A RAT LIVER MICROSOMAL CARBOXYESTERASE AND A BILIRUBIN UDP-GLUCURONYL TRANSFERASE ARE RESPONSIBLE FOR THE FORMATION OF BILIRUBIN GLUCURONIDES FROM BILIRUBIN DIMETHYL ESTER

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1. Introduction

The hepatic clearance of non-conjugated bilirubin from the circulation is an essential homeostatic requirement of mammalian species. Failure of this hepatic function can lead to irreversible brain damage, kernicterus and even death in both the neonatal infant and some adults [1]. The definition of one of the steps in the clearance mechanism, the conjugation of bilirubin to more polar metabolites, is critical for the understanding of certain hereditary hyperbilirubinemias. The lifelong unconjugated hyperbilirubinemia of the Crigler–Najjar syndrome of man and its animal analogue, the Gunn rat, are examples of essentially a complete inability to form the ester glucuronide of bilirubin in vivo and also when hepatic microsomes are assayed in vitro [2,3].

In [4], infusion of the dimethyl ester of bilirubin (DMB) in Gunn rats resulted in the biliary excretion of both mono- and diglucuronides of bilirubin. Bilirubin glucuronides were formed in vitro by liver microsomes from both the Gunn rat and a patient with the Crigler–Najjar syndrome if DMB was used as substrate [4].

The in vitro yields of bilirubin glucuronides from DMB are small and not always reproducible using Gunn rat liver microsomes (G. B. O., B. B., unpublished), therefore we decided to use normal Wistar rat liver to determine what enzymatic pathways are responsible for the biotransformation of DMB to bilirubin glucuronides.

Here, we report that by following the procedure for isolation and purification of Wistar rat liver microsomal bilirubin UDP-glucuronyltransferase in [5] and by further measurement of carboxyesterase activities in these purified fractions, we are able to demonstrate that a microsomal esterase and a bilirubin UDP-glucuronyltransferase catalyse the biotransformation of DMB to bilirubin glucuronides. The relevance of this work to the study of the genetic deficiency of bilirubin UDP-glucuronyltransferase in Gunn rat liver is discussed.

2. Materials and methods

Egg lecithin, grade 1, was purchased from Lipid Products (South Nutfield, Surrey). Bilirubin, 4-nitrophenol, 4-nitrophenyl acetate, bis-(4-nitrophenyl)-phosphate and UDP-glucuronic acid triammonium salt were from Sigma (London). The dimethyl ester of bilirubin (DMB) was synthesized and purified as in [4].

2.1. *Enzyme assays*

UDP-glucuronyl transferase activity towards bilirubin was measured by the methods in [6,7] and towards 4-nitrophenol as in [8]. Glucuronide formation from DMB was measured by a modification of the method in [7]. The incubation mixtures (0.21 ml) contained Tris–maleate buffer (pH 7.4) 0.1 M; DMB, 0.14 mM; UDP-glucuronic acid, 5 mM; $MgCl_2$, 15 mM; KCl, 3.75 mM; 10% DMSO and up to 100 μl of enzyme preparation. Control incubations contained all constituents except the UDP-glucuronic acid. After incubation with shaking (90 cpm) for 30 min at 37°C, 1.3 ml of ice-cold lactic acid/ethyl acetate (8:5) was added, followed rapidly by 1.6 ml chloroform. The mixture was vigorously shaken and then allowed to separate into non-miscible layers prior to layering

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25 μl of diazo-reagent [7] onto the top of the aqueous phase. A final rapid mix was immediately followed by centrifugation for 5 min at 2000 × *g*. The absorbance of the aqueous phase of both control and experimental incubates was measured at 555 nm against a solvent blank. Carboxyesterase activity towards 4-nitrophenyl acetate was assayed by the method in [9].

2.2. *Purification of enzymes*

Wistar rats were pretreated with 2 g phenobarbital/l in their drinking water for 10 days. The enzymes in hepatic microsomal pellets were solubilized and partially purified by ammonium sulphate fractionation as in [10]. Bilirubin UDP-glucuronyl transferase was further purified and reconstituted with lecithin as in [5].

The activity of carboxyesterases isolated in DEAE-cellulose fractions (see section 3) was not further purified.

2.3. *Inhibition studies*

Fifty microliters (containing ~400 μg protein) of the ammonium sulphate fraction or the DEAE-cellulose concentrate of fraction tubes 80–90 were incubated with 0.5 mM bis-(4-nitrophenyl)phosphate for 30 min at 25°C in 10 mM phosphate buffer (pH 8.0) [11]. These mixtures were then assayed for UDP-glucuronyl transferase and carboxyesterase activity.

2.4. *Thin-layer chromatography of DMB and metabolites*

The dimethyl ester and other bilirubin derivatives resulting from carboxyesterase incubations (0.15 ml) were extracted by addition of an equal volume of chloroform and 10 μl glacial acetic acid. After vigorous shaking, the mixture was centrifuged for 10 min at 2000 × *g*. Samples (10 μl) of the lower chloroform layer were spotted onto silica gel sheets (Eastman Kodak Co., Rochester NY), developed using chloro-

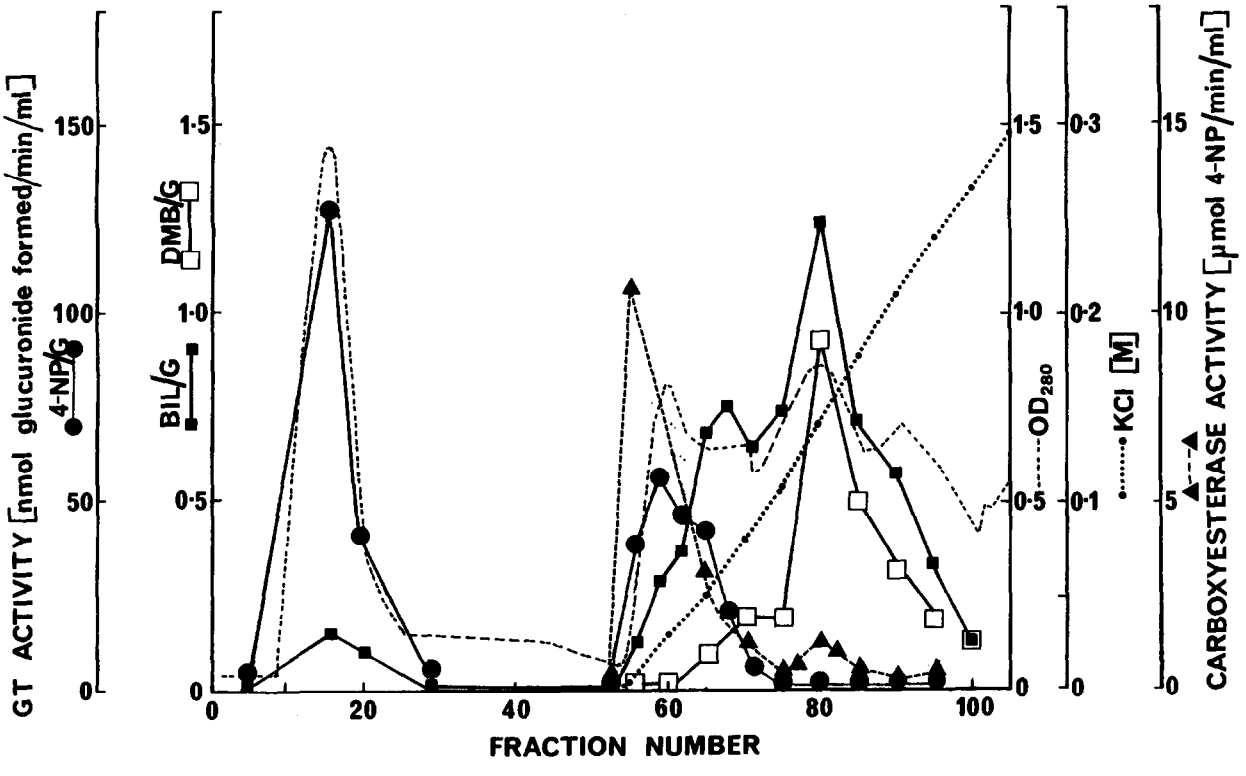

Fig.1. DEAE-Cellulose column chromatography of rat hepatic microsomal carboxyesterases and UDP-glucuronyltransferases. Ammonium sulphate fraction (25 ml) was applied to a DEAE-cellulose column as in [5]. A linear 0–0.3 M KCl gradient was applied to elute activities of interest. Fractions (5 ml) were collected at 15 ml/h and assayed for UDP-glucuronyltransferase activity towards 4-nitrophenol (4-NPG), bilirubin (BIL/G) and carboxyesterase using 4-nitrophenyl acetate. Activity towards DMB was also assayed using the method in the text.

form:glacial acetic acid (98:2), and sprayed with 10 mM diazotized sulfanilic acid in 50% methanol for identification and measurements of their R_F.

3. Results

3.1. *Co-purification of UDP-glucuronyl transferase and carboxyesterase activities involved in the conversion of DMB to bilirubin glucuronides*

Ammonium sulphate fractions containing the required enzyme activity were applied to a DEAE-cellulose column as in fig.1. A sample of each eluted fraction was assayed after mixing with lecithin liposomes (mg lecithin/mg protein, 1:1) [5]. The majority of UDP-glucuronyl transferase activity towards 4-nitrophenol but not towards bilirubin was eluted before the salt gradient was applied to the column (fig.1).

The enzyme activity towards DMB was eluted at 140 mM KCl by the linear salt gradient (fig.1). This enzyme activity coincides with the elution of a large peak of bilirubin UDP-glucuronyl transferase activity which was completely separated from transferase activity towards 4-nitrophenol. Elution of enzyme activity towards DMB also coincides with the elution of the small peak of carboxyesterase activity as determined using the broad spectrum substrate 4-nitrophenyl acetate. It should be noted that the combination eluted in fraction 65, of high levels of esterase and bilirubin UDP-glucuronyl transferase activities was unable to convert DMB into bilirubin glucuronides.

Examination of the action of the carboxyesterases in fraction 56 and 80 by thin-layer chromatography revealed the formation of different DMB metabolites. The C8 monomethyl ester of bilirubin has a larger R_F on silica gel than the isomeric C12 methyl ester [12]. Fraction 80 catalysed the formation of C12 monomethyl ester (R_F 0.47), to a lesser extent C8 monomethyl ester (R_F 0.52) and free bilirubin (R_F 0.78) from residual DMB (R_F 0.25) (fig.2). Fraction 56 could only catalyse the formation of the C12 monomethyl ester. Thus, only a certain fraction of the complex carboxyesterases [13] will form the C8 monomethyl bilirubin and free bilirubin which may preferentially undergo glucuronidation (see section 2.3.).

Further purification of bilirubin UDP-glucuronyl transferase activity in pooled concentrates A and B (A = DEAE-cellulose fractions 65–75; B = DEAE-cellulose fractions 76–90) to near homogeneity using

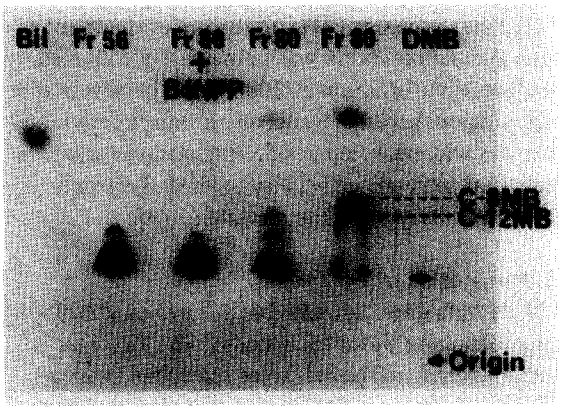

Fig.2. Thin-layer chromatographic analysis of bilirubin derivatives using silica gel. Chloroform extracts (10 μl) from incubation of DEAE-cellulose with DMB (section 2) were applied to silica gel plates (10 cm × 10 cm). Spots were identified as above: B4NPP, bis-(4-nitrophenyl)phosphate; Bil, bilirubin; C-8MB, C8 monomethylbilirubin; C-12MB, C12 monomethylbilirubin.

UDP-hexanolaminesepharose chromatography [5] resulted in complete removal of carboxyesterase activity and DMB could no longer act as substrate for the formation of bilirubin glucuronides (table 1). Thus, UDP-glucuronyl transferase alone does not show activity towards DMB and appears to require prior action from a carboxyesterase. Further evidence to support this hypothesis is described below.

3.2. *Effect of bis-(4-nitrophenyl)phosphate on the formation of bilirubin glucuronides from DMB*

The selective site-specific inhibitor of carboxyesterases bis-(4-nitrophenyl)phosphate [13] was used to confirm that a carboxyesterase demethylates DMB prior to its glucuronidation. This inhibitor selectively binds to the carboxyesterase present in hepatic microsomes [13].

Pre-incubation of either ammonium sulphate fractions or DEAE-cellulose concentrate B (fractions 76–90) with 1 mM bis-(4-nitrophenyl)phosphate (see section 2) inhibited 90% of the carboxyesterase activity towards 4-nitrophenyl acetate (table 2) and thus prevented 85% of the glucuronidation of DMB by either preparation. The UDP-glucuronyl transferase activities towards bilirubin in ammonium sulphate fraction or DEAE-cellulose concentrate B were not inhibited by 1 mM bis-(4-nitrophenyl)phosphate (table 2).

Table 1
Purification of the rat liver enzymes involved in the transformation of dimethyl-bilirubin (DMB) into bilirubin glucuronides (BILG)[Enzyme specific activities (nmol . min^{-1} . mg $protein^{-1}$)]

	Total protein	Carboxyesterase	DMB → BILG	Bilirubin UDP-glucuronyl transferase
25–60% Satn $(NH_4)_2SO_4$ precipitate	238 mg	5200	0.74	0.86
DEAE-cellulose eluate concentrate (fractions 75–90)	23.5 mg	2762	2.16	3.60
UDP-hexanolamine Sepharose/UDP Glucuronic and eluate	0.28 mg	0	0	4.70

The specific activity towards DMB as substrate is expressed as BILG equivalents produced. All activities were assayed after enzyme fractions were reconstituted with lecithin liposomes (1 mg lecithin/mg protein). Results shown were obtained from 3 phenobarbital-treated male Wistar rat livers. Carboxyesterase was assayed using 4-nitrophenyl acetate as substrate and bilirubin UDP-glucuronyltransferase as in [6]

The inhibitory effect of pre-incubation of 100 μl fraction 56 and 80 with 0.5 mM bis-(4-nitrophenyl)-phosphate on the carboxyesterase activity was also examined by thin-layer chromatography. Examination of the bilirubin metabolites showed that only the formation of C8 monomethyl bilirubin and free bilirubin by fraction 80 were prevented by this specific inhibitor (fig.2). Formation of the C12 monomethyl bilirubin by fraction 80 was not significantly inhibited by bis-(4-nitrophenyl)phosphate, although confirmation of this observation requires more precise quantitative measurements. Thus, the C8 monomethyl bilirubin rather than the C-12 monomethyl bilirubin does appear to be preferentially glucuronidated by a bilirubin UDP-glucuronyltransferase in DEAE-cellulose fraction 80.

Table 2
Effect of bis-(4-nitrophenyl)phosphate on the formation of bilirubin glucuronides from dimethyl bilirubin (DMB) by rat liver ammonium sulphate fractions

Enzyme	Substrate	Inhibitor [bis-(4-nitrophenyl)-phosphate]	Specific activity (nmol . min^{-1} . mg $protein^{-1}$)	Inhibition (%)
Carboxyesterase	4-nitrophenyl acetate	+	662	90
		–	6620	–
DMB → BIL/G	DMB	+	0.13	85
		–	0.86	–
UDP-glucuronyl transferase	Bilirubin	+	1.91	0
		–	1.83	–

Results shown are the average obtained using 3 separate preparations of ammonium sulphate fractions from 3 phenobarbital-treated Wistar rat livers; ~50 μl (400 μg protein) of ammonium sulphate fraction was pre-incubated with 50 μl 1 mM bis-(4-nitrophenyl)phosphate for 30 min at 25°C prior to enzyme assays. Bilirubin UDP-glucuronyltransferase was assayed as in [7]

4. Discussion

The above results show that DMB is first demethylated by a carboxyesterase and the C8 monomethyl ester and free bilirubin formed are then glucuronidated by a bilirubin UDP-glucuronyl transferase which is completely devoid of activity towards 4-nitrophenol. Although the DEAE-cellulose concentrate A (fractions 65–75) formed C12 monomethyl bilirubin, this ester was not significantly glucuronidated by the bilirubin UDP-glucuronyltransferase present.

This work suggests that the formation of bilirubin diglucuronide from bilirubin may occur in an ordered sequence rather than by a random mechanism. The C8 monomethyl bilirubin being preferentially glucuronidated indicates that the propionate carboxyl of C12 is the preferred site of attack for either the mono- or diglucuronidation reaction.

These results also delineate a metabolic pathway by which the Gunn rat can metabolize DMB and strongly support the proposal [4] that a functionally defective bilirubin UDP-glucuronyl transferase is present in Gunn rat liver, although this enzyme could not be specifically identified by immunological studies [14].

A number of reasons are possible to explain the complete absence of ester glucuronide formation in Gunn rat liver when bilirubin itself is used as substrate. One of these possibilities must now be seriously considered. As long suspected, bilirubin could be glucuronidated by two enzymes. In Gunn rat liver the first enzyme may be a defective microsomal UDP-glucuronyltransferase which cannot accept free bilirubin, but can accept a bilirubin in which the C8 position is already esterified, as in C8 monomethyl bilirubin. The microsomal carboxyesterase described above would then free the C8 propionate carboxyl group for further conjugation to the diglucuronide. Gunn rats are known to form bilirubin diglucuronide from infused monoglucuronide [15]. Whether this second glucuronidation is also UDP-glucuronic acid-dependent [16] or catalyzed by a dismutase [17] is a subject of current controversy.

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