CCLXXIII. A COMPARISON OF SOME METHODS FOR THE DETERMINATION OF BILE ACIDS IN BILE, AND THE PROPORTION BETWEEN THE DIFFERENT ACIDS.

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(Received 27 June 1936.)

NUMEROUS methods for the determination of the bile acids in bile have been published utilizing for instance the Pettenkofer reaction, determination of amino-groups in the amino-acid components of the conjugated acids, optical properties, determination of taurine-sulphur, fluorescence with sulphuric acid etc. Each of these methods, however, is limited to the determination of particular bile acids.

We therefore thought it worth while by applying selected methods simultaneously to bile to try to determine the proportion between cholic and deoxycholic, conjugated and unconjugated, glycine and taurine acids. At the same time we have tested, compared and partly modified the various procedures.

Methods

The bile was mixed with alcohol, boiled, diluted to 10 vols. and filtered. Aliquot parts were then evaporated to dryness with some NaHCO_3 on the water-bath.

The colorimetric method. As already pointed out by Nakagawa & Fujikawa [1930] and by Reinhold & Wilson [1932], deoxycholic acid and its conjugates do not give the Pettenkofer reaction or its modifications. We have tested some other bile acids with furfuraldehyde and sulphuric acid and found that the following failed to react: cholanic, bilianic, dehydrocholic, chenocholic, α -hyoglycocholic, β -hyoglycocholic acid and "dyslysine";¹ the only positive results were obtained with cholic acid and its conjugates, including scymnol. The reaction was proportional to the molar concentration, whether the substrate was cholic, glycocholic or taurocholic acid and the colour and its absorption spectrum were equal for all these acids. The best way (of many tested) to carry out the reaction proved to be that described by Gregory & Pascoe [1929] as modified by Josephson [1935]. In this form the reaction is sufficiently specific and sensitive for most biles. Cholesterol does not react. The differences between the duplicates from any one bile were less than 1 %.

The gasometric method. Determination of the amino-groups of glycine and taurine after hydrolysis of the conjugated acids, as first described by Schmidt & Dart [1920], does not reveal the presence of the unconjugated acids which, according to Schönheimer *et al.* [1932] may also occur in the bile. For the determination of the conjugated acids the Van Slyke method gave better values than the formaldehyde titration and the colorimetric method of Folin.² Hydrolysis of the conjugated acids is always effected with alkali hydroxide, but the

¹ The substances used in these tests were prepared by the late Prof. O. Hammarsten, Uppsala. His successor, Prof. G. Blix, kindly placed them at our disposal.

² The statement of Rosenthal & Lauterbach [1924] that taurine with this method should give only 2/3 of its N could not be confirmed by Josephson & Swedin. On the contrary, they found that glycine and taurine gave equimolecular amounts of N.

concentration of this and the time of heating employed by different workers vary very much. Table I shows our results for the hydrolysis of glyco- and tauro-cholic acids with KOH of various strengths, according to the different investigators. As the table shows, complete hydrolysis both of glyco- and tauro-cholic acids was obtained only according to Jenke & Steinberg [1930], that is

Table I.

Acid	Conc. of substrate %	KOH %	Hours of heating	NH ₂ -N mg./ml.		Extracted cholic acid % colorimetric		Extracted cholic acid % polarimetric		Method
				Found	Calc.	Found	Calc.	Found	Calc.	
Glycocholic	6.65	5	6	1.26	2.00	4.71	5.83	4.40	5.83	Süllmann & Schaub [1932], Breusch [1934]
"	1.66	5	6	0.35	0.50	1.06	1.46	0.89	1.46	
Taurocholic	7.36	5	6	1.92	2.00	5.51	5.83	4.90	5.85	
"	1.84	5	6	0.45	0.50	1.20	1.46	1.04	1.46	
Glycocholic	6.65	8	8	1.64	2.00	5.24	5.83	5.10	5.83	Foster & Hooper [1915], Rosenthal & v. Falkenhäusen [1923], Schmidt & Dart [1920], Smith <i>et al.</i> [1928], Cuny [1930, 1, 2], Douglas-Sauermann [1935], Rosenthal & Lauterbach [1924]
"	1.66	8	8	0.43	0.50	1.25	1.46	1.12	1.46	
Taurocholic	7.36	8	8	1.93	2.00	5.65	5.83	5.55	5.83	
"	1.84	8	8	0.47	0.50	1.33	1.46	1.20	1.46	
Glycocholic	6.65	15	12	1.96	2.00	5.79	5.83	5.77	5.83	Jenke & Steinberg [1930], Borgatti [1932]
"	1.66	15	12	0.48	0.50	1.46	1.46	1.42	1.46	
Taurocholic	7.36	15	12	2.02	2.00	5.75	5.83	5.73	5.83	
"	1.84	15	12	0.51	0.50	1.49	1.46	1.44	1.46	
Cholic	5.00	15	12	0	0	4.94	5.00	4.95	5.00	
Deoxycholic	5.00	15	12	0	0	0	0	4.93	5.00	
Glycine	0.5357	15	12	0.98	1.00	0	0	0	0	
Taurine	0.8929	15	12	1.00	1.00	0	0	0	0	

with 15 % KOH 12 hours on a boiling water-bath. This was evident not only from amino-N determinations, but also from colorimetric determination of cholic acid in the ether-petroleum extract. Therefore this method was chosen. Even so, however, there often occurred a loss of 1-2 % N. By total N analysis it could always be shown that this loss was due to disappearance of N from the solution, probably as NH₃. In bile this method gave fairly satisfactory values. The results with two or three samples from each bile did not differ more than 1-2 %. Of course the Van Slyke value found before hydrolysis had to be subtracted from that after hydrolysis to obtain the amount of bile acid-N. (With the hypobromite method of Cuny [1930, 1, 2] we did not obtain consistent values.)

The polarimetric method. For polarimetric determination of the acids it was also necessary to obtain complete hydrolysis since the reading of a mixture of several acids would not permit any conclusions. According to Jenke & Steinberg [1930] it is also essential to isolate the unconjugated acids after removal of the cholesterol by extraction with a mixture of ether and light petroleum (4 : 1). Using separating funnels for this extraction we very seldom obtained complete extraction. We therefore let the ether mixture bubble through a long narrow funnel in the acidified hydrolysis solution placed in a wide tube in the middle section of an ordinary Soxhlet apparatus. By extracting in this way for 6 hours, during this time making the aqueous fraction alkaline and again acid four or five times, we always obtained such complete extraction that even the furfuraldehyde reaction became negative in the aqueous phase. (It must however be mentioned that this reaction is much less sensitive in strong salt solutions than in weak ones.) The ether then was evaporated and the residue dissolved in absolute alcohol and determined polarimetrically. As Table I shows we got fairly good

values with this method in standard solutions. In bile the results with this method were somewhat poorer, since the different samples of one bile often differed by 2–3 % and in some cases even much more as shown in Table II. The loss was probably due to destruction of bile acids.

Attempts to separate only the preformed unconjugated acids from the conjugated ones by such extraction did not succeed since occasionally glycocholic acid was somewhat soluble in the ether mixture and taurocholic slightly so. This is the reason why, as shown in Table I, in the incompletely hydrolysed samples the colorimetric values in the ether extract are usually higher than the polarimetric ones. Results obtained from bile by the polarimetric method must be accepted with reserve as in most biles there are small amounts of bile acids other than cholic and deoxycholic, e.g. ursocholic, hyocholic, lithocholic etc., which are optically active. This is of special importance for human bile which contains anthropodeoxycholic and pig's bile containing hyocholic acid with specific rotations differing greatly from those of cholic and deoxycholic acids. Nevertheless we have tried to get an approximate estimate of deoxycholic acid in some biles by subtracting from the total rotation of the mixture the rotation of the colorimetrically determined cholic acid and then calculating from the specific rotation of the deoxycholic acid. We used wave lengths of 6563 and 5893 Å and sometimes of 6867 Å where the absorption was less when much bile pigment was present. The concentrations are calculated from Josephson's [1935] investigation of the optical properties of the bile acids and the same apparatus was used. In most cases the readings were made in 4 dm. tubes, but in some cases, where the extracts were very dark we had to read in 2 dm. tubes. We also tried to make the polarimetric readings in the alkaline hydrolysate after extraction of cholesterol. This would be possible as there could not be present any other optically active substances to count with.

The specific rotation of the bile salts in water solution is well known but, as shown by Josephson, this rotation decreases with increasing concentration of other electrolytes in the solution. In these cases we therefore had to add exactly known amounts of NaHCO_3 (at the stage of evaporation of the alcohol) and KOH. The readings of these solutions, however, often became somewhat inexact, owing to the large amount of light-absorbing bile pigments present. In many biles, especially the human ones, the pigments made the observations in alkaline solution impossible, even if light of wave length 6867 Å was used. Where this method was applicable however it usually gave results which agreed better with those of the other methods than did the results obtained after acid extraction.¹

Sulphur determinations. The S has been determined and the concentration of taurine-N calculated and subtracted from the total amino-N, in order to distinguish between glyco- and tauro-cholic acids [Cuny, 1930, 1, 2; Douglas-Sauermann, 1935; Rosenthal & von Falkenhausen, 1923]. This method, however, is reliable only in those biles where no S-containing substances other than taurocholic

¹ Raue [1925], Borgatti [1932], and Giordano & Levi [1933] have tried to determine bile acids with the aid of the fluorescence reaction with sulphuric acid. Josephson & Swedin [unpublished results] have studied this reaction in standard solutions. Using a quartz lamp and the Stufen-photometer of Zeiss with light filter of euphosglass they found that the fluorescence of cholic acid was about ten times as intense as that of deoxycholic acid. The intensity of the fluorescent light was not directly proportional to the concentration of the acid but followed a logarithmic function of this concentration. The intensity was very dependent on other substances, such as inorganic salts, bile pigments, fatty acids etc., which are always present in bile. Since in addition the reaction was not very specific Josephson & Swedin concluded that it could not be used for quantitative determinations.

Table II.

(1) Species	(2) Cholic acid colorimetric millimol	(3) [α]D of the original bile read in			(4) Calculated α of the cholic acid in the original bile		(5) Deoxycholic acid calc. from [α]D in water millimol	(6) Sum of cholic and deoxycholic acids millimol	(7) Conjugated acid from NH ₃ -N millimol	(8) Unconjugated and deoxycholic acids millimol	(9) Taurine acids from S and N determination millimol	(10) Glycine acids from diff. of N and S determinations millimol
		a	b	c	a	b						
Cow 1	115	Dilution 1 : 2.5	Alkaline water 1.35°, 1.35°, 1.38°, 1.32°, 1.32°, 1.33°	Alcohol 0.84°, 1.02°, 1.09°	Alkaline water 1.36°	Alcohol 1.65°	0	115	70	45	79	Neg.
Cow 2	123	1 : 2.5	0.96°, 1.15°, 1.15°, 1.14°	—	1.44°	1.75°	Neg.	123	82	41	34	48
Cow 3	129	1 : 2.5	1.64°, 1.63°, 1.56°*	—	1.52°	1.84°	5	134	131	3	87	44
Cow 4	112	1 : 2.5	1.52°, 1.52°	—	1.32°	1.60°	10	122	121	1	65	56
Cow 5	101	1 : 2	1.47°, 1.56°, 1.52°, 1.49°	—	1.20°	1.44°	31	132	150	Neg.	62	88
Pig 1	0	1 : 2.5	0.01°, 0.02°	0.30°, 0.72°	0	0	Trace	Trace	118	Neg.	21	97
Pig 2	0	1 : 2.5	0.06°, 0.08°, 0.18°	0.30°, 0.30°	0	0	Trace	Trace	121	Neg.	23	98
Pig 3	0	1 : 2.5	0.09°, 0.01°, 0.11°	—	0	0	Trace	Trace	170	Neg.	19	151
Sheep 1	175	1 : 4.17	2.23°, 2.24°	—	2.03°	2.52°	26	201	185	16	209	Neg.
Sheep 2	266	1 : 2.33	2.65°, 2.56°	1.96°, 2.07°, 1.70°	3.05°	3.79°	Neg.	266	305	Neg.	219	86
Rabbit	55	1 : 12.82 1 : 5.13	3.91° 4.07°	—	0.68°	0.81°	241	296	152	144	111	141
Cat	337	1 : 7.25	—	2.52°, 3.44°	3.80°	4.81°	Neg.	337	295	42	432	Neg.
Dog	165	1 : 5	2.02°, 1.94°, 1.98°, 1.82°	2.37°, 1.64°	1.92	2.37°	3	168	317	Neg.	193	124
Fox	127	1 : 5.56	1.02°	—	1.49°	1.81°	Neg.	127	39	88	126	Neg.
Guinea-pig 1	5	1 : 2.23	0.00°	—	0.07°	0.07°	0	5	23	Neg.	—	—
Guinea-pig 2	5	1 : 5.36	2.59°	—	0.07°	0.07°	227	232	1	231	—	—
Polar bear	219	1 : 4	—	1.41°, 1.39°, 1.68°	2.52°	3.12°	Neg.	219	195	24	244	Neg.
Walrus	360	1 : 4	—	0.84°	—	4.11°	Neg.	360	286	74	—	—

* Not used for the calculation of average.

acid are present. In other biles these acids would have to be isolated before the S determination could be made. We are working on this isolation, and the results obtained with its aid will be published later. Here we report the results of total S determinations and the concentrations of taurine acids calculated from these values. The S determinations were carried out by the method of Josephson [in course of publication].

RESULTS.

The results which were obtained by using the related methods on different animal biles are shown in Table II. In Table III we report the results from some human biles obtained post mortem. It should be pointed out that we have analysed only gall-bladder biles and since we do not know how much water is

Table III.

(1)		(2)	(3)		(4)	(7)	(9)	(10)
<i>a</i>		Cholic acid	[α]D of the original bile read in		Calculated α of the cholic acid in the original bile	Conjugated acid from NH ₂ -N	Taurine acids from S determination	Glycine acids from difference of N and S determinations
Human biles	Age	colorimetric millimol	<i>a</i>	<i>c</i>		millimol	millimol	millimol
Diagnosis	years		Dilution	Alcohol				
Cancer recti	69	25	1:2.49	1.27°	0.36°	96	48	48
Nephrosis acuta	39	10	—	—	0.15°	78	41	37
Sepsis	20	66	1:7.19	1.80°, 2.52°	0.95°	165	98	67
Encephalitis lethargica	29	49	1:4.84	1.97°, 2.09°	0.70°	166	75	91
Agranulocytosis	80	27	1:4.41	0.30°, 0.65°, 0.65°	0.38°	41	—	—
Pneumonia acuta	40	17	1:4.50	0.46°, 0.45°, 0.50°	0.24°	80	18	62
Haemorrhagia cerebri	57	34	1:3.95	1.37°, 1.35°	0.48°	78	32	46
Carcinoma laryngis	60	45	1:3.19	1.08°	0.65°	67	18	49
Vitium organicum cordis	65	61	1:4.35	0.90°, 1.00°, 0.65°	0.87°	80	54	26
Carcinoma recti	71	14	1:3.00	0.17°, 0.15°, 0.12°	0.19°	3	9	Neg.
Arteriosclerosis	70	50	1:2.06	0.80°	0.72°	100	38	62
Nephrosclerosis maligna	22	33	1:3.23	1.31°, 1.26°, 1.09°	0.47°	78	67	11
Pneumonia acuta	3	2	1:3.14	0.00°, 0.02°	0.03°	0.2	4	0
Agranulocytosis	73	81	1:3.13	1.05°, 1.13°	1.16°	129	31	98
Haemorrhagia cerebri	70	23	1:2.86	0.28°, 0.28°, 0.41°	0.33°	44	28	16
Sepsis pneumococci	43	14	1:3.28	0.31°	0.21°	35	—	—

resorbed from the gall-bladder we cannot draw any conclusions about the concentration in liver bile. The proportions between the different acids, however, are probably not altered in the gall-bladder. The biles of the rabbit, guinea-pig, dog and cat were collected from several animals; the results for polar bear and walrus biles were obtained with preparations about 50 years old, precipitated with alcohol in unknown proportions, probably 1/4; these are recorded only as curiosities. All the other biles were from single individuals.

In the colorimetric, gasometric and S determinations only the averages are recorded since the different samples always agreed very closely. On the other hand all the results of polarimetric readings are noted since they usually differed a good deal from one another. Of several biles unfortunately we could not obtain enough for making duplicate analyses by the polarimetric method which requires considerable amounts of material.

Explanation of the tables.

Column 1 in Table II shows the species of animal from which the biles originated. The cow, sheep, pig and fox biles were from single individuals. The rabbit bile was collected from 20 animals, the cat bile from 4, the dog bile from 4, the guinea-pig bile 1 from 40 and the guinea-pig bile 2 from 30 animals.

In Table III column 1 shows the pathological condition and age of the human subjects from which bile samples were taken.

Column 2 shows the results of the colorimetric determinations of cholic acid including the conjugated as well as the unconjugated acids.

Column 3 contains the results of the polarimetric determinations. 3 *a* shows the dilution of the original bile which was used for the determination. 3 *b* shows the optical activity of the original biles calculated from the rotation observed when the bile acids were dissolved in aqueous alkali. 3 *c* gives the activity of the original bile observed when the acids were dissolved in alcohol.

Column 4 indicates the part of the optical activity of the original bile which could be calculated from its colorimetrically determined content of cholic acids only. The difference between these values in aqueous alkali (*a*) and in alcohol (*b*) depends on the different optical activities of cholic acid in these solvents [Josephson, 1935].

Column 5 shows the content of deoxycholic acids, conjugated as well as unconjugated, in the original bile. These values are obtained by subtracting the calculated rotation of the known amount of cholic acids (column 4) from the rotation observed. In this calculation we have used Josephson's [1935] determinations of the specific rotation of deoxycholic acid. In some cases the total rotation of the bile was even smaller than that calculated from the cholic acid content. In these cases we have written Neg. in this column.

Column 6 contains the sums of cholic and deoxycholic acids, conjugated as well as unconjugated. These values represent the sum of those from columns 2 and 5.

Column 7 shows the content of conjugated acids, glyco- as well as tauro-cholic and deoxycholic. These values are calculated from the conjugated amino-N (Van Slyke).

Column 8 shows the amount of free unconjugated acids in the bile, calculated by subtracting the conjugated acids (column 7) from the total conjugated and unconjugated cholic and deoxycholic acids (column 6). In cases where the last value (6) is smaller than that for the conjugated ones (7) we have written Neg. in this column.

Column 9 shows the part of the cholic (or deoxycholic) acid which is conjugated with taurine if the content of this substance is calculated from the observed S content of the bile.

Column 10 shows the part of the cholic (or deoxycholic) acid which is probably conjugated with glycine. These values are calculated by subtraction of the taurine acids (column 9) from the total conjugated acids (column 7). In cases where the S content was higher than corresponded to the amino-N content, it was naturally impossible to calculate the glycine content and here we have written Neg. in this column.

DISCUSSION.

As the tables show, there is usually a considerable difference between the results obtained with the four different methods. The polarimetric method must be regarded as rather unsuitable for biles, since it gives values which do not agree well either with one another or with those of other methods. This is certainly due to destruction of bile acids during hydrolysis and extraction. In many cases we even observed rotations which were lower than could be calculated from the colorimetrically determined cholic acid. In pig and guinea-pig biles the furfuraldehyde reaction was almost or completely negative, indicating the absence of cholic acid. As certain of the hyocholic acids are laevorotatory it is not surprising that the polarimetric values of these biles are so low.

In those biles (cow 1, 2, rabbit, cat, fox, guinea-pig 2), where the gasometric value was much lower than those obtained colorimetrically and polarimetrically, we conclude that there must be a large proportion of unconjugated bile acids. In those where the gasometric value is higher than the colorimetric (cow 5, sheep 2, dog) on the other hand much deoxycholic acid and other acids must be present which do not react colorimetrically. In some biles, especially dog bile, the gasometric value was much higher than the colorimetric and polarimetric values. This indicates the presence of some laevorotatory substance present, or possibly of a nitrogenous compound soluble in alcohol, hydrolysed by alkalis and not reacting like cholic acid.

The S content of the different animal biles varied within wide limits. In sheep, cat and fox biles it was very high, showing without doubt that S-containing substances other than taurine are present (sulphuric esters). In dog bile S was also high but insufficient to account for the total conjugated acids. Contrary to the claims of other investigators, we can thus state that dog bile contains conjugated acids other than taurocholic.

The human biles were all from autopsies conducted not more than 2–3 hours after death. Immediately after collection the biles were precipitated with alcohol. Cases were deliberately selected which had no hepatic lesion. As Table III shows, there is no uniformity at all in the bile acid contents of these biles. This is true for colorimetric as well as polarimetric and gasometric values; in addition to this the values found with the different methods do not agree with one another. With only two exceptions the gasometric values are much higher than the colorimetric, indicating that there must be large amounts of conjugated acids, which do not react colorimetrically. Unfortunately the polarimetric determination of the human biles had to be carried out after acid extraction, since they all were so heavily pigmented that alkaline readings were impossible. Since anthropodeoxycholic acid is usually present in human bile, we have not been able to calculate the deoxycholic content, as this acid has a much lower optical activity than the other bile acids. The S analyses of human biles have shown that the taurocholic acid content is probably of the same order as that of glycocholic acid. Owing to these facts it seems difficult to obtain diagnostically valuable information from bile acid analysis.

REFERENCES.

- Borgatti (1932). *Boll. Soc. ital. Biol. sper.* **7**, 804, 810.
Breusch (1934). *Hoppe-Seyl. Z.* **227**, 242.
Cuny (1930, 1). *Bull. Soc. Chim. biol., Paris*, **12**, 1298.
— (1930, 2). *Pharm. Chim., Paris*, **12**, 485.
Douglas-Sauermann (1935). *Hoppe-Seyl. Z.* **31**, 92.
Foster & Hooper (1915). *J. biol. Chem.* **38**, 355.
Giordano & Levi (1933). *Arch. Sci. méd.* **53**, 599.
Gregory & Pascoe (1929). *J. biol. Chem.* **83**, 35.
Jenke & Steinberg (1930). *Arch. exp. Path. Pharmac.* **153**, 244.
Josephson (1935). *Biochem. J.* **29**, 1484, 1519.
— & Swedin (Unpublished results).
Nakagawa & Fujikawa (1930). *J. Biochem., Tokyo*, **12**, 399.
Raue (1925). *Z. klin. Med.* **102**, 79.
Reinhold & Wilson (1932). *J. biol. Chem.* **96**, 637.
Rosenthal & v. Falkenhausen (1923). *Klin. Wschr.* **2**, 1111.
— & Lauterbach (1924). *Arch. exp. Path. Pharmac.* **101**, 1.
Schmidt & Dart (1920). *J. biol. Chem.* **45**, 415.
Schönheimer, Andrews & Hrdina (1932). *Hoppe-Seyl. Z.* **208**, 182.
Smith, Groth & Whipple (1928). *J. biol. Chem.* **80**, 659.
Süllmann & Schaub (1932). *Biochem. Z.* **251**, 369.