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Isolation and Properties of Conjugated Bilirubin from Bile

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1. A simple, rapid solvent partition method is described for isolation of conjugated bilirubin, free of unconjugated bilirubin, bile salts, phospholipids and cholesterol, from rat bile. Yields are 40–58%. The product is a phosphate-buffered solution containing approx. 0.4mg of bilirubin/ml, principally as mono- and di-glucuronide conjugates. The method may be modified for isolation of conjugates from human bile with 15–22% yield, and for preparation of unconjugated bilirubin from rat or human bile with yields of 55–62%. 2. The conjugated pigment has red–brown fluorescence and an absorption maximum at 450nm with ϵ_{max} 59.8cm^{-1}. Diazotization by the Malloy–Evelyn method gives a direct Van den Bergh reaction (in water) 12% greater than the total reaction (in methanol), with ϵ_{total} 28.4 × 10^3 $l\,mol^{-1}\,cm^{-1}$ at 550nm. After desalting by elution from Sephadex LH-20 in 50%(v/v) ethanol, the product gave water-soluble mustard-yellow crystalline needles. Such desalted conjugates were precipitated by Pb^{2+} but not by Ba^{2+}, Ca^{2+} or Zn^{2+}. 3. At pH7.0 and 37°C the conjugated bilirubin was oxidized at a rate of 1%/h without hydrolysis, whereas 84% was hydrolysed by β-glucuronidase or aqueous alkali. 4. Mono- and di-glucuronides were separated by elution from Sephadex LH-20 in 95% (v/v) ethanol or by extraction with chloroform at pH3.2–3.4. The monoconjugated bilirubin did not become labelled during incubation with unconjugated [^{14}C]bilirubin, and chromatographed as a single spot without dissociating into unconjugated bilirubin and diglucuronide as would be expected of a complex. 5. After intravenous injection of mono- or di-conjugated [^{14}C]bilirubin into normal or Gunn rats, 79–91% was excreted in bile and 2–7% in urine over 2h. In these experiments injected diglucuronide was not hydrolysed whereas 30–41% of injected monoglucuronide was converted into diglucuronide by the normal but not by the Gunn rats. The evidence favours the existence of a true bilirubin monoglucuronide that is not a complex.

The need for a pure preparation of conjugated bilirubin has been stressed in several reviews (Lester & Troxler, 1969; Brodersen & Jacobsen, 1969). Existing methods for the isolation of conjugated bilirubin are often lengthy, low in yield, inappropriate for preparative work, and give products of unspecified purity (Polonovski & Bourrillon, 1952; Najjar & Childs, 1953; Cole, Lathe & Billing, 1954; Sakamoto, Yamamoto, Yahata & Kondo, 1957; Sato & Saitoh, 1965; Brodersen, 1966; Kuenzle, Sommerhalder, Rüttner & Maier, 1966; Fog & Bakken, 1967). Even the two best preparations contain impurities (Lucassen, 1961; Talafant & Appelt, 1968) and may not be free of the other major organic components of bile. The preparation of radioactively labelled conjugated bilirubin has not been reported.

In the present paper a rapid simple procedure is described for isolation of labelled conjugated bilirubin from rat bile with yields averaging 50%. The procedure consists of two major parts. (1) Extraction of the lipid components (unconjugated bilirubin, phospholipid and cholesterol) from bile at pH6.0 by a modification of the solvent partition method of Folch, Lees & Sloane-Stanley (1957), associated with precipitation of proteins at the interphase. (2) Preferential extraction of bilirubin into ethyl acetate at pH3.2–3.4, leaving taurine-conjugated bile salts in the aqueous phase. The product is a solution in 50mM-sodium phosphate buffer, pH6.5, of bilirubin diglucuronide and monoglucuronide. Human bile may be treated by a modified procedure that gives predominantly

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bilirubin diglucuronide. Methods are also described for preparation and crystallization of unconjugated bilirubin from rat or human bile, and for separation of bilirubin monoglucuronide and diglucuronide. The properties of the purified pigments and studies of their interconversion *in vivo* are also reported.

MATERIALS AND METHODS

Preparation of conjugated bilirubin from rat bile

Chemicals. Unconjugated [^{14}C]bilirubin (43.6×10^3 d.p.m./μg) was prepared biosynthetically in the rat from precursor δ-amino[^{14}C]laevulic acid (Tracerlab Inc., Waltham, Mass., U.S.A.) and isolated and crystallized from the bile by the method of Ostrow, Hammaker & Schmid (1961). Unlabelled, unconjugated bilirubin was purchased from the Pfahnstiehl Co., Waukegan, Ill., U.S.A. Sodium [24-^{14}C]taurocholate was purchased from Tracerlab, and when examined by t.l.c. (Kottke, Wollenweber & Owen, 1966) was pure except for 0.2% of the label in unconjugated cholic acid. All other chemicals were purchased from J. T. Baker Co., Phillipsburg, N.J., U.S.A., and were reagent grade except for spectral-quality chloroform, methanol and ethyl acetate.

Animal procedures. Male Sprague–Dawley rats (300–400g body wt.) were anaesthetized with intraperitoneal pentobarbital and provided with external bile fistulae. The rats were infused via a femoral venous catheter with 2.5% (w/v) glucose–0.45% NaCl solution at 1.5–2.0ml/h. To deplete the bile of bile salts, 5% (w/w) cholestyramine (Cuemid; Merck and Co., Rahway, N.J., U.S.A.) was included in the diet for 3 days before surgery. Alternatively, bile drainage with the enterohepatic circulation interrupted was extended over 24h. For each rat 4–5mg of ^{14}C-labelled or unlabelled unconjugated bilirubin was dissolved in 1.0ml of 0.1M-NaOH and diluted to 5.0ml with 4% (w/v) human serum albumin (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) in 50mM-sodium phosphate buffer, pH7.0. The solution, adjusted to pH7.4 and stored on ice in the dark, was administered intravenously in five 1.0ml doses at 90min intervals. Bile (10ml) was collected on ice and in the dark during the 8–10h period after the first injection. A portion was analysed for bilirubin concentration by a micro-modification of the method of Malloy & Evelyn (1937) and radioassay was performed as described below. After addition of a pinch of solid ascorbic acid, the remainder was stored at −15°C.

Isolation of the conjugated bilirubin from rat bile. During development of the procedure, E_{450} values were used to measure bilirubin concentration and [^{14}C]taurocholate was used as a marker for bile salts since rats do not excrete glycine-conjugated bile salts (Haslewood, 1955). Once the definitive procedure had been adopted, yields of bilirubin were determined both from bilirubin radioactivity and by the Malloy & Evelyn (1937) diazo method. For radioassay, 10ml of dioxan-based scintillator (Bray, 1960) was added to: (*a*) 0.1ml of bile blended with 0.2ml of M-Hyamine in methanol (Rohm and Haas Co., Philadelphia, Pa., U.S.A.); (*b*) 0.2–0.5ml of aqueous phases or solutions; (*c*) 0.5ml of organic phases evaporated to dryness.

The isolation was performed in dim light by using 125ml separation funnels adapted for centrifuging in special trunions (International Equipment Co., Needham Heights, Mass., U.S.A.). Emulsions were thus resolved by centrifugation at every stage. Bile, fresh or thawed, was centrifuged for 15min at 4°C and 20000$g_{av.}$ and the supernatant was adjusted to pH7.0 with M-HCl. (1) (Folch extraction): neutralized bile (10ml) was added to 60ml of methanol–chloroform (1:2, v/v) and shaken. Then 5ml of 0.1M-sodium phosphate buffer, pH5.8, was added and the mixture was thoroughly shaken. The lower phase and the 'button' at the interphase were discarded. (2) The upper phase, containing conjugated bilirubin and bile salts, was extracted again with an equal volume of fresh pre-equilibrated lower phase, prepared as above with 0.85% NaCl in place of bile, and the lower phase was again discarded. (3) Methanol remaining in the upper phase from step (2) was extracted into an equal volume of chloroform, which was discarded. (4) The upper phase from step (3) was extracted with an equal volume of light petroleum (b.p. 30–60°C), which was also discarded. (5) The lower (aqueous) phase from step (4) was adjusted to pH3.2–3.4 with M-HCl and shaken with 3vol. of ethyl acetate. The aqueous phase, containing most of the bile salts, was discarded. (6) The upper organic phase from step (5) was shaken with 0.1vol. of 0.1M-sodium phosphate buffer, pH7.4, and the organic phase discarded. (7)–(9) The lower aqueous phase from step (6), which contained almost all remaining conjugated pigment and bile salts, was subjected again to steps (5), (6) and (5). (10) The upper organic phase from step (9) was shaken with 2–3ml of 50mM-sodium phosphate buffer, pH7.4, and discarded. (11) The aqueous phase from step (10) was extracted with 2vol. of chloroform and both phases were kept. (12) The chloroform phase together with all overlying precipitate was extracted with 2ml of 50mM-sodium phosphate buffer, pH5.8, which was then added to the aqueous phase (pH7.4) from step (11). The pooled aqueous phases constituted the final product, which was shown (see below) to be a mixture of bilirubin conjugates in 50mM-sodium phosphate buffer, pH6.5. It was stored under air at −15°C, and is referred to below as 'CBM preparation'.

Isolation of conjugated bilirubin from human bile. Sterile human bile was obtained 5–7 days post-operatively from T-tube drainage of the common duct of patients with normal liver function not receiving drugs excreted as organic anions in the bile. The procedure was the same as for rat bile, except for an extra step in which the aqueous phase from step (4) was adjusted to pH3.5 and glycine-conjugated bile salts, which predominate in human bile (Haslewood, 1955), were extracted twice with 3vol. of chloroform. The aqueous supernatant was then subjected to step (5) without further adjustment of pH. The efficiency of this extraction was monitored by using radiochemically pure [^{14}C]glycodeoxycholate as a tracer (kindly provided by Dr Alan F. Hofmann, Rochester, Minn., U.S.A.). Radioassay was performed as described for rat bile.

Isolation of unconjugated bilirubin from rat and human bile. Rat or human bile was processed as in steps (1)–(4). To the aqueous phase from step (4) a pinch of ascorbic acid and $\frac{1}{7}$ vol. of 4M-NaOH was added. After being stirred in the dark for 30min to hydrolyse bilirubin conjugates, the solution was adjusted to pH6.0 with 12M-HCl and extracted twice with $\frac{1}{4}$ vol. of chloroform to recover the unconjugated bilirubin. The pooled chloroform extracts

were washed once with 10% (w/v) NaCl, thrice with water, and then filtered through chloroform-saturated Whatman no. 40 paper. Unconjugated bilirubin was then crystallized and recrystallized by displacement of the chloroform with methanol over a boiling-water bath (Ostrow *et al.* 1961).

Desalting and crystallization of conjugated bilirubin

Desalting by column chromatography. Columns (1 cm × 13 cm) of gels were prepared in various proportions of methanol, ethanol or propan-2-ol with water. Bio-Rad AG-11A8 (California Biochemicals Corp., Los Angeles, Calif., U.S.A.), Amberlite XAD-2 (Rohm and Haas Co.) and Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) were used as stationary phases, and were packed in columns and eluted with the same solvent. CBM preparation (0.5 ml) was layered on to the gel and eluted in the dark at 20°C. Fractions (1 ml) were collected on ice, the pigment content in each being determined by the diazo method of Malloy & Evelyn (1937); phosphate was detected by precipitation with 10% (w/v) $BaCl_2$. Fractions containing pigment but no phosphate were pooled and vacuum-distilled under N_2 in actinic glass flasks at 30°C.

Desalting by acid-washing. The ethyl acetate phase from step (9) was washed twice with 0.5 vol. of mM-HCl and then vacuum-distilled to a small volume as described above.

Crystallization. This was attempted by gradual addition of acetone to the column eluate or the addition of chlorobenzene to the acid-washed ethyl acetate phase, followed by vacuum-distillation.

Properties and purity of the CBM preparations

Chemical determinations. Bilirubin concentrations were determined by the Malloy & Evelyn (1937) method, with water as diluent for the direct Van den Bergh reaction and methanol for the total reaction. Both were read at 15 min when colour development was completed and stable. Unconjugated bilirubin was also determined as chloroform-extractable pigment at pH 6.5 (Brodersen & Vind, 1963). Cholesterol, bile salt and phospholipid assays were kindly performed by Dr Dewey Neiderheiser, Cleveland, Ohio, U.S.A. (Neiderheiser & Roth, 1968).

Chromatography of pigments. Concentrates of labelled pigments were subjected to ascending paper chromatography as described by Noir, Garay & Royer (1965) but with S & S 598 paper (Carl Schleicher and Schuell, Keene, N.H., U.S.A.) and development for only 8 h. Segments (0.5–1.0 cm) of the dried paper were eluted in a counting vial with 1.0 ml of 50% (v/v) ethanol and their radioactivities counted in 10 ml of Bray's scintillator.

Chromatography of azo-pigments. Ethyl anthranilate azo-pigments were prepared at pH 2.7 and extracted into pentan-2-one–butyl acetate (17:3, v/v) (Van Roy & Heirwegh, 1968). The extract was subjected to ascending t.l.c. on precoated silica gel plates (E. Merck A.-G., Darmstadt, Germany) for 45 min in chloroform–methanol–acetic acid (85:15:3, by vol.). The plates were dried and spots were eluted for 30 min in 1.0 ml of 60 mM-HCl in methanol and silica gel was removed by centrifugation. The E_{535} value of the supernatant was determined and 0.5 ml was added to 1.0 ml of methanol plus 15 ml of toluene-based scintillator (Ostrow *et al.* 1961) for counting of radioactivity. The azo-pigment extract was similarly assayed after appropriate dilution.

Absorption spectra. These were determined from 300–700 nm in spectral-quality solvents with a recording spectrophotometer (Beckman model DB).

Molar extinction coefficients of pigments and azo-pigments. These could not be measured directly since the conjugated pigments were never obtained in pure crystalline form. Values were instead determined on solutions of ^{14}C-labelled conjugates whose exact content of bilirubin was calculated from total radioactivity, corrected for labelled impurities revealed by pigment chromatography, multiplied by the specific radioactivity of the conjugated bilirubin. This last was equal to the specific radioactivity of the crystalline unconjugated [^{14}C]bilirubin obtained by alkaline hydrolysis of the conjugate, with pure crystalline unconjugated bilirubin as reference standard (Ostrow *et al.* 1961).

Stability. The stability of CBM preparations was assessed during storage at −15°C and during incubation at 37°C, both in air and in the dark. Oxidation and hydrolysis were determined from changes in the direct and total diazo reaction (Ostrow, 1967), changes in absorption spectra and changes in the patterns of pigment and azo-pigment chromatograms (see above).

Separation of bilirubin monoglucuronide and diglucuronide

Column chromatography. Columns of Sephadex G-10 or LH-20 (Pharmacia) or Bio-Gel P-2 (Calbiochem) were prepared, eluted, and analysed as described under 'Desalting by column chromatography'. Solutions applied to the columns included 0.5 ml of neutralized bile, the aqueous phase from the second Folch extraction [step (2)] or the purified conjugates in phosphate buffer. With the first two solutions, [^{14}C]taurocholate was added as a bile-salt marker and the radioactivities of the eluted fractions were counted in Bray's scintillator (see above).

Solvent partition. CBM preparation adjusted to pH 3.3 with M-HCl was extracted four times with an equal volume of chloroform. At each extraction the chloroform phase was transferred to a tube containing 1 vol. of 50 mM-sodium phosphate buffer, pH 6.5, and the interphase precipitate was retained with the aqueous phase for re-extraction. The final aqueous phase and precipitate were adjusted to pH 6.5, shaken with an equal volume of chloroform and centrifuged, and the chloroform was discarded. The yellow supernatant contained predominantly bilirubin diglucuronide and is referred to below as 'fraction CB-II'. Pigments from the pooled chloroform extracts, which consisted mainly of bilirubin monoglucuronide, were recovered by two washes with small volumes of 50 mM-sodium phosphate buffer, pH 6.5, and are referred to below as 'fraction CB-I'.

Nature of the conjugated bilirubins

Glucuronic acid. This was determined in solutions and detected in chromatographic spots by the methods of Fishman & Green (1955) and Noir *et al.* (1965) respectively.

Hydrolysis of conjugated bilirubins with β-glucuronidase. To approx. 0.5 mg of ^{14}C-labelled CBM preparation in

4.0ml of 75mM-sodium phosphate buffer, pH6.5, was added 4.0ml of 1% bacterial β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.). At 10min intervals, 1.0ml portions were diazotized successively with ethyl anthranilate at pH5.5 and then at pH2.7. The ethyl anthranilate azo-pigments were extracted, chromatographed and assayed as described above.

Alkaline hydrolysis of conjugated azo-pigments. Ethyl anthranilate azo-pigment extracts were diluted with 9 vol. of M-NaOH in 40% (v/v) methanol containing ascorbic acid [1%] as antioxidant. After 30min in the dark, M-HCl was added to bring the pH to 6.0 and the hydrolysed azo-pigments were extracted completely into pentan-2-one–butyl acetate (17:3, v/v). This azo-pigment extract was washed once with 50mM-sodium phosphate buffer, pH6.5, and chromatographed and analysed as above. The combined buffer wash and initial aqueous phase was extracted twice with chloroform to remove methanol and its content of glucuronic acid, which had been released by azo-pigment hydrolysis, was determined as above.

Exchange of unconjugated [^{14}C]bilirubin with bilirubin monoglucuronide. Unconjugated [^{14}C]bilirubin in a small volume of 0.1M-NaOH was added to equimolar quantities of bilirubin conjugates (one-third monoglucuronide, two-thirds diglucuronide), either in rat bile or as a purified bilirubin-conjugate preparation. After incubation in the dark for 2h at 37°C, one portion was subjected to the Folch solvent partition procedure and the aqueous phase was then diazotized with ethyl anthranilate. The azo-pigments were extracted, chromatographed and assayed as described above. Pigments from a second portion were extracted, concentrated and analysed by radiochromatography as described above. As a control, untreated bile or a solution of bilirubin-conjugate preparation was processed similarly.

Excretion and interconversion of bilirubin conjugates in the rat. Solutions of ^{14}C-labelled fractions CB-I and CB-II were injected intravenously into 300–400g male Wistar or jaundiced Gunn rats with external bile fistulae. Each rat received both fractions of bilirubin conjugate preparation successively with an interval of 4h and with the sequence randomized among the animals studied. For 4h after each injection all bile and urine were collected on ice in the dark, with bile of successive 30, 90 and 120min intervals saved as separate fractions. The radioactivity of one-tenth of each bile fraction warmed with 0.5ml of M-Hyamine, or 0.5ml of urine, was counted after addition of 10ml of Bray's scintillator. The remainder of the first two bile fractions were pooled, and both pigment and ethyl anthranilate azo-pigment extracts were prepared, chromatographed and assayed as described above. Portions of the injected solutions were treated similarly and their total bilirubin concentrations determined by the method of Malloy & Evelyn (1937). Proportions and quantities of bilirubin monoglucuronide and diglucuronide in the injected and excreted pigments were thus determined and compared, allowing their excretion and interconversion to be calculated.

Radioassay techniques. Radioactivities of labelled pigments and azo-pigments were counted in a Nuclear-Chicago model 720-B liquid-scintillation spectrometer. The radioactivity of each sample was counted for 40min or to 10000 counts, and c.p.m. converted into d.p.m. by means of efficiencies determined with added [^{14}C]toluene as internal standard (New England Nuclear Corp., Boston, Mass., U.S.A.). Efficiencies ranged from 42 to 60%.

Statistical methods (Edwards, 1954). Mean values are given with standard errors, calculated with the Yates' correction for small populations. Significance of differences between means was calculated by Student's *t* test. Regression lines in Fig. 9 were calculated by the method of least squares.

RESULTS

Isolation of bilirubin-conjugate preparation from bile

Conditions for Folch extractions [steps (1) and (2)]. Completeness of the extraction of unconjugated [^{14}C]bilirubin from bile into the lower phase increased as pH was decreased (Fig. 1). Less than 6% of the unconjugated bilirubin remained in the upper phase if the buffer pH was 5.8–6.0, and less than 3% if, in addition, the pH of the bile was first adjusted to 7.0. A fivefold increase in the proportion of bile to chloroform–methanol had little effect on the extraction of unconjugated bilirubin but progressively diminished the retention of conjugated bilirubin in the upper phase (Fig. 2), the remainder being lost in the proteinaceous 'button' at the interphase. A ratio of 10ml of bile to 60ml of chloroform–methanol was chosen as the optimum compromise between maximum yields of conjugated bilirubin

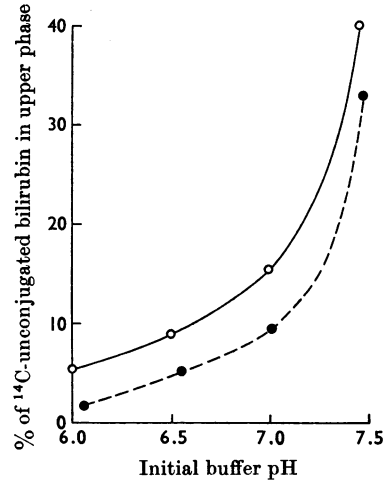

Fig. 1. Effect of buffer pH on the efficiency of the Folch solvent partition procedure in the extraction of unconjugated bilirubin from rat bile (○) and human bile (●). Tracer unconjugated [^{14}C]bilirubin was dissolved in the bile sample, which was then shaken with 20 vol. of methanol–chloroform (1:2, v/v) and 4 vol. of 0.1M-sodium phosphate buffer of the specified pH. After centrifugation, the percentage of unconjugated bilirubin that remained in the upper phase was determined by radioassay.

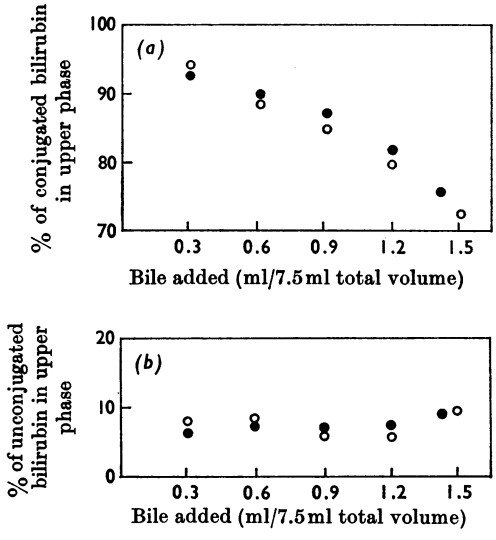

Fig. 2. Effect of the proportion of rat bile (○) and human bile (●) on the efficiency of the Folch solvent partition procedure in separating conjugated and unconjugated bilirubin of bile. Bile (0.3–1.5ml) that contained added tracer unconjugated [^{14}C]bilirubin was shaken with 6.0ml of methanol–chloroform (1:2, v/v), and sufficient 0.1M-sodium phosphate buffer, pH5.8, was added to bring the total volume to 7.5ml. The mixture was shaken and centrifuged and the upper phase was analysed for remaining unconjugated bilirubin by radioassay and for conjugated bilirubin by measurement of E_{450}, corrected for the concentration of unconjugated bilirubin.

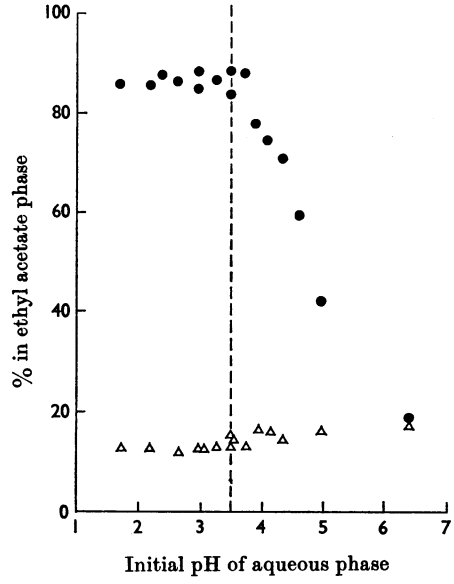

Fig. 3. Effect of pH on the partition of conjugated bilirubin (●) and bile salts (△) into ethyl acetate. One volume of the chloroform-washed aqueous phase from the Folch solvent partition of rat bile was adjusted to the specified pH, shaken with 4 vol. of ethyl acetate and centrifuged. Extraction of conjugated bilirubin was assessed from measurement of E_{450}, and of bile salts from the partition of a tracer [^{14}C]taurocholate marker added before to the Folch aqueous phase. The quantity of pigment or ^{14}C radioactivity in the original Folch extract was taken as 100%.

and minimum ratios of solvent to bile. With the second Folch partition [step (2)] all remaining unconjugated bilirubin but only 2% of remaining conjugated bilirubin entered the lower phase, and no 'button' was formed.

Conditions for ethyl acetate extractions. Extraction of Folch upper phases with 4vol. of ethyl acetate removed 83–87% of the conjugated bilirubin at pH values below 3.6, but the percentage decreased sharply at higher pH values (Fig. 3). Since oxidation to biliverdin was rapid at pH values below 3.0, and only 14–17% of the taurine-conjugated bile salts passed into ethyl acetate over the entire pH range studied (Fig. 3), pH3.2–3.4 appeared to be optimum. Maximal recovery of conjugated bilirubin then occurred with ratios of ethyl acetate to Folch upper phase exceeding 3:1 (v/v), whereas bile salt partition was constant at ratios exceeding 1:1 (v/v) (Fig. 4). After three extraction cycles [steps (5–9)] using 3vol. of ethyl acetate at pH3.2–3.4, over 60% of the bilirubin but less than 0.2% of the bile salts in the Folch upper phase appeared in the final ethyl acetate extract.

Back-extraction from ethyl acetate into neutral

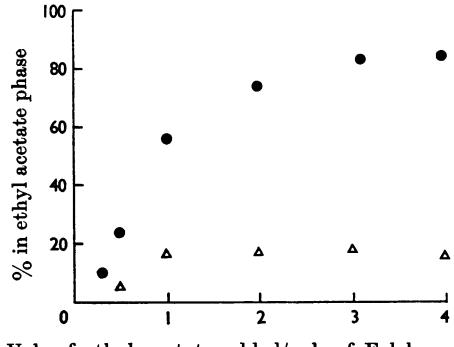

Fig. 4. Effect of the proportion of ethyl acetate on extraction of bilirubin (●) and bile salts (△) from the Folch aqueous phase. The experiment was performed as described for Fig. 3, but the Folch extract was uniformly adjusted to pH3.3 and only the relative volume of ethyl acetate was varied.

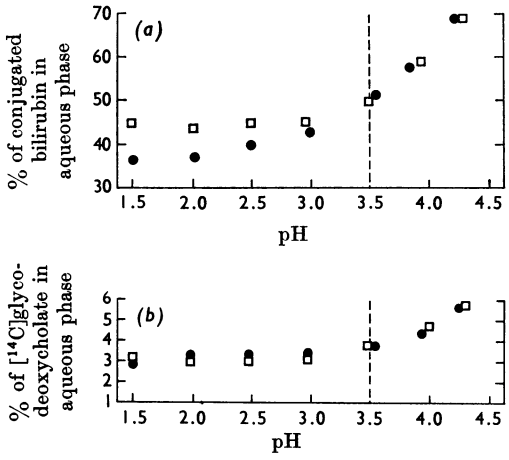

Fig. 5. Effect of pH on chloroform extraction of conjugated bilirubin (*a*) and glycine-conjugated bile salts (*b*) from the Folch aqueous phase extracts of two different samples of human bile (● and □). The experiment was performed as described for Fig. 3, but 3 vol. of chloroform was shaken with Folch aqueous phase from human bile, and [^{14}C]glycodeoxycholate was used as bile salt marker.

phosphate buffer. Over 98% of the conjugated bilirubin and bile salts passed into the buffer at pH values above 7.0 when at least 1 vol. of buffer was used/12 vol. of ethyl acetate. With 1 vol. of pH 7.4 buffer/10 vol. of ethyl acetate, as in the final procedure, each extraction cycle [i.e. steps (5) and (6)] decreased the volume of aqueous phase by approximately half.

Extraction of glycine-conjugated bile salts from human bile. At pH values near 3.5, 50–57% of the conjugated bilirubin but only 3–4% of the [^{14}C]-glycodeoxycholic acid remained in the aqueous phase (Fig. 5). A second chloroform wash removed the remaining bile salts with loss of less than 10% of the remaining bilirubin. Bilirubin oxidation was rapid at pH values below 3.0. Optimum bile salt extraction occurred with ratios of chloroform to Folch upper phase exceeding 2.5:1.

Yields of conjugated bilirubin. These, assessed from both diazo-reactivity and radioassay, ranged from 42 to 60% of the total [^{14}C]bilirubin in the initial rat bile. A 10 ml portion of bile from one rat gave a final CBM preparation containing 2.0–2.5 mg of bilirubin in 5.0 ml. With human bile yields were lowered to 15–22% by losses of pigment during extraction of the glycine-conjugated bile salts, but concentrations of bilirubin in human CBM preparations ranged from 0.6 to 1.0 mg/ml according to pigment concentration in the initial bile.

Preparation of unconjugated bilirubin from bile. With either rat or human bile, 55–61% of the initial bilirubin was recovered as crystalline unconjugated bilirubin, identical in all respects with the material isolated from the same samples by the method of Ostrow *et al.* (1961). The crystalline material migrated as a single spot on pigment chromatography and gave ϵ_{mM} 59.8 cm^{-1} at 450 nm in chloroform. Diazotization with ethyl anthranilate produced a chromatographically homogeneous unconjugated azo-pigment. The Malloy–Evelyn azo-pigment had ϵ_{mM} 24.8 cm^{-1} at 550 nm in chloroform–methanol (1:9, v/v).

Desalting and crystallization of conjugated bilirubin

Desalting on columns. Desalting on columns of Bio-Rad AG-11A8 was precluded by strong binding of pigment to the gel. However, elution with 50% (v/v) ethanol from Sephadex LH-20 or Amberlite XAD-2 separated pigment and phosphate into well-defined but slightly overlapping bands, with the pigment peak in the ninth or tenth ml of effluent and the phosphate peak 3–5 ml earlier. Recovery of applied pigment exceeded 90%, of which 10% was discarded because of overlap of the phosphate band. These desalted conjugated bilirubins were precipitated by Pb^{2+} but not by Ca^{2+}, Ba^{2+} or Zn^{2+} ions.

Desalting by acid-washing. This was complete but 10–14% of the pigment was lost with each wash.

Crystallization. Crystallization was not achieved by addition of up to 10 vol. of acetone to the desalted column eluates, but fine mustard-coloured needles of conjugated bilirubin were obtained by addition of chlorobenzene to the acid-washed ethyl acetate extract from human bile. The yield, however, was less than 10% and the crystals progressively turned green during storage in a vacuum desiccator in the dark. The crystals were completely soluble in water, methanol or ethanol, partially soluble in ethyl acetate and totally insoluble in light petroleum, benzene, or chloroform. Similar treatment of ethyl acetate extracts of rat bile gave only non-crystalline precipitates.

Properties and purity of the conjugates

Chemical analysis. Chemical analysis of six CBM and two crystalline preparations revealed no detectable bile salts, cholesterol or phospholipid, and no chloroform-extractable bilirubin at pH 6.5.

Chromatography of pigments. Freshly prepared ^{14}C-labelled CBM preparations gave only two labelled, mustard-coloured, red-fluorescing spots of R_F 0.65–0.75 and 0.45–0.59. Both yielded glucuronic acid when hydrolysed on paper and rechromatographed in the second dimension. Diazotization of the less mobile spot yielded only conjugated azo-pigments, whereas the faster gave approxi-

Table 1. *Distribution of radioactivity in thin-layer chromatograms of ethyl anthranilate azo-pigments derived from preparations of conjugated* [^{14}C]*bilirubin*

Ethyl anthranilate azo-pigments were prepared at pH2.7 and extracted into a minimal volume of pentan-2-one–butyl acetate (17:3, v/v), as described by Van Roy & Heirwegh (1968). The extract was spotted on a thin-layer plate (0.3mm thick) of silica gel without binder. This was developed by the ascending technique for 45min in chloroform–methanol–acetic acid (85:15:3, by vol.) after the closed tank had been pre-equilibrated with solvent system for 1h. Spots, identified as described by Heirwegh, Van Hees, Leroy, Van Roy & Jansen (1970), were eluted from the dried plate with 60mM-HCl in 50% (v/v) methanol and assayed for radioactivity as described in the text.

Spot	Mean R_F	Colour	Identity	^{14}C radioactivity (% of total) Rat CBM preparation	Human CBM preparation
F	0.95	Yellow–green	Undiazotized pigments	1–3	1–3
α	0.77	Red–purple	Unconjugated azobilirubin	13–30	1–4
β	0.67	Red–brown	Unidentified azo-pigments	2–5	6–10
γ	0.26	Red–purple	Unidentified azo-pigments	4–9	9–15
δ	0.10	Red–purple	Azo-bilirubin glucuronide	55–68	69–76
O	0.00	Brown	Origin material	2–4	2–4

mately equal quantities of unconjugated and conjugated azo-pigments, suggesting that the leading spot was monoglucuronide and the trailing spot diglucuronide. CBM preparations isolated from rat bile after loading with [^{14}C]bilirubin contained 23–66% of the radioactivity in the monoglucuronide whereas the monoglucuronide never exceeded 8% in preparations from human bile. None of the chromatograms revealed a spot of unconjugated bilirubin at the origin. However, owing to losses during preparation of the pigment concentrates, only 72–78% of the radioactivity in the CBM preparations was recovered from the chromatograms.

Chromatography of ethyl anthranilate azo-pigments. This yielded four azo-pigment spots with the mean R_F values given in Table 1. The dominant spot from both human and rat bile CBM preparations was δ-azo-pigment, which is predominantly azobilirubin glucuronide (Compernolle, Jansen & Heirwegh, 1970). Rat CBM preparations yielded large quantities of α-azo-pigment (unconjugated), whereas β- and γ-azo-pigments were more prominent in human CBM preparations. Both rat and human CBM preparations also showed up to 4% of the pigment in a brown spot at the origin (termed O) and up to 3% in a yellow–green spot at the solvent front (termed F). In contrast with plasma, where unconjugated bilirubin is unreactive at pH2.7 (Van Roy & Heirwegh, 1968), up to 20% of unconjugated bilirubin added to bile or CBM preparations was diazotized at that pH. Thus in CBM preparations the α-azo-pigment could be assumed to be derived exclusively from monoconjugated bilirubin only if they were demonstrated by chloroform extraction to be free of unconjugated bilirubin. Over 90% of the radioactivity of ^{14}C-labelled CBM preparations was recovered in the extracted azo-pigments, and 97–104% of this appeared in eluates from the chromatograms. Similarly over 95% of the E_{535} units of the azo-pigment extract was recovered from the eluates, demonstrating excellent stability of ethyl anthranilate azo-pigments during chromatography.

Absorption spectra. All CBM or crystalline conjugated bilirubin preparations showed absorption maxima at 450 and 420nm. The former peak was more intense in aqueous or methanolic solution and the latter more intense in ethyl acetate (Fig. 6). In 50mM-sodium phosphate buffer, pH6.5, ϵ_{mM} at 450nm was $59.8 \pm 0.8 cm^{-1}$. The maxima and extinction coefficients were independent of the proportions of bilirubin monoglucuronide and diglucuronide in the preparations. Freshly made CBM preparations showed no absorption in the 650nm range, but this gradually appeared as biliverdin conjugates formed during storage.

Malloy–Evelyn diazo reaction. The total diazo reaction (in methanol) showed an absorption maximum at 550nm with ϵ_{mM} 28.0–28.8 in nine preparations. The direct reaction (in water) showed an absorption maximum at 560nm with the extinction averaging

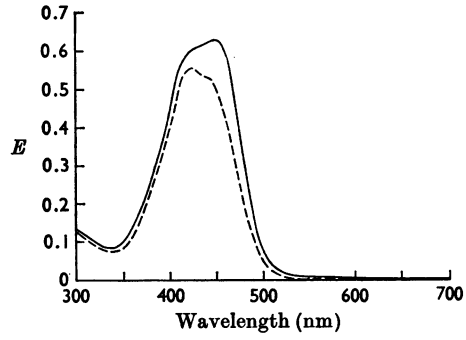

Fig. 6. Absorption spectra of purified mixed conjugates of bilirubin in 50mM-sodium phosphate buffer, pH6.5, after 50-fold dilution with methanol or water (——) or with ethyl acetate (----).

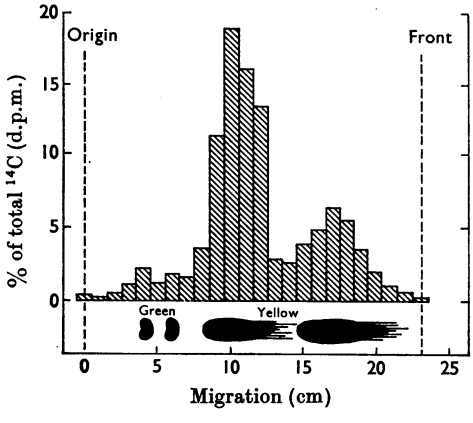

Fig. 7. Ascending paper chromatogram of conjugated [^{14}C]bilirubin preparation (CBM) after storage at −15°C in air for 1 week. The chromatogram was developed for 8h on Schleicher and Schuell 598 paper with butan-1-ol–ethanol–water (6:2:3, by vol.). Visible pigment spots are represented at the bottom, with two bilirubin conjugates on the right and two biliverdin conjugates nearer the origin. The upper panel shows the percentage of total applied radioactivity eluted with 50% (v/v) ethanol from 1.0cm segments of the dried chromatogram.

12±2% higher than that for the total diazo reaction (in methanol) ($P<0.05$). These values were independent of the proportions of bilirubin monoglucuronide and diglucuronide in the preparations, suggesting that both conjugates reacted to the same degree. The direct, but not the total, diazo reaction was suppressed when CBM preparation was added to human or rat serum (Table 2).

Stability of CBM. When stored in the dark under air at −15°C CBM preparations oxidized at a rate of less than 1%/day. When incubated in the dark at 37°C under air at pH7.0 the rate of oxidation was less than 1%/h. Chromatography of the oxidized pigments (Fig. 7) revealed two additional green spots of R_F 0.20–0.33 and 0.30–0.43, corresponding to di- and mono-conjugated biliverdins respectively (Noir *et al.* 1965). Under neither set of conditions was the ratio of direct to total reaction altered, nor was unconjugated bilirubin produced as shown by chromatography or chloroform extraction, indicating that hydrolysis had not occurred.

Separation of bilirubin monoglucuronide and diglucuronide

Column chromatography. This produced two sharply defined bands of pigment only with Sephadex LH-20 eluted with 95% (v/v) ethanol. As judged by diazo-reactivity, over 90% of the applied pigment was recovered intact. Analysis of the leading band showed 83–88% diglucuronide whereas the trailing band was over 90% monoglucuronide. Similar patterns were obtained with whole bile and Folch upper phases from bile (Fig. 8), but CBM preparations from rats usually contained more and those from human beings much less monoglucuronide than the corresponding bile or Folch extract. Unconjugated [^{14}C]bilirubin dissolved in the applied sample adsorbed tightly on the top of the column, with none appearing in the monoglucuronide fraction. Added [^{14}C]taurocholate migrated concurrently with the leading diglucuronide band (Fig. 8).

Solvent partition in chloroform at pH3.3. Some 12–16% of the labelled bilirubin appeared in the chloroform phase (fraction CB-I) and 44–51% in the aqueous phase (fraction CB-II), the rest being lost in the greenish 'button' at the interphase. By chromatography, fractions CB-I and CB-II each contained 8–13% undiazotizable biliverdin conjugates. Fraction CB-I consisted of 60–80% bilirubin monoglucuronide and 7–23% bilirubin diglucuronide, whereas fraction CB-II was 24–33% bilirubin monoglucuronide and 47–63% bilirubin diglucuronide. Neither fraction contained any unconjugated bilirubin. These values were confirmed by analysis of the ethyl anthranilate azo-pigments of fractions CB-I and CB-II (Tables 4 and 5). The calculations were based on the fact that bilirubin diglucuronide gives only δ-azo-pigment whereas the monoglucuronide gives equal quantities of α- and δ-azo-pigments (Heirwegh *et al.* 1970), and on the assumptions that all α-azo-pigment was derived from the monoglucuronide and all remaining δ-azo-pigment was derived from the diglucuronide. The proportions of β- and γ-azo-pigments formed from fractions CB-I and CB-II were similar to those formed from the original CBM preparation.

Table 2. *Millimolar extinction coefficients of azo-bilirubin solutions*

Pure ^{14}C-labelled pigment was dissolved in 0.1 M-NaOH, added to the specified solution and allowed to react with diazotized sulphanilic acid for 15 min with water (direct reaction) or methanol (total reaction) as diluent. E_{560} values for the direct and E_{550} values for the total reaction were then determined, with the diazotized solution containing no added bilirubin being used as a blank. The concentration of added pigment was determined from radioassay of the solution and the known specific radioactivity of the added labelled pigment. Added unconjugated bilirubin gave no direct reaction in any of the solutions.

Solution	CBM preparation of conjugated bilirubin: Direct reaction	CBM preparation of conjugated bilirubin: Total reaction	Unconjugated bilirubin: Total reaction
50 mM-Phosphate buffer, pH 7.4	31.4–32.3 (9)	28.0–28.8 (9)	28.4
Human serum	23.4	28.8	29.9
Rat serum	25.2	29.3	30.3

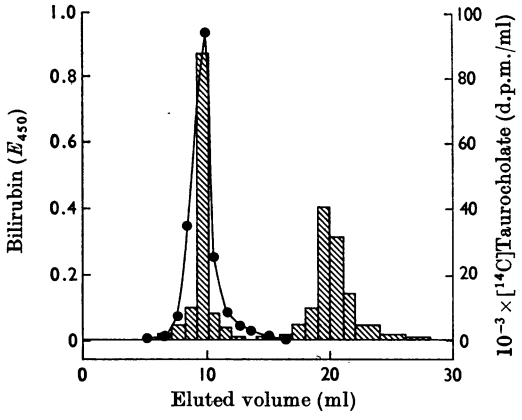

Fig. 8. Chromatography of 0.5 ml of Folch aqueous extract from human bile on a Sephadex LH-20 column (1.0 cm × 13.0 cm) packed and eluted in 95% (v/v) ethanol. ▩, Bilirubin concentration of each 1.0 ml fraction measured by its E_{450}; ●–●, [^{14}C]taurocholate, which had been added as a bile salt marker before application of the Folch extract to the column. The leading peak is mainly bilirubin diglucuronide and the trailing peak is mainly the monoglucuronide. Similar patterns were obtained with whole bile and with purified CBM preparations.

Nature of the bilirubin conjugates

Glucuronic acid/bilirubin ratios. Glucuronic acid/bilirubin ratios for all CBM and fraction CB-I preparations were close to those predicted from the proportions of δ-azo-pigment to total ethyl anthranilate azo-pigments formed from the preparation (Table 3). These findings confirmed the higher proportion of bilirubin monoglucuronide in rat than in human bile preparations, and also the predominance of the monoglucuronide in fraction CB-I. Glucuronic acid/bilirubin ratios for fraction CB-II ranged from 2.78 to 3.53, well above values predicted from azo-pigment assays. However, if the pigments were first diazotized and then extracted into pentan-2-one–butyl acetate, leaving unbound glucuronic acid in the aqueous phase, the glucuronic acid/bilirubin ratio in the azo-pigment extract now concurred with the proportion of δ-azo-pigment in the extract (Table 3). Less than 2% of the original bilirubin colour (E_{450}) remained in the aqueous phase after extraction, indicating that diazotization of the bilirubin conjugates was essentially complete.

Hydrolysis of pigments from ^{14}C-labelled CBM preparations with β-glucuronidase. This was completed by 30 min. On diazotization, it was found that 94% of the ^{14}C radioactivity was lost from the δ-azo-pigment and now appeared in the α-azo-pigment and F spot. The β- and γ-azo-pigments were unaffected.

Hydrolysis of ^{14}C-conjugated azo-pigments with alkali. This treatment eliminated the δ- and γ-azo-pigments. The β-azo-pigments were unaffected and the α-azo-pigment and F spot increased. However, despite addition of ascorbic acid to minimize oxidation, half the diazo-reactivity was lost during hydrolysis.

Rechromatography on paper of [^{14}C]bilirubin monoglucuronide fractions. Rechromatography of [^{14}C]bilirubin monoglucuronide fractions eluted from paper chromatograms or Sephadex LH-20 columns yielded over 90% of the label in the bilirubin monoglucuronide spot and its oxidation product, biliverdin monoglucuronide. No radioactivity was detected at the origin where unconjugated bilirubin normally appears.

Exchange of unconjugated [^{14}C]bilirubin and bilirubin monoglucuronide. Incubation of unconjugated [^{14}C]bilirubin with rat bile or with CBM preparations, followed by chromatography on paper or on Sephadex LH-20 columns, showed that less than 2% of the ^{14}C-radioactivity migrated with

Table 3. *Glucuronic acid/bilirubin molar ratios in purified preparations of conjugated bilirubin*

Values on the left in each column were determined spectrophotometrically by the method of Fishman & Green (1955) for glucuronic acid and the Malloy & Evelyn (1937) diazo method for bilirubin. Values in parentheses are ratios predicted from the proportion of δ-azo-pigment to total azo-pigments formed on diazotization of the preparation with ethyl anthranilate.

Source	CBM preparation (mixed conjugates)	Fraction CB-I (chloroform-extracted, pH 3.3)	Fraction CB-II (water-soluble, pH 3.3)
Rat bile after intravenous bilirubin load	1.61 (1.65)	1.29 (1.20)	3.53 (1.71) 1.77*
	1.55 (1.58)	1.20 (1.27)	3.50 (1.78) 1.73*
	1.40 (1.43)	1.15 (1.20)	2.98
	1.29 (1.34)	1.10 (1.14)	2.78
Human bile, T-tube drainage	2.04 (1.98)		
	1.98 (2.00)		
	1.97 (2.00)		
	1.87 (1.83)		
	1.82 (1.86)		

* These ratios were derived from measurement of the glucuronic acid content of the extracted ethyl anthranilate azo-pigments of the preparation. For details see the text.

Table 4. *Excretion of [^{14}C]bilirubin conjugates by normal and Gunn rats*

Normal and Gunn rats are denoted N and G respectively. The injected dose, given as bilirubin irrespective of conjugated moieties, was determined by the diazo method. The percentages of bilirubin monoglucuronide and diglucuronide refer to the proportion of total injected radioactivity in each conjugate, determined from chromatography of the ethyl anthranilate azo-pigments (see Table 5). Fraction CB-II was obtained as aqueous phase readjusted to pH 7.4 after chloroform extraction of CBM preparations at pH 3.3. Fraction CB-I was the bilirubin extracted into chloroform from CBM preparations at pH 3.3, and subsequently transferred back to 50 mM-sodium phosphate buffer, pH 7.4. Abbreviations: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; N.S., not significant.

		Conjugated bilirubin injected				Cumulative ^{14}C radioactivity excreted (% of injected radioactivity)		
Type of pigment injected	Rat no.	Dose (μg/100 g)	BMG (%)	BDG (%)	BMG/BDG ratio	In 30 min	In 2 h	
						Bile	Bile	Urine
Fraction CB-II	N-1	14.5	33.4	46.6	0.7	70.8	84.6	5.3
	N-2	15.0	33.4	46.6	0.7	75.0	88.8	5.9
	N-3	9.1	30.8	54.3	0.6	73.2	91.4	6.7
	N-4	41.5	24.2	62.9	0.4	77.2	83.8	2.8
	G-1	43.8	24.2	62.9	0.4	68.1	79.1	2.5
	G-2	43.8	24.2	62.9	0.4	79.3	89.7	2.1
				Mean ± S.E.M.	...	73.9 ± 1.8	86.3 ± 2.1	4.2 ± 1.1
Fraction CB-I	N-1	10.9	60.4	22.8	2.7	58.7	83.3	2.4
	N-2	9.6	60.4	22.8	2.7	62.5	86.9	1.7
	N-3	12.7	80.2	6.8	11.8	63.8	90.6	2.7
	N-4	7.8	70.2	10.0	7.0	54.8	84.0	3.8
	G-1	8.1	70.2	10.0	7.0	60.6	80.8	3.4
	G-2	7.9	70.2	10.0	7.0	67.9	81.8	6.3
				Mean ± S.E.M.	...	61.4 ± 2.0	84.6 ± 1.6	3.4 ± 0.6
Significance of difference, fraction CB-II versus fraction CB-I					...	$P < 0.002$	N.S.	N.S.

the bilirubin monoglucuronide fraction which also showed no increase in its proportion of the E_{450}. On subjection of both the incubated mixture and the unincubated control to the Folch solvent portion procedure, 5–7% of the label appeared in the aqueous phase. After diazotization with ethyl

Table 5. *Conversion of* [^{14}C]*bilirubin monoglucuronide into diglucuronide in normal and Gunn rats as determined from analysis of ethyl anthranilate azo-pigments*

Animals and injected solutions were identical with those described in Table 4. The percentage of azo-pigment is the percentage of total ^{14}C radioactivity in the injected solution or in the bile excreted in 2h that was found in α- or δ-azo-pigment on t.l.c. The increment (%) is the percentage of excreted ^{14}C radioactivity in α- (or δ-) azo-pigment minus the percentage of injected ^{14}C radioactivity in that azo-pigment. The mean increment (%) is the average of increments for α- and δ-azo-pigments with the sign reversed for the α-azo-pigment increment. Since there are 2mol of azo-pigment formed/mol of bilirubin, the amount of bilirubin monoglucuronide converted into diglucuronide is twice the mean increment multiplied by the dose of bilirubin injected (μg/100g body wt. from Table 4). For the assumptions involved, see the Discussion section.

		α-Azo-pigment (unconjugated)			δ-Azo-pigment (glucuronide)				
		% from			% from				
Type of pigment injected	Rat no.	Injected solution	Excreted bile	Increment (%)	Injected solution	Excreted bile	Increment (%)	Mean increment (%)	Conversion of BMG into BDG (μg/100g)
Fraction CB-II	N-1	16.7	10.8	−5.9	63.3	67.9	+4.6	5.25	1.52
	N-2	16.7	10.6	−6.1	63.3	70.3	+7.0	6.55	1.96
	N-3	15.4	9.7	−5.7	69.7	76.8	+7.1	6.40	1.16
	N-4	12.1	8.2	−3.9	75.0	80.6	+5.6	4.75	3.94
	G-1	12.1	11.4	−0.7	75.0	75.8	+0.8	0.75	0.66
	G-2	12.1	11.0	−1.1	75.0	74.9	−0.1	0.50	0.44
Fraction CB-I	N-1	30.2	19.8	−10.4	53.0	60.9	+7.9	9.15	2.00
	N-2	30.2	15.9	−14.3	53.0	62.2	+9.2	11.75	2.26
	N-3	40.1	27.5	−12.6	46.9	64.6	+17.7	15.15	3.86
	N-4	35.1	25.5	−9.6	45.1	58.1	+13.0	11.30	1.76
	G-1	35.1	35.4	+0.3	45.1	47.4	+2.3	1.00	0.16
	G-2	35.1	33.4	−1.7	45.1	46.4	+1.3	1.50	0.24
Means±S.E.M.	Normal rats ...		−8.6±1.4				+9.0±1.6		
	Gunn rats ...		−0.8±0.5				+1.0±0.6		
Significance of difference between means for normal versus Gunn rats ...				$P<0.001$			$P<0.002$		

anthranilate at pH2.7, 12% of this radioactivity was found in the α-azo-pigment and 62% in the F spot. The rest was not extracted into pentan-2-one–butyl acetate. No label was detected in β-, γ-, or δ-azo-pigments. Thus the radioactivity in the Folch upper phase was present in unconjugated bilirubin and non-diazotized derivatives rather than monoglucuronide.

Excretion of bilirubin monoglucuronide and diglucuronide by rats (*Table* 4). During a period of 2h after intravenous administration of labelled preparations of fraction CB-I or CB-II, 79–91% of the radioactivity appeared in the bile and 2–7% in the urine irrespective of the proportions of bilirubin monoglucuronide and diglucuronide injected. However, during the first 30min significantly more ^{14}C radioactivity was excreted in the bile when the diglucuronide, rather than the monoglucuronide, was the predominant pigment. With both fractions CB-I and CB-II, another 4–9% of the injected ^{14}C radioactivity appeared in the bile during the third and fourth hour. Excretion rates were the same for Gunn rats as for normal rats.

Interconversion of bilirubin monoglucuronide and diglucuronide in the rat (*Table* 5). The excreted and injected bile pigments exhibited identical distributions of radioactivity in non-diazotizable biliverdin conjugates and in the F, β, γ and O spots of the ethyl anthranilate azo-pigment chromatograms. However, in normal rats there was an increase in the proportion of δ-azo-pigment (glucuronide) and a corresponding decrease in the proportion of α-azo-pigment (unconjugated). By contrast, the proportions of α- and δ-azo-pigments were virtually unchanged by passage through Gunn rats. Duplicate determinations on individual samples never differed by more than 2.2%. Assuming that all the α-azo-pigment was derived solely from bilirubin monoglucuronide, it was calculated that the normal rats converted 36.4±1.7% of the injected bilirubin monoglucuronide into diglucuronide, whereas only 5.0±0.4% was converted by Gunn rats ($P<0.001$). These proportions applied irrespective of the dose of bilirubin monoglucuronide and of the relative amount of bilirubin diglucuronide administered simultaneously (Fig. 9).

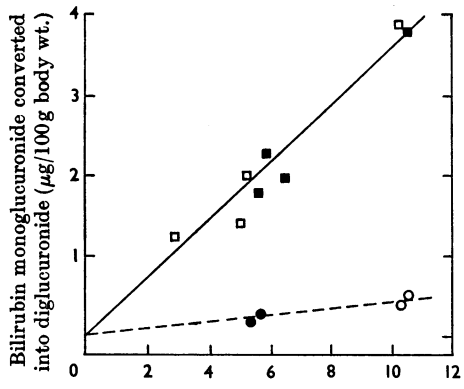

Fig. 9. Conversion of bilirubin monoglucuronide into diglucuronide by normal rats (□ and ■) and Gunn rats (○ and ●). Rats with bile fistulae were given single intravenous injections of solutions of mixed conjugates of [^{14}C]bilirubin in 50mM-sodium phosphate buffer, pH6.5, which contained either monoglucuronide (■ and ●) or diglucuronide (□ and ○) as the predominant conjugate. Ethyl anthranilate azo-pigments were prepared from the injected solutions and from the bile collected for 2h after injection, and the proportions of α-(unconjugated) and δ-(glucuronide) azo-pigments were determined by t.l.c. and radioassay. From these results were calculated the quantity of bilirubin monoglucuronide injected and the amount of this pigment that was converted into and excreted as bilirubin diglucuronide. Normal rats converted 36.4±1.7% whereas Gunn rats converted only 5.0±0.4% (means±S.E.M., $P<0.001$). For details see the text and Tables 4 and 5.

DISCUSSION

Comments on the isolation procedures. The present procedure for the isolation of conjugated bilirubin from bile represents several improvements over the methods of Lucassen (1961) (given in detail in the review by Brodersen & Jacobsen, 1969) and Talafant & Appelt (1968), which are the best yet published. The improvements are as follows. (1) It requires only 3–4h. (2) The product has been shown to be free of bile salt, phospholipid, cholesterol and unconjugated bilirubin. (3) Radioactive labelling of the conjugates has been achieved. (4) The monoglucuronide as well as the diglucuronide has been recovered in relatively pure form, although the two conjugates have not been completely separated. (5) Yields of bilirubin are probably comparable with those of Lucassen (1961) but much superior to the 8% recovery by Talafant & Appelt (1968). [Lucassen (1961) obtained 0.3g of bilirubin from 1 litre of human T-tube bile. He did not state the bilirubin concentration in the initial bile, but it presumably fell in the range 0.5–1.5g/l (Guldhammer & Kjeldsen, 1967).]

Disadvantages of the present method are: (1) large volumes of solvent are required, making it uneconomical for bulk isolation of pigments; (2) failure to obtain adequate quantities of crystalline material, although the CBM preparations are suitable for physiological studies, as was demonstrated; (3) the product slowly oxidizes during storage, whereas Lucassen's (1961) crystalline preparation is stable; both preparations are otherwise similar as to colour, fluorescence, spectra and solubilities. Thus Lucassen's (1961) method seems preferable for the bulk isolation of bilirubin diglucuronide from human bile, whereas the present method is better for rapid isolation of mixed or individual conjugates from multiple small samples of rat bile.

The modified procedure for preparation of unconjugated bilirubin from bile is simpler and more rapid than the generally used method of Ostrow *et al.* (1961) and avoids the troublesome extraction of pigment from a proteinaceous gel that bedevils the older method. Both procedures give comparable yields and are applicable to microquantities of bile and bilirubin.

Separation of bilirubin mono- and di-glucuronide. Significant but incomplete separation of bilirubin monoglucuronide from diglucuronide was achieved by both chloroform extraction at pH3.3 and by elution from Sephadex LH-20 with 95% (v/v) ethanol. The solvent partition procedure involved large losses due to precipitation, hydrolysis and oxidation of pigment, especially of bilirubin monoglucuronide. The column chromatographic procedure gave over 90% recovery of pigment but no more than 1.0g of bilirubin conjugates could be processed on a 1cm×13cm bed of gel. Jacobsen (1970) has reported complete separation of the two conjugates by a similar procedure from a Lucassen (1961) preparation applied to Sephadex LH-20 and eluted with 50% (v/v) ethanol, but further examination of the purity of his two pigment fractions seems warranted before it can be decided whether elution with 95% or 50% ethanol is preferable.

Properties of the conjugated pigments

Spectra. The two-peaked absorption spectrum of conjugated bilirubin has been described by Polonovski & Bourrillon (1952), Cole *et al.* (1954), Lucassen (1961), Maggiore & Giovannetti (1965), Pagliardi & Gaidano (1965), Kuenzle *et al.* (1966) and Fog & Bakken (1967). The two maxima were identical for the purified pigment and for whole bile, indicating that they are not, as suggested by Polonovski & Bourrillon (1952) and Pagliardi & Gaidano (1965), the result of interaction of pigment with other constituents of bile. The maxima were

also unaffected by variations in the proportions of bilirubin monoglucuronide and diglucuronide which supports the conclusion of Cole *et al.* (1954) that these two conjugates have identical spectra. The finding of a more prominent 450nm peak for the diglucuronide and a more prominent 420nm peak for the monoglucuronide reported by Pagliardi & Gaidano (1965) and by Maggiore & Giovannetti (1965) may perhaps be attributed to their use of different solvents for the two pigments. The present study documents solvent effects on the relative intensity of the two maxima and suggests that, in solution, each conjugate may exist in two forms in dynamic equilibrium, dependent on variations of intra- or inter-molecular hydrogen-bonding (Fog & Jellum, 1963) or on keto–enol tautomerism (Gray, Kulczycka & Nicholson, 1961). The extinction coefficient at 450nm is the same for conjugated pigment in methanol or water as for unconjugated bilirubin in chloroform, suggesting that conjugation does not affect the electronic properties of the chromophore.

Malloy–Evelyn diazo reaction. The azo-pigment absorption maxima were at 560nm in aqueous and at 550nm in methanolic solution. Jirsa & Jirsová (1959) presented spectra of the azo-pigment of a synthetic taurobilirubin conjugate that showed a similar shift of the maximum to shorter wavelengths in methanol, although their text stated that the maximum was identical in the two solvents. The millimolar extinction coefficient of $28.4\,cm^{-1}$ for our preparation agrees with values obtained by Overbeck, Vink & Deenstra (1955), and by Lucassen (1961) after correction of the latter's value for the weight of impurities in his crystals.

Although Jirsa & Jirsová (1959) and Billing, Cole & Lathe (1957) noted an increase in the diazo reaction of monoconjugated bilirubin on addition of alcohol, our purified preparations consistently yielded more colour in the aqueous (direct) reaction. However, when CBM preparation was added to human or rat serum, the direct reaction was inhibited with extinction 15% less than the total reaction. However, the total diazo reaction of serum was of similar intensity with CBM preparations and unconjugated bilirubin. Therefore use of unconjugated bilirubin as a standard for conjugated bilirubin is valid only for the total diazo reaction, and the proportion of conjugated bilirubin in serum is consistently underestimated by the direct Malloy–Evelyn diazo reaction currently used in many clinical laboratories.

Solubility. The solubility of the pure conjugated bilirubin in water and its precipitation by Pb^{2+} ions confirms previous findings with impure preparations (Ostrow *et al.* 1961; Talafant, 1956), but the solubility of desalted pure conjugates in the presence of Ca^{2+}, Ba^{2+} and Zn^{2+} differs from the ready co-precipitation of conjugated bilirubin when these cations are added to whole bile (see Ostrow *et al.* 1961, references 51–59). The solubility of the calcium salt of conjugated bilirubin supports the hypothesis that pigmented biliary calculi develop when the conjugate is hydrolysed by bacterial β-glucuronidase, forming the insoluble calcium salt of unconjugated bilirubin (Maki, Sato & Saitoh, 1962; Suzuki & Toyoda, 1966).

Stability. The pure CBM preparations, like Brodersen's (1966) adsorbate of bilirubin diglucuronide to Super-Cel, underwent very slow oxidation to biliverdin conjugates, but no hydrolysis when stored at −15°C and even when incubated at pH 7.0 in the dark in air at 37°C. This contradicts claims (Nosslin, 1960*b*; Lucassen, 1961) that bilirubin conjugates are very unstable in protein-free solutions, and contrasts with the often rapid hydrolysis and oxidation of conjugated bilirubin in bile incubated under similar conditions (Ostrow, 1967). Whether the instability of conjugated bilirubin in bile is due to complex-formation with other constituents or to enzymic action is not yet known. The stability of the pigments in the aqueous CBM preparations is important, since it permits for the first time the use of pure conjugated bilirubin in incubation studies *in vitro*.

Nature of the bilirubin conjugates. CBM preparations and fractions CB-I and CB-II were shown, by paper and column chromatography, by azo-pigment chromatography and by glucuronic acid/bilirubin ratios, to be composed predominantly of mixtures of bilirubin monoglucuronide and diglucuronide. There was a paucity of bilirubin monoglucuronide in human CBM preparations, whereas rat CBM preparations contained up to two-thirds bilirubin monoglucuronide. This is well above values reported for normal rat bile (Hoffman, Whitcomb, Butt & Bollman, 1960), even allowing for the increased proportion of the monoglucuronide that is excreted after intravenous loading with unconjugated bilirubin (Hoffman *et al.* 1960; Schalm & Weber, 1962; Verme & Camarri, 1965; Diaz-Rubio, Diaz-Rubio & Santos, 1967). However, column chromatography of the original bile samples and their Folch extracts showed 25–35% bilirubin monoglucuronide, in agreement with reported values (Billing *et al.* 1957; Hoffman *et al.* 1960; Kuenzle *et al.* 1966; Inoue, 1966). These discrepancies are probably due to preferential extraction of bilirubin monoglucuronide into ethyl acetate [rat bile procedure, steps (5), (7) and (9)] and chloroform [human bile procedure, step (4A)] at pH values near 3.3.

Although murine and human bile contain preponderantly bilirubin diglucuronide and monoglucuronide, azo-pigment chromatography reveals the existence of other conjugates (Tenhunen, 1965; Gaidano, Pagliardi & Fevero, 1965; Inoue, 1966).

Some of these are resistant to β-glucuronidase and some even to alkaline hydrolysis (Isselbacher & McCarthy, 1959; Gaidano *et al.* 1965; Colombo & Bonomo, 1965). Alkali-stability has also been demonstrated directly with undiazotized bilirubin conjugates separated by t.l.c. (Manenti, Solmi & Spanio, 1966). In the present study with ethyl anthranilate azo-pigments, 6% of the δ-fraction and all of the γ- and β-fractions were resistant to β-glucuronidase, and the β-fraction was also resistant to alkali, confirming observations of Heirwegh *et al.* (1970). Some of these resistant azo-pigments, which derive from minor bilirubin conjugates in rat or human bile, are not sulphate conjugates (Isselbacher & McCarthy, 1959; Tenhunen, 1965; Colombo & Bonomo, 1965; Manenti *et al.* 1966; Compernolle *et al.* 1970). Since fractions CB-I and CB-II gave other azo-pigments in addition to the α- and δ-azo-pigments, these preparations of bilirubin monoglucuronide and diglucuronide are contaminated with small proportions of other conjugates of bilirubin.

It is currently believed that bilirubin 'monoglucuronide', represented by the pigment I fraction isolated by reverse-phase partition chromatography of serum from jaundiced patients, is a complex since it partly dissociates into unconjugated bilirubin and pigment II on rechromatography (Nosslin, 1960*a*; Gregory, 1963; Weber, Schalm & Witmans, 1963) or heating (Billing & Lathe, 1958). Nosslin (1960*a*) has also recovered unconjugated bilirubin from pigment I by extraction with chloroform at neutral pH, a procedure designed to extract unconjugated bilirubin completely from complex mixtures (Brodersen & Vind, 1963). Moreover, Nosslin (1960*b*) and Weber *et al.* (1963) have reconstituted a pigment I band by chromatography of mixtures of unconjugated bilirubin, pigment II, and serum.

On the other hand, in conjugated hyperbilirubinaemia the proportion of chloroform-extractable bilirubin is not increased (Tygstrup & Brodersen, 1968) although the proportion of the pigment I fraction is uniformly elevated (Hoffman *et al.* 1960; Schoenfield, Bollman & Hoffman, 1962; Diaz-Rubio & Diaz-Rubio, 1966). Schoenfield & Bollman (1963) were unable to dissociate the pigment I band found in the serum of hepatectomized dogs. Apparently in these two circumstances the pigment I is not a complex of unconjugated bilirubin and bilirubin diglucuronide. This is certainly true of the purified bilirubin monoglucuronide isolated in the present studies, since this was not dissociated by chloroform extraction, the Folch solvent partition procedure or rechromatography, did not exchange with unconjugated [^{14}C]bilirubin and, on oxidation, formed only biliverdin monoglucuronide. Moreover, when administered intravenously to Gunn rats that cannot excrete unconjugated bilirubin (Lester, Klein & Matusen, 1966), [^{14}C]bilirubin monoglucuronide was quantitatively excreted, even when little bilirubin diglucuronide was present. This cannot be accounted for by excretion of a complex of unconjugated bilirubin and bilirubin diglucuronide since large amounts of the diglucuronide have to be injected to carry over a small amount of unconjugated bilirubin (Callahan & Schmid, 1969).

The evidence thus favours the existence of two forms of bilirubin 'monoglucuronide', one a complex of unconjugated bilirubin and bilirubin diglucuronide and the other a true monoglucuronide. It is suggested that the former be referred to as pigment I, the latter as bilirubin monoglucuronide.

Excretion of bilirubin conjugates by the rat. The observed rates of biliary excretion of purified [^{14}C]bilirubin conjugates agree closely with values obtained by Lester & Klein (1966) after intravenous administration of the conjugates as whole bile, and by Lester *et al.* (1966) with comparably low doses of [^{3}H]bilirubin or [^{3}H]mesobilirubinogen. These authors likewise found the percentage of label excreted to be unaffected by wide variations in the load of pigment administered.

Although output of bilirubin monoglucuronide and diglucuronide was similar at 2h, excretion of bilirubin monoglucuronide was initially delayed in both Gunn and normal rats. The delay thus cannot be attributed to a rate-limitation in the conjugation of bilirubin monoglucuronide but probably results from slower excretion of the monoglucuronide *per se*. Urinary excretion of the monoglucuronide and diglucuronide were comparable, indicating that both can pass the glomerulus. Other authors agree, although they differ as to which pigment predominates in icteric urine (Cole *et al.* 1954; Schachter, 1959; Verme & Camarri, 1965; Hoenig & Jirsa, 1966). By using the Schachter (1959) azo-pigment partition method, which unfortunately may not accurately measure proportions of bilirubin monoglucuronide and diglucuronide in urine, Diaz-Rubio, Diaz-Rubio & Agullo (1966) found that renal clearance of the diglucuronide was greater than that of the monoglucuronide. This would be expected from the higher pK and greater lipid-solubility of bilirubin monoglucuronide, which should lead to increased back-diffusion of this conjugate from the tubular fluid at low pH values (Ali & Billing, 1966).

Interconversion of bilirubin monoglucuronide and diglucuronide in the rat. Since neither bilirubin monoglucuronide nor the diglucuronide was administered in pure form free from other conjugates, certain assumptions had to be made in interpreting the results of these studies. (1) It was assumed that differential excretion of the two conjugates did not

account for the increased proportion of bilirubin diglucuronide in the bile. This is validated by two observations: (*a*) the percentage of pigment excreted in 2h and the proportion of administered bilirubin monoglucuronide converted into diglucuronide were unaffected by wide variations in the ratio of the two glucuronides injected; (*b*) in some animals the fraction of injected radioactivity that was not recovered in the excreta was too small to account for the calculated conversion of monoglucuronide into diglucuronide, even if all the unrecovered label were in monoglucuronide. (2) In calculating the absolute conversion of bilirubin monoglucuronide into diglucuronide it was assumed that labelled pigments excreted during the first 2h were representative of the total output of labelled pigment. This seems probable since the distribution of ^{14}C radioactivity in the bile pigments excreted in the third and fourth hour was the same as in the first 2h in the two animals thus examined. (3) It was assumed that the only significant interconversions were of α- and δ-azo-pigments, which is supported by the finding that the proportion of ^{14}C radioactivity in the F, β, γ and O fractions were the same for the excreted and injected pigments of each animal. Moreover, conjugated biliverdins in the injected solutions were recovered intact in the bile, indicating that, unlike unconjugated biliverdin (Goldstein & Lester, 1964), they need not be converted into bilirubin conjugates to be excreted. Apparently the rat cannot conjugate biliverdin.

Since less than 60% of the excreted radioactivity was recovered with pigments extracted by the method of Noir *et al.* (1965), spurious increases in the bilirubin diglucuronide fraction could have been observed if bilirubin monoglucuronide were selectively lost. To circumvent this problem proportions of the two conjugates were determined by chromatography of their ethyl anthranilate azo-pigments, with recovery of over 85% of the excreted radioactivity and almost 100% of the excreted bilirubin conjugates. However, use of the proportions of α- and δ-azo-pigments to calculate the relative amounts of bilirubin monoglucuronide and diglucuronide involved assumptions that all α-azo-pigment was derived from bilirubin monoglucuronide and all δ-azo-pigment came from either bilirubin monoglucuronide (α–δ) or bilirubin diglucuronide (δ–δ). Since no unconjugated bilirubin was demonstrated by chloroform extraction, all α-azo-pigment of the injected and excreted solutions must have been derived from monoconjugated bilirubins. The other assumption is supported by the studies of Heirwegh *et al.* (1970) with rat bile pigments separated on ion-exchange columns with formamide gradients. Their bilirubin diglucuronide fraction yielded only δ-azo-pigments, and δ-azo-pigments were formed only from the bilirubin monoglucuronide and diglucuronide fractions. It thus seems valid in the rat to calculate the relative amounts of bilirubin monoglucuronide and diglucuronide from the proportions of azo-pigments α and δ formed from a mixture of conjugates as follows: % monoglucuronide (= α+equimolar δ) = $2\times\%\alpha$, and % diglucuronide (= residual δ) = % δ − % α.

The above assumptions are further validated by the finding that Gunn rats, which cannot synthesize glucuronide conjugates of bilirubin, showed little increase in the proportion of bilirubin diglucuronide in the excreted as compared with injected pigments, an increment well within experimental error and significantly smaller than the increment found with normal animals. A further safeguard was the use of each rat as its own control, each receiving fractions CB-I and CB-II in a sequence randomized among the six animals. The results demonstrated net conjugation of bilirubin monoglucuronide to the diglucuronide, as shown previously for the intact rat by Schoenfield & Bollman (1963) and in the isolated perfused rat liver by Schoenfield, Grindlay, Foulk & Bollman (1961). These authors have further suggested that the liver cannot make bilirubin monoglucuronide, since none is excreted in the bile of the isolated rat liver perfused with unconjugated bilirubin or bilirubin monoglucuronide and they found only bilirubin diglucuronide in the medium after incubation of rat liver slices or homogenates with unconjugated bilirubin. However, other investigators have detected bilirubin monoglucuronide production by homogenates or partially purified bilirubin glucuronyltransferase from rat liver (Van Roy & Heirwegh, 1968; Mowat & Arias, 1969; Strebel & Odell, 1969), so the question of the hepatic versus extrahepatic origin of bilirubin monoglucuronide remains in dispute. The known increases in the pigment I fraction of plasma in hepatocellular failure (see above) and in bile after intravenous bilirubin loading (see above) can be interpreted to support either view.

The failure to find net conversion of [^{14}C]bilirubin diglucuronide into [^{14}C]bilirubin monoglucuronide or unconjugated [^{14}C]bilirubin in the Gunn rats indicated that significant hydrolysis of conjugated bilirubin did not occur in these experiments. This differs from the conclusion of Okolicsanyi, Magnenat & Frei (1968), who injected much greater doses of conjugated bilirubin in the form of enriched native bile and observed increases in the indirect-reacting bilirubin in the excreted bile. However, they did not use labelled bilirubin to exclude the possibility that these increases came from endogenous unconjugated bilirubin carried into the bile as a complex with administered bile salts or conjugated bilirubin (Callahan & Schmid, 1969). Conversion of conjugated [^{14}C]bilirubin into unconjugated [^{14}C]-bilirubin *in vivo* has, however, been demonstrated

in rats with ligated bile ducts (Acocella, Tenconi, Armas-Merino, Raia & Billing, 1968). Apparently with prolonged retention of large amounts of conjugated bilirubin, as in biliary obstruction, hydrolysis of conjugated pigment occurs, whereas under physiological conditions, as in our studies, hydrolysis is probably minimal.

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REFERENCES

Acocella, G., Tenconi, L. T., Armas-Merino, R., Raia, S. & Billing, B. (1968). *Lancet*, i, 68.

Ali, M. A. M. & Billing, B. (1966). *Clin. Sci.* **30**, 543.

Billing, B. H., Cole, P. G. & Lathe, G. H. (1957). *Biochem. J.* **65**, 774.

Billing, B. H. & Lathe, G. H. (1958). *Am. J. Med.* **24**, 111.

Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.

Brodersen, R. (1966). *Scand. J. clin. Lab. Invest.* **18**, 361.

Brodersen, R. & Jacobsen, J. (1969). In *Methods of Biochemical Analysis*, vol. 17, p. 31. Ed. by Glick, D. New York: Interscience Publishers Inc.

Brodersen, R. & Vind, I. (1963). *Scand. J. clin. Lab. Invest.* **15**, 107.

Callahan, E. W., jun. & Schmid, R. (1969). *Gastroenterology*, **57**, 134.

Cole, P. G., Lathe, G. H. & Billing, B. H. (1954). *Biochem. J.* **57**, 514.

Colombo, B. & Bonomo, E. (1965). *Minerva med., Roma*, **55**, 2529.

Compernolle, F., Jansen, F. H. & Heirwegh, K. P. M. (1970). *Biochem. J.* (in the Press).

Diaz-Rubio, M. & Diaz-Rubio, M., jun. (1966). *Revta clin. esp.* **100**, 20.

Diaz-Rubio, M., Diaz-Rubio, M., jun. & Agullo, J. L. R. (1966). *Revta esp. Enferm. Apar. dig. Nutr.* **25**, 1099.

Diaz-Rubio, M., Diaz-Rubio, M., jun. & Santos, J. M. (1967). *Revta clin. esp.* **104**, 221.

Edwards, A. L. (1954). *Statistical Methods for the Behavioral Sciences*, 1st ed. New York: Holt, Rinehart and Winston Inc.

Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.

Fog, J. & Bakken, A. F. (1967). *Scand. J. clin. Lab. Invest.* **20**, 88.

Fog, J. & Jellum, E. (1963). *Nature, Lond.*, **198**, 88.

Folch, J., Lees, M. & Sloane-Stanley, G. H. S. (1957). *J. biol. Chem.* **226**, 497.

Gaidano, G., Pagliardi, E. & Fevero, A. (1965). *Fegato*, **11**, 384.

Goldstein, G. W. & Lester, R. (1964). *Proc. Soc. exp. Biol. Med.* **117**, 681.

Gray, C. H., Kulczycka, A. & Nicholson, D. C. (1961). *J. chem. Soc.* p. 2268.

Gregory, C. H. (1963). *J. Lab. clin. Med.* **61**, 917.

Guldhammer, E. H. & Kjeldsen, K. (1967). *Acta chir. scand.* **133**, 483.

Haslewood, G. A. D. (1955). *Physiol. Rev.* **35**, 178.

Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970). *Biochem. J.* (in the Press).

Hoenig, V. & Jirsa, M. (1966). *Čslká Gastroent. Výž.* **20**, 379.

Hoffman, H. N., II, Whitcomb, F. F., jun., Butt, H. R. & Bollman, J. L. (1960). *J. clin. Invest.* **39**, 132.

Inoue, T. (1966). *Acta med., Fukuoka*, **36**, 417.

Isselbacher, K. J. & McCarthy, E. A. (1959). *J. clin. Invest.* **38**, 645.

Jacobsen, J. (1970). *Acta chem. scand.* (in the Press).

Jirsa, M. & Jirsová, V. (1959). *Clin. Chem.* **5**, 532.

Kottke, B. A., Wollenweber, J. & Owen, C. A., jun. (1966). *J. Chromat.* **21**, 439.

Kuenzle, C. C., Sommerhalder, M., Rüttner, J. R. & Maier, C. (1966). *J. Lab. clin. Med.* **67**, 282.

Lester, R. & Klein, P. D. (1966). *J. Lab. clin. Med.* **67**, 1000.

Lester, R., Klein, P. D. & Matusen, A. M. (1966). *J. clin. Invest.* **45**, 1839.

Lester, R. & Troxler, R. F. (1969). *Gastroenterology*, **56**, 143.

Lucassen, J. (1961). Doctoral Thesis: University of Utrecht.

Maggiore, Q. & Giovannetti, S. (1965). *Minerva med., Roma*, **55**, 2526.

Maki, T., Sato, T. & Saitoh, T. (1962). *Tohoku J. exp. Med.* **77**, 179.

Malloy, H. T. & Evelyn, K. A. (1937). *J. biol. Chem.* **119**, 481.

Manenti, F., Solmi, G. & Spanio, L. (1966). *Minerva gastroent.* **12**, 53.

Mowat, A. P. & Arias, I. M. (1969). *Pediat. Res.* **3**, 351 (Abstr.)

Najjar, V. A. & Childs, B. (1953). *J. biol. Chem.* **204**, 359.

Neiderheiser, D. H. & Roth, H. P. (1968). *Proc. Soc. exp. Biol. Med.* **128**, 221.

Noir, B. A., Garay, E. R. & Royer, M. (1965). *Biochim. biophys. Acta*, **100**, 403.

Nosslin, B. (1960*a*). *Scand. J. clin. Lab. Invest.* **12**, Suppl. no. 49, p. 8.

Nosslin, B. (1960*b*). *Scand. J. clin. Lab. Invest.* **12**, Suppl. no. 49, p. 27.

Okolicsanyi, L., Magnenat, P. & Frei, J. (1968). *Lancet*, i, 1173.

Ostrow, J. D. (1967). *J. clin. Invest.* **46**, 2035.

Ostrow, J. D., Hammaker, L. & Schmid, R. (1961). *J. clin. Invest.* **40**, 1442.

Overbeek, J. T. G., Vink, C. L. J. & Deenstra, H. (1955). *Recl. Trav. chim. Pays-Bas Belg.* **74**, 85.

Pagliardi, E. & Gaidano, G. (1965). *Minerva med., Roma*, **55**, 2532.

Polonovski, M. & Bourrillon, R. (1952). *Bull. Soc. Chim. biol.* **34**, 973.

Sakamoto, T., Yamamoto, S., Yahata, K. & Kondo, T. (1957). *Acta Med., Okoyama*, **27**, 373.
Sato, T. & Saitoh, T. (1965). *Tohoku J. exp. Med.* **84**, 329.
Schachter, D. (1959). *J. Lab. clin. Med.* **53**, 557.
Schalm, L. & Weber, A. P. (1962). *Ned. Tijdschr. Geneesk.* **106**, 1079.
Schoenfield, L. J. & Bollman, J. L. (1963). *Proc. Soc. exp. Biol. Med.* **112**, 929.
Schoenfield, L. J., Bollman, J. L. & Hoffman, H. N., II (1962). *J. clin. Invest.* **41**, 133.
Schoenfield, L. J., Grindlay, J. H., Foulk, W. T. & Bollman, J. L. (1961). *Proc. Soc. exp. Biol. Med.* **106**, 438.
Strebel, L. & Odell, G. B. (1969). *Pediat. Res.* **3**, 351 (Abstr.).
Suzuki, N. & Toyoda, M. (1966). *Tohoku J. exp. Med.* **88**, 353.
Talafant, E. (1956). *Nature, Lond.*, **178**, 312.
Talafant, E. & Appelt, J. (1968). *Clin. Chem.* **14**, 208.
Tenhunen, R. (1965). *Annls Med. exp. Biol. Fenn.* **43**, Suppl. no. 6, p. 14.
Tygstrup, N. & Brodersen, R. (1968). *Scand. J. clin. Lab. Invest.* **21**, 62.
Van Roy, F. P. & Heirwegh, K. P. M. (1968). *Biochem. J.* **107**, 507.
Verme, G. & Camarri, E. (1965). *Minerva med., Roma*, **55**, 2543.
Weber, A. P., Schalm, L. & Witmans, J. (1963). *Acta med. scand.* **173**, 19.