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UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS OESTRIOL IN FRESH AND CULTURED FOETAL TISSUES FROM MAN AND OTHER SPECIES

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SUMMARY

1. UDP-glucuronyltransferase activity towards oestriol has been demonstrated in liver preparations from foetal and adult human and mouse, and from chick embryo and chick. It was also found in foetal human kidney and gut, but appeared absent from placenta.

2. In human foetal liver and kidney levels of UDP-glucuronyltransferase activity towards oestriol were as low as those towards *o*-aminophenol, and developed with age.

3. No UDP-glucosyltransferase activity towards oestriol was detected in human foetal preparations.

4. UDP-glucuronyltransferase activity towards oestriol develops precociously in cultures of chick embryo liver; in cultures of human foetal liver the transferase activity towards oestriol declines whilst increasing towards *o*-aminophenol.

5. Pre-treatment with phenobarbital increased UDP-glucuronyltransferase activity towards oestriol in chick embryo liver, both *in ovo* and in culture. Pre-treatment with phenobarbital increased this activity in maternal mouse liver but not in foetal liver, *in utero* or in culture, nor in cultured human foetal liver.

6. UDP-glucuronyltransferase activities towards oestriol and towards *o*-amino phenol differ markedly in development, in culture and in response to phenobarbital pre-treatment and activation procedures; heterogeneity of the enzyme is probably responsible.

INTRODUCTION

Developmental studies on UDP-glucuronyltransferase (E.C. 2.4.1.17) have largely concerned laboratory animals and xenobiotic substrates (for reviews see [1–4]). Foetal and perinatal activities of this enzyme towards steroids are less frequently studied. Glucuronides of several steroids, including oestriol, have been isolated from human foetal tissues (for reviews see [5, 6]) and oestriol glucuronide is probably formed in slices of human foetal liver [7]; however, UDP-glucuronyltransferase activity towards steroids does not seem to have been demonstrated in human foetal tissues and its significance there has remained conjectural [8].

This paper reports activity of UDP-glucuronyltransferase towards oestriol in a variety of foetal tissues, including those of man, and compares it with corresponding activity towards xenobiotic substrates. As UDP-glucuronyltransferase activity of chick embryo liver can be precociously induced to very high levels on isolation and culture of the organ or cells [9, 10] and as phenobarbital apparently induces the enzyme's activity both *in vivo* (for reviews see [3, 11]) and in organ culture [12], we have also followed the activity of UDP-glucuronyltransferase towards oestriol in cultured tissues and in those exposed to phenobarbital *in vivo* and *in vitro*.

EXPERIMENTAL

Organ culture

Five liver segments totalling approximately 5 mg wet wt. from human (male) or mouse foetuses, or from 7–13-day-old chick embryos were prepared under sterile conditions and placed on Millipore rafts [25 μm thick, 0·45 μm pore size, Millipore (U.K.) Ltd., London, England] supported by a nylon screen (Nitex, 656 μm grid) over the centre well of an organ culture dish (Falcon Plastics, Oxnard, Calif.) containing 1·2 ml of Eagle's Minimum Essential Medium with phenol red, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid) buffer, 2 mM L-glutamine, 50 units penicillin, 50 μg streptomycin (Flow Laboratories, Irvine, Scotland) and 10% Calf Serum (Biocult Laboratories, Glasgow, Scotland). These preparations were incubated at 37·5°C, atmospheric pressure and 100% humidity; the medium was replaced every 3 days [10, 12].

Animals exposed to phenobarbital

Phenobarbital was supplied to adult male mice, pregnant mice and 4-day-old hatched chicks in drinking water (1 g/l.) for 7 days before sacrifice.

Preparation of tissue for enzyme assay

Microsomal suspensions. Liver samples were homogenized in 4–9 parts (w/v) of ice-cold 0·25 M sucrose by 6 strokes of a Teflon–glass homogenizer. This homogenate was centrifuged for 20 min at 8000 ***g*** av. and the resulting supernatant was recentrifuged for 30 min at 100,000 ***g*** in an M.S.E. Superspeed 50 centrifuge. The "pellet" obtained was resuspended in 0·154 M-KCl brought to pH 7·4 with $KHCO_3$.

Tissue homogenates

Tissue samples were homogenized in 9 parts (w/v) ice-cold 0·154 M-KCl by 6 strokes of the homogenizer, centrifuged at 500 ***g*** for 2 min., and the resulting supernatant was used as the enzyme source.

Enzyme assay

Substrates. [4-^{14}C]-Oestriol [1,3,5(10)-oestratriene-3, 16 α, 17 β-triol], S.A. > 50 mCi/mmol, and UDP-[U-^{14}C]-glucuronic acid, S.A. > 287 mCi/mmol, were from the Radiochemical Centre, Amersham, England. Unlabelled oestriol, oestriol-16α-(β-D-glucuronide), UDP-glucuronic acid (triammonium salt) and UDP-glucose were from Sigma (London) Chemical Co., London, England.

Assay of UDP-glucuronyltransferase activity

(a) *Oestriol as substrate.* The method was basically that of Rao *et al.*[13]: 40 mM-Tris–HCl buffer pH 8·0 (checked as optimal), 16 mM-$MgCl_2$, 3·6 mM-UDP-glucuronic acid, 24 μM-[4-^{14}C]-oestriol and 12 mM-KCl, together with microsomal protein (50–200 μg) or homogenate protein (400–800 μg), were incubated at 37°C. The reaction rate remained linear for the period employed. In controls UDP-glucuronic acid was omitted. After incubation, tubes were cooled in ice-water and free oestriol removed by four extractions with 20 ml water-saturated ethyl acetate. Solid NaCl was added to the aqueous soln. in slight excess. This mixture was then extracted twice with 4 ml water-saturated butan-1-ol; 0·5 ml of the organic layer (conjugate fraction) was counted in 15 ml scintillation fluid and 1 ml of methanol, using a Nuclear Chicago Unilux II liquid scintillation spectrometer, at an efficiency above 90%. The scintillation fluid contained 5 g 2,5-diphenyloxazole and 0·3 g 1,4-bis-(5-phenyloxazolozyl) benzene in 1 l dry sulphur-free scintillation grade toluene.

(b) *o-Aminophenol as substrate.* The method was that of Dutton and Storey [14] as modified by Winsnes [15].

Assay of UDP-glucosyltransferase activity. The assay was identical to that for UDP-glucuronyltransferase except that UDP-glucose replaced UDP-glucuronic acid in test samples and the pH of the incubation mixture was pH 7·0, optimal for *p*-nitrophenyl glucoside synthesis [16]: measurement at pH 8·0 gave less activity.

Protein was determined by Oyama and Eagle's[17] modification of the method of Lowry *et al.*[18]. *β-Glucuronidase* was prepared from female rat preputial gland by the method of Levvy *et al.*[19] as far as the ammonium sulphate precipitation stage. *D-Glucaro-(1→4)-lactone* was prepared in solution by refluxing aqueous potassium hydrogen glucarate (B.D.H.) for 1 h [20].

Chromatographic solvents were as described in Results. Radioactive steroids and their conjugates were detected by photographing the chromatograms in a Radiochromatogram Spark Chamber Scanner (Birchover Instruments Ltd., Hitchin, Herts., England); colorimetric localization also was performed whenever possible, using a spray of 0·4 N Folin–Ciocalteu's Reagent followed by one of 0·44 M ammonium hydroxide solution [21].

RESULTS

Formation of oestriol-β-D-glucuronide

When oestriol was incubated as described above with liver preparations from human foetus, human adult mouse or chick, a radioactive compound was present in the final butan-1-ol extract. The following evidence indicated that this compound was oestriol β-D-glucuronide.

Requirement for UDP-glucuronic acid

UDP-glucuronic acid had to be present during incubation of the enzyme assay mixture before any radioactive oestriol conjugate was obtained.

Presence of β-glucuronyl link

After incubation, 0·25 ml portions of the oestriol-extracted mixture were treated with β-glucuronidase in presence or absence of its specific inhibitor glucaro-(1 → 4)-lactone; control portions were not exposed to the enzyme. Subsequent examination of ethyl acetate extracts of these portions indicated (Table 1) complete hydrolysis of the suspected conjugate by β-glucuronidase and 90–100% inhibition by the lactone of this hydrolysis.

Table 1. Hydrolysis of biosynthesized oestriol conjugate by β-glucuronidase

Source of liver forming conjugate	c.p.m. (a)	(b)	(c)	(d)	Hydrolysis (%)	Inhibition of hydrolysis (%)
Human 10-week foetus	136	56	155	54	114	100
Human 17-week foetus	1043	102	1047	98	100	99
Human, adult	3340	79	3345	164	100	98
Mouse, adult	972	67	972	151	100	91

After the synthetic incubation (see Methods) 0·25 ml aliquots of the oestriol-free conjugate (a) were measured for radioactivity. Other 0·25 ml aliquots were incubated for 2 h at 37°C with (b) 0·25 ml 0·2 M-sodium acetate buffer, pH 5·5, and 0·05 ml H_2O; (c) 0·25 ml of β-glucuronidase preparation in 0·2 M-sodium acetate buffer, pH 5·5, and 0·05 ml H_2O; or (d) contents of (c) but with 0·05 ml 100 mM-*D*-glucaro-(1 → 4)-lactone replacing the added water. The mixtures were heated at 100°C for 2 min, cooled in ice-water, centrifuged to remove protein; the free oestriol was extracted with 1 ml of water-saturated ethyl acetate and measured by radioactivity.

Presence of oestriol

After hydrolysis of the conjugate with β-glucuronidase, thin layer chromatography of the incubation mixture in chloroform–ethanol (9:1 v/v) on silica gel revealed a radioactive spot (R_F 0·5) travelling like free oestriol. Authentic oestriol-16α-(β-D-glucuronide) remained at the origin.

Presence of glucuronic acid

UDP-[U-^{14}C]-glucuronic acid and unlabelled oestriol replaced radioactive oestriol and unlabelled UDP-glucuronic acid in some experiments. Thin layer chromatography of the final butan-1-ol extracts in butanol–acetic acid–water (4:1:1 by vol.) on cellulose revealed a discrete radioactive spot from conjugate-containing incubations; this spot gave a colour reaction for oestriol and possessed the same R_F value (0·8) as authentic oestriol-16α-(β-D-glucuronide); UDP-glucuronic acid and its breakdown products gave an R_F value of (0·1–0·3) in control experiments.

Further comparison with authentic oestriol-16α-β-D-glucuronide

The final butan-1-ol extracts were taken to dryness under N_2 at 40°C, dissolved in dry butan-1-ol and chromatographed on thin layer silica gel in 2-methylpropan-2-ol-ethylenedichloride–acetic acid–water (5:15:6:14 by vol.[13]). The radioactive oestriol conjugate behaved exactly as authentic oestriol-16α-(β-D-glucuronide) in the colorimetric test and in mobility (R_F 0·8).

From the above evidence it may be concluded that oestriol-β-D-glucuronide was the conjugate biosynthesized. The position of the β-glucuronyl link on the oestriol molecule is not relevant to the present study and was therefore not investigated further.

Activity of UDP-glucuronyltransferase in human foetal tissues

Human foetal tissues (10–17-week male) were assayed for UDP-glucuronyl-transferase activity towards oestriol as described. The enzyme was demonstrated in homogenates and microsomal preparations from liver. Its specific activity progressively increased 10-fold between 10–17 weeks of gestation; 16% of the presumed adult level (see Discussion) was detected in the 17-week foetus (Table 2).

UDP-glucuronyltransferase was also observed in foetal human kidney; it developed there similarly

Table 2. Development of UDP-glucuronyltransferase activity towards oestriol in human tissue homogenates

Source of tissue	Specific activity (nmol of oestriol-β-glucuronide formed/mg protein/h) Liver	Kidney	Gut	Placenta (foetal side)
10 Week male foetus	0·14, 0·20 (2·0%)	—	—	0
12 Week male foetus	0·57 (6·7%)	0·56	—	0
14 Week male foetus	0·70 (8·3%)	0·54	0·48	0,0
17 Week male foetus	1·34 (15·8%)	1·58	—	—
Adult male*	8·50			
Adult female*	10·90			
Adult male mouse	8·64			

* Liver samples from adult patients suffering from obstructive jaundice.
Assay as in text. Figures in parentheses indicate the percentage of the adult level (see text).

Table 3. Comparison of the synthesis of oestriol glucoside and oestriol glucuronide by human liver

Source of liver	Preparation	Specific activity (nmol glucuronide formed/mg protein/h) Glucoside	Glucuronide
14 Week foetus	Microsomes	0	1·60
17 Week foetus	Homogenate	0	0·84
17 Week foetus	Microsomes	0	5·17
Adult male*	Microsomes	0·54 (pH 8·0)†	52·04
Adult female*	Homogenate	0·34	17·24

* Liver samples from patients suffering from obstructive jaundice.
† Experiment performed at pH 8·0, not 7·0.
Assays as in text. Simultaneous assays were performed.

(Table 2). Some activity was observed in a gut sample. No activity was detected in tissue from the foetal side of 10, 12 or 14-week placenta (Table 2).

Activity of UDP-glucosyltransferase towards oestriol in human liver preparations

UDP-glucosyltransferase and UDP-glucuronyltransferase activities were measured during parallel incubations from the same tissue source. Table 3 shows that, using either homogenate or microsomal preparations from liver, oestriol glucoside synthesis in the adult was only 1–2% of the corresponding glucuronide synthesis and was not detectable in the foetus.

Activity of UDP-glucuronyltransferase towards oestriol during organ culture of liver

UDP-glucuronyltransferase activity towards *o*-aminophenol develops during organ culture of chick embryo liver from near zero up to five times adult values [10] and this increase requires protein synthesis from amino acids[12]. We examined this system to ascertain whether any similar increase occurred in UDP-glucuronyltransferase activity towards oestriol. Enzyme activity towards oestriol does increase in simple organ culture to about adult values (Table 4), but not as markedly as when *o*-aminophenol is substrate. Presence of 5·5 mM phenobarbital further increases activity, although again less than with *o*-aminophenol as substrate (Table 4).

We repeated this procedure with human foetal liver, to see if its low transferase activity would also develop in culture. The activity towards *o*-aminophenol increased in culture in one case up to 10-fold, yet activity towards oestriol decreased during the same culture period (Table 5). In these human foetal cultures, in contrast to those derived from chick embryo liver, phenobarbital (1·0–5·5 mM) did not further enhance the development of the enzyme activity towards either *o*-aminophenol or oestriol. The same failure of phenobarbital to enhance UDP-glucuronyltransferase activity in cultures was noted with the foetal mouse livers examined.

Effect of exposure to phenobarbital on the development of UDP-glucuronyltransferase activity in vivo

Injection of phenobarbital into eggs increases the UDP-glucuronyltransferase activity of the embryo

Table 4. Development of UDP-glucuronyltransferase in organ cultures of 9-day chick embryo liver and effect of phenobarbital (Pb)

Days in culture	Specific activity (nmol glucuronide formed/mg protein/h) Oestriol as substrate – Pb				Oestriol as substrate + Pb			*o*-Aminophenol as substrate – Pb				*o*-Aminophenol as substrate + Pb		
	A	B	C	D	A	B	C	A	B	C	D	A	B	C
0	1·2	0·8	1·0	—	1·2	0·8	1·0	3·4	1·0	2·4	—	3·4	1·0	2·4
3	2·5	0·5	—		9·3	3·1		111·6	19·0			349·0	81·5	
4		0·9	2·0			8·5			41·0	34·9			130·0	
8				2·6			3·6				107·0			72·2
Fresh liver from hatched chicks*	3·0 ± 0·5				7·3 ± 2·5			17·1 ± 7·8				108·1 ± 23·4		

* Values shows S.D. from the mean of the results.
Methods as in text. The letters A → D indicate separate pooled liver samples.

Table 5. Development of UDP-glucuronyltransferase activity in cultures of male human foetal liver

Source of liver	Substrate	Specific activity (nmol glucuronide formed/mg protein/h) Fresh liver	Liver cultured for 5 days
10 Week foetus	*o*-Aminophenol	0; 1·2	2·9; 1·6
17 Week foetus	*o*-Aminophenol	1·2	12·0
20 Week foetus	*o*-Aminophenol	1·7	4·7
Adult	*o*-Aminophenol	11·0	—
10 Week foetus	Oestriol	0·2	0
12 Week foetus	Oestriol	0·6	0
17 Week foetus	Oestriol	1·3	0·2
Adult	Oestriol	8·5	—

Methods as in text.

Table 6. Effect of phenobarbital on the *in ovo* development of UDP-glucuronyltransferase activity in chick embryo liver

Mg phenobarbital injected into egg	Specific activity (nmol glucuronide formed/mg protein/h) Oestriol as substrate (a)	(b)	*o*-Aminophenol as substrate (a)	(b)
0	1·3; 1·5	0·9	1·7	0·7
10	1·7	1·4; 1·7	2·0	9·4; 21·2
30	3·3	—	—	—
40	8·0	—	34·1	—

(a) Sterile phenobarbital in 0·5 ml balanced salt solution injected into air-space of 8-day-incubated eggs and embryo liver enzyme activity assayed 12 days later. (b) Similar treatment of 11-day-incubated eggs with assay 7 days later. 0·5 ml balanced salt solution was injected into the corresponding control eggs. Activities towards both substrates were assayed simultaneously on each liver sample; methods as in text.

Table 7. Effect of phenobarbital (Pb) pre-treatment on UDP-glucuronyltransferase activity towards oestriol in homogenates of foetal, neonatal and adult mouse liver

Liver source	Specific activity (nmol oestriol β-glucuronide formed/mg protein/h) −Pb	+Pb
14 Days gestation: foetus	0·0 (2)	0·1 (0)
mother	3·1	6·9
17 Days gestation: foetus	1·1	1·1
mother	2·0	3·8
18 Days gestation: foetus	1·1	0·9
mother	2·8	5·7
1 Day neonate	0·7	0·9
Adult male	4·8	7·9
	11·9	20·9
	3·2	5·9

Adult animals were allowed access to phenobarbital in drinking water (see Methods). Foetal and maternal livers from each pregnancy were examined simultaneously.

liver towards xenobiotic substrates [3, 22], overcoming the repression of an *in ovo* environment. Results presented in Table 6 show that injection of phenobarbital into eggs also increased the embryo liver transferase activity towards oestriol, although to a lesser extent than with *o*-aminophenol as substrate.

We then investigated whether phenobarbital would also succeed in overcoming the presumed repression of UDP-glucuronyltransferase activity towards oestriol in foetal ASH/TO mouse liver *in utero*. Administration of the drug to pregnant mice increased this UDP-glucuronyltransferase activity in the maternal liver but it did not appear to alter the activity in livers from the foetus or 1-day neonate (Table 7).

Attempted activation of UDP-glucuronyltransferase activity towards oestriol

UDP-glucuronyltransferase is markedly subject to activation by a variety of procedures [2, 15]. Its activity in mouse foetal liver towards *o*-aminophenol can be increased by prior sonication followed by addition of 16 mM diethylnitrosamine [23]. However this treatment did not significantly increase its activity towards oestriol in homogenates of mouse and human foetal liver.

DISCUSSION

The above results indicate that human foetal liver and kidney exhibit UDP-glucuronyltransferase activity towards oestriol from at least the 10th week of gestation, and that this activity is absent from the foetal side of the placenta.

As human foetal liver possesses both UDP-glucuronic acid [24] and UDP-glucose dehydrogenase [25] its UDP-glucuronyltransferase must contribute to any glucuronidation of oestriol performed by the foeto-placental unit [7], and to the formation of those oestriol glucuronides isolated from amniotic fluid and foetal tissues [5, 6, 8, 26].

Although this study was restricted by a lack of healthy human liver, previous work [24] indicated that UDP-glucuronyltransferase activity towards *o*-aminophenol was roughly similar in adult livers from man and from male mouse; this similarity was confirmed (Table 2) for oestriol using liver from jaundiced human adults of either sex. Our results suggest that in human foetal liver the enzyme's activity towards oestriol is of the same low order as towards the xenobiotics studied, and therefore the human foetus may be as poorly equipped for the glucuronidation of steroids as of xenobiotics.

During current work with ASH/TO strain mice, however, we have noted high foetal levels of the transferase activity towards the xenobiotic *o*-aminophenol, specific activity in 14-day foetal liver being 6·3 nmol glucuronide formed/mg protein/h (maternal level being 6·1 nmol), whereas activity of the same samples towards oestriol was only 0·03 nmol (maternal 4·0 nmol).

This marked difference in behaviour towards the two substrates persists until after birth. These results and those reported above in Tables 5 and 6 and under Activation, would support the heterogeneity of UDP-glucuronyltransferase, a conclusion already suggested from developmental [27, 28] and kinetic [11, 29, 30] work with other substrates.

The precocious and striking increase of UDP-glucuronyltransferase activity towards *o*-aminophenol or *p*-nitrophenol on culture of chick embryo liver [3, 9, 10, 12, 31] so far appears unique; towards oestriol, though precocious, the rise is much smaller. This formation of oestriol glucuronide by chick and chick embryo liver is itself of interest for hitherto (*see* [32]) steroid conjugates from chick tissues have been reported as sulphates, not glucuronides.

In cultured human foetal liver our results indicate a development of UDP-glucuronyltransferase towards *o*-aminophenol which might be considered precocious, but the rate is little greater than that recently reported [33, 34] for two other enzymes in cultured human foetal liver.

As phenobarbital increases UDP-glucuronyltransferase in mammalian liver [11, 35] and in cultured chick embryo liver [12], its lack of effect in cultured mammalian foetal liver is surprising.

Our results also indicate that, for chick, phenobarbital can overcome the *in ovo* repression of transferase activity towards oestriol. In mammals there was no similar effect of the drug *in utero*, even though the maternal enzyme was increased (Table 7) and phenobarbital is reported to cross the placenta and concentrate in the foetal liver [36]. We have obtained broadly similar findings with *o*-aminophenol as substrate. This *in utero* resistance to barbiturates contrasts with the response of the transferase to these drugs in neonatal liver [37, 38] and is consistent with the extremely small increase in xenobiotic metabolism found in human foetal liver and placenta after maternal treatment with phenobarbital [39]; benzpyrene, however, is able to induce hydroxylating enzymes in human placenta [40].

Glucosylation of oestriol by human foetal liver appears absent or negligible. In foetal mouse also the glucosylating enzyme is absent towards oestriol; under optimal conditions *o*-aminophenol glucosylation was detected, but only at 8% of the corresponding rate of glucuronidation.

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