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Changes in Bilirubins in Human Prenatal Development

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1. A densitometric method has been developed for the quantification of azodipyrroles derived from dog bile pigments treated with diazotized ethyl anthranilate. 2. This method was used to estimate the bilirubins in bile and meconium from foetuses of 14–36 weeks gestation. 3. The proportion of the bilirubins in foetal bile changed during gestation. (*a*) No bile pigments were found until 14 weeks. (*b*) Between 14 and 15 weeks bilirubin-IXβ was the only bile pigment detected. (*c*) At 16–17 weeks some unconjugated bilirubin-IXα was found in the bile, but up to 20 weeks bilirubin-IXβ was the predominant bilirubin in the bile. (*d*) At about 20 weeks glucose, xylose, and an unidentified bilirubin-IXα monoconjugate were found in the bile. (*e*) Between 20 and 23 weeks bilirubin-IXα glucuronide appeared in the bile. (*f*) At 30 weeks monoconjugates of bilirubin-IXα were the predominant bilirubins in the bile. (*g*) Only in full-term foetuses was bilirubin-IXα monoglucuronide the major bilirubin derivative.

Virtually all the bilirubins in newborn-human and rhesus-monkey-baby biles were found to be monoconjugates of bilirubin-IXα with GlcA, glucose or xylose (Blumenthal *et al.*, 1979). However, small but variable amounts of bilirubin-IXα diglucuronide were found in virtually all biles collected from newborn rhesus monkey term babies and from many of the newborn human term babies. Unconjugated bilirubin-IXβ was also detected in all of these specimens.

Bile pigments excreted into the bile of foetuses *in utero* accumulate in the intestines of the developing foetus. Bilirubins in the intestinal contents of full-term newborn human and rhesus monkey term babies have been found to be predominantly monoconjugates of bilirubin-IXα. Compared with the bile, the intestinal contents contained proportionally more glucosyl and xylosyl than glucuronyl monoconjugates and more bilirubin-IXβ than bilirubin-IXα (Blumenthal *et al.*, 1979).

The aim of the present investigation was to compare the bilirubin pigments in biles and intestinal contents of foetuses and premature babies with those found in full-term babies and adults.

Abbreviation used: GlcA, glucuronic acid.

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Materials and Methods

General

Dog biles were collected from healthy dogs that had been used for blood collection. Biles were drawn from the gall bladder, stored at −75°C in the dark overnight and used the next day.

Bile and intestinal contents were collected from therapeutically and spontaneously aborted human foetuses and from babies at autopsy. The protocols for these studies were approved by the Committee on Research involving Human Subjects. Permission was obtained from the Human Subjects Committee of the School of Medicine, University of California, Davis, CA, U.S.A. Bile samples (5–50μl) were drawn with a syringe from the gall bladder. The intestines were rinsed with 0.9% NaCl, the mesentery was cut, allowing the intestines to be fully extended. The intestines were then divided into four parts. The contents were gently pushed out of the open ends. In this way, samples (25–175 mg each)

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were collected from the duodenum and upper ileum, middle ileum, lower ileum and large intestines. Samples were kept at −75°C in the dark. Biles were reacted with diazotized ethyl *o*-anthranilate on the day of collection or the next morning. Intestinal contents were extracted a day or two after collection, and the extracts were reacted with ethyl *o*-anthranilate.

All solvents used were freshly distilled. Methanol was redistilled over CaH_2. Chloroform was washed with water, dried and distilled. Butyl acetate was redistilled. All water used was twice-glass-distilled deionized water. All chemicals used were of reagent grade.

For t.l.c., precoated (0.25 mm) silica gel 60 plates (20 cm × 20 cm; Merck, Darmstadt, Germany) were used. The chromatograms were developed at room temperature in the dark.

Preparation and purification of azodipyrroles from dog bile

Pure azodipyrrole-IXα, azodipyrrole-IXα–glucose, azodipyrrole-IXα–xylose, azodipyrrole-IXα–GlcA and azodipyrrole-IXβ were prepared from dog bilirubins by reaction with the diazonium salt of ethyl *o*-anthranilate (Heirwegh *et al.*, 1974). Azodipyrroles were purified by using t.l.c. separations as described previously (Blumenthal *et al.*, 1977).

Reaction of bilirubins from bile and intestinal contents (meconium) with diazotized ethyl anthranilate

Human foetal bile samples were diluted with glycine/HCl buffer, pH 2.7 (0.4 M-HCl solution + glycine to pH 2.7) and ethanol (1:2:2, by vol.) in open glass tubes. Diazotized ethyl *o*-anthranilate was added (4–8 drops) to each tube and mixed. The mixtures were left to react for 60 min at room temperature in the dark. At the end of this time 0.3–0.5 ml of butyl acetate was added to the reaction mixtures and mixed well. The reaction was terminated with 5–10 ml of ascorbic acid (10 mg/ml). The tubes were centrifuged for 2.5 min at 3500 rev./min. The butyl acetate layer containing the azodipyrroles was decanted into disposable glass tubes. These were kept at −20°C under argon.

Bilirubins from intestinal-content specimens were extracted before they were reacted with diazotized ethyl *o*-anthranilate. Intestinal contents (25–200 mg) were suspended in 1.0–2.0 ml of glycine/HCl buffer (0.15 M-HCl + glycine), pH 1.7, and saturated with NaCl and 1.0 ml of ethanol. This suspension was mixed and homogenized by using a Polytron homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.) at a power control setting of 5 for 5 s. The homogenate was then extracted two or three times with 1.0 ml portions of butanol. Mixtures were centrifuged at 3500 rev./min for 5 min and the butanol layer containing the extracted pigments was decanted. Butanol extracts of one sample were pooled and the butanol was removed under vacuum after forming an azeotrope with water. Residues were dissolved in 0.1–0.3 ml of methanol for five min and the supernatant decanted into 10 ml tubes. To buffer the solution, 0.5–1.0 ml of glycine/HCl buffer, pH 2.7, was added. The bilirubins present in the mixture were treated with diazotized ethyl *o*-anthranilate as described above.

The procedures for the t.l.c. separation of the various azodipyrroles in the human samples were identical with those used for the preparation of purified dog-bile azodipyrroles.

Calibration of azodipyrroles from dog bile

T.l.c.-purified azodipyrrole-IXα, azodipyrrole-IXα–glucose, azodipyrrole-IXα–xylose, azodipyrrole-IXα–GlcA and azodipyrrole-IXβ were used for calibration. Calibration curves were obtained by reading the absorbance of azodipyrrole spots on t.l.c. plates. Solutions of 22.5 pmol/μl were prepared. Of these solutions, five spots containing 50, 75, 100, 125 and 150 μl of these azodipyrrole solutions were applied on a line 2.0 cm above the edge of a t.l.c. plate. The diameter of each spot was not larger than 3 mm. Plates were developed with solvent systems I and II as described previously (Blumenthal *et al.*, 1977). The plates were scanned with a Kontes densitometer (model K-495000; Kontes, Vineland, NJ, U.S.A.) with a long-wavelength u.v. source (strong emission at 366, 436 and 546 mm). Peaks were recorded with a linear integrative chart recorder (model 252/A; Linear Instrument Corp., Irvine, CA, U.S.A.). Every spot was scanned twice, once from the bottom to top, and then from top to bottom. Relative absorbance was based on peak areas. The mean-area calculation of each spot was determined, and linear regression analysis was then used to establish the calibration curve for each azodipyrrole.

Quantification of azodipyrroles from foetal biles and meconiums

Azodipyrroles obtained from foetal samples were decreased in volume by using a 40°C water bath and a stream of argon. The concentrated solutions were spotted on t.l.c. plates very carefully so that the diameter of each spot was not greater than 3 mm. Many successive applications of 2 μl each were made. It was necessary to dry each spot with a warm air current between applications. The absorbance of the azodipyrrole spots was determined by densitometric scanning after development, as described above.

Calculation of bilirubin proportions by using azopigments

The results of azodipyrrole quantifications with t.l.c.-purified dog bile azodipyrroles were in turn used for the determination of the following proportions of bilirubins from human samples:

$$\frac{\text{Bilirubin-IX}\alpha}{\text{Bilirubin-IX}\beta} = \frac{\alpha_0+\alpha_2+\alpha_3+\gamma_2+\gamma_3+\delta}{2(\beta_x)} \quad (1)$$

where α_0 is unconjugated azodipyrrole, α_2 is azodipyrrole–xylose, α_3 is azodipyrrole–glucose, δ is azodipyrrole–GlcA (1-*O*-acyl-GlcA), β_x is unconjugated azodipyrrole from bilirubin-IXβ (rings A and B of haem) and γ_2 and γ_3 are rearrangement products of δ (Blankaert *et al.*, 1978), identified as the anomers of 2-*O*-acyl-1-ethyl anthranilate *N*-glucuronide by Compernolle *et al.* (1978).

$$\text{Monoconjugated bilirubin-IX}\alpha = \alpha_2+\alpha_3+\gamma_2+\delta \quad (2)$$

$$\text{Unconjugated bilirubin-IX}\alpha = \frac{\alpha_0+\alpha_3+\gamma_2+\gamma_3+\delta}{2} \quad (3)$$

$$\frac{\text{Monoconjugated bilirubin-IX}\alpha}{\text{Unconjugated bilirubin-IX}\beta} = \frac{2(\alpha_2+\alpha_3+\gamma_2+\gamma_3+\delta)}{\alpha_0-(\alpha_2+\alpha_3+\gamma_2+\gamma_3+\delta)} \quad (4)$$

The amount of bilirubin-IXβ was determined from the amount of azodipyrrole β_x in the sample. The amount of conjugated bilirubin-IXα was determined from the total amount of conjugates of azodipyrrole in the sample. The amount of each conjugate, for instance bilirubin-IXα-glucose, was equivalent to the amount of azodipyrrole, for instance azodipyrrole α_3, in the sample. This simplified treatment is possible because virtually all conjugated bilirubin-IXα in human foetuses and premature newborn babies is in the form of monoconjugates. The amount of unconjugated bilirubin-IXα was determined by subtracting the total amount of conjugated azodipyrroles ($\alpha_2+\alpha_3+\delta+\gamma$'s) from the conjugated azodipyrrole (α_0) of the same sample and then dividing the remaining unconjugated azodipyrrole by 2.

Results

Calibration curves for azodipyrroles α_0, α_2, α_3, β_x and δ

Fig. 1 shows calibration curves for azodipyrroles α_0, α_2, α_3, β_x and δ. The curves obtained for azodipyrroles α_0, α_3 and δ were identical but those obtained for azodipyrroles α_2 and β_x were slightly different due to lower absorbance at 546nm by spots of azodipyrroles α_0 or β_x containing the same molar concentrations as azodipyrroles α_0, α_3 or δ.

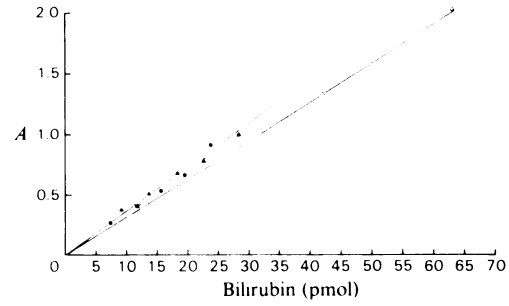

Fig. 1. *Calibration curves prepared for azopigments α_0, α_2, β_x and δ obtained from dog bile bilirubins reacted with diazotized ethyl o-anthranilate*

Absorbance units are arbitrary. They represent the number of integration lines obtained from azopigment spots on t.l.c. plates. Symbols: ●, azodipyrrole α_0; ○, azodipyrrole–xylose α_2; □, azodipyrrole–glucose α_3; △, azodipyrrole β_x; ▲, azodipyrrole–GlcA δ.

Measurements of azodipyrroles α_0 and δ were performed on the same day. Those of azodipyrrole α_3 were performed on different days. Azodipyrroles α_2 and β_x were spotted on different days. Two experiments were done with azodipyrroles α_0, α_2 and δ and three with azodipyrrole β_x.

Gross observations of human foetal biles and meconiums

The bile present in gall bladders of human foetuses was light yellow. No more than 25μl was collected from any one individual. The majority of bile samples were smaller than 15μl per individual bile.

The length of intestine varied according to foetal age. At 20–21 weeks of gestation, the small intestine measured 93–98cm and the large intestine measured 25–26cm. The colour of the small-intestinal contents of human foetuses (ages 15 up to 23 weeks of gestation) was light yellow in the upper third, darker yellow to brownish in the middle third and dark brown to brown–green in the lower third. The large-intestinal contents were colourless or very light yellow in all younger foetuses and colourless with a few specks of brown–green material in foetuses at 12–23 weeks of gestation. The weight of the material collected from the upper third of the small intestines was 25–50mg, and for the middle and lower third it was 100–175mg each. The material collected from the large intestines weighed 50–75mg.

Azodipyrroles in human foetal biles and meconiums

Fig. 2 shows the t.l.c. separations of azodipyrroles obtained from reacting bile and extracts of meconium from a premature baby (*a*) and from a

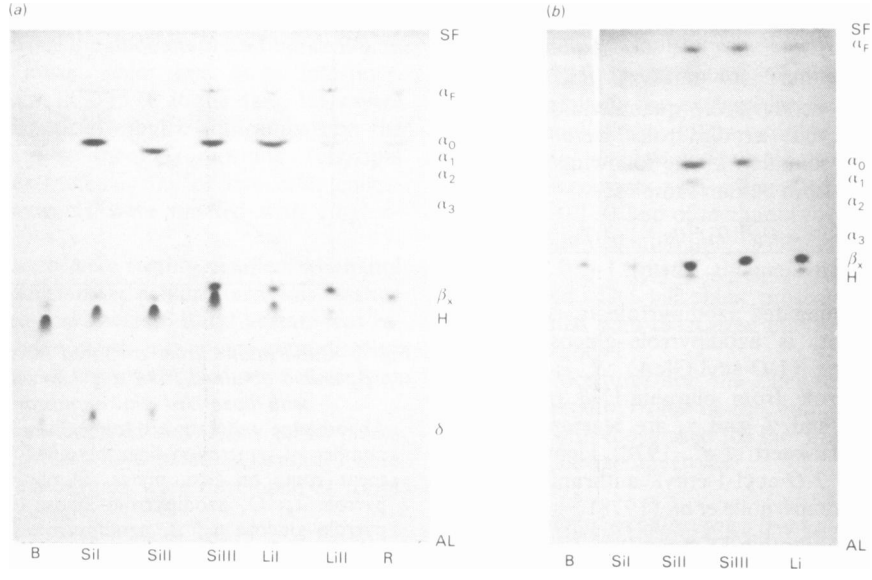

Fig. 2. *T.l.c. separations of azopigments obtained by treatment of human-premature-baby and foetal-baby biles and intestinal contents with diazotized ethyl o-anthranilate*

Gestational ages were (*a*) 23 week-premature baby and (*b*) 16 week foetus. The Greek letters are symbols for the different azopigments found in biles of dog and other animals. Abbreviations: AL, application line; B, bile; Si, small intestine; Li, large intestine; I, upper one-third; II, middle one-third; III, lower one-third; R, rectal; SF, solvent front; H, haem.

foetus (*b*). Azodipyrroles α_0 and β_x were present in bile and intestinal contents of this premature baby. Azodipyrroles γ and α_3 were present in bile and small-intestinal contents of the premature baby, but absent from its large-intestinal contents. Only small amounts of azodipyrrole α_2 were present in the bile and small-intestinal contents of the premature baby. Haem was present in all premature-baby samples, possibly as a result of haemorrhage. Azodipyrrole α_1, as yet unidentified, was present in all the premature-baby samples. Azopigment β_x was the predominant azodipyrrole in the bile, and in small- and large-intestinal contents of the foetus. Azodipyrrole α_0 was present in relatively small amounts in the foetal large-intestinal contents. Azodipyrrole α_3 was present in foetal small- and large-intestinal contents. Azodipyrrole α_2 was not clearly visible, but azodipyrrole α_1 was present in all foetal samples.

Proportions of bilirubin-IXα to bilirubin-IXβ in the biles of foetuses

Fig. 3 shows the proportion of bilirubin-IXα to that of bilirubin-IXβ in the biles of human foetuses aged 16–23 weeks of gestation. Proportions were calculated by using azodipyrroles in separations of biles reacted with diazotized ethyl *o*-anthranilate (see the Materials and Methods section). There was a general correlation between foetal age and the proportion of bilirubin-IXα to bilirubin-IXβ, although the proportions of bilirubin-IXα to bilirubin-IXβ for

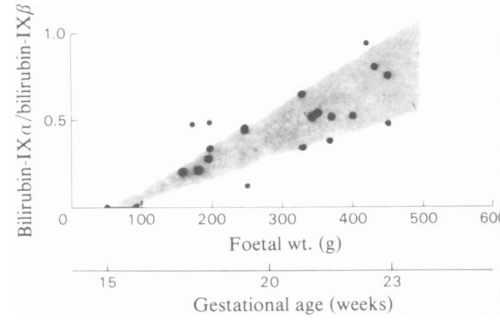

Fig. 3. *Ratio of bilirubin-IXα to bilirubin-IXβ in biles collected from human foetuses at 15–23 weeks of gestation*

Biles were reacted with diazotized ethyl *o*-anthranilate. Azopyrroles were separated on t.l.c. plates. Azodipyrroles-IXα (α_0, α_2, α_3 and δ) and azodipyrrole-IXβ (β_x) were quantified by using the Kontes densitometer. Relative absorbances were based on peak areas. The darker area helps to show the trend obtained.

biles obtained from different individuals of the same age showed considerable variation. The younger the foetus, the higher was the proportion of bilirubin-IXβ in its bile. Biles from foetuses of 14–15 weeks gestation did not contain detectable amounts of bilirubin-IXα.

Proportions of conjugated bilirubin-IXα to unconjugated bilirubin-IXα in the biles of foetuses and premature newborn babies

Fig. 4 shows the proportion of conjugated bilirubin-IXα to unconjugated bilirubin-IXα in biles collected from foetuses, premature babies, and one term newborn baby. Proportions were calculated by using azodipyrroles in t.l.c. separations of biles reacted with diazotized ethyl anthranilate (see the Materials and Methods section). Although there was considerable variation in the proportions of biles collected from individuals of similar ages, there was a general correlation between the age of the individual and the proportion of conjugated bilirubin-IXα to unconjugated bilirubin-IXα in its bile. All biles collected from individuals of less than 20 weeks gestation contained no conjugated bilirubin-IXα. The proportions of conjugated bilirubin-IXα to unconjugated bilirubin-IXα were less than 1 until 29 weeks of gestation (end of third-quarter). In the last 10 weeks of gestation, more conjugated bilirubin-IXα than unconjugated bilirubin-IXα was found in two out of four baby biles tested.

Proportion of bilirubin-IXα to bilirubin-IXβ in the intestinal contents of foetuses

Fig. 5 shows changes in the proportion of bilirubin-IXα to bilirubin-IXβ in intestinal contents collected from different locations of the foetal intestines of five foetuses aged 15–23 weeks of gestation. The proportion of bilirubin-IXα to bilirubin-IXβ was highest in the duodenum and decreased progressively from there. These results also show that the older the foetus, the higher the proportion of bilirubin-IXα to bilirubin-IXβ in its intestinal contents obtained from similar locations.

Bilirubins in the developing foetus

A summary of the various bilirubins in foetal gall-bladder bile at different foetal ages is given in Fig. 6(*a*). The results of this Figure have been given in the synopsis of the present paper. There was a variation of 1 or 2 weeks in the appearance of each new bilirubin in the biles of human foetuses. Therefore the approximate gestational age of the foetus between 15 and 23 weeks gestation can be calculated from the appearance of the bilirubins in the bile. All the bilirubin-IXα conjugates found in foetal biles were in the form of monoconjugated bilirubin-IXα. This was confirmed by preliminary experiments in which bilirubins were extracted and separated from premature stillborn-baby gall-bladder bile and reacted with diazotized ethyl anthranilate. The proportions of bilirubin-IXα to bilirubin-IXβ and of conjugated bilirubin-IXα to unconjugated bilirubin-IXα are given in Fig. 6(*b*). We could not isolate enough t.l.c.-separated bilirubins from any one sample to obtain reliable densitometric measurements of their azodipyrroles. For foetuses under 16 weeks gestation, biles had to be pooled (2–4 biles for each experiment). Extraction of bilirubins from foetal and many premature-baby intestinal contents (upper two-thirds of the small intestines) was complete. Bilirubin-IXβ

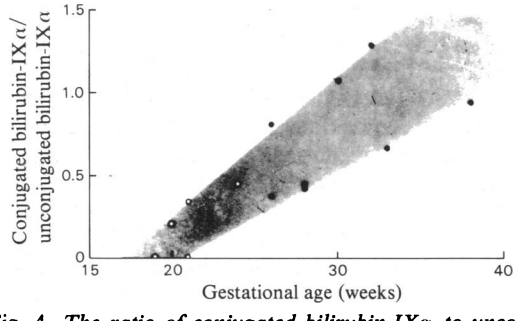

Fig. 4. *The ratio of conjugated bilirubin-IXα to unconjugated bilirubin-IXα in biles collected from human foetuses and premature and term babies*

Biles were reacted with diazotized ethyl *o*-anthranilate. Azodipyrroles were separated on t.l.c. plates. Unconjugated azodipyrrole (α_0) and conjugated azodipyrroles (α_2, α_3 and δ) were quantified by using the Kontes densitometer. Relative absorbances were based on peak areas. Symbols: ○, therapeutically aborted; ●, spontaneously aborted or stillborn. The darker area helps to show the trend obtained.

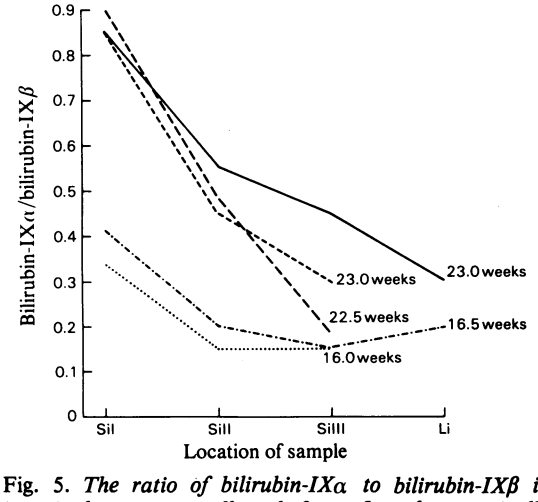

Fig. 5. *The ratio of bilirubin-IXα to bilirubin-IXβ in intestinal contents collected from five therapeutically aborted human foetuses*

Each line depicts the results for one individual foetus. Ages are given in the Figure. Relative absorbance was based on peak areas from densitometry. Abbreviations: Si, small intestine; Li, large intestine; I, upper one-third; II, middle one-third; III, lower one-third.

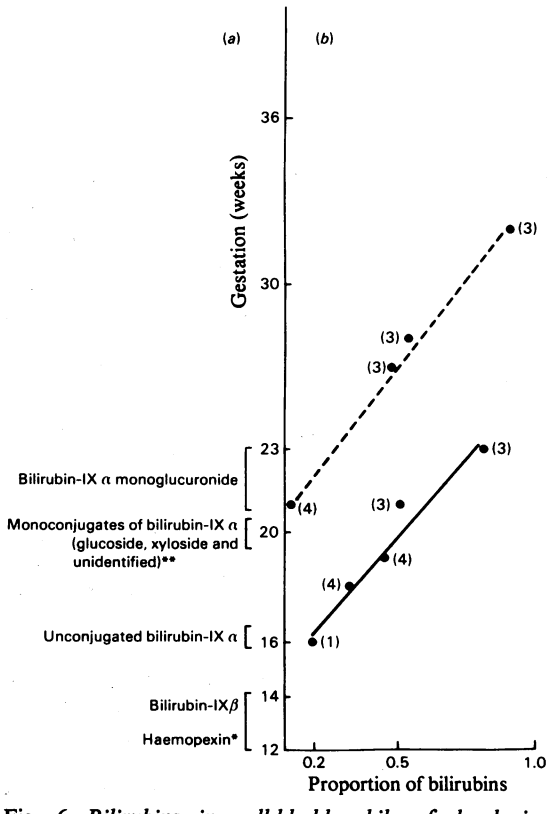

Fig. 6. *Bilirubins in gall-bladder bile of developing human foetuses*

The Figure shows the time at which various bilirubins appear in the bile (*a*) and the changes in ratios of bilirubins during foetal development (*b*). ——, Proportions of bilirubin-IXα to bilirubin-IXβ. – – – –, proportion of monoconjugated bilirubin-IXα to unconjugated bilirubin-IXα. Numbers in brackets represent the number of individual biles used to obtain the mean proportion of the bilirubins. *indicates the results of those of Muller-Eberhardt *et al.* (1975). **indicates an unknown bilirubin-IXα giving rise to azopigments α_0 and α_1 in a ratio of 1 : 1 after treatment with diazotized ethyl *o*-anthranilate.

is virtually the only bile bilirubin in 14–16 week-old foetuses. Bilirubin-IXα was detected in biles of 16 weeks to term babies. By 22 weeks it becomes the predominant bilirubin. Conjugates of bilirubin-IXα were not detected in appreciable amounts until 26 weeks. By 32 weeks they constituted 50% of the total bilirubin-IXα. Points on the curve were obtained by calculating the averages of measurements from three to four individual foetuses.

Discussion

The primary purpose of the present work was to determine the types and relative amounts of unconjugated and mono- and di-conjugated bilirubins excreted in the bile during the development of the human foetus. Determination of absolute amounts of bilirubins was not possible because of the difficulty of quantitative extraction from biological samples and because of differences in concentration of the various bile samples. Neither factor is likely to affect determination of relative amounts of bilirubins. On the other hand two problems, quantitative conversion into azodipyrroles and accurate colorimetric determination [accomplished previously by densitometry on t.l.c. plates (Thompson & Hoffman, 1971, 1973) or elution from silica gel for spectrophotometric determination in solution (Heirwegh *et al.*, 1975)], which could conceivably affect ratios of different bilirubins, have been dealt with successfully. The azodipyrroles were prepared directly from bilirubins in biological samples or their extracts, separated by t.l.c., and their absorbance determined relative to purified standards by densitometry.

The chronological appearance of bilirubins in bile during foetal development (Fig. 6) begins with bilirubin-IXβ, found only in the unconjugated form. It is first observed in foetal bile at 14 weeks gestation. Initially discovered in the bile of adult dogs (Heirwegh *et al.*, 1975), bilirubin-IXβ was subsequently found to occur in adult human and rhesus monkey (Blumenthal *et al.*, 1977), newborn primate baby (Heirwegh *et al.*, 1976; Blumenthal *et al.*, 1979), and newborn pig biles as well as biles of newly hatched chicks and chick embryos (S. G. Blumenthal, R. D. Rasmussen & D. E. Bergstrom, unpublished work). The origin of bilirubin-IXβ has not yet been determined.

At 16 weeks gestation unconjugated bilirubin-IXα appears in human foetal bile. Whether this constitutes the actual production of bilirubin-IXα or whether before 16 weeks bilirubin-IXα is completely transferred through the placenta to the mother (Lester & Troxler, 1969) is not known. The onset of bilirubin-IXα excretion into the bile could represent a maturation of the liver-uptake and biliary-secretion mechanism for bilirubin. This might explain why bilirubin-IXα was not present in bile of foetuses until 16 weeks gestation and why bilirubin-IXα concentration and proportion increase in the bile during foetal development.

Because of the long viability of erythrocytes, not much foetal haemoglobin will be catabolized by week 13 of gestation. Therefore it may be assumed that bilirubins produced up to this time originate mainly from the catabolism of unused haem in haem pools and/or from turnover of haem proteins, but not from haemopoietic haemoproteins. This is supported by our finding that no measurable bilirubins were present in sera collected from foetuses up to 15 weeks gestation, but unconjugated bilirubin-IXα was detected in sera from older foetuses.

The changes observed in the relative amounts of bilirubin-IXα to bilirubin-IXβ in biles of human foetuses are shown in Figs. 3 and 6. A number of observations suggest that the change in the relative proportion of bilirubin-IXα to bilirubin-IXβ with maturation may be the result of other factors besides differential uptake of bilirubin-IXα by the placenta. The ratio of bilirubin-IXα to bilirubin-IXβ was observed to be lower in biles and intestinal contents of sick premature babies that lived for up to 2 weeks than in those obtained from sick term babies that lived for 2 weeks. Furthermore, the proportion of bilirubin-IXα to bilirubin-IXβ was lower in biles and meconiums of newborn puppies and kittens than in biles of adult dogs and cats (S. G. Blumenthal, D. S. Taggart & D. E. Bergstrom, unpublished work). In these mammals the placenta is not permeable to bilirubin-IXα (Bernstein *et al.*, 1969).

The appearance in bile of conjugated bilirubins implies the active functioning of the following: haem oxygenase; biliverdin reductase; plasma transport as well as liver uptake proteins like haemopexin (Muller-Eberhard *et al.*, 1975), albumin, ligandin (Arias *et al.*, 1976), Z protein; UDP-glycosyltransferase (Vaisman *et al.*, 1976). When the first two enzymes are activated the foetus begins to produce bilirubins. The appearance of bilirubins in bile at 14 weeks of gestation indicated that bilirubin was being produced by the foetus and that plasma transport and biliary-secretion mechanisms were active at that time. At 20 weeks the first conjugates of bilirubin-IXα appeared in the foetal bile. The activity of UDP-glycosyltransferase must have been very low during that gestational period because only 10% of the excreted bilirubin-IXα was conjugated. The first conjugates of bilirubin-IXα in biles were monoconjugates of glucose, xylose and another, as yet unidentified, monoconjugate which was the predominant monoconjugate in foetal bile from 20 to 22 weeks gestation. Bilirubin-IXα monoglucuronide was first observed at 22–23 weeks gestation. It was the predominant bilirubin excreted in the bile of term newborn babies. Between 34 and 38 weeks gestation the majority of bilirubin-IXα in foetal bile appeared in conjugated form. These findings suggest that UDP-glycosyltransferase became active in the foetal liver at about 20 weeks and that the activity increased during the next 3 weeks. The changes in the types of the monoconjugates found in foetal bile during development may be a result of a change in the availability of UDP-sugars in the liver or the possible involvement of several specific UDP-transferase enzymes. Fyffe & Dutton (1975) have stated that glucuronidation in intact tissues or cells involves synthesis of nucleotide, and that both the transferase and UDP-glucose dehydrogenase may be considered rate limiting in the natural development of glucuronidation. Vaisman *et al.* (1976) reported that the livers of 1 day-old rats conjugated bilirubin with GlcA at about 66% the rate of that found in the livers of adult rats, and that xylose and glucose were conjugated to bilirubin at about the same rates as GlcA by liver homogenates from 1 day-old rats. However, this proportion changed rapidly to that of the adult rat, which conjugated bilirubin with GlcA three times as actively as with glucose and xylose combined. They suggested that glucose and xylose conjugation compensates, in part, for deficient GlcA conjugation, but they did not clarify whether one or more enzymes were involved in the conjugation process. The present observation that bilirubins excreted in foetal bile change from monoconjugates of glucose, xylose and another ester to monoconjugates of GlcA during the maturation of foetuses agrees well with the findings of Vaisman *et al.* (1976). A very low activity of UDP-glucuronyltransferase was observed in livers of first-trimester and older human foetuses (Felsher *et al.*, 1978) and in livers of rat foetuses (Grimmer *et al.*, 1978). Unfortunately these authors did not test UDP-glycosyltransferase activity for UDP-glucose or UDP-xylose. We suggested previously that only a non-specific transferase enzymes may be actively producing monoconjugates of bilirubin in human and rhesus-monkey foetal livers until birth (Blumenthal *et al.*, 1979).

The relative amounts of the isomers of bilirubin-IXα in biles of foetuses closely resemble the ratios found in the small intestines of older foetuses as well as that in the large intestines of premature newborn and term babies. Therefore, analysis of bilirubins from intestinal contents located at different levels of the intestines may be used to deduce the bilirubin composition of bile at earlier stages of the development of the individual.

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