THE IDENTIFICATION OF “DIRECT-REACTING” BILIRUBIN AS BILIRUBIN GLUCURONIDE*

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It has long been recognized that, with p-diazobenzenesulfonic acid (van den Bergh reagent), bilirubin in serum of patients with jaundice may exhibit two types of reaction (1, 2). If coupling with the diazo reagent occurs promptly in aqueous solution, the reaction is said to be “direct”; if, however, coupling takes place only after prior addition of alcohol, the reaction is said to be “indirect.” The factors responsible for this difference in behavior have been a matter of much controversy (3), but, in recent years, evidence has rapidly accumulated to suggest that the two types of reaction observed reflect the presence of two different forms of the pigment in the serum (4–6). Thus, bilirubin giving an “indirect” diazo reaction appeared to be similar to crystalline bilirubin which is soluble in chloroform, but insoluble in water (7), whereas “direct-reacting” bilirubin is more water-soluble (6). The two pigment fractions could be separated by paper chromatography (5), by column chromatography (8), or by reverse phase column chromatography (6), but the more water-soluble of the two compounds was found to be so unstable that satisfactory purification and isolation could not be achieved (6). A possible way of overcoming this difficulty was indicated by the observation of Ekuni (9) and Kawai (10), later confirmed by Billing (11), that the two forms of bilirubin gave rise to two more stable “azobilirubins,” which could be separated by chromatography. In the present investigation, the bilirubin present in normal bile and in serum and urine of jaundiced patients was converted to the respective hydroxypyrromethene azo derivatives (12), chromatographed on paper, purified, and studied. In preliminary communications, it has been reported that the azo derivative of water-soluble “direct-reacting” bilirubin contains a mole of glucuronic acid, whereas the derivative of water-insoluble “indirect-reacting” bilirubin lacks this component (13, 14).

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Materials and Methods

Fresh human bile was collected from the common bile duct in individuals undergoing laparotomy (liver bile) or from the gallbladder at autopsy (gallbladder bile). Plasma and urine from patients with various disorders associated with jaundice\textsuperscript{1} were frozen immediately after collection. In instances in which the patients were not hospitalized at the National Institutes of Health, the freshly frozen samples were shipped to us packed in dry ice.\textsuperscript{2} All specimens were analyzed as soon as possible after collection.

To prepare the hydroxypyrromethene azo derivatives of bilirubin, the samples were put in an ice bath, and the pH was adjusted to 4.0 with glacial acetic acid. An equal volume of freshly prepared \textit{p}-diazobenzenesulfonic acid (10) was then added, and the mixture was stirred for 15 minutes. After addition of another volume of freshly prepared diazo reagent and 3 volumes of ethanol, stirring was continued for another 30 minutes. The reaction mixture was then washed with large volumes of chloroform until the washes were entirely colorless. After adjustment of the pH to 4.0, the aqueous fraction, containing the azo derivatives, was extracted with \textit{n}-butanol until there was no pink color left in the aqueous phase. The combined butanol extracts were evaporated \textit{in vacuo} at 30\degree, and the dry residue was dissolved in 1 ml. of 1 \textit{N} acetic acid for paper chromatography.

In applying this method to specimens of serum, bile, and urine, the following was found to be of importance: with serum, washing the reaction mixture containing the derivatives with chloroform frequently resulted in precipitation of proteins. Since some of the derivatives remained attached to the precipitate, this was collected by filtration and repeatedly extracted with a 4:1 mixture of \textit{n}-butanol and glacial acetic acid. In this way, most of the azo derivatives could be brought into the solvent mixture which was then separated into a butanol and an aqueous phase by addition of an equal volume of water. The butanol containing the azo derivatives was evaporated as indicated above.

With bile, it was found necessary to remove bile acids by washing the aqueous reaction mixture containing the derivatives with a 7:3 mixture of \textit{n}-heptane and \textit{n}-butanol as well as with chloroform.

With urine, it was observed that the addition of \textit{p}-diazobenzenesulfonic acid resulted not only in formation of hydroxypyrromethene azo derivatives, but also variable amounts of an unknown brown pigment. The

\textsuperscript{1} Included were patients with hemolytic, obstructive and non-hemolytic, non-obstructive jaundice (15).

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formation of this brown pigment could be diminished by acidifying the urine to pH 2.0 prior to the addition of the diazo reagent. It remained in the aqueous phase, when the hydroxypyrromethene derivatives were extracted with n-butanol.

The hydroxypyrromethene azo derivatives were separated and purified by ascending paper chromatography on Schleicher and Schuell paper No. 598, with use of a solvent system consisting of methyl ethyl ketone, propionic acid, and water (75:25:30). After elution of the compounds with 0.05 N hydrochloric acid, their optical density at 560 μm was determined in a Beckman quartz spectrophotometer. The molar concentration was obtained by comparison with a standard curve prepared with crystalline bilirubin (Fisher Scientific Company) by the method of Malloy and Evelyn (16).

For the purpose of identification, purified hydroxypyrromethene azo derivatives were subjected to acid or enzymatic hydrolysis, and the hydrolysates were analyzed for their content of glucuronic acid and of free hydroxypyrromethene derivatives. Acid hydrolysis was carried out in sealed glass tubes in 1 N hydrochloric acid at 100° for 90 minutes. At the end of this period, a small aliquot of the hydrolysate was removed for spectrophotometric estimation and paper chromatographic analysis of the azo derivatives. The major portion of the hydrolysate was decolorized with activated charcoal. The supernatant fluid was filtered through glass wool and then lyophilized. The dry residue was dissolved in 1 ml. of water for estimation of glucuronic acid by the method of Dische (17).

Enzymatic hydrolysis was carried out with beef liver β-glucuronidase (Warner-Chilcott) or with bacterial β-glucuronidase (Sigma, Lot 125-03) at 37° for 24 hours under nitrogen. The incubation mixtures contained 1500 units of animal β-glucuronidase and 750 μmoles of acetate buffer, pH 4.6, or 50 mg. of bacterial β-glucuronidase and 750 μmoles of phosphate buffer, pH 6.2, in a final volume of 3 ml. At the end of the incubation period, a 1.5 ml. aliquot was removed, the pH was adjusted to 4.0, and the hydroxypyrromethene derivatives were extracted with n-butanol. A small portion of the butanol extract was used for paper chromatography and the remainder was evaporated to dryness. The residue was dissolved in 0.05 N hydrochloric acid, and the azo derivatives were estimated spectrophotometrically. The remainder of the incubation mixture was acidified with hydrochloric acid, and then decolorized with activated charcoal, and the glucuronic acid was estimated as described above.

The azo derivatives of crystalline bilirubin (Fisher Scientific Company) and of synthetic neoxanthobilirubinic acid3 and isoneoxanthobilirubinic acid3 were prepared by mixing saturated alcoholic solutions of these compounds available to us.

3 We are grateful to Professor Walter Siedel, Frankfurt-Hoechst (Germany), for making these isomeric hydroxypyrromethene compounds available to us.
pounds with \( p \)-diazobenzenesulfonic acid. The resulting derivatives were extracted with \( n \)-butanol. The latter was evaporated in a vacuum, and the residue was dissolved in 1 ml. of 1 \( N \) acetic acid. The compounds obtained in this way were compared chromatographically and spectroscopically with the derivatives derived from bilirubin in serum, bile, and urine.

**Results**

When the hydroxypyrrromethene azo derivatives, prepared from bilirubin in serum, were studied by paper chromatography, it became apparent that the results depended on the clinical type of jaundice from which the serum specimens were obtained. In instances of hemolytic and of non-hemolytic non-obstructive jaundice, in which most of the serum bilirubin exhibits an "indirect" reaction, paper chromatography with the above solvent system yielded a single band with an \( R_F \) of 0.5 (Type A derivatives). Identical chromatographic findings were obtained with the azo derivatives of crystalline bilirubin, of neoxanthobilirubinic acid, and of isoneoxanthobilirubinic acid. On the other hand, in patients with obstructive jaundice, in whom the serum bilirubin exhibits predominantly a "direct" reaction, paper chromatography yielded, in addition to Type A derivatives, a second and usually much stronger band with an \( R_F \) of 0.25 to 0.30 (Type B derivatives).

In individuals with hemolytic and with non-hemolytic non-obstructive jaundice, bilirubin could not be demonstrated in the urine. In obstructive jaundice, on the other hand, urine regularly contained pigments which on paper chromatography yielded mostly Type B derivatives accompanied only by traces of Type A derivatives. Bilirubin collected from fresh liver bile of individuals without jaundice resulted, after coupling, predominantly in the formation of Type B derivatives while the amounts of Type A derivatives formed were much smaller.

Type A and Type B derivatives, while differing in chromatographic behavior, appeared to have similar absorption spectra in the visible and near ultraviolet range, exhibiting in 0.1 \( N \) hydrochloric acid maximal optical density at 552 \( \text{m} \mu \). Hydrolysis of Type B derivatives resulted in the formation of equimolar amounts of glucuronic acid and of Type A derivatives (Table I). The same molar ratio of glucuronic acid to Type A derivatives was observed, whether hydrolysis was achieved by acid or by \( \beta \)-glucuronidase, and whether the substrate was obtained from normal bile or from serum and urine of patients with obstructive jaundice (Table I). No hydrolysis occurred on incubating Type B derivatives at pH 6.2 with heat-inactivated enzyme (Table I). The Type A derivatives, obtained by hydrolysis of Type B derivatives, exhibited chromatographic and spectroscopic properties identical with those of the azo derivatives.
derived from crystalline bilirubin and from serum bilirubin of patients with hemolytic jaundice. Hydrolysis of Type A derivatives isolated directly from serum and bile failed to yield glucuronic acid (Table I).

If normal bile or urine from individuals with obstructive jaundice exhibiting a pH greater than 7 was incubated for 24 hours at 37°, the amounts of Type B derivatives obtained were often smaller and those of Type A derivatives larger than before incubation. Heating of such bile or urine specimens for 1 hour at 100° produced similar changes. The ratio of Type A to Type B derivatives was frequently found to be smaller in fresh liver bile than in stagnant gallbladder bile. In the latter, it occasionally approached a value of 1. Yellow gallstones, recovered from the gallbladder at autopsy or at cholecystectomy, yielded only Type A derivatives. If undiluted serum containing "direct-reacting" bilirubin or normal bile were buffered at pH 6.2, incubation with bacterial \( \beta \)-glucuronidase did not significantly alter the ratio of Type A to Type B derivatives.

After treating normal human bile with \( p \)-diazobenzenesulfonic acid, paper chromatography occasionally revealed, in addition to Type A and Type B derivatives, small amounts of other azo derivatives, exhibiting a

<table>
<thead>
<tr>
<th>Source of azo derivatives</th>
<th>Hydrolysis</th>
<th>Substrate</th>
<th>Type of azo derivatives</th>
<th>Product</th>
<th>Glucuronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type of azo derivatives</td>
<td>( \mu \text{mole} )</td>
<td>Type of azo derivatives</td>
<td>( \mu \text{mole} )</td>
</tr>
<tr>
<td>Liver bile</td>
<td>Enzymatic</td>
<td>B</td>
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<td>A</td>
<td>0.083</td>
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<tr>
<td></td>
<td></td>
<td>B</td>
<td>0.70</td>
<td>A</td>
<td>0.61</td>
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<td></td>
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<td>B</td>
<td>0.45</td>
<td>B</td>
<td>0.40</td>
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<tr>
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<td>B</td>
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<td>Serum†</td>
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<td>0.72</td>
<td>A</td>
<td>†</td>
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<tr>
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<td>Serum§</td>
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<td>A</td>
<td>0.28</td>
<td>A</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* \( \beta \)-Glucuronidase inactivated by heating at 100° for 30 minutes.
† Acid hydrolysis converted quantitatively Type B to Type A azo derivatives.
‡ Serum and urine from patients with obstructive jaundice.
§ Serum from patient with hemolytic jaundice.
similar absorption spectrum but a different $R_F$. However, the concentration of these compounds was so small that sufficient material could not be obtained for analysis.

**DISCUSSION**

Since it has been demonstrated that an excess of $p$-diazobenzenesulfonic acid converts a mole of bilirubin to 2 moles of hydroxypyrromethene azo derivatives (12), and since bilirubin is asymmetric with regard to its methyl and vinyl side chains (18), the resulting derivatives represent a mixture of two isomeric hydroxypyrromethenes. With paper chromatography in the system used, however, the two isomers could not be separated; this, for the purpose of the present discussion, permits us to regard them as a single compound. The lack of chromatographic separation of the two isomers is further demonstrated by the finding that the azo derivatives of the isomeric compounds neoxanthobilirubinic acid and isoneoxanthobilirubinic acid also had the same $R_F$ value.

The finding that serum from jaundiced patients, exhibiting a "direct" van den Bergh reaction, yielded derivatives which contained an equimolar amount of glucuronic acid, whereas "indirect-reacting" bilirubin yielded only Type A derivatives, indicates that "direct-reacting" bilirubin is a glucuronide. The data obtained in this way, however, do not permit a final decision as to whether the parent "direct-reacting" bilirubin is a mono- or a diglucuronide. Obviously, the former would be expected to yield an equal mixture of Type A and Type B derivatives, whereas the latter would yield only Type B derivatives. The observation that normal liver bile and urine from patients with obstructive jaundice produced much larger amounts of Type B derivatives than of Type A is believed to indicate that water-soluble bilirubin is predominantly a diglucuronide. On the other hand, the occurrence in these instances of a small amount of Type A derivatives along with the Type B derivatives suggests that a minor fraction of the "direct-reacting" bilirubin may also be present as a monoglucuronide, a finding which is in agreement with the conclusions reached by Billing, Cole, and Lathe (19).

Because of the alkali lability of Type B derivatives, it is believed that the glucuronic acid is esterified with the carboxyl groups of bilirubin (19, 20). Such a structure would explain the relatively rapid hydrolysis of bilirubin glucuronide on incubation at 37°, and on heating at 100°, particularly in instances in which the pH tends to be alkaline, as is the case in bile and urine. Partial hydrolysis of bilirubin glucuronide is believed to account for the frequent observation that the ratio of Type A to Type B derivatives is larger in stagnant bile, obtained from the gallbladder, than in fresh liver bile. The finding that yellow gallstones recovered from
the gallbladder contain only free, "indirect-reacting" bilirubin is in agreement with this concept. The possible role of enzymatic hydrolysis of bilirubin glucuronide in serum, bile, and urine is difficult to evaluate because of the frequent presence of \( \beta \)-glucuronidase inhibitors (21, 22). This may explain why in undiluted serum and bile, buffered at pH 6.2, significant hydrolysis of bilirubin glucuronide could not be demonstrated after incubation with bacterial \( \beta \)-glucuronidase.

At neutral pH, bilirubin is almost insoluble in water (7), whereas bilirubin glucuronide is water-soluble over a wide pH range (5). This difference in solubility undoubtedly accounts for the different behavior of the two types of pigment in the coupling reaction with \( p \)-diazobenzenesulfonic acid, as employed in the van den Bergh test (1, 2). As demonstrated by Overbeek et al. (12), however, the kinetics of this reaction are such that difficulties may be encountered in attempting to estimate the actual concentrations of bilirubin glucuronide and of free bilirubin in serum simply by determining the "1 minute direct-reacting" and the "indirect-reacting" bilirubin (23). Furthermore, it would appear that the values obtained for "direct" and for "indirect-reacting" bilirubin in serum may be influenced by the concentrations of such normal blood constituents as bile acids and urea (24-26). Additional studies are therefore necessary to determine to what extent the values obtained for "direct-reacting" bilirubin reflect the exact amounts of bilirubin glucuronide present in the serum and in other biological specimens (27).

The occasional demonstration in normal human bile of small amounts of water-soluble "direct-reacting" pigments, yielding azo derivatives other than Type B, suggests the possibility that some bilirubin may be excreted in water-soluble forms of non-glucuronide nature. This question is presently under study.

SUMMARY

It has recently been shown that the finding of "direct-reacting" and of "indirect-reacting" bilirubin in serum of jaundiced individuals reflects the presence of two different, but closely related, bilirubin fractions, which differ mainly in their solubility properties. The hydroxypyrromethene azo derivatives, prepared from these two pigment fractions with \( p \)-diazobenzenesulfonic acid, were subjected to paper chromatographic analysis. While "indirect-reacting" bilirubin resembled crystalline bilirubin in yielding only Type A derivatives, the water-soluble "direct-reacting" bilirubin gave rise mainly to Type B derivatives along with some of Type A. By acid and enzymatic hydrolysis, Type B derivative was found to be the glucuronide of Type A derivative. This indicates that the water-soluble bilirubin is bilirubin glucuronide. Quantitative considerations...
suggest that the “direct-reacting” bilirubin fraction includes both bilirubin di- and monoglucuronides. In normal bile and in urine, most bilirubin is present in the form of the glucuronide.

**BIBLIOGRAPHY**

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