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GLUCURONIDE FORMATION OF VARIOUS DRUGS IN LIVER MICROSOMES AND IN ISOLATED HEPATOCYTES FROM PHENOBARBITAL- AND 3-METHYLCHOLANTHRENE-TREATED RATS

DIETER ULLRICH and KARL WALTER BOCK*
Department of Pharmacology and Toxicology, University of Göttingen, Kreuzbergring 57, D-3400 Göttingen, Federal Republic of Germany

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Abstract—Various substrates of rat liver microsomal UDP-glucuronosyltransferase were classified *in vitro* as preferred substrates of either 3-methylcholanthrene- or phenobarbital-inducible enzyme forms. Microsomal UDP-glucuronosyltransferase activities towards a third group of substrates (including oestrone, phenolphthalein, paracetamol and oxazepam) are not markedly altered by treatment with either 3-methylcholanthrene or phenobarbital. Some substrates of the 3-methylcholanthrene- and phenobarbital-inducible enzyme activities were selected to evaluate the importance of multiple enzyme forms for glucuronide formation in the intact cell. The metabolism of these compounds was compared in isolated hepatocytes from untreated controls and from rats treated with 3-methylcholanthrene (MC-hepatocytes) or phenobarbital (PB-hepatocytes). Glucuronidation of 1-naphthol and 3-hydroxybenzo[*a*]pyrene was chiefly enhanced in MC-hepatocytes (> 2-fold), whereas glucuronidation of chloramphenicol and bilirubin was chiefly enhanced in PB-hepatocytes. These observations are in agreement with differential induction of UDP-glucuronosyltransferase activities *in vitro* suggesting that, besides other factors such as cofactor supply, physiological activators, etc., the levels of the multiple enzyme forms are critically determining glucuronide formation in the intact cell.

Glucuronide formation is a major pathway in the biotransformation and elimination of a wide variety of lipid-soluble drugs, environmental chemicals and endogenous compounds such as bilirubin and steroid hormones [1]. The products are polar, water-soluble β-D-glucuronides which are efficiently eliminated from the body either via the bile or urine. Accumulating evidence suggests that glucuronidation is catalysed by multiple enzyme forms of GT† [2, 3]. Shortly after its discovery, GT was found to be inducible by xenobiotics [4, 5]. More recently, evidence has been obtained that the multiple GT forms are under different regulatory control [6–9]. Differential inducibility by 3-methylcholanthrene and phenobarbital has been used to classify GT activities [7, 8, 10, 11]. Two groups have been distinguished. Group 1 activities, including those towards planar phenols, were selectively inducible by 3-methylcholanthrene whereas group 2 activities were markedly inducible (>2-fold) by phenobarbital. A similar classification was obtained on the basis of perinatal development of GT activities [12]. Moreover, group 1 activities were copurified during isolation and purification of the 3-methylcholanthrene-inducible GT [10]. Because of the marked phospholipid-dependencies of GT activities, the question remained whether changes in the enzyme activity reflect similar changes in the enzyme level. Using rabbit antibodies against the MC-inducible GT, it was ascertained that increased GT activities towards group 1 substrates reflected an increased enzyme level [13, 14]. However, the physiological implications of enzyme induction remain to be evaluated because of the complexity of factors regulating glucuronide formation in the intact cell [1]. For example, (a) the enzyme level of multiple enzyme forms, (b) the latency of the firmly membrane-bound enzyme, (c) activators and inhibitors, (d) concentration of substrates at the active site of GT and (e) cofactor supply. We therefore investigated glucuronidation of typical substrates of the aforementioned groups 1 and 2 in hepatocytes from untreated controls and from rats treated with MC (MC-hepatocytes) or PB (PB-hepatocytes) in order to elucidate the implications of enzyme induction on glucuronide formation in the intact hepatocyte.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: 1-[1-^{14}C]naphthol, [4-^{14}C]oestrone, D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol and UDP-D-[U-^{14}C]glucuronic acid were from the Radiochemical Centre (Amersham-Buchler, Braunschweig, F.R.G.). Collagenase (Type II) was from Worthington (Freehold, NJ); Brij 58, polyoxyethylene(20)cetyl ether from Atlas (Essen, F.R.G.); 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenzo[*a*]pyrene sulfate from the NCI Chemical Carcinogen Reference Standard Repository (NIH, Bethesda, MD); paracetamol from Thomae (Biberach, F.R.G.); oxazepam from Wyeth (Münster, F.R.G.).

Treatment of animals. Male Wistar rats (160–200 g) were used. Phenobarbital sodium was administered

* To whom correspondence and reprint requests should be addressed.

† Abbreviations: GT, UDP-glucuronosyltransferase; MC, 3-methylcholanthrene; PB, phenobarbital.

by injecting one dose of 100 mg/kg i.p., followed by 0.1% in drinking water for 4 days. 3-Methylcholanthrene was administered as one i.p. dose of 40 mg/kg, dissolved in olive oil. Animals were killed on the fifth day. Liver microsomes were prepared as previously described [15].

Assays of UDP-glucuronyltransferase. Enzyme activities towards various substrates were assayed with the following aglycone concentrations and by methods already described: 0.5 mM 1-naphthol [15], 0.5 mM 4-methylumbelliferone [8], 0.05 mM 3-hydroxybenzo[*a*]pyrene [16], 1.5 mM chloramphenicol [17], 0.1 mM bilirubin [18], 0.5 mM phenolphthalein [19] and 5 mM paracetamol [20]. The glucuronidation of 1.5 mM oxazepam was determined with [^{14}C]UDP-glucuronic acid similar to the method used for calibration of the fluorescence of 3-hydroxybenzo[*a*]pyrene [16].

Care was taken to achieve full enzyme activation and to perform the assay under conditions leading to linear reaction rates with time and protein concentration. The enzyme was fully activated by the addition of Brij 58 (0.5 mg/mg protein), with the exception of bilirubin-GT which was activated by the addition of digitonin (3 mg/mg protein).

Our fluorescence assays are based on monitoring fluorescence of the respective glucuronides. They were carried out using a Perkin–Elmer model 650-10S fluorescence spectrophotometer. Fluorescence intensity was calibrated with quinine sulfate.

Protein was determined according to the method of Lowry *et al.* [21] with bovine serum albumin as protein standard.

Isolation and incubation of hepatocytes. Hepatocytes were isolated by collagenase perfusion as previously described [22] and suspended in Krebs–Henseleit buffer, pH 7.4. The cells excluded trypan blue (> 95%) and NADH (*ca* 80%). NADH exclusion was determined according to Moldéus *et al.* [23]. Incubations were performed at 37° in shaking round-bottomed tubes under an O_2–CO_2 (95:5, v/v) atmosphere at a cell concentration of 2×10^6 cells/ml. The incubation medium was Krebs–Henseleit buffer, pH 7.4. In the experiments with bilirubin, the incubation medium contained bovine serum albumin (2%, w/v) and 4×10^6 cells/ml. Experiments with 3-hydroxybenzo[*a*]pyrene and bilirubin were performed under subdued light. Substrates (with the exception of bilirubin) were dissolved in dimethyl sulfoxide, leading to a final concentration of less than 0.4% of the solvent in the incubation medium.

Analytical methods: 1-naphthol glucuronidation. Incubation of cells with 1-naphthol in a volume of 1.0 ml was terminated with an equal volume of ice-cold acetone. After centrifugation of the precipitated protein, a sample (0.1 ml) of the clear supernatant fluid was applied to TLC plastic sheets (silica gel 60 F_{254}, Merck, Darmstadt, F.R.G.) and chromatographed in 1 M aqueous ammonium acetate–ethanol (1:9, v/v). In this system 1-naphthol glucuronide ($R_f = 0.33$) and sulfate ($R_f = 0.79$) were clearly separated and visualized by UV absorption. The dark spots were cut out of the plastic sheets and extracted with 2 ml methanol. Extracts (1 ml) were added to 0.1 ml 1.6 M glycine/NaOH, pH 10.3, and both conjugates were measured fluorimetrically at 355 nm, with excitation at 288 nm [15]. Fluorescence of zero-time blanks was subtracted. Calibration was carried out with authentic standards. 1-Naphthol could not be completely separated from 1-naphthol sulfate. However, the fluorescence spectrum of 1-naphthol sulfate markedly differs from that of 1-naphthol at pH 10.3. Therefore 1-naphthol did not interfere with the determination of 1-naphthol sulfate. 1-Naphthol glucuronidation was also determined with [^{14}C]-1-naphthol. In this case deproteinized samples (0.5 ml) were extracted first with 5 ml chloroform to remove [^{14}C]-1-naphthol. Samples were chromatographed and conjugates were eluted as described above and quantitated by liquid scintillation counting.

3-Hydroxybenzo[a]*pyrene glucuronidation.* Incubation was terminated by the addition of acetone as described above. Conjugates were chromatographed in ethyl acetate–methanol–H_2O–formic acid, 100:25:10:1 (v/v/v/v). 3-Hydroxybenzo[*a*]pyrene glucuronide ($R_f = 0.4$) and sulfate ($R_f = 0.6$) were visualized by their strong fluorescence. 3-Hydroxybenzo[*a*]pyrene glucuronide was cut out from the plastic sheets, eluted with methanol and quantitated fluorimetrically in glycine–NaOH, pH 10.3, at 425 nm, with excitation at 381 nm. Calibration of the fluorescence of 3-hydroxybenzo[*a*]pyrene glucuronide was carried out with [^{14}C]-3-hydroxybenzo[*a*]pyrene glucuronide as described previously [6] and with a standard of 3-hydroxybenzo[*a*]pyrene sulfate.

Bilirubin glucuronidation. Bilirubin was dissolved shortly before the experiment in 0.1 M Na_2CO_3 containing 2% albumin, and the mixture was neutralized with HCl. Incubations were terminated by the addition of 2 ml 0.4 M glycine–HCl, pH 2.7, and bilirubin conjugates were determined by the formation of ethyl anthranilate azopigments at pH 2.7, according to Heirwegh *et al.* [24]. Bilirubin mono- and di-conjugate formation was estimated from the relative amounts of α_0 (unconjugated azodipyrrole) and δ (azodipyrrole glucuronide) pigments. They were separated on silica-gel plastic sheets in chloroform–methanol–H_2O, 65:25:3 (v/v/v), eluted with methanol and quantified colorimetrically [18].

Chloramphenicol glucuronidation. Incubations were terminated by extraction with 3 ml isoamyl acetate. This extraction was repeated twice to remove unconjugated chloramphenicol completely. Radioactivity in the aqueous phase was used to quantitate chloramphenicol glucuronide [17, 25]. Zero-time blanks were subtracted. To exclude other metabolic products in the aqueous phase, aliquots of the cell-free supernatant fluid were chromatographed on silica-gel plates in ethyl acetate–methanol–H_2O–formic acid, 100:25:10:1 (v/v/v/v). One radioactive peak ($R_f = 0.4$) was found corresponding to the reference sample of chloramphenicol 3-glucuronide. Chloramphenicol 3-glucuronide was synthesized by incubating microsomes with [^{14}C]chloramphenicol and UDP-glucuronic acid [17].

Determination of UDP-glucuronic acid. UDP-Glucuronic acid was determined enzymatically as described [15, 26, 27]. Cell suspensions containing 2×10^6 cells/ml were boiled for exactly 2 min, chilled on ice and centrifuged for 10 min at 10,000 *g*.

UDP-Glucuronic acid was determined in the supernatant fluid by means of the 4-methylumbelliferone-GT assay [8], which contained guinea-pig liver microsomes as the enzyme source to avoid degradation of UDP-glucuronic acid. The assay was started by the addition of 4-methylumbelliferone and stopped after a 10 min incubation by adding $HClO_4$. The recovery of UDP-glucuronic acid was determined in identical cell suspensions to which known amounts of UDP-glucuronic acid had been added before boiling. The recovery of UDP-glucuronic acid was 60%.

RESULTS

Classification of GT activities

Besides group 1 and 2 activities which are chiefly inducible by 3-methylcholanthrene or phenobarbital, respectively, a third group of GT activities can be recognized in the model of differential induction (Table 1). Group 3 activities, including widely used drugs such as paracetamol and oxazepam, are only marginally affected (<2-fold) by the above inducers on the basis of GT activity per mg of microsomal protein. The extent of induction by phenobarbital is underestimated in the above model because the known proliferation of endoplasmic reticulum membranes under the influence of phenobarbital is not taken into account. Although our classification must be somewhat arbitrary, the model illustrates 'functional heterogeneity' of GT and may be helpful in delineating GT forms. Overlapping substrate specificity of a drug for inducible and constitutive enzyme forms has to be taken into account in glucuronidation studies of various compounds. For the studies with hepatocytes we therefore selected substrates which have been classified as typical group 1 or group 2 activities.

Glucuronidation of various compounds in isolated hepatocytes from untreated controls and from MC- and PB-treated rats

As shown in Fig. 1, the rate of glucuronidation of 1-naphthol or 3-hydroxybenzo[*a*]pyrene was chiefly enhanced in MC-hepatocytes (>2-fold). Both phenols are also conjugated with sulfate. In hepatocytes from untreated controls the rate of 1-naphthol glucuronide to 1-naphthol sulfate was *ca* 2:1. In the experiments with 3-hydroxybenzo[*a*]pyrene the ratio of glucuronide to sulfate was *ca* 1:2.

Sulfate formation was unaltered in control-, PB- and MC-hepatocytes, confirming that phenol sulfotransferase is not inducible by xenobiotics [28, 29]. In contrast to the aforementioned group 1 substrates, glucuronidation of chloramphenicol and bilirubin was chiefly enhanced in PB-hepatocytes (>2-fold). Chloramphenicol glucuronidation was slightly enhanced in MC-hepatocytes. Bilirubin monoglucuronide was the major conjugate (>90%) at the high dose of bilirubin (50 nmole/10^6 hepatocytes), shown in Fig. 1. However, at one-tenth of this dose bilirubin was mostly converted to the diglucuronide (56% of total conjugates), in agreement with the results of Blanckaert *et al.* [30].

UDP-Glucuronic acid levels in control-, PB- and MC-hepatocytes

In freshly isolated control- and MC-hepatocytes the level of UDP-glucuronic acid was low. It was higher in freshly isolated PB-hepatocytes (Table 2). However, after a 20 min incubation in the presence of 1-naphthol the UDP-glucuronic acid level had increased in control- and MC-hepatocytes, and a similar cofactor level was found in all three types of hepatocytes investigated. When hepatocytes were incubated in the absence of 1-naphthol the cofactor level was not significantly altered.

Table 1. Classification of rat liver microsomal UDP-glucuronyltransferase activities using the model of differential induction by 3-methylcholanthrene or phenobarbital

	UDP-Glucuronyltransferase activity (nmole/min per mg protein)		
Substrate	Control	3-Methylcholanthrene treatment	Phenobarbital treatment
(1) 1-Naphthol	66 ± 9	226 ± 19 (3.4)	85 ± 10 (1.3)
4-Methylumbelliferone	68 ± 3	284 ± 21 (4.2)	120 ± 14 (1.8)
4-Nitrophenol*	38 ± 5	158 ± 17 (4.2)	61 ± 9 (1.6)
3-Hydroxybenzo[*a*]pyrene	2.3 ± 0.5	11.7 ± 1.3 (5.1)	3.4 ± 0.5 (1.5)
(2) 4-Hydroxybiphenyl*	26 ± 4	33 ± 5 (1.3)	115 ± 11 (4.4)
Morphine*	7.9 ± 0.6	9.9 ± 1.7 (1.3)	18.0 ± 2.8 (2.3)
Chloramphenicol	1.0 ± 0.5	1.5 ± 0.4 (1.5)	5.2 ± 0.0 (5.0)
Bilirubin	1.8 ± 0.2	1.6 ± 0.4 (0.9)	3.6 ± 0.5 (2.0)
(3) Phenolphthalein	24 ± 2	24 ± 2 (1.0)	26 ± 3 (1.1)
Paracetamol	2.9 ± 0.6	3.9 ± 0.8 (1.3)	3.2 ± 0.5 (1.1)
Oxazepam	0.5 ± 0.3	0.7 ± 0.3 (1.4)	0.6 ± 0.2 (1.2)
Oestrone	0.8 ± 0.4	1.0 ± 0.3 (1.3)	0.9 ± 0.2 (1.1)

* Data taken from ref. [10].

Data represent the means ± S.D. of four determinations. Values in parentheses represent the induction factor, i.e. the ratio between GT activity in treated animals vs untreated controls.

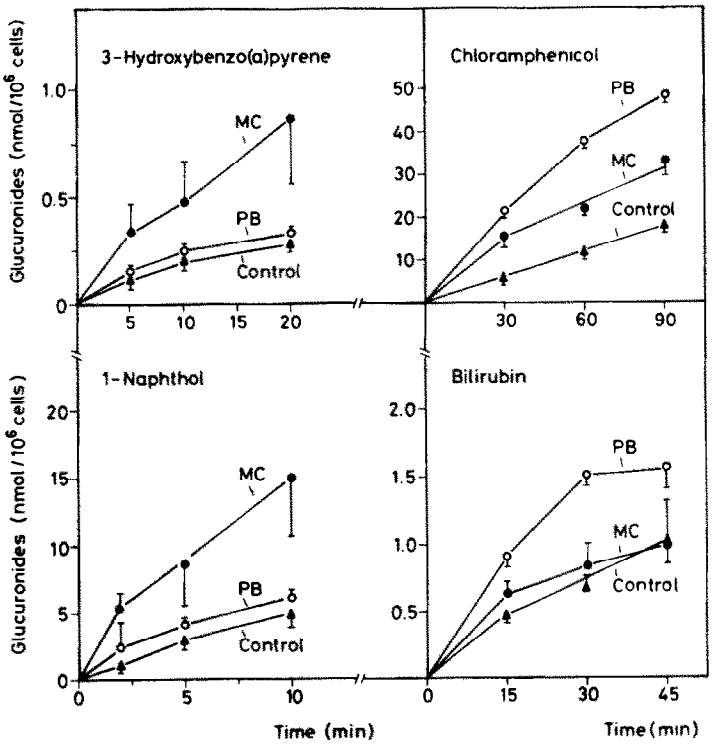

Fig. 1. Glucuronide formation of various substrates in control-, PB- and MC-hepatocytes. Control, PB and MC indicate experiments with control-, PB- and MC-hepatocytes, respectively. Substrates were added to the incubation mixtures at the following doses per 10^6 cells: 10 nmole 3-hydroxybenzo[*a*]pyrene, 25 nmole 1-naphthol, 300 nmole chloramphenicol and 50 nmole bilirubin. Data represent the means ± S.D. of four experiments.

DISCUSSION

In order to evaluate the significance of differential induction of GT activities for glucuronide formation in the intact cell, we compared glucuronidation rates of various substrates in control-, MC- and PB-hepatocytes. Care was taken to select typical group 1 or 2 substrates (induction factor >2). Even for typical group 1 substrates it cannot be excluded that they are substrates for more than one enzyme form. As shown in Fig. 1, the glucuronidation rate of group 1 substrates was selectively increased in MC-hepatocytes whereas the glucuronidation of chloramphenicol and bilirubin was chiefly enhanced in PB-hepatocytes, in agreement with the induction of GT activities *in vitro* (Table 1). This correlation emphasizes the fact that the enzyme level of a particular enzyme form is a major determinant of glucuronide formation in the intact cell. Differential stimulation of cellular glucuronide formation by inducing agents has also been observed by Andersson *et al.* [31], although the substrates investigated had not been classified. In the latter study the glucuronidation of phenolphthalein was unaltered in MC- and PB-hepatocytes whereas the glucuronidation of 4-methylumbelliferone and 2-naphthol was chiefly enhanced in MC-hepatocytes, in agreement with the substrate groups listed in Table 1. 2-Naphthol, which is not included in Table 1, has been classified as a group 1 substrate (W. Lilienblum and K. W. Bock, unpublished results).

Table 2. UDP-Glucuronic acid levels in isolated hepatocytes from untreated controls and from 3-methylcholanthrene- and phenobarbital-treated rats

Treatment *in vivo*	UDP-Glucuronic acid (nmole/10^6 cellls)	
	(2 min)	(20 min)
Untreated controls	0.98 ± 0.2	2.13 ± 0.41 (2.55 ± 0.49)
3-Methylcholanthrene	1.08 ± 0.25	1.80 ± 0.34
Phenobarbital	1.91 ± 0.25	2.10 ± 0.07

UDP-Glucuronic acid was determined shortly after isolation (2 min) and after 20 min incubation in the presence of 1-naphthol under the experimental conditions of Fig. 1. The value in parentheses represents the UDP-glucuronic acid level determined in the absence of 1-naphthol. The means ± S.D. of four experiments are listed.

Although the level of UDP-glucuronic acid in control-, MC- and PB-hepatocytes differed initially, a similar cofactor level was found after 20 min incubation. This level (*ca* 2 nmole UDP-glucuronic acid/10^6 cells) was similar to that described by Moldéus *et al.* [33]. One g liver represents *ca* 127×10^6 liver cells [22], and this gives a cofactor level of 0.25 μmole/g liver, in agreement with studies *in vivo* [15, 32] and in perfused liver [15, 27]. The level of UDP-glucuronic acid is the resultant of both continuous synthesis and degradation or consumption, and it has been demonstrated that the cofactor concentration drops initially after addition of sub-

strate [33]. Nevertheless, the similarity of the cofactor concentration in control-, PB- and MC-hepatocytes as well as in perfused liver and *in vivo* suggests that regulatory mechanisms must exist in the intact cell to maintain a physiological concentration, as has been shown with other cofactors.

Despite the above reasoning, cofactor supply may limit glucuronidation under a variety of conditions: (1) limited carbohydrate reserves in perfused livers from fasted rats [34] and in diabetes [35]; (2) presence of uridylate trapping agents [36] or ethanol [33]. Normally, however, the capacity to regenerate UDP-glucuronic acid appears to be quite high in liver. Therefore in most cases levels of various enzyme forms may be major factors determining glucuronide formation in the intact cell.

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