THE DETERMINATION OF CHOLIC ACID IN BILE*

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In attempting to apply the method described by Gregory and Pascoe (1929) to the determination of bile acids in bile, considerable difficulty was experienced because of the weakness of the blue color developed by cholic acid. Later it was found that because of a misprint in the original paper only one-fifth of the required amount of cholic acid was being used. Before this was known several attempts had been made to increase the color by means of higher concentrations of H₂SO₄ and furfural. The result was a deeper color and a marked rise in absorption of the monochromatic red light with which the colorimetric comparisons were made. The favorable effect of the increased concentration of the regents led to a detailed investigation of principal factors concerned with the development of the color, and when these were adjusted so as to yield maximal values, the sensitivity of the method was more than doubled, while the best results by the new technique are obtained with one-fourth of the amount of bile acid required for the old procedure. The improved sensitivity is particularly advantageous in the analysis of blood and of pathological human bile where low concentrations of cholic acid are frequently encountered. An additional advantage is the reduction in the time of heating from 30 minutes to 8 minutes. Further evidence of the specific nature of the reaction between cholic acid and the furfural-H₂SO₄ reagent is presented, including a comparison of

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desoxycholic acid with cholic acid. The effect of conjugation on the color formed by cholic acid has also been investigated. The results of these studies have been incorporated into the procedure described in the following section.

**Method**

The preliminary treatment of the bile depends on the nature and amount of protein present. Riegel, Johnston, and Ravdin (1932) observed that the small amount of protein in dog bile did not interfere with their modified application of the Gregory and Pascoe procedure, and we have found this to be true also of the technique described in this paper. However, human gallbladder bile contains significant amounts of protein that form an interfering color and cause high results. If this protein is present in human hepatic bile, the amount is too small to have a recognizable effect, and as in dog bile the removal of protein is not essential.

**Procedure for Dog Bile or Human Hepatic Bile**—Gallbladder bile from the dog is diluted 250 to 500 times with distilled water; hepatic bile 50 to 100 times, or less if the rate of flow is rapid. 1 cc. of bile is diluted as described, and 1 cc. of the diluted bile is measured into a test-tube (18 mm. diameter). To this are added 1 cc. of freshly diluted 0.9 per cent (by volume) aqueous furfural and 6 cc. of 16 $\text{N}_2\text{H}_2\text{SO}_4$. The contents of the tube are mixed thoroughly by tapping or by stirring. The resulting solution is 12 $\text{N}_2\text{H}_2\text{SO}_4$ containing 1.3 mg. of furfural per cc. The tubes are then stoppered loosely with rubber stoppers, and are heated in a water bath at 70° for 8 minutes. For the best results, the temperature should not vary more than 1–2°. Cooling is accomplished by placing the tubes in tap water for 2 minutes.

1 cc. of a standard solution of sodium cholate in water, containing 0.215 mg. (0.5 mM solution), is treated like the unknown. Colorimetric comparison is made in monochromatic light of about 6000 to 6900 Å, with the unknown set at 15.0 mm.

**Procedure for Human Gallbladder Bile**—1 cc. of freshly collected bile is added slowly to about 10 cc. of 95 per cent alcohol in a test-tube. After mixing, the contents of the tube are warmed to the boiling point of the alcohol in a water bath. The precipitate of protein is removed preferably by centrifugation since filtration causes a small loss, and the alcohol is decanted into a 25 cc. volumet-
ric flask. The precipitate is extracted with 10 cc. of alcohol by heating, centrifuging, and decanting as before. Usually the precipitate still contains small quantities of cholic acid, and for careful work a second extraction with 5 cc. of alcohol is needed. The volume is then made to 25 cc. with alcohol. Specimens high in cholic acid require additional dilution.

1 cc. of the alcoholic solution of bile is measured into a test-tube (18 mm.), and most of the alcohol is evaporated by heating in a boiling water bath. Generally 7 to 8 minutes are sufficient for the evaporation of the alcohol. About 0.05 cc. of alcohol (enough to moisten the bottom of the tube) should remain or a small loss of taurocholic acid will result. When the tubes have cooled, the evaporated bile is dissolved in 2 cc. of freshly diluted 0.45 per cent (by volume) aqueous furfural solution, and 6 cc. of 16 N H₂SO₄ are added. The remainder of the procedure is the same as that described for dog bile except that in this instance 1 cc. of an alcoholic solution of cholic acid (0.204 mg. per cc.) or the equivalent amount of sodium cholate serves as the standard. The alcohol is removed by evaporation as described above. Because of the insolubility of cholic acid in water, more uniform results are obtained if the H₂SO₄ is added to it before the furfural.

Solutions of human bile sometimes become turbid during the heating with the furfural-H₂SO₄ reagent. When this occurs, 7 cc. of 95 per cent ethyl alcohol are added to both unknown and standard after the tubes have been cooled. After mixing thoroughly, the tubes are allowed to stand for a few minutes until bubbles no longer form before being read in the calorimeter. In rare instances this quantity of alcohol is not enough and a second portion is needed. If the material is pathological human bile, time is saved by preparing duplicate standards, one of which may be diluted with alcohol if required and the other used undiluted. Two precautions must be observed in comparing the alcoholic solutions colorimetrically. The plungers and bottoms of the cups of the calorimeter must be kept free from the minute bubbles that often form in these solutions, and when the unknown is more concentrated than the standard, the readings should be corrected for deviation from Beer's law. This deviation is practically linear, and only two points need to be established, in addition to that representing the standard, usually at 1½ and 2 times the concen-
of the standard. These are plotted against colorimeter readings in the usual manner (see Yoe, 1928), and the corrected values read directly from the curve. In the absence of alcohol, a linear relationship exists between the color formed and the concentration of cholic acid until the readings become difficult because of the depth of color; hence, correction is not required. The results are most satisfactory when the quantity of cholic acid in the unknown is equal to or less than that in the standard if no final dilution with alcohol is made. If alcohol is required to remove turbidity, it is advisable to compensate for the diminished color either by increasing the depth of the solutions read in the colorimeter, or by repeating the experiment with double the original quantities of unknown and standard.

In the analysis of bile, special care is needed in measurement by pipette owing to the high viscosity of many specimens. It is advisable to use pipettes calibrated to contain, and after delivering the bile, to rinse the pipette with the diluting fluid several times. The color of the specimen can generally be used as a guide to the dilution, although exceptions are frequently encountered. Because of the great variations in the composition of bile it is safest to start with comparatively low dilutions.

The sulfuric acid reagent should be standardized by titration. We have encountered one lot of sulfuric acid which was not suitable for use, since it gave a purple tint instead of a clear dark blue with furfural and cholic acid. The blank for this acid was high, and the total color formed by cholic acid was diminished by one-half.

A filtered neon light similar to that described by Gregory and Pascoe was used as a source of illumination for all of the work reported in this paper; however, we have found that equally good results are obtained when an ordinary colorimeter lamp (with the blue daylight filter removed) is used with suitable filters. A red glass disc filter of the type supplied by several manufacturers for use in the colorimeter eyepiece is most convenient, although any type of filter having a sharp cut-off in the 6200 Å. region and a high transmission in the red would be suitable. The spectral transmission of our neon lamp and filters was essentially the same as that reported by Gregory and Pascoe, extending from 6150 Å. to about 6700 Å. with the greatest transmission in the region of 6400 Å.
EXPERIMENTAL

The hue and intensity of the color formed in the reaction depend chiefly on the four factors, time and temperature of heating, and concentrations of $\text{H}_2\text{SO}_4$ and furfural. In testing these variables each was examined in a series of combinations of the other three in order to establish the conditions yielding the greatest amount of color compatible with the practical application of the method.

To evaluate color formation at each concentration of acid properly, it was necessary first to find the optimum time of heating for each normality studied. With the aid of this information various concentrations of $\text{H}_2\text{SO}_4$ were compared, extending from 7.5 $\text{N}$ to 16.8 $\text{N}$. As the concentration is increased, the absorption of the effective wave-lengths rises rapidly to a plateau between 10 and 12 $\text{N}$ and then falls sharply. A second rise occurs with about 14 $\text{N}$ acid, caused for the most part by reaction between the $\text{H}_2\text{SO}_4$ and furfural, and when the blank given by the reagents is subtracted, the color due to cholic acid is much less than at lower acidities. Because it gave maximal color at a comparatively rapid rate with a negligible blank, 12 $\text{N}$ $\text{H}_2\text{SO}_4$ was selected as the best concentration.

Although slightly more color is developed in shorter periods of heating at higher temperatures than 70°, fading occurs more rapidly and the decreased stability of the color more than offsets a possible gain. Besides, if the time is made too short (less than 5 minutes) by the use of higher temperatures, physical factors such as small variations in the diameter of the tubes become significant. The higher concentration of acid and slightly higher temperature make possible the decrease in the time from 30 minutes as recommended by Gregory and Pascoe to 8 minutes. There is little change in the color between 7.5 and 8.5 minutes, but above or below these limits the quantity of color decreases rapidly. If the higher limit is exceeded or if the tubes are overheated, the fading of the color is accompanied by the development of turbidity. The data for time and temperature may be applied directly only if 18 mm. test-tubes are used, although the general relationships are independent of this factor.

Additional furfural heightens the color until the concentration reaches 1.3 mg. per cc. This is about 3 times the amount used in the original method. At higher concentrations the color is
again diminished, while at the same time the blank given by the reagents increases markedly. The blank is under all conditions dependent to a considerable extent upon the state of the furfural, which should be colorless or yellow rather than brown. The blank is small under the conditions selected, but it will rise parallel with the development of brown color in the furfural. When stored in a refrigerator, furfural keeps sufficiently well for several months. Commercial redistilled c. p. furfural gave satisfactory results without further treatment, although samples that had been kept in stock for some time were unsatisfactory. Completely eliminating the blank given by the reagents by lowering the concentration of furfural and H$_2$SO$_4$ greatly diminishes the sensitivity of the method.

Representative results obtained with the modified technique are shown in Table I.

**Color Equivalence of Conjugated Cholic Acids**—One would expect about the same amount of color per mol from the conjugated acids as from cholic acid if the color-producing reaction depends

### Table I

**Recovery of Glycocholic Acid Added to Bile**

<table>
<thead>
<tr>
<th>Cholic acid in bile solution</th>
<th>Cholic acid added</th>
<th>Total cholic acid taken</th>
<th>Total cholic acid found</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 cc. alcoholic extract of hepatic bile of dog, diluted 83.3 times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>0.040</td>
<td>0.022</td>
<td>0.062</td>
<td>0.065</td>
</tr>
<tr>
<td>0.043</td>
<td>0.053</td>
<td>0.083</td>
<td>0.083</td>
</tr>
<tr>
<td>0.086</td>
<td>0.126</td>
<td>0.126</td>
<td>0.121</td>
</tr>
<tr>
<td>0.129</td>
<td>0.169</td>
<td>0.169</td>
<td>0.164</td>
</tr>
<tr>
<td>0.172</td>
<td>0.212</td>
<td>0.212</td>
<td>0.205</td>
</tr>
<tr>
<td>0.215</td>
<td>0.255</td>
<td>0.255</td>
<td>0.252</td>
</tr>
<tr>
<td>0.5 cc. alcoholic extract of gallbladder bile of dog, diluted 250 times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.114</td>
<td>0.021</td>
<td>0.135</td>
<td>0.136</td>
</tr>
<tr>
<td>0.042</td>
<td>0.156</td>
<td>0.156</td>
<td>0.159</td>
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<tr>
<td>0.126</td>
<td>0.240</td>
<td>0.240</td>
<td>0.227</td>
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<td>0.282</td>
<td>0.286</td>
</tr>
<tr>
<td>0.211</td>
<td>0.325</td>
<td>0.325</td>
<td>0.303</td>
</tr>
</tbody>
</table>
on the cholic acid nucleus. It is essential to know if this is true because of the possible existence in bile of unconjugated cholic acid, and also because of the desirability of using cholic acid or sodium cholate as standards (at present these seem to be the only suitable preparations commercially available in pure form). Taurocholic and glycocholic acids were purified according to the method of Hammarsten (1925). Total nitrogen determinations gave for two crystalline preparations of taurocholic acid 2.75 and 2.81 per cent (Samples 3 and 4 respectively). Theoretical is 2.71 per cent. The proportion of bound solvent was determined by heating a portion of each preparation at 100° for 24 hours; and the data for all preparations are corrected for loss of weight under these conditions. The decomposition of taurocholic acid which occurred, shown by the formation of a brown color, was disregarded in calculating the loss of weight. The melting points, specific rotation, and nitrogen contents of two preparations of glycocholic acid were determined. Preparation 1 had the following properties: m.p. 127° corrected, $[\alpha]_{D}^{20} = 33.4^\circ$, N = 3.07 per cent; Preparation 2: m.p. 124–128°, $[\alpha]_{D}^{20} = 32.2^\circ$. Others have found melting points 124–140°, $[\alpha]_{D}^{20} = 32.3^\circ$, N = 3.01 per cent. The cholic acid used was Schuchardt’s Purissima. Its melting point was 196.6° corrected, compared with 196–197° given in the literature, and the $[\alpha]_{D}^{20} = 36.9^\circ$, which agrees well with 37.0°, the figure most frequently cited by other workers. These values did not change appreciably after two recrystallizations from alcohol.

The pure conjugated acids yielded as much color as an equimolar quantity of cholic acid. In terms of per cent of the calculated amount of cholic acid, the two preparations of glycocholic acid gave as an average of six determinations, 98.2 and 99.4 respectively. For taurocholic acid the corresponding figures for the three samples were 97.1, 99.6, and 95.0 per cent. However, this relationship exists only when the acids are treated with aqueous H$_2$SO$_4$. In the presence of more than 0.1 cc. of alcohol, the conjugated acids gave less color than cholic acid, the values decreasing as the amount of alcohol was increased. It was thought at first that this was caused by a process similar to that involved in the formation of choleic acids. Apparently this is not the explanation, for a number of substances known to form choleic acids did not affect the color formed from conjugated acids.
Cholic Acid in Bile

Desoxycholic acid is present in the bile of many species, and it is important to know if it will contribute to the color developed by cholic acid. We were not able to recover a sufficient quantity of the desoxycholic acids of human bile to make purification worth attempting, but through the courtesy of Dr. Carl H. Greene of the Mayo Clinic we were able to compare with cholic acid three samples of desoxycholic acid. The purest of these was a crystalline preparation of the sodium salt from Professor Wieland, and it gave no color. A sample of desoxycholic acetic acid purified by Dr. Greene gave only 7 per cent of the color which an equimolar amount of cholic acid would yield, while the third sample, desoxycholic acetic acid, prepared commercially, gave 15 per cent. It seems probable that this color was due to cholic acid not completely removed during the separation of the desoxycholic acid. Dehydrocholic acid gives a yellow color that is eliminated by the red filter. As desoxycholic acid does not yield color, it is unlikely that lithocholic acid, containing one hydroxyl group less, will do so, and it is probable, therefore, that the method is specific for the cholic acid series. One remaining possible reactant of importance in the analysis of human bile is anthropodesoxycholic acid which has not been tested.

Behavior of Other Constituents of Bile—The following substances neither increased nor inhibited the color developed from cholic acid: cholesterol, ergosterol, cholesteryl oleate, cholesteryl acetate, oleic acid, egg lecithin, tributyrin, bilirubin (crystalline), creatinine, arginine, cystine, glycine, histidine, proline, taurine, tyrosine, adenine nucleotide, uric acid, gelatin, and inositol. Only tryptophane and proteins containing it, phenol, and biliverdin gave color enough to require further consideration. Interfering proteins are removed by alcohol and heat at an early stage in the procedure. Neither tryptophane nor phenol is likely to be present in bile in significant amounts. The biliverdin tested by us yielded about 20 per cent as much color as an equal weight of cholic acid; however, some bile acid was probably present as an impurity. Biliverdin is not formed from bilirubin to any visible extent when the latter is heated with the reagents. Biliverdin is seldom present in sufficient amount in human bile to cause interference. It is frequently present in dog bile, judging from the green color of many specimens, although we have not encountered any in which
the error from this source was significant. It is advisable, of course, to start the analysis as soon as possible after collecting the bile, since in many specimens the oxidation of bilirubin begins as soon as exposure to air has occurred. This change can be retarded by protecting the specimen from oxygen, by storing in the dark and at low temperatures, or by diluting with alcohol.

It is noteworthy that two preparations of cholesteryl oleate gave only a faint pink color, and that barium oleate behaved in like manner. The cholesteryl oleate was synthesized by Mr. H. F. Snider from cholesterol and oleic acid by the method of Hürthle (1895-96). Walker (1930) has recently concluded that it is this substance in blood rather than bile acid which is responsible for the positive Pettenkofer reaction given by alcoholic extracts of blood. This is a sound objection to those methods depending upon comparisons of red or lavender tints, but the use of red monochromatic light removes any possibility of error from this source in the present method since the color given by cholesterol oleate does not absorb light in the wave-lengths used.

A few determinations have been made on the blood of hospital patients with normal serum bilirubin content. After removal of proteins and a portion of the fatty material, alcoholic extracts evaporated nearly to dryness were treated according to the improved procedure. In every case, solutions which absorbed the monochromatic red light were formed, although it was difficult to identify a blue color owing to the development of yellow or buff tints in the solutions. Assuming that this light absorption originated with cholic acid, there was present in the blood of these individuals less than 1 mg. per cent of cholic acid.

**SUMMARY**

By establishing optimal conditions in respect to the four principal variables, the concentrations of H$_2$SO$_4$ and furfural, time and temperature of heating, the sensitivity of the Gregory and Pascoe method for the determination of bile acids was more than doubled.

A comparison of pure conjugated and unconjugated cholic acids showed that equimolar quantities produced equal amounts of color. Cholic acid was found to be best adapted for use as a standard. The reaction appears to be specific for the cholic acid series.
Various substances that might interfere with the application of the method were tested. Only biliverdin has possible significance, and then only under exceptional circumstances. Cholesteryl oleate produced a pink color which did not absorb light of the wave-lengths used; therefore, it is not the substance in blood giving the positive reaction for cholic acid.

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