The Physical Chemistry of Cholesterol Solubility in Bile

RELATIONSHIP TO GALLSTONE FORMATION AND DISSOLUTION IN MAN

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ABSTRACT We determined the maximum solubilities of cholesterol in aqueous conjugated bile salt-egg lecithin-cholesterol systems as a function of several physical-chemical variables including those of physiological importance employing phase equilibria techniques. Equilibration rates are influenced by time and the method of sample preparation in that metastable supersaturation is readily induced at high bile salt: lecithin ratios, and equilibrium saturation by dissolution is achieved sluggishly at low bile salt:lecithin ratios. Equilibrium values for cholesterol saturation vary with the bile salt species, bile salt:lecithin ratio, temperature, ionic strength, and, in particular, with the total concentration of biliary lipids. Within physiological bile salt:lecithin ratios at 37°C the influence of bile salt type and ionic strength is small, whereas the effects of bile salt:lecithin ratio and the total lipid concentration are major factors. We plotted on triangular coordinates a family of cholesterol solubility curves for each total lipid concentration (0.30–30 g/dl) and computed fifth-degree polynomial equations for each curve. With both the curves and the polynomial equations the "percent cholesterol saturation" of fasting gallbladder and hepatic biles from patients with and without gallstones was calculated and both methods gave similar values. These results demonstrate that by employing cholesterol saturation values appropriate to the total lipid concentration (range 0.2–24.9 g/dl) of individual biles, all cholesterol stone patients have supersaturated gallbladder biles, (mean, 132% [normal weight individuals], and 199% [morbidly obese individuals]). With controls and pigment stone patients the mean values were 95 and 98%, respectively, and in both ≈50% of biles were supersaturated. Fasting hepatic biles were significantly more supersaturated than gallbladder biles (means 228–273%). Cholesterol monohydrate crystals were found in the majority of gallbladder (83%) and hepatic (58%) biles of cholesterol gallstone patients but were not observed in pigment stone patients or controls. We conclude that of the several factors in addition to the bile salt:lecithin ratios which can influence the cholesterol saturation of bile the total lipid concentration is the predominant determinant physiologically. Our results demonstrate that (*a*) metastable supersaturation is frequent in both normal and abnormal biles, (*b*) cholesterol gallstone patients have supersaturated gallbladder and hepatic biles without exception, and (*c*) the predominant driving force for cholesterol precipitation appears to be the absolute degree of cholesterol supersaturation.

INTRODUCTION

As cholesterol is the major component of most gallstones and as cholesterol and lecithin are sparingly soluble in water, the nature of the physical-chemical

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interactions between cholesterol and other biliary lipids has been extensively investigated (1–13). After the pioneering work of Isaksson (1) and Nakayama (2) and the systematic phase equilibria studies of Small, Bourgès, and Dervichian (14–16) the importance of mixed micelles containing both bile salts and lecithin in the solubilization of biliary cholesterol was established (15). Molecular models for these micelles were deduced from the fine structure of the liquid crystalline phases and from other empirical physical-chemical considerations (16–19). When it was demonstrated by Admirand and Small (3) that gallbladder bile from cholesterol gallstone patients could be distinguished from controls by relating their relative lipid compositions to the cholesterol solubility limits in a model system, a rational physical-chemical basis for gallstone formation appeared straightforward.

While there is now general agreement that the pathogenesis of cholesterol gallstones is related to the capacity of bile to maintain cholesterol in micellar solution, the precise limits of cholesterol solubility in model bile systems and thus the degree of cholesterol saturation of native bile remains controversial. Hegardt and Dam (7) and Holzbach et al. (9, 10) used longer equilibration times and microfiltration to isolate the micellar phase and found that the maximum cholesterol solubility reported by Admirand and Small was too high. Despite these refinements, the gallbladder biles of stone patients are not always supersaturated or even saturated by Admirand and Small's criteria nor by Holzbach et al.'s criteria (9, 20–27) as would be expected from a priori physical chemical considerations.[1] In other studies (9, 25, 26) it was concluded that fasting gallbladder bile is frequently supersaturated in healthy man when the commonly used cholesterol solubility limits (7, 9) are employed. One possible explanation for these inconsistencies was advanced by Small (28) who showed that the prevalence of gallstones in various populations is closely correlated with the frequency of supersaturated bile in controls. However, this suggestion may not entirely explain the very high percentage (>50%) of supersaturated gallbladder biles in control Caucasians in certain studies (9, 25). For these reasons several authors (9, 20, 25–27) concluded from the scatter in their results that it was impossible to separate patients with cholesterol stones from controls simply by examination of biliary lipid composition. Furthermore, as an appreciable number of bile samples from gallstone patients were unsaturated with cholesterol, it was suggested that supersaturation of bile with cholesterol may not be essential for the production or growth of cholesterol gallstones (26). What all of these authors failed to take into account was that discrepancies in their results might be attributed directly to deficiencies in the data chosen for the physical-chemical limits of cholesterol solubility in model bile systems. It was suggested earlier that the concentration of total lipids (bile salts + lecithin + cholesterol) (2, 3, 28, 29), different bile salts (4, 7, 11), and even temperature (2) or counterion (Na^+) concentration (7) might influence the maximum degree of cholesterol solubility in bile. The importance of total lipid concentration is manifested by the fact that human bile can vary widely in concentration from one collection site to another, from patient to patient and from time to time in the same patient. Published values range from 1 to 4 g/dl for hepatic bile (30) and from 8 to 28 g/dl for gallbladder bile (31). Assuming a linear dependence of cholesterol solubility on total lipid concentration, one of us attempted to calculate directly maximum cholesterol solubilities (29) for total lipid concentrations of 1–10 g/dl from the original Admirand and Small data. Although the direction of the changes was correct, the calculated limits were incorrect because of the uncertainties in the assumptions made (32). Recognizing the complexities of calculating cholesterol solubilities from data at one total lipid concentration and suspecting that variations in cholesterol solubility may not be as simple as assumed for typical micellar systems where the degree of solubilization increases linearly with concentration, it appeared essential to us to determine experimentally the maximum cholesterol solubility in dilute and concentrated biles to express the percentage of saturation of cholesterol in hepatic and gallbladder biles correctly.

This paper, therefore, describes experiments in which the maximum equilibrium cholesterol solubility in bile is measured directly in aqueous bile salt-lecithin-cholesterol model systems. We systematically investigate the effects of variations in total lipid concentration, type of bile salt, temperature, ionic strength (NaCl), dissolution rates, and metastability on cholesterol solubility. Of particular relevance clinically is that a semilogarithmic relationship between cholesterol solubility and total lipid concentration occurs over the physiological range of bile salt:lecithin ratios and total lipid concentrations. These findings, when applied to the relative and total lipid concentrations of gallbladder and hepatic biles of cholesterol gallstone patients, pigment gallstone patients, and controls, clearly demonstrate that no single solubility limit can be employed to define the relative cholesterol content of all individual native biles due to the profound variation in cholesterol saturation with variations in the total lipid concentration.

[1] In any system at constant temperature and pressure a change in the number of phases at equilibrium cannot occur without a change in composition (from the Gibbs Phase Rule). Thus, for bile to undergo a phase change from a one-phase system (single liquid) to a two-phase system (liquid plus stones) the capacity of the liquid phase to solubilize cholesterol must be exceeded.

METHODS

Materials

Cholesterol. Cholesterol (Lipids Preparation Laboratory of the Hormel Institute, University of Minnesota, Austin, Minn.) was recrystallized thrice from hot 95% (vol/vol) ethanol and stored at 4°C under nitrogen. Its purity was checked by thin-layer silica gel chromatography (220 μg spot; hexane, diethyl ether, glacial acetic acid, 7:3:0.1, by vol), hot stage microscopy (melting point 147–149°C uncorrected), and differential scanning calorimetry (5-mg samples) and found to be >99.5% pure. After storage, laser Raman spectroscopy showed that the cholesterol was anhydrous and equivalent to a National Bureau of Standards reference cholesterol.

Lecithin. Egg yolk lecithin (Grade 1; Lipid Products, Surrey England) was judged to be greater than 99% pure by a number of chromatographic methods for polar lipids employing 2-dimensional paper chromatography (33) and neutral lipids by thin-layer silica gel chromatography (200 μg spot; chloroform, methanol, water, 18:6:1 by vol). The lecithin was stored in the dark in chloroform-methanol (2:1 vol/vol) in sealed glass vials at −20°C under nitrogen. The fatty acid composition of the lecithin (principally palmitic, oleic, and linoleic acids) as determined quantitatively by gas-liquid chromatography was similar to literature values (7, 34). The molecular weight calculated from the fatty acid composition corresponded to an average of 765. Complete reanalysis after 6 mo of storage showed no chemical degradation of the lecithin.

Bile salts. The sodium salt of 3α, 7α, 12α-trihydroxy-5β-cholanoyltaurine known trivially as sodium taurocholate (NaTC)[2] was an A-grade product (Calbiochem, San Diego, Calif.). Thin-layer silica gel chromatography (200 μg spot; chloroform, methanol, acetic acid, 13:3:1 by vol) and potentiometric titration (18) with HCl (1 g/dl, wt/vol) revealed 2–3% unconjugated bile acid impurities. The material was therefore recrystallized twice by the method of Pope (35), lyophilized from water, and gave a single spot on repeat thin-layer chromatography. The sodium salts of 3α, 7α-dihydroxy-5β-cholanoyltaurine (Calbiochem) and 3α, 12α-dihydroxy-5β-cholanoyltaurine (Maybridge Company, Tintagel, Cornwall, England) known trivially as sodium taurochenodeoxycholate (NaTCDC) and sodium taurodeoxycholate (NaTDC), respectively, were also found to contain significant (≈5–10%) unconjugated bile acid impurities by thin-layer silica gel chromatography and potentiometric titration and were purified to ≈98–99% purity as previously described (36). All bile salts were hygroscopic and were stored in desiccators over calcium chloride at room temperature.

Reagent grade NaCl was roasted at 600°C in a muffle furnace for 4 h to oxidize and remove organic impurities. Water was triple distilled, the second distillation being from an all-glass Pyrex automatic still (Corning Glass Works, Science Products Div., Corning, N. Y.) and the final distillation was from a seasoned all-Pyrex laboratory distillation assembly over alkaline potassium permanganate. The water was adjusted to pH 7.0 with the addition of a few microliters of 1 N NaOH before use. All glassware was acid/alkali washed by steeping in 1 N HNO_3 and in 2 N KOH/EtOH, 50/50 (vol/vol) consecutively for 24 h. The glassware was oven dried at 100°C after thorough rinsing in distilled water.

[2] *Abbreviations used in this paper*: CMC, critical micellar concentration; NaTC, sodium taurocholate; NaTCDC, sodium taurochenodeoxycholate; NaTDC, sodium taurodeoxycholate; TCC, threshold concentration for cholesterol solubility.

Gallbladder and hepatic duct biles. Surgical bile samples were generously provided by Dr. Eldon A. Shaffer, Montreal General Hospital, Montreal, Quebec, Canada under protocols approved by the Human Studies Committees of Boston City Hospital, The Lahey Clinic, and Veterans Administration Hospital, all of Boston, Mass. and the Montreal General Hospital, Montreal, Canada. All patients had normal liver function tests (including bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase, and serum proteins) and gave informed written consent. Patients were classified (37) on the basis of the analyses of their stones for cholesterol into two categories, those with cholesterol stones (>70% cholesterol by weight) and those with pigment stones (<5% cholesterol by weight). Fasting gallbladder biles were obtained at surgery from 20 patients with cholesterol stones, 4 of whom were morbidly obese; i.e., patients with >150% of ideal weight calculated according to life insurance tables (38), 14 patients with pigment stones, and 12 control patients without gallstones. Fasting common hepatic duct bile was simultaneously sampled at surgery as described (37) from 12 nonobese patients with cholesterol stones, 10 patients with pigment stones, and 8 controls without gallstones. A small number of bile samples not analyzed on the day of aspiration were rapidly frozen and stored at −20°C.

Experimental design

Two independent methods were employed to saturate the micellar solutions with cholesterol. In the multiple mixture method, equilibrium was reached by precipitation from supersaturated solutions and in the second, the dissolution method, equilibrium was approached by dissolution of crystalline cholesterol in lecithin-bile salt solutions. Equilibration of the mixtures was proven by employing a number of classical procedures: (*a*) the upper limit of the micellar phase did not change with time, (*b*) after heating and cooling, the mixtures eventually returned to the original equilibrium values, and (*c*) when the micellar phases were separated by microfiltration from mixtures containing two or three phases, the analytical cholesterol concentrations of the clear micellar phases were the same as values obtained by direct observation of the entire series of tubes.

Preparation of mixtures

For the multiple mixture method, bile salts (NaTC, NaTCDC, NaTDC, or a 4:4:2 molar mixture of NaTC, NaTCDC, and NaTDC) were mixed with lecithin in methanol or in chloroform-methanol 1/1 or 2/1 (vol/vol) in 25-ml volumetric flasks to give a series of stock solutions containing 15 different molar ratios of bile salts and lecithin ranging in composition from the pure bile salt to the bile salt-lecithin phase limit. To aliquots of these stock solutions, varying amounts of cholesterol in chloroform were added to give a series of mixtures with a fixed bile salt-lecithin molar ratio and progressively increasing increments (about 0.16–0.32 mol/100 mol of total lipids) of cholesterol. The total dry weight of the lipids in each tube was 200±10 mg. After mixing, the solvent was partially evaporated under a stream of dry nitrogen at 40°C until the mixtures were reduced to a viscous paste or glass (≈10–30 min). The tubes were then plugged with sterile gauze and taken to dryness by continuous pumping in vacuo over phosphorous pentoxide (24–72 h). As the desiccated mixtures were hygroscopic, extrapolation of 5-min time-weight curves to zero time gave the anhydrous weight. Mixtures were composed with water (no added NaCl) or with 0.05, 0.15, 1.0, and 3.0 M NaCl (all at pH 7.0) to give the appropriate total lipid concentration. After they were flushed with nitrogen,

the tubes were sealed and allowed to equilibrate with gentle shaking for 7–14 days. This procedure was repeated for eight total lipid concentrations (0.16–20 g/dl of solution) at seven temperatures (4–95°C). At temperatures greater than 65°C equilibrium was reached rapidly so that incubation for only a few hours was necessary.

For the dissolution method, ethanolic (95% vol/vol) solutions of NaTC and lecithin were mixed in 250-ml Erlenmeyer flasks to give 21 mixtures containing seven different molar ratios of NaTC and lecithin. The mixtures were then desiccated under nitrogen and in vacuo over phosphorous pentoxide to constant weight as described above. The dried lipids were dissolved in aqueous solvent so that each contained a 10-g/dl bile salt-lecithin solution of fixed molar ratio in H_2O, 0.15 M and 1.0 M NaCl, respectively (total volume 100 ml). After they were sealed with Teflon stoppers, the solutions were sterilized under nitrogen by pasteurization and allowed to equilibrate at room temperature for an additional 24 h. With the use of a sterile technique, twice the weight of the microcrystalline cholesterol sufficient to saturate 10 g/dl of the 7:3 molar bile salt:lecithin mixture was added to each flask. The flasks were then flushed with nitrogen and shaken continuously at a calibrated linear flow rate of 1 cm/s for 35–40 days. 1 ml of the total contents of each flask was sampled with a long wide-bore sterile needle and syringe at 0 and 12 h and subsequently every 24 h for the first 10 days, followed by sampling every 2–3 days for the next 25–30 days. The samples were filtered through 0.22-μm Millipore filters or, in the case of viscous mixtures, were centrifuged at 10,000 *g* for 60 min at the temperature of equilibration to achieve separation of the micellar phase which was aspirated from the top of the tube as described above. Bile salts, lecithin, and cholesterol were measured in triplicate in all samples. A parallel control "unstirred" (no shaking) dissolution study was carried out with a range bile salt-lecithin mixtures in 0.15 M NaCl followed by analysis of the separated micellar phases at 30 days.

Examination of mixtures

Multiple mixture study: optical examination. The appearance of each tube was noted daily during the equilibration period with regard to opacity, fluidity, presence of crystals, and (or) the appearance of a Tyndall phenomenon (14, 15). Each unopened tube was examined for phase separation by employing transmission spectrophotometry (Beckman Acta III spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.) with a wavelength of 750 nm or by measuring the turbidity of light of wavelength 546 nm scattered at an angle of 90° (Brice-Phoenix light scattering photometer, model 200; Phoenix Precision Instrument Div., Virtis Co., Gardiner, N. Y.). As equilibrium was approached, similar examinations were made on the mixtures for several subsequent days to show that no changes in light transmission or turbidity occurred.

Proof of equilibration by lipid analysis. Selected mixtures containing two or more phases after ≈12 days incubation were filtered through 0.22-μm Millipore filters to obtain a sample of the micellar phase. Lipid analysis of this phase was carried out in triplicate as described below.

Determination of metastable zone and the metastable-labile limit. Mixtures containing 20 g/dl total lipid were equilibrated at 95°C until the maximum amount of cholesterol had dissolved. The clear tubes were then rapidly cooled to 24±0.2°C and continuously monitored by optical methods (see above) for the separation of a second phase. For each bile salt-lecithin series the cholesterol content in moles per 100 moles of total lipids was plotted against the time required for precipitation. These mixtures which precipitated fast (seconds or minutes) contained a larger excess of cholesterol compared with those which precipitate slowly (hours or days). The rapidly precipitating mixtures are defined as falling within a subzone of labile supersaturation, whereas the latter mixtures fall in a subzone of metastable supersaturation (39). The interpolated cholesterol concentrations at the boundary corresponds to the metastable-labile limit. The metastable zone is therefore bounded by the metastable-labile limit and the equilibrium solubility of cholesterol (the rationale for this procedure is discussed later).

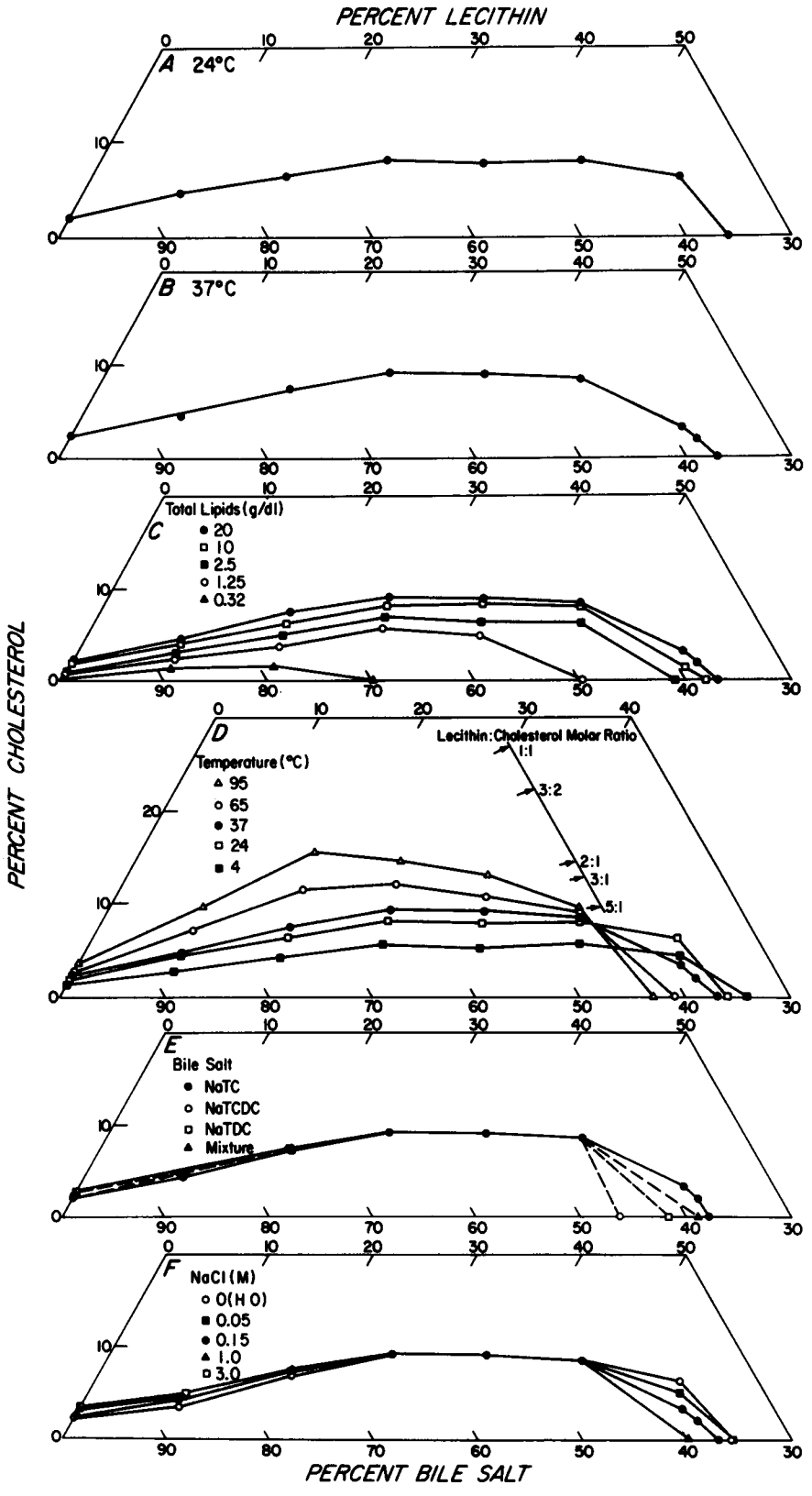
Physical state and characterization of phases. After equilibration, a small quantity (2–5 μl) of each mixture was removed for examination by direct and polarized light microscopy to document the number of phases present and their optical properties (14, 34). At the same time, small samples (~5 μl) were placed in Lindemann glass tubes (Charles Supper Co., Natick, Mass.) for small angle X-ray scattering or diffraction with a focusing camera of the torroidal type utilizing nickel-filtered CuK_α X rays from an Elliot GX6 rotating anode generator (Marconi-Elliot Avionic Systems, Borehamwood, England). Data were recorded on film and analyzed by a Joyce-Loebl densitometer (Joyce-Loebl Manufacturing Co., Gatehead, England) with *'d'* spacings calculated using the Bragg equation. Solution densities were measured in an ultrasonic precision density meter (model DTC 2, Anton Paar, Graz, Austria) and apparent partial specific volumes ($\bar{v}$) calculated as described (40). In all, some 1,000 mixtures were examined by turbidimetric methods at seven different temperatures of which 100 were studied by X-ray and density methods.

Dissolution study. All mixtures were examined daily by gross and microscopic observation to detect microbial contamination or oxidative degradation of the lecithin. Great care was exercised in aspirating samples for analysis to keep the volume aspirated to 1.0 ml and to ensure that the sample was adequately mixed with undissolved cholesterol crystals. The gross changes in viscosity and appearance of the mixtures were documented throughout the dissolution period.

Proof of equilibrium by lipid analysis. Equilibrium was determined by monitoring the micellar solubility of cholesterol as a function of time until it attained a constant value. Samples were harvested periodically and after the excess microcrystalline cholesterol or lecithin-cholesterol liquid crystals were removed as described above, the micellar solution was assayed for all three lipids to obtain the relative cholesterol content. The equilibrium cholesterol solubility was obtained when repeated samples of the micellar phase obtained 2 days apart over the course of at least 8 days showed no further increment in cholesterol content.

Gallbladder and common hepatic duct bile. All bile specimens were immediately examined grossly for the presence of a precipiate and microscopically (direct light and polarizing) for cholesterol crystals, orange-red bilirubinate granules, or lecithin-cholesterol liquid crystals. Aliquots from thoroughly mixed bile specimens were analyzed in duplicate for total bile salts, cholesterol, and phospholipids. Variation of less than 3% was found between duplicate analysis.

Biliary lipid determination. Biliary phospholipids were measured directly in native and artificial bile as inorganic phosphorus by the method of Bartlett (41). Cholesterol was determined with Carr and Drekter's modification (42) of the original method of Abell et al. (43). Total bile salt concentration was measured enzymatically of the 3α-hydroxysteroid dehydrogenase method of Talalay (44) as modified by Admirand and Small (3).


PERCENT LECITHIN
A 24°C
B 37°C
C
Total Lipids (g/dl)
20
10
2.5
1.25
0.32
D
Temperature (°C)
95
65
37
24
4
Lecithin:Cholesterol Molar Ratio
1:1
3:2
2:1
3:1
5:1
E
Bile Salt
NaTC
NaTCDC
NaTDC
Mixture
F
NaCl (M)
0 (H O)
0.05
0.15
1.0
3.0
PERCENT CHOLESTEROL
PERCENT BILE SALT

Data computation

Relative lipid composition is recorded as moles of cholesterol, bile salts, and phospholipids per 100 moles of total lipids (bile salts + lecithin + cholesterol) and total lipid concentration is expressed as grams per deciliter according to the weight of the components and the final volume. Total lipid concentration of native bile is derived from the sum of the concentrations of the components as estimated from the lipid analyses employing molecular weights of 491 for mixed bile salts, 775 for biliary lecithin, and 387 for anhydrous cholesterol. Relative lipid compositions are plotted as moles percent of total lipids on triangular coordinates according to Admirand and Small (3). To facilitate relative composition plots of biliary lipids in native bile according to their total lipid concentrations, the appropriate cholesterol solubility value from the model system is marked on a line describing the bile salt:lecithin ratio of the sample. With the use of rectangular plots (45), cholesterol saturation in mole percent cholesterol, "cholesterol × 100/ bile salt + lecithin + cholesterol (y)", is plotted vs. the molar bile salt:lecithin ratio expressed as "lecithin/bile salt + lecithin (x)", and fifth-degree polynomial regressions were computed for all variations in total lipid concentration and temperature.[3] A family of computerized interpolated curves is given for very small increments in total lipid concentration between 0.30 and 30 g/dl at 37°C (0.15 M NaCl) and for temperatures between 5°C and 95°C (10-g/dl solutions, 0.15 M NaCl) and all coefficients of x are tabulated. In addition these data are plotted in rectangular format with a CALCOMP 960 plotter (California Computer Products, Inc., Anaheim, Calif.). Significance of data was obtained by a two-tailed Student's t test for group comparisons.

RESULTS

Multiple mixture studies

Effects of physical chemical variables on equilibrium micellar solubilities of lecithin and cholesterol. Maximum micellar solubilities of lecithin alone (no cholesterol) in aqueous bile salt solutions as a function of total lipid concentration, temperature, type of bile salt, and added NaCl are shown as individual data points on the baselines of the triangular coordinates in Fig. 1. It is noteworthy that bile salts differ in their capacity to solubilize lecithin, NaTC > mixture > TDC > TCDC (molar lecithin:bile salt ratio varies from 2 → 1.25 depending upon temperature). Solubility is maximal at 4°C and decreases progressively as the temperature is increased (data for NaTC shown in Fig. 1D). Decreasing the total lipid concentration results in dramatic reductions in lecithin solubility which are most pronounced at lipid concentrations less than 10 g/dl (Fig. 1C). Added NaCl also causes a modest reduction in lecithin solubility at 0.15 and 1.0 M compared to H_2O (Fig. 1F). All solutions with high lecithin content were viscous and exhibited a Tyndall phenomenon (bluish opalescence).

The maximum micellar solubilities of cholesterol determined at each bile salt:lecithin ratio (20 g/dl, 0.15 M NaCl, 24°C and 37°C), are illustrated as curves plotted on the triangular coordinates in Figs. 1A and B and as families of curves as a function of total lipid concentration (Fig. 1C), temperature (Fig. 1D), bile salt species (Fig. 1E), and added NaCl (Fig. 1F). In general, for mixtures of the same total lipid concentration and temperature at constant NaCl concentration, cholesterol solubility first increases as the lecithin mole fraction increases, from a low solubility in pure bile salt micelles (data point on bile salt-cholesterol axis). After reaching a plateau at intermediate lecithin contents, cholesterol solubility declines steeply to reach zero values at the limit of the bile salt-lecithin micellar phase (data point on the bile salt-lecithin axis). At constant temperature, increases in total lipid concentration markedly increase maximum cholesterol solubility. When the total lipid concentration is large (>2.5 g/dl), big increases in concentration result in modest but progressive increases in cholesterol solubility, whereas at low concentration the converse is the case. These variations are most marked at high lecithin contents. Temperature elevation at a constant total lipid concentration also increases cholesterol solubility except for micellar mixtures with the highest lecithin contents where a reversed trend is observed (Fig. 1D). In the mixtures with a bile salt:lecithin molar ratio of 4:6, the cholesterol solubility is low at 4°C, peaks at 24°C and decreases again at 37°C, and is insoluble at 55°C. By extrapolating the bile salt apex of the triangle through the maximum cholesterol solubility at each temperature to intersect with the cholesterol-lecithin axis, the maximum molar ratio of lecithin to cholesterol in the saturated micelles is obtained (Fig. 1D). These ratios

[3] Fifth-degree polynomial regression: $y = a + bx + cx^2 + dx^3 + ex^4 + fx^5$ where a–f are constants (see Tables II and III).

FIGURE 1 Triangular coordinate plots of equilibrium micellar cholesterol solubilities in aqueous bile salt-lecithin-cholesterol systems measured by the multiple mixture method (pH 7.0). The percentage of total moles of bile salt, lecithin, and cholesterol constituted by each of the components are shown on the scales along the sides of truncated triangles enclosing the micellar region. A. 24°C (NaTC, total lipid concentration 20 g/dl, 0.15 M NaCl). B. 37°C (NaTC, 20 g/dl, 0.15 M NaCl). C. Variations in total lipid concentration (NaTC, 37°C, 0.15 M NaCl). D. Variations in temperature (NaTC, 20 g/dl, 0.15 M NaCl). Maximum lecithin: cholesterol molar ratios at the temperatures listed are indicated by arrows. E. Variations in bile salt species (20 g/dl, 0.15 M NaCl, 37°C). F. Variations in NaCl concentration (NaTC, 20 g/dl, 37°C). (Tables of individual data points are available to the interested reader upon request).

vary from 5:1 at 4°C to 1:1 at 95°C. In the absence of lecithin, cholesterol solubility in NaTDC was equal to that in the bile salt mixture and was appreciably greater than in NaTCDC which in turn was greater than in NaTC ($P < 0.05$). These differences persist with small additions of lecithin (e.g., 9:1 molar bile salt:lecithin ratio); however no significant variation was found at other ratios ($P > 0.1$). The cholesterol solubility with added NaCl (Fig. 1F) indicates that in NaTC-cholesterol systems (no lecithin) and in NaTC-lecithin systems with molar ratios of 9:1, small solubility increments occur as the NaCl concentration is increased from H_2O to 1.0 M NaCl ($P < 0.01$); however at molar ratios of 8:2 to 5:5 no significant differences are found. Increases in added NaCl significantly ($P < 0.05$) decrease cholesterol solubility at high lecithin contents paralleling the decrease in lecithin solubility.

Metastable-labile limit and metastable zone. The relative lipid compositions of the metastable-labile limit are plotted in Fig. 2. When compared with the relative compositions of the micellar phase boundary, the former are all significantly greater up to a NaTC:lecithin molar ratio of 5:5 where the two sets of values coincide. Thus the metastable zone which is enclosed by the two curves (stippled area in Fig. 2) is most extensive in the physiological range of molar bile salt:lecithin ratios, i.e., 9:1 to 6.5:3.5. The extrapolations on the right of the figure show that the maximum molar ratio of lecithin to cholesterol varies from 3:1 at equilibrium to 3:2 at the metastable-labile limit.

Physical state and characteristics of equilibrated phases. The physical state and characteristics of the equilibrated phases observed both above and below the micellar phase boundary at 24°C (20 g/dl, 0.15 M NaCl) are summarized in Table I. Below the limit of maximum cholesterol solubility the solutions were isotropic by polarizing microscopy and gave a diffuse X-ray scattering and the apparent partial specific volumes ($\bar{v}$) in the absence of cholesterol increase progressively from that of pure bile salts in proportion to the lecithin content, all consistent with the presence of mixed micelles. The micellar solutions near the bile salt-lecithin phase limit (molar NaTC:lecithin ratios of 5:5 and 6:4) exhibited a pronounced viscosity which increased with added NaCl. The viscosity decreased dramatically as the amount of solubilized cholesterol was increased. In the case of the micellar system with the highest lecithin contents, increases in temperature induced precipitation of the micellar phase. In Fig. 3 the temperatures of phase separation or "cloud points" for the 4:6 molar NaTC-lecithin mixtures are illustrated as a function of NaCl concentration and cholesterol content. For micellar solutions of identical lipid composition the cloud points are depressed significantly by added NaCl and by increasing the cholesterol content.

The phases observed at equilibrium (0.15 M NaCl, 24°C) above the limits of maximum cholesterol solubility are illustrated on triangular coordinates in Fig. 4. With mole fractions of lecithin of 0.2 and less, the saturated micellar phase is in equilibrium with a crystalline cholesterol monohydrate phase and its structure was verified by X-ray analysis (strong d[Å] spacings at 33.5, 16.8, 5.9, and 3.8). With mole fractions of lecithin between 0.3 and 0.4, cholesterol crystals and a lamellar liquid crystalline phase are in equilibrium with the micellar phase well above the cholesterol solubility limit, whereas only liquid crystals were observed just above this limit. With mole fractions of lecithin greater than 0.4 liquid crystals of lecithin and cholesterol coexist with an isotropic phase and no cholesterol crystals were detected.

Cholesterol solubility from micellar phase analysis after Millipore filtration (proof of equilibration). The relative compositions (moles/100 moles) of equili-

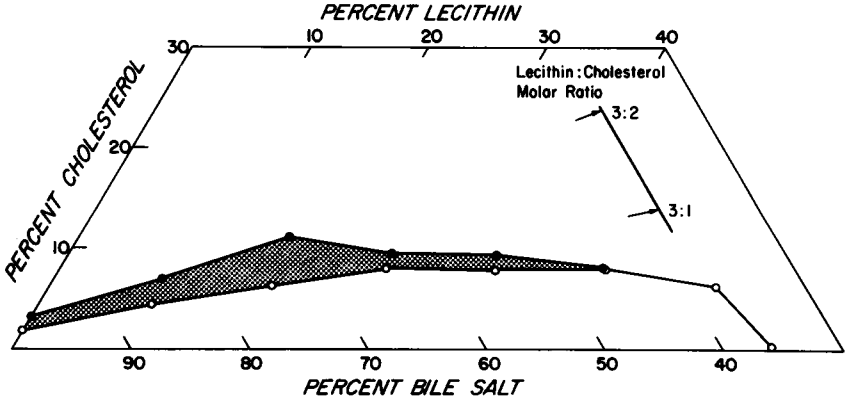


FIGURE 2 Triangular coordinate plot of the metastable-labile limit (● —— ●) and the maximum equilibrium cholesterol solubility (○ —— ○) in NaTC-lecithin-cholesterol systems (20 g/dl, 0.15 M NaCl, 24°C). The stippled area represents the metastable region. Maximum lecithin: cholesterol molar ratios corresponding to each curve are indicated by arrows.

TABLE I

Physical State of Phases above and below Equilibrium Cholesterol Solubility Boundary (20 g/dl Total Lipids, 0.15 M NaCl, 24°C, pH 7.0)

NaTC: lecithin ratio	Less than saturated with cholesterol					Greater than saturated with cholesterol
	Tyndall*	Viscosity‡	Microscopy	$\bar{v}$ (No cholesterol)	X ray	Direct and polarizing microscopy
10:0	(−)	Fluid	Isotropic	0.754	Micellar scattering	Large cholesterol monohydrate crystals.§ After heating and cooling: cholesterol microcrystals. No liquid crystals.
9:1	(−)	Fluid	Isotropic	0.760	Micellar scattering	Cholesterol monohydrate crystals.§ No liquid crystals.
8:2	(−)	Fluid	Isotropic	0.796	Micellar scattering	Cholesterol monohydrate crystals.§ No liquid crystals.
7:3	(−)	Fluid	Isotropic	0.843	Micellar scattering	Liquid crystals of lecithin + cholesterol just above the solubility limit. Well above limit: Liquid crystals + cholesterol crystals. Slight birefringence. (Positive sign).
6:4	(−)	Fluid	Isotropic	0.860	Micellar scattering	Liquid crystals of lecithin + cholesterol.‖ Few cholesterol monohydrate crystals. Birefringence more obvious. (Positive sign).
5:5	(+1)	Slightly viscous	Isotropic	0.881	Micellar scattering	Liquid crystals of lecithin + cholesterol—Maltese crosses obvious. No cholesterol crystals. Birefringence very obvious. (Positive sign).
4:6	(+2)	Very viscous	Isotropic	0.915	Micellar scattering	Liquid crystals of lecithin + cholesterol, Maltese crosses obvious. No cholesterol crystals. Birefringence obvious.
4:6 (H_2O)	(+2)	Viscous	Isotropic	—	Micellar scattering	Liquid crystals of lecithin + cholesterol. Maltese crosses. No cholesterol crystals. Birefringence obvious.
4:6 (0.05 M NaCl)	(+2)	Viscous	Isotropic	—	Micellar scattering	Liquid crystals of lecithin + cholesterol. Maltese crosses. No cholesterol crystals. Birefringence obvious.

* Based on an (1–5) arbitrary scale of blue light scattered at 90°.

‡ Viscosity qualitatively assessed from flow times in unopened tubes.

§ Cholesterol monohydrate structure confirmed by X-ray diffraction.

‖ With prolonged equilibration (1–2 mo) liquid crystals disappear in many of these mixtures probably due to surface adsorption of lecithin onto the cholesterol monohydrate crystals. Liquid crystals reappear upon heating and cooling.

brated 2 or 3 phase mixtures (20 g/dl, 0.15 M NaCl, 24°C) and the final composition of the isotropic phase after Millipore filtration or centrifugation are illustrated on triangular coordinates in Fig. 5. The arrows join the initial compositions to the corresponding relative lipid composition of the separated micellar phases. The maximum solubility of cholesterol thus obtained is indistinguishable from the maximum cholesterol solubility by observation of the entire series of tubes in the multiple mixture method (Fig. 1A). The results also show that during filtration small amounts of lecithin are removed with the insoluble phase when the lecithin content is low but the amounts removed are appreciable when the lecithin content is high, i.e., the direction of arrows in Fig. 5 do not correspond to the interrupted semi-vertical lines of constant bile salt-lecithin composition. This confirms the removal of lecithin-cholesterol liquid crystals which are in equilibrium with the saturated micellar phase.

Thermodynamic analysis of cholesterol solubility

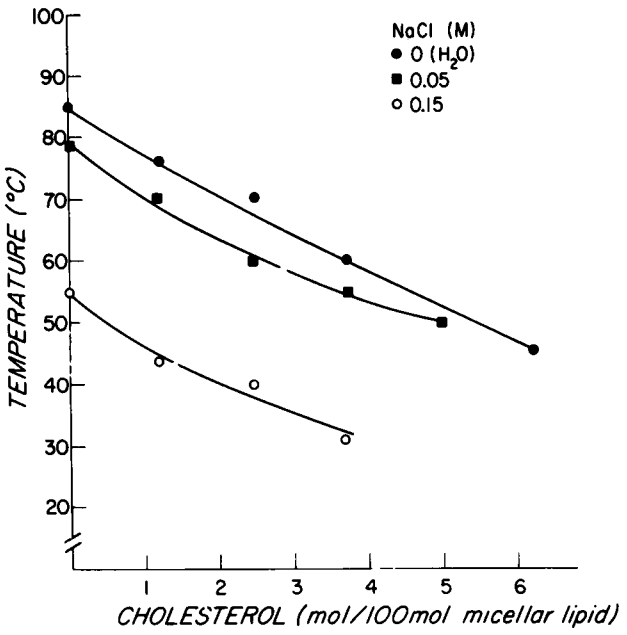


FIGURE 3 Temperature of phase separation ("cloud points") (by light scattering and polarizing microscopy) in 20-g/dl mixed micellar solutions (NaTC:lecithin molar ratio 4:6) as a function of added cholesterol and NaCl (maximum uncertainty ≈±2°C in all points).

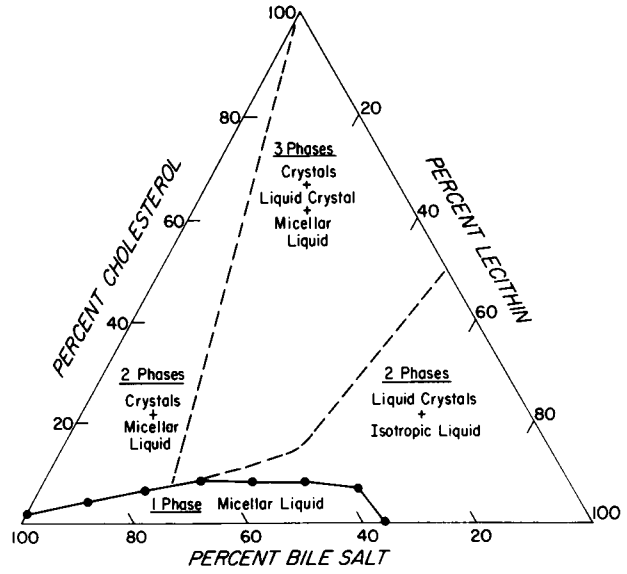


FIGURE 4 Triangular phase diagram showing the physical state of all combinations of NaTC, lecithin and cholesterol as 20-g/dl solutions in 0.15 M NaCl at 24°C (polarizing microscopy and X-ray analysis). The one-phase region gave a diffuse X-ray scattering profile. The two-phase zone on the left gave an X-ray diffraction profile consistent with cholesterol monohydrate crystals in addition to micellar scattering. The three-phase zone in the middle gave a mixed cholesterol monohydrate and lamellar liquid-crystalline pattern and the two-phase zone on the right gave a liquid crystalline profile with isotropic scattering.

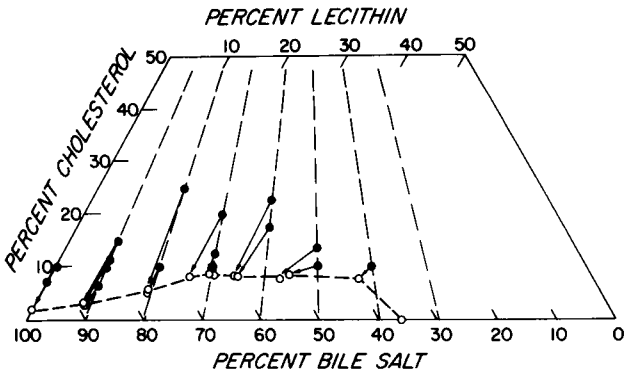


FIGURE 5 Abbreviated triangular coordinate plot of equilibrium cholesterol solubilities (○ —— ○) obtained by micellar lipid analysis of microfiltered two- or three-phase systems (● —— ●) (24°C, 0.15 M NaCl, pH 7.0). Constant NaTC-lecithin compositions are indicated by the dashed lines converging toward the cholesterol apex of the triangle. Arrows which show both direction and change in relative lipid compositions indicate that some lecithin in addition to cholesterol was removed by the filters. The lipid concentration of the initial mixtures was 20 g/dl, but after filtration the concentration was slightly less because of removal of cholesterol and lecithin-cholesterol liquid crystalline phases.

from multiple mixture data. Cholesterol solubilities (37°C, 0.15 M NaCl) as a function of total lipid concentration for each bile salt-lecithin series are plotted semilogarithmically in Fig. 6A. Once above a threshold concentration (TCC), cholesterol solubility increases linearly with the logarithm of the total lipid concentration. The data in Fig. 6A are replotted on Cartesian coordinates in Fig. 6B as cholesterol solubility vs. total lipid concentration minus the TCC. The increments in cholesterol solubility for constant increments in total lipid concentration minus TCC increase progressively with the lecithin content up to a bile salt:lecithin molar ratio of 7:3 and decreases slightly at the higher lecithin contents. The TCC concentration of total lipids expressed as mM NaTC is compared with the apparent critical micellar concentrations (CMCs) of NaTC-lecithin micelles estimated spectrophotometrically (46) and by ultrafiltration[4] at corresponding lecithin mole fractions in Fig. 7. The TCC values are significantly larger than the apparent CMCs at all lecithin mole fractions by the spectrophotometric method ($P < .001$) and coincides with some of the apparent CMC values by ultrafiltration. However, in both the absence of lecithin and as the NaTC-lecithin phase limit is approached the TCC and the CMC values diverge appreciably.

The effects of temperature on equilibrium cholesterol solubility were estimated thermodynamically by

[4] Carey, M. C. Unpublished observations.

employing the integrated form of the van't Hoff equation (47):

$$\log K = -\frac{\Delta H}{2.303\,RT} + \text{constant}, \qquad (1)$$

where K is the cholesterol solubility in moles per 100 moles of total lipids, ΔH is the enthalpy (heat of solution), and R and T are the gas constant and absolute temperature, respectively. In Fig. 8 the cholesterol solubilities at each bile salt:lecithin ratio are plotted logarithmically against the reciprocal of the absolute temperature and the slopes, which are similar for both total lipid concentrations, give the enthalpy values. Employing the formula:

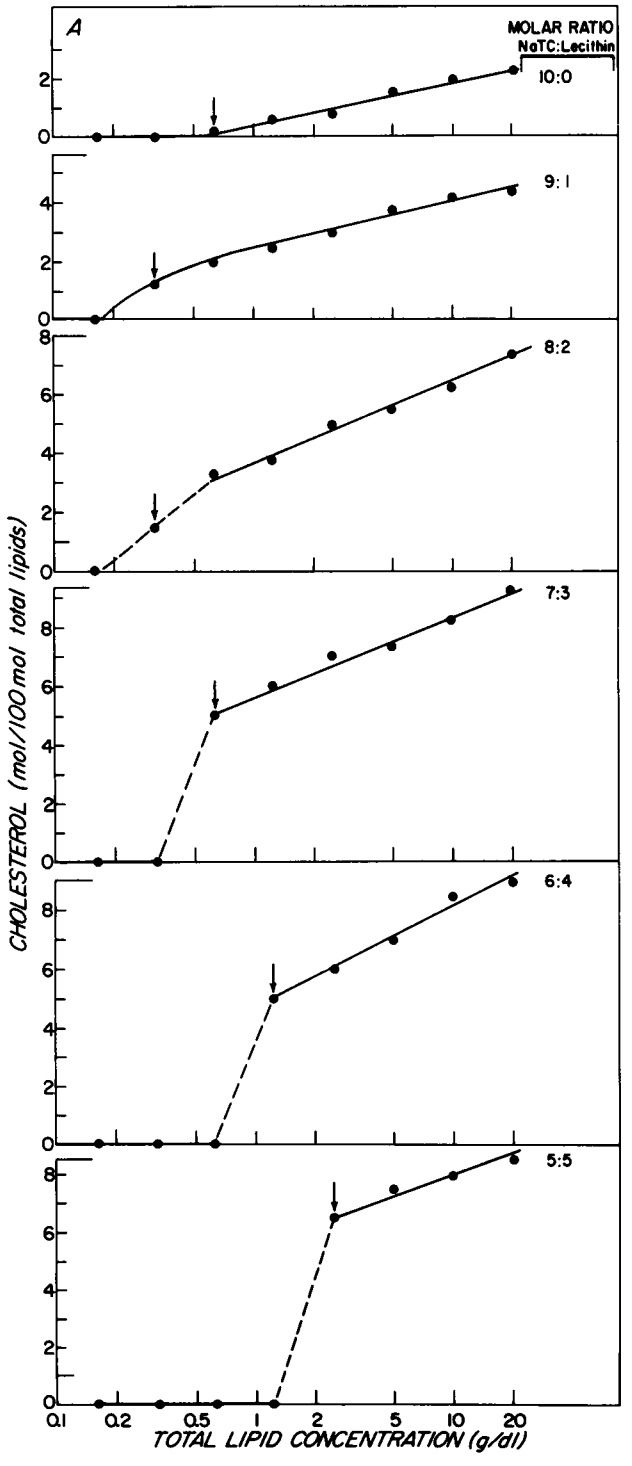
$$\Delta G = -2.303RT \log K^1, \qquad (2)$$

where R and T have the usual meanings and K^1 is the molar solubility of cholesterol per 1,000 moles of total micellar lipids, the free energy changes (ΔG) upon cholesterol solubilization may be derived.

Because

$$T\Delta S = \Delta H - \Delta G, \qquad (3)$$

the entropy changes ($T\Delta S$) at corresponding lecithin mole fractions were also calculated. These thermodynamic data (kJ/mole, 37°C) demonstrate large positive entropy and smaller enthalpy changes which are maximal at a lecithin mole fraction of 0.2 (Fig. 9). The ΔG values are negative and vary slightly with the lecithin content (Fig. 9).



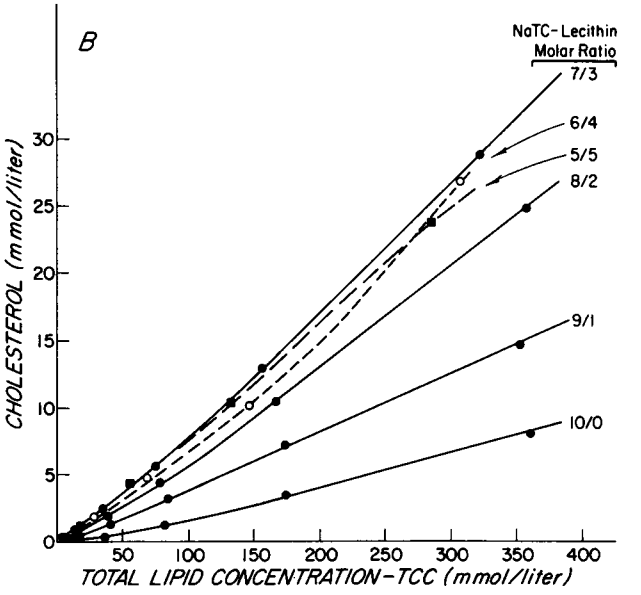


FIGURE 6 A. Maximum micellar solubility of cholesterol (moles/100 moles total lipids) in NaTC-lecithin-cholesterol systems (each with a fixed bile salt:lecithin ratio) as a function of the total lipid concentration (semilogarithmic plots, 37°C, 0.15 M NaCl). The approximate threshold concentrations for cholesterol solubility (TCC) are indicated by vertical arrows. B. Rectangular graphs of cholesterol solubility (millimoles/liter of solution) for each bile salt-lecithin system as a function of the total lipid concentration minus the TCC (millimoles/liter of solution).

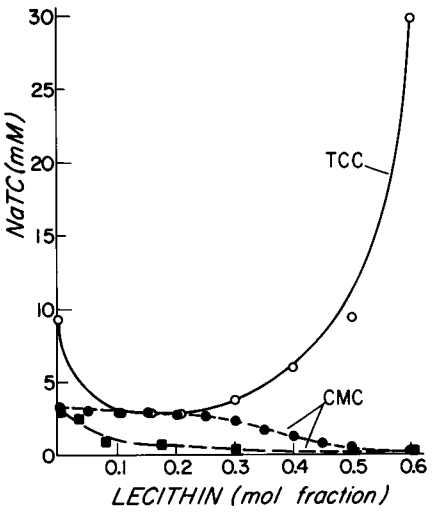


FIGURE 7 TCC values (in millimolars NaTC) for each NaTC: lecithin molar ratio vs. lecithin mole fraction. The critical micellar concentrations (CMC) of NaTC-lecithin micellar solutions estimated by the spectral shift method (■ —— ■) and by ultrafiltration (● —— ●) (M. C. Carey, unpublished observations) are plotted for comparison (0.15 M NaCl, 37°C).

Dissolution studies

Effects of physical-chemical variables on dissolution rates and final cholesterol solubilities. The dissolution rates and final cholesterol solubilities varied with the bile salt:lecithin ratio, NaCl concentration, shaking rate, and time. The percentage of saturation with cholesterol as a function of bile salt:lecithin ratio (0.15 M NaCl) and time is shown in Fig. 10. The time required for 50% saturation (horizontal dashed line) increases progressively with the lecithin content, however saturation was not achieved by 40 days in mixtures with the highest lecithin contents. The effects of added NaCl on the rates of dissolution and the final micellar cholesterol contents as a function of time are illustrated for representative NaTC:lecithin ratios in Fig. 11A, B, and C, respectively. The dissolution rates and the final solubilities of cholesterol increase with NaCl in the order $H_2O < 0.15$ M < 1.0 M. As illustrated by the continued increase in cholesterol solubility during the final week of dissolution in certain mixtures (Fig. 10C), and by plotting the final solubilities at each NaCl concentration on triangular coordinates (Fig. 12A–C), it is apparent that cholesterol solubility in mixtures containing the highest lecithin contents was much less than

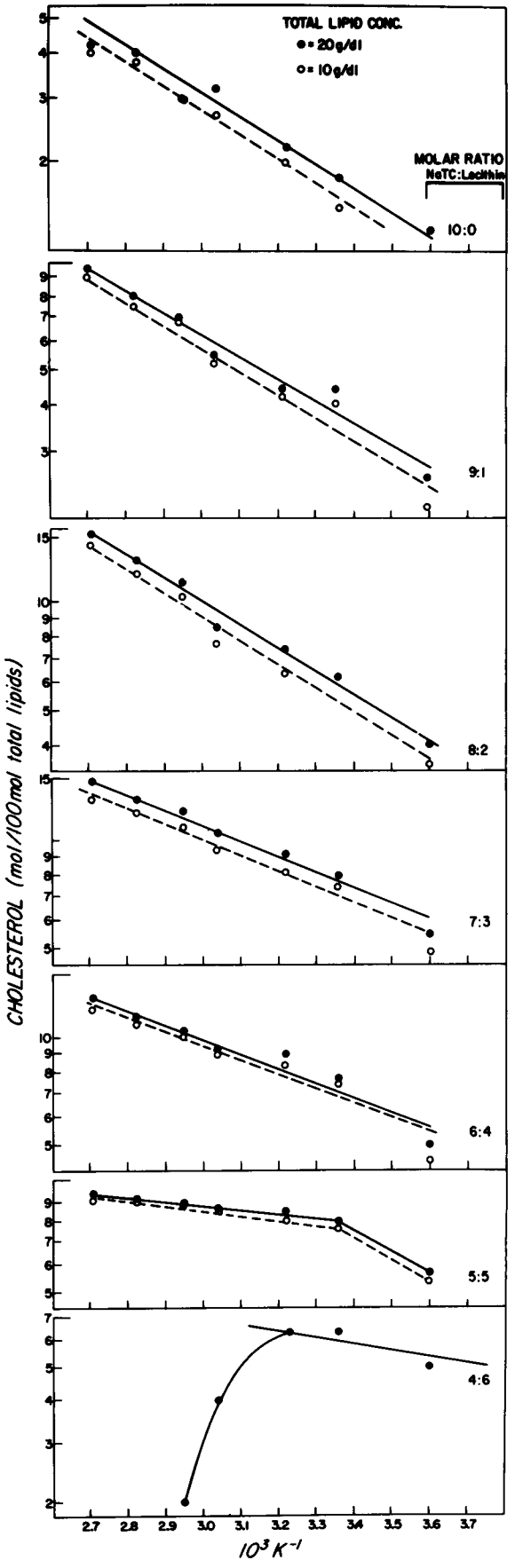


FIGURE 8 Semilogarithmic plots of cholesterol solubility (moles/100 moles total lipid) as a function of the inverse absolute temperature ($10^3 K^{-1}$) for each NaTC:lecithin molar ratio (0.15 M NaCl) (● —— ●, 20 g/dl; ○ —— ○, 10 g/dl). The enthalpy change (ΔH) associated with cholesterol solubilization is derived from the slopes of the curves.

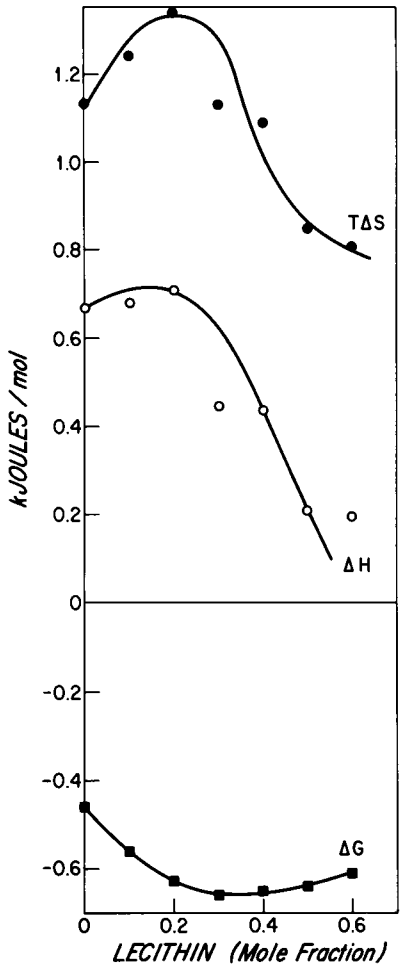


FIGURE 9 Standard thermodynamic functions (in kJoules/mole) for cholesterol solubility in NaTC-lecithin-cholesterol micellar solutions vs. mole fraction of lecithin to bile salts (37°C, 20 g/dl, 0.15 M NaCl). The enthalpy (ΔH) values were calculated from the slopes of the curves in Fig. 8. The free energy (ΔG) values were calculated from molar cholesterol solubilities at 37°C. The entropy ($T\Delta S$) values are the arithmetic differences between ΔH and ΔG values.

that found in the multiple mixture study. These mixtures therefore had not reached equilibrium. Fig. 12D shows the maximum cholesterol solubility achieved without shaking ("unstirred dissolution") for 30 days (10 g/dl, 0.15 M NaCl, 24°C). These values are all significantly less than that achieved in the dissolution studies with continuous shaking or in the multiple mixture methods.

Computed cholesterol solubility curves as a function of total lipid concentration and temperature

Triangular plots of both experimental and interpolated equilibrium cholesterol solubilities (from Fig. 6A) for variations in total lipid concentration (37°C, 0.15 M NaCl) and for variations in temperature (10 g/dl, 0.15 M NaCl) (from Fig. 8) are displayed as families of curves in Fig. 13A, B, and C to encompass the physiological range of molar bile salt:lecithin ratios. The actual total lipid concentrations and temperatures are given in the legend whereas in the figure only the highest and lowest values are marked. The family of curves dramatizes the variations in the cholesterol solubility phase boundary as total lipid concentration or temperature are varied. Fifth-degree polynomial regressions were then computed for these data as described in Methods and these are displayed as rectangular CALCOMP 960 computer plots in Fig. 14A and B (for variations in total lipid concentration and temperatures, respectively), and the corresponding fifth-degree polynomial coefficients of x are listed in Tables II and III. The standard error about the curves from 1 g/dl–30 g/dl varies from 0.1–0.3, depending upon the total lipid concentration with a slightly higher error at concentrations less than this. Therefore a maximum uncertainty of about ±3% occurs between observed (experimental and interpolated) and predicted (from the polynomial regression) values at low total lipid concentration (1–2.5 g/dl) and no more than ±1% at higher concentrations (>2.5 g/dl). The maximum standard error in the polynomial equations for variation in temperature is 0.3.

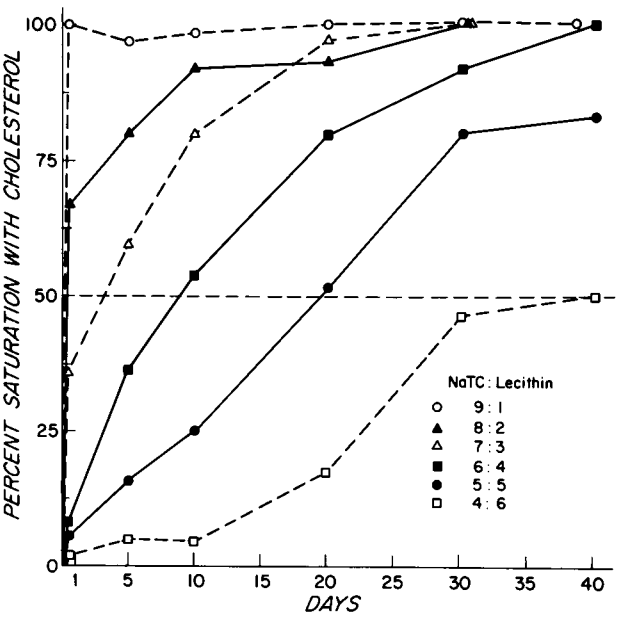


FIGURE 10 Dissolution rates of crystalline cholesterol in 10-g/dl NaTC-lecithin micellar solutions expressed as percentage of saturation vs. time in days (0.15 M NaCl, 24°C). Results for NaTC:lecithin mixtures with a molar ratio of 4:6 are recorded in 1.0 M NaCl. The intersections of a hypothetical vertical line with the curves represents percentage of saturation at 24 h.

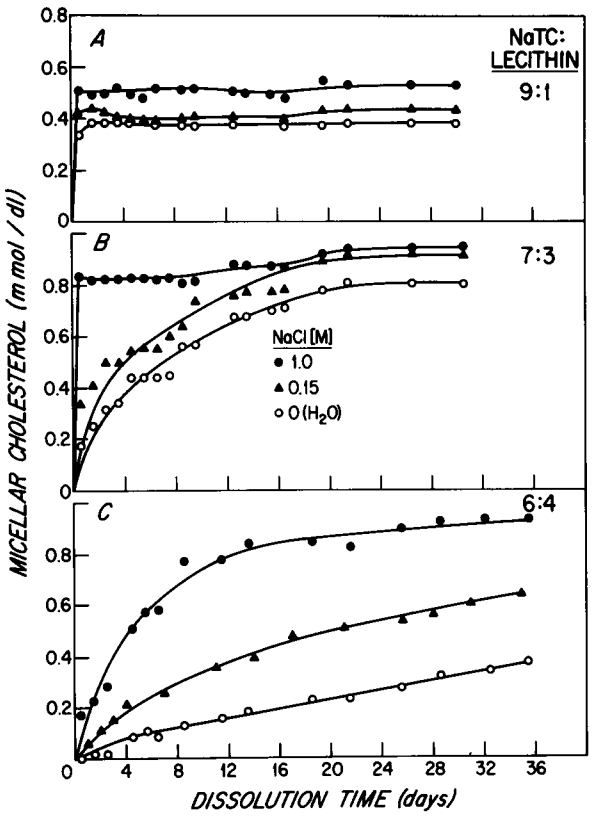


FIGURE 11 (A,B,C) Dissolution rates of crystalline cholesterol in 10-g/dl NaTC-lecithin solutions as a function of NaTC: lecithin molar ratio, added NaCl concentration, and time at 24°C. Data expressed as micellar cholesterol concentration in millimoles per deciliter of solution vs. dissolution time in days. The slopes of the curves can be employed to give the rates of dissolution under the conditions of each experiment.

Relative lipid composition of gallbladder and common hepatic duct biles plotted in relation to maximum cholesterol solubility for each relative and total lipid concentration

The absolute, relative, total lipid concentration, and "percent cholesterol saturation" (48) of surgical biles are tabulated in Table IV (gallbladder biles of cholesterol, pigment stone, and control patients) and Table V (hepatic biles of cholesterol, pigment stone, and control patients). The control data for 18 morbidly obese individuals without gallstones is taken from the recent literature (49). In Fig. 15A–G triangular coordinate plots of the relative lipid composition of each bile is shown as a single data point. Through each point a line is drawn to connect with the cholesterol apex of the triangle thus describing the bile salt:lecithin molar ratio of the sample. From the actual data shown in Fig. 13 the maximum predicted cholesterol solubilities (37°C, 0.15 M NaCl) for the corresponding total lipid concentration were interpolated and plotted as the short horizontal lines to intersect the lines of constant bile salt-lecithin composition in Fig. 15. The continuous phase boundaries are the maximum cholesterol solubilities for the averaged total lipid concentrations of each set of biles and the interrupted phase boundaries represent 10 g/dl solubility limits. The former correspond to the maximum cholesterol solubilities in total lipid concentrations of 2.6, 2.3, and 3.2 g/dl (hepatic biles) and 9.0, 10.2, 10.9, and 14.9 g/dl (gallbladder biles). Of the cholesterol gallstone biles (subjects of normal weight), 16/16 gallbladder biles and 12/12 hepatic biles are supersaturated when analyzed in this fashion. Of the pigment gallstone biles, 7/14 gallbladder biles and 8/10 hepatic biles are supersaturated. Of controls, 5/10 gallbladder biles and 7/8 hepatic biles are supersaturated and of the morbid obese subjects, 4/4 gallbladder biles from the stone group and 17/18 control biles are supersaturated (the latter only compared to average solubility criteria for 10 g/dl total lipids). When the relative compositions are compared to the mean cholesterol solubility limits for the average total lipid concentration in each group or for a constant 10-g/dl line, fewer in each group are supersaturated. From the plots of relative composition of each bile, the percent cholesterol saturation (48) is calculated

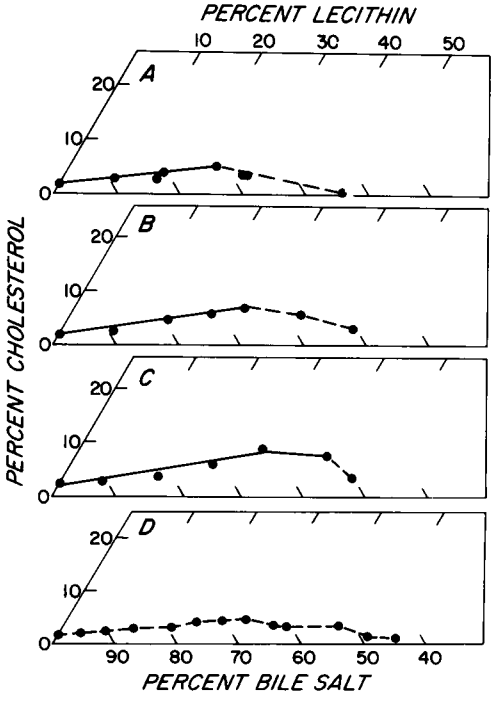


FIGURE 12 Triangular coordinate plots of final cholesterol solubilities in 10-g/dl NaTC-lecithin micellar solutions by dissolution at 24°C; (A) in H_2O (no added NaCl), (B) in 0.15 M NaCl, (C) in 1.0 M NaCl, and (D) "unstirred" dissolution in 0.15 M NaCl.

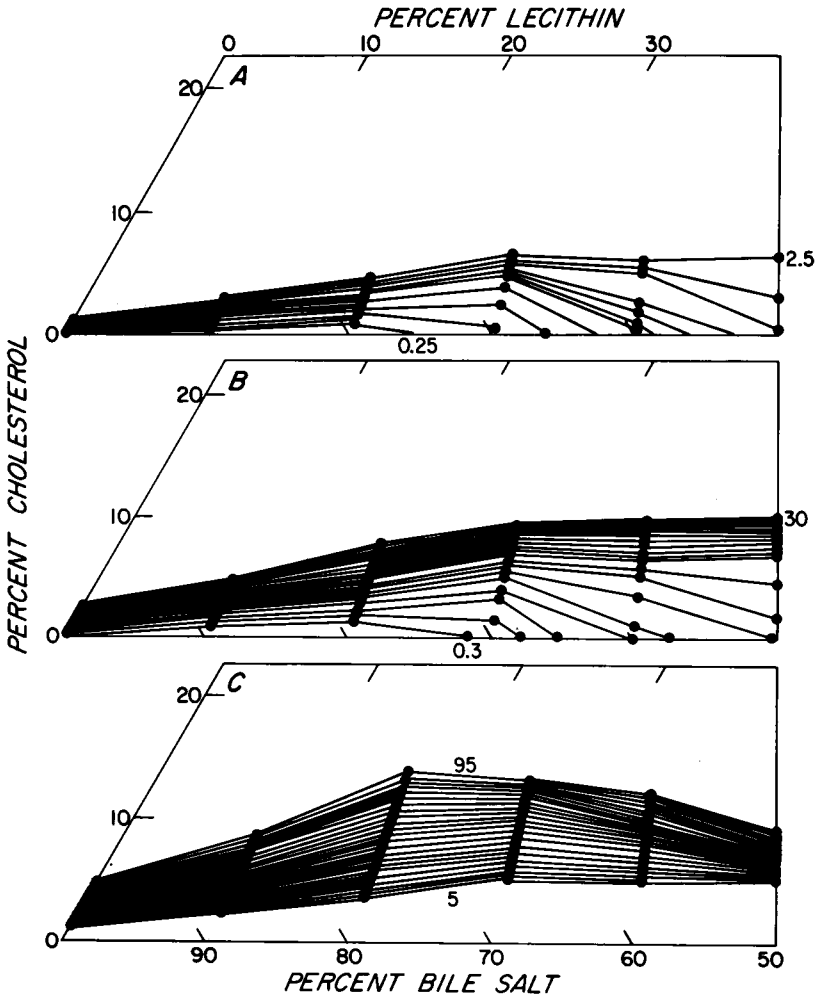


FIGURE 13 Interpolated curves for maximum equilibrium solubility of cholesterol in bile salt-lecithin-cholesterol micellar systems in 0.15 M NaCl for bile salt:lecithin molar ratios between 10:0 and 5:5; (A) As a function of total lipid concentration (from bottom up 0.25, 0.35, 0.45, 0.55, 0.65, 0.70, 0.80, 0.90, 1.25, 1.75, and 2.5 g/dl) at 37°C; (B) as a function of total lipid concentration (from bottom up 0.3, 0.4, 0.5, 0.6, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0, and 30.0 g/dl) at 37°C; (C) As a function of temperature (in 5°C increments between 5° [bottom curve] and 95°C [top curve]) as 10-g/dl solutions (the curves for 24 and 37°C are omitted).

graphically from the experimental curves (Fig. 13) and mathematically from the computed curves (Fig. 14A) with the polynomial equation (Table II) for total lipid concentration closest to that in the actual bile sample (values listed in Tables IV and V). This tabulation shows that the mean errors in percent cholesterol saturation between those calculated by hand measurements from the triangular graphs and by solution of the polynomial equations are ~1% for gallbladder biles and 2–5% for hepatic biles. In gallbladder biles from cholesterol gallstone patients the mean percent cholesterol saturation is 132/131% (normal weight individuals) and 199% (morbid obese individuals) ($P < 0.001$). These values are significantly larger ($P < 0.001$) than the corresponding values for gallbladder bile of the pigment stone group (98%) and normal weight controls (95/94%), and in addition, gallbladder bile of the obese individuals without stones is significantly ($0.001 < P < 0.005$) more supersaturated (139%) than control subjects without stones. Fasting common hepatic duct biles are considerably more supersaturated than gallbladder biles ($P < 0.001$) from cholesterol gallstone patients (273/264%), pigment stone patients (228/211%), and normal weight controls (257/262%). The differences between these groups is only marginally ($P < 0.05$) significant by the triangular graph

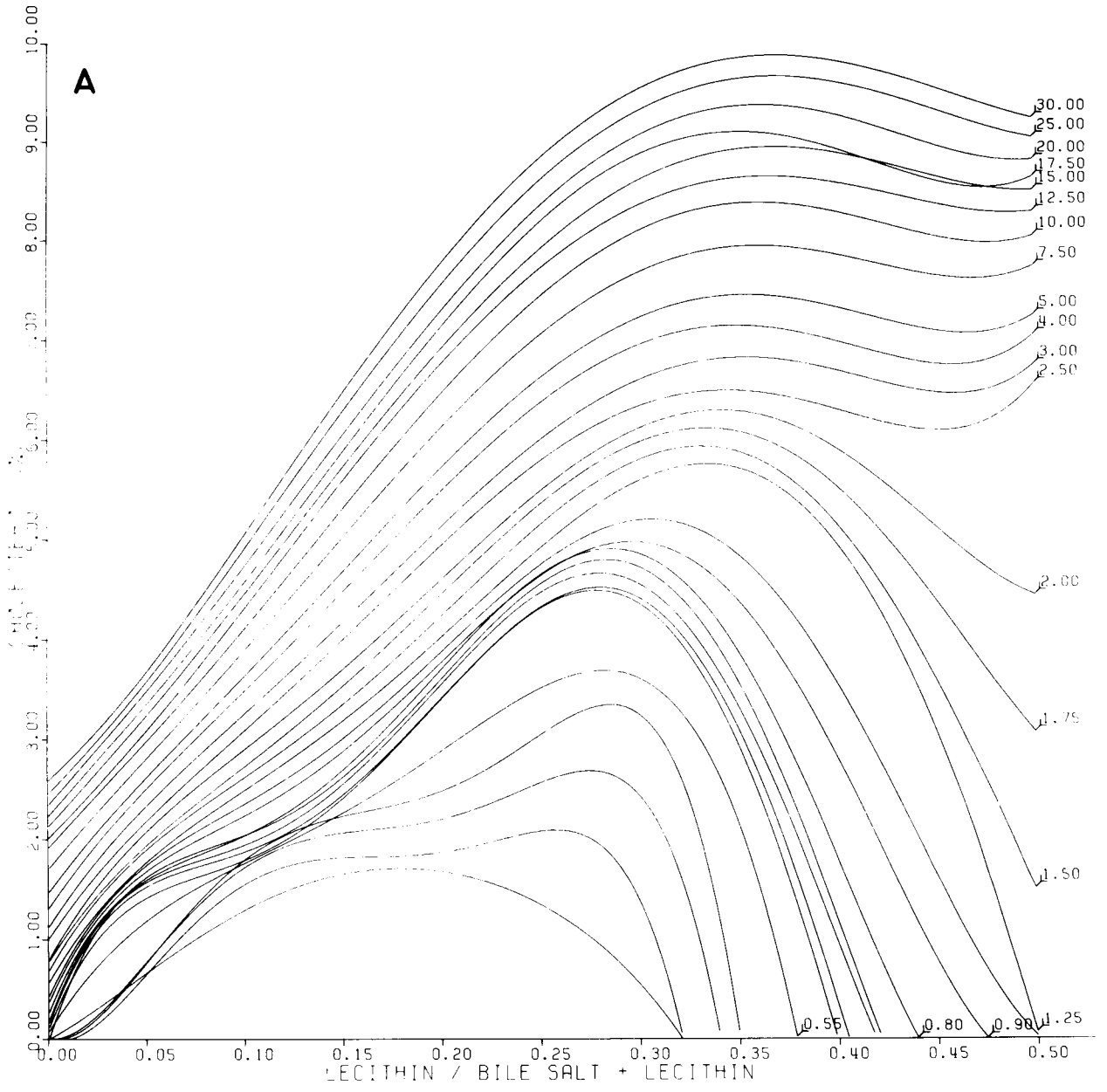
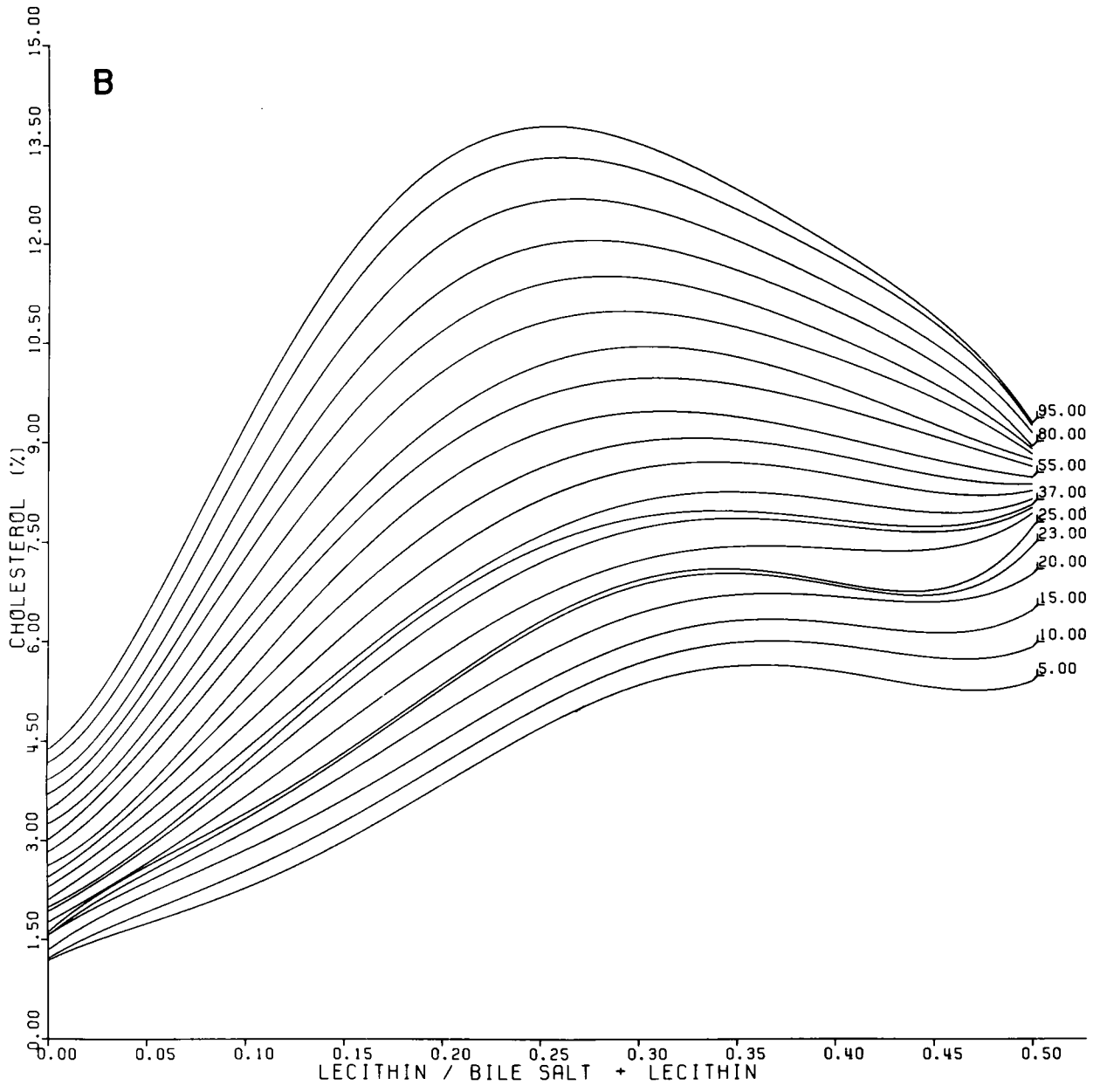


FIGURE 14 (A and B) CALCOMP 960 computer plots of all regressions (except 0.30 g/dl) listed in Tables II and III. The ordinate is percentage of cholesterol (y), i.e., 100 × (cholesterol)/(bile salt) + (lecithin) + (cholesterol) and the abscissa is the lecithin mole fraction (x), i.e., (lecithin)/(bile salt) + (lecithin) as a function of total lipid concentration (A) and as a function of temperature (B). For the purpose of clarity only certain curves in each figure are labeled with the appropriate concentration or temperatures (other physical-chemical conditions are the same as described in Tables II and III).

method with percent cholesterol saturation in cholesterol gallstone patients > normal weight controls > pigment stone patients.

DISCUSSION

These systematic phase equilibria studies were undertaken to fully define the solubility of cholesterol in bile as a function of physical chemical conditions of importance physiologically. In particular the effects of total lipid concentration on cholesterol solubility, a key variable ignored in previous studies, was carefully evaluated. Cholesterol solubility was also studied as a function of temperature and ionic strength to gain insight into the thermodynamics of cholesterol solu-



bility, to investigate metastability in bile, and to explore how variations in ionic strength may have influenced the results of previous studies (1, 3, 4, 6–10, 12, 13). To this end we employed two independent methods to saturate the micellar solutions with cholesterol. In the multiple mixture (coprecipitation) method we approached equilibrium from the side of supersaturation and in the dissolution method we approached equilibrium from the side of unsaturation. We confirmed that equilibrium was achieved and that the final values were unrelated to whether it was approached from above or below. (Exceptions in the dissolution study are discussed below.) In addition, lipid analysis of the separated micellar phases from equilibrated multiphase mixtures provided the same values for cholesterol saturation as obtained by observation of the entire series of tubes. Calculated standard thermodynamic functions reveal that the free energies of cholesterol solubilization are small negative values (i.e., a spontaneous process). This is a result of a large excess entropy term which drives the solubilization process which is counteracted by a large positive enthalpy term. Solubilization is most favorable thermodynamically in the physiological range of bile salt:lecithin ratios.

Species of lipids employed and influence on phase

TABLE II

Fifth-Degree Polynomial Coefficients of x for Equilibrium Cholesterol Solubility as a Function of Total Lipid Concentration (37°C, 0.15 M NaCl)

Concentration		x	x^2	x^3	x^4	x^5
*g/dl**						
0.30	−0.008	13.45	−86.60	990.16	−5,469.71	8,666.69
0.35	−0.002	10.74	93.73	−969.14	2,911.62	−3,510,86
0.40	0.011	−7.67	698.11	−7,009.99	27,021.55	−36,531.01
0.45	0.006	−2.25	605.22	−6,152.51	23,514.46	−31,164.78
0.50	0.008	−4.83	672.23	−6,627.09	25,029.12	−32,769.21
0.55	0.066	31.87	−233.96	931.66	−606.97	−2,143.66
0.60	0.094	50.55	−679.99	4,542.47	−12,025.61	10,307.66
0.65	0.141	53.56	−724.38	4,764.68	−12,522.13	10,769.21
0.70	0.158	54.76	−746.32	4,991.66	−13,415.43	11,939.87
0.75	−0.027	62.14	−810.20	5,188.01	−13,540.30	11,774.33
0.80	0.248	49.91	−648.23	4,333.60	−11,589.00	10,216.55
0.90	0.357	44.73	−505.03	3,206.66	−8,273.45	7,023.95
1.00	0.416	42.64	−441.77	2,707.78	−6,777.98	5,564.11
1.25	0.557	34.56	−272.92	1,513.09	−3,352.69	2,266.67
1.50	0.675	35.21	−292.25	1,715.85	−4,069.94	3,076.93
1.75	0.768	31.93	−234.92	1,438.50	−3,584.50	2,856.41
2.00	0.785	35.54	−264.10	1,543.36	−3,794.87	3,076.92
2.50	1.000	29.84	−175.46	1,138.14	−3,147.43	2,846.15
3.00	1.117	30.68	−177.39	1,111.18	−2,986.25	2,635.90
4.00	1.305	28.90	−141.83	948.25	−2,699.30	2,461.54
5.00	1.464	26.32	−99.24	721.82	−2,177.74	2,025.64
7.50	1.703	23.19	−43.49	439.10	−1,570.28	1,553.84
10.00	1.911	21.28	−12.91	308.51	−1,324.94	1,374.36
12.50	2.001	19.51	41.74	−27.04	−537.99	738.46
15.00	2.121	21.34	14.14	122.81	−845.57	948.72
17.50	2.223	22.39	−12.36	362.51	−1,583.23	1,661.54
20.00	2.334	22.31	3.80	217.99	−1,134.15	1,215.38
25.00	2.450	19.76	61.22	−122.34	−325.17	543.49
30.00	2.580	17.79	95.64	−294.84	29.72	282.05

* Concentration of total lipids in bile.

equilibria. It has been demonstrated previously that the molecular function of bile salts and lecithin in relationship to the micellar solubilization of cholesterol are distinctly different (1, 2–5, 11, 14). By themselves, different bile salts exhibit different though inefficient capacities to solubilize cholesterol (1, 4, 7, 11) a result confirmed in this work (i.e., NaTDC ≈ physiological mixture > NaTCDC > NaTC). Nevertheless, we found in this investigation as had been suggested previously (3), that with the addition of small amounts of lecithin these differences were no longer apparent. We show that a mixture of taurine-conjugated bile salts gave results identical to any one taurine-conjugated bile salt, provided the mole fraction of lecithin to bile salt was at least 0.2 and not greater than 0.5 (i.e., spanning the physiological range of bile salt-lecithin ratios) and provided the total lipid concentration was kept constant. Most of our studies were therefore carried out with NaTC in the place of a bile salt mixture. This approach seemed further validated by earlier phase equilibria studies. McBain et al. (50) had shown that a binary soap-water phase diagram of a commercial soap (a mixture of several soaps) was almost identical to those of pure soaps and Small et al. (34) demonstrated that a mixture of conjugated bile salts acted as a single component giving a ternary bile salt-lecithin-water phase diagram very similar to the one in which sodium cholate was employed. No significant influence of the lecithin species on cholesterol solubility in mixed bile salt micelles has been detected in other studies provided the lecithins contain long chain mixtures of saturated and unsaturated fatty acids (5). Thus, in the present study we employed egg yolk lecithin which contains a fatty acid chain distribution similar to human biliary lecithin (8). Even though egg lecithin (or biliary lecithin) is in reality a mixture of phosphatidylcholines, it nevertheless acts as a single component in excess water (34). In accordance with the

TABLE III

Fifth-Degree Polynomial Coefficients of x for Equilibrium Cholesterol Solubility as a Function of Temperature (10 g/dl, 0.15 M NaCl)

Temperature			x	x^2	x^3	x^4	x^5
°C	10^3K^{-1}*						
5	3.59	1.19	14.36	−102.00	925.13	−2,686.83	2,394.87
10	3.53	1.22	18.02	−118.43	951.10	−2,681.81	2,365.23
15	3.47	1.34	22.19	−154.08	1,110.27	−3,035.31	2,671.79
20	3.41	1.58	18.88	−91.74	799.38	−2,421.09	2,251.28
23	3.37	1.57	23.83	−147.70	1,174.42	−3,477.27	3,246.15
25	3.35	1.62	26.31	−187.78	1,409.33	−4,054.19	3,748.72
30	3.30	1.77	16.91	8.54	231.34	−1,245.92	1,425.64
35	3.24	1.93	16.54	52.37	−26.93	−692.77	1,005.13
37	3.22	2.00	14.49	109.68	−358.49	23.78	471.79
40	3.19	2.11	19.41	39.83	−9.03	−674.00	948.71
45	3.14	2.30	18.25	93.51	−317.33	−10.02	441.02
50	3.09	2.45	17.91	148.72	−679.40	825.76	−220.52
55	3.05	2.63	13.96	250.59	−1,243.96	2,021.45	−1,107.69
60	3.00	2.83	18.07	236.72	−1,249.32	2,115.62	−1,230.77
65	2.96	3.01	20.85	213.78	−1,109.08	1,736.72	−879.44
70	2.91	3.25	15.23	359.11	−1,961.34	3,661.19	−2,415.39
75	2.87	3.46	18.07	372.08	−2,102.18	3,996.97	−2,676.92
80	2.83	3.71	14.89	465.61	−2,650.58	5,228.91	−3,651.28
85	2.79	3.92	14.03	533.07	−3,047.61	6,054.67	−4,241.03
90	2.75	4.18	16.06	570.57	−3,356.31	6,816.20	−4,866.67
95	2.72	4.38	20.54	551.13	−3,320.78	6,756.41	−4,810.26

* Reciprocal degrees Kelvin × 10^3.

principles of the Gibbs phase rule we designed our experiments so that the total lipid concentration (and other variables) were kept constant while the influence of a dependent variable was being systematically examined. We have demonstrated that the solubility of cholesterol, except for mixtures with the highest lecithin content, increases progressively with increases in both the total lipid concentration and temperature. Further the solubility of cholesterol was invariant with added NaCl at intermediate (physiological) bile salt: lecithin ratios but increases at high bile salt:lecithin ratios and decreases at low bile salt:lecithin ratios. As the systems under study are four component systems (three lipids + one solvent) their relative compositions should be graphically represented as points within a regular tetrahedron. However we have appreciably simplified the system by fixing the aqueous solvent concentration and then plotting the molar percentages of bile salt, lecithin, and cholesterol on triangular coordinates (3). Apart from conformity to the phase rule and the increased comprehension of the molecular interactions of the lipids the practical advantages of this method are that (*a*) the effects of bile salts and lecithin are not strictly additive and can be judged separately, (*b*) the composition of the phases in equilibrium with the saturated micellar phase can be plotted, and (*c*) the cholesterol solubility with variations in total lipid concentration (and temperature) can be interpolated graphically for any native bile sample once the bile salt:lecithin ratio and total lipid concentration are known.

Influence of equilibration methods on phase equilibria. A system is defined as being in physical and chemical equilibrium when no further change in the composition or properties of the phases (i.e., phase boundaries) can be detected with time, provided other variables (i.e., temperature and pressure) are not altered. At equilibrium, the free energy is at a minimum as the system is unable to do further work (i.e., the Gibbs free energy per mole is identical throughout the system and the sum of the products of the chemical potentials of the reactant species and their mole fractions is constant). The condition of true equilibrium (in contrast to apparent or pseudoequilibrium) was tested by employing certain classical criteria, (*a*) the equilibrium condition was sensitive to changes in external conditions, thus when the temperature was increased more cholesterol was dissolved and the reverse occurred on cooling, (*b*) the concentrations and physical states were independent of time once equilibrium was reached, and (*c*) the same equilibrium concentrations and physical states were obtained when

TABLE IV

Absolute, Total, and Relative Biliary Lipid Composition Data and Calculated "Percent Cholesterol Saturation" Values of Gallbladder Biles from Gallstone Patients and Controls

Patients	Composition			Total lipids	Composition			Percent cholesterol saturation from	
	Bile salt	Lecithin	Cholesterol		Bile salt	Lecithin	Cholesterol	Triangular graphs	Polynomial equations
	mg/dl			*g/dl*	*% total mol*			*%*	
Gallbladder biles: Cholesterol gallstone patients*									
A. L.	5,450.1	1,863.0	510.0	7.8	74.9	16.3	8.9	159	158
E. S.	7,684.2	4,250.0	905.0	12.8	66.7	23.4	10.0	130	128
M. P.	11,587.6	4,313.0	1,115.0	17.0	73.6	17.4	9.0	130	131
M. W.	4,369.9	2,275.0	680.0	7.3	65.5	21.6	12.9	184	184
K. M.	4,050.8	2,037.5	330.0	6.4	70.3	22.4	7.2	111	105
M. M.	8,960.8	3,825.0	790.0	13.6	72.3	19.6	8.1	114	116
S. K.	6,014.8	2,600.0	475.0	9.1	72.8	19.9	7.3	112	108
C. A.	10,311.0	4,975.0	925.0	16.2	70.4	21.5	8.0	105	105
D. H.	7,144.1	2,512.5	526.7	10.2	76.0	16.9	7.1	118	118
M. F.	3,314.3	1,900.0	368.4	5.6	66.5	24.1	9.4	136	138
J. C.	12,029.5	4,725.0	1,266.7	18.0	72.3	18.0	9.7	137	137
T. T.	5,990.2	4,262.5	996.7	11.3	60.2	27.1	12.7	151	151
R. H.	2,749.6	2,025.0	248.3	5.0	63.2	29.5	7.3	101	100
W. F.	5,017.8	2,468.4	502.0	8.0	70.6	20.9	8.5	129	128
B. R.	10,229.6	4,865.4	1,464.9	16.6	68.6	19.6	11.8	159	155
C. N.	6,840.0	2,858.4	679.5	10.4	72.3	18.7	8.9	139	136
Mean				10.9	69.8	21.1	9.2	132	131
Gallbladder biles: Cholesterol gallstone patients with morbid obesity*									
L. T.	9,882.4	6,878.5	2,139.0	18.9	59.6	24.9	15.5	176	173
J. R.	3,405.9	3,165.4	733.3	7.3	55.0	30.7	14.3	186	181
T. V.	3,423.9	2,473.6	828.3	6.7	58.0	25.1	16.9	220	220
R. S.	1,836.2	1,149.8	338.7	3.3	62.6	23.5	13.9	214	221
Mean				9.0	58.8	26.1	15.2	199	199

* Cholesterol monohydrate crystals were observed microscopically in the fresh gallbladder biles from all patients except D. H. and R. H.

‡ Amorphous bilirubinate granules were observed microscopically in all biles from these patients. No cholesterol crystals were seen.

§ No cholesterol monohydrate crystals or amorphous bilirubinate granules were observed in the fresh biles of any control subjects.

equilibrium was approached from two directions, i.e., from unsaturation and supersaturation, respectively (in the dissolution studies certain mixtures with high lecithin contents never reached equilibrium over the limited period of observation). A distinction must be drawn between real or true equilibrium and the much more common state of pseudoequilibrium such as occurred in most previous studies on these systems (see later discussion). Pseudoequilibrium is a state of quiescence in a system in which all action has apparently ceased. In such cases the system may be regarded formally at least as undergoing change, i.e., moving towards a state of true or real equilibrium, but with such slowness that no change is observed over the finite period of observation. This state, nevertheless, does not satisfy the criteria of true equilibrium outlined above. Unfortunately, in all previous physical-chemical studies on model bile systems (1–13) the state of true equilibrium was never verified by an independent method. Some characteristic reasons for

TABLE IV (*Continued*)

Patients	Composition			Total lipids	Composition			Percent cholesterol saturation from	
	Bile salt	Lecithin	Cholesterol		Bile salt	Lecithin	Cholesterol	Triangular graphs	Polynomial equations
	mg/dl			*g/dl*	*% total mol*			*%*	
Gallbladder biles: Pigment gallstone patients‡									
R. G.	8,396.1	3,600.0	804.0	12.8	71.7	19.5	8.7	124	124
D. M.	1,453.4	1,238.0	88.4	2.8	61.8	33.4	4.7	71	73
E. G.	9,108.1	3,125.0	685.0	9.5	76.2	16.6	7.3	126	122
G. H.	11,906.8	3,975.0	653.4	16.5	78.1	16.5	5.4	84	83
E. D.	9,451.8	2,875.0	513.3	12.8	79.3	15.3	5.5	95	94
M. D.	2,455.0	1,403.2	284.2	4.1	66.3	24.0	9.7	147	148
J. R.	11,661.3	4,387.5	1,003.3	17.1	74.2	17.7	8.1	117	117
G. W.	5,892.0	1,775.0	175.4	7.8	81.4	15.5	3.1	59	59
J. M.	11,833.0	6,862.5	1,333.3	20.0	66.2	24.3	9.5	112	110
P. K.	3,044.2	550.0	86.7	3.7	86.9	9.9	3.2	89	91
D. O'C.	12,078.6	4,150.0	533.3	16.8	78.5	17.1	4.4	67	67
J. H.	2,946.0	1,700.0	158.3	4.8	69.7	25.5	4.8	71	70
R. B.	2,833.1	1,313.0	200.5	4.4	72.4	21.2	6.4	103	107
F. K.	5,451.2	3,022.5	526.5	9.0	69.0	23.0	8.0	110	108
Mean				10.2	73.7	20.0	6.3	98	98
Gallbladder biles: Control subjects without stones§									
H. DeP.	8,003.3	1,525.0	243.8	9.8	86.3	10.4	3.3	79	77
A. D.	8,101.5	2,613.0	590.0	11.3	77.1	15.8	7.1	120	117
J. O.	8,518.9	4,025.0	198.8	12.7	75.2	22.5	2.3	30	31
J. B.	15,466.5	8,100.0	1,370.0	24.9	69.2	23.0	7.8	93	91
F. M.	6,824.9	4,437.0	933.3	12.2	63.0	26.0	10.9	131	132
M. A.	13,207.9	5,887.5	1,080.0	20.2	72.1	20.4	7.5	97	97
F. L.	11,622.0	5,112.5	561.7	17.3	74.6	20.8	4.6	61	61
R. M.	8,396.1	3,400.0	536.7	12.3	74.6	19.3	6.1	90	89
F.B.	7,266.8	4,862.5	793.3	12.9	64.0	27.1	8.9	105	107
M. L.	6,235.7	2,100.0	381.7	8.7	77.5	16.5	6.0	105	107
J. N.	12,373.2	3,712.5	978.3	17.1	77.5	14.7	7.8	130	128
G. C.	11,734.9	6,300.0	1,071.7	19.1	68.7	23.4	8.0	97	95
Mean				14.9	73.3	20.0	6.7	95	94
Gallbladder biles: Control morbid obese subjects without stones									
Bennion and Grundy's 18 patients (49)									
Mean				?	64.9	24.4	10.8	139	139

pseudo- or nonequilibrium in these systems are worth emphasizing. Mufson et al. (13) showed that bile salt-lecithin solutions can become isothermally supersaturated with cholesterol when made by the multiple mixture method and demonstrated that equilibration took as long as 12 days. In the present multiple mixture studies this phenomenon also occurred but most of our systems took no more than the 3–5 days to reach equilibrium at room temperature and less time at higher temperatures. The type of organic solvent employed during coprecipitation of the multiple mixtures may be important. It has been suggested (51) that metastable supersaturation of lecithin-cholesterol liposomes can occur if the lipid mixture is prepared from chloroform but not from ethanol. It is possible that the prolonged supersaturation observed by Mufson et al. (13) and others may have been a result of the use of chloroform or the incomplete removal of other organic

TABLE V

Absolute, Total, and Relative Biliary Lipid Composition Data and Calculated "Percent Cholesterol Saturation" Values of Common Hepatic Duct Biles from Gallstone Patients and Controls

Patients	Composition			Total lipids	Composition			Percent cholesterol saturation from	
	Bile salt	Lecithin	Cholesterol		Bile salt	Lecithin	Cholesterol	Triangular graphs	Polynomial equations
	mg/dl			*g/dl*	*% total mol*			%	
Common hepatic duct biles: Cholesterol gallstone patients*									
A. L.	1,914.9	878.0	255.0	3.1	68.6	19.9	11.6	211	209
E. S.	751.2	513.0	235.5	1.5	54.6	23.6	21.7	402	375
M. P.	4,075.3	1,443.8	355.0	5.9	74.9	16.8	8.3	151	173
M. W.	1,806.9	1,168.0	397.5	3.4	59.2	24.3	16.5	243	254
K. M.	1,129.3	554.2	166.3	1.9	66.8	20.8	12.5	240	238
M. M.	1,291.3	914.6	220.4	2.4	60.1	26.9	13.0	197	204
S. K.	5,234.1	2,257.0	391.3	7.9	73.1	20.0	6.9	106	108
C. A.	883.8	647.5	242.1	1.8	55.2	25.6	19.2	310	317
D. H.	2,617.0	1,122.9	214.6	4.0	72.7	19.8	7.6	135	133
M. F.	1,571.2	1,172.0	408.3	3.2	55.5	26.2	18.3	265	272
J. C.	270.1	170.0	108.2	0.6	52.3	20.9	26.8	705	601
T. T.	1,266.8	1,112.5	378.5	2.8	51.7	28.7	19.6	319	288
Mean				3.2	62.1	22.8	15.2	273	264
Common hepatic duct biles: Pigment gallstone patients‡									
R. G.	438.0	259.5	99.9	0.8	60.1	22.5	17.4	363	222
D. M.	346.2	313.8	60.7	0.7	55.6	32.0	12.4	496	464
E. G.	1,448.5	615.7	146.7	2.2	71.6	19.3	9.2	192	191
G. H.	589.2	362.5	137.9	1.1	59.3	23.1	17.6	344	347
E. D.	5,622.0	1,981.0	367.5	7.8	76.3	17.2	6.4	110	111
M. D.	1,694.0	1,293.8	350.0	3.3	57.3	27.7	15.0	217	223
J. R.	849.4	797.5	113.3	1.8	56.7	33.7	9.6	166	163
G. W.	653.0	246.3	21.3	0.9	78.1	18.7	3.2	90	88
J. M.	967.3	662.5	169.2	1.8	60.4	26.2	13.4	216	225
P. R.	1,964.0	687.5	73.3	2.7	78.8	17.5	3.7	82	81
Mean				2.3	65.4	23.8	10.8	228	211
Common hepatic duct biles: Control subjects, without stones§									
H. DeP.	144.9	42.5	14.4	0.2	76.2	14.2	9.6	640	736
A. D.	392.8	171.9	53.8	0.6	68.9	19.1	12.0	375	320
J. O.	805.2	457.5	104.2	1.4	65.6	23.6	10.8	208	202
F. M.	1,099.8	912.5	290.0	2.3	53.8	28.3	18.0	281	278
F. L.	1,463.2	657.5	75.0	2.2	74.1	21.1	4.8	92	96
R. M.	2,381.4	1,431.3	352.5	4.2	63.8	24.3	12.0	174	179
F. B.	2,258.6	1,503.2	282.9	4.1	63.3	26.7	10.1	140	146
M. L.	2,538.7	950.0	200.0	3.7	74.7	17.7	7.5	143	141
Mean				2.6	66.3	23.0	10.7	257	262

* Cholesterol monohydrate crystals were observed microscopically in the fresh hepatic biles from all patients except M. M., C. A., D. H., J. C., and T. T.

‡ No bilirubinate granules or cholesterol crystals were observed microscopically in any bile sample.

§ No crystals or bilirubinate granules were observed in the bile of any subject.

solvents although the exact conditions were not specified in these reports.

Another important source of error that deserves comment is that individual lipid components in the multiple mixtures may precipitate at different rates during evaporation of the organic solvent. The possibility of this occurring depends upon the differential solubilities of the individual lipids in the organic solvent and on the rates of drying. In a series of preliminary experiments[5] we found that cholesterol may precipitate before the bile salt and lecithin particularly if the rate of initial drying was slow (>1 h). Thus when aqueous solvent was added, the resulting mixture was in fact a system of cholesterol microcrystals undergoing dissolution in bile salt-lecithin micelles. The slow dissolution rates for cholesterol in such a system (see Fig. 11) would obviously delay equilibration for many days or weeks. An important and self-evident point about the multiple mixture method is that one must make enough mixtures with small increments in the amount of cholesterol between mixtures to be sure that a valid equilibrium value has been obtained (see Methods). Our values have an experimental uncertainty of at most ±0.16–0.32 mol % of cholesterol which is well within the experimental error. Finally, the demonstration that the lipid composition of the micellar phase when separated from the solid phases was identical to the equilibrium values obtained from observation of the whole series of mixtures indicates that the micellar phase was indeed in true equilibrium with no net transfer of cholesterol and lecithin from one phase to another. Even though filtration (or ultra-

[5] Carey, M. C. Unpublished observations.

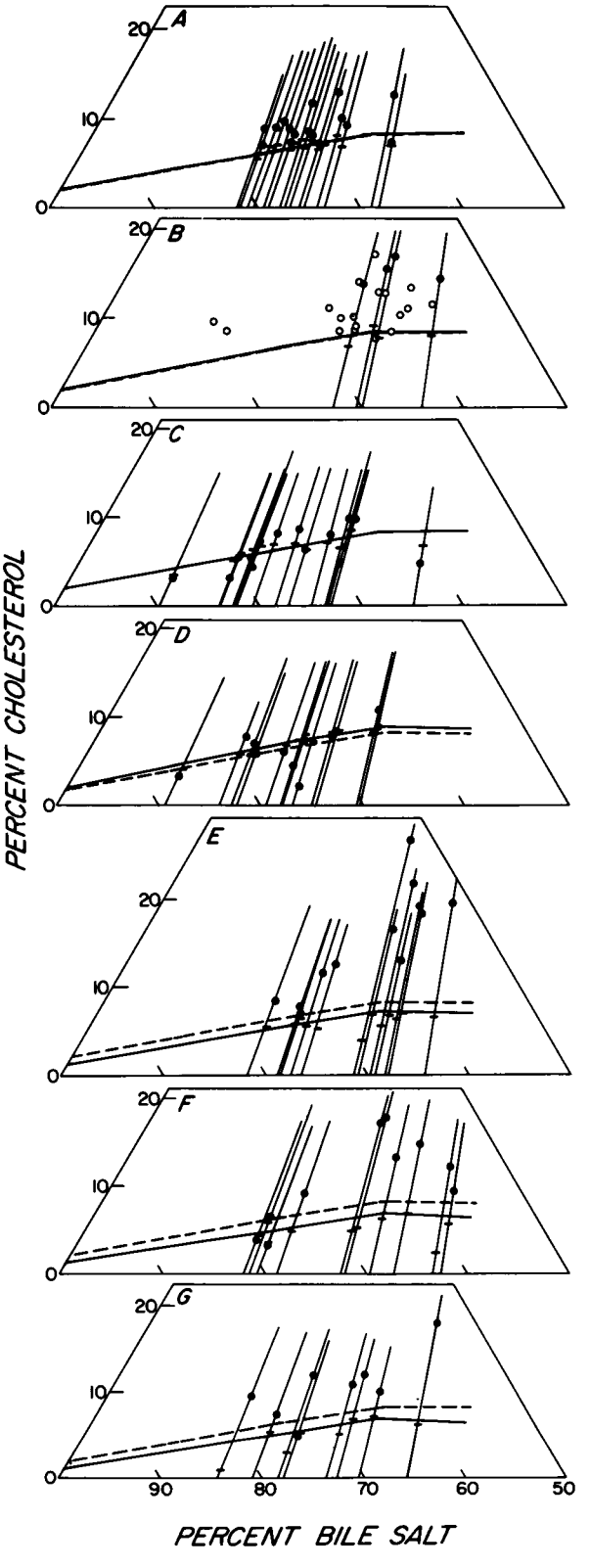


FIGURE 15 Relative lipid compositions of fasting gallbladder (A–D) and common hepatic duct (E–G) biles from patients with cholesterol gallstones, pigment gallstones, and controls. The lines extending to the base of the triangle are the bile salt-lecithin constant composition lines. Horizontal short lines represent the maximum cholesterol solubility for the appropriate total lipid concentration and bile salt: lecithin ratio of the sample. The interrupted phase boundary is drawn for 10-g/dl total lipids and the continuous phase boundary is drawn for the average total lipid concentration in each set of samples. (A) Cholesterol gallstone patients of normal weight:mean total lipid concentration, 10.9 g/dl. (B) Morbid obese patients with (●) and without (○) stones: mean total lipid concentration, 9 g/dl (stone patients). (C) Pigment stone patients of normal weight:mean total lipid concentration, 10.2 g/dl. (D) Normal weight controls (no stones):mean total lipid concentration, 14.9 g/dl. (E) Cholesterol gallstone patients of normal weight:mean total lipid concentration, 3.2 g/dl (hepatic bile). (F) Pigment stone patients of normal weight:mean total lipid concentration, 2.3 g/dl (hepatic bile). (G) Normal weight controls:mean total lipid concentration, 2.6 g/dl (hepatic bile).

centrifugation) and analysis of separated micellar phases has been popular in previous studies most investigators (2, 5–7, 11, 13) assayed for only the cholesterol content whereas in fact the bile salt: lecithin ratio is also altered (see Fig. 5).

In the cholesterol dissoluton studies we attempted to maximize the rates of dissolution by employing a very large surface area of anhydrous cholesterol and rapid flow rates. Equilibration of the systems was judged complete when no further increase in cholesterol solubility occurred over the course of 8–10 days (Fig. 11). The results which satisfied these criteria were indistinguishable from experimental or interpolated data by the multiple mixture method. However, the rates of dissolution were progressively retarded in proportion to the molar ratio of lecithin to bile salt, a phenomenon previously recognized (29, 52) so that systems containing appreciable lecithin contents failed to reach equilibrium as demonstrated by the lack of agreement with the results of the multiple mixture study (Fig. 12) and by the continued increase in micellar cholesterol content up to the end of the experiments (Fig. 11C). It has been suggested that micellar diffusion to and from the crystal interface and an interfacial barrier are the major rate limiting steps in cholesterol dissolution (52). The former should be the most important in our "unstirred" experiments whereas the interfacial resistance is rate limiting in our "stirred" experiments. As shown in Fig. 12D, saturation of the micellar phase did not occur in 30 days under "unstirred" conditions. The fact that the final cholesterol content is maximal at a bile salt:lecithin molar ratio of 7:3 strongly suggests that the linear increases in the amount of cholesterol solubilized with increases in the lecithin content is related to the greater capacities of mixed micelles of intermediate bile salt:lecithin ratios to incorporate cholesterol.

The flow rates in the stirred dissolution experiments were designed to be faster than the estimated diffusion controlled limit (52) so that the overall dissolution rates should only be controlled by the interfacial resistance. Two observations support this possibility. First, the presence of lecithin in the micelles retarded the rates of dissolution (Fig. 10) in proportion to its content. Secondly, dissolution rates were dramatically accelerated with added NaCl concentration for all lecithin contents. The most likely explanation for these findings is that lecithin and bile salt molecules may actually create an inhibitory interfacial monolayer on the crystal surface either by physical adsorption or phase partitioning. Added NaCl by inducing electrostatic shielding may accelerate dissolution by facilitating the approach of highly charged micelles to the negatively charged crystal-solution interface.

Labile and metastable zones and physiological significance of the metastable-labile limit. With increases in temperature, cholesterol solubility in bile salt-lecithin micellar solutions increases dramatically giving micellar cholesterol:lecithin molar ratios which vary from 5:1 at 4°C to 1:1 at 95°C. It is of interest that an equimolar ratio is the maximum equilibrium solubility of cholesterol in the lamellar liquid crystalline phase of egg lecithin in water (53) and is independent of temperature. Upon cooling, the micellar systems become supersaturated and segregate into labile and metastable regions in terms of cholesterol content. This behavior can be rationalized by considering that the driving force of precipitation is the degree of supersaturation and the force opposing it is the energy required for nucleation. If E_1 is the energy level of the supersaturated solution and E_2 the energy level in the precipitate,

$$\therefore E_1 - \Delta E = E_2. \tag{4}$$

For this process to occur spontaneously E_2 must be at a lower energy level than E_1 by the amount of energy ΔE. In practice it is found that this reaction may not occur spontaneously for, as is well known, carefully purified supersaturated solutions can be stored for years without precipitation (39). Nucleation requires that an energy of activation E_3 be gained and the closer E_1 is to E_3, the greater the likelihood of spontaneous precipitation. As a result of spontaneous energy fluctuations in the system, certain nuclei temporarily gain sufficient energy to overcome the activation barrier (supercritical nuclei), with the result that $E_1 \geq E_3$ and spontaneous precipitation occurs.

In the absence of chemical, colloidal, or mechanical impurities, spontaneous precipitation from solutions lying in the metastable region cannot occur as the energy of spontaneous fluctuations can never exceed E_3. In practice, however, it is found that precipitation does occur, as demonstrated in the case of precipitation from the metastable region in the present study. When nucleation in metastable solutions takes place, it is because of "impurities" on whose surfaces favorable conditions exist for an ordered assembly of particles. Thereby the energy of formation of supercritical nuclei is much reduced; i.e., E_3 approaches E_1 and precipitation occurs. This phenomenon is often called heterogeneous nucleation (as opposed to homogeneous nucleation in labile systems) and is probably the dominant form of nucleation in native bile. For the sake of completeness it should be noted that heterogeneous nucleation is a variant of epitaxial nucleation but consideration of this special form of nucleation will not be discussed further.

At the metastable-labile limit the micellar lecithin-cholesterol molar ratio is about 3:2. The significance of this boundary can be understood by considering the areas occupied by lecithin and cholesterol molecules in a lamellar liquid-crystal lattice and the area of

water required for hydration of their polar head groups (54). The minimum cross-section per hydrocarbon chain of lecithin is about 20 Å² and of cholesterol is about 37 Å². The unit area of a 2:1 (molar ratio) structure is therefore $(2 \times 20 + 0.5 \times 37) = 59$ Å². Liquid water approximates hexagonal symmetry with a unit area of 19 Å². Without significant perturbation, three contiguous lattices (3×19 Å²) would be required to fit a 2:1 lecithin-cholesterol head group unit area. If the mole fraction of cholesterol increases further a break in the structure of the polar region would occur (54). Thus at higher temperatures when the cholesterol-lecithin mole fraction in micelles exceeds 0.5 the water lattice is perturbed. This is consistent with our observation that rapid precipitation occurs from these mixtures. Below the metastable-labile limit, the mole fraction of cholesterol to lecithin in micelles is less than 0.5 and the water lattice is unperturbed. Thus the tendency for precipitation is reduced, a fact consistent with the metastability of these mixtures.

Novel variations in cholesterol solubility in systems with high and low lecithin mole fractions. The efficiency of cholesterol solubility by conjugated bile salts can be roughly correlated with the average aggregation number of the pure micelles just above the CMC. The bigger the average micelle, the greater its cholesterol solubilizing efficiency (18). These differences are minimized in the presence of increasing NaCl concentrations, suggesting that even the small micelles including those of NaTC can grow to an optimum size under these conditions. In the presence of small progressive additions of lecithin (provided the total lipid concentration is kept constant) these differences are reduced and eventually eliminated. These results are consistent with the possibility that at high bile salt:lecithin ratios the systems are specifically polydisperse, i.e., simple bile salt micelles co-exist with some mixed bile salt-lecithin micelles. With added NaCl either the simple micelles grow sufficiently in size or a rearrangement of micelles to form a greater number of mixed micelles occurs. Not only are pure bile salt micelles inefficient solubilizers of cholesterol but so are micelles with the highest lecithin contents. Furthermore these micelles are only capable of solubilizing cholesterol at concentrations much above their apparent CMCs (Fig. 7).

The micellar solutions with the highest lecithin contents exhibited reversible "cloud points" characteristic of nonionic detergent systems. Because lecithin is a zwitterion (i.e., effectively nonionic at pH 7.0), its properties predominate when the micellar lecithin content is high. As shown by the effects of added NaCl, the progressive shielding of the anionic charges of the bile salt by counterion allows the mixed micelles to increase their nonionic properties as indicated by the lowering of the "cloud points" analogous to typical nonionic detergents or nonionic-anionic detergent mixtures (55). It is of considerable interest that the Bragg *'d'* spacings in the liquid-crystalline phases of the bile salt-lecithin-cholesterol-water system (14, 16) decrease with additions of cholesterol, suggesting progressive dehydration of the lipid head groups, a finding consistent with the lowering of the "cloud points" by added cholesterol in the micellar phase. The pronounced viscosities and Tyndall effects observed when a mole fraction of lecithin reached 0.5 particularly in the absence of cholesterol, also suggests that the micelles grow enormously in size as the phase limit is approached. Mazer et al. (56), with recently developed techniques of laser light scattering, have demonstrated that these micelles grow in proportion to the lecithin content and exceed a mean hydrodynamic radius of ≈100 Å. Finally, in this region of the micellar phase we also found that the maximum solubility of lecithin (no cholesterol) varied with the bile salt species in the order NaTC > physiological mixture > NaTDC > NaTCDC suggesting subtle differences in the composition of these micelles (56).

Comparison with literature values for cholesterol solubility in bile salt-lecithin model systems. To analyze and compare past studies with the present work we have replotted on triangular coordinates in Fig. 16 (A–J), most of the results on cholesterol solubility in bile-salt lecithin model systems from the literature (shown as solid symbols). We have plotted the equilibrium cholesterol solubilities from the present work (Fig. 13) appropriate to the conditions of temperature, ionic strength, and total lipid concentration employed in each study (shown as open triangles). In some cases it was necessary to plot two equilibrium lines (Fig. 16C, D, E, H) to encompass inadvertent variations in total lipid concentration by previous authors and the metastable-labile limit (Fig. 16B, F), to explain the pseudoequilibrium results obtained by certain investigators. Isaksson (1) carried out a multiple mixture (coprecipitation) study with sequential additions of ethanolic cholesterol to mixed micellar solutions. As shown in Fig. 16A his data for NaTC and NaTDC are in excellent agreement with the present work at low lecithin contents but with increasing lecithin contents his phase limits diverge towards unsaturation. This is consistent with the fact (57) that ethanolic cholesterol when added to aqueous systems leads to microcrystalline precipitation with the result that his mixed micellar solutions with moderate lecithin contents would not have reached equilibrium in 1 h by dissolution (see Figs. 11 and 12).

The deficiencies in Admirand and Small's work (3) relate to the absence of physiologic ionic strength, the short (24 h) equilibration times and the lack of a lipid concentration effect. Their data shown as a single line in Fig. 16B corresponds to the metastable-labile

limit and diverges toward unsaturation at high lecithin contents. As shown in the present work added NaCl dramatically increases the equilibration rates by dissolution and also appears to promote equilibration of bile salt-lecithin systems supersaturated by dilution (46). Although direct proof is lacking the weight of the evidence from our analysis of previous studies (1–13) suggests that supersaturation of bile salt mixed micellar systems by coprecipitation is more prolonged in the absence of physiological concentrations of neutral electrolyte. The recalculated data of Neiderhiser and Roth (4) shown in Fig. 16C span a range from 4.7 to 25 g/dl total lipids. All are appreciably supersaturated with cholesterol when compared to the range of correct equilibrium solubility values for these concentrations (Fig. 16C). It is not obvious how these systems could become supersaturated by dissolution except because of inadvertent filtration of cholesterol microcrystals when the micellar phase was separated for analysis.

The results of Saunders and Wells (5) are of considerable interest as cholesterol saturation was measured in dilute micellar solutions (1–2.5 g/dl) and the results are identical to ours for these concentrations (Fig. 16D). The deficiencies in Hegardt and Dam's study (7) are related to the short dissolution times for intermediate lecithin contents, the intraexperimental variation in total lipid concentration (5–12 g/dl) and the fact that only micellar cholesterol was assayed (7). The cholesterol solubility is higher than for NaTC (Fig. 16E) at high bile:salt lecithin ratio, as would be expected for a bile salt mixture (see Fig. 1E). However with increasing lecithin contents, the results fall on the cholesterol solubility limits expected for 5-g/dl and not the 12-g/dl limit from the present study. Montet and Dervichian's study (8) was designed to be similar to that of Admirand and Small except for a doubling of the equilibration time and increased ionic strength to 0.05 M Na^+. Once again the metastable-labile limit is approximated suggesting that insufficient time was allowed for equilibration at the unphysiological ionic strength employed (Fig. 16F).

The data of Holzbach et al. (9, 10) and our data are similar (Fig. 16G). The expected solubility difference for mixed bile salts in the absence of lecithin are observed and approximates what we find for a similar mixture of taurine conjugated bile salts in Fig. 1E. In the

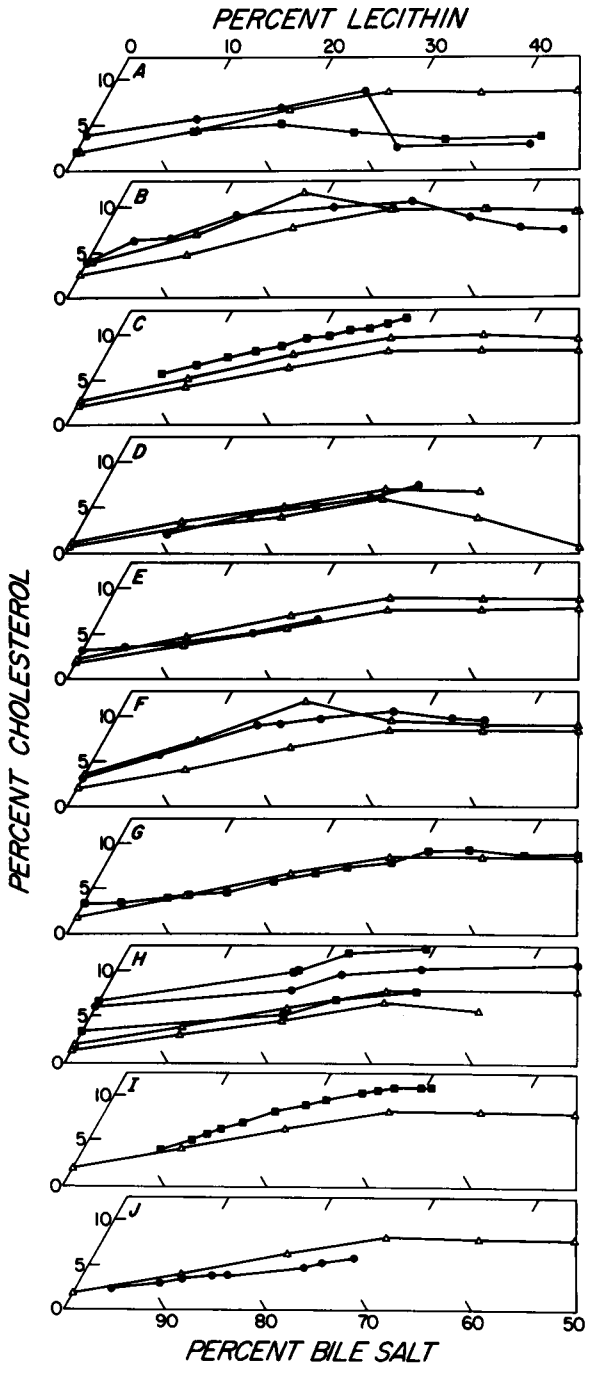


FIGURE 16 Cholesterol solubility values from other authors represented by ● —— ● or ■ —— ■ plotted on triangular coordinates together with the range of equilibrium cholesterol solubility curves for variations in total lipid concentration and the metastable-labile limit from the present work. The lines represented by △ —— △ on each plot represent the correct cholesterol solubility limits or the metastable-labile limit, appropriate to the experimental conditions employed by each investigator. Two equilibrium lines indicate an intraexperimental variation in total lipid concentrations and represents equilibrium cholesterol solubilities at the high and low end of the range (see Discussion for further details). (A) Isaksson, 1953–4(1); (B) Admirand and Small, 1968 (3); (C) Neiderhiser and Roth, 1968 (4); (D) Saunders and Wells, 1969 (5); (E) Hegardt and Dam, 1971 (7); (F) Montet and Dervichian, 1971 (8); (G) Holzbach et al., 1973–75 (9, 10); (H) Tamesue et al., 1973 (11); (I) Swell et al., 1974 (12); and (J) Mufson et al., 1974 (13).

physiologic range of lecithin contents, the phase limit of Holzbach et al. is slightly lower than ours and at higher lecithin contents, their results are slightly higher. The most likely explanations for these discrepancies is that Holzbach et al. carried out their study in water in the absence of physiological ionic strength, the original coprecipitated mixtures contained 10 g/dl, whereas the concentration of the lipids in the separated micellar phase was not documented but must be less. In actual fact their cholesterol solubilities approximate our 8-g/dl equilibrium values in 0.15 M NaCl. Lack of consideration of the effects of variations in total lipid concentration possibly explains why these authors failed to reproduce their in vitro findings with native bile. Tamesue et al. (11) plotted data (intra-experimental variation in total lipid concentration 2.5–13.5 g/dl) that approximates the equilibrium cholesterol solubility in the presence of lecithin for variations in total lipid concentration between 2.5 and 6.0 g/dl. Unaccountably their tabulated data lies in the metastable zone or actually above the metastable-labile limit (Fig. 16H).

For variations in the bile salt:lecithin ratio at a single total lipid concentration (8.5 g/dl) it can be appreciated that the data of Swell et al. (12) falls significantly above the true equilibrium lines and deviates with increasing lecithin contents (Fig. 16I). This study like that of Neiderhiser and Roth (4) provides data by the dissolution technique in which the final solubility of cholesterol is significantly higher than the true equilibrium values. These results are paradoxical when compared with the results of the present experiments employing the dissolution technique in which by 4 h it could be predicted (Fig. 10) that only the mixtures with the smallest amounts of lecithin would be saturated and all others should be appreciably unsaturated.

The cholesterol solubilities of Mufson et al. (13) (by dissolution) are all appreciably unsaturated compared to the correct equilibrium cholesterol solubility values (Fig. 16J) and this discrepancy widens in proportion to the lecithin content. These solutions were not at equilibrium due possibly to the low ionic strength of buffer employed (0.05M Na^+) and the short equilibrium times (14 days). As shown in Fig. 11B the cholesterol solubility in a 7:3 bile salt-lecithin system in water after 14 days dissolution should be 6.5 mol %. This approximates the value of Mufson et al. at 14 days (5.8 mol %) for a bile salt:lecithin molar ratio of 7.3:2.7.

Gallbladder and hepatic biles of cholesterol and pigment stone patients and controls. A large number of studies have now been published in which the relative lipid composition of bile from cholesterol gallstone patients has been compared with controls. In most of these studies the relative lipid compositions have been plotted on triangular coordinates and related to one of the published cholesterol solubility limits (usually from references 3, 7, and 9 or all three) and the degree of cholesterol saturation has been qualified as lithogenic index (58), cholesterol saturation index (12), or percent cholesterol saturation (48), all based arbitrarily on one of these (3, 7, 9) or on a derived third-degree polynomial regression (45). We have shown in this work that two of these phase limits (3, 7) are erroneous and that the third (9) is imprecise probably because the experiments were performed in the absence of physiological concentrations of ionic strength and at an uncertain total lipid concentration. Thus, because the third-degree polynomial equations of Thomas and Hofmann (45) was derived from the pooled data of Hegardt and Dam (7) and Holzbach et al. (9), it contains significant errors (maximum of 8% for a 10-g/dl total lipid concentration) when compared with the present work. Secondly, it is almost a formality to point out that as the total lipid concentration has a profound effect on the cholesterol saturation of bile it is unreasonable to assume that a single cholesterol solubility limit derived for an average lipid concentration in bile can be applied to all individual bile samples. As is apparent from Tables IV and V, the total lipid concentration of bile can vary by two orders of magnitude. We found that the range of lipid concentrations in hepatic duct bile varied between 0.2 and 7.9 g/dl (mean 2.7 g/dl) and in gallbladder bile the range varied between 2.8 and 24.9 g/dl (mean 12 g/dl), variations essentially in agreement with the extensive studies of Tera (30) and Isaksson (31). Therefore in our studies the percent cholesterol saturation (48) of each individual bile sample was obtained by plotting on triangular coordinates its relative composition against the experimental or interpolated equilibrium cholesterol saturation from the model system appropriate to the total lipid concentration of the actual sample (Fig. 15). In addition, the percent cholesterol saturation of these biles are also calculated from concentration-dependent fifth-degree polynomials (Table II) appropriate to the total lipid concentration of each sample and compared to the graph values. The agreement is remarkably good in the case of gallbladder biles and slightly less so in the case of hepatic biles (Tables IV and V). This observation is important as it indicates that the polynomial chosen need only be the computed mathematical function for a total lipid concentration closest to that of the actual sample especially in the case of gallbladder biles. With more dilute biles it is probably necessary to employ both the graph and polynomial methods to obtain the most accurate values for percent cholesterol saturation. The fifth-degree polynomials have a much smaller standard error than sample-computed third-degree polynomials for the same curves (2–3%) and therefore

we believe this increased accuracy justifies the mathematical complexities involved in their use.

One of the more interesting findings in our study is that all gallbladder and hepatic biles from cholesterol gallstone patients are supersaturated with cholesterol. Crystals of cholesterol were seen in 83% of gallbladder and 58% of hepatic biles of patients with cholesterol stones. This means that in well-mixed bile samples we measured some of these in determining the composition of bile so that the percentage of cholesterol saturation is augmented slightly (5–10%)[6] towards higher supersaturation. Even though approximately 50% of pigment stone and control subjects have supersaturated gallbladder biles, the means are unsaturated and no cholesterol crystals were observed. In addition, the fasting hepatic biles of cholesterol gallstone patients are slightly more supersaturated than the hepatic biles from pigment stone patients and controls. Furthermore, the obese individuals with or without cholesterol stones have the most supersaturated gallbladder biles, being significantly more supersaturated than normal weight gallstone patients and controls. These studies clearly demonstrate that supersaturated gallbladder bile accompanies cholesterol gallstones without exception and that in the fasting state at least, gallbladder bile is frequently supersaturated with cholesterol in healthy man, in pigment gallstone patients, and in morbidly obese individuals. Furthermore, gallbladder and hepatic biles from cholesterol gallstone patients were significantly more supersaturated with cholesterol (even when corrected by 5–10% for possible admixture with cholesterol crystals; see above) than controls, confirming the hypothesis that the absolute degree of supersaturation is the important driving force for cholesterol precipitation and gallstone growth.

These results provide a much more accurate pathophysiological basis for gallstone formation and for the high prevalence of gallstones in Western man (59, 60) than previously published and confirm the fact that cholesterol supersaturation of fasting hepatic bile is the rule in both normal individuals and in patients with gallstones (61). As the cholesterol content of a large number of fasting hepatic biles fall well outside the zone of metastability as defined in this work and yet did not precipitate their excess cholesterol as judged microscopically, one is forced to conclude that the metastable region of native bile is much larger than found with the model system. This suggests that the presence of certain other quantitatively minor components in human bile or the absence of nucleating agents maintain cholesterol in metastable supersaturated solution in most individuals. Nevertheless, the greater degree of cholesterol supersaturation in both the gallbladder and hepatic biles of cholesterol gallstone patients when compared to controls strongly suggests that this is the predominant driving force for cholesterol precipitation and gallstone growth.

[6] Carey, M. C. Unpublished observations.

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REFERENCES

1. Isaksson, B. 1953–4. On the dissolving power of lecithin and bile salts for cholesterol in human bladder bile. *Acta Soc. Med. Upsal.* **59:** 296–306.
2. Nakayama, F. 1966. Cholesterol holding capacity of bile in relationship to gallstone formation. *Clin. Chim. Acta.* **14:** 171–176.
3. Admirand, W. H., and D. M. Small. 1968. The physical-chemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* **47:** 1043–1052.
4. Neiderhiser, D. H., and H. P. Roth. 1968. Cholesterol solubilization by solutions of bile salts and bile salts plus lecithin. *Proc. Soc. Exp. Biol. Med.* **128:** 221–225.
5. Saunders, D. R., and M. A. Wells. 1969. The cholesterol solubilizing capacity of lecithin in aqueous solutions of bile salt. *Biochim. Biophys. Acta.* **176:** 828–835.
6. Hardison, W. C. 1971. Metabolism of sodium dehydrocholate by the rat liver: its effect on micellar formation in bile. *J. Lab. Clin. Med.* **77:** 811–820.
7. Hegardt, F. G., and H. Dam. 1971. The solubility of cholesterol in aqueous solution of bile salts and lecithin. *Zeit. Ernaehrungswiss.* **10:** 228–233.
8. Montet, J. C., and D. G. Dervichian. 1971. Solubilization micellaire du cholestérol par les sels biliares et les lecithines extraits de la bile humaine. *Biochimie (Paris).* **53:** 751–756.
9. Holzbach, R. T., M. Marsh, M. Olszewski, and K. Holan. 1973. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* **52:** 1467–1479.
10. Holzbach, R. T., and M. Marsh. 1975. Transient liquid crystals in human bile analogues. *Mol. Cryst. Liq. Cryst.* **28:** 217–222.
11. Tamesue, N., T. Inoue, and K. Juniper. 1973. Solubility of cholesterol in bile sale-lecithin model systems. *Am. J. Dig. Dis.* **18:** 670–678.
12. Swell, L., C. C. Bell, D. H. Gregory, and Z. R. Vlahcevic. 1974. The cholesterol saturation index of human bile. *Am. J. Dig. Dis.* **19:** 261–265.
13. Mufson, D., K. Triyanond, J. E. Zarembo, and L. J. Ravin. 1974. Cholesterol solubility in model bile systems. Implications in cholelithiasis. *J. Pharm. Sci.* **63:** 327–332.
14. Bourgès, M., D. M. Small, and D. G. Dervichian. 1967.

Biophysics of lipid association. III. The quaternary systems lecithin-bile salt-cholesterol-water. *Biochim. Biophys. Acta.* **144:** 189–201.
15. Small, D. M., M. Bourgès, and D. G. Dervichian. 1966. Ternary and quarternary aqueous systems containing bile salt, lecithin and cholesterol. *Nature (Lond.).* **211:** 816–818.
16. Small, D. M., and M. Bourgès. 1955. Lyotropic paracrystalline phases obtained with ternary and quarternary systems of amphiphilic substances in water: Studies on aqueous systems of lecithin, bile salt and cholesterol. *Mol. Cryst.* **1:** 541–561.
17. Small, D. M., S. A. Penkett, and D. Chapman. 1969. Studies on simple and mixed bile salt micelles by nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta.* **176:** 178–189.
18. Small, D. M. 1971. The physical chemistry of cholanic acids. *In* The Bile Acids. P. P. Nair and D. Kritchevsky, editors. Plenum Publishing Corp., New York. **1:** 249–356.
19. Small, D. M. 1967. Physical-chemical studies of cholesterol gallstone formation. *Gastroenterology.* **52:** 607–610.
20. Nakayama, F., and W. van der Linden. 1970. Bile from gallbladder harbouring gallstone: Can it indicate stone formation. *Acta Chir. Scand.* **136:** 605–610.
21. Danzinger, R. G., A. F. Hofmann, L. J. Schoenfield, and J. L. Thistle. 1972. Dissolution of cholesterol gallstones by chenodeoxycholic acid. *N. Engl. J. Med.* **286:** 1–8.
22. Northfield, T. C., N. F. LaRusso, A. F. Hofmann, and J. L. Thistle. 1975. Biliary lipid output during three meals and an overnight fast. II. Effect of chenodeoxycholic acid treatment in gallstone subjects. *Gut.* **16:** 12–17.
23. Iser, J. H., R. H. Dowling, H. Y. I. Mok, and G. D. Bell. 1975. Chenodeoxycholic acid treatment of gallstones, a follow-up report and analysis of factors influencing response to therapy. *N. Engl. J. Med.* **293:** 378–383.
24. Grundy, S. M., A. L. Metzger, and R. D. Adler. 1972. Mechanisms of lithogenic bile formation in American Indian women with cholesterol gallstones. *J. Clin. Invest.* **51:** 3026–3043.
25. Dam, H., I. Kruse, I. Prange, H. E. Kellehauge, H. J. Fenger, and M. K. Jensen. 1971. Studies on human bile. III. Composition of duodenal bile from healthy young volunteers compared with composition of bladder bile from surgical patients with and without uncomplicated gallstone disease. *Zeit. Ernaehrungswiss.* **10:** 160–177.
26. Smallwood, R. A., P, Jablonski, and J. McK. Watts. 1972. Intermittent secretion of abnormal bile in patients with cholesterol gallstones. *Br. Med. J.* **4:** 263–266.
27. Mackay, C., J. N. Crook, D. C. Smith, and R. A. McAllister. 1972. The composition of hepatic and gallbladder bile in patients with gallstones. *Gut.* **13:** 759–762.
28. Small, D. M. 1972. Gallstones: Diagnosis and treatment. *Postgrad. Med.* **51:** 187–193.
29. Small, D. M. 1970. The formation of gallstones. *Adv. Intern. Med.* **16:** 243–264.
30. Tera, H. 1960. Stratification of human gallbladder bile *in vivo*. *Acta Chir. Scand. Suppl.* **256:** 1–85.
31. Isaksson, B. 1954. On the lipid constituents of bile from human gallbladder containing cholesterol gallstones. A comparison with normal human bladder bile. *Acta Soc. Med. Upsal.* **59:** 277–295.
32. Carey, M. C., and D. M. Small. 1972. Micelle formation by bile salts: Physical-chemical and thermodynamic considerations. *Arch. Intern. Med.* **130:** 506–527.
33. Wuthier, R. E. 1966. Two-dimensional chromatography on silica gel loaded paper for the microanalysis of polar lipids. *J. Lipid Res.* **7:** 544–550.
34. Small, D. M., M. Bourgès, and D. G. Dervichian. 1966. Biophysics of lipidic associations. I. The ternary systems lecithin-bile salt-water. *Biochim. Biophys. Acta.* **125:** 563–580.
35. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
36. Carey, M. C., and D. M. Small. 1969. Micellar properties of dihydroxy and trihydroxy bile salts. Effects of counterion and temperature. *J. Colloid Interface Sci.* **31:** 382–396.
37. Shaffer, E. A., J. W. Braasch, and D. M. Small. 1972. Bile composition at and after surgery in normal persons and patients with gallstones. *N. Engl. J. Med.* **287:** 1317–1322.
38. Metropolitan Life Insurance Company. 1959. Statistical Bulletin 40. New York. 1–6.
39. Petrov, T. G., E. B. Treivus, and A. P. Kasatkin. 1969. Growing crystals form solution. English translation by A. Tybulewicz. Consultants Bureau, Inc., New York. 1–106.
40. Carey, M. C., J. C. Montet, and D. M. Small. 1975. Surface and solution properties of steroid antibiotics, 3 acetoxylfusidic acid, cephalosporin P_1 and helvolic acid. *Biochemistry.* **14:** 4896–4905.
41. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
42. Carr, J. J., and I. J. Dreckter. 1956. Simplified rapid technique for the extraction and determination of serum cholesterol without saponification. *Clin. Chem.* **2:** 353–368.
43. 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:** 357–366.
44. Talalay, P. 1960. Enzymatic analysis of steroid hormones. *Methods Biochem. Anal.* **8:** 119–143.
45. Thomas, P. J., and A. F. Hofmann. 1973. A simple calculation of the lithogenic index of bile: Expressing biliary lipid composition on rectangular coordinates. *Gastroenterology.* **65:** 698–700.
46. Carey, M. C., and D. M. Small. 1970. Studies on artificial bile. Influence of composition, counterion concentration and time on the properties of bile salt-lecithin micelles. *Gastroenterology.* **58:** 1057. (Abstr.)
47. Glasstone, S., and D. Lewis. 1960. Elements of physical chemistry. Macmillan & Co. Ltd., London. 2nd edition. 316–321.
48. Redinger, R. N., and D. M. Small. 1972. Bile composition, bile salt metabolism and gallstones. *Arch. Intern. Med.* **130:** 618–630.
49. Bennion, L. J., and S. M. Grundy. 1975. Effects of obesity and caloric intake on biliary lipid composition in man. *J. Clin. Invest.* **45:** 996–1011.
50. McBain, J. W., M. J. Vold, and J. Porter. 1941. A phase study of commercial soap and water. *Ind. Eng. Chem.* **33:** 1049–1064.
51. Freeman, R., and J. B. Finean. 1975. Cholesterol: lecithin association at molecular ratios of up to 2:1. *Chem. Phys. Lipids.* **14:** 313–320.
52. Tao, J. C., E. L. Cussler, and D. F. Evans. 1974. Accelerating gallstone dissolution. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 3917–3921.
53. Lecuyer, H., and D. G. Dervichian. 1969. Structure of aqueous mixtures of lecithin and cholesterol. *J. Mol. Biol.* **45:** 39–57.
54. Forslind, E., and R. Kjellander. 1975. A structure model for the lecithin-cholesterol-water membrane. *J. Theor. Biol.* **51:** 97–109.

55. Shinoda, K., T. Nakagawa, B. Tamamushi, and T. Isemura. 1963. Colloidal surfactants. Academic Press, Inc., New York. 97–178.
56. Mazer, N. A., R. F. Kwasnick, M. C. Carey, and G. B. Benedek. 1977. Quasielastic light scattering spectroscopic studies of aqueous bile salt, bile salt-lecithin, and bile salt-lecithin-cholesterol solutions. *In* Micellization, Solubilization, and Microemulsions. K. L. Mittal, editor. Plenum Publishing Corp., New York. **1**: 383–402.
57. Stoeckenius, W. 1962. Some electron microscope observations of liquid crystalline phases in lipid water systems. *J. Cell Biol.* **12**: 221–229.
58. Metzger, A. L., S. Heymsfield, S. M. Grundy. 1972. The lithogenic index-a numerical expression for the relative lithogenicity of bile. *Gastroenterology.* **62**: 499–501.
59. Friedman, D. K., W. B. Kannel, and T. R. Dawber. 1966. Epidemiology of gallbladder disease: observations in the Framingham study. *J. Chronic Dis.* **19**: 273–292.
60. Heaton, K. 1975. Gallstones and cholecystitis. *In* Refined Carbohydrate Foods and Disease: Some Implications of Dietary Fibre. D. P. Burkitt and H. C. Trowell, editors. Academic Press, Inc., New York. 173–194.
61. Metzger, A., R. Adler, S. Heymsfield, and S. M. Grundy. 1973. Diurnal variation in biliary lipid compositions. *N. Engl. J. Med.* **288**: 333–336.