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Conjugated and Unconjugated Bilirubins in Humans and Rhesus Monkeys

STRUCTURAL IDENTITY OF BILIRUBINS FROM BILES AND MECONIUMS OF NEWBORN HUMANS AND RHESUS MONKEYS

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1. Bilirubin-IXα monoglucuronide was the predominant bilirubin in biles and meconiums of newborn humans and rhesus monkeys. Rhesus-monkey baby biles contained slightly more diglucuronide than did human baby biles. 2. Bilirubin-IXα glucoside, bilirubin-IXα xyloside and bilirubin-IXβ were also constituents of human and rhesus-monkey baby biles and meconiums. Bilirubin-IXα glucuronide glucoside was present in human and rhesus-monkey baby biles but not in meconiums. The identity of the bilirubins was confirmed by u.v.–visible and mass spectroscopy of the azodipyrroles obtained by treating the bilirubins with diazotized ethyl anthranilate. The resulting azodipyrroles were identical with the corresponding azodipyrroles obtained from human adult biles and also from reduced isomers of biliverdin. 3. Bilirubin-IXβ was present in much higher proportions in the extracts of meconiums than in the extracts of biles from the same babies. 4. Oxidation of bilirubins to biliverdins occurs *in utero* to a small but undetermined extent. The resulting green pigments were present in meconiums collected from the lower small and large intestines of newborn babies and rhesus monkeys. 5. Butanol extracted most of the bilirubins present in biles. This modified method proved to be quick and easy. Little hydrolysis of bilirubins took place during extraction or separation by t.l.c.

The azopigments derived from bilirubins in human adult biles have been investigated as previously described (Blumenthal *et al.*, 1977*b*). The principal bilirubins in adult biles are derived mainly from bilirubin-IXα, except for small amounts of bilirubin-IXβ. Bilirubin-IXα is infrequently present in the unconjugated form. The conjugates of bilirubin-IXα found in human and rhesus-monkey adult biles include various esters of glucuronic acid, glucose and xylose. The bile-pigment composition of human and rhesus-monkey baby biles and meconiums is strikingly different from that of adult biles obtained from the same species (Blumenthal *et al.*, 1976). These data strongly suggested that human adult biles contain both diconjugates and monoconjugates of bilirubin, whereas human and rhesus-monkey baby biles and meconiums contain predominantly monoconjugates and only very small amounts of bilirubin diconjugates. Although glucuronides of bilirubin-IXα are the major tetrapyrrolic pigments in adult biles, baby biles and meconiums, a bilirubin giving rise to azopigments αF and βx is found in significant quantities in baby biles and meconiums. Another difference between adult and newborn biles is the frequent occurrence of haemin in baby biles (Blumenthal *et al.*, 1977*a*,*b*). Haemin is found also in meconiums collected from the small intestines of human and rhesus-monkey babies. Human and rhesus-monkey meconiums yield a much higher proportion of azopigments αF and βx than do the biles from the same individuals (Blumenthal *et al.*, 1976). Azopigments αF and βx are products of bilirubin-IXβ (Blanckaert *et al.*, 1976; Blumenthal *et al.*, 1976).

The meconium of newborn babies probably represents a mixture of material swallowed from the amniotic fluid *in utero*, sloughed cells from the gastrointestinal tract and material excreted in the bile during foetal development. This material accumulates in the babies' intestines and is normally passed soon after birth. The colour of meconium is

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dark-green to black and it contains an abundance of pigments originating from the bile. Human and rhesus-monkey foetuses start excreting pigments in bile at the end of the first gestational trimester (S. G. Blumenthal, D. B. Taggart, R. D. Rasmussen, R. M. Ikeda, B. H. Ruebner, D. E. Bergstrom & F. W. Hanson, unpublished work). The extraction of pigments from meconium has been difficult (Blumenthal *et al.*, 1976). The farther from the duodenum the meconium is located, the darker and less fluid it becomes. The pigments in meconium from the upper part of the small intestines are extracted easily. Three consecutive extractions result in a white meconium residue which releases no additional pigments upon further extraction. The large-intestinal meconium also releases no additional pigment after three consecutive extractions, but the meconium residue remains dark-green.

The aims of the present investigation were to confirm by more specific methods that the bilirubins in biles and meconiums of human and rhesus-monkey newborns are chemically identical with those in human adult bile, that newborn bile contains predominantly bilirubin monoglucuronide and that meconium contains significant amounts of bilirubin-IXβ. The methods used were developed by Heirwegh and his colleagues (Heirwegh *et al.*, 1975; Blanckaert *et al.*, 1976). Most of the presently used procedures are detailed in Blumenthal *et al.* (1977*b*).

Materials and Methods

For chemicals and general methods see Blumenthal *et al.* (1977*b*).

Bile samples

Bile samples were collected from the gall bladders of seven full-term babies at autopsy. All the babies died within 2h of birth. Autopsies were performed 12–14h after death. None of the babies had any clinical or morphological evidence of hepatic disease. Adult human bile was collected from two healthy volunteers by a duodenal T-tube. The protocols for these studies were approved by the Committee on Research involving Human Subjects. Rhesus-monkey (*Macaca mulatta*) biles were collected from the gall bladders of seven newborn term monkeys at autopsy. Autopsies were performed 12–24h after death. None of the monkeys showed any morphological evidence of hepatic disease.

All bile samples collected were frozen immediately, stored in dark containers, and kept at −75°C until used. A small sample (0.05–0.10ml) from every bile was treated with diazotized ethyl anthranilate as soon as it arrived from the hospital or the Primate Center. Bilirubins were separated by t.l.c. 1–4 days after collection.

Meconium samples

Intact intestines were removed from newborn humans at autopsy. The ileocaecal junction was removed, cut longitudinally and placed in formalin. The rest of the bowel was wrapped in foil and frozen (−75°C) for a few hours until use. Autopsies were performed 12–14h after death. For the monkey studies a small piece of the lower part of the small intestine (12.5–15cm) was obtained at autopsy. This material was wrapped in foil, frozen (−20°C) and used as soon as possible for analysis.

The intestinal samples were utilized within 12h of receipt from the hospital or Primate Center. The frozen samples were thawed in the dark, then rinsed with 0.1M-NaCl. Gross inspection of the intestine was carried out in dim room light, noting unusual characteristics, including excessive congestion of mesentery, darkened regional lymph nodes, or unusual colour. The mesentery was then cut along the intestines with scissors allowing the intestines to become straightened. Meconium was removed from the intestines by applying gentle pressure along the intestinal wall forcing the meconium out of the cut end into a closed dark container.

In some cases a small quantity of meconium was removed from the intestinal sample immediately upon receipt and allowed to react with diazotized ethyl anthranilate (ethanol/glycine buffer, pH2.7, and ethyl anthranilate diazo reagent added). These azopigments were spotted on silica-gel 60 plates and developed in solvents I and II (see Table 1 of Blumenthal *et al.*, 1977*b*). These direct azo reactions were used primarily to learn which bilirubins were present in the samples and to identify samples that were good sources for azopigments α2 and/or α3. The results of these direct azo reactions were not otherwise used.

Extraction of bile pigments and separation by t.l.c.

For comparison of bilirubins in newborn human and rhesus-monkey biles and meconiums with those of adult human biles we used a modification of K. P. M. Heirwegh's (personal communication) procedure. To 0.5–1.0ml of bile, 2ml of NaCl-saturated glycine/HCl buffer, pH1.8, 1ml of NaCl-saturated ascorbic acid (20mg/ml) and 1ml of ethanol (95%) were added. This mixture was extracted with 1ml of butan-1-ol. The extract was applied to silica-gel G glass plates as a band and the plates were developed with solvent system III (Table 1 of Blumenthal *et. al.*, 1977*b*). The plates were then dried and photographed. Extraction of bilirubins from meconium samples was carried out by the method described previously (Blumenthal *et al.*, 1977*b*). Only 0.5–0.6g of meconium was used for each extraction. The meconium was suspended in 1.0ml of 0.15M-glycine/HCl buffer, pH1.8, saturated with NaCl.

Then 1.0ml of ascorbic acid solution (20mg/ml) saturated with NaCl and 1.0ml of ethanol were added. This mixture was homogenized in either a glass homogenizer or a Willems Polytron (Brinkman Instruments, Westbury, NY, U.S.A.) at a power setting of 6 for 5s. The mixtures were then sonicated in a sonifier cell disrupter (model 350; Branson Sonic Power Corp., Danbury, CT, U.S.A.) at an output control setting of 6 for 15s (continuous mode; 30% duty cycle; micro-tip). The homogenate was extracted with 1.0ml of butan-1-ol. The butanol was removed under vacuum and the residue dissolved in methanol. These suspensions were centrifuged for 5min at 3000rev./min. The clear solutions were applied on silica-gel 60 glass plates. The plates were developed with solvent system III (see Table 1 of Blumenthal *et al.* (1977*b*). The rest of the procedure was as described previously (Blumenthal *et al.*, 1977*b*).

Formation and analysis, absorption spectra, ammonolysis, methanolysis, treatment with diazomethane of azodipyrroles and the identification of the conjugating sugar, identification of azopigments αF and βx, preparation of azopigment *meso* αF, and mass spectrometry of azodipyrroles were performed as described previously (Blumenthal *et al.*, 1977*b*).

Results

Extraction and separation of bile pigments

Butanol efficiently extracts bilirubins from bile, and the different bilirubins can be separated well by t.l.c. However, only a semiquantitative assessment of the bilirubin composition of bile could be obtained. Extracted meconiums retained pigments that were not extractable with chloroform or alcohol. T.l.c. separations of bilirubins from the biles of adult and newborn humans and rhesus monkeys are shown in Fig. 1. Eight to ten yellow bands were present in such chromatographic separations. Fig.

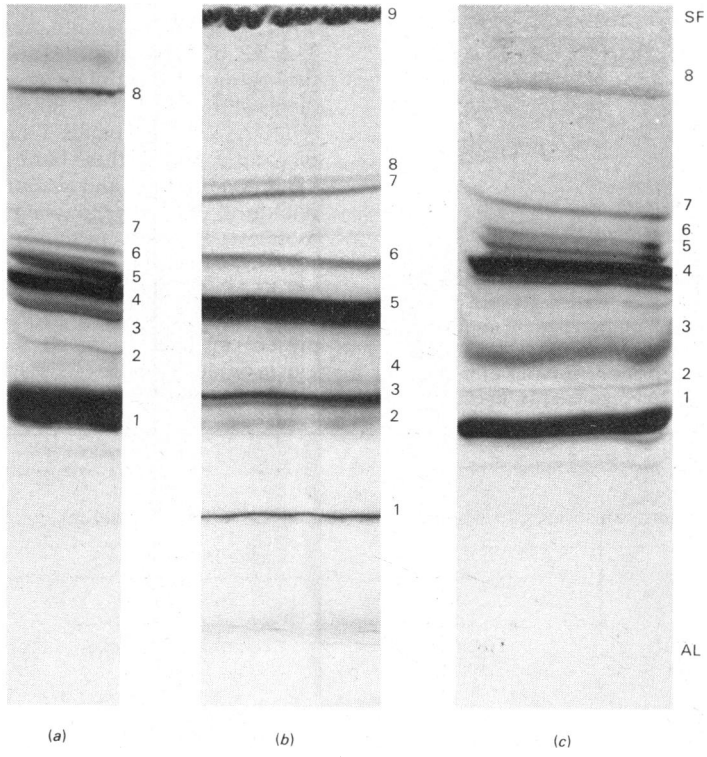

Fig. 1. *Chromatographic separation of bilirubins extracted from human and rhesus-monkey biles*
Bilirubins extracted from normal (*a*) adult human, (*b*) term baby and (*c*) adult rhesus-monkey biles were separated by t.l.c. (to 17–18cm) in solvent system III (see Table 1 in Blumenthal *et al.*, 1977*b*). Bands were numbered from the application line (AL) to the solvent front (SF). The photographs depict one-quarter of a plate. Band 9, representing unconjugated bilirubin-IXα, shows greater hydrolysis of conjugated bilirubin-IXα than usual. These photographs were chosen because they depicted chromatographs in which the bilirubin separation was better than in others.

2 shows a t.l.c. separation of bilirubins extracted from meconium of the lower small intestines of a human baby and a newborn rhesus monkey. The bilirubin bands from all separations were eluted with methanol and each bilirubin reacted separately with diazotized ethyl anthranilate. The bilirubins present in the biles and meconiums of human and rhesus-monkey babies are listed in Table 1. Some yellow pigments present on the t.l.c. separations did not yield azopigments and probably were not bilirubins. In some instances two adjacent well-defined bands yielded the same azopigments. We have no explanation for the fact that some bilirubins will migrate as more than one band. Most of the bilirubin separations on t.l.c. plates yielded a band of bilirubin-IXα. In most cases this band accounted for 1–10% of the total yellow pigments on the plate, and in a few instances for much more.

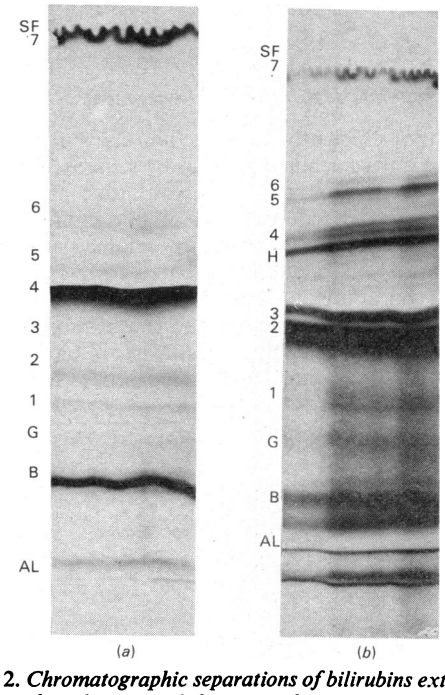

Fig. 2. *Chromatographic separations of bilirubins extracted from human and rhesus-monkey meconiums*
Bilirubins extracted from term baby human (*a*) and rhesus-monkey (*b*) meconiums were separated by t.l.c. (to 17–18cm) in solvent system III (see Table 1 of Blumenthal *et al.*, 1977*b*). Bands were numbered from the application line (AL) to the solvent front (SF). The photographs depict one-quarter of a plate. B, Brown pigment; G, green pigment; H, haem.

Reaction of bilirubins with diazotized ethyl anthranilate

The conversion of bilirubins to azodipyrroles was carried out with the methanol eluate of every bilirubin band present on each t.l.c. plate. The azopigments thus formed were separated by t.l.c. Fig. 3 shows the azopigments derived from the various bilirubins chromatographically separated from the biles of adult and newborn humans and rhesus monkeys. The list of bilirubins in Table 1 was derived from the pattern of azopigments present on the t.l.c. plates shown in Fig. 3. Bilirubin-IXα diglucuronide (GlcUA, GlcUA) bands 1 or 2 produce only azopigment δ. One of these bands was the predominant band in adult biles and accounted for approximately one-third of the yellow pigment in the newborn monkey. In most newborn humans, however, it was present in small amounts only (as in Fig. 1*b* band 2). Bilirubin-IXα glucuronide glucoside (GlcUA, Glc) (yielding azopigments δ and α3) was present in biles in small amounts. Bilirubin-IXα glucuronide xyloside (GlcUA, Xyl) (yielding azopigments δ and α2) was present in most biles extracted,

Table 1. *Bilirubins in human and rhesus-monkey adult and newborn baby biles and meconiums as separated on t.l.c. with solvent system III*
See Figs. 1 and 2. T.l.c. separations were carried out as described in Table 1 of Blumenthal *et al.* (1977*b*) and in the text.

	Band number				
	Adult bile		Newborn bile		
Bilirubins	Human	Monkey	Human	Monkey	Meconium Human and monkey
-IXα GlcUA, GlcUA	1†	1, 2†	2	1	—
-IXα GlcUA, Glc	2	3	3, 4	2	—
-IXα GlcUA, Xyl	3	—	—	—	—
-IXα GlcUA	4	4	5†	3†	1, 2, 3
-IXα GlcUA*, Glc or -IXα GlcUA*	5	5	—	4	—
-IXβ	6	6	5	4	3, 4
-IXα Glc	7	7	6	5	5
-IXα Xyl	—	—	7	6	6
-IXα	8	8	9	7	4, 5, 6, 7

† The predominant bilirubin in this bile.

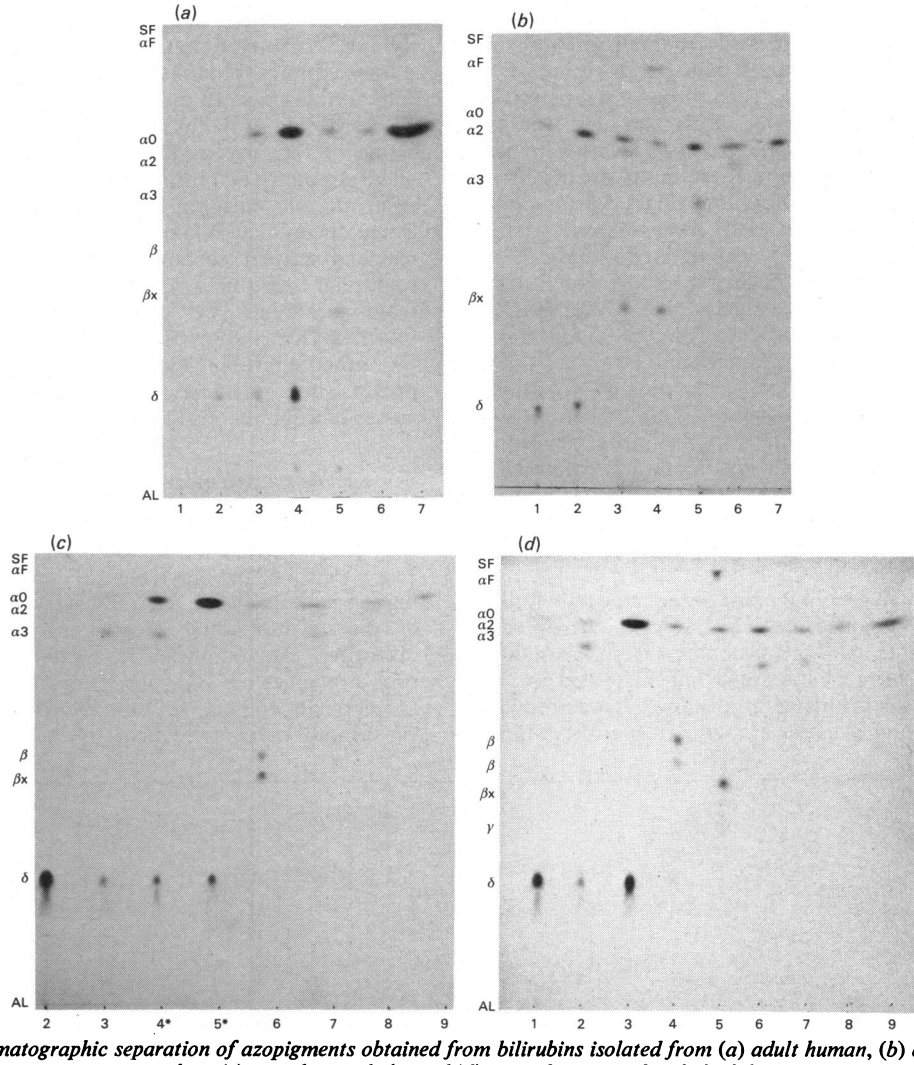

Fig. 3. *Chromatographic separation of azopigments obtained from bilirubins isolated from (a) adult human, (b) adult rhesus-monkey, (c) term human baby and (d) term rhesus-monkey baby biles*

The reference numbers indicated below the application line (AL) denote the parent bilirubin band in Fig. 1. The Greek letters are symbols for the different azopigments in biles. Abbreviation: SF, solvent front. The proportions of azopigments are not representative of the relative amounts of bilirubins in the bile. * The relatively larger amount of α0 in these fractions must be due to the hydrolysis of the bilirubin-IXα glucuronide during the experiments.

but often did not separate well from the lower band of bilirubin-IXα glucuronide glucoside. Bilirubin-IXα monoglucuronide (GlcUA) was present in all biles extracted. In adult biles it was present in appreciable quantities. In newborn biles it was the predominant bilirubin in almost all individuals. Bilirubin-IXα glucuronide* glucoside (glucuronide* indicates that the glucuronic acid is linked to bilirubin at 2′-OH, 3′-OH or 4′-OH rather than at 1′-OH) (Fig. 1*b*, band 4) and bilirubin-IXα glucuronide* (Fig. 1*b*, band 4) were present in small and variable amounts in many of the biles of adults and newborns. Bilirubin-IXβ (yielding azopigments βx and αF) was present in almost all biles but usually in negligible amounts in adult biles and in relatively larger quantities in newborn biles (see Figs. 1*a*, 1*b* and 1*c*). Bilirubin-IXα glucoside (Glc) (yielding α3 and α0) was present in all bile extracts. Bilirubin-IXα xyloside (Xyl) (yielding α2 and α0) was present in smaller amounts. It was not always detectable

(Fig. 3*b*). Unconjugated bilirubin-IXα (yielding azopigment α0) was present in small amounts in almost all the separated bile extracts.

Fig. 4 shows the t.l.c. separation of azopigments obtained from bilirubins of human and rhesus-monkey meconiums. The bilirubins present in human and rhesus-monkey baby meconiums are listed in Table 1. The relative amounts of the various bilirubins in the human and rhesus-monkey baby meconiums were similar. The major bilirubin in all meconiums from the upper and middle small intestines was bilirubin-IXα glucuronide. Bilirubin-IXβ was present in all meconiums from the middle small intestines, lower small intestines and large intestines. Meconiums from the upper part of the small intestines usually also contained some of this bilirubin. The few samples of meconium from the upper part of the small intestines that did not contain bilirubin-IXβ were those collected from a small number of human term babies and some of the rhesus-monkey babies. The proportion of bilirubin-IXβ in the upper small intestine was smaller than that in the lower intestine, but larger than that in the bile of the same individual. Bilirubin-IXα glucoside was present in relatively small amounts in all the meconiums from the middle and lower parts of the small intestines and in the meconiums from the large intestines. It was present in proportionally larger amounts in the meconiums from the upper small intestines. Bilirubin-IXα xyloside was present in very small amounts in almost all meconiums obtained from the small or large human intestines. In monkey meconiums, bilirubin-IXα xyloside was found in trace amounts only. Some of the meconiums extracted contained very small amounts of bilirubin-IXα glucuronide glucoside and/or bilirubin-IXα glucuronide xyloside. Some unconjugated bilirubin-IXα was present in all chromatographic separations. During the chromatographic separation of the bilirubins extracted from meconium we often observed that some bilirubin-IXα and/or bilirubin-IXβ was present in the other bilirubin bands. This resulted in the presence of more than two azopigments in the various eluted bilirubin bands (see Fig. 4).

U.v.–visible spectra of azopigments

U.v.–visible spectra of azopigments obtained from human adults and newborn babies were recorded. The spectra of azodipyrroles αF, α0 and βx from human adult biles are given in Fig. 5(*a*). The spectra of the corresponding azopigments purified from diazotized ethyl anthranilate-treated human baby biles are given in Fig. 5(*b*). For these and other azopigments, the $\lambda_{max.}$ and the absorbance at higher-wavelength maxima relative to that at the lower

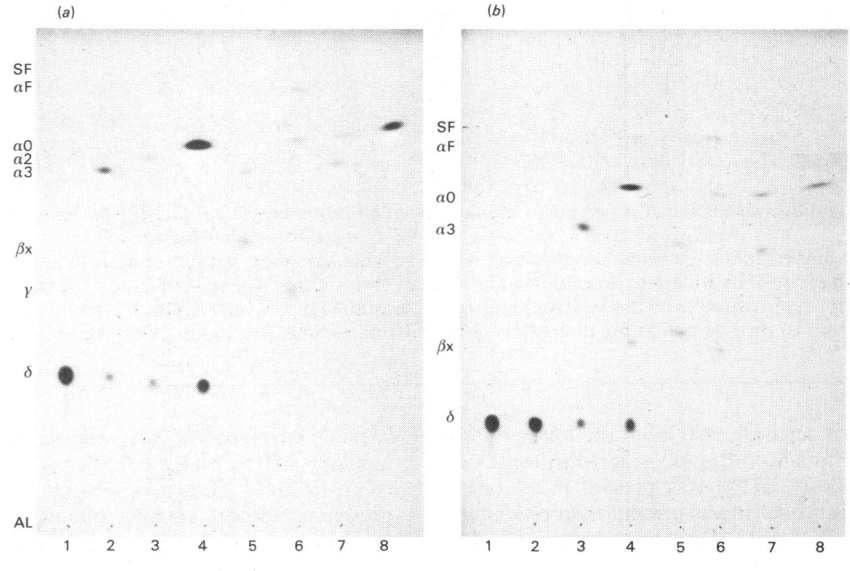

Fig. 4. *Chromatographic separation of azopigments obtained from bilirubins isolated from term baby (a) human and (b) rhesus-monkey meconiums*

The reference numbers indicated below the application line (AL) denote the parent bilirubin band in Fig. 1. The Greek letters are symbols for the different azopigments found in biles. Abbreviation: SF, solvent front. Amounts of azopigments spotted do not represent the relative amounts of bilirubins in the meconiums.

wavelength (symbolized by R) are given in Table 2. The $\lambda_{max.}$ and R of azopigments αF and βx obtained from bilirubin-IXβ, separated from biles of human adults and human and monkey babies, were the same as those of azopigments αF and βx obtained from synthetically prepared bilirubin-IXβ. The $\lambda_{max.}$ and R of azopigment α0 obtained from bilirubin-IXα separated from biles of human adult and human and monkey babies and synthetically prepared bilirubin-IXα were identical. Azopigments α2, α3 and δ obtained from bilirubin-IXα conjugates present in biles of human and monkey adults and babies are also compared in Table 3. The $\lambda_{max.}$ of the same azopigment obtained from different biles was identical. The R values of the conjugated azopigments α2, α3 and δ were higher for the azopigments from baby biles. This is due to the fact that the concentration of those azopigments is much higher in adult biles and can consequently be purified more readily.

The spectra of azodipyrroles αF, α0, α2, α3, βx and δ from human and rhesus-monkey baby meconiums had the same $\lambda_{max.}$ as the corresponding azodipyrroles purified from human adult biles.

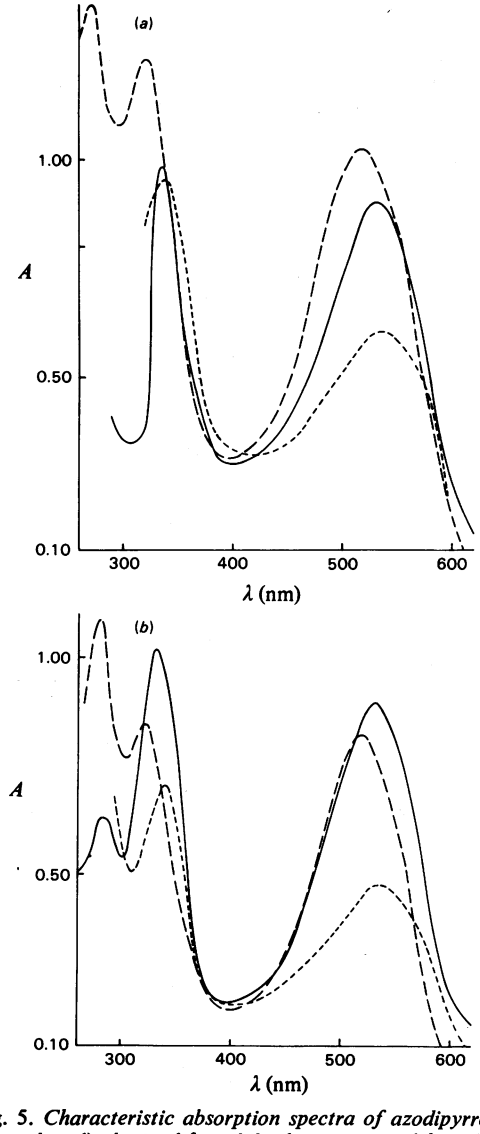

Fig. 5. *Characteristic absorption spectra of azodipyrroles (in methanol) obtained from bilirubins separated from biles of (a) human term baby and (b) human adult*
——, Azopigment αF; – – – –, azopigment α0; ------, azopigment βx.

Table 2. *Properties of various azodipyrroles obtained from bilirubins separated from human adult and human and rhesus-monkey newborn*
Spectra of azodipyrroles in methanol were recorded with a Cary 17 spectrophotometer. Values given in parentheses were obtained from spectra run on very dilute samples. $R = A_{333-340}/A_{520-537}$

		R		
Azodipyrrole	$\lambda_{max.}$ (nm)	Human adult	Human baby	Monkey baby
αF	537, 340	1.44	1.27	(1.77)
α0	530, 335	1.09	1.03	1.15
α2	530, 334	1.18	(1.51)	(1.46)
α3	530, 334	1.21	(1.44)	1.26
βx	520, 324	1.09	1.04	1.03
δ	532, 333	1.04	1.15	1.09

Table 3. *Mass spectra of azopigment meso αF prepared from adult human and newborn baby biles and from commercial haemin*
Values are relative intensities. The instrument's sensitivity at lower values of m/e was decreased in order to enhance the spectrum of fragments at higher m/e values.

	meso αF		
m/e	Synthetic*	Human baby	Human adult
420	100	67	100
405	4	2	4
391	7	4	6
375	4	2	4
374	8	4	8
347	42	21	39
256	22	12	19
165	24	78	72
120	26	38	28
119	35	77	67
43	14	29	10

* *meso* αF prepared from synthetic bilirubin-IXβ.

These azodipyrrole absorption spectra were virtually superimposable on the spectra obtained from the corresponding azodipyrroles purified from human adult bile.

Ammonolysis of azopigments

The following purified unconjugated and conjugated azopigments from human adult, human baby and rhesus-monkey baby biles and meconiums were subjected to ammonolysis: αF, α0, α1, α2, α3, βx and δ. All azopigments except αF, α0 and βx yielded azodipyrrole amide and various small amounts of azodipyrrolic acid. The propionic ester bond of conjugated azodipyrroles was cleaved and the corresponding amides were obtained. No chemical change occurred in the unconjugated dipyrroles αF, α0 and βx owing to the lack of an ester linkage in those dipyrrolic acids.

Methanolysis of azopigments

The following purified unconjugated and conjugated azopigments from human adult, human baby, rhesus-monkey baby biles and meconiums were subjected to methanolysis by treatment with sodium methoxide in methanol: αF, α0, α2, α3, βx and δ. All azopigments except αF, α0 and βx yielded azodipyrrole monomethyl esters. Azopigments αF, α0 and βx were therefore methylated with diazomethane. Azodipyrrole α0 reacted to give azodipyrrole monomethyl ester. Azopigment βx gave its corresponding azodipyrrole dimethyl ester. Azopigment αF did not change under these methylation conditions.

Mass spectra

Mass-spectral data for azopigment *meso* αF and the dimethyl ester of azopigment βx derived from human adult, human baby and synthetic bilirubin-IXβ are shown in Table 4. They clearly indicate that the azopigment *meso* αF from human biles results from bilirubin-IXβ. The synthetic azopigment αF prepared from bilirubin-IXδ is an isomer of αF prepared from bilirubin-IXβ and gives a slightly different mass spectrum (Heirwegh *et al.*, 1975; Blumenthal *et al.*, 1977*b*). Mass-spectral data for βx dimethyl ester from human adult, human baby and synthetically prepared bilirubin-IXβ are given in Table 4. The mass spectra of the βx methyl esters were identical. Mass-spectral data for α0 methyl ester derived from azopigments α0 and δ prepared from adult and baby human bilirubins and α0 methyl ester synthesized from chemically prepared bilirubin-IXα are given in Table 5. The mass-spectral data clearly indicate that α0 methyl ester

Table 4. *Mass spectra of azopigment βx dimethyl ester prepared from human adult and newborn baby biles*

Values are relative intensities. The higher-mass fragments are reported to hundredths of a percent because of the low sensitivity of the instrument at higher *m/e* values.

	βx dimethyl ester		
m/e	Synthetic	Human baby	Human adult
536	8.41	2.15	4.36
505	0.43	0.08	0.18
463	1.18	0.24	0.49
449	0.55	0.12	0.18
299	0.26	0.15	0.10
285	0.34	0.28	0.18
239	0.45	0.45	0.31
109	9	9	7
69	35	36	41
69	35	36	41
57	45	44	50
55	64	69	61
43	100	100	100

Table 5. *Mass spectra of azopigment α0M prepared from azopigments α0 and δ derived from bile bilirubins of human adult and newborn baby biles and from commercial bilirubin-IXα*

Values are relative intensities.

	α0M from α0			α0M from δ	
m/e	Synthetic	Human baby	Human adult	Human baby	Human adult
476	50	8	68	37	16
445	3		4	2	1
403	5	1	12	4	3
389	1		6	2	2
312	31	8	100	23	32
311	6	3	32	14	10
280	11	4	40	10	12
252	6	3	36	8	14
165	47	18	70	21	26
120	33	12	44	11	24
119	76	28	57	20	37

derived from azopigments α0 and δ prepared from human adult and baby bile bilirubins are identical with those prepared from synthetic azopigment α0.

Analysis of the conjugating groups

R_F values for glucose, xylose, glucuronic acid and for these sugars treated with sodium methoxide were given previously (Blumenthal *et al.*, 1977*b*). There was a marked change in R_F values of sodium methoxide-treated glucuronic acid and xylose, but almost no change in R_F value of sodium methoxide-treated glucose. Chromatographic separation of the sugars cleaved from azopigment α2 prepared from human and rhesus-monkey baby biles resulted in xylose. The sugar cleaved from azopigment α3 prepared from human and rhesus-monkey baby biles was glucose, and the sugar cleaved from azopigment δ prepared from human and rhesus-monkey baby biles was glucuronic acid. The sugar cleaved from azopigment δ purified from human meconium was also glucuronic acid. The sugar cleaved from azopigment α3 purified from human and rhesus-monkey meconiums was also glucose. We could not obtain positive sugar identification with azopigment δ from rhesus-monkey meconium using the treatment with sodium methoxide. We did not have enough azopigment α2 from human or rhesus-monkey meconiums to test for its conjugating group.

Discussion

The methods used here are based on those of Heirwegh *et al.* (1975) with modifications, most of which were described previously (Blumenthal *et al.*, 1977*b*). In this investigation we have employed a new modification which involved the use of butanol instead of chloroform as the solvent to extract the bile pigments. By this method we were able to extract 90–95% of the bilirubins in biles, and the extracts obtained were suitable for t.l.c. separation of the bilirubins. This quick and easy extraction procedure resulted in an excellent representation of the actual bilirubin composition in biles and only some slight hydrolysis of glycosidic linkages. The extraction of bilirubins from meconium and baby stools is tedious. Extraction procedures using ethanol/butanol (1:1, v/v) were found to give more complete extraction of bilirubins from meconium than did the corresponding chloroform/ethanol (1:1, v/v) mixture (Heirwegh *et al.*, 1975). Extraction of bilirubins from the contents of the upper third of the small intestine was complete in most experiments. However, we were unable to devise a method for complete extraction of the pigments from the contents of the middle and lower third of the small intestine or the large intestine. We tried sonicating the homogenate, treating the homogenate with proteinase and/or lipase (pig pancreas; United States Biochemicals) and both methods combined. The results did not justify sonic or enzymic pretreatment of the homogenates. It is important to mention here that the pigments extracted from the upper third of the small intestinal material were yellowish, whereas those from the middle intestinal material were yellow to brown. Extracts obtained from the lower third of small intestines and the large-intestinal material were brown to dark-green. These changes in colour are the result of: (1) larger concentrations of bilirubins present in the lower intestinal contents and (2) the occurrence of green pigments, which are probably oxidation products derived from bilirubins that had been in the large intestine for prolonged periods (Noir *et al.*, 1965). Extracts were separated on silica-gel 60 plates into one or more green bands. These bands were probably biliverdin-IXα monoglucuronide (Noir *et al.*, 1965) and other unknown green pigments. Green pigments were found more frequently and/or more abundantly in extracts of lower small and large intestines than in the upper or middle part of the small intestine or bile of the same individual. They were also found more frequently in meconium of rhesus monkeys than in human meconiums. The amounts of green pigment found in bile and in meconium extracts from different individuals of the same species varied but never exceeded 10% of total pigments extracted from human meconiums and 15% of total pigments extracted from rhesus-monkey meconiums.

The t.l.c. separations of the azopigments obtained from the diazocoupling reaction between t.l.c.-separated bilirubins and diazotized ethyl anthranilate were generally quite satisfactory. We made a particular effort during this investigation to identify azopigments αF, α0, α2, α3, βx and δ isolated from human and rhesus-monkey newborn baby biles and meconiums. Results from t.l.c. strongly suggested that baby bile and meconium azopigments were identical with azopigments obtained from adult human and rhesus-monkey biles (Blumenthal *et al.*, 1976). Chemical and spectroscopic evidence confirmed this suggestion and showed that the azopigments and, therefore, also the bilirubins from which they were derived, were identical (Blumenthal *et al.*, 1976, 1977*b*).

The results of this investigation establish conclusively our previous suggestion (Blumenthal *et al.*, 1976) that bilirubin-IXα monoglucuronide is the predominant bilirubin in the biles and meconiums of human and rhesus-monkey babies. Bilirubin-IXα diglucuronide is the predominant bilirubin in adult bile (Blanckaert *et al.*, 1976; Blumenthal *et al.*, 1976, 1977*b*). This is one of the major differences between adult and newborn-baby biles. Adult human and monkey biles also contain more of the other bilirubin-IXα diesters than

do biles or meconiums from newborn humans and monkeys. The difference in bilirubin composition between newborn baby biles or meconiums and adult biles is not unexpected in view of previous work (Strebel & Odell, 1971) describing metabolic changes during maturation of the baby liver. It has been suggested that bilirubin conjugation occurs in two steps, the first in the endoplasmic reticulum, the second at the canalicular membrane (Chowdury *et al.*, 1976). Preliminary work in our laboratory suggests that conjugation of bilirubin-IXα in human and rhesus-monkey foetuses starts early in development. Our observation that most of the bilirubins of the newborn human and monkey are monoconjugates suggests that one of the conjugation sites may still be immature at birth. Complete maturation of the human baby liver appears to occur soon after birth when the proportion of bilirubin diglucuronide in the bile increases, with a decreased proportion of bilirubin monoglucuronide (Strebel & Odell, 1971).

Although glucuronides regularly predominate in the baby biles and meconiums of newborn infants and adults, there is considerable variation between different individuals in the proportions of the other bilirubin-IXα conjugates. In general, the same pigments are found in human and rhesus-monkey babies as in adults. Contrary to our previous findings (Blumenthal *et al.*, 1977*b*), bilirubin-IXα glucuronide glucoside was present in all the biles from human and rhesus-monkey adults and in most biles from human and monkey babies, when butanol extracts were employed. Small amounts of bilirubin-IXα glucoside xyloside were present in most biles from human and rhesus-monkey adults and monkey baby biles, but rarely in biles from human babies. Human and rhesus-monkey meconiums did not contain bilirubin-IXα glucuronide glucoside or bilirubin-IXα glucoside xyloside. Bilirubin-IXα glucoside was present in almost all biles from human and rhesus-monkey adults and in most human and monkey baby biles or meconiums. The relative amounts of this bilirubin were very small in biles but larger in meconiums from the same individual. In many of the human adult biles and in a few samples of the rhesus-monkey adult biles other bilirubins were present. Treatment of these bilirubins with diazotized ethyl anthranilate produced azopigments λ1, λ2*, and in some cases λ3* and λ4*. (λ2*–λ4* are azopigments with an azodipyrrole glucuronic acid in which the sugar is linked to the dipyrrole at a non-C-1 linkage.) Azopigments λ2*, λ3* and λ4* are non-C-1 glucuronide isomers (Compernolle *et al.*, 1977; Blanckaert *et al.*, 1978). We have also had some adult and baby biles containing bilirubin-IXα glucuronide, λ2*, and bilirubin-IXα glucuronide, λ2. Human baby biles and meconiums contained some bilirubin-IXα xyloside. Rhesus-monkey baby biles and meconiums contained extremely low concentrations of bilirubin-IXα xyloside or none. The presence of some unconjugated bilirubin-IXα in all the butanol extracts was certainly a result of hydrolysis during extraction and/or the chromatographic separation of the bilirubins. Bilirubin-IXβ was present in newborn-baby biles. However, it was never a major bile constituent in full-term babies. All meconium extracts contained a substantially larger proportion of bilirubin-IXβ than did the biles of the same individuals. Bilirubin-IXβ was present in the unconjugated form only. We have not found any evidence for the presence of bilirubin-IXγ or bilirubin-IXδ in any of the biles or meconiums tested.

Bilirubins present in meconiums of newborn babies must have been excreted by the individual during foetal life. Therefore the meconium may be used to indicate which bilirubins were excreted in the bile of that individual *in utero*. However, it must be admitted that some of the bilirubins present in meconium may have been chemically altered while in the intestines of the developing foetus. Many types of changes might occur: (1) hydrolysis of the sugar from conjugated bilirubin with the production of unconjugated bilirubin, which may occur enzymically (β-glucuronidase) or non-enzymically; (2) exchange of the sugar molecule from one bilirubin to another, thus producing bilirubins that may differ from those excreted in the bile; (3) rearrangement of pyrrole rings and production of bilirubin-IIIα and/or bilirubin-XIIIα (Bonnett & McDonagh, 1970; McDonagh & Assisi, 1971); (4) oxidation of bilirubins with the production of biliverdins (Noir *et al.*, 1965). Our results suggest that, except for oxidation of bilirubins, chemical changes seem to be slight. The proportional amount of unconjugated bilirubin is very similar in small-and large-intestinal meconiums Therefore hydrolysis of the sugars from conjugated bilirubins seems unlikely. Sugar transfer from one bilirubin molecule to another may occur in the meconium during foetal development. However, we have no evidence for such a rearrangement, since the azopigments we obtained from meconium were not different from the azopigments obtained from the same individual's bile. Azodipyrrole glucuronide prepared from bilirubin-IXα glucuronide isolated from bile and meconium of one human individual was treated with sodium methoxide. Separation of the resulting azodipyrrole α0M isomers on t.l.c. in benzene/ethyl acetate (17:3, v/v) showed that they occurred in equal amounts. Therefore glucuronic acid is conjugated to rings C and D of the bilirubin in equal amounts. Some green pigments were formed by oxidation of bilirubins to biliverdins. These biliverdins have not been identified by us. It was therefore concluded that the bilirubins extracted from intestinal contents of newborn humans and rhesus monkeys were mostly unchanged bilirubins which had been excreted by the foetus *in utero*. This

has been confirmed by later experiments in which we extracted bilirubins from biles and intestinal contents of human foetuses of 16–30 weeks' gestation (S. G. Blumenthal, D. B. Taggart, R. D. Rasmussen, R. M. Ikeda, B. H. Ruebner, D. E. Bergstrom & F. W. Hanson, unpublished work).

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References

Blanckaert, N., Heirwegh, K. P. M. & Compernolle, F. (1976) *Biochem. J.* **155**, 405–417

Blanckaert, N., Compernolle, F., Leroy, P., Van-Houtte, R., Fevery, J. & Heirwegh, K. P. M. (1978) *Biochem. J.* **171**, 203–214

Blumenthal, S. G., Ikeda, R. M. & Ruebner, B. H. (1976) *Pediatr. Res.* **10**, 664–668

Blumenthal, S. G., Ruebner, B. H., Ikeda, R. M., Hanson, F. W. & Bergstrom, D. E. (1977*a*) *Experientia* **33**, 592–593

Blumenthal, S. G., Taggart, D. B., Ikeda, R. M., Ruebner, B. H. & Bergstrom, D. E. (1977*b*) *Biochem. J.* **167**, 535–548

Bonnett, R. & McDonagh, A. F. (1970) *J. Chem. Soc.* 238–239

Chowdury, T. R., Jansen, P. L. M., Fischberg, E., Daniller, A. I. & Arias, I. M. (1976) *Gastroenterology* **71**, 901

Compernolle, F., Blanckaert, N. & Heirwegh, K. P. M. (1977) *Biochem. Soc. Trans.* **5**, 317–319

Heirwegh, K. P. M., Fevery, J., Michiels, R., Van Hees, G. P. & Compernolle, F. (1975) *Biochem. J.* **145**, 185–199

McDonagh, A. F. & Assisi, F. (1971) *FEBS Lett.* **18**, 315–317

Noir, B. A., Garay, E. R. & Royer, M. (1965) *Biochim. Biophys. Acta* **100**, 403–410

Strebel, L. & Odell, G. B. (1971) *Pediatr. Res.* **5**, 548–559