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Identification of bilirubin reduction products formed by *Clostridium perfringens* isolated from human neonatal fecal flora

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Abstract

Urobilinoids belong to the heterogenous group of degradation products of bilirubin formed in the gastrointestinal tract by intestinal microflora. Among them urobilinogen and stercobilinogen with their respective oxidation products, urobilin and stercobilin, are the most important compounds. The aim of present study was to analyze the products of bacterial reduction of bilirubin in more detail. The strain of *Clostridium perfringens* isolated from neonatal stools, capable of reducing bilirubin, was used in the study. Bacteria were incubated under anaerobic conditions with various native as well as synthetic bile pigments, including radiolabeled unconjugated bilirubin (UCB). Their reduction products were extracted from media and separated following thin layer chromatography. Pigments isolated were analyzed by spectrophotometry, spectrofluorometry and mass spectrometry. In a special set of experiments, bilirubin diglucuronide was incubated with either bacterial lysate or partially purified bilirubin reductase and β-glucuronidase to reveal whether bilirubin glucuronides may be directly reduced onto conjugated urobilinoids. A broad substrate activity was detected in the investigated strain of *C. perfringens* and a series of bilirubin reduction products was identified. These products were separated in the form of their respective chromogens and further oxidized. Based on their physical–chemical properties, as well as mass spectra, end-catabolic bilirubin products were identified to belong to urobilinogen species. The reduction process, catalyzed enzymatically by the studied bacterial strain, does not proceed to stercobilinogen. Bilirubin diglucuronide is not reduced onto urobilinoid conjugates, glucuronide hydrolysis must precede double bond reduction and thus UCB is reduced much faster.

Keywords: Bilirubin; Bacterial reduction; Intestinal microflora; Urobilinoids; Bile pigments

1. Introduction

Microbial catabolism of bilirubin in the gut lumen contributes importantly to serum bilirubin homeostasis [1]. In the absence of bilirubin-reducing microflora, such as in the early newborn period, or in patients treated with systemic antibiotics, unconjugated bilirubin (UCB) may undergo substantial enterohepatic and enterosystemic circulation. Despite the importance of the intestinal catabolic pathway of bilirubin, only little is known about precise mechanisms of bilirubin reduction onto urobilinoids and the particular microbial species involved in this process.

It is generally believed that negligible amounts of fecal urobilinoids are present in the intestinal lumen of infants during the first months of their life, due to undeveloped intestinal microflora capable of reducing bilirubin [1,2–4]. This presumably contributes importantly to the pathogenesis of neonatal jaundice [1]. In adults, the urobilinoid production is highly efficient. Under normal conditions only small amounts of bilirubin can be found in stools of adults (5–20 mg/day) while urobilinoids are predominant bile pigments (50–250 mg/day) [2]. Nevertheless, only few bacterial strains have been isolated, that are unequivocally

Abbreviations: C., *Clostridium*; BDG, bilirubin diglucuronide; BDM, bilirubin dimethylester; BDT, bilirubin ditaurate; MB, mesobilirubin; UCB, unconjugated bilirubin

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capable of reduction of bilirubin to urobilinogens: *Clostridium ramosum* [5], *Clostridium perfringens* and *Clostridium difficile* [1] and *Bacteroides fragilis* [6]. Previously, such catalytic activity had been already suggested to be an attribute of the *Clostridium* genus [7–9]. This is consistent with previous findings by Weimer et al. mentioned by Watson et al. [10] who isolated pure strains of *C. perfringens*, capable of reducing bilirubin, from ileostomy contents and other human fluids.

It is generally accepted [11,12] that bilirubin is reduced by multiple sequential reactions, into a series of urobilinogens; these colorless chromogens may in turn be oxidized to respective yellow oxidation products, urobilins (Fig. 1). It is also believed

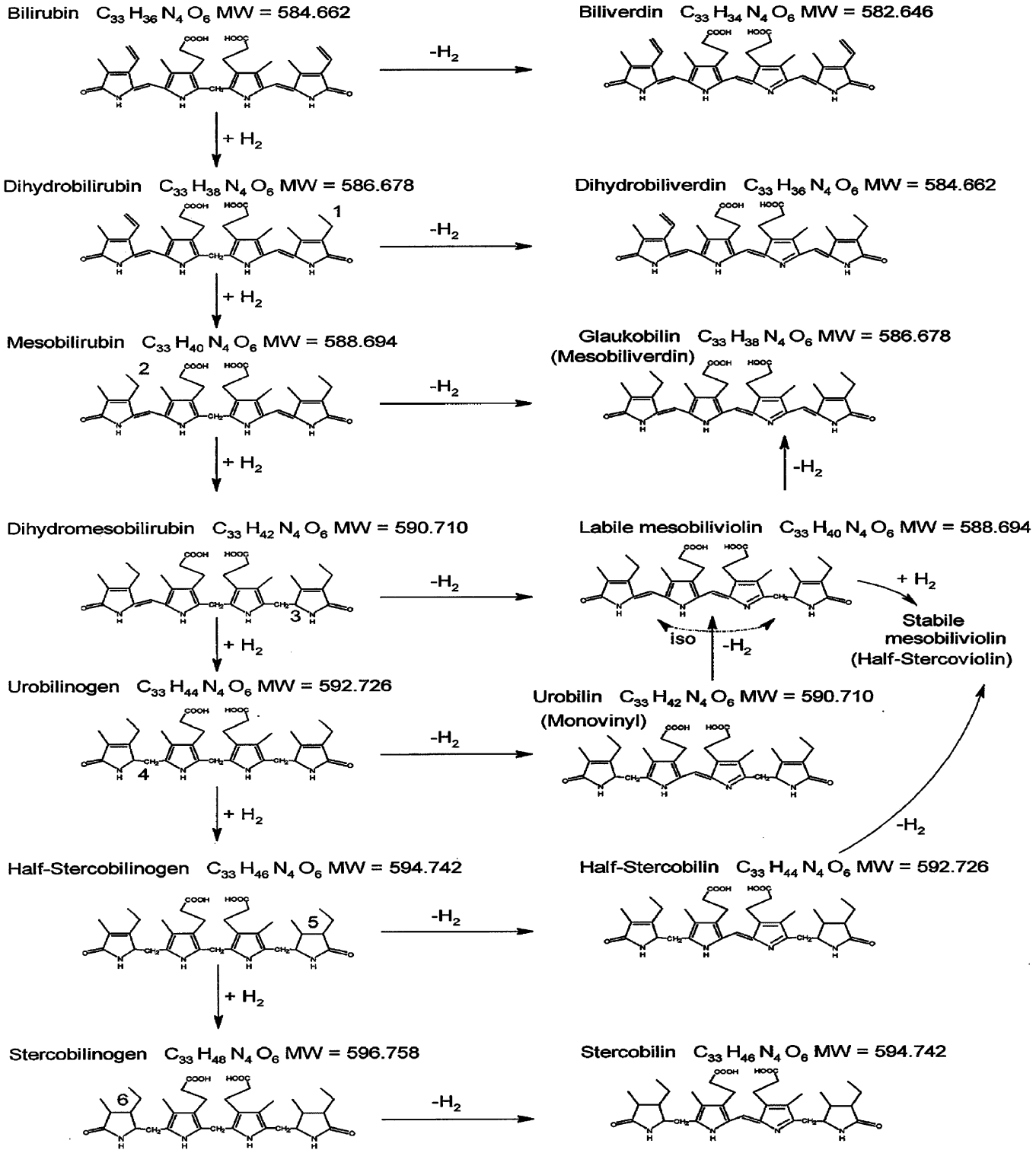

Fig. 1. Multiple steps in the reduction of UCB by intestinal microflora showing the chemical structure of the products formed.

that vinyl side-chains of the A and D rings of bilirubin are reduced prior to the reduction of any double bonds within the end pyrrolic rings or the 1- and 3-methene bridges [11,12]. However, there is some evidence that monovinyl urobilinoids might occur naturally [13,14].

Another important issue is whether or not bilirubin diglucuronide may be reduced without prior deconjugation. Watson et al. [15] observed that conjugated bilirubin, when administered intra-duodenally to normal subjects, was extensively converted to fecal urobilinoids, whereas this was not the case when UCB was administered. Similarly, normal fecal flora in broth cultures in vitro quantitatively reduced bilirubin glucuronide, but not UCB [15]. However, no attempt was made to determine whether urobilinoids were present, even in part, as conjugated compounds. On the other hand, Noro had reported earlier that a part of the urinary urobilinoids could be extracted with chloroform only after hydrolysis with alkali providing an indirect evidence for the presence of conjugated urobilinoids [16]. Kahan and Kahan [17] detected urobilinoid glucuronide, as well another sugar-linked form, by electrophoretic analysis of urine. No subsequent evidence for the natural occurrence of conjugated urobilinoids has been published, so this concept remains to be verified.

The aim of present study was to undertake a more detailed analysis of the products of bacterial reduction of bilirubin, formed by a novel strain of *C. perfringens* isolated from neonatal feces. Of particular interest were the unsettled issues regarding the relative reduction rates of conjugated versus unconjugated bilirubins and the major products resulting therefrom, including the possible formation of conjugated urobilinoids.

2. Experimental

The strain of *C. perfringens* [1], isolated from neonatal stools, was incubated with either native bile pigments in human bile or purified bile pigments in broth (2% yeast extract, Difco, USA). Depending on their polarity, purified pigments were dissolved either in dimethylsulfoxide (Sigma, St. Louis, MO, USA) or distilled water and added to the broth at a final concentration of 50 μmol/l. Natural in vitro studied bile pigments (Table 1) included: UCB IXα (Sigma), UCB IXβ (prepared and purified according to ref. [18]), mesobilirubin (MB) (Porphyrin Products, UT, USA), bilirubin diglucuronide (BDG) (prepared according to ref. [19]) and biliverdin (Sigma). Also studied (Table 1) were synthetic bilirubin derivatives, including: bilirubin diamide (a kind gift of Professor Milan Jirsa), bilirubin dimethylester (BDM) (Porphyrin Products), bilirubin diethylester (prepared according to ref. [20] with modification), and bilirubin ditaurate (BDT) (Porphyrin Products). All prepared pigments used in the study had at least 90% purity as determined by HPLC (Agilent 1100 Series, CA, USA) (UCB IXβ) or TLC with densitometric analysis (bilirubin diamide, bilirubin diethylester, BDG) (CAMAG TLC Scanner II, CAMAG, Muttenz, Switzerland).

Table 1
Reduction of bile pigments by a strain of *C. perfringens* isolated from neonatal stools

Bile pigment	Conversion rate (%/24 h)
Mesobilirubin	24.1
Bilirubin diamide	22.1
UCB IXα	16.8
Bilirubin dimethylester	14.9
Bilirubin diglucuronide	13.8
Bilirubin diethylester	8.3
Bilirubin ditaurate	5.9
UCB IXβ	3.3
Biliverdin	1.0

All pigments were incubated with *C. perfringens* in broth, experiments were carried in duplicates and values represent means of two determinations.

After 24 h of incubation at 37 °C in Anaerostat (Oxoid, GB), the concentration of urobilinoids was measured and normalized to the mass of bacteria. The optical density of the inoculated media after 24 h incubation was approximately 0.8. The bile pigment conversion rate was calculated as a proportion of urobilinoid production to the initial bile pigment concentration. Urobilinoids were determined spectrophotometrically as oxidation products of zinc complexes of urobilinoids [21]. The selected bile pigments produced by a strain of *C. perfringens* were extracted in a native form, or in specific cases submitted to alkaline methanolysis according to Blanckaert [20] and then separated by TLC and examined under visible and UV light. Products of reduction of UCB were extracted from silica gel with methanol (urobilinoids) or chloroform (UCB, MB) or with mixtures of both solvents and further analyzed by spectrophotometry (UV/Vis Spectrophotometer Lambda 20, Perkin-Elmer, USA), spectrofluorometry (Luminiscence Spectrometer LS 55, Perkin-Elmer, USA) and mass spectrometry (ZAB-EQ, Micromass, Manchester, UK). Reaction of bile pigment reduction products with zinc salts and products of oxidation with $FeCl_3$ (mesobiliviolin reaction) [22] were also determined. Products of mesobiliviolin reaction were analyzed spectrophotometrically. MB was used for time-dependent analysis of reduction process. The possible effect of bacterial microflora represented by an above described strain of *C. perfringens* on reduction of urobilin and stercobilin (Porphyrin Products) to their respective chromogens was also analyzed as described above, including separation by TLC under conditions described below. Bile pigment reduction products, formed by action of the bacterium on bile pigments from human bile (obtained by external biliary drainage), was similarly analyzed.

Urobilinoids produced by *Clostridia* were separated as their respective chromogens, using HPTLC plates coated with silica gel (HPTLC silica gel 60, Merck, Germany) (solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.)). Separated chromogens were either eluted with methanol, or oxidized in situ under UV light, followed by development in the second dimension (solvent system B: $CHCl_3$:MeOH:CH_3COOH (80:19:1, by vol.)). Possible enol/keto isomers were separated by TLC according to Kahan and Szepesy [23]. In some cases bilirubin reduction products were reacted with zinc salts (1% zinc acetate, Sigma, MO, USA) both in situ on the TLC plate and after elution, and both absorption and emission fluorescent maxima were determined.

Direct identification of reduction products of bilirubin was performed using [^{14}C]-labeled bilirubin as a substrate followed

with visualization of labeled TLC bands on Phosphorimager Bas 5000 (Fuji, Japan). [^{14}C]-bilirubin was labeled biosynthetically from [^{14}C]-δ-aminolevulinic acid according to Ostrow et al. [24].

To determine the presence of urobilinoids conjugated with glucuronic acid, reduction products of bilirubin diglucuronide were: (a) converted to ethylantranilate azopigments [25], that were then separated by TLC, and (b) hydrolyzed in an ammonia atmosphere and the released sugar moieties analyzed by TLC [26]. The effect of D-saccharic acid 1,4-lacone (saccharonolactone) (Sigma) (10 mM, [27]), a potent β-glucuronidase inhibitor, on the reduction of BDG incubated with *C. perfringens* was also investigated. In this case incubation was carried out for 8 h to minimize spontaneous hydrolysis of BDG. In another set of experiments selected bile pigments (UCB, BDG and BDM) were incubated with partially purified bilirubin reducing protein fraction. Purification was carried out as follows. *C. perfringens* was incubated in 5 l of 2% yeast extract as described above. Cells were centrifuged (2000 × *g*, 30 min, 4 °C), collected and disintegrated using French press (6 cycles, 1500 psi, SLM-Aminco, USA). Cell debris was removed by centrifugation (20,000 × *g*, 30 min, 4 °C) and supernatant containing active protein was precipitated by ammonium sulphate (Sigma). Forty to seventy percent precipitate was dissolved in the phosphate buffer (0.025 M, pH 7.5) containing 5 mM $MgCl_2$, 2 mM EDTA and 10% glycerol and dialyzed against this buffer overnight (dialyzation membrane with cut-off 10 kDa, Sigma). Dialysate was concentrated using Ultrafree 15 centrifugal filter (cut-off 10 kDa, Millipore, USA) and loaded on the ionex column (Fast Flow Q, Amersham Biosciences, USA, column dimensions 1.6 cm × 30 cm). Active fraction was eluted with linear gradient of sodium chloride (0–0.6 M NaCl). Using this purification procedure 95% enrichment of bilirubin reducing activity could be obtained. To confirm whether BDG must be hydrolyzed prior its reduction, this pigment was incubated with supernatant containing crude protein extract after French press disintegration followed with centrifugation as described above. Furthermore, β-glucuronidase derived from *Escherichia coli* (β-glucuronidase Type IX-A, 17000 units, Sigma) was added to the final incubation of BDG and bilirubin reductase partially purified as described above.

Mass spectra were measured on a mass spectrometer ZAB-EQ (Micromass, Manchester, UK) using fast atom bombardment ionization with Xe as a bombarding gas at 8 kV. 3-Nitrobenzyl alcohol was the best matrix found. High resolution measurement of protonated molecules was provided at a resolving power 5000 using polyethylenglycol 600 as a standard. A MALDI-TOF mass spectrometer (REFLEX IV, Bruker Daltonics (Saxonia Analytik), Leipzig, Germany) was used for a comparison measurement.

3. Results

3.1. Reduction of bile pigments by C. perfringens

A broad range of bile pigments was reduced by the investigated strain of *C. perfringens* (Table 1). The highest rate was seen for MB, in which the vinyl groups at both end pyrrolic rings are already reduced to ethyl groups (Fig. 1). Slower rates were observed for UCB IXα and BDG, as well as BDM. The rates for BDT and bilirubin diethylester were much lower.

The strain of *C. perfringens* that reduced bilirubin was also able to reduce urobilin and stercobilin back to colorless urobilinogens as proved by TLC analysis of chromogens (data not shown). No reduction of bilirubin could be detected in the supernatant after incubation of broth with bacteria, whereas French press disintegration of the bacterial cells resulted in release of enzymatic activity, indicating that the bilirubin reductase is not secreted into the medium.

3.2. Analysis of bilirubin reduction products

As can be seen in Fig. 2, natural biliary bile pigments, comprised predominantly of BDG, were reduced by *C. perfringens* into several species of urobilinoids. The urobilinoids, separated as respective chromogens, rapidly turned yellow, with resultant loss of mobility in solvent system A. That this was due to spontaneous oxidation to more polar urobilin and/or stercobilin was

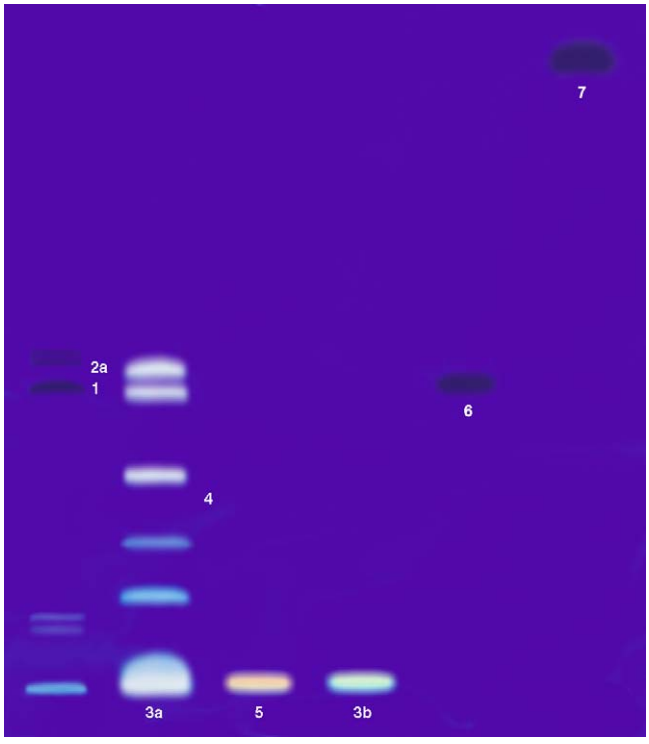

Fig. 2. TLC of reduction products formed from bile pigments in bile by anaerobic incubation with *C. perfringens* for 24 h. The biliary bile containing about 74% BDG and 26% bilirubin monoglucuroniodes was added to the broth at a final concentration of 50 μmol/l. Bile pigment substrates were methyl esterified according to ref. [20]. TLC system specification: HPTLC silica gel 60 plate (Merck, Germany), solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.). Track 1: Human biliary bile. Track 2: Human biliary bile incubated with a strain of *C. perfringens* reducing bilirubin. Tracks 3, 4, 5, 6: Standards. 1: BDM. 2a: Bilirubin C8 monomethylester. 2b: Bilirubin C12 monomethylester. 3a: Urobilin formed from bile pigments contained in human bile by *C. perfringens*. 3b: Urobilin (standard). 4: Advanced reduction products of bile pigments. 5: Stercobilin (standard). 6: BDM (standard). 7: UCB IXα (standard).

supported by the immobility of pure urobilin and stercobilin in this solvent system.

Since the peptide bond of BDT is presumably resistant to hydrolysis by the enzymes of intestinal microflora, reduction of this pigment might result in the formation of the corresponding urobilinoid tauroconjugate. Similarly, the avid reduction of BDG, the natural bilirubin conjugate, might result in formation of conjugated urobilinoids. However, the eluted reduction products of BDG were neither diazotized with ethyl anthranilate, nor released glucuronic acid on ammonolysis, indicating that they were not conjugates of urobilinoids with glucuronic acid. The addition of the β-glucuronidase inhibitor saccharonolactone to the bacterial culture did not lead to the production of conjugated urobilinoids and chromatography of the reduction products of BDG revealed identical urobilinoid profile to that of UCB reduction products, strongly suggesting that saccharolactone did not inhibit β-glucuronidase activity. When incubating BDG with *C. perfringens* no UCB was formed during 8-h incubation indicating that under conditions used spontaneous hydrolysis of BDG had not occurred. Interestingly, chromatography profile of BDM reduction products was almost identical to that of UCB suggesting that BDM must have been hydrolyzed prior to reduction, while BDT reduction products were not extracted into chloroform, an indirect evidence that more polar tauro-conjugates of urobilinoids had been formed (data not shown). On the other hand, when UCB, BDG and BDM were used as substrates for partially purified bilirubin reductase, only UCB was reduced (16% conversion rate in 8 h), while BDG and BDM could not be reduced at all. However, when BDG was in the same experiment co-incubated with β-glucuronidase hydrolyzing bond between bilirubin and the sugar moiety the substantial conversion of the substrate onto urobilinoids was achieved (8% conversion rate in 8 h). Similar conversion rate of BDG to that of UCB was detected when BDG was used as a substrate for crude

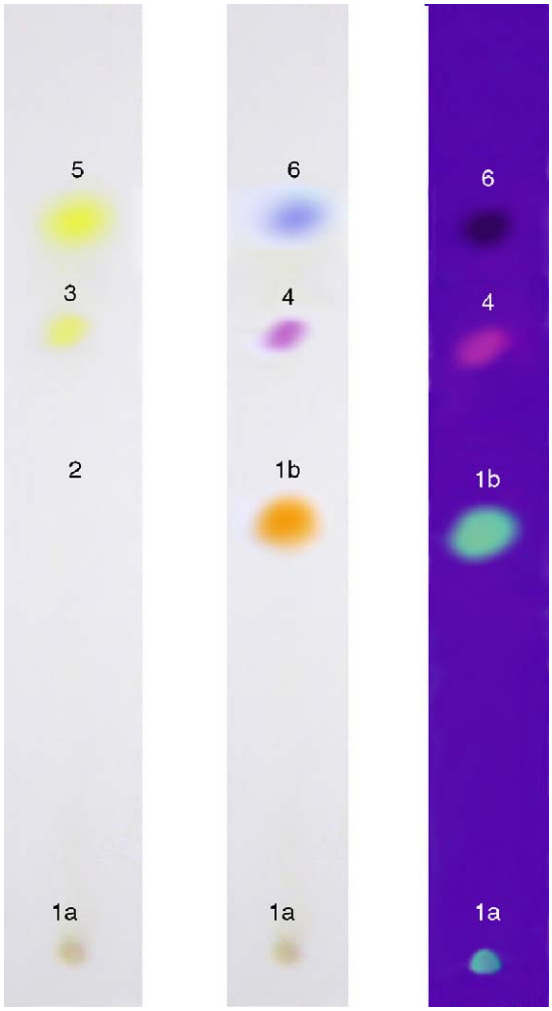

Fig. 3. TLC of reduction products formed from mesobilirubin (MB) by anaerobic incubation with *C. perfringens* in broth culture for 24 h. The initial bile pigment concentration in the broth was 50 μmol/l. TLC system specification: HPTLC silica gel 60 plate (Merck), solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.). Left track, reduction products. Center and right tracks, after oxidation of the middle methylene bridge under UV light. Center track, color observed under visible light. Right track, fluorescence observed under UV light. 1a: Urobilin IXα. 1b: Urobilin IXα oxidized from urobilinogen IXα after development in the first dimension. 2: Urobilinogen IXα, not visible. 3: Dihydromesobilirubin. 4: Labile mesobiliviolin. 5: MB IXα. 6: Glaukobilin IXα.

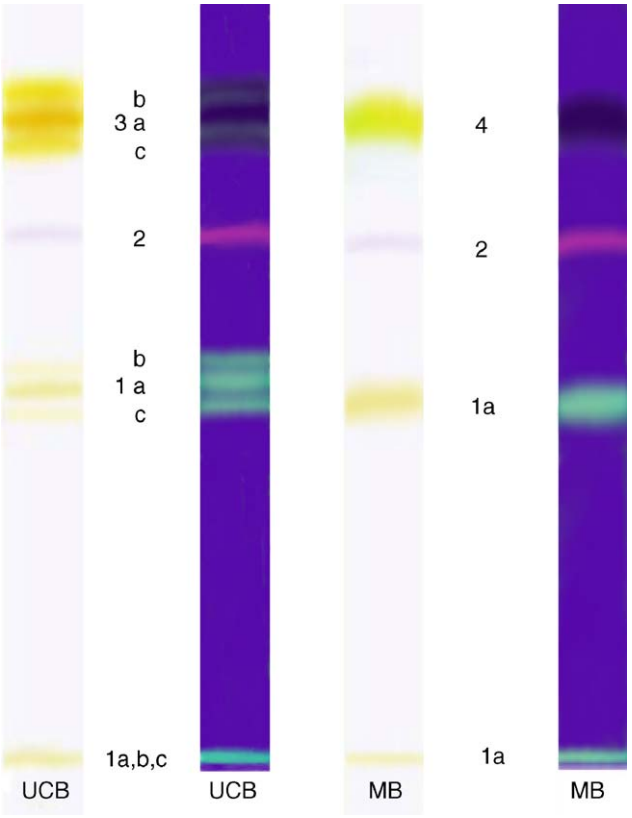

Fig. 4. TLC of reduction products formed from UCB (Tracks A and B) and mesobilirubin (Tracks C and D), by anaerobic incubation with *C. perfringens* in broth culture for 24 h. The central methylene bridge of the separated urobilinogens was oxidized under UV light after TLC development in the vertical dimension. Tracks 1 and 3, color under visible light. Tracks 2 and 4, fluorescence under UV light. The initial bile pigments concentration in the broth was 50 μmol/l. TLC system specification: HPTLC silica gel 60 plate (Merck), solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.). 1a: Urobilin IXα. 1b: Urobilin IIIα. 1c: Urobilin XIIIα. 2: Labile mesobiliviolin. 3a: UCB IXα. 3b: UCB IIIα. 3c: UCB XIIα. 4: MB IXα.

unpurified protein extract from *C. perfringens* (24% versus 22%, respectively).

Exposure of the multiple, individually separated, reduction products of MB to UV light, which oxidizes the central methene bridge, yielded distinctive products (Fig. 3), all of which were very unstable and degraded rapidly into other compounds. A colorless chromogen was converted to a yellow pigment with a greenish UV fluorescence, characteristic of urobilin, identifying the chromogen as urobilinogen. A light yellow pigment was converted to a purple compound with a pink/red UV fluorescence, characteristic of mesobiliviolin, identifying the yellow pigment as dihydromesobilirubin. A darker yellow pigment was converted into a blue compound without fluorescence, characteristic of glaucobilin, characterizing the yellow pigment as unmetabolized MB. As shown in Fig. 4, identical products were derived from incubation of UCB and MB with *C. perfringens*, but each product showed three bands. This experiment suggested that MB was an intermediate in the reduction of UCB, but that the substrate UCB had contained the IIIα and XIIIα as well as IXα isomers and yielded the corresponding isomers of the reduction products. The three yellow bands (1a, 1b and 1c in Fig. 4) exhibited identical absorption maxima at 495 nm, and emission fluorescent maxima at 501.5 nm, which shifted to 512 nm in the presence of Zn ions (data not shown). Molecular mass of three isolated urobilins was determined by mass spectrometry and revealed identical $[M+H]^+$ ions at *m/z* 591 for all three pigments confirming thus occurrence of different isomers of one compound. The measured accurate mass 613.3024 corresponds to the elemental composition of sodium salt of urobilin IXα molecule $C_{33}H_{42}N_4O_6Na$ (calculated exact mass 613.3002).

Ferric chloride oxidation of the reduction products yielded identical derivatives, i.e. only urobilin-originated mesobiliviolins were produced (data not shown). The possibility that these three urobilin bands represented keto/enol tautomers was excluded using a TLC system capable of separating these forms of isomers [23] (data not shown).

When the dominant IXα isomer was eluted from the TLC plate and reincubated with *C. perfringens*, only IXα isomers of the reduction products were detected (not shown). Combined with the result with incubation of MB IXα (Fig. 3), this indicated that scrambling of the dipyrrinone halves of the IXα isomers did not occur during incubation (Fig. 5). In no instance were reduction products detected corresponding to half-stercobilinogen/half-stercobilin or stercobilinogen/stercobilin.

In order to identify whether urobilinoids detected were produced from UCB [^{14}C]-labeled bilirubin was used as a substrate. The 2D TLC results clearly demonstrated presence of [^{14}C]-labeled bilirubin reduction products produced by *C. perfringens* (Fig. 6). The pigment on the start represents most likely urobilin, since stercobilin is not produced in substantial amounts (see above), whereas pigments within the path represent urobilinogen, MB and dihydromesobilirubin oxidized onto respective oxidation products (Fig. 6). Although it is hard to quantify urobilinoid production, which is certainly affected by cultivation conditions as well as substrate concentration, it is apparent that majority of the substrate was converted onto more or less reduced products (Fig. 6).

In a further study, the formation of urobilinoids from MB was found to be time-dependent. As shown Fig. 7, production of urobilin is preceded by formation of an intermediate, dihydromesobilirubin, which is gradually consumed as urobilin is produced. As in previous experiments no production of stercobilinogens/stercobilins could be detected.

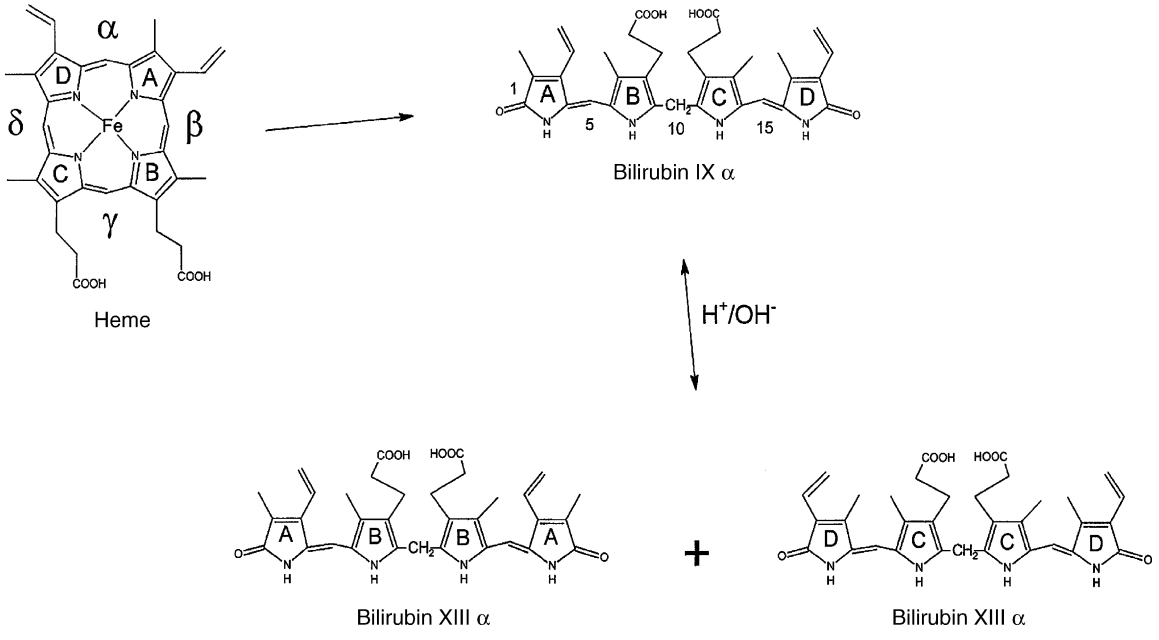

Fig. 5. Bilirubin isomers formed by molecular scrambling.

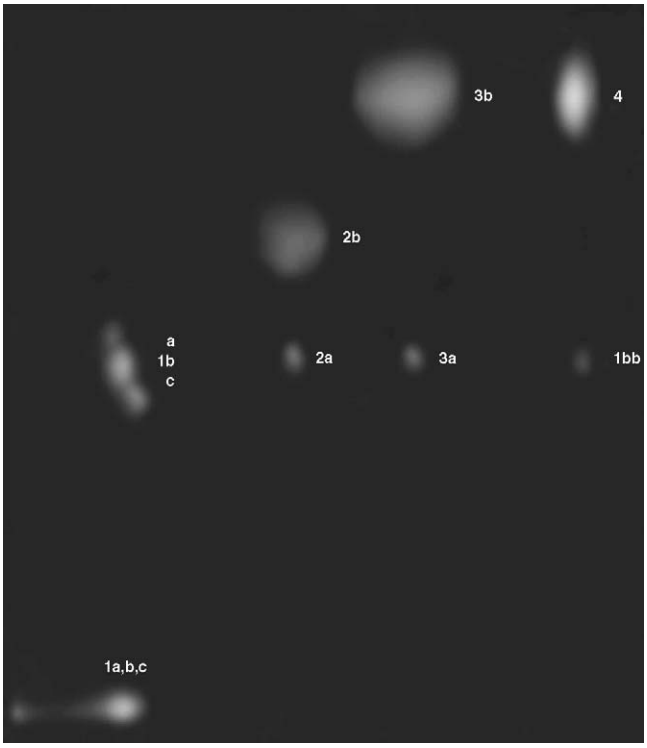

Fig. 6. 2D TLC of reduction products formed by anaerobic incubation of ^{14}C-UCB with *C. perfringens* in broth culture for 24 h. The central methylene bridge of the separated isomers was oxidized from urobilinogen isomers after TLC development in the vertical dimension. The initial bile pigment concentration in the broth was 50 μmol/l. TLC system specification: HPTLC silica gel 60 plate (Merck), 1st dimension: solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.). 2nd dimension: solvent system B: $CHCl_3$:MeOH:CH_3COOH (80:19:1, by vol.). Specific activity of the ^{14}C-UCB was 13000 dpm/nmol. 1a: Urobilin IIIα. 1b: Urobilin IXα. 1c: Urobilin XIIIα. 1bb: Urobilin IXα oxidized from urobilinogen IXα after development in the second dimension. 2a: Labile mesobiliviolin produced by advanced oxidation of urobilinogen IXα after development in the first dimension. 2b: Labile mesobiliviolin. 3a: Glaukobilin produced by advanced oxidation of urobilinogen IXα after development in the first dimension. 3b: Glaukobilin produced by advanced oxidation of MB after development in the first dimension. 4: UCB mixture of all three isomers.

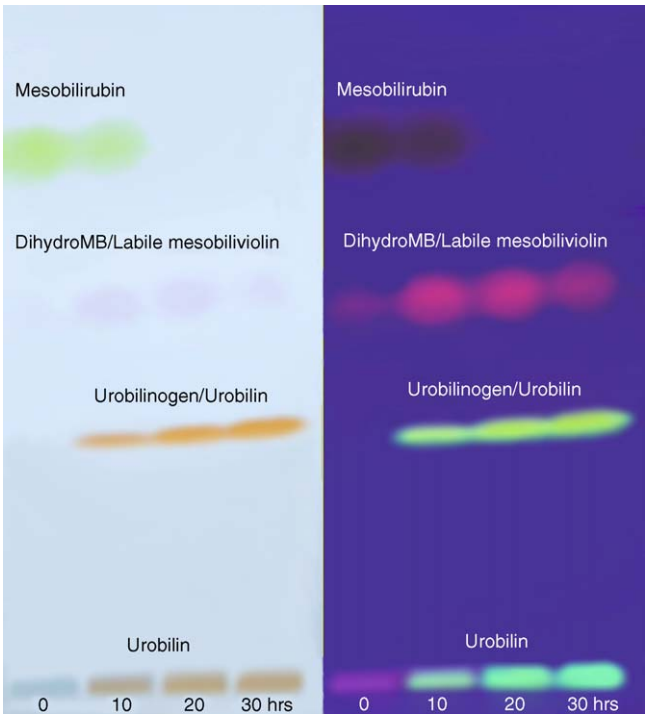

Fig. 7. Time dependency of formation of MB reduction products. The initial bile pigments concentration in the broth was 50 μmol/l. TLC system specification: HPTLC silica gel 60 plate (Merck), solvent system A: $CHCl_3$:CH_3COOH (99:1, by vol.).

4. Discussion

In this study, we focused on the process of bilirubin reduction catalyzed by a single intestinal strain of *C. perfringens*. Surprisingly, our clostridial strain could reduce a wide variety of bile pigments, that differ substantially in their polarity and secondary structure, including: UCB, MB, bilirubin glucuronides, amides, alkyl esters, and taurates, and even urobilin and stercobilin. Since bacteria are not altruistic, it seems that this broad substrate specificity of bilirubin reducing enzyme(s) could be an effective tool for disposal of electrons produced by fermentolytic processes within these anaerobic bacteria. This has been described also for substrates other than bilirubin, which are present physiologically in the gut lumen, such as bile salts [28] and their sulfate esters [29].

As we confirmed, bilirubin reductase is not secreted by this bacterium, so reduction of bilirubin must occur intracellularly. Although the weakly polar UCB, MB and its alkyl esters should readily diffuse into the bacteria, the polar amides, glucuronide and especially taurine conjugates would not [30], suggesting that there must be carrier-mediated bacterial uptake of the bile pigments, as there are for bile salts [28]. Indeed, despite its ready diffusibility, carrier-mediated uptake of UCB is present not only in mammalian cells [31], but also in simpler organisms such as *Caenorhabditis elegans* [32]. Even more interestingly, it was demonstrated that the beta-subunit of the inner mitochondrial membrane protein of the rat liver, F_1-ATPase, is identical to an organic anion binding protein, a putative bilirubin transporter at the sinusoidal hepatocyte membrane [33]. However, this β-subunit of F_1-ATPase is prevalent in many prokaryotes [34] suggesting possible role in active transport of bile pigments of this or related ABC proteins [35].

It is highly likely that hydrolysis bilirubin diglucuronide by β-glucuronidase of either bacterial [36] or human origin [37] precedes bilirubin reduction, compatible with the finding that natural unconjugated bile pigments, UCB and MB, are more efficiently reduced than conjugated pigments, and *Clostridia* secrete β-glucuronidase [36]. Thus, our studies did not detect conjugated urobilinoids as products of the microbial reduction of bilirubin conjugates. The possibility that urobilinoid conjugates might have been formed but then hydrolyzed by bacterial β-glucuronidase was excluded since BDG could not be reduced with partially purified bilirubin reductase, whereas unpurified protein lysate avidly reduced BDG to urobilinoids presumably due to presence of clostridial β-glucuronidase abundant

in this bacterial strain [36]. On the other hand, addition of bacterial β-glucuronidase to the partially purified bilirubin reductase restored its capacity to reduce this bile pigment to urobilinogen providing strong evidence that BDG must be deconjugated prior its reduction. Conflicting results by Watson and colleagues [15], who postulated some decades ago that conjugated bilirubins are preferentially reduced to urobilinoids, were most probably related to the very low water-solubility of UCB, limiting reduction of UCB in their aqueous systems. It is interesting to note that an addition of β-glucuronidase inhibitor saccharonolactone did not influence production of urobilinoids indicating that saccharolactone was either not internalized by *C. perfringens* or more likely consumed by rapidly growing bacteria. Another important finding is that bilirubin alkyl esters (BDM) were hydrolyzed prior reduction suggesting that other hydrolases must have been present to first deconjugate these bilirubin esters. This is not surprising since esterases are common in clostridia [38]. On the other hand, indirect evidence suggests that reduction of BDT might result in formation of more polar taurin conjugated urobilinoids.

The strain of *C. perfringens* used in our study was found to reduce UCB only to the level of urobilinogen. The three urobilin bands found upon reduction of commercial UCB were proven, by UV–vis and fluorescence spectroscopy, ferric chloride oxidation (mesobiliviolin reaction), mass spectrometry, and elemental composition to be the oxidation products of different isoforms of urobilinogens III, IX and XIII, formed from the respective isomers of UCB. When an isolated UCB IXα isomer or pure MB IXα were used as substrates, only one urobilinogen species was produced. Similarly, TLC of the reduction products of [^{14}C]-UCB (Fig. 6) and MB (Fig. 3), demonstrated the production of three urobilin species, whereas in case of MB only one urobilin could be detected. Thus, under in vitro conditions used molecular scrambling (i.e. dipyrrolic exchange) (Fig. 5) did not occur and the initial configuration was maintained. The commercial MB was, therefore, apparently much purer than the UCB supplied by a different vendor. This is in accord with results by McDonagh and Assisi, who observed marked differences in the content of these isomers in bile pigments supplied by various vendors [39]. The possibility of keto/enol tautomers to account for the trios of detected pigments is unlikely since only keto isoforms are physiologically important [40]. This was confirmed by our failure to detect keto/enol isoforms on chromatographic separation. Unfortunately, due to low amounts of material further analyses such as NMR measurement, or analyses of other isolated pigments were not possible.

It is interesting that bile pigments are converted only to the level of urobilinogens and reduction does not proceed to stercobilinogens. This is in accord with previous suggestions indicating that reduction to levorotary stercobilinogens may require an additional specific isomerase [12]. It should also be stressed that, although our clostridial strain was isolated from an urobilinoid-positive neonatal stool, these cases are rare since colonization of neonatal gastrointestinal tract with bacteria reducing bilirubin is very slow and accounts for the low levels of urobilinoids in neonatal feces [1].

Major findings of the present study may be summarized as follows:

(1) Novel methods for detection and isolation of a wide range of bile pigment reduction products have been developed, including those separating colorless chromogens.
(2) Intestinal anaerobes may reduce a variety of different bile pigments, suggesting metabolic importance of these enzymatic processes for homeostasis of intestinal microflora.
(3) A *C. perfringens* isolated from neonatal stools reduced preferentially UCB, but only to the level of urobilinogen. Other bacterial strains and species, absent in neonates, are presumed to be essential for catabolism to the level of stercobilinogen.
(4) The study proved definitely that hydrolysis of bilirubin conjugates precedes reduction to urobilinoids.

Further studies are needed to fully elucidate details of bilirubin catabolism in the intestinal lumen, in particular by indigenous intestinal microflora. Our study was carried out with only one microbial strain, which might oversimplify the situation within the intestinal lumen. Intestinal catabolism of bilirubin is likely of clinical importance, as evidenced by our recent study on the effect of alterations in intestinal microflora on serum bilirubin levels in rats [41], an approach that might be applied therapeutically to jaundiced infants.

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