Purification and Analysis of Bilirubin

John T. Clarke

Pure crystalline bilirubin was isolated from human, calf, hog, and dog gallbladder bile and compared with commercially-available materials. All may be purified by recrystallization from pyridine. The extinction coefficient in chloroform is 60,000-61,000 at 450-453 mµ.

Standardization of the analytical "azobilirubin" (van den Bergh) reaction may be based on aqueous alkaline solutions of bilirubin.

Paper chromatography of azobilirubins derived from various sources of bilirubin showed identical patterns.

Unconjugated bilirubin due to hemolytic jaundice can be both quantitated and distinguished from conjugated (digluconuronide) bilirubin, due to obstructive jaundice or related conditions, by means of the highly specific van den Bergh "azobilirubin" reaction (1, 2). Diazotized sulfanilic acid in the absence of methanol reacts rapidly and nearly completely with "direct-reacting" (conjugated) bilirubin, whereas the presence of organic compounds (50% methanol is preferred) is required for the complete reaction of "indirect" (unconjugated) bilirubin, although a number of coupling agents will react in aqueous solutions. Recent studies of inconsistency in determinations of bilirubin levels in jaundiced sera (3-5) have concluded that two factors have been primarily responsible: variability in purity of the primary standard, and improper standardization conditions. In this communication is described the ready isolation of purified crystalline bilirubin, as well as an acceptable standardization technic.

Bilirubin is the primary product of the biochemical cleavage of protoporphyrin in unspecified modes, and is excreted in conjugated form in the bile primarily as bilirubin digluconuride (6-13). Stereobilin, biliverdin, etc., apparently are secondary decomposition products (14) which may be present in bile. Unconjugated bilirubin, such as evidenced in
erythroblastosis fetalis, circulates as a serum-bound protein complex and may be deposited in basal ganglia, as in kernicterus (15). The unusually low solubility of bilirubin in water and most organic solvents and its ready crystallizability permits its isolation from alkali-hydrolyzed gallbladder or fistula bile (16, 17) or its extraction from beef gallstones; it is the only bile pigment that has been isolated in consistently pure condition.

**Isolation from Gallbladder Bile**

Fresh human gallbladder bile is a reddish-yellow concentrate of liver (fistula) bile, with a wide range of composition. It is usually believed to contain approximately 1% bile salts (principally glycocholic acid), 1% mucins, 0.7% electrolytes, 0.3% lipids, and up to 1.5% bile pigments (18). Bilirubin diglucuronide, recognized to be the principal (70%) pigment of human bile (6–12), is a compound evidently readily oxidizable under ordinary conditions. Crystalline bilirubin may be isolated after removal of the glucuronic acid moiety by alkaline hydrolysis at room temperature, provided that the alkaline concentration is maintained at a minimum of 1 N. Prior precipitation (12, 16) of conjugated bilirubin is believed to be unnecessary. Ordinarily, crude crystalline bilirubin is isolated after acidification by means of its limited solubility in chloroform (0.1%) and complete insolubility in methanol and water. It may be isolated in a highly pure state after thermal recrystallization from pyridine.

**Procedure**

Pooled human gallbladder bile (100 ml.) obtained at necropsy and frozen is diluted with an equal volume of water, centrifuged, and the supernatant mixed with 50 ml. of 50% (w/v) KOH. After 10 min., the mixture is acidified to about pH 4 (incipient precipitation) with HCl, and after cooling, extracted with 450 ml. of chloroform. Practically all of the saponified pigment is in the chloroform layer, which is washed with water and the solvent removed under reduced pressure. The dark residue is triturated with methanol, and the crude product recovered by centrifugation. It is redissolved in 50 ml. of warm chloroform, filtered, concentrated to about 10 ml., and 50 ml. of methanol added. Crude bilirubin readily appears. After methanol washing, the product is dried to a greenish powder yielding about 50% of the original bilirubin content of the bile. As its purity is 80–90%, the product may be dissolved in gently boiling anhydrous pyridine sufficient to make a saturated (0.6%) solution. This solution is filtered, and upon cooling to room temperature there are deposited well-formed crystals of bilirubin. These crystals are recovered by decantation of the dark mother liquors (solubility at room temperature, 0.06%), washed with methanol, and dried in vacuo. The
reecrystallization may be repeated, to give large, well-formed, flat, brick-red rhombs (Fig. 1). Yield: 25–30%, based on original bilirubin content of the bile.

Bilirubin may be isolated from hog bile by essentially the same procedure. Omission of the chloroform extraction step yielded a red precipitate that was of only 20% purity, but pyridine recrystallization gave bilirubin of excellent quality. Calf bile apparently contains alkali-labile pigments of unknown constitution, and the yield of bilirubin was low. Dog bile resembles human bile more closely, except for its lack of precipitable mucins (Table 1).

**Results**

Paper chromatography, with Whatman No. 1 paper, of recrystallized bilirubins from all sources failed to reveal significant differences (Fig. 2), as also did X-ray crystallography. Elemental analysis agrees well with \( C_{33}H_{36}N_4O_8 \), the structure of bilirubin: the theoretical percentages of constituent elements are C, 67.71; H, 6.21; and N, 9.58; found by analysis were C, 67.85; H, 5.96; and N, 9.37. Nevertheless, paper chromatography in alkaline solvents has suggested that two closely-related forms might possibly coexist in most samples. Impurities have a marked influence upon the migration characteristics of bilirubin or its derivatives, a fact that heretofore has prevented the resolution of bile pigments without preliminary purification.

In chloroform at 25°, purified bilirubin (mol. wt., 584) was found to have an extinction coefficient of 60,000–61,000 at the maximum of 450–452 m\( \mu \). In 1962, the Committee on Bilirubin Standards of the American
Table 1. SOLUTION OF BILIRUBIN FROM VARIOUS SOURCES

<table>
<thead>
<tr>
<th>Source</th>
<th>Original bile</th>
<th>Percent yield</th>
<th>In chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Bilirubin (μg./ml.)</td>
<td>Crude</td>
</tr>
<tr>
<td>Pooled human bile, crude</td>
<td>6.5</td>
<td>6.2</td>
<td>50</td>
</tr>
<tr>
<td>Commercial, as received</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Commercial, as received</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Commercial*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Commercial*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hog bile†</td>
<td>6.7</td>
<td>0.56</td>
<td>—</td>
</tr>
<tr>
<td>Calf bile†</td>
<td>7.5</td>
<td>0.22</td>
<td>—</td>
</tr>
<tr>
<td>Dog bile†</td>
<td>7.8</td>
<td>1.4</td>
<td>—</td>
</tr>
</tbody>
</table>

*Recrystallized 3 times from pyridine.
†Recrystallized twice from pyridine.

Academy of Pediatrics tentatively adopted a value of 60,700 ± 800 at 453 mμ in chloroform at 25°C (19). In M/10 sodium carbonate, it has an extinction coefficient of 49,000–51,000 at the maximum of 435–436 mμ. As determined by solvent partition with ethyl acetate, the pK is about 7.8.

Azobilirubin

The reaction of bilirubin (sodium salt) in 50% methanol with diazotized sulfanilic acid under acidic conditions forms a mixture of pigments termed "azobilirubin," which is readily soluble in water or in n-butanol.
Procedure

To 0.1 ml. of bilirubin (up to 40 μg.) in M/10 sodium carbonate or of conjugated bilirubin (diluted bile) is added 1.9 ml. of water and 3.0 ml. of methanol, followed by 1.0 ml. of 13 mM diazotized sulfanilic acid in 0.8 N hydrochloric acid. The latter is prepared by mixing 1 ml. of 1% (w/v) sodium nitrite with 10 ml. of a sulfanilic acid solution (1.0 g/ml. sulfanilic acid [p-aminobenzene sulfonic acid monohydrate] in 15 ml. of concentrated HCl and 190 ml. of water). After 10 min. at room temperature (found adequate for complete reaction), the purple reaction mixture is extracted with two volumes of n-butanol. After the extract is washed, the butanol is removed under reduced pressure and the residue extracted with 2 ml. of boiling water for 10 min. to hydrolyze any esters.

Colors of the water-soluble derivative range from deep violet to bright pink, depending upon pH (Table 2).

Results

Paper chromatography of the azobilirubin prepared from all pyridine-recrystallized samples and from a number of commercially-available unrecrystallized samples (Fig. 2) invariably showed identical patterns, with the presence of a major component at Rf 0.43, a minor component at Rf 0.55, and a trace component at Rf 0.75 when developed with isopropanol-0.1 M sodium bicarbonate. For reasons that are not understood, azobilirubin prepared from bilirubin obtained by hydrosulfite reduction of biliverdin (vide infra), or from alkali-hydrolyzed human bile without the isolation of pigment, gave a proportionately higher content of the Rf 0.55 component.

Biliverdin

Oxidation of bilirubin to biliverdin under acidic conditions has been accomplished with ferric chloride, nitric acid, iodine, benzoquinone, copper salts, and by autoxidation (20). Impure biliverdin has been isolated, with a low yield, upon oxidation of bilirubin with hydrogen peroxide (14, 20), and its methyl ester synthesized and reduced (21). Under alkaline conditions, however, biliverdin apparently is not detected as an intermediate, and the oxidation of bilirubin proceeds beyond it (16). Microcrystalline biliverdin dihydrochloride of about 85% purity may be produced by oxidation of bilirubin with two equivalents of ferric chloride in methanolic hydrogen chloride.

Procedure

Recrystallized bilirubin (200 mg.) is dissolved in 20 ml. of M/10 sodium carbonate (20 min.) and the solution dispersed in 400 ml. of methanol.
Table 2. Spectral Characteristics of Azobilirubin (5.5 μg./ml., as bilirubin)

<table>
<thead>
<tr>
<th>pH</th>
<th>Abs. max. (μμ)</th>
<th>Absorbance at max. (1 cm.)</th>
<th>Ext. coef.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% Methanol Solvent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>562</td>
<td>0.77</td>
<td>85,700 (0.13 N HCl)</td>
</tr>
<tr>
<td>2.0</td>
<td>542</td>
<td>0.67</td>
<td>73,000 (0.014 N HCl)</td>
</tr>
<tr>
<td>3.0</td>
<td>520</td>
<td>0.64</td>
<td>—</td>
</tr>
</tbody>
</table>

Water Solvent

<table>
<thead>
<tr>
<th>pH</th>
<th>Abs. max. (μμ)</th>
<th>Absorbance at max. (1 cm.)</th>
<th>Ext. coef.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>510</td>
<td>0.58</td>
<td>—</td>
</tr>
<tr>
<td>9.0</td>
<td>510</td>
<td>0.57</td>
<td>—</td>
</tr>
<tr>
<td>11.0</td>
<td>530</td>
<td>0.54</td>
<td>—</td>
</tr>
<tr>
<td>13.0</td>
<td>580</td>
<td>0.82</td>
<td>—</td>
</tr>
</tbody>
</table>

To the clear yellow solution is added 200 mg. of ferric chloride hexahydrate dissolved in 5 ml. of N HCl. The resultant suspension is stirred for 2 hr. at room temperature; there is rapid production of green color and gradual disappearance of precipitate. The methanol is removed under reduced pressure, the compact green residue repeatedly dispersed in 30 ml. of N HCl, and the liquors discarded after centrifugation. The residue is dissolved in 10 ml. of methanol, and after a few hours at 5°, the unreacted bilirubin (25 mg.) is removed by centrifugation. The solution is evaporated to dryness. The residue dissolved in 15 ml. of M/10 sodium carbonate, centrifuged to remove small amounts of biliverdin esters, and then acidified to about N HCl. The microcrystalline precipitate is repeatedly washed with N HCl and dried in vacuo. Yield: 145 mg. (64%). The theoretical percentage of chloride is 10.82; 8.96 was found by analysis.

Results

Paper chromatography (Fig. 2) of biliverdin prepared as above shows that the principal spot (Rf 0.37–0.45) is accompanied by some monomethyl (Rf 0.88) and dimethyl (Rf 0.98) esters. Attempts to hydrolyze the esters with sodium carbonate were complicated by extensive losses due to polymerization, as also was the case when reduced to bilirubin in 10% yield by sodium hydrosulfite or 25% yield by sodium borohydride.

Standardization of Bilirubin

Stability

Crystalline bilirubin appears to be stable indefinitely. Alkaline aqueous or organic solutions deteriorate rapidly in the presence of air, particularly if illuminated (16), to colorless oxidation products. The decomposition rates of bilirubin (0.3 mg./ml.) in sodium carbonate and in
sodium hydroxide in loosely-stoppered 50-ml. flasks in the absence of light were followed by means of the azobilirubin reaction. Under these conditions, the steady deterioration in M/10 sodium carbonate (2%/hr.) shows first-order kinetics, with a half-time of 39 hr. at room temperature (Fig. 3).

Kinetics of decomposition in N/10 sodium hydroxide are more complicated: after a fast initial rate of about 10%/hr., about half is decomposed in 20 hr. Products of oxidation are believed to be poorly-defined unreactive water-soluble dioxydipyrrolmethenes that have practically no absorption in the visible range (16). On the other hand, chloroform solutions tend to be considerably more stable, especially if protected from light, and biliverdin is an important intermediate. The “instability” of bilirubin in chloroform-methanol that has been previously reported (22) is unquestionably due to the tendency of bilirubin to form colloidal suspensions in methanol, in which it is completely insoluble.

Method

Customarily, chloroform solutions of bilirubin have been used for standardization of the van den Bergh analytical reaction (1, 22). Bilirubin forms a complex with serum proteins under acidic or neutral conditions, with an absorption maximum at 462 m\(\mu\) (23), and hence by virtue of the protein complex, the transfer of bilirubin from the chloroform phase to the aqueous phase is expedited. Complete transfer to the aqueous phase therefore would be expected to depend on both the type and concentration of the protein. Suitable standard mixtures of protein and bilirubin already have been proposed (3).

As an alternative, aqueous alkaline solutions of crystalline bilirubin

![Graph showing first-order plot of decomposition rates of 0.2 mg./ml. bilirubin in M/10 sodium carbonate and in N/10 sodium hydroxide, stored in dark in loosely-stoppered flask.](image-url)
may be used. At a selected concentration of 0.3 mg./ml. of bilirubin, it is suggested that bilirubin should be dissolved in M/10 sodium carbonate, but with exclusion of light, minimum exposure to air, and the time between initial mixing and withdrawal of aliquots for standardization purposes not to exceed ½ hr. at room temperature. Under these conditions, the analytical error introduced should not exceed 1%. The rate of solution of bilirubin is significantly affected by crystal size, the larger crystals dissolving more slowly; but 25-ml. batches always have become homogeneous within ½ hr., with occasional gentle swirling.

Analysis

Malloy and Evelyn (1) found that the presence of 50% methanol markedly enhanced the color yield in the case of hemolytic-jaundice (unconjugated bilirubin) serum, but had little effect upon the color yield of obstructive-jaundice (conjugated bilirubin) serum. Dueci and Watson (2) suggested a "combined" procedure by which both "direct" (conjugated) and "total" (unconjugated plus conjugated) bilirubin might be determined on the same sample. Lathe and Ruthven (24) re-investigated the effects of pH, solvent, protein, and concentration of reagent, and concluded that linear responses and high precision are possible with 0.052% diazotized sulfanilic acid and with a 1-min. reaction time for the "direct" reaction. The direct reaction for conjugated bilirubin was found in this laboratory to be 70-75% complete in 1 min. under these conditions, whereas only 2.6% of the unconjugated bilirubin reacted. Probably due to the catalytic effect of excess diazo reagent (24), the direct (methanol-free) reaction for unconjugated bilirubin continues with time and approaches "total" levels after a few hours. Increases of the direct reaction time to 5 min. (24) or of the concentration of the diazo reagent five-fold were found to cause unexplained marked curvature in the subsequent total bilirubin concentration curves. After addition of methanol, the reaction for total bilirubin was determined in this laboratory to be complete within 20 min.

As evaluated with 78 separate human gallbladder bile specimens obtained at necropsy (average, 0.5 gm./100 ml. conjugated bilirubin), the direct/total ratio was observed to average 0.60 (S.D. ± 0.10), when the 1-min. reaction that employs 0.052% diazotized sulfanilic acid was used and the samples read at 545 m. Consequently there is little question that the high direct reaction in cases of obstructive jaundice must be due to the presence of conjugated bilirubin, although hemolytic jaundice may occasionally be complicated by obstructive jaundice (26). Normal serums are reported (25) to contain an average of 0.3 mg./100 ml. (range, 0.1-1.1) of bilirubin.
Alternative Methods

Although a large number of methods have been described (27–30), they generally are not regarded with favor because of low sensitivity or because only total bilirubin is measured. The interference of oxymoglobin in the suggested spectrophotometric technic (27) may be eliminated by a correction at 575 m\(\mu\), but there are complications due to turbidity, nonlinear response to bilirubin concentration, and variations of the spectra of obstructive-jaundice serums. The quantitation of biliverdin as produced by ferric chloride oxidation also has been explored in this laboratory, inasmuch as the intensity of the absorption maximum of biliverdin at 694 m\(\mu\) is proportional to initial bilirubin concentration, but the considerably lower precision of this approach certainly excludes it as a sensitive analytical method.

References