Congenital jaundice in rats due to the absence of hepatic bilirubin UDP-glucuronyltransferase enzyme protein

Isobel Scragg, Chantal Celier+ and Brian Burchell

Department of Biochemistry, University of Dundee, DD1 4HN, Scotland and +*Inserm U-36, 17 Rue du Fer-a-Moulin, 75005 Paris, France*

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Three major UDP-glucuronyltransferase isoenzymes (50–54 kDa) have been identified by immunoblot analysis. Bilirubin UDP-glucoronyltransferase (54 kDa) was specially induced by treatment of the rats with clofibrate. This isoenzyme was not detectable in liver microsomal extracts from congenitally jaundiced Gunn rats and was not induced by treatment of these animals with clofibrate. Phenol UDP-glucuronyltransferase, the only isoenzyme determined to be present in foetal Wistar rat liver microsomes was not detected by enzyme assay or immunoblot analysis of foetal Gunn rat liver microsomal extracts. These results provide the first indication that bilirubin UDP-glucuronyltransferase and possible phenol UDP-glucuronyltransferase proteins are not present in the congenitally jaundiced Gunn rat.

Gunn rat *Purified isoenzyme* *Immunoblot analysis* *Clofibrate* *Fetal rat*

1. INTRODUCTION

Liver microsomal bilirubin UDP-glucuronyltransferase is the rate limiting enzyme in the excretion of bilirubin and failure of this hepatic function results in irreversible cerebral dysfunction, kernicterus and even death in both the newborn infant and some adults [1]. Gunn described a mutant strain of Wistar rat, which has a hereditary hyperbilirubinaemia [2] due to a complete inability to glucuronidate bilirubin [3]. This mutant rat is a good animal model for the study of human Crigler-Najjar syndrome [4]. The hereditary defect in the Gunn rat decreases the rate of glucuronidation of various substrates other than bilirubin, which has led to much confusion concerning the exact nature of the molecular disturbances [5] for more than 25 years [6]. Recent advances in the purification of UDP-glucuronyltransferase(s) have determined that conjugation of endogenous compounds and xenobiotics is catalysed by a family of isoenzymes [5,7–11]. Previously, we have determined that the level of total UDP-glucuronyltransferase proteins present in Gunn rat liver microsomes is only 64% of the level in Wistar rat liver microsomes, which indicated that certain isoenzymes may be missing from Gunn rat liver [12]. Here, we report that the biochemical lesion in the Gunn rat results in the absence of bilirubin UDP-glucuronyltransferase and possibly phenol UDP-glucuronyltransferase.

Abbreviations: UDPGT, UDP-glucuronyltransferase; FITC, fluorescein isothiocyanate

2. MATERIALS AND METHODS

Nitrocellulose was made by Schleicher and Schull and obtained from Anderman & Co, London. Anti-sheep IgG precipitating serum (donkey) was obtained from the Scottish Antibody Production Unit, Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire. Polyoxyethylene sorbitan monolaurate (Tween 20) was from Sigma.

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Gunn and Wistar rats were from the colony maintained in the Institute.

2.1. *Pretreatment of animals*

Male rats (6–8 weeks old) were given clofibrate intraperitoneally twice daily (200 mg/kg in corn oil) for 4 days before being killed and the livers removed.

2.2. *Enzyme assays*

UDP-glucuronyltransferase activity towards various substrates was assayed by previously described methods: 2-aminophenol [13], testosterone [14], androsterone [11], bilirubin [15] and 1-naphthol [16], [4-^{14}C]testosterone and 1-[1-^{14}C]-naphthol were purchased from Amersham. [9,11-^{3}H]Androsterone was from New England Nuclear. Protein concentrations were measured by the Biuret method [17] with bovine serum albumin as standard.

2.3. *Purification of enzymes*

UDP-glucuronyltransferases were purified by conventional procedures as previously described [5].

2.4. *Antibodies specific for UDPGT*

Sheep anti-rat UDPGT antiserum was prepared using testosterone/4-nitrophenol UDPGT as antigen as described [18]. IgG was purified by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and bound to CNBr-activated Sepharose 4B [19].

2.5. *Immunoaffinity purification of enzymes*

Ammonium sulphate fractions, of equal protein concentrations, prepared from Wistar and Gunn rat livers [20] were applied to antibody-Sepharose columns [19], washed with 5 column volumes of phosphate-buffered saline to remove non-specifically bound material and purified isoenzymes were eluted with 0.1 M glycine-HCl (pH 3.0).

2.6. *Immunoblot analysis*

Slab gel electrophoresis was performed on 8% separating gels by the method of Laemmli [21] in the presence of 0.1% SDS. The proteins were electrophoretically transferred from the gel to a nitrocellulose filter at 12 volts, overnight as described by Towbin et al. [22]. After transfer, nitrocellulose sheets were soaked in 2 changes of 0.05% polyoxyethylene sorbitan monolaurate (Tween 20)/0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl (TBS) at 37°C for 2 h to block reactive sites. The filters were then incubated with sheep anti-rat UDPGT IgG in a minimal volume of TBS, for 2 h at room temperature. Sheets were washed 3 times for 10 min each with 0.05% Tween/TBS, then incubated for 1 h with FITC labelled donkey anti-sheep IgG [23]. The sheets were then washed as before and photographed on a black background under UV light with Kodak Technical Pan film and an exposure time of 15–30 min.

3. RESULTS AND DISCUSSION

3.1. *Comparison of UDP-glucuronyltransferase activities towards various substrates in Wistar and Gunn rat liver microsomes*

Rat liver microsomes were assayed for UDP-glucuronyltransferase (UDPGT) activities towards various substrates to illustrate the existence of multiple defective glucuronidation reactions in the Gunn rat (table 1). These results show the complete absence of UDP-glucuronyltransferase activity towards bilirubin. In addition, levels of activity towards 2-aminophenol, 1-naphthol and androsterone are very reduced, whereas activity towards testosterone, morphine and aniline are present at nearly normal levels, as previously reported [5].

3.2. *Examination of microsomal extracts from Wistar and Gunn rat livers by SDS gel electrophoresis and immunoblot analysis*

We have developed an immunoblot analysis procedure which enables identification of 3 different isoenzymes of UDP-glucuronyltransferase in crude solubilised Wistar rat liver microsomal extracts which exhibit slightly different molecular masses (50–54 kDa). We have compared extracts from Wistar and Gunn rat liver microsomes using this technique (fig.1). These isoenzymes have been further characterised by co-migration with individually purified isoenzymes [24]. Purified bilirubin UDPGT (54 kDa, see fig.2) is the largest of 3 isoenzymes and the smallest isoenzyme is

Table 1

UDP-glucuronyltransferase activities in Wistar and Gunn rat liver microsomes

Substrate	Wistar	Gunn	Gunn/ Wistar (%)	Foetal Wistar	Foetal Gunn
2-Aminophenol	1.8 ± 0.1	0.2 ± 0.1	10	2.6 ± 0.3	0
2-Aminophenol + 10 mM pentan-3-one	3.4 ± 0.4	1.6 ± 0.4	47	2.6 ± 0.3	0
1-Naphthol	13.9 ± 5.0	4.2 ± 1.3	30	20.5 ± 6.6	0
Bilirubin	0.7 ± 0.3	0	0	0	0
Testosterone	2.9 ± 1.6	1.9 ± 1.8	66	0	0
Androsterone	1.2 ± 0.2	0.5 ± 0.4	42	0	0

Activities are expressed as nmol glucuronide formed/min per mg microsomal protein, and are means ± SD for at least 5 experiments. Liver microsomes, 0.8 mg protein, were assayed in the presence of 0.02% Brij 58 in the steroid assays or 0.2% digitonin for all other substrates. Pentan-3-one, 10 mM final concentration, was added to the assays with 2-aminophenol as substrate

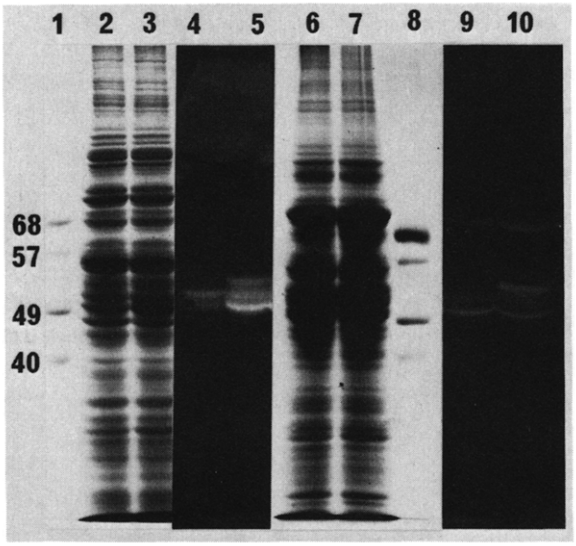

Fig.1. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of the ammonium sulphate fractions prepared from Wistar and Gunn rat liver microsomes. Tracks 1 and 8, protein standards, 2 μg of each protein (albumin (68 kDa), pyruvate kinase (57 kDa), fumarase (49 kDa) and aldolase (40 kDa)). Ammonium sulphate fractions of liver microsomes (140 μg protein) prepared from: untreated Wistar rats (3 and 5); untreated Gunn rats (2 and 4); clofibrate treated Wistar rats (7 and 10); clofibrate treated Gunn rats (6 and 9). One set of samples were stained with Coomassie blue (tracks 2,3,6 and 7) and a duplicate set of samples were immunoblotted and proteins visualised with FITC labelled antibodies.

testosterone UDPGT (50 kDa) [11,24]. The isoenzyme of molecular mass 52 kDa is tentatively identified as the UDPGT isoenzyme which glucuronidates either androsterone [11] or oestrone. These proteins are apparently the 3 most abundant UDP-glucuronyltransferases identified by this method. Comparison of extracts from Wistar and Gunn rat liver microsomes indicates the absence of the 54-kDa isoenzyme, bilirubin UDPGT from the Gunn rat microsomes (fig.1).

3.3. *Clofibrate induction of bilirubin UDP-glucuronyltransferase*

We have emphasised the loss of bilirubin UDP-glucuronyltransferase in Gunn rat liver microsomes by using microsomal extracts from rats treated with clofibrate, which specifically induces bilirubin UDPGT [25,26]. When Wistar rats were treated with clofibrate, microsomal bilirubin UDPGT activity was increased 2.5-fold whereas other UDPGT activities were either unchanged or slightly decreased [24] as expected from earlier studies [25,26]. Bilirubin UDPGT activity was still not detectable in liver microsomes from Gunn rats treated with clofibrate. When bilirubin UDPGT activity has been induced 2.5-fold by clofibrate this isoenzyme is a more prominent species, molecular mass 54 kDa, in the immunoblot of Wistar rat liver microsomal extract, but the isoenzyme is still not detectable in liver microsomes from clofibrate-treated Gunn rats (fig.1).

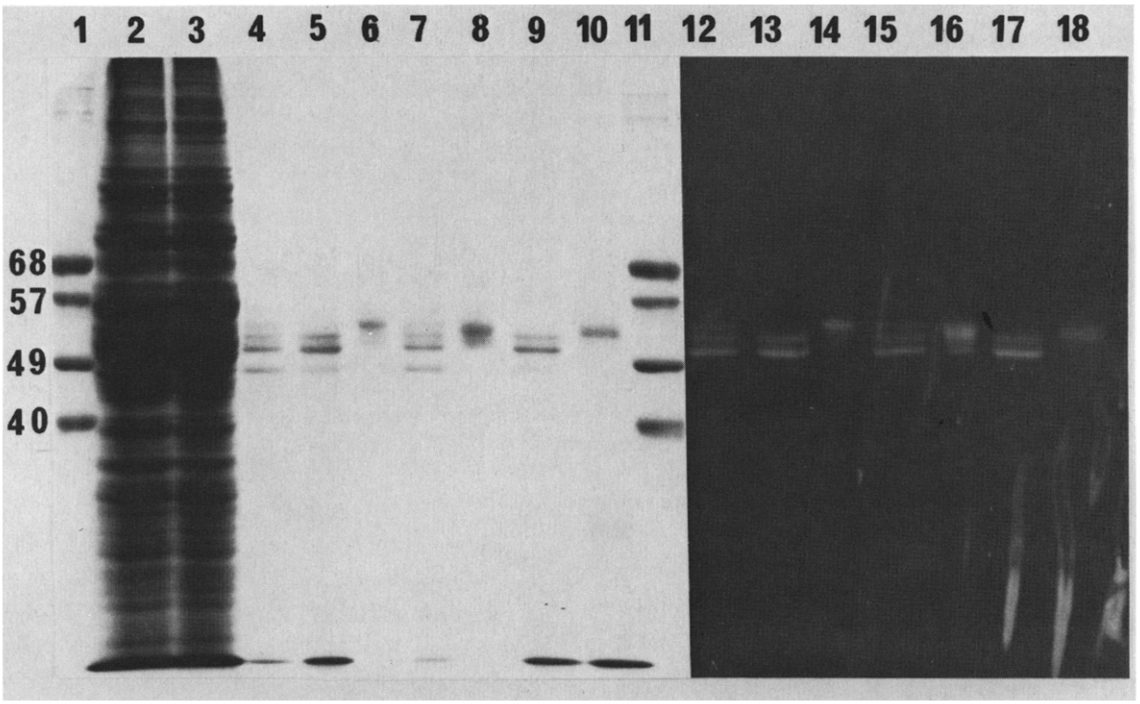

Fig.2. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of immunoaffinity purified and conventionally purified UDPGT isoenzymes. Tracks 1 and 11, M_r standards as fig.1; 2, ammonium sulphate fraction prepared from solubilised Wistar rat liver microsomes (200 μg protein); 3, ammonium sulphate fraction from Gunn rats (200 μg protein); 4,7,12 and 15, immunoaffinity purified UDPGTs from Wistar rat liver ammonium sulphate fraction (5 μg protein); 5,9,13 and 17, immunoaffinity purified UDPGTs from Gunn ammonium sulphate fractions (5 μg protein); 6 and 14, conventionally purified bilirubin UDPGT (1.5 μg protein); 8 and 15, purified 4-nitrophenol UDPGT (8 μg protein); 10 and 18, immunoaffinity purified UDPGT from Wistar foetal rat liver ammonium sulphate fractions (7 μg protein). Tracks 1–11, microsomal protein fractions separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Tracks 12–18, duplicates of samples 4–10 immunoblotted and visualised with FITC labelled antibodies.

3.4. *Immunoblot examination of purified UDP-glucuronyltransferase isoenzymes*

To obtain further confirmation of the above results and to avoid non-specific blotting, such as the background seen with the clofibrate-induced enolase, molecular mass 70 kDa (fig.1), we have immunoaffinity purified UDPGTs from microsomal extracts using antibody-Sepharose prior to immunoblot analysis. The immunoaffinity purified proteins were run on parallel SDS polyacrylamide gels, one of which was stained with Coomassie blue, and the other immunoblotted. These gels and immunoblots show the prominent isoenzymes present in Wistar and Gunn rat liver microsomes, compared to purified bilirubin UDPGT and phenol UDPGT. Examination of these results clearly shows that the protein in the 54-kDa region which co-migrates with bilirubin UDPGT is not detectable in the Gunn rat liver isolate (fig.2). Phenol UDPGT is also not apparently detectable in affinity purified isoenzyme preparations from Wistar or Gunn rat livers.

3.5. *Possible absence of phenol UDP-glucuronyltransferase from Gunn rat liver microsomes*

The recent separation and purification of testosterone UDPGT from phenol UDPGT has indicated that these two isoenzymes are both able to catalyse the glucuronidation of 4-nitrophenol [11]. Phenol UDP-glucuronyltransferase activity is known to appear at the late foetal stage (19–20

days gestation) during development, whereas testosterone UDPGT and many other UDP-glucuronyltransferase activities appear in a postnatal cluster [27]. Thus, examination of the UDPGT isoenzyme content of late foetal liver should provide further discriminative evidence concerning the phenol UDPGT in Gunn rat livers. Only one UDPGT was isolated from late foetal Wistar rat liver by immunoaffinity chromatography (fig.2). Assay of microsomal enzyme activities revealed the existence of phenol UDPGT in foetal Wistar rat liver (table 1). The phenol UDPGT enzyme exhibiting its characteristic activities has also been purified from foetal Wistar rat liver by conventional procedures (not shown). Phenol UDPGT activity was not detectable in foetal Gunn rat liver microsomes (table 1) and the phenol UDPGT protein was not detected by immunoblot analysis of crude extracts or immunoaffinity purified extracts from foetal Gunn rat livers. This study of late foetal liver microsomes from Wistar and Gunn rats indicates that phenol UDPGT might not be present in Gunn rat liver.

Phenol UDPGT, which is the only isoenzyme observed in foetal Wistar rat liver, does not appear to be a major isoenzyme in adult Wistar rat liver (fig.2) as it does not co-migrate with any of the 3 major isoenzymes. Testosterone UDPGT appears to be able to compensate for the reduction in the adult levels of this isoenzyme, although low levels of the hepatic phenol UDPGT activity are induced by 3-methylcholanthrene [11] or -naphthoflavone [26] treatment of rats. However, we have not been able to clearly examine the induction of phenol UDPGT protein in adult rat liver microsomal extracts using our 1-D immunoblot analysis due to the similar sizes of the UDPGT isoenzymes and low abundance of phenol UDPGT [24]; this problem should be resolved by using a more sophisticated 2-D immunoblot analysis.

One curiosity which has confused the analysis of the enzymological disturbance in this genetic deficiency is the specific activation of Gunn rat liver UDP-glucuronyltransferase activity towards 2-aminophenol by diethylnitrosamine and pentan-3-one [28] (see table 1). It is now apparent that these compounds might exert their effect on testosterone UDP-glucuronyltransferase, because phenol UDP-glucuronyltransferase may not be present in Gunn rat liver. Further indications which support this conclusion are that (a) phenol UDPGT activity towards 2-aminophenol in foetal Wistar rat liver is not activated by pentan-3-one (table 1) and (b) purified Gunn rat liver testosterone UDPGT activity towards 2-aminophenol is activated by pentan-3-one (not shown).

4. CONCLUDING REMARKS

These results have provided the first insight into the molecular disturbances which have occurred in the congenitally jaundiced Gunn rat. The detailed analysis of individual UDPGT isoenzymes in foetal and adult liver indicates that bilirubin UDPGT is absent from adult Gunn rat liver microsomes and that a low abundance phenol UDPGT may also be absent. The methods for identification of UDPGT isoenzymes will be greatly improved by the use of 2-D gel electrophoretic analysis [29] prior to immunoblotting. This approach should expand our preliminary studies on human liver extracts where we have been able to detect UDPGT isoenzymes [24] and facilitate our studies of the human genetic deficiency. Studies of the genetic deficiency of UDPGT not only provide information of the nature of the genetic lesion, they also provide useful insights into the natural heterogeneity of UDPGT isoenzymes and their regulation during development in rats and humans.

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