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REVIEW

CHROMATOGRAPHIC ANALYSIS AND STRUCTURE DETERMINATION OF BILIVERDINS AND BILIRUBINS

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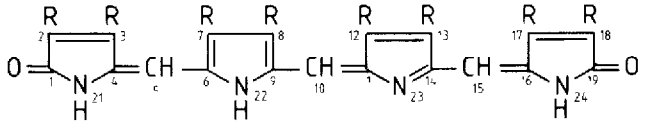
1. INTRODUCTION

In the late sixties, Van Roy and Heirwegh [1] developed the ethyl anthranilate diazo cleavage procedure for analysis of bilirubins [2]. Application of this reagent to biological samples proved to be of value in confirming [3–5] the disputed structures [6] of the glucuronic acid ester conjugates of bilirubin-IXα. In addition, the novel procedure led to the discovery of glucose and xylose ester conjugates of bilirubin-IXα [7–9]. Thin-layer chromatographic (TLC) procedures for separation of ethyl anthranilate azo derivatives were refined in the early 1970s and led to the demonstration of a large number of ester glycosides of bilirubin-IXα in native dog bile [10]. Such heterogeneity of the natural bilirubins is even more pronounced under pathological conditions. In cholestasis, formation of acyl-shifted conjugates [11–13] and of bilirubin–protein conjugates occurs [14–17]. Under phototherapy, geometric isomers of bilirubin-IXα are formed [18–20]. Considerable effort has therefore been devoted to develop TLC and high-performance liquid chromatographic (HPLC) procedures for separation of the full range of natural bilirubins. Recent advances in HPLC have been of key importance in tackling the separation problems involved because, apart from the increased sensitivity and speed of separation, HPLC permits easy exclusion of oxygen and light, which both are instrumental in modification and destruction of bilirubins [18,19,21–24].

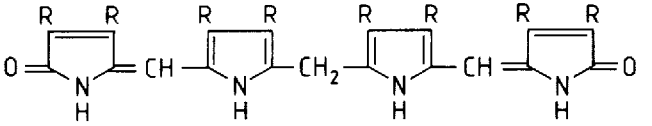
The purpose of the present review is to evaluate recent developments in chromatographic analysis of bilirubins, of azo pigments derived from bilirubins and of biliverdins. Because the composition of bile pigments present in biological specimens may be very complex, chromatographic bands or peaks often cannot be identified without verification of band or peak homogeneity. Hence, micro techniques for structure elucidation are also discussed. Earlier reviews [2,18,22–25] should be consulted for more detailed information and references relating to techniques published before 1972.

2. NOMENCLATURE

The collective term 'bile pigments' is used to refer to biliverdins and bilirubins, which are linear open-chain tetrapyrroles with the basic tetrapyrrole skeletons shown in Fig. 1. Natural bile pigments contain a characteristic set of β-substituents (Table I) and are formed from heme by α-cleavage of the protoporphyrin-IX ring structure. The numbering system for the atoms of the tetrapyrrole skeleton of bile pigments is the same as the one applied to the parent protoporhyrin-IX ring. The phantom atom 20 represents the carbon atom lost during opening of the ring structure [18]. In agreement with a proposal of the IUPAC–IUB Commission on Biochemical Nomenclature [26], the trivial names biliverdin-IXα and bilirubin-IXα are used to indicate the two principal natural bile pigments. For other, quantitatively less important nat-



biliverdin



bilirubin

Fig. 1. Biliverdin and bilirubin tetrapyrrole skeletons. The structures of the β-substituents indicated by R are given in Table 1.

TABLE 1

NATURAL BILIRUBINS AND BILIVERDINS

For the corresponding biliverdins replace the core name bilirubin by biliverdin

Trivial name	Semi-systematic name	β-Substituent (R) and location							
		2	3	7	8	12	13	17	18
Bilirubin-IXα	3,18-Vn_2-bilirubin-8,12	Me	Vn	Me	Cet	Cet	Me	Me	Vn
Bilirubin-IXβ	13,18-Vn_2-bilirubin-3,7	Me	Cet	Cet	Me	Me	Vn	Me	Vn
Bilirubin-IXγ	8,13-Vn_2-bilirubin-2,18	Cet	Me	Me	Vn	Me	Vn	Me	Cet
Bilirubin-IXδ	12,17-Vn_2-bilirubin-3,7	Me	Cet	Cet	Me	Vn	Me	Vn	Me

ural bile pigments, a semi-systematic nomenclature is adopted which is based on retaining the trivial core names 'biliverdin' and 'bilirubin' in conjunction with designations referring to the locations of the vinyl (*Vn*) and propionic acid β-substituents [19,27] (Table 1). For example, biliverdin-IXβ is named 13,18-Vn_2-biliverdin-3,7. The numbers 13 and 18 indicate the positions of the vinyl groups, the subscript the number of such groups and the numbers 3 and 7 the positions of the propionic acid side-chains. The system also permits easy naming of the ester conjugates of bile pigments (Fig. 2). The nature of the conjugating residue(s) is indicated between brackets at the end of the name. The position number of the propionic acid side-chain is italicized if it is involved in linkage of the conjugating group. For example, a diester of bilirubin-IXγ with glucose attached at C-2 and glucuronic acid at C-18 is denoted as 8,13-Vn_2-bilirubin-*2,18*(Glc;GlcU). The C-8 monoglucuronide of bilirubin-IXα is termed bilirubin-*8*,12(GlcU). A monoglucuronide of bilirubin-IXα in which the position of esterification is unknown is indicated as bilirubin-8,12(GlcU). The same nomenclature system can conveniently be applied to other deriva-

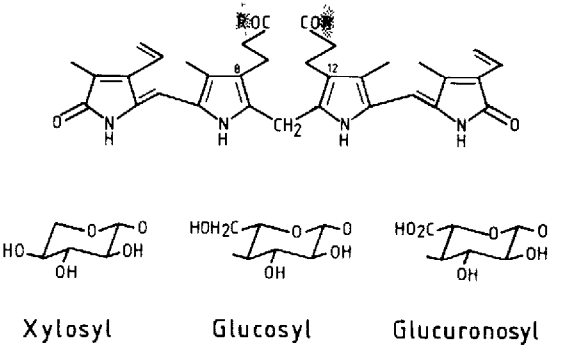
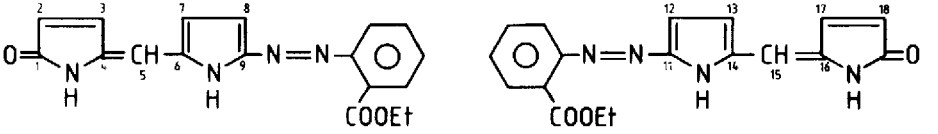


Fig 2. Structures of bilirubin-IXα ester conjugates. Mono-β-D-glycosidic conjugates: one of the two R groups is OH, the other one being any of the sugar moieties shown, di-β-D-glycosidic conjugates: each R group is one of the sugar moieties shown. (Reproduced by permission from ref 146.)

TABLE 2

AZOPYRROMETHENES DERIVED FROM REACTION OF FOUR BILIRUBIN ISOMERS WITH DIAZOTIZED ETHYL ANTHRANILATE



Parent pigment	Semi-systemic name	β-Substituent (R) and location							
		2	3	7	8	12	13	17	18
IXα	Azpm-8	Me	Vn	Me	Cet				
	Azpm-12					Cet	Me	Me	Vn
IXβ	Azpm-3,7[a]	Me	Cet	Cet	Me				
	13,18-Vn_2-Azpm					Me	Vn	Me	Vn
IXγ	Azpm-2	Cet	Me	Me	Vn				
	Azpm-18					Me	Vn	Me	Cet
IXδ	Azpm-3,7	Me	Cet	Cet	Me				
	12,17-Vn_2-Azpm					Vn	Me	Vn	Me

[a]Can be obtained from either the IXβ or the IXδ isomer.

tives of the propionic acid groups, such as amides or methyl esters, and to synthetic bile pigments for which the nature and sequence of β-substituents differ from those typical of natural bile pigments.

The nomenclature of dipyrrolic azo derivatives obtained by diazo cleavage of the tetrapyrrolic bilirubins is based on use of the abbreviation 'Azpm' (for azopyrromethene) and on retaining the numbering of the parent tetrapyrroles (Table 2). In the course of the diazo cleavage reaction, carbon atom 10 is ex-

pelled. Hence, this carbon atom is absent in the Azpm compounds. A more detailed discussion of this nomenclature system [19] with an explanation of the possible geometric and conformational isomers of the tetrapyrroles can be found in recent reviews [18–20].

Following a proposal of Blanckaert et al. [28] the term 'bilirubin–protein conjugate' will be used to denote the serum bilirubin fraction tentatively identified as bilirubins covalently linked to serum albumin. Two types of natural bilirubin–protein conjugate exist. An esterified bilirubin–protein conjugate corresponds to pigment that is covalently bonded to protein (probably at a propionic acid side-chain) and that is also esterified, at the other propionic acid side-chain, with a sugar moiety. An unesterified bilirubin–protein conjugate consists of bilirubin with one free propionic acid. The term 'esterified bilirubins' denotes bilirubins in which one or both propionic acid side-chains are esterified and covalent linkage to protein is absent. The term conjugated bilirubins comprises monoesterified and diesterified bilirubins, as well as the two types of bilirubin–protein conjugate described above. The older term [29], δ-bilirubin, which is currently being used for denoting bilirubin–protein conjugates, is non-systematic, is likely to create confusion with bilirubin-IXδ (Table 1) and will not be employed.

3. NATURAL OCCURRENCE OF BILIVERDINS AND BILIRUBINS

Natural bile pigments contain predominantly the IXα skeleton [18]. In humans and animals, they are derived largely from senescent red blood cells. Metabolic breakdown of hemoglobin heme results in removal of the α-meso bridge carbon atom and formation of CO and an open-chain tetrapyrrole [18,30–32]. This process generates biliverdin-IXα, which in mammals is further reduced to bilirubin-IXα [18,30,32]. The occurrence and metabolism of bile pigments in non-mammalian species has been reviewed [33].

Before excretion in bile, bilirubin-IXα is rendered more water-soluble, predominantly by esterification of either one or both carboxyl groups with the C-1′ atom of β-D-glucopyranuronic acid, β-D-glucopyranose or β-D-xylopyranose [5] (Fig. 2). The sugar esters of bilirubin-IXα comprise C-8 and C-12 monoconjugates of the three sugars, and diconjugates of either two identical or two different sugars [5,10,34–40]. The composition of bilirubin-IXα conjugates is greatly species-dependent [33]. For example, in humans, rats and guinea pigs, bilirubin-IXα is esterified predominantly with glucuronic acid, whereas in dogs and cats conjugates with glucose and xylose are important [34–40]. The ratio of the C-8 and C-12 monoester conjugates also depends on the species [41]. The compositional complexity of bilirubin conjugates is further enhanced during acquired or experimental bilirubinostasis or cholestasis (e.g. in partial or total obstruction of the common bile duct), by non-enzymic acyl-shifting of the bilirubin aglycone to form the 2′-O-acyl, 3′-O-acyl and 4′-O-acyl posi-

tional isomers [11–13] (Fig. 3) and by formation of covalently linked bilirubin–protein conjugates [14–17]. It is a long-standing observation that part of the bilirubin pigment in blood is firmly associated with protein [29,42–45]. The conjugates are formed from monoesters and, if present, from diesters of bilirubin-IXα [17,46–48] and a serum protein, probably serum albumin [15,16]. McDonagh et al. [17] have suggested that the covalent binding of bilirubin to albumin occurs via intermolecular migration of a bilirubin acyl group from the oxygen atom of a sugar ester residue to a free NH_2 group on the protein. If this mechanism is correct, the chromophore linkage is a propionamide. Depending on pathology, bilirubin–protein conjugates may constitute a major fraction of total serum bilirubin [28,46,49]. In the recovery phase, the relative importance of the bilirubin–protein conjugate fraction increases temporarily [16,17,46,50,51] because bilirubin–protein conjugates are cleared from the blood with about the half-life of serum albumin, whereas the other forms of bilirubin disappear from the blood much more rapidly. Covalently linked biliverdin–protein complexes have been detected in the blood of some fishes [52].

Bilirubin-IXα ditaurate, which is available in synthetic form [53], is used as a secondary standard in assays of conjugated bilirubin-IXα [54]. This is present in bile from some marine teleosts [55]. Studies with dipyrrolic azo derivatives have provided evidence for the existence of sulphate conjugate(s) of bilirubin-IXα, but this is unconvincing [5] and requires confirmation by analysis of the parent tetrapyrrole(s). Future studies on such types of hydrophilic compound should not be done solely on an organic extract but, at least, also involve the remaining aqueous phase.

Biliverdin-IXγ has been detected in Lepidoptera [56] and in the water beetle *Laccophilus minutus* [57]. Biliverdin-IXβ is excreted in the urine of rabbits following administration of phenylhydrazine or hemoglobin [58]. Bilirubin-

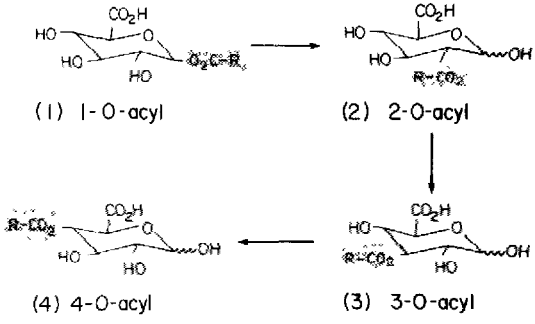


Fig. 3. Bilirubin-IXα and azopyrromethene 1-, 2-, 3- and 4-O-acylglucuronides produced by sequential non-enzymic migration of the 1-O-acyl group to position 2, 3 or 4 of glucuronic acid (R = bilirubin-IXα or azopyrromethene) Similar migrations are observed with glucose and xylose ester conjugates of the aglycones. (Reproduced by permission from ref. 147.)

IXβ occurs in small amounts in body fluids of adult mammals [10,35,38,59] and may be quantitatively more important in neonates and fetuses [36,37]. Indirect evidence based on the isolation of the dipyrrolic azopigment Azpm-3,7 following diazo treatment of biological fluids suggests that bilirubin-IXβ and/or bilirubin-IXδ occur widely distributed in the animal kingdom and comprise an important fraction of the natural bile pigments in some species [33]. All dipyrrolic azo pigments corresponding to the four isomers of bilirubin-IX (Table 2) have been obtained from bile of the R/APfd-j/j rat [60], a mutant with a complete defect of esterification of bilirubin-IXα [61,62] similar to the Gunn vat.

4. LABILITY OF BILIVERDINS AND BILIRUBINS

Biliverdins and bilirubins are labile and particularly sensitive to light and oxidizing agents, such as molecular oxygen. Chemical reactions with bile pigments should be carried out in the dark or in subdued light under anaerobic conditions [18,19,21]. Information on the stability of these bile pigments refers mainly to studies with the unconjugated pigments. Observations relevant to work with conjugated bilirubins [22–24] are summarized in Table 3.

Apart from oxidative and light-induced decomposition, which affects all bile pigments, bilirubins are prone to left-half–right-half disproportionation (intermolecular dipyrrole exchange) [18,40,63–68]. For example, disproportionation of bilirubin-IXα produces a mixture of the IIIα, IXα and XIIIα isomers (Fig. 4). The reaction is catalysed by protic acids in organic solutions and by free radicals, such as molecular oxygen, in aqueous solutions [18,65]. Since high pigment concentration promotes disproportionation, and complete deoxygenation of liquids is virtually impossible, disproportionation of non-α isomers of bilirubin-IX and of ester conjugates of bilirubin-IXα [40,67,68], which

TABLE 3

CHEMICAL CHANGES OF BILIRUBIN AND OF SUGAR ESTERS OF BILIRUBIN

	Chemical changes
Tetrapyrrole moiety	Decomposition (i.e. autoxidation)
	Disproportionation resulting e.g. in formation of IIIα and XIIIα isomers from bilirubin-IXα
	Formation of geometric isomers (e.g. 4*Z*,15*E*)
Ester linkage	Hydrolysis in slightly alkaline medium
	Transesterification (alcoholysis) in alkaline medium
	Positional isomerization (acyl-shifting) in neutral to slightly alkaline medium
	Formation of bilirubin–protein conjugates with serum albumin at pH 7–9

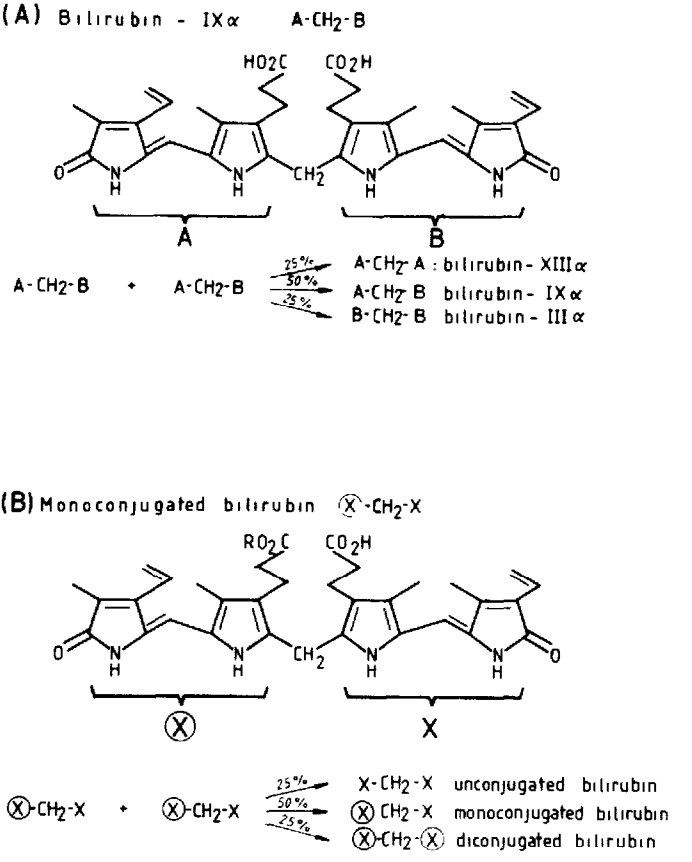


Fig. 4. Disproportionation of unconjugated and monoconjugated bilirubins. As shown in (A) for unconjugated bilirubin-IXα, disproportionation of bilirubin of the IXα-type produces IIIα and XIIIα isomers. With monoconjugated bilirubins (B), this reaction results in the formation of unconjugated and diconjugated bilirubins (Reproduced by permission from ref. 146.)

are rather water-soluble, is likely to occur. Thus the possibility of interfering dipyrrole exchange should always be considered when interpreting results on such pigments. Disproportionation of bilirubin-IXα monoglucuronide not only yields bilirubin-IXα and its mono- and diglucuronide but also leads to the formation of mixtures of the IIIα, IXα and XIIIα isomers [67,68] for each pigment (Fig. 4). In aqueous medium, under aerobic conditions, the reaction occurs most rapidly at pH 6.5 [67,69] and is effectively inhibited by low concentrations of ascorbate [67,70].

The relatively high chemical reactivity of the linkage in ester conjugates can give rise to a number of artifacts. In aqueous solution in the absence of protein, reactions of the ester linkage to be considered are base-catalysed positional isomerization of the sugar residue(s) (Fig. 3) and hydrolysis of the ester bond.

In neutral to slightly alkaline medium, acyl shifting occurs sequentially from the 1′ position of the sugar residue to the 2′, 3′ and 4′ positions [11,13] (Fig. 3). After incubation of 3 h at 37°C, the 1′-O-acyl isomer of azopyrromethene glucuronide was unchanged at pH 6 but had completely disappeared at pH 9 [13]. Whereas the ester bond is stable at pH 2.7 [70], non-enzymic hydrolysis is already noticeable at pH 7.1 [17,71] and is rapid at pH 11–11.5 [25,72,73]. In alkaline medium containing ammonia, both the carboxylate and the amide of the aglycone are formed [2,74]. Finally, base-catalysed alcoholysis of ester conjugates can occur, in particular in solutions containing methanol [25,75]. Several of these reactions, which are characteristic of sugar–carboxylic ester conjugates, have been observed for ester glucuronides of drugs [76–78].

Apart from hydrolysis and positional isomerization of the sugar residue(s), incubation of bilirubin-IXα mono- or diglucuronide with serum albumin at pH 7–9 leads to the formation of covalent protein–bile pigment conjugates with release of a glucuronic acid residue [17]. Depending on whether the reacting pigment is the mono- or diglucuronide, the complex contains bilirubin-IXα or its monoglucuronide. It is likely that, in both cases, isomeric mixtures of the complex are formed with the bilirubin acyl group bound in amide linkage at either C-8 or C-12 to an ϵ-amino group [17]. Enzymatic hydrolysis of β-D-1′-O-acyl conjugates, which may occur in serum, is inhibited by addition of structurally related aldonolactones [79,80].

Several specific measures can be taken to prevent artifactual changes during isolation of conjugated bilirubins from bile. Disproportionation and formation of acyl-shifted isomers of the various ester conjugates are avoided by collecting freely flowing duct bile, in the dark at 0°C, into a solution buffered at ca. pH 6 [13] containing 1–5 m*M* ascorbic acid [67,70]. Depending on the type of anesthetic used for surgical preparation of the animal, the composition of bile collected under anesthesia generally differs from that of native bile [40,81,82]. For determination of the natural composition of bile, bile collection should be started when recovery from anesthesia is complete.

5. SEPARATION AND ANALYSIS OF THE FREE ACIDS AND THE DIMETHYL ESTERS OF BILIVERDINS AND BILIRUBINS

5.1. Biliverdins

The dimethyl esters of the biliverdin-IX isomers are usually isolated by sequential TLC with the solvent mixture of Bonnett and McDonagh [83] for separation of the β and δ isomers, and with the acidic mixture of O'Carra and Colleran [59] for the α and γ isomers. Alternatively, the latter isomers can be

separated on acid-pretreated neutral silica [84]. The biliverdins are stabilized if, immediately after separation, the plates are sprayed with a methanolic solution of ascorbic acid [84]. This permits densitometric analysis of the separated pigments.

A variety of HPLC procedures permits analysis of the dimethyl esters of biliverdin-IX. For preparative HPLC, gradient elution on silica can be used [85]. On an analytical scale, the four isomers can be separated isocratically on either a silica column [85,86] or a reversed-phase column [58,85,87]. Separation by gradient elution on a silica column has also been reported [88]. Braslavsky et al. [89] have warned against possible formation of strong solvent adducts giving rise to artifactual migration of pure biliverdin dimethyl esters as two chromatographic bands on silica. The isocratic elution procedure of Rasmussen et al. [86] has been validated analytically, and yields baseline separation of the four biliverdin-IX dimethyl esters within ca. 16 min. An additional potentially useful feature is detection of biliverdin-XIIIα, since it permits detection of disproportionation in preparations derived from bilirubin-IXα.

Before analysis, the free acids of biliverdin usually are first converted into their methyl esters. The commonly used methylation procedures (5% H_2SO_4–CH_3OH or BF_3–CH_3OH) often yield coloured side-products. This complication and the ensuing losses can be avoided by application of the isocratic HPLC method of Cole and Little [90] for separation of the free acids of biliverdin-IX. The procedure of Fevery et al. [38] is probably suitable for extraction of biliverdins from biological media.

Beer's law is obeyed for the dimethyl esters and free acids of biliverdin-IX in several solvent systems [84]. The molar absorption coefficients for two absorption maxima in the visible area are available for the free acids and dimethyl esters in methanol or acidified methanol [84,91], and also for the dimethyl esters in chloroform [86,92] (Table 4). The values for the free acids and dimethyl esters are identical in methanolic solvents [84]. The individual biliverdin-IX dimethyl esters have identical molar absorption coefficients in both methanol and chloroform. Upon addition of HCl to a methanolic solution of a biliverdin the typically broad band at 650 nm becomes sharp and asymmetrical, with λ_{max} between 691 and 698 nm, depending on the isomeric type [84]. Extreme sensitivity of the absorption spectra to trace amounts of acid may have led to anomalies in published spectra [93]. The use of acidified methanol as a solvent increases the sensitivity of assay of biliverdins at the long-wavelength absorption maximum about two-fold. In addition to increased sensitivity, such a procedure also renders the assay less vulnerable to inadvertent contamination by traces of acid or heavy metal ions [84].

Reference dimethyl esters of biliverdin-IX can be obtained from hemin [83], the free acid isomers being prepared by subsequent alkaline hydrolysis [84] (Fig. 5). The total synthesis of biliverdins differing from the natural pigments

TABLE 4

SPECTROSCOPIC DATA FOR THE ISOMERS OF BILIVERDIN-IX AT THE WAVELENGTH MAXIMA 1 AND 2

Solvent	Isomer	λ_1 (nm)	ϵ_1 ($mM^{-1}cm^{-1}$)	λ_2 (nm)	ϵ_2 ($mM^{-1}cm^{-1}$)	Remarks[a]	Ref.
CH_3OH	IXα	375	51	664	14.7	A	84
		376	50.8	-	14.4	B	91
	IXβ	376	52	663	15.2	A	84
	IXγ	369	55	647	16.3	A	84
	IXδ	372	47	655	15.5	A	84
$CHCl_3$	IXα	380	52.0	-	15.6	C	86
				-	14.3	C	92
	IXβ	382	52.4	-	15.4	C	148
				-	15 4	C	86
	IXγ	376	52.8	-	16.8	C	148
				-	17 7	C	86
	IXδ	378	45.2	-	15.4	C	148
				-	15.6	C	86
CH_3OH/HCl	IXα	374	67	698	31	A, D	84
		377	66.2	696	30.8	B, E	91
	IXβ	374	55	698	30	A, D	84
	IXγ	358	48	695	29	A, D	84
	IXδ	365	49	691	33.5	A, D	84

[a]A = Pooled data for the free acid and dimethyl ester; B = biliverdin free acid; C = bilirubin dimethyl ester; D = CH_3OH–HCl (40:1, v/v); E = 5% (w/v) HCl in CH_3OH.

with respect to the sequence and nature of the β-substituents has been reported [94–96].

5.2. *Bilirubins*

5.2.1. *Bilirubin-IIIα, -IXα and -XIIIα*

The IIIα and XIIIα isomers have not been observed to occur naturally, but often appear as contaminants in bilirubin-IXα obtained commercially or prepared in the laboratory [97]. The isomers can be separated analytically by TLC on polyamide or silica [18], by isocratic [98,99] or gradient [100] normal-phase HPLC and by reversed-phase procedures [101]. Pronounced lability of the IIIα isomer probably leads to selective underestimation. Recovery of each individual isomer in these methods has not yet been evaluated. In some procedures for HPLC of the photoproducts of bilirubin-IXα [102], separation of the IIIα, IXα and XIIIα isomers of bilirubin is achieved but necessitates excessively long elution times.

TABLE 5

CHARACTERISTICS OF THE VISIBLE ABSORPTION SPECTRA OF SOME BILIRUBINS

Pigment	Solvent	λ_{max} (nm)	ϵ (at λ_{max}) ($mM^{-1}\,cm^{-1}$)	Ref.
Bilirubin IXα	$CHCl_3$	453–455	62.6	97
	$CHCl_3$–CH_3OH(1 1)	450	61.5	75
Bilirubin IIIα	$CHCl_3$	455–458	65.2	97
Bilirubin XIIIα	$CHCl_3$	449–453	52.2	97
Mesobilirubin IXα	$CHCl_3$	434	57.8	6
Bilirubin-*8*,12(Me)	$CHCl_3$	438	59.3	75
	$CHCl_3$–CH_3OH(1.1)	450	58.4	75
Bilirubin-8,*12*(Me)	$CHCl_3$	442	55.2	75
	$CHCl_3$–CH_3OH(1 1)	450	57.0	75
Bilirubin-*8*,12(Me;Me)	$CHCl_3$	397	60.5 (14 μmol/l)[a]	75
			71.7 (104 μmol/l)[a]	75
	$CHCl_3$–CH_3OH(1:1)	448	60.8	75

[a]Bilirubin dimethylester does not obey Beer's law in this solvent.

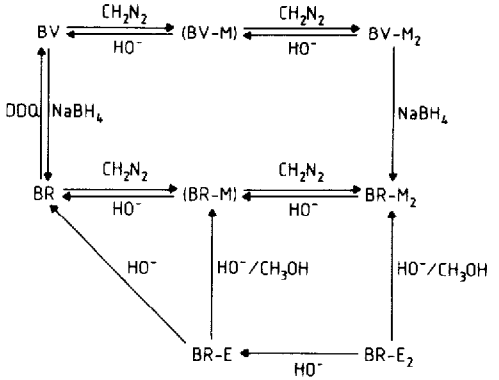


Fig. 5. Interconversion of unconjugated and conjugated biliverdins and bilirubins. The naturally occurring bilirubin-IXα mono- and disugar esters are indicated by BR-E and $BR\text{-}E_2$, respectively. The analytically important mono- and dimethyl esters of biliverdin (BV) and bilirubin (BR) isomers are denoted by BV-M and $BV\text{-}M_2$, and BR-M and $BR\text{-}M_2$, respectively. In the presence of ascorbate and Na_2EDTA as protective agents, alkaline hydrolysis [25,75,84] and, in particular, methanolysis in alkaline medium [75] provide useful near-quantitative routes for deconjugation and transmethylation, respectively. Semi-quantitative reduction of biliverdins to bilirubins is possible by treatment with $NaBH_4$ [75,84].

The IIIα and XIIIα isomers of bilirubin can be prepared by brief treatment of bilirubin-IXα with strong acid [18,64] and separated by preparative TLC [97] or isocratic HPLC on silica [98,99]. The molar absorption coefficients in chloroform of the free acids have been reported [18,97] (Table 5).

5.2.2. Bilirubin-IXβ, -IXγ and -IXδ

The presence of non-α isomers of bilirubin-IX can conveniently be tested for by formation and TLC analysis of ethyl anthranilate azo derivatives [103].

TABLE 6

SPECTROSCOPIC DATA IN METHANOL FOR AZOPYRROMETHENES DERIVED FROM PURIFIED BILIRUBIN-IX ISOMERS (FROM REF. 84)

Compound	λ_{max} (nm)	ϵ ($mM^{-1}\,cm^{-1}$)
Azpm-8	526	26,6
Azpm-12	526	26.6, 28.2
Azpm-2	526	26.7
Azpm-3,7	516	26.1
13,18-Vn_2-Azpm	532	25.0, 26.9
2,17-Vn_2-Azpm	532	27.0, 27.3

Detection of azo pigments Azpm-3,7 and 13,18-Vn_2-Azpm and/or 12,17-Vn_2-Azpm strongly supports the presence of bilirubin-IXβ and/or -IXδ. Likewise, bilirubin-IXγ is probably present if Azpm-2 and Azpm-18 are detected. When any of these tests are positive, it is necessary to confirm the presence of the isomers at the tetrapyrrole level. In contrast to bilirubin-IXα, which can be extracted from aqueous solution at pH 8.15 with chloroform [104], extraction of the non-α isomers of bilirubin-IX requires acidification and addition of a salting-out reagent [103]. Extraction with chloroform–ethanol (1:1, v/v) in the presence of a salting-out reagent and ascorbic acid at pH 1.8, as described by Fevery et al. [38], is probably satisfactory for the non-α isomers of bilirubin-IX. Semi-quantitative analysis can then be performed by TLC on silica gel by development with chloroform–methanol–water (48:9:1, v/v) [103]. Densitometric analysis can be improved by spraying the chromatograms with an ethanolic solution of ascorbic acid immediately after development [84] in order to stabilize the separated pigments. Data on the analytical recovery of each individual isomer and molar absorption coefficients are not available.

Synthesis, isolation and partial purification of the non-α isomers have been reported [84,103,105]. Assay of the sum of the IXβ and IXδ isomers can be based on quantitation of the derived azopigment, Azpm-3,7 (Table 6).

6. SEPARATION AND ANALYSIS OF THE SUGAR-ESTER CONJUGATES OF BILIRUBIN-IXα

6.1. Ester conjugates of bilirubin

TLC separations of bilirubins are generally performed on silica. This implies prior extraction with a solvent that can be evaporated without decomposition or alteration of the bile pigments when the concentrated extract is applied to the thin-layer plate. Near-quantitative extraction of the ester conjugates of bilirubin-IXα is achieved with chloroform–ethanol (1:1, v/v) at pH 1.8, con-

taining ascorbic acid as an antioxidant (extraction yield, 90–100% for urine, liver homogenate, bile, stools and serum) [38]. With chloroform containing the ion-pair reagent tetraheptylammonium chloride, overall recovery of bile pigment is 90% [34], but yields for individual bilirubins are lacking. The latter procedure is probably adequate for samples containing predominantly glucuronic acid conjugates of bilirubin-IXα (e.g. as obtained from humans, guinea pigs and rats) but may lead to selective losses of the less polar xylose and glucose conjugates [23].

TLC on silica of extracted bile pigments from dog bile with chloroform–ethanol–water mixtures permits separation of about twenty chromatographic bands [10]. The major bands have been identified by analysis of their dipyrrolic azo derivatives (Section 7.2). For example, the bands identified as bilirubin-IXα, bilirubin-IXβ and bilirubin-IXα diglucuronide yielded, respectively, the azo pigment pairs Azpm-8/Azpm-12, Azpm-3,7/13,18-Vn_2-Azpm and Azpm-*8*,12 (GlcU)/Azpm-8,*12* (GlcU). Separation of isomeric sugar conjugates was not achieved. The monoconjugate bands each consisted of two positional isomers, e.g. bilirubin-*8*,12 (Xyl) and bilirubin-8,*12* (Xyl) for monoxyloside. Similarly, each of the conjugates that contained two different conjugating sugars were present as the mixture of positional isomers, e.g. bilirubin-*8,12* (GlcU,Xyl) and bilirubin-*8,12* (Xyl,GlcU). Artifactual formation of conjugates, such as those containing two different sugars, by dipyrrole exchange has been excluded on the basis of TLC analysis of IIIα, IXα and XIIIα bilirubin obtained from isolated conjugates by saponification [10]. With the improved assay of Fevery et al. [38], artifactual formation of methyl esters by transesterification [106] is avoided and quantitative conversion of separated tetrapyrroles into azo derivatives was obtained for quantitation and identification purposes. For bile analysis, results of this method agree well with those obtained with the alkaline methanolysis procedure (Section 6.2).

A variety of reversed-phase HPLC procedures has been developed for separation of native bilirubins present in bile [40,68,102,107–111] or synthesized in vitro [39,109,111,112]. Except for those procedures applied exclusively to bile [40,68,102,107,110] the samples are either deproteinized or extracted before application to the column [39,102,111–113]. Separation of the isomeric pairs of monosugar conjugates and of mixed diconjugates is achieved with some systems [39,68,102,109–111]. The methods of Onishi et al. [102] and of Uesugi and co-workers [109,114] provide excellent resolution, at the cost of excessively long retention times. Similar resolution at somewhat shorter elution times (ca. 60 min) is achieved in the methods of Gordon and Goresky [39] and of Yamashita et al. [111]. In some procedures [39,68,102,109] heterogeneity due to the presence of triplets of aglycones (IIIα, IXα, XIIIα) is detected. Disproportionated bilirubins in biological specimens or in isolated chromatographic bands of ester conjugates can be detected conveniently by

TLC or HPLC of the methyl ester derivatives prepared by alkaline methanolysis (Section 6.2).

In general, the reported HPLC procedures have not been validated completely as methods for determination of individual bilirubins in natural specimens, since complete data on recovery of injected material and on accuracy, precision, interference and sensitivity are lacking. Some methods have been validated partially [39,40,68].

For reference purposes, mono- and diester conjugates of glucuronic acid, glucose and xylose can be synthesized by incubation of bilirubin-IXα and the appropriate UDP sugar with a microsomal preparation from liver as the source of bilirubin-IXα UDP-glycosyltransferase [61,115]. Rat bile is a useful source of mono- and diglucuronide of bilirubin-IXα and dog bile provides additional xylose and glucose conjugates. The chemical synthesis of bilirubin-IXα diglucuronide has been reported [5,116].

6.2. Bilirubin-IXα, its C-8 and C-12 monomethyl esters and its dimethyl ester obtained by alkaline methanolysis from ester conjugates of bilirubin

By brief (60 s) base-catalysed methanolysis ('alkaline methanolysis'), in the presence of ascorbate and Na_2EDTA as protective agents, the sugar moieties bound in ester linkage are replaced quantitatively by methyl residues [75] (Fig. 6). Unconjugated bilirubin is not esterified and disproportionation does not occur under the applied reaction conditions. Procedures for TLC [75] and

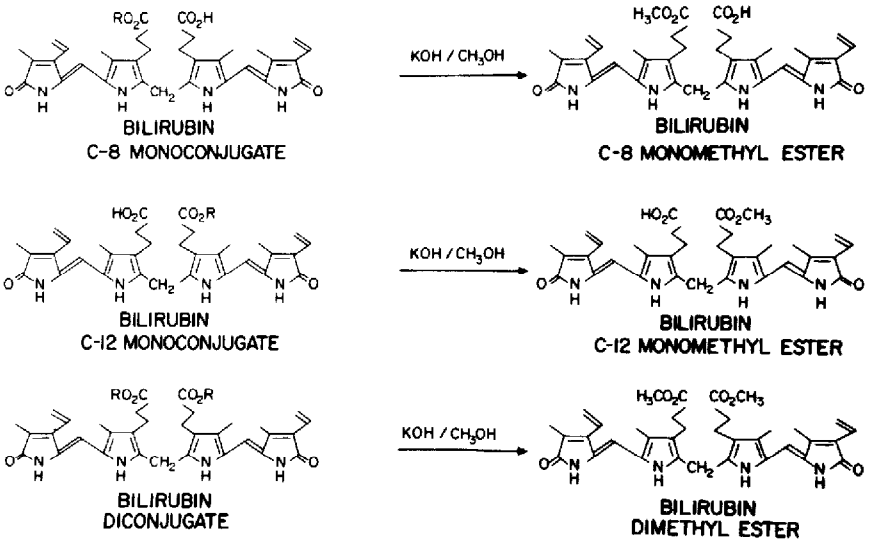


Fig. 6. Conversion of bilirubin monosugar and disugar esters into the corresponding monomethyl and dimethyl esters by base-catalysed methanolysis (R=carbohydrate residue). (Based on ref. 75.)

HPLC [117,118] are available for separation of bilirubin (IIIα, IXα and XIIIα), bilirubin-IIIα monomethyl ester, bilirubin-IXα C-8 monomethyl ester, bilirubin-IXα C-12 monomethyl ester and bilirubin dimethyl ester (IIIα, IXα, XIIIα) (Fig. 7). Addition of an internal standard to the sample prior to alkaline methanolysis permits determination of the various pigment fractions [117–121]. Apart from protein–bilirubin conjugates, which escape detection (Section 6.3), unconjugated bilirubin (150 nM) and its C-8 and C-12 monoesters (10 nM) and diesters (20 nM) can be measured in biological specimens with the limits of detection indicated in parentheses. At present, the alkaline methanolysis–HPLC procedure is the only well validated analytical method for determination of bilirubin-IXα and its esters in serum. For samples containing only bilirubin (IXα and possibly also IIIα and XIIIα) and the mono- and/or diesters of a given sugar, interpretation of the chromatographic results in terms of the parent bilirubins is straightforward. This applies to biological samples from rodents [40] and, to a good approximation, to blood and bile of humans [40]. For more complex samples, some chemical information is lost as a consequence of replacement of several types of sugar moiety by a methyl

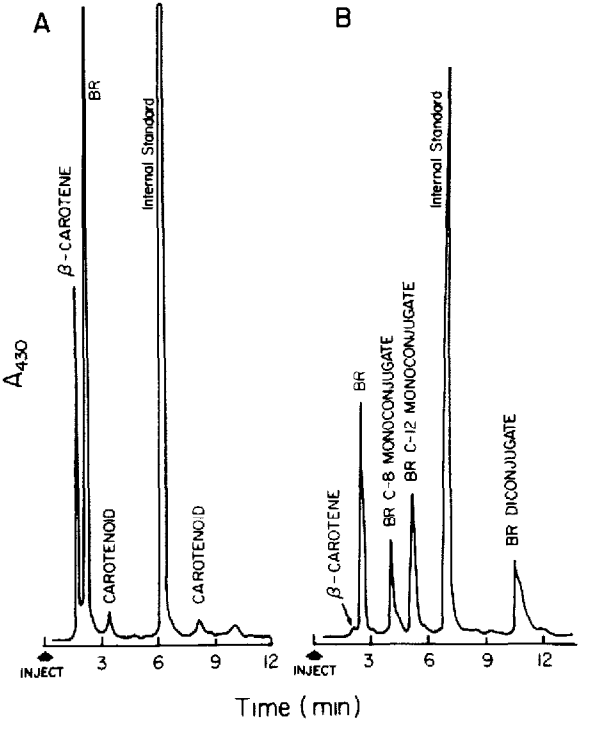


Fig. 7. Chromatograms obtained by HPLC of bilirubins from serum of (A) a healthy adult (total bilirubin, 10 μM) and (B) a patient with obstructive jaundice (total bilirubin, 166 μM). BR = bilirubin. (Reproduced by permission from ref. 147.)

ester group. The same principle of analysis is likely to be applicable to other types of sugar esters [25]. A more exhaustive discussion of the alkaline methanolysis–HPLC procedure is available elsewhere [24].

The monomethyl [75,122] and dimethyl esters [75,123] of the bilirubin-IX isomers can be synthesized for reference purposes. The molar absorption coefficients of the free acids [18,97] and of the mono- and dimethyl esters [75] have been reported (Table 5).

6.3. Bilirubin–protein conjugates

Bilirubin–protein conjugates in serum can be detected and assayed semi-quantitatively by open-column chromatography [29] and reversed-phase HPLC [17,124] (Fig. 8). The quantitative HPLC procedure of Lauff and co-workers [15,50] possibly overestimates bilirubin–protein conjugate because during the 20-min pre-incubation of the samples at 37°C protein–bilirubin conjugates could be formed [17,28]. Pre-incubation of serum is avoided in more recently reported HPLC procedures [125,126]. Relatively simple assays are based on measurement of bilirubin–protein conjugates by a diazo method after

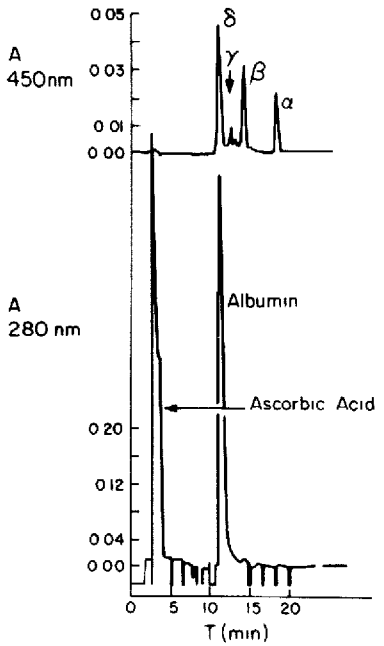


Fig. 8. Separation of bilirubins from the serum of a patient with conjugated hyperbilirubinemia by HPLC according to Lauff et al. [15]. The δ peak corresponds to bilirubin covalently bound to protein, and the γ, β and α peaks to di-esterified, mono-esterified and unconjugated bilirubins, respectively.

selective removal of non-covalently bound pigment [28,127,128]. An automated assay has been developed for the Kodak Ektachem® analyser [129]. The absorption characteristics and diazo reactivity of bilirubin–protein conjugates have been reported [130].

7. STRUCTURE ELUCIDATION OF BILIVERDINS AND BILIRUBINS

Natural biliverdins and bilirubins are labile and often are obtainable only in small amounts from biological specimens. Therefore, structure verification is based mainly on preparation of suitable, more stable derivatives and use of micro methods for analysis. The present availability of sensitive TLC and HPLC procedures for separation of bile pigments and a number of their derivatives is helpful. However, it should be remembered that identity of the R_F value of an unknown compound with that of an appropriate reference substance supports, but does not prove, chemical identity. A useful approach is to subject the unknown pigment to several structural tests aimed at verifying the presence or absence of different functional groups. These tests accomplish identification of the basic tetrapyrrole skeleton and of the sequence and nature of the β-substituents. For conjugated bile pigments one must further determine the nature of the conjugating residue(s) and the linkage(s) connecting them to the tetrapyrrole skeleton.

7.1. Structure elucidation at the tetrapyrrole level

Several reactions can be exploited to prepare derivatives for further analysis (Fig. 5). For example, the structure of an unconjugated biliverdin isomer can be verified by chromatography of both the free acid and the corresponding methyl ester derivatives against appropriate references, thus limiting the range of candidate structures that need to be considered.

The isomeric IIIα and XIIIα bilirubins formed by disproportionation of bilirubin-IXα can be detected and quantified by TLC and HPLC (Section 5.2.1). Disproportionation of bilirubin-IXβ and -IXδ yields bilirubins of widely different polarities [75]. Separation of biliverdin and bilirubin isomers deserves special attention, since various structures with different sequence and nature of the β-substituents have found increasing application in chemical and biochemical studies [41,131–135].

The aglycone structure of isolated ester conjugates of bilirubin-IXα can conveniently be determined by HPLC of mono- and/or dimethyl esters [117] obtained by alkaline methanolysis [75] (Section 6.2). Formation of an alkyl ester derivative by alkaline alcoholysis is diagnostic for ester linkage of a sugar to the aglycone, and for monoconjugates it indicates the carboxyl group involved. For example, from a monoester of bilirubin-IXα, one obtains the C-8 or C-12 monoalkyl ester of bilirubin-IXα. The position labelled with an alkyl

group then indicates the original point of attachment of the sugar residue. Methods for determination of the ester bond type and of the ring structure of the conjugating residue have been reviewed [5]. These analyses are conveniently performed at the stage of the dipyrrolic azo derivatives (Section 7.2).

Full structure determination of the bile pigment moiety in bilirubin–protein conjugates has not yet been reported. The nature of the sugar residue present in an esterified bilirubin–protein conjugate can be determined by the procedures mentioned above. Treatment with specific glycosidases in the presence and absence of aldonolactone inhibitors may further yield information about the nature of the ester bond (Section 7.2). However, the same information can be obtained more elegantly by formation and analysis of the extractable esterified azo pigment (Section 7.2) released from the conjugate by diazo cleavage.

7.2. Structure elucidation at the dipyrrole level

7.2.1. Formation of dipyrrolic azo derivatives

The diazo cleavage reaction, which is typical of compounds with a bilirubin skeleton, provides a powerful tool for structure elucidation. First, we consider bilirubins that are not covalently linked to protein. Reaction of a natural asymmetric unconjugated (BR), monoconjugated (BR-C) or diconjugated bilirubin (BR-C_2) with a diazonium salt leads to cleavage of the tetrapyrrole at the central methylene bridge with the formation of formaldehyde [136] and a typical pair of dipyrrolic azo pigments [2,10,19,24]:

$$\left.\begin{matrix}\text{BR}\\ \text{BR-C}\\ \text{BR-C}_2\end{matrix}\right\} \rightarrow \text{Azpm}_1 + \text{Azpm}_2$$

For example, bilirubin-IXα yields Azpm-8 (with *endo* vinyl group) and Azpm-12 (with *exo* vinyl group) (Table 2). Bilirubin-8,12 (Xyl,Xyl) is converted into Azpm-8 (Xyl) and Azpm-12 (Xyl). In contrast, a symmetric bilirubin such as bilirubin-IIIα (2,18-*Vn*$_2$-bilirubin-8,12) gives a single derivative, Azpm-12.

Diazo cleavage of a bilirubin–protein conjugate yields one azo pigment, Azpm_1, which remains attached to the protein moiety, and another dipyrrolic half, Azpm_2, which is free.

bilirubin–protein conjugate $\rightarrow \text{Azpm}_1$–protein + Azpm_2

Azpm_2 may be either esterified with a sugar or unesterified. Only the structure of Azpm_2 can be established by current methods.

For isolated bilirubins without covalent protein linkage, structure elucidation of the separated azo derivatives Azpm_1 and Azpm_2 permits identification of the dipyrrolic halves in the parent bilirubin. Because the dipyrrolic halves are linked by a CH_2 bridge in all natural bilirubins, the above-mentioned approach permits reconstruction of the parent pigment. Diazo-positive bilirubin-

like compounds with a CHR bridge can be synthesized under nearly physiological conditions by an addition reaction involving the central –CH= group of biliverdin-IXα and a thiol compound [137]. Diazo cleavage yields the same pair of dipyrrolic azo pigments as would be obtained from a similar bilirubin with central CH_2 bridge. Bilirubins with a CHR bridge have not yet been found occurring naturally but the possibility that such compounds exist should be kept in mind.

The ethyl anthranilate diazo reagent [1,2,5,22] has been used extensively for preparation of azopyrromethenes. With this reagent, the diazo cleavage reaction can be performed under controlled, very mild conditions. For example, in aqueous solution diazo cleavage of conjugated bilirubins proceeds to completion, apparently without side-reactions, within 30 min at 25 °C and pH 2.7 [1,2]. At this pH, no hydrolysis occurs of the sugar ester bonds [70]. The seven ethyl anthranilate azo pigments that can be obtained from the isomers of bilirubin-IX (Table 2) have been characterized in underivatized and in various derivatized forms by mass spectrometry [5], NMR [5] and spectrophotometry (Table 6). They are more stable than aniline azo pigments [138].

7.2.2. Chromatographic separation of dipyrrolic azo pigments

A number of TLC systems are available for separation of conjugated and unconjugated azopyrromethenes in underivatized and in various derivatized forms [2,22–24]. The conjugated and unconjugated forms of Azpm-8 and Azpm-12 can be separated without derivatization by HPLC [90,111,139,140]. Reference unconjugated azopyrromethenes can be obtained from natural bilirubin-IXα [1,40] and from the non-α isomers of bilirubin [103]. Reference azopyrromethenes conjugated with xylose [115], glucose [115] or glucuronic acid [61] can be isolated from bile and from incubation mixtures containing bilirubin-IXα, a microsomal preparation from liver and the appropriate UDP sugar.

7.2.3. Characterization of the sugar–ester conjugates

Apart from the ring structure of the sugar residue (furanosidic or pyranosidic), methods applicable at the micro scale are available for determination of the nature of the sugar, and of the nature (e.g. C-1′ or C-2′) and anomeric character of the carbon atom connecting the sugar to the aglycone [2,25]. Diagnostic tests for verifying whether an azopyrromethene is esterified with a sugar have been reported [25].

Sugars conjugated in ester linkage to an aglycone can be liberated by gentle procedures and subsequently identified [2,8,9,25,73,75]. In particular, treatment with ammonia vapour of a sugar conjugate applied to a thin-layer plate followed by TLC of the liberated sugar permits sensitive identification of the sugar moiety [25].

In general, natural glucuronides have a pyranose ring structure [141]. Or-

ganic analytical techniques that are generally applicable to sugar–ester conjugates permit assessment of the sugar residue [7] and of the sugar carbon atom to which the aglycone is attached (1′, 2′, 3′ or 4′ carbon atom) [11]. The 1′-, 2′-, 3′ and 4′-O positional isomers of the acylglucuronides of azopyrromethene can be separated by TLC of the methyl ester derivatives [13]. The anomeric character of the 1′ carbon atom of glycosides, which generally has the β-D configuration, can be demonstrated by hydrolytic removal of the sugar residue by the action of specific glycosidases, provided care is taken to check for inhibition of the process by specific aldonolactone inhibitors [79,80]. The β-D-glycosidic linkage was confirmed by NMR for azopyrromethene glucuronide [11]. That the site of attachment to the aglycone is the carboxyl group can be verified as follows. Treatment of the conjugate, applied to a thin-layer plate, with ammonia vapour [2,25] or of a solution of the conjugate with alkaline alcohol [2,25,75] should yield, respectively, the amide or alkyl ester of the aglycone. For alcoholysis of sugar esters of ethylanthranilate azo pigments, ethanol is preferable to methanol since, in alkaline medium, the ethyl group of the ethylanthranilate residue is replaced to some extent by a methyl group [9]. These various aspects of structure determination of sugar esters conjugates have been covered in detail elsewhere [2,5,22–25].

7.3. Structure elucidation at the monopyrrole level

Oxidative degradation to monopyrrolic derivatives provides useful structural information and, in principle, is applicable to any pyrrole-containing compound. The dichromate–chromic acid procedure [56,142] is particularly useful. Alternative oxidative procedures are based on the use of alkaline permanganate [143,144] or paraperiodic acid [145]. Compared with the formation and analysis of dipyrrolic azo derivatives, derivation of monopyrrolic oxidation products is elaborate and yields only partial information about the monopyrrolic building blocks of the parent tetrapyrrole skeleton.

8. SUMMARY

Recent applications of thin-layer chromatographic (TLC) and high-performance liquid chromatographic (HPLC) procedures has revealed an unexpected wide variety of naturally occurring unconjugated and conjugated bilirubins. Biliverdins seems to occur only in unconjugated forms, mainly as the IXα isomer. Several synthetic biliverdins and bilirubins present interesting models for biochemical and metabolic studies. Owing to recent recognition of the astounding heterogeneity of natural bilirubins and to the various artifactual changes that bile pigments can undergo, considerable confusion has existed, and still exists, with regard to the nomenclature of the bile pigments and their derivatives. To set a background for further discussion, the present re-

view starts with a brief discussion of nomenclature and of the various characteristic forms of lability of the bile pigments. TLC and HPLC procedures for preparation and analysis of unconjugated biliverdins and bilirubins and their methyl ester and sugar ester conjugates, as well as procedures for analysis of bilirubin–protein conjugates, are then discussed. Since, in view of the lability and pronounced heterogeneity of bile pigments, it is important to assess the composition and nature of chromatographically isolated pigments, the review is concluded by a brief evaluation of various structural tests.

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