Preparation and Properties of Crystalline Biliverdin IX α

SIMPLE METHODS FOR PREPARING ISOMERICALLY HOMOGENEOUS BILIVERDIN AND [^{14}C]BILIVERDIN BY USING 2,3-DICHLORO-5,6-DICYANO BENZOQUINONE

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Amorphous isomerically pure biliverdin IX α is readily prepared in more than 70% yield by dehydrogenation of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone in dimethyl sulphoxide under carefully controlled conditions. Crystalline biliverdin IX α and amorphous [^{14}C]biliverdin can be obtained similarly in more than 40% yield. The pure crystalline pigment was characterized by elemental analysis, methylation, chemical and enzymic reduction to bilirubin, i.r.- and u.v.-visible-absorption spectroscopy, n.m.r. spectroscopy and field-desorption mass spectrometry, and its solubility was determined. Under certain conditions, dehydrogenation gave biliverdin contaminated with III α and XIII α isomers as a result of disproportionation of bilirubin. Formation of non-IX α isomers depends on the concentrations of the reagents and the order in which they are mixed, and occurs under neutral anaerobic conditions. Free-radical reactions probably are responsible, suggesting that the first step in the dehydrogenation of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone in dimethyl sulphoxide is formation of a bilirubin cation radical, rather than hydride ion abstraction.

Biliverdin is a well-known compound of established structure. [The names biliverdin and bilirubin, used without any suffix, refer to the IX α isomers, as recommended by Bonnett (1978). Other structural isomers are indicated by a suffix, e.g. XIII α .] It occurs widely in Nature, either free or complexed with protein, and it is a major product of haem catabolism in all vertebrates (McDonagh, 1979). Structurally similar pigments occur in plants and algae, where they act as photoregulators of plant growth and development and as accessory pigments in photosynthesis. Biliverdin is a useful model for these pigments, and a potentially useful hydrophilic quencher of singlet oxygen (Stevens & Small, 1976; Matheson & Toledo, 1977) and molecular excited states (Land, 1979). Surprisingly, crystalline biliverdin of well-established purity has not been prepared and is not readily available (Lee & Cowger, 1973). Although the less biologically useful dimethyl ester has been fully characterized (Bonnett & McDonagh, 1970, 1973; Manitto & Monti, 1974; Lehner *et al.*, 1978), the crystalline free acid has not, presumably because of difficulties in making it. Commonly used methods involve dehydrogenation

of bilirubin, which is commercially available, with FeCl_3 , benzoquinone or H_2O_2 under acidic conditions (Scheme 1; for a review see McDonagh, 1979). These methods have several drawbacks. Yields are often low, work-ups are difficult and the products are amorphous and highly impure. In addition, disproportionation of the starting material to bilirubin III α and bilirubin XIII α leads to isomerically heterogeneous products (Bonnett & McDonagh, 1970; McDonagh & Assisi, 1972a). Since purification of the free acid by chromatography is difficult, crude preparations are sometimes methylated, purified as the dimethyl ester and then hydrolysed back to the free acid. Purification by this method is inconvenient, suitable only for small-scale work and has never been shown to give a pure product.

A need for pure biliverdin and a convenient method for making isotopically labelled biliverdin for biological studies led us to develop the methods described in the present paper. These methods use oxidation of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone under controlled conditions. They lead to isomerically pure biliverdin in high yield, are

Scheme 1. Dehydrogenation of bilirubin to biliverdin

readily adaptable for preparing isotopically labelled biliverdin, and provide a simple route to crystalline biliverdin.

Experimental

Materials

Bilirubin (Koch–Light Laboratories, Colnbrook, Bucks., U.K., BDH Biochemicals, Poole, Dorset, U.K., and Sigma Chemical Co., St. Louis, MO, U.S.A.) was purified, crystallized, and assayed for isomeric purity before use (McDonagh & Assisi, 1972*b*). 2,3-Dichloro-5,6-dicyanobenzoquinone (98%; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) had m.p. 213–216°C. Tetramethylammonium hydroxide pentahydrate, NaBH₄, Tris base (Trizma), bovine serum albumin, BF₃/methanol (14%, w/v), NADPH and biliverdin dihydrochloride were purchased from Sigma Chemical Co., argon (99.999% purity) from Matheson Co., Newark, CA, U.S.A., and Celite analytical filter aid from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Silica gel H and silica gel G (both type 60 and for t.l.c.), aluminium oxide (Activity I, neutral) and sand were obtained from E. Merck Laboratories, Elmsford, NY, U.S.A. Dimethyl sulphoxide (ACS reagent grade; Eastman Kodak Co., Rochester, NY, U.S.A.) and chloroform (analytical-reagent grade containing about 1% ethanol) were used as supplied. Water and dimethyl sulphoxide were purged vigorously with argon for about 10 min before use in reactions. Light petroleum (b.p. 60–80°C) was redistilled. Beckman Ready-Solv HP was purchased from Beckman Instruments, Fuller-

ton, CA, U.S.A. Guinea-pig serum was obtained from adult males by cardiac puncture under ketamine anaesthesia, and rat serum from adult Sprague–Dawley males by exsanguination under ether anaesthesia. Biliverdin reductase, isolated from rat liver by the method of Tenhunen *et al.* (1970), was purified by (NH₄)₂SO₄ precipitation and dialysis to step 3 as described in that paper. Other reagents were commercially available analytical reagent grades.

General methods

Visible- and u.v.-absorption spectra were recorded on a Cary 118C or Aminco model DW2 spectrophotometer, standardized with holmium oxide, with 1 cm-path-length cuvettes. The i.r. spectrum of biliverdin was measured in dry air on a Perkin–Elmer 225 instrument by courtesy of Professor R. Bonnett, Queen Mary College, London, U.K. Electron-impact mass spectra were determined on an AEI MS12 mass spectrometer, and the field-desorption mass spectrum of biliverdin on a Varian CH5D spectrometer fitted with a field-desorption source (emitter current 18 mA, source temperature 70°C, sample applied in dimethyl sulphoxide) by courtesy of Professor A. H. Jackson, University College, Cardiff, Wales, U.K. The proton n.m.r. spectrum of biliverdin was measured on a Bruker HP90 instrument by Dr. M. Chedekel, Ohio State University, Columbus, OH, U.S.A. Melting points were determined in open capillary tubes, with caffeine (observed m.p. 235–237°C) as standard. Elemental analyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of

California, Berkeley, CA, U.S.A. Work with bile pigments was done in a windowless room under low-intensity light. The composition of solvent mixtures is given in ratios by volume.

Analytical chromatographic procedures

Polyamide layer sheets (15 cm $\times$ 50 cm, 50 μ m layer; Cheng Chin Trading Co., Taipeh, Taiwan) and silica gel G plates (20 cm $\times$ 20 cm, 250 μ m layer; Analtech, Newark, DE, U.S.A.) were used as supplied. Silica gel H (E. Merck Laboratories) plates were prepared according to the manufacturer's directions, activated for 1 h at 110°C, and used after equilibration in air at room temperature. Chromatograms were generally developed for 1 h in the dark. Analysis of bilirubin isomers was carried out as previously described (McDonagh & Assisi, 1971). Qualitative analysis was done by t.l.c. on polyamide with aq. NH₃ (sp.gr. 0.880)/methanol (1:99) (system A) and on silica gel H with chloroform/acetic acid (99:1) (system B). Biliverdin from oxidation of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone was chromatographed on silica gel G plates, with development first in acetone (20 min) and then in chloroform/methanol/acetic acid (900:100:1) (system C). For routine t.l.c., pre-development with acetone is unnecessary; it was included to detect fluorescent impurities derived from 2,3-dichloro-5,6-dicyanobenzoquinone that are less polar than biliverdin and are easier to detect by pre-development with acetone. Biliverdin samples were also analysed by reverse-phase high-pressure liquid chromatography (Fu *et al.*, 1979) by Dr. H. W. Siegelmann, Brookhaven National Laboratory, New York, NY, U.S.A. [column, E. Merck RP-18 (5 μ m), 0.4 cm $\times$ 15 cm; solvent, acetone/water (3:1) containing H₃PO₄ (2 mM) and NaH₂PO₄ (0.05 mM); flow rate, 1.5 ml/min; pressure, 13.1 MPa (1900 lb/in²); detector, absorption set at 365 nm]. Biliverdin dimethyl ester preparations were analysed qualitatively by t.l.c. on silica gel G with acetone/chloroform (3:97) (system D) (Bonnett & McDonagh, 1970, 1973). Biliverdin dimethyl ester from crystalline biliverdin also was analysed by high-pressure liquid chromatography by Dr. T. Wooldridge, University of Nevada, Reno, NV, U.S.A., on a silica column with a toluene/ethanol solvent (Wooldridge & Lightner, 1978).

Determination of absorption coefficients for biliverdin/serum solutions

For solutions in guinea-pig serum, biliverdin (0.2 mg) in 0.1 M-NaOH (0.05 ml) was diluted to 2 ml with a mixture (1:1) of guinea-pig serum and 0.1 M-Tris/HCl buffer, pH 7.4. Portions were diluted further with the same serum/buffer mixture to give 13 solutions of final pH 7.5 containing 2.15–172 μ M-biliverdin. Absorption spectra were run with serum/

buffer mixture (1:1) as reference, and ϵ was computed from the slope of a plot of concentration versus maximum absorbance between 665 and 690 nm. Although this plot was linear ($r^2 = 0.999$) over the concentration range studied, the wavelength of maximum absorbance (λ_{max}) in the 665–690 nm region was not constant. For biliverdin concentrations between 43.0 and 172 μ M, λ_{max} remained essentially constant (665–670 nm), but as the concentration was decreased from 43.0 to 2.15 μ M λ_{max} shifted from 670 to 685 nm.

For solutions in diluted rat serum, biliverdin (1 mg) was dissolved in 0.1 M-NaOH (0.2 ml), and rat serum (0.2 ml) was added. This solution was made up to 20 ml with 0.1 M-Tris/HCl buffer, pH 7.4, and further dilutions made with the same buffer. The absorption coefficient was calculated from a plot of absorbance at 675 nm versus concentration, which was linear ($r^2 = 0.9999$) over the concentration range 17.2–86 μ M.

Radioisotope procedures

[¹⁴C]Bilirubin prepared biosynthetically in rats (Lester & Klein, 1966) from 5-amino[4-¹⁴C]laevulinic acid hydrochloride (Amersham/Searle, Arlington Heights, IL, U.S.A.) was isolated and crystallized as previously described (Ostrow *et al.*, 1961). Specific radioactivities were determined as follows (Helman *et al.*, 1974). Samples (2–5 μ l) of [¹⁴C]bilirubin in chloroform or of [¹⁴C]biliverdin in 0.1 M-NaOH were treated for 30 min in low-potassium glass counting vials with aq. 1 M-tetramethylammonium hydroxide (0.3 ml) and 30% (v/v) H₂O₂ (0.3 ml). Then 3 M-HCl (0.3 ml) and 15% (w/v) ascorbic acid (0.3 ml) were added with thorough mixing, followed by Beckman Ready-Solve HP (15 ml). Radioactivities of samples were counted on a Beckman LS-250 or LS-350 liquid-scintillation spectrometer, with [¹⁴C]toluene (New England Nuclear Corp., Boston, MA, U.S.A.) as internal standard. The counting efficiency was 67%.

Preparation of crude biliverdin

A freshly prepared solution of bilirubin (200 mg, 340 μ mol) in dimethyl sulphoxide (250 ml) was poured rapidly into a freshly prepared solution of 2,3-dichloro-5,6-dicyanobenzoquinone (163 mg, 720 μ mol) in dimethyl sulphoxide (250 ml). The mixture was stirred and purged continuously with argon during the addition. After 5 min water (1500 ml) was added, and the precipitate was collected by filtration through a thin pad of Celite layered onto a Whatman no. 4 filter paper in a Buchner funnel (10 cm internal diam.). The solid was washed with water (2 $\times$ 10 ml) and redissolved by adding 0.1 M-NaOH to the filter cake in three 25 ml portions, the filtrate being collected each time. The combined basic filtrates were centrifuged, if neces-

sary, to remove traces of Celite particles, and biliverdin was re-precipitated by adding acetic acid (0.5 ml). The product was collected by centrifugation, washed three times with an equal volume of water and freeze-dried to give crude biliverdin, containing fluorescent impurities plus 2,3-dichloro-5,6-dicyanobenzoquinone and/or its hydroquinone.

Purification of crude biliverdin

A slurry of silica gel G (15 g) in methanol/acetone (1:1) (45 ml) was poured into a 60 ml glass Buchner funnel with a fritted sinter-glass disc (porosity 60C; funnel dimensions 40 mm internal diam. $\times$ 50 mm high) and allowed to settle by gravity. The solvent was then drained to the top of the column under low vacuum [1.6–1.9 kPa (12–14 mmHg) below atmospheric pressure] and a further 20 ml of methanol/acetone (1:1) was sucked through the column. Crude biliverdin (100 mg) in methanol/acetone (1:1) (30 ml) was added carefully and evenly to the top of the column with a Pasteur pipette, and light vacuum was applied. The adsorbed material was eluted under light vacuum with methanol/acetone (1:1) (10 ml), followed by chloroform/methanol/acetic acid (700:300:3). Yellow-green or green eluates were discarded and the main blue-green band was collected and evaporated to dryness under reduced pressure. The residue in 0.1 M-NaOH (4 ml) was filtered through a thin layer of Celite in a small (2 ml) Buchner funnel containing a fritted sinter-glass disc, and the Celite was rinsed through with 0.1 M-NaOH (3 $\times$ 1 ml). 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 (4:1) (40 ml). An equal volume of water was added, the mixture was shaken, and acetic acid (5 μ l) was added. The mixture was shaken, and left to stand for 10 min. The precipitate was collected by centrifugation, washed twice with water, and freeze-dried, giving purified biliverdin (55 mg, 55%) as an amorphous green powder; λ_{max} in methanol 377 and 666 nm (ϵ 49 700 and 13 900 M⁻¹·cm⁻¹ respectively).

Crystallization of biliverdin

Purified biliverdin (100 mg) was mixed to a slurry with cold methanol (1–2 ml) in an Erlenmeyer flask (125 ml) containing a magnetic stirrer. 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 boil for about 30 s and then was filtered, while hot, through a fluted filter paper into a flask containing a few millilitres of boiling methanol. As soon as filtration was completed the filtrate was removed from the hot-plate, cooled to room temperature, seeded and left at 4°C overnight. The product was dried at 65°C under

reduced pressure overnight to give biliverdin (78 mg, 78%) as needles with m.p. > 290°C (Found: C, 67.8; H, 5.9; N, 9.5%; M^+ , m/e 582. Calc. for C₃₃H₃₄N₄O₆: C, 68.0; H, 5.9; N, 9.6%; M^+ , m/e 582). Additional physical data are given in Table 3 and the Results and Discussion section. The overall yield from bilirubin was 43%.

Preparation of reagent-grade biliverdin

Crude biliverdin was prepared as described above except that the final freeze-drying step was omitted. The moist product was mixed with acetone/water (4:1) (80 ml) and an equal volume of water was added. The mixture was shaken and after 10 min the precipitate was collected, washed twice with water and freeze-dried. 'Reagent-grade' biliverdin was obtained as an amorphous green powder (73%) with λ_{max} in methanol 376 and 666 nm (ϵ 46 700 and 12 900 M⁻¹·cm⁻¹ respectively). T.l.c. (system C; Fig. 1) revealed trace amounts of polar by-products at the origin and the absence of fluorescent impurities. Crystallization from methanol gave short stubby needles mixed, at times, with amorphous aggregates.

Methylation of crystalline biliverdin and other biliverdin preparations

Crystalline biliverdin (60 mg, 103 μ mol) in 7% (w/v) BF₃/methanol (12 ml) was refluxed under

Fig. 1. T.l.c. of biliverdin preparations (1) and (7) Crystalline biliverdin; (2) amorphous purified biliverdin before crystallization; (3) 'reagent-grade' biliverdin; (4) biliverdin prepared by oxidation of bilirubin with benzoquinone; (5) biliverdin prepared by FeCl₃ oxidation of bilirubin; (6) biliverdin from Sigma Chemical Co. The t.l.c. system used was silica gel G with chloroform/methanol/acetic acid (900:100:1). The faint high- R_F spot in each channel is an artifact introduced during chromatography.

Fig. 2. T.l.c. of biliverdin dimethyl ester preparations (1) Ester prepared from Sigma biliverdin; (2) ester prepared from biliverdin obtained by benzoquinone oxidation of bilirubin; (3) ester prepared from pure crystalline biliverdin; (4) crude mixture known to contain the III α , IX α and XIII α isomers of biliverdin dimethyl ester. The t.l.c. system used was silica gel G with acetone/chloroform (3:97). The plates were overloaded for photographic purposes.

argon for 15 min, stirred overnight at room temperature, diluted with water (72 ml) and extracted with chloroform (1 $\times$ 50 ml, 2 $\times$ 10 ml). The extracts were washed with water (2 $\times$ 50 ml) and 0.1 M-NaHCO₃ (1 $\times$ 50 ml), dried and evaporated to dryness under reduced pressure. The residue, in chloroform/benzene (1:9) (20 ml), was chromatographed on alumina (Activity V, neutral; 80 g) and the main blue band, eluted with chloroform/benzene (1:1), was collected and crystallized from chloroform/light petroleum (Bonnett & McDonagh, 1973) to give biliverdin dimethyl ester (27 mg, 42%), with m.p. 213–214°C [literature values: 207–208°C (Lemberg, 1932), 206–207°C (Manitto & Monti, 1974), 208–209°C (Bonnett & McDonagh, 1970), 201–203°C (Lehner *et al.*, 1978)], $M^+ = 610$ m/e and λ_{max} in chloroform 380 and 660 nm (ϵ 54 600 and 14 700 M⁻¹·cm⁻¹ respectively). This material showed only traces of non-IX α isomers on t.l.c. (system D; Fig. 2) and co-chromatographed with an authentic sample (Bonnett & McDonagh, 1970). On high-pressure liquid chromatography the ester showed one main peak flanked by smaller peaks due to III α and XIII α isomers; the proportion of isomers was similar to that in the biliverdin sample from which the biliverdin dimethyl ester originated.

Dimethyl esters of crude biliverdin samples for t.l.c. analysis were purified only by filtration through silica gel G (Bonnett & McDonagh, 1973).

Reduction of crystalline biliverdin

Methanol (5 ml) was added to a mixture of biliverdin (20 mg, 34 μ mol) and NaBH₄ (200 mg, 5.3 mmol) with rapid stirring and cooling in an ice bath. After 1 min, water (10 ml) was added and bilirubin was precipitated by adding acetic acid dropwise. The mixture was extracted with chloroform (1 $\times$ 75 ml, then 2 $\times$ 10 ml) and the extracts were washed with water (2 $\times$ 50 ml) and 0.1 M-NaHCO₃ (1 $\times$ 50 ml), filtered and evaporated to give 12 mg (58%) of bilirubin. Crystallization from chloroform/methanol gave 8 mg (39%) of bilirubin identical with authentic bilirubin on t.l.c. (systems A and B), with λ_{max} in chloroform 453 nm (ϵ 57 300) and $M^+ = 584$ m/e. The product contained 95% bilirubin, 1% bilirubin III α and 4% bilirubin XIII α . (Analysis of the original bilirubin from which the crystalline biliverdin had been made gave 93% bilirubin, 3% bilirubin III α and 4% bilirubin XIII α .) On re-oxidation the product gave crude biliverdin that was chromatographically inseparable (system C) from the biliverdin used as starting material.

Reaction of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone

Effect of excess quinone. (1) 2,3-Dichloro-5,6-dicyanobenzoquinone (1, 2, 3 or 4 mol.-equiv.) in dimethyl sulphoxide (5 ml) was added to bilirubin (20 mg, 1 mol.-equiv.) in dimethyl sulphoxide (5 ml) with continuous mixing. After 5 min a sample (5 ml) was added to water (15 ml), and the precipitated product was collected, washed with water and freeze-dried. T.l.c. (systems A and B) revealed bilirubin in the 1:1 reaction, complete reaction in the 1:2 system and purple by-products in the 1:3 and 1:4 reactions.

(2) Bilirubin (10 mg, 1 mol.-equiv.) in dimethyl sulphoxide (12.5 ml) was added to 2,3-dichloro-5,6-dicyanobenzoquinone (1.1, 1.3 or 1.5 mol.-equiv.) in dimethyl sulphoxide (12.5 ml) with continuous stirring. After 5 min water (75 ml) was added. The precipitated products, washed twice with water, were re-precipitated from 0.1 M-NaOH (2 ml) with acetic acid and examined by t.l.c. (systems A and B). All products contained bilirubin. When equimolar amounts of bilirubin and quinone were used at a final bile pigment concentration of 2 mg/ml, the recovery of bilirubin was 20% (two experiments).

Effect of concentration. Solutions of bilirubin (5 mg, 8.6 μ mol) in 0.75, 1.25 or 7.5 ml dimethyl sulphoxide were added, respectively, to solutions of 2,3-dichloro-5,6-dicyanobenzoquinone (4.1 mg, 1.8 μ mol) in 0.50, 1.25 or 5.00 ml of dimethyl sulphoxide under argon to give final bilirubin concentrations of 4, 2 or 0.4 mg/ml. After 5 min, the reactions were worked up as in the preparation of

crude biliverdin, and a portion of each product was methylated. Traces of biliverdin XIII α dimethyl ester were detectable (system D) in the ester derived from the two more concentrated reaction solutions (4 and 2mg/ml) but were undetectable in the product from the most dilute reaction (0.4mg/ml). The proportion of polar by-products in the crude biliverdin was least in the most dilute reaction (system C).

In two further experiments, 2,3-dichloro-5,6-dicyanobenzoquinone (16.3mg, 7.18 μ mol) in dimethyl sulphoxide was added to an equal volume of bilirubin (20mg, 34.2 μ mol) in dimethyl sulphoxide so that the final bile pigment concentrations were 0.4 and 4.0mg/ml respectively. T.l.c. of the crude biliverdin and corresponding dimethyl ester showed traces of biliverdin XIII α and more polar by-products in the most concentrated mixture (4mg/ml).

Effect of addition sequence and prolonged reaction times. Solutions of bilirubin and 2,3-dichloro-5,6-dicyanobenzoquinone in dimethyl sulphoxide

(equal volumes) were mixed under argon, or oxygen, with stirring and kept at room temperature for 5, 60 or 180 min. In some experiments the bilirubin solution was added to the quinone solution; in others, vice versa. The molar ratios of bilirubin to quinone 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 water (3 vol.), shaken and centrifuged. The precipitate was washed twice with water and then twice with methanol to remove biliverdin, and dissolved in chloroform. The chloroform solutions, containing predominantly bilirubin, were washed with water and 0.1 M-NaHCO₃, filtered and evaporated to dryness. The residues were analyzed by t.l.c. (systems A and B) (Table 1).

Solubility of biliverdin

Crystalline biliverdin (0.5mg, 0.9 μ mol) and solvent (0.5ml) were shaken at 22°C for 1h, then vortex-mixed and centrifuged. The solubility of biliverdin was estimated from the colour of the

Table 1. *Formation of III α and XIII α isomers in the reaction of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone* Solutions of bilirubin and 2,3-dichloro-5,6-dicyanobenzoquinone in dimethyl sulphoxide were mixed in the sequence given and the mixtures were worked up after 5, 60 or 180 min. For reactions with a bilirubin/2,3-dichloro-5,6-dicyanobenzoquinone molar ratio of 1:2.1, the formation of III α and XIII α isomers was determined by qualitative t.l.c. of the esterified biliverdin product. For reactions with a larger bilirubin/2,3-dichloro-5,6-dicyanobenzoquinone molar ratio, isomer formation was determined by t.l.c. of recovered bilirubin. Isomer formation was indicated by the presence of larger amounts of the III α and/or the XIII α isomer than were present in the original bilirubin starting material. In most experiments where isomers were formed the amount of XIII α isomer exceeded the amount of III α . N.D. indicates that non-IX α isomers were not detectable. For further methodological details see the text.

Bilirubin/2,3-dichloro-5,6-dicyanobenzoquinone molar ratio	Total volume of reaction mixture (ml)	Bilirubin concentration* (mg/ml)	Reaction time (min)	Isomer formation
2,3-Dichloro-5,6-dicyanobenzoquinone added to bilirubin				
1:2.1	50.0	0.4	5	Trace
1:2.1	5.0	4.0	5	Yes
1:0.8	30.0	0.4	5	Yes
1:1	12.5	2.0	5	Yes†
1:1	12.5	2.0	180	Yes†
Bilirubin added to 2,3-dichloro-5,6-dicyanobenzoquinone				
1:2.1	50.0	0.4	5	N.D. or trace
1:2.1	10.0	2.0	5	N.D. or trace
1:2.1	5.0	4.0	5	Trace
1:0.1	5.0	1.0	5	N.D.
1:0.8	30.0	0.4	5	N.D.
1:1.1	25.0	0.4	5	N.D.
1:1.3	25.0	0.4	5	N.D.
1:1.5	25.0	0.4	5	N.D.
1:1.1	50.0	0.4	60	Yes
1:1.0	12.5	2.0	5	N.D.†
1:1.0	12.5	2.0	180	Yes†

* Amount of bilirubin added, divided by the total volume after mixing the reagent solutions.

† Identical results for experiments run under argon and oxygen.

supernatant and the amount of undissolved solid. The mixtures were then shaken at 50°C for 1 h and the estimation was repeated.

Preparation of biliverdin by oxidation of bilirubin with benzoquinone and ferric chloride

Bilirubin (250 mg, 428 μ mol) and *p*-benzoquinone (250 mg, 2.31 mmol) or FeCl₃·6H₂O (250 mg; 0.925 mmol), in dimethyl sulphoxide/acetic acid (9:1) (125 ml), were heated on a steam bath under argon for 30 min. The mixture was cooled, added to water (500 ml), and the pH adjusted to 4.3 with solid NaHCO₃. After 1 h at 4°C the precipitate was collected, washed with water, re-precipitated from 0.1 M-NaOH with acetic acid, and then washed again and freeze-dried.

Biliverdin III α and biliverdin XIII α

Biliverdin III α and biliverdin XIII α were prepared by oxidation of the corresponding rubins (McDonagh & Assisi, 1972a) with 2,3-dichloro-5,6-dicyanobenzoquinone, and their identities were confirmed by methylation.

Preparation of [¹⁴C]biliverdin

[¹⁴C]Bilirubin (2.1 mg, 3.6 μ mol) in dimethyl sulphoxide (2.6 ml) was added rapidly with a Pasteur pipette to 2,3-dichloro-5,6-dicyanobenzoquinone (1.7 mg, 7.5 μ mol) in dimethyl sulphoxide (2.6 ml) with continuous argon purging. After 5 min water (15.7 ml) was added, and the precipitate collected, washed three times with water and freeze-dried. A slurry of silica gel G (0.5–0.6 g) in methanol/acetone (1:1) was poured into a 2 ml glass Buchner funnel with fritted sinter-glass disc (porosity 10M; funnel dimensions 10 mm internal diam. $\times$ 35 mm high), and the product was purified as described above for purifying crude biliverdin with the following changes: the volume of methanol/acetone (1:1) was decreased to 2 ml; volumes of 0.1 M-NaOH were 2 ml and 0.5 ml; the volumes of acetic acid were 10 ml and 1 μ l; the volume of acetone/water (4:1) was 0.4 ml; the volume of water was 0.4 ml. [¹⁴C]Biliverdin (41%) was obtained as an amorphous green powder showing one spot on t.l.c. (system C) with no baseline residue, λ_{max} in 0.1 M-NaOH at 380 and 720 nm, and a specific radioactivity similar to that of the starting material.

Results and Discussion

Preliminary studies

Before developing a preparative procedure we attempted to develop a sensitive t.l.c. system for product analysis by using amorphous biliverdin containing biliverdins III α and XIII α and other impurities (Bonnett & McDonagh, 1970). We examined many systems, with silica gel and poly-

amide as stationary phases. Solvents used included methanol/chloroform, methanol/chloroform/acetic acid, methanol/chloroform/aq. NH₃, methanol/ethyl acetate, methanol/ethyl acetate/acetic acid, methanol/acetone, methanol/acetone/acetic acid and butan-2-one/water/acetic acid mixtures. A major drawback with many of these and many of the t.l.c. systems that have been published (McDonagh, 1979) is severe tailing of the biliverdin band. Silica gel G proved to be the most useful adsorbent, and methanol/chloroform/acetic acid and methanol/chloroform/aq. NH₃ the most useful solvents. For routine use we chose silica gel G with methanol/chloroform/acetic acid (100:900:1). This system was not completely satisfactory for three reasons. First, slight decomposition of biliverdin to yellow pigments occurred during chromatography, due, apparently, to some factor in the adsorbent rather than solvent or adsorbent activity. Secondly, the R_F of biliverdin was very sensitive to humidity and the activity of the silica gel, and tended to be low. The R_F could be increased by changing the solvent composition, but this caused increased tailing. Thirdly, the system did not clearly separate the III α , IX α and XIII α isomers of biliverdin, and we were unable to find a t.l.c. system that would. To detect III α and XIII α isomers, biliverdin preparations were reduced to bilirubin or methylated, and these derived products were examined by t.l.c. (Bonnett & McDonagh, 1970; McDonagh & Assisi, 1971). Unmethylated III α and XIII α isomers of biliverdin did separate on silica gel with chloroform/methanol/acetic acid (1800:200:1) and rather better on polyamide with methanol/aq. NH₃ (sp.gr. 0.880) (99:1) when only the two isomers were present, but not when the IX α isomer also was present. In both of these systems R_F values decreased in the order XIII α > IX α > III α .

As starting material for preparing biliverdin, we chose bilirubin because of its availability. This limits the potential purity of the end product, because commercial bilirubin preparations generally contain non-IX α isomers (McDonagh & Assisi, 1971). However, the proportion of these is usually small and can be readily monitored. Choice of oxidant was restricted by the need to avoid acidic conditions that could isomerize the starting material and lead to a heterogeneous product. Benzoquinone oxidizes bilirubin to biliverdin readily in the presence of acid, but not under neutral conditions at room temperature. Therefore we selected 2,3-dichloro-5,6-dicyanobenzoquinone, a quinone with a higher redox potential (Walker & Hiebert, 1967) that oxidizes bilirubin rapidly at room temperature (Pleninger *et al.*, 1972; Stoll & Gray, 1977). Treatment of bilirubin with 2,3-dichloro-5,6-dicyanobenzoquinone in chloroform gave a green precipitate, possibly a charge-transfer complex (Walker & Hiebert,

1967), that yielded mainly unchanged bilirubin on work-up. With dimethyl sulphoxide as solvent, oxidation was rapid and the product, biliverdin, was precipitated when the reaction mixture was diluted with water. However, preliminary studies indicated that the yield and purity of the product were highly dependent on the reaction conditions and that III α and XIII α isomers of biliverdin were sometimes formed in addition to the IX α isomer. Therefore a more detailed study of the reaction was carried out.

In theory, 1 mol.equiv. of 2,3-dichloro-5,6-dicyanobenzoquinone is required for complete dehydrogenation of bilirubin. In practice, unchanged bilirubin was recovered in about 20% yield when equimolar amounts of reagents were used. At least 2 mol.equiv. of quinone was required for complete reaction, and with 2.1 mol.equiv. the reaction was complete in 5 min at room temperature. Larger excesses of quinone (e.g. 3 or 4 mol.equiv.) or longer reaction times led to increased formation of impurities. Since 2,3-dichloro-5,6-dicyanobenzoquinone gradually decomposes in dimethyl sulphoxide, solutions should be used at once, otherwise unchanged bilirubin will be recovered from the reaction. Although reactions were carried out routinely under argon, air or even oxygen caused little, if any, increase in by-product formation. However, formation of non-biliverdin impurities was somewhat concentration-dependent and tended to increase as the concentration of the reagents increased.

Formation of non-IX α isomers was monitored in two ways: (1) by methylating the product and analysing the dimethyl ester by t.l.c. (Bonnett & McDonagh, 1970); (2) by carrying out reactions with insufficient quinone and examining the recovered excess bilirubin for III α and XIII α isomers produced as artifacts. Formation of non-IX α isomers depends on the sequence in which the reagents are mixed and, to a smaller extent, on concentration and reaction time (Table 1). When 2,3-dichloro-5,6-dicyanobenzoquinone solutions were added to bilirubin solutions, isomerization occurred except under the most dilute conditions. But, when bilirubin solutions were added to 2,3-dichloro-5,6-dicyanobenzoquinone solutions under comparable conditions, isomers were detectable only when concentrations were high or a long reaction time was used. Under the reaction conditions used, biliverdin did not disproportionate to non-IX α isomers. Therefore all of the observed non-IX α isomer formation was due to disproportionation of bilirubin before it was oxidized to biliverdin.

Preparation of biliverdin

In the optimized method that was eventually chosen, bilirubin (1.36 mM) in dimethyl sulphoxide was added to an equal volume of 2,3-dichloro-5,6-dicyanobenzoquinone (2.88 mM) in dimethyl

sulphoxide. These were the highest concentrations at which isomerization was undetectable. The reaction was complete after 5 min, and the product was easily recovered by precipitation with water and centrifugation. This material contained fluorescent impurities, but these could be removed partly by re-precipitation from NaOH and more completely by further re-precipitation from aqueous acetone. This gave, in 73% yield, 'reagent-grade' biliverdin that showed a single peak on high-pressure liquid chromatography and had a u.v.-visible-absorption spectrum similar to that of pure crystalline biliverdin. This material was fairly pure by t.l.c. (Fig. 1), but contained a small proportion of fluorescent impurities and polar material and did not give well-formed crystals from methanol. However, it was far purer than commercial biliverdin or biliverdin prepared with benzoquinone or FeCl₃ (Fig. 1).

Attempts to purify crude biliverdin by silica-gel and alumina column chromatography were unsuccessful owing 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 t.l.c.-grade silica gel was used as the adsorbent, the eluent was sucked through under slightly reduced pressure [1.6–1.9 kPa (12–14 mmHg) 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 removed yellow and fluorescent impurities and dark-green polar materials. Biliverdin from the column was re-precipitated from 0.1 M-NaOH with acetic acid and from its colloidal suspension in aqueous acetone with very dilute acetic acid, and freeze-dried. The overall yield from bilirubin was 55%. The two precipitation steps may seem redundant. They were included because they lead to a bulky product that dissolves readily in solvents, is easy to handle and can be crystallized readily. The amorphous product crystallized from methanol in well-formed birefringent needles and the overall yield of crystalline product from bilirubin was 43%. Prolonged heating of the solution during crystallization led to formation of insoluble amorphous material. This was avoided, at the expense of yield, by carrying out the crystallization rapidly. Crystalline biliverdin was indistinguishable from uncrystallized amorphous biliverdin on t.l.c. and high-pressure liquid chromatography and had a similar u.v.-visible absorption spectrum. For most applications crystallization probably is unnecessary.

Structure and isomeric purity

The structure and isomeric homogeneity of the crystalline product were established as follows. (1) A

Fig. 3. Field-desorption mass spectrum of biliverdin

compatible elemental analysis was obtained. (2) The base peak in the field desorption mass spectrum was the molecular ion at 582 m/e (Fig. 3). (3) Methylation gave biliverdin dimethyl ester containing no more than trace amounts of the III α and XIII α isomers by t.l.c. (Fig. 2) and high-pressure liquid chromatography. (4) Reduction with NaBH₄ in methanol yielded bilirubin containing 95% bilirubin IX α , 1% III α and 4% XIII α . This analysis was comparable with that obtained for the original bilirubin (93% IX α , 3% III α and 4% XIII α) from which the crystalline biliverdin had been prepared. (5) Reduction with NaBH₄ followed by re-oxidation of the product gave a substance identical with the crystalline biliverdin starting material on t.l.c. (6) Treatment with NADPH in the presence of biliverdin reductase (EC 1.3.1.24) from rat liver (Tenhunen *et al.*, 1970) led to rapid and complete reduction to bilirubin (Fig. 4).

Fig. 4. Enzymic reduction of biliverdin to bilirubin

The reference cuvette contained 1M-potassium phosphate buffer, pH 7.4 (0.1 ml), water (0.75 ml) and step-3 partially purified biliverdin reductase (0.1 ml) (Tenhunen *et al.*, 1970). The sample cuvette contained phosphate buffer (0.1 ml), water (0.7 ml), biliverdin reductase (0.1 ml) and biliverdin solution (0.05 ml). The cuvettes (1 cm path length) were maintained at 30°C. At zero time the absorption spectrum was run. Then 2 mM-NADPH solution (0.05 ml) was added to the sample cuvette, the contents were rapidly mixed, and spectra were run repeatedly every 2.72 min. The biliverdin solution was made up as follows. Biliverdin (57.2 μ g) was dissolved in 0.1 M-NaOH (0.05 ml) and the solution was diluted with 1M-potassium phosphate buffer, pH 7.4 (0.95 ml), containing bovine serum albumin (20 mg). This experiment was carried out by Dr. T. Yoshida.

Fig. 5. Electronic absorption spectra of biliverdin (a) In 0.1M-NaOH (----) and 1.0M-NaOH (—); (b) in methanol (----) and 5% (w/v) HCl in methanol (—).

Properties of crystalline biliverdin

Crystalline biliverdin is insoluble at 22 or 50°C in water, dilute aqueous acids (e.g. 0.1 M-HCl or 50 mM-Tris/HCl, pH 4.5) and non-polar organic solvents (e.g. benzene, light petroleum). It is slightly soluble in chloroform and ethyl acetate, sparingly soluble in methanol, acetone, 5% (w/v) HCl in methanol and 50 mM-Tris/HCl buffer, pH 7.5, and soluble in pyridine, dimethyl sulphoxide, acetic acid and aqueous base (e.g. 0.1 M-NaHCO₃, 0.1 M-NaOH and 50 mM-Tris/HCl buffer, pH 8.6). Solutions of biliverdin vary in colour from blue through green to nearly yellow, depending on the solvent, concentration and pH. In methanol the pigment is weakly thermochromic. Interestingly, biliverdin is more soluble in some binary solvent mixtures (e.g. acetone/methanol) than in the individual solvents. The solubility properties of biliverdin show that it is much less lipophilic than bilirubin and, at physiological pH, much more hydrophilic.

The electronic absorption spectrum of biliverdin in methanol at room temperature (Fig. 5) has maxima at 376 and 666 nm. Absorption coefficients in methanol and acidified methanol are considerably higher than those previously reported (Tables 2 and 3), reflecting the poor quality of previous preparations. However, the absorption coefficients for biliverdin in methanol and chloroform/methanol are similar to those reported (Table 3) for pure crystalline biliverdin dimethyl ester in chloroform. This attests to the purity of the biliverdin preparation, and also indicates that the free acid and its dimethyl ester have similar conformations in solution in these solvents.

The short-wavelength absorption of biliverdin is relatively insensitive to solvent and pH, but the long-wavelength band varies considerably (Table 2). On acidification, methanol solutions of biliverdin turn green and the absorption coefficient of the long-wavelength band increases markedly (Fig. 5) as

Table 2. *Electronic absorption spectra for biliverdin*

Solvent	$\lambda_{\max.}$ (nm)	ϵ^* (M ⁻¹ ·cm ⁻¹)	$\lambda_{\max.}$ (nm)	ϵ^* (M ⁻¹ ·cm ⁻¹)	Colour of solution
Methanol	376	50 800	666	14 400	Turquoise blue
Acetone/methanol (1:1)	377	52 500	656	15 000	Turquoise blue
Chloroform/methanol (1:1)	380	51 600	666	15 100	Turquoise blue
5% (w/v) HCl in methanol	377	66 200	696	30 800	Turquoise blue†
0.1 M-NaOH‡	381	44 200	720	10 700	Green
0.5 M-NaOH	384	44 300	760	13 500	Lime green
1.0 M-NaOH	384	45 800	760	13 900	Lime green
0.05 M-Tris/HCl buffer, pH 8.58	376	45 500	680	12 400	Emerald green
Diluted guinea-pig serum, pH 7.5	—	—	665–690§	12 900	Green
Diluted rat serum, pH 7.4	—	—	674	12 800	Green

* Except for the measurements in dilute serum, all values are the mean of six determinations.

† Concentrated solutions (1 mg/ml) are emerald green, dilute solutions turquoise blue.

‡ No deviations from Beer–Lambert Law over the concentration range 3.43–34.3 μ M.

§ Concentration-dependent (see the text for details).

Table 3. *Previously reported electronic absorption data for biliverdin and biliverdin dimethyl ester*

Solvent	$\lambda_{\max.}$ (nm)	ϵ (M ⁻¹ ·cm ⁻¹)	$\lambda_{\max.}$ (nm)	ϵ (M ⁻¹ ·cm ⁻¹)	Reference
Biliverdin					
Methanol	375–380	40 500	640–650	12 900	Gray <i>et al.</i> (1961)
Methanol	392	25 000	640	10 400	Lemberg & Legge (1949)
5% (w/v) HCl in methanol	373	47 300	665–670	23 300	Gray <i>et al.</i> (1961)
5% (w/v) HCl in methanol	377	48 000	680	28 000	Lemberg & Legge (1949)
Chloroform	378	41 700	645	13 400	Gray <i>et al.</i> (1961)
Chloroform	384	52 500	665	15 800	Tixier (1945)
Biliverdin dimethyl ester					
Chloroform	379	51 800	656–664	15 100	Bonnett & McDonagh (1970)
Chloroform	380	44 384	660	12 570	Manitto & Monti (1974)
Chloroform	380	55 700	657	15 500	Stoll & Gray (1977)

a result of protonation of the weakly basic chromophore. In alkaline solutions the long-wavelength bands shows a progressive shift to the red as pH increases, up to a pH of about 13 (Fig. 5). This undoubtedly reflects ionization of a lactam NH group of the biliverdin dicarboxylate dianion (Scheer, 1976). Biliverdin obeyed the Lambert-Beer Law in 0.1M-NaOH over the range 3.4–34 μM , suggesting that aggregation does not occur, and, when fresh solutions in 1.0M-NaOH were neutralized, unchanged biliverdin was recovered quantitatively. However, in NaOH biliverdin does undergo very slow decomposition, manifested by loss of absorption at 381–384 nm and 720–760 nm and development of fluorescence. For air-saturated solutions in 0.1M- or 1.0M-NaOH kept in the dark the decomposition rate was less than 1%/h. Contrary to a previous report (Gray *et al.*, 1961), the spectrum of biliverdin in 5% HCl/methanol is stable for at least 21 h.

The molar absorption coefficients of biliverdin in the biliverdin reductase assay mixture (Fig. 4) were estimated to be 14 100 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 674 nm and 49 300 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 377 nm, and the value for the enzymically produced bilirubin, assuming quantitative conversion, to be 58 700 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 464 nm. Although these values were not determined accurately, they are similar to the corresponding values of 12 900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (674 nm) for biliverdin in diluted rat and guinea-pig serum (Table 2) and 57 000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (463 nm) for bilirubin in bovine serum albumin at pH 7.4 (Chen, 1973). However, they are considerably different from the corresponding values of 7300–8000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (650–660 nm) for biliverdin and 27 500–32 700 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (460 nm) for bilirubin determined by Tenhunen *et al.* (1970) and subsequently used in many studies of biliverdin reductase and microsomal haem oxygenase. This discrepancy is probably due, at least in part, to the use of impure pigments in earlier studies.

The 'biliverdin' used by Tenhunen *et al.* (1970) was prepared by a method (Gray *et al.*, 1961) that yields impure biliverdin dimethyl ester and little, if any, biliverdin (Bonnett & McDonagh, 1970). Furthermore, the bilirubin from which it was prepared probably contained substantial quantities of non-IX α isomers (McDonagh & Assisi, 1971). Therefore the previously reported absorption coefficients and derived data are unreliable. The quantitative physiological relevance of the biliverdin reductase studies (Tenhunen *et al.*, 1970) also becomes questionable since a non-physiological substrate was apparently used throughout.

The i.r. spectrum of crystalline biliverdin (Fig. 6) shows clear evidence of hydrogen-bonding, but does not indicate whether this is intramolecular or intermolecular. The broad absorption extending upward from about 2600 cm^{-1} probably is due to hydrogen-bonded carboxylic O–H stretching vibrations. The two weak broad absorption bands at 3420 and 3200 cm^{-1} are assigned respectively to free and hydrogen-bonded N–H vibrations. A similar, though stronger, pair of bands (3412 and 3265 cm^{-1}) occurs in the spectrum of bilirubin (McDonagh, 1979). The spectrum is poorly resolved in the double-bond stretching region, making interpretation difficult. The main band at 1695 cm^{-1} could be due to hydrogen-bonded carboxylic C=O or lactam C=O absorption, and the shoulder at 1725 cm^{-1} is due, most probably, to non-hydrogen-bonded carboxylic C=O. The band at 1645 cm^{-1} , which does not appear in the spectrum of the corresponding dimethyl ester (Nomura & Tsuchiya, 1966; R. Bonnett & A. F. McDonagh, unpublished work), is reminiscent of the lactam C=O band in bilirubin (McDonagh, 1979) and mesobilirubin (Fog & Jellum, 1963), and may be due to a hydrogen-bonded lactam C=O.

The proton n.m.r. spectrum of biliverdin (Fig. 7) can be readily interpreted by reference to other

Fig. 6. I.r.-absorption spectrum of biliverdin (KBr disc)

Fig. 7. P.m.r. spectrum (90 MHz) of biliverdin in $[^2\text{H}_6]$ dimethyl sulphoxide

bilatrienes (McDonagh, 1979), especially the dimethyl esters of biliverdin and its isomers. The C-5 and C-15 protons appear as a pair of singlets at δ 6.09 and 6.11 p.p.m. and the central C-10 proton as a singlet at δ 7.01 p.p.m. The broad double-humped peak near δ 10 p.p.m. is assigned to lactam and possibly pyrrole NH absorption. Carboxylic protons were not detectable. Vinylic protons on the C-3 and C-18 substituents appear as multiplets in the region δ 5.60–6.85 p.p.m., and the eight $-\text{CH}_2-$ protons of the propionic acid side chains give rise to an A_2B_2 multiplet between δ 2.41 and 2.53 p.p.m. The *exo* (C-18) β -methyl protons absorb at δ 1.79 p.p.m. and the three *endo* β -methyl groups appear as well-resolved singlets at δ 2.05, 2.07 and 2.15 p.p.m. Corresponding values for the β -methyl protons of biliverdin dimethyl ester in $[^2\text{H}]$ chloroform are δ 1.80 (*exo*), 2.04, 2.07 and 2.13 p.p.m. (Nomura *et al.*, 1966; Cole *et al.*, 1968; Manitto & Monti, 1974). In IX α bile pigments the *exo* β -methyl resonance is a useful sensor of conformational rigidity and conformational preference (McDonagh, 1979). The remarkably similar frequencies for the β -methyl resonances of biliverdin in $[^2\text{H}_6]$ dimethyl sulphoxide and its dimethyl ester in $[^2\text{H}]$ chloroform indicate that, despite the difference in solvents, both compounds have similar conformations in solution, as suggested by their electronic absorption spectra and as concluded by Chae & Song (1975). The similarity also indicates that alkylation of the carboxy groups does not provoke such a radical conformational change in biliverdin as in bilirubin.

Biliverdin was too involatile for electron-impact or chemical-ionization mass spectrometry, but gave a satisfactory spectrum showing a molecular ion and little fragmentation by the field desorption technique (Fig. 3). The small $M + 14$ peak could be an artifact, or it could reflect the presence of a trace of biliverdin monomethyl ester that might have been introduced

during crystallization. The only other naturally occurring 1,19-dihydroxybilin free acid for which a mass spectrum has been reported is phycocyanobilin (Crespi *et al.*, 1968; Schram & Kroes, 1971; Dougherty, 1972). Rather surprisingly, phycocyanobilin gave a well-defined electron-impact spectrum with strong fragment ions at 288 and 301 m/e , as in IX α rubinoid pigments (McDonagh, 1979), and a weaker peak at 588 m/e , 2 units higher than expected for the molecular ion. It is unlikely that the reported spectrum is actually the spectrum of phycocyanobilin itself. More probably, it is the spectrum of a more volatile adventitious reduction product ('phycocyanorubin') formed by addition of two hydrogen atoms/molecule during mass spectrometry (Crespi *et al.*, 1968). This is supported by measurements of phycocyanobilin dimethyl ester, which predict a mol.wt. of 586 for the free acid. The unambiguous spectrum obtained for biliverdin demonstrates the advantage of field-desorption mass spectrometry for studying non-volatile bile pigments in the free acid form.

Mechanism of bilirubin dehydrogenation

Dehydrogenations with 2,3-dichloro-5,6-dicyanobenzoquinone generally are ionic reactions (for a review see Walker & Hiebert, 1967) in which the initial rate-limiting step is abstraction of a hydride ion (eqn. 1):

For bilirubin (Scheme 2), hydride ion abstraction would lead first to a resonance-stabilized protonated biliverdin cation, which could lose a proton to give biliverdin. This mechanism explains the ready formation of biliverdin, but it does not explain why isomers are formed (Table 1), why their formation depends on the sequence in which the reagents are

Scheme 2. Ionic mechanism for the dehydrogenation of bilirubin to biliverdin with 2,3-dichloro-5,6-dicyanobenzoquinone

mixed, or why more than 1 mol.equiv. of 2,3-dichloro-5,6-dicyanobenzoquinone is required for complete reaction. Therefore, although the simple ionic mechanism cannot be ruled out entirely, additional or alternative mechanisms must be considered.

Disproportionation of bilirubin to III α and XIII α isomers can occur by ionic or free-radical processes (McDonagh, 1975, 1979). The ionic reaction is catalysed by acids. The free-radical reaction is initiated by molecular oxygen and other radical species. As observed in the 2,3-dichloro-5,6-dicyanobenzoquinone reaction (Table 1), formation of isomers by a radical process is concentration-dependent and the degree of isomer formation diminishes with increasing dilution (McDonagh, 1975). In free-radical isomerization it is believed that initiation generates resonance-stabilized dipyrromethane radicals (eqns. 2 and 3; I $\cdot$ = initiator; A-CH $_2$ -B = bilirubin)

that react with other bilirubin molecules (eqns. 4 and 5) by a bimolecular substitution reaction to give III α (A-CH $_2$ -A) and XIII α (B-CH $_2$ -B) isomers (Pryor, 1976; McDonagh, 1979):

In this way the IX α isomer is converted into a random mixture of III α , IX α and XIII α isomers. Since the 2,3-dichloro-5,6-dicyanobenzoquinone oxidation of bilirubin was carried out in a neutral solvent, the acid-catalysed ionic mechanism can be ruled out as a source of non-IX α isomers. A more likely source is a free-radical process (eqns. 2–5), in which the initiating radicals are species derived from bilirubin and the quinone.

There are several possible radical routes for the dehydrogenation of bilirubin to biliverdin. One

pathway is shown in Scheme 3 and summarized in eqns. (6)–(9):

According to this scheme, the first step is electron transfer from bilirubin to the quinone (eqn. 6) to give a bilirubin cation radical (2), followed by proton transfer (eqn. 7) to give a resonance-stabilized tetrapyrrolic radical (3). One-electron oxidation of this radical (eqn. 8) would form protonated biliverdin (4), and then biliverdin (5) by loss of a proton (eqn. 9). If the proposed intermediate bilirubinoid radical (3) is sufficiently long-lived, it could also be generated by the reactions shown in eqns. (10) and (11) (Reid *et al.*, 1961):

Conceivably, the intermediate (3) (eqns. 6 and 7) could be formed in a single step by hydrogen atom abstraction, as proposed for the conversion of perinaphthene into perinaphthyl radical by 2,3-dichloro-5,6-dicyanobenzoquinone (Reid *et al.*, 1961). However, electrochemical oxidation of bilirubin (Slifstein & Ariel, 1973) and model compounds (Falk *et al.*, 1978) indicates that initial formation of the bilirubin cation radical (2) is more likely.

Conversion of the cation radical (2) into biliverdin could occur via several routes, of which only one is shown in Scheme 3. Alternative pathways are shown in Scheme 4. One of these proceeds via a bilirubin radical dication (6), a mechanism suggested by Slifstein & Ariel (1973) for the electrochemical oxidation of bilirubin. Another involves the formation of intermediates (7 and 8) that are simply tautomers of the corresponding inter-

Scheme 3. *Proposed mechanism for the oxidation of bilirubin to biliverdin by 2,3-dichloro-5,6-dicyanobenzoquinone*

Scheme 4. *Alternative pathways for the oxidation of bilirubin cation radical to biliverdin*

mediates in Scheme 3. On the basis of the available data it is impossible to choose between these alternative pathways. However, regardless of which is correct, removal of a second electron (eqn. 8) or hydrogen atom probably requires an oxidation potential at least as high as, if not higher, than that required for removal of the first (eqn. 1) (Slifstein &

Ariel, 1973; Falk *et al.*, 1978). Therefore 2,3-dichloro-5,6-dicyanobenzoquinone itself, rather than the derived semiquinone radical, may be required for this stage of the reaction, as indicated in eqn. (8). This would explain why more than 1 mol.equiv. of the quinone is necessary for quantitative oxidation of bilirubin to biliverdin.

The proposed mechanism (Scheme 3) also explains why the formation of non-IX α isomers depends on the order in which the reagents are mixed. When bilirubin is added to excess 2,3-dichloro-5,6-dicyanobenzoquinone, the quinone is in excess throughout favouring the overall reaction sequence (6)–(8) and preventing accumulation of unchanged bilirubin or derived radicals. Under these conditions there should be little isomerization, as observed (Table 1). However, when the quinone is added to bilirubin, the bile pigment is initially in excess, favouring reactions (6)–(7), (10) and (11). In this situation semiquinone radicals (QH $\cdot$), the bilirubin cation radical (2) and derived radicals (e.g. 3) would be formed in the presence of unchanged bilirubin, and these radicals would initiate the isomerization process summarized in eqns. (2)–(5). Therefore addition of quinone to bilirubin should tend to cause isomer formation, as observed (Table 1).

Formation of non-IX α isomers during the preparation of biliverdin with hydrogen peroxide, benzoquinone or FeCl $_3$ has previously been attributed to acid-catalysed disproportionation of the starting material, bilirubin (Bonnett & McDonagh, 1970; McDonagh & Assisi, 1972a). The present observations suggest that radical-induced isomerization also may be involved. When bilirubin is heated in dimethyl sulfoxide/acetic acid (9:1) at 85°C for 60 min, about 20% of the bilirubin is converted into non-IX α isomers by acid-catalysed disproportionation (McDonagh & Assisi, 1972a). But an even greater proportion of non-IX α biliverdin isomers is formed when bilirubin is heated for 30 min in the same solvent in the presence of FeCl $_3$ or benzoquinone (Bonnett & McDonagh, 1970, and unpublished work). Therefore the presence of the oxidant increases the amount of isomerization, and this may now be attributed to radical-induced reactions of the type suggested above for 2,3-dichloro-5,6-dicyanobenzoquinone.

Conclusion

Crystalline biliverdin was first obtained by Lemberg & Legge (1940), after a tedious and difficult work-up, by oxidation of bilirubin with H $_2$ O $_2$ /HCl. Their material was undoubtedly heterogeneous and probably contained a substantial proportion of biliverdin XIII α , as indicated by chromatography of biliverdin dimethyl ester prepared by the same route (Redshaw *et al.*, 1971). Most other biliverdin preparations that have been reported also were grossly impure and contaminated with non-IX α isomers, biliverdin esters, higher oxidation products and other impurities. This includes many of the ersatz preparations that have been used as chromatographic standards, as enzyme substrates and in physicochemical and metabolic studies. Commer-

cial biliverdin (Figs. 1 and 2) actually contained very little biliverdin, and that was a mixture of isomers.

The procedure described in the present paper is simple, though time-consuming and somewhat laborious. Provided that the starting material is isomerically pure, it gives isomerically pure crystalline biliverdin in good yield. A disadvantage is that the method cannot be carried out on greater than a 100 mg scale, since overloading or increasing the size of the column during chromatographic purification leads to inefficient and poor purification. However, on a 100 mg scale yields of about 40% were obtained reproducibly, and scaled down the method gave amorphous [14 C]biliverdin from [14 C]bilirubin in similar yield. When highly pure crystalline material is not required, the method can be modified to give amorphous isomerically pure biliverdin rapidly and in high yield (73%). This modification avoids chromatography, takes less than a day and is not limited to a 100 mg scale.

Since the present work was completed, Manitto & Monti (1979) have reported an alternative procedure, which takes somewhat longer to complete, for making isomerically pure amorphous biliverdin in 65% yield. The spectroscopic properties of their preparation are similar to those reported in this paper.

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