The Physicochemical Basis of Cholesterol Gallstone Formation in Man

WILLIAM H. ADMIRAND and DONALD M. SMALL

From Boston University Medical School, Department of Medicine, Section of Gastroenterology, Boston, Massachusetts 02118

ABSTRACT The concentrations of bile salt, lecithin, and cholesterol were determined on each of 66 samples of gall bladder bile from patients with cholesterol gallstones and 25 samples of normal gall bladder bile. When these three constituents were plotted simultaneously on triangular coordinates, a complete separation of the normal and "abnormal" bile was achieved. This separation was the result of an increase in the quantity of cholesterol relative to the amounts of bile salts and lecithin contained in the bile from patients with cholesterol gallstones.

An in vitro model system was constructed (on triangular coordinates) that allows prediction of the maximum amount of cholesterol that can be solubilized in solutions containing varying proportions of bile salt and lecithin. When the bile data were compared with the solubility of cholesterol derived from the model system, normal biles were found to be less than saturated with cholesterol, whereas biles from patients with cholesterol gallstones were saturated and in some cases contained insoluble cholesterol in the form of microcrystals.

It is suggested that the physical state of bile (i.e., the presence or absence of insoluble cholesterol) is determined by the relative concentrations of bile salt, lecithin, and cholesterol, and the other biliary constituents do not appear to significantly effect the solubility of cholesterol in bile.

An abstract of this work has appeared in 1967 *J. Clin. Invest.* **46**: 1032.

Dr. Small is a Markle Scholar in Academic Medicine.

Dr. Admirand's present address is Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass. 02118. Address requests for reprints to Dr. Donald M. Small, Department of Medicine, Boston University School of Medicine, Boston, Mass. 02118.

Received for publication 31 August 1967 and in revised form 4 December 1967.

INTRODUCTION

Purpose. The formation of cholesterol gallstones can be separated conceptually into two steps (1). In the first step, the capacity of bile to solubilize cholesterol is exceeded; thus part of the cholesterol precipitates, presumably in the form of microcrystals. During the second step in gallstone formation microcrystals of cholesterol unite or grow to form macroscopic gallstones. These two steps probably proceed simultaneously. We here consider the first step of cholesterol gallstone formation, namely the conditions that bring about the precipitation of cholesterol in bile.

Theory. Pure cholesterol is almost totally insoluble in water (2, 3), yet the cholesterol in normal human bile is completely soluble. It is assumed that cholesterol is solubilized in bile in the form of mixed micelles of bile salt and lecithin (1, 4–8). In this case, the formation and persistence of insoluble cholesterol in bile could be the result of an increase in the quantity of cholesterol, a decrease in the quantity bile salt or lecithin, or a combination of these factors.

Previous studies have shown wide variations in the concentrations of bile salt, lecithin, and cholesterol in normal human gall bladder bile. Consistent differences between normal bile and bile from patients with cholesterol gallstones have been difficult to demonstrate (9–17). Although

some of this difficulty is due to differences in the analytical methods used, some confusion may have arisen from the way in which the data have been expressed. For example, when the quantities of bile salt, lecithin, and cholesterol per 100 ml of bile were compared, no significant differences were found in the concentration of any of these substances between the bile from normal subjects and from patients with cholesterol gallstones (13). This method of expressing the quantity of bile constituents per unit volume of bile might be expected to produce a wide range of values since it is dependent upon the degree to which the bile has been concentrated at the time the sample is obtained. Further, the quantity of any single constituent (e.g., cholesterol) has little meaning without its being compared to the quantities of both other major components. The use of binary ratios such as cholesterol : bile salt or cholesterol : lecithin failed to show consistent differences between normal bile and bile from patients with cholesterol gall stones (17).

Isaksson used an in vitro system to determine the maximum solubility of cholesterol in mixtures of bile salt and lecithin (18). If the ratio of cholesterol to bile salt plus lecithin was above 1 : 11, cholesterol precipitated out of solution. Applying the results of the in vitro system to the clinical situation, he found that in 70–95% of the patients with cholesterol gallstones the ratio was above the 1 : 11 critical level. This method of comparison was not entirely satisfactory since occasionally normal biles had high ratios, and some biles from patients with cholesterol gallstones had normal ratios. Isaksson's ratio does not distinguish between the individual effects of bile salt and lecithin. It has been demonstrated previously that the molecular function of bile salt and lecithin in relation to the micellar solubilization of cholesterol are distinctly different (3, 19–22) and, as will be shown in the present paper, not strictly additive. Therefore, the effects of each component must be judged separately.

In the present study we have expressed the relative quantity of bile salts, lecithin, and cholesterol in each bile sample as a percentage of the total quantity of all three components. To compare the composition of normal biles with that of biles from patients with cholesterol gallstones, each bile was plotted as a single point on triangular coordinates. This method permits simultaneous representation of the relative quantities of bile salts, lecithin, and cholesterol in any bile sample. When the data are expressed in this fashion, a clear-cut separation between normal and abnormal biles is achieved. Abnormal biles have more cholesterol relative to the quantities of bile salt and lecithin than do normal biles.

To determine if the basis of this separation was related to the limits of cholesterol solubility in pure lecithin and bile salt solutions, the results obtained in human bile were compared to an in vitro model system. The model system was derived from previous studies by Small, Bourges, and Dervichian (3, 19–23) in which the physical state of all possible mixtures of four components (bile salt, lecithin, cholesterol, and water) was determined and the fine structure of the different phases (i.e., crystal, liquid crystal, and micellar) was defined. The techniques for representing this quaternary system as a regular tetrahedron[1] have been described elsewhere (21). In the present paper, these techniques have been modified to show the effects of changes in composition of the three solid components (bile salt, lecithin, and cholesterol) at constant water concentrations similar to that found in gall bladder bile. This technique permits one to examine the individual effects of bile salts and lecithin on cholesterol solubilization.

A comparison of the results obtained in human bile and from the in vitro system showed that the solubility of cholesterol in both was similar. Since the in vitro system contained only bile salt, lecithin, and cholesterol, it is suggested that only these three substances determine the solubility of cholesterol in bile.

METHODS

Clinical Studies. Bile specimens were obtained from 91 subjects by needle aspiration of the gall bladder during abdominal surgery. All gall bladders were aspirated as completely as possible to exclude the possibility of unrepresentative sampling due to stratification of bile (25). The subjects were divided into two groups: (*a*) 25 subjects in whom there was no history of biliary tract disease, and in whom (after aspiration of the gall bladder) there were no palpable gallstones, and (*b*) 66 patients with cholesterol or mixed gallstones classified according to the scheme of Rains (26). The mean ages of both

[1] A general discussion of the representation of four component systems is given in reference 24, p. 403.

groups were similar, 52 years in normals and 57 in gallstone patients. 3 of the 25 normal subjects and 7 of the 66 patients with gallstones were female. The relatively small percentage of females in both groups was accounted for by the fact that all of the specimens were obtained at Veteran Administration Hospitals.

The bile samples were sealed and refrigerated within minutes after collection and were warmed to room temperature before analysis. An aliquot of each bile was centrifuged, and the sediment was observed under a polarizing microscope for the presence of crystals. When crystals were found, their melting point was determined on a microscope heating stage. Flat parallelogram-shaped crystals, similar to those described by Juniper and Burson (27), having melting points between 146 and 149°C, were considered to be cholesterol crystals. When present in adequate amounts, these crystals were isolated and run on silica gel thin-layer chromatography as described by Hofmann (28). In all cases the crystals had the same R_f as the pure cholesterol standard. No crystals were observed in the 25 normal biles.

All chemical determinations were performed in duplicate on aliquots of uncentrifuged bile. The per cent solids was calculated by dividing the dry weight by wet weight. All weighings were timed and extrapolated to time zero to correct for errors due to evaporation or hydration. Specimens that were obviously white bile or contained less than 4% total solids were not included in the study. The per cent solids varied between 5 and 25% with an average of 10%.

Total bile salts were enzymatically determined by a modification of the method of Talalay (29). The reagents were similar with the exception that hydroxysteroid dehydrogenase [2] solution was made with 0.03 M tris buffer (pH = 7.2) containing 0.001 moles of disodium ethylenediaminetetraacetate per liter. Methanolic bile solutions were prepared by diluting 0.1–0.5 ml of bile to a volume of 10 ml with methanol. The reaction mixture contained: 1.0 ml of pyrophosphate buffer; 0.5 ml of distilled water; 0.3 ml of β-diphosphopyridine nucleotide solution; 1.0 ml of hydrazine hydrate solution; 0.1 ml of hydroxysteroid dehydrogenase solutions; and 0.1 cc methanolic bile solution. The OD of the reaction mixtures were measured at 340 mμ, and the quantity of bile salt determined from a standard curve (obtained with pure bile salts) which was run with each series of determinations. The accuracy of the method was ± 3%. A complete description of the method will be published elsewhere.

Cholesterol was measured by the method of Abell, Levy, Brodie, and Kendall (30) and the method of Schoenheimer and Sperry (31), and there was no significant differences between the values obtained with either technique. Phospholipid, which in bile is almost entirely lecithin (9, 12), was determined by the method of Fiske and Subbarow (32).

In Vitro Studies. To determine the limits of cholesterol solubility in pure bile salt–lecithin solutions in vitro, a large number of dry mixtures of bile salt, lecithin, and cholesterol were prepared as previously described (20, 21). The bile salt (a mixture of conjugated taurine and glycine dihydroxy and trihydroxy bile salts) and the purified egg lecithin used in this experiment were the same as described previously (20). Water was added to each dry mixture so that the final solutions contained 20% solids by weight. The mixtures were allowed to equilibrate and then were examined grossly and microscopically for the presence of crystals and (or) liquid crystalline phases (20). Similar mixtures were diluted with water so that their final concentrations were 5 and 10% solids by weight. Those mixtures which contained both cholesterol crystals and an aqueous solution, or a mixture of cholesterol crystals, liquid crystals, and an aqueous solution were designated two- or three-phase systems, whereas those mixtures which contained only a single solution were considered one aqueous phase [micellar (20, 21)].

[2] Worthington Biochemical Corp., Freehold, N. J.

TABLE I

Composition of Normal Gall Bladder Bile and Gall Bladder Bile from Patients with Cholesterol Gallstones

	Millimoles per liter				Per cent total millimoles		
	Bile salt	Lecithin	Cholesterol	Total	Bile salt	Lecithin	Cholesterol
Normal biles, n* = 25							
Mean	135.2	38.0	11.1	184.3	73.5	20.4	6.0
SD	64.6	19.6	5.9	87.3	3.9	3.6	1.3
Abnormal biles, without cholesterol microcrystals, n* = 38							
Mean	87.9	15.9	11.0	114.8	76.2	14.2	9.6
SD	59.7	11.5	7.8	76.2	6.9	6.3	1.9
Abnormal biles, with cholesterol microcrystals, n* = 28							
Mean	97.6	17.7	21.9	137.2	70.6	13.0	16.4
SD	59.5	14.8	12.6	84.0	8.0	6.4	3.9

* n, number of patients in each group.

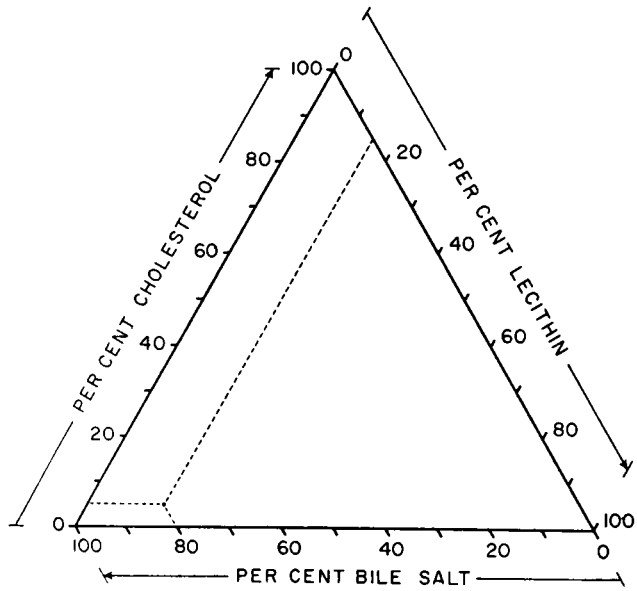

FIGURE 1 The method of representing bile composition on triangular coordinates. The per cent of the total moles of bile salt, lecithin, and cholesterol constituted by each of these components are shown on the scales along the sides of the triangle. Since the sum of bile salt, lecithin, and cholesterol equals 100%, the composition of any bile containing these components can be represented as a single point within triangular coordinates (24). Thus, a bile containing 80% bile salt, 15% lecithin, and 5% cholesterol is represented by a single point formed at the intersection of the dashed lines extended from the 80% level on the bile salt scale at the base of the triangle, the 15% level on the lecithin scale at the right of the triangle, and the 5% level on the cholesterol scale at the left of the triangle.

RESULTS

Clinical Studies. The mean values for all determinations on normal gall bladder biles and gall bladder biles from patients with cholesterol gallstones are shown in Table I. The millimolar concentrations of bile salt, lecithin, and cholesterol are shown in columns 1, 2, and 3, and the sum of these three components is shown in column 4. In columns 5, 6, and 7 are shown the per cent of total millimolar concentration contributed by each component. Since the sum of the three components in any one sample is equal to 100%, *each bile* can be represented by a single point within triangular coordinates (see reference 24, p. 277). The position of this point within the triangle is dictated by the relative concentrations of each of the components. Thus, a bile containing 80% bile salt, 15% lecithin, and 5% cholesterol would be plotted as is shown in Fig. 1. When the results from each of the 91 bile samples were plotted on one graph, there was a complete separation between the normal and abnormal biles (Fig. 2). This separation

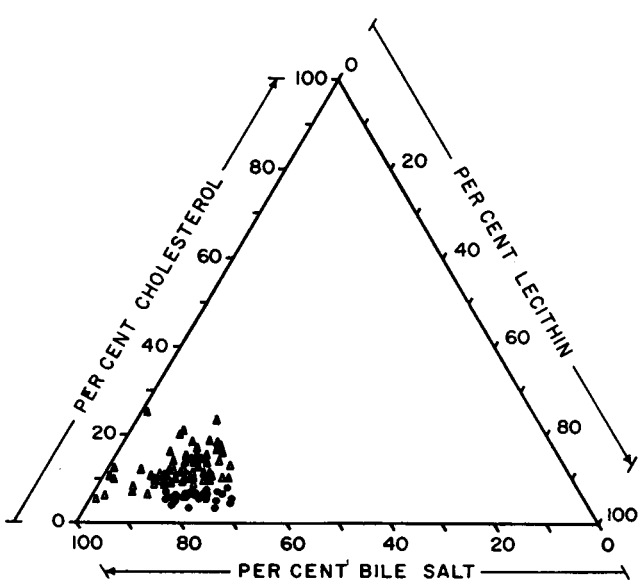

FIGURE 2 The composition of gall bladder bile from normal subjects and patients with gallstones. The composition of gall bladder bile, in terms of bile salts, phospholipids, and cholesterol from each of 25 normal subjects and 66 patients with cholesterol or mixed gallstones are plotted on triangular coordinates. The closed circles represent biles from normal subjects. The triangles represent biles from gallstone patients. The closed triangles indicate the presence of cholesterol microcrystals, whereas the open triangles represent biles without microcrystals of cholesterol.

TABLE II

Physical State of In Vitro Mixtures of Bile Salt, Lecithin, and Cholesterol as 10% Solutions by Weight

Mixture number	Per cent total moles: Bile salt	Lecithin	Cholesterol	Physical state of mixture
1	90.3	3.5	6.2	L*
2	89.9	3.5	6.7	L+C‡
3	86.6	6.8	6.6	L
4	86.3	6.7	7.0	L+C
5	78.7	12.3	9.0	L
6	78.4	12.2	9.3	L+C
7	68.8	21.5	9.7	L
8	68.4	21.4	10.1	L+C
9	60.9	28.7	10.3	L
10	60.8	28.7	10.5	L+C+LC§
11	56.3	35.5	8.2	L
12	56.2	35.5	8.3	L+C+LC
13	51.9	40.7	7.4	L
14	51.8	40.6	7.6	L+C+LC
15	48.0	45.2	6.9	L
16	47.9	45.1	7.0	L+C+LC
17	44.9	49.2	5.8	L
18	44.8	49.1	6.1	L+C+LC
19	43.9	55.0	3.1	L
20	43.8	54.8	3.5	L+LC

* L, the liquid phase.
‡ C, cholesterol crystals present.
§ LC, liquid crystal phase present.

appears to be the result of an increase in the quantity of cholesterol relative to the quantities of bile salt and lecithin present in gall bladder bile from the patients with cholesterol or mixed gallstones. Further, nearly all of the abnormal biles having relatively high cholesterols contained small cholesterol crystals (closed triangles, Fig. 2). These biles are two-phase systems containing liquid bile and microscopic cholesterol crystals. No liquid crystals were noted.

In Vitro Studies. The physical state of cholesterol in pure bile salt–lecithin systems containing 90% water and 10% solids are given in Table II. The results were identical when the experiment was performed on a series of mixtures containing 5% solids or a series containing 20% solids. More dilute solutions (below 3% solids) became unstable with time, whereas concentrated solutions with greater than 25% solids contained liquid crystalline phases which altered the solubility characteristics of both lecithin and cholesterol. In Fig. 3 each of the mixtures listed on Table II has been plotted according to its composition on triangular coordinates (similar to those shown in Fig. 1). The physical state of each mixture is indicated: closed circles to represent a single liquid phase and open circles to indicate 2- or 3-phase systems (i.e., mixtures containing insoluble cholesterol). A line has been drawn by inspection separating the open and closed circles, and it thus represents the maximum solubility of cholesterol in varying proportions of lecithin and bile salt. Therefore, any mixture beneath the line is less than saturated. Mixtures falling on the line are saturated, and any mixture above the line contains more cholesterol than can be maintained in solution.

In the absence of lecithin, approximately 97 molecules of bile salt are necessary to solubilize three molecules of cholesterol. As the proportion of lecithin increases, progressively more cholesterol is solubilized to maximum of about 10 molecules per 60 of bile salt and 30 molecules of lecithin. This amount is similar to the maximum solubility of cholesterol determined by Isaksson (18). Further increase in the proportion of lecithin results in decreased cholesterol solubilization in the micellar solution. At a molecular ratio of 40 molecules of bile salt to 60 of lecithin almost no cholesterol is solubilized in a micellar solution. Note that the state of insoluble cholesterol is crystalline at low lecithin concentrations but is liquid crystalline at higher lecithin concentrations.

The correlation of the findings in human bile with those of the model system are shown in Fig. 4. It is significant that all normal biles have compositions within micellar zone, whereas biles from patients with cholesterol or mixed gallstones fall on, or above the line of maximum saturation with cholesterol. The biles of those patients that have cholesterol values clearly above the maximum saturation of cholesterol in the micellar solution nearly all contained microscopic crystals of cholesterol (closed triangles, Fig. 4). These biles are two-phase systems: cholesterol crystals[8] and a micellar solution. Biles from gallstone patients falling very close to the line did not contain cholesterol crystals and are one-phase systems. Therefore, they can be considered to be saturated with cholesterol. These facts suggest that the

[8] The fact that no liquid crystals were observed in any bile sediments could have been predicted by the phase relationships shown in Fig. 3 since none of the biles contain adequate lecithin for liquid crystal formation.

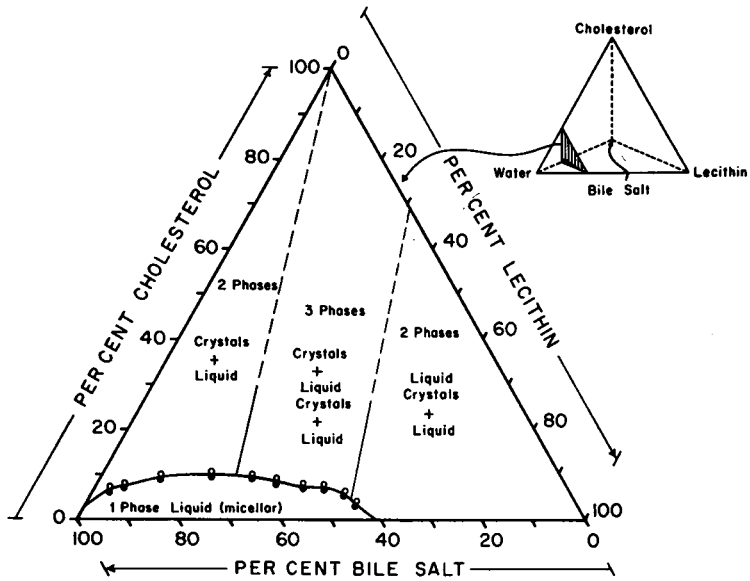

FIGURE 3 The model system. The tetrahedron shown in the upper right corner has been used to represent the physical state of all possible combinations of bile salt, lecithin, cholesterol, and water (21). The section in the tetrahedron taken at 90% water results in a triangular phase diagram which has been enlarged and is shown on the left. This diagram shows the physical state of all possible combinations of bile salts, lecithin, and cholesterol in aqueous solutions containing a total of 10% solids and 90% water. The composition and physical state of the mixtures from which the diagram was constructed are listed in Table II. Closed circles represent mixtures forming one liquid phase. Open circles represent mixtures forming two- or three-phase systems (clear liquid plus cholesterol crystals and (or) islets of lamellar liquid crystal). The line separating the open and closed circles indicates the maximum amount of cholesterol solubilized by any mixture of lecithin and bile salt.

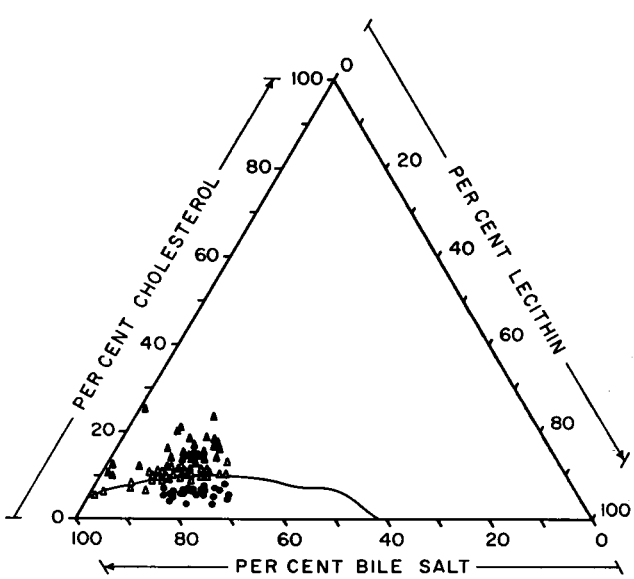

FIGURE 4 The composition of gall bladder bile from normal subjects and patients with gallstones compared with the limits of cholesterol solubility as determined from a model system. The diagram is the same as Fig. 2 except that the line representing maximal cholesterol solubilization, as defined by Fig. 3, has been superimposed. The composition of bile from normal subjects (represented by closed circles) is such that all circles fall within the micellar zone. The bile samples from patients with cholesterol or mixed gallstones in which no microcrystals were present (open triangles) fall on or very near the line, indicating maximum cholesterol solubilization. The biles from gallstone patients, which contain microcrystals of cholesterol (closed triangles), fall well above the line of maximum saturation.

TABLE III

Compositon of Human Gall Bladder Bile as Reported in Previous Studies

	Millimoles per liter				Per cent of total millimoles			
	Bile salt	Lecithin	Cholesterol	Total	Bile salt	Lecithin	Cholesterol	Ref. No.
Normal biles								
1	143.9	30.4	11.2	185.5	77.6	16.4	6.0	9–11
2	149.1	29.1	7.2	185.4	80.4	15.7	3.9	12
3*‡	222.2	54.7	19.0	295.9	75.1	18.5	6.4	13
4§	136.0	36.0	9.6	181.6	74.9	19.8	5.3	14
5	137.0	37.0	4.7	178.7	76.7	20.7	2.6	14
6*	255.3	44.2	20.9	320.4	79.7	13.8	6.5	13
7	140.0	33.5	10.0	183.5	76.3	18.3	5.4	16
8	142.6	37.7	12.8	193.1	73.9	19.5	6.6	17
Mean	165.8	37.8	11.9	215.5	76.8	17.8	5.3	
SD	46.1	8.3	5.6	57.7	2.3	2.3	1.4	
Abnormal biles								
9‖	128.3	33.4	25.3	187.0	68.6	17.9	13.5	9–11
10¶	133.9	31.9	21.2	187.0	71.6	17.1	11.3	9–11
11**	153.6	60.8	28.0	242.4	63.4	25.0	11.6	13
12**	110.5	46.3	22.2	179.0	61.7	25.9	12.4	15
13‡‡	129.0	37.4	16.0	182.4	70.7	20.5	8.8	16
14§§	127.0	39.1	15.5	181.6	70.0	21.5	8.5	16
15‖‖	103.2	32.8	10.8	146.8	70.3	22.3	7.4	17
16	64.6	24.9	8.8	98.3	65.7	25.4	8.9	17
Mean	118.8	38.3	18.5	175.6	67.8	22.0	10.3	
SD	26.6	11.0	6.8	40.7	3.7	3.4	2.2	

* Recalculated for total bile salts. See text.
‡ Pregnant females without biliary tract disease.
§ Patients with cancer of the liver but no biliary tract disease.
‖ Males with cholesterol gallstones.
¶ Females with cholesterol gallstones.
** Humans with cholesterol gallstones.
‡‡ Humans with "pure" cholesterol stones.
§§ Humans with mixed stones.
‖‖ Complicated by biliary obstruction.

physical state of bile is determined by the relative concentrations of lecithin, bile salt, and cholesterol present in the bile and not by other components. The model system serves to predict the maximum amount of cholesterol that can be solubilized by any bile, and thus the degree of cholesterol saturation.

In order to correlate our findings with those of other laboratories, we have taken from the literature mean values for bile salt, lecithin, and cholesterol in normal biles and biles from patients with cholesterol or mixed gallstones (Table III). The methods of chemical analysis for each of the three components frequently differed, but we have taken the values as reported with the exception of one study in which only "cholate" was measured (13). We have corrected this value by assuming that cholate represents 45% of total bile salts (33, 34). The correlation of the values from Table III with the model system are shown in Fig. 5.[4] Again it is noted that the composition of normal gall bladder bile is such that all the plotted values fall within the micellar zone. The biles from patients with cholesterol gallstones again contain more cholesterol relative to the amounts of lecithin and bile

[4] A similar diagram has appeared in reference 35; however, no corrections were made for "cholate" in that paper.

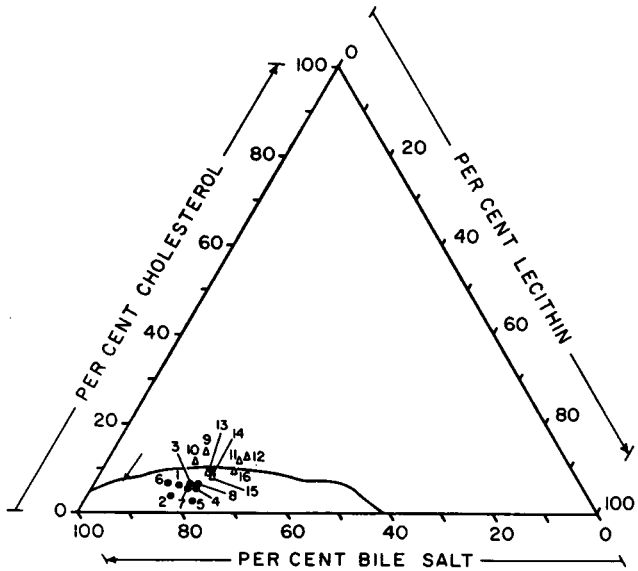

FIGURE 5 The results of previous studies of the composition of gall bladder bile from normal subjects and patients with gallstones represented on triangular coordinates and compared with the limits of cholesterol solubility as determined from a model system. The values for bile salt, lecithin, and cholesterol, as shown in Table III, have been plotted on triangular coordinates. Normal biles are represented as closed circles, and bile samples from patients with cholesterol or mixed gallstones are shown as open triangles. The number beside each of the symbols corresponds to the numbers found in column 1 of Table III. The line representing the limit of cholesterol solubility in the in vitro system, as defined by Fig. 3, is superimposed on this diagram.

salt and fall, in general, on or above the line marking the maximum solubilization of cholesterol as determined from the model system (Fig. 3).

DISCUSSION

The model system defines the maximum quantity of cholesterol that can be solubilized in mixed micelles of bile salt and lecithin. The triangular diagram shown in Fig. 3 was constructed by determining the physical state of a large number of mixtures of bile salt, lecithin, and cholesterol in varying proportions. The total quantity of these three substances (i.e., total solids) was the same in all mixtures. Each mixture contained 10% solids and 90% water by weight. This concentration was selected because it approximated the mean concentration total solids in the 91 samples of gall bladder bile. It might be argued that the findings of the model system containing 10% solids are not applicable to the more dilute or concentrated bile samples. This possibility can be excluded since the limits of the micellar zone at 10% solids (Fig. 3) are the same at 5 or 20% solids. Thus, in terms of water and total solids the model system is similar to the 91 samples of human gall bladder bile included in the present study. There are changes in cholesterol solubilization in the in vitro system when the total solids are less than 3%. Therefore, the limits of cholesterol solubilization are probably not applicable to very dilute biles. For this reason, dilute biles have been excluded from the present study.

A mixture of taurine and glycine conjugated bile salts was used to construct the model system. Almost identical results have been obtained when the free bile salt sodium cholate was used at an alkaline pH (21). Alterations in pH do not affect the physical state of mixtures containing conjugated bile salts until the pH falls below about 4.5, at which point glycine conjugates tend to precipitate. In contrast, the solubilizing capacity of the systems using free sodium cholate is dependent upon the maintenance of an alkaline pH. At a pH above 9.5 sodium cholate is completely ionized. Under this condition sodium cholate is as effective as conjugated bile salts in solubilizing lecithin and cholesterol. At somewhat lower pH levels, where a portion of free bile salt is in the form of protonated bile acid, solubilization is less efficient. Below pH 6 no solubilization occurs since cholic acid precipitates from solution.

The model system contained only bile salts, lecithin, cholesterol, and water and thus was devoid of many of the normal biliary constituents such as bilirubin, inorganic ions, mucus, and protein. Despite the absence of these substances the model system apparently predicts which bile samples will contain insoluble cholesterol crystals (Fig. 4). It therefore may be suggested that the solubility of cholesterol in bile is determined solely

by the amount of cholesterol relative to the quantities of bile salts and lecithin present.

We should like to emphasize that all of the findings discussed in the present study deal only with one step in the formation of cholesterol gallstones, namely the precipitation of cholesterol microcrystals. The process by which these microcrystals unite or grow to form macroscopic gallstones is unknown. Factors such as inflammation, stasis, mucus content, divalent cation concentration, bile pigment, pH etc., which do not appear to be significant in the precipitation of cholesterol microcrystals, may be important in the process by which these microcrystals form macroscopic cholesterol gallstones.

ACKNOWLEDGMENTS

The authors are grateful to Professor Franz J. Ingelfinger for his suggestions and guidance during the performance of these studies and to Miss Betty Egan and the surgical staff at the Boston Veterans Hospital for the collection of bile samples. One of the authors (D. M. S.) wishes to thank Dr. D. G. Dervichian for his tutelage and guidance.

This work was supported by NIH Grant AM-055-89-06. Dr. Admirand is a postdoctoral research fellow, supported by NIH training grant TI-AM-5025-11.

REFERENCES

1. Small, D. M. 1967. Physicochemical studies of cholesterol gallstone formation. *Gastroenterology.* **52:** 607.
2. Ekwall, P., and L. Mandell. 1961. The solubility of cholesterol in sodium caprylate solutions at 20°C. *Acta Chem. Scand.* **15:** 1404.
3. Bourges, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipidic associations. II. The ternary systems cholesterol-lecithin-water. *Biochem. Biophys. Acta.* **137:** 157.
4. Isaksson, B. 1954. On the lipids and bile acids in normal and pathological bladder bile. Doctoral thesis. Lund, Sweden.
5. Verschure, J. C. M., and P. F. Mijnlieff. 1956. The dominating macromolecular complex of human gallbladder bile. *Chin. Chim. Acta.* **1:** 154.
6. Furusawa, T. 1962. Surface-chemical studies on the stability of human bile. *Fukuoka Acta Med.* **53:** 124.
7. Juniper, K. 1965. Physicochemical characteristics of bile and their relation to gallstone formation. *Am. J. Med.* **39:** 98.
8. Tamesue, N., and K. Juniper. 1967. Concentrations of bile salts at the critical micellar concentrations of human gallbladder bile. *Gastroenterology.* **52:** 473.
9. Isaksson, B. 1952. On the lipid constituents of normal bile. *Acta Soc. Med. Upsalien.* **56:** 177.
10. Isaksson, B. 1954. On the lipid constituents of bile from human gallbladder containing cholesterol gallstones. *Acta Soc. Med. Upsalien.* **59:** 277.
11. Isaksson, B. 1954. On the main cholanic acids of human bile from normal gallbladder and from gallbladder associated with cholesterol stones. *Acta Soc. Med. Upsalien.* **59:** 307.
12. Polonovski, M., and R. Bourrillon. 1952. Les phospholipides de la bile. *Bull. Soc. Chim. Biol.* **34:** 712.
13. Large, A., C. G. Johnston, T. Katsuke, and H. L. Fachnie. 1960. Gallstones and pregnancy: the composition of gallbladder bile in the pregnant woman at term. *Am. J. Med. Sci.* **239:** 713.
14. Mirvish, S. S. 1964. Bile acids and other lipids in the gall-bladder biles of Africans with primary cancer of the liver. *Brit. J. Cancer.* **18:** 478.
15. Tera, H. 1963. Sedimentation of bile constituents. *Ann. Surg.* **157:** 468.
16. Burnett, W. 1965. Pathogenesis of gallstones in the biliary System. *In* NATO Advanced Study Institute on the Biliary System, Newcastle-on-Tyne. 1963. W. Taylor, editor. F. A. Davis Co., Philadelphia. 601.
17. Dam, H., I. Kruse, H. E. Kallehauge, O. E. Hartkoff, and M. K. Jensen. 1966. Studies on human bile. I. Composition of bladder bile from cholelithiasis patients and surgical patients with normal bile compared with data for bladder bile of hamsters on different diets. *Scand. J. Clin. Lab. Invest.* **18:** 385.
18. Isaksson, B. 1954. On the dissolving power of lecithin and bile salts for cholesterol in human bladder bile. *Acta Soc. Med. Upsalien.* **59:** 296.
19. Small, D. M., M. Bourges, and D. G. Dervichian. 1966. Ternary and quaternary aqueous systems containing bile salt, lecithin and cholesterol. *Nature.* **211:** 816.
20. Small, D. M., M. Bourges, and D. G. Dervichian. 1966. Biophysics of lipidic associations. I. The ternary systems lecithin-bile salt-water. *Biochim. Biophys. Acta.* **125:** 563.
21. Bourges, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipidic associations. III. The quaternary systems lecithin-bile salt-cholesterol-water. *Biochim. Biophys. Acta.* **144:** 189.
22. Small, D. M., and M. Bourges. 1966. Lyotropic paracrystalline phases obtained with ternary and quaternary systems of amphiphilic substances in water. *Mol. Crystals.* **1:** 541.
23. Small, D. M. 1967. Observations of lecithins. Phase equilibria and structure of dry and hydrated egg lecithin. *J. Lipid Res.* **8:** 551.
24. Findlay, A. 1951. The Phase Rule and Its Applications. A. N. Campbell and N. O. Smith, editors. Dover Publications, Inc., New York. 9th edition.
25. Tera, H. 1960. Stratification of human gallbladder bile *in vivo*. *Acta Chir. Scand. Suppl.* **256:** 1.
26. Rains, A. J. 1964. Gallstones (Causes and Treatment). Charles C Thomas, Publisher, Springfield. 11.
27. Juniper, K., Jr., and E. N. Burson, Jr. 1957. Biliary

tract studies. II. The significance of biliary crystals. *Gastroenterology.* **32:** 175.
28. Hofmann, A. F. 1963. Thin layer absorption chromatography of lipids. *In* Biochemical Problems in Lipids. A. C. Frazer, editor. Elsevier, Amsterdam. 1.
29. Talalay, P. 1960. Enzymatic analysis of steroid hormone. *Methods Biochem. Analy.* **8:** 119.
30. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357.
31. Schoenheimer, R., and W. M. Sperry. 1934. A micro-method for the determination of free and combined cholesterol. *J. Biol. Chem.* **106:** 745.
32. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375.
33. Sjovall, J. 1960. Bile salts in man under normal and pathological conditions. *Clin. Chem. Acta.* **5:** 33.
34. Wootton, I. D. P., and H. S. Wiggins. 1953. Studies in the bile acids. II. The non-ketonic acids of human bile. *Biochem. J.* **55:** 292.
35. Hofmann, A. F., and D. M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. *Ann. Rev. Med.* **18:** 333.