A NEW PROCEDURE FOR THE ESTIMATION OF BILE SALTS IN BODY FLUIDS BASED ON BILE SALT HEMOLYSIS*

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The present method was devised to estimate minute amounts of all types of bile salts in small quantities of body fluids.

Only conjugated bile salts are determined (1), or desoxycholic acid (2) or minimal amounts of bile salts (3) fail to be estimated, by established procedures. Colorimetric methods based on the original Pettenkofer reaction (4) have been criticized for lack of specificity (5).

Studying the effect of sodium citrate on bile salt hemolysis, Williams (6) noted that this agent rendered erythrocytes more susceptible to the hemolytic action of bile salts. He suggested the possibility of application of this principle to the estimation of bile salts. The present study has established definite quantitative relationships between concentrations of citrate, bile salts, and erythrocytes. Furthermore, the demonstration that sodium citrate exercises a dual rôle, on the one hand enhancing bile salt hemolysis, on the other deterring sodium oleate hemolysis, has made possible the application of hemolysis in citrate solution to bile salt analysis in body fluids.

Principle

The hemolytic activity of bile salts upon a standardized erythrocyte suspension is measured. A constant relationship between the hemolytic activity of the various common bile salts has been established.

Desoxycholic acid was selected as a standard because of its high hemolytic activity. Furthermore, it is procurable in pure state.

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The results are expressed in terms of desoxycholate or "hemolytic equivalents" of the other bile salts.

The minimal amount of bile salts necessary to effect complete hemolysis in a given time under specified conditions is defined as the threshold hemolytic reaction. Varying subthreshold amounts of desoxycholate are added to a test solution which contains less than the necessary minimal concentration. The content of the test solution is thus determined.

EXPERIMENTAL

Standardization of Erythrocyte Suspension and Bile Acid Mixture.

Erythrocyte Suspension—The serum of defibrinated sheep blood is removed completely by centrifugation for 15 minutes at moderate speed. The cells are washed for 10 minutes in 5 volumes of 0.1 M phosphate buffer solution, then suspended in 10 volumes of fresh buffer, and stored in the refrigerator. Immediately before each test, an adequate portion of the stock suspension is centrifuged at moderate speed for 10 minutes, the supernatant discarded, and the cells suspended in 1.74 M sodium citrate solution to make a 5 per cent suspension. The preliminary treatment with phosphate buffer stabilizes the erythrocyte, the phosphate ion combining firmly with the stroma (7).

The erythrocyte reagent preserved in the refrigerator keeps for 1 week. In many hundred determinations the threshold hemolytic reaction occurred at 9 to 10 mg. per cent, most commonly at 9.3 or 9.6 mg. per 100 cc.

Bile Acid Mixtures—The hemolytic activity of the bile salts was compared. Desoxycholate (Riedel-de Haen) was found to be the most active, being hemolytic in concentration of 10 mg. per 100 cc. or 1 part in 10,000, taurocholate1 22 mg. per 100 cc., glycocholate2 50 mg. per 100 cc., and cholate in concentrations of 70 mg. per cent.3

1 Taurocholate (Merck) claimed by the manufacturers to contain 48 to 55 per cent of taurocholate, most of the remainder being glycocholic acid.
2 Glycocholic acid (Reidel-de Haen) and a sample prepared by E. U. Still, University of Chicago, recrystallized several times.
3 Anthropodesoxycholic acid, isomer of desoxycholic acid which occurs in human bile, and the choleic acids which consist essentially of desoxycholic acid were not studied for their hemolytic properties. It may safely be assumed that they are hemolytic.
In mixtures the hemolytic effect of subthreshold fractions of individual bile salts was found to be precisely additive; e.g., 50 per cent of the desoxycholate threshold combined with 50 per cent of the cholate threshold produced complete hemolysis (Table I). Thus by the addition of subthreshold amounts of any bile salt to any unknown mixture, its bile salt content may be determined and expressed in terms of equivalents of the known salt.

**Table I**

*Summation of Hemolytic Activity of Mixtures of Subthreshold Fractions of Bile Salts*

<table>
<thead>
<tr>
<th>Bile salt mixture No.</th>
<th>Subthreshold fractions</th>
<th>Aggregate thresholds</th>
<th>Hemolysis</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Desoxycholate</td>
<td>Cholate</td>
<td>Glycocholate</td>
</tr>
<tr>
<td></td>
<td>Mg. %</td>
<td>mg. per cent</td>
<td>Mg. %</td>
</tr>
<tr>
<td>1</td>
<td>4.8</td>
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<td>40</td>
</tr>
<tr>
<td>2</td>
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<td>47</td>
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</tr>
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</tr>
<tr>
<td>6</td>
<td>3.3</td>
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<td>24</td>
</tr>
</tbody>
</table>

*Standard Hemolytic Thresholds—Desoxycholate, 9.6 mg. per 100 cc.; glycocholate, 50.0 mg. per 100 cc.; cholate, 80.0 mg. per 100 cc.; oleate, no hemolysis in 15 minutes.

+ signifies that the sheep erythrocyte suspension in standard buffered citrate is completely hemolyzed at the end of 15 minutes; — signifies the absence of, or partial hemolysis at the end of 15 minutes.

* The fraction of a threshold in per cent of any component in the mixture of bile salts is determined by dividing its concentration in the mixture by the concentration of this bile salt giving the standard threshold reaction multiplied by 100.

Hemolytic equivalents have been established for cholate and glycocholate by comparing minimal concentrations necessary to effect complete hemolysis in 15 minutes. Desoxycholate in mg. per cent may be translated approximately into cholate equivalents by multiplying by 7.6, into glycocholate by multiplying by 5.0. Determinations made on sixteen different sheep bloods for cholate...
hemolytic equivalents gave results varying between 6.6 and 8.6. In ten determinations made for the "glycocholate equivalent" the results varied between 4.5 and 5.7. For exact quantitative studies expressing results in terms of glycocholate or cholate their hemolytic equivalents must be determined on the same blood simultaneously with the desoxycholate threshold.

![Figure 1](http://www.jbc.org/)  

**Fig. 1.** The effect of sodium citrate concentration on the minimal bile salt and sodium oleate concentrations necessary to effect complete hemolysis in 15 minutes. 0.07 M concentration phosphate buffer, pH 7.0, in each tube; 0.3 cc. of 5 per cent sheep erythrocyte suspension in varying citrate concentrations. Total volume of each tube, 1 cc.

In conjunction with other established methods, the hemolytic equivalents may assist in the determination of bile salt partitions of body fluids.

**Velocity of Hemolysis**—The rate of hemolysis is influenced by the concentration and type of bile and other salts. 0.07 M con-
centration of phosphate buffer solution preserves erythrocytes.

With increasing concentrations of sodium citrate, lower concentrations of bile salts are necessary to effect complete hemolysis in 15 minutes (Fig. 1).

For estimation of bile salt hemolysis the following concentrations of citrate and phosphate have been found optimal: 0.52 m sodium citrate and 0.07 m phosphate buffer pH 7.0. Under these conditions bile salt hemolysis approaches completion while sodium oleate hemolysis has not begun in 15 minutes (Fig. 1). Oleate permitted to act 30 minutes or more begins to produce partial hemolysis.

Influence of Hydrogen Ion Concentration on Bile Salt Hemolysis—Sheep erythrocytes washed in phosphate buffer solutions pH 7.0, 7.3, 7.6, respectively, and suspended in 20 volumes of 1.74 m sodium citrate were then added to phosphate buffer solutions of corresponding hydrogen ion concentration to make a final 0.07 m phosphate and 0.52 m citrate concentration. Decrease in hydrogen ion concentration markedly accelerated bile salt hemolysis; with a concentration of 9.6 mg. per 100 cc. of desoxycholate complete hemolysis occurred in 25 seconds at pH 7.6, in 45 seconds at pH 7.3, in 10 minutes at pH 7.0 (Fig. 2).

At pH 7.0, however, phosphate-citrate mixtures are more effectively buffered than at pH 7.6. Furthermore, too rapid hemolysis makes readings impossible where several determinations are made simultaneously. Moreover, erythrocytes are better preserved with less clumping at pH 7.0 than at 7.6. For these reasons pH 7.0 was adopted.

Temperature—The entire procedure is carried out at ordinary room temperature.

Specificity—Substances present in body fluids or those added during the procedure have been eliminated as hemolytic agents. Urea, glucose, sodium chloride, cholesterol, uric acid, phenol, cresol, and acetone within concentrations present in normal and pathological fluids have been found to be non-hemolytic.

The differential effect of citrate between bile salt and oleate hemolysis however eliminates soaps as sources of error. Quantitative readings, at 15 minutes precisely, determine only the bile salt content of a mixture which contains even an overwhelming amount of sodium oleate.
Quantitative studies of the bile salt content of body fluids on the basis of the Neufeld phenomenon (8), i.e. lysis of the pneumococcus by bile salt, gave essentially the same results. It required the addition of analogous subthreshold amounts of bile salts to those already present in the body fluids to produce the lysis of either erythrocytes or pneumococci.

A standardized pneumococcus Type I suspension was employed. For the determination of bile salt content of body fluids by the bacterial method, advantage was taken of the observations of

![Graph showing the influence of hydrogen ion concentration on the rate of bile salt hemolysis.](image)

**Fig. 2.** The influence of hydrogen ion concentration on the rate of bile salt hemolysis. 0.52 M concentration of sodium citrate in each tube; 0.07 M concentration of phosphate buffer, pH 7.0, 7.3, 7.6 respectively; 0.3 cc. of 5 per cent sheep erythrocyte suspension in final volume of 1 cc.
Bayer (9) and of Falk and Yang (10) that increasing concentra-
tions of sodium chloride accelerate bile salt lysis of pneumococci 
and the observation of Falk and Yang (11) that 0.85 per cent saline 
inhibits oleate lysis of pneumococci.

Sensitivity—Protein diminishes or prevents bile salt lysis. 
Body fluids must be rendered protein-free. In quadruplicate 
determinations, the hemolytic threshold for desoxycholic acid 
checked to 0.1 mg. per cent. In body fluids duplicate tests checked 
to 0.3 mg. per cent.

Procedure

Reagents

Erythrocyte Suspension—The stock suspension of sheep erythro-
cytes prepared as described above is centrifuged in two lots of 
about 10 cc. The packed cells of one are then suspended in 1.74 M 
sodium citrate solution to make a 5 per cent suspension. This is 
used for the threshold and preliminary test fluid titrations. When 
the final titration of the test solution is made, the second lot of 
packed cells, after removal of the supernatant phosphate buffer 
solution, is freshly treated with sodium citrate. In the interim, 
the buffered packed cells are kept in the refrigerator.

Sodium Citrate Solution, 1.74 M—51.2 gm. of Na₃C₅H₇O₇·2H₂O 
are dissolved in 70 cc. of water with the aid of heat and then made 
up to 100 cc.

1.53 mm Desoxycholate Solution—60 mg. of desoxycholic acid 
(Riedel-de Haen) are dissolved in a slight excess of 0.1 M sodium 
hydrate, 2.1 cc., and the volume made up to 100 cc. with phosphate 
buffer solution pH 7.0.

0.1 M Phosphate Buffer Solution, pH 7.0—5.29 gm. of KH₂PO₄ 
and 21.89 gm. of Na₂HPO₄·2H₂O are dissolved in a liter of dis-
tilled water.

Determination of Standard Threshold Hemolytic Reaction

The minimal volume of 1.53 mm sodium desoxycholate solution 
which will effect complete hemolysis is determined. Tubes, size 
10 × 77 mm., are filled as follows: 0.17 to 0.14 cc. of 1.53 mm 
desoxycholate solution, representing concentrations between 10.2 
and 8.4 mg. per cent, are added with a pipette calibrated to 0.001 
cc. Buffer solutions, pH 7.0, are then added to 0.7 cc. in each
tube (Table II). After the addition of 0.3 cc. of erythrocyte suspension, the mixtures are agitated vigorously and the tube containing the minimal amount of desoxycholate with complete hemolysis in exactly 15 minutes is determined. Tubes in which complete hemolysis has taken place are easily recognized when viewed against transmitted daylight or a daylight electric bulb by their perfect transparency and absence of sediment of erythrocytes. The transition between tubes with complete and partial lysis is sharp and offers no difficulty.

A more definitive threshold is finally determined within a closer range in tubes with gradations of 0.1 mg. per cent desoxycholate when a less concentrated (0.51 mM) solution is used.

Bile Salts in Blood

Extraction of Blood—A minimum of 3 cc. of oxalated whole blood is necessary; 6 to 9 cc. are preferable. The proteins are precipitated by the slow addition of 8 volumes of alcohol to 1 volume of blood. Chilling for 30 minutes at freezing temperature and the use of barium hydroxide assure clear filtrates. The chilled mixture is carefully filtered. The precipitate is washed with about 5 cc. of alcohol. 0.5 cc. of saturated barium hydroxide is added to the filtrate and then evaporated on the water bath to complete dryness. The residue in the evaporating dish is washed with 5 cc. of petroleum ether, of high boiling point, just brought to a boil on the water bath. The ether washing is decanted, avoiding loss of residue. The mixture is just evaporated to dryness in the same dish. The residue is now recovered quantitatively in as many cc. of phosphate buffer pH 7.0 as cc. of blood were extracted if 6 cc. or more were originally used. Otherwise recovery is made in twice as many cc. of buffer.

The buffered extract is further chilled for 15 minutes, then centrifuged, and the supernatant bile salt extract is ready for determination. Repeated chilling and centrifugation may be necessary if the filtrate is not perfectly clear.

The extract is added in 0.5 cc. amounts to a series of tubes to which 0.170 to 0.105 cc. of 1.53 mM solution of desoxycholate has been added, representing concentrations between 10.2 and 6.3 mg. per cent. The volume in each tube is made up to 0.7 cc. with phosphate buffer before 0.3 cc. of 5 per cent erythrocyte suspension
Table II

Outline of Procedure

| Tube No. | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   | 19   |
|----------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Na       | mg. %| 10.2 | 9.9  | 9.6  | 9.3  | 9.0  | 8.7  | 8.4  | 8.1  | 7.8  | 7.5  | 7.2  | 6.9  | 6.6  | 6.3  | 5.4  | 4.2  | 3.0  | 1.8  | 0.6  |
| desoxy-  | mM,  | 0.170| 0.165| 0.160| 0.155| 0.150| 0.145| 0.140| 0.135| 0.130| 0.125| 0.120| 0.115| 0.110| 0.105| 0.090| 0.070| 0.050| 0.030| 0.010|
| cholate  | cc.  | 0.170| 0.165| 0.160| 0.155| 0.150| 0.145| 0.140| 0.135| 0.130| 0.125| 0.120| 0.115| 0.110| 0.105| 0.090| 0.070| 0.050| 0.030| 0.010|

0.1 m phosphate buffer of pH 7.0 was added to make a total volume of 0.7 cc. in each tube after the addition of body fluid extract or desoxycholate or both as required. 0.3 cc. of 5 per cent sheep erythrocyte suspension in 1.74 m sodium citrate was added to each tube. Complete hemolysis was read precisely in 15 minutes after mixture of cells with the contents of each tube.

**Standard Hemolytic Threshold Determination**—Tubes 1 to 7 are set up for the preliminary determination of the standard threshold. In the final determination 0.5 mM desoxycholate is used. This permits readings within a narrow range accurate to 0.1 mg. per cent. For example, if the preliminary titration indicates complete hemolysis in 15 minutes produced by the minimum concentration of 9.3 mg. per 100 cc. of desoxycholate, the final determination is carried out with 0.5 mM desoxycholate between 9.3 and 9.0 mg. per 100 cc. at intervals of 0.1 mg. per 100 cc.

**Addition of Subthreshold Concentrations of Desoxycholate to Blood, Urine, and Bile Extracts**—For blood and urine determinations, Tubes 1 to 14 are set up. 0.5 cc. of blood and urine extracts is added to each tube. For bile requiring the addition of subthreshold concentrations of desoxycholate to produce complete hemolysis, bile extract diluted 1:10 with buffer is added in 0.5 cc. amounts to Tubes 1, 5, 9, and 13 to 19.

**Direct Action of Bile Extracts without Addition of Subthreshold Concentrations of Desoxycholate**—For bile extracts rich in bile salts, the extract diluted 1:20 with buffer is added to make the volume in each tube up to 0.7 cc. A final determination is made within a narrow range. For example, if complete hemolysis is obtained with a minimum of 0.4 cc. of bile extract (1:20) then 0.38, 0.36, 0.34, 0.32 cc. are added and the tube in which complete hemolysis occurs discovered.
is added (Table II). The mixtures are agitated vigorously and the results read precisely in 15 minutes. Complete hemolysis is noted. In doubtful tubes comparison with the tube containing the threshold concentration of desoxycholate added to the unknown is made.

In pathological bloods with unusually high bile salt content further dilution of the extract with buffer solution may be necessary to bring the determination within the desired range. Such concentrations have not been encountered by us thus far in pathological bloods.

Calculation—(Desoxycholate concentration of standard threshold reaction) − (subthreshold concentration which just gives complete hemolysis in unknown) × dilution factor = concentration of bile salts expressed as desoxycholate (mg. per 100 cc.). The dilution factor is 2 if the bile salts are taken up in a volume of buffer equal to the original volume of blood used, since 0.5 cc. of extract is diluted to 1 cc. The dilution factor is 4 if buffer equal to twice the original volume of blood is used.

Bile Salt Content of Normal Blood—The bile salt content of whole blood has been found to vary between 0.5 and 4.0 mg. per 100 cc. in terms of sodium desoxycholate. Examinations were made in twenty-five normal donors or individuals with minor medical ailments and free of liver disease. In a limited number of observations on whole blood from subjects with liver disease the maximum bile salt content thus far encountered has been 10 mg. of desoxycholate per 100 cc.

Determination of Bile Salts in Urine

The procedure is identical with that in blood. 8 to 10 cc. of urine are extracted with alcohol. The bile salts are recovered in the same amount of buffer solution (Table II).

Calculation—The method is the same as for blood. The factor of dilution, the standard threshold concentration of desoxycholate, and the subthreshold amounts added enter into the calculation.

Bile Salts in Normal Urine—The bile salt content of normal urine has been found to vary between 0.5 and 4.0 mg. per 100 cc. in terms of desoxycholate. The urines of twenty-five normal subjects were examined. In a limited number of observations on urines from patients with liver disease increases in bile salt content
as high as 8 mg. per 100 cc. of desoxycholate equivalents have been encountered thus far.

_Determination of Bile Salts in Bile_

The procedure is slightly different from that in urine and blood because of the high bile salt content of bile. 1 cc. of bile is carefully measured and slowly precipitated with 30 to 40 cc. of alcohol. The mixture is chilled for 30 minutes at freezing temperature and then filtered clear. 3 cc. of saturated barium hydroxide are added to the filtrate which is evaporated to complete dryness on the water bath. The residue is extracted with 15 cc. of high boiling point petroleum ether just brought to a boil on the water bath. The ether washing is decanted. Care is exercised to avoid loss of residue. The residue is thoroughly mixed in the evaporating dish with 10.5 cc. of 1 per cent sodium sulfate solution, then evaporated just to dryness. The residue is quantitatively recovered in 10 cc. of phosphate buffer solution pH 7.0. After centrifugation, the supernatant extract is ready for the determination.

If 0.7 cc. of extract (1:10 dilution) or 0.1 cc. of this diluted extract further diluted to 0.7 cc. with buffer solution hemolyzes 0.3 cc. of erythrocyte suspension, the 1:10 extract is diluted to 1:20 with buffer and the procedure carried on as in Table II. If no hemolysis occurs in 15 minutes in this preliminary orientation test, subthreshold concentrations of desoxycholate are added as with blood and urine (Table II). This is necessary only in pathological biles devoid of or poor in bile salts. The final estimation in any event is made in a more narrowly restricted range of dilution (Table II).

_Calculation_—As in blood, the factor of dilution, the standard threshold desoxycholate concentration, the subthreshold concentrations of desoxycholate, if added, enter into the calculation. Results are expressed in gm. per cent.

_Bile Salt Content of Normal Bile_—The content of cadaver gallbladder bile in thirty subjects without liver and gallbladder disease varied between 0.96 and 4.80 per cent of desoxycholate equivalents. In pathological cases marked decreases were found.
Recovery of Bile Salts Added to Body Fluids

When varying concentrations of desoxycholate, cholate, and glycocholate were added to normal and pathological blood, urine, and bile over 90 per cent could be recovered (Table III). Varying concentrations of sodium oleate added did not influence the quantitative recovery of bile salts.

<table>
<thead>
<tr>
<th>Bile salt added</th>
<th>Body fluid</th>
<th>Desoxycholate present</th>
<th>Desoxycholate equivalents added</th>
<th>Amount recovered</th>
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<tbody>
<tr>
<td>Desoxycholate</td>
<td>Blood</td>
<td>4.2</td>
<td>1.7</td>
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<tr>
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<td>4.2</td>
<td>3.4</td>
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<td>120.0</td>
<td>265.0 – &lt;280.0</td>
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<td>Urine</td>
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<td>1.3</td>
<td>4.2 – &lt;4.5</td>
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<td>2.4</td>
<td>6.6 – &lt;6.9</td>
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<tr>
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<td>&quot;</td>
<td>3.0</td>
<td>3.6</td>
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<td>2.1</td>
<td>5.0 – &lt;5.3</td>
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</tbody>
</table>

* Cholate and glycocholate added were translated into desoxycholate by means of hemolytic equivalents determined on the same sheep erythrocyte suspensions.

Comment

The demonstration of bile salts in concentrations varying between 0.5 and 4.0 mg. of desoxycholate equivalents per 100 cc. in normal whole blood merits comment. Evidence for the presence of bile salts in normal blood could not be adduced by Walker (5) nor by Jenke and Steinberg (12). However, the possible influence of desoxycholic acid by itself or in mixture with other bile salts on colorimetric and spectroscopic determinations was ignored. Approximately a 5-fold concentration of desoxycholic acid is required for spectroscopic demonstration with the Pettenkofer reaction as compared with cholic acid.
The concentrations of bile salts found in the blood are not toxic. Even higher concentrations are rendered non-toxic in the presence of blood proteins (13-16). In the presence of plasma approximately 15 times the concentration of desoxycholate is necessary to effect complete hemolysis.

The bile salts found in blood and urine, though expressed in terms of desoxycholate equivalents, represent all types of bile salts. Since desoxycholate is over 7 times as hemolytic as cholate, it must be appreciated that pathological increases in these latter salts may only appear as slight increases in terms of desoxycholate. Therefore, such slight increases must be regarded as significant. Should unusually high figures be obtained, the increase may actually be mainly in the form of desoxycholate, as such an increase translated into terms of the other bile salts might give figures incredibly high. Unusual increases of this type of bile salt in body fluids may prove to be of physiological and clinical significance especially since desoxycholic acid is highly reactive both chemically and biologically.

**SUMMARY**

A procedure for the estimation in terms of desoxycholate of minute amounts of all types of bile salts in body fluids has been devised based on the hemolytic property of bile salts. The results expressed as desoxycholate can be translated into cholate and glycocholate by means of hemolytic equivalents.

The bile salt content of normal blood, urine, and bile has been determined. Normal blood and urine contain concentrations between 0.5 and 4.0 mg. of desoxycholate per 100 cc. or its equivalent. Normal cadaver gallbladder bile contains between 1 and 5 per cent desoxycholate or its hemolytic equivalent.

The interfering hemolytic influence of fatty acids and soaps has been obviated.

Expert technical assistance was rendered by Mr. A. L. Lichtman and Miss Leah Kaplan.

**BIBLIOGRAPHY**

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