THE DETERMINATION OF BILIRUBIN WITH THE PHOTOELECTRIC COLORIMETER

By HELGA TAIT MALLOY* AND KENNETH A. EVELYN*

(From the Department of Medicine, McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

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The main source of error in the determination of bilirubin in serum by the diazo reaction has been the loss caused by adsorption on the protein precipitate. In addition, the lack of an accurately matching artificial standard and the sensitivity of the azobilirubin color to changes in pH have made accurate colorimetric determinations impossible, without the aid of some type of objective photometer such as the spectrophotometer, the Pulfrich photometer, or the photoelectric colorimeter (1-4).

In this paper we shall describe a method for the quantitative determination of both direct and indirect bilirubin in serum, in which protein precipitation and consequent loss of bilirubin have been eliminated. By a slight modification of the method, a quantitative study of the behavior of the direct reaction has also been made possible. Artificial standards have been eliminated in the colorimetric determinations by the use of the photoelectric colorimeter (5) with a specially selected light filter.

Selection of Color Filter

The spectrophotometric curve of the rose-mauve color of the azobilirubin solutions obtained by the method to be described below has a single broad absorption band at 540 mµ (Fig. 1). We have therefore chosen a filter which transmits a narrow spectral band in the vicinity of 540 mµ, so that light which has passed through the filter is readily absorbed by solutions of azobilirubin. This filter has the further advantage of being unaffected by the

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presence of the yellow serum pigments whose absorption at 540 $m\mu$ is negligible. The only other interfering color is that due to the possible presence of hemoglobin in the serum. Although this pigment does absorb light in the vicinity of 540 $m\mu$, any error from this source is obviated by the use of a blank tube in the initial adjustment of the instrument.

**Indirect Reaction**

Van den Bergh (6) assumed that indirect bilirubin in serum would only react with diazo reagent in the presence of alcohol,

![Spectrophotometric curves of azobilirubin, Filter 540, and jaundiced serum.](http://www.jbc.org/)
after the proteins had been removed by precipitation. Our experiments, however, have proved that complete coupling of indirect bilirubin will take place in the presence of serum proteins provided the alcohol concentration is of the order of 50 per cent. This concentration can be achieved without protein precipitation if the serum is first diluted with water, and this procedure forms the basis for our technique for indirect bilirubin. This not only eliminates loss of bilirubin on the protein precipitate, but also provides a buffer substrate sufficient to stabilize the pH-sensitive color of the azobilirubin.

Method

Reagents—
1. Solution A, 1.0 gm. of sulfanilic acid dissolved in 15 cc. of concentrated HCl, and diluted to 1 liter with water.
2. Solution B, 0.5 per cent sodium nitrite.
3. Diazoreagent freshly prepared by adding 0.3 cc. of Solution B to 10 cc. of Solution A.
4. Hydrochloric acid for blank tubes (diazo blank), 15 cc. of concentrated HCl in 1 liter of water.
5. Absolute methyl alcohol. The use of absolute methyl alcohol is recommended, since it yields clearer solutions than 95 per cent ethyl alcohol. The amount of color produced is, however, unaltered.

Procedure—Two colorimeter tubes are set up as follows: Tube 1, indirect blank, 5 cc. of absolute methyl alcohol and 1 cc. of diazo blank solution; Tube 2, indirect sample, 5 cc. of absolute methyl alcohol and 1 cc. of diazo reagent.

1 cc. of serum or plasma is diluted to 10 cc. with distilled water, and 4 cc. of the diluted material are added to each tube. The contents are mixed by inversion, care being taken to handle both tubes in the same way, so that any turbidity which may result from too vigorous shaking will be the same in both tubes. If bubbles form they are best removed by gentle tilting and rotation of the tubes.

Tube 2 is read in the colorimeter with Filter 5401 30 minutes.

1 This filter is one of the set of eight filters which have been selected for use with the photoelectric colorimeter. Any of these filters may be obtained from the Rubicon Company, 29 North 6th Street, Philadelphia. The complete colorimeter may also be obtained from the Rubicon Company.
after addition of the serum, Tube 1 being used for the initial adjustment of the galvanometer. If the galvanometer reading is less than 10, it is advisable for the sake of greater accuracy to dilute both tubes with 10 cc. of 50 per cent methyl alcohol and read again immediately. In this case, the final answer in mg. per 100 cc. must be multiplied by 2.

**Calculation**—The bilirubin concentration in mg. per 100 cc. of serum is obtained from the formula

\[
x = \frac{2 - \log G}{6.72} \times 100
\]

where \(X\) is mg. of bilirubin per 100 cc. of serum, and \(G\) is the galvanometer reading.

For routine determinations, a calibration curve may be made from this formula from which the values for \(X\) may be read directly (Fig. 2).

**Calibration**—Since the concentration of azobilirubin in a solution is proportional to the negative logarithm of the light transmission, the following formula is valid

\[
C = \frac{2 - \log G}{K_1}
\]

where \(C\) is the concentration of azobilirubin (expressed as bilirubin) in mg. per cc. of colored solution, \(G\) is the galvanometer reading, and \(K_1\) is a constant.

Conversion of the bilirubin concentration in terms of mg. per cc. of colored solution to mg. per 100 cc. of serum is made by means of the formula

\[
x = \frac{2 - \log G}{K_1} \times \frac{V}{A} \times 100
\]

where \(X\) is mg. of bilirubin per 100 cc. of serum, \(V\) is the volume of colored solution, and \(A\) is the amount of serum used.

Thus in the method described above

\[
x = \frac{2 - \log G}{K_1} \times \frac{10}{0.4} \times 100
\]

Calibration of the instrument consists essentially in determining the value of \(K_1\) by obtaining the galvanometer readings for known
concentrations of bilirubin. For this purpose about 10 mg. (accurately weighed) of pure bilirubin$^2$ were dissolved in 100 cc. of chloroform. Portions were withdrawn from this solution and diluted with ethyl alcohol to a final concentration of 0.01 mg. per cc. Varying amounts of the alcoholic solutions were placed in a series of colorimeter tubes to which were added 1 cc. of diazo reagent and sufficient ethyl alcohol to make the final volume 10 cc. The tubes were read at 5 minute intervals until the color was at a maximum. The known values for $C$ and $G$ were then substituted in Equation 1, and the value of $K_1$ was found to be 168.0. Since all photoelectric colorimeters of this type are interchangeable with respect to calibration, we recommend that this value of $K_1$ be generally adopted to insure uniformity of results.

$^2$ We have tested both Eastman Kodak and Hoffmann-La Roche bilirubin, but have used the latter for calibration, since the yield of color is 10 per cent higher. Moreover, van den Bergh and Grotepass (7) state that they have found Hoffmann-La Roche bilirubin to be identical with the chemically pure bilirubin prepared by Professor Hans Fischer.
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Substituting $K_1$ in Equation 3 above we obtain

$$x = \frac{2 - \log G}{6.72} \times 100 \text{ mg. bilirubin per 100 cc. serum}$$

The value 6.72 we have termed $K_2$.

Results

Effect of Alcohol Concentration upon Color Development—The curve in Fig. 3 marked 0 per cent shows the color produced when diazo reagent, without alcohol, is added to serum. By definition, this must be due to direct bilirubin only. The addition of alcohol in increasing amounts causes more and more of the indirect bilirubin to take part in the reaction, until at concentrations above 40 per cent the indirect reaction is complete. An alcohol concentration of 50 per cent was chosen for the method, since it affords an adequate margin of safety, yields perfectly clear solutions, and produces maximum color in a shorter time than 40 per cent.
Recovery of Bilirubin Added to Serum—Table I shows that bilirubin added to serum is recovered with a maximum error of

| Table I |

Recovery of Pure Indirect Bilirubin Added to Normal and Jaundiced Sera

0.4 cc. of serum was used in each case. The figures in the second column represent the amount of bilirubin present in this 0.4 cc., as measured directly on the instrument. The first five experiments were made on normal sera. (Normal values obtained by our method are between 0.2 and 0.8 mg. per 100 cc.)

<table>
<thead>
<tr>
<th>Bilirubin per 100 cc. serum</th>
<th>Bilirubin in 0.4 cc. serum</th>
<th>Bilirubin added to 0.4 cc. serum</th>
<th>Total bilirubin present</th>
<th>Amount of bilirubin measured</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg. × 10⁻⁴</td>
<td>mg. × 10⁻⁴</td>
<td>mg. × 10⁻⁴</td>
<td>mg. × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>1.0</td>
<td>4.4</td>
<td>5.4</td>
<td>5.5</td>
<td>101.8</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>8.8</td>
<td>10.8</td>
<td>10.6</td>
<td>98.1</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>50.0</td>
<td>52.0</td>
<td>50.0</td>
<td>96.1</td>
</tr>
<tr>
<td>0.75</td>
<td>3.0</td>
<td>34.0</td>
<td>37.0</td>
<td>36.4</td>
<td>97.7</td>
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<td>80.0</td>
<td>83.0</td>
<td>80.0</td>
<td>96.3</td>
</tr>
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<td>4.0</td>
<td>43.6</td>
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<td>92.0</td>
<td>95.0</td>
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<td>31.2</td>
<td>38.0</td>
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<td>2.7</td>
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<td>40.0</td>
<td>98.0</td>
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<td>3.2</td>
<td>12.8</td>
<td>50.0</td>
<td>62.8</td>
<td>63.4</td>
<td>101.0</td>
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<tr>
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<td>50.0</td>
<td>66.0</td>
<td>67.4</td>
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<td>96.5</td>
</tr>
<tr>
<td>8.0</td>
<td>32.0</td>
<td>40.0</td>
<td>72.0</td>
<td>73.2</td>
<td>101.9</td>
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<td>20.0</td>
<td>84.0</td>
<td>83.6</td>
<td>99.7</td>
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</table>

| Table II |

Comparison of Determinations Made on Same Sera by Different Techniques

All the figures represent mg. per 100 cc. of serum. All the final colorimetric measurements were made on the photoelectric colorimeter.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
<th>Experiment 5</th>
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</thead>
<tbody>
<tr>
<td>Van den Bergh and Grotpass (7) (indirect reaction)</td>
<td>2.0</td>
<td>4.2</td>
<td>3.1</td>
<td>7.8</td>
<td>12.4</td>
</tr>
<tr>
<td>Thannhauser and Andersen (8)</td>
<td>3.3</td>
<td>4.9</td>
<td>4.6</td>
<td>9.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Jendrassik and Czike (9)</td>
<td>3.4</td>
<td>4.9</td>
<td>5.2</td>
<td>10.5</td>
<td>15.1</td>
</tr>
<tr>
<td>Malloy and Evelyn (indirect reaction)</td>
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<td>6.5</td>
<td>9.0</td>
<td>15.2</td>
<td>22.8</td>
</tr>
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</table>

±4 per cent, the average error being only 2 per cent. Since the calibration curve used in these experiments was made with known
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amounts of pure indirect bilirubin in alcoholic solution, the results prove that the presence of serum proteins does not interfere with the diazo reaction of bilirubin, or with the quantitative determination of the resulting azobilirubin by means of the photoelectric colorimeter.

Duplicate determinations on serum will usually agree within \( \pm 1 \) per cent, with a maximum variation of \( \pm 2 \) per cent.

Comparison with Other Methods—The results of duplicate analyses on the same sera by different methods are shown in Table II. All the final colorimetric measurements were made on the photoelectric colorimeter so that any discrepancies which occurred could only have been due to differences in the preliminary treatment of the serum. From Table II we conclude that (1) precipitation of proteins before addition of the diazo reagent, as recommended by van den Bergh and Grotepass (7), causes a large and variable loss of bilirubin (30 to 60 per cent); (2) this loss can be decreased but not eliminated by the technique of Thannhauser and Andersen (8), in which the diazo reagent is added before the proteins are precipitated; (3) the addition of caffeine sodium benzoate, as suggested by Jendrassik and Czike (9), is a further slight improvement, but the recovery of azobilirubin is still seldom more than 70 per cent, as compared with our method.

Direct Reaction

Van den Bergh and Grotepass (7), have recommended a quantitative method for the direct reaction, but, since 25 per cent alcohol is used, the method measures not only direct bilirubin but also a fraction of the indirect (see Fig. 3). The results obtained are therefore too high, and since the amount of direct bilirubin in any serum bears no constant relationship to the amount of indirect bilirubin, the error involved is variable.

For routine determinations in which a clinical interpretation is required, we recommend that the direct reaction be carried out in the accustomed manner. For a more quantitative study of the behavior of the direct reaction, we have, however, adopted the following procedure: Two tubes are set up as in the method for the indirect reaction except that 5 cc. of water are substituted for 5 cc. of methyl alcohol. Readings are made on the photoelectric colorimeter at 10, 30, 60, and 120 minutes, and the corre-
sponding bilirubin concentrations, obtained from the calibration curve, are plotted to show development of color with time.

Curve C of Fig. 4 shows the slow development of color from a serum of the "delayed" type, in which the true end-point of the reaction is not attained for several hours. Curve A shows the rapid development of maximum color typical of sera of the "prompt" type. Curve B shows a reaction intermediate between these extremes, which corresponds to what is usually termed the "biphasic" reaction.

In addition, the final galvanometer reading is used to determine the concentration of direct bilirubin in the serum. Sera have been found to differ not only in the shape of their color development curves, but also in the ratio of the amounts of direct and indirect bilirubin. The clinical significance of these two variables will be discussed elsewhere.

SUMMARY

1. A method has been described for the accurate photoelectric determination of both direct and indirect bilirubin in serum, in
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which protein precipitation and consequent loss of bilirubin by adsorption have been eliminated.

2. The interfering effect of yellow serum pigments in the color determination has been overcome by the use of a specially selected light filter, which also eliminates the necessity for artificial color standards.

3. It has been shown that all the bilirubin in serum will react with diazo reagent even in the presence of serum protcins, provided a sufficiently high concentration of alcohol (50 per cent) is present.

4. The new method has been found to give higher and consistently more accurate results than any of the older methods with which it has been compared. Bilirubin added to serum is recovered with an average error of ±2 per cent.

5. By a modification of the method a quantitative study of the behavior of the direct reaction of bilirubin in serum has been made possible.

BIBLIOGRAPHY

6. van den Bergh, A. A. H., Der Gallenfarbstoff im Blute, Leipsic (1918).
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Helga Tait Malloy and Kenneth A. Evelyn