CHEMISTRY OF THE VAN DEN BERGH REACTION

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(Received for publication, October 23, 1939)

The form in which bilirubin exists in the blood is of considerable interest because of the observed differences in the van den Bergh reaction in disease and because of the relation of bilirubin to hemoglobin metabolism.

All of the bilirubin in plasma reacts in 30 minutes with diazonium salts in acid 50 per cent methyl alcohol to give a red dye (1). A variable fraction of the total reacts with varying rapidity in aqueous acid ("direct" reaction). Clinically the size of this fraction is of some diagnostic value in distinguishing obstructive from hemolytic jaundice.

Nearly all of the many theories propounded to explain the differences among plasmas from cases of jaundice in the percentage of the total bilirubin which reacts in aqueous acid can be divided into two classes, (1) those which postulate that the bilirubin not reacting in aqueous acid is prevented from doing so by a definite valence bond to a fraction of the plasma proteins presumably derived from hemoglobin, and (2) those which postulate that catalytic or inhibiting substances present in various amounts produce variations in the per cent of bilirubin giving the direct (aqueous) reaction. Most of the experiments designed to support the latter type of theory have been synthetic, involving the construction of models apparently duplicating, by the use of catalysts, the effects observed in naturally occurring jaundiced plasmas.

The following experiments are an attempt at an analytical solution of the problem by means of the accurate and convenient plasma bilirubin method of Malloy and Evelyn (1). They appear to show, to the exclusion of the second type of theory, that in the plasma from jaundiced patients the bilirubin not reacting in
aqueous acid is attached by a definite valence linkage to a fraction of the plasma proteins.

Cataphoresis—The following experiments show quantitatively that all of the bilirubin is attached to the plasma albumin in jaundiced plasma and moves with it in an electric field.

It has been shown qualitatively that the plasma bilirubin is attached to the plasma albumin (2, 3). Table I shows the results of quantitative experiments in which 2.00 cc. samples of oxalated human plasma of high bilirubin content are subjected to cata-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original plasma</th>
<th>Cataphoresis fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin to globulin ratio</td>
<td>Total bilirubin</td>
</tr>
<tr>
<td>A</td>
<td>1.01</td>
<td>9.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.01</td>
<td>9.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.01</td>
<td>9.0</td>
</tr>
<tr>
<td>B</td>
<td>0.87</td>
<td>9.05</td>
</tr>
<tr>
<td>&quot;</td>
<td>Same cataphoresis as above</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>1.4</td>
<td>7.71</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.4</td>
<td>7.71</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.4</td>
<td>7.71</td>
</tr>
<tr>
<td>&quot;</td>
<td>Same cataphoresis as above</td>
<td>26.2</td>
</tr>
</tbody>
</table>

phoresis with varying voltages and distances of migration in the apparatus of Coolidge (4) and fractions analyzed after cataphoresis. The bilirubin reacting in aqueous acid in 2 hours is called "direct" reacting. Globulin was estimated after separation from albumin by precipitation in 2.02 M phosphate buffer, pH 7.0 (5). This separation was shown to be unaltered by increasing the plasma dilution 1:120, as was necessary in this case.

As can be seen from Table I the recovery of the total bilirubin parallels that of the albumin within the limits of accuracy of the method. The agreement is very satisfactory, when one considers
the lability of plasma bilirubin in the absence of the reducing substances present in normal plasma. One can conclude that practically all the bilirubin is bound to the plasma albumin. This has been confirmed by ultrafiltration of plasma through cellophane membranes. No bilirubin passes through, in agreement with the results of Gregory and Andersen (6) and contrary to earlier observations.

A comparison of the per cent of direct reacting bilirubin in the original plasma with that in the cataphoresed fluid shows both increases and decreases. These are difficult to interpret, as the data are obtained on such small amounts of material that the limit of accuracy of the method for direct reacting bilirubin is approached. They indicate, however, that if the ability to react in aqueous solution is conditioned by factors other than bilirubin or plasma albumin, such factors are not completely removed by several hours of cataphoresis in a strong electric field and are therefore strongly bound to the albumin.

**Reaction of Mixed Plasmas**—That the differences in reactivity of direct and indirect reacting bilirubin are due to structural rather than catalytic causes is shown by the effect of mixing equal volumes of two plasmas having very different proportions of direct reacting bilirubin and treating the mixture with diazotized sulfanilic acid. The results are shown in Table II.

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Bilirubin reacting in aqueous acid</th>
<th>Mixture of Plasmas A and B × 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma A</td>
<td>Plasma B</td>
</tr>
<tr>
<td>min.</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>10</td>
<td>1.15</td>
<td>1.18</td>
</tr>
<tr>
<td>30</td>
<td>1.45</td>
<td>1.46</td>
</tr>
<tr>
<td>60</td>
<td>1.65</td>
<td>1.62</td>
</tr>
<tr>
<td>120</td>
<td>2.10</td>
<td>1.68</td>
</tr>
</tbody>
</table>

Plasma A contains 8.60 mg. per cent of total bilirubin; 25 per cent direct reacting. Plasma B contains 2.22 mg. per cent of total bilirubin; 76 per cent direct reacting.
The rate of the reaction of the mixture is equal to the sum of the rate of reaction of the bilirubin in the two plasmas. If either catalyst or inhibitor were present in excess in one of the plasmas (as the "catalytic" type of theory would assume, since the plasmas are quite different in their reactivity), such catalyst or inhibitor would, in the mixture, alter the reactivity of the bilirubin in both plasmas and thus change the reactivity of the mixture from that of the sum of the rates of component plasmas. This is not the case. Hence this experiment is evidence against the "catalytic" type of theory.

The summative effect of the individual constituents is the same even if the mixture stands overnight.

Rôle of Bile Salts—That the bile acids might be involved in the reaction with diazonium salts in aqueous solution has been suggested by several writers (7). The following experiments show that, with the analytical methods available, there is nothing to support this suggestion.

The only method sufficiently sensitive and accurate for an investigation of this theory is that of Josephson (8), which gives the sum of free and conjugated cholic acid. This method was tested by analyzing samples of plasma to which known amounts of cholic acid had been added and consistently gave an accuracy of better than 20 per cent. Table III shows that there is no correlation between this sum and the character of the van den Bergh reaction.

Extraction with Organic Solvents—That organic liquids capable of dissolving bilirubin extract the direct reacting bilirubin alone from the plasma has been known in a qualitative way for some time. Table IV shows quantitatively the effect of extracting plasma with butyl alcohol. In these experiments 1 cc. of plasma was run into about 50 cc. of butyl alcohol, containing 1.0 cc. of 0.18 N HCl, with stirring. The precipitate was washed twice with methyl alcohol and any remaining alcohol removed under reduced pressure. The precipitate was taken up in 20 cc. of water and the total bilirubin determined.

The amount of bilirubin extracted by alcohol appears to be about equal to that which reacts with diazotized sulfanilic acid in aqueous acid in the first 10 minutes. The simplest explanation of the data appears to be that the bilirubin reacting in the first
10 minutes is held to the albumin in a complex from which it can be separated by alcohol, that the remainder of the bilirubin is

**Table III**

*Relationship between Character of van den Bergh Reaction and Plasma Cholic Acid Level*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Albumin</th>
<th>Globulin</th>
<th>Bilirubin</th>
<th>Plasma cholic acid</th>
<th>Van den Bergh reaction (qualitative)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>Direct</td>
<td>Indirect</td>
</tr>
<tr>
<td>Leucemia, obstructive jaundice</td>
<td>3.4</td>
<td>3.2</td>
<td>8.06</td>
<td>4.8</td>
<td>Direct</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>3.3</td>
<td>2.7</td>
<td>10.3</td>
<td>12.9</td>
<td>&quot;</td>
</tr>
<tr>
<td>Subsiding obstructive jaundice</td>
<td>2.8</td>
<td>4.1</td>
<td>0.86</td>
<td>6.8</td>
<td>Indirect</td>
</tr>
<tr>
<td>Biliary cirrhosis (autopsy)</td>
<td>1.2</td>
<td>4.7</td>
<td>2.07</td>
<td>4.5</td>
<td>Direct</td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>1.6</td>
<td></td>
<td>8.3</td>
<td>&quot;</td>
<td>Indirect</td>
</tr>
<tr>
<td>Icterus neonatorum</td>
<td>3.6</td>
<td>1.7</td>
<td>5.7</td>
<td>9.1</td>
<td>&quot;</td>
</tr>
<tr>
<td>Normal infant</td>
<td></td>
<td></td>
<td>7.0</td>
<td>10.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>Cirrhosis with ascites</td>
<td></td>
<td></td>
<td>0.95</td>
<td>2.7</td>
<td>&quot;</td>
</tr>
<tr>
<td>Catarrhal jaundice</td>
<td></td>
<td></td>
<td>13.5</td>
<td>4.7</td>
<td>Direct</td>
</tr>
<tr>
<td>Septicemia, 2 wks. jaundice</td>
<td>2.9</td>
<td>3.8</td>
<td>2.3</td>
<td>6.8</td>
<td>&quot;</td>
</tr>
<tr>
<td>Sickle-cell anemia</td>
<td>3.9</td>
<td>3.66</td>
<td>1.33</td>
<td>5.0</td>
<td>Indirect</td>
</tr>
<tr>
<td>Secondary</td>
<td>4.45</td>
<td>2.93</td>
<td>1.3</td>
<td>5.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hyperemesis gravidarum</td>
<td>2.46</td>
<td>2.04</td>
<td>0.71</td>
<td>5.0</td>
<td>Direct</td>
</tr>
<tr>
<td>Familial congenital jaundice</td>
<td>4.8</td>
<td>2.3</td>
<td>1.9</td>
<td>3.0</td>
<td>Indirect</td>
</tr>
<tr>
<td>Congenital heart failure, syphilis</td>
<td>2.25</td>
<td>3.10</td>
<td>1.4</td>
<td>3.5</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The van den Bergh reactions in this table were carried out by the method of McNee (9).

**Table IV**

*Amount of Bilirubin Reacting in Various Times Compared with Amount Extracted by Butyl Alcohol (Mg. Per Cent)*

<table>
<thead>
<tr>
<th>Plasma No.</th>
<th>Bilirubin</th>
<th>Total</th>
<th>Reacting in 10 min.</th>
<th>Reacting in 30 min.</th>
<th>Reacting in 120 min.</th>
<th>Extracted by butyl alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.2</td>
<td>8.39</td>
<td>9.77</td>
<td>10.0</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15.8</td>
<td>9.43</td>
<td>10.7</td>
<td>11.8</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11.82</td>
<td>6.97</td>
<td>7.17</td>
<td>7.31</td>
<td>7.7</td>
<td></td>
</tr>
</tbody>
</table>
at a variable, slow rate determined by unknown factors. This explanation is in accord with the observed shapes of the curves expressing the rates of development of color in the van den Bergh reaction in clinical cases. In these there is usually a fairly rapid development of color in the first 10 minutes, followed by a slower change at a rate varying in different plasmas.

_Rôle of Alcohol_-That the rôle of the 50 per cent methyl alcohol in causing the reaction of the total bilirubin is an entirely reversible one appears in Table V which shows the effect of adding excess methyl alcohol and subsequently removing it by evaporation _in vacuo_ below 0°. The course of the reaction before and after this treatment is the same. In these experiments 0.8 cc. of plasma was run into 16 cc. of methyl alcohol, chilled in a dry ice-toluene bath, 4 cc. of water were added, and the chilled mixture evaporated at about 0° to about 3 cc. It was then made up to a volume of 16.00 cc. on aliquots of which the van den Bergh reaction was run. Both ultrafiltration and cataphoresis indicate that in mixtures of equal volumes of plasma and methyl alcohol, such as are used in determining the total bilirubin, there is no separation of the bilirubin from the albumin. From these facts it appears that the rôle of the alcohol in enabling all the bilirubin to react can be looked on as a purely catalytic one.

_Fractionation of Plasma Proteins and Determination of Bilirubin Distribution_-If, as appears above, the indirect reacting bilirubin is firmly bound to a fraction of the plasma albumin, it should, theoretically, be possible to fractionate the latter and obtain a protein fraction containing all the indirect reacting bilirubin.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Original plasma</th>
<th>Methyl alcohol-treated plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>61</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>65</td>
<td>64</td>
</tr>
<tr>
<td>60</td>
<td>68.2</td>
<td>65.4</td>
</tr>
<tr>
<td>120</td>
<td>70.0</td>
<td>66.5</td>
</tr>
</tbody>
</table>

(Table V)
An attempt has been made to fractionate the plasma albumin and to follow the van den Bergh reaction of the fractions. This is rendered extremely difficult by the fact that plasma bilirubin, especially the direct reacting fraction, is sensitive to oxidation and to sunlight, especially in acid solution.

Two sheep and a dog underwent ligation of the common duct and were killed when jaundice developed. Because of the rapid oxidation of the bilirubin the only conclusions that can be drawn from the experiments on the sheep are that much of the bilirubin is associated with the proteins precipitated by ammonium sulfate, pH 6.8, between 61.0 and 72.5 per cent saturation at 30°. The green color of the biliverdin which eventually appears in plasma albumin fractions precipitated three times with ammonium sulfate by the method of McMeekin (10) is confined to the above fraction except for a faint blue-green color associated with the fractions separating near the half saturation point.

The experiment on the dog plasma (Table VI) was carried out with a minimum of exposure to light and air and as rapidly as possible, without reprecipitation of the fractions. The order of accuracy is low because of the difficulty in removing the ammonium sulfate from the precipitated proteins. The recovery of total bilirubin is only 12.5 per cent and of indirect reacting bilirubin only 55 per cent. In the dark 200 cc. of the dog plasma were poured into a cylinder containing a mixture of 800 cc. of water and 1000 cc. of saturated ammonium sulfate through which hydrogen was bubbling. After 7 hours the mixture was filtered through asbestos wool. The filtrate was placed in a cylinder containing 800 cc. of saturated ammonium sulfate and nitrogen passed through for 12 hours. The mixture (sp. gr. 1.165) was

| TABLE VI |
| Distribution of Total Bilirubin in Ammonium Sulfate-Precipitated Plasma Protein Fractions (Mg. Per Cent) |
| Original plasma | Fractions precipitated by ammonium sulfate at saturations of |
|                 | 50 per cent and below | 50-62.5 per cent | Above 62.5 per cent |
| 2.69 (77.3% direct reacting) | 0.08 | 0.07 | 0.34 |
filtered. The filtrate received 1.5 liters of saturated ammonium sulfate, and nitrogen was bubbled through it for 12 hours. It was filtered, and the clear, colorless filtrate discarded. The three precipitates obtained in the above filtrations were washed with 4.05 m phosphate buffer, pH 7.0, and dissolved in 250 cc. of water each. The solutions gave a precipitate in 50 per cent methyl alcohol and the total bilirubin was determined in about 40 per cent phosphoric acid. The agreement of this method of determination with that carried out in the usual way was within 1 per cent when applied to the original plasma. Reliable determinations of the direct reacting bilirubin were not possible. It can probably be safely assumed that all the bilirubin recovered was originally indirect reacting, as the direct reacting bilirubin is much more susceptible to chemical destruction than the indirect reacting. The results of this experiment confirm those obtained on sheep plasma.

**SUMMARY**

All of the bilirubin in human plasma of high bilirubin content is bound to the plasma albumin.

The plasma bilirubin which gives a direct reaction in 10 minutes when the procedure of Malloy and Evelyn is used is attached to the plasma albumin as a dissociable complex.

That which does not give a direct reaction is attached to a fraction of the plasma albumin precipitated by ammonium sulfate at pH 6.8 between 61 and 72.5 per cent saturation—probably by a valence bond.

The rôle of the methyl alcohol in causing all the bilirubin to react is purely catalytic.

The character of the van den Bergh reaction cannot be correlated with the concentration of cholic acid in the plasma.

To Dr. Anne Yates Graves of the Department of Biochemistry, Duke University School of Medicine, to Dr. T. L. McMeekin of the Department of Physical Chemistry, Harvard Medical School, and to Dr. Harold Finkelstein and Dr. Joseph W. Beard of Duke Hospital, Department of Experimental Surgery, the author expresses his thanks for their help.
BIBLIOGRAPHY

7. Barron, E. S. G., Medicine, 10, 77 (1931).
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