

Physicochemical characterization of a model digestive mixture by ^2H NMR

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Abstract ^2H nuclear magnetic resonance (NMR) spectra were obtained at 30.87 MHz for 8% (w/v) aqueous dispersions of mixtures of bile salts (MBS), mixed intestinal lipids (MIL; myristic acid, monomyristoylglycerol, dimyristoylphosphatidylcholine = 5:1:1), and cholesterol, in which a single lipid component is selectively ^2H -labeled. Using the observation that the time-averaged quadrupole splitting of a C^2H_3 group varies according to whether it exists in a micellar, multilamellar or solid phase, one-, two-, and three-phase regions in the equilibrium phase diagram have been identified. From the intensities of the singlets and powder patterns in the wide-line ^2H NMR spectra, the relative amounts of these organized molecular assemblies were determined. With different C^2H_3 -labeled components in samples of identical total composition, the chemical composition of each phase was calculated for one point (20 mol % cholesterol; 50 mol % MIL, and 30 mol % MBS) in a two-phase region of the phase diagram where the ^2H NMR spectrum displayed both a sharp spectral component and a broad uniaxial powder pattern. X-ray diffraction measurements on this sample confirmed that the uniaxial powder pattern in the NMR spectra can be assigned to multilamellar vesicles. At this same point in the phase diagram with the ^2H label on the α -methylene site of myristic acid, both narrow and broad ($\Delta\nu = 37$ kHz) spectral components were again observed. Relaxation time (T_1 and T_2) measurements of the sharp spectral component indicate that this peak arises from rapidly tumbling aggregates which, at a total lipid concentration of 8% (w/v), are micellar particles and not unilamellar vesicles. These experiments demonstrate the feasibility of structural investigations of model digestive mixtures by ^2H NMR.—Westerman, P. W. Physicochemical characterization of a model digestive mixture by ^2H NMR. *J. Lipid Res.* 1995. **36**: 2478-2492.

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The physical state of lipids and lipolytic products in the upper gastrointestinal tract during fat digestion was viewed for many years as a two-phase lipid system (1–5): an oil phase containing triacylglycerols, diacylglycerols, monoacylglycerols and fatty acids, and a micellar phase (6, 7) containing primarily bile salts, fatty acids, and monoacylglycerols. Subsequent experiments showed that this oil-micellar view of fat digestion is an oversim-

plification and other phases probably occur during hydrolysis of triacylglycerols under physiological conditions (8). In ultracentrifuged human upper intestinal contents collected during rapid fat lipolysis, a viscous gel-like phase was observed between the oil and micellar phases (9). The birefringence of this phase suggested it was liquid-crystalline. A light microscopy study (10) of the hydrolysis of triacylglycerol emulsions in the presence of pancreatic lipase, colipase, and bile salts showed, initially, formation of a birefringent lamellar liquid crystalline phase containing calcium and ionized fatty acid, and then a “viscous isotropic phase” composed predominantly of monoacylglycerols and fatty acids. Freeze-fracture electron microscopy on similar preparations showed a variety of liquid crystalline phases, including transient hexagonal and tubular lamellar phases, depending on the pH and bile salt concentration in the surrounding medium (11, 12).

The traditional approach to physically characterize aqueous dispersions of amphiphiles, such as are found in bile and intestinal contents, has been to determine phase diagrams for model systems of similar composition (13, 14). Knowledge of the phase diagram and the constraints of the Gibbs phase rule enables one to determine the distribution of lipid components between the various phases. To better understand model systems resembling lipid intestinal contents, Stafford and Carey (15) and Stafford et al. (16) used light scattering and chemical analysis to define the phase equilibria of model systems of the aqueous intestinal lipids for typical physi-

Abbreviations: NMR, nuclear magnetic resonance; T_1 , spin-lattice relaxation time; T_2 , spin-spin relaxation time; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; MLV, multilamellar vesicles; ULV, unilamellar vesicles; TLC, thin-layer chromatography; QELS, quasi-elastic light scattering; MIL, mixed intestinal lipids; MBS, mixed bile salts.

ological conditions. They determined that the so-called "micellar" phase described by Hofmann and Borgström (4, 5) is a two-phase system consisting of bile salt mixed micelles and liquid crystalline liposomes. Recently, the same group reported (17) a complete phase diagram for the ternary lipid system, composed of a physiological mixture of bile salts, mixed intestinal lipids (MIL; oleic acid/monooleoylglycerol/dioleoylglycerol/egg-yolk lecithin = 5/1/0.2/1), and cholesterol, all at fixed aqueous electrolyte concentrations, pH, and temperature. The authors identified two one-phase zones composed of mixed micelles and lamellar liquid crystals, respectively, and two two-phase zones, one composed of solid cholesterol and cholesterol-saturated micelles and the other of physiological relevance composed of cholesterol- and MIL-saturated mixed micelles and unilamellar vesicles. In addition, a single large three-phase zone in the system was composed of cholesterol-saturated micelles, solid cholesterol, and liquid crystals. In these studies the lipid compositions of the various aggregates were determined chemically after their separation by ultracentrifugation, ultrafiltration, or gel filtration. Each of these techniques suffers from the disadvantage that artifacts may be created by demulsification and the spontaneous transfer of lipids among the several aggregate structures during the separation process.

Based on the many NMR investigations of triacylglycerols, monoacylglycerols, bile salts and phospholipids individually (18–22), this technique should be an attractive method for characterizing aggregate organization and dynamics in model digestive mixtures. However, unlike the better characterized model bile systems (23–32), there have been few NMR studies of these mixtures. Recently, high resolution ^{13}C NMR was used to quantitate methanolic and aqueous mixtures of sodium taurocholate, 1-monocapryloyl-*rac*-glycerol, and caprylic acid (33). The techniques in this work are of limited use for more physiological systems composed of higher fatty acid and monoacylglycerol homologs where multilamellar, cubic, hexagonal, and solid phases could exist.

We report here wide-line NMR techniques that enable both the physical state and chemical compositions of aggregate states that form in an aqueous medium to be directly determined without separation of the different phases. The procedure utilizes ^2H NMR to measure, orientational ordering of a non-perturbing $-\text{C}^2\text{H}_3$ group on one of the lipid components in the mixture, and the molecular dynamics of a $-\text{C}^2\text{H}_2$ group on the fatty acid component of the mixture. The lipid dispersions chosen for physicochemical characterization were selected from representative one-, two-, and three-phase zones in the phase diagram of analogous unsaturated lipid mixtures (15–17).

MATERIALS AND METHODS

Unlabeled myristic acid, cholesterol, monomyristoyl-*rac*-glycerol, DMPC, and DMPE were purchased from either Sigma Chemicals (St. Louis, MO) or Avanti Polar Lipids, Inc. (Birmingham, AL) and stored at -20°C prior to use. $[26,26,26,27,27,27\text{-}^2\text{H}_6]$ cholesterol (chol- d_6) was obtained from Medical Isotopes, Concord, NH. Conjugated bile salts were obtained commercially (Sigma) and stored desiccated at low temperature. Solvents and reagents of highest purity were purchased and purified by recrystallization or distillation before use (34). Bile salt mixtures were patterned after the average composition of human bile (17). Mixed bile salt (MBS) stock solutions were prepared gravimetrically and dissolved in methanol, whereas stock solutions of all other lipids were prepared individually in chloroform. Mixed intestinal lipid (MIL) solutions were prepared volumetrically from stock solutions to give defined molar ratios of the principal lipids (fatty acid, phosphatidylcholine, monomyristoylglycerol).

Synthesis of ^2H -labeled lipids

[14,14,14- $^2\text{H}_3$]myristic acid. $[14,14,14\text{-}^2\text{H}_3]$ myristic acid was prepared by a synthetic route (35) utilizing the half ester of tetradecanedioic acid which was prepared from commercially available diacid (Aldrich Chemical Co., Milwaukee, WI) by the method of Durham, McLeod, and Cason (36). Terminally labeled myristic acid has also been prepared from deuterated acetic acid and the monoester of tetradecanedioic acid by Kolbe electrolysis according to the method of Greaves et al. (37). A specially designed electrolytic cell and platinum electrodes were used for this conversion. The purity of the fatty acid was confirmed by gas chromatography, the position of the ^2H label by ^{13}C NMR, and the extent of isotopic incorporation by mass spectrometry.

[2,2- $^2\text{H}_2$]myristic acid. ^2H exchange at the α -position of myristic acid was accomplished using the method of Aasen, Lauer, and Holman (38).

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine- N^+ (C^2H_3) $_3$. A solution of DMPE (0.2 g) in chloroform (1.5 ml) was added to a mixture of 2-propanol (4 ml), dimethylformamide (2.5 ml), and 1 M sodium carbonate (5 ml) in a 50-ml flask equipped with a reflux condenser through which circulated ice-cold water. Methyl- $^2\text{H}_3$ iodide (0.2 ml) was added via the condenser to the single-phase reaction mixture using a pre-cooled syringe. The solution was stirred at room temperature overnight and then mixed with water (4 ml) and chloroform (4 ml) in a separating funnel. The lower layer was collected and filtered to remove sodium iodide. Solvent was removed by rotary evaporator and high-vacuum pump. The re-

sulting oil was chromatographed on silica gel (Bio-Rad; 200–400 mesh). The product was eluted by chloroform:methanol 7:3 to yield a white powder (after several triturations with ethyl ether) in 80–90% yield. TLC of the product, when developed with molybdenum blue or iodine, showed it to be pure phosphatidylcholine.

1-Myristoyl-2-[14,14,14-²H₃]myristoyl-sn-glycero-3-phosphocholine. DMPC, ²H-labeled on the terminal methyl group of the *sn*-2 acyl chain, was prepared and purified from egg yolk lecithin as described elsewhere (39).

1-Mono [14,14,14-²H₃]myristoyl-rac-glycerol. Monomyristoyl-rac-glycerol was prepared as it has been demonstrated (40) that under the conditions to which the NMR samples were subjected (at least 7 days equilibration at 37°C), 1- and 3-monoacyl-*sn*-glycerols isomerize to an equilibrium mixture of 90% monoacyl-rac-glycerol and 10% 2-monoacylglycerol. Mono [14,14,14-²H₃]myristoyl-rac-glycerol was synthesized by condensation of [14,14,14-²H₃]myristoyl chloride with the acetone derivative of glycerol, followed by removal of the protecting group by mild acid treatment. The procedure was as follows.

[14,14,14-²H₃]myristic acid (1 mol) and oxalyl chloride (1.2 mol) were heated at reflux in dry benzene for 4 h before the solvent was removed by distillation. The product [14,14,14-²H₃]myristoyl chloride (23.6 mmol) was added slowly with shaking to a cooled mixture of DL- α,β -isopropylidene-glycerol (2.09 g; 15.8 mmol), chloroform (60 ml), and pyridine (2.1 ml; 25.1 mmol). After allowing the reaction mixture to stand at room temperature for 15 h, absolute ethanol (3.84 g; 0.079 mmol) was added and the solvent was removed at 30°C. The residue was dissolved in ethyl ether (100 ml), washed with water (5 × 20 ml), dried, and the solvent was removed. The isopropylidene ester was dissolved in 2-methoxyethanol (45 ml), boric acid (15 g) was added, and the reaction mixture was heated on a steam bath for 3 h. On cooling, ethyl ether was added and the resulting solution was washed with water (4 × 50 ml) and dried. The volume was reduced to 30 ml by evaporation at 30°C and hexane (45 ml) was added. The product crystallized on cooling. Crystals were recovered by filtration and washed with cold hexane; m.p. 59–62°C, lit. (41) 62–64°C.

Glycodeoxycholic acid-(glycine-2-²H₂). This deuterated bile salt was prepared by reaction of deoxycholic acid with ethyl [2,2-²H₂]glycinate hydrochloride in the presence of the peptide coupling reagent, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), according to the method of Tserng, Hachey, and Klein (42). Ethyl [2,2-²H₂]glycinate hydrochloride was prepared by bubbling dry HCl gas through a stirred ethanolic solution of [2,2-²H₂]glycine. A suspension of ethyl [2,2-²H₂]glycinate hydrochloride (0.5 g) in 35 ml of ethyl acetate

containing 0.5 ml of triethylamine was stirred at 25°C for 0.5 h. Deoxycholic acid (2.5 mmol) and EEDQ (0.86 g) were added to the solution. After stirring at 25°C for 10 min, the suspension was refluxed on a steam bath overnight. The resulting suspension was cooled to room temperature and worked up as described (42). The product was obtained in 90% yield; m.p. 135–137°C, lit. (42) 140–142°C. Qualitative analysis of the product was performed by thin-layer chromatography (43).

Unilamellar vesicle preparation (44)

Egg yolk PC (200 mg) and [2,2-²H₂]myristic acid (14.69 mg; 20 mol %) were mixed in chloroform and the solvent was evaporated under N₂. The sample was hydrated with ²H-depleted buffer (3 ml) and ultrasonically irradiated using an Ultrasonics Heat System W-255 Sonifier in a pulsed mode, 35–65% duty cycle, and 4–6 output level. The sonication vial was suspended in an ice bath to keep the sample temperature at 15–20°C. Sonication was carried out for 2 h, titanium was removed by low speed centrifugation, and the translucent supernatant was transferred to an NMR tube. The size of the vesicles was measured by negative stain electron microscopy using 1% ammonium molybdate or 1% phosphotungstic acid on carbon-coated Formvar grids at 25,000 × magnification (45).

NMR sample preparation

Samples containing all three components (cholesterol/MIL/MBS) with a single ²H-labeled constituent were prepared by coprecipitation of the lipid mixtures from CHCl₃-CH₃OH mixtures. Organic solvents were evaporated in a 5 mm o.d. NMR tube under a stream of dry nitrogen and then under high vacuum for 48 h. An aliquot of aqueous buffer was added and the lipids were dispersed by vortex stirring. The aqueous buffer was 33 mM NaH₂PO₄/Na₂HPO₄ (pH 6.5) with 3 mM NaN₃ (to prevent microbial growth) and sufficient NaCl to give a final [Na⁺] concentration of 150 mM. ²H-depleted H₂O (Aldrich) was used in buffer preparation. The concentration of total lipids in the aqueous medium was approximately 8% (w/v). The NMR tubes were flame sealed and the lipids were further mixed by repeated centrifugation through a constriction in the tube. After equilibration for at least 7 days (17), the tube was cut open at a point 1.25" from the bottom. It is essential that the NMR tube be 1" or shorter so that the whole sample is confined within the receiver coil of the probe and signal obtained from the entire heterogeneous sample. A few microliters of the sample was examined for the presence of crystals, liquid crystals, and oils by direct and polarizing light microscopy (Leitz Laborlux 12 POL fitted with a modified and calibrated Mettler FP-2 heat-

ing stage). Textures observed under the polarizing light microscope were compared with published textures for well-defined lyotropic liquid crystals (46, 47). Finally, the NMR tube was sealed with epoxy glue.

Acquisition of NMR spectra

High resolution ^1H and ^{13}C NMR spectra were recorded at ambient temperature on a Varian Gemini-200 instrument. ^2H NMR spectra were recorded at 30.87 MHz on a home-built spectrometer equipped with a 4-inch bore superconducting solenoid (Nalorac Inc., Concord, CA). Sample temperatures are regulated to an accuracy better than 0.5°C with a liquid-flow circulation unit. Further details of the instrument, temperature control unit, and probehead are published elsewhere (39, 48). ^2H NMR spectra of the samples were obtained on-resonance, using a modified quadrupole echo sequence (49). With a 25-mg lipid sample, specifically labeled on a single component in a single position, adequate signal-to-noise for the purpose of spectral fitting was obtained from 5,000–40,000 transients depending on the site labeled and the concentration of the labeled component. Only a slight apodization was applied to the accumulated free induction decay. A dwell time of $5\ \mu\text{s}$ or $10\ \mu\text{s}$ was used for data acquisition. These times are suitable compromises between the requirements to obtain enough points to define the shape of the sharp spectral component and at the same time acquire a broad spectral component. Data were obtained at 37°C after allowing the sample 30 min to equilibrate in the instrument. Each sample was kept in an oven at 37°C for at least 4 days before recording its spectrum. Several samples were re-run several weeks after the initial data acquisition to confirm that equilibrium had been established. Further high resolution NMR and chromatographic analyses were performed on each sample after recording its wide-line NMR spectra to prove the absence of lipid degradation.

Instrumental limitations precluded the use of our home-built spectrometer for relaxation measurements which were performed with a Chemagnetics CMX-300 NMR spectrometer operating at 46.005 MHz. The instrument was equipped with a high power transmitter and solid-state probe. Several samples that needed greater sensitivity or dispersion were recorded at 92.069 MHz on a Varian Unity Plus 600 MHz spectrometer located at The University of Akron (Akron, OH). A Doty ultra-high speed magic angle spinning probe equipped for 5 mm o.d. tubes, and run in the static mode, was used to acquire spectra. The 90° pulse of 3.1 μsec was generated by a 1 KW linear amplifier. The same modified quadrupole echo sequence as above was used for data acquisition.

Theory of ^2H NMR

The theory of ^2H NMR is well established and a fuller discussion may be found elsewhere (49, 50). The ^2H nucleus ($I = 1$) has three allowed nuclear spin states separated equally in energy in an external magnetic field (H_0). The interaction of the nuclear quadrupole moment with an electric field gradient at the nucleus produced by surrounding electrons (usually in the same molecule) perturbs the energies of the three spin states so that they are unequally separated. Transitions between these states, induced with electromagnetic radiation in the radio-frequency region, give rise to a quadrupole coupling, which, in the case of a C- ^2H bond, depends on its orientation with respect to H_0 . A spherical or random distribution of an ensemble of immobilized C- ^2H bonds yields a Pake doublet or powder-pattern spectrum with 90° edge-singularities separated by approximately 128 kHz. This splitting is reduced by molecular motions, both intramolecular and movement of the molecule as a whole. If the label is on a molecule in a micellar aggregate or small unilamellar vesicle, the movement is so rapid on the NMR time-scale that the electrons are effectively smeared out around the nucleus during the measurement and the quadrupole splitting is zero. The NMR signal appears as a singlet. If the ^2H -labeled molecule is in a multilamellar or hexagonal phase, molecular motions are not sufficiently rapid to eliminate the quadrupole coupling and a residual splitting is observed. The same is true for the solid state, only a larger splitting is observed. If a ^2H -labeled molecule coexists in micellar, multilamellar, and solid forms and exchange between the aggregates is slow on the NMR time-scale, it is possible to observe all three and quantitatively determine (by peak-area integration or spectral simulation) the contribution from each. It is not possible using only the quadrupole splitting to distinguish between micellar and small unilamellar forms as both aggregates tumble rapidly on the NMR time-scale at ambient temperatures, and produce single peaks (51). In the estimation of the relative contributions of sharp and broad components by integration, it was sometimes necessary to apply a correction (18% of the integrated area of the powder pattern) to the contribution from the broad component because the "shoulders" in the powder patterns (49, 50) could not be observed.

Spectral simulations of ^2H NMR line-shapes

Experimental spectral patterns were fitted by computer-generated simulations made up of spherically distributed powder patterns (39, 49). In the multilamellar phase, where intramolecular fluctuations about the time-averaged director axis are fast on the NMR time-scale, line shapes can normally be well approximated. In this case, the fitting parameters associated with each

powder pattern are the time-averaged coupling constant and the motionally induced asymmetry parameter, which in these systems is zero. Rounding of the edges and singularities of the patterns is accomplished by including a dipolar line broadening contribution. The dipole-dipole contribution to line-broadening is included in the standard manner by taking the line shape to be Gaussian with a frequency-dependent line-width.

Measurement of deuterium relaxation times

Spin-lattice relaxation times (T_1) for deuterons attached to the 2-position of myristic acid in MIL-containing systems were determined by a modified inversion recovery technique (52). T_1 was calculated from a non-linear least square fit of the equation $M_z(\tau) = A[1 - ke^{\tau/T_1}]$ to the data. $M_z(\tau)$ is the peak amplitude at delay time τ between the 180° and 90° rf pulses; A and k are adjustable parameters. The waiting time between pulse sequences is at least $5 \cdot T_1$. The spin-spin relaxation time (T_2) was determined using 1) a $90^\circ - \tau - 180^\circ - \tau$ "acquire" pulse sequence and measuring the peak height of the echo as a function of τ ; 2) a $90_x^\circ - \tau - 90_y^\circ - t$ "acquire" pulse sequence and measuring the peak height of the echo as a function of τ ; and 3) the half-height line width ($\Delta\nu_{1/2}$) of the NMR signal after correcting for magnetic field inhomogeneity by using the relationship $\pi T_2^{-1} = \Delta\nu_{1/2}(\text{measured})\Delta\nu_{1/2}(^2\text{H}_2\text{O})$ where $\Delta\nu_{1/2}(^2\text{H}_2\text{O})$ is the line width of a sample of buffer made from $^2\text{H}_2\text{O}$. Very good agreement was found between the T_2 values determined by these three methods, a result not unexpected in light of the observation by Stockton et al. (51) that the line width measurements of ^2H NMR signals yield good estimates of spin-spin relaxation times (T_2) in single bilayer vesicles of egg yolk lecithin.

Analysis of deuterium relaxation times (53, 54)

For a quadrupolar nucleus, the spin relaxation is dominated by the interaction between the electric quadrupole moment of the nucleus and the electric field gradient at the nucleus. For a nucleus with a spin of one, such as ^2H , the spin-lattice (T_1) and spin-spin (T_2) relaxation times are given by (55):

$$\frac{1}{T_1} = \frac{3\pi^2}{40} \chi^2 (2J(\omega_0) + 8\tilde{J}(2\omega_0))$$

$$\frac{1}{T_2} = \frac{3\pi^2}{40} \chi^2 (3\tilde{J}(0) + 5\tilde{J}(\omega_0) + 2\tilde{J}(2\omega_0))$$

where $\chi = eQV_{zz}/h$, eQ is the electric quadrupole moment of the nucleus, V_{zz} is the largest component of the electric field gradient tensor in its principal axis system at the nucleus, ω_0 is the Larmor frequency, h is the Planck constant, and $\tilde{J}(\omega)$ is the reduced spectral

density of the electric field gradient fluctuations at the nucleus.

The above equations show that T_1 is sensitive to motions occurring at ω_0 and $2\omega_0$, while T_2 is also influenced by very slow motions through the term ($\tilde{J}(0)$) (54). It has been shown in a lipid aggregate that, as the size grows, molecular motions at rates comparable to ω_0 and $2\omega_0$ remain unaltered (i.e., T_1 is constant) while the slow motions get slower (T_2 gets shorter) (54). It is reasonable to view the molecular motion in micellar and liquid crystalline systems as being composed of two independent motions: one very fast (correlation times, $\tau_c^f \sim 10^{-10}\text{s}$) consisting of fluctuations of the molecular orientation relative to the local director of the aggregate, and a slower one ($\tau_c^s \sim 10^{-8}$ – 10^{-9}s) consisting of diffusion of molecules along the aggregate surface and/or overall rotation of the aggregate. It was shown (51, 54) in this simplified case that:

$$\frac{1}{T_2} - \frac{1}{T_1} \approx \frac{9\pi^2}{20} (\Delta\nu)^2 \tau_c^s$$

where $\Delta\nu$ is the quadrupole splitting in the multilamellar phase; τ_c^s is the correlation time characteristic for the slow motion and can be related to the size and shape of the particle at a given temperature. If the size of the particle grows, τ_c^s would get longer and the difference $1/T_2 - 1/T_1$ would increase, as $1/T_1$ is independent of particle size.

$1/T_2 - 1/T_1$ was measured for the α -deuterons in [2,2- $^2\text{H}_2$] myristic acid in the MIL/MBS/cholesterol/systems at point B (see Fig. 1) of the phase diagram. With a knowledge of $\Delta\nu_{\text{CD2}}$ for [2,2- $^2\text{H}_2$]myristic acid in the one-phase region of the phase diagram, τ_c^s was determined.

One way of interpreting τ_c^s is to assume that spherical aggregates are present. The correlation time for the slow motion is given (54) by:

$$1/\tau_c^s = 1/\tau_c^d + 1/\tau_c^r$$

where τ_c^d and τ_c^r describe the translational diffusion of amphiphiles along the surface and rotation of the whole aggregate, respectively. Using $\tau_c^d = R^2/6D$ and $\tau_c^r = 4\pi\eta R^3/3kT$ (where D is the translational diffusion coefficient on the surface taken (29) as $5 \cdot 10^{-12}\text{m}^2/\text{s}$, η is the viscosity taken as being that of pure water at 24°C , k is the Boltzmann constant, and T the absolute temperature) the value of R , the radius of the lipid aggregate, can be determined. τ_c^s can also be interpreted in terms of disc-shaped particles and a radius and hydrodynamic radius calculated for that shape (54).

X-ray scattering measurements

X-ray scattering experiments are carried out on a Siemens X-1000 X-ray scattering system incorporating a two-dimensional, position-sensitive area detector for measurement of diffracted X-rays. It uses a 2.0kW sealed-tube X-ray generator with a 3-axis goniometer, and includes General Area Detector Diffraction Software (GADDS) for data collection and processing. The two-dimensional nature of the detector permits the rapid collection of X-ray scattering data, and also provides a unique capability of directly imaging diffraction patterns on the controlling computer screen. All experiments are conducted in transmission mode. The monochromator receives X-rays produced at the tube anode, diffracts the $\text{CuK}\alpha$ portion of the primary beam with a fire-hardened flat pyrolytic graphite crystal, and precisely directs monochromatic X-rays into the incident beam collimator. Samples are mounted on the 3-axis goniometer, permitting precise movement and positioning of the samples in the phi and omega axes. The detector is also mounted on the 2-theta arm of the goniometer, and is positioned under computer control. The samples were sealed in glass capillaries, and placed inside an oven with precision temperature control. The sample chamber (oven) openings were covered with thin mylar windows to allow the X-ray beam to pass with minimal attenuation. The temperature was controlled by a computer with stability of ± 15 mK.

RESULTS

The equilibrium phase diagram for aqueous model lipid mixtures resembling those found in the upper small intestine during lipid digestion and absorption in adult human beings was developed by Staggers et al. (17) and is shown in modified form in Fig. 1. Their ternary lipid system was composed of a physiological mixture of bile salts (MBS), mixed intestinal lipids (MIL; oleic acid-monooleoylglycerol-dioleoylglycerol-egg yolk lecithin 5:1:0.2:1, and cholesterol, all at fixed aqueous electrolyte concentrations, pH, and temperature. The justification for regarding the mixed intestinal lipids as a single component was that partially ionized fatty acid and monoacylglycerols behave as a single component in dilute systems as both are swelling amphiphiles. The bile salt mixture behaves as a single component at high dilution because the bile salts are members of a closely related family of molecules.

Samples corresponding in composition to selected points in each of the one-, two-, and three-phase regions of this phase diagram (Fig. 1) were prepared. The initial set of samples contained 20 mol % cholesterol and varying ratios of MIL/MBS (points labeled A, B, C, and

D). A single ^2H -labeled component was incorporated into each lipid mixture, and the sample was examined by ^2H NMR. Several ^2H -labeled myristoyl derivatives (myristic acid, monomyristoylglycerol, and DMPC) had been prepared earlier in this laboratory for other purposes, so model systems incorporating as their MIL component, myristic acid-monomyristoylglycerol-DMPC = 5:1:1, were examined first, rather than the corresponding oleoyl analogs used by Staggers et al. (17). Much higher concentrations [8% (w/v)] were used in this study than in the more physiological systems (1% w/v) examined by these workers. The limited sensitivity of our spectrometer required these higher concentrations. Another difference from the earlier study is that diacylglycerol was omitted from the MIL component. Its presence has been shown to have little effect on the phase properties of the MIL/MBS/cholesterol/ H_2O system.

^2H NMR spectra of mixtures of 20 mol % cholesterol- d_6 and varying ratios of mixed bile salts (8 conjugated bile acids in physiological proportions (17)) and mixed intestinal lipids (myristic acid-monomyristoylglycerol-dimyristoylphosphatidylcholine = 5:1:1) in ^2H -depleted water (92%; pH 6.5; $[\text{Na}^+] = 150$ mM) are shown in Fig. 2. In Fig. 2A, a uniaxial powder pattern with a motionally reduced quadrupole splitting of 2.7 kHz is observed. The signal arises from cholesterol- d_6 which is

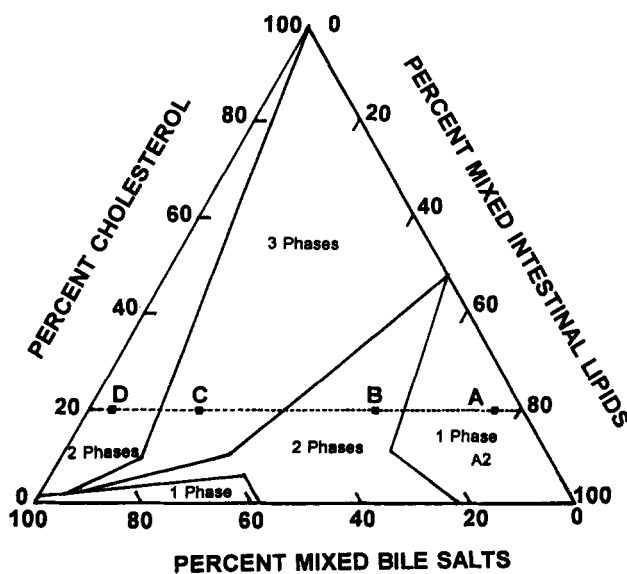


Fig. 1. Equilibrium phase diagram of the MBS-MIL-cholesterol system in which the fatty acid is oleic acid, the lecithin is egg yolk lecithin, and the monoglyceride is monoolein (reproduced with modifications from ref. 17). The spectra of the samples that were examined by ^2H NMR in the analogous system containing myristoyl derivatives, are labeled A, B, C, and D.

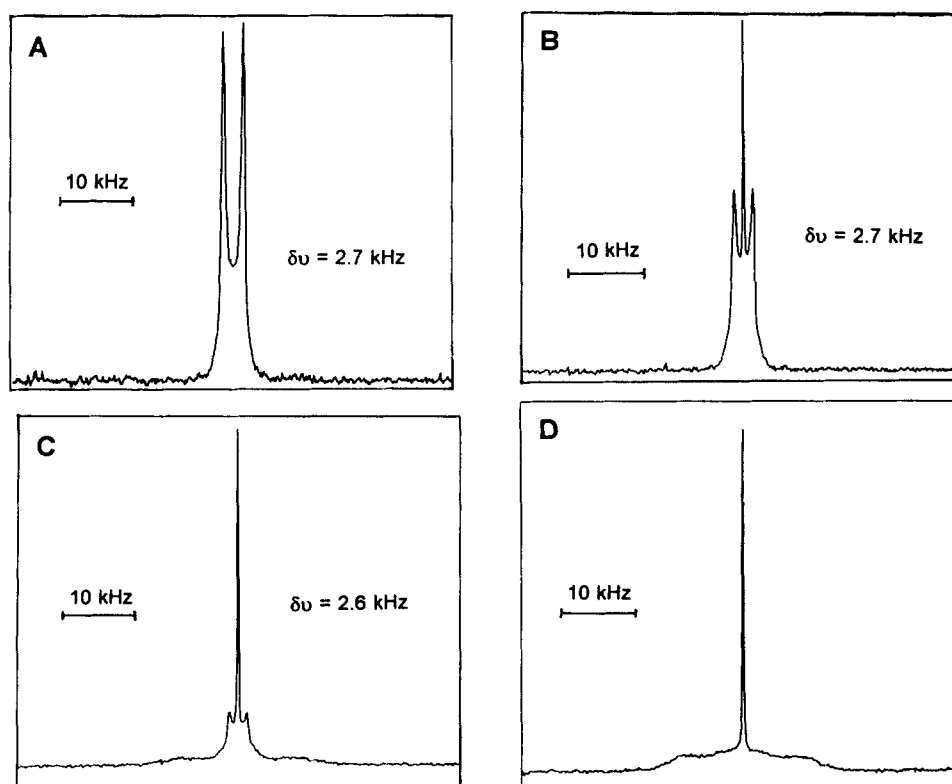


Fig. 2. The 30.87 MHz ^2H NMR spectra of 8% (w/v) aqueous mixtures of cholesterol- $^2\text{H}_6$ (20 mol %) in the presence of A) 75 mol % mixed intestinal lipid (MIL) and 5 mol % mixed bile salts (MBS), B) 50 mol % MIL and 30 mol % MBS, C) 20 mol % MIL and 60 mol % MBS, and D) 5 mol % MIL and 75 mol % MBS. The lipid mixtures were dispersed in phosphate buffer (pH 6.5) prepared from ^2H -depleted water. Spectra were obtained at 37°C from the averaged accumulations of 20,000 pulses. In each case the spectral width is 60 kHz.

incorporated into large multilamellar vesicles (MLV) with tumbling times long on the NMR time scale. The size of the splitting is comparable with that observed in egg yolk PC multibilayers containing cholesterol- d_6 (56). Low angle X-ray diffraction of the pellet obtained by centrifugation of this sample as well as the sample of higher bile salt concentration (point B, Fig. 1), revealed two lamellar reflections with Bragg spacings of 82Å and 41Å, and a broad scattering fringe. These dimensions suggest a bilayer structure for the lamellae. Polarizing light microscopy of the same material showed optical textures consistent with a lamellar structure (57). Unlike phospholipids whose phase behavior has been extensively studied, lamellar phases consisting primarily of monoacylglycerols and fatty acids have received little attention. In an X-ray and microscopic study, Lindström et al. (58) showed that mixtures of oleic acid and monooleoylglycerol display a complex polymorphism that depends on the ionization state of the oleic acid and the proportion of oleic acid in the mixture.

At a higher mole fraction of MBS (Fig. 2B), both a uniaxial powder pattern and sharp peak in the center of the spectrum are observed. The size of the splitting in the uniaxial powder pattern is identical to that shown in

Fig. 2A. If egg yolk PC multibilayers containing cholesterol have a structure similar to MIL/cholesterol multibilayers, then the size of the splitting ($\Delta\nu = 2.7$ kHz) and the observed single, rather than double, splitting for the motionally inequivalent 26 and 27-deuteriomethyl groups suggest that the content of cholesterol in these MLVs is approximately 40–50% (56). The sharp spectral component in Fig. 2B arises from either unilamellar vesicles and/or mixed micelles that are undergoing rapid isotropic tumbling in the aqueous medium. To determine whether cholesterol undergoes exchange between MLVs and the unilamellar vesicles (ULV)/micellar components of this sample, spectra were recorded at several temperatures. At 37°C, the sharp spectral component accounts for 12% of the total integrated signal while at 26°C and 50°C, it comprises 5% and 16%, respectively. All the observed spectral changes are readily reversible, confirming cholesterol exchange between MLVs and micelles/ULVs, and showing that the rate is slow on the ^2H NMR time-scale.

The chemical composition of each phase in this sample was determined using samples of the same total composition (20 mol % cholesterol; 50 mol % MIL and 30 mol % MBS) in which each of the components,

myristic acid, DMPC, or monomyristoylglycerol was ^2H -labeled on a terminal methyl group, in turn. ^2H NMR spectra are shown in Fig. 3. The sample with composition B, in which the myristic acid is ^2H -labeled on the terminal methyl group, has a spectrum consisting of a sharp isotropic peak superimposed on a broad Pake doublet with 90° edge singularities separated by 7.3 kHz. This splitting is much larger than that shown by the terminal methyl group of the *sn*-2 chain of DMPC in the same sample (vide infra). The larger value probably reflects the lower depth of penetration into the bilayer of this site in myristic acid, compared with the corresponding site in DMPC. If the label is located on the three methyl groups of the quaternary nitrogen in the headgroup of DMPC, then the spectrum shows a sharp singlet with two distinct shoulders, which are the edge singularities of the powder pattern arising from DMPC in multilamellar vesicles (Fig. 3). Although it is difficult to assess the relative contributions of the two spectral

components by integration, fairly reliable values were obtained by comparison of experimental and simulated spectra. To confirm these results, the spectrum of a sample of identical composition was obtained, in which DMPC was ^2H -labeled on the terminal methyl of the *sn*-2 chain. A time-averaged quadrupole splitting of 2.8 kHz at 37°C was measured, which facilitated the accurate determination by integration of the relative contributions of the narrow and wide-line spectral components. Finally, a spectrum of a sample with identical composition was recorded in which the terminal methyl group of monomyristoylglycerol was ^2H -labeled (Fig. 3). The wide-line component in this spectrum was of poor quality because of the small amount of labeled component in the MLV phase. The observed quadrupole splitting of 7.3 kHz is identical to that of the terminal methyl group in myristic acid in the same sample. With the judicious choice of labeled sites, it should be possible to determine the relative lipid composition of the MLV

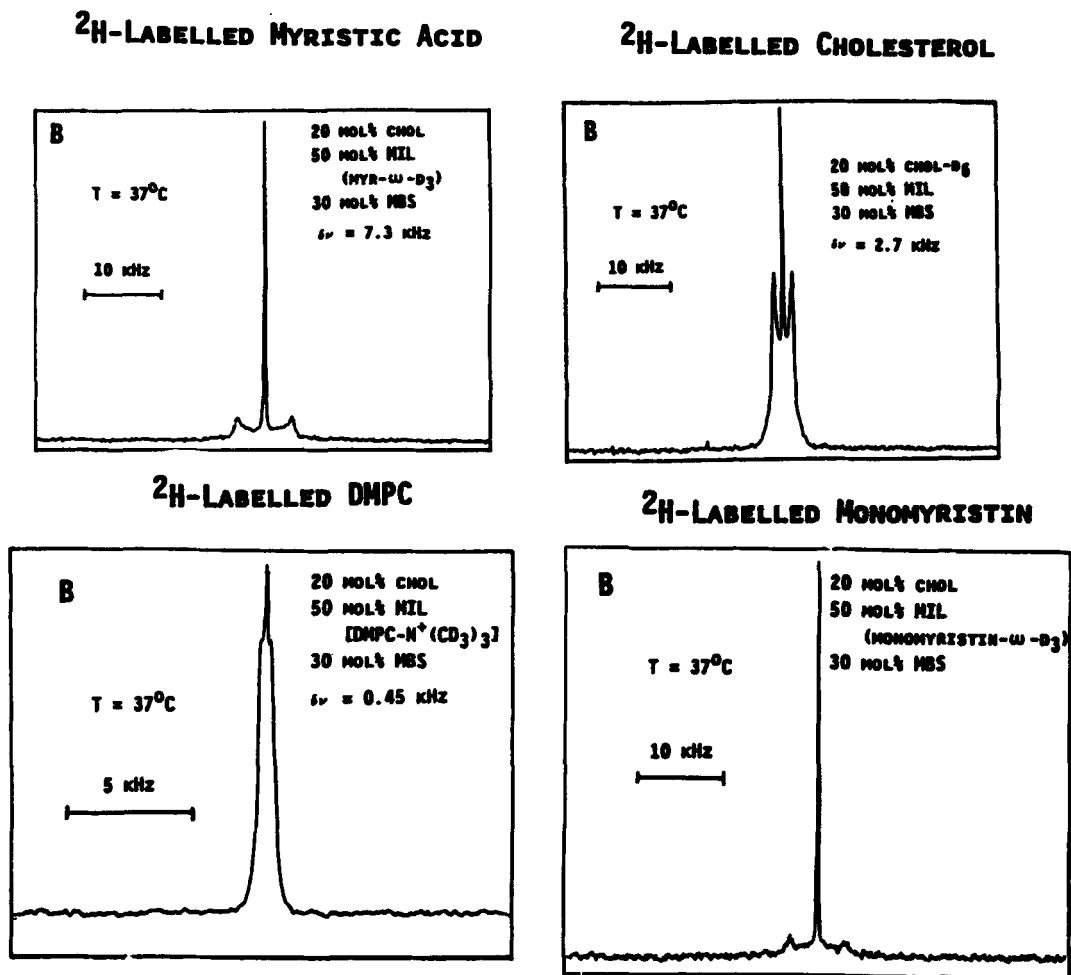


Fig. 3. Four ^2H NMR spectra of 8% (w/v) aqueous mixtures of cholesterol (20 mol %) in the presence of mixed intestinal lipids (50 mol %) and mixed bile salts (30 mol %) (composition B). The deuterium label has been placed, in turn, on myristic acid, cholesterol, DMPC, and monomyristoylglycerol.

phase in a single experiment, without having to record four spectra with each of the lipids separately labeled. The total chemical analysis of the two-phase mixture would require a spectrometer of sufficiently high field to resolve the resonances in the central isotropic peaks arising from different chemical shifts of the methyls in cholesterol, DMPC, myristic acid, and monomyristoylglycerol. This has been shown to be possible in model lipid systems containing cholesterol- d_6 and DMPC- d_9 , whose ^2H NMR spectra recorded at 92.069 MHz show central peaks separated by 2.35 ppm, a value consistent with the chemical shift difference between the $\text{N}^+(\text{CH}_3)_3$ and 26,27- CH_3 groups in the ^1H NMR spectra of model bile systems (28). Using the data in Fig. 3, the compositions of the MLV and ULV/micellar phases were estimated from the known quantities of each lipid in the mixture and the relative proportions of the various phases as determined by peak area integration and spectral simulation. The results (Table 1) show that the micellar/ULV particles are relatively enriched in myristic acid and monomyristoylglycerol. DMPC and cholesterol prefer the bilayers of the MLV phase. These results are not unexpected given the proposed wedge-like shape of hydrated monoacyl chain amphiphiles, such as myristic acid and monomyristoylglycerol, and the approximately cylindrical shape of cholesterol and lecithins (59).

In Fig. 2C the presence of three spectral components is evident, indicating the coexistence of three phases: a micellar phase, a multilamellar liquid-crystalline phase, and a solid phase. The broadest spectral component, with a width of approximately 25 kHz at the baseline and with poorly defined singularities, was assigned to solid cholesterol because it has features in common with the published ^2H NMR spectrum of polycrystalline $[26,26,26,27,27,27\text{-}^2\text{H}_6]$ cholesterol (60). We have confirmed this assignment by recording the ^2H NMR spectrum of solid cholesterol- d_6 . If all motions about the carbon-carbon bonds of the alkyl chain in cholesterol were frozen in the solid state, except for rotation about the C(25)- CD_3 (26) and C(25)- CD_3 (27) linkages, then the spectrum would be a Pake doublet with a splitting of approximately 36 kHz (39). The observed envelope is

narrower, indicative of additional side-chain motions in the solid state. Finally, at the highest mole fraction of MBS, (5 mol % MIL; 75 mol % MBS), only a singlet and broad powder pattern are present in the spectrum (Fig. 2D), indicating that solid cholesterol and cholesterol-saturated mixed micelles of MIL/MBS/cholesterol are the only aggregate structures present.

To gain an insight into the role of DMPC and cholesterol in stabilizing the liquid crystalline phase of myristic acid/monomyristoylglycerol systems, we have examined one other composition on the MIL/MBS/ H_2O phase diagram. Where the 20 mol % cholesterol tie-line intersects the MIL edge of the diagram represents a lipid mixture of 80 mol % MIL (myristic acid-monomyristoylglycerol-DMPC = 5:1:1) and 0 mol % MBS. The ^2H NMR spectrum at 37°C of this mixture in which the myristic acid is ^2H -labeled at its terminal methyl group is shown in Fig. 4A. A quadrupole splitting of 7.3 kHz is observed. Removal of DMPC from this mixture changes a single-phase system to a three-phase system whose ^2H NMR spectrum (Fig. 4B) shows the coexistence of solid $[14,14,14\text{-}^2\text{H}_3]$ myristic acid ($\Delta\nu \approx 34$ kHz), MLVs containing monomyristoylglycerol, $[14,14,14\text{-}^2\text{H}_3]$ myristic acid ($\Delta\nu = 7.3$ kHz) and cholesterol, and a small amount of a phase showing a sharp singlet in the spectrum (mixed micelles or dissolved monomeric lipid). The cholesterol in the MLVs of this cholesterol/myristic acid/monomyristoylglycerol system plays an important structural role because its removal from the lipid mixture produces a spectrum (Fig. 4C) in which the Pake doublet from the ^2H -labeled myristic acid is considerably narrowed from 7.3 to 1.3 kHz.

To more completely characterize these lipid systems, we have attempted to use ^2H NMR to define the relative amounts of micelles and ULVs that contribute to the sharp spectral components observed in Figs. 2 and 3, as well as determine the sizes of these lipid aggregates. The coexistence of both micelles and vesicles in model gastrointestinal lipid dispersions (1% w/v total lipid) has been demonstrated by Staggers et al. (17), who also measured their mean particle hydrodynamic radii using quasi-elastic light scattering (QELS). Mixed micelles and vesicles in bile salt-lecithin systems have been characterized by QELS, NMR, and several other methods under a variety of dilutions and compositions (24, 26, 27, 61-67). Several ^2H NMR studies of model bile systems have been reported (25, 30, 31). In one report, it was found that ^2H spin-lattice relaxation rates in deoxycholate/palmitoylphosphatidylcholine (POPC) micelles were 40-70% faster than in POPC vesicles (25), suggesting that it may be possible to show the coexistence of micelles and vesicles by demonstrating that the spin-lattice relaxation is governed by two time constants. Thus, in analogous MIL/MBS/cholesterol systems, we

TABLE 1. Lipid composition of spectral components in 20 mol % cholesterol, 50 mol % MIL, and 30 mol % MBS mixtures^a

	MLV	Micelles
	mol%	
Cholesterol	41	8
Myristic acid	39	71
Monomyristoylglycerol	6	17
DMPC	14	4

^aThe bile salt composition of micellar particles is unknown.

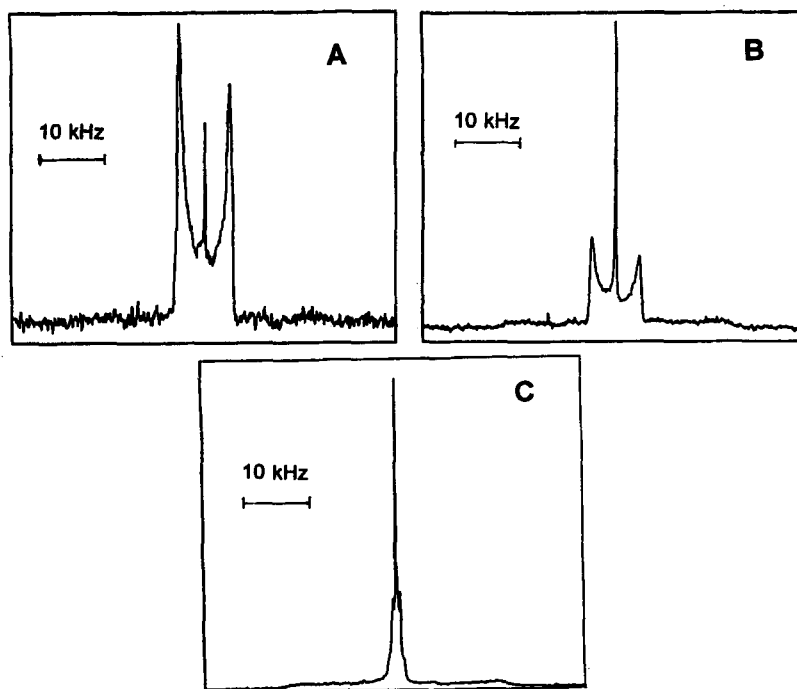


Fig. 4. ^2H NMR spectra of 8% (w/v) aqueous dispersions of A) cholesterol (20 mol %) in the presence of mixed lipids (80 mol %; myristic acid- α - $^2\text{H}_3$ /monomyristoylglycerol/DMPC = 5/1/1), B) cholesterol (20 mol %) in the presence of mixed lipids (80 mol %; myristic acid- α - $^2\text{H}_3$ /monomyristoylglycerol = 5/1), C) 100 mol % MIL (myristic acid- α - $^2\text{H}_3$ /monomyristoylglycerol = 5/1). Spectra were recorded at 37°C from the averaged accumulations of 20,000 pulses. In each case the spectral width is 60 kHz.

have characterized the relaxation behavior of the lipid component which gives rise to the sharp peak in Fig. 2B. The α -methylene site of myristic acid rather than the terminal methyl group of a lipid component was chosen for these measurements because a site was needed where $T_1 \neq T_2$ so that particle sizes could also be calculated (vide infra) (54). At the terminal methyl group of an acyl chain in both micelles and ULVs, T_1 has been shown to be equal to T_2 (25). In the T_1 determination at the α -methylene site, a plot of peak amplitude as a function of the delay time τ , between the 180° and 90° rf pulses in the modified inversion-recovery pulse sequence (52) is shown in Fig. 5. Included on the same graph is a plot of the peak height of the echo as a function of τ , the delay time between the 90_x and 90_y pulses in a quadrupole echo pulse sequence. These data were used to determine T_2 . The values of $T_1 = 30$ ms and $T_2 = 6.7$ ms for a temperature of 37°C and a field strength of 46.05 MHz, determined from the data in Fig. 5, are consistent with results from analogous bile salt/phosphatidylcholine mixed micelle systems (25). The straight lines in Fig. 5 indicate that relaxation is governed by single time-constants, suggesting that the myristic acid exists either in a single population of rapidly tumbling particles or in a mixture of micelles and ULVs where the ^2H -labeled myristic acid is undergoing fast exchange between these aggregates.

To distinguish between these possibilities, we have prepared by ultrasonication, ULVs of egg yolk lecithin containing 20 mol % [2,2- $^2\text{H}_2$]myristic acid. Negative stain electron microscopy of the preparation revealed particles 230 \pm 50 Å in diameter. The ^2H NMR spectrum at 30.87 MHz of these ULVs was a broad singlet which could be fitted to a single Lorentzian line shape with a width at half-height of 436 \pm 10 Hz, after correcting for the width of the $^2\text{H}_2\text{O}$ signal in a deuterated buffer sample. The line shape and width indicate that spin-spin relaxation is governed by a single T_2 value of 730 \pm 17 μ s. Similar measurements, recorded under identical conditions, of a mixed micellar preparation consisting of mixed bile salts (80 mol %) and [2,2- $^2\text{H}_2$]myristic acid (20 mol %) gave a single ^2H T_2 value of 4.8 \pm 0.1 ms. The similarity of T_2 values obtained from model mixed micellar systems and the MIL/MBS/cholesterol sample (Fig. 5), after allowing for the different frequencies (30.87 vs. 46.05 MHz), provides evidence that the small particles in the MIL/MBS/cholesterol sample (20 mol % cholesterol/50 mol % MIL/30 mol % MBS) are predominantly micellar.

^2H spin-lattice relaxation times (T_1) have also been measured in the above model micellar and ULV systems. T_1 values at 30.87 MHz for the α -deuterons of myristic acid in the micellar and ULV states were 16 \pm 1 ms and 18 \pm 1 ms, respectively. Thus, particle size has little effect

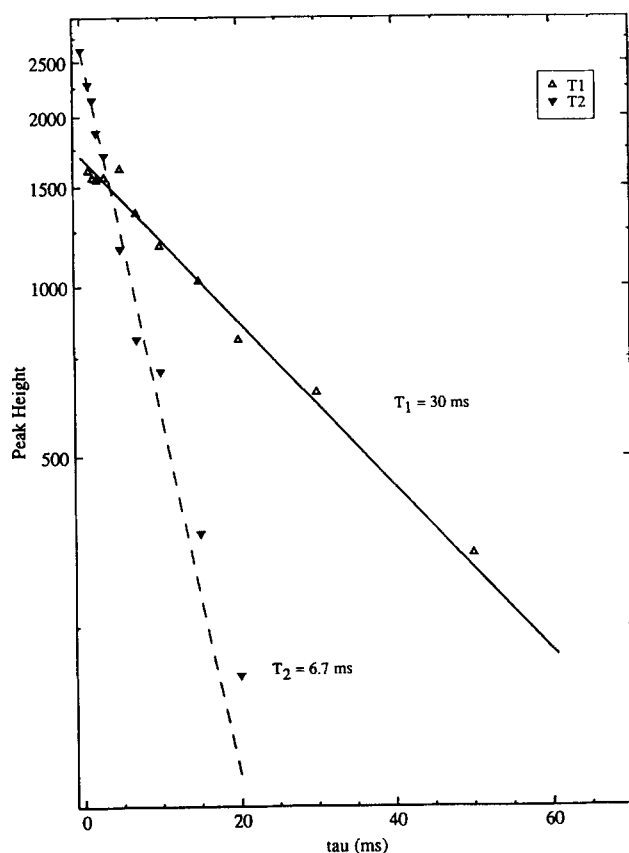


Fig. 5. Relaxation times (T_1 and T_2) determination of the sharp singlet in the ^2H NMR spectrum of an 8% (w/v) aqueous mixture of cholesterol (20 mol %) in the presence of mixed intestinal lipids (50 mol %) and mixed bile salts (30 mol %) (composition B). The myristic acid component of the mixed intestinal lipids is selectively ^2H -labeled on its alpha carbon. Measurements were obtained at 46.05 MHz at a temperature of 37°C. In the determination of T_1 , peak amplitude is plotted as a function of the delay time, τ between the 180° and 90° rf pulses in the modified inversion-recovery pulse sequence (52). In the T_2 determination, the peak height of the echo is plotted as a function of τ , the delay time between the 90_x and 90_y pulses in the solid echo pulse sequence.

on ^2H spin-lattice relaxation, an observation previously noted (51) in a study of ^2H and ^{13}C T_1 values in MLVs and ULVs (68, 69). Comparisons of T_2 and T_1 values show that the former is approximately 25 times smaller than the latter for myristic acid in ULVs, a result consistent with earlier relaxation studies of ^2H -labeled stearic acid dispersed in egg yolk ULVs (51). There is only a threefold difference between T_2 and T_1 values in the case of micelles. All these relaxation data are consistent with the idea that fast internal motions, which should be similar for a ^2H -labeled fatty acid in micelles, ULVs or MLVs, are the predominant relaxation mechanism for T_1 , whereas slower motions such as vesicle tumbling and lateral diffusion play a more significant role in determining T_2 .

^2H NMR relaxation times have also been used to determine the sizes of lipid particles in an aqueous medium. Ericksson, Arvidson, and Lindblom (54) developed a treatment for using the differences between ^2H T_1 and T_2 values at the $\alpha\text{-C}^2\text{H}_2$ site in the palmitoyl moiety of POPC in sodium cholate/POPC micelles to calculate their size. The results accorded well with measurements using light-scattering techniques. When this treatment was used to analyze ^2H relaxation times from MIL/MBS/cholesterol mixtures, we calculated that the rapidly tumbling particles responsible for the sharp peak in Fig. 2B were predominantly micelles of radius 3.1 nm, assuming a spherical shape. To perform this calculation (see Materials and Methods) it was necessary to determine the time averaged quadrupole splitting for the α -methylene deuterons of myristic acid in a lamellar phase. This quantity is 37.6 kHz at 37°C and was obtained from a sample of composition identical to that at point A in the phase diagram where the only labeled component was $[2,2\text{-}^2\text{H}_2]$ myristic acid. It is important to emphasize the assumptions and simplifications used in the method of Ericksson et al. (54). The difference between T_1 and T_2 values cannot be used to determine the shape or polydispersity of the particles. It is only possible to estimate the approximate average size of these particles after an assumption is made about whether they are spherical or disc-like. If it is assumed that the aggregates are oblate ellipsoids with a long semi-axis (a) as the radius of the disc, then the same experimental values of T_1 and T_2 give a calculated (54) value of $a = 3.4$ nm.

DISCUSSION

Understanding the factors controlling the absorption of dietary fats requires a knowledge of their physical and chemical states in the upper gastrointestinal tract. To date, these states have been inadequately defined. A simple methodology, based on ^2H NMR, for characterizing the physicochemical state of model aqueous lipid dispersions is described above. The technique avoids the lengthy separation and analytical procedures usually required for determining the chemical composition of these mixtures, but requires the synthesis of specifically ^2H -labeled chemicals.

The potential of ^2H NMR to construct phase diagrams analogous to that reported by Stammers et al. (17) is clearly demonstrated, even though there are insufficient data reported here to construct a complete phase diagram for the ternary MBS/MIL/cholesterol system, in which the MIL component consists of myristic acid and its derivatives. The utility of our technique for estimating the chemical composition of each phase is demonstrated for one point (B) on the phase diagram. Errors

in determining particle composition at this point are small but will be larger at the edges of the phase diagram and near phase boundaries, when one component or phase is present in small amounts and gives a weak NMR signal. This problem could be alleviated by using magnetic fields larger than 4.70 Tesla.

One apparent discrepancy between the results reported by Staggers et al. (17) and those described here, is the nature of the phases in the two-phase zone of the phase diagram (point B, Fig. 1). Our data indicate that micelles exist in equilibrium with MLVs, whereas the earlier light scattering study showed the co-existence of micelles and ULVs and the absence of MLVs. The probable reasons for this absence are the difference between the total lipid concentrations used in the two studies, and perhaps the different degrees of unsaturation of the lipid components. Carey and coworkers (15–17, 70) have shown that increased total lipid concentrations in both model bile and lipid digestive mixtures favor the formation of MLVs. Most of their studies on model digestive mixtures were performed on the more physiological 1% (w/v) lipid dispersions whereas our data are for 8% (w/v) dispersions. To explore the role of total lipid concentration in the formation of MLVs, we recorded ^2H NMR spectra of samples with composition B (Fig. 2), in which the water content was between 92% and 99.5% (w/v), and the ^2H -label was on the α -methylene site of myristic acid. At a total lipid concentration of approximately 1% (w/v), it was found with decreasing lipid concentration, that the sharp spectral component broadened appreciably (width at half-height of 320 Hz). Based on the line-width measurements of model [2,2- $^2\text{H}_2$]myristic acid/egg yolk lecithin ULVs reported above, this observation is consistent with the conversion of micellar particles to unilamellar vesicles.

The possibility of using time-averaged quadrupole splittings ($\Delta\nu$) to reveal structural features of the lamellar phase in aqueous dispersions of MIL/MBS/cholesterol is demonstrated in the following two cases. As the composition of a sample with 80 mol % MIL and 20 mol % cholesterol is changed by the addition of mixed bile salts, it was found that the quadrupole splitting of the terminal methyl group in myristic acid ($\Delta\nu = 7.3$ kHz) remained unchanged, even up to 60 mol % MBS. Similarly, the splittings for monomyristoylglycerol ($\Delta\nu = 7.1$ kHz) and cholesterol ($\Delta\nu = 2.7$ kHz) are independent of the amount of MBS. In contrast, the splitting in the spectrum of the sample where DMPC is ^2H -labeled on its quaternary nitrogen methyl groups changes from 0.59 ± 0.01 kHz to 0.30 ± 0.05 kHz as the mole percentage of MBS increases from 0 to 60 (point C in Fig. 1). These data suggest that almost no glycodeoxycholate is intercalated in bilayers as the ordering of the terminal

methyl groups of myristic acid and monomyristoylglycerol in the center of the bilayer is unperturbed. Most disordering caused by this bile salt to the MLVs occurs at the headgroup, a finding consistent with a model for MIL-bile salt interactions, analogous to the radial shell model of Ulmius et al. (29) and Lindblom, Eriksson, and Arvidson (30) for lecithin-bile salt interactions. It has been confirmed that most of the added bile salts are present in mixed micelles and not in the lamellar phase, as examination of a lipid system of composition B (Fig. 1) in which glycodeoxycholic acid was ^2H -labeled on its glycine methylene group reveals only a sharp singlet in the ^2H NMR spectrum, even with the accumulation of a very large number of free-induction decays (data not shown). Further study of this sample by ^2H NMR spectroscopy at 92.069 MHz failed to identify a spectral component with a non-zero quadrupole splitting. These results show that exchange of bile salt between the bilayer surface and mixed micelles in the aqueous medium is fast on the NMR time-scale.

The second example where a consideration of $\Delta\nu$ reveals structural features of the lamellar phase is provided by a comparison of $\Delta\nu$ for the terminal methyl groups of myristic acid and monomyristoylglycerol in samples containing 20 mol % cholesterol and varying ratios of MIL/MBS. In the three cases examined, $\Delta\nu$ was almost the same in myristic acid and its monoacylglycerol analog. It has been shown (48) that for saturated straight-chain hydrocarbons intercalated in lamellar systems, the quadrupole splitting of the terminal methyl group is a measure of its depth of penetration in the bilayer. Therefore, the terminal methyl groups of myristic acid and monomyristoylglycerol have the same disposition in the bilayer, and it follows that, if their acyl chains are equally ordered, the carboxylate (or carboxylic acid) group of myristic acid should be level in the bilayer with the carbonyl group of 1-monomyristoylglycerol.

The potential of ^2H NMR relaxation measurements to determine approximate particle size, but not shape, is also demonstrated. There is agreement between the size of the mixed micelles measured by ^2H NMR and the QELS results reported by Staggers et al. (17). For mixed micelles of a different cholesterol content, they determined a mean hydrodynamic radius (R_h) of 32 Å at a MIL/MBS ratio of 0.7, whereas we report a value of 31 Å for an MIL/MBS ratio of 1.6 (point B), assuming a spherical geometry. The scope of this methodology for lipid mixtures of other compositions is currently being further explored.

The one-phase liquid-crystalline (A2) phase observed by Staggers et al. (17) was not studied systematically in their work, and also needs further characterization for the analogous myristate derivative system examined

here. At the two points in the A2 region studied by ^2H NMR and X-ray diffraction, it is evident that lamellar phases exist. The data in Fig. 4 show that systems containing a 5/1 molar ratio of myristic acid/monomyristoylglycerol require both DMPC and cholesterol to remain totally in a lamellar phase. Removal of DMPC from the MIL created a two-phase system consisting of solid myristic acid ($\Delta\nu = 34$ kHz) in equilibrium with a lamellar phase containing myristic acid, monomyristoylglycerol, and cholesterol (Fig. 4B). Preliminary ^2H NMR measurements and titration curve determinations of fatty acid/ H_2O and fatty acid/monoacylglycerol/ H_2O systems suggest that myristic acid at pH 6.5 forms lamellar phases with monomyristoylglycerol optimally at a molar ratio of two fatty acids to one monomyristoylglycerol. When the fatty acid/monoacylglycerol molar ratio is 5/1, too much fatty acid is present to form a stable lamellar phase in the absence of DMPC, so myristic acid or its acid-soap dimer separates out as a solid. If cholesterol is also removed from the MIL, the ^2H NMR spectrum of the myristic acid/monomyristoylglycerol/ H_2O system, with the ^2H label on the fatty acid, consists of the superposition of a broad uniaxial powder pattern from solid myristic acid, a uniaxial powder pattern with a much smaller splitting ($\Delta\nu = 1.3$ kHz), and a sharp singlet (Fig. 4C). It is not possible to interpret these results unequivocally, but it has been noted that dilute dispersions of 5:1 molar fatty acid:monoacylglycerol systems at pH 6.5 form an L-2 phase (58). According to Ekwall (71) this phase is an isotropic solution in organic solvents of micelles of the inverse type. They have a core composed of polar groups and water that is surrounded by a layer of hydrocarbon chains of the micelle forming substances, in this case excess melted fatty acids or acid-soap complexes acting as an intermicellar liquid. It is also possible that the myristic acid/monomyristoylglycerol/ H_2O system is not the L-2 phase but the poorly characterized "viscous isotropic" phase described by Lindström et al. (58). It is apparent that the monomyristoylglycerol/myristic acid/ H_2O system needs to be better characterized and the effect of added cholesterol and lecithin should be determined. In general, monoacylglycerol/fatty acid/ H_2O systems require further study because of their importance in lipid digestion in the upper gastrointestinal tract.

The experiments on model lipid mixtures described above are non-physiological in several respects (apart from the fact that myristic acid derivatives are not major dietary constituents). First, the total lipid concentrations are much higher than those found in the upper gastrointestinal tract (72). Second, no carbohydrates or proteins are present that could alter the physical state of the lipids in the model systems. Third, the results describe a system at equilibrium when the time taken to achieve

equilibrium is longer than the time that lipids are present in the gastrointestinal tract. Fourth, 20 mol % levels of cholesterol are never found in the mixed lipids of the upper gastrointestinal tract. Finally, 1-monoacylglycerols, rather than the 2-monoacylglycerols formed by hydrolysis in the intestine of triacylglycerols, are used in the preparation of model systems. The 1-isomer was used in our experiments because of its availability and greater stability. Previous studies (73) have shown that 1-monooleoylglycerol and 2-monooleoylglycerol behave identically in bile salt solutions.

Because of these limitations, and to further explore the scope of this technique, we are currently examining additional model digestive systems in which the fatty acid constituents are unsaturated, the cholesterol and total lipid contents are lower, the bile salt composition is altered, the fatty acid to monoacylglycerol ratio is changed, and other gastrointestinal lipid constituents such as triglycerides, lysophosphatidylcholine, and diacylglycerols are present. ■

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REFERENCES

1. Carey, M. C., D. M. Small, and C. M. Bliss. 1983. Lipid digestion and absorption. *Annu. Rev. Physiol.* **45**: 651-677.
2. Tso, P. 1985. Gastrointestinal digestion and absorption of lipid. *Adv. Lipid Res.* **21**: 143-185.
3. Shiau, Y-F. 1987. Lipid digestion and absorption. *In Physiology of the Gastrointestinal Tract*. Second ed. L. R. Johnson, editor. Raven Press, New York. 1527-1556.
4. Hofmann, A. F., and B. Borgström. 1962. Physicochemical state of lipids in intestinal content during their digestion and absorption. *Fed. Proc.* **21**: 43-50.
5. Hofmann, A. F., and B. Borgström. 1964. The intraluminal phase of fat digestion in man: the lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. *J. Clin. Invest.* **43**: 247-257.
6. Porter, H. P., and D. R. Saunders. 1971. Isolation of the aqueous phase of human intestinal contents during the digestion of a fatty meal. *Gastroenterology.* **60**: 997-1007.

7. Mansbach, C. M., R. S. Cohen, and P. B. Lief. 1975. Isolation and properties of the mixed lipid micelles present in intestinal content during fat digestion in man. *J. Clin. Invest.* **56**: 781-791.
8. Carey, M. C., and O. Hernell. 1992. Digestion and absorption of fat. *Semin. Gastrointest. Dis.* **3**: 189-208.
9. Holt, P. R., B. M. Fairchild, and J. Weiss. 1986. A liquid crystalline phase in human intestinal contents during fat digestion. *Lipids*. **21**: 444-446.
10. Patton, J. S., and M. C. Carey. 1979. Watching fat digestion. *Science*. **204**: 145-148.
11. Rigler, M. W., R. E. Honkanen, and J. S. Patton. 1986. Visualization by freeze fracture, in vitro and in vivo, of the products of fat digestion. *J. Lipid Res.* **27**: 836-857.
12. Rigler, M. W., and J. S. Patton. 1983. The production of liquid crystalline product phases by pancreatic lipase in the absence of bile salts. A freeze-fracture study. *Biochim. Biophys. Acta*. **751**: 444-454.
13. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61**: 998-1026.
14. Carey, M. C. 1983. Measurement of physical-chemical properties of bile salt solutions. In *Bile Acids in Gastroenterology*. L. Barbara, R. H. Dowling, A. F. Hofmann, and E. Roda, editors. MTP Press, Boston. 19-56.
15. Stafford, R. J., and M. C. Carey. 1981. Physical-chemical nature of the aqueous lipids in intestinal content after a fatty meal: revision of the Hofmann-Borgström hypothesis. *Clin. Res.* **29**: 511A.
16. Stafford, R. J., J. M. Donovan, G. B. Benedek, and M. C. Carey. 1981. Physical-chemical characteristics of aqueous duodenal content after a fatty meal. *Gastroenterology*. **80**: 1291A.
17. Staggars, J. E., O. Hernell, R. J. Stafford, and M. C. Carey. 1990. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. I. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry*. **29**: 2028-2040.
18. Rabiller, C., and F. Maze. 1989. Quantitative analysis and determination of the enantiomeric purity of glycerides by ^{13}C NMR spectroscopy. Application to the lipase-catalyzed transesterification of triacylglycerides. *Magn. Reson. Chem.* **27**: 582-584.
19. Dawe, R. D., and J. L. C. Wright. 1988. A new quantitative method for the analysis of monoacylglycerol isomers using ^{13}C nuclear magnetic resonance spectroscopy. *Lipids*. **23**: 355-358.
20. Waterhous, D. V., S. Barnes, and D. D. Muccio. 1985. Nuclear magnetic resonance spectroscopy of bile acids. Development of two-dimensional NMR methods for the elucidation of proton resonance assignments for five common hydroxylated bile acids, and their parent bile acid, 5 β -cholanoic acid. *J. Lipid Res.* **26**: 1068-1078.
21. Stevens, R. D., A. A. Ribeiro, L. Lack, and P. G. Killenberg. 1992. Proton magnetic resonance studies of the aggregation of taurine-conjugated bile salts. *J. Lipid Res.* **33**: 21-29.
22. Chan, S. I., D. F. Bocian, and N. O. Petersen. 1981. Nuclear magnetic resonance studies of the phospholipid bilayer membrane. In *Membrane Spectroscopy*. E. Grell, editor. Springer-Verlag, Berlin. 1-50.
23. Small, D. M., S. A. Penkett, and D. Chapman. 1969. Studies on simple and mixed bile salt micelles by nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta*. **176**: 178-189.
24. Lichtenberg, D., Y. Zilberman, P. Greenzaid, and S. Zamir. 1979. Structural and kinetic studies on the solubilization of lecithin by sodium deoxycholate. *Biochemistry*. **18**: 3517-3525.
25. Stark, R. E., J. L. Manstein, W. Curatolo, and B. Sears. 1983. Deuterium nuclear magnetic resonance studies of bile salt/phosphatidylcholine mixed micelles. *Biochemistry*. **22**: 2486-2490.
26. Stark, R. E., and M. F. Roberts. 1984. 500 MHz ^1H NMR studies of bile salt-phosphatidylcholine mixed micelles and vesicles. Evidence for differential motional restraint on bile salt and phosphatidylcholine resonances. *Biochim. Biophys. Acta*. **770**: 115-121.
27. Stark, R. E., G. J. Gosselin, J. M. Donovan, M. C. Carey, and M. F. Roberts. 1985. Influence of dilution on the physical state of model bile systems: NMR and quasi-elastic light-scattering investigations. *Biochemistry*. **24**: 5599-5605.
28. Ellul, J. P. M., G. M. Murphy, H. G. Parkes, R. Z. Slapa, and R. H. Dowling. 1992. Nuclear magnetic resonance spectroscopy to determine the micellar cholesterol in human bile. *FEBS Lett.* **300**: 30-32.
29. Ulmius, J., G. Lindblom, H. Wennerstrom, L. B-A. Johansson, K. Fontell, O. Söderman, and G. Arvidson. 1982. Molecular organization in the liquid-crystalline phases of lecithin-sodium cholate-water systems studied by nuclear magnetic resonance. *Biochemistry*. **21**: 1553-1560.
30. Lindblom, G., P-O. Eriksson, and G. Arvidson. 1984. Molecular organization in phases of lecithin-cholate-water as studied by nuclear magnetic resonance. *Hepatology*. **4**: 129-133S.
31. Ram, P., and J. H. Prestegard. 1988. Magnetic field induced ordering of bile salt/phospholipid micelles: new media for NMR structural investigations. *Biochim. Biophys. Acta*. **940**: 289-294.
32. Groen, A. K., B. G. Goldhoorn, P. H. M. Egbers, R. A. F. M. Chamuleau, G. N. J. Tytgat, and W. M. M. J. Bovée. 1990. Use of ^1H -NMR to determine the distribution of lecithin between the micellar and vesicular phases in model bile. *J. Lipid Res.* **31**: 1315-1321.
33. Wang, D., N. L. Hadipour, E. A. Jerlin, and R. E. Stark. 1992. Quantitation of model digestive mixtures by ^{13}C NMR. *J. Lipid Res.* **33**: 431-439.
34. Vogel, A. I. 1956. *A Textbook of Practical Organic Chemistry*. 3rd. ed. John Wiley, New York.
35. Westerman, P. W., and N. Ghayeb. 1981. Synthesis of methyl α -deuterated tetradecanoate and hexadecanoate. *Chem. Phys. Lipids*. **29**: 351-358.
36. Durham, L. J., D. J. McLeod, and J. Cason. 1963. Methyl hydrogen hendecanedioate (undecanedioic acid, methyl ester). *Org. Synth. Col.* **IV**: 635-637.
37. Greaves, W. S., R. P. Linstead, B. R. Shephard, S. L. S. Thomas, and B. C. L. Weedon. 1950. Anodic syntheses. Part I. New syntheses of stearic, myristic, and other acids. *J. Chem. Soc.* 3326-3330.
38. Aasen, A. J., W. M. Lauer, and R. T. Holman. 1970. Mass spectrometry of triglycerides. II. Specifically deuterated triglycerides and elucidation of fragmentation mechanisms. *Lipids*. **5**: 869-877.
39. Westerman, P. W., M. J. Vaz, L. M. Strenk, and J. W. Doane. 1982. Phase transitions in phosphatidylcholine multibilayers. *Proc. Natl. Acad. Sci. USA*. **79**: 2890-2894.

40. Jensen, R. G., and R. E. Pitas. 1976. Synthesis of some acylglycerols and phosphoglycerides. *Adv. Lipid Res.* **14**: 213-247.
41. Baer, E., and H. O. L. Fischer. 1945. Synthesis of homologous series of optically active normal aliphatic α -monoglycerides (L-series). *J. Am. Chem. Soc.* **67**: 2031-2037.
42. 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.
43. Batta, A. K., G. Salen, and S. Shefer. 1979. Thin-layer chromatography of conjugated bile acids. *J. Chromatogr.* **168**: 557-561.
44. Hamilton, J. A., and D. M. Small. 1981. Solubilization and localization of triolein in phosphatidylcholine bilayers: a ^{13}C NMR study. *Proc. Natl. Acad. Sci. USA.* **78**: 6878-6882.
45. Tickner, A. L., E. J. A. Lea, and R. W. Horne. 1990. Electron microscopic studies of negatively stained liposomes and membrane intrinsic polypeptide. *Micron Microsc. Acta.* **21**: 91-99.
46. Rosevear, F. B. 1954. The microscopy of the liquid crystalline neat and middle phases of soaps and synthetic detergents. *J. Am. Oil Chem. Soc.* **31**: 628-639.
47. Rosevear, F. B. 1968. Liquid crystals: the mesomorphic phases of surfactant compositions. *J. Soc. Cosmet. Chem.* **19**: 581-594.
48. Westerman, P. W., J. M. Pope, N. Phonphok, J. W. Doane, and D. W. Dubro. 1988. The interaction of n-alkanols with lipid bilayer membranes: a ^2H NMR study. *Biochim. Biophys. Acta.* **939**: 64-78.
49. Davis, J. H. 1983. The description of membrane lipid conformation, order and dynamics by ^2H NMR. *Biochim. Biophys. Acta.* **737**: 117-171.
50. Cohen, M. H., and F. Reif. 1957. Quadrupole effects in nuclear magnetic resonance studies of solids. *Solid St. Phys.* **5**: 321-438.
51. Stockton, G. W., C. F. Polnaszek, A. P. Tulloch, F. Hasan, and I. C. P. Smith. 1976. Molecular motion and order in single-bilayer vesicles and multilamellar dispersions of egg lecithin and lecithin-cholesterol mixtures. A deuterium nuclear magnetic resonance study of specifically labelled lipids. *Biochemistry.* **15**: 954-966.
52. Frye, J. S. 1989. Comparison of Inversion-recovery methods for measuring longitudinal relaxation rates. *Concepts Magn. Reson.* **1**: 27-33.
53. Eriksson, P-O., A. Khan, and G. Lindblom. 1982. Nuclear magnetic resonance studies of molecular motion and structure of cubic liquid crystalline phases. *J. Phys. Chem.* **86**: 387-393.
54. Eriksson, P-O., G. Arvidson, and G. Lindblom. 1983. NMR studies of sodium cholate-lecithin mixed micelles. *Israel J. Chem.* **23**: 353-355.
55. Abragam, A. 1961. *The Principles of Nuclear Magnetism.* Oxford University Press, Oxford.
56. Taylor, M. G., T. Akiyama, H. Saito, and I. C. P. Smith. 1982. Direct observation of the properties of cholesterol in membranes by deuterium NMR. *Chem. Phys. Lipids.* **31**: 359-379.
57. Small, D. M., M. Bourges, and D. G. Dervichian. 1966. The biophysics of lipidic associations. The ternary systems lecithin-bile salt-water. *Biochim. Biophys. Acta.* **125**: 563-580.
58. Lindström, M., H. Ljusberg-Wahren, K. Larsson, and B. Borgström. 1981. Aqueous lipid phases of relevance to intestinal fat digestion and absorption. *Lipids.* **16**: 749-754.
59. Israelachvili, J. N., S. Marcelja, and R. G. Horn. 1980. Physical principals of membrane organization. *Q. Rev. Biophys.* **13**: 121-200.
60. Akiyama, T., S. Takagi, U. Sankawa, S. Inari, and H. Saito. 1980. Saponin-cholesterol interaction in the multibilayers of egg yolk lecithin as studied by deuterium nuclear magnetic resonance: digitonin and its analogues. *Biochemistry.* **19**: 1904-1911.
61. Narayanan, R., R. Paul, and P. Balaram. 1980. Fluorescent probe studies of mixed micelles of phospholipids and bile salts. Effect of cholesterol incorporation. *Biochim. Biophys. Acta.* **597**: 70-82.
62. Claffey, W. J., and R. T. Holzbach. 1981. Dimorphism in bile salt/lecithin mixed micelles. *Biochemistry.* **20**: 415-418.
63. Müller, K. 1981. Structural dimorphism of bile salt/lecithin mixed micelles. A possible regulatory mechanism for cholesterol solubility in bile? X-ray structure analysis. *Biochemistry.* **20**: 404-414.
64. Hjelm, R. P., M. H. Alkan, and P. Thiagarajan. 1990. Small-angle neutron scattering studies of mixed bile salt-lecithin colloids. *Mol. Cryst. Liq. Cryst.* **180A**: 155-164.
65. Spink, C. H., V. Lieto, E. Mereand, and C. Pruden. 1991. Micelle-vesicle transition in phospholipid-bile salt mixtures. A study by precision scanning calorimetry. *Biochemistry.* **30**: 5104-5112.
66. Mazer, N. A., G. B. Benedek, and M. C. Carey. 1980. Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochemistry.* **19**: 601-615.
67. Brouillette, C. G., J. P. Segrest, T. C. Ng, and J. L. Jones. 1982. Minimal size phosphatidylcholine vesicles: effects of radius of curvature on head group packing and conformation. *Biochemistry.* **21**: 4569-4575.
68. Sears, B. 1975. ^{13}C nuclear magnetic resonance studies of egg phosphatidylcholine. *J. Membr. Biol.* **20**: 59-73.
69. Godici, P. E., and F. R. Landsberger. 1974. The dynamic structure of lipid membranes. A ^{13}C nuclear magnetic resonance study using spin labels. *Biochemistry.* **13**: 362-368.
70. Mazer, N. A., and M. C. Carey. 1983. Quasi-elastic light scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile solutions. *Biochemistry.* **22**: 426-442.
71. Ekwall, P. 1975. Composition, properties, and structures of liquid crystalline phases in systems of amphiphilic compounds. *Adv. Liq. Cryst.* **1**: 1-142.
72. Hernell, O., J. E. Stagers, and M. C. Carey. 1990. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry.* **29**: 2041-2056.
73. Hofmann, A. F. 1963. The behavior and solubility of monoglycerides in dilute, micellar bile-salt solution. *Biochim. Biophys. Acta.* **70**: 306-316.