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Synthesis and placental transport of biliverdin IX-alpha

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SYNTHESIS AND PLACENTAL TRANSPORT OF BILIVERDIN IX-ALPHA

by
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THESIS

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To my loving Gus, my darling Aileen, and to

my dearest parents

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CHAPTER 1

GENERAL INTRODUCTION

Bilirubin, the orange bile pigment and the major end-product of heme catabolism in mammals,[1] is principally derived from the degradation of the hemoglobin of senescent erythrocytes by the reticulo-endothelial cells of the spleen and liver, and also from the catabolism of other heme proteins. The sequence of reactions which probably is involved in the formation of bilirubin from heme in vivo starts with hydroxylation of heme at the α-bridge. This is then followed by autoxidation of the resulting α-hydroxy heme to carbon monoxide and a biliverdin-iron complex; hydrolysis of the iron complex to biliverdin IXα; and enzymatic reduction of biliverdin IXα to bilirubin IXα (indirect reacting, unconjugated bilirubin) by biliverdin reductase.[2] Unconjugated bilirubin, a nonpolar lipid-soluble pigment that is capable of diffusing easily across lipoid membranes, cells,[3] and intestinal mucosa,[4] is converted in the liver by the bilirubin glucuronyl transferase system into conjugated bilirubin. Conjugated bilirubin (direct reacting bilirubin) is a polar water-soluble pigment which is rapidly excreted into the bilecannaliculus, probably by an energy-dependent mechanism, and normally eliminated into the intestine. Its polar form interferes with its passage across lipoid membranes and prevents its reabsorption in the intestinal mucosa.[5,6] Figure 1 shows the biochemical steps in the catabolism of heme and bilirubin.[1,3]

Bilirubin is toxic to tissues of the central nervous system.[3,7] Under physiological conditions it is tightly bound to albumin in

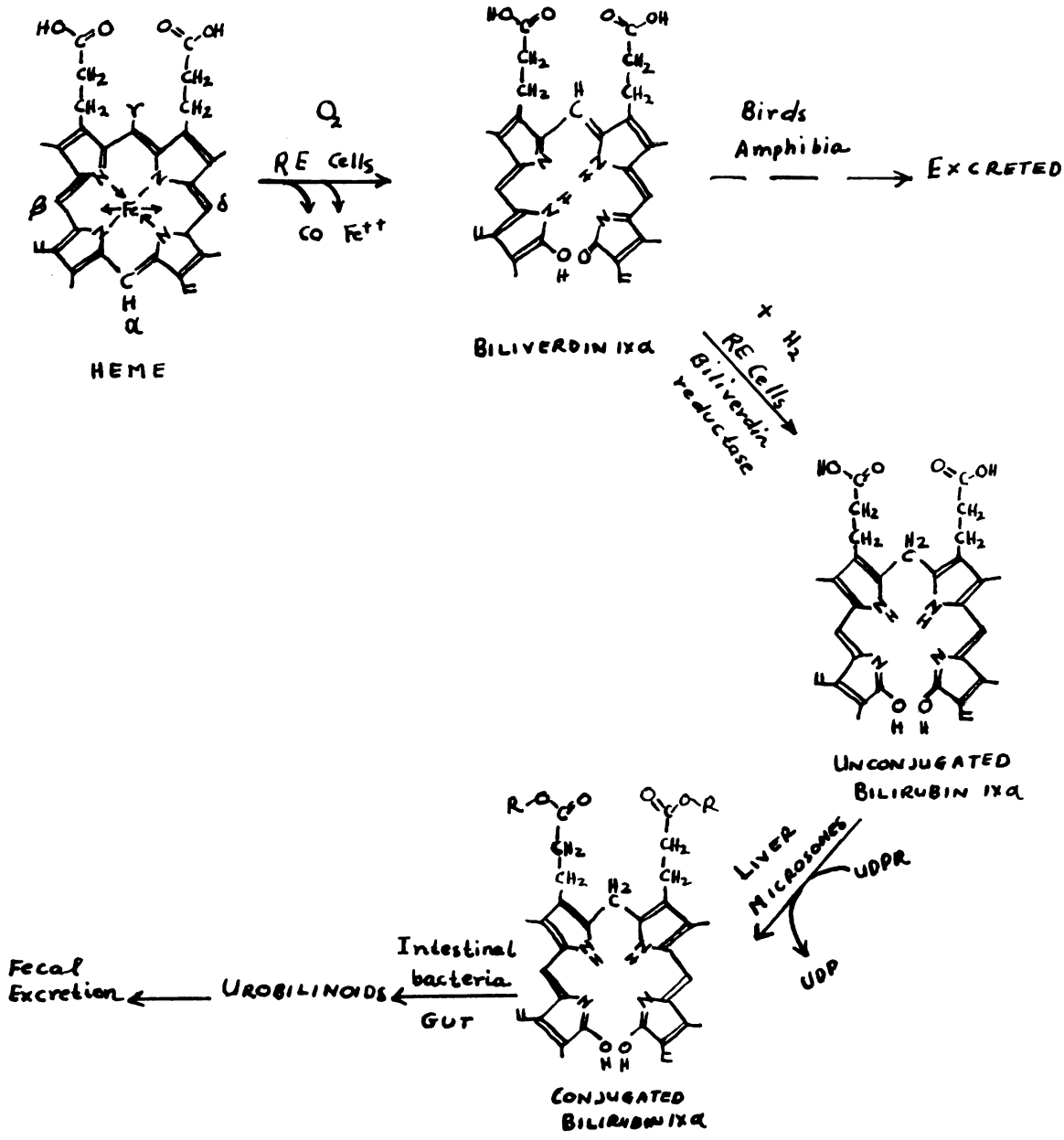

Figure 1.[1,3] Biochemical steps in the formation of bilirubin from heme. UDP = uridine diphosphate; R = glucuronyl or oligosaccharide group.

the circulation and rapidly excreted, and thus the body is protected. But when serum levels of bilirubin become high for prolonged periods, as in neonatal jaundice or the Criglar-Najjar Syndrome,[3] bilirubin can escape to the central nervous system and cause irreversible damage. In view of this, the need for enzymatic conversion of biliverdin, which is apparently non-toxic,[3,8] into bilirubin is quite puzzling; particularly since bilirubin is not only cytotoxic but also requires a complicated conjugation and transport mechanism for excretion. The heme degradation of most birds, amphibia, reptiles, and some fishes ends with the formation of biliverdin and its excretion by the liver.[9,10] Why then should mammals have to reduce biliverdin, which probably can be excreted without conjugation, into bilirubin which is difficult to excrete and potentially toxic?

In pregnant mammals, bilirubin formed in the fetus does not accumulate in the fetal circulation. It is thought that this is due mainly to clearance of the pigment through the placenta to the maternal circulation rather than to fetal conjugation. The placenta is permeable only to the unconjugated lipid-soluble bilirubin and not to the conjugated water-soluble form. Conjugation is low in the fetus and newborn due to an inadequate hepatic enzyme system. Any conjugated bilirubin that is formed in the fetus is hydrolyzed by the beta-glucuronidases present in the fetal intestine to the unconjugated form. This can then be returned to the liver across the intestinal mucosa by the enterohepatic shunt and from there to the circulation for clearance by the placenta.[5] Biliverdin, on the other hand, is more polar than bilirubin and would therefore not be expected to cross the placenta. These considerations led to the hypothesis that perhaps biliverdin is reduced to bilirubin in

mammals (i.e. in species with a placenta) solely to facilitate excretion of bile pigment by the fetus. The aim of this investigation was to test this hypothesis by examining the placental transport of biliverdin *in vivo* in guinea pigs and comparing it with that of bilirubin. This required a convenient source of pure biliverdin and isotopically labelled biliverdin, which are not commercially available. Therefore, in preliminary work, a synthetic method was devised and cystalline biliverdin was fully characterized for the first time.

This thesis is divided into two parts. The first part deals with the preparation of biliverdin IXα and ^{14}C-biliverdin IXα and their characterization, and the second part describes studies on the placental transport of biliverdin IXα and bilirubin IXα in guinea pigs.

CHAPTER 2

PREPARATION AND CHARACTERIZATION OF BILIVERDIN IXα AND ^{14}C-BILIVERDIN IXα

I. INTRODUCTION

Biliverdin, the pigment precursor of bilirubin, was discovered by Berzelius in ox bile in 1841.[11] It was later synthesized by Kuster in 1909 in an impure form,[11] prepared by Lemberg[12] from oxidation of bilirubin by ferric chloride in glacial acetic acid, and isolated by Lemberg and Barcroft[13] as its crystalline dimethyl ester from the placenta of a dog. Biliverdin IXα is not commonly available commercially. However, when obtainable, it usually contains isomeric impurities (biliverdin IIIα and biliverdin XIIIα) and highly polar impurities such as polymeric materials, verdins with modified side chains, and oxidation products of biliverdin.[14,15]

The most commonly used methods for preparing biliverdin IXα or its dimethyl ester involve oxidation of bilirubin IXα, which is commercially available, with ferric chloride, benzoquinone or hydrogen peroxide under acidic conditions. Problems associated with these methods include formation of non-IXα isomers by isomerization of the starting material, difficult work-ups, low yields and non-crystalline products. Often crude products are converted to the dimethyl esters for purification and then hydrolyzed back again because of difficulties in chromatography of biliverdin itself. The ferric chloride-acetic acid and benzoquinone-acetic acid methods give products containing substantial amounts of isomeric impurities.[15] A crystalline product has been obtained, after a tedious and difficult work-up, by oxidation with hydrogen peroxide in hydrochloric acid:

methanol[16] but this material was heterogeneous and probably contained a substantial proportion of biliverdin XIIIα as shown by chromatography of its dimethyl ester.[17] Oxidation of bilirubin with ferric chloride in HCl-MeOH[18] yields a "biliverdin" that has been used as a standard, as a chromatographic marker, and an enzyme substrate.[19,20,21] However, this material contains little, if any, biliverdin IXα.[12,15,22] In a recent paper,[22] after surveying published methods, the authors concluded that "at present absolutely pure biliverdin seems to be unobtainable". In the light of current knowledge, it is now clear that biliverdin used in previous metabolic studies invariably contained green polar impurities, isomers, and often biliverdin methyl esters.

Because of the deficiencies in existing methods and the need for pure biliverdin for the placental transport studies, a new method had to be developed for synthesizing biliverdin from bilirubin. In devising a suitable method, the main problems that we encountered were isomerization of starting material, removal of by-products and crystallization of the purified product. Acidic conditions and elevated temperatures were avoided because previous work had shown that they favor formation of non-IXα isomers.[15,23] In the procedure that was eventually chosen biliverdin IXα was prepared by dehydrogenation of bilirubin IXα with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dimethyl sulfoxide (DMSO) at room temperature. DDQ had previously been used for dehydrogenation of hydrogenated porphyrins (chlorins)[24] but not for the preparation of biliverdin IXα. The reaction is rapid and, provided that the initial

concentration of biliverdin IXα is less than 1 mg/ml, formation of non-IXα isomers is negligible. DMSO was chosen as a solvent because DDQ and bilirubin are readily soluble in it, and because the product, biliverdin, can be easily isolated from it by precipitation, thereby avoiding the difficult and tedious solvent extractions used in some earlier methods.

II. MATERIALS AND METHODS

Reagents: Bilirubin samples purchased from Koch Light Laboratories, Colnbrook, Bucks., U.K., BDH Biochemicals, Poole, Dorset, U.K., and Sigma Chemical Co., St. Louis, Mo. were purified, crystallized, and assayed for isomeric purity before use.[25] 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 98%, Aldrich Chem. Co. Inc., Milwaukee, Wisconsin) had mp 213-216° C and was used without further purification. Tetramethylammoniumhydroxide pentahydrate, sodium borohydride, Trizma base, and boron trifluoride in methanol (14% w/v) were purchased from Sigma Chem. Co. Argon (99.999% purity) was obtained from Matheson Co., Newark, California. Celite analytical filter aid was from J.T. Baker Chem. Co., Phillipsburg, N.J. Silica gel H and silica gel G (both Type 60 and for tlc), aluminum oxide (neutral, Activity I for column chromatography), and sand were obtained from E. Merck Laboratories Inc., Elmsford, N.Y. All other reagents were either analytical or "Baker analyzed" reagent grades.

Solvents: Methyl sulfoxide (DMSO, ACS reagent grade) was purchased from Eastman Kodak Company, Rochester, N.Y. Beckman Ready - Solv™ HP was purchased from Beckman Instrument Inc., Fullerton, California. All other reagents were analytical reagent grade purchased from Mallinckrodt Inc., St. Louis, Mo. Water was distilled

from glass and petroleum ether was redistilled (bp 60-80°C). Argon-purged DMSO was prepared by bubbling DMSO with argon for about 10 minutes.

Spectra: Visible and ultraviolet spectra were recorded on a Cary 118 C or Aminco model DW2 spectrophotometer. Wavelength calibration was done using a holmium oxide standard from Arthur H. Thomas Co., Philadelphia, Pa. Extinction coefficients (ε) are given in 1 $mole^{-1}$ cm^{-1}. Infrared spectra were determined in KBr discs on a Perkin-Elmer instrument. Mass spectra were determined by field desorption and electron impact methods (A.E.I. MS12).

Miscellaneous: Melting points (uncorrected) were done on a Thomas-Hoover Capillary melting point apparatus. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Ca. Fluorescent spots on tlc plates were located using UV lamps from Ultraviolet Products Inc., San Gabriel, Ca. All work with bile pigments was done in a windowless room under low intensity light.

Isotopic Procedures: ^{14}C-bilirubin was prepared biosynthetically by infusion of 5-aminolevulinic acid-4-^{14}C hydrochloride (Amersham/Searle, Arlington Heights, Illinois) into male Sprague-Dawley rats fitted with external biliary fistulas.[26] ^{14}C-bilirubin was isolated and crystallized as previously described.[27] For specific activity determination, ^{14}C-bilirubin and ^{14}C-biliverdin were treated and counted as follows. Aliquots (2-5 µl) of ^{14}C-bilirubin and ^{14}C-biliverdin in $CHCl_3$ and 0.1N NaOH, respectively, were treated for 30 minutes with aqueous tetramethylammoniumhydroxide (1M, 0.3 ml) and hydrogen peroxide (30%, 0.3 ml) solutions in low

potassium glass vials fitted with polyseal cone-lined screw caps (Packard Instrument Co. Inc., Donners-Grove, Illinois). Hydrochloric acid (3M, 0.3 ml) and ascorbic acid (15 grams/100 ml, 0.3 ml.) solutions were added with thorough mixing, followed by addition of Beckman Ready-Solv ^{Tm}HP (15 ml).[28] Samples were counted on a Beckman LS-250 or LS-350 Liquid Scintillation spectrometer. Toluene-^{14}C, purchased from New England Nuclear Corp., Boston, Mass., was used as an internal standard for determining quenching corrections. Results were expressed as disintegrations per minute (dpm) per microgram (µg). Counting efficiencies were 67%.

Thin Layer Chromatography: Polyamide layer sheets (15 x 50 cm, 50 µ layer) were purchased from Cheng Chin Trading Co. Ltd., Taiwan. Silica gel G plates (20 x 20 cm, 250 µ) were purchased from Analtech Inc., Newark, Delaware. Silica gel H (E. Merck Laboratories Inc.) plates were made according to the manufacturer's directions, activated for one hour at 110^{o}C and used after equilibration in air at room temperature. Quantitative analysis of mixtures of bilirubin isomers was carried out by preparative tlc and spectrophotometry.[29] Qualitative analysis of bilirubin samples was by tlc on polyamide sheet using 1% ammonia-methanol (1 hr., System A) and on silica gel H with 1% acetic acid-chloroform (1 hr., System B). Biliverdin was chromatographed on silica gel G tlc plates, developing first in acetone (20 minutes), then in chloroform:methanol:acetic acid (9:1:0.1%, v/v) (1 hr., System C).* Qualitative analysis of the dimethyl esters of

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* The predevelopment step with acetone is not necessary for routine tlc of biliverdin since biliverdin remains at the baseline during the acetone run. This step was included to detect fluorescent impurities derived from DDQ. These are less polar than biliverdin and are easier to detect if the plate is first run with acetone.

biliverdin was by tlc on silica gel G using 3% acetone-chloroform (1 hr., System D).[15,30]

Preparation of crude biliverdin IXα: A freshly prepared solution of bilirubin (200 mg, 3.4 x 10^{-4} mole) in argon-purged DMSO (250 ml) was poured rapidly into a freshly prepared solution of DDQ (163 mg, 7.2 x 10^{-4} mole) in argon-purged DMSO (250 ml). The mixture was stirred and continuously purged with argon during the addition. After 5 minutes argon-purged glass distilled water (1500 ml) was added and the mixture was stirred for a few minutes. The precipitate was collected by filtration through a thin pad of celite layered onto a Whatman #4 filter paper in a Buchner funnel (10 cm i.d.) and washed twice with water (2 x 10 ml). The biliverdin precipitate was redissolved by the addition of three portions of 0.1N NaOH (3 x 25 ml) to the filter cake, collecting the filtrate each time. The combined basic filtrates were centrifuged to remove traces of celite particles and acetic acid (0.5 ml) was added to the supernatant to reprecipitate the biliverdin. The product was collected by centrifugation, washed three times with an equal volume of water and freeze-dried. Crude biliverdin IXα, containing fluorescent impurities plus DDQ and/or its hydroquinone, was obtained in quantitative yield as a voluminous green amorphous powder.

Purification of crude biliverdin IXα: A slurry of silica gel G (EM reagents, Type 60, for making tlc plates, 15 g) in methanol: acetone (1:1, v/v, 45 ml) was poured into a 60 ml glass Buchner funnel with a fritted sinter glass disc (porosity 60 C, funnel dimensions 40 mm ID x 50 mm high). The slurry was allowed to settle by gravity and then the solvent was drained to the top of the column under low

vacuum (12-14 mm Hg below atmospheric pressure). A further 20 ml of methanol:acetone (1:1) was similarly sucked through the column. A solution containing crude freeze-dried biliverdin (100 mg) in methanol:acetone (1:1, 30 ml) was then added carefully and evenly to the top of the column with a Pasteur pippette and light vacuum was applied. The adsorbed material was then eluted under light vacuum with methanol:acetone (1:1, 10 ml), followed by chloroform: methanol:acetic acid (7:3:0.3%). Yellow-green or green eluates were discarded and the main blue green band was collected and evaporated to dryness under reduced pressure. The residue was dissolved in 0.1N NaOH (4 ml), filtered through a thin layer of celite in a small (2 ml) fritted glass Buchner funnel, and the celite was rinsed through with 0.1N NaOH (3 x 1 ml). Glacial acetic acid (3 drops) was added to the combined filtrate and rinsings and the precipitated product was collected by centrifugation, washed three times with an equal volume of water, and mixed with acetone:water (8:2, v/v, 40 ml). To this was added an equal volume of water, and the mixture was shaken. Then acetic acid (5 µl) was added. The mixture was shaken, and allowed to stand for 10 minutes. The precipitated biliverdin was collected by centrifugation, washed twice with an equal volume of water, and freeze-dried. The purified biliverdin IXα (t.l.c. Fig. 3; 55 mg, 55%) was obtained as an amorphous green powder.

Crystallization of purified biliverdin IXα: Pure freeze-dried biliverdin (100 mg) in an Erlenmeyer flask (125 ml) containing a magnetic stirrer was slurried with cold methanol (1-2 ml). The flask was then placed on a heated hot plate and boiling methanol (50 ml) was added with rapid magnetic stirring. The solution was allowed to reflux for

about 30 seconds and then filtered, while hot, through a fluted filter paper into a flask containing a few ml of boiling methanol. The solution was removed from the hot plate as soon as filtration was completed and allowed to cool to room temperature. The solution was seeded, kept at 4°C overnight and filtered. The product was dried at 65°C under reduced pressure overnight to give biliverdin IXα (78 mg, 78%) as microscopic, long thin needles (Fig. 4), (Found 67.78% C, 5.86% H, 9.51% N; calculated for $C_{33}H_{34}N_4O_6$ 68.02% C, 5.88% H, 9.62% N) with mp > 290°C; mass spectrum (FD) M^+ 582; $\varepsilon^{MeOH}_{376nm}$ = 50,800; $\varepsilon^{MeOH}_{666nm}$ = 14,400; $\varepsilon^{5\%HCl:MeOH}_{376nm}$ = 66,000; $\varepsilon^{5\%HCl:MeOH}_{696nm}$ = 30,800. The overall yield from bilirubin was 43%.

Preparation of practical grade biliverdin IXα: Crude biliverdin IXα was prepared as described previously but the freeze-drying was omitted. The moist product was mixed with acetone:water (8:2, 80 ml), followed by addition of an equal volume of water. The mixture was shaken and then allowed to stand for 10 minutes. The biliverdin precipitate was collected by centrifugation, washed twice with water and freeze-dried. "Practical grade" biliverdin IXα was obtained as amorphous green powder (73%). Tlc (System C, Fig. 3) showed the absence of fluorescent impurities, but traces of baseline by-products. Crystallization from methanol gave short, stubby needles mixed, at times, with amorphous aggregates.

Esterification of crystalline biliverdin IXα[30]: A solution of biliverdin (60 mg, 1.03 x 10^{-4} mole) in argon-purged methanol (12 ml) and BF_3/MeOH (12 ml) was refluxed on a steam bath with continuous purging of argon for 15 minutes, stirred overnight at room temperature, and diluted with water (72 ml). The mixture was extracted with chloroform (1 x 50 ml, 2 x 10 ml) and the chloroform extracts were washed

with water (2 x 50 ml) and 0.1N $NaHCO_3$ (1 x 50 ml), filtered through fluted filter paper, and evaporated to dryness under reduced pressure. The residue was chromatographed on alumina (Activity V, neutral, 80 grams, column dimensions 44 mm ID x 150 mm high). The material was applied to the column in chloroform:benzene (1:9, 20 ml) and eluted with chloroform:benzene (1:1). The main blue band was collected and evaporated to dryness under reduced pressure. A chloroform solution (5 ml) of the residue was filtered, concentrated by boiling, followed by addition of methanol (3 drops). Crystallization of the ester was initiated by addition of petroleum ether (10 ml). The suspension was kept in the refrigerator overnight, filtered, washed once with petroleum ether, and dried under reduced pressure to give 27 mg (42%) of dark green prisms, with mp 213 - 214°C (literature: 207-208°C;[12,36] 206 - 207°C;[14] 208 - 209°C[15]); $\varepsilon^{CHCl_3}_{380nm}$ = 54,600; $\varepsilon^{CHCl_3}_{660nm}$ = 14,700; M^+ (E.I.) 610. This material showed a single spot on tlc (System D; Fig. 5) and co-chromatographed with an authentic sample of biliverdin IXα dimethyl ester.[15]

Reduction of crystalline biliverdin IXα: Methanol (5 ml) was added to a mixture of crystalline biliverdin IXα (20 mg, 3.4×10^{-5} mole) and sodium borohydride (200 mg, 5.3×10^{-3} mole) with rapid stirring and cooling in an ice bath. After one minute, water (10 ml) was added and bilirubin was precipitated from the solution by dropwise addition of glacial acetic acid. The mixture was extracted with chloroform (1 x 75 ml, 2 x 10 ml) and the extracts washed with water (2 x 50 ml) and 0.1M $NaHCO_3$ (1 x 50 ml), filtered, and evaporated to dryness under reduced pressure to give 12 mg (58%) of bilirubin. Crystallization[25] gave 8 mg (39%) of a product that was identical with authentic bilirubin IXα on tlc (Systems A and B) and had $\lambda^{CHCl_3}_{max}$

453 nm (ε = 57300), mass spectrum (E.I.) M^+ 584. On isomer analysis,[29] this material was found to contain 95% bilirubin IXα, 1% bilirubin IIIα, and 4% bilirubin XIIIα. (Isomer analysis of the original bilirubin from which the biliverdin was made gave 93% bilirubin IXα, 3% bilirubin IIIα and 4% bilirubin XIIIα). On oxidation with DDQ as described earlier, this product gave crude biliverdin that was chromatographically unseparable (System C) from the biliverdin that was used as starting material.

Reaction of bilirubin with DDQ: Effect of excess DDQ.

a) A solution of DDQ (1,2,3,4 mole equivalents) in DMSO (5 ml) was added to a solution of bilirubin (20 mg; 1 mole equivalent) in DMSO (5 ml) with continuous mixing. After 5 minutes, an aliquot (5 ml) of the reaction mixture was added to water (15 ml), mixed, and centrifuged. The precipitate was washed three times with water, then freeze-dried. The products were examined by tlc (System A & System B). This showed unreacted bilirubin in the 1:1 reaction, complete reaction in the 1:2 system and purple byproducts in the 1:3 and 1:4 reactions.

b) A solution of bilirubin (10 mg; one mole equivalent) in DMSO (12.5 ml) was added to a solution of DDQ (1.1, 1.3, 1.5 mole equivalents) in DMSO (12.5 ml) with continuous magnetic stirring. After 5 minutes, water (75 ml) was added. The mixture was stirred for a few minutes, then centrifuged. The precipitate was washed twice with water, redissolved in 0.1N NaOH (2 ml), acidified to pH 4.3 with glacial acetic acid, centrifuged, washed three times with water and freeze-dried. The products were examined by tlc (System A & System B). All reaction products contained unreacted bilirubin. In analogous experiments using equimolar amounts of bilirubin and DDQ and a final pigment concentration of 2 mg/ml, the recovery of unreacted bilirubin was 20%

(mean of two experiments).

Reaction of bilirubin with DDQ: Effect of time and air.

Crude biliverdin was prepared as previously described except the reaction was allowed to proceed for various periods of time (1 minute, 5 minutes, 15 minutes, and 60 minutes) and in some instances was run without excluding air. The products were examined by tlc (System C). The reaction went to completion within 5 minutes and the exclusion of air did not cause any marked diminution in the proportion of pigmented impurities.

Reaction of bilirubin with DDQ: Effect of Concentration.

a) Solutions of bilirubin (5 mg, 8.6×10^{-6} mole) in 0.75, 1.25, or 7.50 ml DMSO were added, respectively, to solutions of DDQ (4.1 mg, 1.8×10^{-5} mole) in 0.50, 1.25, or 5.00 ml of DMSO under argon to give final bilirubin concentrations of 4,2, or 0.4 mg/ml. After 5 minutes, the reactions were worked up as described in the preparation of crude biliverdin IXα and a portion of each crude product was examined by tlc (System C). The remainder was methylated as previously described and the product examined by tlc (System D). Traces of biliverdin XIIIα dimethyl ester were detectable in the ester derived from the two most concentrated reactions but were undetectable in the product from the most dilute reaction. The proportion of polar byproducts in the crude biliverdin was least in the most dilute reaction.

b) In two further experiments, DDQ (16.3 mg, 7.18×10^{-5} mole) in DMSO was added to bilirubin (20 mg, 3.42×10^{-5} mole) in DMSO so that the final pigment concentrations were 0.4 and 4.0 mg bilirubin per ml DMSO. Crude products were isolated and methylated as described in (a). Qualitative tlc of the crude biliverdin and corresponding dimethyl ester showed that the product of the reaction using the most

concentrated solution (4 mg bilirubin per ml DMSO) contained traces of biliverdin XIIIα and more of the polar byproducts that remain at the baseline. The product from the reaction using 0.4 mg bilirubin per ml DMSO contained less polar byproduct and biliverdin XIIIα was not detectable in its ester.

Reaction of bilirubin with DDQ: Effect of addition sequence and prolonged reaction times. Equal volumes of bilirubin and DDQ in DMSO were mixed under argon, or in some instances under oxygen, and allowed to react for 5, 60, or 180 minutes. In some experiments, the bilirubin solution was added to the DDQ solution with continuous magnetic stirring; in others, the DDQ solution was added to the bilirubin solution. The mole ratios of bilirubin to DDQ that were used ranged from 1:1.5 to 1:0.1 and the volumes of the reagent solutions ranged from 2.5 ml to 25 ml (Table 1). At the end of each reaction the solution was diluted with three volumes of water and the mixture was shaken and centrifuged. The precipitated product was washed twice with water, twice with methanol to remove biliverdin, and dissolved in chloroform. The chloroform solutions, which contained predominantly bilirubin, were washed once with water, twice with 0.1M $NaHCO_3$, filtered, and evaporated to dryness under reduced pressure. The residues were qualitatively analyzed by tlc (Systems A & B). Results are included in Table 1.

Solubility of biliverdin IXα: Mixtures of biliverdin (0.5 mg, 8.55×10^{-7} mole) with each solvent (0.5 ml) were prepared and then shaken in a water bath at $22^{\circ}C$. After one hour, the mixtures were vortexed and centrifuged. The solubility of biliverdin in each solvent was noted based on the visual intensity of the color of the supernatant as well as the amount of undissolved solid. The mixtures

were then shaken in a water bath at 50°C. After one hour, they were vortexed, and centrifuged, and the solubility of biliverdin in each solvent was noted. Results are summarized in Table 2.

Determination of extinction coefficients: Duplicate samples of biliverdin (0.5 mg) were dissolved in a measured volume of solvent (10-50 ml). Three aliquots of each solution were diluted in volumetric flasks to give a concentration equivalent to about 0.9 absorbance units at the most intense peak and spectra were recorded. Thus six determinations of extinction coefficient data were made for each solvent; data reported on Table 3 are mean values of these six determinations.

Preparation of crude biliverdin by oxidation of bilirubin IXα with benzoquinone:[15] A solution of bilirubin (250 mg, 4.28×10^{-4} mole) and p-benzoquinone (250 mg, 2.31×10^{-3} mole) in DMSO: acetic acid (9:1, v/v) was refluxed on a steam bath with continuous purging with argon. After 30 minutes, the reaction mixture was cooled, added to water (500 ml) and the pH was adjusted to 4.3 with solid sodium bicarbonate. The suspension was kept in the refrigerator for an hour, centrifuged, and the precipitate washed three times with water and freeze-dried. A solution of the freeze-dried crude product in 0.1N NaOH (25 ml) was adjusted to pH 5.0 with glacial acetic acid and then centrifuged. The residue was washed three times with water and freeze-dried. The crude biliverdin was obtained as a fluffy green amorphous powder which showed substantial quantities of baseline residue on tlc (System C, Fig. 3).

Preparation of ^{14}C-biliverdin IXα: A solution of ^{14}C-bilirubin IXα (2.1 mg, 3.58×10^{-6} mole, SA = 0.9 - 1.5 x 10^4 dpm/μg) in argon-purged DMSO (2.6 ml) was added rapidly with a pasteur pipet to a

solution of DDQ (1.7 mg, 7.52 x 10^{-6} mole) in argon-purged DMSO (2.6 ml), The reaction mixture was continuously purged with argon. After five minutes water (15.7 ml) was added and the mixture was centrifuged. The precipitate was washed three times with water and freeze-dried. A slurry of silica gel G (0.5 - 0.6 gram) in methanol:acetone (1:1) was poured into a 2 ml glass Buchner funnel with fritted sinter disc (porosity 10 M, funnel dimensions 10 mm ID x 35 mm high). Then the procedure described previously for the purification of crude biliverdin IXα was followed with the following changes: The volume of methanol:acetone (1:1) was reduced to 2 ml; volumes of 0.1N NaOH were 2 ml and 0.5 ml; glacial acetic acid, 10 and 1 microliter; acetone:water (8:2, v/v, 0.4 ml); and water, 0.4 ml. The ^{14}C-biliverdin IXα obtained was an amorphous green powder which showed a single spot on tlc (System C) with no baseline residue. Spectrophotometric analysis of a 0.1N NaOH solution of ^{14}C-biliverdin IXα gave λ_{max} 380,720 nm. The yield was 41 percent (estimated spectroscopically) and the specific activity of the product was 2.1 x 10^{4} dpm/μg.

III. RESULTS AND DISCUSSION

Prior to devising a suitable preparation of biliverdin IXα, a sensitive and discriminating thin layer chromatographic system for qualitative product analysis had to be developed. For this purpose, amorphous biliverdin was prepared by oxidation of bilirubin with benzoquinone using an adaptation of the method of Bonnett and McDonagh.[15] This material contains biliverdin IIIα and XIIIα as well as IXα[15] and other impurities but was pure enough for surveying tlc systems. A number of solvent systems were examined using silica gel G tlc plates. These included methanol:chloroform, methanol:chloroform:acetic acid, methanol:chloroform:ammonium hydroxide, methanol:ethyl acetate, meth-

anol:ethyl acetate:acetic acid, methanol:acetone, methanol:acetone: acetic acid, and methyl ethyl ketone:water:acetic acid mixtures. Of these systems studied, the most useful mixtures were found to be those containing methanol:chloroform:acetic acid and methanol:chloroform:ammonium hydroxide. Of these two, the former ($MeOH:CHCl_3$:HOAc, 1:9:0.1%) was adopted for routine assay. This system was not completely satisfactory for three reasons. First, some decomposition of biliverdin to yellow pigments occurred during the chromatography. This seemed to vary with each batch of chromatography plates and with one batch it was particularly marked. However, generally it was noticeable, but not marked. This decomposition was apparently due to some factor (metal impurities?) in the plates rather than solvent or plate activity. Second, the Rf of biliverdin was rather low and sensitive to the activity of the silica gel and the relative humidity of the ambient atmosphere. The Rf could be increased by varying the solvent composition but this then resulted in increased tailing. Third, the system did not give a clear separation of isomeric III, IX, and XIIIα biliverdins. For detection of these isomers the pigments had to be converted to the corresponding biliverdin dimethyl esters which are more readily separable.[15] Despite the disadvantages of the chosen system it was found to be superior, particularly with respect to tailing, to systems described in the literature.

Preliminary studies and published work[35] had indicated that DDQ oxidizes bilirubin rapidly at room temperature and, therefore, might be a suitable reagent for biliverdin preparation. With chloroform as a solvent, there was rapid reaction with formation of a green precipitate. Work-up of the product gave mainly unreacted bilirubin.

Possibly the initial green precipitate was due to charge-transfer complex formation which has been observed before with DDQ in chloroform.[24] With DMSO as solvent, the reaction was rapid and the product was easily recovered simply by diluting with water. Therefore, DMSO was chosen as the solvent for the reaction. In preliminary work it became evident that the yield and purity of the biliverdin formed was very dependent on the experimental protocol. Therefore, the effect of different reaction conditions was examined in detail to optimize the method.

In theory, one mole of DDQ is required for complete reaction (Fig. 2). However, it was found experimentally that with equimolar amounts of reagents (bilirubin and DDQ), not all of the bilirubin reacted and about 20 percent of it was recovered unchanged. At least two moles of DDQ were required for complete reaction. Increasing amounts of impurities were obtained when larger excesses of DDQ (e.g. 1:3, 1:4) were used. The reason why more than stoichiometric amounts are needed is not clear. It may be due to decomposition of DDQ in DMSO[35] since unreacted bilirubin was also observed with two moles of DDQ when the DDQ solutions were allowed to stand before use. For this reason, the DDQ solutions used in the reaction should be prepared freshly and used without delay.

Complete reaction was observed in five minutes at room temperature using 2.1 mole equivalent of DDQ. Longer reaction times led to formation of impurities. Although reactions were commonly carried out under argon this precaution was subsequently found to be unnecessary. The presence of air or oxygen caused little if any, by-product formation. However, increasing dilution diminished the formation of non-biliverdin

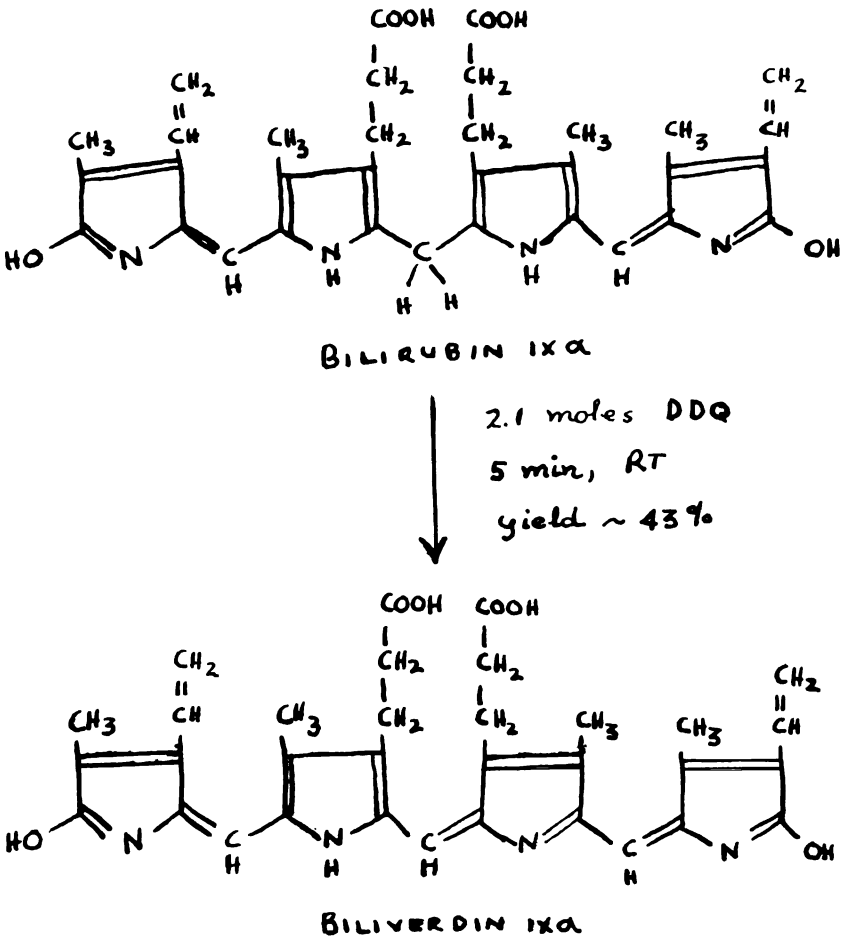


Figure 2. Reaction of bilirubin IXα with DDQ.

impurities.

In previous methods for the preparation of biliverdin, notably those carried out under acidic conditions,[12,15,16,18] isomerization of the starting material to bilirubin IIIα and XIIIα resulted in the formation of a heterogeneous product. It was anticipated that isomerization would not be a problem with the DDQ reaction because of the mild conditions and absence of a protic acid. Since biliverdin IIIα and XIIIα, the possible isomer contaminants, have not been characterized and methods for their separation by thin layer chromatography were not available, formation of these isomers was checked indirectly in two ways: 1) by esterification of the product and qualitative analysis on tlc using authentic standards[15] and systems known to separate the dimethyl esters of the biliverdin isomers; and 2) by carrying out reactions with insufficient DDQ and analyzing the recovered excess starting material for the presence of artifactual bilirubin IIIα and XIIIα isomers. The starting bilirubin sample used in all these studies was shown by analysis to contain at least 93-96 percent of the bilirubin IXα isomer. By the use of these methods it was found that the formation of isomers depended on: 1) the sequence of addition of the reagents, and 2) to a lesser extent on the concentration and reaction time (Table 1). For example, in reactions in which DDQ solution was added to the bilirubin solution isomerization occurred except under very dilute conditions, but when the bilirubin solution was added to the DDQ solution isomers were observed only when concentrations were high or a long reaction time was used. In general when isomers were formed the XIIIα exceeded the IIIα isomer.

DDQ has been used for dehydrogenation of a variety of compounds including hydrogenated porphyrins (chlorins).[24] Dehydrogenation can

occur via ionic or radical mechanisms.[32] In the ionic mechanism the initial step is abstraction of a hydride ion (Equation 1). Loss of a proton then yields product (Equation 2).

$$RH_2 + Q \longrightarrow RH^+ + QH^- \quad (1)$$

$$RH^+ + QH^- \longrightarrow R + QH_2 \quad (2)$$

In the radical mechanism, abstraction of a hydrogen atom yields a free radical derived from the substrate and a semiquinone radical (Equation 3).

$$RH_2 + Q \longrightarrow RH^{\cdot} + QH^{\cdot} \quad (3)$$

Electron transfer (Equation 4) followed by proton transfer (Equation 2) then yields products.[32]

$$RH^{\cdot} + QH^{\cdot} \longrightarrow RH^+ + QH^- \quad (4)$$

If the $RH^{\cdot}$ is a stable long-lived radical, it may also be generated by reactions (5) and (6).[32]

$$RH_2 + QH^{\cdot} \longrightarrow RH^{\cdot} + QH_2 \quad (5)$$

$$2RH_2 + Q \longrightarrow 2RH^{\cdot} + QH_2 \quad (6)$$

Bilirubin is known to undergo isomerization via a radical mechanism.[33,34] In water (but not organic solvents) the reaction is initiated by atmospheric oxygen. The mechanism is thought to be as follows (where $A\text{-}CH_2\text{-}B$, $A\text{-}CH_2\text{-}A$, and $B\text{-}CH_2\text{-}B$ represent bilirubin IX, III, and XIIIα respectively).

$$A\text{-}CH_2\text{-}B \xrightarrow[\text{Initiator}]{I^{\cdot}} A\text{-}\dot{C}H\text{-}B + IH \quad (7)$$

$$A\text{-}\dot{C}H\text{-}B + A\text{-}CH_2\text{-}B \rightleftharpoons \underline{A\text{-}CH_2\text{-}A} + B\text{-}\dot{C}H\text{-}B \quad (8)$$

$$A\text{-}\dot{C}H\text{-}B + A\text{-}CH_2\text{-}B \rightleftharpoons \underline{B\text{-}CH_2\text{-}B} + A\text{-}\dot{C}H\text{-}A \quad (9)$$

In this way the IXα isomer is converted to a mixture containing the III, IX, and XIIIα isomers. The reaction is concentration dependent and isomerization diminishes with increasing dilution. The observation of isomer formation under certain conditions during the oxidation of bilirubin with DDQ strongly suggests that this reaction occurs at least in part via a radical rather than an ionic mechanism. The observed isomer formation was not caused by oxygen initiation since it

Table 1. Formation of isomers in the reaction of bilirubin and DDQ

Bilirubin:DDQ mole ratio	Total volume of reaction mixture (ml)	Bilirubin concentration[a] (mg/ml)	Reaction time (min)	Isomer Formation[e]
		DDQ added to Bilirubin		
1:2.1	50.0	0.4	5	Trace[b]
1:2.1	5.0	4.0	5	Yes[b]
1:0.8	30.0	0.4	5	Yes[c]
1:1	12.5	2.0	5	Yes[c,d]
1:1	12.5	2.0	180	Yes[c,d]
		Bilirubin added to DDQ		
1:2.1	50.0	0.4	5	ND or trace[b]
1:2.1	10.0	2.0	5	ND or trace[b]
1:2.1	5.0	4.0	5	Trace[b]
1:0.1	5.0	1.0	5	ND[c]
1:0.8	30.0	0.4	5	ND[c]
1:1.1	25.0	0.4	5	ND[c]
1:1.3	25.0	0.4	5	ND[c]
1:1.5	25.0	0.4	5	ND[c]
1:1.1	50.0	0.4	60	Yes
1:1.0	12.5	2.0	5	ND[c,d]
1:1.0	12.5	2.0	180	Yes[c,d]

[a] Amount of bilirubin added divided by total volume after mixing of reagent solutions.

[b] Determined by qualitative tlc analysis of esterified biliverdin product.

[c] Determined by qualitative tlc analysis of unreacted bilirubin.

[d] Same results for experiments run under argon or oxygen.

[e] Isomer formation was indicated by the presence of the IIIα and/or the XIIIα isomer together with the IXα. In most experiments where isomers were formed the amount of XIIIα isomer exceeded the amount of IIIα.

was found to be independent of the oxygen concentration. A radical mechanism is consistent with the observation that isomer formation diminishes with dilution (Table 1) and that the sequence in which the reagents are mixed is critical. Thus, when bilirubin is added to excess DDQ the latter is in excess throughout the addition and the reaction sequence (3), (4) should be favored. Under these conditions there should be little isomerization as observed. However, when DDQ is added to bilirubin the bile pigment is in excess during the early stages of the addition and reactions (3), (5) and (6) should be favored. In this situation the formation of RH$^{\bullet}$ ($\equiv$ A-$\dot{C}$H-B) in the presence of RH_2 ($\equiv$ A-CH_2-B) would tend to facilitate reactions (8) and (9) which would lead to isomerization as observed. The finding that this dehydrogenation proceeds via a radical mechanism and is accompanied by isomer formation suggests that the formation of isomers during the preparation of biliverdin with hydrogen peroxide[16] and benzoquinone[15] may be due to radical reactions too, rather than acid-catalyzed isomerization as previously thought.

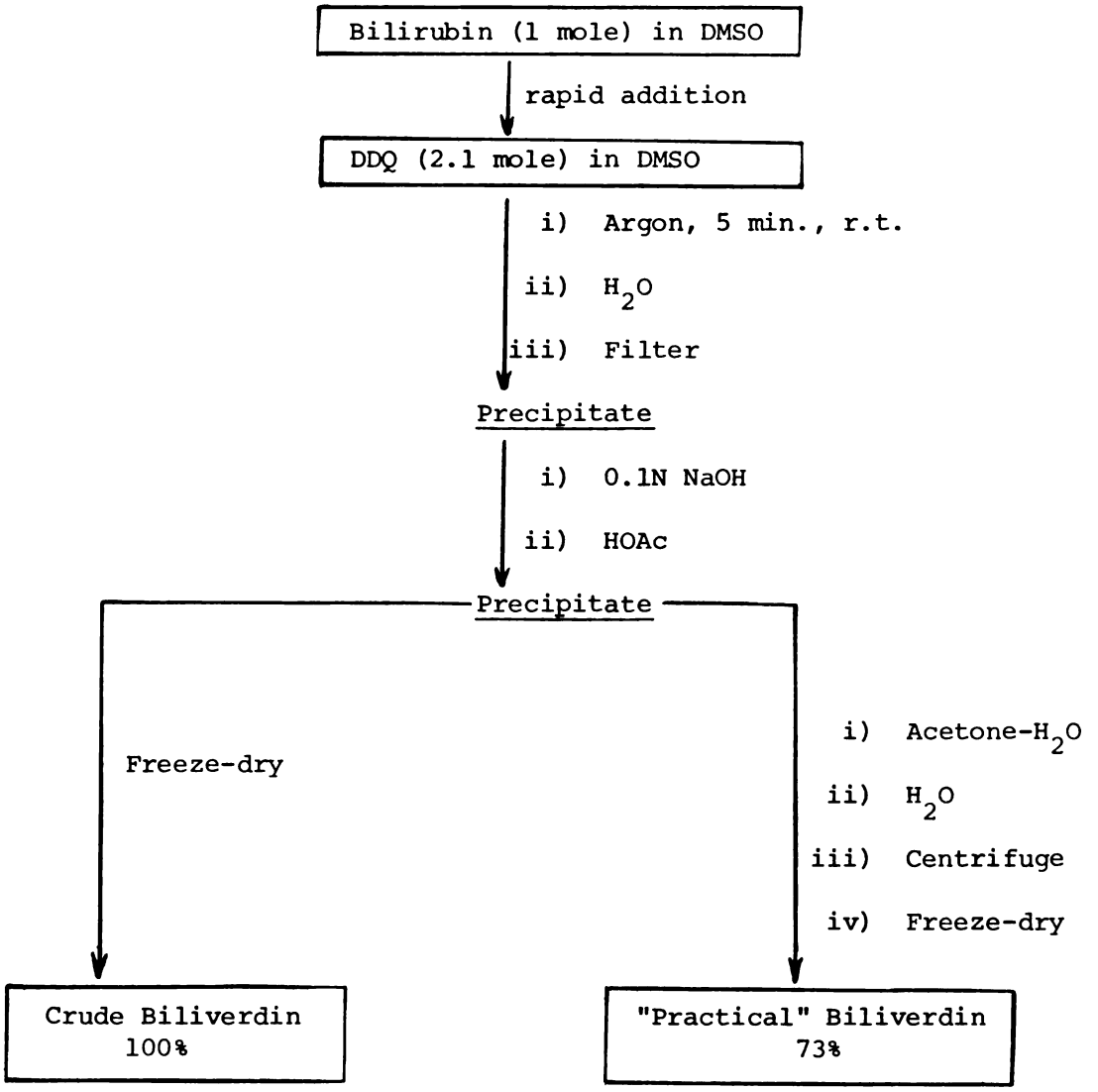
Based on the above findings the method outlined in Scheme 1 was devised for the preparation of biliverdin. Bilirubin (1.20×10^{-3} M) in DMSO was added to an equal volume of DDQ in DMSO (2.88×10^{-3} M). These were the highest concentrations at which isomerization was considered to be negligible. The reaction was complete after five minutes and the product was easily recovered in quantitative yield by precipitation with water and centrifugation. This material contained fluorescent impurities derived from DDQ. These could be removed partly by reprecipitation from base to give a quantitative yield of

crude biliverdin and more completely by suspending the crude biliverdin IXα product in acetone:water (8:2,v/v) followed by precipitation with water. This gave a "practical grade" biliverdin in 73 percent yield. This material appeared to be fairly pure by tlc (Fig. 3) but it contained a small amount of fluorescent impurities and polar material and did not give well formed crystals on crystallization from methanol.

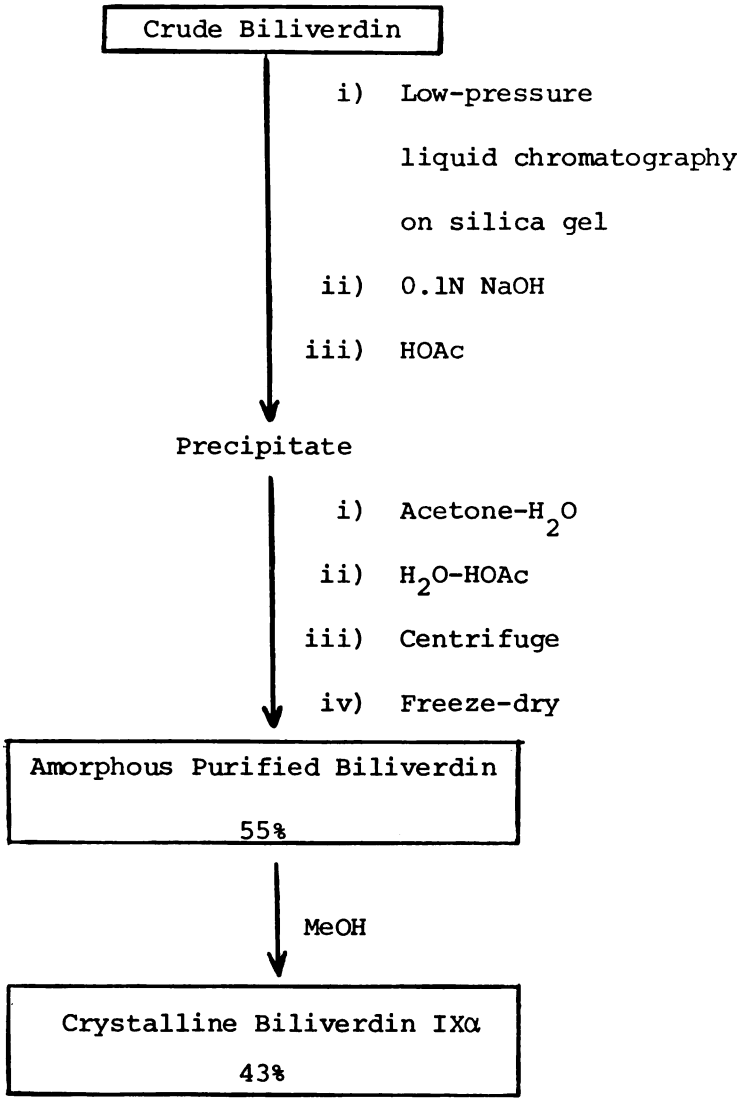
Attempts to purify crude biliverdin by silica gel and alumina column chromatography were unsuccessful due to tailing and extensive decomposition as the biliverdin passed down the column. A low pressure liquid chromatographic technique was therefore developed to avoid these difficulties. In this method, small particle size tlc grade silica gel was used as the adsorbent, the eluting solvent was sucked through under slightly reduced pressure (12-14 mm Hg below atmospheric pressure), and the height to width ratio of the column was kept small to minimize contact time and maximize column capacity. This technique provided efficient removal of the yellow and fluorescent impurities and dark green polar materials. The product from the column was reprecipitated from 0.1N NaOH with acetic acid and from its colloidal suspension in aqueous acetone with very dilute acetic acid. This yielded a pure product that was obtained as a green powder by freeze-drying. Overall yield of this material was 55 percent. The last two precipitation steps may appear to be redundant, but it was found empirically that they lead to a bulky product that dissolves readily in solvents, is easy to handle, and can be crystallized readily. This amorphous material crystallized from methanol in well-formed birefringent needles (Fig. 4). The overall yield of crystalline product from bilirubin was 43 percent.

SCHEME I. PREPARATION OF BILIVERDIN IXα

A. Preparation of Crude and "Practical grade" Biliverdin IXα



B. PREPARATION OF PURIFIED AND CRYSTALLINE BILIVERDIN IXα



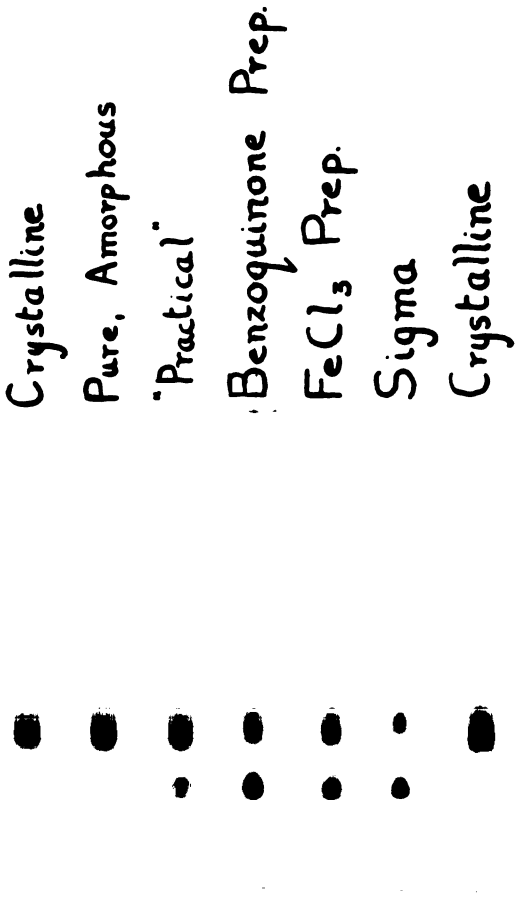


Figure 3. Thin layer chromatography of biliverdin samples.

Prolonged heating of the solution during crystallization leads to formation of insoluble amorphous material, presumably polymeric. This was avoided, at the expense of yield, by carrying out the crystallization rapidly. The amorphous freeze-dried material was indistinguishable from the crystalline product by tlc. For most applications recrystallization probably is not necessary.

The structure and isomeric homogeneity of the crystalline product was established as follows:

1) Elemental analysis

2) Field desorption mass spectrometry

3) Esterification with BF_3/MeOH gave biliverdin IXα dimethyl ester (characterized by melting point, mass spectrum, electronic absorption spectrum) that co-chromatographed with authentic samples and was found to be free of the IIIα and XIIIα isomers (Fig. 5).

4) Reduction with sodium borohydride in methanol gave bilirubin IXα, (characterized by mass spectrum, electronic absorption spectrum and tlc in two adsorbents). Isomer analysis of this material gave 95% bilirubin IXα, 1% IIIα, and 4% XIIIα, which was comparable to the analysis obtained for the original bilirubin (93% IXα, 3% IIIα, 4% XIIIα) from which the biliverdin had been prepared.

5) Reduction with sodium borohydride in methanol followed by reoxidation of the product with DDQ/DMSO gave a product that was identical by tlc to the crystalline biliverdin starting material.

6) The crystalline biliverdin was reducible by the substrate specific and isomer-selective enzyme biliverdin reductase.*

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* I thank Dr. Eugene Hrycay for examining the reaction of the crystalline biliverdin IXα with the enzyme, biliverdin reductase.

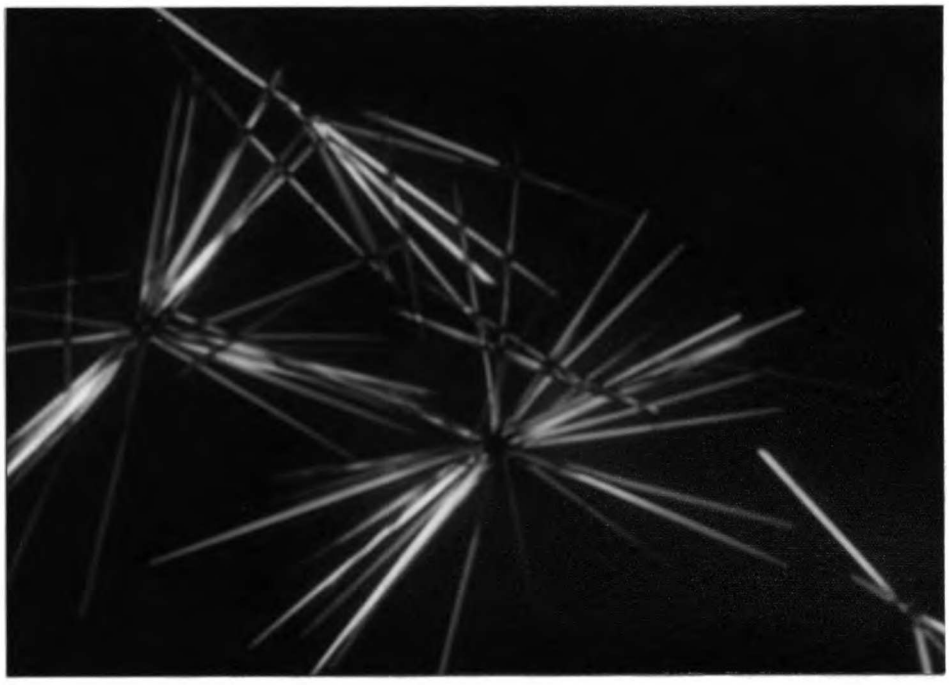

Figure 4. Biliverdin IXα crystals.

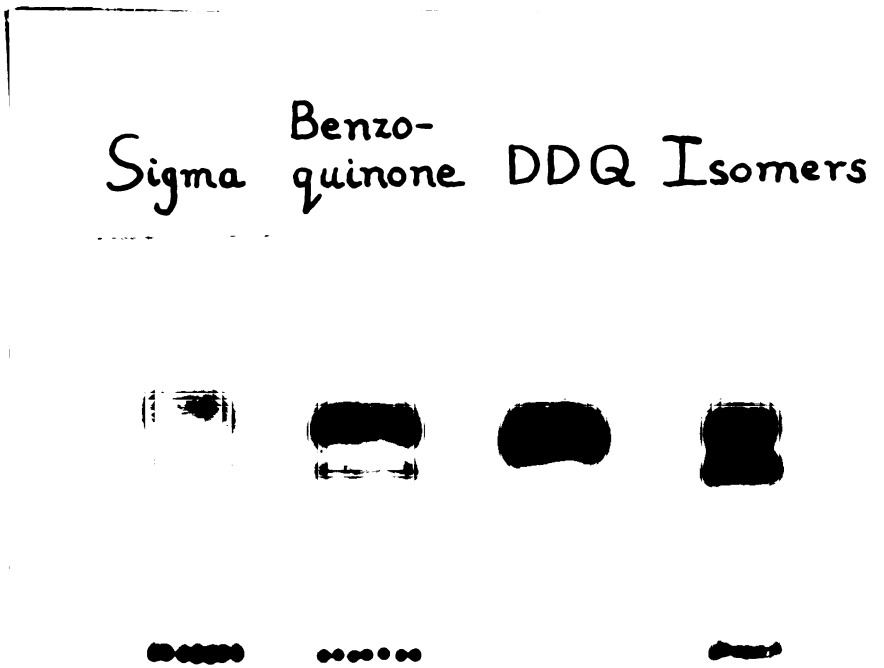

Figure 5. Thin layer chromatography of biliverdin IXα dimethyl ester.

Lane 1. Sigma biliverdin after esterification.

Lane 2. Esterified biliverdin prepared by benzoquinone oxidation of bilirubin.

Lane 3. Esterified biliverdin prepared by DDQ oxidation.

Lane 4. Mixture known to contain biliverdin IXα and XIIIα dimethyl ester and a trace of biliverdin IIIα dimethyl ester.

The procedure devised for the preparation of crystalline biliverdin IXα is simple, though time consuming and somewhat laborious. It cannot be carried out on greater than a 100 mg scale since overloading or increasing the size of the column during the chromatographic purification step leads to inefficient and poor purification. However, yields of about 40% (40 mg) of crystalline material were obtained reproducibly and the product was isomerically homogeneous and far purer than commercial biliverdin or biliverdin prepared by other common procedures ($FeCl_3$ or benzoquinone; Fig. 3). If highly pure crystalline material is not required, the method is readily adaptable for the rapid preparation of an amorphous "Practical grade" biliverdin in good yield (73%); in this case the only limit on the scale is the size of the available centrifuge. Scaled down, the method was used for the preparation of ^{14}C-biliverdin IXα from ^{14}C-bilirubin IXα in 41 percent yield.

The solubility of crystalline biliverdin IXα in a range of solvents is shown in Table 2. The molecule is insoluble in nonpolar organic solvents, in water, and in dilute aqueous acids. It is soluble in the more polar organic solvents and in aqueous base. It is much less lipophilic than bilirubin as shown by its insolubility in chloroform and benzene in which bilirubin is soluble. Solutions of biliverdin vary in color from blue through green to nearly yellow depending on the solvent and/or the pH and in methanol the pigment is somewhat thermochromic.

The absorption spectrum of biliverdin IXα in methanol is shown in Fig. 6; maxima were observed at 376 and 666 nm. Values obtained for the extinction coefficients were higher than previously determined [18,36]

Table 2. Solubility of biliverdin IXα[a]

Solvent	Solubility[b]		Color of Supernate
	22°C	50°C	
Methanol	sparingly soluble	sparingly soluble	green blue
Acetone	sparingly soluble	sparingly soluble	blue
Chloroform	slightly soluble	slightly soluble	blue
Benzene	insoluble	insoluble	colorless
Ethyl acetate	slightly soluble	slightly soluble	blue
Pyridine	soluble	soluble	green blue
DMSO	soluble	soluble	green blue
Acetic acid	soluble	soluble	green blue
Acetone:MeOH (1:1)	soluble	soluble	blue
$CHCl_3$:MeOH (1:1)	sparingly soluble	sparingly soluble	green blue
5% HCl:MeOH (w/v)	slightly soluble	sparingly soluble	emerald green
0.1N HCl	insoluble	insoluble	colorless
0.05M Tris buffer, pH4.5	insoluble	insoluble	colorless
0.05M Tris buffer,pH 7.5	sparingly soluble	sparingly soluble	emerald green
0.05M Tris buffer,pH 8.58	soluble	soluble	emerald green
0.1M $NaHCO_3$	soluble	soluble	emerald green
0.1N NaOH	soluble	soluble	emerald green
1.0N NaOH	soluble	soluble	olive green
Water	insoluble	insoluble	colorless

[a] For method see Materials and Methods.

[b] Soluble - all solid dissolved; sparingly soluble - intense colored solution with little undissolved solid; slightly soluble - pale colored solution with most solid undissolved; insoluble - no visible color in supernate.

(Tables 3 and 4) and higher[14] or similar[15] to values published for crystalline biliverdin IXα dimethyl ester (Table 4). The short wavelength absorption is relatively insensitive to solvent and pH but the long wavelength band varies considerably (Table 3). On acidification, methanol solutions of biliverdin IXα turn green and the extinction coefficient of the long wavelength band increases markedly (Fig. 6) presumably because of protonation of the weakly basic chromophore. The long wavelength band is very sensitive to pH in basic media showing a progressive shift to the red as pH increases, up to a pH of about 13 (Fig. 6). It seems unlikely that this marked shift is due solely to ionization of the carboxyl groups. The ionization of an imide -NH may well be responsible at high pH. Biliverdin IXα obeyed Beer-Lambert's law in 0.1N NaOH over a concentration range of 3.43×10^{-6} to 3.43×10^{-5} M.*

The infrared spectrum of biliverdin IXα is shown in Fig. 7. The compound did not give a meaningful electron impact or chemical ionization mass spectrum due to its involatility. But a satisfactory spectrum showing a molecular ion and little fragmentation was obtained by the field desorption technique. This is the first mass spectrum of a natural bilatriene pigment in the free acid form that has been recorded (Fig. 8).

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*Contrary to previous reports[18] biliverdin in 5% HCl-MeOH remained stable for 21 hours. Observations of Gray[18] et al that the spectrum of biliverdin in methanol or 5% HCl-MeOH changes with time were probably due to impure pigment or (less likely) failure to maintain the solutions at constant temperature.

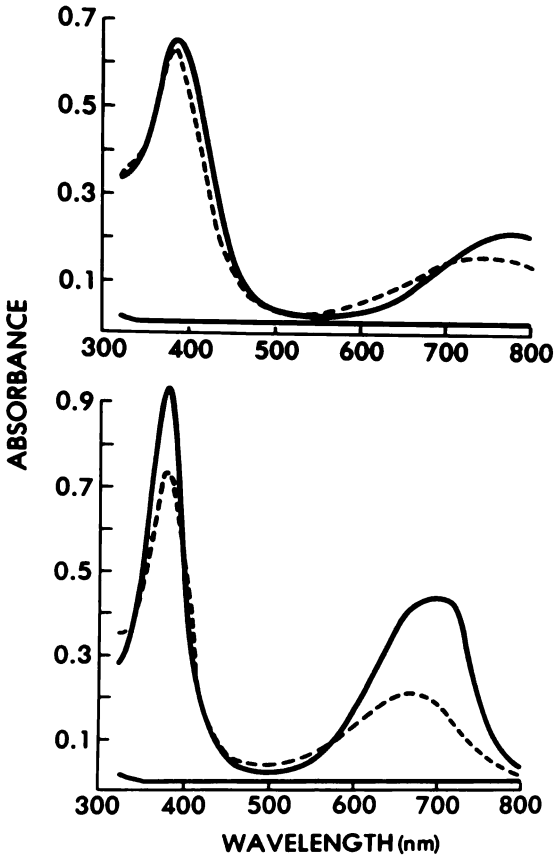

Figure 6. Absorption spectra of biliverdin IXα in (top) 0.1N NaOH (---), 1.0N NaOH (——), (bottom) methanol (---), and 5% HCl-MeOH (w/v) (——).

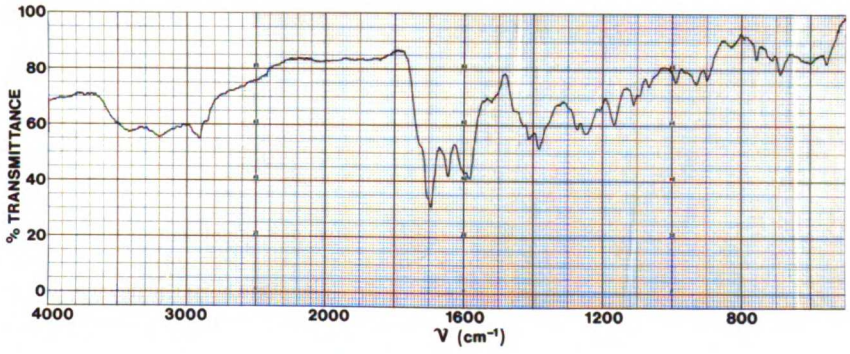

Figure 7. Infrared spectrum of biliverdin IXα.

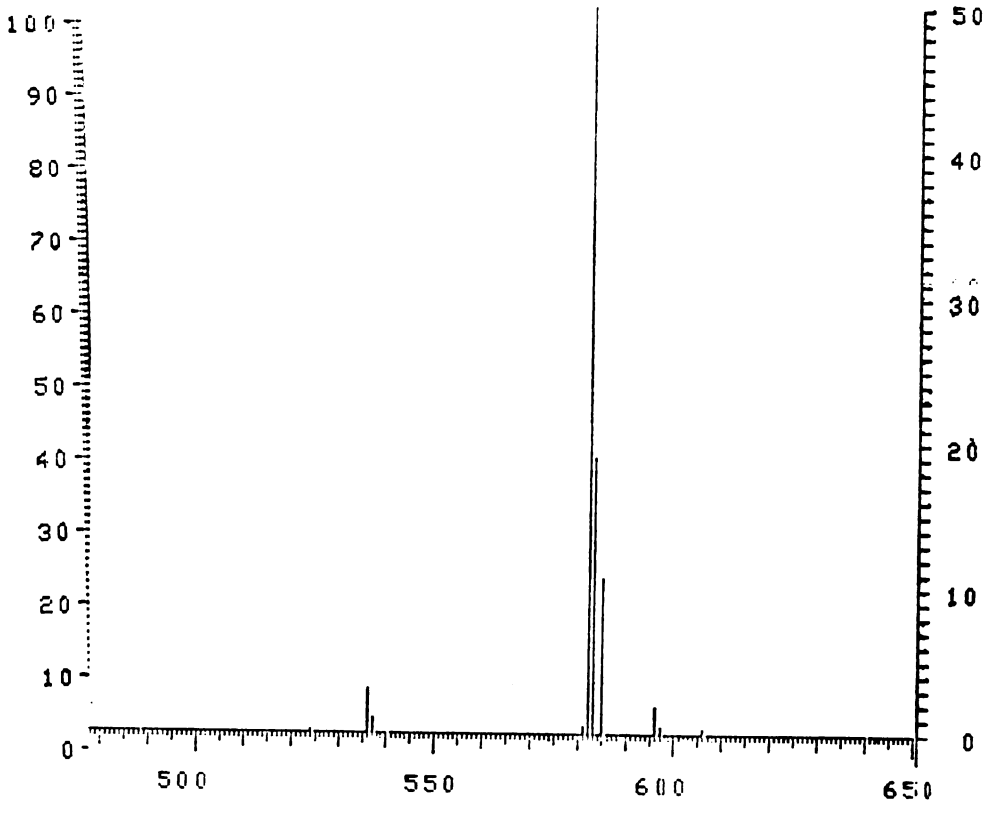

Figure 8. Mass spectrum of biliverdin IXα (Field desorption technique).

Table 3. Extinction coefficient of biliverdin IXα

Solvent	λ(nm)	ε^{a}(l-mole^{-1}cm^{-1})	λ(nm)	ε^{a}(l-mole^{-1}cm^{-1})	Color of Solution
Methanol	376	50800	666	14400	Turquoise blue
Acetone:MeOH(1:1)	377	52500	656	15000	Turquoise blue
$CHCl_3$:MeOH(1:1)	380	51600	666	15100	Turquoise blue
5% HCl-MeOH(w/v)	377	66200	696	30800	Turquoise blue[b]
0.1N NaOH	381	44200[c]	720	10700[c]	Green
0.5N NaOH	384	44300	760	13500	Lime green
1.0N NaOH	384	45800	760	13900	Lime green
0.05M Tris buffer pH 8.58	376	45500	680	12400	Emerald green

[a] Mean of six determinations. See Materials and Methods for details.
[b] In 1 mg/ml solution, biliverdin IXα in 5% HCl-MeOH solution is emerald green whereas dilute solutions are turquoise blue in color.
[c] No deviation from Beer-Lambert law over a concentration range of 3.43×10^{-6} to 3.43×10^{-5}M.

Table 4. Previously reported extinction coefficient values of biliverdin IXα and biliverdin IXα dimethyl ester.

Solvent	λ_{max}(nm)	ε(l-mole^{-1}cm^{-1})	λ_{max}(nm)	ε(l-mole^{-1}cm^{-1})	Reference
I. Biliverdin IXα					
MeOH	375-380	40500	640-650	12900	18
MeOH	392	25000	640	10400	36
5% HCl-MeOH (w/v)	373	47300	665-670	23300	18
5% HCl-MeOH (w/v)	377	48000	680	28000	36
$CHCl_3$	378	41700	645	13400	18
$CHCl_3$	384	52500	665	15800	37
II. Biliverdin IXα dimethyl ester					
$CHCl_3$	379	51800	656-664	15100	15
$CHCl_3$	380	44384	660	12570	14

CHAPTER 3

PLACENTAL TRANSPORT OF BILIVERDIN AND BILIRUBIN IXα

I. INTRODUCTION

Bilirubin, the nonpolar, lipid-soluble pigment and end product of heme catabolism in mammals, is normally conjugated by a glucuronyl transferase into polar, water-soluble pigment conjugates that are then readily excreted by the liver. Bilirubin is also formed in the fetus by heme catabolism, but this conversion is virtually unstudied and the metabolism of bilirubin in the fetus is less clear than in the adult. In the near-term fetal dog[38,39] and possibly fetal sheep,[40,41] the primary mechanism of bilirubin metabolism is via fetal hepatic conjugation and excretion. In fetal monkeys,[38,42,43,44] rats,[45,46] rabbits,[46] guinea pigs,[46-50] possibly younger fetal sheep,[40,41] and very likely the human fetus, the fetal hepatic bilirubin conjugation mechanism is deficient. Therefore it can be assumed that in these animals elimination of fetal bilirubin via placental transport to the maternal circulation is important, especially since healthy newborns in these species don't have excessively high bilirubin levels at birth.

Studies of the placental transport of bile pigment have been carried out on guinea pigs, dogs, monkeys, rats, rabbits, sheep, and humans as follows:

a) Guinea pigs: In 1959, Schmid and co-workers[47,48] demonstrated the transport of unconjugated bilirubin through the placenta from the fetal to the maternal circulation. However, the system employed was unphysiologic due to the use of high initial pigment concentrations and exclusion of the fetal circulation by direct injection into the umbilical artery. In subsequent more physiologic studies Schenker

and co-workers[49,50] found support for the concept of bidirectional transfer of unconjugated bilirubin across the guinea pig placenta and showed that conjugated bilirubin does not cross freely. Simultaneous studies with ^{131}I-labelled guinea pig albumin indicated that bilirubin has to be detached from the serum albumin for placental passage. Their studies suggested that bilirubin is not excreted by the fetal guinea pig liver and therefore, the primary mechanism of fetal bilirubin elimination is by placental transfer.

b) Dogs: Bernstein and co-workers[38] and Lester et al[39] found that only a small amount of unconjugated bilirubin crossed the placenta of a near-term dog fetus and therefore fetal hepatic conjugation and excretion was suggested as the primary mechanism for fetal bilirubin elimination.

c) Monkeys: Studies on fetal monkeys[38,42,43,44] indicated that the primate placenta transfers unconjugated bilirubin bidirectionally but not conjugated bilirubin. These studies also suggested that the primary mechanism of fetal bilirubin elimination is by placental transfer since bilirubin is not excreted by the fetal monkey liver.

d) Rabbits: In 1963, Wynn[46] studied the placental transport of bilirubin in rabbits by using pregnant rabbits made jaundiced by bile duct ligation and complete or almost complete hepatectomy. He concluded that the placenta is relatively impermeable to bile pigment since a significant equilibration of the fetal-maternal serum bilirubin levels was not observed.

e) Rats: Grodsky and co-workers[45] failed to observe significant placental transfer of unconjugated bilirubin from fetus to mother in

rats. This may be attributed to a species difference or methodologic difficulties. Tritiated bilirubin prepared by the Wilzbach procedure was used and this may have contained much exchangeable tritium, which would invalidate the technique. Wynn[46] also reported that there is little transfer of bilirubin in either direction across the placenta in rats, guinea pigs, and rabbits. He injected the bilirubin solution into the fetal jugular vein to study the fetal-maternal transfer of the bile pigment and used pregnant animals made jaundiced by bile duct ligation and complete or almost complete hepatectomy to study maternal-fetal placental transfer of bilirubin. Thaler[51] and Johnson[52] found that Gunn rats, which have a genetic defect in bilirubin excretion, are not jaundiced at birth despite high levels of unconjugated bilirubin in the maternal serum. This suggests that bilirubin does not equilibrate between the maternal and placental circulations, but does not necessarily mean that bilirubin can't cross the placenta.

f) Sheep: Jackson and co-workers[41] noted placental transfer of ^{14}C after injection of ^{14}C-heme into fetal sheep. They postulated that the heme was degraded in the fetus and then transported across the placenta as bilirubin. Alexander and co-workers[40] suggested that the near-term sheep fetus handles bilirubin both by hepatic conjugation and excretion like the near-term dog fetus. On the other hand the younger sheep fetus seemed to be similar to guinea pigs, rats, rabbits, monkeys, and possibly humans in that hepatic excretory capacity was limited and placental transport seemed to play an important role in the fetal clearance of bilirubin.

g) Man: Study of the placental transport of bilirubin in humans is limited for obvious reasons. Indirect evidence for the permeability

of the placenta to bilirubin has been obtained by comparing bilirubin levels in the umbilical vein and umbilical artery of full term infants at the time of birth as well as by comparing bilirubin levels in maternal and placental blood.[53,56] The concentration of bilirubin in the umbilical artery was found to be slightly higher than in the umbilical vein in all these studies, implying removal of bilirubin during circulation of blood through the placenta. Brown and co-workers[56] also reported that the concentration of bilirubin in the maternal peripheral and placental (intervillous) blood is lower than the concentration of bilirubin in fetal cord blood.

Shier and co-workers[57] studied the bidirectional placental transport of unconjugated bilirubin using an isolated human placenta in a modified placental perfusion device. They reported that bilirubin transferred readily from the fetal to the maternal circulation but not the reverse. However, the conditions were unphysiologic due to the use of protein-free saline for the perfusion and the infusion of bilirubin in sodium hydroxide.

Observations on children born to jaundiced mothers are confusing. Weech,[58] Smith,[59] and Slater[60] have reported cases in which jaundiced mothers gave birth to infants with serum bilirubin levels lower than the mother. Hibbard[61] also reported a case where no gross evidence of jaundice or kernicterus was observed at autopsy in a fetus delivered to a jaundiced mother whose unconjugated serum bilirubin was 20 mg%. These studies do not support the concept of rapid maternal to fetal tranplacental transfer of bilirubin. On the other hand Lipsitz and co-workers[62] observed that the cord blood bilirubin level of an infant born to a mother with unconjugated hyperbilirubinemia was simi-

lar to the maternal serum bilirubin level on the day of delivery. They suggested that this case documents evidence of a maternal to fetal placental transfer of unconjugated bilirubin in man.

Under normal conditions healthy full term infants have cord blood bilirubin levels of 2-3 mg%[55,56] which is much higher than the normal maternal serum bilirubin concentration (0.3 mg%). While this does not show that bilirubin cannot cross the placenta, it does suggest that the placental transport mechanism is not a rapid dynamic equilibrium. From all of these previous studies, despite discrepancies and possible species differences, it appears that placental transport of bilirubin from the fetal to the maternal circulation can occur and that it is an important route of excretion particularly in guinea pigs and monkeys. Furthermore, although the evidence is less convincing, bilirubin may also be able to cross the placenta from the maternal to the fetal circulation.

The metabolism of biliverdin, in contrast to bilirubin, has been studied very little and its transport across the placenta has not been studied at all. Barry and Levine,[63] who studied the reduction of biliverdin by liver and yeast cells probably were the first to suggest that the reduction of biliverdin to bilirubin in tissues is enzymatic. This was supported by Lemberg and Wyndham[64] who showed that minced or sliced preparations of many tissues reduce biliverdin to bilirubin in vitro. They found that whole blood did not reduce biliverdin, but that reducing activity was particularly high in minced mammalian liver. The enzymatic nature of the reduction was confirmed subsequently by Singleton and Laster[65] and by Tenhunen and co-workers[66] by the isolation of a soluble enzyme from guinea pig and rat liver that catalyzed the reaction in vitro in the presence of NADPH or NADH.

This enzyme, named biliverdin reductase, was detected in many tissues with highest concentrations in the liver, kidney, and spleen. Reduction of biliverdin *in vivo* has been studied by only two groups. Goldstein and Lester,[20] using ^{14}C-labelled biliverdin, found that when the pigment was injected intravenously into rats very little was excreted in bile; most was slowly reduced to bilirubin which was then excreted in conjugated form. Unfortunately this finding is not unequivocal since the labelled pigment that was used, judging by the method of preparation, probably contained mostly methyl esters and little, if any, unesterified biliverdin. This conclusion is supported by the observation that the labelled pigment was unstable and largely insoluble in neutral albumin which is not true for biliverdin. The behavior of biliverdin *in vivo* was also studied in the rat by Rodriguez Garay and co-workers[11,67] using biliverdin prepared by oxidation of bilirubin with hydrogen peroxide. They found that after intraperitoneal injection biliverdin was disposed of by two routes. Some was excreted as biliverdin in the bile; the remainder was reduced to bilirubin by the liver and then excreted in the usual way. In 5 hours 48% of the injected pigment was excreted in the bile, 18% as biliverdin and 30% as bilirubin.

The aim of the present experiments was to see whether biliverdin can be transported from the maternal to the fetal circulation and vice-versa, and to compare this with the placental transport of bilirubin. The guinea pig was chosen because it is readily available and easier to handle than the rat, less expensive than primates, and because several previous studies using bilirubin had been done on the guinea pig.*

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* The guinea pig placenta is discoid and of the hemomonochorial type.[67a] In these respects, it is similar to the human placenta.

During the course of the placental transport studies, it became apparent that biliverdin is reduced in vivo much faster than had been expected. Therefore the experiments were extended to cover this aspect of biliverdin metabolism.

The maternal-fetal transfer of biliverdin and bilirubin was examined by injecting the bile pigments into pregnant animals and examining pigment concentration in the serum of their fetuses. Fetal to maternal transfer was studied by injecting isotopically labelled pigments into a fetus and monitoring isotope levels in the mother. The reduction of biliverdin to bilirubin, following parenteral administration, was studied in vivo in fetal, pregnant, and adult male guinea pigs.

II. MATERIALS AND METHODS

General methods and reagents: Crystalline biliverdin and amorphous biliverdin-^{14}C were prepared as described in Chapter 2. Bilirubin-^{3}H was prepared biosynthetically from δ-aminolevulinic-3,5-$^{3}H(N)$ acid hydrochloride.[26,27] Toluene-^{3}H, used for quenching corrections, and Aquasol were purchased from New England Nuclear Corp., Boston, Mass. In single label experiments bilirubin-^{3}H or biliverdin-^{14}C were dissolved in 0.1N NaOH (~60 µg pigment per 0.1 ml 0.1N NaOH) and diluted with guinea pig serum to give a pigment concentration of 26.8 µg per ml. Samples (0.75 ml; 20 µg; SA = 0.3 - 8.5 X 10^{-4} dpm/µg) of these solutions were used for injections into the umbilical vein of the guinea pig fetuses. In the dual labelled experiments, 0.75 ml of a solution containing bilirubin-^{3}H (20 µg; SA = 8.5 X 10^{4} dpm/µg) and biliverdin-^{14}C (20 µg; SA = 1.65 X 10^{4} dpm/µg) similarly prepared in guinea pig serum was used for injection into the umbilical vein. The unlabelled

biliverdin IXα and bilirubin IXα solutions used for injection in the maternal-fetal transfer experiments were prepared by dissolving the crystalline pigment in 0.1N NaOH (27 mg per ml of 0.1N NaOH) and adding this solution dropwise to guinea pig serum to give a final concentration of 4-8 mg bile pigment per ml solution.

Albino guinea pigs (pregnant gp = 1-2 kg; male gp = 1.25-1.28 kg) were obtained from Cloverdale Farms, Hollister, California and Simonsen Laboratories, Gilroy, California. Pregnant guinea pigs were estimated, but not guaranteed, by the suppliers to be at a late stage of gestation and most of those used did appear to be late term. However, judging by fetal development, a few of the animals used were probably late mid-term. In most experiments anesthesia was induced by ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.; dose = 40 mg/kg) in conjunction with acepromazine maleate (Ayerst Lab. Inc., N.Y.; dose = 2 mg) administered as separate injections into the quadriceps femoris muscle of each animal.[68] This resulted in an average unconciousness time of about 4-5 hours. In a few early experiments anesthesia was induced by ether inhalation and intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, Illinois; dose 37.5 mg/kg). Xylocaine hydrochloride (1%) (Astra Pharmaceutical Products Inc., Worcester, Mass.) was administered subcutaneously at the incision site before surgical operations to minimize reflex movements. PE-10 and PE-90 polyethylene tubing (Clay Adams, Inc., New York, N.Y.) were used as femoral vein and biliary catheters, respectively. The abdomen and left leg of pregnant and male guinea pigs used in all these studies were shaved prior to surgery. Surgical silk thread size 000, Ethicon, was used for all sutures.

Guinea pig serum for preparing sample solutions was either fresh or had been stored at 4°C for not more than three days. Isotonic saline (0.89% sodium chloride) was used. Other methods and reagents were as described in the previous chapter.

Maternal-fetal transfer of bilirubin IXα: An indwelling PE 10 polyethylene catheter was inserted (to about 5 inches insertion length) into the femoral vein of the left leg of a pregnant guinea pig and patency was checked using a small volume of saline. A control sample of maternal blood (1-2 ml) was collected by cardiac puncture. An abdominal incision was made and a single fetus with intact placental circulation was exteriorized through small incisions in the uterus and amniotic sac. The fetus was wiped dry with cotton gauze and a control blood sample (~1.0-1.5 ml) was collected by cardiac puncture. The umbilical cord was clamped in two places close to the fetus, the cord was cut between the clamps and the fetus was removed and discarded. The bilirubin in guinea pig serum (see below) was infused into the mother at a rate of about 1 ml/min followed by saline solution (1 ml) as a chaser. At timed intervals after the infusion, blood samples (1-2 ml) were collected from the mother and the remaining fetuses as described above. In a few instances cardiac puncture of the fetus was unsuccessful or did not yield sufficient blood; in these cases the umbilical cord was rapidly clamped and cut and the head of the fetus was cut off with a pair of scissors and blood that dripped from the carcass was collected. Maternal and fetal blood samples were allowed to clot, serum was collected by centrifugation, and the bilirubin concentrations were determined (see below). Following each experiment maternal and fetal tissues were examined for hyperpigmentation.

Experiments were carried out using the following doses of bilirubin:

10 mg (in 2.5 ml serum)

20 mg (in 5.0 ml serum)

30 mg (in 5.0 ml serum) - animal died during experiment.

40 mg (in 5.0 ml serum) - animal died shortly after infusion.

For results see Figure 12 and Table 8.

Maternal-fetal transfer of biliverdin IXα: The experimental procedure was as described above for bilirubin. Maternal and fetal serum samples were analyzed for biliverdin and bilirubin (see below).

Experiments were carried out using the following doses of biliverdin:

10 mg (in 2.5 ml serum; 3 experiments)

20 mg (in 5.0 ml serum; 2 experiments)

20 mg (in 2.5 ml serum; 2 experiments)

40 mg (in 10 ml serum; 2 experiments)

Results are given in Figure 20 and Table 8.

Fetal-maternal transfer of ^{3}H-bilirubin and ^{14}C-biliverdin. (Single and double labelling experiments): This procedure was based on the procedure of Schenker, Dawber, and Schmid[50] with minor modifications (Fig. 9).

Through a small midline epigastric incision, the distal end of the maternal common bile duct was ligated, and a PE 90 polyethylene catheter was inserted into the proximal portion and secured with a ligature. The cystic duct was tied and the contents of the gall bladder were removed with a hypodermic syringe. The catheter was brought through the skin to the outside near the left collarbone to enable bile to be collected. The epigastric incision was then closed with sutures. A second abdominal incision just above the pubis was made and loosely

tied with surgical silk thread (Fig. 10). The animal was then placed in a specially-constructed wire basket, its feet secured with rubber bands, and in this cradle was submerged to the neck in a vertical position in a glass tank containing saline thermostatically maintained at 37°C. The loose abdominal sutures were removed, a portion of the uterus was extruded and an incision (3 cm) was made, taking care to cut as few marginal blood vessels as possible. A fetus was then delivered into the the bath. The amnion was cut away from the fetus and umbilical cord. The fetus was loosely secured with a silk thread in a submerged cradle (Figures 11a, b). The vitelline circulation was tied by a ligature placed close to the umbilical cord. The heart beat of the fetus and pulse of the umbilical cord were checked by gentle palpation.* The radioactive pigment (0.75 ml) was then injected slowly and very carefully over a period of 1-2 minutes through an umbilical vein in the direction of the fetus using a 27 gauge needle bent at a right angle close to the syringe. After withdrawal of the needle, bleeding and loss of labelled pigment was prevented by application of gentle pressure at the site of injection with the thumb and the forefinger for not more than 60 seconds. Collection of maternal bile in 15 minute batches on ice in the dark was begun immediately following the injection. Bile flow was measured by weighing and was constant to within 0.1 ml per hour in all experiments. Throughout each experiment the viability of the fetus was monitored by palpation of the heart and observation of the pulsating umbilical artery. After two hours, the mother and fetus were removed from the saline bath. The umbilical

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* In two experiments, the first fetus exposed had a weak heart beat and so a second fetus with a stronger heart beat was exteriorized and used for the experiments.

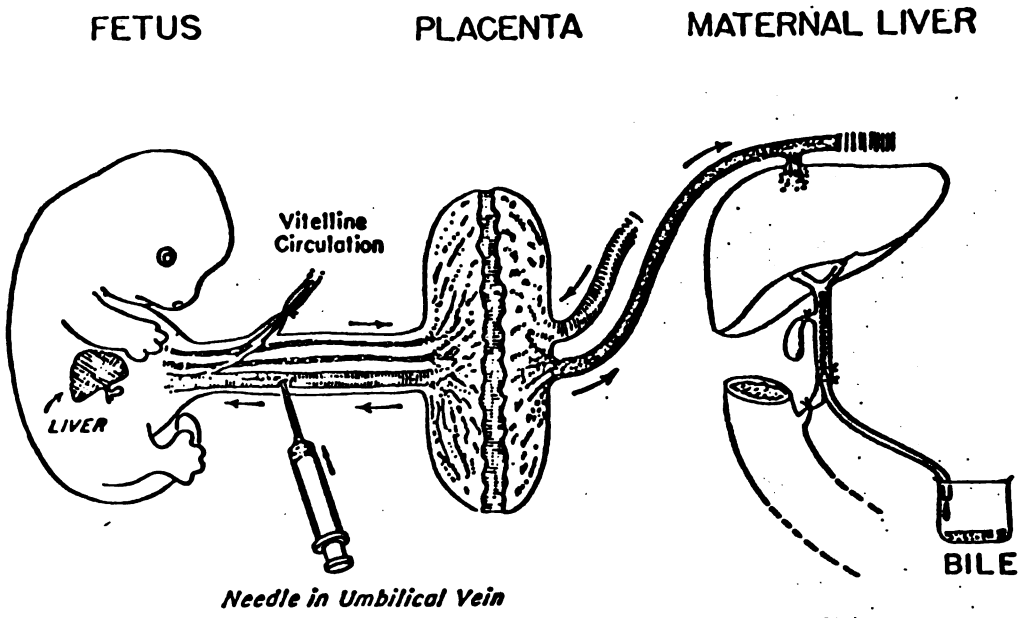

Figure 9. Experimental technique for studying fetal-maternal transport of bile pigments. Fetus, placenta, and uterus were submerged in isotonic saline bath at 37°C.[50]

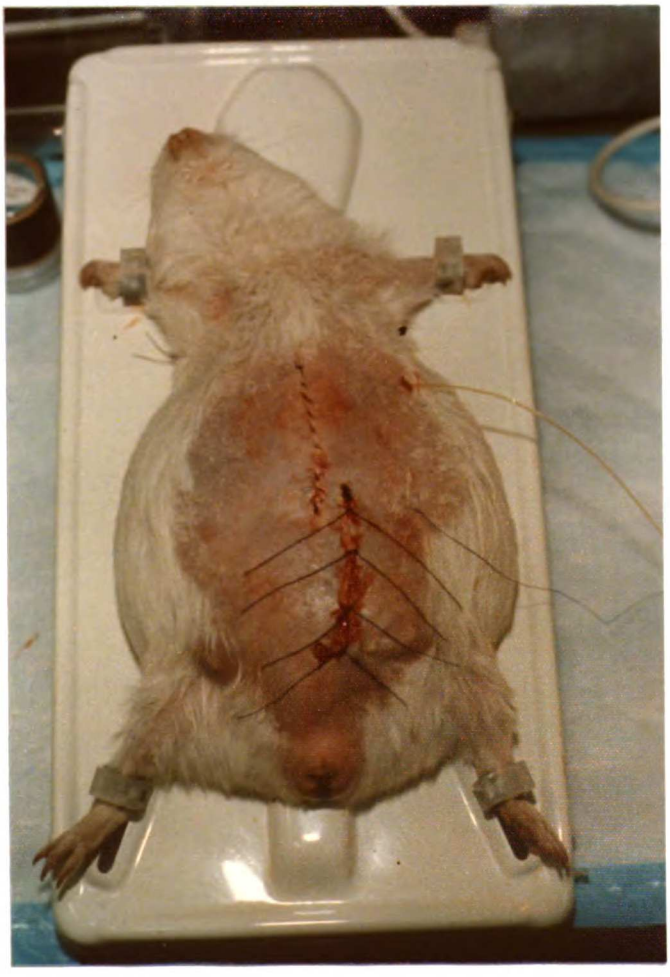

Figure 10. Pregnant guinea pig after insertion of bile duct catheter and laparotomy in preparation for isotopic labelling experiments.

Figure 11a. The mother and fetal guinea pig submerged and held in position in the isotonic saline bath at 37^{o}C.

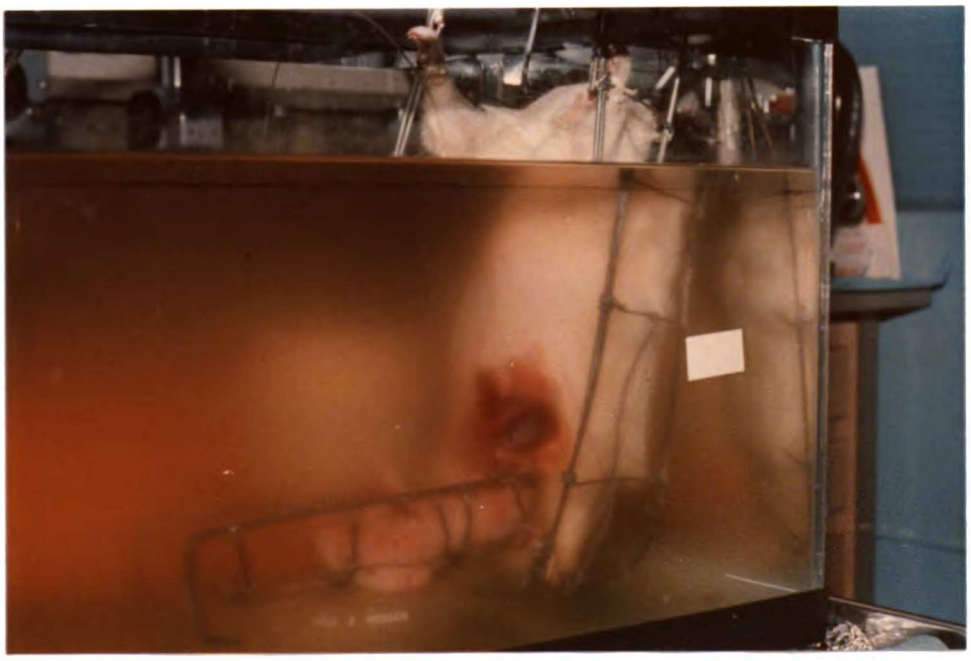

Figure 11b. The mother and fetal guinea pig submerged and held in position in the isotonic saline bath at 37°C.

cord was clamped in two places close to the fetus and the fetus was separated by cutting the cord between the clamps. The placenta was removed in a similar way from the uterus. In two experiments, blood was collected from the mother and a second, noninjected, fetus at the end of the experiment by cardiac puncture. Aliquots (0.25-0.50 ml) of the bile samples and aliquots (0.50 ml) of the serum samples were taken immediately for counting. The fetus and placenta were frozen at once and kept at -12°C until analyzed. The syringe used for the injection of the isotopically-labelled pigment was rinsed thoroughly with 0.3 ml of 1M tetramethylammonium hydroxide and the solution set aside for counting.

Six experiments were carried out using ^{3}H-bilirubin IXα, three with ^{14}C-biliverdin IXα and two with ^{3}H-bilirubin and ^{14}C-biliverdin IXα combined.

Metabolism of bilirubin and biliverdin IXα in male guinea pigs:

1. Bilirubin IXα: A male guinea pig (1.28 kg) was anesthetized and an indwelling PE 10 polyethylene catheter was inserted (to a 5 inch length) into the femoral vein of the left leg and patency was checked with saline solution. A control blood sample (1-2 ml) was collected by cardiac puncture and then bilirubin IXα in guinea pig serum (20 mg in 5 ml) was infused via the femoral vein at ~ 1 ml per minute followed by a saline chaser solution (1 ml). Blood samples were collected by cardiac puncture at different time intervals and serum concentrations of bilirubin (direct and indirect) were determined. After the last blood sample collection, the color of the skin and internal organs were examined.

2. Biliverdin IXα: The experimental procedure was as for bilirubin above but serum samples were analyzed for biliverdin as well as

for direct and indirect bilirubin.

Characterization of bilirubin formed by reduction of biliverdin in vivo: Bilirubin was extracted by the method of Kirshenbaum and co-workers[69] from pooled serum samples obtained from a guinea pig injected with biliverdin as above. Bilirubin (0.055 mg) was obtained from 3.5 ml of pooled serum. This was identified as bilirubin IXα by spectrophotometry and co-chromatography with an authentic sample using tlc systems A and B.

Reduction of biliverdin IXα in the fetus: A pregnant guinea pig was anaesthetized and a midline abdominal incision (5-6 cm) was made upwards from just above the pubis. The incision was stitched together loosely and the animal was submerged to the neck in an isotonic saline bath at 37°C as described for the labelling studies. One fetus was exteriorized as before and biliverdin in guinea pig serum (1.5 mg biliverdin, 0.5 ml) was injected under saline through the umbilical vein in the direction of the fetus. After 6 minutes, blood was obtained from the fetus by cardiac puncture under saline. The umbilical cord was tied with silk thread in two places close to the fetus and then cut between these ties. The separated fetus, still alive, was taken out of the bath and a second blood sample was obtained by cardiac puncture. This procedure was repeated with two more fetuses except that 16 and 18 minutes, respectively, were allowed to elapse before doing the first cardiac puncture. The last fetus in the uterus was used as control. After delivery into the bath, the control fetus was injected with 0.5 ml guinea pig serum. Blood was obtained by cardiac puncture under saline at 14 minutes and then again at 19 minutes after the fetus had been separated from the placenta and removed from the bath. Fetal tissues were examined for pigmentation

and fetal serum samples were examined spectrophotometrically (800-350 nm) and analyzed for fetal bilirubin content.

Reduction of biliverdin IXα in whole blood:

1) A solution of biliverdin IXα (0.91 mg) in 0.1N NaOH (1.0 ml) was prepared. Fresh whole blood was obtained from a pregnant guinea pig by cardiac puncture using a heparinized syringe. To 5 ml of fresh blood was added 250 µl of the biliverdin solution. The mixture was shaken gently for a few seconds and centrifuged and the plasma was examined spectrophotometrically (800-350 nm). Another 5 ml fresh blood was mixed gently with 250 µl of biliverdin solution and incubated at 37°C for one hour. This sample was then centrifuged and the plasma was analyzed spectrophotometrically. To a third 5 ml of fresh blood was added 250 µl of 0.1N NaOH as a control. The sample was shaken gently, incubated at 37°C for one hour, centrifuged and the plasma was again examined spectrophotometrically.

2) Frozen guinea pig serum from a pregnant female was thawed. To one ml of this was added 5 µl of the biliverdin solution used in (1) above. The spectrum of the resulting solution was scanned repetitively at 37°C for 45 minutes.

No reduction of biliverdin IXα as measured by change in absorbance was observed in either of these studies.

Determination of bilirubin: The total and direct bilirubin content of serum samples was measured by a micro-modification of the method of Malloy and Evelyn.[70] In some selected instances (see Results) the presence of bilirubin also was evaluated by spectrophotometric examination of a 1:10 dilution of the serum with 0.1M Tris buffer pH 7.4; the presence of bilirubin was indicated by a peak or shoulder

in the 470 nm region.

Effect of turbidity on bilirubin determination: Three frozen turbid fetal guinea pig serum samples were thawed and 0.1 ml aliquots were placed in separate tubes. The remaining portions were centrifuged (Sorvall centrifuge, 0-4°C, 20 minutes) and aliquots of the clear supernates were removed. The bilirubin content of the supernates and the original samples was determined. There was no significant difference in the bilirubin concentration of the turbid serum samples and the corresponding supernates.

Effect of biliverdin on bilirubin determination: Crystalline biliverdin IXα (1.96 mg) was dissolved in 0.1N NaOH (0.15 ml) and then added dropwise to guinea pig serum (0.85 ml). Aliquots of this stock solution were diluted with guinea pig serum to give six solutions with biliverdin concentrations of 1.96, 9.8, 19.6, 39, 65, and 98 mg%. Aliquots (0.100 ml) of each of the biliverdin solutions prepared as described above were analyzed by the Malloy and Evelyn[70] diazo method to determine any possible interference of biliverdin in the bilirubin determination of serum samples by this method. It was found that at 40 mg% biliverdin, "apparent bilirubin" was 0.3 mg% as shown in Figure 17.

Determination of biliverdin: Serum samples were diluted with 0.1M Tris buffer pH 7.4 and examined spectrophotometrically (800-400 nm). Biliverdin concentrations were calculated from the absorbance reading at λ_{max} (~680 nm) taking $\varepsilon_{\lambda_{max}} = 1.28 \times 10^4$. Qualitative detection of biliverdin in some early experiments was done by visual inspection of serum samples for green coloration.

Liquid scintillation counting of samples:

a) Serum, bile: 0.25-.50 ml of bile or serum was pipetted into

a low-potassium glass vial fitted with a poly-seal cone-lined screw cap. The sample was treated with tetramethylammonium hydroxide, hydrogen peroxide, hydrochloric acid, and ascorbic acid as described in chapter 2. Toluene-^{3}H and toluene-^{14}C (50 µl each) were added as internal standards for determining quenching corrections. Results were expressed as total disintegrations per minute (dpm), and percent radioactive recovery based on the injected dose. Counting efficiencies ranged from 57-67 percent for ^{14}C and 26-37 percent for ^{3}H.

b) Fetus: Each fetus was homogenized with 80 ml of water in a Waring blender, then diluted to 300 ml. A one ml aliquot was treated as for the serum and bile samples except that 20 ml of Aquasol was used as the scintillation solvent instead of Ready-Solv^{Tm}HP. Counting efficiencies ranged from 27-28 percent for ^{3}H and 57-59 percent for ^{14}C. Results were expressed as for the serum or bile samples.

Recovery experiment: Three unlabelled fetuses were homogenized as previously described and to each homogenate (100 ml) was added a serum solution of bilirubin-^{3}H (400 µl, 1.52-1.85 X 10^{5} dpm). Duplicate 1 ml aliquots of each homogenate were counted as previously described. The efficiency was 26-29 percent and the recovery of bilirubin-^{3}H was 89.40 percent (average of three determinations).

c) Placenta: Each placenta was homogenized in a Waring blender and made up to 50 ml with water. A 3 ml aliquot was treated and counted as the fetus homogenates described above. Efficiencies ranged from 28-29 percent for ^{3}H and 53-59 percent for ^{14}C. Results were expressed as total disintegrations per minute (dpm) and percent of the injected dose recovered.

Recovery experiment: Three unlabelled control placentas were homogenized and made up to 50 ml with water. Each homogenate was

fortified with bilirubin-^{3}H in serum (200 µl, 7.76 x 10^{4} dpm) and three ml aliquots of each were counted in duplicate. Efficiency was 28 percent and recovery of bilirubin-^{3}H was 89.36 percent (average of three determinations).

III. RESULTS

After intravenous injection of bilirubin into pregnant guinea pigs at doses of 10, 20 and 40 mg there were marked increases in fetal serum bilirubin levels within less than 10 minutes as determined by the diazo reaction (Fig. 12). This was in general agreement with the results of Schenker and co-workers.[50] The animal injected with 30 mg died before completion of the experiment as did two other animals injected with 40 mg. Therefore, it was not possible to repeat the work of Schenker and co-workers at these higher doses. In the reported work the volume of the artificially jaundiced serum samples that was injected was not given. In our studies, it was felt wise to restrict the volume of the injected samples to between 2.5-5.0 ml. With these volumes and high amounts of pigment the solutions are undoubtedly colloidal or supersaturated and the observed toxicity at the higher doses is probably related to this. Blanc and Johnson[71] have reported that large doses of bilirubin given intravenously to albino rats caused massive pulmonary hemorrhage.

Figures 13 and 14 show the maternal serum biliverdin levels following intravenous injection of 10 mg and 40 mg biliverdin into late-term pregnant animals. Biliverdin concentrations were determined spectrophotometrically at λ_{max} (680 nm) after diluting serum samples at least 1:10 with 0.1M Tris buffer pH 7.4. This method was validated by showing that biliverdin obeyed the Beer-Lambert law within the concentration ranges observed and that mild hemolysis or the presence

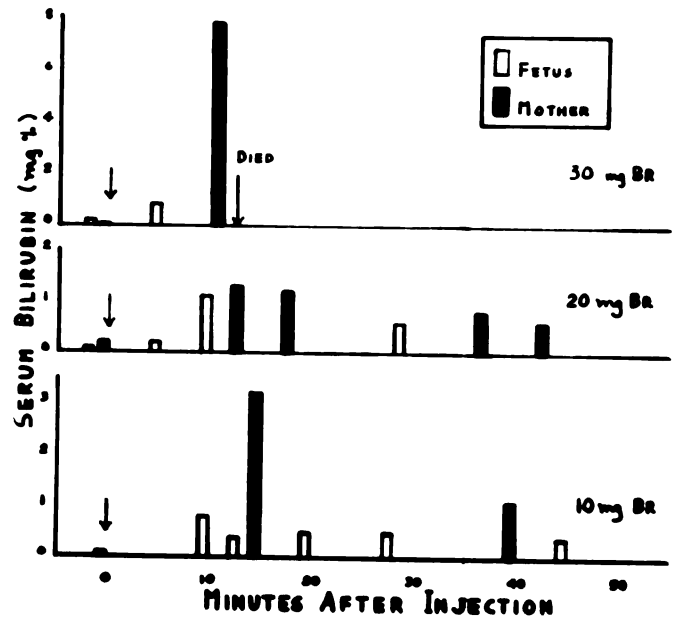

Figure 12. Maternal and fetal serum bilirubin concentrations following injection of bilirubin into the mother. Pregnant guinea pigs were injected intravenously with bilirubin (10 mg, 20 mg, 30 mg) in serum and the bilirubin concentration in maternal and fetal serum was measured. Blood samples taken shortly before injection served as t=0 controls. ■ Serial samples from the mother; □ samples from individual sibling fetuses

of bilirubin did not cause significant interference. Maternal serum samples were markedly green shortly after injection of the biliverdin and progressively became olive green and yellow. The absorption spectra of the samples that were visibly green after dilution with 0.1M Tris buffer pH 7.4 showed a pronounced peak at 680 nm corresponding to the absorption maximum of biliverdin. None of the fetal serum samples obtained after injection of the mother with biliverdin (Figures 15 and 16) were green. Most were milky due to high lipid concentrations and some were yellow due to the presence of bilirubin (see below). But none were green, even those taken at times when biliverdin concentrations in the maternal serum were high (Figure 16). Altogether nine experiments were carried out analogous to those shown in Figures 15 and 16 using biliverdin doses of 10 mg (3 experiments), 20 mg (4 experiments) and 40 mg (2 experiments). In none of these did the fetuses develop even faintly green serum following injection of biliverdin into the mother although in all of them biliverdin was easily visible in the maternal serum for at least 12 minutes after injection. Accurate determination of fetal biliverdin levels by spectrophotometry was impossible due to the high amount of lipid in most fetal serum samples which was probably an indirect effect of laparotomy.[72] However in two experiments, one with a dose of 10 mg, the other with a dose of 20 mg (Figure 14) all fetal serum samples were scanned spectrophotometrically, and no biliverdin band was detected at a serum dilution of 1:10 and an absorbance scale expansion of 0-0.2 using 1 cm cells. This rules out concentrations of biliverdin in excess of 0.03 mg%.

Early experiments showed that serum and in some instances mesenteric and subcutaneous fat rapidly developed a yellow color following

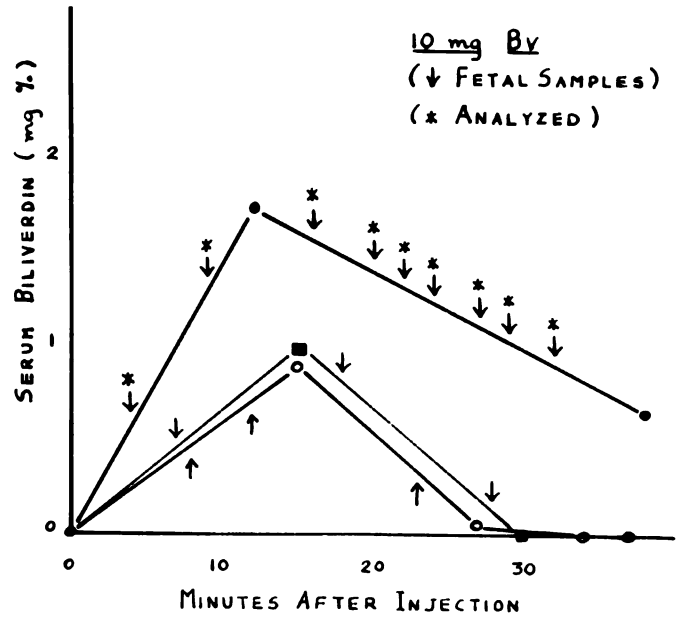

Figure 13. Serum biliverdin levels in 3 pregnant guinea pigs following injection of 10 mg biliverdin. At the times marked ↓ a blood sample was taken from a different fetus. (Also see Figure 15) *Indicates fetal serum samples that were scanned for biliverdin by spectrophotometry.

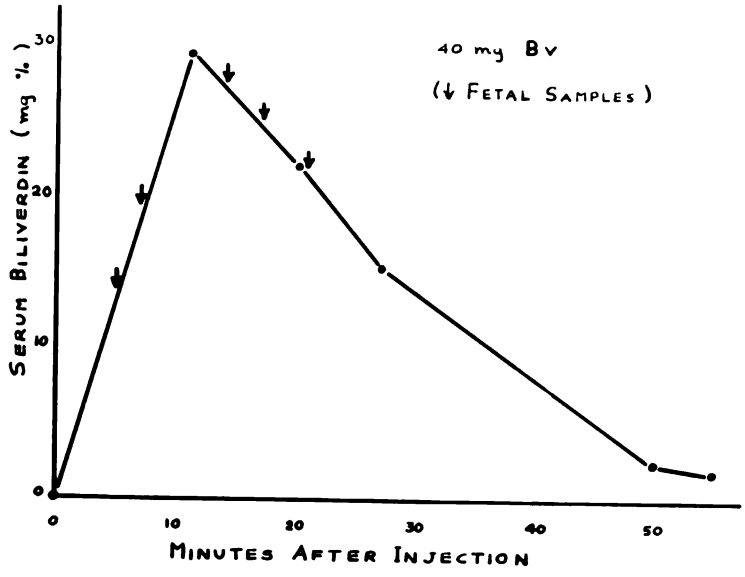

Figure 14. Serum biliverdin levels following intravenous injection of 40 mg biliverdin into pregnant guinea pig. At the times marked ↓ a blood sample was taken from a different fetus.*

* Results shown in Figure 16.

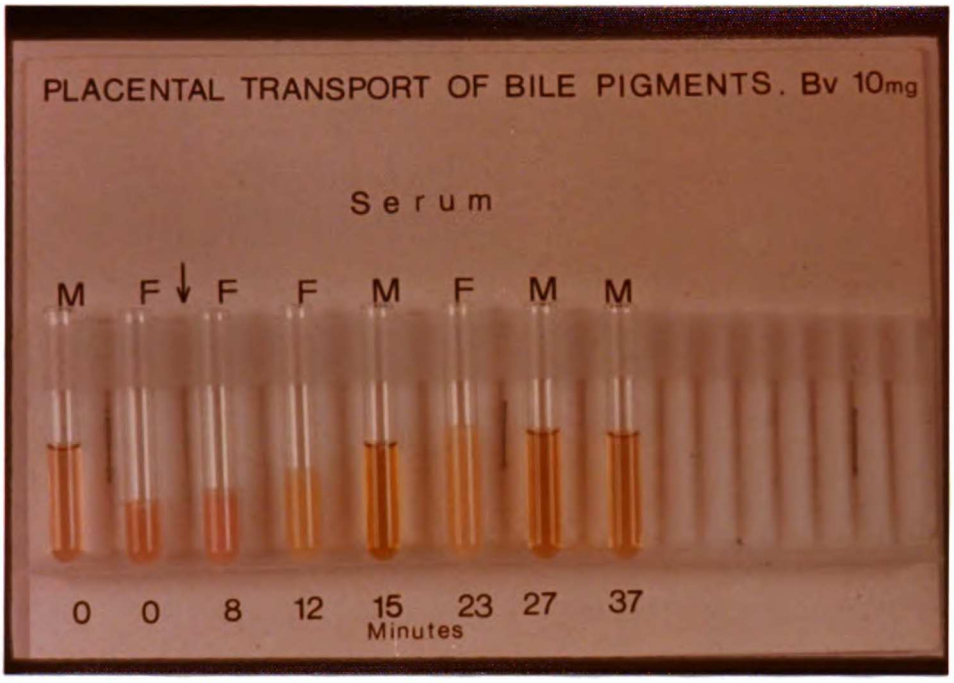

Figure 15. Maternal and fetal sera obtained after injection of 10 mg biliverdin into pregnant guinea pig.

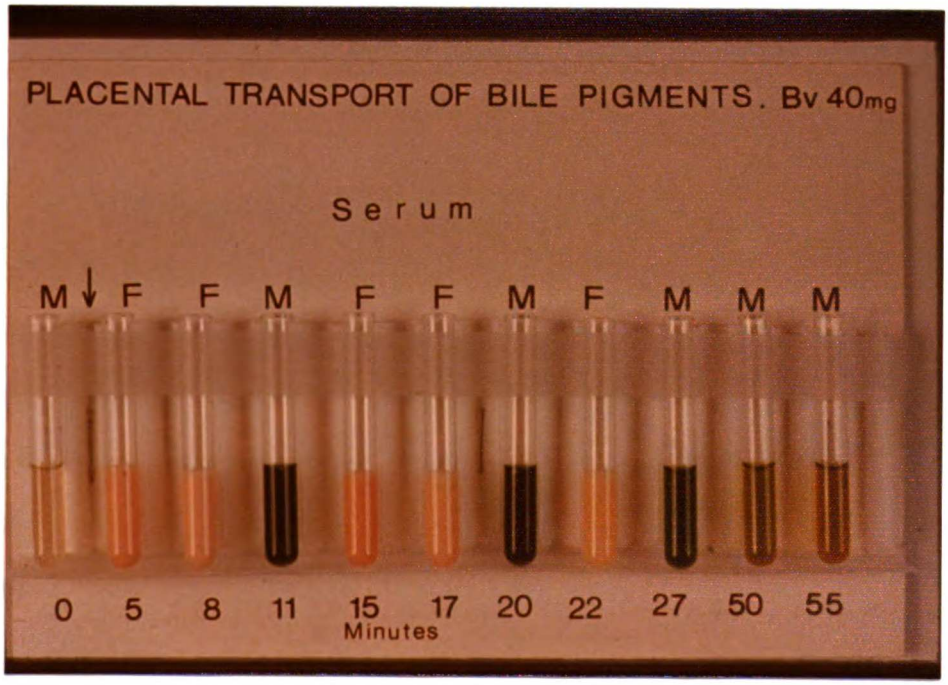

Figure 16. Maternal and fetal sera obtained after injection of 40 mg biliverdin into pregnant guinea pig.

injection of biliverdin. Spectrophotometric examination of the serum revealed a distinct peak or shoulder at ~470 nm and the serum showed a positive diazo reaction. Addition of bilirubin IXα to the serum *in vitro* resulted in enhancement of the absorption band at 470 nm. These observations suggested that the yellow pigment was bilirubin, formed by the reduction of parenteral biliverdin *in vivo*. Because of its bearing on the placental transport studies this reduction of biliverdin was studied in more detail.

The kinetics of the reduction were studied in 3 systems; pregnant guinea pigs, adult males, and fetuses. Biliverdin was injected intravenously at various doses and serum bilirubin levels following the injection were determined by the diazo technique. Control studies showed that the interference of biliverdin in the determination of bilirubin by the diazo method was negligible. For example, when the concentration of biliverdin in nonjaundiced serum was adjusted to 40 mg% the apparent bilirubin concentration was only 0.3 mg% (Figure 17). Measured serum biliverdin concentrations did not exceed 40 mg% in any of the animal studies and in most samples were considerably below this.

After intravenous injection of 40 mg of biliverdin into a pregnant animal, maternal serum bilirubin concentrations rose to 5.4 mg% within 10 minutes and then remained elevated and essentially constant for almost an hour (Figure 18). By the end of this period, when the experiment was terminated, the mesenteric and subcutaneous fat of the mother was yellow. A rapid elevation of serum bilirubin followed by a drop to a lower plateau was also observed with lower doses of biliverdin (Figure 19). In all cases maternal serum bili-

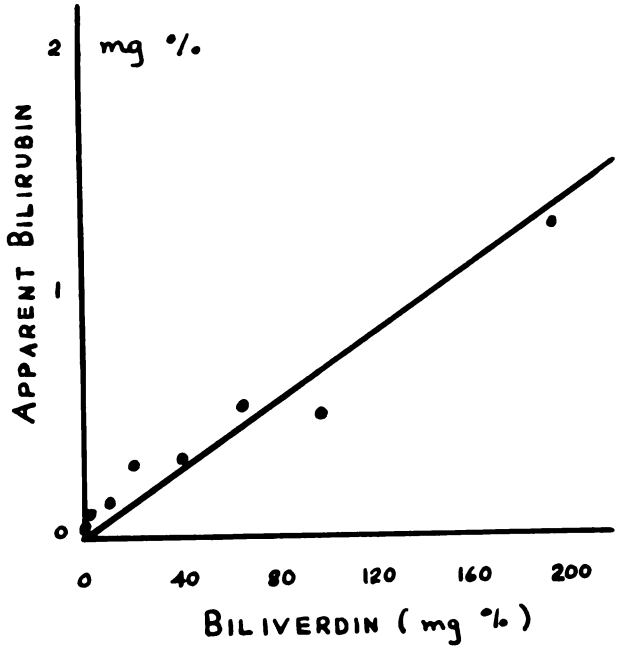

Figure 17. Effect of biliverdin on the determination of total bilirubin by the diazo method.

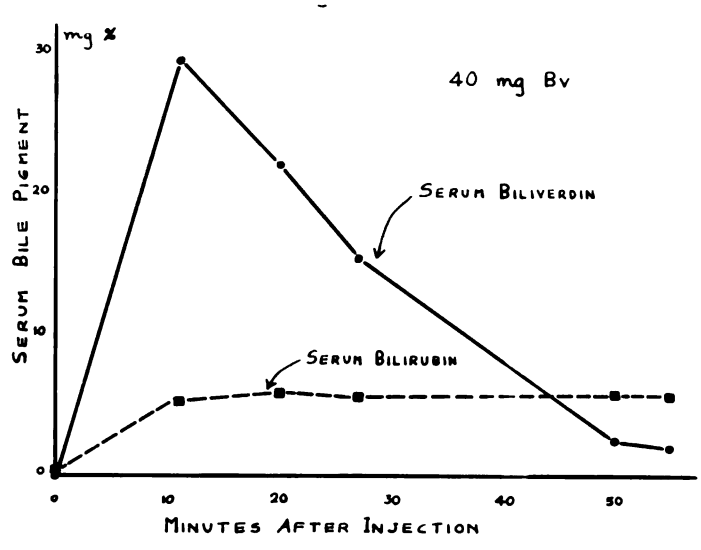

Figure 18. Serum biliverdin and bilirubin levels following injection of biliverdin (40 mg) into a pregnant guinea pig. —— Serum biliverdin levels; ---serum bilirubin levels.

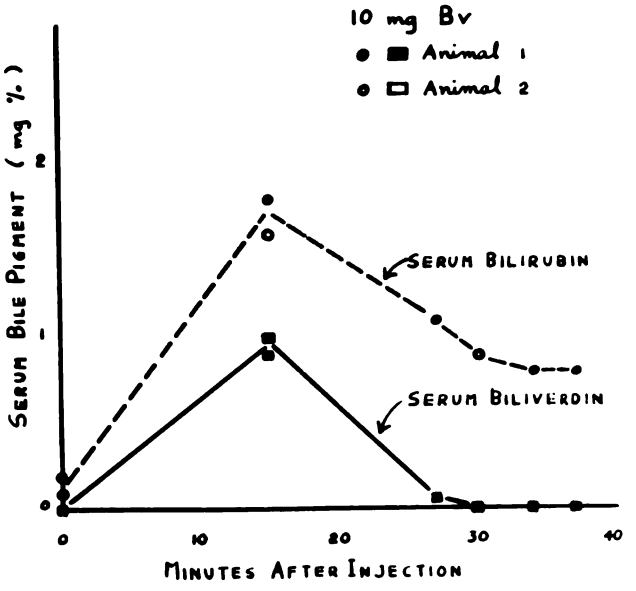

Figure 19. Serum biliverdin and bilirubin levels following intravenous injection of biliverdin (10 mg) into pregnant guinea pigs. —— Serum biliverdin; ---serum bilirubin; • and o represent values from two different animals.

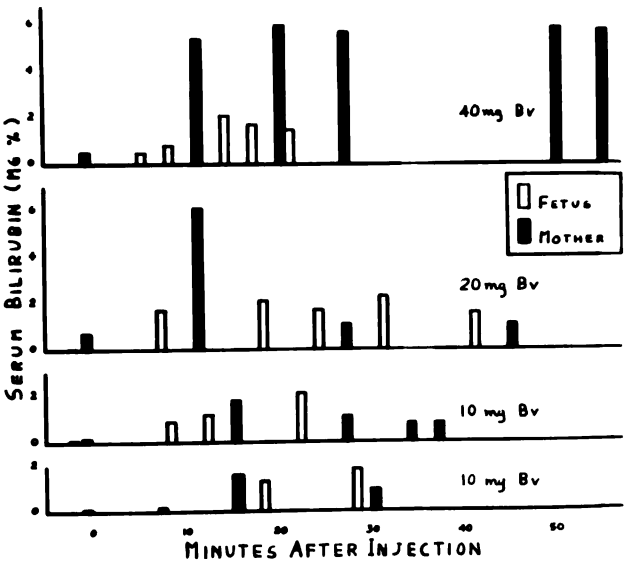

Figure 20. Maternal and fetal serum bilirubin concentrations following injection of biliverdin into the mother. Pregnant guinea pigs were injected intravenously with biliverdin (10 mg, 20 mg, 40 mg) in serum. Bilirubin concentrations in maternal and fetal serum samples were measured. Blood samples taken shortly before the injection served as t=0 controls. ■ Serial samples from mother; □ samples from individual sibling fetuses.

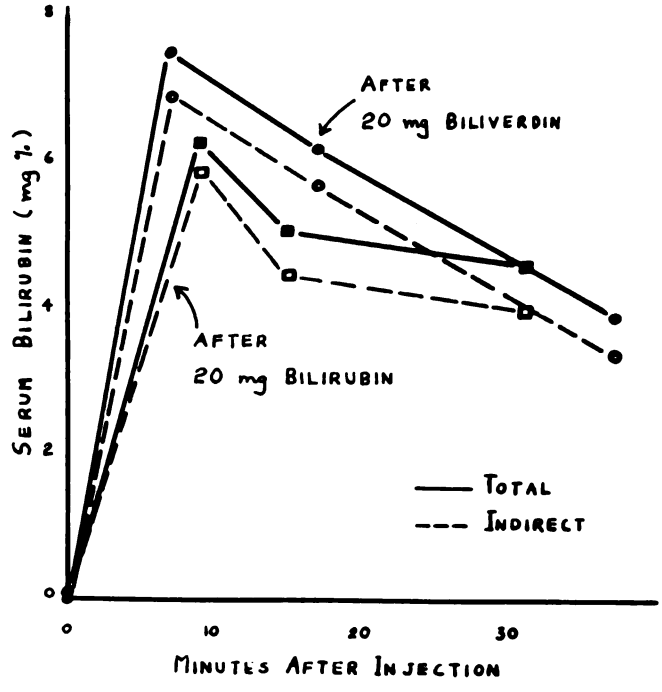

Figure 21. Serum bilirubin levels following injection of biliverdin (20 mg) and bilirubin (20 mg) into male guinea pigs.—— Total bilirubin; --- indirect bilirubin.

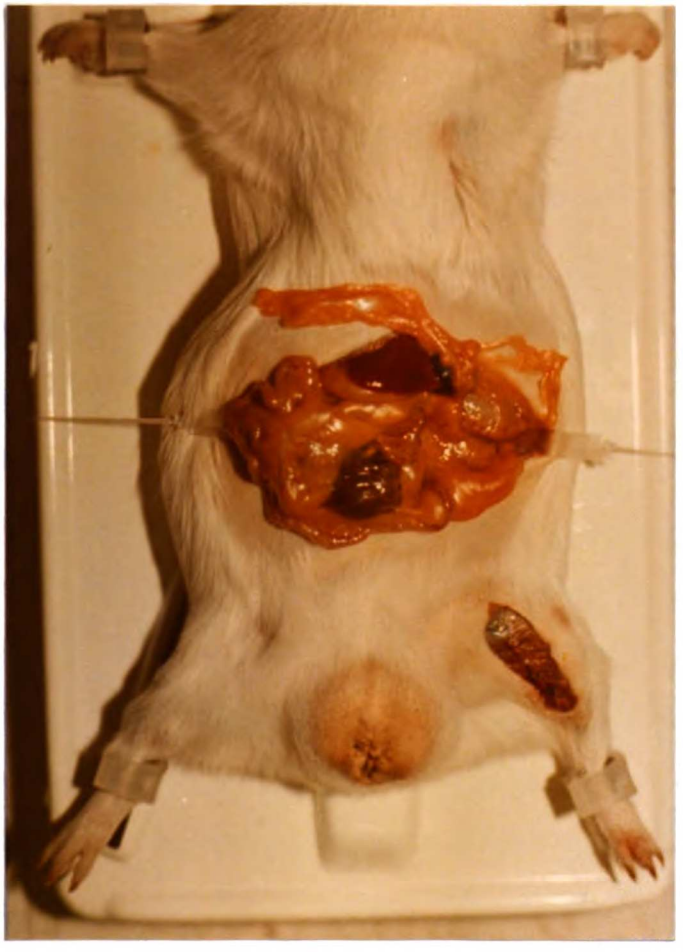

Figure 22. Pigmentation of adipose tissues of a male guinea pig 40 min after injection of biliverdin (20 mg).

verdin levels rose rapidly and then gradually declined (Figures 18 and 19). Concomittant with the elevation of bilirubin levels in the mother, there was a pronounced increase in the level of bilirubin in the fetal serum at all doses of biliverdin studied (Figures 15, 16 and 20).

Rapid reduction of biliverdin also was observed in a male guinea pig (Figure 21) along with marked pigmentation of fat (Figure 22). The serum levels of bilirubin that were reached following injection of 20 mg biliverdin were of the same order of magnitude as those reached in a control animal that was injected with 20 mg bilirubin (Figure 21). Only a small fraction of the bilirubin in the serum following the biliverdin injection gave a direct diazo reaction showing that virtually all of the pigment, essentially, was in the unconjugated form. The pigment obtained by chloroform extraction of a serum sample taken from the male guinea pig 37 minutes after injection of biliverdin was confirmed to be bilirubin by tlc on two different adsorbents and by its visible absorption spectrum in chloroform.

Reduction of biliverdin in fetal guinea pigs was studied in one experiment. In this experiment, the fetuses were delivered by Caesarian section in a saline bath. With the fetal-placental-maternal circulation still intact, biliverdin (1.5 mg) in serum was injected into the umbilical vein of each fetus and one or more blood samples were obtained by cardiac puncture. The first sample from each fetus was collected while the fetal-maternal circulation was still intact; the second was obtained while the fetus was still alive, but after the cord had been severed and the fetus had been removed from the saline bath. One fetus was injected only with serum

as a control.* This experiment was not easy, largely because of the difficulty in obtaining blood samples from each fetus by cardiac puncture while the fetus was submerged in the saline bath. Results of the experiment are shown in Table 5 and Figure 23. Serum samples from the control fetus were colorless, had no detectable absorption bands at 680 nm (biliverdin) or 470 nm (bilirubin) and had a low concentration ($\leq$ 0.2 mg%) of bilirubin. On the other hand the fetuses injected with biliverdin had serum that was green or yellow, showed absorption bands at 680 nm and 470 nm and had elevated serum bilirubin levels (1.0-1.7 mg%). Slight yellow pigmentation of subcutaneous fat was visible at the end of the experiment in the test fetuses but not in the control fetus.

Fetal-maternal transfer of bile pigment was studied by injecting small quantities of isotopically labelled pigments into the umbilical vein of a fetus and following the appearance of the isotope in the maternal bile.[50] Three series of experiments were carried out; single-pigment studies with ^{3}H-bilirubin, single-pigment studies with ^{14}C-biliverdin, and double pigment studies with ^{3}H-bilirubin and ^{14}C-biliverdin administered simultaneously. In some experiments, the fetus and its placenta were homogenized and counted. For counting, the method of Shucard and co-workers[68] was used in which the sample is dissolved in tetramethylammonium hydroxide and bleached with hydrogen peroxide. Complete bleaching and solubilization of the homogenized fetus and placenta was not effected with this technique. However, recovery experiments determined with ^{3}H-bilirubin were high (89.40 ±

* The dose of biliverdin injected was roughly equivalent to a dose of 20 mg in the mother based on 1250 grams body weight (guinea pig fetus weighs 94 grams).

6.09 for fetus and 89.36 ± 1.03 for placenta).

Six experiments were performed using ^{3}H-bilirubin. One of these was disregarded since the total recovery of isotope was low (32%). In the remaining five (Table 6) the total recovery of injected isotope varied between 75-101 percent. In all the experiments, the isotope appeared in the maternal bile within 15-30 minutes. The pattern of excretion of the radioisotope in the maternal bile in experiments 1-3 is shown in Figures 24a, 24b. The output of isotope peaked at 30-45 minutes after injection and then slowly declined. This pattern is similar to that reported by Schenker and coworkers.[50] In two experiments (#4 & 5, Table 6), the proportion of radioactivity excreted in the maternal bile was low and hold-up in the fetus was high. In these experiments, the isotope was present in all of the maternal bile samples collected but the pattern was irregular and did not conform to the pattern observed in Experiments 1-3 (Figure 24a). This could perhaps have been due to some variation in technique during the rather difficult intravenous injection, or due to immaturity of the fetus injected. However the actual reason was not ascertained.

In three experiments, ^{14}C-biliverdin was injected at the same doses as for the ^{3}H-bilirubin experiments. In one of these experiments, the total recovery of radioactivity was low and the results were discarded. In the other two (#6 & 7 and Table 6), total recovery was high and the pattern of isotope excretion in the maternal bile (Figures 25a and 25b) was qualitatively similar to that observed with ^{3}H-bilirubin (Figures 24a and 24b). Quantitatively, the proportion of radioactivity in the maternal bile following

Table 5. Reduction of biliverdin in fetal guinea pigs.

Fetus No.	Time after biliverdin injection (min)	Serum bilirubin concentration (mg%)	Serum color	Serum absorption[a] 680nm	470nm
1[b]	6	1.2	green	very strong	shoulder
	21	1.5	yellow	strong	shoulder
2[b]	16	1.7	green-yellow	strong	shoulder
3[b]	18	1.0	green-yellow	--	--
	35	1.0	yellow	weak	shoulder
4[c]	14	0.2	colorless	none	none
	19	0.1	colorless	none	none

[a] After 1:10 dilution with 0.1M Tris buffer, pH 7.4. Solutions in 1 cm path length cells were scanned from 800-400 nm at absorbance ranges 0-0.2 or 0-0.5.

[b] Injected intravenously with 1.5 mg biliverdin in 0.5 ml guinea pig serum.

[c] Injected with 0.5 ml guinea pig serum alone.

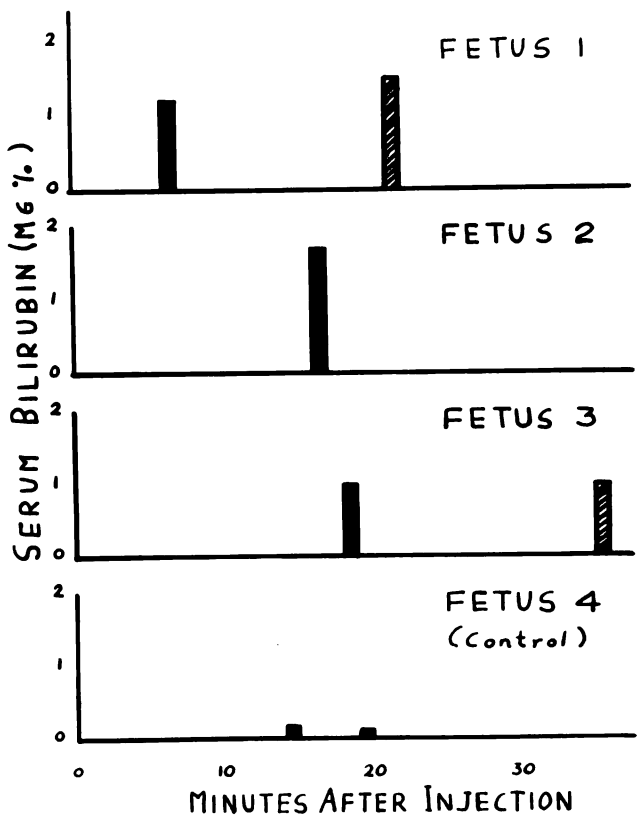

Figure 23. Serum bilirubin in 3 sibling fetuses following injection of biliverdin (1.5 mg). ■ Indicates blood samples taken with placental circulation intact; ▨ indicates blood samples taken from the same fetus after severing the cord and removal of the fetus from the saline bath.

Table 6. Recovery of radioactivity following injection of labelled bile pigment into the circulation of fetal guinea pigs.

Bile pigment injected	Experiment #	Recovered counts expressed as % of injected counts				
		Maternal bile	Fetus[a]	Placenta[b]	Syringe hold-up[c]	Total
20 µg ^{3}H-bilirubin	1	51.0	36.0	0.5	1.0	88.5
20 µg ^{3}H-bilirubin	2	60.0	32.0	0.8	8.0	100.8
20 µg ^{3}H-bilirubin	3	27.0	39.0	9.0	--d	75.0
20 µg ^{3}H-bilirubin	4	17.0	68.0	1.0	--d	86.0
20 µg ^{3}H-bilirubin	5	8.0	89.0	--d	--d	97.0
20 µg ^{14}C-biliverdin	6	23.0	66.0	0.5	2.0	91.5
20 µg ^{14}C-biliverdin	7	22.0	67.0	0.4	3.0	92.4
20 µg ^{3}H-bilirubin plus	8	25.0	67.0	0.2	2.8	95.0
20 µg ^{14}C-biliverdin		25.0	63.0	0.2	2.7	90.9
20 µg ^{3}H-bilirubin plus	9	50.0	45.0	0.6	3.8	99.4
20 µg ^{14}C-biliverdin		54.0	(27.0)[e]	0.7	3.8	85.5

a Corrected figures based on 89.40% recovery of counts.
b Corrected figures based on 89.36% recovery of counts.
c Percentage of label remaining in syringe after infusion of isotope.
d Not determined
e This number appears to be anomalously low.

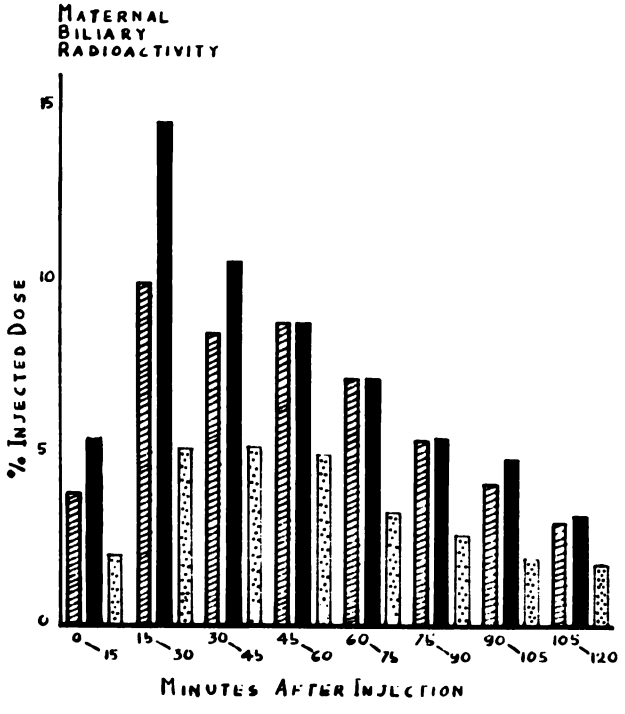

Figure 24a. Maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin. ■ Expt.1; ▨ expt. 2; ⊡ expt. 3 as referred to in Table 6.

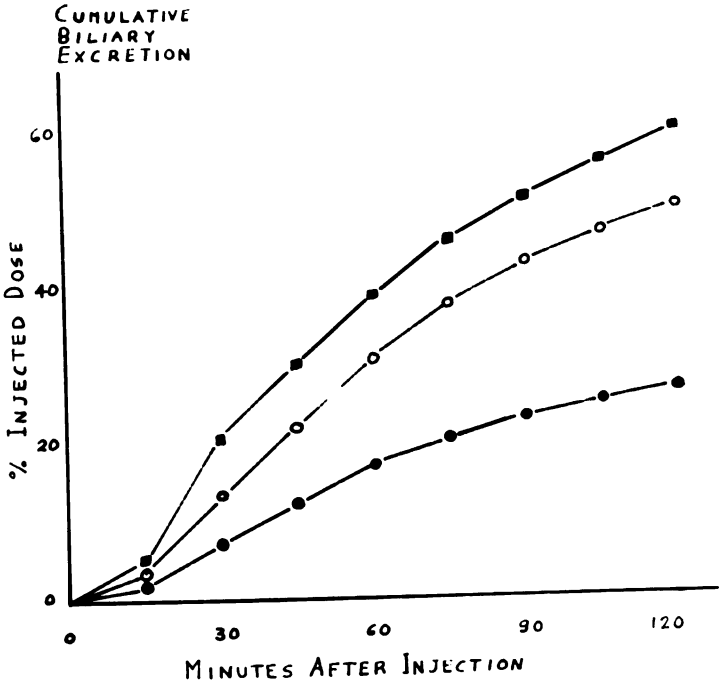

Figure 24b. Cumulative maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin. O Expt. 1; ■ expt. 2; ● expt. 3 of Table 6.

^{14}C-biliverdin injection was somewhat lower than after ^{3}H-bilirubin injection. The double-labelling experiments described below suggest that this was probably due to individual animal variation. Individual variation may have been magnified because the fetuses used were not of identical gestational ages.

The effect of simultaneous injection of ^{14}C-biliverdin and ^{3}H-bilirubin into the fetal umbilical vein was followed in two pregnant animals. The results of these experiments (#8 & 9) are given in Table 6 and the distribution of counts in the maternal bile following injection of the fetus is shown in Figures 26a, 26b, 27a, and 27b. The two experiments show that the two isotopes were excreted at comparable rates by the maternal liver. Furthermore in each experiment, the ratio of ^{3}H:^{14}C in the maternal bile remained essentially constant and comparable to the ^{3}H:^{14}C ratio in the original bile pigment solution that was injected into the fetal circulation (Table 7).

IV. DISCUSSION

These studies show that the guinea pig placenta is permeable to bilirubin in both directions <u>in vivo</u> as reported by others.[49,50] After injection of ^{3}H-bilirubin into the fetal circulation, isotope excretion in the maternal bile started within 15 minutes and peaked between 15-45 minutes. The proportion of radioactivity excreted into the maternal bile was different in each experiment but in all cases, at the end of the experiment, most of the unexcreted isotope was found in the fetal carcass and there was little in the placenta. Variations in gestational age or individual differences may account for the variation in the rate of transplacental excretion of isotope. It is relevant to note that the amount of bile pigment injected was not

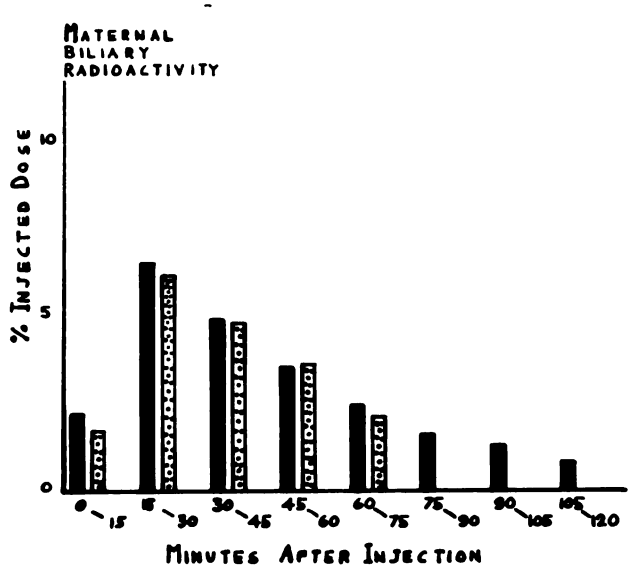

Figure 25a. Maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{14}C-biliverdin. ■ Expt. 6; ⊡ expt. 7 as referred to in Table 6.

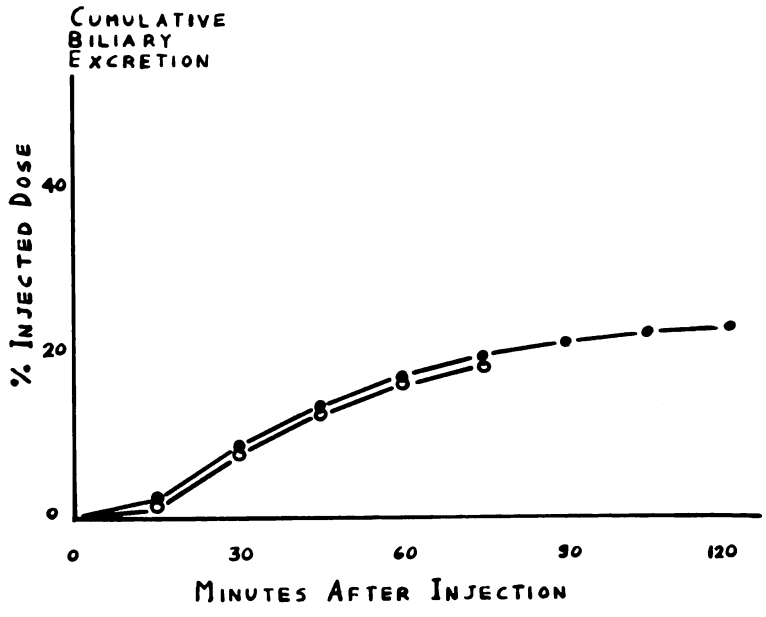

Figure 25b. Cumulative maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{14}C-biliverdin. ● Expt. 6; o expt. 7 in Table 6.

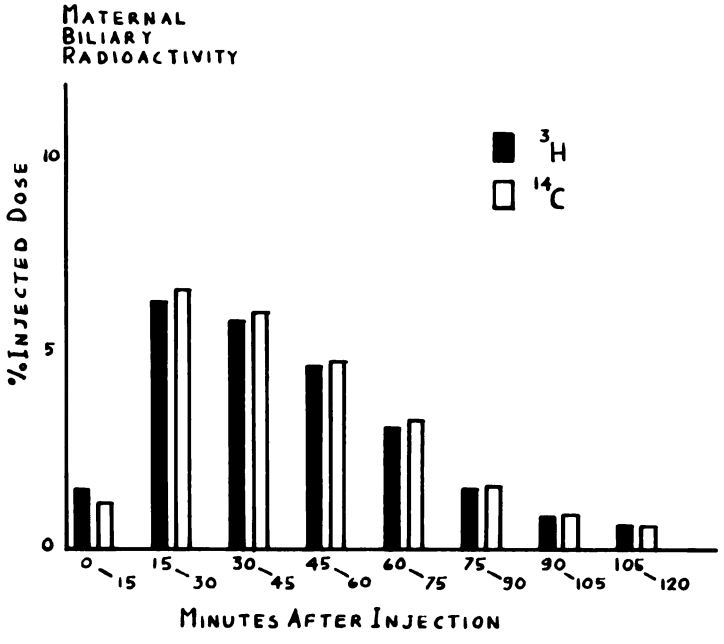

Figure 26a. Maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin and ^{14}C-biliverdin. ■ ^{3}H; □ ^{14}C. Expt. 8 in Table 6.

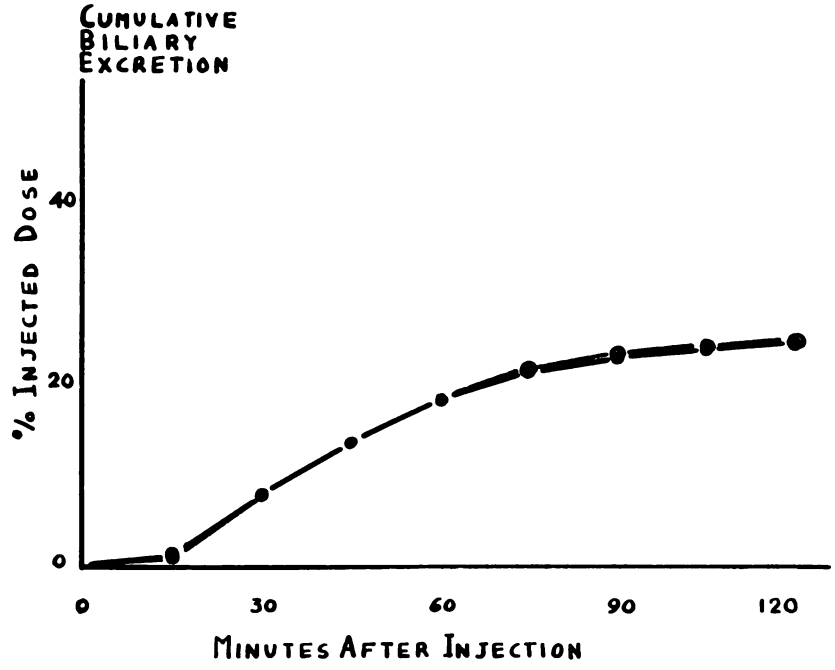

Figure 26b. Cumulative maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin and ^{14}C-biliverdin. ● ^{3}H; ○ ^{14}C. Expt. 8 in Table 6.

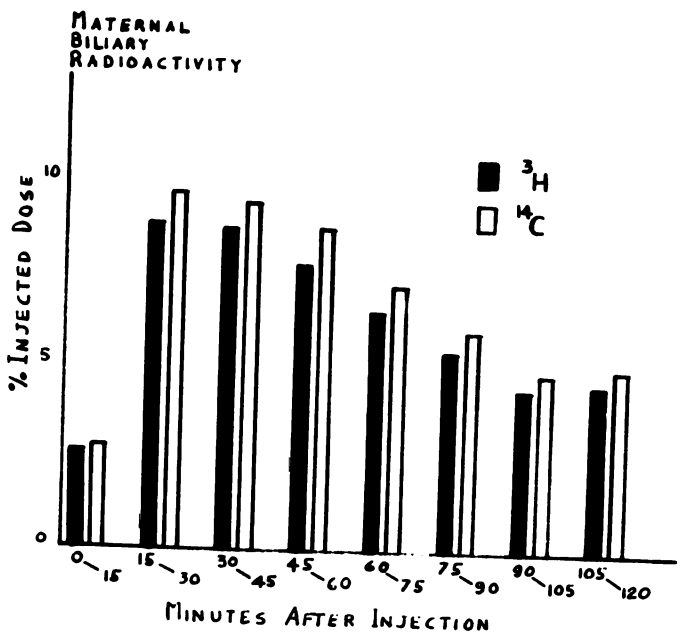

Figure 27a. Maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin and ^{14}C-biliverdin. ■ ^{3}H; □ ^{14}C. Expt. 9 in Table 6.

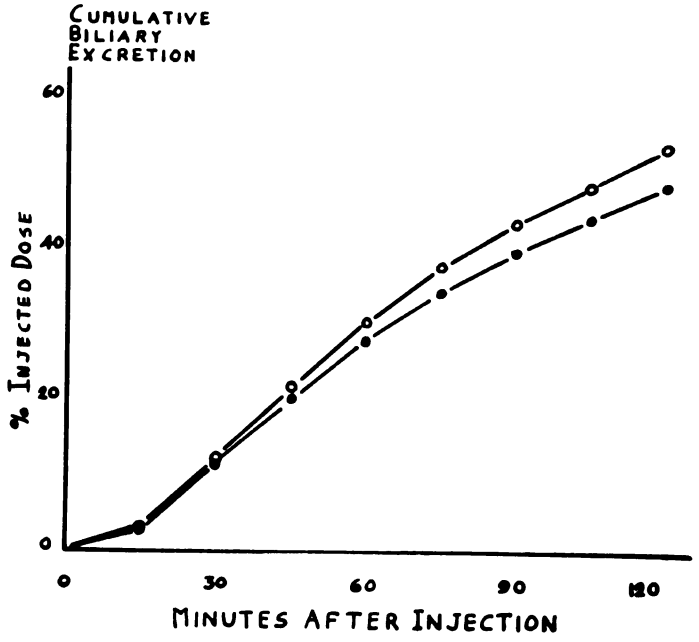

Figure 27b. Cumulative maternal biliary excretion of isotopically labelled bile pigments following injection of a fetal guinea pig with ^{3}H-bilirubin and ^{14}C-biliverdin. ● ^{3}H; o ^{14}C. Expt. 9 in Table 6.

Table 7. Measured $^{3}H:^{14}C$ isotope ratios for double-labelling experiments using ^{3}H-bilirubin and ^{14}C-biliverdin.

	$^{3}H:^{14}C$	
	Experiment 8	Experiment 9
Infusate (calcd)	5.25	5.25
Infusate (detd)	5.45	5.19
Bile, 0-15 min	6.81	4.98
Bile, 15-30 min	5.02	4.79
Bile, 30-45 min	5.10	4.71
Bile, 45-60 min	5.10	4.66
Bile, 60-75 min	4.95	4.72
Bile, 75-90 min	4.99	4.75
Bile, 90-105 min	5.18	4.79
Bile, 105-120 min	5.29	4.79
Fetus	5.58	8.97[a]
Placenta	5.35	4.92

[a] This value appears to be too high, possibly because of an error in the ^{14}C determination. See Table 6.

really a trace amount. It amounted to roughly 4% of the daily bile pigment production.* Nevertheless the amount of bilirubin injected (20 μg; 3.42 X 10^{-8} mole) was far less on a molar basis, than the amount of albumin in the fetal circulation (2.89 X 10^{-6} mole)** and presumably insufficient to saturate the high-affinity bilirubin-binding site on the albumin.

Maternal to fetal transfer of bilirubin was demonstrated by injecting large amounts of bilirubin into the maternal circulation. The doses used (10-40 mg) were massive compared to the estimated daily endogenous production of bile pigment (6 mg),*** but were necessary in order to overcome the high excretory capacity of the mother and produce high maternal serum bilirubin levels. However, with such large doses it was difficult to ensure that the bilirubin was truly in solution in the infusate. In three experiments the animals died of respiratory failure following doses of 40 mg (2 animals) and 30 mg (1 animal). Probably this was caused by colloidal pigment in the infusate. Despite these difficulties, the data from animals that did not die (Figure 12) show clearly that unconjugated hyperbilirubinemia in the mother causes the fetal serum bilirubin to increase to moderately elevated levels.

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* Calculated on the assumption that fetal guinea pigs make bile pigment at the same rate per gram body weight as human babies. The weight of a guinea pig fetus was taken as 94 g (average of 7 fetuses) and the bilirubin production rate of a 3000 g infant was taken as 17 mg per day.[73,74]

** Calculated for a 94 g fetus, assuming a blood volume of 7.14 ml/100 g body weight and an albumin concentration of 3.03 g/100 ml blood.[75,76]

*** This rough figure was estimated as follows: In man, the daily production of hemoglobin is 6.25 grams.[74] One gram of hemoglobin produces 35 mg of bilirubin.[5] Therefore, the total daily production of bilirubin in man is 218.75 mg. If the kinetics of hemoglobin metabolism in the guinea pig are similar to man the equivalent daily production for a two kg guinea pig would be 6 mg.

Taken together, the studies with labelled and unlabelled bilirubin confirm the experience of others[46-50] and show that in the guinea pig: 1) Bilirubin can cross the placenta in both directions. 2) Passage of the pigment is rapid and begins within minutes of loading the fetal of maternal circulation with exogenous pigment. However, these studies, which were done under nonphysiological conditions, give no clue as to the normal capacity or Tm of the placenta for passage of bilirubin. Nor does observation of rapid bidirectional flow imply that pigment concentrations in maternal and fetal plasma reach a concentration equilibrium. Indeed, the data indicate that they do not. For example, in some of the experiments where biliverdin was injected, despite bilirubin concentrations of up to 6 mg% in the mother, fetal levels of greater than 2.5 mg% were not observed (Figure 20). On the other hand, in other experiments the fetal serum bilirubin concentration clearly rose above the maternal level at some point during the experiment (Figure 20). Such inbalance and lack of equilibration between maternal and fetal bilirubin levels has been ascribed to rapid clearance of bilirubin from the maternal circulation.[50] Rapid clearance undoubtedly is important in preventing an equilibrium from being established but probably it is not the only reason why the fetal bilirubin levels do not become approximately equal to the maternal levels. In the experiment with 40 mg biliverdin (Figure 20) bilirubin clearance clearly was not interfering because the maternal serum bilirubin remained at about 6 mg% for a considerable length of time. Yet, the fetal serum bilirubin still barely exceeded 2 mg%. Furthermore, in none of the studies did the fetal serum bilirubin concentration rise above 2.5 mg%. This suggests that there is an upper limit to the concen-

tration of serum bilirubin that can be achieved in fetal guinea pig blood in vivo and that the maximum bilirubin-binding capacity of fetal guinea pig serum is considerably lower than that of maternal serum. Since there is no reason to suppose that the levels of albumin (which binds bilirubin) in fetal and maternal serum are markedly different, this apparent discrepancy between the binding capacity of fetal and maternal blood is puzzling. Possibly it is related to the high lipid levels that were observed in the fetal blood, which in turn may have been a result of laparotomy.[72]

From these and other studies[38,42-50] it is clear that in mammals, including man, bilirubin can pass from fetus to mother, and that elevation of serum bilirubin in the mother leads to elevated serum bilirubin in the fetus. It is also clear that, without additional data, it is impossible to predict from the maternal serum bilirubin level alone what the fetal serum bilirubin will be.

Studies on the placental transport of biliverdin were complicated by the rapid reduction of the pigment in vivo. Although reduction was expected, the enormous capacity and the speed of the process had not been anticipated because previous studies had indicated reduction and excretion to be rather slow,[11,20,67] observations that are in conflict with the present findings. After intravenous injection of biliverdin into pregnant guinea pigs the pigment concentration in serum declined to undetectable levels within 30-60 minutes depending on the dose (10-40 mg dose) (Figures 13 & 14). Part of this disappearance was due to rapid biliary excretion of biliverdin judging by the color and visible absorption spectra of the gall bladder bile samples. The injection of biliverdin also was followed by the rapid appearance of a yellow-diazo positive (indir-

ect reacting) substance in the serum. This was subsequently identified unambiguously as bilirubin IXα by chromatographic and spectroscopic analyses. The serum concentration of bilirubin peaked about 10-12 minutes after the biliverdin injection and then either remained at a plateau level or declined at a slower rate than the serum biliverdin concentration. Simultaneously, fatty tissues became jaundiced without becoming initially green. Therefore, it seems that biliverdin can be excreted by the guinea pig liver and yet can also undergo rapid reduction to bilirubin. After injection, there is a competition between direct excretion and reduction. Both of these pathways contribute to the rapid elimination of biliverdin from the serum but the reduction mode results in transient hyperbilirubinemia. The bilirubin thus formed presumably is excreted in the normal way by hepatic uptake and conjugation. However, the transient hyperbilirubinemia can be of such degree that bilirubin leaves the circulation and becomes deposited in adipose tissues. This extravascular bilirubin fraction eventually returns to the blood after the injected biliverdin has been eliminated and formation of bilirubin has ceased. It is noteworthy that in the experiment shown in Figure 18 the serum bilirubin maintained a constant concentration for 55 minutes. This steady state value of serum bilirubin (~6 mg%) may well represent the maximum amount of bilirubin that the blood can accomodate in this animal without "leakage" of the pigment to the extravascular compartments. If this interpretation is correct, 6 mg% must reflect the maximum serum albumin bilirubin-binding capacity for this animal in vivo. Injection of biliverdin may, therefore, provide a simple method for measuring bilirubin binding capacities in vivo.

The reduction of biliverdin to bilirubin was observed in preg-

nant guinea pigs given doses of 10, 20, and 40 mg biliverdin. The effect was not caused by the method of anesthesia (ketamine plus acepromazine maleate given as an intramuscular injection) since it was also observed with animals anesthetized with sodium pentobarbital and ether. This process was not limited to the pregnant state or to females because it was observed also in males (Figure 21). Although only a single experiment with a matched pair of males was carried out, it is interesting that the serum bilirubin level achieved 15 minutes after injecting biliverdin was approximately the same as that in the control animal which was injected with bilirubin. Furthermore, the straining of adipose tissue was much more marked in the animal injected with biliverdin (Figure 22) than in the control animal which showed little or no visibly detectable yellow staining of fat. The reason for this is not clear. Reduction and fat staining also occurred rapidly in the fetus (Figure 23, Table 5) following injection of 1.5 mg biliverdin. This dose is equivalent to a dose of 20 mg biliverdin in a 1250 grams adult animal on a body weight basis (guinea pig fetus 90-95 grams). Bilirubin levels of 1-2 mg% were reached in the fetus within 5-20 minutes compared to 0.1-0.2 mg% in the control fetus which had been injected only with serum. These bilirubin values were lower than those reached after injecting a comparable dose into an adult male and much lower than those expected if the injected pigment was all reduced to bilirubin and remained in the fetal circulation. Several factors (e.g. more fat, different rates of reduction) could contribute to this but the most likely ones are lower bilirubin binding capacity of fetal blood plus transport of bilirubin out of

the fetal blood across the placenta. However, the fact that hyperbilirubinemia is seen at all suggests that transport of bilirubin across the placenta, though rapid, can be rate-limiting when the load is large.

The reduction of biliverdin to bilirubin in vivo has been studied previously by only two groups. Goldstein and Lester[20] found slow excretion of radioactivity after intravenous injection of ^{14}C-biliverdin in the rat. The isotope was excreted mostly in the form of conjugated bilirubin and biliary excretion of biliverdin was not observed. However, the labelled biliverdin used in their studies probably contained substantial amounts of dimethyl ester[15] and this presumption is supported by their observation that the labelled biliverdin that they used was unstable and poorly soluble in serum. Garay, Royer, and co-workers[11,67] studied the excretion of biliverdin in the rat after intraperitoneal injection of the pigment. They observed that a portion of the injected pigment was excreted unchanged via the liver and that much of the remainder was reduced to bilirubin and then excreted in the bile. After injection of biliverdin the serum level of the pigment rose and there was also a rise in the serum level of bilirubin. Their experiments suggested that the reduction of biliverdin to bilirubin occurred mainly in the liver. The observations made in the present studies are in general agreement with those made by the Argentinians except that the elimination of biliverdin occurred more rapidly. This was probably due to the use in these studies of a different animal species, a different route of administration, and purer biliverdin. The site of reduction of biliverdin in the guinea pig cannot be determined from the present work. But it is reasonable to assume that it occurs in the liver as reported for the rat.[11,67] Of the major organs, the liver has the highest concentration

of biliverdin reductase[65] and liver homogenates reduce biliverdin faster than homogenates of other tissues *in vitro*.[64] Reduction does not occur in the blood since incubating biliverdin with fresh whole blood or serum did not produce bilirubin, in confirmation of Lemberg's findings.[64] Since yellow pigmentation of adipose tissues occurred after injection of biliverdin it might be supposed that this portion of the bilirubin had been formed by extrahepatic reduction. However, the fat went from its normal light off-white color to yellow without becoming at all green at any intermediate stage. Therefore, it seems likely that the bilirubin that goes into the fat is formed elsewhere (probably the liver) and is secreted from the circulation into the lipocytes.

After injection of biliverdin into the late-term pregnant guinea pigs at high doses the pigment could not be detected in fetal serum (Figures 15 and 16) even when the maternal serum biliverdin was as high as 20-30 mg%. However, fetal serum bilirubin levels increased sharply and even appeared to exceed maternal serum bilirubin values in three experiments (Figure 20). Curiously the fetal bilirubin levels that were reached were somewhat higher than those reached following injection of comparable doses of bilirubin (Compare Figure 12 and 20). These observations can be interpreted in two ways. 1) That biliverdin can cross the placenta but is reduced to bilirubin in the fetus so fast that it never becomes detectable; or 2) that biliverdin cannot cross the placenta and that the elevated bilirubin concentrations in the fetus result from maternal to fetal transport of bilirubin. The first possibility cannot be eliminated completely, but the evidence favors the second. As shown above, reduction of biliverdin to bilirubin occurred very rapidly in the fetus following injection of 1.5 mg

biliverdin. Nevertheless biliverdin was still detectable in the fetal serum after about half an hour (Table 5). Therefore, it is unlikely that biliverdin could cross the placenta in sufficient amounts to markedly enhance the fetal serum bilirubin and yet remain undetectable itself. Secondly, biliverdin is polar and less lipophilic than bilirubin and therefore less likely to cross the placenta. In this respect biliverdin is probably more like conjugated bilirubin which does not cross the mammalian placenta.[38,43,48,50] The guinea pig placenta cannot have a large capacity for excreting or transporting biliverdin from fetus to mother otherwise biliverdin would have disappeared via that route when it was injected into the fetal circulation (Table 5).

The experiments on the fetal to maternal transport of biliverdin also gave equivocal results. In two experiments, after injection of isotopically labelled biliverdin into the fetal umbilical vein, there was rapid excretion of radioactivity in the maternal bile (Figure 25) and the excretory pattern was similar to that obtained with labelled bilirubin (Figure 24). In each experiment, the percentage of the injected dose excreted in the bile was lower than in the comparable experiments with bilirubin but this was probably due to the characteristics of the individual animal and variation in gestational age. This conclusion was supported by duplicate double-labelling experiments where equal weights of ^{14}C-biliverdin and ^{3}H-bilirubin were injected simultaneously into a fetus (Figures 26 and 27). Here the excretion of each isotope in each experiment was almost identical. Qualitatively similar results were obtained from both experiments, yet there were quantitative differences between them. In these two experiments not only were the two isotopes excreted via the mother

at identical rates but, except for two values that may be ascribed to experimental errors, the ratio of the two isotopes in the excreted bile remained fairly constant and was close to the ratio in the mixture injected (Table 7). Again, these findings can be interpreted in two ways: Either biliverdin can cross the placenta just as fast as bilirubin or biliverdin is reduced very rapidly in the fetus at these low doses and then crosses the placenta as bilirubin. The second interpretation is most plausible. Even if it is assumed that biliverdin can cross the placenta, it would not be expected to cross at exactly the same rate as bilirubin, as observed, because of the differences in polarity and affinity for albumin[22] between the two pigments. The fact that they cross the placenta at the same rate suggests that they are identical as they cross. It may be argued that if the second interpretation of the data is correct and biliverdin has to be reduced before it can cross to the maternal circulation then the reduction would take a finite time and then would result in a delay in the excretion of the biliverdin isotope and differences between the isotope ratios in the bile and in the injected material. This may well be. However in the experiments maternal bile samples were collected in 15 minute batches. This time period is probably long compared to the half-life for the reduction process and, therefore,the experiments probably would not detect early or fine distinctions between the excretion patterns of the two labelled bile pigments.

CHAPTER 4

CONCLUDING REMARKS

The overall goal of these studies was to find out why mammals convert biliverdin to bilirubin; why there has evolved a specific enzyme, biliverdin reductase, that converts an apparently innocuous and easily excreted compound to one that is potentially toxic and cannot be excreted without modification. More specifically the experiments were designed to investigate whether only bilirubin and not biliverdin can cross the placenta. If this were so it would make reduction an obligatory step for the excretion of bile pigment during gestation, and it would explain the requirement for a specific biliverdin reducing enzyme in the fetus. The presence of the enzyme in the adult could then be rationalized simply as a harmless carry over from life in the womb. Unfortunately, the unexpectedly rapid reduction of biliverdin in vivo that was found in the guinea pig frustrated attempts to determine unequivocally whether or not biliverdin can cross the placenta. However, the studies did indicate that the placenta is impermeable to biliverdin from both the maternal and fetal sides and they were at least consistent with the hypothesis that reduction of biliverdin is necessary for its disposal across the placenta.

Very rapid reduction of biliverdin to bilirubin occurs in adult and fetal guinea pigs. The capacity of the reducing system appears to be far in excess of the physiological rate of biliverdin production. At higher doses, reduction of biliverdin is accompanied by development of hyperbilirubinemia and yellow pigmentation of fatty

tissue. Following very high doses of biliverdin (20 mg/kg) hyperbilirubinemia is prolonged, seems to reach a steady-state, and lasts well after all traces of biliverdin have disappeared. Thus, injection of biliverdin appears to afford a method for making an animal temporarily hyperbilirubinemic and may yield a convenient animal model for studying the jaundiced neonate and patients with Gilberts disease.

The site of biliverdin reduction was not determined in these studies but the liver seems likely on the basis of previous studies on hepatectomized rats.[67] Pigmentation of fatty tissue seems to be caused by migration of preformed bilirubin from plasma rather than reduction of biliverdin in the fat itself since after an intravenous pulse of biliverdin the fat gradually becomes yellow without becoming green first. Paradoxically, staining of fatty tissue with bilirubin was more pronounced after injection of biliverdin than after injection of the same weight of bilirubin (Table 8). This suggests that bilirubin formed endogenously and secreted into the plasma may behave differently than exogenous bilirubin injected directly into the plasma, perhaps because the latter may be colloidal and not in solution at high doses. This point merits further investigation because of its obvious relevance to the design and interpretation of metabolic experiments with bilirubin especially those dealing with the development of bilirubin encephalopathy.

If it is assumed that the observed reduction of injected biliverdin occurs mainly in the liver, then it follows that the hepatic uptake of biliverdin from plasma must be rapid. Furthermore, the rapid appearance of unconjugated bilirubin in serum after injection of biliverdin shows that conjugation of bilirubin is saturable and

Table 8. Yellow pigmentation of adipose tissues[a] following intravenous injection of biliverdin and bilirubin into guinea pigs.[b]

Dose	Pigment	Pigmentation
10	biliverdin	yes, pale
20	bilirubin	slight
20	biliverdin	yes
20[c]	bilirubin	very slight
20[c]	biliverdin	yes, intense
40	bilirubin	yes, pale
40	biliverdin	yes

a Mesenteric and subcutaneous fat was examined 30-60 minutes after injection of the pigment.
b All animals were late-term pregnant except where noted.
c Male guinea pigs.

is rate-determining for bilirubin excretion. The use of pulse doses of biliverdin may offer a novel tool for studying the kinetics of distribution of bilirubin between various compartments within the hepatocyte and within the body.

These studies provided additional evidence that bilirubin can pass from the fetal to maternal plasma via the placenta and vice versa in the guinea pig. Transfer of the bilirubin across the placenta from either side occurs within minutes with the greatest rate of transport occurring within 15-45 minutes. Although rapid transport of pigment across the placenta can occur, this does not necessarily mean that equilibration of bilirubin between maternal and fetal plasma is rapid too because the capacity of the placental membrane may be limited and also dependent on the concentration of bilirubin on either side. In the present studies fetal serum bilirubin levels in some instances became higher than maternal levels measured just previously or at about the same time. Furthermore, it is well known that the concentration of bilirubin in the cord blood in humans is about 2-3 mg%[55,56] whereas the mother has a much lower bilirubin level of about 0.3 mg%. Therefore, it seems that only relatively small amounts of bilirubin can pass the placenta rapidly. Probably the transport capacity of the placenta is adequate to cope with the normal daily production of bilirubin which is small, but is insufficient to handle a sudden relatively large load of pigment. It is interesting to note that there seems to be a maximum concentration of bilirubin of about 2 mg% that can be achieved in the fetal guinea pig circulation whether this arises from the biliverdin injected into the fetus or from biliverdin or bilirubin injected into the mother. This is similar to the situ-

ation in Gunn rats where newly-born offspring of jaundiced mothers with bilirubin concentrations of about 10 mg% have serum concentrations of only about 1 mg%.[51,52] The observed maximum fetal serum bilirubin concentration cannot readily be explained just in terms of serum albumin concentrations since there are no marked differences between the albumin concentrations of maternal and fetal serum.[77] Therefore it appears that fetal blood becomes saturated with bilirubin at lower concentrations than maternal blood. This point deserves further investigation since it is relevant to bilirubin encephalopathy and the rate of development of neonatal jaundice.

Lastly, these studies have shown that oxidation of bilirubin IXα with DDQ under carefully controlled conditions gives biliverdin IXα, thereby providing a convenient method for preparing isotopically labelled and crystalline biliverdin IXα in good yield and purity, and suitable for research purposes.

CHAPTER 5

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