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Research Article

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Cholesterol Solubility in Bile

EVIDENCE THAT SUPERSATURATED BILE IS FREQUENT IN HEALTHY MAN

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ABSTRACT The development and validation of a direct method for measuring maximum cholesterol solubility in bile is described. Application of this method to five large mammalian species, including man, produced a micellar zone significantly smaller than that previously reported. Further studies on in vitro model solutions patterned after bile confirmed this new micellar zone. Thus, direct evidence demonstrates that the micellar zone boundary derived in vitro from model solutions is applicable to human gallbladder bile. Using the present criteria, normal human bile, in contrast to bile from other mammalian species, is commonly supersaturated with cholesterol. A male-female difference in bile composition is not demonstrable despite the well-established female preponderance of cholelithiasis. Bile from patients with cholesterol cholelithiasis has a micellar zone similar to normals but differs compositionally in that there is a greater excess of cholesterol above saturation. We conclude that cholesterol supersaturation may be a necessary but not solely sufficient cause for gallstone formation.

INTRODUCTION

In developed countries the major component of human gallstones is cholesterol (1, 2). Of importance in understanding gallstone formation is how cholesterol, which is normally dissolved in bile, precipitates out of solution.

The reported average composition of normal human gallbladder bile is about 84% water, 11.5% bile salts, 3% lecithin, 0.5% cholesterol, and only 1% of other components (bile pigments, proteins, and inorganic ions) (3, 4). Since cholesterol is virtually insoluble in water, its biliary solubility appears to depend solely upon its concentration relative to the molar concentrations of bile salts and lecithin, the other two very different lipid species present. There are two reasons for this hypothesis: (*a*) bile salts and lecithin have cholesterol solubilization properties that are markedly enhanced by their combination in mixed micelles, and (*b*) the remaining solid constituents present in small quantities are water soluble and based on known physicochemical properties are believed to have little capability to effect aqueous lipid solubilization. Bourgès, Small, and Dervichian have studied the quarternary system (bile salts, lecithin, cholesterol, water) using phase diagrams to determine the micellar zone of cholesterol solubilization (4). The micellar zone in this system was defined as an area bounded by a derived line. Any combination of the three major components producing a point outside of this zone would be characterized by the presence of a maximally solubilized system plus a variable amount of insoluble cholesterol. This definition of maximum cholesterol solubility was then applied to a large number of human biles. Good separation of normal biles from those associated with cholesterol gallstones was achieved (5). This derived definition of cholesterol solubility in bile has become widely accepted as a reference standard for lithogenic bile. However, several recent studies (6–8) have failed to confirm separation of normal from abnormal biles on this basis.

The present work deals with two important issues arising out of the above work: first, the validity of the in vitro micellar zone boundary for cholesterol solubility, and second, whether or not the in vitro boundary has in vivo applicability.

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As a result of the present study the following important conclusions are drawn: first, the micellar zone of cholesterol solubility for bile and model solutions is significantly less than previously reported. Second, bile from humans and all other mammalian species examined has a micellar zone closely resembling that derived from model solutions. This finding provides the first *direct* support for the concept that maximum cholesterol solubility in such solutions is a valid indicator of the limit of cholesterol saturability in mammalian bile. Finally, normal human gallbladder bile is commonly saturated or supersaturated with cholesterol. These observations suggest that cholesterol supersaturation of human gallbladder bile may be a necessary but is not a solely sufficient cause for gallstone formation.

METHODS

Maximum cholesterol solubility measurement method

Canine bile was used throughout the standardization experiments because of its availability in adequate quantities for pooling. A sufficient excess of anhydrous cholesterol[1] (General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio, recrystallized from absolute ethanol, tested for purity by thin-layer chromatography (TLC) and dried at 37° – 40°C in a vacuum oven) was added to the sample to ensure that its capacity for micellar solubilization was exceeded. Saturation of previously assayed, pooled canine bile was attempted by the addition of cholesterol based on a previous report (9) of 4 mg cholesterol per ml, but resulted in an undersaturated solution. Further studies indicated that 20 mg cholesterol per ml canine bile was necessary to exceed saturation. This amount was used in all succeeding experiments on bile specimens and in in vitro model solutions.

A mixing step was used to achieve maximal solubilization as rapidly as possible. Two methods were tested: (*a*) gentle shaking at 37°C in a shaker incubator (Gyrotory shaker, model G-76, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) and (*b*) sonication under nitrogen (Sonifier Cell Disruptor, model W-140 D, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.).

The final step was removal of the nonmicellar cholesterol. Two methods for removal of excess cholesterol were studied: (*a*) centrifugation at 25,000 *g* for 1 h at 25°C (International centrifuge, model PR-2, International Equipment Co., Needham Heights, Mass.) and (*b*) microfiltration using 0.10 and 0.22-μm millipore filters (Millipore Corp., Bedford, Mass.). After removal of nonmicellar cholesterol, the samples were assayed for the three components: bile acids, phospholipid, and cholesterol. Comparisons were made between the two methods of mixing and between the two methods of removing excess cholesterol. The assay methods were as previously described (10). Total bile acids were enzymatically determined by a modification of the 3α-hydroxysteroid dehydrogenase method (11); phospholipid was determined by Bartlett's modification (12) of the Fiske-Subbarow total phosphorus determination (13); and total cholesterol was measured according to the method of Abell, Levy, Brodie, and Kendall (14).

In vitro model solution studies

Purpose. In vitro solution studies were designed to determine the micellar boundary zone for cholesterol solubility by approaching the boundary from two directions. Solutions of varying composition were constructed that were either undersaturated or supersaturated with cholesterol. Cholesterol was either added or removed and the resulting solutions were considered as fully saturated. The final composition of each solution was represented by a point on triangular coordinates, and the area bound by these points was designated the micellar zone.

Procedure. Dry mixtures of bile salts, lecithin, and cholesterol were prepared as previously described by Small, Bourgès and Dervichian (15). Bile salts consisted of a proportionate mixture of six pure conjugates comparable with the bile salt composition of human bile described by Neiderhiser and Roth (16). Egg lecithin was purified chromatographically from two sources: commercial crude egg lecithin (ICN Nutritional Biochemical Div., International Chemical & Nuclear Corp., Cleveland, Ohio) and fresh egg yolks by a modification of previously described methods (17). Molecular weights for these lecithins determined by vapor pressure osmometry were 785 and 777, respectively (18).[2] No cholesterol solubilization differences were observed with lecithin isolated from either source. Water was added to each dry mixture so that all of the final solutions contained 10% solids by weight. The solutions were shaken for 24 h and incubated for 72 h at 37°C. Three experiments each utilizing a separate series of in vitro solutions were carried out.

(*a*) Three undersaturated solutions were made up to contain 5% total mol cholesterol with the remaining 95% total mol the summation of reciprocally paired amounts of bile salts (77.5, 72.5, 67.5%) and lecithin (17.5, 22.5, and 27.5%), respectively. Saturation of these solutions was achieved by the maximum cholesterol solubility method.

(*b*) Three dry mixtures were prepared consisting of exactly the same proportions of solid components as used in the previous series. But before aqueous suspension, an amount of excess crystalline cholesterol equivalent to that delivered by the maximum cholesterol solubility method was brought into solution with the other components using pure methanol as a common solvent. After dessication water was added to each dry mixture, and the solutions were shaken for 24 h and incubated for 72 h at 37°C. These supersaturated solutions were then processed by the maximum cholesterol solubility method, omitting excess cholesterol addition. Thus, the second in vitro experimental solution series was entirely comparable to series *a* except for the coprecipitation method of combining excess cholesterol with the other components.

(*c*) The final in vitro study was designed to observe the physical state and filtration characteristics of a series of solutions chosen so that the resulting points could be joined to describe a curvilinear line near the previously reported micellar zone boundary (4, 5). According to the previous in vitro

[1] Characterization kindly performed in the laboratory of Daniel Mufson, Ph.D., Smith Kline & French Laboratories, Philadelphia, Pa. Methods included: Differential thermal analysis; Thermogravimetric analysis; and X-ray diffractometry.

[2] Kindly performed in the laboratory of Edward Cussler, Ph.D., Department of Chemical Engineering, Carnegie-Mellon University, Pittsburgh, Pa.

micellar zone, these solutions should be slightly undersaturated (0.5 − 1.0% total mol) in cholesterol concentration and a line joining the points would be parallel to and within the boundary of the former micellar zone.

These solutions were divided to make two compositionally identical series that were studied by the following two techniques: In one series, portions were filtered at 37°C immediately after 72 h incubation. Some showed visible turbidity both before and after filtration. Those filtrates that were turbid were further incubated and periodically refiltered until clear. In the other series portions were similarly filtered but instead of further incubation the turbid filtrates were immediately frozen for 24 h, thawed and equilibrated for 24 h at 37°C, and then refiltered, producing clear filtrates. All solutions were examined with a polarizing microscope immediately following initial incubation, after initial and subsequent filtrations and at differing periods of sterile incubation at 37°C up to 21 days. Prefiltered solutions and the isotropic filtrates of both series were assayed. The isotropic filtrates under these conditions were considered as fully saturated with cholesterol.

In vitro studies of natural bile

Fresh canine, bovine, porcine, and ovine gallbladder bile samples were obtained in the fasting state by direct puncture aspiration at sacrifice.

Human bile specimens were obtained from 68 subjects by needle aspiration of the gallbladder during abdominal surgery. Gallbladder aspiration was as complete as possible to avoid sampling error due to stratification (19). Two groups of subjects were studied. In one group of 33 patients, there was no history of biliary tract disease and an absence of palpable gallstones. The remaining group of 35 patients had cholesterol or mixed gallstones according to the classification of Rains (20). The mean ages of the two groups were comparable, 52.5 yr in normals and 53.3 in patients with gallstones. Females composed 60% of the normal group and 54% of the patients with gallstones.

A portion of each of the freshly obtained bile samples was centrifuged, and the sediment was examined under a polarizing microscope for the presence of cholesterol microcrystals. The criteria of Juniper and Burson (21) as well as morphology observed in aqueous suspensions of pure crystalline cholesterol were used for identification. The remainder of each sample was immediately sealed and quick-frozen for storage for periods of up to 6 mo. No significant compositional changes or differences in maximum cholesterol solubility were observed when analyses of fresh specimens were compared in the same specimens after frozen storage for as long as 1 yr. Specimens to be filtered were thawed and equilibrated at 37°C for 24 h. Assays were performed in duplicate on portions of carefully mixed uncentrifuged bile to determine initial composition. Percent solids were calculated gravimetrically by dividing the dry weight by the wet weight with corrections as previously described (5). Specimens containing less than 4% total solids were excluded from further analysis. After maximum cholesterol solubility measurement, all assays were performed in duplicate. Lipid constituents in sequential micropore filtrates were analyzed to exclude significant lipid adherence. In addition, the maximum cholesterol solubility method was applied to fresh and frozen bile specimens from the same source to exclude artifact from freezing.

RESULTS

Maximum cholesterol solubility measurement method

Achievement of equilibrium. While sonication at 66 power output watt equivalents for 5 min was found to be as effective as shaking for 16 h with a 24 h equilibration at 37°C, the spontaneous dissolution rate with equilibration *alone* under these conditions was found sufficient to saturate canine bile. Sonication appeared to reduce viscosity and enhance filtration and was thus retained in the standardized measurement method. The sonication dose used produced no evidence of degradation by TLC in canine lecithin. The spontaneous dissolution rate for exogenous cholesterol was further examined by varying the equilibration period from 24 h to 7 days. No enhancement in the measured cholesterol saturability was observed with prolonged incubation. The minimum equilibration period was therefore used in all subsequent experiments.

Removal of excess cholesterol. Fig. 1 shows mean cholesterol values during various stages of the method development. Moderately high speed centrifugation was used on canine bile specimens from a single pool, and the cholesterol concentration in relative percent total moles observed in the supernate after 24 h of equilibration was consistently higher than after microfiltration of the centrifuged supernate. Furthermore, it was observed that

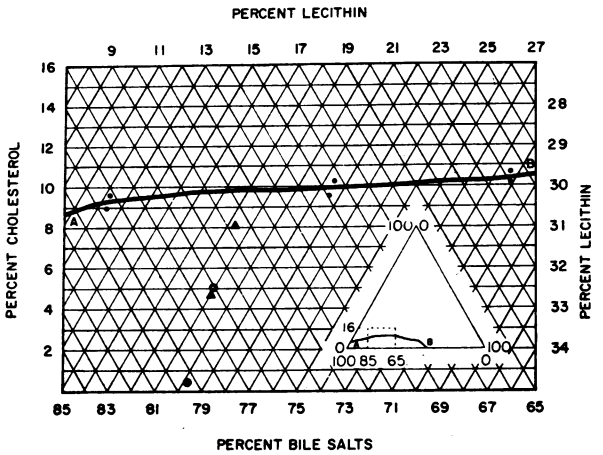

FIGURE 1 Triangular coordinate plot of representative observations from a single pool of canine bile using the maximum cholesterol saturability measurement method. Key inset indicates the portion of the triangular coordinate system depicted. Line AB represents the micellar zone in model solutions as previously defined (4, 5). The number of observations and standard deviations are shown in parenthesis: ●, initial value (untreated) (3, ±0.01); △, excess cholesterol removal by centrifugation alone (6, ±2.11); ○, excess cholesterol removal by centrifugation followed by filtration (8, ±0.18); ▲, excess cholesterol removal by filtration alone (2, ±0.01).

sedimentation of cholesterol was apparent by accumulation of visible precipitate of crystalline cholesterol during the equilibration period. Results with microfiltration alone or microfiltration of the centrifuged supernate were identical by assay.

Thus, it was clear that under these conditions, microfiltration was more effective than centrifugation in removal of excess cholesterol. Results with 0.10 μm millipore filtration were compared with 0.22 μm filtration. No difference in excess cholesterol removal was observed and the 0.22 μm size was thereafter used in the standardized method.

Reproducibility. 12 samples of a single pool of canine bile were tested. Assays for each of the three components were performed before and after maximum cholesterol solubility measurement. The coefficient of variation for solubilized cholesterol in the maximum cholesterol solubility method was 2.0% expressed as millimoles per liter or 1.6% expressed as relative percent total moles. Values for bile salts and lecithin were comparable. Sonication and filtration resulted in a mean increase of about 3.0% in the absolute concentration of bile salts and lecithin. This was attributed to slight dehydration occurring during handling since it was observed when either step alone was performed.

In vitro model solutions

The initial and postfiltration composition of the nine in vitro solutions from series *c* selected for definition of the micellar zone of cholesterol solubility are shown in Fig. 2. Postfiltration results from other methods used to achieve maximum cholesterol solubility as described in series *a* and *b* were identical. Polarizing microscopy at 37°C of each solution in series *c* before and after filtration over a period of 21 days incubation revealed that the initial solutions consisted of two or more phases and contained cholesterol microcrystals. Effective total removal of excess cholesterol by microfiltration from those solutions with higher relative lecithin concentration was achieved after prolonged incubation or freeze-thaw nucleation. Duration of sterile incubation required to reach equilibrium correlated directly with increasing relative lecithin concentrations. For example, in solutions (left to right) 1–5, microscopically clear filtrates were observed within 72 h; in solutions 6–7, within 14 days and in solutions 8–9, up to 21 days. Taken together, these in vitro solution experiments demonstrate that either sufficient equilibration or induced rapid nucleation followed by microfiltration are necessary for accurate boundary definition by removal of all excess cholesterol above saturation. The experiments also support the validity of the cholesterol addition step used in the maximum cholesterol

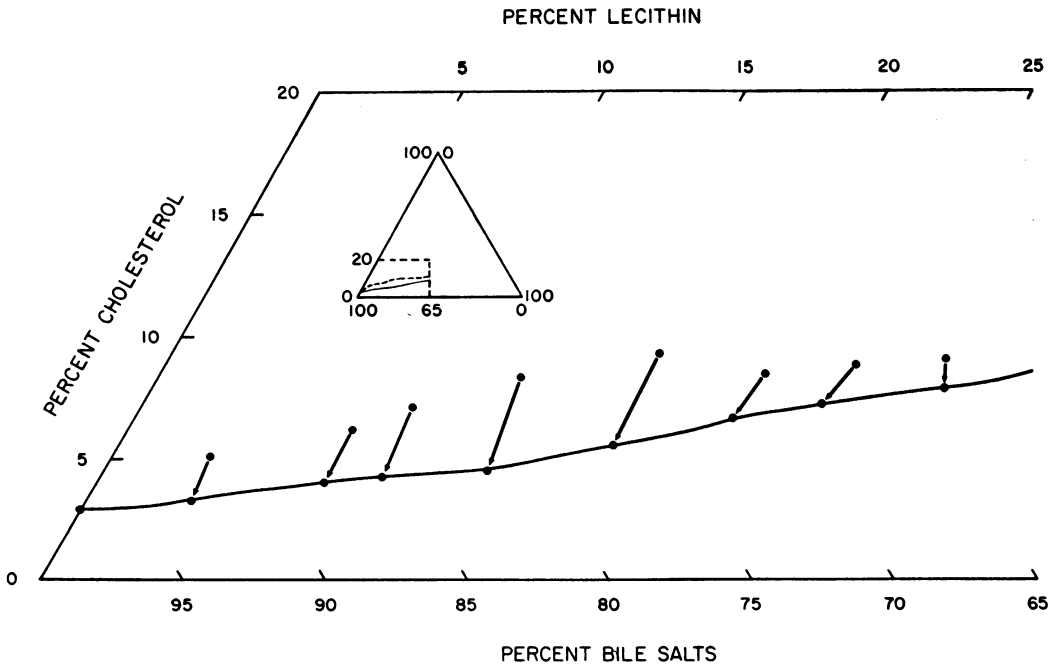

FIGURE 2 Postfiltration definition of the in vitro model solution micellar zone. Dotted line within the key inset represents the micellar zone as previously reported; solid line within the key inset indicates the present definition.

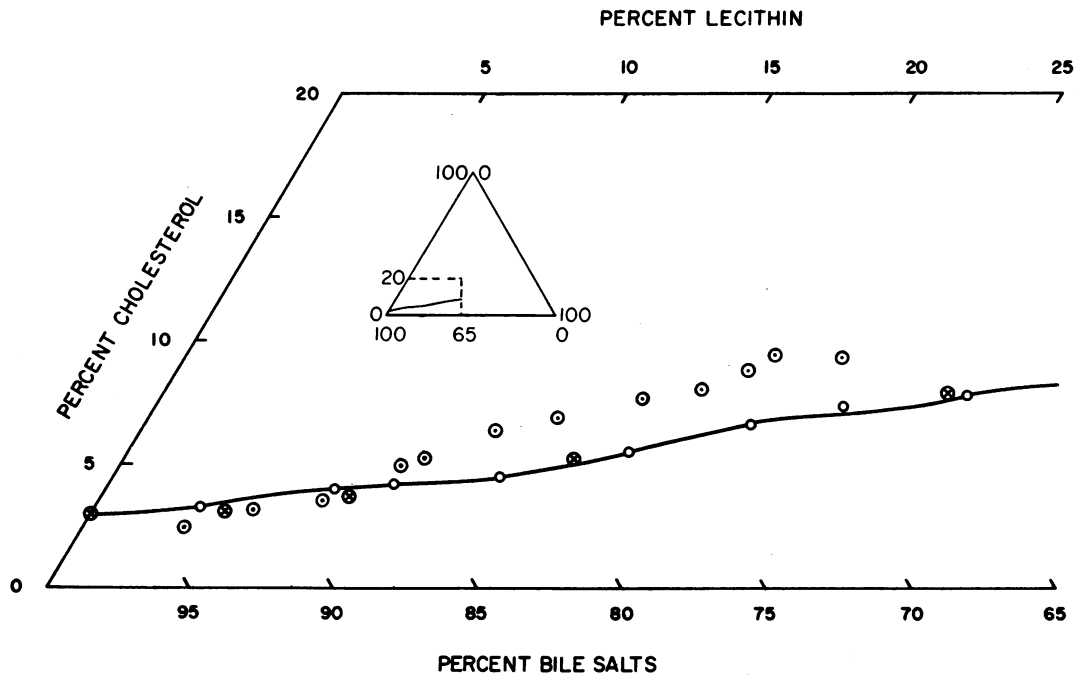

FIGURE 3 Comparison of postfiltration definition of in vitro model solution micellar zone with data from recent studies. Key inset as in Fig. 2. ○, data from this study; ⊗, data from Hegardt and Dam (22); ⊙, data from Niederhiser and Roth (24).

saturability method as applied to natural bile specimens. Fig. 3 shows a comparison of the present micellar zone with similarly plotted data from two other published studies in which filtration was also applied to in vitro model solutions.

In vitro natural biles

Biological characteristics of the various normal bile sources are listed in Table I. These data consist of one specimen each, obtained from a male and female source in the mammalian species indicated, as well as pooled

TABLE I

Characteristics of Various Mammalian Bile Specimen Sources and Comparative Gallblader Bile Lipid Data

Species	Sex	Weight	Age	Initial pretreatment samples composition			Maximum cholesterol solubility composition		
				C	L	BS	C	L	BS
				percent total moles			*percent total moles*		
Human	Male	140–200 lb	51.2 yr	8.0	20.6	71.3	6.7	21.1	72.2
	Female	100–200 lb	52.3 yr	7.4	20.4	72.2	7.0	20.2	72.8
Porcine	Male	200 lb	4–6 mo	2.2	15.1	82.7	3.6	13.8	82.6
	Female	200 lb	4–6 mo	2.8	15.0	82.2	4.2	13.5	82.3
Ovine	Male	100 lb	12–18 mo	0.5	7.3	92.2	3.0	4.7	92.3
	Female	100 lb	12–18 mo	0.7	6.0	93.3	2.7	5.5	91.8
Bovine	Male	1000 lb	3 yr	0.8	5.8	93.4	2.8	5.3	91.9
	Female	600 lb	2 yr	0.7	7.9	91.4	3.1	7.5	89.4
Canine	Both (pooled)	40–60 lb	3–7 yr	0.5	18.6	80.9	4.8	17.7	77.5

C, cholesterol; L, lecithin; BS, bile salt.

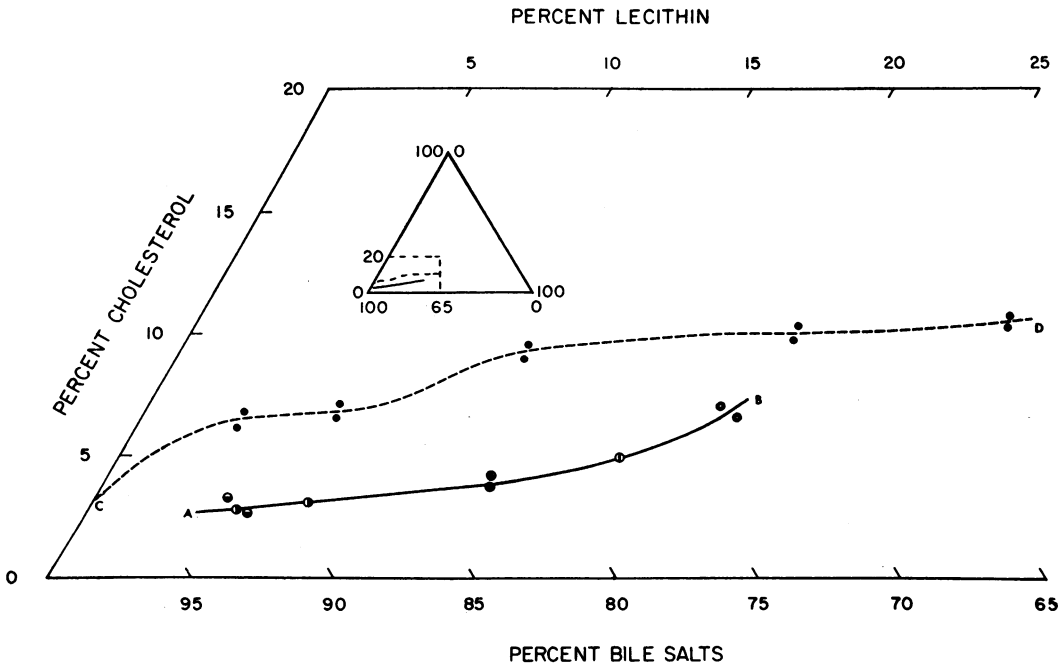

FIGURE 4 Probable micellar zone as derived from maximum cholesterol solubility measurement method in five mammalian species. AB represents visual line of best fit. CD represents the micellar zone as previously defined (4, 5). Key inset as in Fig. 2. ◫, Pooled canine; ◒, Ovine; ○, Human; ◑, Bovine; ⊙, Porcine.

canine biles and biles obtained from the 33 humans without biliary tract disease.

The percent of total solids present in the gallbladder bile of each of these mammalian species is comparable with that of human bile and ranged from 11 to 18%.

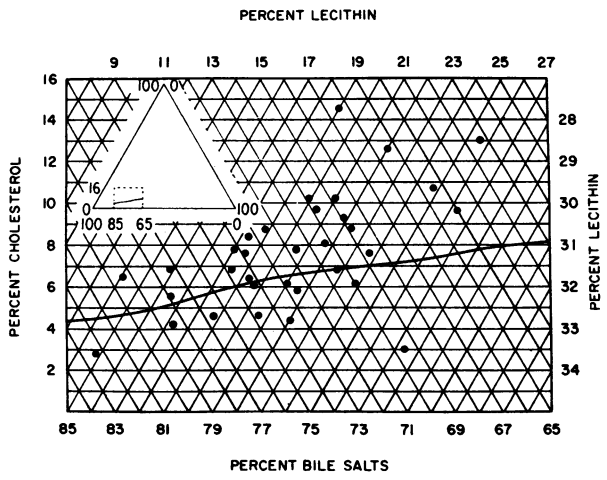

FIGURE 5 Initial composition of 33 normal human gallbladder biles with superimposed in vitro model solution micellar zone boundary. Key inset indicates the portion of the triangular coordinate system; solid line indicates the present micellar zone boundary.

An intraspecies sex difference in either initial composition or maximum cholesterol solubility was not discernible. Table I shows that, of the various nonhuman species in this comparison, porcine and canine gallbladder bile are more nearly comparable with human bile in lipid composition except for marked cholesterol undersaturation. In Fig. 4, a visual curvilinear line of fit (AB) is drawn between points derived from the maximum cholesterol solubility measurement in each of the species studied indicating the probable micellar zone observable by direct assay as compared with the in vitro derived line (CD) as previously reported (4, 5). The present in vitro solution micellar zone boundary is in excellent agreement with line AB.

Data obtained from the 33 patients in the present study who did not have biliary tract disease are shown in Fig. 5. With reference to the present revised in vitro micellar zone for cholesterol solubilization, it can be seen that 25 (three-fourths) of these untreated "normal" specimens are saturated or supersaturated with cholesterol. Results after application of the maximum cholesterol solubility measurement method in 30 of these normal bile samples are shown in Fig. 6*a* and 6*b*. Good agreement is seen with the in vitro micellar zone boundary derived from model solutions. Since the variation coefficient for this method is approximately ±1.5% total mol for cholesterol, 27 of these 30 normal gallbladder bile specimens

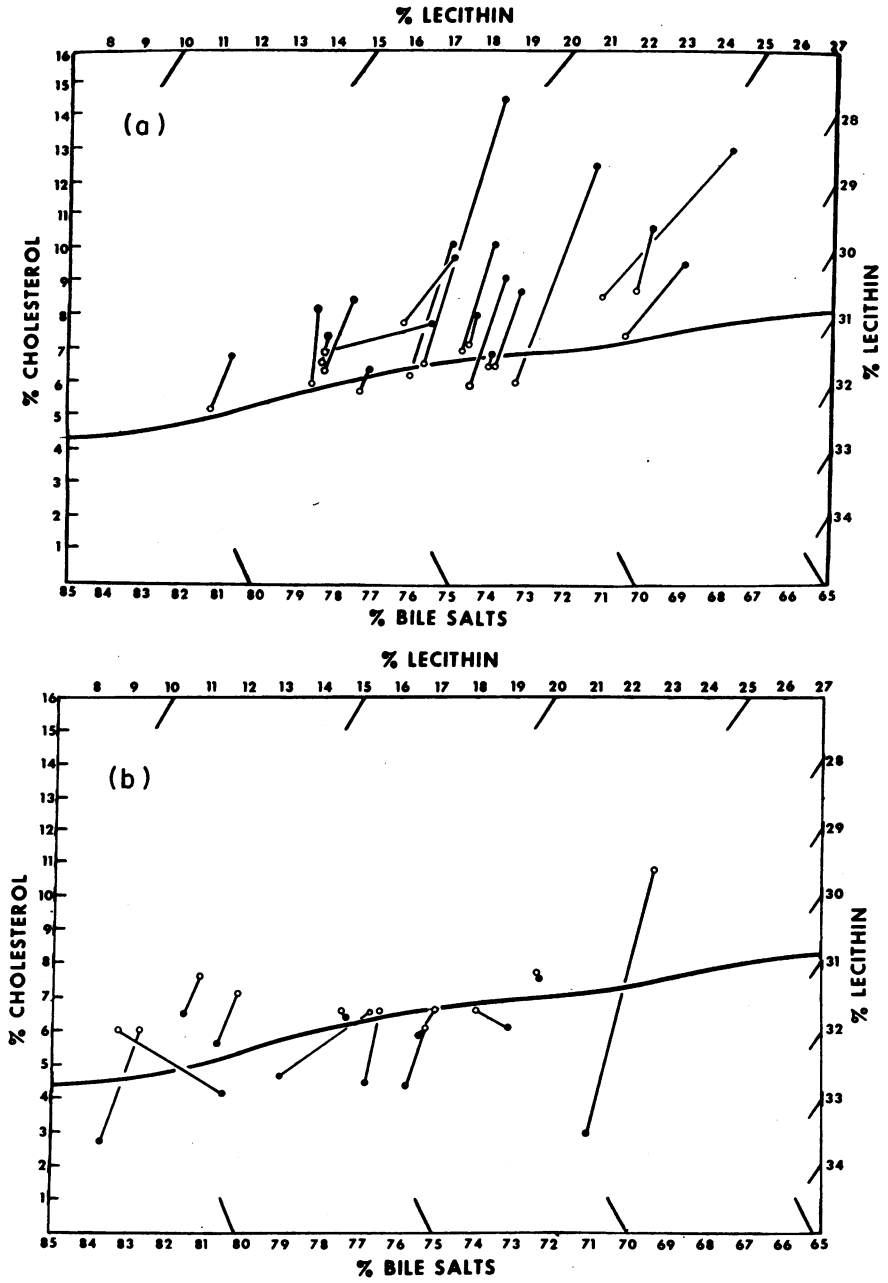

FIGURE 6 Compositions of paired initial and maximum cholesterol solubility measurements from 30 normal human gallbladder biles with superimposed in vitro model solution micellar zone boundary. Portion of the triangular coordinate system as in Fig. 5. (*a*) Data from 18 specimens whose maximum cholesterol solubility ○ was *less than* initial composition values ●. (*b*) Data from 12 specimens whose maximum cholesterol solubility ○ was *greater than* initial composition values ●.

tested revealed a cholesterol saturability value compatible with the in vitro model solution micellar zone definition.

For a comparison with normals in Fig. 5, compositional data from the 35 patients in the present series with the cholesterol gallstones are similarly displayed in Fig. 7. In reference to the variation coefficient for the micellar zone boundary, all but one of the bile specimens are either saturated or supersaturated with cholesterol.

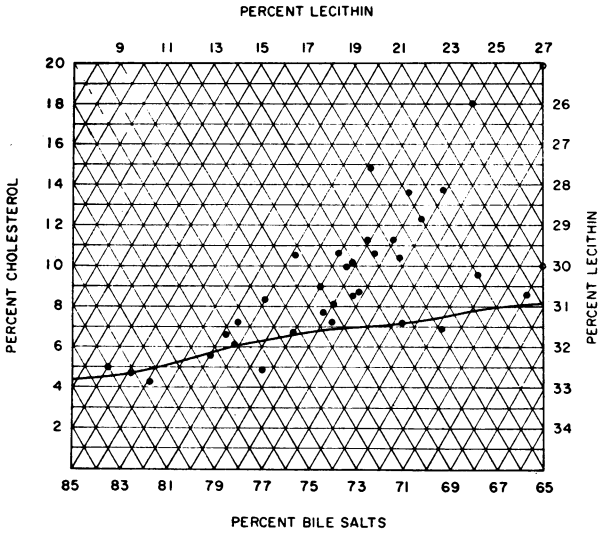

FIGURE 7 Initial composition of 35 gallstone-associated human gallbladder biles with superimposed in vitro model solution micellar zone boundary. Portion of the triangular coordinate system as in Fig. 5. Open circles did not fit within the area of the graph. Cholesterol coordinates for these are correct; upper circle: bile salts, 40%; lecithin, 40%; lower circle: bile salts, 49.2%; lecithin, 40.8%.

In the abnormal group, cholesterol microcrystals were observed in 15 of the 35 specimens, but in contrast to a previous report (5), there was no correlation between the presence of microcrystals and the relative cholesterol concentration. Since cholesterol microcrystals were not detected in any fresh normal specimens in the present study and have not been reported in previous studies, cholesterol supersaturation in normal human gallbladder bile must be a common occurrence (Fig. 5).

Results following application of the maximum cholesterol solubility measurement method in 28 of the abnormal bile samples are shown in Fig. 8*a* and 8*b*. 8 of the 28 abnormals had a value exceeding the maximal cholesterol solubility variation coefficient (±1.5% total mol for cholesterol). In neither the normal or the abnormal group was a correlation found between the maximum cholesterol solubility and percent solids.

DISCUSSION

A significant reduction in the micellar zone for cholesterol solubility is one of several important results of this study. The greater saturability limit for in vitro solutions observed by Bourgès et al. (4), and Admirand and Small (5) could be explained by failure of microscopic scanning to detect cholesterol crystals in the higher portion of the previously defined micellar zone. The study of Hegardt and Dam (22, 23) shows excellent agreement with present data whereas that of Neiderhiser and Roth (24) shows a somewhat higher level of in vitro cholesterol saturability. It should be noted that the micellar zone in all of these three studies, which utilized microfiltration for boundary definition, was significantly reduced from the previous definition obtained without similar assurance of complete removal of excess cholesterol. No disproportionate microfilter lipid adherence was observed by analysis of sequential filtrates, and no difference could be demonstrated in the maximum cholesterol solubility of previously frozen as compared with paired fresh human bile specimens (Table II). Also, it has recently been independently shown (25) that microfiltration under similar conditions does not disrupt the micellar association between labeled cholesterol and bile salts. In addition, the use of coprecipitation in solution preparation and relatively brief incubation in the earlier studies could have produced transiently supersaturated solutions as recently shown by Mufson, Meksuwan, Zarembo, and Ravin (26).

The present and other recent studies do not explain the failure to observe cholesterol microcrystals in a large number of human gallbladder biles that fell outside of the micellar zone, yet were not associated with gallstones. If any physical form of excess cholesterol were the sole important initiating factor in gallstone formation, one might expect that in a large series of such observations some normal subjects would also have cholesterol microcrystals. Several bile specimens found to contain liquid crystals (*27*) were maintained at 37°C for up to 3 wk. Periodic polarizing microscopy provided evidence for a spontaneous phase transition from the liquid crystalline to the cholesterol crystallite state. The lack of correlation between the degree of excess cholesterol and the presence of microcrystals may in part be explained by such phase transitions.

The greater frequency with which maximum cholesterol solubility values in the abnormal group exceeded the in vitro model solution limit as compared with normals is perhaps more likely an experimentally induced rather than a real difference. A possible explanation for the elevated apparent maximum cholesterol solubility could be the formation of liquid crystals during the measurement procedure. Recent in vitro model solution experiments[3] have revealed the presence of liquid crystals in solutions of lipid composition comparable with human biles containing excess cholesterol. Additional observations show that the liquid crystals can be extruded through microfilter pores and that freezing induces a liquid to solid crystalline phase transition. Although this occurrence was observed in about 19% of the total number of human maximum cholesterol solu-

[3] Unpublished observation.

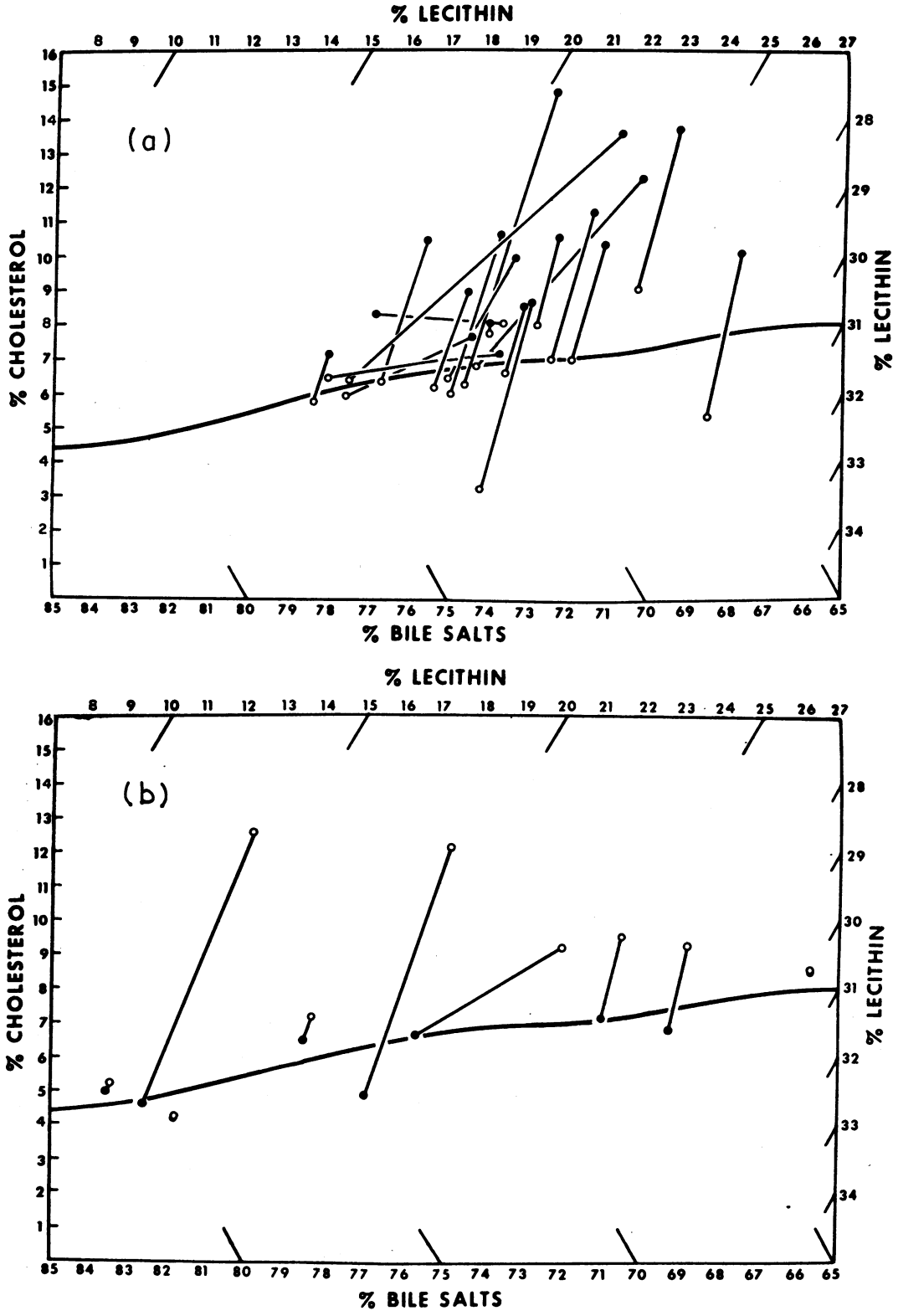

FIGURE 8 Compositions of paired initial and maximum cholesterol solubility measurements from 28 abnormal human gallbladder biles with superimposed in vitro model solution micellar zone boundary. Portion of the triangular coordinate system as in Fig. 5. (*a*) Data from 19 specimens whose maximum cholesterol solubility ○ was *less than* initial composition values ●. (*b*) Data from nine specimens whose maximum cholesterol solubility ○ was *greater than* initial composition values ●.

TABLE II

Compositional Data. Sequential Filtration and Maximum Cholesterol Solubility from Paired Fresh and Frozen Normal Human Gallbladder Biles

No.	Sample tested	Composition			Total millimoles	Composition		
		Cholesterol	Lecithin	Bile salt		Cholesterol	Lecithin	Bile salt
		mmol/liter				*percent total mol*		
1	Fresh	5.8	24.4	111	141.2	4.1	17.3	78.6
	Filtrate 1	5.6	24.7	103	133.3	4.2	18.5	77.3
	Filtrate 2	5.6	22.6	112	140.7	4.0	16.1	79.9
	MCS	10.0	23.5	138	171.5	5.8	13.7	80.5
	Frozen	5.4	21.9	118	145.3	3.7	15.1	81.2
	Filtrate 1	4.7	22.9	118	145.6	3.2	15.7	81.0
	Filtrate 2	4.7	22.6	119	146.3	3.2	15.4	81.3
	MCS	9.6	21.8	128	159.4	6.0	13.7	80.3
2	Fresh	28.4	56.2	134	218.6	13.0	25.7	61.3
	Filtrate 1	21.1	55.3	132	208.4	10.1	26.5	63.3
	Filtrate 2	20.2	54.6	124	198.8	10.2	27.5	62.4
	MCS	21.2	58.2	160	239.4	8.9	24.3	66.8
	Frozen	27.8	55.2	139	222.0	12.5	24.9	62.6
	Filtrate 1	17.4	49.0	138	204.4	8.5	24.0	67.5
	Filtrate 2	17.3	48.9	141	207.2	8.3	23.6	68.1
	MCS	20.1	57.8	157	234.9	8.6	24.6	66.8
3	Fresh	14.4	31.4	125	170.8	8.4	18.4	73.2
	Filtrate 1	14.4	31.2	125	170.6	8.4	18.3	73.3
	Filtrate 2	14.3	32.4	128	174.7	8.2	18.5	73.3
	MCS	12.1	35.3	144	191.4	6.3	18.4	75.2
	Frozen	14.8	32.7	124	171.5	8.6	19.1	72.3
	Filtrate 1	10.6	31.1	124	165.7	6.4	18.8	74.8
	Filtrate 2	10.7	30.3	128	169.0	6.3	17.9	75.7
	MCS	14.3	35.9	152	202.2	7.1	17.8	75.2
4	Fresh	14.1	36.6	128	178.7	7.9	20.5	71.6
	Filtrate 1	13.9	37.5	125	176.4	7.9	21.3	70.8
	Filtrate 2	14.0	35.8	126	175.8	8.0	20.4	71.6
	MCS	14.1	38.9	160	213.0	6.6	18.3	75.1
	Frozen	14.4	38.9	130	183.3	7.9	21.2	70.9
	Filtrate 1	12.0	34.6	120	166.6	7.2	20.8	72.0
	Filtrate 2	12.1	36.3	124	172.4	7.0	21.1	71.9
	MCS	12.7	36.2	144	192.9	6.6	18.8	74.6
5	Fresh	20.7	43.1	148	211.8	9.8	20.3	69.9
	Filtrate 1	20.4	43.0	149	212.4	9.6	20.2	70.2
	Filtrate 2	19.1	42.7	144	205.8	9.3	20.7	70.0
	MCS	17.2	43.0	150	210.2	8.2	20.4	71.4
	Frozen	20.8	42.8	148	211.6	9.8	20.2	69.9
	Filtrate 1	15.6	40.3	147	202.9	7.7	19.9	72.4
	Filtrate 2	15.7	40.2	149	204.9	7.7	19.6	72.7
	MCS	16.2	40.2	147	203.4	7.9	19.8	72.3

MCS, Maximum cholesterol solubility.

bility measurements, an additional step to ensure nucleation or liquid crystal to solid crystalline phase transition before microfiltration must now be recommended. Since this phenomenon was largely confined to the atypical subgroup of abnormals that were initially at or below saturation (Fig. 8*b*), one may speculate that an unusual

TABLE III

Lithogenic Index of Normal and Cholesterol Gallstone-Associated Human Gallbladder Bile from the Present and Previously Published Studies*

No.	Source	Reference	No. studied	CHa	CHs	Lithogenic index
Normals						
1.	Sweden	(7)	20	13.1	6.4	2.1
2.	Sweden	(32)	19	12.6	6.9	1.8
3.	Finland	(29)	11	9.6	6.2	1.6
4.	USA	(30)	2	11.1	7.9	1.4
5.	USA	(8)	11	9.0	6.4	1.4
6.	New Zealand	(29-NZ)	13	6.1	4.4	1.4
7.	USA	(33)	9	8.4	7.0	1.2
8.	USA	(P)	33	7.6	6.5	1.2
9.	USA	(29)	19	7.1	6.2	1.2
10.	Japan	(32)	20	6.1	5.4	1.1
11.	Denmark	(6)	14	6.7	6.2	1.1
12.	USA	(5)	25	6.6	6.3	1.1
13.	Masai-Africa	(29)	9	3.9	4.4	0.9
Abnormals						
1.	New Zealand	(29-NZ)	27	12.0	4.4	2.7
2.	USA	(5)	66	12.5	4.7	2.7
3.	Sweden	(7)	20	14.6	6.0	2.4
4.	Finland	(29)	34	11.7	5.0	2.3
5.	USA	(29)	27	10.1	5.4	1.9
6.	USA	(32)	7	14.8	8.1	1.8
7.	USA	(8)	10	13.1	7.4	1.8
8.	USA	(30)	14	12.5	7.1	1.8
9.	USA-Indian	(33)	6	11.5	7.0	1.6
10.	USA	(P)	35	9.4	6.6	1.4
11.	USA-Indian	(34)	11	8.6	6.4	1.3
12.	Denmark	(6)	26	8.2	7.0	1.2

CHa = Molar percentage for cholesterol.
CHs = Maximum molar percentage for solubilized cholesterol.
(P) = Present study.
* Lithogenic Index = 1.0 indicates saturation.

predisposition to liquid crystal formation may be present in these specimens.

The present study shows a considerable overlap (Figs. 5 and 7) between the normal and abnormal groups, both of which demonstrate a high incidence of biliary cholesterol in excess of saturation. Strong support for the wide applicability of this observation is provided in Table III using the recently described lithogenic index (28) to compare data in the present and previous studies with ranking in decreasing order of magnitude. Assay methods, specimen sources, and calculation assumptions were carefully scrutinized for comparability before inclusion. In the 10 studies included where paired data are available, the difference between abnormals and normals ranges widely. In two studies (5, 29-NZ), there is about a twofold difference, but, in six (6–8, 30, 33, and present study), abnormals exceed normals by no more than 33%. Since fresh normal biles are usually purely isotropic solutions, whereas abnormals often contain at least one other cholesterol containing phase, it is not certain from present data whether this apparent difference in supersaturation between the two groups is real or possibly insignificant. Where the degree of biliary cholesterol supersaturation seems important experimentally (31), duration is likewise important. Since biliary cholesterol supersaturation is frequent in healthy man, this alone no longer seems the central issue in gallstone pathogenesis. Rather, now required is identification and explanation of those factors that promote precipitation of cholesterol from *abnormal* supersaturated biles and those which inhibit precipitation from equally supersaturated *normal* biles.

In the present study we failed to find any correlation in the wide variety of specimen sources examined between the maximum cholesterol solubility and percent solids of the various groups. This suggests that in the range

of values studied the other nonlipid components present in bile do not exert a significant influence on this property. Not excluded, however, is their possible effect on the *rate* of cholesterol precipitation from supersaturated systems.

In summary, the following has been established: the micellar zone boundary, as derived by direct measurement of biliary cholesterol saturability, is nearly identical in all mammalian species examined and approximates that derived from model solutions; human bile from patients without biliary tract disease is commonly supersaturated with cholesterol; a male-female difference is not demonstrable despite the well-established female preponderance of cholelithiasis; and finally, bile from most patients with cholesterol cholelithiasis appears to have a micellar zone similar to that of healthy individuals.

Note added in proof: Following final acceptance of this manuscript, we received an unpublished paper from Dr. Kerrison Juniper, Jr., University of Arkansas Medical Center, which showed in vitro cholesterol solubility data essentially in agreement with the present work.

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