Sedimentation of Bile Constituents *

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THE LITERATURE on the clinical pathology of gallstone disease is too extensive to readily summarize. Investigations relative to the composition of bile and its normal physiology appear rather infrequently and data concerning the physical and chemical properties of bile are rare indeed. For example, the specific weights of the most important components of bile—bile acids, lecithin, bilirubin—are still unknown.[3, 7] Similarly, their relative solubilities have not been quantitatively determined.

Cholesterol is completely insoluble or at least very poorly soluble in water. According to Isaksson[5] it is quite insoluble in water but according to Hodgman 260 mg. cholesterol can be dissolved in 100 ml. water. The current concept is that cholesterol is held in solution in gallbladder bile in the high concentrations which normally occur with the help of lecithin and bile acids, which act as emulsifying agents.[5, 6, 8, 11] Thus bile is a colloidal solution of cholesterol. It has been suggested that during the formation of gallstones, there is a selective decrease of these emulsifying factors, mainly as a result of increased absorption in a pathological gallbladder,[10] which results in the precipitation of cholesterol. These authors postulated that, "the problem of gallstone formation is the problem of precipitation of cholesterol out of the bile" and in support of this they have quoted their own chemical investigations. Supported by clinical and surgical experience, Hultén[4] expressed the same points of view. He stated that during the formation of gallstones, "the cholesterol is precipitated as a large quantity of fine microscopic grains which combine to form larger particles, visible to the naked eye as golden specks. These in their turn join to become still larger particles, etc. so that finally all the cholesterol appears as a mulberry shaped, yellow-white, single stone. Superficially at least, the process resembles the churning of butter."

If bile is to be considered as a colloidal solution for cholesterol, it should be possible to predict, at least theoretically, that cholesterol is precipitated from the solution without change in the lecithin-bile acid concentration. Colloidal systems themselves *age* with time and the suspension or emulsion is broken—a well known problem in manufacturing ointments and liquid drugs.[9, 14] To judge from the available literature, this aspect of the problem in the formation of gallstones has not previously been investigated.

On the basis of these latter premises, the present investigation of the stability of bile as a suspension in general and of cholesterol in particular was initiated. Thirty-six specimens of bile were allowed to stand for varying periods of time in test tubes. The superficial and bottom layers were then analyzed for their content of cholesterol, bilirubin, calcium, lecithin and bile acids as well as certain physical properties (specific weight, pH, relative viscosity and dry substance). The problem was whether any separation of bile components occurred, with special reference to cholesterol. How fast and to what extent do these changes occur? Finally, is cholesterol singularly precipitated without a concomitant and consecutive precipitation of the emulsifying agents lecithin and bile acids?

Methods

Human gallbladder bile was used in all the experiments. This was obtained during cholecystectomy and only from those patients whose preoperative cholecystography had shown one or two large stones in a

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well visualized gallbladder. Only bile which had a macroscopically normal appearance was used. The definitive experimentation started the same day that the samples of bile were obtained. To promote uniformity, a container was completely filled with bile and shaken mechanically for some minutes. The bile was then transferred to a series of from two to six test tubes, depending on the availability of material. The test tubes were completely filled with bile and then sealed with a smooth stopper without any surface layer of air.

The test tubes were allowed to stand undisturbed in a vertical position at a room temperature of 22 to 23° C. In the experimental Series 1 and 2, cylindrical test tubes 15 cm. high, containing 5.5 ml. bile were used, while in the other test tubes were 10 cm. high and contained 8.0 ml.

After a period of time varying from one half to 33 hours, a 2.0 ml. superficial layer and a 2.0 ml. bottom layer were carefully sucked out using a syringe with a connecting piece of polyethylene tubing leaving behind a middle column of 1.5 ml. in the first two series. In the following series wider and shorter test tubes were employed and 2.5 ml. of bile was taken from the superficial and bottom layers, respectively. Somewhat schematically the samples consisted of the upper and lower thirds of the column of bile in the test tube, leaving the middle third intact.

These samples were maintained at room temperature and airtight until the series was completed. They were then analyzed for substances and physical properties previously mentioned. Analytical technics, calculations of error, etc., have been considered in detail in a previous publication.[13]

Results

A total of ten series (each series from one gallbladder) of sedimented bile samples were examined at time intervals varying from one-half to 33 hours. The results are shown in Table 1, where one of the series is given in full, while in other series only a number of isolated representative time intervals are provided.

In the majority of cases, it was possible to see a whitish amorphous bottom layer with the naked eye, which was not, however, clearly demarcated or delineated from the remaining bile. In Series 3 a similar amorphous substance collected on the surface like cream on milk. It can be seen from the table that cholesterol is most unstable and its sedimentation increases with time. In Series 1 there was no sedimentation within the first two hours, but after four hours the cholesterol content was twice as high in the bottom layer as in the surface layer and after 28 hours the difference was three times as great.

In Series 2 only the whitish amorphous bottom layer was taken from one of the test tubes after 33 hours' sedimentation since the remaining bile had been discarded. In this 0.4 ml. bottom layer the cholesterol concentration was 2.2 per cent.

Series 3 differed from the others by the absence of sedimentation and a creaming up of cholesterol instead. After seven hours the concentration in the surface layer was nearly three times that in the bottom layer. Another difference in this series was that the bilirubin also showed the creaming up phenomenon, while on the other hand, calcium sank to the bottom layer. In addition, there was a certain separation of lecithin which did not occur in the other series.

The table also shows that the bile acids did not participate in the sedimentation and creaming up phenomenon. In every instance the bilirubin followed the cholesterol fraction although separation occurred to a considerably lesser degree, about 110 to 120 per cent in the thickest layer as compared with the original unsedimentated value of 100 per cent. The bottom layer always contained 20 to 40 per cent more calcium than the initial unsedimentated value. These figures are valid for about 12 hours' sedimentation.

The pH showed very small and rather insignificant variations. The relative viscosity, specific weight and content of dry substance showed a slight increase towards the bottom layer on sedimentation. In ref-

TABLE 1

Series No.	Time for Sedimentation in Hours	Layer	Cholesterol, mg./100 Gm.	Bilirubin mg./100 Gm.	Calcium, mg./100 Gm.	Bile Acids, Per Cent	Lecithin, Per Cent	Total Solids, Per Cent	Spec. Gravity	pH	Relative Viscosity (Water = 1.0)
I	1	surface	667	154	21.2	3.91	2.56	10.9	1.0231	7.02	2.0
		bottom	686	155	27.7	3.99	2.60	11.0	1.0236	6.94	2.2
	2	surface	684	154	24.2	3.82	2.56	10.7	1.0229	6.97	2.1
		bottom	693	155	26.1	4.01	2.37	11.0	1.0234	6.94	2.1
	4	surface	427	140	24.4	4.02	2.56	10.7	1.0230	6.94	2.0
		bottom	935	163	28.4	3.98	2.67	11.6	1.0237	6.97	2.5
	9	surface	420	142	20.5	4.00	2.47	10.8	1.0232	6.94	2.0
		bottom	933	170	28.3	3.67	2.57	11.1	1.0234	6.91	2.3
	28	surface	293	136	23.4	4.18	2.52	10.4	1.0230	6.98	1.9
		bottom	1,044	176	26.1	3.68	2.65	11.1	1.0234	6.88	2.5
II	27	surface	520	288	28.8		2.61	11.7	1.0234	7.26	3.3
		bottom	762	306	35.6		2.29	12.6	1.0236	7.32	3.0
	33	sediment only	2,212	348			2.41				
III	7	surface	1,111	233	14.3	3.63	3.15	12.7	1.0224	7.39	2.0
		bottom	427	184	16.0	3.92	2.00	10.5	1.0230	7.57	1.7
V	24	surface	951	314	35.8	5.01	4.54	16.8	1.0296	7.04	3.3
		bottom	1,397	351	43.5	5.11	5.25	19.7	1.0342	6.84	4.5
VII	10	surface	248	79	10.6	2.32	1.20	6.5	1.0139	7.01	1.3
		bottom	360	86	11.1	2.25	1.10	6.7	1.0142	7.13	1.3

erence to the creaming up phenomenon both the relative viscosity and content of dry substance were highest in the superficial layer.

In each series culture samples were sent for bacteriological investigation. Bacteria were not found in any of the bile samples.

Discussion

The physico-chemical properties of suspension stability and emulsion stability are given by Stokes' Law, which states that the sedimentation rate of a spherical particle in a viscous fluid is determined by the formula: [1]

$$u = \frac{2\,g\,r^2\,(d_1 - d_2)}{\eta}$$

u = rate of sedimentation
g = acceleration of gravity
r = drop radius
d_1 = density of the sphere
d_2 = density of the liquid
η = viscosity of the liquid.

Other factors of importance are the forces of cohesion for both phases, the forces of adhesion between both types of molecule, the electric charge of the drops and the resistance of the emulsifying film.[9]

As stated earlier, emulsions are by nature more or less unstable. The changes which occur may be of several types: the dispersal phase can move upwards (= creaming up) or form a sediment. The degree of dispersion can be decreased by flocculation and coalescence. Finally, the emulsion can be broken through inversion, which occurs during the churning of butter.[12]

If an attempt is made to regard bile from the above viewpoint, it is obvious that our knowledge is extremely rudimentary. Apparently, measurements of drop or particle size in bile have never been performed. Differences in specific weight cannot be given since the specific weight of the majority of bile components is unknown. Cholesterol and calcium are the only important bile constitutents whose specific weights are given in the literature. The specific gravity of cholesterol is 1.052 [2] and according to Hodgman [3] the corresponding figure is 1.067 at 20° C. Calcium compounds found in bile have the following specific weights: [7] calcite 2.6–2.7; aragonit 2.9 and apatite 3.2. Thus it is impossible even to attempt to analyze the results obtained in this investigation from a purely physico-chemical point of view.

In the present experiments bile was derived from gallbladders containing calculi. According to a previous investigation of bile stratification,[13] the concentrations of the components of such bile do not differ appreciably from the normal and as previously mentioned, bile was taken only from well visualized gallbladders containing one or two large stones. The gallbladders under consideration had normal emptying characteristics according to the preoperative cholecystograms.

With the knowledge of the specific weight of cholesterol above and since that of bile was only 1.023 it is surprising that creaming up of cholesterol was obtained in Series 3. Since this was the only case where lecithin and cholesterol paralleled each other, the explanation may be that lecithin has a lower specific weight and formed some compound with cholesterol having together a specific weight lower than 1.023.

Small quantitative variations in the values for the same series of the upper and lower layers can be explained by consideration of the following factors: 1) The test tubes were not precision made, a slight geometrical difference could occur; 2) The quantities removed could not be kept exact but could vary by 0.1 ml.; 3) When samples were taken a certain amount of stirring was unavoidable and, more important, could not be kept constant; and 4) Small particles of sediment could possibly adhere to the wall of the test tube.

If the lecithin-bile acid concentration, in comparison with the cholesterol concentration, is calculated as a percentage of dry substance, quotients obtained in unsedimentated material were not less than the critical value 11:1 given in the literature.

The experiments described here were performed at room temperature, which theoretically encourages precipitation partly

because of reduction in Brownian molecular movement.

Apart from the sedimentation and creaming up of cholesterol as found in this particular investigation, the separation probably may occur in an intermediate layer analogous to the floating stones as seen in cholecystography.

Conclusions

Sedimentation of bile components has been investigated in 36 bile samples representing ten different series, each series representing bile from one gallbladder. The bile was allowed to stand at room temperature in upright test tubes, which had been completely filled with gallbladder bile taken during cholecystectomy from gallbladders which contained only one or two stones and were well visualized at cholecystography. After periods of time varying from one-half to 33 hours, samples were taken from the superficial and bottom layers and then analyzed for: cholesterol; bilirubin; calcium; bile acids (glycocholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, taurocholic acid and taurochenodeoxycholic together with taurodeoxycholic acid); lecithin; dry substance; specific weight; pH; and relative viscosity.

The investigation indicates that cholesterol is very unstably *dissolved* in bile. After only four hours a clear sedimentation occurred (in one series creaming up), with concentration about twice as high in the bottom layer than in the superficial layer. The bilirubin concentration always paralleled that of cholesterol but sedimentation (and creaming up in one case) was, however, considerably less, on the average approximating 110 to 120 per cent of the initial value (100%) after 12 hours. Calcium always sedimented to the bottom layer, on the average 120 to 140 per cent of the initial value (100%). Lecithin was associated with cholesterol in the creaming up phenomenon only in one series, while in the other nine series lecithin was evenly and similarly distributed in the superficial and bottom layers in all samples. Bile acids did not take part in sedimentation or creaming up. Physical properties: pH, content of dry substance, specific weight and relative viscosity showed slight changes which completely correlated with above findings.

The most interesting point in this investigation is that a high degree of separation of cholesterol from bile can occur without a simultaneous change in lecithin-bile acid concentration.

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