Cholesterol gallstone dissolution in bile. Dissolution kinetics of crystalline cholesterol monohydrate by conjugated chenodeoxycholate-lecithin and conjugated ursodeoxycholate-lecithin mixtures: dissimilar phase equilibria and dissolution mechanisms

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Abstract Using compressed discs and microcrystals of cholesterol monohydrate, we evaluated the mechanisms and kinetics of dissolution in conjugated bile salt-lecithin solutions. In stirred conjugated ursodeoxycholate-lecithin and chenodeoxycholate-lecithin solutions, dissolution of 10,000-psi discs was micellar and linear with time for 10 hours. The dissolution rate constants (k) decreased in proportion to the lecithin content and dissolution rates and k values were appreciably smaller in conjugated ursodeoxycholate-lecithin solutions. After dissolution for 5 to 10 days the discs incubated with ursodeoxycholate-lecithin systems became progressively transformed into macroscopic liquid crystals. Unstirred dissolution of 3,000-psi discs in "simulated" human bile containing physiological lecithin concentrations gave apparent k values that decreased in the following order: ursodeoxycholate-rich $\geqslant$ chenodeoxycholate-rich > normal. In most cases the discs incubated with ursodeoxycholate-rich bile became covered with a microscopic liquid-crystalline layer. With 20–25 moles % lecithin, these layers eventually dispersed into the bulk solution as microscopic vesicles. During dissolution of microcrystalline cholesterol in conjugated ursodeoxycholate-lecithin systems, a bulk liquid-crystalline phase formed rapidly (within 12 hours) and the final cholesterol solubilities were greater than those in conjugated chenodeoxycholate-lecithin micellar systems. Prolonged incubation of cholesterol microcrystals with pure lecithin or lecithin plus bile salt liposomes did not reproduce these effects. Condensed ternary phase diagrams of conjugated ursodeoxycholate-lecithin-cholesterol systems established that cholesterol-rich liquid crystals constituted an equilibrium precipitate phase that coexisted with cholesterol monohydrate crystals and saturated micelles under physiological conditions. Similar phase dissolution-relationships were observed at physiological lecithin-bile salt ratios for a number of other hydrophilic bile salts (e.g., conjugated ursocholate, hyocholate, and hyodeoxycholate). In contrast, liquid crystals were not observed in conjugated chenodeoxycholate-lecithin-cholesterol systems except at high (nonphysiological) lecithin contents. Based on these and other results we present a molecular hypothesis for cholesterol monohydrate dissolution by any bile salt-lecithin system and postulate that enrichment of bile with highly hydrophilic bile salts will induce crystalline cholesterol dissolution by a combination of micellar and liquid crystalline mechanisms. Since bile salt polarity can be measured and on this basis the ternary phase diagram deduced, we believe that the molecular mechanisms of cholesterol monohydrate dissolution as well as the in vivo cholelitholytic potential of uncommon bile salts can be predicted.—**Salvioli, G., H. Igimi, and M. C. Carey.** Cholesterol gallstone dissolution in bile. Dissolution kinetics of crystalline cholesterol monohydrate by conjugated chenodeoxycholate-lecithin and conjugated ursodeoxycholate-lecithin mixtures: dissimilar phase equilibria and dissolution mechanisms. *J. Lipid. Res.* 1983. **24:** 701–720.

Supplementary key words static discs • microcrystalline cholesterol • dissolution rate constant (mass transfer coefficient) • ternary phase diagrams • time-lapse photomicrography • liquid-crystalline phase • hydrophilic bile salts • lecithin liposomes • simulated bile • hydrophilic-hydrophobic balance

In man, oral ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid, UDC) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid, CDC) induce cholesterol gallstone dissolution with similar efficacy (1–8). We demonstrated in earlier studies (9–12) that the ca-

Abbreviations: CDC, chenodeoxycholate; UDC, ursodeoxycholate; C, cholate; DC, deoxycholate; T-, G-, prefixes indicate taurine and glycine conjugates, respectively; ChA, anhydrous cholesterol; ChM, cholesterol monohydrate; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; Cs, equilibrium micellar solubility of cholesterol monohydrate.

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pacity of micellar solutions of the sodium salt of UDC and its conjugates to solubilize cholesterol is considerably poorer than that of the common bile salts. We showed further that micellar dissolution of crystalline cholesterol monohydrate (ChM) in pure UDC solutions was appreciably slower than in equimolar CDC micellar solutions (11). Based on these results we suggested (11) that the phospholipid lecithin (13) and/or the coexisting common bile salts (14, 15) in native bile may be important in promoting the dissolution efficacy of UDC. Despite the fact that conjugated UDC-lecithin micellar solutions also solubilize ChM poorly (10) and dissolution of ChM discs by the common bile salts is actually retarded with added lecithin (16-21), Corrigan and associates (22) noted that micellar dissolution of microcrystalline ChM in tauro- (T-) UDC-lecithin systems was followed by development of a liquid-crystalline phase that dramatically enhanced ChM dissolution.

The purpose of the present work is: *1*) to examine systematically the influence of lecithin on the dissolution kinetics of ChM by CDC and UDC conjugates; *2*) to define the respective roles of mixed micelles and liquid crystals in ChM dissolution and their relationships to condensed phase diagrams of model bile systems; *3*) to determine the roles of lecithin and common bile salts in ChM dissolution with UDC-rich bile; and *4*) to develop a simple conceptual framework to facilitate a molecular understanding of ChM dissolution by any bile salt-lecithin system. Our findings confirm the results of Corrigan et al. (22); however, a ChM-induced liquid crystalline phase transition is not unique to UDC-containing biles but is a characteristic of all bile salt-lecithin-crystalline ChM systems where the bile salt is more hydrophilic than cholate and its conjugates (23). Further, in simulated UDC-rich bile, ChM dissolution is also accelerated without a macroscopic bulk phase change; however, an interfacial and in some cases a bulk accumulation of microscopic lecithin-ChM liquid crystals occurs at physiological lecithin contents. Based on these and other results, we offer a unified molecular mechanism for ChM dissolution by bile salt-lecithin systems based on the hydrophilic-hydrophobic balance of the molecules. We also suggest that both lecithin and coexisting common bile salts are important for ChM dissolution in UDC-rich bile, and show that the potential for in vivo gallstone dissolution by an uncommon bile salt can be predicted on the basis of the appropriate bile salt-lecithin-ChM phase diagram.

EXPERIMENTAL PROCEDURE

Materials

Cholesterol (Nu-Chek Prep, Austin MN) was recrystallized from ethanol to achieve >99% purity and cholesterol monohydrate (ChM) and anhydrous cholesterol (ChA) were prepared as described (11). Radiolabeled [1-2^{3}H]ChM (New England Nuclear, Boston MA) was identical to that employed in our earlier study (11). Grade I egg yolk lecithin (lecithin) (Lipid Products, Surrey, U.K.) was chromatographically pure (>99%) by TLC. Common and uncommon bile salts were either purchased or received as gifts from Calbiochem (San Diego CA), Steraloids (Meriden NH), Tokyo Tanabe Co. (Tokyo, Japan), and Gipharmex S.P.A. (Milan, Italy), and were recrystallized (11–13) to attain a purity of 98–99% by TLC and HPLC (23). Other reagents were Fisher Certified Grades (Fisher Scientific, Pittsburgh PA) and solvents and buffers were identical to those described previously (11–13).

Methods

1. *Phase equilibria*

a. *Preparation and equilibration of aqueous lipid mixtures.* Lipid mixtures containing bile salts, lecithin, and cholesterol were coprecipitated from methanol (13) and a buffered NaCl solution was added to give a total lipid concentration of 10 g/dl, total ionic strength (Na^+) of 0.2 M, and final pH 7.4 (taurine-conjugated bile salts, 5 mM Tris), pH 8–9 (glycine-conjugated bile salts, 0.1 M phosphate), and pH 9–10 (unconjugated bile salts, 0.01 M carbonate/bicarbonate) (24, 25). An antimicrobial agent (NaN_3, 0.2 mg/ml) and antioxidant (butylated hydroxytoluene, 0.05 mg/ml) were added to the final mixtures (10 ± 1 ml). The tubes were sealed under N_2 and equilibrated for 14 days at 37°C.

b. *Gross and microscopic examination of mixtures.* After equilibration (13), tubes were opened and well-mixed samples (5 μl) were examined at 37°C (Mettler FP5 Heat-Exchanger, Mettler Instruments, Nutley NJ) for crystals, micellar liquid, and liquid crystals by direct and polarized light microscopy (Zeiss Photomicroscope III) (13). Bulk mixtures were then separated into individual phases and chemically analyzed as described below.

c. *Separation and analyses of phases.* After centrifugation (100,000 *g*, 90 min) at 37°C the liquid-crystalline and micellar phases separated and were aspirated into prewarmed hypodermic syringes. Each micellar phase was then microfiltered several times through 0.22-μm Millipore filters (Millipore Corporation, Bedford MA) at 37°C to remove contaminating liquid crystals as judged microscopically. To separate buoyant liquid crystals from solid ChM crystals, the aspirates were resuspended, washed thrice with buffer, and recentrifuged (100,000 *g*, 60 min). With lecithin-rich (>25 mol %) systems, liquid crystals and ChM crystals co-sedimented, and were separated by multiple microfiltrations through 0.5-μm Millipore filters. Phases were identified and their homogeneity was checked microscopically;

following this the relative lipid compositions of the bulk phases were determined as described below. Since percent aqueous buffer was fixed at 90%, the ternary phase diagrams were plotted on triangular coordinates with each axis representing a solid component (13).

2. *Dissolution experiments*

a. *Mixed micellar solutions.* Bile salt-lecithin micellar solutions were prepared on a wt/vol basis in 50-ml volumetric flasks generally by the method described in 1 (a) and in some cases by bile salt dissolution (1–4 days) of a lecithin film dried from $CHCl_3$–CH_3OH 1:1 (vol/vol). Bile salt concentrations were 100 mM or 200 mM, NaCl concentration was 0.2 M with or without 10 mM $CaCl_2$, and lecithin concentrations were 0, 10, 25, and 75 mM. To simulate normal, chenodeoxycholate-rich (cheno-rich) and ursodeoxycholate-rich (urso-rich) biles, mixed T- and G-conjugated bile salts (molar ratio 3.5:1) containing physiological ratios (6, 9, 14, 15) of deoxycholate (DC), CDC, and UDC and cholate (C) were prepared. These were coprecipitated with lecithin to give a series of mixed micellar solutions whose final total lipid concentration was 10 g/dl (5 mM Tris, 150 mM NaCl, pH 7.4 ± 0.1) with lecithin concentrations of 0–25 mol per 100 mol of bile salt.

b. *Liposomal dispersions.* Dry films of lecithin and lecithin plus bile salt (9:1 molar ratios) were prepared by coprecipitation as described under 1(a). Multilamellar vesicles were formed by 30-min vortex mixing of the lipid films in 150 mM NaCl plus 5 mM Tris buffer at pH 7.4. Small unilamellar liposomes were prepared by 30–min sonication of the multilamellar vesicles at 4°C (Branson Model W185 Sonifier, Heat-Systems, Inc., Plainview NY), followed by fractionation from larger vesicles by ultracentrifugation (100,000 *g*, 1 hr) (26). Quasielastic light scattering of the dispersions (courtesy of Dr. George B. Benedek, M.I.T.) indicated that the liposomal vesicles were small and relatively monodisperse (hydrodynamic radii, 250 ± 20 Å). Vesicle preparations were aged at 37°C for 24 hr.

c. *Static disc dissolution.* The apparatus, methods, and data analysis employed have been described in our earlier publication (11). ChM discs (30 mm in diameter) were compressed at either 3,000 or 10,000 psi (Carver Laboratory Press, Fisher Scientific, Medford MA) and stirring rates were either 300 rpm or 0 rpm (unstirred). Prior to sampling, the unstirred solutions were stirred at 50 rpm for 20 sec. The dissolution rate constant (k) was calculated from the initial (linear) dissolution rates (11) and the equilibrium micellar cholesterol solubilities (Cs). The Cs values were either directly measured (see below) or interpolated from appropriate bile salt-lecithin-ChM phase diagrams[2] or calculated from the critical tables of Carey (10) with appropriate ChM solubility corrections for the micellar content of GUDC and TUDC.

d. *Dissolution of microcrystalline ChM.* Ten-ml mixed micellar solutions or liposomal dispersions were added to ≃500 mg of microcrystalline ChM or ChA and incubated at 37°C with intermittent shaking. Well-mixed samples (100 μl) were aspirated at 0.25- to 4-day intervals for chemical and radiochemical analysis. At the end of each experiment, micellar, liquid-crystalline (when present), and solid phases were separated by microfiltration and/or ultracentrifugation (see 1(c)), examined microscopically to verify homogeneity, and their individual chemical and radiochemical compositions were determined. An estimate of Cs was derived from the plateau portions of the appropriate ChM micellar solubility curves.

e. *Time-lapse photomicrography of ChM dissolution.* Approximately 25 μl of 10 g/dl TUDC-, TCDC-, and TUDC/TCDC (molar ratios 1:1, 2:1, and 3:1) -lecithin micellar solutions (bile salt-lecithin molar ratios 9:1, 4:1) were added to a few ChM crystals in wells of hanging-drop slides. The wells were purged with N_2 and the coverslips were hermetically sealed with molten plastic. Polarized light microscopy was carried out at 37°C for 7 days and time-lapse photomicrographs were taken through a first order quartz compensator. Other slide-dissolution studies were carried out with mixed micellar solutions of lecithin and GUDC, GCDC, several uncommon bile salts (see Results), and cheno-rich and urso-rich simulated biles.

f. *Bile salt dissolution of interfacially adsorbed lecithin.* Microcrystalline ChM (1000 mg) was incubated for 3 days at 37°C with 10-ml mixed micellar solutions of TCDC (100 mM) plus lecithin (43 mM) or TUDC (100 mM) plus lecithin (43 mM) (5 mM Tris, 0.15 M NaCl, pH 7.4). After centrifugation (10,000 *g*, 60 min), the supernatants were decanted and the crystals were washed thrice by resuspension in Tris-NaCl and recentrifugation. Crystals were filtered once through a 10–15 mesh glass filter and remaining solvent was removed by overnight evaporation at 37°C. Cholesterol crystals with adsorbed lecithin were harvested and then divided into equal parts by weight (350 mg). Five ml of 100 mM TUDC was added to one set, and 5 ml of 100 mM TCDC was added to the other set. Each tube was incubated (unstirred) at 37°C for 24 hr and every 2 hr the *lecithin* concentration in well-mixed portions (0.2 ml) of the supernatants was assayed.

g. *Bile salt dissolution of multilamellar lecithin vesicles.* Appropriate amounts of dry TCDC, TUDC, TCDC/TUDC (1:1), TC, and TDC were added to 10-ml portions of multilamellar dispersions of lecithin to give lecithin to bile salt ratios of 0.1 to 1.0 and final total lipid

[2] Carey, M. C., and G. Ko. Unpublished observations.

concentrations of 10 g/dl (0.15 M Na^+, pH 7.0). The mixtures were then incubated (unstirred) at 37°C for 10 days. The time required for the mixtures to achieve optical clarity was determined with the aid of a high intensity light source. Micellar solubilization was confirmed when 750-nm absorbances measured against water were less than 0.05 (Cary-Varian 118C Spectrophotometer).

h. *Miscellaneous dissolution experiments.* (i) To study the effects of calcium on stirred (300 rpm) dissolution rates, GCDC-lecithin and GUDC-lecithin (100 mM:25 mM) micellar solutions with NaCl concentrations of 200 mM were incubated with 10,000-psi ChM discs at 37°C (pH 8.0, (24)) with and without 10 mM $CaCl_2$. The ChM contents of the bulk mixtures were assayed every 2 hr.

(ii) To test whether preincubation of ChM with a TCDC-lecithin mixed micellar solution subsequently influenced dissolution in a TUDC-lecithin mixed micellar solution and vice versa, we measured unstirred dissolution rates of 3,000 psi ChM discs in TUDC-lecithin or TCDC-lecithin (4:1 molar) solutions for 5 hr, respectively. The ChM discs were then carefully removed, washed twice with NaCl-Tris buffer, and their ChM dissolution rates were measured for the same time period in the crossover solution.

(iii) To evaluate whether preincubation of microcrystalline ChM with a TUDC-lecithin mixed micellar solution influenced subsequent dissolution in an identical TCDC-lecithin mixed micellar solution, we preincubated 500-mg lots of ChM for 12 hr in a TUDC-lecithin (4:1 molar) solution and in a TUDC solution, respectively. The microcrystals were then removed, washed twice with NaCl-Tris buffer, and the quantity of ChM dissolved in a TCDC-lecithin (4:1 molar) solution was measured for 22 hr. Control preincubations of ChM were carried out in solutions of TCDC and lecithin (4:1) and TCDC alone followed by washing and monitoring ChM solubilities in TCDC-lecithin solutions.

i. *Microscopy of ChM discs and microcrystals after dissolution.* At termination of sampling during dissolution experiments, incubation was continued for several days to a week thereafter. The surfaces of compressed ChM discs and microcrystals were then inspected with a dissecting microscope (×40) and their physical textures were determined by scraping a fine spatula across the surface. When this procedure removed a surface layer, the identification of liquid crystals and their microscopic textures were verified by polarized light microscopy (see 1(b) above).

3. *Chemical determinations*

Micellar phases were prepared for analysis as described (13); liquid crystalline and crystalline phases were analyzed after solubilization in methanol–benzene 8:2 (vol/vol). Bile salts, lecithin, and cholesterol were assayed in duplicate by methods summarized earlier (13). The measurement of [1,2-^{3}H]cholesterol was by scintillation counting (11). Most final cholesterol concentrations were confirmed by the cholesterol oxidase method (27). When a phosphate buffer was employed (G-conjugated bile salts), lecithin was assayed by the choline oxidase method (28).

RESULTS

1. The phase diagrams

Fig. 1 depicts the condensed phase diagrams of 10 g/dl TUDC-, TC-, and TCDC-lecithin-ChM systems at 37°C as a function of hydrophilicity of bile salt species (23). The limits of micellar ChM solubility (solid lines) decrease slightly between TCDC and TC, and appreciably between TC and TUDC. The upper part of each triangle is divided by interrupted lines into two 2-phase areas enclosing a central 3-phase area. The 2-phase areas on the left are composed of ChM crystals and a micellar solution saturated with ChM; the 3-phase areas are composed of ChM crystals, liquid crystals, and a micellar solution saturated with ChM; and the 2-phase areas on the right are composed of liquid crystals and mixed micelles saturated with lecithin *and* ChM. With increasing bile salt hydrophilicity, the 3-phase area expands at the expense of the 2-phase region on the left, and in the TUDC-lecithin-ChM system the latter becomes quite small. The 2-phase area on the right is not appreciably modified; however, the maximum micellar solubility of lecithin is largest in the TC system (baseline data points).

The condensed phase diagrams of equimolar TCDC/TUDC- and GCDC/GUDC-lecithin-ChM systems are displayed in **Fig. 2.** The micellar area for the taurine conjugates (solid lines) is appreciably larger than that for the glycine conjugates (interrupted lines). As noted before, the glycine conjugates solubilize slightly more ChM than do the taurine conjugates in the absence of lecithin (11, 12). Above the micellar zone, the interrupted phase boundary (labeled AB) between the left 2-phase and 3-phase areas is intermediate to that for pure TCDC- and TUDC- and lies slightly to the left of that for TC-lecithin-ChM systems (Fig. 1). Phase boundary AB is shifted slightly to the right in the GCDC/GUDC 1:1 system (not plotted), and progressively to the left with *a*) increasing conjugated UDC to CDC ratio, *b*) increases in total lipid concentration, and *c*) in the metastable state during equilibration of all these systems. The number and physical states of the phases observed are the same as those in Fig. 1.

Phase boundary AB was also displaced to the left in

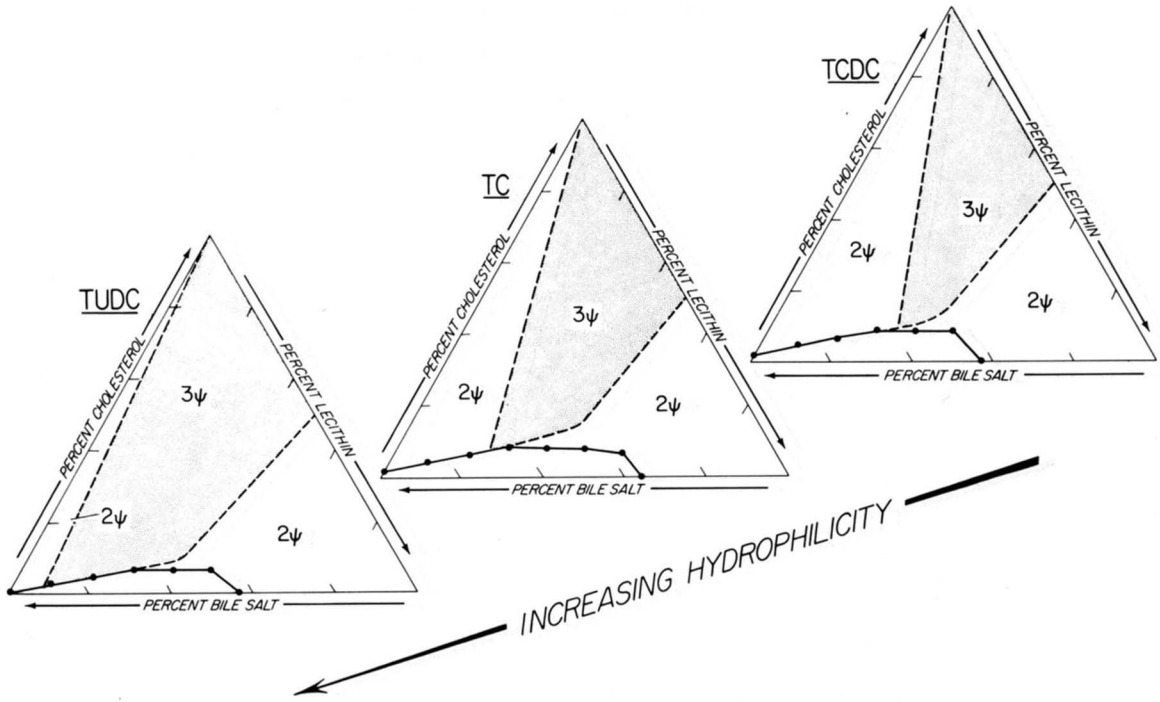

Fig. 1. Condensed phase diagrams of aqueous taurine-conjugated bile salt-lecithin-cholesterol monohydrate (ChM) systems. The three solid components are expressed in moles percent for total lipid concentrations of 10 g/dl. Other conditions were 0.20 M Na^+, pH 7.4, 37°C. Micellar phases are enclosed by solid lines. Symbol ψ and dashed lines represent the number of phases and phase boundaries, respectively. With increases in bile salt hydrophilicity from TCDC (taurochenodeoxycholate) to TC (taurocholate) to TUDC (tauroursodeoxycholate), the three-phase area (shaded) expands at the expense of the two-phase area on the left.

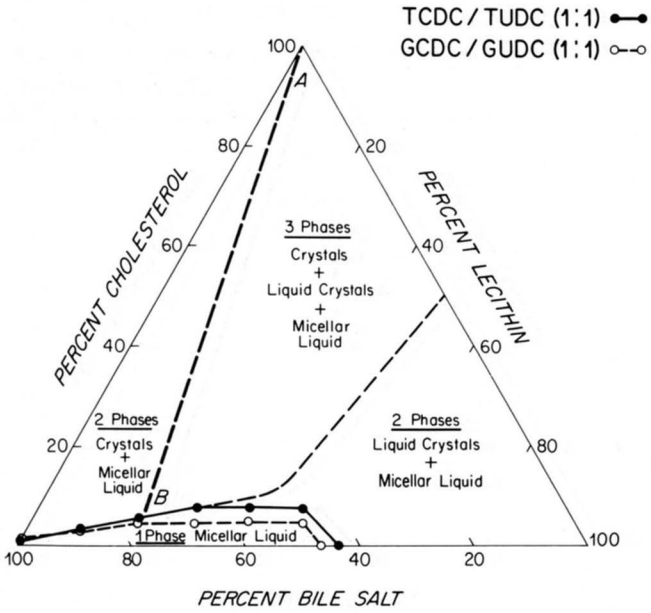

Fig. 2. Condensed phase diagrams of equimolar TCDC/TUDC- and GCDC (glycochenodeoxycholate)/GUDC (glycoursodeoxycholate)-lecithin-ChM systems (conditions as in Fig. 1, except pH 8.0–9.0 for the glycine conjugates). Phase boundary AB is markedly influenced by the hydrophilic-hydrophobic balance of a bile salt (or a bile salt mixture) moving to the left with increasing hydrophilicity and to the right with decreasing hydrophilicity. The relationship of this boundary to the physical mechanisms of ChM dissolution is discussed in the text.

bile salt-lecithin-ChM systems containing the following bile salts: sulfotaurolithocholate (3α-sulfate), taurohyocholate (3α,6α,7α-trihydroxy), taurohyodeoxycholate (3α,6α-dihydroxy), tauroursocholate (3α,7β,12α-trihydroxy), ursocholate (3α,7β,12α-trihydroxy), 12-oxocholate (3α,7α dihydroxy,12-oxo), and unconjugated (free) UDC. With sulfotaurolithocholate (5 g/dl systems, see ref. 29) and 12-oxocholate (10 g/dl systems), phase boundary AB (Fig. 2) extrapolates to a relative composition on the base line of 85% bile salt–15% lecithin, whereas with taurohyodeoxycholate (10 g/dl systems) AB intersects the baseline at ≃90% bile salt. These bile salts, as inferred by reverse phase HPLC, are more hydrophilic than TC, but less so than TUDC (23). With taurohyocholate, tauroursocholate, and ursocholate (10 g/dl systems), the 3-phase region was so expanded that phase boundary AB (Fig. 2) was closely aligned with the percent cholesterol axis; these bile salts are more hydrophilic than TUDC and UDC respectively (23 and footnote 3). The phase relations for urso-rich simulated bile (50% UDC conjugates) were similar to that for TCDC/TUDC (1:1) systems (Fig. 2) in that boundary AB extrapolated to ≃20% lecithin. The phase relations

[3] Salvioli, G., and M. C. Carey. Unpublished observations.

TABLE 1. Composition of initial mixtures and liquid-crystalline phases formed in TUDC-lecithin-cholesterol (monohydrate) systems[a]

No.	Initial Relative Composition			Composition of Liquid-Crystalline Phases[b]				
	TUDC	Lecithin	ChM	TUDC	Lecithin	ChM	ChM/Lecithin Molar Ratio	TUDC
	Mol %			*μmol/ml*				*Mol %*[c]
1[d]	87.5	2.5	10	0.003	0.034	0.03	0.9	4.2
2	85.0	5.0	10	0.81	3.38	5.5	1.6[e]	8.4
3	82.5	7.5	10	0.40	1.43	2.3	1.6[e]	9.8
4	80.0	10	10	1.60	5.83	10.7	1.6[e]	8.8
5	72.0	18	10	trace	0.09	0.1	1.1	trace
6	50.0	40	10	0.13	0.19	0.07	0.4	4.6
7	40.0	50	10	trace	0.43	0.04	0.1	trace

[a] Ten g/dl, 37°C, 5 mM Tris, 0.15 M NaCl, pH 7.4, equilibration time 14 days.
[b] Aspirated after centrifugation (100,000 *g*) at 37°C for 90 min.
[c] Percent of total lipids in separated liquid-crystalline phases.
[d] Liquid-crystalline phases floated in tubes 1–5 but sedimented in tubes 6 and 7.
[e] Possibly nonequilibrium values.

for simulated cheno-rich and normal biles were similar to those for TCDC and TC, respectively, in Fig. 1.

Tabulations of the compositions of the liquid-crystalline phases in the 3-phase region of aqueous TUDC-lecithin-ChM systems containing 10 mol % ChM (Fig. 1) show that the phases were composed of ChM and lecithin in ratios that varied from 0.9 to 1.6 (**Table 1**). When adjusted for possible contamination with the micellar phase (30), the corrected ChM/lecithin ratios for the larger values approximated 2:1 (see footnote *e*. Table 1). Compositions of the liquid-crystalline phases in the 2-phase region of TUDC-lecithin-ChM systems (Fig. 1) showed that they contained ChM/lecithin ratios of <0.4 (Table 1). In all purified liquid-crystalline phases TUDC was present in amounts that varied from traces to 10 mol %.

2. Dissolution studies

a. *Static ChM disc dissolution experiments.* In **Table 2,** dissolution data for 10,000-psi ChM discs in stirred (300 rpm) micellar solutions of GCDC, and GCDC-lecithin, GUDC, and GUDC-lecithin and equimolar GCDC/GUDC plus lecithin mixtures are listed. Selected results for bile salt-lecithin ratios of 4:1 are displayed in **Fig. 3.** The quantity of ChM dissolved increases as a linear function of time (11), and the dissolution rates, as inferred from the slopes of the curves, are markedly slower in GUDC-lecithin systems than in GCDC-lecithin systems (Table 2, Fig. 3). Equimolar GCDC/GUDC mixtures give intermediate values and doubling the total lipid concentration substantially increases the dissolution rate (Table 2, Fig. 3). With added lecithin, most initial dissolution rates are faster than observed with pure bile salts (Table 2) and the percent increase is greater for GUDC-lecithin mixtures than for GCDC-lecithin mixtures. These differences are related in part to the larger percent increment in equilibrium ChM solubilizing capacity (Cs) in the case of GUDC-lecithin systems compared with GCDC-lecithin systems (Table 2). **Fig. 4** displays curvilinear decreases in ChM disso-

TABLE 2. Dissolution of cholesterol monohydrate discs[a]

	GCDC[b]					GUDC[c]					GCDC + GUDC[d]	
Bile salt concentration (mM)	100	100	100	100	100	100	100	100	100	100	100	200
Lecithin concentration (mM)	0	10	25	50	75	0	10	25	50	75	25	50
Equilibrium ChM solubility (mM)[e]	2.2	5.4	9.1	10.9	11.3	0.19	1.33	3.2	3.86	3.88	6.1	11.7
Moles of bile salt/mole of ChM at saturation	45:1	19:1	11:1	9:1	9:1	562:1	75:1	31:1	26:1	26:1	16:1	17:1
Initial dissolution rate ($mmole \cdot cm^{-2} \cdot sec^{-1} \cdot 10^7$)	2.5	4.2	4.2	2.9	0.4	0.09	0.36	0.35	0.32	0.08	0.98	3.0
Dissolution rate constant (k)[f] ($cm \cdot sec^{-1} \cdot 10^4$)	1.14	0.73	0.46	0.22	0.03	0.46	0.26	0.12	0.08	0.02	0.17	0.25

[a] Conditions were 0.20 M Na^+, 37°C, pH 8–9, 25 ml total volume, stirring rate 300 rpm, 10,000-psi discs, 30-mm diameter.
[b] Glycochenodeoxycholate.
[c] Glycoursodeoxycholate.
[d] Equimolar mixtures.
[e] Taken from unpublished phase equilibria observations of M. C. Carey and G. Ko (symbol, Cs).
[f] Approximates the initial dissolution rate divided by the equilibrium ChM solubility (Cs).

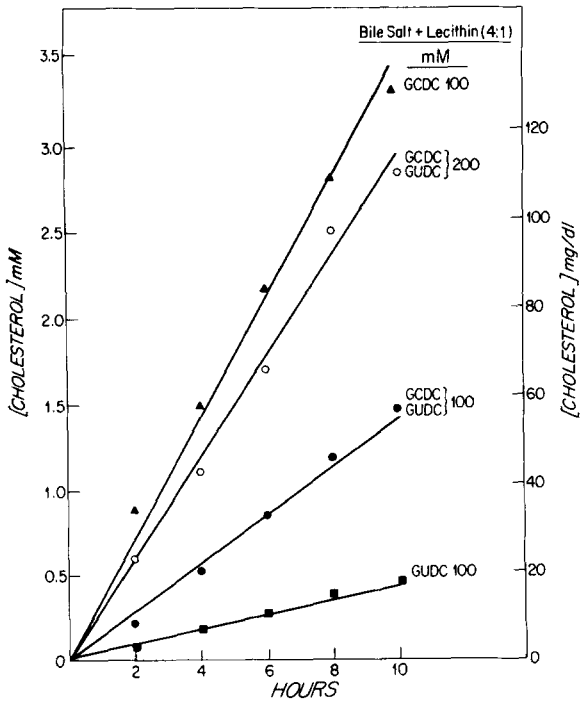

Fig. 3. Initial dissolution rates of ChM discs (10,000 psi) in 25 ml of GCDC-, GUDC-lecithin, and equimolar GCDC/GUDC-lecithin mixtures (molar ratios 4:1) at two different total liquid concentrations (stirring rate 300 rpm, pH 8.0–9.0, 37°C, 0.2 M Na^+).

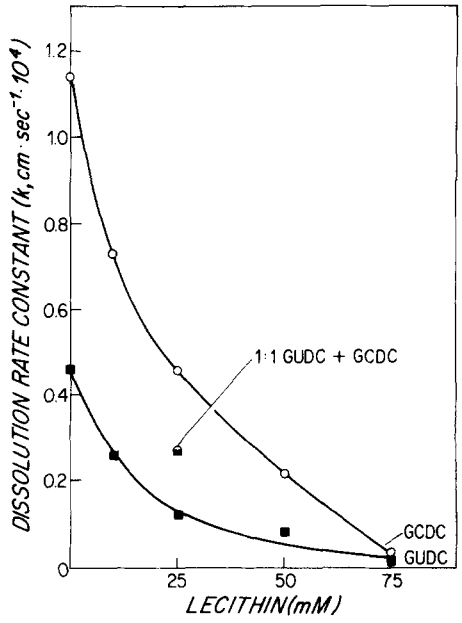

Fig. 4. Influence of added lecithin on the ChM dissolution rate constant (k) in 100 mM GCDC, GUDC, and an equimolar GCDC/GUDC mixture. (Conditions as in Fig. 3; data employed in the calculations of k (see ref. 11) are listed in Table 2.)

lution rate constants (k, Table 2)[4] as functions of added lecithin. The values converge and become quite small at bile salt-lecithin ratios close to the micellar phase limit (Fig. 4). At a physiological lecithin concentration (25 mM), equimolar ratios of GUDC and GCDC give intermediate k values (Table 2, Fig. 4). By the end of these dissolution periods (10 hr) no liquid crystals were detected microscopically on the discs or in the bulk solution. However, all GUDC- and GUDC/GCDC-lecithin systems formed bulk liquid crystals after several days of incubation with 10,000-psi ChM discs. Ten mM $CaCl_2$ with an excess of NaCl (200 mM) reduced the dissolution rates of 4:1 bile salt-lecithin systems in the absence of calcium by 4% (GCDC) and 7% (GUDC), respectively, and did not lead to micro- or macroscopic liquid-crystalline phase transformation in 10 hr.

Unstirred dissolution rates of 3,000-psi ChM discs in normal, cheno-rich, and urso-rich biles together with the dependence of apparent[5] k values on percent lecithin are shown in **Fig. 5.** The bile salt compositions employed, Cs[6] values, and calculated initial dissolution rates and rate constants are listed in **Table 3.** In the absence of lecithin the initial dissolution rates follow the order normal > cheno-rich > urso-rich biles. However, with added lecithin, initial dissolution rates are fastest in cheno-rich biles. The apparent dissolution rate constants (k, Fig. 5 inset, Table 3) indicate that without lecithin and with 25% lecithin the values for all systems are similar. However, in the physiological ranges (10 and 20% lecithin) the apparent k values for urso-rich bile are appreciably larger than the values for normal biles and cheno-rich biles (with 10% lecithin). In the presence of 20% and 25% lecithin, a thin liquid-crystalline film was observed microscopically on the surfaces of the discs in urso-rich bile after several days; however, the bulk solutions remained optically clear. By the end of 2 weeks of incubation, liquid-crystalline vesicles were

[4] To calculate k, the initial dissolution rates must be linear and Cs must be known. When a liquid-crystalline phase forms in a micellar system, accurate Cs values are difficult to estimate experimentally because the relative lipid composition in the micellar phase is not the same as the starting lipid composition of the dissolution medium. With this problem in mind, we interpolated Cs values from equilibrium bile salt-lecithin-ChM phase diagrams for the G-conjugates (see footnote 2). Further, since no liquid-crystalline phase formed during the time course of these experiments, we employed the Cs value appropriate to the bile salt-lecithin ratio in the initial dissolution medium.

[5] "Apparent," in that initial bile salt-lecithin ratios may have been altered slightly by the formation of liquid crystals on the ChM discs in urso-rich bile (see footnote 6).

[6] The Cs values used in the calculations show excellent concordance between critical table calculations (10) utilizing the initial bile salt-lecithin ratios and those directly measured by dissolution to equilibrium (Table 3). As a microscopic bulk liquid-crystalline phase formed only in mixtures with the highest lecithin contents during the time period of dissolution, the precision of these estimates of k should be high. Further, surface deposition of lecithin on ChM discs should not have altered the relative bulk lipid composition appreciably.

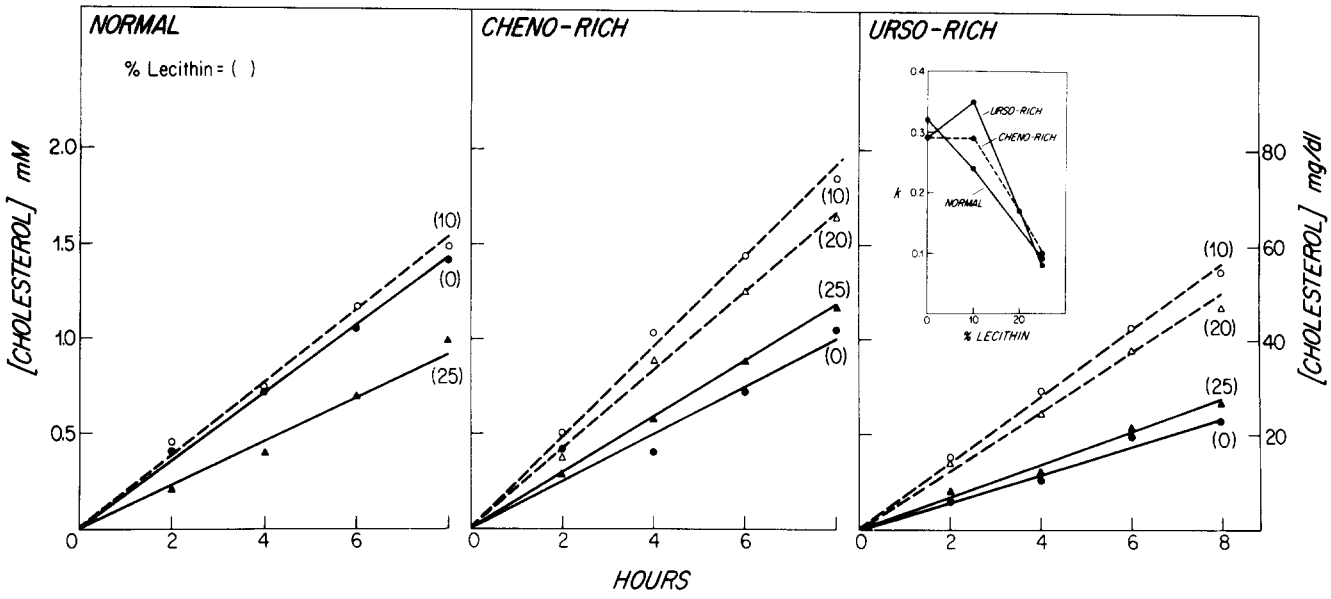

Fig. 5. Initial unstirred dissolution of ChM discs (3,000 psi) in 25 ml of simulated bile mixtures (bile salt compositions are listed in Table 3) as functions of moles percent lecithin (total lipid concentrations, 10 g/dl, 0.15 M Na^+, 5 mM Tris, pH 7.4, 37°C). Inset shows the dissolution rate constants (k) as functions of percent lecithin.

visualized by freeze-fracture electron microscopy in the bulk urso-rich systems containing 20–25% lecithin.[7]

When ChM discs (3,000 psi) were incubated (unstirred) with a 4:1 molar TUDC-lecithin or TCDC-lecithin solution followed by reversal of the dissolution medium, the dissolution rates in the crossover solutions were not different from those in the initial dissolution solutions (**Fig. 6**). Even though the slopes of each set of dissolution curves are identical, the mass of ChM solubilized during the second dissolution period in TUDC-lecithin systems is somewhat greater at each time point (Fig. 6B).

b. *Microcrystalline ChM and ChA dissolution with mixed micellar solutions.* The photographs in **Fig. 7** show that during dissolution of microcrystalline ChM, pure GCDC-lecithin (molar ratio 7:3) -ChM systems displayed only two phases: micellar liquid and excess ChM crystals. In contrast, GUDC-lecithin solutions incubated with solid ChM formed three phases which by chemical and phase analysis were micellar liquid, ChM crystals (bottom), and ChM-lecithin liquid crystals (buoyant phase).

Fig. 8 shows the results of microcrystalline ChM dissolution in pure GCDC-, TUDC-lecithin, and TCDC-, TUDC-lecithin systems over the course of 7 days. During the first 12 hr dissolution was slower in the GUDC-lecithin and TUDC-lecithin systems compared with the GCDC-lecithin or TCDC-lecithin systems. Once the arrowed points were reached, the conjugated UDC-lecithin systems became turbid and thereafter the concentrations of ChM dispersed greatly exceeded those dissolved in conjugated CDC-lecithin mixtures. All conjugated CDC-lecithin systems reached equilibrium Cs values by 1–2 days as inferred from the plateau portions of the curves. In contrast, conjugated UDC-lecithin systems showed continued dissolution/dispersion for an additional 2–6 days. In other studies (not plotted) the solubilities of ChA were appreciably higher than those of ChM. Upon phase separation and analysis at 7 days, the ChM concentrations in conjugated CDC-lecithin systems remained unaltered (closed circles and triangles, Fig. 8). This verified that the GCDC- and TCDC-lecithin systems corresponding to the plateau regions of the curves were micellar and saturated with ChM. In contrast, the ChM content of conjugated UDC-lecithin micellar phases was much less than that solubilized in the total mixtures (closed squares and diamonds, Fig. 8). This indicates that most of the "solubilized" ChM in the final conjugated UDC-lecithin-ChM systems was dispersed in liquid-crystalline form. These results also show that micellar Cs values for conjugated UDC-lecithin systems are appreciably smaller than for conjugated CDC-lecithin systems as noted earlier (10). Since the final micellar ChM values correspond closely with those at the appearance of visible turbidity in the bulk conjugated UDC-lecithin systems (arrowed Fig. 8), this suggests that liquid-crystalline dispersion of ChM only becomes the predominant dissolution mechanism once the micellar phase is saturated.

Fig. 9 depicts the dissolution of micro-crystalline ChM in mixed micellar solutions of constant bile salt-

[7] Igimi, H., and M. C. Carey. Unpublished observations.

lecithin ratio (4:1) as functions of systematic variations in the GUDC to GCDC ratio. By analogy with the data in Fig. 8, the dissolution of ChM with GUDC:GCDC ratios of 0:5, 1:4, 2:3, and 3:2 suggests that only micellar dissolution occurs. This is further verified by the fact that Cs, as inferred from the plateau portions, decreases with increases in the GUDC:GCDC ratio (see ref. 10). With GUDC/GCDC ratios of 5:0 and 4:1, initial micellar dissolution during the first 24 hr is superseded by rapid liquid-crystalline dispersion of ChM (dashed curve, Fig. 9). However, since initial micellar dissolution rates for these ratios are not as low as expected (10), we conclude that during the 0–24 hr interval liquid crystalline dissolution may in part be responsible for ChM solubilization.

Microcrystalline ChM dissolution by normal, cheno-rich, and urso-rich biles as functions of percent lecithin composition is displayed in **Fig. 10.** Each curve rises sharply during the first few days and then attains a plateau level, consistent with saturation of micellar phases. In each case, progressive increments in lecithin composition give rise to higher ChM solubilities. In UDC-rich bile no rapid takeoff of the curves is noted and the dissolution medium did not become macroscopically turbid, suggesting that a bulk liquid-crystalline phase did not develop. For equivalent lecithin contents, the Cs values (plateau regions) in urso-rich bile are significantly lower than in cheno-rich or normal biles. Only with 20% and 25% lecithin were liquid crystals noted microscopically on the surfaces of the ChM microcrystals and at the end of the experiments were detected in both systems as microscopic vesicles by freeze fracture electron microscopy.[7] The results of the crossover dissolution study (see Methods) with microcrystalline ChM are shown in **Fig. 11** (AB). Preincubation of ChM with a TUDC-lecithin system, but not with TUDC alone, enhanced subsequent dissolution with an equimolar TCDC and lecithin (4:1) solution (Fig. 11 (A)). The control study which involved preincubation in either a TCDC-lecithin system or TCDC alone (Fig. 11B) did not enhance subsequent dissolution in the TCDC plus lecithin system as inferred from the fact that both curves in panel B are, within experimental error, similar to curve b in panel A. All systems remained optically clear and the final ChM solubilities were similar.

c. *Time-lapse photomicrography of ChM crystal dissolution.* To assess the time course of liquid crystal formation induced by ChM in pure bile salt-lecithin systems, time-lapse photomicrography of TUDC-lecithin-solid ChM and TCDC-lecithin-solid ChM systems was carried out (**Plate 1**). Microscopic liquid crystals developed rapidly (0.5 hr) on the surface of ChM crystals in the TUDC-lecithin (4:1) system and their birefringence and optical textures were consistent with lamellar (bilayer) packing

TABLE 3. Dissolution of ChM discs using simulated bile mixtures[a]

	Normal				Cheno-rich				Urso-rich			
Bile salt composition[b] (mol %)	(31% DC, 35% CDC, 34% C, 0% UDC)				(9% DC, 80% CDC, 9% C, 2% UDC)				(14% DC, 21% CDC, 15% C, 50% UDC)			
Lecithin composition (mol %)	0	10	25		0	10	20	25	0	10	20	25
Observed equilibrium ChM solubility, mg/dl (mM)	214 (5.3)	284 (7.1)	524 (13.0)		172 (4.3)	310 (7.7)	488 (12.1)	548 (12.9)	83 (2.1)	202 (5.0)	329 (8.2)	378 (9.4)
Calculated[c] equilibrium ChM solubility, mg/dl (mM)	213 (5.3)	316 (7.8)	548 (13.6)		168 (4.2)	320 (7.9)	484 (12.0)	548 (13.6)	83 (2.1)	180 (4.5)	335 (8.3)	418 (10.4)
Initial dissolution rate[d] ($mmol \cdot cm^{-2} \cdot sec^{-1} \cdot 10^7$)	1.69	1.81	1.21		1.25	2.26	2.03	1.35	0.69	1.67	1.42	0.82
Dissolution rate constant[d] ($cm \cdot sec^{-1} \cdot 10^4$)	0.32	0.24	0.09		0.29	0.29	0.17	0.10	0.32	0.35	0.17	0.08

[a] Conditions were: total lipid concentration 10 g/dl, Tris-buffer 5 mM, 0.15 M NaCl, pH 7.4, 37°C, 3,000-psi discs, 30 mm in diameter. Unstirred for 8 hr.
[b] Glycine/taurine ratio was 3.5:1; DC, deoxycholate; CDC, chenodeoxycholate; C, cholate; UDC, ursodeoxycholate.
[c] As described in Ref. 10 (critical tables plus correction factors for % TUDC and % GUDC).
[d] See Ref. 11 for methods of calculation.

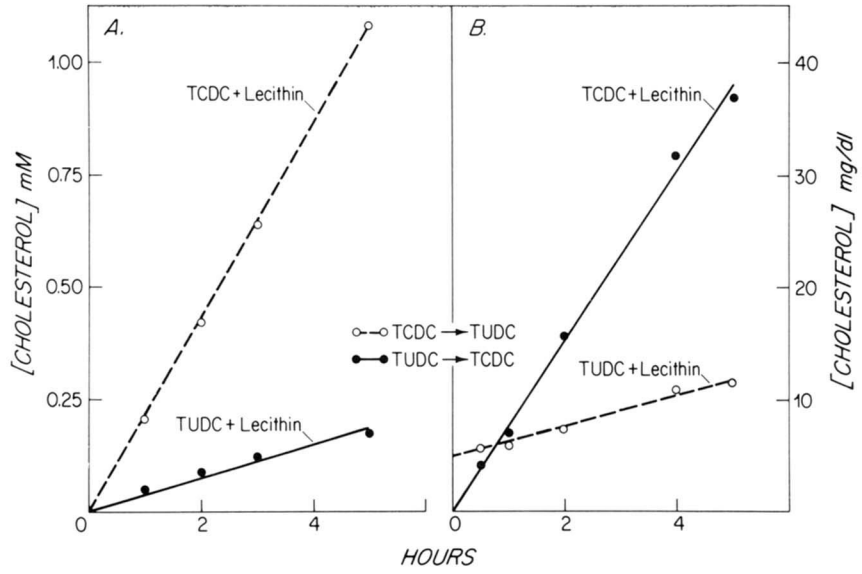

Fig. 6. Initial unstirred dissolution rates of ChM discs (3,000 psi) in 4:1 molar TCDC-lecithin and TUDC-lecithin systems (left panel) and of the same discs after washing and reversal of the dissolution solutions (right panel). Bile salt concentrations were 100 mM and lecithin concentrations were 25 mM (pH 7.4, 5 mM Tris, 0.15 M Na^+, 37°C).

of the molecules (31). During ChM dissolution in the TCDC-lecithin (4:1) system (Plate 1), no liquid crystals formed but sawtooth etching of the crystals became microscopically evident after 24 hr. With passage of time the ChM crystals in the TCDC-lecithin solution became considerably reduced in size but true dissolution did not take place in the TUDC-lecithin system.

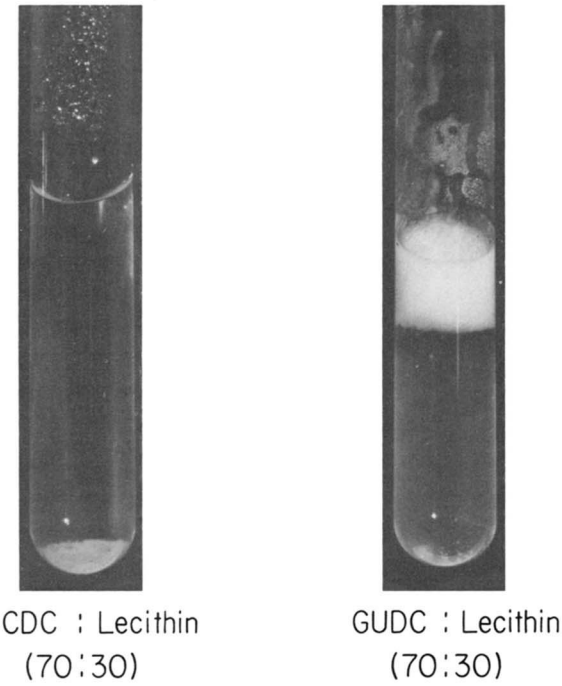

Fig. 7. Gross appearance of GCDC-lecithin (left) and GUDC-lecithin (right) systems during dissolution of microcrystalline ChM (pH 8.0–9.0, 37°C, 0.2 M Na^+). The systems on the left contains two phases, micellar liquid and ChM crystals (bottom). The system on the right contains three phases, micellar liquid, liquid crystals (floating), and ChM crystals (bottom).

A microscopic surface liquid crystalline phase was also observed when ChM crystals were incubated with 10 g/dl TUDC/TCDC (molar ratios 3:1 and 2:1) plus 20 mol % lecithin. No liquid crystals formed with equimolar TUDC/TCDC ratios containing 20 mol % lecithin nor in pure TUDC-lecithin systems with 10% lecithin. With urso-rich bile (Table 3) at lecithin contents of more than 20 mol %, a transient surface liquid-crystalline phase appeared in 2 hr. This phase became permanent with higher lecithin contents (>25 mol %) or with small increases in percent UDC conjugates (50–>60%) in the bile salt mixture. Microscopy of ChM crystals in 10 g/dl TCDC-lecithin systems containing in excess of 40 mol lecithin revealed slow formation (24–48 hr) of similar liquid crystals. All of these observations were consistent with the phase diagram relationships described in Part 1 of Results.

d. *Microcrystalline ChM and ChA dissolution with liposomal dispersions.* **Fig. 12** shows that dilute (10 mM) dispersions of small unilamellar liposomes dispersed ChM and ChA very slowly. After 24 hr average lecithin-cholesterol molar ratios in the liposomes were 40:1 (ChA) and 80:1 (ChM), respectively. As the solubility curves tended to approach saturation, mean lecithin-cholesterol molar ratios were reduced to 6 → 7:1 for both ChM and ChA. Liposomes containing 10% bile salts

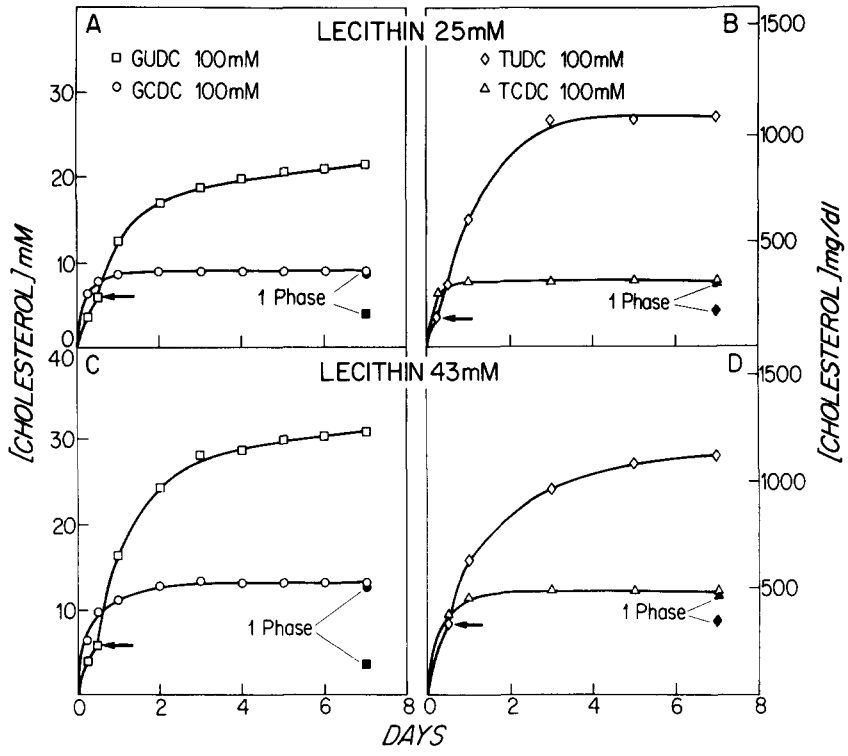

Fig. 8. Dissolution of microcrystalline ChM in bile salt-lecithin solutions with intermittent stirring [37°C, 0.15 M Na^+, pH 7.0 (taurine conjugates), pH 8.0–9.0 (glycine conjugates)]. Open symbols in A and C give the ChM concentrations solubilized by 100 mM GUDC and GCDC solutions with lecithin concentrations as shown. Panels B and D give similar data for the taurine conjugates. Closed symbols give the final concentrations of ChM in each *micellar* phase. Arrows correspond to the points where conjugated UDC-lecithin systems became turbid. The ChM concentrations at these points approximate those at micellar saturation (closed squares and diamonds).

incorporated slightly less ChM (Fig. 12A), but about the same amount of ChA (Fig. 12B). ChM was also poorly solubilized during 10-hr stirred (300 rpm) incubations of 3,000-psi discs with unilamellar and multilamellar liposomes and with liquid-crystalline phases harvested during dissolution of ChM in 100 mM GUDC and 43 mM lecithin (final lecithin-cholesterol ratios of 40:1 and 134:1, respectively, not shown). By microscopy, incubation with preformed liposomes did not induce liquid-crystalline transformation of the ChM crystals.

e. *Bile salt dissolution of multilamellar lecithin vesicles.* As shown in **Fig. 13,** bile salts dissolved multilamellar lecithin vesicles (final total lipid concentration, 10 g/dl) with rates that decreased in the order TDC > TCDC > TC > TCDC/TUDC (1:1) > TUDC. This sequence varies inversely with the hydrophilicity of the bile salt (TDC, most hydrophobic; TUDC, most hydrophilic) as inferred by reverse phase HPLC elution profiles (11, 23). As was found with TUDC, other hydrophilic bile salts (e.g., ursocholate, taurohyocholate, taurolithocholate sulfate) also induced slow bulk lecithin dissolution rates.

f. *Bile salt dissolution of lecithin adsorbed to ChM interfaces.* As shown in **Fig. 14,** TCDC and TUDC induced dissolution of lecithin films (32) adsorbed to microcrystalline ChM from TCDC-lecithin and TUDC-lecithin

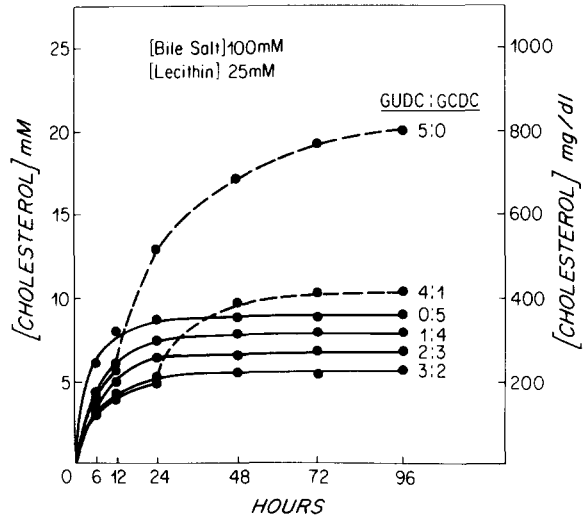

Fig. 9. Dissolution of microcrystalline ChM in GUDC/GCDC plus lecithin systems (bile salt-lecithin ratio 4:1) in which the GUDC/GCDC ratio is varied systematically. (Other conditions as in legend to Fig. 8.)

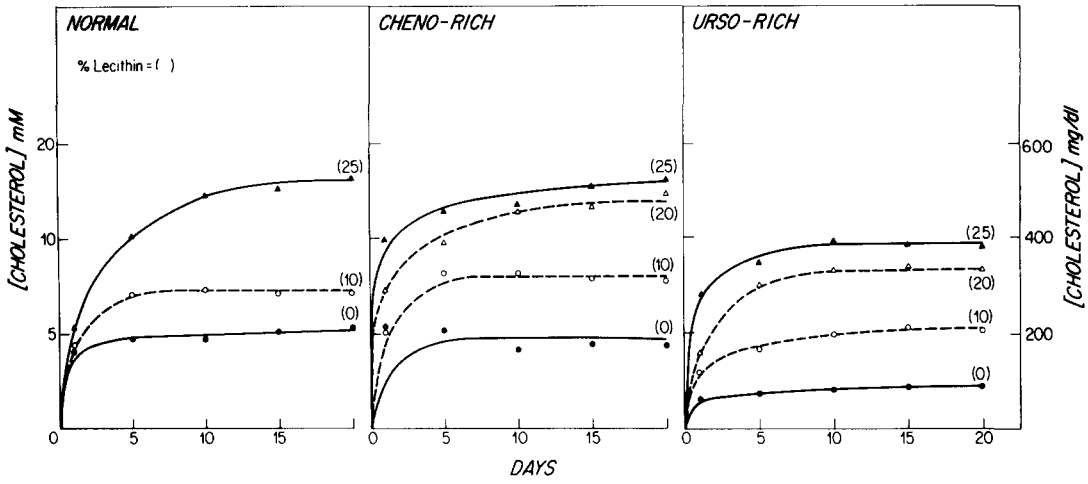

Fig. 10. Dissolution of microcrystalline ChM in simulated bile mixtures (compositions given in Table 3) as functions of moles percent lecithin (bracketed values) and time (pH was 7.4; other conditions are given in legend to Fig. 8).

micellar solutions (see Methods). The total amount of lecithin solubilized by 24 hr was about 5-fold greater after preincubation with TUDC-lecithin micellar solutions (Fig. 14A) than with TCDC-lecithin micellar solutions (Fig. 14B), suggesting that more lecithin was adsorbed to ChM crystals in the former case. In addition, TCDC solubilized interfacially adsorbed lecithin at an appreciably faster rate than TUDC. These results and those in (e) above indicate that both bulk and interfacial lecithin are solubilized more slowly by TUDC than by TCDC.

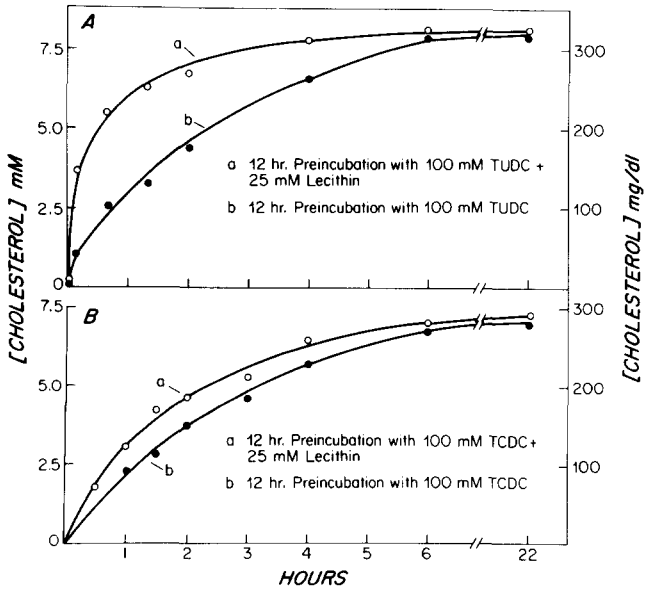

Fig. 11. Panel A. Unstirred dissolution of microcrystalline ChM with 100 mM TCDC and 25 mM lecithin after (a) 12-hr preincubation with 100 mM TUDC plus 25 mM lecithin and (b) 12-hr preincubation with 100 mM TUDC alone. Panel B. Dissolution in the same medium after (a) 12-hr preincubation with 100 mM TCDC plus 25 mM lecithin and (b) 12-hr preincubation with 100 mM TCDC alone. Other conditions as in Fig. 8.

DISCUSSION

1. Overview

The results of the present investigation demonstrate the complex complementary roles played by lecithin and the polarity of bile salts in the physical chemistry of ChM dissolution. In the case of the pure bile salt-lecithin systems, our results are generally consistent with initial micellar dissolution followed by liquid-crystalline dispersion only in the case of conjugated UDC (and other hydrophilic bile salt) -lecithin systems. With simulated biles the mechanisms become more complex, particularly in the case of urso-rich systems. We have attempted in these experiments to determine under what conditions micelles or liquid crystals dominate the dissolution mechanisms and whether surface and/or bulk liquid crystal formation is necessary for urso-rich bile to act as it does. The following discussion also explores the relationship of ChM dissolution in bile salt-lecithin systems to the appropriate equilibrium bile salt-lecithin-ChM phase diagrams.[8]

2. General physical-chemical principles in ChM dissolution

Dissolution of ChM in vitro and in vivo necessitates that solid ChM be bathed in bile unsaturated with ChM.

[8] This treatment is arbitrary since the phase relations above the micellar zone are altered in the nonequilibrium kinetic state. See earlier results in relation to phase boundary AB in Fig. 2.

LIQUID-CRYSTALLINE AND MICELLAR DISSOLUTION OF CHOLESTEROL·H_2O CRYSTALS

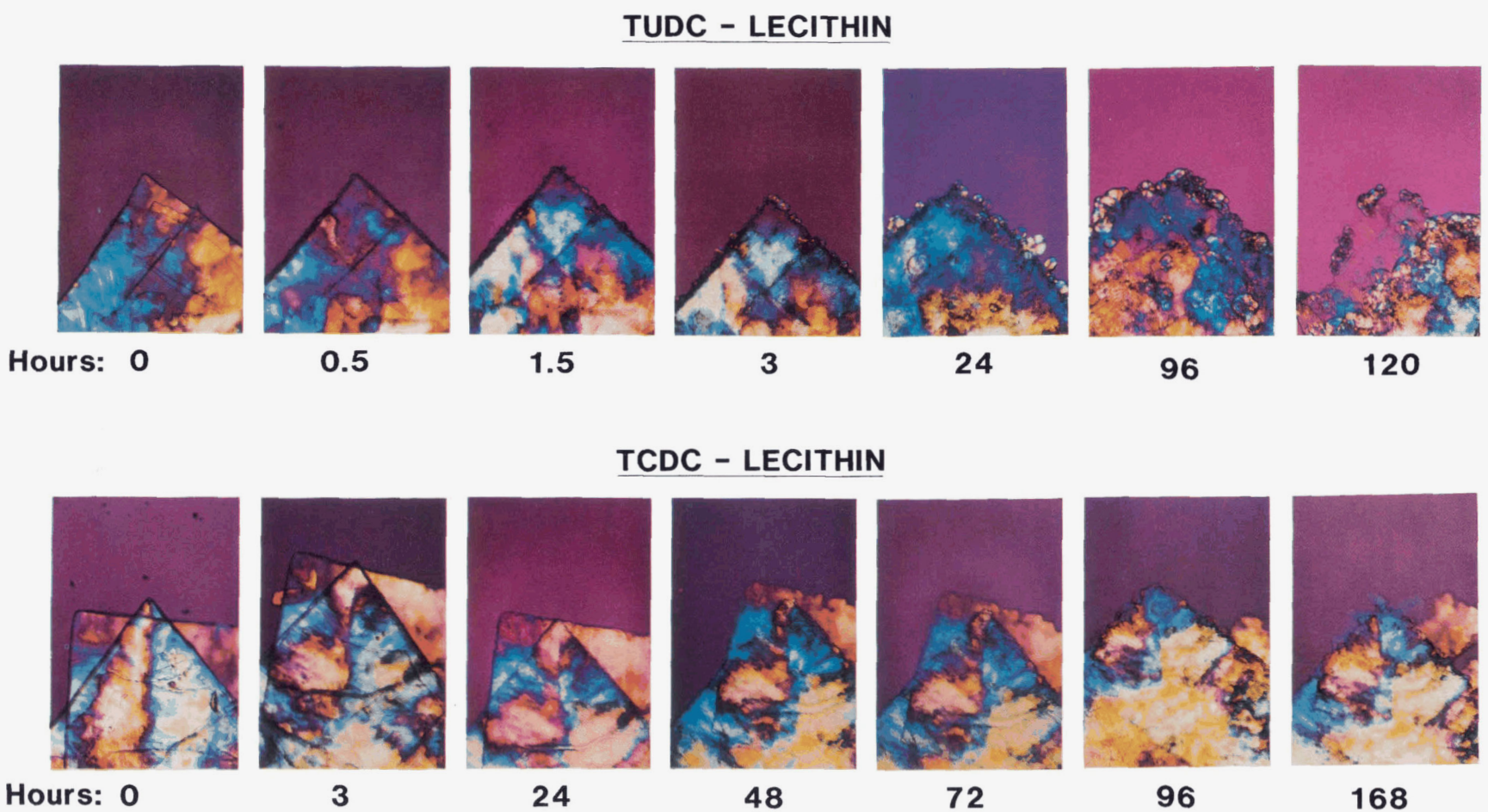

Plate 1. Time-lapse photomicrographs (crossed Nicols, first order quartz compensator, ×40) of single ChM crystals bathed in 4:1 molar TUDC plus lecithin solutions (top panels) and 4:1 molar TCDC plus lecithin solutions (bottom panels). Five μl of a 10 g/dl micellar solution was added to ChM crystals at time zero. (Other conditions were 37°C, 0.15 M Na^+, pH 7.4.)

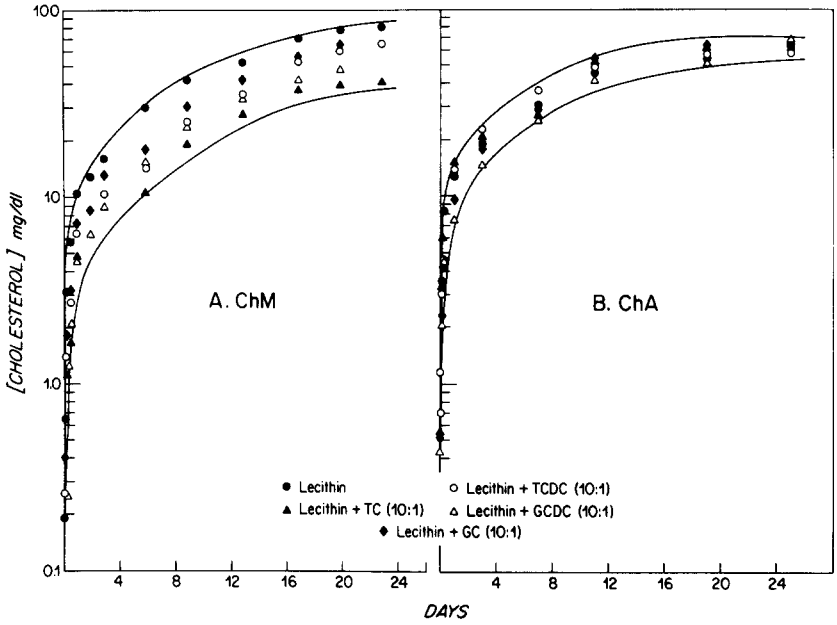

Fig. 12. Unstirred dissolution of (A) microcrystalline ChM and (B) microcrystalline anhydrous cholesterol (ChA) by unilamellar liposomes of 10 mM lecithin and lecithin plus common bile salts (TC, GC, TCDC, GCDC) in 10:1 molar ratios. (Other conditions as in legend to Fig. 8. See list for bile salt abbreviations.)

This has generally implied that, during dissolution, gallstones or solid ChM attempt to equilibrate with native or model bile whose relative lipid compositions lie within the *micellar zone* of an appropriate phase diagram (e.g., Figs. 1 and 2). By relating this concept to the ternary phase diagrams of the systems it becomes apparent that, at any time point during dissolution, the path over which equilibration takes place is an imaginary tie-line connecting the relative biliary lipid composition to the ChM apex of the triangular plot (see Figs. 1 and 2). Since native bile usually contains between 15 to 25 mol % lecithin (13), this tie-line in normal or cheno-rich bile (containing 70 → >90% CDC conjugates, 14, 15) will under all conditions transect a 2-phase area where micelles and ChM crystals coexist (Fig. 1). In the case of urso-rich bile, the analogous tie-line transects either a 2-phase (ChM crystals and micellar liquid) or a 3-phase (ChM crystals plus micellar liquid plus liquid crystals) area, depending on the position of phase boundary AB (Fig. 2). This phase boundary varies, at equilibrium, with the percent UDC conjugates, the glycine-taurine ratio, the lecithin-bile salt ratio, and the total lipid concentration. Obviously, if bile could be enriched with UDC conjugates to the same degree as CDC conjugates, this tie-line would cut across the 3-phase region at all physiological lecithin contents (Fig. 1). Assuming, as a first approximation, that the equilibrium phase relations (Figs. 1 and 2) can be employed as a conceptual framework to understand the kinetic situation, then an unsaturated micellar phase containing physiological amounts of lecithin should become progressively saturated with ChM during dissolution. At micellar saturation one of the following schemata may occur: *1*) With the common bile salts as in normal or cheno-rich bile, dissolution will stop at micellar saturation because only one phase is in equilibrium with saturated micelles, that is, ChM crystals (Fig. 1). As shown in Fig. 8, no further ChM can be solubilized at micellar saturation in TCDC-

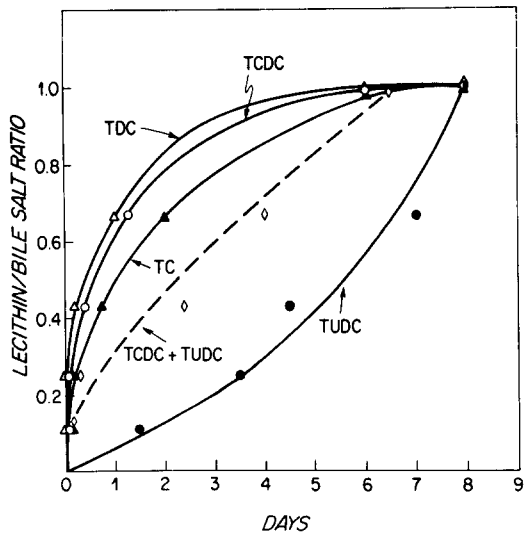

Fig. 13. Unstirred rates of micellar solubilization of multilamellar lecithin liposomes by taurine-conjugated bile salts. Final total lipid concentration at each data point was 10 g/dl (37°C, pH 7.4, 0.15 M Na^+).

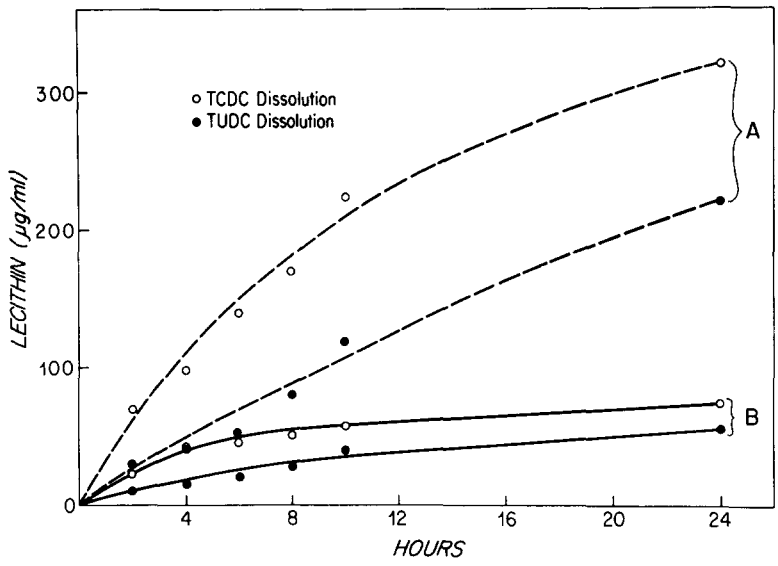

Fig. 14. Rates of solubilization of lecithin adsorbed to ChM crystal interfaces by TCDC (100 mM) and TUDC (100 mM). In data set A, lecithin was adsorbed to microcrystalline ChM (350 mg) from a mixed micellar solution containing TUDC (100 mM) and lecithin (43 mM). In data set B, lecithin was adsorbed to microcrystalline ChM (350 mg) from a mixed micellar solution containing TCDC (100 mM) and lecithin (43 mM). (Other conditions as in legend to Fig. 13.)

and GCDC-lecithin systems, and none can be removed by ultracentrifugation. *2*) If the bile salt in the system is GUDC or TUDC, the initial dissolution mechanism also appears to be micellar;[9] once micellar saturation is achieved (demonstrated by the breaks in the curves, Fig. 8), a bulk liquid-crystalline phase forms and continues ChM dissolution in conformity with the phase relations, since liquid crystals in addition to solid ChM constitute equilibrium phases with saturated micelles (Figs. 1 and 2). Not only can this liquid-crystalline phase disperse large quantities of ChM compared with the micellar solubilizing capacity of either species (Fig. 8), but it can become supersaturated with ChM (ChM-lecithin ratio ≃2:1) when compared to equimolar lecithin-cholesterol ratios at equilibrium (33–36).

Both sequences in the mechanism of ChM dissolution are shown in Fig. 9 where GUDC/GCDC-lecithin systems with bile salt ratios of 0–1.5 give rise to micellar dissolution, whereas bile salt ratios higher than this give rise to predominant[10] micellar dissolution followed by liquid-crystalline dispersion. Thus micellar dissolution alone or micellar dissolution plus liquid-crystalline dispersion can occur at the *same* bile salt-lecithin ratio simply by altering bile salt composition, to induce a shift in boundary AB (Fig. 2). In the case of simulated biles (Figs. 5 and 10), it is apparent that *bulk* micellar dissolution occurs in all cases initially. However, since the ChM microcrystals and discs become coated with liquid crystals at 20 and 25% lecithin, and in the case of prolonged incubation form a bulk vesicle phase, this strongly suggests that liquid crystals are first an intermediate-interfacial phenomenon and then a bulk phenomenon. However, dissolution in urso-rich bile was also accelerated without lecithin (Fig. 5) and dramatically accelerated with only 10% lecithin (see k values, Fig. 5). Neither interfacial nor bulk liquid crystals were observed in these systems. We therefore conclude that not only are the coexisting common bile salts important in ChM dissolution with urso-rich biles but, in addition, mixed micelles are important in dissolution, and operate apart from visible lecithin disposition.

3. Dissolution within the micellar zone: effects of lecithin

Micellar dissolution was observed when 10,000-psi ChM discs were dissolved at stirring rates of 300 rpm (Figs. 3 and 4, Table 2). The dissolution rates in conjugated UDC-lecithin systems were considerably slower than in equimolar conjugated CDC-lecithin systems, in agreement with what was found earlier for the pure bile salts (11). The influence of increasing amounts of leci-

[9] The absolute purity of the initial mechanism of dissolution appears to depend, in addition, on the ChM surface area, the compression pressure, and, perhaps, the stirring rate. The influences of these variables have not been systematically evaluated in this work.

[10] "Micellar dissolution" is a term employed cautiously since true micellar dissolution with GUDC/GCDC ratios of 4:1 and 5:0 (Fig. 9) should have fallen below that for the 3:2 ratio. Since the curves are higher than predicted, it is likely that a surface liquid-crystalline phenomenon is playing a role in dissolution before micellar saturation is achieved. Also, see Plate 1, where interfacial liquid crystals were observed in TUDC-lecithin systems in 30 min, a time when micellar saturation is unlikely, as inferred from the data in Fig. 8B.

thin is typical of that described previously for bile salt-lecithin mixed micelles (16, 17) in that, despite more rapid initial dissolution rates (Table 2), the dissolution rate constant (k) decreases in proportion to the added lecithin concentration (Fig. 4). Since the systems were rapidly stirred, the k values reflect principally the interfacial resistance to dissolution (11), which in the case of the common bile salts is known (16–21) to increase in proportion to the lecithin content.

Since the simulated bile systems (Fig. 5) were unstirred, the rate constant (k) contains contributions from convection/diffusion of micelles as well as the interfacial resistance to ChM dissolution (17, 18). The high k value for urso-rich bile with 10% lecithin (where no microscopic liquid crystals were detected) may be attributed to accelerated micellar convection/diffusion or to a decrease in interfacial resistance to dissolution. Since convection/diffusion factors are probably similar (as simple and mixed conjugated UDC and CDC micelles are of similar size, 12, 37, 38) we believe that accelerated dissolution is attributable to a fall in interfacial resistance. Our data in Fig. 14 suggest that in the urso-rich system, the fall in intrinsic interfacial resistance may be due to a greater amount of interfacially adsorbed lecithin which is not microscopically visible. The force employed to compress discs of ChM may also be of crucial importance since over the time course of dissolution 10,000-psi discs did not demonstrate a liquid-crystalline transformation whereas in the presence of physiological lecithin contents 3,000-psi discs did. Despite the fact that calcium binds to bile salts and lecithin (25, 39), it did not appreciably influence the dissolution kinetics of 10,000-psi ChM discs, nor did it induce the formation of surface or bulk liquid crystals in these systems.

4. Dissolution outside the micellar zone: liquid-crystalline dispersion

Upon the basis of these studies we can divide liquid-crystalline dispersion into two types: *1*) that which cooperates with micelles while dissolution continues within the micellar phase, i.e., an interfacial phenomenon; and *2*) that which forms a separate bulk phase when micelles are saturated. The first situation is exemplified by dissolution in simulated urso-rich bile (Fig. 10). The bulk phase remained micellar throughout dissolution and only in the case of the higher lecithin contents were dispersed liquid crystals detected after prolonged incubation. The second situation is typically observed in pure or highly enriched TUDC-lecithin or GUDC-lecithin micellar systems in the presence of microcrystalline ChM (Figs. 7–9).

The presence of conjugated UDC-lecithin micelles is obviously essential for the catalysis of these phenomena. Dissolution of solid ChM with small unilamellar liposomes (Fig. 12) did not reproduce the high ChM-lecithin ratios attained in the liquid-crystalline phase of conjugated UDC-lecithin systems. Liposomes took 24 days to reach saturation at a ChM-lecithin molar ratio of 1:6–7, whereas in conjugated UDC-lecithin-ChM systems the corresponding ratio was 2:1 in a few days. Microscopically, none of the ChM crystals incubated with liposomes showed a liquid-crystalline transformation.

5. Dissolution in simulated biles

Under unstirred conditions, using soft 3,000-psi ChM discs, cheno-rich and normal biles gave slower overall dissolution rates than did the urso-rich systems (Fig. 5). In all, bulk dissolution was micellar but in the case of the urso-rich system, dissolution was accelerated with as little as 10 mol % cholesterol without the appearance of liquid crystals. This suggests a predominant role of mixed UDC-lecithin micelles in dispersing cholesterol from the solid form. Only at higher lecithin contents were liquid crystals detected on the disc's surface and freeze-fracture electron microscopy[7] revealed no liquid crystalline vesicles until the system had been incubated for many days. These results suggest that in urso-rich bile time factors may be of the utmost importance for the appearance of bulk liquid crystals. In contrast, cheno-rich or normal biles remain micellar upon incubation with ChM for 2 weeks. We have recently demonstrated (30) that supersaturated model hepatic biles (3 g/dl) containing TC with relative lipid compositions lying outside the metastable zone consist of small stable liquid-crystalline aggregates (mean hydrodynamic radii = 300–400 Å) which contain ChM and lecithin in a ratio of 2:1. These appear to be the earliest precipitation nuclei and, when they agglomerate in concentrated bile, are slowly transformed into ChM crystals. It is likely that in urso-rich bile this sequence is reversed with solid ChM being transformed into ChM-lecithin liposomes of the same lipid ratio as the aggregates in lithogenic bile. Since these aggregates are stable they should be observable by quasielastic light scattering spectroscopy of native bile and should phase-separate upon centrifugation.

6. Hydrophilic-hydrophobic balance of bile salts, lecithin deposition, and ChM dissolution

It has been appreciated for some time that lecithin is adsorbed from mixed micellar solutions (TCDC-lecithin) onto solid ChM crystals (32). On this basis it has been suggested (32) that adsorbed lecithin increases interfacial resistance to micellar dissolution (i.e., k decreases (Fig. 4)) (16–20). However, in the case of UDC systems, the opposite conclusion may be made, especially in unstirred systems with soft ChM discs. The fact

that more lecithin is adsorbed from conjugated UDC-lecithin systems than conjugated CDC-lecithin systems (Fig. 14) is likely to be important in the acceleration of dissolution at low lecithin contents and the explanation of the early appearances of interfacial liquid crystals at high lecithin contents especially in pure systems (Plate 1). Not only was the dissolution rate of these interfacially adsorbed lecithin films much faster with 100 mM TCDC than with 100 mM TUDC (Fig. 14), but multilamellar lecithin liposomes were also more quickly dissolved (Fig. 13). Hence, the apparent rates of micellar solubilization of lecithin decrease as the hydrophilicity (23) of the bile salt decreases, even though the final lecithin solubilities are about the same (Fig. 1). The dissolution rates of ChM also decrease in the same order, but here the final micellar solubilities are different (11). These results once again underscore the importance of the hydrophilic-hydrophobic balance of a bile salt (23) in governing its lipid-binding functions. That hydrophobic and hydrophilic bile salts may have synergistic functions in the control of dissolution is suggested by the results in Fig. 11 where preincubation with TUDC-lecithin but not TCDC-lecithin micelles accelerated subsequent dissolution with TCDC, presumably by induction of an alteration in the ChM surface from lecithin deposition. It is unlikely that another conclusion, such as altered crystal surface area or morphology, is appropriate, since preincubation with TCDC-lecithin and TCDC alone did not appreciably accelerate subsequent dissolution rates.

7. A general molecular model for ChM dissolution

Mazer, Benedek, and Carey (38) demonstrated that at low lecithin-to-bile salt (TC, TDC, TUDC, and TCDC) ratios (0 to ~0.4–0.6), simple bile salt micelles and small bile salt-lecithin mixed micelles coexist. Only at high lecithin-to-bile salt ratios (>0.6) (outside the physiological range) do mixed micelles alone occur. Higuchi et al. (40), utilizing this coexistence model, quantitatively analyzed their results for ChM dissolution in TC-lecithin micellar solutions and concluded that only the simple bile salt micelles were involved in the rate-determining step. Our 10,000-psi ChM disc dissolution data (Fig. 4) indirectly confirm this suggestion since at the highest lecithin contents the dissolution rate constant (k) is quite small. Obviously, in pure conjugated UDC-lecithin systems simple UDC micelles will not be that effective in a collision with solid ChM (11). However, it is apparent that coexisting simple micelles of the common bile salts and UDC-lecithin mixed micelles may play important roles in ChM dissolution in urso-rich bile and give rise to the high k values initially observed (Fig. 5). Much more work will be necessary to provide a rigorous quantitative interpretation of the fast micellar dissolution in urso-rich bile.

Based on the present and earlier work, a dynamic schema for gallstone dissolution by mixed micelles of lecithin and a pure hydrophobic (TCDC) or a hydrophilic (TUDC) bile salt can be proposed (**Fig. 15**). With physiological compositions, simple and mixed micelles coexist, and both collide with solid ChM and remove ChM molecules. Based on the results in ref. 11, simple CDC micelles are more successful during a collision than UDC micelles. Mixed micelles unfold during a collision with ChM, depositing lecithin on the surface into which ChM from the solid phase can be incorporated. Owing to the poorer detergency of TUDC (Fig. 13), the lecithin (+ cholesterol) on the crystal surface may accumulate to a greater extent (Fig. 14) and remain for a longer period than in the case with TCDC. The longer interfacial residence time probably leads to an incorporation of more ChM molecules and eventually the mixed surface layers detach as liquid-crystalline lipo-

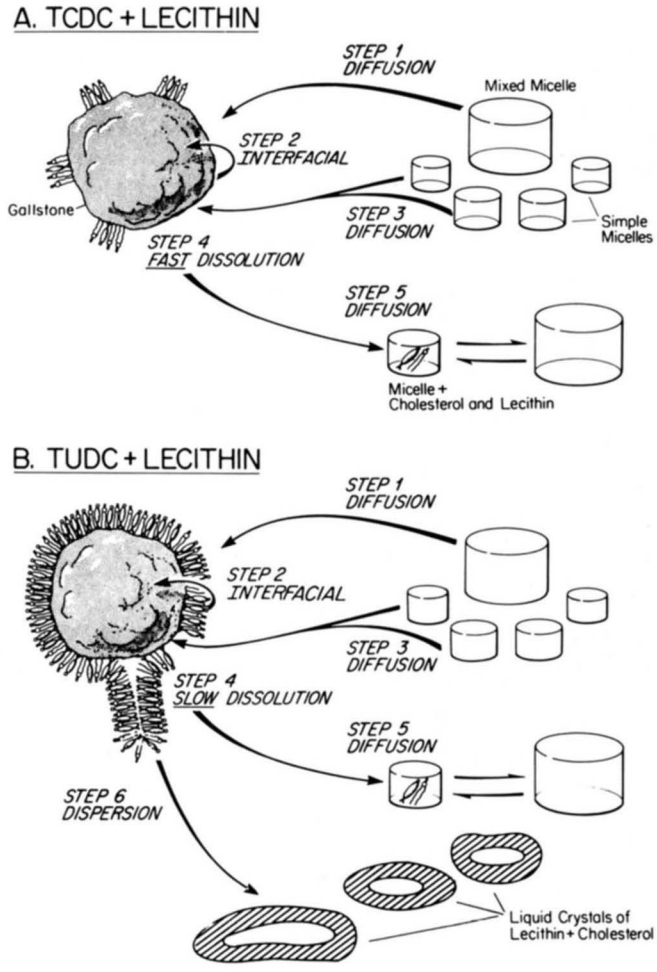

Fig. 15. Highly schematized diagram for the rate-limiting steps and proposed molecular mechanisms of ChM gallstone dissolution in (A) TCDC plus lecithin and (B) TUDC plus lecithin micellar solutions of physiological bile salt-lecithin composition (discussed in text).

somes. The liposomes may persist for two reasons: *1*) if the micellar phase is already saturated, the phase relations of the system above the micellar zone will preclude solubilization (Fig. 1); and *2*) the slow kinetics of lecithin dissolution into unsaturated UDC-lecithin systems (Fig. 13) may be further retarded if the liposomes are supersaturated with ChM. The catalysis of dissolution by interfacial or bulk lecithin liquid crystals is not a specific effect of UDC (41) but may represent the predominant mechanism of dissolution with other hydrophilic bile salts as inferred from the phase equilibria of these systems in the presence of excess ChM (see Results). When hydrophilic plus hydrophobic bile salts exist as mixtures with lecithin, both micellar and liquid crystalline mechanisms probably occur as simultaneous events, such as in urso-rich bile. We now know that liquid crystals accumulate in urso-rich model bile after prolonged incubation with solid ChM. This suggests that the coexisting common bile salts may not be capable of dispersing ChM-rich liquid crystals into *supersaturated* micelles.

8. Pathophysiological implications

Dual (micellar and liquid crystalline) dissolution mechanisms appear to be operative in urso-rich bile at physiological lecithin concentrations. This is the most likely explanation as to why dissolution rates and efficacies in gallstone patients on "urso-therapy" appear to be similar to those on "cheno-therapy" (1–8). We may predict therefore that liquid crystalline vesicles should be present in these biles and should be capable of centrifugal separation, light and electron microscopic visualization, and analysis. Perhaps many patients with cholesterol gallstones that are found to be resistant to dissolution in urso-rich biles have highly compressed gallstones analogous to our 10,000-psi ChM discs (Fig. 3, 4). In this situation, surface or bulk liquid crystals may not form efficiently and therefore only slow micellar dissolution into urso-rich bile can take place.

Several interesting structure-function relationships concerning the lipid binding properties of bile salts can be deduced from these and earlier studies (10–13, 23, 29). As hydrophilicity of bile salts increases (see 23), the micellar solubility of ChM (including that in the presence of lecithin) decreases (10, 11, 29) and the 3-phase region above the micellar zone of the ternary bile salt-lecithin-ChM phase diagram expands (Figs. 1 and 2). The position of boundary AB (Fig. 2) is, therefore, critically dependent on the overall hydrophilicity of a bile salt mixture, moving to the left as hydrophilicity increases, and to the right as hydrophobicity increases. Thus, hydrophilic bile salts (free and conjugated ursocholate, hyocholate, hyodeoxycholate, UDC, etc.) which exhibit a high reverse phase HPLC mobility (23) will induce the formation of liquid crystals with ChM in the presence of physiological lecithin contents, whereas hydrophobic bile salts (C, DC, CDC) will not. In this regard, some earlier pathophysiological observations are pertinent.

Dam and Christensen (42) inhibited formation of cholesterol gallstones in the lithogenic hamster model by feeding hyodeoxycholic acid. This observation was confirmed by Wheeler (43), who found that in hyodeoxycholate-enriched hamster gallbladder bile most of the cholesterol and lecithin was present in liquid-crystalline dispersions. Our phase equilibria studies with taurohyodeoxycholate (see Results) show that the ternary taurohyodeoxycholate-lecithin-ChM phase diagram is rather similar to that of TUDC-lecithin-ChM (Fig. 1). Hence, Dam and Christensen's (42) and Wheeler's (43) observations are consistent with the transformation of solid ChM into liquid crystals or a prevention of a liquid crystal → solid ChM transition which occurs as part of initial nucleation events (30). Dam and Christensen's results (42) led Thistle and Schoenfield (44) to feed hyodeoxycholic acid to gallstone patients in an attempt to reproduce the results that occurred in hamsters, but only 3% of conjugated hyodeoxycholate appeared in human bile (44). However, this result may in part be attributable to the high critical micellar temperature (>37°C, 23) of glycohyodeoxycholate (the major conjugate in man) and infused glycohyodeoxycholate is cholestatic in the hamster (43). If Thistle and Schoenfield had fed taurohyodeoxycholate to humans, we believe that gallstone dissolution would have occurred.

A second pertinent point relates to the importance and interpretation of the cholesterol saturation index during UDC therapy (3, 45–49). In spite of successful dissolution of both gallbladder and common duct stones (48, 49), many biles remain supersaturated, particularly when the saturation index is corrected for the diminished micellar ChM-solubilizing capacity of bile enriched with UDC conjugates (10). This is strong indirect evidence that a phase transformation is operative clinically and that a liquid-crystalline phase is dissolving stones. Liquid crystalline phases that form in lithogenic bile will always be initially unsaturated with ChM since ChM-lecithin ratios greater than 0.5 are rarely observed (13), even in the most lithogenic human biles (e.g., in morbid obesity). Even though the micellar phase may be saturated, the liquid-crystalline phase will be initially unsaturated, so gallstone dissolution should theoretically be possible without a significant change in biliary ChM content. When gallstone dissolution *appears* to occur in supersaturated bile, we believe that accurate measurements of the ChM saturation index may provide an extremely useful clue as to the predominant mechanism of ChM dissolution in the biles of such patients.

The results of the present work strongly suggest that

the efficiency of liquid-crystal formation during dissolution will depend on the position of phase boundary AB (Fig. 2). Whereas the bile salt-lecithin ratio of bile is not readily manipulated, the position of this line for constant (physiological) bile salt-lecithin compositions can be manipulated most effectively by increasing the hydrophilicity of the bile salt pool. As noted in our results, UDC and several other very hydrophilic bile salts will induce this change; however, this could be augmented by an increase in taurine-glycine ratio of bile salts and the total lipid concentration of bile. Hence the results of human studies, in which TUDC or taurine plus UDC is fed at bedtime, are eagerly awaited.

Finally, a number of miscellaneous predictions are pertinent. Since the hydrophilicity of any bile salt can now be accurately determined by reverse phase HPLC (23), the phase relations with lecithin and ChM can be roughly predicted on the basis of Fig. 1. Thus the gallstone dissolution potential of an uncommon or synthetic bile salt may be inferred from its HPLC retention time, provided the critical micellar temperature of both its conjugates is <37°C. Our results also suggest that an alternating enrichment of the bile salt pool with hydrophilic and hydrophobic bile salts may act synergistically to accelerate ChM dissolution (see Fig. 11). Indeed, feeding CDC and UDC together or in tandem (UDC at night, CDC in the morning) might accelerate dissolution by facilitating the formation of liquid crystals in the UDC-rich stagnant bile during the overnight fast and then inducing their rapid clearance during the day by CDC enrichment. Most important of all, low dose UDC may be much more effective than CDC in preventing stone formation and recurrence. It is now virtually certain that the initial gallstone nucleus in labile bile is a ChM-lecithin liquid-crystalline precipitate (30). Owing to the distinct phase relations of UDC-rich bile (Figs. 1 and 2), low biliary levels of UDC may prevent or retard the liquid crystal → ChM transformation that gives rise to solid ChM crystals (30).

We are grateful to Professor William I. Higuchi (University of Michigan, Ann Arbor) for important comments, to Ms. Grace Ko for skillful technical assistance, and to Ms. Elizabeth Steeves, Ms. Claire Supple, and Ms. Rebecca Ankener for typing the manuscript. Dr. Igimi's permanent address is First Department of Surgery, Fukuoka University School of Medicine, Fukuoka, Japan. Dr. Salvioli's permanent address is Scuola di Geriatria e Gerontologia, Ospedale Estense, Modena, Italy. During the tenure of the work, Dr. Carey was in receipt of a Research Career Development Award (AM 00195) from the National Institutes of Health. Supported in part by Research Grant AM 18559 and a grant from Zambon Farmaceutici S.p.a., Bresso-Milano, Italy.

Manuscript received 18 May 1982 and in revised form 31 January 1983.

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