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Formation of Bilirubin Glucoside

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1. Rat liver microsomal preparation can effect the transglucosylation from UDP-glucose to bilirubin in the presence of Mg^{2+}. 2. Other nucleotides, namely CDP-glucose, ADP-glucose and GDP-glucose, were not active as glucosyl donors. 3. Only trace amounts of galactose, galacturonic acid and *N*-acetylglucosamine were conjugated to bilirubin when their respective UDP derivatives were used in the reaction mixture. 4. The azobilirubin glucosides produced by coupling with *p*-diazobenzenesulphonic acid and diazotized ethyl anthranilic acid were separable from the corresponding azobilirubin glucuronides by t.l.c. 5. The glucoside was, however, hydrolysed by both β-glucosidase and various preparations of β-glucuronidase; azobilirubin and glucose were liberated in the process. 6. Kinetic studies showed that the effects of pH and Mg^{2+} on the two conjugating systems were similar. 7. The specific activities of hepatic bilirubin UDP-glucosyltransferase, expressed as μg of bilirubin 'equivalents' conjugated/h per mg of protein, are respectively 1.7 and 2.4 for male and female rats. 8. The K_m values for bilirubin and UDP-glucose are 5.7×10^{-5} M and 1.6×10^{-3} M respectively. 9. The glucoside and glucuronide conjugations of bilirubin are discussed in relation to the availability of the conjugating agents and aglycone in the liver.

Bilirubin is excreted in normal bile essentially as the glucuronide (Dutton, 1966*a*). A fraction is believed to be sulphated (Isselbacher & McCarthy, 1959; Noir, Dewalz & Garay, 1970). However, because of the discovery of bilirubin glucoside in dog bile (Heirwegh, van Hees, Compernolle & Fevery, 1970) the concept that the glucuronide is the predominant excretory product of bilirubin has been modified. Kuenzle (1970) has proposed that the major bilirubin conjugates in human bile occur not as glucuronides but as the acyl glycosides of aldobiouronic acid, pseudoaldobiouronic acid and hexuronosylhexuronic acid. These observations suggest that sugars other than glucuronic acid may be conjugated to bilirubin by hepatic cells. In the present paper the biosynthesis of bilirubin glucoside is shown to occur in rat liver microsomal fraction. From kinetic considerations, it is conceivable that the glucosylation of bilirubin may be of considerable importance in this animal.

MATERIALS AND METHODS

Chemicals. The following were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.: bilirubin, UDP-glucose (sodium salt), CDP-glucose (sodium salt), GDP-glucose (sodium salt), ADP-glucose (disodium salt), UDP-galactose (sodium salt), UDP-*N*-acetylglucosamine (sodium salt), UDP-galacturonic acid (tripotassium salt), UDP-glucuronic acid (ammonium salt), β-glucosidase (emulsin) from almonds, and β-glucuronidase from *Helix pomatia* (type H-1), limpets (type L-1) and ox liver (type B-3).

Analytical procedures. The preparations of microsomal enzyme from liver and the analysis of the products of the enzymic reaction were done by the procedure of Wong (1971) with slight modifications: UDP-glucuronic acid in the medium was replaced by UDP-glucose (final concentration, 3.3 mM). In most experiments the reaction was terminated after 60 min of incubation. A portion (0.75 or 1 ml) of the 1.5 ml of butanol extract was evaporated and quantitatively applied to cellulose-coated plates. Treatment with diazotized ethyl anthranilate (van Roy & Heirwegh, 1968) was also done, then the azo-pigments were extracted with butanol and identified by t.l.c.

Hydrolysis of azobilirubin glucoside. The azo-pigments of the bilirubin glucoside that were formed enzymically in various reaction tubes by treatment with *p*-diazobenzenesulphonic acid were pooled and isolated by t.l.c. The products were subjected to hydrolysis by β-glucosidase (emulsin) from almonds at pH 5 or by preparations of β-glucuronidase from *H. pomatia* at pH 4, from limpets at pH 4 and from ox liver at pH 5. In all instances, 0.2 M-sodium acetate buffer was used. The aglycone and the sugar liberated were identified by t.l.c. as described by Wong (1971).

Protein determination. Protein in the microsomal preparations was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Formation of bilirubin glucoside. The azo-pigment of bilirubin glucoside was produced in the reaction after treatment with *p*-diazobenzenesulphonic acid (Fig. 1). It is distinct from the corresponding glucuronide synthesized in an analogous reaction (Wong, 1971). When diazotized ethyl anthranilate was employed as the reagent, the azobilirubin glucoside and glucuronide that were produced exhibited essentially similar chromatographic behaviour with respect to their relative mobilities in the various solvent systems (Table 1). In contrast to the bright red coloration of the azobilirubin glucuronide formed with diazotized sulphanilic acid, that of the glucoside has a brownish tinge. However, on extraction with butanol and subsequent chromatography, the eluted azobilirubin glucoside has an absorption spectrum similar to that of the glucuronide, with a maximum at 530nm.

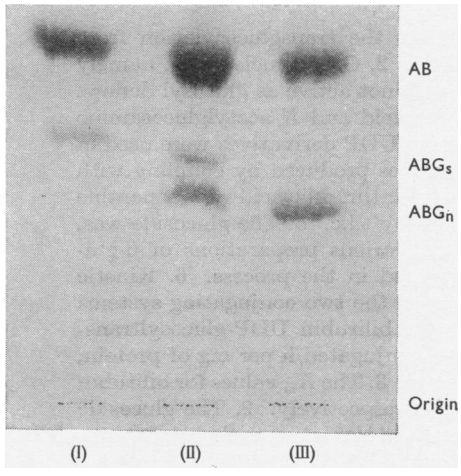

Fig. 1. T.l.c. of azo-pigments. Azobilirubin glucoside (ABG_s) and azobilirubin glucuronide (ABG_n) were formed in reaction mixtures containing UDP-glucose (I) and UDP-glucuronic acid (III) respectively. These reaction mixtures were co-chromatographed (II). Separation of these azo-pigments from azobilirubin (AB) was accomplished on t.l.c. plates (20cm×20cm) coated with silica gel G and developed in butan-1-ol–acetic acid–water (4:1:4, by vol.).

Specificity. The synthesis of bilirubin glucoside is specific with respect to the nucleotide donor, UDP-glucose. Other nucleotides, namely CDP-glucose, ADP-glucose and GDP-glucose were inactive. UDP-galactose, UDP-*N*-acetylglucosamine and UDP-galacturonic acid showed only minor transference of their sugar moieties to bilirubin when added in concentrations comparable to that of UDP-glucose.

Kinetic studies. These were done by using female rat liver microsomal fractions that had been dialysed against 10mM-EDTA (disodium salt) adjusted to pH8.2. The enzyme activity curves obtained for different pH and Mg^{2+} concentrations exhibited similar profiles to those of the glucuronyltransferase (Wong, 1971). The quantity of bilirubin glucoside formed increased with the time of incubation and the enzyme concentration. From the double-reciprocal plots of Lineweaver & Burk (1934), the K_m values for bilirubin and UDP-glucose are respectively 5.7×10^{-5}M and 1.6×10^{-3}M (Figs. 2 and 3). For comparison, the K_m values for

Table 1. *Thin-layer chromatography of azobilirubin, azobilirubin glucoside and azobilirubin glucuronide*

Experimental details are given in the text. Microscope slides (25mm×75mm) were used for t.l.c. The solvent systems used were: I, butan-1-ol–acetic acid–water (4:1:4, by vol.); II, butan-1-ol–acetic acid–water (4:1:5, by vol.); III, ethyl methyl ketone–propan-1-ol–water (15:5:6, by vol.). SA, diazotized sulphanilic acid; EA, diazotized ethylanthranilate.

Solvent systems	Adsorbents	Diazo reagents	R_F values: Azobilirubin	Azobilirubin glucoside	Azobilirubin glucuronide
I	Silica gel G	SA	0.61*	0.36	0.30*
	Silica gel G	EA	0.63	0.40	0.28
	Cellulose	SA	0.67*	0.56	0.56*
	Cellulose	EA	0.68	0.59	0.59
II	Silica gel G	SA	0.56	0.34	0.28
	Silica gel G	EA	0.69	0.49	0.37
	Cellulose	SA	0.63	0.50	0.50
	Cellulose	EA	0.63	0.53	0.53
III	Silica gel G	SA	0.56*	0.61	0.47*
	Cellulose	SA	0.83*	0.68	0.61*

* R_F values taken from Wong (1971) for comparison.

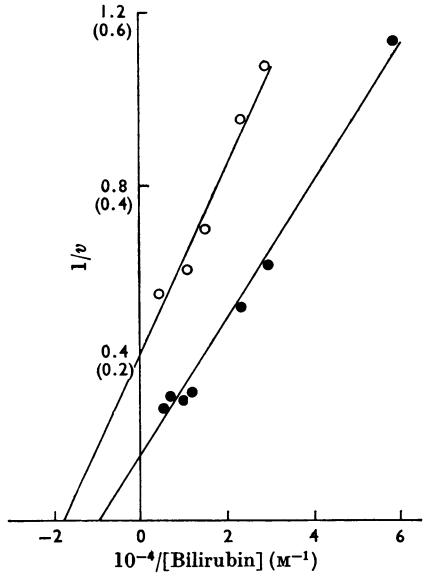

Fig. 2. Double-reciprocal plots of velocity (μg of bilirubin 'equivalents'/h per mg of protein) against concentrations of bilirubin. UDP-glucose (○) and UDP-glucuronic acid (●) were used in the reactions; values in parentheses on the ordinate were obtained for the latter conjugating agent.

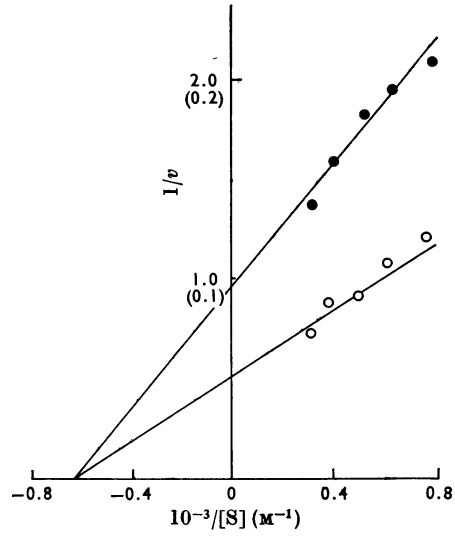

Fig. 3. Double-reciprocal plots of velocity (μg of bilirubin 'equivalents'/h per mg of protein) against concentrations of UDP-glucose (○) and UDP-glucuronic acid (●). Liver microsomal preparation of female rats was used as the enzyme source. Values in parentheses on the ordinate were obtained with UDP-glucuronic acid as the conjugating agent.

bilirubin and UDP-glucuronic acid were determined with the same enzyme preparation under the same experimental conditions; these were 1.25×10^{-4} M and 1.6×10^{-3} M respectively. These results showed that the affinity of glucosyltransferase for bilirubin is higher than that of glucuronyltransferase.

Hydrolysis of azobilirubin glucoside and glucuronide. When present in low concentration, azobilirubin glucoside was completely hydrolysed by both β-glucosidase and β-glucuronidase, but partial hydrolysis by the latter enzyme was observed when the conjugate was added in high concentrations. Azobilirubin and glucose were detected in the hydrolysate by t.l.c. Because of the non-specificity of the above hydrolases (cf. Levvy & Conchie, 1966), azobilirubin glucuronide was subjected to similar hydrolytic treatments. It was susceptible to attack by β-glucosidase as well, glucuronic acid being released in this case. For the detection of these two sugars, chromatography of the hydrolysates was done on a t.l.c. plate (20cm × 20cm) coated with silica gel G prepared with 0.1M-boric acid. In the solvent system benzene–acetic acid–methanol (1:1:3, by vol.) glucose and glucuronic acid have R_F values of 0.72 and 0.65 respectively.

Measurement of bilirubin glucosyl- and glucuronyl-transferase activities. Bilirubin UDP-glucosyl- and UDP-glucuronyl-transferase activities of rat liver microsomal preparations were measured under the same conditions (Table 2). Both activities were higher in the preparation from female rats, with a female/male activity ratio of 1.4–1.5:1. In this connexion, it is noteworthy that this ratio was reversed for the amount of free bilirubin in blood (Broderson, Sparre & Vind, 1963). This unique reciprocal relationship seemed more than coincidental. However, on the basis of the present findings, it is difficult to explain the inhibitory action of oestrogens on the glucuronyltransferase system (Hsia, Riabov & Dowben, 1963; Adlard & Lathe, 1970).

DISCUSSION

The biosynthesis of glucosides was thought to be limited to plants (Dutton, 1966*a*; Parke, 1968), insects (Dutton & Duncan, 1960; Dutton, 1962; Smith & Turbert, 1964) and molluscs (Dutton, 1965, 1966*b*; Illing & Dutton, 1970). The absence of glucosyltransferase would preclude the formation of glucosides in mammals. However, the glucosides of steroids have been synthesized (Williamson, Collins, Layne, Conrow & Bronstein, 1969; Williamson, Polakova & Layne, 1971; Collins, Williamson & Layne, 1970) and those of bilirubin and 3-hydroxykynurenine (van Heyningen, 1971) have been shown

Table 2. *Activity of bilirubin UDP-glucosyl- and glucuronyl-transferase of rat liver*

Experimental details are given in the text. Assays were for 30 min for glucuronyltransferase and 60 min for glucosyltransferase.

Enzyme preparation		Specific activity (μg of bilirubin 'equivalents'/h per mg of protein)	
Sex of rat	Dialysis against EDTA (10 mM)	Glucosyltransferase	Glucuronyltransferase
Female	—	1.6	7.7
*Female	Dialysed	2.4	9.4
Male	—	1.0	4.2
*Male	Dialysed	1.7	6.2

* The female/male enzyme activity ratio is 1.41:1 for glucosyltransferase and 1.52:1 for glucuronyltransferase.

Table 3. *Participation of UDP-glucose in biochemical reactions*

Enzyme	Product of reaction	K_m value for UDP-glucose	Reference
UDP-glucose dehydrogenase (EC 1.1.1.22)	UDP-glucuronic acid	2.0×10^{-5} M to 2.5×10^{-4} M	Maxwell, Kalckar & Strominger (1956)
UDP-glucose–glycogen glucosyltransferase (EC 2.4.1.11)	Glycogen	1.1×10^{-3} M (I-form) 9.0×10^{-4} M (D-form)	Salitis & Oliver (1964); Steiner, Younger & King (1965); Villar-Palasi, Rosell-Perz, Hizukuri, Huijing & Larner (1966)
UDP-glucose 4-epimerase (EC 5.1.3.2)	UDP-galactose	9.0×10^{-5} M 8.8×10^{-6} M	Maxwell (1957) Goldberg, Dahl & Parke (1961)
Bilirubin UDP-glucosyltransferase	Bilirubin glucoside	5.7×10^{-5} M	This paper

to occur physiologically in mammalian systems.

The present paper shows that bilirubin glucoside is biosynthesized *in vitro* by rat liver microsomal fraction. Like the glucuronyltransferase, the enzyme that catalysed the transglucosylation is found in the microsomal fraction. Both exhibited similar pH optima and have a requirement for Mg^{2+} for their activity (cf. Wong, 1971). Because of their similar features, it is essential to ensure that no conversion of UDP-glucose into its oxidized analogue occurs under the assay conditions. Analysis for UDP-glucuronic acid by the specific fluorimetric procedure of Wong & Sourkes (1967) showed no enzymic or spontaneous dehydrogenation of the added nucleotide after an initial incubation period of 60 min.

To evaluate the relative importance of the glucosyl- and glucuronyl-transferases, kinetic results of the two enzymic reactions were obtained simultaneously and compared. The lower K_m of glucosyltransferase for bilirubin suggests that glucosylation may be the favoured route of conjugation in the rat. This would be further enhanced by the higher hepatic concentration of UDP-glucose (about 100 μmol/100 g of liver) compared with that of UDP-glucuronic acid, which is only one-third of this value (Wong & Sourkes, 1967; Muller-Oerlinghausen & Kunzel, 1969). It is not known if these nucleotides in the tissues are compartmentalized or present in a homogeneous pool. On the basis of the latter assumption, their concentrations would be 1.4 mM and 0.4 mM respectively. Since the K_m values for these substrates are higher than the probable hepatic tissue concentrations of these nucleotides, it is conceivable that these nucleotides may be rate-limiting, and particularly that of UDP-glucuronic acid.

From the above considerations, it is envisaged that glucosylation of bilirubin may be the more probable pathway for bilirubin conjugation in the rat. However, the accessibility of this uridine nucleotide *in situ* must be taken into account. UDP-glucose is involved in yet another important pathway: it is the immediate precursor of glycogen. It can also be oxidized to UDP-glucuronic acid or epimerized to UDP-galactose in the liver. For comparison, the K_m values for UDP-glucose for these biochemical reactions are shown in Table 3.

With these diverging pathways and dissimilar K_m values, some metabolic control at this locus seems critical. Such regulation of the uridine nucleotides has been demonstrated in plant tissues (Neufeld & Hall, 1965).

Besides the uridine nucleotides, the concentration of the aglycone may also determine the rate of the transglucosylation reaction. The concentration of unconjugated bilirubin in serum varies from 2.8 to 4mg/l (Broderson *et al.* 1963), i.e. 5–7μM. Assuming that free bilirubin in plasma is in equilibrium with bilirubin in hepatic cells, these concentrations are well below the K_m values of bilirubin measured *in vitro* for both the conjugating enzymes. Thus the transglucosylation and transglucuronylation processes may also be very dependent on the availability of bilirubin.

The specific activities of the two transferases of rat liver microsomal preparations, measured at the same time, are shown in Table 2. The activity of glucuronyltransferase appears to be higher, an observation that contradicts the conclusions derived from the kinetic results: the K_m values for the conjugating agents being equal, the lower K_m of glucosyltransferase for bilirubin infers a higher affinity for this aglycone. The discrepancy may be attributable to (a) a lower molar extinction coefficient of the azobilirubin glucoside as compared with that of the glucuronide and (*b*) incomplete or differential diazotization in the absence of an accelerator. From this, it is clear that quantitative comparison in this instance needs to be interpreted with caution. It is recommended that until these conjugates are available for standardization, experimental results should be more appropriately expressed as μg of bilirubin 'equivalents' conjugated rather than as μg or μmol of bilirubin conjugated. The term 'equivalent' implies 'with reference to a standard of bilirubin'; no direct molar proportionality between the aglycone and conjugate being implied, unless otherwise stated. The stoicheiometric relationship established in the colorimetric and radioisotopic procedures for bilirubin glucuronyltransferase (Wong, 1971) suggests that such a proportionality may exist between azobilirubin and azobilirubin glucuronide.

The study of this alternative pathway of conjugation of bilirubin should be extended to newborn animals where a deficiency of UDP-glucuronic acid and UDP-glucuronyltransferase has been shown to be the cause of neonatal jaundice (Lathe & Walker, 1957; Dutton & Greig, 1957; Brown, Zueler & Bollet, 1958). Preliminary experiments showed that the hepatic glucosyltransferase activity of 6-day-old litters of rats was too low to be measured by the procedure described in the present paper. It would seem that bilirubin UDP-glucosyltransferase is also deficient in the newborn animal.

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