Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 867–872. Pergamon Press. Printed in Great Britain

STEROID AND NON-STEROID UDP GLUCURONYLTRANSFERASE: GLUCURONIDATION OF SYNTHETIC ESTROGENS AS STEROIDS

GEORGE W. LUCIER and OTELIA S. MCDANIEL
Environmental Toxicology Branch, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

(*Received* 30 *November* 1976)

SUMMARY

The hepatic microsomal glucuronidation profiles of eight substrates were investigated; *p*-nitrophenol (PNP), 4-methylumbelliferone (MUB), 1-naphthol (N), diethylstilbestrol (DES), β-estradiol (E_2), estrone (E_1), testosterone (T), and phenolphthalein (P). Three differences between microsomal steroid and non-steroid glucuronidations were characterized: (1) non-steroid uridine diphosphoglucuronyltransferase (UDPGT) exhibited a developmental peak about the time of birth such that hepatic activity in one-day-old animals exceeded adult levels whereas this peak was not observed for steroid UDPGT, (2) non-steroid UDPGT was TCDD-inducible whereas steroid UDPGT was not, and (3) non-steroid UDPGT exhibited significant activity in kidney and uterus whereas steroid glucuronidation rates were low or non-detectable in these tissues. PNP, MUB, and N were glucuronidated as non-steroids and E_2, E_1, T, DES, and P were clearly in the steroid class. A positive correlation existed between the existence of steroidal activity and the occurrence of steroid glucuronidation characteristics. No apparent qualitative differences were evident between glucuronidation of E_2 at the 3- and 17-positions.

INTRODUCTION

Uridine diphosphoglucuronyltransferase (UDPGT) is predominantly a microsomal enzyme, which plays a role in the detoxication of foreign chemicals [1] and is also an important steroid metabolic pathway [2]. Considerable evidence has been produced recently to demonstrate the multiplicity of UDPGT [3–7], including differences between steroid and non-steroid glucuronidation [8–11].

Many of the foreign chemicals contaminating the environment exhibit significant steroidal properties which could result in a variety of toxicological symptoms. Diethylstilbestrol (DES), a potent synthetic estrogen, has been used as an anti-abortive drug and cattle food additive. DES has been implicated as a transplacental carcinogen, and it can also affect fertility in both male and female offspring exposed to DES during gestation [12, 13]. Since the major metabolic pathway of DES is glucuronidation [14], we were interested in determining whether DES glucuronidation characteristics resembled steroid or non-steroid substrates. To answer this question, we first attempted to clearly define differences between steroid and non-steroid glucuronidation according to three criteria. The first criterion involved the characterization of perinatal developmental patterns, which have been reported to differ between testosterone and *p*-nitrophenol [10]. The second criterion was UDPGT inducibility following treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD induces *p*-nitrophenol glucuronidation but has no effect on testosterone glucuronidation [9]. The third criterion involves relative tissue distributions of steroid and non-steroid UDPGT, which could differ if multiple forms of UDPGT are present.

In the studies described here, we selected several steroid and non-steroid substrates; β-estradiol (E_2), estrone (E_1), testosterone (T), DES, 1-naphthol (N), phenolphthalein (P), *p*-nitrophenol (PNP), and 4-methylumbellifone (MUB). The primary goals were to characterize clear and consistent differences between steroid and non-steroid UDPGT in rat microsomes and then to determine if foreign chemicals possessing steroidal activity are glucuronidated as steroids or non-steroids.

MATERIALS AND METHODS

Chemicals. TCDD (Lot No. 851-144-II; purity > 99%) was supplied as a gift by the Dow Chemical Company (Midland, MI). E_2, E_1, T, MUB, N, PNP, DES, and P were obtained from Sigma Chemical Company, St. Louis, MO. Respective glucuronic acid metabolites with the exception of DES monoglucuronide were also obtained from Sigma. DES monoglucuronide was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI.

Abbreviations: PNP = *p*-nitrophenol, MUP = 4-methylumbelliferone, N = 1-naphthol, DES = diethylstilbestrol, E_2 = β-estradiol, E_1 = estrone, T = testosterone, P = phenolphthalein, UDPGT = uridine diphosphoglucuronyltransferase, TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

^{14}C-labeled substrates (purity > 98%) had the following specific activities: DES (monoethyl-^{14}C), 52 mCi/mmol; [4-^{14}C]-estrone, 52 mCi/mmol; [4-^{14}C]-estradiol, 54 mCi/mmol; [4-^{14}C]-testosterone, 59 mCi/mmol, [1-^{14}C]-1-naphthol, 20 mCi/mmol.

Animals and tissue preparation. Pregnant rats (CD-stock, Sprague–Dawley derived) were bred at the NIEHS. The sperm positive day was designated as day 0 and the average length of gestation was 22 days. Adult CD-1 male and female rats were obtained from Charles-River Breeding Company (Wilmington, MA). The animals were maintained on synthetic diets (Wayne Sterilizable Lab-Blox, Allied Mills, Inc., Chicago, IL.) and allowed free access to water. Bedding areas for rat litters and adult animals were filled with hardwood chips.

Pregnant rats were administered 3 μg TCDD/kg as a single oral dose (in 0.5 ml corn oil) on the 15th day of gestation. This dose did not result in measurable fetotoxicity. Control animals received 0.5 ml corn oil. Fetuses and offspring were sacrificed by decapitation at designated ages. Microsomes from liver and kidney tissues were prepared as described previously [15] using 150 mM Tris–HCl (pH 7.4) and resuspended in buffer so that one ml suspension contained microsomes from 0.5 g tissue. Uteri were minced in four vol. of Tris buffer, subjected to three 15-sec bursts from a Brinkman Polytron at 30 Hz and microsomes prepared as described previously [16].

Enzyme assays. The standard incubation medium used to measure glucuronidation contained 10 μmol $MgCl_2$ and 2 μmol UDPGA in a total incubation vol. of 1.0 ml. Substrate concentrations were as follows: PNP, 0.8 mM; MUB, 1.2 mM; N, 1.2 mM; P, 0.8 mM; DES, 0.4 mM; E_2, 0.3 mM; E_1, 0.3 mM; T, 0.3 mM. These concentrations included the addition of approximately 0.01 μCi of ^{14}C-labeled substrate when activities were determined by radioassay (DES, E_2, E_1, T, and N). After the incubation mixtures were warmed at 37° for three min., 0.3–1.0 mg microsomal protein was added. The reaction time was 5–20 min. Incubation conditions were designed so that glucuronidation rates were linear for the duration of the incubation period. In assays utilizing colorimetric techniques (PNP, MUB, P), the reaction was stopped by the addition of 5.0 ml of 0.2 M glycine buffer (pH 10.4) and substrate disappearance measured; PNP at 405 nm, MUB at 365 nm, and P at 550 nm. N, E_2, E_1, DES, and T glucuronidations were measured radiometrically and these reactions were stopped by the addition of 10 ml of non-aqueous scintillation fluid. Substrate disappearance or product appearance was measured as described previously [16]. Separate measurements of glucuronidation rates at the 3- and 17-positions were made by resolving E_2, E_2-3β-D-glucuronide, and E_2-17β-D-glucuronide on DEAE-sephadex A-25 columns [17]. Five ml fractions were collected at a flow rate of approximately one ml/min.

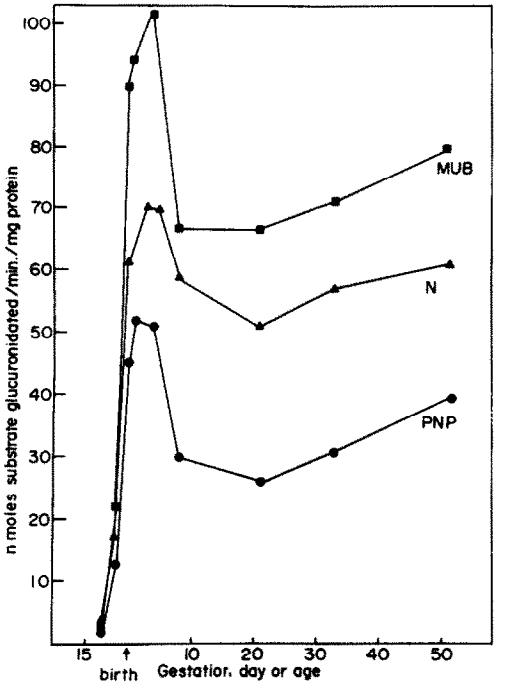

Fig. 1. Perinatal development of non-steroid UDPGT conjugating PNP (●), MUB (■), and N (▲) in rat liver. Each litter was pooled and data for each developmental stage represents the average of three separate litters.

Protein determinations were made by the method of Lowry *et al.* [18].

RESULTS

Perinatal development of UDPGT

Developmental patterns for MUB, N, and PNP in rat liver microsomes are illustrated in Fig. 1. None of the three substrates have reported steroidal activity. The developmental patterns represent an average of male and female values. Previous studies have indicated that UDPGT activities from male and female rat microsomes are similar [7]. Each litter was pooled and each value represents an average of at least three separate litters. Onset of enzyme activity for MUB, N, and P glucuronidations was the 18th day of gestation with a limit of detection of approximately 0.1 nm/min/mg protein using the assay conditions described in the Materials and Methods. A characteristic developmental peak in activity was observed about the time of birth such that UDPGT activity one day after birth exceeded adult levels. Newborn activities decreased from the 4th to the 21st day after birth by approximately 30–40% and then gradually increased to adult levels. Relative fetal, newborn, and adult glucuronidation rates for the three substrates were consistent and in the following order; MUB > N > PNP.

The developmental patterns of steroid glucuronidations are presented in Fig. 2. Like non-steroid

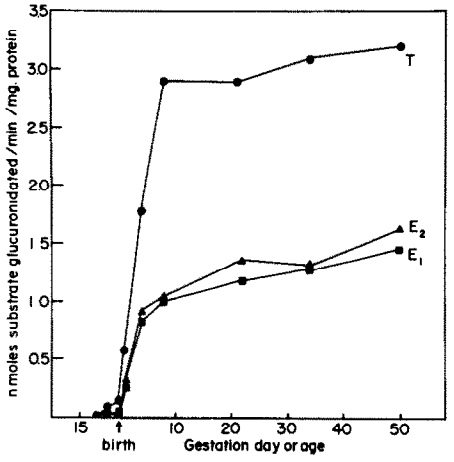

Fig. 2. Perinatal development of steroid UDPGT conjugating T (●), E_2 (▲), and E_1 (■) in rat liver. Each litter was pooled and data for each developmental stage represents the average of three separate litters.

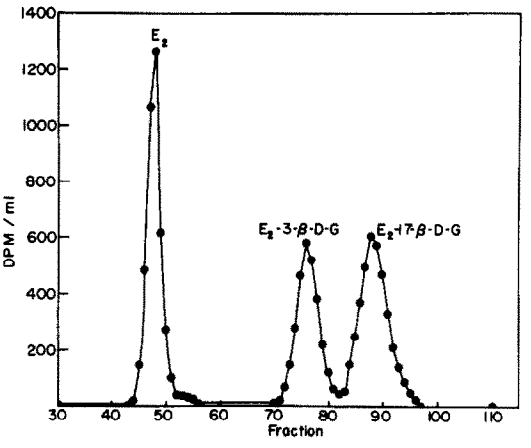

Fig. 3. Resolution of E_2 from its glucuronides on DEAE Sephadex A-25 columns (1.5 × 70 cm) using a gradient of 0–0.8 M NaCl in a total vol. of 500 ml.

UDPGT, activities were not detectable prior to the 18th day of gestation and dramatic increases were evident around parturition. In contrast to non-steroid UDPGT, there was no developmental peak in activity immediately after birth such that newborn glucuronidation rates exceeded adult levels. Relative fetal, newborn, and adult activities were consistently $T > E_2 > E_1$, although E_2 and E_1 differences were not statistically significant.

E_2 can be glucuronidated at either the 3- or 17-position of the steroid molecule. We were therefore, interested in determining the relative contributions of E_2-3-β-D-glucuronide and E_2-17-β-D-glucuronide to total E_2 glucuronide formation in newborn and adult animals. Resolution of E_2 and its respective glucuronides on DEAE Sephadex columns following incubation of ^{14}C-E_2 (0.1 mM) with hepatic microsomes from adult female rats is illustrated in Fig. 3 and indicate that the contributions of the two glucuronides are similar. Data presented in Table 1 demonstrates that the relative rates of formation of the 3-β-D-glucuronide and 17-β-D-glucuronide do not change markedly with development, although specific activities are 7–8 fold greater in adult females than in one-day old rats (average of males and females). The ratios were 0.69 (1 day), 0.92 (8 days), and 0.85 (52 days).

P and DES are non-steroidal xenobiotics that possess significant estrogenic activities. Since glucuronidation is a major metabolic pathway for these two compounds, we characterized their developmental patterns for the purpose of determining possible similarities with steroid or non-steroid substrates. The patterns revealed that both P and DES exhibit the characteristics of steroid UDPGT rather than non-steroid UDPGT (Fig. 4).

Effects of TCDD

TCDD inducibility was the second characteristic used to differentiate steroid from non-steroid UDPGT. Table 2 indicates the effects of administering 5 μg TCDD/kg to adult non-pregnant female rats. One week after treatment, hepatic PNP, MUB, and N glucuronidations were induced 6–7 fold whereas the induction factors were 0.9 (P), 1.0 (T), 1.1 (E_1), 1.3 (E_2), and 1.6 (DES) for substrates apparently conjugated by steroid UDPGT. DES was the only substrate in the steroid class exhibiting significantly higher ($P < 0.05$) glucuronidation rates in the TCDD-treated group compared to controls. The levels of significance were $P < 0.01$ for the non-steroid substrates. Neither the rates of formation of 17-β-D-glucuronide nor 3-β-D-glucuronide was increased in the TCDD-treated group.

Table 1. Microsomal glucuronidation of β-estradiol at the 3- and 17-positions*

Animal age (days)	Nmol glucuronide formed per min per mg protein†		
	3-β-D-Glucuronide	17-β-D-Glucuronide	Ratio
1	0.09 ± 0.03	0.13 ± 0.02	0.69
8	0.57 ± 0.07	0.62 ± 0.15	0.92
52	0.79 ± 0.09	0.93 ± 0.14	0.85

* Each value represents the average ±S.D. derived from at least four animals from four separate litters. † Estradiol concentration in the incubation medium was 0.1 mM and glucuronide quantitations made as described in the Materials and Methods.

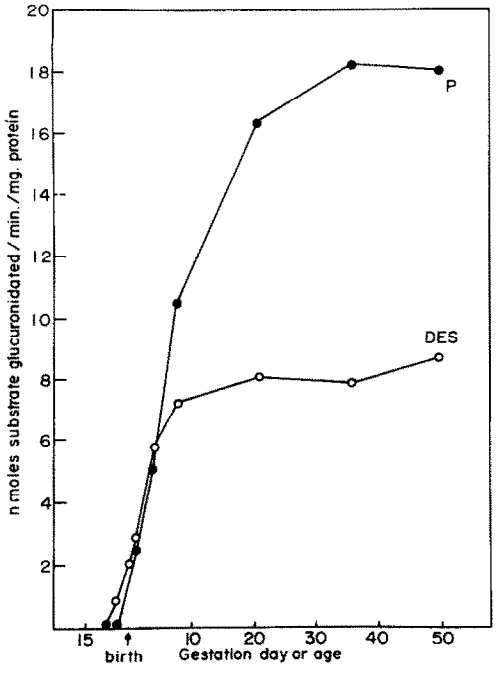

Fig. 4. Perinatal development of the UDPGT conjugating DES (○) and P (●) in rat liver. Each litter was pooled and data for each developmental stage represents the average of three separate litters.

Relative tissue distributions

The third differentiating criteria involved tissue distributions of steroid and non-steroid UDPGT in adult female rats. Liver, kidney, and uterine specific activities for the eight substrates tested are given in Table 3. The liver to kidney specific activity ratios were 1.3–1.4 for PNP, MUB, and N. In contrast, the ratios were >16 for E_1, E_2, P, and T. DES was intermediate between the two groups exhibiting a liver to kidney ratio of 4.3. Liver to uterine ratios, although consistently higher than liver to kidney values, indicated the same groupings: PNP, MUB, and N (5.3–7.9); DES (18.2); E_1, E_2, T, and *P* (>21–>269). E_1, E_2, T, and P were not detectable in uterine microsomes using the incubation conditions described in the Materials and Methods. Hepatic specific UDPGT activities were in the following order N > MUB > PNP > P > DES > T > E_2 > E_1.

DISCUSSION

Previous reports have provided evidence for the enzyme multiplicity of UDPGT [3–7]. Studies conducted in this laboratory and others have suggested that non-steroid UDPGT differs from steroid

Table 2. Induction of hepatic microsomal glucuronidation pathways by oral TCDD administration (5 μg/kg) to female rats*

Substrate†	Nmol per min per mg protein		
	Control	TCDD-treated	Induction factor
p-nitrophenol	38.7 ± 5.9	246.4 ± 34.5	6.4†
4-methylumbelliferone	63.5 ± 12.4	439.0 ± 33.9	6.9†
1-naphthol	77.0 ± 5.0	465.3 ± 69.7	6.0†
diethylstilbestrol	9.5 ± 2.6	14.8 ± 1.7	1.6‡
β-estradiol	2.4 ± 0.3	3.1 ± 0.8	1.3
estrone	2.0 ± 0.6	2.2 ± 0.2	1.1
testosterone	3.9 ± 0.3	3.8 ± 1.2	1.0
phenolphthalein	22.8 ± 2.1	19.5 ± 3.8	0.9

* Rats sacrificed 7 days after TCDD administration and each value represents the mean ± S.D. derived from at least four rats. † Significantly different from controls at least at $P < 0.01$. ‡ Significantly different from controls at least at $P < 0.05$.

Table 3. Tissue distributions of UDP glucuronyltransferase activities in female rats*

Substrate	Nmoles glucuronidated per min per mg protein			Tissue activity ratio	
	Liver	Kidney	Uterus	Liver / Kidney	Liver / Uterus
p-nitrophenol	33.9	27.0	6.4	1.3	5.3
4-methylumbelliferone	54.2	36.6	4.8	1.4	7.9
1-naphthol	83.9	63.0	12.7	1.3	6.6
diethylstilbestrol	10.1	1.8	0.4	4.3	18.2
β-estradiol	3.3	0.2	ND	16.5	>33.0
estrone	2.1	0.1	ND	21.0	>21.0
testosterone	4.1	ND	ND	>41.0	>41.0
phenolphthalein	26.9	0.7	ND	37.2	>269.0

* Each value represents an average of six determinations using microsomes derived from four female rats.

UDPGT based on differences in TCDD-inducibility [9] and perinatal development [8, 10], although only two substrates were used in each of these studies. In addition, there are apparent differences in activations of steroid and non-steroid UDPGT *in vitro* [11].

Data presented in this manuscript characterize clearly some of the differences between steroid and non-steroid glucuronidation. Substrates were selected according to three groups: (1) non-steroid (MUB, N, PNP), having no reported steroidal activity; (2) endogenous steroids (T, E_2, E_1) that are metabolized through glucuronidation pathways; and (3) xenobiotics (DES, P) having estrogenic activity. We first established that glucuronidation characteristics of group I were clearly different from group II. Hepatic glucuronidation rates of group I substrates were TCDD-inducible and were higher one day after birth than in adults. In contrast, group II substrates were not TCDD inducible and did not exhibit a developmental peak immediately after birth. Estrogenic properties of DES are greater than E_2 whereas the estrogenicity of P is several orders of magnitude less than that of endogenous steroids [19, 20]. Nevertheless, both substrates were glucuronidated by steroid UDPGT.

A third criteria was selected involving tissue distribution of UDPGT and again clear differences were evident between steroid and non-steroid UDPGT. Comparing liver activities to those of kidney and uterus, characteristic profiles of different UDPGTs were defined. Liver, kidney, and uterus non-steroid UDPGT activities were relatively high for all substrates. In contrast, glucuronidation rates of steroidal substrates were low or non-detectable in kidney and uterus. Tissue distributions of DES glucuronidation were intermediate between the two groups. For example, the liver to kidney ratios for MUB, PNP, and N were 1.3–1.4 and >16.5 for E_2, E_1, T, and P compared to 4.3 for DES. A possible explanation for low uterine steroid UDPGT levels could involve the evolution of cell biochemistry designed to prevent rapid steroid deactivation in the reproductive tract. In addition, steroid sulfates are important to steroidogenesis [21] and the rapid formation of steroid glucuronides would inhibit sulfation since both enzymes systems compete for the same sites.

Literature data on the perinatal development of UDPGT activity towards different substrates provide supportive evidence for the distinction between steroid and non-steroid UDPGT. Developmental peaks in UDPGT activity were reported in perinatal rat liver for PNP [7, 22, 23] and bilirubin [22, 24], indicating that bilirubin, like PNP, is glucuronidated as a non-steroid substrate. However, the perinatal development of hepatic *o*-aminophenol glucuronidation in a variety of species [25–27], morphine glucuronidation [7], and P glucuronidation [26] more resembled steroid glucuronidation in that no developmental peak was evident. P exhibits estrogenic activity [20]; the steroidal activity of morphine and *o*-aminophenol has not been reported.

In contrast to our studies and other reports, Mulder [3] has postulated that PNP, P, OAP, and MUB are glucuronidated by the same enzyme as evidenced by kinetic constants and inhibition data. A later report [4] suggested that the apparent competitive inhibition exhibited by these substrates could be explained by one enzyme forming a dead-end complex with a form of UDPGT catalyzing the glucuronidation of a different substrate or there could be separate binding sites for different substrates and a single site for UDPGA.

There are several possible explanations for the observed differences in UDPGT characteristics for steroid and non-steroid substrates. One involves the fact that UDPGT is a phospholipid-dependent enzyme system [28–29] and differences could exist in the phospholipid compliment of the two sets of enzymes. A second possibility is that there are different protein moieties or multiple active sites. Since UDPGT is difficult to solubilize and has not been purified to homogeneity these possibilities cannot be easily investigated. A third explanation could be that steroid and non-steroid UDPGT have different membrane environments on the endoplasmic reticulum. This possibility is supported by recent studies demonstrating that membrane-perturbing agents activate non-steroid UDPGT *in vitro* but have no significant effects on steroid UDPGT [11]. However, solubilization and partial purification of microsomal UDPGT from control and TCDD-treated animals did not change the magnitude of PNP UDPGT induction or change the lack of induction of T glucuronidation [9].

Table 4. Summary of glucuronidation characteristics of selected substrates

Substrate	Developmental peak	TCDD induction	Kidney and Uterus activity
p-nitrophenol	+	+++	+++
4-methylumbelliferone	+	+++	+++
1-naphthol	+	+++	+++
diethylstilbestrol	–	+	+
β-estradiol	–	–	–
estrone	–	–	–
testosterone	–	–	–
phenolphthalein	–	–	–

A summary of the glucuronidation characteristics of the substrates used in these studies are presented in Table 4 indicating the differences between steroid and non-steroid UDPGT with the exogenous estrogens (DES and P) clearly in the steroid UDPGT class. These studies categorize two types of UDPGT activity although it is likely that there are several non-steroid UDPGTs and several steroid UDPGTs. In regards to steroid UDPGT, it might be of interest to determine possible multiplicity using progestins and corticosteroids as well as estrogens and androgens.

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