Physicochemical and Physiological Properties of Cholylsarcosine

A Potential Replacement Detergent for Bile Acid Deficiency States in the Small Intestine

Jan Lillienau, Claudio D. Schteingart, and Alan F. Hofmann
Division of Gastroenterology, Department of Medicine, University of California, San Diego, La Jolla, California 92093

Abstract

The properties of cholylsarcosine (the synthetic *N*-acyl conjugate of cholic acid with sarcosine [*N*-methylglycine]) were examined to determine its suitability as a bile acid replacement agent for conditions of bile acid deficiency in the small intestine, which causes fat malabsorption. Previous studies in rodents had shown that the compound was well transported by the liver and ileum and underwent neither deconjugation nor dehydroxylation during enterohepatic cycling. By ^{1}H-nuclear magnetic resonance, cholylsarcosine was found to exist in dilute aqueous solution as an almost equimolar mixture of two geometric isomers—*cis* and *trans* (around the amide bond)—in contrast to cholylglycine, which was present entirely in the *trans* form. The critical micellization concentration was 11 mmol/liter, similar to that of cholylglycine (10 mmol/liter). By nonaqueous titrimetry, the pK'_a of cholylsarcosine was 3.7, only slightly lower than that of cholylglycine (3.9). Cholylsarcosine was poorly soluble below pH 3.7, but highly soluble above pH 4. In vitro, cholylsarcosine behaved as cholylglycine with respect to promoting lipolysis by lipase/colipase. There was little difference between cholylsarcosine and cholylglycine in their solubilization of an equimolar mixture of oleic acid, oleate, and monoolein (designed to simulate digestive products of triglyceride) or in their solubilization of monooleyl-glycerol alone. When a [^{3}H]triolein emulsion with either cholylsarcosine or cholyltaurine was infused intraduodenally in biliary fistula rats, recovery of ^{3}H in lymph was 52±10% (mean±SD) for cholylsarcosine and 52±11% for cholyltaurine. When perfused into the colon of the anesthetized rabbit, cholylsarcosine (5 mmol/liter) did not influence water absorption or permeability to erythritol, in contrast to chenodeoxycholate, which induced vigorous water secretion and caused erythritol loss. We conclude that cholylsarcosine possesses the physicochemical and physiological properties required for a suitable bile acid replacement in deficiency states. (*J. Clin. Invest.* 1992. 89:420–431.) **Key words: malabsorption • steatorrhea • fat digestion • micelles**

Introduction

Bile acids function as biological detergents that form mixed micelles with lipids such as fatty acids and monoglycerides, thereby promoting lipid absorption (1). If the intestinal concentration of bile acids is below the critical micellar concentration (CMC),[1] dietary triglyceride is more slowly absorbed and malabsorption of fat-soluble vitamins occurs (1–7). Bile acid deficiency occurs when bile acid secretion into the proximal intestine is decreased because of hepatobiliary obstruction or biliary diversion or when active bile acid absorption by the distal ileum is severely impaired. When such conditions induce fat malabsorption, unabsorbed fatty acids pass into the colon and may contribute to diarrhea because of their secretory effects (8, 9). Therapy for bile acid deficiency due to bile acid malabsorption has included restriction of dietary fat (10), the administration of desiccated animal bile (11), or the administration of pure bile acids in unconjugated (12, 13) or conjugated (2) form. Low-fat diets are disliked by patients; and desiccated bile preparations may worsen diarrhea, even if they improve steatorrhea (2). Attempts to use synthetic detergents such as nonionic surfactants have had only modest success (14–18).

We reasoned that there was a continuing need for satisfactory bile acid replacement therapy and that the clinical utility of restoring bile acids to a concentration well above their CMC had not been given an adequate evaluation because of lack of a suitable bile acid substitute. We judged that a suitable molecule for bile acid replacement would be required to fulfill a number of physicochemical and physiological criteria: (*a*) it would be insoluble at gastric pH and thus noninjurious to the gastric mucosa; (*b*) it would dissolve rapidly at small intestinal pH; (*c*) in the presence of colipase it would not inhibit pancreatic lipase; (*d*) it would solubilize the products of triglyceride digestion rapidly and efficiently; (*e*) it (and its bacterial metabolites) would not induce colonic secretion; and (*f*) it would be inexpensive and well tolerated, since large quantities (up to 10 mmol/meal) would be required if oral supplementation were to replace the normal secretion of bile acids into the small intestine.

A candidate molecule that might fulfill these criteria is cholylsarcosine, the synthetic sarcosine (*N*-methylglycine) conjugate of cholic acid (Fig. 1). Previous studies from this laboratory, using rodents, have shown that this compound was well transported by the ileal and hepatic transport systems and underwent neither deconjugation nor dehydroxylation during en-

This work was presented in part at the May 1990 meeting of the American Gastroenterological Association and published in abstract form (1990. *Gastroenterology.* 98:A661).

J. Lillienau was a Fellow of the Swedish Medical Research Council, and this paper is in partial fulfillment of the requirements for his doctoral thesis. His present address is Department of Physiological Chemistry, University of Lund, P.O. Box 94, S-22100 Lund, Sweden.

Address correspondence to Alan F. Hofmann, M.D., Ph.D., Division of Gastroenterology, 0813, Department of Medicine, University of California, San Diego, La Jolla, CA 93093.

Received for publication 19 October 1990 and in revised form 10 October 1991.

J. Clin. Invest.

0021-9738/92/02/0420/12 $2.00
Volume 89, February 1992, 420–431

1. *Abbreviations used in this paper:* CMC, critical micellar concentration; CMpH, critical micellization pH; CP, concentration product; IP, ion product; K_{sp}, solubility product; NMR, nuclear magnetic resonance.

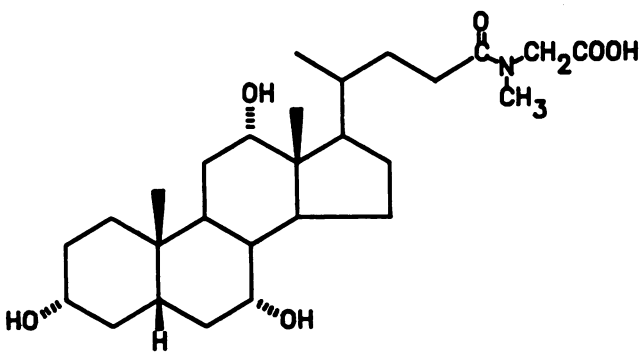


Figure 1. Chemical structure of cholylsarcosine in the protonated form. The A/B ring junction is *cis*. Molecular weight = 479.7 g/mol.

terohepatic cycling (19). The compound was nontoxic and did not induce changes in biliary lipid composition or liver tests when administered chronically (19).

In this paper, we have extended our studies on cholylsarcosine to test whether this synthetic conjugated bile acid fulfills the physicochemical and physiological requirements for a satisfactory bile acid replacement for bile acid deficiency states caused by bile acid malabsorption.

Methods

Chemicals

Bile acids. Cholylsarcosine was synthesized in this laboratory from cholic acid (Fluka Chemie AG, Buchs, Switzerland) and sarcosine ethyl ester hydrochloride (Aldrich Chemical Co., Milwaukee, WI) using the method of Tserng et al. (20). The material was purified by solvent extraction and crystallized in the form of the sodium salt. Chromatographic properties (HPLC and TLC) and structure characterization (^{1}H-nuclear magnetic resonance [NMR], mass spectrometry) have been reported previously (19). Additional material of high purity was provided by Diamalt AB, Raubling, FRG (courtesy of A. Vogt, A. Aigner, and T. Messerschmidt). Cholylglycine was prepared in this laboratory by the method of Tserng et al. (20). Chenodeoxycholic acid was a gift of Diamalt. All bile acids were ≥ 98% pure by TLC and HPLC.

Other chemicals. Human lipase and colipase were prepared and provided by Berit Sternby (Department of Physiological Chemistry, University of Lund, Sweden), who also provided tributyrin (tributyrylglycerol), which was 99.5% pure. Oleic acid (99% pure) and 1-monooleyl-glycerol (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO).

Polymeric dyes (Poly R-478 and Poly B-411) were purchased from Aldrich Chemical Co. The spectroscopic and physiological properties of these dyes have been reported previously (21). Erythritol was obtained from Sigma Chemical Co. and [^{14}C]erythritol was purchased from Amersham Corp. (Arlington Heights, IL).

Triolein was obtained from Nu-chek Prep (Elysian, MN) and [^{3}H]triolein from Amersham Corp. Phosphatidylcholine was purchased from Sigma Chemical Co.

Physiochemical properties

^{1}H-NMR spectra were determined at 360 MHz at 21°C; chemical shifts were referred to tetramethylsilane. In the spectrum in D_2O, the signal of HDO was used as reference δ_{HDO} 4.63.

The acidity constant, or the apparent pK_a, was determined by nonaqueous titrimetry as described by Fini and Roda (22), with some modifications. Experimental details are presented here; the theoretical treatment is given in the Appendix.

The pH* was measured using a combination pH calomel electrode and a pH meter (Accumet model 915; both from Fisher Scientific Co., Pittsburgh, PA), calibrated against standard aqueous buffer solutions. Solutions of the bile acid (acid form), 2 mmol/liter in methanol/water mixtures of composition 10:90, 30:70, and 50:50 wt/wt were prepared. Each solution was titrated with a 20.0 mmol/liter NaOH methanol/water solution of the same composition, with continuous pH* monitoring. The pH* at one-half neutralization was considered to be the pK_a^* for the particular methanol/water composition. The pK_a^*s were plotted versus the mole fraction of methanol (n_{MeOH}) in the solvent (calculated according to Eq. 1), and a regression line was calculated by the method of least squares. Extrapolation to zero mole fraction of methanol afforded the corresponding pK_a in water solution. A control experiment with acetic acid gave pK_a 4.8, in agreement with the reported value (23).

$$n_{MeOH} = (\%/\text{mol wt}_{MeOH})/[(\%/\text{mol wt}_{MeOH}) + (\%/\text{mol wt}_{water})]$$

(% = wt/wt% of methanol in solvent mixture) (1)

The apparent aqueous solubility of the protonated form of cholylsarcosine and cholylglycine was determined by incubating 20 or 100 μmol of the bile acid in 4 ml water or saline at room temperature. The pH of the water or saline was adjusted to < 2 or 3.0 with an appropriate volume of 1.0 mol/liter HCl. After 3, 7, or 60 d of incubation, a small sample was taken and filtered through a 0.45-μm filter (model HV; Millipore Corp., Bedford, MA). Some samples were centrifuged at 14,000*g* for 15 min to remove any microcrystals that might have passed through the filter. The bile acid concentration in the samples was determined by HPLC (24), as well as by enzymatic assay using a 3-hydroxysteroid dehydrogenase assay (25).

Interaction with calcium. The solubility of the calcium salt of cholylsarcosine was determined according to Jones et al. (26). Samples of cholylsarcosine or cholylglycine solutions ranging in concentration from 25 to 75 mmol/liter were mixed with 10–75 mmol/liter $CaCl_2$ and 0.15 mol/liter NaCl in a total volume of 4 ml. The samples were thoroughly mixed, incubated at room temperature for 1 wk, and thereafter centrifuged. The total calcium concentration in the supernatant was determined by a fluorescent dye-binding technique (Calcette model 4009, Precision Systems, Inc., Natick, MA). In addition, in a few experiments the Ca^{++} activity ($a_{Ca^{++}}$) was determined using an $a_{Ca^{++}}$-specific electrode (Orion Research, Inc., Boston, MA) in the laboratory of E. W. Moore (Medical College of Virginia, Richmond, VA) (27). A 3-hydroxysteroid dehydrogenase method was used to determine bile acid concentration ([BA]) in the supernatant (25). The concentration product (CP) was calculated using the equation $CP = [Ca^{++}] \cdot [BA]^2$, assuming that the insoluble calcium salt had this stoichiometric composition. In addition, the $a_{Ca^{++}}$ was measured in two samples to give the ion product (IP), which is not very different in value from the solubility product (K_{sp}). The IP was calculated using the equation $IP = a_{Ca^{++}} \cdot [BA]$ (26). Since the bile acid concentration rather than activity was measured, the calculated value for the IP might be slightly higher than the true value for the K_{sp}.

Critical micellization pH (CMpH). The CMpH was measured in a manner similar to that reported by van Berge Henegouwen et al. (28). Stepwise increasing amounts of 1 mol/liter HCl were added to a series of bottles containing 25 mmol/liter of a solution of the sodium salt of cholylsarcosine or cholylglycine; the solutions also contained 0.15 mol/liter NaCl. The samples were incubated for 3 d at 37°C, after which the pH was measured in each sample, using a combination pH calomel electrode and a pH meter (both from Fisher Scientific Co.). The samples were then centrifuged (10,000*g* for 15 min), and the bile acid concentration in the supernatant was measured, using an endpoint 3-hydroxysteroid dehydrogenase–based assay (Sigma Chemical Co.), as described by Turley and Dietschy (25). The bile acid concentration (ordinate) was plotted against pH, and a vertical line was drawn through the vertical part of the curve toward the *x*-axis. The intercept of this line with the abscissa was defined as the "CMpH."

The CMCs of the sodium salts of cholylsarcosine, cholylglycine, cholyltaurine, and cholic acid were determined using a dynamic surface tension device based on the maximum bubble pressure method. The device, which was designed by K. J. Mysels, has been described in

detail (29, 30); and the method has been fully validated (31). It has been used previously by this laboratory to measure the CMC of bile acids (31). For the present studies, bile acids were repurified by column chromatography to an HPLC purity exceeding 99%, and great care was taken to keep all surfaces uncontaminated by surface-active impurities. The surface tension was measured at different concentrations at bubble intervals of 1 and 2 s. The surface tension (ordinate) was plotted against the logarithm of the bile acid concentration. This gave two lines: one above the CMC, which showed little change in surface tension with increasing concentration, and one below the CMC, which showed a near-linear concentration-dependent decrease in surface tension. The linear regressions of the two lines were calculated using the method of least squares and the intersection of the two lines was taken as the CMC. For bile acids tested, the difference in CMC between two bubble intervals (1 and 2 s) was < 5%; accordingly, all bile acids were considered to be uncontaminated significantly by surface-active impurities. All measurements were done with a total Na^+ concentration of 0.15 mol/liter. The surface pressure (i.e., the decrease in surface tension) at 1 mmol/liter and at the CMC was calculated by subtracting the measured surface tension at 1 mmol/liter and at the CMC from the surface tension of water at 37°C (70.1 dyn/cm).

CMC of cholylsarcosine in the presence of (1)-monooleyl-glycerol. Ekwall and Danielsson showed in 1951 (32) that detergents such as sodium laurate will form mixed micelles in the presence of a long chain alkanol at a concentration well below the CMC of the detergent alone. An experimental method similar to that used by Hofmann (33) was used to define the CMC for cholylsarcosine or cholylglycine in the presence of (1)-monooleyl-glycerol. Cholylsarcosine or cholylglycine was incubated at concentrations ranging from 2 to 10 mmol/liter with (1)-monooleyl-glycerol at concentrations ranging from 0.5 to 15 mmol/liter. Bile acids and (1)-monooleyl-glycerol were dispersed in a phosphate buffer at pH 6.3 and physiological NaCl concentration. The samples were placed on a mixing table at 37°C overnight. Solubility was measured turbidimetrically by the optical density at 550 nm. At an OD < 0.020, the samples were considered to be in a (isotropic) micellar phase. The samples with the highest (1)-monooleyl-glycerol concentration in a micellar phase were plotted against the bile acid concentration.

Micellar solubilization of a fatty acid and monoglyceride mixture. Equimolar amounts of oleic acid, oleate, and monoolein (termed an oleyl mixture) were mixed with different proportions of bile acid to make a total substance concentration of 20 mmol/liter (cf. reference 34). After overnight incubation, the turbidity (indicated by absorbance at 400 nm) was measured using a spectrophotometer (Uvikon model 860, Kontron Instruments, Milan, Italy).

In vitro lipolysis. The effect of cholylsarcosine, cholylglycine, cholyltaurine, or deoxycholyltaurine on the activity of pure human pancreatic lipase/colipase, carboxyl ester lipase, as well as on the lipolytic activity present in aspirated human pancreatic juice, was determined using the assay system described by Erlanson and Borgström (35). Samples were acquired at the Lund University Hospital some years ago from patients without pancreatic disease; informed consent was obtained. The substrate consisted of 0.5 ml tributyryl-glycerol that was emulsified in 10 ml buffer (consisting of 1 mmol/liter Tris-maleate, 1 mmol/liter $CaCl_2$, and 0.15 mol/liter NaCl at pH 7.0). Hydrolysis was induced by the addition of the following: (*a*) 25 μl lipase (100 μg/ml) and 5 μl colipase (100 μg/ml), (*b*) 50 μl cholesterol ester lipase (1 mg/ml), or (*c*) 10 μl pancreatic juice. The bile acid concentration was 4.0 mmol/liter, except for lipase/colipase, where 10 mmol/liter also was tested. The assay was performed at room temperature; for lipase/colipase, the assay was also performed at 37°C. A video titrator (model VIT 90; Radiometer, Copenhagen, Denmark) was used to measure the rate of fatty acid release. The last 2 min of a 5-min incubation were used to calculate the activity of the enzymes, which was expressed as μkatal/mg protein (katal = amount of enzyme activity that transforms 1 mol/s of substrate). The activity of pancreatic juice was expressed in percentage of cholylsarcosine rather than μkatal/mg protein, since pancreatic juice contains multiple lipolytic enzymes.

Physiological experiments

Triolein absorption in lymph fistula rats. These experiments were done in the laboratory of B. Borgström (Dept. of Physiological Chemistry, University of Lund, Sweden) using a technique developed by Tso et al. (36). Sprague-Dawley rats (ALAB, Södertälje, Sweden), weighing 230–240 g, were fasted overnight, anesthetized with ether, and a midline abdominal incision was made. A segment of vinyl tubing (Dural Plastics and Engineering, Dural, Australia, 0.5 mm i.d.), with a PP-10 (Portex) tip, was inserted in the proximal biliary duct, and the thoracic duct was cannulated using identical tubing. Another segment of tubing was passed through the stomach wall and the tip positioned 30 mm into the duodenum. The animals were housed at 30°C in Bollman restraining cages; chow was withheld but water was allowed ad lib. A solution of 140 mmol/liter glucose, 85 mmol/liter NaCl, and 6.7 mmol/liter KCl was infused overnight into the intestine in order for the animals to recover from surgery and stabilize the lymph flow. The following morning a fat emulsion consisting of 5 mmol/liter triolein, [^{3}H]triolein (0.17 MBq), 1 mmol/liter phosphatidylcholine, and 15 mmol/liter of cholylsarcosine or cholyltaurine was infused. Cholyltaurine was used as a control in this study, since it is more commonly used in this type of experiment. The fat was emulsified in the glucose solution described above with the help of ultrasound. The emulsion with one of the bile acids was infused at a rate of 3.0 ml/h over 8 h the day after surgery. The other bile acid was infused the following day. Lymph and bile were collected in 1-h aliquots for 20 h. Since the emulsions were not completely stable and emulsion droplets collected at the top of the tube, a sample of the fat emulsion was taken at the beginning and end of the infusion to correct for a decrease in the concentration of dispersed triglyceride droplets. Although this is an experimental problem, the results still allow a comparison of the two bile acids.

For analysis, lymph flow was measured gravimetrically and radioactivity was measured in 0.5 ml of lymph. Scintillation cocktail (10 ml) was added and the samples were counted in a liquid scintillation counter.

Perfusion of rabbit colon with bile acid solutions. A technique previously described by Chadwick et al. (37) was used. Male New Zealand rabbits (Holbert's Rabbitry, Spring Valley, CA), weighing 1.45–1.75 kg, were housed in the animal quarters ≥ 1 wk before use. The animals were anesthetized intraperitoneally with ketamine after an overnight fast, and a midline abdominal incision was made. A PE-160 catheter was inserted through the cecal wall and pushed 1 cm into the colon. The tubing was secured with a purse-string suture in the cecal wall, and the colon was tied loosely around the tubing as it entered the colon. The entire colon was rinsed with 100–150 ml saline at 37°C or until the effluent was clear. To collect effluents, a 24-Fr Foley silicone catheter was inserted 3 cm into the rectum and the balloon inflated. Body temperature was maintained at 38±0.5°C with a heating lamp connected to a temperature probe. Before bile acid perfusion, the colon was perfused at a rate of 1.3 ml/min for 60 min with a perfusion solution prepared to simulate colonic contents (120 mmol/liter NaCl, 5 mmol/liter KCl, and 25 mmol/liter $NaHCO_3$, pH 8.0). The perfusate was preheated to a temperature of 38°C. A nonabsorbable polymeric dye marker, Poly-R or Poly-B, was added to quantify water absorption/secretion (21) and [^{14}C]erythritol (18 kBq, together with 1 mmol/liter erythritol as carrier), was added to allow assessment of epithelial permeability. Each animal was perfused for 60 min with 5 mmol/liter of cholylsarcosine, cholyltaurine, or chenodeoxycholate perfusion solution. Either cholylsarcosine or cholyltaurine was perfused after the control period, whereas chenodeoxycholate always was perfused as the last bile acid since it is known to be cytotoxic, and the changes induced might not be rapidly reversible. 10-min aliquots of the colonic effluent were taken during the entire experiment.

Examination of the effluent by TLC showed that it took 20–40 min to completely replace one bile acid by the following one (38). Accordingly, the fourth to the sixth collection period and the first of the following collection period were considered to represent steady-state intervals for water secretion/absorption and erythritol loss determination.

For analysis of radioactivity, 500 μl of each aliquot was pipetted into a scintillation vial and 10 ml of Scinti-Verse BD (Fisher Scientific Co.) was added. For analysis of absorbance, 2 ml was taken off and used for determination of the absorbance at 520 nm (Poly-R) or 595 nm (Poly-B) in a spectrophotometer (Gilford, Instrument Laboratories, Inc., Oberlin, OH). Calculations were done as follows. Water absorption/secretion was calculated using Eq. 2:

$$A_w = F[(OD_s/OD_o) - 1], \quad (2)$$

where A_w is absorption in ml/U, F is perfusate flow in ml/h, OD_s is optical density of sample, and OD_o is optical density of unused perfusate. Percent loss of [^{14}C]erythritol was calculated using Eq. 3:

$$L_e = [1 - (DPM_s/DPM_o)/(OD_s/OD_o)]\ 100, \quad (3)$$

where L_e is loss of [^{14}C]erythritol in percent, DPM_s is DPM in sample, DPM_o is DPM in infused perfusate, OD_o is optical density of infused perfusate, and OD_s is optical density of sample.

Statistics

All values are mean ± standard deviation. When needed, the Student's *t* test was used to determine statistical differences using the SOLO Statistical System Version 2.0 from BMDP Statistical Software, Inc. (Los Angeles, CA).

Results

Physicochemical properties

The ^{1}H-NMR spectrum of the acid form in d_6-dimethylsulfoxide (Table I) showed a *trans/cis* ratio of 3, that is, 75% of the compound was present as the *trans* conformer. This behavior parallels that of a simpler model compound, acetylsarcosine, where 70% is in *trans* form for the acid form and 48% is in the *trans* form when the anion is present (39). The ^{1}H-NMR spectrum of the sodium salt of cholylsarcosine in deuterated water at a concentration below the CMC (3 mmol/liter) (Fig. 2*a* and Table I) showed two sets of peaks for many hydrogens. This is in sharp contrast to the ^{1}H-NMR spectra of glycine or taurine (40) conjugates of bile acids, where only one resonance is found for each type of hydrogen. A typical example, the ^{1}H-NMR spectrum of the sodium salt of cholylglycine, under the same conditions is presented in Fig. 2 *b*, and Table I. The two sets of peaks in the case of cholylsarcosine are due to the presence of two isomers, *trans* and *cis* (see Fig. 9), by rotation around the amide bond. The two isomers interconvert slowly on the NMR time scale and are therefore observed as two sets of resonances. The assignment of the *trans* and *cis* resonances for the *N*-methyl group and the α-CH_2 group of glycine was based on well-known chemical shift differences for groups E or Z to the amide carbonyl (41). Cholylglycine presents only one set of resonances, which can be safely attributed to the almost exclusive presence of the *trans* isomer. It has been found that amides with *one* alkyl substituent on the nitrogen atom exist in solution preponderantly in the *trans* form; in most cases, the *cis* form is not detectable (39, 41).

The cholylsarcosine *trans/cis* ratio in water was 0.83 and was not altered at 15 mmol/liter (above CMC) or in the presence of 0.15 M NaCl. (The spectra were essentially identical to Fig. 2a and are not shown.)

Acidity constant. The pH* measured when 50% of the acid had been titrated was plotted against mole fraction of organic solvent for each of the bile acids as well as for acetic acid (Fig. 3), and the regression line was calculated for the three points. The pK_a calculated from the regression lines were 3.7 for cholylsarcosine and 3.9 for cholylglycine. Thus, cholylsarcosine is a stronger acid than cholylglycine, its pK_a being 0.2 pK_a units lower than that of cholylglycine. The pK_a of acetic acid was also measured by this technique, and the expected value of 4.8 was obtained.

The apparent aqueous solubility of the protonated form for cholylsarcosine was 2.4±0.0 mmol/liter and that of cholylglycine 0.6±0.0 mmol/liter when incubated for 60 d in 0.15 mol/liter NaCl at a pH < 2 with 25 μmol bile acid per ml solution. In incubations set at pH 3, a slightly higher solubility of the bile acids was measured: cholylsarcosine, 3.1±0.0 mmol/liter and cholylglycine, 0.7±0.0 mmol/liter. The higher solubility at this pH is explained by the additional presence of cholylsarcosine anions. Cholylsarcosine, having a lower pK_a than cholylglycine, will display a greater increase in solubility at this pH. Determination of the bile acid concentration with 3-hydroxysteroid dehydrogenase confirmed the HPLC results.

Interaction with calcium and solubility of the calcium salt. Table II shows the concentration of cholylsarcosine, cholylgly-

Table I. ^{1}H-NMR Data of Bile Acid Conjugates According to Conformation of the Amide Bond

Peak assignment	Cholylsarcosine in d_6-DMSO (10 mmol/liter)		Cholylsarcosine sodium salt in D_2O (3 mmol/liter)		Cholylglycine sodium salt in D_2O (3 mmol/liter)
	cis	*trans*	*cis**	*trans**	*trans**
Me-18	0.580	0.592	0.537	0.550	0.513
Me-19	0.809	0.809	0.735	0.738	0.709
Me-21	0.902 d, 6.5 Hz	0.945 d, 6.5 Hz	0.787 d, 6.5 Hz	0.825 d, 6.1 Hz	0.775 d, 6.1 Hz
N-CH_3	2.790	2.999	2.735	2.906	—
H-3	3.18 m	3.18 m	3.320 m	3.320 m	3.296 m
H-7	3.61 m	3.61 m	3.723 bs	3.723 bs	3.692 bs
N-CH_2-COO	3.92–4.00 m	3.92–4.00 m	3.800	3.723 bs	3.526 d, 2.5 Hz
H-12	3.79 m	3.79 m	3.86–3.91 m	3.86–3.91 m	3.863 m
Molar ratio	1	3	1	0.83	—
form present (%)	25	75	55	45	100

* The assignment of *cis* and *trans* has been made on the basis of the behavior of the model compounds discussed, but has not been confirmed for these compounds by an independent method. Singlets are not indicated. *Trans/cis* ratio calculated by integration of the N-CH_3 signals. Me, methyl group; m, multiplet; d, doublet; bs, broad singlet.

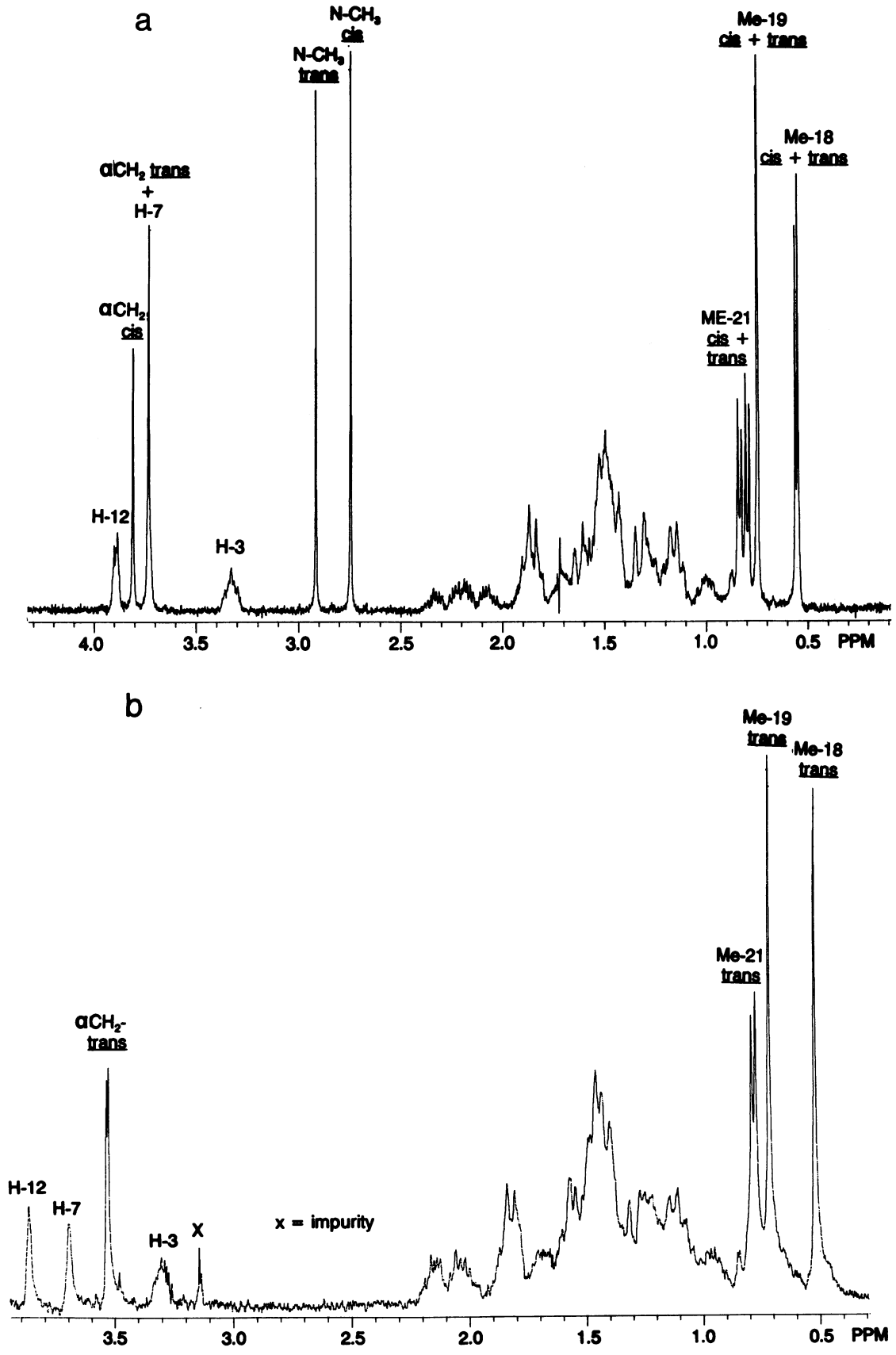


Figure 2. ^1H-NMR of the sodium salt of cholylsarcosine (*a*) and the sodium salt of cholylglycine (*b*) (3 mmol/liter in D_2O, 360 MHz, HDO = 4.63 ppm).

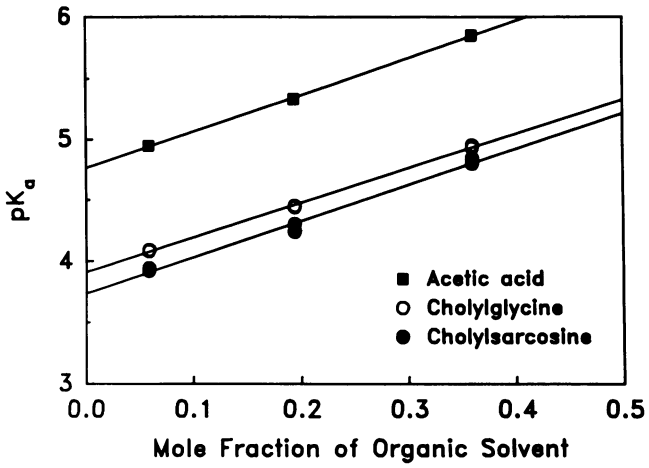


Figure 3. Determination of the acidity constant (pK_a) by plotting the pH^* measured when 50% of the acid was titrated against mole fraction of organic solvent. The regression line was calculated for the three points and the pK_a determined by extrapolation to a mole fraction of zero.

cine, and $CaCl_2$ in the incubations, whether a precipitate was formed, the concentration product of the bile acid and Ca^{++} in the supernatant, and the ion product based on measurement of $a_{Ca^{++}}$ and bile acid concentration in the supernatant. Precipitates occurred with cholylsarcosine only at a concentration of 10 mmol/liter $CaCl_2$ and did not occur with cholylglycine. The solubility product for cholylglycine could not be obtained in these experiments since it did not precipitate from solution; solutions of cholylglycine that are supersaturated with respect to the calcium salt are known to manifest remarkable metastability (26). Thus, although the solubility product of calcium salt of cholylsarcosine is quite high, its supersaturated solutions, in contrast to those of cholylglycine, do not inhibit metastability. The incubations were in 0.15 mol/liter NaCl, which will decrease the activity coefficient of cholylsarcosine. Thus, the calculated IP of $1.8 \pm 0.4 \times 10^{-7}$ (mol/liter)^3 for cholylsarcosine is approximately twice the true value of the K_{sp} which is $\sim 1 \times 10^{-7}$ (mol/liter)^3.

CMpH. Fig. 4 illustrates the bile acid concentration in supernatant in relation to final supernatant pH. The bile acid concentration increased very sharply over a narrow pH range, which indicates the CMpH. The CMpH value for cholylsarcosine was 3.7, a value considerably lower than that of cholylglycine, which was 4.8.

The CMCs for the sodium salts of cholylsarcosine, cholylglycine, cholyltaurine, and cholic acid ranged from 9 to 11 mmol/liter, respectively, indicating that all three cholic acid conjugates had a CMC similar to that of the unconjugated bile acid sodium cholate (Table III). The surface activity, as evidenced by the decrease in surface tension with increasing concentration, was similar for all cholic acid conjugates. Typical curves for cholylsarcosine and cholylglycine are shown in Fig. 5.

CMC of cholylsarcosine in the presence of 1-monooleyl glycerol. Fig. 6 shows the maximum solubility of (1)-monooleylglycerol for a given bile acid concentration. It also shows that, although the CMCs of cholylsarcosine and cholylglycine are around 10 mmol/liter, these bile acids form mixed micellar solutions at a concentration > 5 mmol/liter when an amphiphile, such as (1)-monooleyl-glycerol, is also present. The CMC for cholylsarcosine and cholylglycine in the presence of such a C_{18} amphipathic additive is 5 mmol/liter.

Micellar solubilization of fatty acids and monoglycerides. The turbidity at different concentrations of an oleyl mixture and cholylsarcosine or cholylglycine is presented in Table IV. There was only a slightly lower solubilization capacity for cholylsarcosine at a ratio of 3:2 oleyl additives/bile acid.

In vitro lipolysis. Table V summarizes the results from the lipolysis experiments with human pancreatic lipase/colipase, cholesterol ester lipase, or pancreatic juice. No statistical differences were seen between the trihydroxy bile acids in incubations with lipase/colipase; however, the activity of cholesterol ester lipase was significantly higher in the cholylglycine incubations and significantly lower in the cholyltaurine and deoxycholyltaurine incubations. Pancreatic juice showed a higher activity with cholylglycine compared with cholylsarcosine. Lipase/colipase was also incubated with 10 mmol/liter of each

Table II. Interaction of Cholylsarcosine Solutions with Ca^{2+} Ions

	Bile acid concentration	$CaCl_2$ concentration	Cholylsarcosine precipitate present	Cholylglycine precipitate present	Concentration product $[Ca^{2+}][CS^-]^2$	Ion product
	mmol/liter	*mmol/liter*			*(mol/liter)^3*	*(mol/liter)^3*
1	25	25	+	−	1.3×10^{-7}	1.6×10^{-7}
2	25	10	−	−	−	
3	50	50	+	−	3.1×10^{-7}	
4	50	25	+	−	1.8×10^{-7}	2.1×10^{-7}
5	75	75	+	−	4.6×10^{-7}	
6	75	50	+	−	3.4×10^{-7}	
7	75	25	+	−	2.9×10^{-7}	
8	75	10	−	−	−	

The concentration product was calculated from the Ca^{++} ion concentration and bile acid concentration in the supernatant of the incubations. The value for the ion product was based on Ca^{++} activity rather than concentration and should give a value about twice that of the true solubility product. The approximate solubility product for the calcium salt of chenodeoxycholylglycine is 0.2×10^{-7}; that of ursodeoxycholylglycine is 0.4×10^{-7}, and that of cholylglycine is $\sim 6 \times 10^{-7}$ (mol/liter)^3 (32).

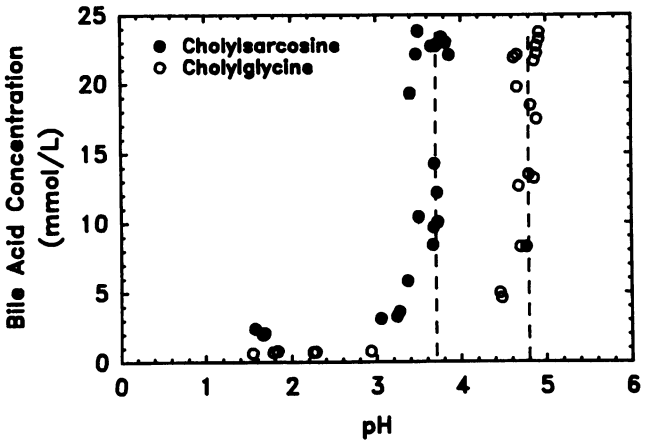


Figure 4. Solubility of cholylsarcosine in relation to pH. The critical micellar pH is defined as a pH value within the narrow range of pH over which solubility increases markedly and is indicated by the intersection of the dashed vertical line with the *x*-axis.

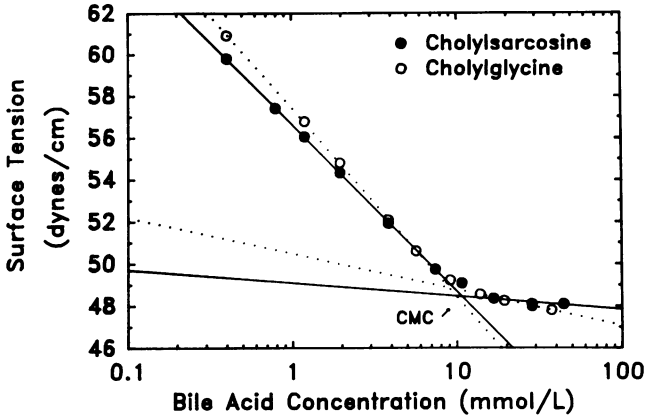


Figure 5. Determination of critical micellization concentration (CMC) for cholylsarcosine and cholylglycine in 0.15 mol/liter NaCl by a maximum bubble pressure technique. Surface tension is shown in relation to aqueous concentration (logarithmic scale). The CMC is defined as the intercept of the lines given by the surface tension above and below the CMC. The CMC for cholylsarcosine was 11 mmol/liter and for cholylglycine 10 mmol/liter.

bile acid. Observed catalytic activities were similar with all bile acids (data not presented).

Physiological properties

Triolein absorption in lymph fistula rats. The total recovery of infused [^{3}H]triolein (15 μmol/h) in lymph was 52±10% for cholylsarcosine and 52±11% for cholylglycine. There was no significant difference between the two bile acids. Fig. 7 shows a graph of the time course of ^{3}H excreted in lymph. Both bile acids gave a similar pattern of ^{3}H recovery, with a plateau reached after 5 h of infusion. Fat output in lymph dropped sharply as soon as infusion of the fat/bile acid emulsion was stopped. (Unpublished observations from Dr. Patrick Tso, Shreveport, LA, showed no difference between cholylsarcosine and cholyltaurine when a dose of 40 μmol/h triolein was infused in the rat. This dose is closer to the normal fat intake of the rat.)

Effect of cholylsarcosine on water movement in the perfused rabbit colon. Fig. 8, *top,* indicates that cholylsarcosine did not induce colonic secretion; nor did cholylglycine at a concentration of 5.0 mmol/liter. In contrast, when chenodeoxycholate was infused, absorption changed to marked secretion ($P < 0.0001$). There was no change in permeability to erythritol during cholylsarcosine (or cholylglycine) infusion, indicating that these conjugated trihydroxy bile acids did not induce mucosal damage (Fig. 8, *bottom*). In contrast, chenodeoxycholate caused considerable loss of erythritol (22.8±5.8%) ($P < 0.0001$).

Table III. Critical Micellization Concentration and Surface Pressure for Sodium Salts of Cholylsarcosine, Cholylglycine, Cholyltaurine, and Cholic Acid as Measured by a Maximum Bubble Pressure Method

Bile acid	Critical micellization concentration*		Surface pressure‡	
	1-s bubble interval	2-s bubble interval	at 1 mmol/liter	at CMC
	mmol/liter		*dynes/cm*	
Cholylsarcosine	11	10	13.5	21.0
Cholylglycine	9	9	12.8	20.9
Cholyltaurine	10	11	9.8	18.4
Cholate	11	10	9.5	21.2

* There are no statistical differences between any of the measured critical micellization concentrations. ‡ Surface pressure = surface tension$_{solvent}$ − surface tension$_{solvent + bile\ acid}$ at the indicated concentrations.

Discussion

These experiments suggest that cholylsarcosine possesses the requisite physicochemical and physiological properties of an

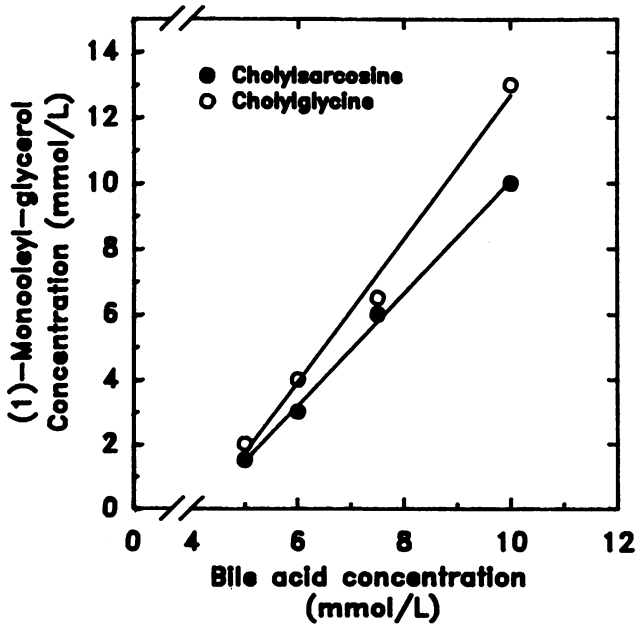


Figure 6. Incubation of cholylsarcosine or cholylglycine with (1)-monooleyl-glycerol at pH 6.3. Graph shows highest concentration of (1)-monooleyl-glycerol for each bile acid concentration where the amphiphiles were in an isotropic micellar phase.

Table IV. Solubilization of Oleyl Mixture by Cholylsarcosine Solutions

Oleyl* mixture concentration	Bile acid concentration	Absorbance at 400 nm	
		Cholylsarcosine	Cholylglycine
mmol/L			
0	20	0.011	0.004
4	16	0.018	0.018
8	12	0.043	0.038
12	8	1.635	1.536
16	4	2.812	2.853

* Oleyl mixture = 1/3 oleic acid, 1/3 oleate, and 1/3 mono-olein. This mixture corresponds approximately to the products formed by the action of pancreatic lipase on triolein at pH 6.5 (33).

oral replacement agent for conditions of bile acid deficiency in the proximal small intestine. Cholylsarcosine had physicochemical properties that clearly differed from those of cholylglycine, indicating that the presence of an *N*-methyl group in the amino acid moiety influences ionization, apparent aqueous solubility, and interaction with Ca^{++} ions. Nonetheless, the magnitude of these differences was small and probably of little biological significance. The physiological properties of cholylsarcosine and cholylglycine, such as hepatic and ileal transport, have also been shown previously to be quite similar (19). Thus, the only major effect of the *N*-methyl group is to greatly inhibit deconjugation-dehydroxylation by bacterial enzymes during enterohepatic cycling.

Physicochemical properties

^{1}H-NMR and pK_a values. In nature, bile acids are conjugated with taurine or glycine through an *N*-acyl (amide) linkage. Because of the partial double bond character of the amide C—N bond, two conformers are possible: *cis,* where the larger group (glycine, taurine) on the nitrogen atom is close to the bile acid side chain; and *trans,* where the bulky group is on the same side as the amide carbonyl (Fig. 9). Both isomers interconvert easily at room temperature by rotation around the amide C—N bond, but the *trans* conformer appears to be the only one found in water solutions of common bile acids (Fig. 2 *b*) (40). The significant steric interference between the 23-methylene group and the methylene group of glycine (or β methylene of taurine) present in the *cis* isomer makes this conformer less stable than the *trans* isomer, where the 23-methylene group is close to the sterically less demanding hydrogen atom.

In *N,N*-dialkyl amides, both isomers are generally present in solution in proportions that depend on the relative free energy of the two isomers (41). Sarcosine conjugates have two groups of similar size on the nitrogen atom: the methylene group of glycine and the *N*-methyl group; the *cis* (glycine close to side chain) and *trans* (*N*-methyl close to side chain) isomers have, therefore, similar energies (Fig. 9). Accordingly, the water solution of the sodium salt of cholylsarcosine contained a 0.83:1 mixture of the *trans* and *cis* isomers (Table I).

Although the rate of interconversion is fast at room temperature, the presence of a significant proportion of *cis* isomer provides an explanation for sarcosine conjugates possessing physicochemical properties slightly different from those of natural bile acids. The pK_a of cholylsarcosine provides an example: cholylsarcosine is slightly more acidic (pK_a 3.7) than cholylglycine (pK_a 3.91). This difference also parallels that of the model compounds acetylsarcosine, pK_a 3.34 (calculated from reference 39), and acetylglycine, pK_a 3.67 (42). Evans and Rabenstein (39) found that the *trans* conformer of acetylsarcosine is less acidic (pK_a 3.5) than the *cis* conformer (pK_a 3.1). The evidence seems to indicate that in the *trans* form the acidic proton forms an intramolecular hydrogen bond with the amide carbonyl (Fig. 10), whereas in the *cis* form this is not possible. This renders the *trans* conformer of the protonated acid more stable, thus decreasing its acidity compared with the *cis* isomer. In accordance with these observations, the ^{1}H-NMR spectra of both cholylsarcosine (Fig. 1) and acetylsarcosine (39) showed a high *trans/cis* ratio (3 and 2.3, respectively) for the acid forms; and acetylglycine, which should exist almost exclusively in the *trans* form, has a pK_a (3.67) very close to the *trans* conformer (3.5) of acetylsarcosine. Our pK_a and ^{1}H-NMR measurements suggest that this description can be safely extended to the cholylsarcosine case. Thus, the presence of a substantial proportion of the more acidic *cis* conformer in sarcosine conjugates will contribute to an increase in the overall macroscopic acidity constant compared with all *trans* glycine conjugates.

The rather high apparent aqueous solubility of the protonated form of cholylsarcosine was quite unexpected. The protonated form of cholylsarcosine forms a hydrated gum in water, which may promote aqueous solubility or may have led to contamination of the aqueous phase by hydrated microcrystals that were not removed by filtration or centrifugation; in contrast, cholylglycine remains present as discrete crystals.

Table V. Hydrolysis of Tributyrylglycerol by Human Pancreatic Lipase/Colipase, Cholesterol Ester Lipase, or Human Pancreatic Juice

	Temperature	Bile acid (4 mmol/l)			
		Cholylsarcosine	Cholylglycine	Cholyltaurine	Deoxycholyltaurine
	°C				
Lipase/colipase (*μkatal/mg protein*)	23	51.2±0.3	52.0±6.4	50.1±1.2	36.3±1.3§
Lipase/colipase (*μkatal/mg protein*)	37	73.6±4.4	69.4±5.6	71.8±7.0	50.0±5.7‡
Cholesterol ester lipase (*μkatal/mg protein*)	23	1.00±0.01	1.11±0.02§	0.81±0.02§	0.88±0.03‡
Human pancreatic juice (Percent of rate observed for CS; CS = 100%)	23	100±2	128±11‡	107±5	116±5‡

The assay was performed at pH 7.0. Statistical difference relative to cholylsarcosine is indicated by: * $P < 0.05$; ‡ $P < 0.01$; § $P < 0.001$.

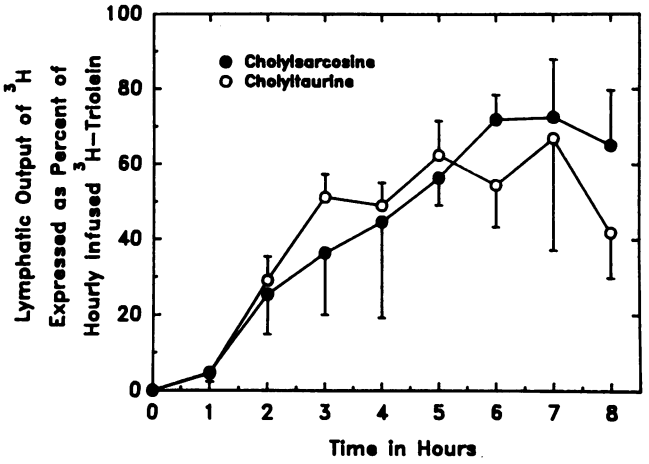


Figure 7. Recovery of [^{3}H]triolein in lymph after intraduodenal infusion of an emulsion of [^{3}H]triolein, phosphatidylcholine and bile acid in rats with both a lymph and biliary fistula. There is no statistical difference between the two bile acids (mean ± SD, n = 3).

Cholylsarcosine differed from cholylglycine in having a slightly lower solubility product of its calcium salt. Nonetheless, the K_{sp} of the calcium salt of cholylsarcosine was one to two orders of magnitude higher than the corresponding calcium salts of deoxycholylglycine or chenodeoxycholylglycine (26). Accordingly, formation of the calcium salt in the gallbladder is unlikely, since the ionized calcium concentration in gallbladder bile is usually < 2 mmol/liter (43) and the monomeric concentration of cholylsarcosine will be in the range of 1–2 mmol/liter (44).

Micellar aggregation. The CMpH was considerably lower for cholylsarcosine than for cholylglycine. Probably two factors are responsible. The first is the greater solubility of the protonated form of cholylsarcosine at low pH. When the protonated species is more soluble, there is a corresponding increase in the concentration of the ionized species at acidic pH. As the pH is increased, there is an exponential increase in the concentration of the ionized form; and when it reaches the CMC (at the CMpH), micelles are formed. A higher concentration of protonated species may promote the formation of mixed micelles containing both species at a lower bulk pH. A second factor contributing to the lower CMpH of cholylsarcosine is the slightly lower pK_a of cholylsarcosine, resulting in greater ionization of cholylsarcosine for a given bulk pH.

The CMC of cholylsarcosine was very close to its glycine and taurine congeners, which implies that it will form mixed micelles in the jejunum. The average concentration of bile acids in the human jejunum is 5–10 mmol/liter (6), which is about the CMC of cholylsarcosine. However, the CMC for cholylsarcosine in the presence of a C_{18} 1-monoglyceride was 5 mmol/liter, which indicates that mixed micelles form, well below the CMC of cholylsarcosine alone. Whether a bile acid will form mixed micelles below its CMC in vivo will depend on its interaction with other biological amphiphilic molecules such as monoolein, oleic acid, and phosphatidylcholine, which all are present in jejunum during digestion (32, 44, 45). The measured CMC for cholylglycine in the presence of a C_{18} 1-monoglyceride was in good agreement with literature data (33).

The surface pressure of cholylsarcosine was also similar to cholylglycine and cholyltaurine. Recent results have shown that dihydroxy bile acids with high surface pressure, such as deoxycholyl and chenodeoxycholyl conjugates, can cause histamine release from mast cells (46) or worsen *Escherichia coli*–induced peritonitis (47). This is not observed with trihydroxy conjugates and is unlikely to occur with cholylsarcosine. The pH in the stomach at the start of a meal is ~ 3, rising to 4–5 during digestion (48). Thus, cholylsarcosine might cause gastric irritation since it is soluble at a pH > 3.7.

Solubilization of the oleyl mixture was very similar for cholylsarcosine and cholylglycine. Catalysis and hydrolysis of tributyrin by lipase and colipase, or by carboxyl ester lipase, as well as lipolytic activity of human pancreatic juice, were very similar for cholylsarcosine and common natural bile acids. Thus, the presence of the *N*-methyl group had little effect on pancreatic lipolytic activity.

Physiological properties

The results from the intestinal perfusion studies in lymph fistula rats indicated that cholylsarcosine functions as well as cholyltaurine as an enhancer of triolein absorption in this particular animal model. The steady-state lymphatic output of 65% of the triglyceride infused (Fig. 7) during triolein/cholylsarcosine administration was similar to results from other investigators using the same experimental model (49). Incomplete recovery may indicate incomplete collection of abdominal lymph, incorporation of fatty acid into enterocyte membrane lipids, or

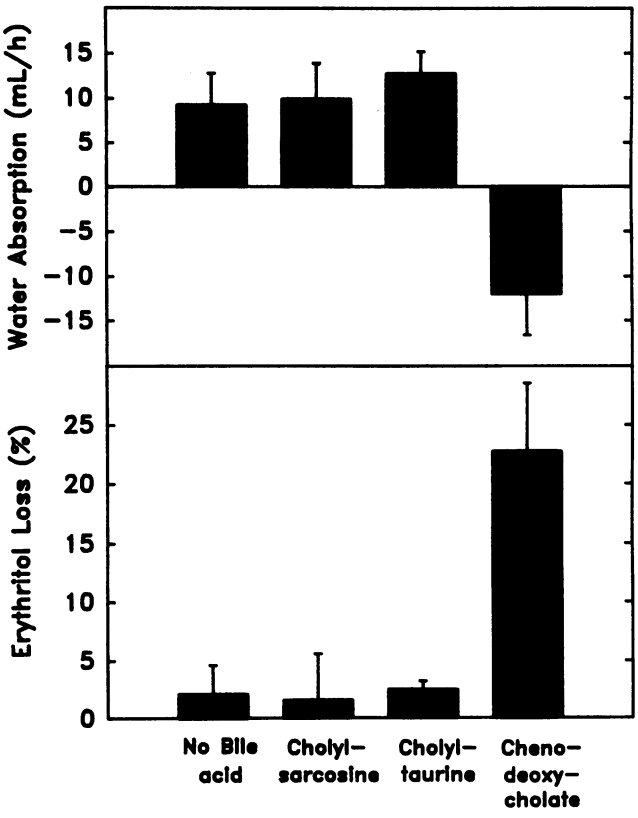
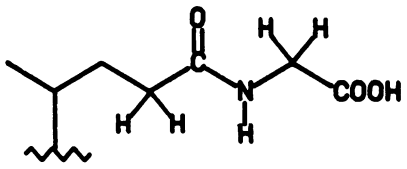
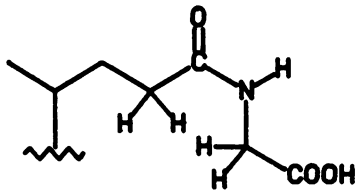


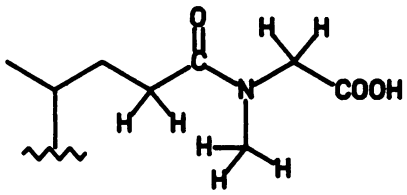
Figure 8. Water absorption (*top*) and erythritol loss (*bottom*) during colonic perfusion of the anesthetized rabbit with solutions (5 mmol/liter) of different bile acids (mean ± SD, n = 8).



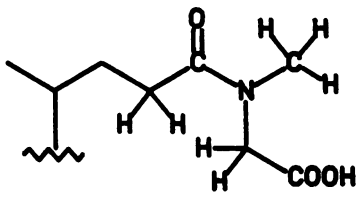
trans



cis



trans



cis

Figure 9. Side chain conformation of glycine conjugates; the *cis* conformation causes more steric crowding than the *trans* isomer (*top*). Side chain conformation of sarcosine conjugates; both isomers have similar stability (*bottom*).

portal absorption of unesterified fatty acids or any or all of these (50).

Cholylsarcosine and cholyltaurine did not cause any colonic secretion, in contrast to chenodeoxycholate which was a potent secretagogue. The lack of secretory effect of cholyl conjugates is in agreement with several other studies in both human (51) and rabbit (37, 52) in which endogenous bile acids have been perfused. In addition to cholyl conjugates, hydrophilic trihydroxy- and tri-oxo bile acids—as well as hydrophilic dihydroxy bile acids such as ursodeoxycholate—do not induce secretion by the colon, at least not at concentrations < 10 mmol/liter. In contrast, lipophilic dihydroxy bile acids, such as deoxycholate or chenodeoxycholate, evoke secretion of water in the colon at concentrations above the CMC (~ 3 mmol/liter).

Clinical implications

Cholylsarcosine is potentially useful as an exogenous surfactant to promote micelle formation in patients with resection of most of the terminal ileum or with an external biliary fistula. Such patients are likely to require a large amount of cholylsarcosine, 4–6 g/meal, since there is a greatly impaired enterohepatic circulation in such patients and orally administered cholylsarcosine will not be actively absorbed. Cholylsarcosine is not absorbed passively from the small or large intestine because of its low pK_a and its resistance to bacterial deconjugation and dehydroxylation (19).

Cholylsarcosine might also be useful to enhance cyclosporin absorption in liver transplant patients in whom bile is diverted for up to 1 wk after transplantation. Absorption of orally administered Cyclosporin A, which is a very lipophilic drug, increases severalfold when bile is returned to the duodenum (53, 54). Cholylsarcosine is not likely to be used for patients with retained bile acids, such as biliary atresia, because of the potential risk of inducing additional hepatic injury.

We conclude that cholylsarcosine fulfills the physicochemical and physiological requirements for promoting lipid absorption when there is a deficiency of natural bile acids. Cholylsarcosine has good pH-solubility properties and is a potent enhancer of lipid absorption in lymph fistula rats. It is neither secretory in the colon nor metabolized by intestinal bacteria to secretory metabolites. It is nontoxic and, in principle, is prepared synthetically at low cost and in high purity. Studies to assess its clinical utility in bile acid deficiency states appear indicated.

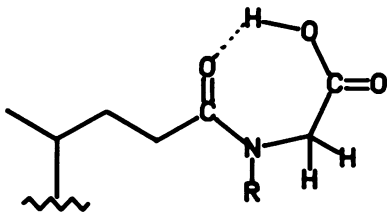


Figure 10. Formation of intramolecular hydrogen bond in *trans* glycine or *N*-methyl glycine conjugates of bile acids (R = H, CH_3).

Appendix

The determination of the pK_a of a bile acid by titration of an aqueous solution is not feasible because the protonated form is, in most cases, insoluble in water. In addition, water solutions of bile salts contain aggregates and micelles, a complex system that gives a composite pK_a different from the true one. These problems can be avoided by titrating the bile acid in different mixtures of water and an organic solvent and extrapolating the pK_a^*s measured to zero concentration of organic solvent (22).

For a given water/organic solvent composition (methanol in our case), the equilibrium relationship gives:

$$pK_a^* = pH^* - \log \frac{[A^-]}{[AH]} - \log \gamma_{A^-}^*,$$

where the asterisks denote values in the particular methanol/water composition, pH* is the pH referred to standard solutions in the same solvent system, and $\gamma_{A^-}^*$ is the activity coefficient of the anion in the solvent mixture. Since the activity coefficient of the ionized bile acid is close to 1 at the low ionic strength used, and the correction for hydrolysis is small (55), pK_a^* can be approximated to $pH_{1/2}^*$ at one-half neutralization ($[A^-]/[AH] = 1$).

The measurement of pH* requires that the glass electrode/pH meter be calibrated with standard solutions in the particular water/methanol mixture used. Although these buffers have been described

(56), they are not necessary if what is desired is a value calculated from extrapolation to zero organic solvent concentration. If the pH meter is instead calibrated with standard aqueous solutions, the measurement carried out in a solvent mixture will give pH#, different from pH*. However, the difference between pH# and pH* is an approximately linear function of the methanol concentration and extrapolates to zero for pure water (56). With the electrode calibrated with aqueous solutions, for a particular solvent mixture, at one-half neutralization, we obtain:

$$pK_a^{\#} = pH_{1/2}^{\#},$$

where $pK_a^{\#}$ is a "mixed" constant that includes pK_a^{*} and the difference between pH# and pH* for that particular solvent mixture. Since the relationships between both quantities and methanol concentration are linear, and the pH difference extrapolates to zero for pure water, we conclude that $pK_a^{\#}$ extrapolates to pK_a. However, the plot of $pK_a^{\#}$ versus molar fraction of methanol will have a slope different from the one obtained from pH* measurements. To test its suitability, we determined the pK_a of acetic acid by this method. We obtained a good linear correlation between $pK_a^{\#}$ and the molar fraction of methanol (r = 0.999) and an extrapolated pK_a in excellent agreement with the reported value (23).

Acknowledgments

We wish to thank Dr. Berit Sternby for providing pure human pancreatic lipase, colipase, cholesterol ester lipase, and pancreatic juice, as well as advise on how to measure lipolysis. We also wish to thank Dr. Mats Lindström for advice regarding an animal model for intestinal uptake of fat and Professor Bengt Borgström for reading the manuscript. Vicky Huebner prepared the manuscript.

This work has been supported in part by NIH grants DK-21506 and DK-32130 (principal investigator: Dr. E. W. Moore, Medical College of Virginia, Richmond, VA). Grants-in-aid were also received from the Falk Foundation e.V and Diamalt AG, West Germany.

A patent for the use of this compound in bile acid deficiency states has been applied for by the University of California.

References

1. Hofmann, A. F., and K. J. Mysels. 1988. Bile salts as biological surfactants. *Colloids Surf.* 30:145–173.

2. Hardison, W. G. M., and I. H. Rosenberg. 1967. Bile-salt deficiency in the steatorrhea following resection of the ileum and proximal colon. *N. Engl. J. Med.* 277:337–342.

3. Austad, W. I., L. Lack, and M. P. Tyor. 1967. Importance of bile acids and of an intact distal small intestine for fat absorption. *Gastroenterology.* 52:638–646.

4. Maislos, M., and S. Shany. 1987. Bile salt deficiency and the absorption of vitamin D metabolites. *Isr. J. Med. Sci.* 23:1114–1117.

5. McLeod, G. M., and H. S. Wiggins. 1968. Bile-salts in small intestinal contents after ileal resection and in other malabsorption syndromes. *Lancet.* 873–881.

6. Van Deest, B. W., J. S. Fordtran, S. G. Morawski, and J. D. Wilson. 1968. Bile salt and micellar fat concentration in proximal small bowel contents of ileectomy patients. *J. Clin. Invest.* 47:1314–1324.

7. Poley, J. R., and A. F. Hofmann. 1976. Role of fat maldigestion in pathogenesis of steatorrhea in ileal resection. Fat digestion after two sequential test meals with and without cholestyramine. *Gastroenterology.* 71:38–44.

8. Bright-Asare, P., and H. J. Binder. 1973. Stimulation of colonic secretion of water and electrolytes by hydroxy fatty acids. *Gastroenterology.* 64:81–88.

9. Ammon, H. V., and S. F. Phillips. 1973. Inhibition of colonic water and electrolyte absorption by fatty acids in man. *Gastroenterology.* 65:744–749.

10. Hofmann, A. F., and J. R. Poley. 1972. Role of bile acid malabsorption in pathogenesis of diarrhea and steatorrhea in patients with ileal resection. I. Response to cholestyramine or replacement of dietary long chain triglyceride by medium chain triglyceride. *Gastroenterology.* 62:918–934.

11. Fordtran, J. S., F. Bunch, and G. R. Davis. 1982. Ox bile treatment of severe steatorrhea in an ileectomy-ileostomy patient. *Gastroenterology.* 82:564–568.

12. LaRusso, N. F., and J. L. Thistle. 1981. Ursodeoxycholic acid ingestion after ileal resection. Effect on biliary bile acid and lipid composition. *Dig. Dis. Sci.* 26:705–709.

13. Huijbregts, A. W. M., T. M. Cox, J. Hermsen, G. P. van Berge Henegouwen, A. Van Shaik, and V. S. Chadwick. 1981. Micellar solubilization of intestinal lipids after ursodeoxycholic acid therapy in short bowel patients and healthy controls. *Neth. J. Med.* 24:108–113.

14. Jones, C. M., P. J. Culver, G. D. Drummey, and A. E. Ryan. 1948. Modification of fat absorption in the digestive tract by the use of an emulsifying agent. *Ann. Intern. Med.* 29:1–10.

15. Boyd, H., and F. Helfrick. 1951. Celiac disease treated with polyoxyethylene sorbitan monooleate. *J. Pediatr.* 38:493–497.

16. Du Bois, J. J., P. R. Holt, G. W. Kuron, S. A. Hashim, and T. B. Van Itallie. 1964. Effect of Tween 80 on cholestyramine-induced malabsorption. *Proc. Soc. Exp. Biol. Med.* 117:226–229.

17. Sheltawy, M. J., and M. S. Losowsky. 1975. Effect of non-ionic detergents on the absorption of oleic acid in the anaesthetised rat. *Biomedicine (Paris).* 22:468–472.

18. King, R. F. G. J., P. D. Howdle, J. Kelleher, and M. S. Losowsky. 1979. Synthetic detergents in bile-salt-deficient steatorrhoea. *Clin. Sci. (Lond.).* 56:273–281.

19. Schmassmann, A., M. A. Angellotti, H. T. Ton-Nu, C. D. Schteingart, S. N. Marcus, S. S. Rossi, and A. F. Hofmann. 1990. Transport, metabolism and effect of chronic feeding of cholylsarcosine, a conjugated bile acid resistant to deconjugation and dehydroxylation. *Gastroenterology.* 98:163–174.

20. Tserng, K.-Y., D. L. Hachey, and P. D. Klein. 1977. An improved procedure for the synthesis of glycine and taurine conjugates of bile acids. *J. Lipid Res.* 18:404–407.

21. Dupas, J.-L., M. Moreau, and A. F. Hofmann. 1985. Polymeric dyes: useful nonabsorbable reference markers for intestinal perfusion studies in animals. *J. Pharm. Sci.* 74:328–330.

22. Fini, A., and A. Roda. 1987. Chemical properties of bile acids. IV. Acidity constants of glycine-conjugated bile acids. *J. Lipid Res.* 28:755–759.

23. Serjeant, E. P., and B. Dempsey. 1979. Ionization Constants of Organic Acids in Aqueous Solution. Pergamon, New York. 19–20.

24. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* 28:589–595.

25. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3α-hydroxy steroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* 19:924–928.

26. Jones, C. A., A. F. Hofmann, K. J. Mysels, and A. Roda. 1986. The effect of calcium and sodium ion concentration on the properties of dilute aqueous solutions of glycine conjugated bile salts: phase behavior and solubility products of the calcium salts of the common glycine conjugated bile acids. *J. Colloid Interface Sci.* 114:452–470.

27. Moore, E. W. 1984. The role of calcium in the pathogenesis of gallstones: Ca^{++} electrode studies of model bile salt solutions and other biologic systems. *Hepatology.* 4:228S–243S.

28. van Berge Henegouwen, G. P., A. F. Hofmann, and T. S. Gaginella. 1977. Pharmacology of chenodeoxycholic acid. I. Pharmaceutical properties. *Gastroenterology.* 73:291–299, 1977.

29. Mysels, K. J. 1986. Improvements in the maximum-bubble-pressure method of measuring surface tension. *Langmuir* 2:428–432.

30. Mysels, K. J. 1989. Some limitations in the interpretations of the time dependence of surface tension measured by the maximum bubble pressure method. *Langmuir.* 5:442–447.

31. Roda, A., A. F. Hofmann, and K. J. Mysels. 1983. The influences of bile salt structure on self-association in aqueous solutions. *J. Biol. Chem.* 258:6362–6370.

32. Ekwall, P., and I. Danielsson. 1951. The solubilization of long-chain alcohols in association colloid solutions below the critical concentration for micelle formation. *Acta Chem. Scand.* 5:973–975.

33. Hofmann, A. F. 1963. The function of bile salts in fat absorption. The solvent properties of dilute micellar solutions of conjugated bile salts. *Biochem. J.* 89:57–68.

34. Hofmann, A. F. 1968. Molecular association in fat digestion. *Adv. Chem. Ser.* 84:53–66.

35. Erlansson, C., and B. Borgström. 1972. Purification and further characterization of colipase from porcine pancreas. *Biochim. Biophys. Acta.* 271:400–412.

36. Tso, P., M. B. Lindström, and B. Borgström. 1987. Factors regulating the formation of chylomicrons and very-low-density lipoproteins by the rat small intestine. *Biochim. Biophys. Acta.* 922:304–313.

37. Chadwick, V. S., T. S. Gaginella, G. L. Carlson, J.-C. Debongnie, S. F. Phillips, and A. F. Hofmann. 1979. Effect of molecular structure on bile acid–induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. *J. Lab. Clin. Med.* 94:661–674.

38. Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *J. Lipid Res.* 3:127–128.

39. Evans, C. A., and D. L. Rabenstein. 1974. Nuclear magnetic resonance studies of the acid-base chemistry of amino acids and peptides. II. Dependence of the acidity of the C-terminal carboxyl group on the conformation of the C-terminal peptide bond. *J. Am. Chem. Soc.* 96:7312–7317.

40. Stark, R. E., R. W. Storrs, S. E. Levine, S. Yee, and M. S. Broido. 1986. One- and two-dimensional NMR relaxation studies of dynamics and structure in bile salt–phosphatidylcholine mixed micelles. *Biochim. Biophys. Acta.* 860:399–410.

41. Stewart, W. E., and T. H. Siddall III. 1970. Nuclear magnetic resonance studies of amides. *Chem. Rev.* 70:517–551.

42. Weast, R. C. 1977. CRC Handbook of Chemistry and Physics. CRC Press, Inc., Cleveland, OH. D-147.

43. Shiffman, M. L., J. M. Kellum, H. J. Sugerman, and E. W. Moore. 1990. Gallstone (GS) formation following rapid weight loss. II. Free Ca^{++} ion in gallbladder (GB) bile increases prior to GS appearance. *Gastroenterology.* 98:A262. (Abstr.)

44. Duane, W. C. 1975. The intermicellar bile salt concentration in equilibrium with the mixed micelles of human bile. *Biochim. Biophys. Acta* 398:275–286.

45. Ekwall, P. 1954. Concentration limits in association colloid solutions. *J. Colloid Sci.* 1(Suppl.):66–80.

46. Quist, R. G., H.-T. Ton-Nu, J. Lillienau, A. F. Hofmann, and K. E. Barrett. 1991. Activation of mast cells by bile acids. *Gastroenterology.* 101:446–456.

47. Andersson, R., K.-G. Tranberg, J. Lillienau, C. Schalen, U. Srinivas, L. Larsson, A. Sonesson, and S. Bengmark. 1990. Influence of individual bile acids in *Escherichia coli* peritonitis. *Scand. J. Gastroenterol.* 25:1129–1136.

48. Meldrum, S. J., B. W. Watson, H. C. Riddle, R. L. Bown, and G. E. Sladen. 1972. pH profile of gut as measured by radiotelemetry capsule. *Br. Med. J.* 2:104–106.

49. Bergstedt, S. E., H. Hayashi, D. Kritchevsky, and P. Tso. 1990. A comparison of absorption of glycerol tristearate and glycerol trioleate by rat small intestine. *Am. J. Physiol.* 259(*Gastrointest. Liver Physiol.* 22):G386–G393.

50. Simmonds, W. J. 1974. Absorption of lipids. *In* Gastrointestinal Physiology. Physiology Series One, Volume 4. A. C. G. Guyton, E. D. Jacobson, and L. L. Shanbour, editors. Butterworths, London. 343–376.

51. Mekhjian, H. S., S. F. Phillips, and A. F. Hofmann. 1971. Colonic secretion of water and electrolytes induced by bile acids: perfusion studies in man. *J. Clin. Invest.* 50:1569–1577.

52. Chadwick, V. S., S. F. Phillips, and A. F. Hofmann. 1977. Measurements of intestinal permeability using low molecular weight polyethylene glycol (PEG 400). II. Application to normal and abnormal permeability states in man and animals. *Gastroenterology.* 73:247–251.

53. Mehta, M. U., R. Venkataramanan, G. J. Burckart, R. J. Ptachcinski, B. Delamos, S. Stachak, D. H. Van Thiel, S. Iwatsuki, and T. E. Starzl. 1988. Effect of bile on cyclosporin absorption in liver transplant patients. *Br. J. Clin. Pharmacol.* 25:579–584.

54. Naoumov, N. V., J. M. Tredger, C. M. Steward, J. G. O'Grady, J. Grevel, A. Niven, B. Whiting, and R. Williams. 1989. Cyclosporin A pharmacokinetics in liver transplant recipients in relation to biliary T-tube clamping and liver dysfunction. *Gut* 30:391–396.

55. Cookson, R. F. 1974. The determination of acidity constants. *Chem. Rev.* 74:5–28.

56. Alfenaar, M., and C. L. DeLigny. 1967. The universal pH-scale in methanol and methanol-water mixtures. *Recl. Trav. Chim. Pays-Bas Belg.* 86:1185–1191.