New Tests for Bile and Detection of Bile in Serum and Urine

M. Z. Barakat, S. K. Shehab, and M. M. El-Sadr

The purposes of the present paper are to describe some new sensitive tests suitable for the detection of bile in biologic fluids which overcome the defects demonstrated in procedures described in most standard texts, and also to confirm the experiments of C. E. May et al. (1). The sensitivity of the proposed oxidation tests to detect bilirubin, viz. ferricyanide, molybdate, N-bromosuccinimide (2, 3), N-chlorosuccinimide (4), and dichromate tests, is compared with the previously known Gmelin, Fouchet, Obermeyer, Nakayama, iodine and nitrite tests. Similarly a comparison of the proposed diazo tests, i.e., diazochlorides obtained from various members of sulfonamides, as well as from the aromatic amines previously tested by May et al. (1), with the previously known diazotized sulfanilic acid test is reported.

Materials

1. Potassium ferricyanide reagent: To 12.5 Gm. of trichloroacetic acid in 50 ml. of distilled water add 5 ml. of 10 per cent (w/v) potassium ferricyanide aqueous solution and mix well. The reagent is kept in a dark bottle.
2. Saturated aqueous ammonium molybdate solution.
3. 0.1% (w/v) N-bromosuccinimide aqueous solution.
4. 0.1% (w/v) N-chlorosuccinimide aqueous solution.
5. 7% (w/v) aqueous potassium dichromate solution.
6. Stock Solutions:

   Diazo I: 0.1 Gm. of the aromatic amino compound, e.g., anthranilic acid, etc., was dissolved in 1.5 ml. of concentrated HCl and some

From the Biochemistry Department, Faculty of Medicine Abbassia, Ein-Shams University, Cairo, Egypt.
Received for publication June 29, 1956.
distilled water or 20 ml. of alcohol in the case of α- or β-naphthylamine and diluted with distilled water to 100 ml. This solution should be prepared fresh daily or refrigerated at 4° for storage.

**Diazo II**: 0.5% (w/v) aqueous sodium nitrite solution. *Diazo Reagent* was freshly prepared as follows:

To 25 ml. of Diazo I, add 0.75 ml. of Diazo II and mix well. The diazo reagent must be kept in a dark bottle, especially in the case of α-naphthylamine diazo reagent which becomes a strong purple on standing exposed to light.

7. Bilirubin Solution is prepared by dissolving 5 mg. of bilirubin in cold, neutral alcohol, then add 2 ml. of 2% (w/v) NaOH aqueous solution and diluted with neutral alcohol to 100 ml. One milliliter of this solution contains 50 μg. of bilirubin.

8. The previously known Fouchet’s, Nakayama’s, and Obermeyer’s reagents have been prepared according to the literature.

**METHODS AND EXPERIMENTAL RESULTS**

**Experiments on Bilirubin Test Solutions**

**Oxidation Tests**

Starting with 3 ml. of the bilirubin solution, i.e., containing 150 μg. of bilirubin, various dilutions were prepared by mixing 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 ml. of the bilirubin solution with 1, 2, 2.2, 2.4, 2.6, 2.8, and 2.9 ml. of distilled water, respectively, to obtain the various concentrations of bilirubin in 3 ml. solution (Table 1), on which the proposed oxidation tests were carried out at the same time with those previously known.

**Table 1. Comparative Sensitivity of the New Oxidation Tests with Some Previous Tests**

<table>
<thead>
<tr>
<th>Test</th>
<th>Bilirubin content in μg./3 ml. of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Gmelin’s</td>
<td>-</td>
</tr>
<tr>
<td>Fouchet’s</td>
<td>+</td>
</tr>
<tr>
<td>Obermeyer’s</td>
<td>+</td>
</tr>
<tr>
<td>Nakayama’s</td>
<td>+</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>+</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>+</td>
</tr>
<tr>
<td>N-Bromoeuconimide</td>
<td>+</td>
</tr>
<tr>
<td>N-Chloroeuconimide</td>
<td>+</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>+</td>
</tr>
</tbody>
</table>

All tests gave positive results on bilirubin solutions containing 50 μg./3 ml. or higher.
Table 2. Comparison of the Sensitivity of the New Coupling Tests with Ehrlich's Diazo Test

<table>
<thead>
<tr>
<th>Diazo reagent of</th>
<th>No. drops of diazo reagent</th>
<th>Bilirubin content in μg./ml. of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Benzidine</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Aniline</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>α- or β-Naphthylamine</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Sulfathioisole</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>5</td>
<td>+</td>
</tr>
</tbody>
</table>

All tests were positive when bilirubin concentration was 15 μg./ml. or higher.

Diazo Tests

Coupling tests for bilirubin were carried out by adding 2 to 5 drops of the diazo reagent to 1 ml. of the bilirubin solution, i.e., containing 50 μg. of bilirubin or of the various dilutions such as those obtained by mixing 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 ml. of the bilirubin solution with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 ml. of distilled water, respectively (Table 2). A purple-violet color was formed with the diazochlorides investigated.

The previously tested diazochlorides prepared from, e.g., aniline, α- and p-toluidine, benzidine, etc., did not give any color when bilirubin was dissolved in neutral alcohol; the purple-violet color appeared only on the addition of one drop of 2% (w/v) NaOH.

In the case of low concentrations of bilirubin, starting from 15 μg./ml., one or two drops of the diazo reagent were only added to detect bilirubin.

The blank test was always colorless or pale yellow even upon addition of 2 drops of 2% NaOH, except in the case of the α-naphthylamine diazo reagent. When 1 ml. of neutral alcohol was treated with 5 drops of this latter diazo reagent, a faint purple color appeared.

Experiments on Bile

The following new tests have been applied on fresh ox bile.

Tests for Bile Pigments

I. (a) To 5 ml. of diluted bile (5%) in a test tube add 2 ml. of the potassium ferricyanide reagent, a bluish-green color appears.
(b) To 5 ml. of the diluted bile add 0.5 ml. of 10% potassium ferricyanide solution and add 3 ml. of conc. $\text{H}_2\text{SO}_4$ down the side of the test tube. At the point of contact green and red rings appear. A blank shows no colored rings.

II. To 5 ml. of the diluted bile add 2 ml. of saturated ammonium molybdate solution and then 3 ml. of conc. $\text{H}_2\text{SO}_4$ added down the side of the test tube; a blue ring appears at the interface of the two liquids. On shaking, the solution assumes a blue color. A control, using water alone, is colorless.

III. To 5 ml. of the diluted bile add 2 drops of 0.1% (w/v) $N$-bromosuccinimide or $N$-chlorosuccinimide aqueous solution; a blue color is produced. A blank is colorless.

IV. To 5 ml. of the diluted bile add 0.5 ml. of 7% potassium dichromate solution, then carefully add 3 ml. of conc. $\text{H}_2\text{SO}_4$ so that the two fluids do not mix. At the point of contact a blue ring appears. A blank, using water alone, shows an orange-red color.

V. To 5 ml. of the diluted bile, add 2 ml. of freshly prepared diazo reagent of any of the following aromatic amino compounds: aniline, $p$-toluidine, anthranilic acid, etc. In this test the compound azobilirubin is formed either directly or after the addition of 2 to 4 drops of 2% NaOH as indicated in the following table.

<table>
<thead>
<tr>
<th>Diazo Reagent of</th>
<th>2% NaOH</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Aminobenzoic acid</td>
<td>—</td>
<td>Purple-violet</td>
</tr>
<tr>
<td>$p$-Nitroaniline</td>
<td>—</td>
<td>Pink</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>—</td>
<td>Purple-violet</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>—</td>
<td>Purple-violet</td>
</tr>
<tr>
<td>Sulfathiazone</td>
<td>—</td>
<td>Purple-violet</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline</td>
<td>+</td>
<td>Purple</td>
</tr>
<tr>
<td>$p$-Toluidine</td>
<td>+</td>
<td>Purple</td>
</tr>
<tr>
<td>$\alpha$- or $\beta$-Naphthylamine</td>
<td>+</td>
<td>Pink</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>+</td>
<td>Purple</td>
</tr>
</tbody>
</table>

A blank using water instead of bile solution, is either colorless or very faint purple, e.g., in the case of the diazo reagent prepared from $p$-toluidine or $\alpha$- and $\beta$-naphthylamines.

Interfering Substances

Various substances and, in particular, reducing substances that occur in normal and pathologic serum and urine and which might interfere with the proposed tests have been investigated.

With the exception of the molybdate test, no interference occurs with
the proposed tests for bile pigments in the presence of the following substances: Glucose and lactose; ascorbic acid, thiamine hydrochloride, riboflavin, and nicotinic acid; oxalates, tartrates, and citrates; urea and uric acid; formaldehyde, acetone, and phenol; creatine and creatinine; amino-acids such as alanine and cysteine hydrochloride; chloroform, ethyl alcohol, and ether.

The molybdate test is interfered with only in the presence of glucose in excess, ascorbic acid, cysteine hydrochloride, and hydroquinone.

Detection of Bilirubin in Pathologic Serum (Bilirubinemia)

All the following tests are carried out on a white porcelain slab.

I. To 2 drops of the pathologic serum add 2 drops of the potassium ferricyanide reagent; a blue or green precipitate appears according to the bilirubin content in the sample. With normal serum a white precipitate is obtained.

II. To 2 drops of the pathologic serum add 2 drops of saturated ammonium molybdate solution and 2 drops of 60% (v/v) conc. H₂SO₄; a blue color appears. Normal serum gives a white precipitate.

III. To 2 drops of the pathologic serum add 2 drops of the diazo reagent prepared from any of the following aromatic amino compounds and mix well with a clean glass rod. A pink or purple color which may show a violet tint is immediately observed or appears after the addition of 2 drops of 2% NaOH solution as shown below. With normal serum no color develops or only a yellow color appears upon the addition of 2% NaOH.

When 2 drops of the diazo reagent are treated with 2 drops of 2% NaOH no color or a yellow color only develops.

<table>
<thead>
<tr>
<th>Diazo Reagent of</th>
<th>2% NaOH</th>
<th>Pathologic Serum</th>
<th>Normal Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitroaniline</td>
<td>—</td>
<td>Pink</td>
<td>Colorless</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>—</td>
<td>Purple-violet</td>
<td>Colorless</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>2 drops</td>
<td>Purple-violet</td>
<td>Yellow</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>2 drops</td>
<td>Purple-violet</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>—</td>
<td>Brilliant pink</td>
<td>Colorless</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>—</td>
<td>Purple-violet</td>
<td>Colorless</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>—</td>
<td>Purple-violet</td>
<td>Colorless</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>—</td>
<td>Purple-violet</td>
<td>Colorless</td>
</tr>
</tbody>
</table>

In the case of the diazo reagent prepared from sulfa drugs, if 2 drops of 2% NaOH are added, a blue color is obtained with pathologic serum while a yellow color is observed with normal serum.

Comparative sensitivity of the proposed oxidation and diazo tests, to
Table 3. COMPARATIVE SENSITIVITY OF PROPOSED AND SOME PREVIOUSLY KNOWN TESTS AFTER 5 MINUTES ON PATHOLOGIC SERUM

<table>
<thead>
<tr>
<th>Tests</th>
<th>1/50</th>
<th>1/20</th>
<th>1/10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fouchet’s test</td>
<td>+</td>
<td>Trace</td>
<td>—</td>
</tr>
<tr>
<td>Nitrite test</td>
<td>+</td>
<td>Trace</td>
<td>—</td>
</tr>
<tr>
<td>Potassium ferricyanide test</td>
<td>+</td>
<td>+</td>
<td>Trace</td>
</tr>
<tr>
<td>Ammonium molybdate test</td>
<td>+</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Diazo Reagent of:—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>+</td>
<td>Trace</td>
<td>—</td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>+</td>
<td>Trace</td>
<td>—</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>+</td>
<td>+</td>
<td>Trace</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>+</td>
<td>+</td>
<td>Trace</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>+</td>
<td>Trace</td>
<td>—</td>
</tr>
</tbody>
</table>

All tests gave positive results on dilutions of serum less than 1/50.

detect bilirubin in pathologic serum, with the previously known Fouchet, nitrite, and Ehrlich diazo tests respectively is shown in Table 3.

Detection of Bilirubin in Pathologic Urine (Obstructive Jaundice)

I. To 2 ml. of pathologic urine in a clean test tube add 2 ml. of the potassium ferricyanide reagent, and mix well by shaking; a greenish-blue color develops immediately. To 2 ml. of normal urine, add 2 ml. of the reagent and shake well; a yellow color appears.

II. To 2 ml. of pathologic urine, add 1 ml. of saturated ammonium molybdate solution and add carefully down the side of the test tube, 3 ml. of 60% (v/v) H₂SO₄. A green ring appears at the interface of the two liquids and the green color gradually spreads in the upper aqueous layer. On shaking, the whole solution assumes a dull green color, and a bluish-green precipitate deposits on standing.

With normal urine, under parallel conditions, a violet ring appears gradually after a while at the interface of the two liquids; on shaking a violet color appears in the whole solution, and on standing a violet precipitate is deposited.

III. To 2 ml. of pathologic urine, add from 3 to 12 drops of 1% (w/v) aqueous N-bromosuccinimide (2, 3) or N-chlorosuccinimide (4) solution according to the bilirubin content, drop by drop. With shaking a bluish-
green color develops and changes to olive green on standing. With normal urine, under parallel conditions, no change in the color occurs.

IV. To 2 ml. of pathologic urine, add 3 to 10 drops of the diazo reagent prepared from any of the aromatic amino compounds, drop by drop, with shaking. A purple or reddish-purple color is formed, either immediately or after the addition of 10 drops of 2% NaOH as stated below; the color does not change on standing. With normal urine, under parallel conditions, the color is unaffected or becomes deep yellow or faint orange.

A blank using water instead of urine, treated with the diazo reagent and 2% NaOH, remains colorless or assumes a yellow, pale pink, or faint orange color.

<table>
<thead>
<tr>
<th>Diazo Reagent of</th>
<th>No. of drops</th>
<th>% NaOH</th>
<th>Pathologic Urine</th>
<th>Normal Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic acid</td>
<td>3</td>
<td>–</td>
<td>Purple</td>
<td>No change</td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>5</td>
<td>–</td>
<td>Reddish purple</td>
<td>Deep yellow</td>
</tr>
<tr>
<td>m-Nitroaniline</td>
<td>5</td>
<td>–</td>
<td>Reddish purple</td>
<td>Faint orange</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>No change</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>No change</td>
</tr>
<tr>
<td>α- or β-Naphthylamine</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>Orange &amp; turbid</td>
</tr>
<tr>
<td>Aniline</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>Yellow &amp; turbid</td>
</tr>
<tr>
<td>α-Toluidine</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>Yellow &amp; turbid</td>
</tr>
<tr>
<td>m-Toluidine hydrochloride</td>
<td>10</td>
<td>+</td>
<td>Reddish purple</td>
<td>Pale yellow and turbid</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>10</td>
<td>–</td>
<td>Reddish purple</td>
<td>Orange</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>10</td>
<td>–</td>
<td>Reddish purple</td>
<td>Orange yellow</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>10</td>
<td>–</td>
<td>Reddish purple</td>
<td>Orange yellow</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>10</td>
<td>–</td>
<td>Reddish purple</td>
<td>Orange yellow</td>
</tr>
</tbody>
</table>

Comparison of the sensitivity of the proposed oxidation and diazo tests, to detect bilirubin in pathologic urine, with the previously known Fouchet, Obermeyer, iodine test, and diazotized sulfanilic acid test respectively is described in Table 4.

Application of Spot Tests for Bile Pigments on Urine

Spot tests based on oxidation or coupling are proposed to detect bilirubin in urine. Precipitation of the bile pigments is effected by adding, to 25 ml. of the urine sample, a saturated solution of barium chloride (about 5 ml.) and then filtering. Two drops of the reagent are sufficient to detect bilirubin.

<table>
<thead>
<tr>
<th>Test</th>
<th>Pathologic Urine</th>
<th>Normal Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium ferricyanide</td>
<td>Blue-green</td>
<td>–</td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>Olive green</td>
<td>–</td>
</tr>
<tr>
<td>N-Chlorosuccinimide</td>
<td>Olive green</td>
<td>–</td>
</tr>
<tr>
<td>p-Nitroaniline (diazochloride)</td>
<td>Purple</td>
<td>–</td>
</tr>
<tr>
<td>p-Aminobenzoic acid (diazochloride)</td>
<td>Purple after 5 minutes</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 4. COMPARISON OF THE SENSITIVITY OF PROPOSED TESTS WITH SOME PREVIOUSLY ACCEPTED TESTS AFTER 5 MINUTES ON PATHOLOGIC URINE (OBSTRUCTIVE JAUNDICE)

<table>
<thead>
<tr>
<th>Tests</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation</td>
<td></td>
</tr>
<tr>
<td>Fouchet's test</td>
<td>+ + Tr.</td>
</tr>
<tr>
<td>Obermeyer's test</td>
<td>+ + Tr.</td>
</tr>
<tr>
<td>Iodine test</td>
<td>+ + +  Tr.</td>
</tr>
<tr>
<td>Potassium ferricyanide test</td>
<td>+ + +  Tr.</td>
</tr>
<tr>
<td>Ammonium molybdate test</td>
<td>+ + + +  Tr.</td>
</tr>
<tr>
<td>N-Bromosuccinimide test</td>
<td>+ +  Tr.</td>
</tr>
<tr>
<td>Diozo Reagent of:—</td>
<td></td>
</tr>
<tr>
<td>Sulphanilic acid</td>
<td>+ + + + + +  Tr.</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>+ + + + + +  Tr.</td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>+ + + + + +  Tr.</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>+ + + + + +  Tr.</td>
</tr>
<tr>
<td>Anthraquinone acid</td>
<td>+ + + + + +  Tr.</td>
</tr>
<tr>
<td>a- &amp; p-Naphthylamine</td>
<td>+ +  Tr.</td>
</tr>
<tr>
<td>Aniline</td>
<td>+ + + + +  Tr.</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>+ + + + + + +  Tr.</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>+ + + + + + +  Tr.</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>+ + + + + + +  Tr.</td>
</tr>
<tr>
<td>Sulfaflazole</td>
<td>+ + + + + + + +  Tr.</td>
</tr>
</tbody>
</table>

Urine from patient with intense obstructive jaundice was diluted with mixed routine hospital urine.

When 2 drops of 7% potassium dichromate solution and 2 drops of 60% (v/v) H$_2$SO$_4$ are added to the precipitate, both the pathologic and normal samples show the same play of colors (green, blue, and purple).

**DISCUSSION**

For the detection of bilirubin by the proposed tests two principles are involved: (a) the controlled oxidation of bilirubin to biliverdin and (b) the formation of azobilirubin.

The controlled oxidation of bilirubin to biliverdin by ferric chloride in trichloroacetic acid (Fouchet's reagent) (5) or hydrochloric acid (Obermeyer's reagent) (6) has proved superior to the older method of progressive oxidation by nitric acid or iodine of Foord (7). Several tests have already been reported depending on the oxidation principle, but in this investigation only the following common tests have been considered, viz. the well-known Gmelin, Fouchet, Nakayama (8), and Obermeyer (6) tests. Comparative sensitivity of these tests and the proposed tests towards the detection of bilirubin in pure solution is shown in Table 1.
New tests based on the formation of azobilirubin by various modifications of Ehrlich's diazo reagent have been devised. Some of these surpass the most sensitive and highly specific tests available at present (see Table 2), except the diazo tablet test which requires no special equipment.

We have tried again the diazochlorides of the amines previously tested by Clarence E. May et al. (1), as well as those of other aromatic amines, e.g. sulfonamides, and were able to obtain stable and highly colored products. When bilirubin was dissolved in neutral alcohol, the color did not appear with the diazochlorides of any of the previously tested amines; the color appeared only after adding one drop of 2% NaOH. In a blank experiment, when 1 ml. of neutral alcohol was treated with 2 drops of the diazochloride of either, for example, p-toluidine or anthranilic acid, even on adding one drop of 2% NaOH, the solution remained colorless; with other diazochlorides, the solution assumed a yellow or very faint purple color.

It has already been reported that bilirubin dissolves only in alkaline solution (pH 8.9–14); in neutral and acid media it is precipitated. The diazo reaction therefore is positive only in alkaline solution; in acid solution no compound with the diazo reagent is formed. The reaction with bilirubin of bile or urine, on the other hand, occurs at all pH values (1.0–14) except in solutions highly acid with inorganic acids (9).

Comparison of the sensitivity of the proposed tests with some previously and most generally accepted tests for bile pigments on pathological serum and urine is reported in Tables 3 and 4.

SUMMARY

1. New tests proposed for the detection of bilirubin have been described.
2. Application of some of the proposed tests on pathologic and normal serum and urine have been demonstrated.
3. The diazochlorides of certain amines previously reported as failures to detect bilirubin have been shown to be successful and have been applied to bile, serum, and urine.
4. Comparative sensitivity of the proposed tests and the most generally accepted tests for bilirubin in test solutions, pathologic serum, and urine have been given.
5. Spot tests suitable for the detection of bilirubin in pathologic urine have been presented.
REFERENCES

5. Fouchet, A., *Compt. rend. soc. biol.* 80, 326 (1917); *J. pharm. chim.* 18, 19 (1918).