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ORIGINAL PAPER

Bilirubin photo-isomers: regiospecific acyl glucuronidation in vivo

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Abstract (4*Z*,15*Z*)-Bilirubin-IXα, the end product of heme catabolism, requires uridine glucuronosyl transferase 1A1 (UGT1A1)-catalyzed glucuronidation for elimination in bile, where it appears as two isomeric monoglucuronides and a diglucuronide. When people are exposed to light, endogenous bilirubin is converted partly to photo-isomers that are produced in greater abundance during treatment of jaundiced babies with phototherapy. Little is known about the metabolism of the photo-isomers, other than that they appear not to require glucuronidation for elimination in bile. Studies have been hampered by their unavailability and instability, as well as confusion about the identity, structures, preparation, and purity of bilirubin photoproducts. This paper outlines methods for preparing photo-isomers of bilirubins in sufficient quantity and purity for metabolic studies in rats and reappraises the composition of some previous preparations. The studies show that (*Z*,*E*)-isomers of bilirubins and the structural isomer (*Z*)-lumirubin undergo glucuronidation in the rat, but unlike (4*Z*,15*Z*)-bilirubin, form only monoglucuronides. Moreover, glucuronidation is regiospecific for just one of the two propionic acid groups, the one attached to the isomerized half of the molecule. This unusual stereoselectivity appears to be dictated by intramolecular hydrogen bonding. Formation of hydroxylated bilirubins was not detected. During phototherapy, photo-isomers will compete with endogenous (4*Z*,15*Z*)-bilirubin for glucuronidation by nascent hepatic enzyme UGT1A1.

Keywords Bile pigments · Enzymes · Photochemistry · Jaundice

Introduction

Bilirubin-IXα (BR) is a yellow-orange bichromophoric tetrapyrrole present in blood as a reversible non-covalent complex with serum albumin. Cleared efficiently from the circulation by the liver, it is eliminated in bile as two isomeric monoglucuronides and a diglucuronide whose formation is catalyzed by a specific hepatic glucuronosyl transferase isozyme, UGT1A1 [1]. The need for diglucuronidation, when monoglucuronidation would suffice, has long been a puzzle [2]. When the activity of UGT1A1 is impaired, as in certain genetic disorders, BR accumulates in the circulation, resulting in hyperbilirubinemia, which may be manifested as jaundice. In newborn infants, UGT1A1 is developmentally deficient while BR production is increased (relative to adults) and this combination can result in neonatal jaundice, which is common and which in severe cases can cause irreversible brain damage [3, 4]. Phototherapy with visible (blue) light is the most widely used first-line therapeutic defense against severe neonatal jaundice [5].

BR absorbs visible light over a broad band of wavelengths centered around 460 nm and loses photoexcitation energy primarily by radiationless processes, including photo-isomerization reactions. When people are exposed to light, a fraction of BR in the circulation is converted to two

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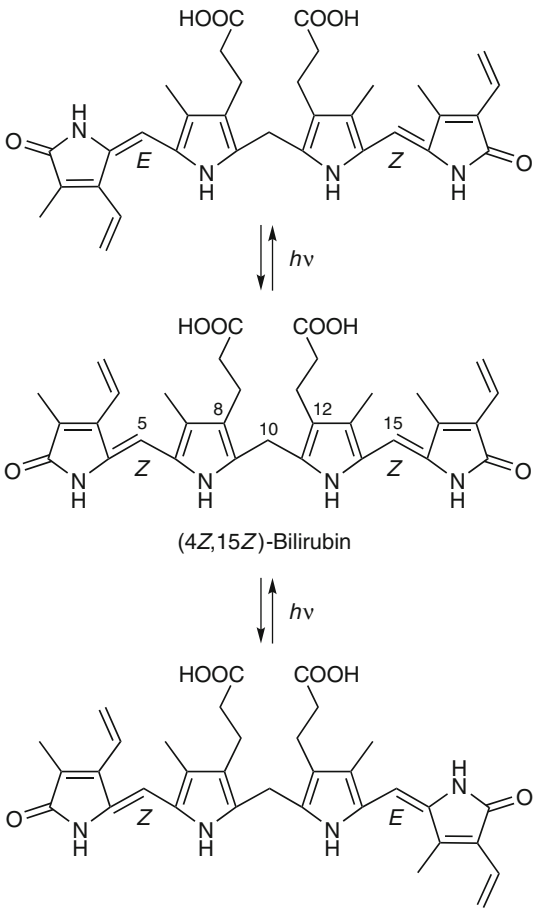

Fig. 1 Configurational photo-isomerization of (4*Z*,15*Z*)-bilirubin to its (4*Z*,15*E*)- and (4*E*,15*Z*)-isomers. A third photo-isomer, (4*E*,15*E*), is not shown

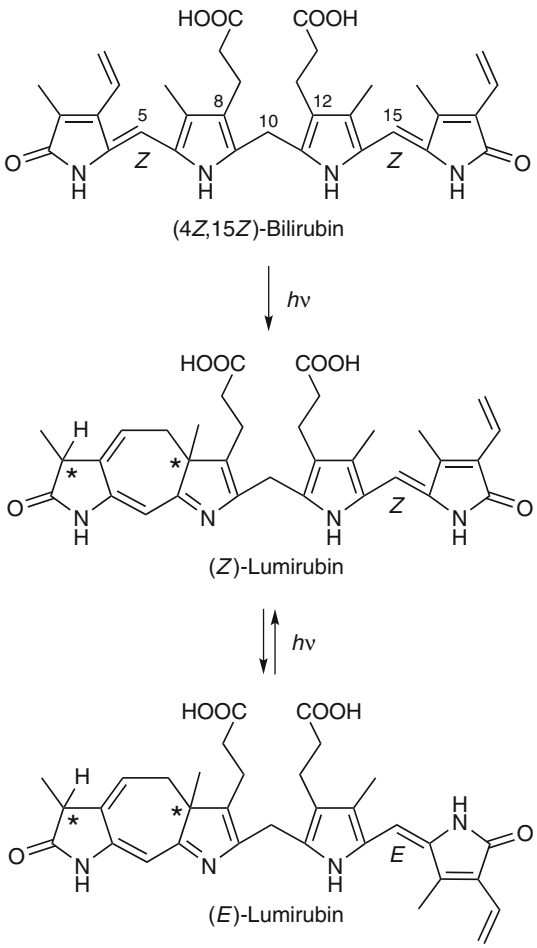

Fig. 2 Structural photo-isomerization of (4*Z*,15*Z*)-bilirubin to (*Z*)-lumirubin and configurational isomerization of the latter to (*E*)-lumirubin. *Stereogenic centers

families of isomers, namely configurational isomers and structural isomers [6–8]. The former result from reversible twisting of the molecule about one or both of the unsymmetrically substituted double bonds at C(4) and C(15) (Fig. 1); the latter from irreversible[1] intramolecular cyclization involving the *endo* vinyl group (Fig. 2). Of the two competing processes, configurational isomerization is the fastest, quantum yields for ($Z \rightarrow E$) isomerization of BR bound to human serum albumin (HSA) being ~0.1, some 50 times greater than for intramolecular cyclization [9–12]. Because configurational isomerization is photochemically reversible, a photostationary state mixture of all four isomers is reached when BR is irradiated in vitro, the composition of which depends on the solvent and wavelength of irradiation but in which the (4*Z*,15*Z*)-isomer invariably predominates. [A claim that (4*E*,15*E*)-BR can be separated chromatographically into two stable atropisomers [13, 14] is not plausible (see "Results and discussion")].

Photo-isomerization reactions of BR in humans are normally inconsequential but they play an essential role in phototherapy of neonatal jaundice because the photo-isomers, unlike the parent (4*Z*,15*Z*)-isomer, do not appear to require glucuronidation for excretion into bile [5, 7, 8]. Thus, their formation facilitates the biliary elimination of bilirubin pigments at a time when the normal pathway of hepatic glucuronidation is inadequate. Formation of photo-isomers during phototherapy also may act as a detoxification process, rapidly reducing the concentration of the more lipophilic (4*Z*,15*Z*), and presumably most toxic, isomer in the circulation together with the attendant risk that it will enter the brain and cause encephalopathy [15–17].

[1] Lumirubin can be partially reconverted back to (4*Z*,15*Z*)-BR on irradiation with UV light or prolonged irradiation with blue light [13, 26], but its formation is irreversible on the time scale required to effect complete equilibration of (*Z*)-lumirubin and (*E*)-lumirubin. Thus, irradiation of (*Z*)-lumirubin with blue light leads rapidly to a photoequilibrium mixture of (*Z*)- and (*E*)-isomers, but not to a photoequilibrium mixture with (4*Z*,15*Z*)-BR which appears only along with overall pigment decomposition (McDonagh AF, unpublished observations). Therefore on the time scales of configurational isomerization and structural isomerization it is not inappropriate to describe lumirubin formation as irreversible.

Although hepatic UGT1A1 activity is relatively low at birth and shortly thereafter, the enzyme is not totally deficient and its activity increases during the first ~10 postnatal days [3, 4]. Whether photo-isomers of BR are substrates for UGT1A1 or other hepatic enzymes is unknown and has been difficult to determine because of their instability and unavailability, as well as confusion in the literature regarding their structures, preparation, and identification. However, as will be shown, their instability can be turned to advantage in the identification of their metabolites. This paper investigates the glucuronidation and phase II metabolism of BR photo-isomers in the rat. It outlines simple methods for preparing BR photo-isomers in sufficient purity and quantity for in vivo studies and reappraises earlier structural assignments and methods for the preparation of "purified" photo-isomers and BR photoproducts.[2] The studies show that the principal BR photo-isomers are substrates for a uridine glucuronosyl transferase (UGT)1 enzyme in vivo but, in striking contrast to the parent (4*Z*,15*Z*)-isomer, undergo highly regioselective monoglucuronidation, with no diglucuronidation. The stereoselectivity of glucuronidation seems to be dictated by the ability to form intramolecular hydrogen bonds, as demonstrated by studies on a simple stable model compound. In addition to their relevance to neonatal jaundice and phototherapy, the studies provide an explanation for the formation of BR diglucuronide in normal metabolism and question the structures and importance of various hydroxylated bilirubins [18–21] proposed as products of BR metabolism and phototherapy.

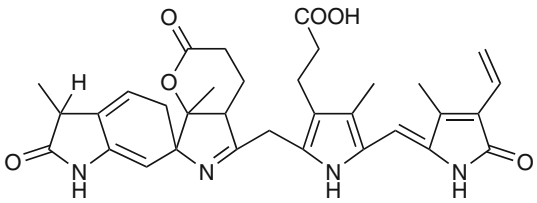

Fig. 3 Spirolactone structure assigned to photobilirubin II and cyclobilirubin [23, 26]

Results and discussion

For metabolism studies, solutions of pure individual photo-isomers or, at least, solutions highly enriched in the content of specific isomers are essential. In preliminary work the methods of Stoll and colleagues for preparing "purified" photo-isomers of BR-IXα were examined, beginning with photobilirubins IA and IB, which had been assigned structures (4*Z*,15*E*)-BR and (4*E*,15*Z*)-BR, respectively [13], followed by photobilirubin II, originally identified as a separable mixture of two stable conformational isomers of (4*E*,15*E*)-BR but later assigned other structures, including that of (*Z*)-lumirubin [23].

Photobilirubins IA and IB

Samples were prepared by irradiation of BR in $CHCl_3$ with a mercury lamp [13]. The final solution after irradiation and the crude product obtained after extraction of photo-isomers into acetone were dark green, indicating substantial by-product formation. Reversed-phase high-performance liquid chromatography (HPLC) of the acetone extract (Fig. 4) showed a complex mixture and revealed that partial metathesis of the starting material had occurred. Photobilirubin IA, isolated by thin-layer chromatography (TLC) as described [13], which had been assigned the (4*Z*,15*E*) structure, was found by HPLC to be a mixture containing none of that isomer and with the approximate bilirubin composition (based on relative peak areas) of 8 % (4*E*,15*Z*)-BR-XIIIα, 10 % (4*E*,15*Z*)-BR-IXα, 23 % (4*Z*,15*Z*)-BR-XIIIα, 47 % (4*Z*,15*Z*)-BR-IXα, and 11 % (4*Z*,15*Z*)-BR-IIIα. Photobilirubin IB, which had been assigned the (4*E*,15*Z*)-BR-IXα structure [13], contained very small amounts of both (*Z*,*E*)- and (*E*,*Z*)-isomers of BR and had the approximate composition (by HPLC) of 3 %

[2] The nomenclature and identification of BR photoisomers in the literature is confusing. The photoisomer structures shown in Figs. 1 and 2 were established unambiguously by NMR and other spectroscopic and chemical methods published in 1982 and, for simplicity, the trivial name "lumirubin" (CAN 83664-21-5 and 83729-98-0) was assigned to structural isomers with the novel cycloheptadienyl ring (Fig. 2) [7, 8]. Previously the term "photobilirubin" had been used to describe early photoproducts of BR [22], but that term became redundant once the individual photoisomer chemical structures had been established. Yet the term was not completely abandoned, appearing in the literature as photobilirubins IA and IB, photoproducts isolated by Stoll et al. who thought them to be (4*E*,15*Z*) and (4*Z*,15*E*)-BR, respectively [13, 14]. However, as shown in this paper those structure assignments are incorrect. Another photoproduct, photobilirubin II, initially thought to contain two stable atropisomers of (4*E*,15*E*)-BR [13, 14] was subsequently assigned either the lumirubin structure (Fig. 2) or spiro-lactone structure (Fig. 3) [23], and finally the lumirubin structure [24]. The lumirubin structure in Fig. 2 is sometimes called (*E*,*Z*)-cyclobilirubin. Cyclobilirubin, originally called "unknown pigment", was initially assigned the (4*E*,15*E*)-bilirubin structure [25], then the spiro-lactone structure [26], and eventually, in 1984 [27], the lumirubin structure elucidated earlier [7]. The name is confusing because it implies that a (*Z*,*Z*)-cyclobilirubin isomer could exist; however, such a structure is stereochemically impossible. In 1987, Bonnett and Ioannou published a table of structure/name correlations for the photoproducts isolated by different investigators [28]. Unfortunately, there are errors in that table. Adding further confusion, structures for several photoisomers and for bilirubin glucuronides depicted in a more recent review [29] are incorrect. The current paper uses unambiguous chemical nomenclature for configurational isomers and the trivial name lumirubin for isomers with a cycloheptadienyl ring system linking two adjacent pyrrolic rings formed by intramolecular cyclization of an *endo* vinyl group. There is no longer a need for the ambiguous and confusing photobilirubin or cyclobilirubin nomenclature.

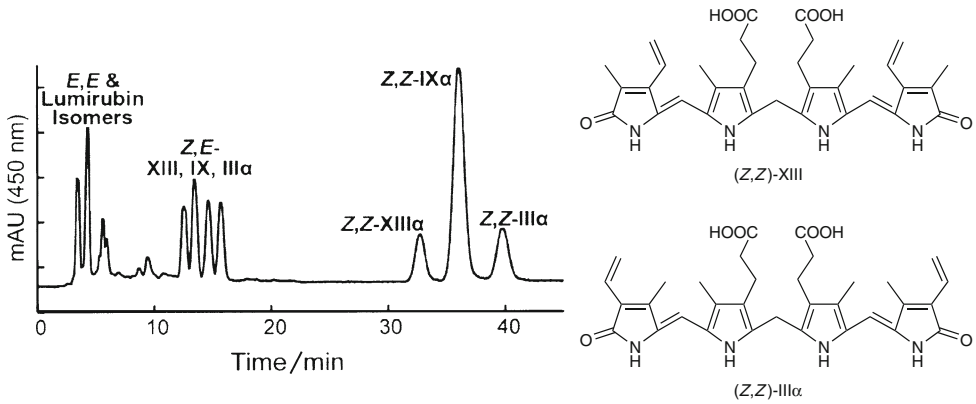

Fig. 4 *Left* HPLC chromatogram of partially purified photobilirubins IA and IB. *Right* Constitutional structures of (4*Z*,15*Z*)-bilirubins-XIIIα and -IIIα

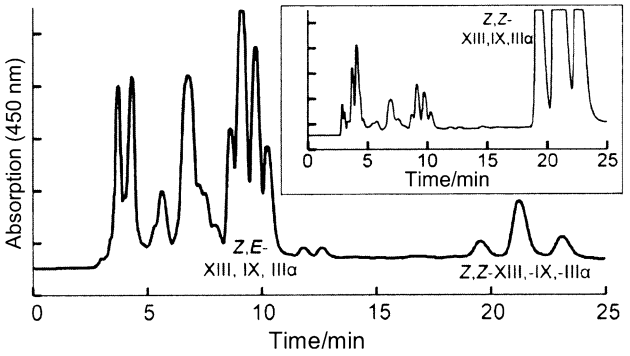

Fig. 5 Partially purified product from the preparation of photobilirubin II as described by Stoll et al. [13, 23]. The *inset* shows a chromatogram of the final dark green $(CH_3)_2SO$ solution after irradiation of BR and before CH_3OH extraction of photo-isomers. The chromatograms show only pigments with absorption at 450 nm

(4*E*,15*Z*)-BR-XIIIα, 2 % (4*E*,15*Z*)-BR-IXα, 3 % (4*Z*,15*E*)-BR-IXα, 3 % (4*Z*,15*E*)-BR-IIIα, 8 % (4*Z*,15*Z*)-BR-XIIIα, 38 % (4*Z*,15*Z*)-BR-IXα, 25 % (4*Z*,15*Z*)-BR-IIIα, and 17 % unidentified peaks. Thus, aside from questions of purity, the structures originally assigned to photobilirubins IA and IB [13] are incorrect. Contrary to the supposition of Stoll et al. [13], the (4*E*,15*Z*)-isomer of BR-IXα precedes the (4*Z*,15*E*)-isomer on silica TLC, which is consistent with their relative mobilities on HPLC [8].

Photobilirubin II

Samples were prepared by irradiation of BR in $(CH_3)_2SO$ with a mercury lamp as described [13, 23]. The solution was dark green at the end of the reaction indicating extensive verdinoid by-product formation. HPLC revealed a complex mixture containing less than 3 % lumirubin with extensive disproportionation of the starting material to BR-IIIα and -XIIIα (Fig. 5, inset). HPLC of the crude product, after CH_3OH extraction and before TLC purification (Fig. 5) showed numerous components, with (*Z*)-lumirubin-IXα (photobilirubin II) as a minor constituent. Repeated preparative TLC of this mixture yielded (*Z*)-lumirubin-IXα in exceedingly low yield.

"Photobilirubin" preparations

Careful repetition of the published procedures [13, 23, 30] showed them to be unsatisfactory for the practical preparation of BR photo-isomers. Yields were exceedingly low and products impure. This can be attributed to over-irradiation of solutions, use of unsuitable solvents, and an inappropriate intense UV–Vis mercury light source with strong emission within the main absorption band of BR in the visible. Under these conditions BR-IXα undergoes dismutation to -IIIα and -XIIIα isomers and isomerization of the *exo* vinyl group to a highly reactive photo-isomer [31], as well as free radical reactions to verdinoid pigments. Stoll et al. [13] originally "purified" photobilirubins IA and IB [(*Z*,*E*)-isomers] by TLC on silica, which, as noted earlier, catalyzes ($E \rightarrow Z$) reversion. Consequently, the isolated products invariably contained (4*Z*,15*Z*)-BR which was interpreted as evidence that "photobilirubins IA and IB exist in solution as complexes with bilirubin" [13], a speculation for which there is no experimental support. $CHCl_3$ was used as solvent for the preparation of photobilirubins IA and IB, and $(CH_3)_2SO$ for preparation of photobilirubin II. The choice of $(CH_3)_2SO$ was based on the unfounded speculation that the solvent would break hydrogen bonds in BR and favor formation of the (4*E*,15*E*)-photo-isomer [13]. Comparative solvent studies showed this not to be the case and that $CHCl_3$ and $(CH_3)_2SO$ are poor solvents for preparing photo-isomers, as borne out in part by quantum yield measurements [10].

Ostrow et al. reported separation of (4*E*,15*E*)-BR by TLC on silica into two stable "rotamers" termed photobilirubins IIA and IIB [13, 14]. These were depicted as conformational isomers (atropoisomers) that differed only by rotation of the end rings about single bonds and were supposedly stabilized by intramolecular hydrogen bonding. Considering the low energy barrier for conformational

interconversion in bilirubins [32–34], and the instability of (*E*)-isomers during chromatography [35], that claim is chemically implausible. The HPLC analyses in the present paper show that all of the photo-isomer structural assignments in the paper by Stoll et al. [13] are incorrect. Consequently, the absorbance data assigned to particular photo-isomers in the original paper are unreliable. (Confusingly, several photo-isomer structures and the structure of bilirubin diglucuronide in a more recent review [29] are also incorrect.) In follow-up studies, Stoll et al. [30] investigated the excretion of ^{14}C-radiolabelled photobilirubins I and II in Gunn rats. The composition of injectates was not analyzed. The present studies, and data in the published studies, show that the "purified" photo-isomers injected were not, in fact, pure and were undoubtedly complex mixtures which undermines the scientific basis for the original conclusions.

Preparation of photo-isomers

Although photo-isomers of BR have yet to be isolated in pure crystalline form, the present work shows that they can be easily prepared photochemically in adequate quantity and purity for metabolic studies in the rat by using isomerically pure starting material, visible light of appropriate wavelength, a suitable deoxygenated solvent, and exclusion of light during product work-up. In preparing configurational isomers, solutions should be irradiated only until a photostationary mixture of photo-isomers has formed, as determined by preliminary difference spectra or HPLC measurements, to avoid formation of by-products. Yields are limited because the parent (4*Z*,15*Z*)-isomer invariably predominates at photoequilibrium. $CHCl_3/Et_3N$ was used as solvent for preparing (*Z*,*E*)-isomers of BR-IXα, BR-IIIα, BR-XIIIα, and dihydrobilirubin-VIIIα, this solvent being chosen because it readily dissolves BR, is easily removed under vacuum at room temperature, provides a satisfactory quantum yield for ($E \rightarrow Z$) isomerization, and inhibits acid-catalyzed reversion of (*E*)- to (*Z*)-isomers. Separation of photo-isomers from starting material relied on the differential solubility of BR and its photo-isomers in CH_3OH [8, 35] and serum, which do not dissolve solid BR. Configurational isomers of BR undergo slow spontaneous thermal ($E \rightarrow Z$) reversion in CH_3OH at room temperature, so that CH_3OH extractions must be done at low temperature and rapidly. Similar to the experience of others [35, 36], we found it impossible to purify (*E*)-isomers by TLC on silica as reported by Stoll et al. [13, 30], or by HPLC on normal phase columns because of ($E \rightarrow Z$) reversion. However, the photo-isomers separate well on reversed-phase HPLC with the methanolic di-*n*-octylamine acetate solvent used in these studies. This solvent inhibits acid-catalyzed reversion of configurational isomers and has the advantage that it is used isocratically and can be recycled. Unfortunately, it is not useful for preparative purification of photo-isomers because of the di-*n*-octylamine acetate content.

Injectate solutions highly enriched in (4*Z*,15*E*)-BR, the isomer formed fastest during phototherapy of neonatal jaundice [17], were prepared via photo-isomerization of BR bound to HSA, which is highly regioselective for the (4*Z*,15*E*)-isomer [8].

Preparation of (*Z*)-lumirubin was achieved by irradiation of BR in $CHCl_3/Et_3N$ beyond the initial photostationary state, followed by brief treatment with acid to revert all (*E*)-isomers to the corresponding (*Z*)-isomers, CH_3OH extraction, and TLC purification. Some decomposition of lumirubin occurs during application of the pigment to the TLC plate, but this can be minimized by working rapidly. Much greater yields of lumirubin were obtained by irradiation of BR bound to HSA or rabbit serum albumin. Since lumirubin formation is irreversible under the irradiation conditions used in these studies, it should be possible in theory to convert BR quantitatively to lumirubin. In practice, however, unwanted secondary reactions set in before complete conversion occurs, and optimal irradiation times need to be determined in preliminary HPLC/UV–Vis experiments. Since bilirubin photo-isomers are yellow, final solutions after irradiation should also be yellow. Green solutions indicate over-irradiation and formation of unwanted by-products. No advantage was found to adding EDTA to solutions [13].

Exclusion of a spirolactone structure for lumirubin

A spirolactone structure (Fig. 3), with just one free carboxyl group, has at times been suggested for a photo-isomer of BR [23, 26, 37]. This structure had been ruled out on the basis of NMR studies [7], but because the present studies were investigating glucuronidation, it was important to definitively rule out the lactone structure by an alternative method. Photochemical formation of the lactone ring would be impossible if the C(8)-propionic acid were methylated and, therefore, irradiation of BR-dimethyl ester should not lead to photocyclization, whereas formation of lumirubin dimethyl ester would rule out the cyclic structure. Accordingly, BR-dimethyl ester was irradiated in 1 % NH_4OH/CH_3OH solution and the crude product treated briefly with trifluoroacetic acid (TFA) to convert (*E*)-isomers to (*Z*)-isomers. TLC showed the presence of only one main product, which, after purification by TLC, showed an absorbance spectrum ($\lambda_{max} = 429$ nm) in CH_3OH similar to that of lumirubin. This presumptive lumirubin dimethyl ester was not characterized further, but on saponification with NaOH was converted to lumirubin, which was identified by TLC comparison with authentic material using

three different solvent systems and by HPLC. Thus, methylation of the COOH side chains of BR does not prevent intramolecular photocyclization, ruling out the spirolactone structure (Fig. 3) for lumirubin.

(Z,E)-Isomer injectates

Highly enriched solutions of (*Z*,*E*)-isomers of bilirubins-IIIα, -IXα, and -IIIα in rat serum were easily prepared. Irradiation of the corresponding (*Z*,*Z*)-isomers in $CHCl_3$/Et_3N to near-photoequilibrium was followed by removal of solvent and extraction of (*Z*,*E*)-isomers into CH_3OH and then rat serum. The time to reach photoequilibrium was determined by preliminary absorbance difference measurements. Final solutions were yellow, indicating no formation of verdinoid by-products. (Looking ahead, typical photo-isomer enrichments can be seen by HPLC of the injectates of Figs. 8 (inset), 12 (inset), 14a, and 16a.) Product yields were limited by the isomer composition at photoequilibrium, in which the (*Z*,*Z*)-isomer invariably predominates. Common impurities in the preparations were the parent (*Z*,*Z*)-isomer and, for pigments containing *endo*-vinyl groups, lumirubin isomers. The proportion of (*Z*,*Z*)-isomer in final product depends to a large degree on the speed of work-up of the crude product, particularly during the CH_3OH extraction step and evaporation steps. For glucuronidation studies, the presence of a small proportion of the (*Z*,*Z*)-isomer in (*Z*,*E*)-preparations proved advantageous because it facilitated the eventual identification of (*Z*,*E*)-glucuronides in bile. In the preparation of BR-IXα photo-isomers, it is essential to use isomerically pure starting material free of BR-IIIα and -XIIIα to ensure that irradiations are not prolonged beyond the photostationary state and do not cause disproportionation of the starting material, to use narrow-band blue light, and to rigorously maintain safelight precautions during extractions and manipulation of product solutions. Consistent with the experience of others [35, 36], attempted purification of CH_3OH-extracted products by TLC [13] failed because of (acid-catalyzed) reversion of (*E*)- to (*Z*)-isomers.

Irradiation of BR-IXα in organic solvents and isolation of the initially formed photo-isomers yields a mixture highly enriched in both possible (4*Z*,15*E*)- and (4*E*,15*Z*)-isomers. In contrast, photo-isomerization of the BR–HSA complex in aqueous buffer is highly regioselective for the (15*Z*) double bond of BR [8, 38]. Injectate solutions highly enriched in only (4*Z*,15*E*)-BR, the most rapidly formed isomer in humans, were readily prepared in good yield by irradiation of (4*Z*,15*Z*)-BR in HSA solution followed by removal of HSA and extraction with CH_3OH and serum. Contaminants in the product were (*Z*)-lumirubin, (4*E*,15*Z*)-BR, and (4*Z*,15*Z*)-BR. The proportion of (4*Z*,15*Z*)-BR is minimized by expeditious work-up of the product, and the proportion of (*Z*)-lumirubin is small if solutions are not over-irradiated.

(Z)-Lumirubin injectates

Irradiation of BR in $CHCl_3$/Et_3N, followed by brief treatment with TFA to revert all (*E*)-configuration isomers to the corresponding (*Z*)-isomers and preparative TLC, afforded homogeneous (*Z*)-lumirubin, but yields were low (∼2 %) because of the low quantum yield for photocyclization in that solvent [10]. Longer irradiation led to unwanted side-reactions and products. However, quantum yields for photocyclization of BR in the presence of excess human or rabbit serum albumin in aqueous buffer are much higher [11, 12, 38] and irradiation of BR in those solvents led to much higher yields (∼30–50 %) of (*Z*)-lumirubin, albeit at the cost of a slightly more complicated work-up. Millimolar extinction coefficients for (*Z*)-lumirubin were found to be 33.0 in 1 % ammonia/CH_3OH (λ_{max} = 437 nm) and HSA solution (pH 7.4, λ_{max} = 453 nm) and 39.0 ± 2.6 in the 0.1 M methanolic di-*n*-octylamine acetate HPLC solvent (λ_{max} = 440 nm); corresponding values for (*E*)-lumirubin ($\varepsilon_{mM}/\lambda_{max}$) were 24.4/446–450, 24.1/446–456, and 23.3/449, respectively.

Excretion of photo-isomers in homozygous Gunn rats

Homozygous Gunn rats lack UGT1 activity and do not glucuronidate BR [1, 2]. When subjected to phototherapy with blue light the concentration of pigment in bile immediately begins to increase, reaching a steady-state level after about 2–3 h. Figure 6 shows typical HPLC chromatograms (450 nm), on the same scale, of equal volumes of Gunn rat bile collected before and after 90 min of phototherapy. Before phototherapy the bile shows only very minor peaks. One corresponds to a trace of (4*Z*,15*Z*)-BR. The other two more polar peaks are acid labile and correspond to (4*E*,15*Z*)- and (4*Z*,15*E*)-BR, probably generated during surgical preparation of the animal. During irradiation of the animal there was copious excretion of (*Z*)-lumirubin, (4*E*,15*Z*)-BR, and (4*Z*,15*E*)-BR, along with

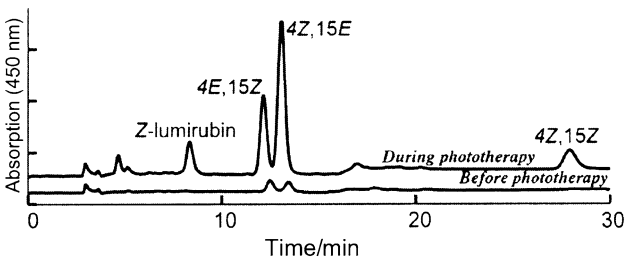

Fig. 6 HPLC chromatograms of bile collected from a Gunn rat before and 90 min after beginning phototherapy

lesser amounts of (*E*)-lumirubin, (4*E*,15*E*)-BR, and (4*Z*,15*Z*)-BR. Most of the (4*Z*,15*Z*)-BR is an artifact generated by thermal reversion of (*E*)-isomers as they exit slowly through the biliary cannula; its proportion increased with increasing length of the cannula.

Previous investigators have reported that Gunn rat bile contains substantial quantities of poorly characterized hydroxylated bile pigments whose concentration increases during phototherapy or on induction of hepatic enzymes [18–21]. Extensive HPLC and TLC of concentrated extracts of Gunn rat bile and of extracts treated with alkaline methanolysis failed to reveal the presence of significant quantities of such pigments in bile collected before or during phototherapy. Trace amounts of bilirubins IX-β, -γ, and -δ [39] were detected in bile collected in the dark, consistent with the findings of Blanckaert et al. [40], along with two pigments with bilirubinoid absorbance spectra (λ_{max} = 443 and 449 nm, respectively). These may correspond to the side chain hydroxylated BR derivatives found by Blanckaert et al. [40] which are structurally different from the hydroxylated compounds proposed by Ostrow et al. [18–21].

When (*Z*)-lumirubin-IXα, (*Z*)-lumirubin-XIIIα, and the (*Z*,*E*)-isomers of BR-IIIα, -IXα and -XIIIα were injected separately in Gunn rats the individual pigments were excreted rapidly in bile, with no HPLC evidence of metabolism to other rubins. To illustrate relative rates of excretion, a synthetic mixture of (*Z*)-lumirubin, (4*E*,15*Z*)-BR, and (4*Z*,15*E*)-BR-IXα in rat serum showing roughly similar HPLC peak heights for each isomer was injected intravenously as a bolus in a Gunn rat in the dark and bile, collected at frequent intervals, was chromatographed (Fig. 7). The chromatograms confirm that all three photo-isomers are excreted unchanged in bile and show that their relative rates of excretion correspond to their order of elution on reversed-phase HPLC. Thus, whether photo-isomers are generated endogenously or are injected intravenously, they do not appear to undergo significant phase I or II metabolism in the absence of UGT1 enzymes.

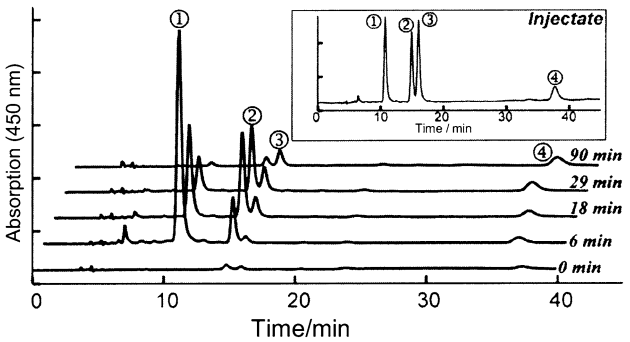

Fig. 7 Successive HPLC chromatograms of bile excreted by a Gunn rat kept in the dark at various times after intravenous injection of the pigment mixture shown in the *inset*. *1* (*Z*)-lumirubin, *2* (4*E*,15*Z*)-BR, *3* (4*Z*,15*E*)-BR, *4* (4*Z*,15*Z*)-BR

Metabolism of (Z,E)-isomers in wild-type rats

Initial studies were done with solutions of the (*Z*,*E*)-isomers of the symmetrically substituted BR analogues (4*Z*,15*Z*)-BR-IIIα and (4*Z*,15*Z*)-BR-XIIIα in rat serum. On intravenous (i.v.) administration to wild-type Sprague–Dawley (SD) rats, each is rapidly eliminated in bile as a mono- and a diglucuronide (MG and DG) whose HPLC and spectral characteristics were determined in preliminary experiments (not shown). The BR-IIIα photo-isomer injectate contained, in addition to (*Z*,*E*)-BR-IIIα, relatively small amounts of the (4*E*,15*E*)-isomer and the parent (4*Z*,15*Z*)-isomer (Fig. 8, inset) which facilitated subsequent metabolite identification. Figure 8 shows HPLC chromatograms of SD bile before and 9 min after administration of the injectate. Comparison of the chromatograms reveals that (*Z*,*E*)-BR-IIIα was excreted partly unchanged but largely as a more polar metabolite. On treatment of bile samples containing the metabolite with acid or on brief exposure to blue light (Fig. 9), to convert (*E*)-isomers to the corresponding (*Z*)-isomers, the metabolite peak and the (*Z*,*E*)-BR-IIIα peak disappeared and the (*Z*,*Z*)-BR-IIIα monoglucuronide and (*Z*,*Z*)-BR-IIIα peaks increased with no increase in the (*Z*,*Z*)-BR-IIIα diglucuronide peak. This identified the metabolite peak as a monoglucuronide of (*Z*,*E*)-BR-IIIα. Consistent with this, on treatment of the bile with β-glucuronidase all glucuronide peaks and the metabolite peak disappeared, and a relatively large (*Z*,*Z*)-BR-IIIα peak appeared (Fig. 10). During glucuronidase treatment (*E*)-isomers revert to (*Z*)-isomers and glucuronides are hydrolyzed to the parent aglycones.

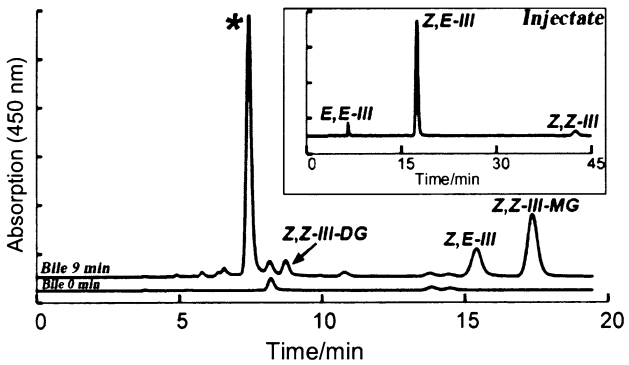

Fig. 8 Metabolism of (4*Z*,15*E*)-BR-IIIα in a wild-type rat. The *inset* shows an HPLC chromatogram of the highly enriched injectate and the *main panel* shows chromatograms of bile taken just before and 9 min after injection. Most of the injected photo-isomer was excreted in bile as a monoglucuronide metabolite (*), the remainder being excreted unchanged

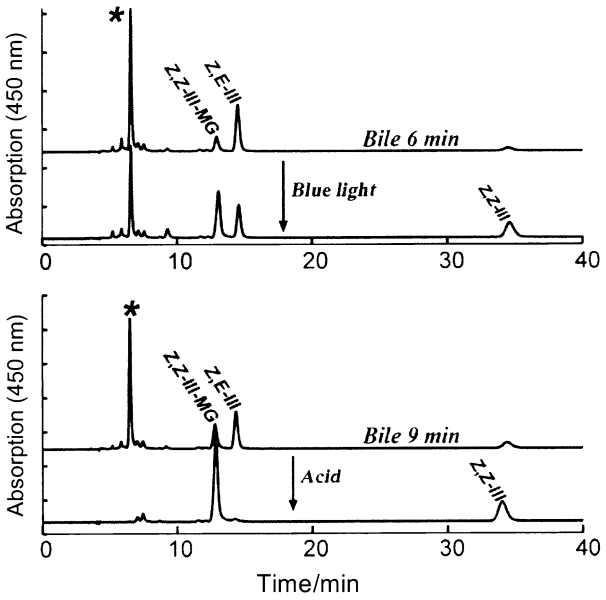

Fig. 9 HPLC chromatograms of bile containing the metabolite (*) of (4*Z*,15*E*)-BR-BR-IIIα (*top*) after brief exposure to blue light and (*bottom*) after treatment with acid. Light exposure led to loss of the metabolite peak and growth in (4*Z*,15*Z*)-BR-IIIα monoglucuronide, along with the expected loss of (4*Z*,15*E*)-BR-IIIα and growth of (4*Z*,15*Z*)-BR-IIIα. Acid treatment resulted in loss of the metabolite peak, a BR-IIIα monoglucuronide peak, and conversion of unchanged photo-isomer to the parent isomer

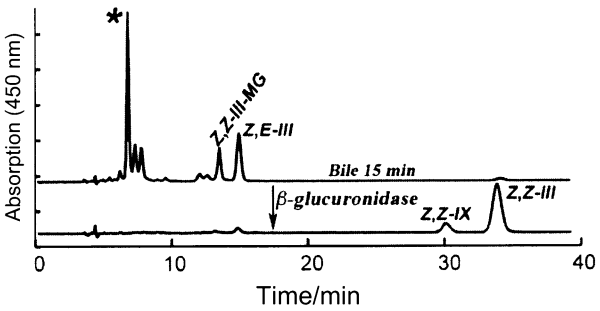

Fig. 10 Effect of β-glucuronidase on bile collected 15 min after injecting enriched (4*Z*,15*E*)-BR-IIIα into a UGT1A1-competent rat. All peaks disappeared and were replaced by (4*Z*,15*Z*)-BR-IXα and -IIIα. The two minor peaks running close to the (4*Z*,15*E*)-BR-IIIα metabolite (*) are the diglucuronides of (4*Z*,15*Z*)-BR-IXα and -IIIα

Thus, (*Z*,*E*)-BR-IIIα is metabolized in part by a UGT1 enzyme to a monoglucuronide ((*Z*,*E*)-BR-IIIα monoglucuronide) that elutes close to endogenous (4*Z*,15*Z*)-BR-IXα diglucuronide. This (*Z*,*E*)-monoglucuronide has a spectrum ($\lambda_{max} = 425$ nm) different from that of the injected (*Z*,*E*)-BR-IIIα isomer (Fig. 11) and rather similar to those of the mono- and diglucuronides of (4*Z*,15*Z*)-BR-IIIα (not shown).

Similarly to (*E*,*Z*)-BR-IIIα, (*E*,*Z*)-BR-XIIIα was also excreted partly unchanged and partly as a more polar metabolite with $\lambda_{max} = 424$ nm (Figs. 11, 12). On

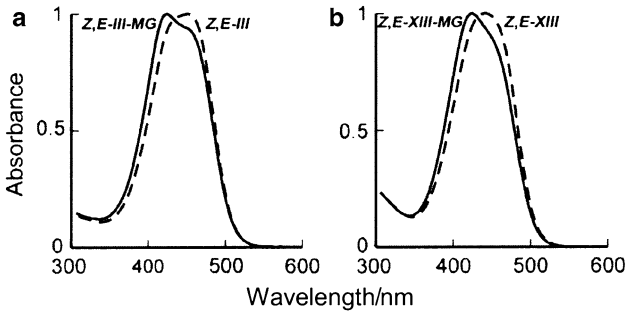

Fig. 11 Normalized absorbance spectra of **a** (4*Z*,15*E*)-BR-IIIα (*dashed line*) and its monoglucuronide (*solid line*) and of **b** (4*Z*,15*E*)-BR-XIIIα (*dashed line*) and its monoglucuronide (*solid line*)

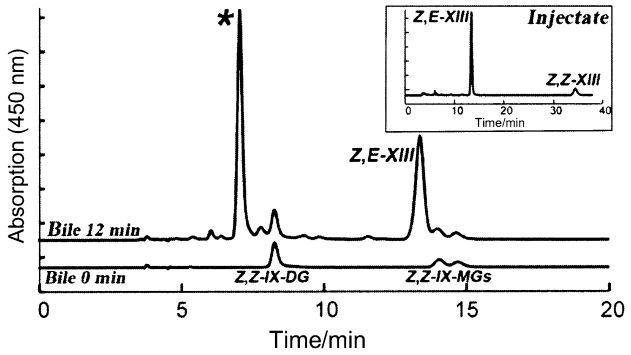

Fig. 12 HPLC chromatograms of bile from a wild-type rat before and 12 min after injecting a solution of (4*Z*,15*E*)-BR-XIIIα (*inset*) intravenously as a bolus. The photo-isomer was excreted partly unchanged and partly as a metabolite (*)

exposure to light or acid (Fig. 13) the metabolite peak disappeared, a peak corresponding to (*Z*,*Z*)-BR-XIIIα appeared, and there remained a strong peak at the retention time of (*Z*,*E*)-BR-XIIIα. However, the absorption spectrum of this strong peak was different from that of the (*Z*,*E*)-BR-XIIIα photo-isomer and identical to that of a (*Z*,*Z*)-BR-XIIIα monoglucuronide standard. In the HPLC system used, the (*Z*,*E*)-BR-XIIIα photo-isomer and (*Z*,*Z*)-BR-XIIIα monoglucuronide have similar retention times and do not separate.

These observations provided strong evidence that the (*Z*,*E*)-photo-isomers of both BR-IIIα and BR-XIIIα are excreted partly unchanged and partly as monoglucuronides in the rat, but they did not show which of the two non-equivalent propionic acid groups in each photo-isomer had undergone glucuronidation, i.e., whether just monoglucuronide was formed. That question was answered by studying the metabolism of (4*Z*,15*E*)-BR-IXα, the most rapidly formed photo-isomer in humans.

The (4*Z*,15*E*)-BR-IXα injectate was contaminated with unchanged (4*Z*,15*Z*)-BR-IXa and (*Z*)-lumirubin, which proved difficult to avoid (Fig. 14). However, as shown in

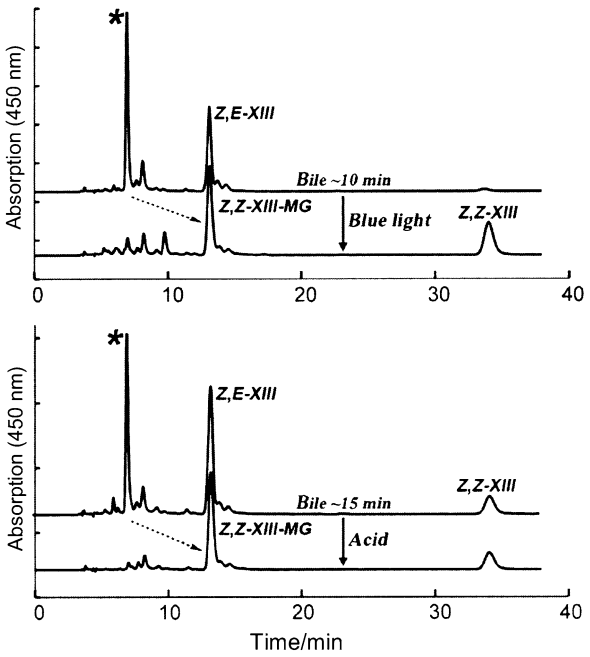

Fig. 13 Chromatograms of bile containing the metabolite (*) of (4*Z*,15*E*)-BR XIIIα (*top*) after brief exposure to blue light and (*bottom*) after treatment with acid. Light exposure led to loss of the metabolite peak and growth in (4*Z*,15*Z*)-BR-XIIIα monoglucuronide which has the same retention time as (4*Z*,15*E*)-BR-XIIIα but is readily distinguished from it by its absorption spectrum. Acid treatment resulted in loss of the metabolite peak and a marked increase in the (4*Z*,15*Z*)-XIIIα monoglucuronide

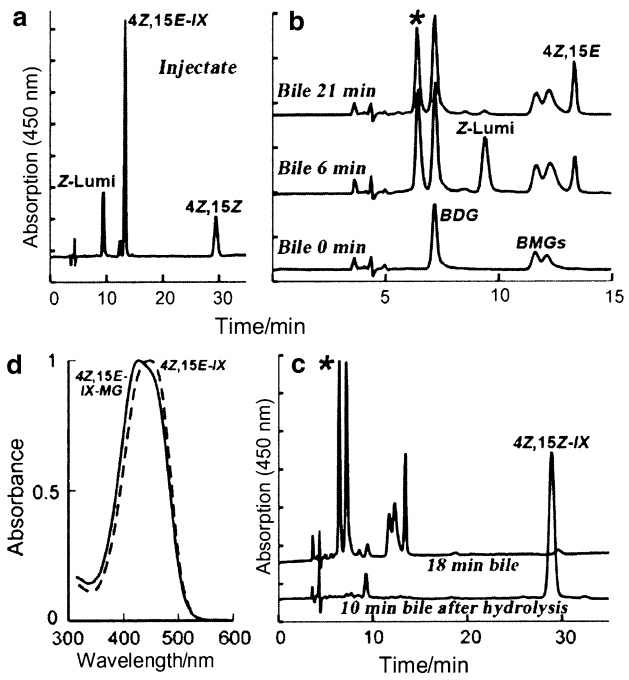

Fig. 14 **a** (4*Z*,15*E*)-BR injectate. **b** HPLC chromatograms of bile collected before and at 6 min and 21 min after administration of the (4*Z*,15*E*)-BR injectate as a bolus in a wild-type rat. **c** HPLC of bile collected 18 min after injection of (4*Z*,15*E*)-BR (*top*) and of bile collected 10 min after injection and then subjected to β-glucuronidase hydrolysis. **d** Normalized absorbance spectra of (4*Z*,15*E*)-BR and its (monoglucuronide) metabolite. *Indicates the major (monoglucuronide) metabolite

Fig. 7, (*Z*)-lumirubin is excreted much faster than (4*Z*,15*E*)-BR. HPLC of bile collected after the more rapidly excreted lumirubin had disappeared showed unchanged (4*Z*,15*E*)-BR and a large metabolite peak with a retention time close to that of BR diglucuronide (Fig. 14b) and (λ_{max} = 426–428 nm, Fig. 14d). On treatment of a bile sample with β-glucuronidase all peaks (except for a small lumirubin peak) disappeared and there was a marked increase in the (4*Z*,15*Z*)-BR peak (Fig. 14c). This, along with the fact that no metabolite is formed in Gunn rats, is consistent with a glucuronide structure for the metabolite. On brief treatment of bile samples with TFA or on brief exposure to blue light the metabolic peak partially disappeared along with growth of the (4*Z*,15*Z*)-BR C(12)-monoglucuronide peak, loss of the (4*Z*,15*E*)-BR peak, and growth of the (4*Z*,15*Z*)-BR peak (Fig. 15). From these observations it can be concluded that (4*Z*,15*E*)-BR is metabolized in the rat to a monoglucuronide and that glucuronidaton is regioselective for the C(12)-propionic acid side chain.

These conclusions were supported by similar experiments with injectates containing both (4*Z*,15*E*)-BR and (4*E*,15*Z*)-BR. lnjectates highly enriched in those two photo-isomers, without significant lumirubin contamination, were easily prepared by irradiation of (4*Z*,15*Z*)-BR in $CHCl_3/Et_3N$ to a photostationary state followed by extraction into CH_3OH and then rat serum (Fig. 16a). After injection of this mixture of photo-isomers two overlapping metabolite peaks, slightly more polar than BR diglucuronide, were observed in bile (Fig. 16b). The ratio of these two metabolites changed with time; the least polar, longer retention time peak appeared first, eventually giving way to the most polar (Fig. 16c). This allowed absorption spectra for each component to be determined (Fig. 16d). These were similar, but not identical. The least polar metabolite had λ_{max} = 422–426 nm; the most polar, λ_{max} = 426–430 nm; with a spectrum that closely resembled that of (4*Z*,15*E*)-C(12)-glucuronide determined in the previous experiment. This suggested that the least polar, but most rapidly excreted, pigment might be (4*E*,15*Z*)-C(8)-monoglucuronide. That was confirmed by brief exposure of an early bile sample to blue light to convert (*E*)-isomers to (*Z*)-isomers. Exposure resulted in a marked increase of (4*Z*,15*Z*)-C(8)-monoglucuronide (Fig. 17), as well as an increase in (4*Z*,15*Z*)-BR.

These experiments show that (*Z*,*E*)-isomers of BR and other rubins are substrates for a UGT1 (presumably UGT1A1) enzyme in the rat that catalyzes the coupling of glucuronic acid stereoselectively to the propionic acid side

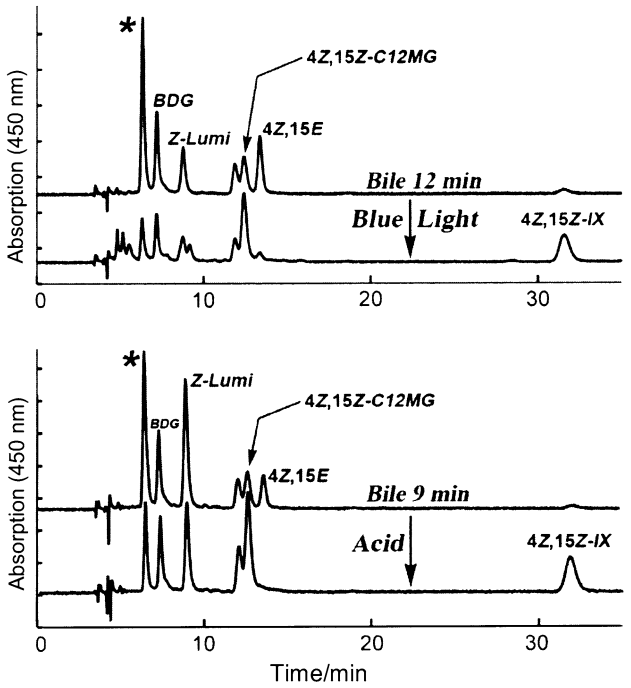

Fig. 15 HPLC chromatograms of bile collected 12 or 9 min, respectively, after injection of (4*Z*,15*E*)-BR into a wild-type rat before and after brief exposure to blue light (*top panel*) or acid (*bottom panel*). *Indicates the major (monoglucuronide) metabolite of (4*Z*,15*E*)-BR

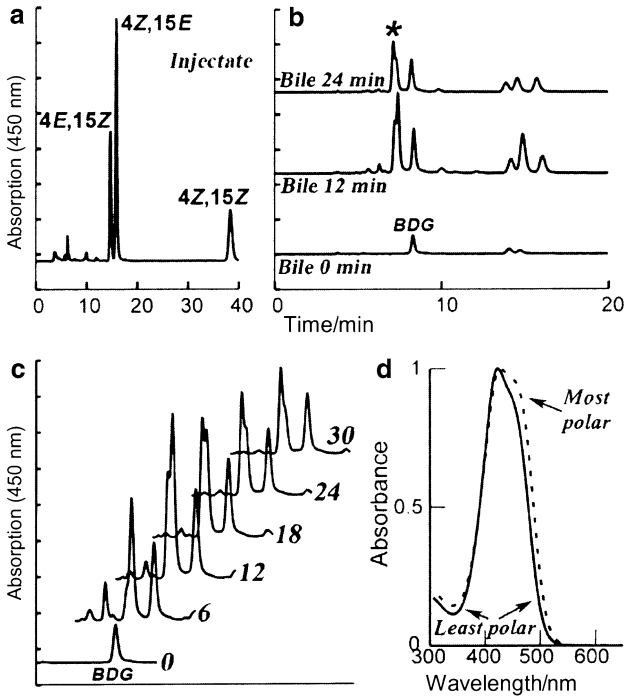

Fig. 16 **a** HPLC chromatogram of injectate enriched in (4*E*,15*Z*)- and (4*Z*,15*E*)-photo-isomers of BR. **b** Chromatograms of bile collected before, and 12 and 24 min after injecting the mixture of photo-isomers shown in *panel a*. **c** Time evolution of the metabolite peaks following injection of a mixture of (4*E*,15*Z*)- and (4*Z*,15*E*)-photo-isomers of BR into a wild-type rat. **d** Absorbance spectra of the most and least polar components of the partially resolved metabolite (monoglucuronide) peak shown in *panel c*. *Indicates two overlapping metabolite peaks: less polar, more rapidly excreted (4*E*,15*Z*)-C(8)-monoglucuronide and more polar, less rapidly excreted (4*Z*,15*E*)-C(12)-monoglucuronide

chain attached to the dipyrrinone moiety containing the isomerized (*E*) double bond.

Metabolism of (Z)-lumirubin

lnjectate solutions of (*Z*)-lumirubin were isomerically homogeneous and showed a single peak on HPLC (Fig. 18a). HPLC of bile samples collected after injection of (*Z*)-lumirubin as a bolus in rat serum showed rapid excretion of unchanged pigment, accompanied by a more polar metabolite that eluted close to BR diglucuronide (Fig. 18a). The absorption spectrum of this metabolite (λ_{max} = 435–441 nm) was identical in shape to that of the parent (*Z*)-lumirubin but shifted by ~2 nm to shorter wavelength. On treatment of an early bile sample with β-glucuronidase the metabolite peak disappeared and the unchanged (*Z*)-lumirubin peak increased (Fig. 18b). On treatment with NaOH the metabolite disappeared and the (*Z*)-lumirubin peak was retained, but underwent partial decomposition (Fig. 18c). Both treatments resulted in hydrolysis of endogenous BR glucuronide; and appearance of (4*Z*,15*Z*)-BR as expected. Thus, (*Z*)-lumirubin is partly metabolized in the rat to a glucuronide with an HPLC retention time consistent with that of a monoglucuronide. Similar results were obtained with (*Z*)-lumirubin-XIIIα.

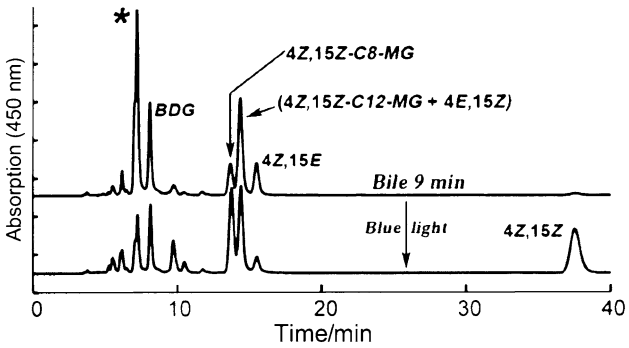

Fig. 17 Effect of blue light exposure of bile collected 9 min after injecting a mixture of (4*E*,15*Z*)- and (4*Z*,15*E*)-photo-isomers of BR as a bolus in a wild-type rat. *Indicates unresolved mixture of diastereoisomeric monoglucuronides containing predominantly the least polar diastereoisomer. Light exposure led to a marked increase in (4*Z*,15*Z*)-C(8)-monoglucuronide

The strategy used to confirm monoglucuronidation of lumirubin and determine whether a particular one of the two propionic acid side chains is selectively glucuronidated is summarized in Fig. 19. The two isomeric C(8)- and

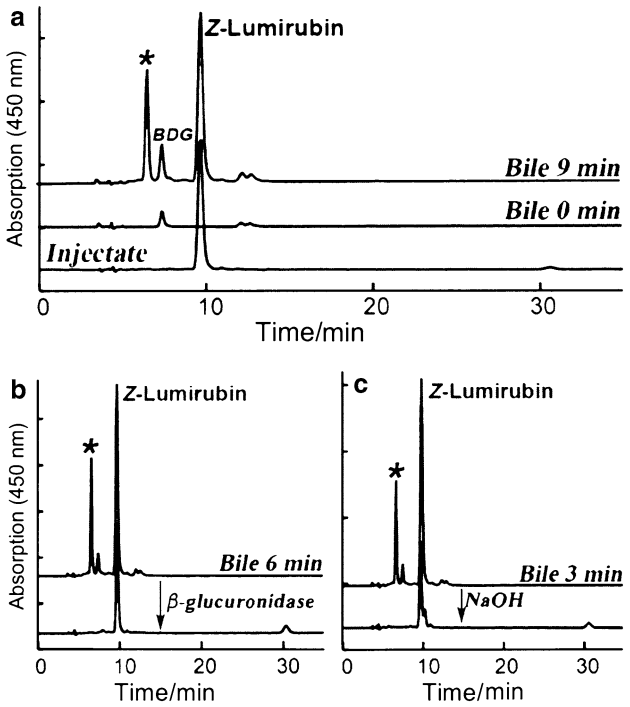

Fig. 18 **a** HPLC chromatograms of purified (*Z*)-lumirubin solution in rat serum and chromatograms of bile collected before and 9 min after injecting the solution as a bolus into a wild-type rat. **b**, **c** Effects of β-glucuronidase and NaOH on bile samples collected 6 and 3 min, respectively, after (*Z*)-lumirubin injection. *Indicates (monoglucuronide) metabolite

C(12)-monomethyl esters of BR were prepared and irradiated to generate the corresponding isomeric lumirubin monomethyl esters. After treatment with TFA to convert (*E*)-isomers to (*Z*)-isomers, each reaction product showed two major peaks on HPLC (Fig. 20a)—a peak corresponding to unchanged BR monomethyl ester and a peak with a lumirubin absorption spectrum corresponding to one of the two possible lumirubin monomethyl esters. These two presumptive isomeric lumirubin monomethyl esters were widely separated on HPLC as expected from 3D models, the more polar being the lumirubin monomethyl ester derived from BR C(8)-monomethyl ester. These were compared by HPLC to a bile sample containing the lumirubin metabolite that had been treated with alkaline methanolysis to convert glucuronides to the corresponding methyl esters (Fig. 20a). The lumirubin methyl ester from methylated bile was indistinguishable on HPLC from the lumirubin derived from BR C(8)-monomethyl ester. Although the absorbance spectra of the two synthetic isomeric lumirubin monomethyl esters are almost identical (Fig. 20b), they can be distinguished from each other, and the absorbance spectrum of the lumirubin derived from rat bile was identical to that of the synthetic lumirubin monomethyl ester. Thus, (*Z*)-lumirubin, despite having two carboxyl groups, is metabolized specifically to a monoglucuronide in which the glucuronic acid is conjugated with the propionic acid attached to the photocyclized moiety of BR (Fig. 21).

Metabolism of dihydrobilirubin-VIIIα

Dihydrobilirubin-VIIIα (Fig. 22) is an analogue of (4*Z*,15*Z*)-BR with the (C)12 propionic acid side chain moved to C(13). When injected intravenously as a bolus in a Gunn rat in the dark, unlike (4*Z*,15*Z*)-BR, the pigment was excreted rapidly in bile (data not shown). In contrast, in SD rats it was excreted partly in unchanged form, but predominantly as a single more polar metabolite (Fig. 22a) with an absorption spectrum that was consistent with a glucuronide (Fig. 22b).

Metabolism of photo-isomers: summary

As illustrated in Fig. 7, (4*Z*,15*E*)-BR, (4*E*,15*Z*)-BR, and (*Z*)-lumirubin are excreted rapidly in bile without conjugation after intravenous injection in homozygous Gunn rats deficient in UGT1 isozymes [7, 8]. In wild-type rats, the three isomers, as well as (4*Z*,15*E*)-isomers of BR-IIIα and BR-XIIIα, were excreted into bile partly in unchanged form and partly as glucuronides, whose formation was catalyzed presumably by UGT1A1. Strikingly, and in contrast to the corresponding (4*Z*,15*Z*) parent isomers, the photo-isomers formed only monoglucuronides. Furthermore, monoglucuronidation did not occur more or less randomly on either of the two propionic acid side chains, as with (4*Z*,15*Z*)-BR, but was specific for only one of the two carboxyl groups, the one appended to the dipyrrinone moiety in which photo-isomerization had occurred. In linear molecular representations, as in Figs. 1 and 2, there appears to be little difference between the two carboxyl groups of each photo-isomer. However, 3D representations of the photo-isomers (Fig. 23), based on the preferred conformation of BR in organic solvents [41] or the crystalline state [42, 43], reveal that the carboxyl group that becomes glucuronidated is the only one of the two that can undergo intramolecular hydrogen bonding similar to that in BR. Although the preferred conformations of BR photo-isomers in vivo are not known, the observations suggest that the catalytic site on UGT1A1 binds the photo-isomers in a folded (*P*)- or (*M*)-helical[3] conformation with the almost planar, but chiral, intramolecularly hydrogen-bonded moiety (Fig. 23) inserted into the active site rather than the more exposed carboxyl group attached to the half that

[3] (*P*) = plus, (*M*) = minus define the helical sense of the molecular conformation and thus the chirality of the molecule, with reference to the relative orientation of the component two dipyrrinone chromophores and their long wavelength electric transition dipole moments lying along the long axis of each dipyrrinone [34].

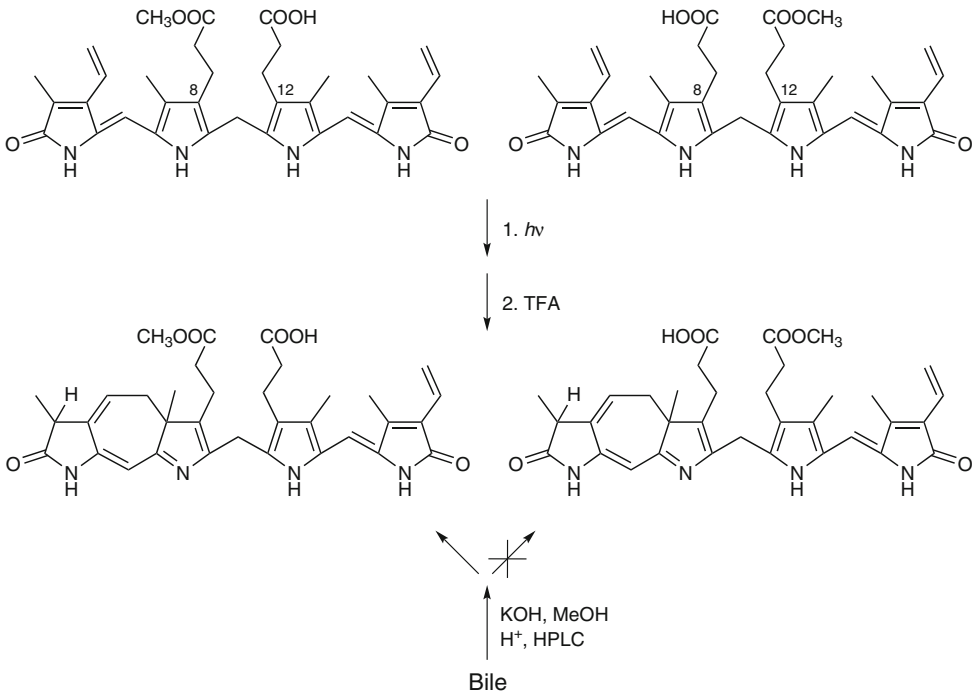

Fig. 19 Strategy for determining which propionic acid side chain of (*Z*)-lumirubin undergoes acyl glucuronidation in vivo in the rat. Individual diastereoisomers of (4*Z*,15*Z*)-BR-IXα monomethyl ester were converted photochemically to the corresponding (*Z*)-lumirubin monomethyl esters and these were compared chromatographically to the product obtained by alkaline methanolysis of bile collected from a wild-type rat after injection of (*Z*)-lumirubin

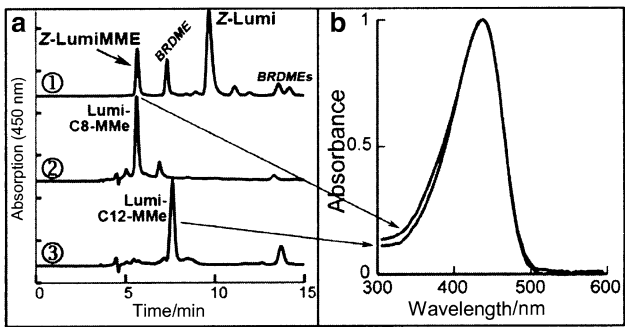

Fig. 20 **a** HPLC chromatograms of ① product obtained by methanolysis of bile collected from a wild-type rat after intravenous injection of (*Z*)-lumirubin, ② synthetic (*Z*)-lumirubin C(8)-monomethyl ester, ③ synthetic (*Z*)-lumirubin C(12)-monomethyl ester. **b** Absorbance spectra of (*Z*)-lumirubin C(8)- and C(12)-monomethyl esters

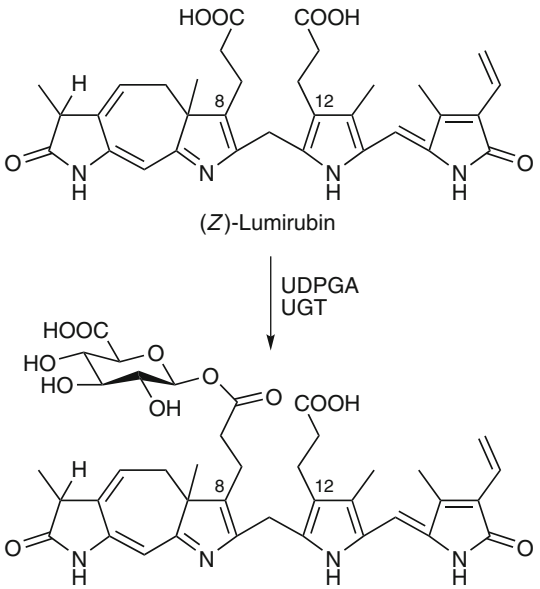

Fig. 21 Regioselective acyl glucuronidation of (*Z*)-lumirubin in vivo

has not undergone photo-isomerization. If this is correct, then (4*Z*,15*Z*)-bilirubin analogues bearing one propionic acid located on C(8), as in BR itself, and a second propionic acid located at a position other than C(8) or C(12) which are sterically restricted from intramolecular hydrogen bonding should be excreted intact in Gunn rats and excreted partly unchanged, and partly as a monoglucuronide in wild-type rats. Studies on dihydrobilirubin-VIIIα (Fig. 22), along with previous studies on synthetic bilirubins [44], bear this out. In Gunn rats dihydrobilirubin-VIIIα was excreted unchanged, but in wild-type rats it was excreted partly as a metabolite. Although the metabolite was not fully characterized, there is little doubt, on the basis of its absorbance spectrum, HPLC retention time, and the fact that it was not formed in Gunn rats, that it is a monoglucuronide.

These results are relevant to neonatal jaundice and the mechanism of phototherapy. There is no reason to believe that glucuronidation of BR photo-isomers in humans and rats will be qualitatively different. Since (4*Z*,15*E*)- and (*Z*)-lumirubin undergo glucuronidation in vivo by the same enzyme that catalyzes glucuronidation of the (4*Z*,15*Z*)-isomer it is likely that they will compete with BR for glucuronidation as the enzyme activity increases during

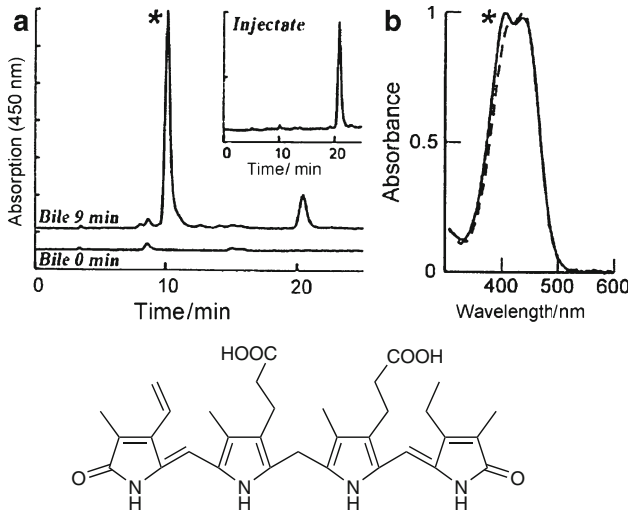

Fig. 22 Metabolism of dihydrobilirubin-VIIIα in the rat. **a** HPLC chromatograms of bile before and 9 min after intravenous injection. **b** Normalized absorbance spectra of metabolite (*solid line*) and parent pigment (*dashed line*). *Indicates (monoglucuronide) metabolite

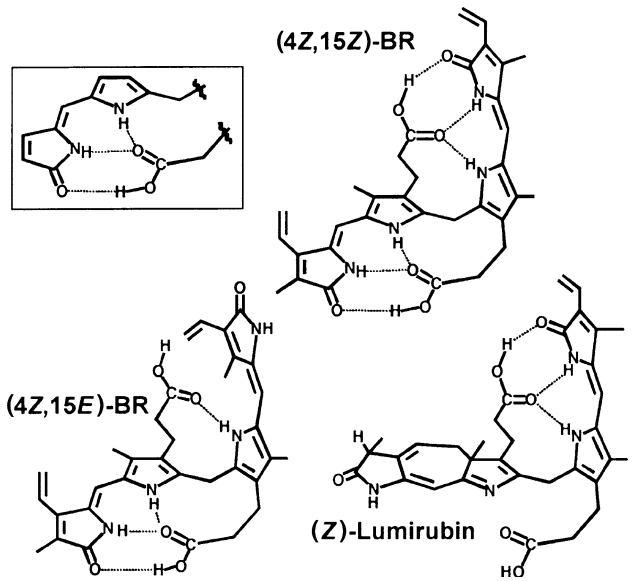

Fig. 23 Preferred conformation of (4*Z*,15*Z*)-BR stabilized by intramolecular hydrogen bonding and hydrogen-bonded conformations of BR photo-isomers based on this. Note: (1) Each conformer can exist as a pair of mirror-image (*M*)- or (*P*)-chirality enantiomers which interconvert rapidly in solution [33, 34]. Only a single enantiomer is shown for each structure. The structure depicted for (4*Z*,15*Z*)-BR is an (*M*)-chirality enantiomer. The *inset* shows the structural motif that seems to be preferred by UGT1A1. (2) If monoglucuronidation of BR involves preferential binding of, say, a (*P*)-chirality conformer, then the monoglucuronide (and the photo-isomers) will have to maintain the same chirality in order to undergo further glucuronidation

developmental maturation of the neonatal liver. In this way, phototherapy might retard the normal excretion of BR as the liver matures. However, this is unlikely to be a major effect because BR is always in excess in the circulation and because competition between photo-isomers and BR for conjugation will favor the latter statistically because it carries two carboxyl groups that undergo glucuronidation. Second, the observations strongly suggest that (4*E*,15*E*)-BR and (*E*)-lumirubin, which lack carboxyl groups that can form the hydrogen-bonded motif shown in Fig. 23, will be completely excreted in bile unchanged and will not undergo conjugation to glucuronides in vivo. These two photo-isomers, which do not accumulate in serum during phototherapy, may make a greater contribution to the effectiveness of the treatment than generally thought. Third, the pigment composition of bile of infants undergoing phototherapy is likely to be more complex than previously believed. Initially, the bile will contain principally photo-isomers of BR along with relatively low concentrations of (4*Z*,15*Z*)-BR formed by thermal reversion of its configurational isomers in bile. However, as the glucuronidating system in the liver becomes active these pigments will be accompanied, not only by mono- and diglucuronides of BR, but by monoglucuronides of (*Z*)-lumirubin, (4*E*,15*Z*)-BR, and (4*Z*,15*E*)-BR.

The observations lead to a stereochemical explanation for the formation of BR diglucuronide in normal BR metabolism. Monoglucuronidation is adequate to ensure rapid biliary elimination of BR and diglucuronidation appears to be redundant and unnecessary. For a time it was thought that the two processes, mono- and diglucuronidation, were catalyzed by two different enzymes [45], a view no longer current. In its preferred conformation, BR has two intramolecularly hydrogen-bonded dipyrrinones (Fig. 23). These are structurally similar, but not identical because of differences in the vinyl substitution sequence on the two lactam end rings. There is little selectivity in binding of one or the other of these to the UGT1A1 active site because the C(8)- and C(12)-monoglucuronides are formed in similar amounts when BR undergoes glucuronidation. Glucuronidation converts BR into a product that, like BR photo-isomers, still has a single dipyrrinone moiety capable of intramolecular hydrogen bonding. Once glucuronidation has occurred and the monoconjugate leaves the active site it can be transported into bile or, maintaining the same (*M*)- or (*P*)-chirality, it can undergo a rotation of 180° around an axis bisecting the C(9)–C(10)–C(11) bond angle and present the remaining dipyrrinone moiety to the active site for conjugation and formation of the diglucuronide. Thus, it is likely that BR monoglucuronides compete with unconjugated BR for glucuronidation and formation of BR diglucuronide can be seen to be an inevitable and accidental consequence of the 3D structure and symmetry of the BR molecule rather than a "purposeful" process. The final ratio of BR diglucuronide to monoglucuronides excreted into bile, which shows a wide species variation [46], will depend on intracellular transport dynamics.

Alternate pathways of BR metabolism

There are several reports that Gunn rats metabolize BR to hydroxylated pigments whose formation is augmented by inducers of cytochrome P450, such as tetrachlorodibenzo-dioxin, and phototherapy. Although often referred to as having established structures, these hydroxylated pigments have not been well characterized. Three pathways have been proposed to explain their genesis (Fig. 24) [18–21]. One pathway (Fig. 24a), suggested as important in phototherapy and in the metabolism of BR in the Gunn rat in the dark [18], involves initial "phototautomerization" of BR followed by regioselective reaction with singlet oxygen to give a dioxetane which undergoes rearrangement to a *vic*-diol. "Phototautomerization" of the type proposed has not been observed in BR photochemistry and is energetically unlikely not least because it involves migration of a hydrogen from one saturated carbon center to another. The source of the singlet oxygen implicated in the second step was not explained and the cleavage of the 1,2-dioxetane in the final step, which involves dissociation of a hydrogen from a saturated carbon center, is highly improbable. A second pathway (Fig. 24b) invokes addition of water to a bis-lactim tautomer of BR and also dehydrogenation to biliverdin followed by addition of water. However, water does not add to BR or biliverdin as suggested, nor does dehydrogenation of BR to biliverdin occur under physiological conditions. And if it did, the biliverdin would be reduced back to BR by biliverdin reductase in vivo. The third pathway (Fig. 24c) postulates addition of hydrogen peroxide, of unexplained origin, to the C(4) (or C(15)) double bond of BR, yielding a *vic*-diol, which spontaneously loses water to yield a C(5)- (or C(15)-)hydroxy-BR. However, hydrogen peroxide does not normally add to double bonds to give *vic*-diols, except under UV light [47]. Thus, the mechanisms proposed for the formation of hydroxylated bilirubins in homozygous Gunn rats are all highly unlikely.

During the present studies and our extensive studies on the excretion and metabolism of BR photo-isomers and BR model compounds in wild-type and Gunn rats and on the effects of phototherapy on biliary pigment excretion in Gunn rats, we have observed no evidence for the excretion in bile of significant quantities of hydroxylated pigments of the type proposed by Ostrow and co-workers [18–21]. As can be seen in Figs. 6 and 7, BR photo-isomers are excreted unchanged in Gunn rats and are the only significant products absorbing near 450 nm excreted in bile during phototherapy. We have found no evidence that "phototherapy simply accelerates the alternate pathway(s) of bilirubin catabolism which exist normally in the Gunn rat" [48]. Possibly the putative hydroxylated pigments are non-physiological artifacts generated during their extraction, chromatography, and isolation. Until their structures, mechanism of formation, and physiological relevance have been substantiated they should be viewed with skepticism.

Experimental

In general, solvents and solutions were purged with Ar (>99.9 % purity) before use. Isomerically pure BR-IIIα, -IXα, and -XIIIα, used for synthesis of the corresponding photo-isomers, were isolated by preparative HPLC on silica, crystallized from $CHCl_3/CH_3OH$, and dried overnight at 65 °C under vacuum. Commercial BR (Porphyrin Products, Logan, UT, and Koch-Light Laboratories, Colnbrook, Bucks., UK, containing 2 % BR-IIIα, 95 % BR-IXα, and 3 % BR-XIIIα by HPLC) was used for preparation of photobilirubins I and II. Rat serum was isolated from fresh rat blood, collected by venisection under ether anesthesia, briefly evaporated on a rotary evaporator to remove ether anesthetic, and frozen at below −50 °C until needed. HSA and rabbit serum albumin were from Sigma-Aldrich. Blue light refers to Westinghouse Special Blue fluorescent lamps (F20T12/BB); 20 W lamps were used for in vitro irradiation of solutions and 40 W lamps for irradiating Gunn rats. All work, except for irradiation experiments, was done under safelights in a darkroom. Where indicated, solutions were evaporated rapidly at room temperature on a rotary evaporator fitted with a vacuum pump and the final residues evacuated to less than 13 mbar before being stored under Ar at below −20 °C. For flash-freezing, bile samples in 6 × 50 mm glass Pasteur tubes were plunged into powdered dry-ice. Preparative and analytical TLC were run on 20 × 20 cm × 250 mm silica gel G (Analtech, Newark, DE, USA) or homemade silica gel H (E. Merck Laboratories) plates activated at 110 °C for 1 h and used after equilibration in air at room temperature. Silica K6 and silica gel D-O TLC plates, used as described [13], were also investigated for the preparation and separation of photobilirubins I and II. Developed plates were viewed under blue light to improve visualization of yellow bands or spots. Essential details of the reversed-phase HPLC analyses have been described previously [44]. Eluted peaks were detected at 450 nm and relative peak areas measured with Hewlett-Packard HP ChemStation software. The isocratic HPLC eluent was 0.1 M di-*n*-octylamine acetate in CH_3OH containing from 2 to 8 % water at a flow rate of 0.75–1.00 cm^3/min. Used solvent can be exposed to blue light and recycled. In a few early studies less than 0.1 M di-*n*-dodecylamine acetate was used in place of or in admixture with di-*n*-octylamine acetate to improve peak resolution. Metabolism studies were done in male homozygous Gunn rats or wild-type SD

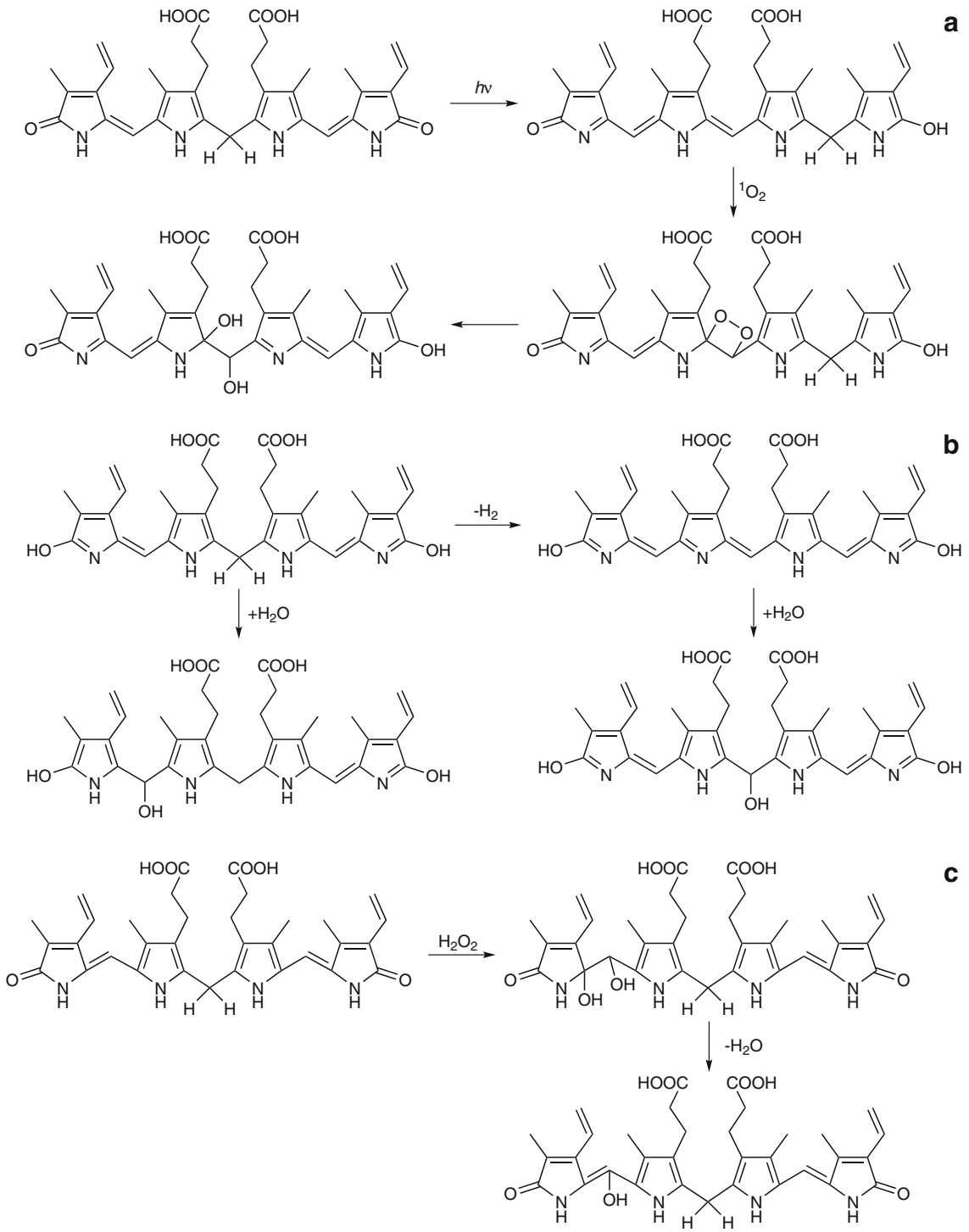

Fig. 24 Mechanisms proposed for formation of hydroxylated bilirubins in vivo in homozygous Gunn rats during phototherapy and in the dark [18–21] (redrawn from original figures)

rats weighing more than 250 g fitted with a short (∼7.5 mm) indwelling biliary cannula for bile collection and a femoral venous line for administration of samples as previously described [39, 44]. The external segment of biliary cannula was painted black or red with nail varnish and experiments were done under safelights in a darkroom. Surgery was done in the dark or under safelights with only the small area of the abdomen and abdominal cavity being operated on illuminated with light from a fiber-optic halogen-lamp illuminator. For generation of photo-isomers

in vivo, homozygous Gunn rats, with dorsal hair removed with a surgical depilatory cream, were exposed in restraining cages to 40-W Special Blue lights for ~4 h and bile was collected.

(Z,E)-Bilirubin-IIIα, (Z,E)-bilirubin-XIIIα, and (4E,15Z)/(4Z,15E)-bilirubin IXα injectate

Bilirubin-IIIα, -XIIIα, or -IXα (1.3 mg) was dissolved in 10 cm^3 Ar-purged $CHCl_3/Et_3N$ (1:1) in a 15-cm^3 Erlenmeyer flask and the solution irradiated from below with blue light with continuous bubbling of Ar for 10 min (photostationary state). The solution was flash-evaporated under vacuum and the residue rapidly rinsed thrice with ice-cold CH_3OH (2, 2, 1 cm^3). The rinsings were decanted, briefly centrifuged, combined, and flash-evaporated under vacuum in a 25-cm^3 round-bottom flask, and stored until required at below −50 °C under Ar. Residues from two preparations were rinsed with 1.5 cm^3 rat serum and the liquid phase decanted and centrifuged. Samples of the clear supernate were taken for HPLC and UV–Vis analyses and 0.5-cm^3 portions were for i.v. injections in metabolism studies. Pigment concentrations in the injectates, determined spectroscopically using $\varepsilon_{max}/\lambda_{max}$ for BR in rat serum (50,000/400 nm; A.F. McDonagh and L.A. Palma, unpublished observations) were in the range 0.1–0.8 mg/cm^3. Typical enrichments are shown in the insets of Figs. 8 and 12 and in Fig. 16a. Injectate solutions contained predominantly (*Z*,*E*)-photo-isomers along with a relatively small proportion of the corresponding (*Z*,*Z*)- and (*E*,*E*)-isomers.

(4Z,15E)-Bilirubin-IXα injectate

BR (2.5–2.7 mg) was dissolved in 0.2 cm^3 Ar-degassed 0.1 M NaOH and the solution added at once to 0.28 g HSA in 10 cm^3 Ar-purged 0.1 M potassium phosphate buffer, pH 7.4 in a 15-cm^3 Erlenmeyer flask. The solution was purged slowly with Ar gas (beware of frothing) for 10 min and then, with continued Ar bubbling, irradiated from below with blue light for 10 min. The solution was shaken with 50 cm^3 0.1 M ammonium acetate (NH_4OAc) in CH_3OH and centrifuged for 1 min. The supernate was rapidly evaporated on a rotary evaporator to a moist residue (speed is essential at this step to avoid poor yields and enrichments). The residue was mixed with 10 cm^3 $CHCl_3/CH_3OH$ (9:1, v/v) and the organic phase washed with water (2 × 10 cm^3) using brief centrifugation to separate phases. The organic phase was evaporated to dryness on a rotary evaporator and then on a high-vacuum line. The residue was rinsed rapidly with 5 cm^3 ice-cold Ar-purged CH_3OH in three portions and the combined rinsings were centrifuged. The supernate was flash-evaporated and the residue further dried under vacuum. Three such preparations were mixed with 1.5 cm^3 rat serum and the solution centrifuged. Samples of the clear supernate were taken for HPLC and UV–Vis analysis and 0.5- to 1.0-cm^3 portions for i.v. injections in metabolism studies. These injectates contained predominantly (4*Z*,15*E*)-BR along with lesser amounts of (4*Z*,15*Z*)-BR and (4*E*,15*Z*)-BR and (*Z*)-lumirubin as seen in Fig. 14a. The final pigment concentration in injectates, estimated spectroscopically, varied from 0.3 to 1.0 mg/cm^3. Substitution of rabbit or pigeon serum albumin for HSA in the above preparation gives final solutions containing both (4*E*,15*Z*)- and (4*E*,15*Z*)-stereoisomers of BR, with the former predominating.

(Z)-Lumirubin-IXα injectate

A fresh solution of BR (2.5–2.7 mg) in 0.2 cm^3 Ar-degassed 0.1 M NaOH was diluted into a solution of 0.28 g rabbit serum albumin in 10 cm^3 Ar-purged 0.1 M potassium phosphate buffer, pH 7.4 in a 15-cm^3 Erlenmeyer flask. The solution was purged slowly with Ar for 10 min in the dark and then, with continued Ar bubbling, irradiated from below with blue light for 4 h. The yellow solution was then added slowly to 50 cm^3 0.1 M NH_4OAc in CH_3OH and the mixture centrifuged. The supernate was decanted into a 250-cm^3 round-bottom flask and the residue rinsed with 5 cm^3 methanolic NH_4OAc. The combined supernate and rinsings were evaporated to a moist residue on a rotary evaporator. This was mixed with 10 cm^3 CH_3Cl/CH_3OH (9:1, v/v) and the organic phase washed with water (2 × 10 cm^3), clarified by centrifugation, and transferred to a 25-cm^3 round-bottom flask. To this solution was added 0.020 cm^3 TFA, to convert exocyclic (*E*) to (*Z*) double bonds, and the solution flash-evaporated at once. The residue was dried under high vacuum, rinsed with 2–5 cm^3 ice-cold Ar-purged CH_3OH in three portions, and the rinsings decanted into a 12-cm^3 centrifuge tube and centrifuged. The clear supernate was flash-evaporated to dryness and the residue stored at below −50 °C under Ar. For metabolism experiments this was dissolved in 1.5 cm^3 of rat serum to give an absorbance of ~2 in a 1-mm-pathlength cell; 0.5-cm^3 aliquots were used for injectates. HSA can be substituted for rabbit albumin, but yields are somewhat lower. Yields were in the range 35–50 %, measured spectrophotometrically in NH_4OH/CH_3OH (1:99, v/v). (*Z*)-Lumirubin-XIIIα injectates were prepared similarly.

The following simpler, lower yield, procedure not involving protein was also used. BR (2.5 mg) in 10 cm^3 Ar-purged $CHCl_3/Et_3N$ (1:1) in a 15-cm^3 Erlenmeyer was irradiated with blue light with continuous bubbling of Ar for 4 h and the solution evaporated to dryness under vacuum. The residue was dissolved in 10 cm^3 $CHCl_3/CH_3OH$ (9:1, v/v), 0.020 cm^3 TFA was added, and the solution flash-evaporated. Crude products from two such preparations were mixed with 5 cm^3 ice-cold Ar-purged CH_3OH and the mixtures decanted and centrifuged to remove

insoluble bilirubin. The CH_3OH solution was evaporated to dryness and the residue purified by preparative TLC on silica gel G. Pigment was applied to the plates in Ar-purged $CHCl_3/CH_3OH$ (1:1) and plates developed with 1 % acetic acid (HOAc) in $CHCl_3/CH_3OH$ (9:1). The isolumirubin and lumirubin bands were collected [7], combined, and eluted with developing solvent and the solution evaporated. Yield: 1.7 %. For animal studies the residue was rinsed with 1.5 cm^3 rat serum and the liquid phase decanted and centrifuged. Samples of the clear supernate were taken for HPLC and UV–Vis analysis and 0.5-cm^3 portions were used for i.v. injections.[4]

Photobilirubins IA and IB

The published procedure [13] was followed exactly on a smaller scale, starting with 20 mg rather than 200 mg commercial BR. Briefly, BR was irradiated in $CHCl_3$, under N_2 for 2 min/mg BR with an unfiltered 100-W Hg spotlight, $CHCl_3$ was removed by evaporation and the residue extracted with acetone. Evaporation of the acetone extract gave crude product to which TLC on silica was applied, using $CHCl_3/CH_3OH/H_2O$ (40:9:1, v/v/v) as developing solvent. Bands corresponding to photobilirubins IA and IB (R_f values $\sim$0.42 and 0.52) were collected and pigments eluted.

Photobilirubin II

The published procedures [13, 23] were followed exactly on a smaller scale. BR (20 mg) was irradiated in $(CH_3)_2SO$ containing ethylenediaminetetraacetic acid (EDTA) with a 100-W Hg spotlight as outlined above for the preparation of photobilirubins IA and IB. The solution was diluted with $CHCl_3$ and water and the $CHCl_3$ extracts washed with water, filtered, and evaporated. The residue was extracted with CH_3OH, the extracts evaporated, and the residue applied to a TLC plate in $CHCl_3$. The plate was developed and the photobilirubin II band(s) collected and eluted. Two slightly different variations of the procedure have been published. In the first, the UV light was unfiltered, $(CH_3)_2SO$ was purged with N_2, $CHCl_3/CH_3OH$/formic acid (30:3:1) was used for TLC development, and eluted pigments in $CHCl_3$ were washed with 0.1 M $NaHCO_3$ and water to remove formic acid [13]. In the second, the spotlight was filtered through a Wratten 2A filter (to cut out Hg emission at 365 and 405 nm), $(CH_3)_2SO$ was purged with Ar, and $CHCl_3/CH_3OH/H_2O$ (40:9:1) used for TLC development [23]. Both variants were investigated. A third procedure [24] using ammoniacal CH_3OH as solvent and blue fluorescent lights was not investigated because preliminary studies indicated relatively low quantum yields for lumirubin formation in that solvent.

Isomeric (Z)-lumirubin-IXα monomethyl esters

Isomeric C(8)- and C(12)-BR-monomethyl esters [50] were separated and purified by preparative TLC on 250-μm silica G plates using $CHCl_3/CH_3OH$/glacial acetic acid (HOAc) (97:2:1, v/v/v) as developing solvent and eluent. The C(8)-isomer runs ahead of the C-12) isomer. The identity of the products was confirmed by HPLC and TLC comparison with authentic samples (kindly provided by Dr. D.A. Lightner, University of Nevada, Reno) and literature data [50, 51]. Individual purified isomers were dissolved in Ar-saturated CH_3OH ($\sim$0.35 cm^3) to give an absorbance of $\sim$2 in a 1-mm-pathlength quartz cuvette and each solution was irradiated with blue light through a Lucite filter. Changes were followed by absorbance difference measurements and 0.040-cm^3 samples were taken at intervals for HPLC. By 60 min most of the starting material had been converted to photo-isomers. The solution remaining after 60 min irradiation was evaporated to dryness, the residue dissolved in 0.2 cm^3 $CHCl_3$, and 0.0020 cm^3 TFA added. This solution was immediately evaporated to dryness and the residue taken in 0.080 cm^3 CH_3OH for HPLC.

Preparation and hydrolysis of lumirubin dimethyl ester

BR dimethyl ester (13 mg; Porphyrin Products/Frontier Scientific, Logan, UT) in 100 cm^3 Ar-purged 1 % NH_4OH/CH_3OH in a 500-cm^3 Erlenmeyer flask was irradiated from below with continuous Ar bubbling for 4 h. The solution was evaporated to dryness and the residue dissolved in 50 cm^3 $CHCl_3$. The solution was washed with 3 × 50 cm^3 water, filtered through $CHCl_3$-moistened filter paper, saturated with Ar, and 0.1 cm^3 TFA added. The solution was immediately evaporated and the residue chromatographed on four CH_3OH-washed silica gel G plates (analytical thickness) with $CHCl_3/CH_3OH$/HOAc (94:5:1, v/v/v) as irrigant. The main yellow product band (which turned brown if not eluted at once) was collected and eluted with the developing solvent. The residue after evaporation of solvent was rechromatographed on three plates and the main yellow band collected and eluted. This showed a single yellow spot on analytical TLC and one peak on HPLC. The purified product was dissolved in 1 cm^3 degassed CH_3OH and 0.1 cm^3 of this was diluted to 5 cm^3 for UV–Vis absorbance measurements (λ_{max} = 429 nm). To the remaining 0.9 cm^3 of solution was added 0.5 cm^3 1 M NaOH and the solution kept at 37 °C under Ar for 5 min, followed by addition of 5 cm^3 water and adjustment

[4] (*Z*)-Lumirubin has two stereogenic centers, denoted by *in Fig. 2. Prepared by irradiation of BR-HSA, (*Z*)-lumirubin is not identical to (*Z*)-lumirubin prepared by irradiation of BR in $CHCl_3/Et_3N$. Because of chiral induction by the protein the former is chiral and optically active whereas the latter is a racemate and optically inactive [49]. Both preparations behaved identically in the in vivo studies and are not distinguished in this paper.

of the pH to 4 with HOAc. This solution was extracted with 5 cm^3 $CHCl_3$ and the extract washed with 5 cm^3 water and evaporated to dryness. The residue was analyzed by TLC and HPLC.

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