The Formation and Distribution of Bilirubin Monoglucuronide and Diglucuronide in Rat Liver Slices

By MARY T. CAMPBELL and GEOFFREY J. DUTTON*

Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

(Received 6 November 1978)

1. Bilirubin conjugation in rat liver slices was reassessed by using analysis of ethyl anthranilate azopigments to estimate separately the formation of bilirubin mono- and di-glucuronides. 2. Conjugation in slices resembles the situation *in vivo* more closely than does microsomal conjugation, in that diglucuronide is formed in appreciable quantity. 3. Both bilirubin mono- and di-glucuronides were present in slices in approximately equal amounts, but the monoglucuronide was the major product found in the incubation medium. 4. These results are discussed in relation to recent theories on the relationship between bilirubin mono- and di-glucuronide formation *in vivo*.

In man and rat bilirubin is excreted in bile predominantly as diconjugates of glucuronic acid (Fevory *et al.*, 1972a, 1977a; Noir, 1976), but synthesis *in vitro* of bilirubin conjugates by using broken-cell preparations from human and Wistar rat liver, with UDP-glucuronic acid as sugar donor, predominantly produces bilirubin monoglucuronide (Van Roy & Heirwegh, 1968; Black *et al.*, 1970; Fevory *et al.*, 1972b), a presumed intermediate in formation of diglucuronide (Heirwegh *et al.*, 1973). This reaction is catalysed by microsomal UDP-glucuronosyltransferase (EC 2.4.1.17). Liver slices provide a practical, but little used, alternative for investigating the products of bilirubin conjugation *in vitro*. One report has suggested that rat liver slices produce only bilirubin diglucuronide (Schoenfield & Bollman, 1963), thus closely reflecting bilirubin conjugation *in vivo*, and cultures of hepatoma MH₁C₁ cells may also synthesize both mono- and di-glucuronides (Rugstad *et al.*, 1970). Since these reports, more reliable techniques have become available for identification and assay of conjugated bilirubin pigments (see Heirwegh *et al.*, 1974). We have reinvestigated formation of bilirubin mono- and di-conjugates in rat liver slices by using these methods and find that both types of conjugates are formed. Diconjugate, however, was found mainly within the slices and appeared to have been converted into monoconjugate during or after excretion from them.

Materials and Methods

Bilirubin, glucaro-1,4-lactone and bovine serum albumin (fraction V) were from Sigma Chemical Co.,

Poole, Dorset, U.K., and silica-gel 60 precoated glass plates or plastic sheets were from E. Merck, Darmstadt, Germany. Fresh male serum was obtained from healthy human volunteers and frozen male and female serum was provided by the Department of Virology, Ninewells Hospital, Dundee. Rat serum was obtained by heart puncture under diethyl ether anaesthesia. Adult male Wistar R rats, fed *ad libitum*, were used throughout.

Preparation and incubation of liver slices

This was carried out as described by Lathe & Walker (1958), except that the incubation medium contained 2.2ml of the phosphate/bicarbonate solution, 0.3ml of whole serum or bovine serum albumin solution (final albumin concentration approx. 0.12mM) and 30 μ l of bilirubin solution (final concentration 0.21mM). Each incubation flask contained two or three liver slices [total 200 ± 20 (mean $\pm$ S.D.)mg wet wt./flask] and control flasks were either incubated at 37°C without bilirubin, which was added at the end of incubation, or kept on ice with bilirubin added. Incubation began within 30min of the death of the animals and all experiments with dissolved bilirubin were performed in dimmed light.

After incubation, the flasks were chilled on ice and the contents homogenized. In experiments requiring separation of slices and medium the contents of the flasks were centrifuged at 600g for 2min, the supernatant (incubation medium) was removed and the slices were homogenized in 2.5ml of phosphate/bicarbonate solution.

Identification and quantification of bilirubin conjugates

Bilirubin conjugates were detected by formation of ethyl anthranilate azopigments at pH2.7 and

* To whom reprint requests should be addressed.

separated by t.l.c. (see Heirwegh *et al.*, 1974). To 1 ml of sample was added 5 ml of glycine/HCl buffer, pH 2.7, and 2.5 ml of ethyl anthranilate reagent (Heirwegh *et al.*, 1974). After 45 min at room temperature (18–20°C) the azopigments were extracted twice into a total of 4 ml of pentan-2-one, concentrated to a convenient volume by repeated washing with glycine/HCl buffer, and the A_{546} was determined. Azopigment formation was maximal under these conditions.

Bilirubin mono- and di-conjugate formation was estimated from the relative amounts of α_0 (unconjugated azodipyrrole), δ (azodipyrrole glucuronoside) and α_3 (azodipyrrole glucoside) pigments. These were separated and identified by t.l.c. on silica-gel 60 plates with benzene/ethyl acetate (85:15, v/v) and then chloroform/methanol/water (65:25:3, by vol.) as solvent systems. Reference pigments were (a) diazotized rat bile and (b) authentic δ and α_3 azopigments, obtained by incubation of bilirubin with rat liver microsomal preparations and UDP-glucuronic acid or UDP-glucose respectively.

The separated azopigments were eluted completely from the silica with 2 ml of dimethylformamide/pentan-2-one (1:1, v/v) and 1 ml of water, the organic phase containing the pigments was separated by addition of 5 ml of glycine/HCl buffer, and the A_{546} was determined.

Incubated samples produced mainly α_0 and δ azopigments (see the Results section). Although only conjugated bilirubin should be diazotized at pH 2.7 (Van Roy & Heirwegh, 1968), controls containing high concentrations of unconjugated bilirubin yielded some α_0 azopigment (see also Ostrow & Boonyapisit, 1978). Attempts to decrease this blank value by pre-extraction with chloroform were unsuccessful. Blank values were therefore subtracted and the amount of monoconjugated bilirubin estimated as equivalent to $2\alpha_0$, the amount of diconjugated bilirubin being equivalent to the total azopigment formation minus $2\alpha_0$. This method for measuring monoconjugate formation has been extensively discussed (Heirwegh *et al.*, 1974; Fevery *et al.*, 1977a,b).

Bilirubin monoglucuronide and diglucuronide preparations

These were obtained by extraction from Wistar rat bile collected under anaesthesia by catheterization of the common bile duct. The tetrapyrroles were extracted into chloroform at pH 1.8 (J. Fevery, personal communication) and separated by t.l.c. on silica-gel 60 plates with chloroform/methanol/water (30:15:2, by vol.) as developing solvent. They were eluted with methanol, rapidly dried by rotary evaporation and redissolved in water for immediate use. The conjugates were identified from their azopigments, the bilirubin monoglucuronide preparation producing

54% α_0 and 46% δ azopigments, the bilirubin diglucuronide preparation 7% α_0 and 93% δ azopigments.

Preparation of cell fractions derived from smooth and rough endoplasmic reticulum

These preparations were obtained from rat liver by the discontinuous sucrose gradient method of Dallner (1974), the concentration ratio of RNA/protein being used to check their identity. Ratios were determined as 0.036 and 0.044 for two preparations derived from smooth endoplasmic reticulum and 0.24 and 0.26 for those derived from rough endoplasmic reticulum (Dallner, 1974). RNA was determined by the method of Wool & Cavicchi (1967) and protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Assay of bilirubin-UDP-glucuronosyltransferase

This was assayed in digitonin-activated microsomal preparations by the method of Heirwegh *et al.* (1972).

Results

Formation of monoconjugates of bilirubin by rat liver microsomal preparations

Preliminary experiments confirmed that microsomal preparations from livers of Wistar rats in our colony produced almost exclusively bilirubin monoglucuronide. When digitonin-activated preparations, derived from either smooth or rough endoplasmic reticulum, were assayed for bilirubin-UDP-glucuronosyltransferase activity, the bilirubin conjugates yielded only α_0 and δ azopigments in approximately equal amounts. In two experiments, monoglucuronide produced was 91 and 87% of total conjugate with smooth microsomal preparations and 94 and 91% with rough microsomal preparations. These findings do not support previous suggestions that diglucuronides are formed primarily by microsomal fractions derived from rough endoplasmic reticulum (Halac *et al.*, 1972).

Formation of monoconjugates and diconjugates of bilirubin by rat liver slices

(a) Formation of total conjugate. Adult rat liver slices conjugated bilirubin at a linear rate over a 2 h incubation period (Fig. 1). Conjugation in the presence of male human serum was consistently higher [89.9 ± 7.3 (mean $\pm$ s.d.) nmol of conjugate formed/h per g of liver, $n = 23$] than with female human or male rat serum or bovine serum albumin. With constant serum concentrations and variable bilirubin concentrations, conjugate formation was only detected when the molar ratio of bilirubin/albumin was greater than 0.3, thereafter increasing with increasing bilirubin concentrations (Fig. 2). Without serum albumin, bilirubin tended to precipi-

Fig. 1. Time course of bilirubin conjugation and distribution between slices and medium

Liver slices (200 ± 20 mg wet wt.) were incubated with bilirubin (0.25 mM) and male human serum (350 μ l/flask) as described in the Materials and Methods section for the times shown; total conjugate formation in slice and medium was measured (●). In another series of experiments, slices were separated from the medium after incubation and the bilirubin conjugates in slices (▲) and in medium (△) were determined, the range shown being the s.e.m. for four samples.

Fig. 2. Effect of bilirubin concentration on rate of conjugation in liver slices

Liver slices were incubated as described in the Materials and Methods section except that bilirubin concentrations were varied over the range shown, whereas human serum albumin concentration was maintained constant at approx. 0.14 mM (350 μ l of serum/flask).

tate and conjugation rates were widely variable. We selected 0.21 mM-bilirubin in an approximate molar ratio of 2:1 to albumin for optimal conjugation rate and minimal interference with azopigment analysis.

We found total conjugate formed during slice incubations to be distributed approximately equally between tissue and incubation medium (Fig. 1).

(b) Estimation of monoconjugates and diconjugates. The α_0 and δ azopigments contributed 90–97% of total azopigments. Thus glucuronides were virtually the only products of slice incubations. The remaining 3–10% of pigments were mainly α_3 azopigments, with traces of a band tentatively identified as β azopigments, derived from non- α isomers of bilirubin-IX (Blanckaert *et al.*, 1976). Both these pigments were difficult to quantify and were found mainly in the incubation medium. For practical purposes the β azopigments have been disregarded and the α_3 azopigments included with the δ azopigments for estimating mono- and diconjugate formation.

Both bilirubin monoglucuronide and diglucuronide were formed by liver slices, in contrast with the almost exclusive formation of monoconjugates by microsomal preparations. However, monoglucuronides were predominant, being 60–75% of total conjugates. Decreasing the bilirubin concentration to 60 μ M did not increase the proportion of diglucuronide.

The distribution of the two conjugates between tissue and medium after incubation is given in Table 1 for several experimental conditions. Approximately equal amounts of mono- and di-glucuronides were found within the liver slice, but the incubation medium contained 70–100% monoconjugates. This distribution was the same after 1 and 2 h incubations. The low amount of diconjugate in the medium was highest when the contribution of α_3 azopigments was highest.

In spite of an apparently decreased excretion of diconjugates into the medium, they did not accumulate within the slice, suggesting their hydrolysis might be occurring either in the medium or in transit from the cell. However, glucaro-1,4-lactone, a specific inhibitor of β -glucuronidase, did not increase the proportion of diconjugate (Table 1). The possibility of hydrolysis was therefore tested more directly. Authentic bilirubin diglucuronide (see the Materials and Methods section) was incubated in the liver slice system, instead of bilirubin, with the appropriate controls. After 60 min incubation 75% of the total pigment was recoverable, but half of this was now detected as monoglucuronide, strongly suggesting that part of any diglucuronide synthesized during the original slice incubations had also been converted into monoglucuronide. This transforma-

Table 1. *Percentage distribution of bilirubin mono- and di-conjugates between slice and incubation medium*

Liver slices were incubated with bilirubin for the times given and the presence of bilirubin mono- and di-conjugates in slice and incubation medium was determined as described in the Materials and Methods section. Results are given as mean percentage of total conjugate formed $\pm$ S.E.M. for four samples in Expts. 1 and 2, and three samples in Expt. 3.

Expt. no.	Incubation time (min)	Proportion of total conjugate formed (%)			
		Slice		Medium	
		Monoconjugate	Diconjugate	Monoconjugate	Diconjugate
1	60	25.0 $\pm$ 1.6	20.7 $\pm$ 1.6	43.6 $\pm$ 3.5	10.7 $\pm$ 3.5
	120	22.9 $\pm$ 2.6	20.8 $\pm$ 3.5	42.5 $\pm$ 1.2	13.8 $\pm$ 1.5
2	90	25.4 $\pm$ 2.4	13.5 $\pm$ 0.7	63.1 $\pm$ 2.0	0
	90*	23.5 $\pm$ 2.2	20.2 $\pm$ 0.8	52.1 $\pm$ 2.7	4.2 $\pm$ 2.3
3	90	21.7 $\pm$ 2.9	17.0 $\pm$ 1.9	44.1 $\pm$ 8.1	17.2 $\pm$ 4.3
	90†	21.2 $\pm$ 3.7	19.9 $\pm$ 4.2	41.3 $\pm$ 6.1	17.6 $\pm$ 2.6

* 0.4 mM-Glucaro-1,4-lactone in incubation medium.

† 10.0 mM-Glucaro-1,4-lactone in incubation medium.

tion was apparently dependent on intact slices, since it did not occur during microsomal incubations with diglucuronide. In contrast, when authentic bilirubin monoglucuronide was incubated with liver slices, it was wholly recoverable and thus remained as monoglucuronide. This latter finding also indicated that both α_0 and δ azopigments were being extracted equally from the incubation samples.

Discussion

Glucuronidation of bilirubin by rat liver slices should largely reflect its overall conjugation *in vivo*, a function of several processes: uptake of bilirubin from serum albumin, conjugation with glucuronic acid by microsomal UDP-glucuronosyltransferase, transport of conjugates to bile canaliculi membranes and excretion across these membranes (see Fleischer & Arias, 1970). Formation of diglucuronides is arguably the least understood of these processes.

Our overall rates of conjugation in liver slices are comparable with those of Lathe & Walker (1958), but much lower (5% or less) than rates with fully activated UDP-glucuronosyltransferase assayed in cell extracts (Heirwegh *et al.*, 1972; M. T. Campbell, unpublished work) or than maximum excretion rates *in vivo* (Van Damme & Desmet, 1969). A similar wide discrepancy between rates of bilirubin conjugation in slices and homogenates was observed by Lathe & Walker (1958). In spite of the high extracellular concentration of bilirubin and the high molar ratio of bilirubin to albumin, saturating concentrations were not reached intracellularly (Fig. 2), indicating that uptake of bilirubin is probably rate limiting; high concentrations of bilirubin may also be toxic to slices. The approximately equal distribution of total conjugated bilirubin between slices and medium at 1 and 2 h (Fig. 1) indicates that

excretion is not limiting here. This excretion may, however, be due to 'leakage' of conjugates across plasma membranes as well as to normal transit across bile canaliculi membranes, such a 'leakage' being observed *in vivo* during infusion of rats with high bilirubin concentrations (Arias *et al.*, 1961; Snyder *et al.*, 1967).

The high proportion of monoconjugates formed by the liver slice system reflects the excess of monoconjugate found in the medium and seems to have been due, at least partly, to conversion of diconjugates into monoconjugates, presumably in the medium or at the cell surface. The approximately equal amount of mono- and di-conjugates within the slice may reflect more closely the immediate intracellular products. Preliminary work suggests that in hepatocyte suspensions, also, diconjugates occur mainly within cells and not medium (M. T. Campbell, unpublished work). If we allow for some likely selective loss of diconjugate within the slice, either by the mechanism responsible for this loss in the medium or because of the demonstrated less complete extraction of diglucuronide than of monoglucuronide, then the proportion of diconjugate initially formed in our slices reaches the 60–75% normally excreted in Wistar rat bile (e.g. Fevery *et al.*, 1972a; Noir, 1976; Jansen *et al.*, 1977).

The presence of diglucuronide within liver slices is also interesting because of recent suggestions that it is formed in rat at the bile canaliculi membrane by transesterification between two molecules of bilirubin monoglucuronide, possibly as the conjugate is excreted from the cell (Jansen *et al.*, 1977; Wolkoff *et al.*, 1978). By this theory monoglucuronide, but not diglucuronide, should be found within the hepatocyte (Wolkoff *et al.*, 1978). In our experiments diglucuronide may be formed at the bile canaliculi membrane, but, unlike the situation *in vivo*, may not

be excreted from slices by the canalicular mechanism: it may accumulate and be de-esterified as it 'leaks' across plasma membranes. Diglucuronide formation and transport of conjugates across canalicular membranes are separable events, since many species excrete predominantly monoconjugates (Fevery *et al.*, 1977a) and in man and rat the biliary monoconjugate/diconjugate ratio is altered drastically under a variety of conditions (Fevery *et al.*, 1972b, 1977b; Noir, 1976). However, our results also suggest that diglucuronide is indeed formed at the canalicular membrane, but in the neighbourhood of high concentrations of bilirubin (i.e. at the periphery of the slice) a reversal of the reaction described by Jansen *et al.* (1977) occurs, so that diglucuronide and unconjugated bilirubin react there to form two molecules of monoconjugate. Such a reversal could explain the higher proportion of monoconjugate excreted in rats loaded with bilirubin (Fevery *et al.*, 1972a; Noir, 1976) and would ensure the maximum efficiency of bilirubin excretion desirable in such circumstances and not otherwise explicable by the reaction of Jansen *et al.* (1977).

The authors thank Dr. S. Scott, Department of Virology, Ninewells Hospital, for providing human sera, Mrs. A. Donald for skilled technical assistance and the Scottish Hospitals Endowment Research Trust for financial assistance.

References

- Arias, I. M., Johnson, L. & Wolfson, S. (1961) *Am. J. Physiol.* **200**, 1091–1094
- Black, M., Billing, B. H. & Heirwegh, K. P. M. (1970) *Clin. Chim. Acta* **29**, 27–35
- Blanckaert, N., Heirwegh, K. P. M. & Compennolle, F. (1976) *Biochem. J.* **155**, 404–417
- Dallner, G. (1974) *Methods Enzymol.* **21**, 191–201
- Fevery, J., Van Damme, B., Michiels, R., De Groote, J. & Heirwegh, K. P. M. (1972a) *J. Clin. Invest.* **51**, 2482–2492
- Fevery, J., Leroy, P., Van de Vijver, M. & Heirwegh, K. P. M. (1972b) *Biochem. J.* **129**, 635–644
- Fevery, J., Van de Vijver, M., Michiels, R. & Heirwegh, K. P. M. (1977a) *Biochem. J.* **164**, 737–746
- Fevery, J., Blanckaert, N., Heirwegh, K. P. M., Preaux, A. M. & Berthelot, P. (1977b) *J. Clin. Invest.* **60**, 970–979
- Fleischer, G. & Arias, I. M. (1970) *Am. J. Med.* **49**, 576–589
- Halac, E., DiPiazza, M. & Detwiler, P. (1972) *Biochim. Biophys. Acta* **279**, 544–553
- Heirwegh, K. P. M., Van de Vijver, M. & Fevery, J. (1972) *Biochem. J.* **129**, 605–618
- Heirwegh, K. P. M., Meuwissen, J. A. T. P. & Fevery, J. (1973) *Adv. Clin. Chem.* **16**, 239–389
- Heirwegh, K. P. M., Fevery, J., Meuwissen, J. A. T. P., De Groote, J., Compennolle, F., Desmet, V. & Van Roy, F. P. (1974) *Methods Biochem. Anal.* **22**, 205–250
- Jansen, P. L. M., Chowdhury, J. R., Fischberg, E. & Arias, I. M. (1977) *J. Biol. Chem.* **252**, 2710–2716
- Lathe, G. H. & Walker, M. (1958) *Biochem. J.* **70**, 705–712
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Noir, B. (1976) *Biochem. J.* **155**, 365–373
- Ostrow, J. D. & Boonyapisit, S. T. (1978) *Biochem. J.* **173**, 263–267
- Rugstad, H. E., Robinson, S. H., Yannoni, C. & Tashjian, A. H. (1970) *J. Cell Biol.* **47**, 703–710
- Schoenfield, L. J. & Bollman, J. L. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 929–932
- Snyder, A. L., Saterlee, W., Robinson, S. H. & Schmidt, R. (1967) *Nature (London)* **213**, 93–94
- Van Damme, B. & Desmet, V. (1969) *Experientia* **26**, 27–28
- Van Roy, F. P. & Heirwegh, K. P. M. (1968) *Biochem. J.* **107**, 507–518
- Wolkoff, A. W., Ketley, J. N., Waggoner, J. C., Berk, P. D. & Jakoby, W. B. (1978) *J. Clin. Invest.* **61**, 142–149
- Wool, I. G. & Cavicchi, P. (1967) *Biochemistry* **6**, 1231–1242