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Analysis of Bilirubin and Bilirubin Mono- and Di-conjugates

DETERMINATION OF THEIR RELATIVE AMOUNTS IN BIOLOGICAL SAMPLES

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1. A novel method for determination of the relative amounts of unconjugated bilirubin and sugar mono- and di-conjugates of bilirubin in biological samples, including serum, is described and illustrated by its application to the analysis of bilinoids in rat bile. 2. The method is based on specific conversion of the carbohydrate conjugates of bilirubin into the corresponding mono- or di-methyl esters by base-catalysed transesterification in methanol. Under the selected reaction conditions, unconjugated bilirubin remains intact and no dipyrrole exchange in the bilinoids is detectable; transesterification of bilirubin mono- or di-glucuronide is virtually complete (approx. 99%), and saponification is negligible (less than 1%); recovery of the pigments is approx. 95%. 3. The reaction products bilirubin and its methyl esters are separated by t.l.c. and determined spectrophotometrically; the two isomeric bilirubin-IXα monomethyl esters are separated and therefore can be determined individually. 4. Reference bilirubin mono- and di-methyl esters have been synthesized and characterized, and the two isomers of bilirubin-IXα monomethyl ester and bilirubin dimethyl ester were obtained individually, in crystalline form. 5. With this new method, virtually all bilinoids (over 99%) in normal rat bile have been found to be conjugated, with diconjugates (71%) predominating. A significantly increased proportion of monoconjugates is present in bile collected from heterozygous Gunn rats or from normal rats that were infused with large amounts of bilirubin.

In humans and most mammals degradation of haemoproteins leads to formation of bilirubin.* Biliary excretion of this molecule occurs after esterification in the liver of one or both propionic acid side-chains with a carbohydrate residue to form polar derivatives. Conjugates with glucuronic acid, glucose and xylose have been demonstrated and the relative abundance of these glycosides in bile has been found to be species-dependent (Compernolle *et al.*, 1971, 1978; Gordon *et al.*, 1976, 1977; Fevery *et al.*, 1977*a*). In most species thus far examined virtually all bilinoids excreted in bile are in conjugated form, and over 90% of these pigments are bilirubin glucuronides, both mono- and di-conjugated.

A major limitation in studies of the metabolism of bilirubin is the lack of methods for the specific determination of unconjugated bilirubin and its mono- and di-conjugates. In general, three different approaches can be discerned. (1) Several solvent-partition methods (Weber & Schalm, 1962; Brodersen & Vind, 1963) have been devised to determine unconjugated and conjugated bilinoids, but these methods lack specificity and do not permit determination of mono- and di-conjugates individually (Bratlid & Winsnes, 1971; Fevery *et al.*, 1972; Ostrow & Boonyapisit, 1978). (2) Spectrophotometric measurement of 'direct'- and 'indirect'-reacting pigments by diazo-methods with diazotized sulphanilic acid is the most widely used procedure for quantification of the bilinoid fractions. Although these procedures have been found to be very useful for clinical diagnosis, the 'direct' or 'indirect' reaction does not accurately reflect conjugated or unconjugated bilirubin (Michaelsson, 1961; Lucassen, 1961; Jacobs *et al.*, 1964). More specific determination of conjugated bilinoids is possible by the diazotized-ethyl anthranilate method (Van Roy & Heirwegh, 1968), which also permits measurement of the mono- and di-conjugated fraction by t.l.c. of the azo derivatives (Heirwegh *et al.*, 1974). Rigorous adherence to the originally described reaction conditions, including sufficient dilution of the sample, is a prerequisite to achieve selective and complete

* In agreement with the recommendations of the IUPAC/IUB Commission on Biochemical Nomenclature the name bilirubin represents bilirubin-IXα (see also Bonnett, 1978).

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coupling of diazotized ethyl anthranilate with conjugated pigments (Fevery *et al.*, 1977*a*; Ostrow & Boonyapisit, 1978). However, even under these reaction conditions, values for unconjugated azodipyrrole (azopigment α_0) may exceed 50% of total azo derivatives from bile (Fevery *et al.*, 1977*a*,*b*), thus indicating that pigments different from monoester conjugates may contribute to formation of unconjugated azodipyrrole and thereby lead to overestimation of monoconjugates. (3) The ideal approach would be to separate all pigment fractions and to directly measure each isolated bilinoid. Such an assay requires determination of the amount, nature and homogeneity of each isolated pigment band and necessitates the availability of many unstable reference pigments, with knowledge of their molar absorption coefficients. None of the bilirubin conjugates, however, has yet been completely characterized in its tetrapyrrolic form or isolated as a pure stable compound. Several procedures for such 'direct' analysis of bilinoids have been described, but none has been adequately validated as an analytical method. Except for the reversed-phase column-chromatography technique used in the classical studies of Cole *et al.* (1954) and Billing *et al.* (1957), all published methods involve separation of the pigments by t.l.c. Direct application of bile to silica gel (Thompson & Hofmann, 1971; Noir, 1976; Boonyapisit *et al.*, 1976) limits the applicability of the method to concentrated bile samples. On the other hand, extraction of the pigments before t.l.c. (Heirwegh *et al.*, 1975; Gordon *et al.*, 1977) probably results in differential loss of polar conjugates, and recovery of individual pigment fractions with these procedures has not been evaluated. Another shortcoming of these methods is that the problem of direct measurement and identification of separated bilinoid fractions has been circumvented by preparing azo derivatives of the isolated pigments. This approach, however, renders the method elaborate and less accurate and, even more important conceptually, implies that these 'direct' methods end up as 'indirect' ones, since measurement and identification of the isolated bilinoids again completely depend on diazo-methods.

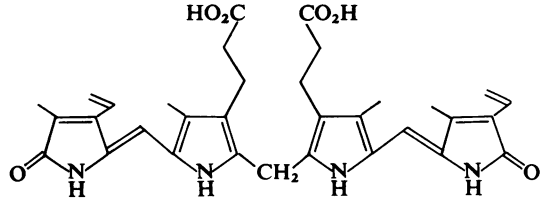
A novel method, based on a different approach, has now been developed and validated for specific determination of bilirubin, its two isomeric monoconjugates and its diconjugates in biological specimens. The procedure is based on the selective conversion of mono- and di-conjugates to the corresponding mono- and di-methyl esters by alkali-catalysed transesterification (Scheme 1). Polar bilirubin conjugates are thereby quantitatively converted into non-polar derivatives, which are easily extractable into chloroform. The reaction products are analysed by t.l.c. and the relative amounts of bilirubin, the two isomeric bilirubin monomethyl esters and bilirubin dimethyl ester are determined spectrophotometrically. The appropriate reference pigments have been synthesized and characterized.

Materials and Methods

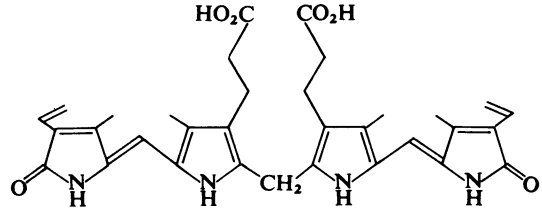
Chemicals

Bilirubin (ε_{452} in chloroform 61.0 litre·mol^{-1}·cm^{-1}) was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Analysis of the pigments by t.l.c. (McDonagh & Assisi, 1971) showed that this bilirubin preparation contained 1% IIIα, 93% IXα and 6% XIIIα isomers (Fig. 1). Whereas bilirubin-IXα is the natural isomer, commercial bilirubin preparations invariably contain IIIα and XIIIα isomers, formed by dipyrrole exchange* of bilirubin-IXα during isolation of the pigment (McDonagh, 1975).

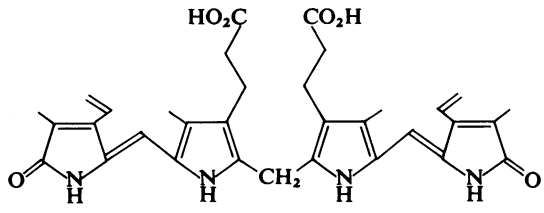
* Dipyrrole exchange is used to denote disproportionation of bilinoids with a central methylene bridge. This reaction involves cleavage of each molecule at either side of the central C-10 bridge into two dipyrrolic fragments, followed by random recombination of dipyrrole moieties from different molecules.



Bilirubin-IXα



Bilirubin-IIIα



Bilirubin-XIIIα

Fig. 1. *Structures of bilirubin-IXα, -IIIα and -XIIIα*

δ-Amino[4-^{14}C]laevulinic acid (sp. radioactivity 58mCi/mmol) was from New England Nuclear (Boston, MA, U.S.A.) and bovine serum albumin (fraction V) from Sigma (St. Louis, MO, U.S.A.). Chloroform (containing 0.75% ethanol as stabilizer) and pentan-2-one (dried over $CaSO_4$ and redistilled) were from Mallinckrodt (St. Louis, MO, U.S.A.). All other reagents were of analytical reagent grade.

Collection of bile samples

Bile was collected from 350–450g male Sprague–Dawley rats ('normal rats') or heterozygous Gunn rats as described by Blanckaert *et al.* (1978) and only animals producing at least 1ml of bile/h, after complete recovery from anaesthesia (2–3h after surgery), were used. Bilinoid-enriched bile ('enriched bile') was obtained from rats infused intravenously with bilirubin (195nmol/min per 100g body wt.) after an initial priming dose of 4.1μmol. For infusion, a solution of bilirubin (15.4μmol/ml) was prepared by first dissolving the pigment in 0.1M-NaOH, then followed by dilution with a solution of bovine serum albumin (20g/litre in 0.15M-NaCl) and adjustment of the pH to 7.8 with 0.15M-HCl. Bile samples were collected in tared tubes placed on ice and protected from light; bile flow was determined gravimetrically. Dog bile was gall-bladder bile obtained from anaesthetized dogs during surgery.

Spectrophotometry

All photometric measurements were carried out at ambient temperature (20–25°C) with a Cary model 118 spectrophotometer (Varian, Palo Alto, CA, U.S.A.), calibrated with a holmium oxide filter. For determination of molar absorption coefficients, observed absorbancies were plotted against molar concentrations of pigment for a series of dilutions (0.6–130μM) and the ε values were calculated from the slope of the regression line.

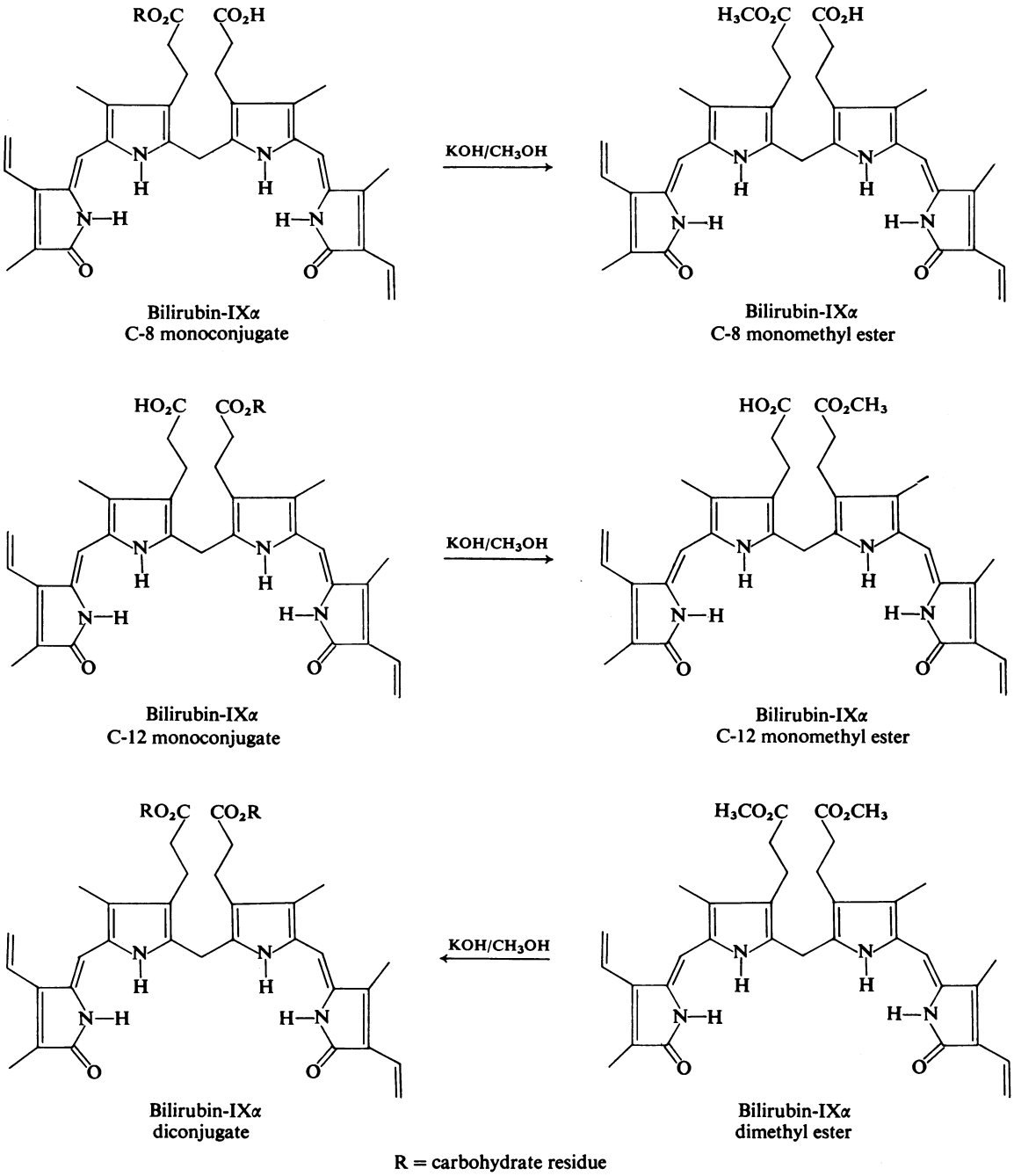
Methods

All work with bile pigments was done under dim light. Centrifugation was carried out at room temperature in an ordinary laboratory centrifuge at 3000rev./min ($1000g_{av.}$). The glycine/HCl buffer was 0.4M-HCl adjusted at 20–25°C to pH2.7 with solid glycine. Except where stated otherwise glass plates precoated with silica gel (60, 5762/0025 or 5763/0025 from Merck, Darmstadt, Germany, or E.M. Laboratores, Elmsford, NY, U.S.A.) were used for t.l.c.; the pigments were applied to the plates under a stream of N_2 while blowing warm air on the backside of the plate. Elementary analyses were done in Galbraith Laboratories, Knoxville, TN, U.S.A.

The alkaline-methanolysis procedure. Base-catalysed methanolysis of bilirubin conjugates in aqueous samples and t.l.c. of the reaction products were carried out as follows. Methanol (2ml), about 20mg of ascorbic acid and a trace of EDTA (disodium salt) were added to 0.2ml of sample (containing up to 120nmol of pigment). The mixture was treated with 2ml of 2% (w/v) KOH in methanol and thoroughly vortex-mixed. After 1min at 20–25°C, 2ml of chloroform and 4ml of glycine/HCl buffer, pH2.7, were added sequentially, the mixture was shaken and centrifuged and the extract was evaporated under N_2 at 30°C. The dried pigments could then be stored under argon at −15°C. For chromatographic analysis, the pigment residue was redissolved in a portion of chloroform and applied to a t.l.c. plate. To avoid breakdown of the bilinoids, which occurs particularly fast when the pigment is adsorbed to dry silica gel, only one sample was applied to each t.l.c. plate (5cm × 20cm) and development of the chromatogram over 18cm with chloroform/methanol/acetic acid (97:2:1, by vol.) was started immediately. For quantification, pigment bands were scraped from the plates, eluted with a known volume of chloroform/methanol (1:1, v/v) and the A_{450} values of the eluates were determined.

Preparation of reference bilirubin mono- and di-methyl esters (*Scheme* 1). Bilirubin (120mg) was dissolved by brief refluxing in chloroform (150ml). The solution was cooled, treated for 2min at 20–22°C with excess ethereal diazomethane and evaporated to dryness in a rotary evaporator at 35°C. The residue was dissolved in 3–5ml of chloroform/light petroleum (b.p. 35–60°C) (1:1, v/v) and applied to a column (45cm × 2.0cm internal diameter) of neutral alumina (120g) (Neutral Alumina AG7, 100–200mesh, activity III–IV; Bio-Rad Laboratories, Richmond, CA, U.S.A.). With the chloroform/light petroleum mixture as eluent, di- and mono-methoxy derivatives of bilirubin dimethyl ester were eluted sequentially. Bilirubin dimethyl ester was then eluted with chloroform/methanol (20:1, v/v) and purified by preparative t.l.c. on glass plates coated with silica gel (silica gel G, type 60; E.M. Laboratories; layer thickness 0.5mm) by developing the chromatogram with benzene/ethanol (25:2, v/v). The purified ester (R_F approx. 0.6) was eluted from the silica gel with chloroform/methanol (20:1, v/v) and, after removal of the eluent, crystallized from methanol. Bilirubin dimethyl ester, containing 5% IIIα, 88% IXα and 7% XIIIα isomers, m.p. 198°C (decomp.) (Found: C, 68.3; H, 6.7; N, 9.0; $C_{35}H_{40}N_4O_6$ requires C, 68.6; H, 6.6; N, 9.1%) was obtained as bright orange birefringent platelets; this material was probably partially crystalline.

Bilirubin-IXα monomethyl esters were prepared by partial saponification of the dimethyl ester as follows. Bilirubin dimethyl ester (100μmol, 61mg) was dissolved in 500ml of methanol and, after addition of 50mg of ascorbic acid and 1–2mg of EDTA (disodium salt), the mixture was treated with 250ml of 1M-NaOH (final pH13.0–13.5) and shaken



Scheme 1. *Base-catalysed transesterification of bilirubin mono- or di-conjugates in methanol*
Bilirubin sugar mono- or di-conjugates are converted into the corresponding methyl ester derivatives by alkaline methanolysis. Chosen trivial names and corresponding systematic names of the bilirubin methyl esters are: bilirubin-IXα C-8 monomethyl ester, 12-(2-carboxyethyl)-10,21,23,24-tetrahydro-8-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-3,18-divinylbilin-1,19-dione; bilirubin-IXα C-12 monomethyl ester, 8-(2-carboxyethyl)-10,21,23,24-tetrahydro-12-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-3,18-divinylbilin-1,19-dione; bilirubin-IXα dimethyl ester, 10,21,23,24-tetrahydro-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-3,18-divinylbilin-1,19-dione.

for 2min at 37°C under argon in a closed vessel. After acidification with 32ml of acetic acid, 500ml of chloroform and 1000ml of glycine/HCl buffer, pH2.7, were added. The extract was then washed with 0.1M-$NaHCO_3$ (50ml) and water (2×100ml) and filtered on chloroform-moistened filter paper. After removal of the solvent *in vacuo* at 35°C, the residue was dissolved in chloroform and applied to t.l.c. plates (approx. 1.5μmol of pigment per plate). Development of the chromatogram with chloroform/methanol/acetic acid (97:2:1, by vol.) yielded four major and two minor yellow bands, corresponding to bilirubin (R_F 0.69), the two isomeric bilirubin-IXα monomethyl esters (R_F 0.49 and 0.45), bilirubin dimethyl ester (R_F 0.26) and bilirubin-IIIα monomethyl ester (R_F 0.52) and XIIIα monomethyl ester (R_F 0.41) (Fig. 2). Approx. 35 and 28% of the starting dimethyl ester was converted by the saponification procedure into monomethyl esters and unconjugated bilirubin respectively. Bilirubin-IXα monomethyl esters were eluted from the adsorbent with chloroform/methanol (40:1, v/v) and, after removal of the eluent, crystallized from methanol; both isomers were individually obtained as bright orange birefringent needles. *Bilirubin-IXα C-8 monomethyl ester*, m.p. 245°C (decomp.), $\lambda_{max.}$ (chloroform) 438nm (ε 59.3×10^3 litre·mol^{-1}·cm^{-1}) (Found: C, 68.0; H, 6.6; N, 9.2. $C_{34}H_{38}N_4O_6$ requires C, 68.2; H, 6.4; N, 9.4%). *Bilirubin-IXα C-12 monomethyl ester*, m.p.168°C (decomp.), $\lambda_{max.}$ (chloroform) 442nm (ε 55.2×10^3 litre·mol^{-1}·cm^{-1}) (Found: C, 67.9; H, 6.6; N, 9.0. $C_{34}H_{38}H_4O_6$ requires C, 68.2; H, 6.4; N, 9.4%).

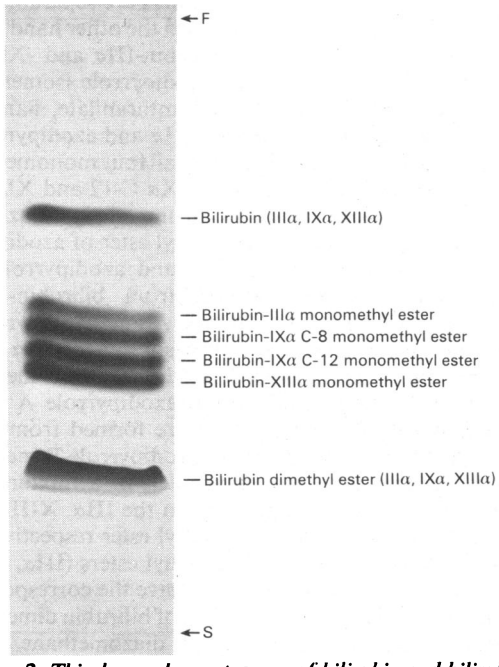


Fig. 2. *Thin-layer chromatogram of bilirubin and bilirubin methyl esters*

Reference pigments were chromatographed on silica gel by development with chloroform/methanol/acetic acid (97:2:1, by vol.). The application line is indicated by S, the solvent front by F. The chromatogram was photographed through a blue filter (Kodak Wratten C5 no. 47).

Reference bilirubin-IIIα or -XIIIα mono- and di-methyl esters were synthesized from bilirubin-IIIα or -XIIIα by procedures identical with those used for preparation of the corresponding IXα isomers, but not crystallized. Bilirubin-IIIα and -XIIIα were prepared by acid-catalysed isomerization of bilirubin-IXα and isolated by t.l.c. (McDonagh & Assisi, 1971).

Determination of bilirubin-IIIα, -IXα and -XIIIα. Bilirubin conjugates and methyl esters were saponified as previously described (Blanckaert *et al.*, 1976*a*) and the extracted pigments were subjected to t.l.c. with authentic bilirubin-IIIα, -IXα and -XIIIα as reference compounds (McDonagh & Assisi, 1971). For quantification, the pigments were eluted in chloroform and their relative amounts were calculated from the A_{452} values.

Preparation of bilirubin-IXβ, -IXγ, -IXδ, [^{14}C]bilirubin and unlabelled and [^{14}C]bilirubin mono- and di-glucuronide. The non-α-isomers of bilirubin-IX were synthesized as described (Blanckaert *et al.*, 1976*a*). Glucuronides (labelled or unlabelled) were prepared biosynthetically and isolated by t.l.c. as previously reported (Blanckaert *et al.*, 1979). The specific radioactivity of the [^{14}C]bilirubin glucuronides was 730–2350d.p.m./nmol. Unconjugated [^{14}C]bilirubin (sp. radioactivity 800–3100d.p.m./nmol) was prepared biosynthetically from δ-amino[4-^{14}C]laevulinic acid and isolated from bile as described by Ostrow *et al.* (1961). Except when used for injection in rats to prepare [^{14}C]bilirubin glucuronides, [^{14}C]bilirubin was further purified by t.l.c. with chloroform/methanol/acetic acid (97:2:1, by vol.). For addition of [^{14}C]bilirubin to aqueous samples, the pigment was dissolved in dimethyl sulphoxide and 10μl of this solution was added to 0.2ml of aqueous sample. Dried [^{14}C]bilirubin glucuronides were dissolved immediately in the aqueous samples.

Radioassays. For determination of ^{14}C radioactivity, samples were pipetted in counting vials and organic solvents were evaporated under a stream of N_2. The residue was dissolved in 1ml of Soluene-350 (Packard Instrument Co.) Downers Grove, IL, U.S.A.) and 0.2ml of propan-2-ol and 0.2ml of aq. 30% H_2O_2 were added. The vials were mixed and kept overnight at 20–25°C or heated to 40°C during 2h, and 10ml of scintillation fluid (Dimilume-30; Packard Instrument Co.) was then added. After capping, the vials were shaken and kept overnight in

darkness at 4°C. The samples were counted for radioactivity for 10min or for 100000 counts in a Beckman (Fullerton, CA, U.S.A.) liquid-scintillation spectrometer model LS-250, and the observed radioactivity-count rates were corrected by use of an internal standard ([^{14}C]toluene, 3.66×10^5 d.p.m./ml; New England Nuclear). The standard error of the radioactivity-count rates varied between 0.5 and 7.5% and the efficiency of counting was 81–89%. For estimation of radioactivity in chromatograms, pigment bands were scraped from the plates, transferred to counting vials and further processed in the same way as pigments in solutions.

Preparation and analysis of azo derivatives. Total bilinoids in bile were determined by the *p*-iodoaniline method (Van Roy *et al.*, 1971). Formation of ethyl anthranilate azo derivatives from the organic or aqueous phase obtained after alkaline methanolysis was done as follows (addition of ascorbic acid to the reaction mixture for methanolysis was omitted in these experiments). Propan-2-ol (2.4ml) and 0.3ml of ethyl anthranilate diazo-reagent (Van Roy & Heirwegh, 1968) were added to 0.3ml of chloroform extract. After 5min at 20–25°C, 6ml of glycine/HCl buffer, pH2.7, was added and the pigments were extracted with 2ml of pentan-2-one. Azopigment formation from the aqueous phase was as described (Heirwegh *et al.*, 1970). The A_{530} values of the organic phases were immediately determined and a value of 22.2×10^3 litre·mol^{-1}·cm^{-1} was used as molar absorption coefficient of the azopigments for calculation of their concentration. Formation of derivatives and chromatographic analysis of the ethyl anthranilate azopigments were done as described previously (Blanckaert *et al.* 1976*a*, 1978). Azopigments are denoted by letters, as adopted previously (Blanckaert *et al.*, 1977); structures of these pigments were given in Scheme 2 of the same paper.

Results

The method reported in the present paper is based on (i) complete conversion of the bilirubin mono- and di-ester conjugates to the corresponding mono- and di-methyl ester derivatives by alkaline methanolysis, i.e. base-catalysed transesterification and on (ii) subsequent separation and individual quantification of bilirubin and its mono- and di-methyl esters. In validating the method, particular attention has been paid to the following questions. (i) Are mono- and di-conjugates selectively converted into respectively mono- and di-methyl esters and does unconjugated bilirubin remain unchanged during treatment with alkaline methanol? (ii) Does hydrolysis or dipyrrole exchange occur? (iii) Are the recoveries of the various bilinoid fractions complete?

Separation of bilirubin and its methyl esters by t.l.c. and characterization of the reference compounds

Adequate separation of unconjugated bilirubin and its mono- and di-methyl esters could be achieved on an analytical and preparative scale by t.l.c. (Fig. 2). Bilirubin-IIIα, -IXα and -XIIIα consistently chromatographed as a single band (R_F 0.69) and so did the IIIα, IXα and XIIIα isomers of bilirubin dimethyl ester (R_F 0.26) in most chromatograms; occasionally, the XIIIα dimethyl ester moved slower than the IIIα and IXα isomers. The monomethyl ester fraction was resolved in four distinct bands, corresponding to IIIα (R_F 0.52), IXα C-8 (R_F 0.49), IXα C-12 (R_F 0.45) or XIIIα (R_F 0.41) isomers of bilirubin monomethyl ester.

Structural assignments to the reference bilirubin methyl esters were partly based on chromatographic analysis of their ethyl anthranilate azo derivatives. Because of its unsymmetrical substitution sequence (Fig. 1), bilirubin-IXα yields two isomeric dipyrrolic azopigments on reaction with diazotized ethyl anthranilate. Azodipyrrole isomer A corresponds to the dipyrrole methine moiety of bilirubin-IXα that contains an *endo*-vinyl β-substituent and isomer B corresponds to the dipyrrole methine moiety with an *exo*-vinyl group. These two isomers are separable by t.l.c. (Blanckaert *et al.*, 1976*a*). On the other hand, the symmetrically substituted bilirubin-IIIα and -XIIIα isomers each give a unique azodipyrrole isomer on reaction with diazotized ethyl anthranilate, namely azodipyrrole isomer A from XIIIα and azodipyrrole isomer B from IIIα. As expected, all four monomethyl ester fractions (IIIα, IXα C-8, IXα C-12 and XIIIα) yielded equimolar amounts of unmethylated azodipyrroles A or B and of the methyl ester of azodipyrroles A or B; azodipyrrole B and azodipyrrole B methyl ester were obtained from bilirubin-IIIα monomethyl ester, azodipyrrole A and azodipyrrole A methyl ester from the XIIIα isomer; the IXα C-8 monomethyl ester yielded azodipyrrole A methyl ester and azodipyrrole B, and azodipyrrole A and azodipyrrole B methyl ester were formed from the IXα C-12 monomethyl ester; azodipyrrole B methyl ester, azodipyrrole A methyl ester, or a mixture of both isomers were obtained from the IIIα, XIIIα or IXα isomers of bilirubin dimethyl ester respectively. The isolated bilirubin monomethyl esters (IIIα, IXα C-8, IXα C-12 and XIIIα) each gave the corresponding isomer (IIIα, IXα and XIIIα) of bilirubin dimethyl ester on treatment with ethereal diazomethane, and saponification of isolated mono- or di-methyl ester in each case yielded the corresponding isomer (IIIα, IXα and XIIIα) of unconjugated bilirubin. These structural assignments were further confirmed by elementary analysis (see above) and by mass-spectrometric analysis of the bilirubin dimethyl ester and four isomeric monomethyl esters, each of which gave

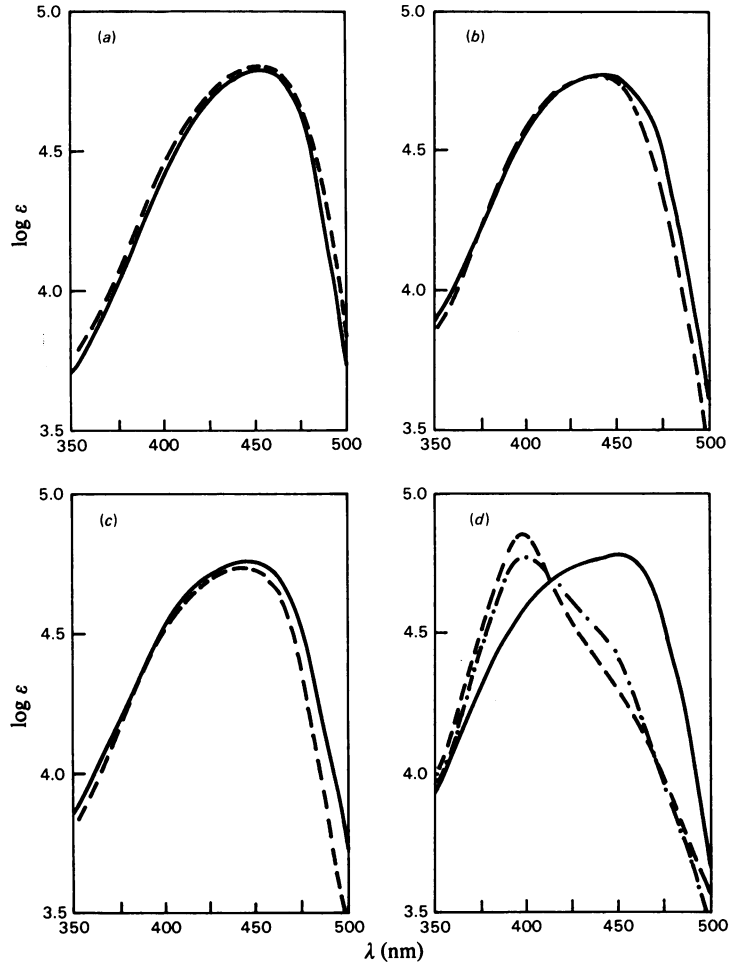


Fig. 3. *Characteristic absorption spectra of bilirubin and bilirubin methyl esters*
The spectra were obtained either in chloroform (----) or in chloroform/methanol (1 : 1, v/v) (——). The concentration of bilirubin dimethyl ester in chloroform was 104 μM (----) or 14 μM (-·-·-·-). (*a*), (*b*), (*c*) and (*d*) are absorption spectra of respectively bilirubin, bilirubin-IXα C-8 monomethyl ester, bilirubin-IXα C-12 monomethyl ester and bilirubin dimethyl ester.

the expected molecular ion (F. Compernolle & N. Blanckaert, unpublished work).

Spectrophotometric determination and absorption spectra of bilirubin and methyl ester derivatives

For calculation of the relative amounts of bilirubin and its mono- and di-methyl esters in the chromatogram from the A_{450} values of the chloroform/methanol eluates, absorption spectra of the individual crystalline pigments in chloroform/methanol (1 : 1, v/v) were recorded and molar absorption coefficients were determined (Fig. 3). The ε_{450} values were (litre·mol^{-1}·cm^{-1}): bilirubin, 61.5×10^3; bilirubin-IXα C-8 monomethyl ester, 58.4×10^3; bilirubin-IXα C-12 monomethyl ester, 57.0×10^3; bilirubin dimethyl ester, 60.8×10^3. Elution of pigment from silica gel was complete and Lambert–Beer's law was obeyed up to 100 μM for all four fractions.

As reported (Kuenzle, 1970; Lightner *et al.*, 1979), the absorption spectrum of bilirubin dimethyl ester was solvent-dependent [in chloroform; $\lambda_{max.}$ 397nm, inflexion 445nm; in chloroform/methanol (1 : 1, v/v) $\lambda_{max.}$ 448nm, inflexion 410nm] and, in chloroform,

Table 1. *Recoveries of bilirubin and bilirubin mono- and di-glucuronide added to biological samples and carried through the alkaline-methanolysis procedure*
^{14}C-labelled unconjugated bilirubin or bilirubin mono- or di-glucuronide was added to either normal rat bile or serum from patients with obstructive jaundice or aqueous solutions containing rat microsomal fraction (4.7 mg of protein/ml). To all samples except human serum 20 μl of a concentrated solution of unlabelled carrier pigments (unconjugated bilirubin only for normal rat bile, a mixture of unconjugated bilirubin and both glucuronides for microsomal preparations) in dimethyl sulphoxide was added. Alkaline methanolysis was performed and the two phases were radioassayed. A known amount of the extract was subjected to t.l.c., and radioactivity was determined in the bilirubin (B), bilirubin monomethyl ester (BMM) and bilirubin dimethyl ester (BDM) fractions. Distribution of ^{14}C label is given as percentages of total ^{14}C label (mean ± S.D.).

^{14}C-labelled pigment added to reaction mixture	Type of sample	Recovery of ^{14}C label (% of added radioactive label)		Number of experiments	Recovery of ^{14}C label in chromatogram (% of applied radioactive label)			Number of experiments
		In extract	In aq. phase		B	BMM	BDM	
[^{14}C]Bilirubin	None*	98.0 ± 2.0	0.6 ± 0.3	3	94.2 ± 1.9	2.1 ± 0.7	1.0 ± 0.9	3
[^{14}C]Bilirubin	Bile	97.7 ± 4.0	0.8 ± 0.7	8	95.1 ± 3.3	1.3 ± 0.4	1.5 ± 0.7	8
[^{14}C]Bilirubin	Serum	98.9 ± 1.5	0.3 ± 0.1	8	97.3 ± 0.6	1.2 ± 0.3	0.7 ± 0.2	3
[^{14}C]Bilirubin	Microsomal fraction	99.3 ± 3.1	0.5 ± 0.1	3	95.7 ± 1.0	2.4 ± 0.6	1.4 ± 0.5	4
[^{14}C]Bilirubin monoglucuronide	Bile	92.7 ± 3.1	1.0 ± 0.1	3	4.7 ± 0.4	75.6 ± 2.2	11.7 ± 2.4	3
[^{14}C]Bilirubin monoglucuronide	Serum	98.9 ± 4.4	0.8 ± 0.1	7	5.0 ± 1.1	78.7 ± 5.8	9.9 ± 2.8	3
[^{14}C]Bilirubin monoglucuronide	Microsomal fraction	99.0 ± 1.7	0.9 ± 0.3	3	4.5 ± 0.8	78.5 ± 4.4	13.6 ± 2.9	3
[^{14}C]Bilirubin diglucuronide	Bile	96.5 ± 1.0	1.4 ± 0.2	4	0.3 ± 0.1	4.7 ± 0.4	93.2 ± 2.8	8
[^{14}C]Bilirubin diglucuronide	Serum	97.0 ± 4.8	1.4 ± 0.1	7	0.4 ± 0.2	4.6 ± 0.1	94.7 ± 0.6	3
[^{14}C]Bilirubin diglucuronide	Microsomal fraction	96.0 ± 1.8	1.3 ± 0.3	4	0.4 ± 0.1	5.0 ± 0.2	94.1 ± 1.0	3

* Control experiment in which radioactively labelled bilirubin (dissolved in dimethyl sulphoxide, 20 μl) was added to 4 ml of methanol and extracted with 4 ml of glycine/HCl buffer, pH 2.7, and 2 ml of chloroform, which contained unlabelled bilirubin and bilirubin monomethyl and dimethyl ester as carrier pigments.

also concentration-dependent (at 104μM, ε 71.7 $\times 10^3$ litre·mol^{-1}·cm^{-1}; at 14μM, ε 60.5$\times 10^3$ litre·mol^{-1}·cm^{-1}) (Fig. 2*d*).

Is unconjugated bilirubin affected by treatment with alkaline methanol?

Bilirubin (added to the reaction mixture either as dried pigment or in aqueous solution) remained unchanged when carried through the methanolysis procedure, and recovery was virtually complete (Tables 1 and 5). Chromatography of the pigment showed that neither methylation nor methoxylation had occured and that the relative amounts of IIIα, IXα and XIIIα isomers were identical before and after treatment with alkaline methanol, indicating that dipyrrole exchange had not occurred either. Analysis of ethyl anthranilate azo derivatives from the extracted pigment revealed only authentic unconjugated azodipyrroles A and B. When Gunn-rat serum was subjected to the procedure, unconjugated bilirubin-IXα was recovered intact (Table 5).

Base-catalysed methanolysis of bilirubin glucuronides: specificity and completeness of the reaction and recovery studies

When tested with methanolic solutions of purified bilirubin mono- and di-glucuronides, either in the presence or absence of 0.2ml of water, methanolysis was virtually complete after 30s (Table 2). Less than 2% of starting pigment remained in the aqueous phase and over 99% of extracted pigments were in the form of methyl ester derivatives. Similar results were obtained when longer reaction times, up to 5min, were used. After 5min only a trace amount of glucuronide (approx. 0.5%) was still present in the reaction mixture, as indicated by structural analysis of the azo derivatives from the extract. Virtually complete transesterification and comparable extraction yields were obtained with bile as the source of bilirubin conjugates (Table 2). In rat bile samples containing up to 4mM bilinoid, 99% or more of the extracted pigments had been transesterified after reaction for

Table 2. *Completeness of the alkaline methanolysis*

Methanolic solutions of bilirubin mono- or di-glucuronide (2ml) were subjected to alkaline methanolysis by mixing with 2ml of 2% (w/v) KOH/methanol. After 1min at 20–25°C, the pigments were extracted with 4ml of glycine/HCl buffer, pH2.7, and 2ml of chloroform. Rat bile samples containing various concentrations of bile pigments were prepared by mixing normal rat bile with bile from rats infused intravenously with bilirubin. These bile samples were then subjected to alkaline methanolysis as described under 'Methods', except for addition of ascorbic acid to the reaction mixture. Bile pigments in the starting material were determined by the *p*-iodoaniline procedure (Van Roy *et al.*, 1971). Assay of the pigment concentration in the extract and aqueous phase, and chromatographic analysis of ethyl anthranilate azo derivatives from the extract, were done as described under 'Methods'. Results given are values from individual experiments.

Sample			Recovery of pigment (% of starting material)		
Type	Amount of pigment (nmol)	Reaction time	In extract	In aq. phase	Conjugated azodipyrrole from extract (% of total azopigment)
Bilirubin monoglucuronide in methanolic solution	175	0.5	93.3; 96.0	0.8; 0.5	0.7; 0.5; 0.1*
		1.0	94.5; 93.4	0.8; 0.4	0.5; 0.5; 0.5*
		1.5	95.2; 93.2	0; 0.6	0; 0.6; 0*
		2.0	96.1; 95.0	0.9; 0.8	0.9; 0.2
		5.0	93.2; 94.1	0.4; 0.5	1.2; 0.3
Bilirubin diglucuronide in methanolic solution	164	0.5	94.0; 93.2	1.8; 1.4	0; 0.2; 0*
		1.0	97.4; 95.0	1.7; 1.4	0.5; 0.2; 0*
		1.5	97.3; 97.0	1.5; 1.6	0; 0.4; 0.5*
		2.0	97.5; 94.8	1.7; 1.5	0.5; 0.1
		5.0	92.2; 95.1	1.4; 1.3	0; 0.2
Rat bile	48	1.0	90.7; 99.0	0.5; 0.6	0.4; 0.8; 0.5
	124	1.0	95.2; 105.0	0.6; 0.7	0.3; 0.6; 0.7
	840	1.0	98.3; 107.2	1.9; 1.5	0.6; 1.0; 1.1
	2000	1.0	97.5; 89.0	1.8; 2.0	1.3; 2.4
	4680	1.0	85.4; 82.9	2.1; 2.4	3.4; 4.9
Dog bile	119	1.0	90.8; 102.0	1.3; 0.4	1.9; 0.5
	198	1.0	99.1; 91.7	0.8; 1.6	1.5; 3.3
	397	1.0	93.4; 97.2	3.0; 1.1	3.8; 1.2

* With 0.2ml of water added to the methanolic solution before the alkaline methanolysis was carried out.

1 min, and less than 1% of the pigments remained in the aqueous phase with bilinoid concentrations in bile up to 600 μM. Comparable results were obtained with dog bile (Table 2) in which glucose and xylose account for approx. 40% of the conjugating carbohydrate residues of the bilirubin conjugates (Fevery *et al.*, 1977*a*). This suggested that these non-glucuronide conjugates also undergo rapid transesterification. Virtually complete extraction and/or transesterification was also found when [^{14}C]bilirubin or [^{14}C]bilirubin mono- or di-glucuronide was added to rat bile, human serum or incubation mixtures containing rat liver homogenate or microsomal fraction (Table 1). Negligible amounts (less than 2%) of the ^{14}C radioactivity remained in the aqueous phase, and losses in the protein layer at the interphase, which was white, were insignificant, as indicated by recovery of at least 93% of added radioactivity in the chloroform extract. With [^{14}C]bilirubin added to the samples, 94% or more of extracted ^{14}C radioactivity co-chromatographed with bilirubin-IXα. Small amounts of radioactivity were found also in the mono- and di-methyl ester band; this was probably due to some degradation during chromatography since comparable results were obtained when a control extract of bilirubin was chromatographed (Table 1). With extracts from samples containing [^{14}C]bilirubin monoglucuronide, approximately 5% and 10–15% of extracted radioactive label was recovered in the bilirubin and dimethyl ester fraction respectively; since dipyrrole exchange could be excluded (see below), this was probably due to contamination of the parent monoglucuronide preparation with unconjugated bilirubin and bilirubin diglucuronide. Some dipyrrole exchange does indeed occur during isolation of bilirubin glucuronides by t.l.c. (Blanckaert *et al.*, 1979).

In developing this method for aqueous samples, it was important to define reaction conditions that did not lead to saponification. An excess of methanol relative to water in the reaction mixture was maintained throughout the whole procedure by mixing the sample with methanol before the methanolysis reaction was started by addition of alkaline methanol. With methanolic solutions of bilirubin diglucuronide, hydrolysis of glucuronide was less than 1% when 0.2 ml of water per 4 ml of methanol was present in the reaction mixture (Table 3). With 0.4 and 1.0 ml of water added, up to 4% and 6% of the pigment respectively were saponified. Slightly more hydrolysis occurred when the reaction was allowed to proceed for 3 min. With a reaction time of 1 min, hydrolysis was further evaluated for bile samples with added [^{14}C]bilirubin mono- or di-glucuronide as sources of bile pigment (Table 4). As in methanolic solutions with added water, saponification of less than 1% of mono- or di-glucuronide occurred under the finally adopted reaction conditions for the alkaline-methanolysis procedure.

Does dipyrrole exchange occur?

Dipyrrole exchange in monoester conjugates would result in artificial formation of unconjugated and diconjugated pigments and therefore invalidate the method. Bile samples enriched with bilirubin-IXα were subjected to alkaline methanolysis and bilirubin-IIIα, -IXα and -XIIIα isomers in the parent bile samples and in the isolated bilirubin, bilirubin monomethyl and dimethyl ester fractions were determined (Table 5). No differences were found when the isomeric composition of the three fractions obtained by alkaline methanolysis was compared with the pattern from the parent bile sample, thus indicating that dipyrrole exchange was insignificant or absent. This was confirmed by the absence of appreciable amounts of IIIα and XIIIα isomers in

Table 3. *Saponification of bilirubin diglucuronide in the alkaline-methanolysis procedure*

Various amounts of water were added to methanolic solutions of bilirubin diglucuronide (2 ml). After addition of about 20 mg of ascorbic acid and a trace of EDTA (disodium salt) the mixtures were immediately subjected to methanolysis by mixing with 2 ml of 2% (w/v) KOH/methanol. The reaction was allowed to proceed for 1 min at 20–25°C, after which 4 ml of glycine/HCl buffer, pH 2.7, and 2 ml of chloroform were added. Chromatographic analysis of the extracted pigments was done as described under 'Methods'. Unconjugated bilirubin was undetectable in all chromatograms. Results shown are values from individual experiments. Values in parentheses are results obtained when methanolysis was allowed to proceed for 3 min.

Amount of pigment (nmol)	Water added to samples (ml) ...	Bilirubin monomethyl ester (% of total pigment in chromatogram)			
		0	0.2	0.4	1.0
107		1.3	2.0	4.2	5.9
47		1.6	2.3	5.3	7.1
105		0.9	1.8	2.8	5.2
40		0.9	1.9	2.7	4.0
96		0.3 (0.3)	0.9 (1.4)	1.5 (3.8)	2.3 (3.8)
90		0.7 (0.9)	1.3 (1.9)	1.3 (2.0)	1.5 (4.0)

Table 4. *Saponification of bilirubin mono- and di-glucuronide in bile when subjected to alkaline methanolysis*
^{14}C-labelled bilirubin mono- or di-glucuronide was added to normal rat bile that was enriched with unlabelled unconjugated bilirubin by addition of 5μl of a solution of bilirubin-IXα in dimethyl sulphoxide (500μM) to each bile sample (0.2ml). These samples were then subjected to alkaline methanolysis as described under 'Methods'. As controls, anhydrous methanolic solutions of the same ^{14}C-labelled mono- or di-glucuronide preparation were treated with alkaline methanol as described in the legend to Table 3, except that chloroform used for extraction contained unlabelled bilirubin and bilirubin monomethyl and dimethyl esters as carrier pigments. Extracted pigments were analysed by t.l.c. and radioactivity was determined in the bilirubin (B), bilirubin monomethyl ester (BMM) and bilirubin dimethyl ester (BDM) fractions. Results given are values from individual experiments.

Sample	Recovery of ^{14}C label in extract (% of added radioactive label)	Distribution of ^{14}C label in reaction products (% of total ^{14}C label)		
		B	BMM	BDM
[^{14}C]Bilirubin monoglucuronide				
Without bile	95.0; 95.2	3.8; 3.2	82.2; 82.8	14.0; 14.0
With bile added	92.9; 93.7	5.3; 4.2; 4.0	80.5; 80.8; 81.0	14.2; 15.0; 15.0
[^{14}C]Bilirubin diglucuronide				
Without bile	90.5; 95.5	0.4; 0.2	3.1; 4.3	96.5; 95.5
With bile added	93.3; 93.9; 91.9	0.2; 0.3; 0.2; 0.4	4.3; 4.8; 3.5; 4.7	95.5; 94.9; 96.3; 94.8

Table 5. *Comparision of isomeric composition of bile pigments in bile and Gunn-rat serum and in their methanolysis products*
Normal rat bile was enriched with unconjugated bilirubin by addition of 10μl of a solution of bilirubin-IXα to 0.5ml of bile; 0.2ml of this sample was subjected to alkaline methanolysis as described under 'Methods', and the remaining part was saponified for determination of bilirubin-IIIα, -IXα and -XIIIα in the parent sample. Pigments extracted after methanolysis were separated by t.l.c. and the bilirubin, bilirubin monomethyl ester and dimethyl ester fractions were saponified individually and assayed for their isomeric composition. Serum from homozygous Gunn rats (0.2ml) was also subjected to the methanolysis procedure; another 0.2ml of the sample from which bilirubin was extracted by mixing with 4ml of methanol, 2ml of chloroform and 4ml of glycine/HCl buffer, pH2.7, served as a control. Both extracts were immediately subjected to t.l.c. for determination of bilirubin-IIIα, -IXα and -XIIIα. The results are shown as percentages of total pigment in the chromatogram (means $\pm$ S.D.).

Sample	Isomeric composition (% of total pigment)		
	IIIα	IXα	XIIIα
Bile ($n = 3$)			
Parent sample	0.6$\pm$0.2	94.2$\pm$1.0	5.1$\pm$1.1
after methanolysis			
Bilirubin	0.7$\pm$0.4	95.0$\pm$1.0	4.3$\pm$0.8
Bilirubin monomethyl ester	1.0$\pm$0.7	96.2$\pm$2.2	2.8$\pm$1.6
Bilirubin dimethyl ester	0.9$\pm$0.3	93.5$\pm$3.6	5.5$\pm$3.6
Gunn-rat serum ($n = 4$)			
Parent serum	0.2$\pm$0.2	98.6$\pm$0.2	1.2$\pm$0.3
after methanolysis			
Bilirubin	0.1$\pm$0.1	98.9$\pm$0.4	1.0$\pm$0.3

extracts obtained by alkaline methanolysis of Gunn-rat serum or human serum from jaundiced patients (Table 5).

Identification of reaction products from alkaline methanolysis of biological samples or purified mono- and di-glucuronides

With chloroform/methanol/acetic acid (97:2:1, by vol.) as solvent, the reaction products co-chromatographed with reference bilirubin-IXα and/or with synthetic mono- and/or di-methyl esters. The identity of the reaction products with the corresponding reference pigments was further demonstrated by their co-chromatography in three other solvent systems [chloroform, chloroform/acetic acid (99:1, v/v), benzene/ethanol (25:2, v/v)] and by chromatographic analysis of the ethyl anthranilate azo derivatives. Bilirubin-IXβ, -IXγ or -IXδ were immobile on the silica gel plates when developed with chloroform/methanol/acetic acid (97:2:1, by vol.).

Table 6. *Precision of the alkaline-methanolysis procedure*

The alkaline-methanolysis procedure as described under 'Methods' was applied to a variety of biological samples. Results shown are mean values for percentages of total bile pigments in the sample and the coefficients of variation are given in parentheses. Normal rat bile was enriched with unconjugated bilirubin as described in the legend to Table 5. Serum (total bilinoid concn., 210 μM) was taken from a patient with obstructive juandice. Mixtures containing liver homogenate or microsomal fraction from Sprague–Dawley rats (8.6 or 5.2mg of protein/ml of incubation mixture respectively), 33 or 164 μM-bilirubin-IXα, 6.15mM-$MgCl_2$, 1.64mM-NAD^+, 8.2mM-glucaro-1,4-lactone and 2.8mM-UDP-glucuronic acid were incubated at pH7.6 and at 37°C for 20min (Blanckaert *et al.*, 1979) and reaction products were assayed by the methanolysis procedure.

Sample		Composition of bile pigments (% of total pigment)		
Number of experiments	Type	Unconjugated bilirubin	Monoester conjugates	Diester conjugates
10	Rat bile with added bilirubin	76.6 (1.0)	10.5 (1.0)	12.9 (4.6)
10	Human serum	30.7 (3.5)	36.5 (2.4)	32.8 (3.5)
5	Homogenate (164 μM-bilirubin)	74.6 (1.0)	24.2 (2.3)	1.2 (15.3)
5	Homogenate (33 μM-bilirubin)	19.7 (3.8)	72.0 (0.8)	8.3 (3.5)
5	Microsomal fraction (164 μM-bilirubin)	5.6 (5.9)	80.2 (0.4)	14.2 (1.1)
5	Microsomal fraction (33 μM-bilirubin)	1.0 (12.9)	32.0 (2.7)	67.0 (1.3)

Table 7. *Composition of bilinoids in rat bile*

Bile was collected for three consecutive periods of 20min (periods *a*, *b* and *c*) from three heterozygous Gunn rats and from Sprague–Dawley (SD) rats; three rats (group B) of the latter strain were infused intravenously with bilirubin during periods *b* and *c*, and three other animals (group A) were further infused with 0.9% NaCl. Alkaline methanolysis was carried out immediately after collection of bile, and reaction products were analysed by t.l.c. and determined spectrophotometrically. Values are means ± S.D. Significance of differences between means was calculated by Student's *t* test for paired and unpaired data; a *P* value of less than 0.05 was considered significant. Abbreviations used: B, bilirubin; BMC, bilirubin monoconjugate; BDC, bilirubin diconjugate.

Rats			Bilinoids in bile			
				Composition (% of total pigment)		
Number	Strain	Collection period	Conc. (μM)	B	BMC	BDC
3	SD, group A	*a*	217 ± 31	0.3 ± 0.3	30.6 ± 1.2	68.8 ± 1.4
		b	248 ± 53	0.3 ± 0.4	29.3 ± 1.7	70.3 ± 2.0
		c	282 ± 123	0.2 ± 0.2	27.5 ± 1.7	72.4 ± 1.4
3	SD, group B	*a*	179 ± 47	0.2 ± 0.3	28.7 ± 1.2	71.2 ± 1.4
		b	$(21 \pm 1.8) \times 10^3$	1.2 ± 0.4	52.2 ± 0.9	46.4 ± 0.7
		c	$(19.4 \pm 5.0) \times 10^3$	2.0 ± 0.4	45.6 ± 3.1	49.0 ± 6.3
3	Heterozygous Gunn	*a*	180 ± 46	0.4 ± 0.5	36.4 ± 0.9	63.2 ± 1.3
		b	171 ± 10	0.3 ± 0.1	36.3 ± 2.1	63.4 ± 2.0
		c	179 ± 38	0.3 ± 0.4	36.0 ± 1.8	63.8 ± 1.7

Precision of the alkaline-methanolysis procedure

The reproducibility of the method was investigated by repeatedly assaying the same bile sample, incubation mixture (containing rat liver homogenate or microsomal fraction) or human serum (Table 6). For fractions below 10%, the values for the coefficient of variation were 4–15%. Higher fractions in the ranges of 10–30% and 30–80% yielded coefficients of variation below 5 and 1% respectively.

Composition of bilinoids in rat bile

Similar to reported results in Wistar rats (Noir, 1976; Fevery *et al.*, 1977*a*), bilinoids in bile samples ($n = 12$) of our strain of Sprague–Dawley rats were almost exclusively (99.3–100%) in conjugated form, with 29 ± 1.7% (mean ± S.D.) present as monoconjugates and 71 ± 1.9% as diconjugates (Table 7). A significant increase in the ratio of mono- to diconjugates (49 ± 4.1% mono- and 49 ± 3.6% diesters) and a slightly higher proportion of unconjugated bilirubin (0.8–2.4%) were found in bile samples ($n = 6$) from normal rats loaded with bilirubin. These results are in agreement with those of Billing (1965) and Noir (1976), but at variance with the findings of Jansen *et al.* (1977), who reported a significant decrease in the proportion of bilirubin

monoglucuronide in bile of Wistar rats infused with comparable amounts of bilirubin. In agreement with a previous report (Van Steenbergen & Fevery, 1978), heterozygous Gunn rats, which are partially deficient in hepatic bilirubin UDP-glucuronyltransferase, were found to excrete a significantly decreased proportion of diconjugates ($63 \pm 1.5\%$; $n = 9$) in bile.

Analysis of ethyl anthranilate azo derivatives indicated that glucuronides were the major ester conjugates of bilirubin in all bile samples analysed, since 95–98% of the conjugated ethyl anthranilate azo derivatives were found to be glucuronides; the proportion of mono- to di-ester conjugates therefore closely reflects the relative amounts of mono- and di-glucuronide in rat bile.

The ratio of C-8 isomer to C-12 isomer of bilirubin-IXα monoesters, determined ten times by individual quantification of the two monomethyl ester isomers, ranged from 1.3 to 2.2 in normal rat bile ($n = 6$) and from 1.7 to 2.0 in 'enriched' bile ($n = 6$). A similar predominance of C-8 isomer over C-12 isomer has been found in monoglucuronide synthesized *in vitro* by rat liver preparations (Jansen, 1974; Blanckaert *et al.*, 1979), but had not been documented for bile (Jansen & Billing, 1971).

Discussion

The alkaline methanolysis method described in the present paper is useful for determining the relative amounts of bilirubin and of its mono- and di-ester conjugates in biological samples. The assay is precise and accurate and is simple to perform. Conversion of the polar bilirubin conjugates to methyl ester derivatives before extraction and t.l.c. has many advantages. The pigments are readily extractable into chloroform and the extract can be rapidly concentrated and applied to a t.l.c. plate in a short period of time. This expedites the procedure and minimizes breakdown of the unstable tetrapyrroles. The pigment pattern obtained by chromatographic analysis is relatively simple, well-characterized reference pigments are available and, in contrast with bilirubin and bilirubin glucuronides, the pigments can be eluted from the silica gel with a single solvent. Moreover, the method allows individual determination of the two isomeric bilirubin-IXα monoester conjugates. Another advantage of the method is that haemin, which is a common contaminant in biological samples, is immobile in the chromatographic system used, and therefore does not interfere. In addition, use of an internal standard and high-pressure liquid chromatography for analysis of the pigments has allowed us to refine the procedure to a very sensitive and precise method for direct determination of the concentration of the various pigment fractions in serum (N. Blanckaert, F. A. Farina & P. M. Kabra, unpublished work).

A slight disadvantage of the alkaline-methanolysis procedure is that complete elucidation of the composition of the parent bilirubin sugar conjugates cannot be achieved, because no information can be obtained on the nature of the conjugating groups. However, the relative abaundance of the various carbohydrate moieties, which is fairly constant in a given species, and of the positional isomers with respect to attachment of the acyl group can easily be determined by the ethyl anthranilate diazo method (Heirwegh *et al.*, 1974; Compernolle *et al.*, 1978; Blanckaert *et al.*, 1978).

The present results, obtained with the alkaline-methanolysis method, on the composition of bilinoids in rat bile confirm conclusions reached by most other investigators who used more indirect and/or less validated analytical methods (Billing, 1965; Noir, 1976; Jansen *et al.*, 1977; Fevery *et al.*, 1977*a*). When compared with normal Sprague–Dawley rats, a significantly higher proportion of monoglucuronides was excreted in bile of animals with an increased bilirubin load relative to the conjugating enzyme activity, as in Sprague–Dawley rats that had been infused with large amounts of bilirubin or in heterozygous Gunn rats, which have a deficient hepatic bilirubin UDP-glucuronyltransferase. A similar increase in the proportion of monoconjugates in bile has been reported in humans with decreased hepatic bilirubin UDP-glucuronyltransferase activity, as in newborns, patients with Gilbert syndrome or Crigler–Najjar disease (Blanckaert *et al.*, 1976*b*; Fevery *et al.*, 1977*b*; Goresky *et al.*, 1978). It has been reported that in normal rat liver the capacity of the microsomal UDP-glucuronyltransferase system that converts bilirubin monoglucuronide into diglucuronide is considerably lower than that for monoglucuronide synthesis (Blanckaert *et al.*, 1979). This observation therefore offers a plausible explanation for the observed increase in the proportion of biliary monoconjugates in conditions associated with an increased load of monoglucuronide that exceeds the capacity of the microsomal fraction to convert monoconjugates into diconjugates.

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