Azorubin in Aqueous and Methanolic Solutions

Spectrophotometric Studies

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DEVELOPMENTAL STUDIES

The original quantitative procedure for bilirubin of Van den Bergh (1) was based upon comparison of the color of an ether extract, first with ferric thiocyanate solutions and later with cobalt sulfate standards. This inaccuracy of standardization has been eliminated by the availability of purified bilirubin in both solid form and in comparatively stable solutions, and by the adaptation of the procedure to spectrophotometers and photoelectric colorimeters, both of which isolate narrow bands of light as compared to the entire spectrum used with visual colorimeters.

The second problem was that of precipitation of the proteins by the addition of ethanol in the determination of total bilirubin, accompanied by subsequent loss of some bilirubin by adsorption, and by incomplete dissociation from the assumed protein-bilirubin complex. The solution to this problem was worked out by Malloy and Evelyn (2), who found that the serum proteins did not precipitate in 50% methanol provided the final protein concentration was below a certain level. At this concentration of methanol, full color development of the azorubin takes place. The final dilution in the original method was about 1 to 40 or 50, with a protein concentration of 300–400 mg. per 100 ml. Observations during the present study indicate that protein precipitation in methanol does not occur under the proposed test conditions, if the serum dilution is 1 to 8 or more—that is, a protein concentration of not more than 1%. Above this protein concentration precipitation almost invariably occurs.

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The division of serum bilirubin into prompt, biphasic, and delayed reactions has been shown by Gray and Whidborne (3) to depend in large part upon the total concentration of direct bilirubin. They pointed out that when the serum direct bilirubin concentration was greater than 10 mg. per 100 ml., a "prompt" reaction always occurred. When the concentration was less than 5 mg./100 ml., the formation of azorubin is so slow that the reaction is called "delayed." With concentrations between 5 and 10 mg./100 ml., the "biphasic" reaction is most commonly observed. These concentration ranges were determined with rather large dilutions of serum. It is quite likely that with a dilution of 1 to 8 biphasic reactions will be observed in concentrations as low as 2 mg./100 ml.

Several workers (4, 5, 6) have pointed out that Watson's (7) 1-minute direct bilirubin is merely an arbitrary point on a time rate-color development curve. It takes about 7 minutes to develop 95% of the color of azorubin, regardless of the concentration of direct bilirubin. Therefore, since the shape of the curve is determined by the total direct bilirubin concentration, and since the 1-minute concentration is merely a point on this curve, the 1-minute bilirubin has no more clinical significance than the total direct bilirubin, upon which it depends.

It remained, therefore, to determine whether "direct" and "indirect" bilirubin actually exist in serum, or whether they are artefactual.

EXPERIMENTAL AND RESULTS

The apparatus used was the Coleman Junior Spectrophotometer, Model 6A, with 12 × 75 mm. cuvets.

The reagents were those of Malloy and Evelyn (2). The bilirubin stock standard solution (Hartmann-Leddon Company) contained 10 mg. of bilirubin in 100 ml. of chloroform. This solution was kept in the refrigerator when not in use. The factor determined from this standard, which has been used for calibration of the spectrophotometer for routine bilirubin determinations in this laboratory, has not changed in more than 3 years, using the same bottle of stock standard.

**Methanol Dilution**

Dilute standard solutions of bilirubin were prepared by diluting 1, 2, 3, 4, and 5 ml. of the chloroform solution to 10 ml. with methanol. These solutions contained 1, 2, 3, 4 and 5 mg. per 100 ml., respectively. A reagent blank was prepared using chloroform instead of the stock standard.
Table 1. **Absorbence of Azorubin with Methanol Dilution**

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>MeOH conc. (g%)</th>
<th>Concentration of standard (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
</tr>
<tr>
<td>1/4</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>1/8</td>
<td>87.5</td>
<td>0</td>
</tr>
<tr>
<td>1/12</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>1/16</td>
<td>94</td>
<td>0</td>
</tr>
</tbody>
</table>

**Procedure**

Half-milliliter aliquots of each standard were placed in a series of 12 × 75 mm. cuvettes. To each tube was added 1 ml. of methanol. After mixing by tapping the tubes 0.5 ml. of diazo working reagent (2) was added. Thus each test solution contained 75% methanol. After the solutions were mixed and allowed to stand for 15 minutes at room temperature, the absorbences were read at 540 mµ, with the absorbence of the blank tube set at 0. Two milliliters of methanol were then added to each tube, the tubes mixed by inversion, and the absorbences read again. Two milliliters of each solution were transferred to a second series of cuvettes, 1 ml. of methanol added, the tubes mixed, and the absorbences read as before. The absorbences were again determined after adding another 1 ml. of methanol. All tubes were run in triplicate. The results are shown in Table 1.

**Water Dilution**

Another set of solutions was prepared exactly as above. After the solutions were allowed to stand 15 minutes, the absorbences were read. Distilled water was added to each tube in 2-ml. aliquots, the solutions mixed, and the absorbences read again. Two milliliters of each solution were then added to 2 ml. of distilled water in another series of cuvettes, the tubes mixed, and the absorbences read. One milliliter of each solution was then transferred to 2 ml. of distilled water, the tubes mixed, and the absorbences read again. All tests were run in triplicate. The results are shown in Table 2.

**Methanol-Water Dilution**

Similar experiments were carried out with serum specimens from which 0.5 ml. of serum was added to 1.5 ml. of diazo working reagent and allowed to stand at room temperature for 15 minutes after mixing. The absorbence was read against a similarly prepared blank. Two
ml. of methanol were then added to each tube, and the absorbences read again after mixing and standing for 15 minutes. Two milliliters of the mixture were then added to 2 ml. of distilled water, bringing the methanol concentration down to 25%. In each case, as seen in Table 3, the final absorbence was less than one half that in 50% methanol, and very close to one quarter that of the first absorbence reading in water alone.

**DISCUSSION**

The small amount of chloroform present in the standard solutions did not cause precipitation or cloudiness when distilled water was added to any of the test mixtures.

For the purpose of interpretation, one must assume that after 15 minutes all of the bilirubin is converted to azorubin, and that this dye is comparatively stable—that is, it does not decompose or dissociate appreciably upon dilution with methanol or distilled water. From Table
1, it is obvious that no decomposition occurs upon dilution with methanol.

In Table 1, all absorbences were proportional in each tube to the final dilution with methanol, as well as proportional to the concentration in each tube, up to an absorbence of more than 1.000. Thus, Beer's law is followed over absolute bilirubin concentrations from 0.001 to 0.25 mg. per 2 ml. of test solution.

When the original test solutions are diluted 1:12 with water, in successive steps, to a final dilution of 1 to 48, as shown in Table 2, the decrease in absorbence is not proportional to the dilution. This is observed even in a final dilution of only 1 to 8, in 37.5% methanol. However, the absorbence is proportional to the concentration for any given concentration of methanol. Theoretically, the absorbence of Tube 5, (5 mg./100 cc.) should decrease from 1.080 to 0.090 in a 1:48 dilution. The actual absorbence is 0.070, or 78 per cent of the theoretical. Thus, of 5 mg./100 cc., only 3.9 mg./100 cc. are recovered in aqueous solution. Of 4, 3, 2 and 1 mg./100 cc., only 3.4, 2.5, 1.8 and 0.8 mg./100 cc. are recovered, or 78, 85, 83, 90 and 80 per cent, respectively.

Most clinical laboratory workers find that these figures represent approximately the percentages of direct bilirubin out of the total serum bilirubin which are determined in almost every range of concentration except the low range below about 1.5 mg./100 cc. Also excluded are instances of bilirubinemia due to hemolytic causes, for it is fairly well established that in these cases we are dealing with a more or less definitely defined excess of bilirubin-globin complex, which does not diazotize completely until alcohol is added.

CONCLUSIONS

Thus, the author feels that it is reasonable to conclude that a large part, if not all, of the differences in values observed between direct and total bilirubin in serum is artefactual, and due to the difference in absorbence of azorubin in aqueous and alcoholic solutions.

Although at first glance it is difficult to reconcile this conclusion with the observed renal threshold for bilirubin, which is about 1.4 mg. of direct bilirubin per 100 ml., one soon realizes that the 1.4 mg./100 ml. may actually be 1.6 to 1.9 mg./100 ml. of a single type of bilirubin. It also becomes apparent that, whereas in obstructive and regurgitative types of jaundice only one type of bilirubin is probably present, in hemolytic jaundices the existence of two types may still be required to explain the observed Van den Bergh reactions.
SUMMARY

A study of the spectrophotometric characteristics of azorubin in varying proportions of methanol and water has been presented.

The absorbance of azorubin in water was found to vary from 73 to 90 per cent of that in methanol, over a concentration range of 0.001 to 0.025 mg. per 2 ml. of test solution.

The absorbance of azorubin was found to be linear over the same range of concentration, and over the range from 0.1 to 10 mg. per 100 ml. of serum, using a final test dilution of 1 to 8.

The strong probability of the artefactual nature of "direct" and "total" serum bilirubin values has been demonstrated.

REFERENCES