Inaccuracies in Measurement of Conjugated and Unconjugated Bilirubin in Bile with Ethyl Anthranilate Diazo and Solvent-Partition Methods

By J. DONALD OSTROW* and SASIPRAPA T. BOONYAPISIT†

Gastrointestinal Section, University of Pennsylvania Medical Division, Veterans Administration Hospital, Philadelphia, PA 19104, U.S.A.

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A critical evaluation was made of the ethyl anthranilate diazo and two solvent-partition methods for the determination of conjugated and unconjugated bilirubin in human and rat bile. The ethyl anthranilate diazo reagent, which reacts only with conjugated bilirubin in serum, also diazotized a variable proportion of unconjugated bilirubin in bile and thus overestimated the concentration of monoconjugates. With the Weber–Schalm and modified Folch solvent-partition methods applied to human or rat bile, 4–9% of added ^{14}C -labelled unconjugated bilirubin partitioned with the conjugated bilirubin in the upper phase, and 4–9% of added ^{14}C -labelled conjugated bilirubin partitioned into the lower phase. With dog bile, the spill-over of ^{14}C -labelled conjugated bilirubin into the lower phase was 9–11%. Analysis of azopigments from the Weber–Schalm partition confirmed that over two-thirds of the bilirubin in the lower phase represents monoconjugates, principally the less-polar monoxylsides and monoglucosides. These solvent-partition methods thus overestimate the concentration of unconjugated bilirubin in bile.

In attempting to elucidate the aetiology of the pigment gallstones, it became important to measure accurately the low concentrations of unconjugated bilirubin in bile, which contains predominantly conjugated bilirubin (With, 1968; Royer & Dubin, 1970; Thompson & Hofmann, 1973). To this end, a critical analysis of published methods was undertaken, all of which were found wanting. The present paper delineates the inaccuracies of the ethyl anthranilate diazo method (Heirwegh *et al.*, 1974) and two solvent-partition methods (Weber & Schalm, 1962; Ostrow & Murphy, 1970) that are most often used currently for this determination.

Materials

Bile

Hepatic-fistula bile was collected from 350–400g male Sprague–Dawley rats during the first 6h after catheterization of the common duct under pentobarbital anaesthesia. Uninfected human bile was obtained from T-tube drainage of the common duct of patients with normal liver functions who had undergone cholecystectomy 5–7 days previously, or from aspiration of the gall bladder at surgery, after informed consent. Dog biles were obtained by direct

puncture of the gall bladder, or by common-duct drainage of unanaesthetized cholecystectomized animals via a Thomas duodenal cannula (Thomas *et al.*, 1934). All biles were collected and other bilirubin-containing solutions were prepared and stored on ice, protected from light.

Unconjugated bilirubin

Unlabelled unconjugated bilirubin was from BDH Chemicals, Poole, Dorset, U.K. (lot 1423960), and used without further purification. By t.l.c. (Berry *et al.*, 1972), it was 99.8% pure, with $\epsilon_{453} = 59.8 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$. ^{14}C -labelled unconjugated bilirubin of specific radioactivity $43.6 \times 10^3 \text{ d.p.m./}\mu\text{g}$ was prepared biosynthetically in the rat from 5-amino[4- ^{14}C]laevulinic acid (New England Nuclear Corp., Boston, MA, U.S.A.). It was isolated, crystallized, and its specific radioactivity determined by the methods of Ostrow *et al.* (1961). For addition to bile, ^{14}C -labelled unconjugated bilirubin was dissolved quickly in 0.1M-NaOH, and immediately diluted with 4 vol. of 0.05M-sodium phosphate buffer, pH6.5, to a final concentration of 0.4–0.5mg/ml. Up to 0.1 ml of this solution was then added per ml of bile.

Mixtures of labelled unconjugated and conjugated bilirubin

Enriched rat bile, collected after intravenous administration of ^{14}C -labelled unconjugated bilirubin dissolved in rat serum, was used for this without further processing. Pure ^{14}C -labelled conjugated bilirubin was isolated from such enriched bile by the

* Present address and address for reprints: Gastroenterology Section, Northwestern University Medical School, Veterans Administration Lakeside Hospital, Chicago, IL 60611, U.S.A.

† Present address: Department of Medicine, Siriraj Hospital, Mahidol University, Bangkok 7, Thailand.

method of Ostrow & Murphy (1970). The product, which was used on the day of preparation, consisted of 0.4–0.5 mg of mixed bilirubin conjugates/ml in 0.05 M-sodium phosphate buffer, pH 6.5, of which approximately two-thirds was diglucuronide and one-third monoglucuronide. Its specific radioactivity ranged from 10.6×10^3 to 14.2×10^3 d.p.m./ μ g of bilirubin, as determined from the specific radioactivity of ^{14}C -labelled unconjugated bilirubin released by hydrolysis in 1.0 M-NaOH in the presence of ascorbate (Ostrow & Murphy, 1970). These preparations were free of unconjugated bilirubin, bile acids, phospholipids and cholesterol, and had $\epsilon_{453} = 59.8 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (Ostrow & Murphy, 1970).

Chemicals

Diphylline was from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade from J. T. Baker Co., Phillipsburg, NJ, U.S.A.

Methods

Diazo reactions

These were performed by the method of Michaelsson (1961), with half as much bile as the recommended volume of serum. Diazotization of bilirubin conjugates with ethyl anthranilate at pH 2.7 was performed as described by Heirwegh *et al.* (1974).

Folch *et al.* (1957) solvent partition

This procedure for extraction of total lipids from tissue homogenates was adapted for partition of conjugated and unconjugated bilirubins in bile as described by Ostrow & Murphy (1970). First, 0.3 ml of bile was shaken with 6.0 ml of chloroform/methanol (2:1, v/v) and then 1.2 ml of 0.1 M-sodium phosphate buffer, pH 5.8, was added. After shaking and centrifugation at 900g for 10 min at 20°C, total bilirubin in each phase was determined by the method of Malloy & Evelyn (1937). To increase the accuracy of determination of low concentrations of unconjugated bilirubin, 3 vol. of lower phase was diazotized by using proportionally less methanol, a procedure that did not alter the values obtained per unit weight of unconjugated bilirubin.

Weber & Schalm (1962) solvent partition

This was performed as originally described. To determine the proportion and types of conjugates extracted into the lower phase, parallel partitions were run on six dog and seven human bile samples by using HCl in place of diazo reagent. To 2.4 ml of this 'blank' lower phase was added 0.2 ml of 1.0 mM-EDTA, adjusted to pH 7.4, and the organic solvent was evaporated under a stream of N_2 at 40°C. The residual 0.4 ml of solution was then diazotized with ethyl anthranilate reagent at pH 2.7 (Heirwegh *et al.*,

1974), and the azopigments were extracted into 0.5 ml of chloroform. After twice washing with water to remove salts, the extract was diluted to 1.0 ml with chloroform and the A_{530} determined. The azopigments were then evaporated under N_2 at 40°C, and chromatographed on precoated silica-gel G thin layers (IB2; J. T. Baker Co.) in chloroform/methanol/water (65:25:3, by vol.) (Heirwegh *et al.*, 1974). Azopigments prepared with unextracted dog bile and from unconjugated bilirubin were run in adjacent channels as standards. The α_0 (unconjugated) and pooled conjugated azopigments were scraped from the gels, eluted with 0.06 M-HCl in methanol, and the A_{530} values of the eluates determined. Recoveries from the chromatograms exceeded 90%.

Radioassay

^{14}C -labelled unconjugated bilirubin was blended with Hyamine (Hyamine hydroxide, 1.0 M in methanol; New England Nuclear) and a toluene-based scintillator (Ostrow *et al.*, 1961). ^{14}C -labelled conjugated bilirubin solutions, and bile containing labelled bilirubin(s) (0.05–0.10 ml), were warmed (10 min, 70°C) with 0.5 ml of Hyamine, and 10 ml of Bray's (1960) scintillator was added. To avoid quenching by azobilirubin, radioassay of Weber-Schalm partition phases was performed on blanks prepared with NaNO_2 omitted from the diazo reagent. Portions (0.5–1.0 ml) of upper phases from solvent partitions were blended directly with 10 ml of Bray's (1960) scintillator. Lower phases (0.5–2.0 ml) were evaporated to 0.1 ml under a stream of warm air, and then 1.0 ml of methanol and 15 ml of toluene-based scintillator added (Ostrow *et al.*, 1961).

All samples were assayed to 10^4 counts in a Beckman LS-200 liquid-scintillation spectrometer, and corrected for counting efficiencies measured with [^{14}C]toluene (New England Nuclear Corp.) as internal standard. Efficiencies ranged from 45 to 58%.

Results

Ethyl anthranilate diazo reaction (Fig. 1)

Diazotization of bile with added conjugated bilirubin revealed a linear relationship between the increase in absorbance (ΔA) of the ethyl anthranilate diazo reaction and the concentration of added conjugates (mg/dl) determined by the Michaelsson (1961) method (x). As calculated by the method of least squares, $\Delta A = 0.023 + 0.665x$; $r = 0.989$, $P < 0.001$. By contrast, addition of unconjugated bilirubin to the biles produced a definite but highly variable increment in the absorbance of the ethyl anthranilate reaction, which ranged from 0.01 to 0.30 unit per mg of unconjugated bilirubin/dl, equal to 1–44% of the absorbance of azopigments derived from conjugated bilirubin. pH values during

diazotization, determined with a pH-meter, were always less than 2.8. This reactivity of unconjugated bilirubin in bile was evident even at pH values as low as 2.0.

Solvent-partition methods

Table 1 shows the partition of labelled bilirubin added in tracer quantities to fresh bile samples from three species. With human or rat bile 4-9% of added ¹⁴C-labelled unconjugated bilirubin partitioned into the upper phase, and 4-9% of added ¹⁴C-labelled conjugated bilirubin appeared in the lower phase. With dog bile contamination of the lower phase with

Fig. 1. Reaction of bile bilirubin with diazotized ethyl anthranilate at pH2.7 (Heirwegh *et al.*, 1974) —, Relationship between the change in absorbance at 530nm of the ethyl anthranilate diazo reaction (ΔA) and the increase in total bilirubin concentration produced by addition of pure conjugated bilirubin to 21 bile samples. All samples contained at least 97% of the total bilirubin as conjugates. ▲, Increment in absorbance (ΔA) of the ethyl anthranilate diazo reaction produced by addition of pure unconjugated bilirubin to the same bile samples. Concentrations of added bilirubin (abscissa) were determined by the Michaelsson (1961) diazo reaction.

Table 1. Solvent partition of labelled bilirubin added to bile samples ¹⁴C-labelled conjugated or unconjugated bilirubin was added to fresh biles in tracer quantities, solvent partition performed, and the two phases were radioassayed. Recoveries of radioactivity from the two phases consistently exceeded 93.8% of the ¹⁴C initially added to bile. Each value is the mean of duplicate determinations which differed by less than 0.6%.

Method ...	Modified Folch partition		Weber-Schalm partition	
	¹⁴ C-labelled conjugated bilirubin in lower phase (%)	¹⁴ C-labelled unconjugated bilirubin in upper phase (%)	¹⁴ C-labelled conjugated bilirubin in lower phase (%)	¹⁴ C-labelled unconjugated bilirubin in upper phase (%)
Type of bile				
Human	4.7, 6.3, 6.4	3.7, 4.8, 6.2	5.9, 7.3, 8.9	4.5, 4.6, 7.2
Rat	4.4, 4.5, 4.8	6.0, 6.3, 6.6	6.5, 7.3, 7.5	7.2, 8.4, 9.0
Dog	Not done	Not done	9.6, 10.1, 11.1	5.4, 6.2, 6.7

¹⁴C-labelled conjugated bilirubin was somewhat greater (9-11%). T.l.c. (Berry *et al.*, 1972) of the phases with radioassay of the pigment bands showed that during partition less than 2% of the labelled bilirubin had oxidized to more polar derivatives and that no hydrolysis of ¹⁴C-labelled conjugated bilirubin had occurred.

Diazotization of the lower phase of the Weber-Schalm partition revealed significant quantities of conjugated azopigments (Table 2). The proportion of endogenous conjugated bilirubin that partitioned into the lower phase was 2-4% with fresh human bile and 7-9% with fresh dog bile, values slightly lower than those found by using tracer ¹⁴C-labelled conjugated bilirubin. This contamination of lower phase with conjugated bilirubin was more marked if previously frozen-and-thawed bile was used. In all biles tested, at least two-thirds and usually more than three-quarters of the bilirubin in the lower phase consisted of conjugated pigments.

T.l.c. of the ethyl anthranilate azopigments (Heirwegh *et al.*, 1974) from the lower phases revealed almost equal amounts of α₀ (unconjugated) and conjugated azopigments, indicating that mostly monoconjugates were extracted (Ostrow & Murphy,

Table 2. Extraction of conjugated bilirubin into lower phase of Weber-Schalm partition

Total bilirubin in lower phase, and total amount of conjugated bilirubin in sample, were determined from diazo partition (method of Weber & Schalm, 1962). Conjugated bilirubin in lower phase was determined from ethyl anthranilate diazo reaction (Heirwegh *et al.*, 1974) of a blank Weber-Schalm partition.

Bile samples	Total conjugates in lower phase (%)	Lower-phase bilirubin that is conjugated (%)
Human, fresh	2.1, 2.7, 3.4	67, 87, 87
Human, thawed	11.1, 12.3, 14.2, 18.0	90, 92, 93, 97
Dog, fresh	7.6, 8.3, 9.1	76, 80, 88
Dog, thawed	11.7, 16.5, 20.8	92, 96, 98

1970). In all bile samples, most of the conjugated azopigments in the lower phase were of the α_2 (xyloside) or α_3 (glucoside) classes (Heirwegh *et al.*, 1974).

Discussion

Ethyl anthranilate diazo method

We found that a variable and often large proportion of unconjugated bilirubin added to undiluted bile reacted directly with the ethyl anthranilate diazo reagent at pH 2.7. This differs from reports that this reagent reacts exclusively with conjugated bilirubin in serum (Heirwegh *et al.*, 1974) and bile (Heirwegh *et al.*, 1970) at this pH value. The discrepancy may well result from their use of bile diluted 20-fold, thus diluting out an endogenous accelerator of the unconjugated bilirubin reaction, such as bile acids (Yamamoto, 1958). Our finding precludes both the use of the ethyl anthranilate reaction as a specific assay for conjugated bilirubin in bile and the assumption that all α_0 - (unconjugated) azopigment formed represents bilirubin monoconjugates (Ostrow & Murphy, 1970; Billing *et al.*, 1973).

Even if the ethyl anthranilate diazo reaction accurately measured conjugated bilirubin in bile, it is inherently inaccurate to determine unconjugated bilirubin from the difference between this value and that obtained in the total diazo reaction. Although the relative errors in these two determinations are individually less than 5% (Michaelsson, 1961; Billing *et al.*, 1971), the cumulative absolute error of their differences is quite large compared with the small absolute value for unconjugated bilirubin. Only by prior separation and subsequent direct measurement of the small unconjugated bilirubin fraction is any degree of accuracy likely to be achieved in estimating this fraction in bile. Solvent-partition methods would therefore theoretically provide a more valid approach to the measurement of conjugated and unconjugated bilirubin in bile.

Solvent-partition methods

Since its first publication in 1962, the Weber-Schalm method has been widely applied to serum, even though it was validated initially only by comparison with the direct diazo reaction, which does not accurately measure conjugated bilirubin (Michaelsson, 1961; Lucassen, 1961; Jacobs *et al.*, 1964). It is noteworthy that even the original report (Weber & Schalm, 1962) found that pigment I, eluted from a silicone-treated kieselguhr column (Cole *et al.*, 1954), partitioned equally into both phases. Though it was thought that this indicated that pigment I was a complex of conjugated and unconjugated bilirubin (Weber & Schalm, 1962), the data could have been equally interpreted to indicate that pigment I was a true monoglucuronide (Ostrow &

Murphy, 1970) that partitioned as such into both phases.

Subsequent experiments involving addition of pure conjugated and unconjugated bilirubin to plasma have revealed that approx. 5% of unconjugated bilirubin was extracted into the upper phase (Berk *et al.*, 1969; Perrelli & Watson, 1970; Brodersen, 1972), and approx. 3% of added conjugated bilirubin was extracted into the lower phase (Perrelli & Watson, 1970). The contamination of upper phase after addition of unconjugated bilirubin was attributed to extraction of polar products formed by oxidation of unconjugated bilirubin (Brodersen, 1972). The pigment found in the lower phase after addition of conjugated bilirubin was considered to represent unconjugated bilirubin formed by hydrolysis of conjugated bilirubin (Upson *et al.*, 1970) or isomerization of monoconjugates (Jansen, 1973) during processing. The validity of these explanations is doubtful, since even chloroform alone extracts some conjugated bilirubin from plasma, mostly in the form of monoconjugates (Ostrow & Murphy, 1970; Bratlid & Winsnes, 1971).

Despite these documented inaccuracies in the measurement of plasma conjugated bilirubin and unconjugated bilirubin by the Weber-Schalm partition, this method has been applied to bile without verification (Berthelot, 1967; Billing *et al.*, 1973). As with serum (Berk *et al.*, 1969; Perrelli & Watson, 1970; Brodersen, 1972), both the Weber-Schalm and Folch partition methods suffered from significant cross-extraction of ^{14}C -labelled conjugated and unconjugated bilirubin into the inappropriate phase, especially if the bile had been previously frozen and thawed. Chromatography of the azopigments from the lower phase revealed that this contamination represented true extraction of monoconjugates, and not extraction of unconjugated bilirubin formed by hydrolysis or isomerization of conjugates. These monoconjugates were principally xylosides and glucosides; therefore the greater extraction of conjugated bilirubin into the lower phase for dog than for human bile was probably related to the higher proportion of these polar glycoside conjugates in dog bile (Fevry *et al.*, 1971).

Since bilirubin in bile is mostly conjugated (With, 1968; Royer & Dubin, 1970), extraction of even a small proportion of conjugated bilirubin into the lower phase causes gross overestimation of unconjugated bilirubin. Our data revealed that at least two-thirds, and usually over three-quarters, of the lower-phase pigments were conjugates. These solvent-partition methods must thus overestimate the proportion of unconjugated bilirubin in bile by 3-4-fold (Berthelot, 1967; Ostrow & Murphy, 1970; Billing *et al.*, 1973). Our results also require re-evaluation of previous publications in which the

Weber-Schalm method was used to estimate unconjugated bilirubin in bile (Schalm & Weber, 1962; Berthelot, 1967; Billing *et al.*, 1973). Thus it is now unclear whether, as reported (Schalm & Weber, 1962; Berthelot, 1967), an intravenous load of unconjugated bilirubin led to augmented excretion of unconjugated bilirubin in bile, or whether it was actually excretion of monoconjugates that had increased.

In conclusion, the currently available diazo and solvent-partition methods inaccurately measure the proportions of conjugated and unconjugated bilirubin in bile. Clearly, the new t.l.c. method developed in this laboratory (Boonyapisit *et al.*, 1976) is preferable for assessment of the concentration of unconjugated bilirubin in bile, a key factor in the pathogenesis of pigment gallstones (Soloway *et al.*, 1977).

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