STUDIES IN THE METABOLISM OF THE BILE.

I. A QUANTITATIVE PETTENKOFER TEST APPLICABLE TO THE DETERMINATION OF BILE ACIDS IN THE BLOOD.*

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(Received for publication, February 27, 1928.)

The striking progress in our knowledge of the origin and fate of the bile pigments in recent years has been due in great part to the elaboration of methods for studying changes in the bilirubin content of the blood (2, 22, 38, 43). The deficiencies in our knowledge of the physiology of the bile acids by contrast are even more striking. Progress in this field of physiology and medicine may be facilitated by a satisfactory method for determining bile acids, particularly in small amounts of blood.

Methods have been devised for determining glycocholic and taurocholic acids in the bile or urine, and Foster and Hooper (7), Symth and Whipple (39), Brakefield and Schmidt (5), and Rosenthal and his associates (32-36) have studied the effect of various physiologic influences and experimental conditions on the excretion of these compounds in the bile or urine. Unfortunately, the procedures used by these authors are not sufficiently sensitive to be readily applicable to blood.

Kühne (17) and Huppert (15) reported the isolation of bile acids from the blood following both biliary obstruction and their intravenous injection. Various other experimenters have obtained qualitative color tests for bile acids in the blood. Moleschott (26), Lehmann (18), Blankenhorn (4), Gilbert, Chabrol, and Rénard (8), and Petren (30), and others (14, 18) found
positive Pettenkofer tests under appropriate conditions. Recently Tas-
hiro (41), and Hersfeld and Haemmerli (12, 13) reported partially successful
attempts to adapt this test to quantitative determinations. Perlzweig
and Barron (29) reported a new colorimetric test for bile acids using acetic
anhydride and sulfuric acid; Szilard (40) precipitates the bile acids with
ferric chloride and determines the iron colorimetrically; Rosenthal and Wis-
lucki (36) use a modification of the gasometric method for the determination
of amino acids in the bile; McNee (25) has suggested a similar method.

Because of the difficulties in the application of chemical tests, Adler (1)
and others turned to physical methods and have reported extensive studies
on the surface tension of serum. A review of these methods, however,
showed that they were unsuitable for our proposed study because they
either were not sensitive or specific enough or were not applicable clinically.

The Pettenkofer (31) test is probably the most sensitive reaction
known at present for the bile acids. As such it offers the best
basis for a method which can be readily adapted for use with small
amounts of blood. One of us (Aldrich) has been able to develop
a quantitative modification of the Pettenkofer test suitable for
such use. The quantities of bile acids found in the blood even
under experimental conditions are too small to permit a satisfac-
tory comparison of this method with those previously used for the
analysis of bile. The modified Pettenkofer reaction is equally
applicable to bile and a comparison with these methods is reported
later.

### Quantitative Adaptation of the Pettenkofer Test.

The method here described is a quantitative adaptation by
which the maximal Pettenkofer value obtainable from the blood
is determined. Since the technique is comparatively simple and
only 5 cc. of blood are used, it is capable of direct clinical applica-
tion. We have carried out more than 2000 determinations on
the blood in various clinical and experimental conditions during
the last 2 years.

5 cc. of oxalated whole blood are added with shaking to 35 cc. of
redistilled 95 per cent alcohol in a 50 cc. volumetric flask. The
contents are made to volume with alcohol and filtered. From 40
to 50 mg. of norit are added to 35 cc. of the slightly colored filtrate,

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1 This standardization of the Pettenkofer reaction was undertaken at
the suggestion of Dr. L. G. Rowntree and carried out under the supervision
of Dr. C. H. Greene.
shaken well, and filtered at once. The resulting solution should be clear and colorless.

30 cc. of this filtrate are transferred to a 100 cc. beaker, 1 cc. of a saturated solution of barium hydroxide is added, and the mixture evaporated rapidly to a volume of 3 or 4 cc. on a hot electric plate. Overheating must be avoided and the final drying is best accomplished with an electric fan. To remove cholesterol and fat, the thoroughly dried residue of barium salts is then extracted three times with 5 cc. portions of redistilled anhydrous ether brought to the boiling point on the hot plate each time. Fragments of the residue loosened during this extraction are recovered by centrifuging the ether washings. Recrystallized glycocholic acid is used as a standard. A 0.1 per cent alcoholic stock solution is diluted 10 times with alcohol, so that the resulting standard solution contains 0.1 mg. of glycocholic acid for each cc. Five standards containing 0.1, 0.15, 0.2, 0.3, and 0.4 mg., respectively, are prepared, and the solutions evaporated to dryness by placing the beakers before an electric fan.

To the dried unknown and to each standard is added 0.4 cc. of a 1 per cent solution of cane sugar which, in the unknown, should be rubbed up with the barium precipitate. 8 cc. of 60 per cent (by volume) sulfuric acid are added to each beaker and the contents mixed. The loosened precipitate recovered from the ether washings is washed from the centrifuge tube to the respective beaker at this time. The beakers are then placed in an oven or water bath at approximately 37° for 1 hour. It is during this period that the solution becomes pink. The solutions are then cooled in the ice box for a few minutes to retard further development of color. During the development of the Pettenkofer color, and thereafter, the unknown and standard solutions must be treated alike with respect to light and heat. The barium sulfate precipitate in the unknown is removed by centrifuging at high speed for 15 minutes. The supernatant solution is then read in a colorimeter against the standard most nearly corresponding in intensity.

The color developed in the Pettenkofer reaction varies from a faint pinkish yellow to a deep purplish pink as the amount of bile acids in the solution increases. Because of this change in hue, direct colorimetric comparison is difficult. Satisfactory results have been obtained with a Duboseq colorimeter, fused glass cups
and a compensating wedge being used. The standard which most nearly corresponds in intensity to the unknown is set at 15 mm. in the left hand cup, and the unknown matched with it. The compensating wedge is used to secure an exact correspondence in hue of the two solutions.

The strength of the Pettenkofer reaction is reported in terms of mg. of glycocholic acid for each 100 cc. of blood calculated by the formula:

\[
\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{strength of standard in mg.} \times \frac{100}{3} = \text{mg. of glycocholic acid for each 100 cc. of blood.}
\]

Preparation of Crystalline Glycocholic Acid.\(^2\)

Commercial preparations of glycocholic acid sufficiently pure for use as a standard have been difficult to obtain and we have found it necessary to make our own preparations. For this purpose, a 5 per cent solution of a commercial preparation of the bile salts obtained from Armour and Company was used. The glycocholic acid in this solution was then precipitated by the addition of an excess of a 10 per cent ferric chloride solution according to the directions of Hammarsten (11). The curdy precipitate was filtered off in a Buchner funnel and washed once with water. The iron salt was then decomposed with 5 per cent sodium carbonate solution. Care must be taken to rub up the precipitate thoroughly with several changes of the carbonate solution; otherwise there will be considerable loss at this stage. The solution containing the sodium glycocholate was then neutralized to litmus with hydrochloric acid and evaporated on a steam bath to a small volume. Ether was poured on the solution to a depth of 1 to 2 cm., and dilute hydrochloric acid added slowly, with vigorous shaking, until permanent opalescence was produced. It was then placed on ice until crystallization started. Once this was started more acid was added and the solution almost immediately became transformed into a firm mass of fine needle-like crystals. The mother liquor was removed by a suction filter. The crystals were redissolved with the aid of sodium carbonate to form approximately a 10 per cent solution and recrystallized by the addition of ether

\(^2\) This has been prepared by Dr. C. H. Greene.
and acid. In crystallization an excess of acid is to be avoided, and
the process is greatly facilitated by "seeding" with crystals of
glycocholic acid. The first crop of crystals was usually white;
if not, the crystals were dissolved in alcohol and decolorized by
charcoal. The material was recrystallized three times as outlined.
It was then recrystallized twice from absolute alcohol by the
addition of distilled water. The final product was of acceptable
purity as judged by the analytic figures given in Table I. Letsche
(19) found that the melting point is not sharp and depends on the
rate of heating. The values reported are for the temperature at
which the material sinters. He has pointed out that care must be
used in the final drying of the crystals to avoid the formation of
paraglycocholic acid, which is present in all specimens which have

<table>
<thead>
<tr>
<th>Table I. Analysis of Preparations of Crystalline Glycocholic Acid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theory for C_{54}H_{43}O_{25}N_2</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Preparation 1 (used as</td>
</tr>
<tr>
<td>standard)</td>
</tr>
<tr>
<td>Preparation 2</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*Uncorrected.

stood for some time. The formation of the latter markedly ele-
vates the melting point but it gives the same reaction with the
Pettenkofer test as glycocholic acid and its presence in a sample of
glycocholic acid does not interfere with the use of the latter as a
standard.

Phosphatides are reported to give a positive Pettenkofer reac-
tion, and Long and Gephart (21) have pointed out the difficulty in
eliminating all traces of them from preparations of the bile acids.
The recrystallized glycocholic acid used by us as a standard con-
tained only 0.027 per cent of total phosphorus. This is equivalent
to less than 1 per cent of lecithin, a negligible amount so far as this
method is concerned.
Control Experiments on Method.

Development of Color.—The accuracy of any colorimetric method depends on the uniformity of the color and on the correctness of the proportionality between the color and the amount of material to be determined. In the Pettenkofer test the sulfuric acid and sugar form a furfural derivative, which, under the influence of the sulfuric acid, reacts with cholic acid to produce a purplish pink color. The development of the color is a progressive reaction, the speed of which can be controlled by temperature. For the quantitative test this is most satisfactorily controlled by first evaporating the solution to be tested to dryness; sugar in a small volume of solution (4 mg. in 0.4 cc.) and 60 per cent sulfuric acid are then added to the residue at room temperature and the temperatures of all the unknown and standard solutions are raised simultaneously.

Different degrees of color are developed by different degrees of heat but, whatever the condition of heat, it must be exactly the same for all tests, in order to secure proportionality in the intensity of the color. Variations in the temperature of different sections of a drying oven or incubator may lead to considerable variation in the color developed. The optimal conditions of heating were secured in a water bath of uniform temperature. To standardize the heating more accurately the same size and shape of receptacle should be used in all tests. 100 cc. beakers are convenient for the volumes recommended.

With the same amount of bile acids the hue and intensity of color are dependent not only on the temperature but on the concentration of the reagents. Sugar was found to be a more satisfactory reagent than furfural, which has been used by certain investigators, since furfural did not increase the sensitivity of the test and is more difficult to standardize. 4 mg. of sugar are suitable for routine use, since the color is good and the intensity adequate for colorimetry. The solution containing 60 per cent sulfuric acid was found to be the most satisfactory for colorimetry in regard to both the hue and intensity of the color produced.

The uniformity of the color developed by this technique and the proportionality in intensity between the color and the amount of bile acids used is shown by the comparison of various standard
solutions in Table II. This table, which gives a typical series of results, shows variations in most cases not exceeding 3 per cent. These are due in part to variations in the colors developed because of unequal heating and in a slight degree to the difficulties of the colorimetric comparison of solutions of varying hue. Slight changes in the concentration of bile acids produce marked changes in the hue. It is essential, therefore, that the standard and unknown be of approximately the same concentration, but it is manifestly impossible to prepare standards which will match all concentrations of bile acids. Furthermore, the blood tests are slightly yellower than the corresponding standard solutions.

| TABLE II. |
| Uniformity and Proportionality of Color Developed by Pettenkofer Reaction Under Standardized Conditions. |

Observed Colorimetric Readings of Standard Solutions of Varying Concentrations.

<table>
<thead>
<tr>
<th>0.1 mg. glycocholic acid.</th>
<th>0.15 mg. glycocholic acid.</th>
<th>0.20 mg. glycocholic acid.</th>
<th>0.25 mg. glycocholic acid.</th>
<th>0.30 mg. glycocholic acid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Against 0.1 mg. at 15 mm. (theory, 15.0)</td>
<td>Against 0.15 mg. at 15 mm. (theory, 22.5)</td>
<td>Against 0.20 mg. at 15 mm. (theory, 18.5)</td>
<td>Against 0.25 mg. at 15 mm. (theory, 18.75)</td>
<td>Against 0.30 mg. at 15 mm. (theory, 20.0)</td>
</tr>
<tr>
<td>15.5</td>
<td>23.5</td>
<td>15.0</td>
<td>20.0</td>
<td>15.0</td>
</tr>
<tr>
<td>15.0</td>
<td>23.4</td>
<td>15.5</td>
<td>20.4</td>
<td>15.0</td>
</tr>
<tr>
<td>14.8</td>
<td>22.2</td>
<td>15.0</td>
<td>20.0</td>
<td>14.7</td>
</tr>
<tr>
<td>14.7</td>
<td>22.5</td>
<td>15.0</td>
<td>20.0</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Especially attention, therefore, must be given to the colorimetric readings, which should be made within an hour after the development of the color. On standing at room temperature, the solutions slowly become browner, the rapidity of the change depending on the temperature.

Colorimetric Comparison.—For an accurate comparison of intensity of colors by colorimetry the hue and transparency of the solutions to be compared should correspond exactly. In the case of the Pettenkofer reaction it is essential that full compensation for the yellow be made if the intensity of the pink is to be determined quantitatively. This is especially necessary in blood tests.
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in which unavoidably the tint is yellower than the corresponding standards. Satisfactory readings were obtained with a Duboscq calorimeter by means of a compensating wedge of the type used in the Autenrieth-Hellige calorimeter. The wedge was filled with a solution prepared by heating 0.4 cc. of the sugar solution and 8 cc. of 60 per cent sulfuric acid for 1 hour at 37°. A second wedge was filled with this solution diluted from one-third to one-half with water. These colors are quite stable and can be used over a long period of time.

Because of the strong sulfuric acid a calorimeter equipped with fused glass cups is essential. The unknown solution is then read against the standard which most nearly corresponds in color. The yellow wedge is held beneath the pinker of the two solutions (usually the standard) and so regulated as to secure an exact equivalence in hue. The colorimetric reading is laborious but after some practice a fair degree of speed and accuracy can be obtained. Satisfactory readings can also be obtained by the use of a bicolorimeter such as that described by Myers (27) or by Wu (44).

Comparison of the Pettenkofer Test with Previously Accepted Methods for Determination of Bile Acids in Bile.—The Pettenkofer reaction provides a most sensitive test for the bile acids but because this reaction is not entirely specific its use for the determination of these substances in biologic fluids has been criticized. While the method reported here was developed primarily for the analysis of blood it is equally applicable to bile (23, 24). As such it is possible to control the results obtained by comparison with the accepted methods for the determination of the bile acids in bile.

Samples of bile were extracted with boiling alcohol, cooled, and made up to a volume corresponding to a 1:200 dilution. The filtered solution was then decolorized by adding norit in the proportion of 50 mg. for each 35 cc. An aliquot supplying 0.2 to 0.4 mg. of bile acids was then evaporated to dryness and treated with acid as were the blood samples. The norit removes nearly all the cholesterol from the bile sample and in consequence treatment by barium hydroxide and subsequent extraction with ether are unnecessary.

For comparison with the Pettenkofer reaction in the latter case
we have used the method of Schmidt and Dart (37), a modification of the original method of Foster and Hooper (7), in which the bile acids are hydrolyzed by alkali and the liberated taurine and glycine determined by the gasometric amino acid method of Van Slyke. The method of Rosenthal and Lauterbach (35) is similar except that the β-naphthoquinone-sulfonic acid reagent of Folin (6) is used for the colorimetric determination of the liberated amino acids. In each case we have determined the neutral sulfur content of the protein-free bile and calculated the taurocholic acid present by the method of von Bergmann (3). By making appropriate corrections in each case, it is then possible to calculate the glycocholic acid present.

The comparison of the three methods as applied to the analysis of commercial preparations of bile salts and specimens of bile from different sources is shown in Table III. In each group, one set of analyses in triplicate is given to show the variations of each method. To simplify the presentation of this material only the averages of such determinations are given in the remainder of the table. The composition of the different specimens of bile agrees with the usually accepted values while the extreme variability in the composition of commercial preparations of bile salts, a point emphasized by Lewis (20), is well illustrated.

The gasometric method for the determination of amino nitrogen can be used on colored solutions such as the alcoholic extract of bile, but the pigment must be removed if the amino nitrogen liberated by hydrolysis is to be determined colorimetrically or if the Pettenkofer test is to be used for analysis. Charcoal has been used commonly to decolorize the bile. Table III shows that charcoal, when used in excess, may cause a loss of from 2 to 10 per cent in the amino nitrogen liberated by hydrolysis in bile samples. The sulfur content of dog bile can be used as an index to the taurocholic acid present, and sulfur determinations show a corresponding loss when the bile is treated with charcoal. An excess of charcoal, therefore, may be a source of loss, but if minimal amounts are used, this does not introduce an error greater than is inherent in the method, and the analytic results obtained by the use of the modified Pettenkofer test generally agree with those obtained by the gasometric method which does not require the use of charcoal.
### TABLE III.
Comparative Analyses of Bile and Preparations of Bile Salts.

<table>
<thead>
<tr>
<th></th>
<th>Gasometric determination of amino N (Schmidt and Dart), per cent.</th>
<th>Colometric determination of amino N (Rosenthal and Lauterbach), per cent.</th>
<th>Quantitative Pettenkofer test (Aldrich), per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycocholic acid.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparation 1</td>
<td>2.98</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>(standard)</td>
<td>2.76</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td>Preparation 2</td>
<td>3.25</td>
<td>1.40</td>
<td>1.40</td>
</tr>
<tr>
<td>Commercial bile salt preparations.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.21</td>
<td>1.88</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>3.24</td>
<td>1.90</td>
<td>0.50</td>
</tr>
<tr>
<td>3</td>
<td>3.57</td>
<td>1.55</td>
<td>2.08</td>
</tr>
<tr>
<td>4</td>
<td>2.45</td>
<td>1.07</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>0.48</td>
<td>0.21</td>
<td>1.09</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Bile samples (from hospital patients).</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallbladder...</td>
<td>0.106 0.047 0.171 0.047 0.124 0.120 1.72 3.98 5.70 0.113 0.033 0.026 1.72 2.69 4.41 5.17 1.55 1.72 3.62 5.31</td>
</tr>
<tr>
<td>Bile fistula...</td>
<td>0.113 0.074 0.127 0.123 4.08 5.80 0.113 0.096 2.69 4.41 5.25 3.70 5.42</td>
</tr>
<tr>
<td>Gallbladder...</td>
<td>0.102 0.172 0.125 0.121 4.02 5.74 0.119 0.096 2.90 4.62 5.08 3.53 5.25</td>
</tr>
<tr>
<td>a. Gallbladder untreated...</td>
<td>0.098 0.043 0.125 0.043 0.082 0.070 1.67 2.62 4.19 0.113 0.029 0.048 1.57 2.80 4.37 3.88 1.41 1.57 2.47 4.04</td>
</tr>
<tr>
<td>b. After treatment with charcoal...</td>
<td>0.90 0.039 0.123 0.039 0.084 0.081 1.45 2.65 4.10 0.102 0.026 0.076 1.45 2.52 3.97 3.70 1.31 1.45 2.39 3.84</td>
</tr>
<tr>
<td>Bile samples (from dogs).</td>
<td></td>
</tr>
<tr>
<td>Gallbladder...</td>
<td>0.90 0.40 0.35 0.40 14.8 0.0 12.9 0.26 0.27 14.8 0.0 14.4 13.1 13.4 14.8 14.5 0.8 15.6</td>
</tr>
<tr>
<td>Bile fistula...</td>
<td>0.94 0.35 12.9 0.25 13.8 14.2</td>
</tr>
<tr>
<td>a. Gallbladder untreated...</td>
<td>0.91 0.35 12.9</td>
</tr>
<tr>
<td>Gallbladder...</td>
<td>0.85 0.37 0.33 0.37 13.6 0.0 12.2 0.15 0.24 13.6 0.0 7.8 13.4 12.3 13.6 1.1 14.7</td>
</tr>
<tr>
<td>Bile fistula...</td>
<td>0.101 0.043 0.039 0.043 1.61 0.0 1.44 0.026 0.029 1.61 0.0 1.44 1.45 1.45 1.61 1.61</td>
</tr>
<tr>
<td>a. Bile fistula untreated...</td>
<td>0.336 0.146 0.144 0.146 5.40 0.0 5.31 0.053 0.097 5.40 0.0 2.92 4.92 4.87 5.40 0.05 5.45</td>
</tr>
<tr>
<td>b. After treatment with charcoal...</td>
<td>0.329 0.143 0.132 0.143 5.28 0.0 4.86 0.047 0.095 5.28 0.0 2.60 4.73 4.76 5.28 5.25</td>
</tr>
</tbody>
</table>
Rosenthal and Lauterbach (35) report that the reaction of taurine with the Folin amino acid reagent produces only two-thirds of the expected amount of color. We have found this reaction to be much more irregular. The color is yellower than that obtained from alanine or glycine standards, and the intensity also seems to vary with the quantity of taurine present and its relationship to the total amino acids. We used the same correction factor in calculating the taurine color as did Rosenthal and Lauterbach but believe that it is but an approximation at best. The analysis of dog bile in particular emphasizes the disparity between the taurocholic acid so determined and that calculated from the sulfur present.

In general these three methods give concordant results, especially in the analysis of bile obtained from man. The method of Rosenthal and Lauterbach is more variable than the other two and is not reliable in the analysis of dog bile. The difference between the results obtained with the modified Pettenkofer test and the Foster and Hooper (7) method as modified by Schmidt and Dart (37) are within the limits of error of either. As such they would seem to be equally useful for the proximate analysis of bile. The assumed lack of specificity of the Pettenkofer test will always cause adverse criticism, yet comparison of the results obtained by the Pettenkofer method and that of Foster and Hooper indicates that, in the alcoholic extract of bile, extraneous substances do not interfere to an appreciable extent with the determinations of bile acids by this modification of the Pettenkofer test.

Application of Method to Blood.

Extraction of Bile Acids from Blood.—The bile acids occur in the blood as alkaline salts which are readily soluble in water or alcohol. Redistilled alcohol was used in the method to precipitate the blood proteins and extract the bile salts. Similar values were obtained both with hot and cold alcohol extraction; heating is therefore unnecessary.

The Pettenkofer reaction is not a specific test for bile acids. Von Udrenszy (42), Mylius (28), and others, have pointed out numerous other compounds which give a similar color, among them lecithin, cholesterol, and oleic acid which may be present in an alcoholic extract of blood. As shown later, the Pettenkofer
reaction of these substances under the conditions of our test is minimal, but we have not been able to extract bile acids from the blood quantitatively without having to include some of these substances in the extract. The method, therefore, has been developed to give the maximal Pettenkofer test in the protein-free blood filtrate.

The alcoholic filtrate may be colored even when no bile pigment is present and this coloring matter interferes with the subsequent Pettenkofer test. No solvent was found that would extract bile acids without likewise extracting considerable quantities of pigment. Various methods of decolorizing the alcoholic filtrate were tried; the results indicate that the activated carbon, norit, could be used more satisfactorily than the others tested.

The optimal amount of norit seems to be the smallest quantity that will free the filtrate of interfering pigment. Norit will remove bile acids from pure solutions and, as we have pointed out, its use in excess in decolorizing bile causes a slight loss. However, charcoal is less active in removing bile salts from blood filtrates containing pigments and other readily adsorbable substances. On an average more than 90 per cent of bile acids added to blood filtrates can be recovered after clearing with 50 mg. of norit and for routine use this amount of norit added to 35 cc. of blood filtrate is optimal.

The clear alcoholic filtrate, evaporated to dryness, treated with sugar and sulfuric acid, and heated, causes the pink color characteristic of the Pettenkofer reaction. With this technique some lecithin, cholesterol, and fat are to be found in the solution. These substances have been reported as giving a positive Pettenkofer test; they were, therefore, studied.

Fatty acids and cholesterol, in amounts equivalent to those present in the extract of a blood sample containing 400 mg. of the former and from 200 to 900 mg. of the latter for each 100 cc., respectively, when evaporated to dryness and treated with sugar and sulfuric acid under the conditions of this test did not give a positive test. Samples of commercial lecithin gave a slight color, the purest sample producing about one three-hundredths of the color which would be developed by the same weight of bile acids. The only other substance which gave a positive Pettenkofer test was a sample of cholesterol which had been in the laboratory for
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several years. The introduction of a Pettenkofer color developed by such substances apparently is not a serious source of error. They do, however, add to the organic substances which, when charred by the sulfuric acid, produce an interfering yellow tint in

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Initial blood sample, mg. per cent.</th>
<th>Acid added, mg. per cent.</th>
<th>Total theoretic values, mg. per cent.</th>
<th>Observed values, mg. per cent.</th>
<th>Difference between observed and theoretic values, mg. per cent.</th>
<th>Difference, mg. per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>4.0</td>
<td>8.0</td>
<td>7.9</td>
<td>-0.1</td>
<td>-1.2</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>4.0</td>
<td>8.2</td>
<td>7.7</td>
<td>-0.5</td>
<td>-6.1</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>4.0</td>
<td>8.0</td>
<td>7.9</td>
<td>0.1</td>
<td>+1.1</td>
</tr>
<tr>
<td>4</td>
<td>4.7</td>
<td>4.0</td>
<td>8.7</td>
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</table>

Difference.

the test solution. This makes the colorimetric reading difficult, particularly in blood giving only a faint Pettenkofer test. Their removal was therefore attempted by ether extraction.

Although pure bile salts are relatively insoluble in ether,
amounts equivalent to those studied here are readily soluble in ether containing fats or other lipoids. The addition of a small amount of barium hydroxide does not prevent this loss. Petrén cleared an alcoholic blood filtrate with dry powdered barium hydroxide and evaporated it to dryness. After extracting with ether to remove the fats he found that the ether extract gave a positive Pettenkofer reaction, and so questioned the use of this test. These experiments were repeated and confirmed by us. Furthermore we found that bile acids added to the alcoholic filtrate prepared according to Petrén’s method are largely removed when the dried residue is extracted with ether. The addition of 1 cc. of a saturated solution of barium hydroxide to the blood filtrate prevented any such loss of bile acids during the extraction with ether. Analysis showed this ether extract to contain cholesterol and some fats but no lecithin. No further separation of the bile acids from the organic substances that escaped ether extraction was attempted. Even with this degree of separation the color was improved in quality and higher Pettenkofer values were obtained. The addition of baryta and the washing with ether had no effect on the Pettenkofer reaction of the standard glycocholic acid.

Recovery of Bile Acids Added to Blood.—The accuracy of the method as a whole is shown in Table IV, which gives the results obtained in the recovery of known quantities of bile acids added to blood. The average loss was about 0.5 mg. for each 100 cc.

Comment.

We fully realize the difficulties which arise in interpreting data obtained by means of a non-specific color reaction such as the Pettenkofer test for bile acids. Although, so far as possible, interfering substances have been removed, we cannot attach a precise chemical identity to all the material responsible for the Pettenkofer reaction, especially in normal blood. Only the isolation and chemical identification of pure bile acids from normal blood will conclusively prove their presence. By the technique described, the maximal reading in the alcoholic extract of the blood is obtained. In man the blood normally gives a positive Pettenkofer reaction equivalent in intensity to that which would be
produced by the presence of from 3 to 6 mg. of glycocholic acid for each 100 cc. Increased values have been found in abnormal blood. Table V shows the values obtained in a variety of clinical and experimental conditions.

In conditions in which it is definitely accepted that bile acids are present in the blood in increased amounts, as following their intravenous injection, or in obstructive jaundice, the method is of value in the quantitative study of the changes which occur. Since bile acids added to the blood may be recovered with an accuracy of 90 per cent or more, a normal Pettenkofer value assuredly excludes any significant increase in the blood. Keeping

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glycocholic acid (mg. per cent)</th>
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<tbody>
<tr>
<td>Normal blood (dog)</td>
<td>6.5</td>
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<tr>
<td>Experimental obstructive jaundice in dog</td>
<td>16.3</td>
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<tr>
<td>Following intravenous injection of bile acids in dog</td>
<td>75.0</td>
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<tr>
<td>Normal blood (man)</td>
<td>5.1</td>
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<tr>
<td>&quot; &quot; &quot;</td>
<td>3.2</td>
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<tr>
<td>Obstructive jaundice</td>
<td>8.3</td>
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<tr>
<td>&quot; &quot; &quot;</td>
<td>16.7</td>
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<tr>
<td>Carcinoma of liver</td>
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<tr>
<td>Portal cirrhosis</td>
<td>7.2</td>
</tr>
<tr>
<td>Biliary &quot;</td>
<td>14.3</td>
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</table>

in mind the limits to the interpretation of the values, one may use this modification of the Pettenkofer test to obtain valuable information on the metabolism of the bile acids by the quantitative study of the changes in the blood in various experimental and clinical conditions.

SUMMARY.

The Pettenkofer reaction can be used to determine pure bile acids quantitatively in amounts of from 0.10 to 0.50 mg. with an accuracy of ±5 per cent.

A series of analyses of commercial preparations of bile salts and of bile from different sources, made to compare this method with
that of Schmidt and Dart, shows that the results obtained by the modified Pettenkofer test agree within the limits of error with those obtained by the gasometric determination of the amino nitrogen liberated by alkaline hydrolysis. Because of the rapidity and ease of the determination by the new colorimetric method and because it can be applied in the analysis of small amounts of material, it is the method of choice when these factors enter into the selection of a method.

The reaction was applied to an alcoholic extract of 5 cc. of blood and a method of extraction which permits the determination of maximal amounts of Pettenkofer-reacting material is described. Recovery of bile acids added to the blood is made by this method with an average loss of about 0.5 mg. for each 100 cc. With few exceptions the recovery is greater than 90 per cent.

When interfering substances are removed as completely as possible, normal blood yields a Pettenkofer value equivalent to from 3 to 6 mg. of glycocholic acid for each 100 cc. of blood. Increased values have been found under certain clinical and experimental conditions.

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Martha Aldrich and Mary Sue Bledsoe


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