL) which is not a monoterpenoid alcohol but which showed 2.3-fold induction.

Some structural characteristics of aglycones

In attempting to account for the differences in induction and activity among the different groups of compounds, we examined the structural characteristics of some of these compounds.

Dipole moment. We plotted (Fig. 5) the V_{max} versus μ (dipole moment) for the aglycones given in Table 2. The dipole moment was calculated by the experimental geometry obtained by the complete neglect of the differential overlap (CDNO/2) method [30] using calculation based on energetic and structural criteria (ESC). Although the data do not give a consistent correlation ($r = 0.2$), there are certain areas of correspondence between V_{max} and μ; for example, hydroxylated coumarins ($r = 0.76$) or monoterpenoid alcohols ($r = 0.80$).

Orientation of dipole moment and dimensions of the aglycones. In Fig. 6, we examined the orientation of these dipole moments, on the assumption that this could influence the orientation of the molecule inside the active site of the enzyme as shown for cytochrome P-450 [31]. The orientation of the dipole moment seems to be the same for a given chemical family of compounds. Thus, the orientation of an aglycone inside the active site might be always the same for this chemical family of compounds. Therefore, as indicated for the hydroxycoumarins (II and VI), the location of the hydroxyl group (which will be conjugated to glucuronic acid moiety) is of great importance. If the hydroxyl group is in the area of the transfer site of the GT_1,* V_{max} would be significant [as observed for 7-hydroxycoumarin (II)]. In contrast, V_{max} would be low [as observed for 4-hydroxycoumarin (VI)] if the hydroxyl group is not located in the same transfer area. This is in agreement with the data of Table 1 for these two series of coumarins.

In Fig. 7, we have compared the dimensions of some aglycones which are basically aromatic derivatives. According to previous findings [9], these compounds, which are flat, show increased conjugation after 3-methylcholanthrene induction; that is, typical GT_1 behavior, as exemplified by 4-nitrophenol (X). Examining the dimensions inside the aglycones, one can see that the length of the 3-nitro-4-cresol [from oxygen (O) to the farthest carbon] is 5.6 Å for this flat molecule. 2-Naphthol (IX), another typical GT_1 compound, has a distance O–a (see Fig. 7) of 6.3 Å and is also a flat compound. If the distance O–a is 6.3 Å, but the "a" carbon out of the plane of the molecule (e.g. 4-isopropylphenol, XXI), the molecule is bulkier and it behaves like a GT_1/GT_2 compound, namely its activity is enhanced by both 3-methylcholanthrene and phenobarbital. If an even bulkier compound is considered, 4-hydroxybiphenyl (XXII), this overlapping behaviour disappears and the compound is conjugated strictly as a GT_2 (inducible by phenobarbital). The last compound examined was 7-hydroxy-4-phenylcoumarin (V). This aglycone behaved strictly like a GT_1 compound, despite its length (O–a = 8.6 Å) and the bulkiness of the phenyl substitute (O–b = 6.4Å). This suggests that the two comparable compounds (XXII and V) are conjugated by active sites with different conformation or topology.

* In this study because of the broad specificity of UDPGT, GT_1 refers to GT(phenols) and GT_2 to GT(morphine), according to Bock *et al.* [32].

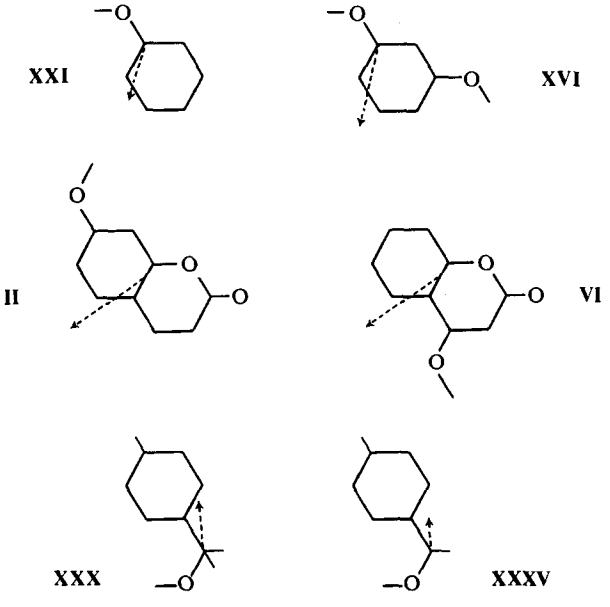
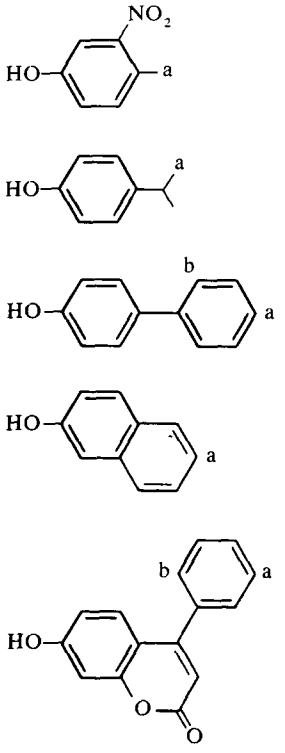


Fig. 6. Orientation of the dipole moments of six model substrates of UDP-glucuronosyltransferase(s). The orientation of the dipole moment (μ) was calculated from SCF by experimental geometry obtained with the CNDO/2 method [30]. The skeletons of the molecules are depicted with orientation and amplitude of the dipole moment represented by the arrow.



	Thickness		O-a	O-b
XIII	F	GT_1	5.6	—
XXI	B	GT_1-GT_2	6.3	—
XXII	B	GT_2	8.4	6.3
IX	F	GT_1	6.3	—
V	B	GT_1	8.6	6.4

Fig. 7. Comparison of the dimensions of some typical aglycones, substrates of UDP-glucuronosyltransferase(s). The molecules are classified GT_1 or GT_2 whether their activities in rat are induced by either 3-methylcholanthrene or phenobarbital, respectively, according to Wishart [5] and to Bock *et al.* [32]. The molecules are classified as flat, < 4.5 Å (F), or bulky, > 4.5 Å (B). "a" is the furthest carbon from the oxygen. O–a and O–b refer to the corresponding distances expressed in angstroms, and calculated from SCF by using the CNDO/2 method [30].

DISCUSSION

From our data it appears that, in Wistar rats, three families of aglycones can be distinguished: hydroxylated coumarins, phenols, and monoterpenoid alcohols. This idea is supported by the following data obtained in Gunn rat and in guinea pig. In the former, which is a mutant strain of Wistar rat, partially deficient in glucuronidation [26], some of these activities reached a "normal" level (i.e. the level of Wistar rat) after induction by phenobarbital. In guinea pig, phenobarbital specifically induced the activities toward a chemically defined family of aglycones (monoterpenoid alcohols).

The presence of a 2-methoxy or 2-methyl substituent on the phenol ring appeared to determine whether the corresponding aglycone is detoxified by GT_1 (phenol) or GT(monoterpenoid alcohols). Similarly, 4-alkyl substituents (isopropyl or *n*-propenyl) may also play a role in orienting the aglycone into the GT(monoterpenoid alcohol) active site. Substitution by an aryl group impairs the aglycone to be substrate of both GT_1 and GT(monoterpenoid alcohols). Similar results were observed in guinea pigs. Different behavior of the three structural familes of monoterpenoid alcohols was noted, even though it was not possible to determine whether they were related to different molecular forms of UDPGT. Our purpose was to determine whether a relationship exists between activities in rats (Wistar and Gunn) and guinea pigs and the structure of the considered aglycones.

We first considered flat aromatic molecules, in particular 4- and 7-hydroxycoumarins, for which the *in vitro* conjugations have not been carefully studied, but which are known to be eliminated *in vivo* as glucuronides [25]. Because they are flat, we expected them to behave like GT_1 aglycones [9]. Although 7-hydroxycoumarins behaved as expected, 4-hydroxycoumarins did not; the activities toward these compounds were low and were not induced by 3-methylcholanthrene. Nevertheless, their K_m values were low, similar to those for 7-hydroxycoumarins (Table 2) and were similar in controls and after induction. We therefore checked the possible influence of the direction of the dipole moment (μ) on the fixation of these molecules (Fig. 6). We showed that the low activity could be due to the position of the hydroxyl group on the coumarin skeleton.

It has been suggested that whether phenolic compounds are detoxified by GT_1 or GT_2 could depend on the nature, the hindrance, and the number of

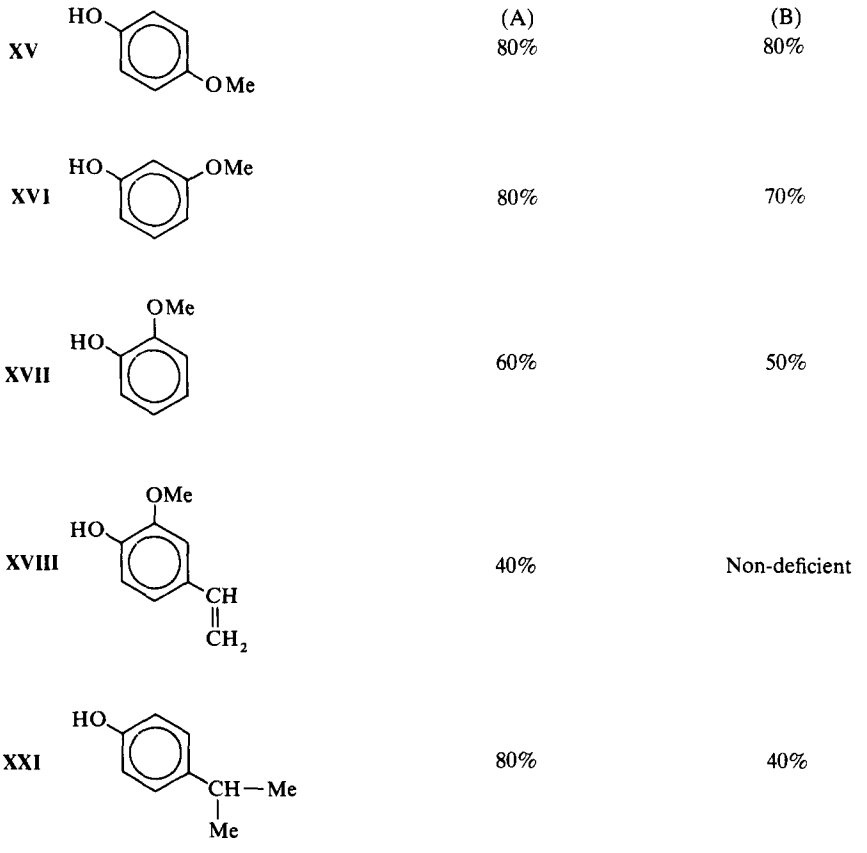


Fig. 8. Deficiencies of the hepatic microsomal UDP-glucuronosyltransferase activities toward five phenolic compounds in control (A) and phenobarbital-induced (B) Gunn rats. The values are given as the ratio to the corresponding activity in control or phenobarbital-induced Wistar rats taken as non-deficient.

carbons of their substituents [10, 11, 33, 34]. As shown in Fig. 7, certain characteristics define the behavior of these phenols. Thus, a carbon in an aglycone at 6.3 Å or more out of a plane imposes on the molecule a GT_2 behavior. Nevertheless, 4-phenyl-7-hydroxycoumarin showed strictly GT_1 behavior, even though the distance to the nearest carbon out of the plane was 6.4 Å. We therefore suggest that this compound may be conjugated by a specialized enzymatic form. This has been confirmed by recent findings [8, 14, 22] showing differential behavior of coumarin and phenol conjugations. Therefore, it can no longer be claimed that all the flat molecules such as benzo[a]pyrene [35] are conjugated by GT_1, irrespective of their structure. A similar situation has been observed for cytochromes P-450 in which dealkylation of anisole (phenol) and ethoxycoumarin are not catalyzed by the same enzymatic isoform [36, 37].

Gunn rats with deficient UDPGT activities and induction [14, 38] provide us with an even better model to define UDPGT heterogeneity. On the basis of the extent of deficiency, we suggest that the aglycones can be subdivided into three groups: planar compounds, deficient, mainly conjugated by the GT_1 form; activities towards monoterpenoid alcohols, less deficient after phenobarbital induction; and GT_2 conjugating bulky aglycones, half deficient [9, 26]. This is in agreement with other studies on different aglycones [14].

In the present studies we have examined in greater detail GT(monoterpenoid alcohol) compounds (Table 3) and some interesting phenolic aglycones. The variation of both deficiency and phenobarbital inducibility of the activities toward phenolic compounds (XVI to XXI) shows (Fig. 8) that the movement of methoxy group from position 4 or 3 (XV, XVI, deficient) to position 2 (XVII, less deficient), would permit the compound to fit better in the GT(monoterpenoid alcohols) active site. Either stabilization of the hydroxy group or a spatial conformation could give to the aglycone a GT(monoterpenoid alcohol)-like behavior (Fig. 8). Since there is no GT_1 activity in Gunn rats [13–15] we assume that phenobarbital induces GT(monoterpenoid alcohol), so that the structure of a compound could give information on the GT(monoterpenoid alcohols) site structure. This was the case for 4-isopropylphenol (XXI) activity, which was deficient in control Gunn rats, but was enhanced 4.5 times after phenobarbital induction, thus resulting in only a 40% deficiency. Therefore, the 4-alkyl substitution seems to fit in the GT(monoterpenoid alcohols) active site, since no other compounds behaved in this manner. For example, the very low activity toward carvacrol (XLVI) suggests that, if the alkyl substituent is in

the 3-position, the aglycone is not able to fit to this GT(monoterpenoid alcohols) active site. The behavior of two other aryl phenols (XXII, XXIV) leads us to conclude that these compounds are neither conjugated by GT_1(phenol) nor GT(monoterpenoid alcohol). Most of the monoterpenoid alcohol activities were not significantly deficient in induced or control Gunn rats. This clearly suggests that heterogeneity is not limited to the five isoforms [6, 7] but depends to a greater extent on chemical structure of the substrates.

In a preliminary investigation of guinea pigs, we found a specific UDPGT activity towards some monoterpenoid alcohols. In extending these studies, the present values obtained in the guinea pig were slightly lower than those found previously.* However, we studied a different strain obtained from a different company and we believe that strains are of importance in guinea pig levels of UDPGT activities as they are in mouse [39] and rat [40].

In guinea pig, phenobarbital induction led to a very selective increase of the activities toward monoterpenoid alcohols. The induction towards aromatic planar aglycones was limited (II, VI, X, XLVIII) and not statistically significant. From XXV to XLV, all the monoterpenoid alcohol activities were increased about 1.8, 2 or 2.5 over control, depending on the chemical nature of the monoterpenoid alcohols. The overlapping aglycone activities were similar to those in Gunn rats (*vide supra*); after induction, eugenol (XVIII) and isoeugenol (XIX) gave a limited increase (1.8), which was between GT_1 (1.2) and monocyclic monoterpenoid alcohols (2.8). These two phenolic aglycones have structures comparable with aliphatic monoterpenoid alcohols. They could be studied with a more specific method of measurement [41].

It has been suggested [42] that a study of the way in which kinetic constants, and particularly the K_m values, are changed might aid evaluation of the heterogeneity of UDPGT. Our results indicate, however, that apparent K_m values do not provide any new data on heterogeneity. Even the determination of kinetic constants in rat [43] and in guinea pig after phenobarbital induction,* does not give new information, particularly if more than a single binding site for UDP-glucuronic acid exists [43, 44].

We suggest that further examination of UDPGT heterogeneity will be greatly aided by studies on induction assessed with chemically related families of aglycones rather than single compounds. Currently we are investigating the isolation of UDPGT based on these approaches.

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