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Reduction of biliverdin and placental transfer of bilirubin and biliverdin in the pregnant guinea pig

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Biliverdin was reduced to bilirubin in pregnant and foetal guinea pigs, and the 100000 ***g*** supernatant from homogenates of foetal liver, placenta and maternal liver showed high biliverdin reductase activity. The placental transport of unconjugated bilirubin and biliverdin was compared by injecting unlabelled and radiolabelled pigments into the foetal or maternal circulation and analysing blood collected from the opposite side of the placenta. Injected bilirubin crossed the placenta from foetus to mother and vice versa, but injected biliverdin did not appear to cross without prior reduction to bilirubin. The guinea-pig placenta is apparently more permeable to bilirubin than biliverdin. Reduction of biliverdin to bilirubin in the foetus may, therefore, be essential for efficient elimination of haem catabolites from the foetus in placental mammals.

All vertebrates degrade haem to biliverdin. In some, the biliverdin is excreted in bile unchanged (Colleran & O'Carra, 1977). In others, notably mammals, the biliverdin is reduced enzymically to bilirubin. This reduction is rather curious. It involves conversion of a readily excretable and apparently harmless metabolic waste-product to a substance that is potentially toxic (McDonagh, 1979), that requires further metabolic alteration for its efficient excretion and that has no known biological function. Why it occurs is unknown.

One of us has suggested that enzymic reduction of biliverdin to bilirubin in mammals may have evolved to facilitate excretion of bile pigments by the foetus (Schmid, 1976). This hypothesis was based on the assumption that only bilirubin, the more lipophilic of the two pigments (McDonagh, 1979), can cross the placenta. Several studies have shown that the placenta is permeable to bilirubin and, indeed, in some mammals transfer of bilirubin from foetus to mother appears to be an important route for the disposal of haem catabolites formed in the foetus (Lester *et al.*, 1963; Schenker *et al.*, 1964; Bashore *et al.*, 1969; Bernstein *et al.*, 1969). However, the metabolism of biliverdin in the foeto-placental unit and the placental transport of biliverdin have not been examined. The present studies were undertaken to investigate biliverdin reduction in the foetus and, by comparing the permeability of the placenta to biliverdin and bilirubin, determine whether, in fact, the placenta represents a real barrier to biliverdin.

The species chosen was the guinea pig, which has been used previously for studying the placental transport of bilirubin (Schenker *et al.*, 1964). In the initial studies the rate of biliverdin reduction in the full-term foetus and the activity of biliverdin reductase (EC 1.3.1.24) in foetal liver and placenta were examined. Then the transfer of biliverdin and bilirubin from mother to foetus was studied by injecting large doses of each compound into the maternal circulation and analysing foetal serum samples during the subsequent brief period that the pigment concentrations in the maternal plasma remained high. Transport in the reverse direction, foetus-to-mother, was investigated by three methods. (1) Unlabelled pigments were injected into the foetal circulation and serial blood samples collected rapidly from the maternal side of the placenta were analysed. (2) [^{14}C]Biliverdin and [^{3}H]bilirubin were injected separately or together into the foetal circulation and the subsequent appearance of the radioisotopes in maternal bile was monitored. (3) [^{3}H]Bilirubin and [^{14}C]biliverdin were injected simultaneously into the foetal circulation and the relative rate at which the two isotopes crossed the placenta was determined by measuring the $^{3}H/^{14}C$ ratio in whole blood or plasma collected directly from the placenta on the maternal side.

The comparative placental transport studies were greatly complicated by the reduction of biliverdin to bilirubin *in vivo*, which turned out to be much more rapid than suggested by previous work (Goldstein & Lester, 1964). This reduction made it difficult to design unequivocal experiments. Nonetheless, collec-

tively our findings show that the guinea-pig placenta, which is permeable to bilirubin (Schenker *et al.*, 1964), is relatively impermeable to biliverdin. They also indicate that, if placental elimination of biliverdin occurs at all, it is slow compared with the rate at which the pigment is reduced.

Materials and methods

Materials, instrumentation, and analyses

Crystalline bilirubin, crystalline biliverdin and [^{14}C]biliverdin were prepared as previously described (McDonagh & Assisi, 1972; McDonagh & Palma, 1980). Crystalline [^{3}H]bilirubin was prepared biosynthetically in rats from 5-amino[3,5-^{3}H]laevulinic acid hydrochloride (Amersham/Searle, Arlington Heights, IL, U.S.A.) (McDonagh, 1979). Tetramethylammonium hydroxide pentahydrate, Trizma base, bovine serum albumin and NADPH (tetrasodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{14}C]Toluene and [^{3}H]toluene (internal standards for liquid-scintillation counting), Protosol (a 0.5 M quaternary ammonium hydroxide solution) and Aquasol were from New England Nuclear Corporation (Boston, MA, U.S.A.). Beckman Ready-Solv HP was obtained from Beckman Instruments Inc. (Fullerton, CA, U.S.A.). Water was distilled from glass and saline refers to 0.89% (w/v) NaCl in water. Other chemicals were analytical-reagent grade from Mallinckrodt Inc. (St. Louis, MO, U.S.A.). Guinea-pig serum, obtained from adult male animals, was used at once or after storage at 4°C for not more than 3 days. Spectrophotometric measurements were made in 1 cm path-length quartz cuvettes with a Cary 118 C (Varian Associates, Palo Alto, CA, U.S.A.) or Aminco DW2 (American Instrument Company, Silver Springs, MD, U.S.A.) spectrophotometer.

Serum bilirubin and biliverdin estimations

Bilirubin was detected qualitatively in serum samples by a peak or shoulder near 470 nm in the absorption spectrum after 1:10 dilution with 0.1 M-Tris buffer, pH 7.4. The total bilirubin concentration of serum samples was measured by a modified Malloy–Evelyn diazo procedure (McDonagh, 1979). Interference by biliverdin was negligible at the concentrations encountered. Serum samples for biliverdin analysis, clarified by ultracentrifugation if turbid, were diluted at least 1:10 with 0.1 M-Tris buffer, pH 7.4, and scanned spectrophotometrically from 800 to 400 nm. Biliverdin concentrations were calculated from the absorbance at $\lambda_{max.}$ (670–685 nm) with $\varepsilon = 12.8 \times 10^3$ litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} (McDonagh & Palma, 1980). Interference due to mild haemolysis or the presence of bilirubin was negligible.

Preparation of radioactive samples for counting

Radioactive bilirubin (in chloroform), biliverdin (in 0.1 M-NaOH), serum, plasma, bile and tissue homogenates were bleached by treatment with aqueous tetramethylammonium hydroxide (1 M, 0.3 ml) and H_2O_2 (30%, v/v; 0.3 ml) for 30 min in count vials by the procedure of Helman *et al.* (1974). Sample volumes were 2–5 μl for pure pigment solutions, 0.25–0.5 ml for serum, plasma and bile, 1 ml for foetal homogenates and 3 ml for placental homogenates. HCl (3 M, 0.3 ml) and aqueous ascorbic acid solution (150 g/litre, 0.3 ml) were added with thorough mixing, followed by Beckman Ready-Solve HP (15 ml) or, for foetal and placental homogenates, Aquasol (20 ml) and, if necessary, 0.5–1.0 ml of water to clarify the mixture. Whole blood required more vigorous digestion and decolorization. Protosol/ethanol (1:2, v/v; 1 ml) was added to the blood sample in a counting vial and the mixture was incubated in a shaking water bath at 60°C for 1 h. The vial was cooled and 30% (v/v) H_2O_2 (0.5 ml) was added dropwise. After further incubation at 60°C for 30 min, Biofluor (15 ml) and 0.5 M-HCl (0.5 ml) were added in sequence, with vigorous mixing after each addition.

Radioactive samples were counted for at least 10 min with [^{3}H]- or [^{14}C]-toluene as internal standard. Counts less than twice the background were considered insignificant. Counting efficiencies were 57–67% for ^{14}C and 26–37% for ^{3}H. Count recoveries, determined by adding pure radioactive pigments to unlabelled samples *in vitro*, were quantitative except for foetal and placental homogenates, for which the recovery (based on added [^{3}H]bilirubin) was 89%. Count recoveries for whole-blood samples varied according to the sample size. With 0.1 ml samples recovery was quantitative, but with 0.5 ml samples it ranged from 80 to 90% for both ^{3}H and ^{14}C.

Animal studies

Late-term pregnant albino guinea pigs (1–2 kg) (Cloverdale Farms, Hollister, CA, U.S.A., and Simonsen Laboratories, Gilroy, CA, U.S.A.) were used. Anaesthesia was induced by ketamine hydrochloride (Bristol Laboratories, Syracuse, NY, U.S.A.) (40 mg/kg body wt.) in conjunction with acepromazine maleate (Ayerst Laboratories, New York, NY, U.S.A.) (dose, 2 mg), administered as separate intramuscular injections into a hind leg (Shucard *et al.*, 1975). This anaesthetized the animals for 4–5 h. Before surgery xylocaine hydrochloride (1%) (Astra Pharmaceutical Products, Worcester, MA, U.S.A.) was administered subcutaneously at the incision site to minimize reflex movements (Shucard *et al.*, 1975). In maternal-to-foetal transfer experiments, bile-pigment solutions were infused at 1 ml/min through an indwelling

PE10 catheter inserted (12–13 cm insertion length) into a femoral vein of the mother. Blood samples from pregnant and foetal guinea pigs were obtained by cardiac puncture. In a few instances, when cardiac puncture of the foetus was unsuccessful or yielded insufficient blood, the umbilical cord was rapidly clamped and cut, the head of the separated foetus was cut off with scissors, and blood dripping from the head and carcass was collected.

Solutions of unlabelled bilirubin or biliverdin for intravenous injection were prepared by dissolving the crystalline pigment in 0.1 M-NaOH (27 mg of pigment/ml) and adding this solution dropwise and with gentle agitation to guinea-pig serum to give a final pigment concentration of 3–8 mg/ml. For single-label radioisotope experiments [^{3}H]bilirubin or [^{14}C]biliverdin [$(0.3–8.5) \times 10^4$ d.p.m./μg] was dissolved in 0.1 M-NaOH (about 60 μg of pigment/0.1 ml) and diluted at once with guinea-pig serum to give a pigment concentration of 26.8 μg/ml; 0.75 ml portions were injected. For dual-label experiments, solutions containing equimolar quantities of [^{3}H]bilirubin [20 μg, $(0.85–2.6) \times 10^5$ d.p.m./μg] and [^{14}C]biliverdin [20 μg, $(1.7–3.1) \times 10^4$ d.p.m./μg] in guinea-pig serum (0.75 ml) were prepared as for single-label studies and 0.3–0.75 ml portions were injected. Freshly prepared pigment solutions were used in all experiments. Solutions of bile pigments were protected from light and experiments were carried out under low-intensity diffuse artificial light.

Foetal guinea pigs were exteriorized and injected by a modification of the technique devised by Schenker *et al.* (1964). In this technique the foetus to be injected is delivered by Caesarian section under saline without disrupting the placental circulation. Provided that the foetus is kept submerged and not allowed to breathe the placental circulation remains intact and the foetus remains viable throughout the experiment. The abdomen of the anaesthetized pregnant mother was shaved from the pubis to just below the rib case and a midline abdominal incision, about 5 cm long, was made starting about 2.5 cm above the pubis. This incision was then drawn together with three or four loose sutures, and the animal was placed in a specially constructed wire basket, its feet secured to the sides of the basket with rubber bands. The basket was designed so that the guinea pig could be held recumbent at an approximately 45° angle with its limbs spread apart allowing easy access to the abdominal region. The guinea pig in the basket was then lowered into a saline bath at 37°C until all of the body except the head, neck and forelimbs was submerged. The loose abdominal sutures were removed, and a foetus was delivered into the bath through an incision in the uterus. With the foetus completely submerged, the amnion was cut away from the foetus and umbilical cord, and the vitelline circulation was interrupted by tying a ligature around the vitelline stalk close to the umbilical cord (Kaufmann & Davidoff, 1977). Bile-pigment solutions were infused over 30–90 s into the umbilical vein in the direction of the foetus by using a 27-gauge needle bent at a right angle close to the syringe. After withdrawal of the needle, leakage from the site of injection was prevented by applying gentle pressure with the thumb and forefinger for not more than 1 min. During each experiment the condition of the foetus was monitored by gentle palpation of the umbilical cord or precardial region.

Biliverdin reductase in placenta and foetal liver

Two foetuses and their placentas were removed from a pregnant female. Each foetus was decapitated and, after blood had drained from the carcass, the liver was excised. The placenta was trimmed free from attached amnion and umbilical cord. The mother was exsanguinated by cutting the hepatic artery and vein and a sample of liver was removed. The tissues were immersed at once in ice-cold potassium phosphate buffer (0.02 M, pH 7.4) containing 0.134 M-KCl, and weighed samples (5 g for liver, 4 g for placenta) were homogenized with KCl/phosphate buffer in a glass homogenizer with a Teflon pestle to give a 20% homogenate, which was then centrifuged (12000 *g* for 10 min, supernatant then re-centrifuged at 100000 *g* for 60 min). Biliverdin reductase activity of the final supernatant was followed at room temperature by absorbance difference spectroscopy from 350 to 800 nm, using an assay mixture containing potassium phosphate buffer (0.1 M, pH 7.4), biliverdin (5 μM), bovine serum albumin (14.5 μM), NADPH (100 μM) and supernatant (0.1 ml, diluted 1:1 for maternal liver) in a final volume of 1 ml (Noguchi *et al.*, 1979). Because of the complex kinetics of biliverdin reductase (Colleran & O'Carra, 1977; Noguchi *et al.*, 1979), enzyme activity in the three tissues was compared by using $t_{\frac{1}{4}}$ values, where $t_{\frac{1}{4}}$ is the time required for reduction of 50% of the biliverdin in the assay mixture expressed per mg of supernatant protein. The validity of this method has been substantiated by Noguchi *et al.* (1979). Values of $t_{\frac{1}{4}}$ were computed from the loss of biliverdin in the difference spectrum at 675 nm. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Reduction of biliverdin to bilirubin in the foetus

Biliverdin (1.5 mg) in serum (0.5 ml) was injected into a foetus and two timed blood samples were removed by cardiac puncture for bile-pigment analyses, the first with the placental circulation intact, the second after cutting the cord and removing the foetus from the bath. Two other sibling

foetuses were treated similarly, and a fourth was injected with 0.5 ml of serum containing no biliverdin as a control. In two additional experiments the dose of biliverdin was doubled (3.0 mg/0.5 ml) and pigment reduction was determined in a single foetus per uterus.

Maternal-to-foetal transfer of bilirubin and biliverdin

After a control blood sample had been obtained from the mother, a single foetus was exposed and a control foetal blood sample was drawn. The umbilical cord was cut and the foetus discarded. Bilirubin or biliverdin in guinea-pig serum was then infused into the mother and blood samples, for bilirubin and biliverdin determination, were collected at timed intervals from the mother and from each of the remaining foetuses in sequence. The following doses (mg of pigment/ml of serum) were used: bilirubin, 10/2.5, 20/5.0; biliverdin, 10/2.5 (three experiments), 20/5.0 (two experiments), 20/2.5 (two experiments), 40/10 (two experiments).

Transfer of bilirubin and biliverdin from foetal to maternal blood

A foetus with intact placental circulation was exteriorized under saline and the placenta was positioned so that its maternal side was just above the surface of the saline bath. A short length of surgical thread was looped loosely around the principal maternal intramural vein of the placenta, and auxiliary intramural veins were ligated to ensure that all blood flowing from the placenta to the mother passed through the unblocked vein. The flow of blood through this vein was transiently interrupted by raising the thread looped around it and a 5–6 cm length of PE90 tubing moistened internally with heparin was inserted through a small transverse incision in the vessel wall. The thread was then released and blood was collected continuously in 20–60 s batches into sampling tubes or directly into count vials containing Protosol/ethanol. If blood clotted in the cannula, the cannula was replaced. If blood clotted in the vein, the ligature around an adjacent ligated intramural vein of the same placenta was removed and this vein was cannulated, allowing blood collection to be continued with little interruption. After control samples had been collected, bile pigment solutions were injected into the foetal circulation and collection of blood from the maternal intramural vein was continued for 10–20 min. Then the umbilical cord was clamped and cut and a blood sample obtained from the separated foetus. Blood or plasma samples were analysed for bilirubin and biliverdin or counted for ^{3}H and ^{14}C as appropriate. Unlabelled biliverdin (3 mg in 0.5 ml of serum) was injected in two experiments, unlabelled bilirubin (3 mg/0.5 ml) in one, and serum (0.75 ml) containing [^{3}H]bilirubin (20 μg) and [^{14}C]biliverdin (20 μg) was injected in five experiments. As a control for the dual-labelled experiments, the post-injection isotope ratio in foetal plasma was determined in separate experiments as follows. Labelled pigments were injected simultaneously into an exteriorized foetus under saline and blood was collected from the submerged foetus at a timed interval after injection for measurement of the plasma $^{3}H/^{14}C$ ratio.

Transfer of bilirubin and biliverdin from foetal blood to maternal bile

The common bile duct of the mother was cannulated (Schenker *et al.*, 1964) and a foetus was exteriorized under saline. Radiolabelled bilirubin (20 μg) and/or biliverdin (20 μg) was injected into the umbilical vein, and maternal bile was collected continuously in 15 min batches for 2 h. At the end of the experiment the umbilical cord was clamped and cut, and the foetus and placenta were removed and homogenized in a blender with water (80 ml and 5–10 ml respectively). The foetal and placental homogenates were diluted with water to 300 ml and 50 ml respectively, and portions of bile and tissue homogenates were counted.

Results

Reduction of biliverdin to bilirubin

Supernatant fractions prepared from homogenates of maternal liver, foetal liver and placenta exhibited pronounced biliverdin reductase activity in the presence of NADPH. Difference spectra run during the enzyme assay showed a broad biliverdin loss peak at 675 nm, a sharp bilirubin synthesis peak at 466 nm, and tight isosbestic points at 405 and 525 nm (Fig. 1). Characteristically (Colleran & O'Carra, 1977; Noguchi *et al.*, 1979) the rate of bilirubin formation by each preparation increased with time until the substrate had disappeared. Formation of bilirubin was quantitative with placental and maternal liver preparations, but with foetal liver, only about 75% of the substrate was reduced to bilirubin, the remainder apparently being degraded by competing NADPH-independent side reactions of unknown nature. The enzyme activity of maternal liver, expressed per mg of soluble protein, was approximately twice that of placenta and foetal liver.

Biliverdin also was reduced to bilirubin in foetal guinea pigs *in vivo* (Table 1). Injected biliverdin (1.5–3.0 mg) gradually disappeared from foetal serum with the concomitant appearance of bilirubin, identified by its diazo reactivity and absorbance near 470 nm. Foetal hyperbilirubinaemia was detectable 6 min after injecting biliverdin and persisted for at least 35 min.

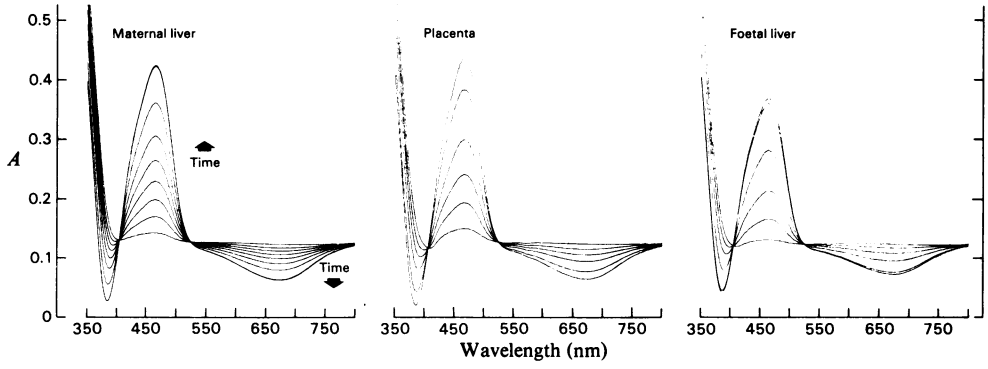

Fig. 1. *Reduction of biliverdin by biliverdin reductase preparations from maternal and foetal liver and placenta*
Sample and reference cuvettes contained biliverdin (5 μM), bovine serum albumin (14.5 μM), phosphate buffer (0.1 M, pH 7.4) and 100000 *g* supernatant from the homogenized tissue (0.1 ml, diluted 1:1 with phosphate buffer for maternal liver) in a final volume of 1 ml. At zero time 2 mM-NADPH solution (0.05 ml) was added to the sample cuvette and spectra were scanned repetitively from 800 to 350 nm (cycle time 3 min).

Table 1. *Reduction of biliverdin to bilirubin in foetal guinea pigs*
Foetal guinea pigs with the placental circulation intact were exteriorized under saline at 37°C and biliverdin (1.5–3.0 mg in 0.5 ml of serum) was injected into the umbilical vein. At the times indicated blood samples were drawn from the foetus by cardiac puncture. Foetal serum was assayed for total bilirubin by a diazo procedure and, after dilution with 0.1 M-Tris buffer, pH 7.4, examined spectroscopically for biliverdin and bilirubin.

Biliverdin injected (mg)	Time after injection (min)	Serum bilirubin concentration (mg/100 ml)	Serum colour	Spectroscopic analysis	
				Biliverdin	Bilirubin
3	17	3.6	Green	+	+
3	22	3.2	Green	+	+
1.5	6	1.2	Green	+	+
	21*	1.5	Yellow	+	+
1.5	16	1.7	Green–yellow	+	+
1.5	18	1.0	Green–yellow		
	35*	1.0	Yellow	Weak	+
0	14	0.2	Colourless	–	–
	19*	0.1	Colourless	–	–

* Blood samples drawn after cutting the umbilical cord and removing the foetus from the saline bath.

Maternal-to-foetal transfer

Increased concentrations of bilirubin were detectable in foetal serum less than 13 min after bilirubin infusion was begun in the mother (Fig. 2). In contrast, when similar or even greater doses of biliverdin were infused into the mother, biliverdin remained undetectable in foetal serum even though the maternal biliverdin concentration remained high for more than 30 min (Fig. 3). However, in these experiments reduction of biliverdin in the mother led to maternal hyperbilirubinaemia and eventually there was an increase in the concentration of bilirubin in foetal serum (Fig. 4).

Foetal-to-maternal transfer

When bilirubin (3 mg) was injected into a foetus with an intact placental circulation and maternal blood was collected directly from an intramural vein of the placenta, bilirubin became spectroscopically detectable in maternal serum within 7 min after injection (Table 2). In contrast, when the same amount of biliverdin was injected, biliverdin remained undetectable in maternal serum even though the concentration of biliverdin in foetal serum remained high for the duration of the experiment.

When equimolar quantities of [^{3}H]bilirubin and [^{14}C]biliverdin were injected simultaneously into the foetus, both isotopes were detectable in maternal blood collected from a placental vein within 4 min of starting the injection. Invariably ^{3}H was detectable first, 30 s to 2.5 min after the injection, and there was

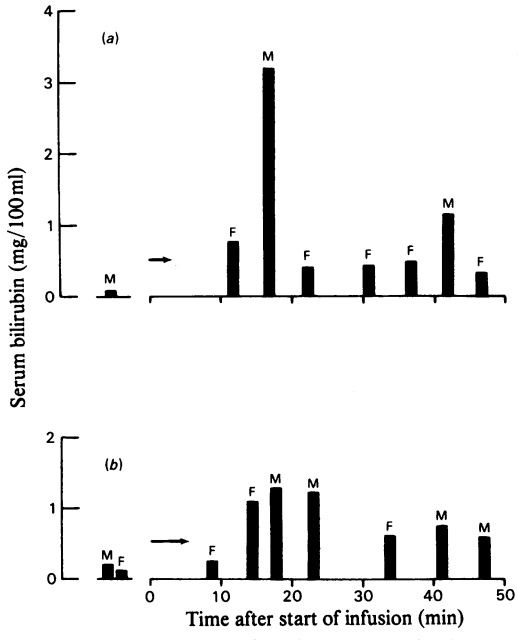

Fig. 2. *Maternal and foetal serum bilirubin concentrations before and after intravenous infusion of bilirubin into pregnant guinea pigs*
The arrows indicate the duration of each infusion; M and F represent mother and foetus respectively. The doses of bilirubin were 10mg (*a*) and 20mg (*b*).

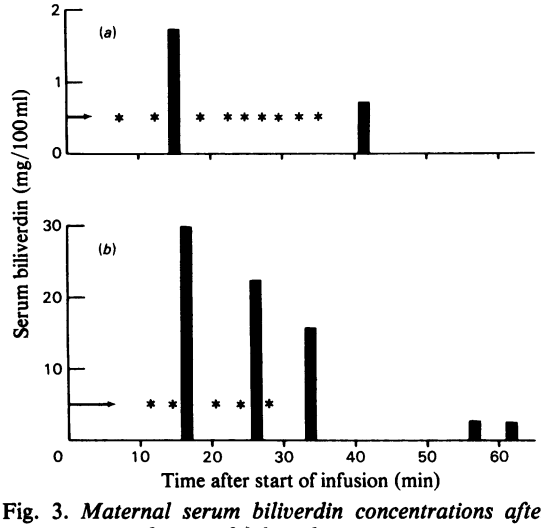

Fig. 3. *Maternal serum biliverdin concentrations after intravenous infusion of biliverdin into pregnant guinea pigs*
The arrows indicate the duration of each infusion. At the times marked by asterisks, foetal serum samples were collected and analysed spectroscopically for biliverdin; none was detectable. The doses of biliverdin were 10mg (*a*) and 40mg (*b*).

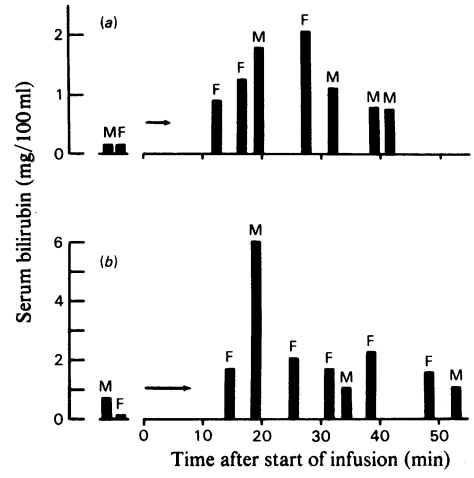

Fig. 4. *Maternal and foetal serum bilirubin concentrations before and after intravenous infusion of biliverdin into pregnant guinea pigs*
The arrows indicate the duration of each infusion; M and F represent mother and foetus respectively. The doses of biliverdin were 10mg (*a*) and 20mg (*b*).

a delay of 30s to 3min before significant ^{14}C radioactivity was detectable. The isotope ratio (d.p.m. of ^{3}H/d.p.m. of ^{14}C) in blood or plasma collected from the maternal side of the placenta was much larger than in the injected mixture shortly after injection, but decreased rapidly with time (Fig. 5). By the end of each experiment, the ratio was still somewhat larger than in the injected solution and considerably larger than the ratio in foetal plasma. Control studies showed that the ratio of $^{3}H/^{14}C$ in foetal whole blood within 1–24min of injecting [^{3}H]bilirubin and [^{14}C]biliverdin into the foetal circulation remained comparable with that in the injected mixture, but the ratio in foetal plasma was substantially smaller (Table 3) due to preferential uptake of bilirubin over biliverdin by foetal erythrocytes (A. F. McDonagh & L. A. Palma, unpublished work).

After the injection of [^{3}H]bilirubin and [^{14}C]biliverdin into the foetal circulation ^{3}H and ^{14}C were excreted in maternal bile (Table 4). The excreted labelled material, which was not isolated or identified, was assumed to be predominantly conjugated bilirubin (Schenker *et al.*, 1964). When the two pigments were injected simultaneously and serial bile samples were analysed at 15min intervals, there was no detectable difference in the excretion rate of the two isotopes (Fig. 6). The $^{3}H:^{14}C$ ratio in maternal bile remained essentially constant throughout the experiment and similar to the ratio in the injected solution.

Table 2. *Transfer of biliverdin and bilirubin from foetal to maternal blood*

Foetal guinea pigs with an intact placental circulation were exteriorized under saline at 37°C and bilirubin or biliverdin (3 mg in 0.5 ml of serum) was injected into the umbilical vein. Foetal and maternal blood samples were collected at the times indicated and analysed spectrophotometrically for biliverdin and by a diazo procedure for bilirubin. Foetal blood samples were collected by cardiac puncture; maternal blood samples were collected from a cannulated intramural placental vein.

Pigment injected	Serum	Time after injection (min)	Colour of serum	Bilirubin concentration (mg/100 ml)	Biliverdin concentration (mg/100 ml)
Biliverdin	Foetal	17	Green	3.6	40.2
	Maternal	0–12*	Colourless	<0.14	Undetectable
Biliverdin	Foetal	22	Green	3.2	16.6
	Maternal	0–18†	Colourless	<0.13	Undetectable
Bilirubin	Foetal	14	Yellow	1.8	
	Maternal	0–2	Colourless	0.23	
		2–4	Colourless	0.20	
		4–6	Colourless	0.25	
		6–7	Yellow	0.81	
		7–8	Yellow	0.92	
		8–9	Yellow	1.03	
		9–10	Yellow	0.65	

* Twelve individual samples were collected at 1 min intervals; alternate samples were assayed for bilirubin.

† Individual 1 min samples were collected; quantitative bilirubin determinations were performed on samples collected between 1 and 2, 3 and 4, 11 and 12 and 17 and 18 min.

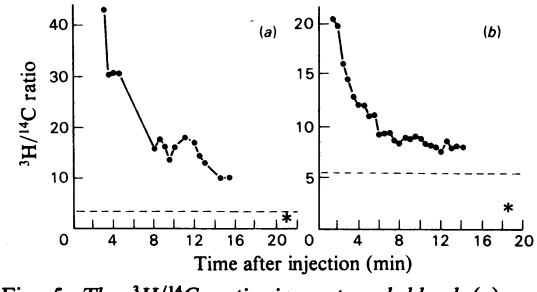

Fig. 5. *The $^3H/^{14}C$ ratio in maternal blood (a) and maternal plasma (b) after injecting [^3H]bilirubin and [^{14}C]biliverdin simultaneously into the foetal circulation*

Maternal blood was collected directly from an intramural vein of the placenta. The broken line indicates the $^3H/^{14}C$ ratio in the injected solution. The asterisk indicates the $^3H/^{14}C$ ratio in plasma collected from the injected foetus at the end of the experiment. Each curve represents a separate experiment.

Table 3 *Isotope ratio in foetal blood and plasma after injecting mixtures of [^3H]bilirubin and [^{14}C]biliverdin intravenously into foetal guinea pigs*

Foetal guinea pigs with an intact placental circulation were exteriorized under saline at 37°C and equal amounts (20 μg) of [^{3}H]bilirubin and [^{14}C]biliverdin of different specific radioactivities were injected into the umbilical vein. At the times indicated a blood sample was collected from the injected foetus and the $^3H/^{14}C$ ratio in foetal blood and plasma was measured.

Time after injection (min)	$^3H/^{14}C$ ratio		
	Injected	Foetal blood	Foetal plasma
1.3	6.6	6.0	1.9
1.5	6.6	3.8	1.6
2–3	5.7	5.8	1.9
5	4.9	4.2	2.1
14	4.9	3.6	1.6
21	3.6	5.5	2.0
22	5.2	—	1.7
24	4.5	5.2	2.0

Discussion

Biliverdin reductase was present in foetal liver and placenta, and exogenous biliverdin was reduced rapidly to bilirubin in the foeto-placental unit *in vivo*. The enzyme activities of placenta and foetal liver were less than that of maternal liver, yet of the same order of magnitude. The detection of high enzyme activity in guinea-pig placenta confirms a previous report by Colleran & O'Carra (1977) for human placenta, but differs from the observations of Krasner *et al.* (1971), who were unable to detect enzyme activity in murine and human placenta.

Despite the reduction of biliverdin to bilirubin in foetus and mother and the rapid disappearance of

Table 4. *Isotope recovery 2 h after injecting radiolabelled bile pigments into foetal guinea pigs*

The common bile duct of a late-term pregnant guinea pig was cannulated and a foetus was exteriorized under saline at 37°C without interrupting the placental circulation. Radiolabelled bilirubin (20 μg) and/or biliverdin (20 μg) were injected into the umbilical vein and bile was collected for 2 h. The foetus and attached placenta were removed and homogenized in water and the total radioactivity in bile, foetus and placenta was measured.

	Percentage recovery of injected dose			
Pigment injected	Maternal bile	Foetus	Placenta	Total
[^{3}H]Bilirubin	52	36	0.5	88
[^{3}H]Bilirubin	65	35	0.9	101
[^{3}H]Bilirubin	18	70	1.0	89
[^{14}C]Biliverdin	23	67	0.5	90
[^{14}C]Biliverdin	23	69	0.4	92
[^{3}H]Bilirubin and [^{14}C]biliverdin	26	69	0.2	95
	26	65	0.2	91
[^{3}H]Bilirubin and [^{14}C]biliverdin	52	47	0.6	100
	56	28	0.7	85

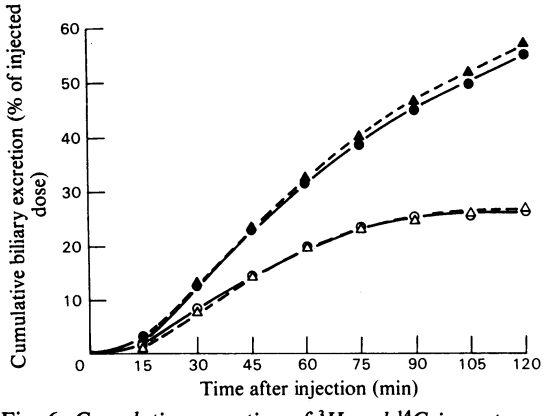

Fig. 6. *Cumulative excretion of ^3H and ^{14}C in maternal bile after injecting foetal guinea pigs with a mixture of [^3H]bilirubin and [^{14}C]biliverdin*

Results from two experiments are shown (▲ and △, ^{3}H; ● and ○, ^{14}C).

the two pigments from the maternal circulation, the transplacental transfer of biliverdin and bilirubin could be distinguished by injecting large doses of unlabelled pigments into the maternal or foetal circulation. Biliverdin remained undetectable in foetal or maternal serum when hyperbiliverdinaemia was induced on the opposite side of the placenta (Table 2, Fig. 3), but when hyperbilirubinaemia was induced, bilirubin was detectable on the opposite side within minutes (Table 2, Fig. 2). In addition, when the mother was given biliverdin, bilirubin gradually appeared in foetal serum (Fig. 4). These observations provide clear evidence that, of the two pigments, only bilirubin undergoes rapid bidirectional transfer.

Direct confirmation of this conclusion by radiotracer techniques was hampered by the rapid enzymic reduction of biliverdin in the foetus at low doses (Table 1). When [^{3}H]bilirubin and [^{14}C]biliverdin were injected simultaneously into a foetus, both isotopes became detectable on the maternal side of the placenta within a few minutes. However, there was a distinct delay in the appearance of ^{14}C relative to ^{3}H. Furthermore, the ^{3}H/^{14}C ratio in the initial maternal blood samples was much larger than in those collected later and larger than the ratio in the injected solution (Fig. 5). The increased ratio was not an artefact caused by the dissimilar tissue distribution of the injected pigments within the foetus, because control studies showed that the ^{3}H/^{14}C ratio in foetal plasma soon after injecting the pigment mixture was actually smaller than in the injected solution and remained so for the duration of the experiment (Table 3). The most likely explanation is that bilirubin, and therefore ^{3}H, starts to cross the placenta almost immediately after injection, in contrast with the ^{14}C label of biliverdin, which only begins to cross, in the form of [^{14}C]bilirubin, after reduction has begun to occur. This would explain the observed delay in the appearance of ^{14}C and the early transient increase in the ^{3}H/^{14}C ratio in maternal blood. Whether reduction occurs in the foetal liver, in the placenta, or in both cannot be determined on the basis of the present data; all are possible. This explanation may appear to be inconsistent with the additional observation (Fig. 6) that ^{3}H and ^{14}C were excreted in maternal bile at similar rates after injecting [^{3}H]-bilirubin and [^{14}C]biliverdin into the foetus. However, it is likely that reduction of the administered [^{14}C]biliverdin to [^{14}C]bilirubin occurred at such a rate that analysis of maternal bile in 15 min batches was too insensitive to reveal subtle differences in the placental transfer of the two injected isotopes. Presumably, counting bile more frequently would have revealed a difference in the appearance of the two isotopes during the first 15 min, but this could not be checked because the radioactivity of the maternal bile was too low.

These findings are consistent with previous reports that bilirubin readily crosses the placenta in several species (Döring, 1958; Lester *et al.*, 1963; Schenker *et al.*, 1964; Bashore *et al.*, 1969; Bernstein *et al.*, 1969; Shier *et al.*, 1971). Moreover, they show that the guinea-pig placenta is considerably less permeable to biliverdin than to bilirubin. This distinction between the two pigments is

compatible with their physicochemical properties. At physiological pH biliverdin is hydrophilic and much less lipophilic than bilirubin (McDonagh, 1979) and it is well known that for compounds with similar molecular weights, low lipophilicity is associated with slow placental transfer (Mirkin & Singh, 1976). Although placental structures differ according to species, the guinea-pig placenta is not atypical (Flexner & Gellhorn, 1942; Ramsey, 1975) and, therefore, the placental membrane probably is a true barrier to biliverdin in other mammals.

The general belief that bilirubin is merely a metabolic waste product, useful only in diagnosing liver disease, tends to obscure the fact that the evolutionary reasons for its formation are unknown. Bilirubin or its conjugates are the predominant bile pigments in the bile and serum of all mammals that have been studied (With, 1968), with the possible exception of the sloth (Barac, 1963). They also occur to a lesser degree in certain fish (Colleran & O'Carra, 1977; Jansen & Arias, 1977), amphibia (Lester & Schmid, 1961), reptiles (Otis, 1973; Cornelius *et al.*, 1975; Noonan *et al.*, 1979), birds (Israels *et al.*, 1966; Lind *et al.*, 1967; Sova *et al.*, 1971) and non-placental mammals (Parsons *et al.*, 1970). However, enzymic formation of bilirubin is clearly not essential for bile-pigment excretion, since biliverdin reductase is undetectable in the liver and spleen of several birds, amphibia and reptiles (Colleran & O'Carra, 1977; Tenhunen, 1971) and biliverdin is the main pigment in their bile (Tenhunen, 1971; Lin *et al.*, 1974; Himes & Cornelius, 1975). The evolutionary advantage of biliverdin reduction and bilirubin formation is, therefore, puzzling. The present studies provide the first evidence for a specific biological advantage to bilirubin formation. They show that reduction of biliverdin in a mammalian foetus converts the primary product of haem degradation into a form that can be eliminated readily via the placenta. Whether this is the sole reason for the enzymic formation of bilirubin in mammals is unknown, and probably will remain so until more information is available about the phylogenetic distribution of biliverdin reductase. However, the persistence of this enzyme in mammals after birth and its presence in certain fish (Colleran & O'Carra, 1977) make it seem likely that there are additional reasons for bilirubin formation. What these may be is not clear. One old, not very convincing, suggestion (Najib-Farah, 1937, 1948) is that bilirubin may be a natural protective agent, since it exhibits antioxidant and bactericidal properties *in vitro*. Whatever the reason, our results show that by facilitating bile-pigment elimination, biliverdin reduction is advantageous to foetal mammals. They also suggest that were it not for biliverdin reductase all men would be born, not only equal, but green.

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