CLXXXII. THE DETERMINATION OF CHOLIC ACIDS IN BLOOD.

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MANY methods for the determination of bile acids in blood have been published. Most of them have been tested here and have all been found to give bad results, when small amounts of bile acids were added to the blood. This is due chiefly to two circumstances which interfere with the exact determination; one is the difficulty of separating the bile acids from other blood constituents, and the other is the lack of a specific reaction for the former.

General remarks.

In all methods so far described the proteins are precipitated by treating the blood with neutral alcohol, after which the bile acids are separated from the filtrate and the washings. This precipitation however entails a considerable loss of bile acids, which are carried down with the precipitate and cannot be washed out. This loss is greater for taurocholic and glycocholic acids than for cholic acid. I have been able to avoid loss by precipitating the blood with alcohol to which a certain amount of saturated $\text{Ba}(\text{OH})_2$ solution has just been added; in this way an alkalinity was reached which was strong enough to prevent precipitation of the bile acids with the proteins but not so strong as to cause solution of alkaline hematin. Barium has also an advantage in its well-known capacity to precipitate the colouring matter of the blood. It is therefore possible even to boil the alcoholic solution containing Ba without obtaining a pigmented filtrate. This ensures a much better extraction of the blood corpuscles and the precipitate than the use of cold alcohol as described by most of the previous investigators. When using $\text{Ba}(\text{OH})_2$ and alcohol there will appear now and then a colloidal protein-containing suspension (of Ba protein?), which cannot be filtered off. This trouble can be avoided by increasing the Ba concentration, for instance by adding some Ba acetate to the $\text{Ba}(\text{OH})_2$ solution. Decoloration by aid of $\text{Ba}(\text{OH})_2$ has been used by several investigators [Aldrich and Bledsoe, 1928; Charlet, 1929; Süllman and Schaub, 1932; Scheinfinkel, 1933; Scott, 1934; Lichtman, 1934], but they all use it after the precipitation with alcohol, when the loss of bile acids has already occurred.

For the final determination of the bile acids different methods have been used. In most methods some modification of the Pettenkofer test is used, that with furfuraldehyde and sulphuric acid having proved to be the best. If the readings are made in more or less monochromatic light in the neighbourhood of the absorption maximum of the characteristic colour (6200 Å. [Gregory and Pascoe, 1929]), the reaction may be considered as fairly specific. Thus Süllman and Schaub used the Pulferich "Stufenphotometer" of Zeiss, and Scheinfinkel spectrographic readings. I have found the use of the "Stufenphotometer" to be the most convenient way for this determination in routine work. If in rare cases the bile acid-containing extract is coloured, or gives a colour with sulphuric acid, this does not prevent the determination, if the same solution without

furfuraldehyde is put into the comparison cup. The slight absorption of the furfuraldehyde itself can be corrected for by subtraction of the reading of a pure furfuraldehyde solution, treated in the same way as the extract. Unspecific absorption with furfuraldehyde generally has little or no effect if the right filter is chosen. I found the most suitable filter to be the S 61, not, as Süllman and Schaub assert, the S 57.

One disadvantage of these colour reactions must be specially pointed out. They are absolutely negative for deoxycholic acid and its conjugates, as has previously been shown by Nakagawa and Fujikawa [1930] and by Reinhold and Wilson [1932]. The numerous statements in the literature that this acid also gives a colour with furfuraldehyde and sulphuric acid must be due to insufficient purification. For this reason it is possible to determine only cholic acid and its conjugates in blood, since all methods of determining the bile acids other than the modified Pettenkofer test give very inaccurate values. For cholic acid and its conjugates, however, the furfuraldehyde test gives very good values. With some modifications the procedure of Gregory and Pascoe proved to be the best. If the readings were made in the "Stufenphotometer", the extinction coefficients found were fairly proportional to the concentration between about 0.1 and 100 mg./100 ml. cholic acid. For cholic, glycocholic and taurocholic acids the extinction coefficient is proportional to the bile acid content (Table I).

The usual statement in the literature, that the reaction is not equimolecular for these different acids, is not correct.

Table I.

2 ml. of	Concentration of acid in mg./100 ml.	Milli- molarity	Extinction coefficient S 61	Extinction coefficient bile salt - extinction coeffi- cient furfuraldehyde
				molarity bile salt
1 % furfuraldehyde	—	—	0.046	—
Na cholate	0.1	0.0025	0.051	2.00
	5	0.1225	0.288	1.89
	100	2.4510	4.697	1.90
Na glycocholate	0.1	0.0022	0.050	1.82
	5	0.1075	0.248	1.88
	100	2.1505	4.107	1.89
Na taurocholate	0.1	0.0019	0.040	2.11
	5	0.9709	0.232	1.93
	100	1.9417	3.759	1.91

EXPERIMENTAL.

Reagents.

- (a) Absolute alcohol.
- (b) Saturated $\text{Ba}(\text{OH})_2$ solution in water with 0.4 % Ba acetate. The bottle should contain some crystalline $\text{Ba}(\text{OH})_2$.
- (c) Quartz sand.
- (d) H_2SO_4 about 2*N*.
- (e) NaOH about 2*N*.
- (f) Ethyl acetate, dried and neutralised if acid.
- (g) Water saturated with ethyl acetate; must be freshly prepared every time, as otherwise the ester will soon be hydrolysed.
- (h) H_2SO_4 55 vol. %.
- (i) Furfuraldehyde 1 vol % in water. The furfuraldehyde must be freshly distilled.

Pipettes, carefully calibrated with 5 ml. blood or an exact 5 ml. "Record" syringe, a water-bath kept at a constant temperature of $65 \pm 1^\circ$, a good centrifuge, 100 ml. volumetric flasks, an ordinary water-bath *etc.*

Finally special tubes for evaporation and extraction are needed, consisting of an upper part 10–8 cm. long and about 2–4 cm. in diameter connected through a thick-walled constriction with a bulb to hold about 5 ml., the exact 5 ml. mark being in the constricted portion. The whole tube must hold 25–30 ml. The mouth of the tube is provided with a ground glass stopper. The tubes should be made of thick, good glass, so that they stand heating in a boiling water-bath as well as centrifuging at 2000–3000 r.p.m. They must be made to fit in a good centrifuge with about this speed.

Into a 100 ml. volumetric flask containing about 50 ml. absolute alcohol are introduced 2 ml. of the $\text{Ba}(\text{OH})_2$ -Ba acetate solution; immediately thereafter exactly 5 ml. of the blood which is to be examined are added and the flask is thoroughly shaken. The blood must not contain oxalate or citrate, as these salts precipitate the barium; it is of no importance however whether the blood is defibrinated and taken with pipette or injected with an exact syringe as it comes from the blood vessel. A few grains of quartz sand are then added, and the flask is placed in a boiling water-bath for about 5 minutes. During the first 1–2 minutes it should be shaken to prevent the precipitated protein from sticking to the walls. Neutral alcohol is added after the boiling to nearly 100 ml., and the flask is kept for one day or until the supernatant fluid is clear.

The volume is then brought exactly to 100 ml. and the alcohol filtered off, the first part of the alcohol being allowed to pass the filter two or three times until quite clear and colourless. The precipitate must not be washed, since the bile acids are evenly distributed between the precipitate and the fluid.

The excess of barium is then precipitated with about 0.3 ml. 2 N sulphuric acid. When the barium sulphate has flocculated, the solution is again made alkaline to litmus with about 0.5 ml. 2 N NaOH. The alcoholic solution is filtered or decanted from the precipitates. The quantity of the filtrate usually reaches 80 ml.

Two amounts of this solution of exactly 30 ml. each must then be evaporated in two of the special tubes and treated as duplicates. The best way is to measure 15 ml. into each tube and to evaporate this amount together with some grains of quartz sand in a boiling water-bath to a few ml. Then the remaining 15 ml. should be added and evaporated until only a few drops remain. Octyl alcohol must not be added during the evaporation, since it will dissolve some bile salt, which then can scarcely be got into aqueous solution.

After the evaporation about 4 ml. of water are added, and the tube is heated again in the water-bath for 10–15 minutes to secure complete solution of the bile salts. The walls of the tubes must be thoroughly washed with the hot water from the lower part of the tubes. When the tubes have cooled, the contents are made strongly alkaline by about 0.5 ml. 2 N NaOH. The lipoids must be extracted with ethyl acetate. The alkalisation is necessary to prevent extraction of the bile acids. The ethyl acetate often contains free acetic acid and as the unconjugated bile acids are rather weak, they will be extracted at a slightly alkaline reaction.

To each tube about 10 ml. ethyl acetate are added; the tubes are stoppered and thoroughly shaken three or four times. The stopper is then removed, without unnecessary loss, and the tubes are centrifuged 10–20 minutes until complete separation of the water and ethyl acetate layers is obtained. The lower aqueous layer is then diluted to exactly 5 ml. with water saturated with ethyl

acetate introduced by means of a fine pipette. The tubes are again shaken and centrifuged, and the ethyl acetate together with the thin surface-layer on the water-fraction, consisting of insoluble substances, is sucked off. The aqueous solution fraction is then filtered off or decanted. A slight turbidity does not matter, as it will disappear on treatment with H_2SO_4 . 2 ml. of the solution are measured into each of two ordinary test tubes. Into a third tube 2 ml. of a known standard solution of bile acid are introduced (*e.g.* 5 mg./100 ml. cholic acid in diluted NaOH). A fourth tube should contain 2 ml. of pure water. To all the test-tubes 5 ml. 55 % sulphuric acid are added. To one of the two tubes containing blood extract 1 ml. of water is added, and to the other tubes 1 ml. of 1 % furfuraldehyde solution. The tubes are vigorously shaken and placed in a water-bath at 65°. After exactly 20 minutes they are removed and quickly cooled in tap-water. A violet-blue colour in the tubes with furfuraldehyde indicates the presence of bile acids. Very rarely there appears a turbidity, caused by fatty acids, but even if this occurs it does not prevent the reading, since it is similar in the furfuraldehyde tube and in the control.

The solutions are then to be read in the photometer. This should be done within about 30 minutes, as the colour then begins to fade. The blood extract solution with furfuraldehyde should be read against the one without furfuraldehyde as control, the non-significant colours thus being eliminated. The standard and the furfuraldehyde blank are to be read against water. The concentration of bile acids, expressed as cholic acid, may then be calculated according to the usual formula:

$$\frac{\text{conc. of unknown}}{\text{conc. of standard}} = \frac{\text{ext. coeff. of the unknown} - \text{ext. coeff. of the furfuraldehyde}}{\text{ext. coeff. of the standard} - \text{ext. coeff. of the furfuraldehyde}}$$

For larger series it is most convenient to plot the extinction coefficient values of the furfuraldehyde and of the standard in a coordinate system. The concentrations of the unknown may then be read by plotting their extinction coefficient values on the straight line connecting these two points. The concentration found is 3/10 of that in the original blood sample.

DISCUSSION.

To test the accuracy of the method a large number of determinations have been made on defibrinated blood, to which known concentrations of different bile acids had been added. Some few examples of these experiments are given in Table II.

There is a possibility that the distribution of the bile salts between plasma and corpuscles might be different in the addition experiments and in blood from real cases of jaundice. To make sure if the bile acids in the corpuscles were quantitatively extracted by the alcohol and evenly distributed between precipitate and filtrate, the blood from two cases of jaundice, one mild and one severe, was tested. One part of each sample was treated in the usual way, another part was haemolysed with alkali, and thereafter treated like the first. Before and after precipitation with $\text{Ba}(\text{OH})_2$ and alcohol the excess of alkali was neutralised with the calculated amount of HCl. A third part was also treated like the first, but the precipitate was extracted with alcohol in a Soxhlet apparatus for 5 hours. The extract was added to the first filtrate and the solution then treated in the usual way. Within the limits of the ordinary errors of the method all gave the same value for the respective original blood (Table III).

At concentrations exceeding about 5 mg./100 ml. the method gave values with at the most 6–8 % errors, which were always losses. In most cases the

Table II.

Blood from	Na salt added of acid	Concentration of cholic acid part from added bile		Cholic acid found	Cholic acid calculated
		salt	mg./100 ml.		
Man I	0	0	1.3	—	—
"	0	0	1.5	—	—
"	Glycocholic	5.0	6.2	6.4	6.4
"	"	5.0	6.0	6.4	6.4
"	"	25.0	26.0	26.4	26.4
"	"	25.0	25.1	26.4	26.4
"	"	100.0	98.2	101.4	101.4
"	"	100.0	96.7	101.4	101.4
"	Taurocholic	5.0	6.5	6.4	6.4
"	"	5.0	6.1	6.4	6.4
"	"	50.0	49.0	51.4	51.4
"	"	50.0	48.1	51.4	51.4
"	Cholic	5.0	6.4	6.4	6.4
"	"	5.0	6.0	6.4	6.4
"	"	10.0	11.0	11.6	11.6
"	"	10.0	11.8	11.6	11.6
Man II	0	0	1.8	—	—
"	0	0	0.6	—	—
"	Glycocholic	2.5	3.0	3.3	3.3
"	"	2.5	2.9	3.3	3.3
"	Taurocholic	2.5	2.2	3.3	3.3
"	"	2.5	2.9	3.3	3.3
"	Cholic	2.5	3.3	3.3	3.3
"	"	2.5	3.0	3.3	3.3
"	"	100.0	98.0	100.8	100.8
"	"	100.0	95.2	100.8	100.8
Cow I	0	0	2.7	—	—
"	0	0	3.0	—	—
"	Glycocholic	5.0	7.2	7.85	7.85
"	"	5.0	7.3	7.85	7.85
"	Taurocholic	5.0	7.8	7.85	7.85
"	"	5.0	7.5	7.85	7.85
"	Cholic	5.0	7.8	7.85	7.85
"	"	5.0	7.3	7.85	7.85
Sheep I	0	0	1.5	—	—
"	0	0	1.6	—	—
"	Glycocholic	10.0	11.2	11.55	11.55
"	"	10.0	11.4	11.55	11.55
Cat I	0	0	0.9	—	—
"	0	0	1.1	—	—
"	Glycocholic	5.0	6.0	6.0	6.0
"	"	10.0	11.6	11.0	11.0
"	Taurocholic	5.0	5.8	6.0	6.0
"	"	10.0	10.8	11.0	11.0
Rabbit I	0	0	0.3	—	—
"	0	0	0.2	—	—
"	Glycocholic	5.0	5.1	5.25	5.25
"	"	25.0	25.0	25.25	25.25
"	Taurocholic	5.0	4.8	5.25	5.25
"	"	25.0	24.7	25.25	25.25

errors were much less. They were chiefly due to decomposition of bile acids during the evaporation. This could be shown by adding a known amount of bile acids immediately before and immediately after the evaporation to two different tubes with the same extract. The difference between the two tubes was as a rule equal to the usual errors. At lower concentrations however the proportional

Table III.

Jaundice case no.	Treatment	mg./100 ml. cholic acid average
I	As usual	4.9
I	Haemolysed	5.0
I	Soxhlet extract	5.0
II	As usual	13.1
II	Haemolysed	12.9
II	Soxhlet extract	13.2

errors increased with decreasing concentration, and in normal blood they might be 20 % or even more.

It might be doubted whether the low values found for normal blood really corresponded to bile acids, or whether they were due to some unspecific reaction with the furfuraldehyde. There are, however, some facts indicating that bile acids really were determined. One is the very low normal value for rabbit. It is well known that rabbit bile contains mostly deoxycholic acid and proportionally little cholic acid. It may perhaps be supposed that the same proportions occur in the blood (as mentioned above, deoxycholic acid does not give any colour reaction). There is no reason why rabbit blood should contain less of the substances, giving an unspecific reaction, than blood from other species.

Another circumstance pointing in the same direction is the fact that, if the final aqueous fraction of the blood was treated with lanthanum acetate, every trace of the chromogenic matter was removed. This was not the case if for instance iron, lead or mercury ions were used. This accords with the observation that of numerous metallic ions tested only lanthanum was able to precipitate the bile acids completely both from slightly acid and from alkaline solution.

As a preliminary observation some results may be recorded which were obtained from 19 healthy men, 19–25 years of age. The cholic acid content of the blood varied from 0.6 to 2.2 mg./100 ml., average 1.6 mg./100 ml.

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