It is generally accepted that in serum two types of bilirubin may exist, the so called direct and indirect. The differentiation is based upon the van den Bergh (1) reaction which is essentially a coupling reaction between the pigments and freshly diazotized sulfanilic acid. The indirect bilirubin is the normally occurring pigment and does not couple readily with the diazo reagent except upon the addition of alcohol to the reactants. The direct, on the other hand, couples with the reagent rapidly and completely within 30 minutes and produces the same color without the addition of alcohol. This latter appears in serum only when the bile duct system is blocked and the pigment is thereby reintroduced into the blood stream. The importance of these two types of pigments lies in the fact that they indicate different types of pathology and therefore assume diagnostic significance in clinical medicine. The results of the chemical and clinical studies on these pigments are still controversial and have been adequately reviewed recently by Lemberg and Legge (2). The difficulty was largely due to the fact that it was not possible to obtain native pure bilirubin from serum for comparative study with that available from bile. We have recently succeeded in obtaining indirect serum bilirubin in crystalline form (3) with relative ease and in yields as high as 85 per cent of that present originally in serum.

The purpose of this report is to describe in detail the method of crystallization and the properties of the direct and indirect pigments. A brief report on this work was presented earlier (3).

**EXPERIMENTAL**

Method of Crystallization of Indirect Serum Bilirubin

Samples of icteric serum were obtained from patients with hemolytic jaundice, sickle-cell anemia, hematoma fluid, jaundice of the new-born, kernicterus, and congenital familial non-hemolytic jaundice with kernicterus (4). All these diseases yield indirect bilirubin. 5 ml. of serum were

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brought to about pH 5.0 with 0.3 M acetate buffer, pH 5. This was extracted in a separatory funnel with 40 volumes of peroxide-free ether by shaking vigorously for 5 to 10 minutes. The extraction procedure was repeated two or more times when necessary for complete transfer of the pigment into the ether phase. In this procedure about 2 per cent of the direct pigment is extracted (3). The ether was then washed four times by shaking with an equal volume of distilled water. Hemoglobin does not contribute to the extractable material. One can therefore extract bilirubin directly from whole blood. The ether extracts were pooled in a large separatory funnel and shaken with 5 ml. of 0.01 to 0.02 M sodium pyrophosphate. When the pigment was not completely transferred into the aqueous phase, successive amounts of 0.1 M sodium hydroxide were introduced dropwise into the funnel and the contents shaken after each addition, until a complete transfer of the pigment was obtained. The aqueous extract was then placed in a test-tube and 0.2 M phosphate buffer, pH 7.0, added dropwise with stirring until the pH reached 7.6 to 7.8, at which time crystals began to form. The early formation of these microcrystals could be detected by the greenish fluorescent-like Tyndall effect that was produced when the sample was exposed to white light. This phenomenon was usually observed between pH 7.6 to 8.0, depending on the concentration of bilirubin in the solution, and can be used as an end-point for the addition of the phosphate buffer. The sample was then allowed to stand for an hour at room temperature. The dropwise addition of the phosphate buffer was resumed until the pH was gradually brought down to 7.0 to 7.2. The sample was then left in the ice box for 4 to 6 hours, after which it was kept in the freezing compartment overnight for complete crystallization.

In the early stages crystals always appeared as needles, mostly arranged together in bundles (Fig. 1, a). As the process continued, plates with well defined edges were formed. These increased in number, while the needle-shaped crystals slowly disappeared until nothing but plates (Fig. 1, b) remained. It is necessary to stress that the two seemingly different forms of crystals may both be plates at different stages of formation but do not represent two different kinds of pigment. The yellow crystals were spun down and washed with cold distilled water in which they are insoluble. Recrystallization was accomplished by redissolving the crystals in 0.01 M sodium pyrophosphate with alkali addition, if necessary, as before. Again the pH was brought down to 7.4 gradually by dropwise addition of 0.1 M phosphate buffer, pH 7.0, and the procedure continued as before. Although the first crystallization is almost always successful, recrystallization is more difficult. It is more likely to succeed if the pH is brought down very gradually and the temperature lowered slowly.

The washed second crystals were then dissolved in chloroform. This
was shaken a few times with an equal volume of water to wash off the contaminating salts. The chloroform was then evaporated in vacuo at 4–10° outside temperature. The pigment so prepared was compared with commercially available bilirubin crystallized from chloroform. In both preparations the following identical characteristics were obtained: (1) Absorption curves in chloroform (Fig. 2) showed a maximum at 450 mp with a molar extinction\(^1\) of 5.63 \(\times\) 10\(^4\). In 0.05 M pyrophosphate the maximum was found, in fresh preparations, to be at 440 mp with an extinction\(^1\) of 4.2 \(\times\) 10\(^4\). In concentrations of 4 to 25 γ per ml, this solution obeyed Beer's law. When bilirubin in 0.05 M pyrophosphate was fully combined with serum albumin by the addition of equimolar amounts of the crystalline protein, the maximum was found at 460 mp and the extinction\(^1\) at this maximum was 4.33 \(\times\) 10\(^4\), indicating a shift in the maximum with little interference in the over-all absorption. (2) The absorption curves of the diazonium complex (5) gave a maximum at 548 mp. (3) The pigments were slowly oxidized in 0.1 N NaOH (6). This oxidation was markedly enhanced by the bivalent cations Fe\(^{++}\), Cu\(^{++}\), Co\(^{++}\), Mn\(^{++}\), and Ca\(^{++}\). The pigments did not melt at temperatures up to 250° but charring was observed around 195–200°.

\(^1\) The absorption of a molar solution in a 1 cm. light path in the Beckman spectrophotometer model DU.
It thus appears that there is no difference in the chemical and physical behavior of the so called indirect bilirubin isolated from indirect reacting serum and that commercially available from bile.

Properties of Bile Pigments

The combination of bilirubin with serum protein was studied spectrophotometrically with the Beckman model DU. As stated above, bilirubin in 0.01 M pyrophosphate buffer, pH 8.4, has an absorption maximum at 440 m\(\mu\). When fully combined with protein, the maximum shifts to 460 m\(\mu\). By adding increasing amounts of protein to bilirubin dissolved in pyrophosphate buffer, pH 8.4, and following the shift of the maximum until it reached 460 m\(\mu\), we were able to show that under these conditions 2 moles of pigment combine with each mole of crystalline human serum albumin, 3 \(\gamma\) with 1 mg. of Cohn fraction (No. IV-4) containing \(\alpha\)- and \(\beta\)-globulin mainly, and no combination took place with \(\gamma\)-globulin.\(^2\) These results are similar to those obtained earlier by Martin (7). There was also no qualitative or quantitative difference here between the pigment isolated from serum and that available commercially from bile. Furthermore, we have obtained human bile from duodenal juice and subjected it

\(^2\) Crystalline serum albumin, \(\gamma\)-globulin, obtained from E. R. Squibb and Sons; Cohn fraction (No. IV-4) obtained through the courtesy of Dr. M. Mayer.
to the same extraction and crystallization procedure. The crystals were identical with those obtained from serum although they did not form as readily. There was also no difference observed in the chemical and physical properties described above.

It is thus evident that essentially the same type of pigment is present in both serum and bile and in either case the van den Bergh reaction is indirect, necessitating the addition of alcohol before coupling can occur when the reaction takes place below pH 3.0. There is little doubt, nevertheless, that in obstructive jaundice, in which bile is reintroduced into the blood stream, the serum shows a direct reaction. This is distinctly different from the pigment that merely accumulates in serum from excessive hemolysis or from an inability of the liver to excrete it into the bile (4). One can safely assume, therefore, that in bile the molecular species of the pigment is different from that observed elsewhere and that it is altered by the process of isolation such as we have used or is employed commercially.

Further studies on these two types of pigments were carried out in the native state. The sera used were from two cases of congenital obstructive jaundice having the direct pigment and from four cases of congenital familial jaundice with kernicterus containing the indirect (4). In order to study the attachment of the pigments to serum proteins, fractionation of the two types of sera with ammonium sulfate was instituted. For that purpose virtually hemoglobin-free serum was used. The procedure consisted essentially of using the appropriate dilution of serum and adding a sufficient amount of saturated ammonium sulfate to obtain the desired concentration of the salt and a final dilution of serum of 1:25. In practice 0.4 ml. of serum was pipetted into celluloid centrifuge tubes. The calculated amount of water necessary was then added, followed by the ammonium sulfate solution saturated at room temperature. The addition of the salt was done dropwise while stirring so that adequate mixing could take place. The final volume in all tubes was 10 ml. The samples were then placed in the cold room at 4° for 1 hour for equilibration, then centrifuged at that temperature at high speed until the supernatant solution was completely clear.

Either the precipitate or the supernatant solution was used for bilirubin measurement. The former was dissolved in water to an appropriate volume, cleared by centrifugation, and measured either by the van den Bergh reaction as described by Malloy and Evelyn (5) or spectrophotometrically at 460 mμ in the Beckman. The supernatant solution when used was also measured spectrophotometrically. With appropriate standardization the amount of bilirubin precipitated could be calculated in each instance.

As is shown in Fig. 3, the fractionation pattern differed markedly with the two types of pigment. The patterns shown by the bilirubin from all
the four sera containing the indirect pigment were similar and followed the distribution shown for globulin rather closely (8). On the other hand, the patterns shown by the bilirubin from the two sera of obstructive jaundice followed that of albumin (8). This was further confirmed by the use of brom phenol blue. It was shown (9) that the dye combined only with the albumin fraction of serum when added in appropriate amounts enough to

![Ammonium sulfate fractionation pattern of serum with indirect bilirubin (from a patient with congenital familial jaundice with kernicterus) (▲) and the fractionation pattern of serum with direct bilirubin (from a patient with congenital atresia of the bile ducts) (●) to which brom phenol blue was added (○) in 0.06 mg. per ml. of serum. The precipitate from each fraction was dissolved in water, and bilirubin was measured at 460 mλ and brom phenol blue at 605 mλ in the Beckman spectrophotometer model DU.](http://www.jbc.org/)

stain serum blue. We found that the dye in serum has a sharp absorption maximum at 605 mλ, at which wave-length bilirubin shows practically no measurable absorption. Furthermore, at 460 mλ the absorption by the dye is insignificant. This made it possible to measure quantitatively both bilirubin and brom phenol blue in the same sample of serum without resort to corrections. Brom phenol blue was added in amounts of 0.04 to 0.08 mg. per ml. of serum and ammonium sulfate fractionation carried out in the usual manner. Fig. 3 shows that the pattern exhibited by the dye was similar to that of the bilirubin in the direct serum and different from that
of the bilirubin in the indirect serum. The distribution maximum of the bilirubin in fresh direct serum fell to about 0.54 to 0.58 saturation and that of the indirect to about 0.44 to 0.48.

When direct serum was diluted with distilled water 1:5 and incubated under toluene for 4 to 16 hours at 37°, some of the pigment became indirect and, upon fractionation with ammonium sulfate, there was found a definite shift in the fractionation pattern towards the lower salt concentrations. Furthermore, that portion that had become indirect could be extracted with ether at pH 5.0 and crystallized as described earlier. Aging of direct sera in the ice box for a few weeks also showed a similar shift to the indirect. It appears, then, that by such means one can effect a change from direct to indirect. For this reason we have found it necessary to keep direct sera at 4° as soon as possible after the blood is drawn and perform our studies on the same day.

In an earlier report (10) the possibility was suggested that direct bilirubin is a metal complex of the pigment stabilized by attachment to serum albumin. This was based on indirect evidence. We have attempted to study this aspect by direct observation of a possible metal complex formation with bilirubin which might show a shift in the titration curve and absorption spectrum. In the former, the fact that bilirubin was insoluble in water below pH 8.0 made the result difficult to interpret. We have not been able to find a good solvent for the pigment that is water-miscible and therefore suitable for titration. In either method there is the added complication that bilirubin at high pH is oxidized to biliverdin. This we found to be particularly rapid in the presence of bivalent cations such as Co++, Fe++, Cu++, Ca++, and Mn++. The formation of biliverdin would in itself show an alteration of the absorption spectrum, thereby nullifying any effect the metal may have on the spectrum of bilirubin.

The fact that heavy metal ions catalyze the oxidation of the pigment is presumptive evidence of complex formation during the process. On that basis the existence of a metal complex in the direct pigment may well be responsible for the strikingly rapid oxidation of that pigment to biliverdin (2) as compared to the indirect. Furthermore, when thoroughly dialyzed icteric serum is precipitated with trichloroacetic acid, the pigment from direct serum is quickly oxidized and imparts a deep green color to the precipitate, whereas that from indirect serum remains yellow and stable under these conditions. The oxidation of the direct pigment in alkali and that in trichloroacetic acid are counteracted by ethylenediaminetetraacetic acid (Versene) and 8-hydroxyquinoline, both metal-binding agents. These observations lend further support to the suggestion that the direct pigment is a metal complex combined with albumin.
SUMMARY

1. A method for the crystallization of indirect bilirubin from serum is described.

2. With ammonium sulfate fractionation of serum the direct pigment is precipitated with the albumin fraction and the indirect with the globulin fraction.

3. There is strong suggestive evidence that the direct bilirubin is a metal complex although direct and conclusive evidence is still lacking.

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