The ionization behavior of bile acids in different aqueous environments

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Abstract  The ionization behavior of cholic acid, deoxycholic acid, and chenodeoxycholic acid in a variety of physiologically important molecular environments was studied using $^{13}$C NMR spectroscopy. The apparent pKa of the carboxyl group was determined from titration curves obtained from the dependence of the carboxyl carbon chemical shift on pH. Using 90% $^{13}$C isotopic substitution of the carboxyl carbon, a complete titration curve was obtained for cholate at a concentration below its critical micelle concentration and solubility limit in water. Incorporation of 12 mole % bile acid into mixed micelles with its taurine conjugate prevented precipitation of the unconjugated bile acid, and titration curves for cholic, deoxycholic, and chenodeoxycholic acids in the mixed micelles were obtained. The apparent pKa was also determined for $^{13}$C-enriched bile acids complexed with bovine serum albumin and in egg phosphatidylcholine vesicles. For monomers, micelles, and BSA complexes of all three bile acids and for deoxycholic and chenodeoxycholic acid in vesicles, one magnetic environment was observed. In contrast, two environments, both titratable, were detected for cholic acid in phosphatidylcholine vesicles. The apparent pKas of the bile acids in the different environments ranged from 4.2 to 7.3. At pH 7.4, as monomers or bound to albumin, the bile acids were fully ionized, but when associated with phosphatidylcholine vesicles they were only partially ionized. In addition, aspects of the molecular motion and relative hydrophobicity of the bile acid carboxyl group in the environments studied were discerned from chemical shift, linewidth, and lineshape data. — Cabral, D. J., J. A. Hamilton, and D. M. Small. The ionization behavior of bile acids in different aqueous environments. J. Lipid Res. 1986. 27: 334–343.

Supplementary key words  bile salts • $^{13}$C NMR spectroscopy • apparent pKa • monomers • micelles • phospholipid vesicles • protein complexes

Bile salts are important physiologic solubilizers of other lipids (1–3). Bile salts readily dissolve in aqueous media while the corresponding bile acids have limited solubility and must be solubilized by other molecules. Bile acids and salts may exist in monomeric form, in simple and mixed bile salt micelles, in mixed micelles with other lipids, in phospholipid vesicle bilayers (membranes), or bound to proteins (4–6). In most species the bile salts in hepatic and gallbladder bile exist mostly as taurine and glycine conjugates. The bile salts are secreted into the duodenum where they encounter large changes in pH (pH 3–8) and aid in the digestion and absorption of fat (7). In man and some animals, some of the bile salts are hydrolyzed by intestinal bacteria to the corresponding unconjugated bile salt. These are reabsorbed by the intestine and transported in the portal vein bound to albumin to the liver where they are effectively extracted and taken up by hepatic cells (4). The free bile salts must then traverse cellular membranes and interact with enzymes which re conjugate them with glycine or taurine. Finally, the conjugated bile salts are resecreted into hepatic bile where they may be present as monomers or in mixed micelles or even in vesicles. Thus, both free and conjugated bile salts may encounter different pHs during their enterohepatic recycling and also interact with proteins. Because of possible variation in the carboxyl group microenvironment, it may not be accurate to extrapolate the ionization behavior of monomeric bile acids to the more complex systems. It is important, therefore, to determine the ionization state of the unconjugated bile acids in these different molecular environments.

The ionization behavior of many bile acids has been studied potentiometrically (8–11). However, it has been difficult to assess the ionization state of bile acids when present in small amounts in other environments, particularly when these environments have other titratable groups. $^{13}$C NMR spectroscopy is well suited for studying the ionization states of carboxylic acids by measurement of the carboxyl $^{13}$C chemical shift versus pH (12). The use of $^{13}$C enrichment of the carboxyl carbon provides a

Abbreviations: NMR, nuclear magnetic resonance; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (5α,7α-dihydroxy-5β-cholanoic acid); PG, phosphatidylcholine; BSA, bovine serum albumin; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; NaTDCD, sodium taurochenodeoxycholate; 24-13C, a bile acid 90% isotopically enriched in $^{13}$C at carbon 24 (standard numbering); NaC, cholic acid, sodium salt; NaD, deoxycholic acid, sodium salt; NaCD, chenodeoxycholic acid, sodium salt; CMC, critical micelle concentration; S/N, signal to noise ratio; TLC, thin-layer chromatography.
means of monitoring small amounts of the acids in solution alone (12) or with other molecules (13-15). In this study we have used $^{13}$C NMR spectroscopy to study aqueous carboxyl (C24) $^{13}$C-enriched cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA) below the critical micelle concentration (CA only), in mixed micelles with their taurine conjugates, in egg phosphatidylcholine (PC) unilamellar vesicles, and in complexes with bovine serum albumin (BSA). The apparent pKa's of the bile acids were determined in each environment. In addition, features of the bile acid molecular environments were assessed from the carboxyl $^{13}$C chemical shift, linewidth, and lineshape.

**MATERIALS AND METHODS**

**Materials**

Sodium taurocholate (NaTTC), sodium taurodeoxycholate (NaTDC), and sodium taurochenodeoxycholate (NaTCDC) were purchased from Calbiochem-Behring (San Diego, CA). Egg PC was obtained from Lipid Products (South Nutley, Surrey, UK), and crystalline fatty acid-free BSA (fraction V) was from Sigma Chemical Company (St. Louis, MO). CDCA and methyl cholate with 90% $^{13}$C isotopic substitution at carbon 24 were gifts from Dr. Peter Klein. Ninety percent [24, $^{13}$C]DCA was purchased from Merck & Company (St. Louis, MO). All non-enriched bile salts were >95% pure by TLC. NMR spectra of $^{13}$C-enriched bile salts with signal to noise ratios >20:1 (in H$_2$O at pH 10.0) showed only a single carboxyl peak. D$_2$O, CDCl$_3$, and (CH$_3$)$_4$Si were obtained from Stohler Isotope Chemicals (Waltham, MA).

**NMR spectroscopy**

Proton-decoupled Fourier transform $^{13}$C NMR spectra were obtained at 50.3 MHz with a Bruker WP-200 spectrometer equipped with a Bruker B-VT:1000 variable temperature unit and an Aspect 2000 data system. Aqueous samples were placed in 10-mm NMR tubes with D$_2$O included as an internal lock for the PC and BSA samples. For monomeric and micellar samples, CDCl$_3$ with (CH$_3$)$_4$Si in a coaxial insert served as an external lock and reference for chemical shift assignments. The terminal methyl of the PC acyl chains at 14.10 ppm (16) or the relatively narrow amino acid resonance at 39.84 ppm (17) in the BSA spectra was used as an internal chemical shift reference. Chemical shifts were accurate to ±0.05 ppm except for BSA spectra (±0.1 ppm). Broad-band proton decoupling (1.0 W) centered 3.4 ppm downfield from (CH$_3$)$_4$Si was used. Spectra were obtained at 35°C using 16K data points and a pulse interval of 2s or 3s.

**Sample preparation**

To prepare [24-$^{13}$C]sodium cholate (NaC), [24-$^{13}$C]methyl cholate was hydrolyzed in excess NaOH with heating. After 2 hr the reaction mixture was cooled to 20°C and spun at 2000 rpm for 15 min, and the clear supernatant was separated from any unreacted crystalline methyl cholate. The cholate preparation showed a single spot by thin-layer chromatography (cyclohexane-ethyl acetate-acetic acid 7:23:3). A $^{13}$C NMR spectrum at pH 10 gave a single resonance in the carboxyl region at the expected chemical shift (18, 19). Stock solutions of [24-$^{13}$C]NaC, [24-$^{13}$C]sodium deoxycholate (NaDC), and [24-$^{13}$C]sodium chenodeoxycholate (NaCDC) at 15 mg/ml, pH 10, were used for sample preparation.

An aliquot of the [24-$^{13}$C]NaC stock was diluted with H$_2$O to yield aqueous cholate (0.2 mM) below its critical micelle concentration (CMC) and below the solubility limit of CA. For mixed bile salt micelles, 10 mg of the appropriate bile salt (natural abundance) and 90 mg of its taurine conjugate were dissolved in 2.0 ml of H$_2$O and the pH was adjusted to 11. This 7:1 NaTTC-NaC mol ratio adequately maintains the cholic acid in solution at low pH (8, 9). All micellar and monomeric solutions were clear and colorless at pH 11.

Bile acid-BSA complexes were prepared by the procedure of Parks et al. for the preparation of fatty acid-BSA complexes (13). An aliquot (1.3 ml; 1.6 × 10^{-5} mmol) of aqueous 8% (w/v) BSA (pH 7.4) was added with mixing to 0.1 ml (3.2 × 10^{-3} mmol) [24-$^{13}$C]NaC, NaDC, or NaCDC stock solution in an NMR tube. Samples with higher mol ratios of bile acid/BSA were prepared for DCA. The pH was adjusted to 10; the sample was clear and slightly viscous.

Unilamellar vesicles were prepared by two methods. In the first method, described previously (20), 97 mg of egg PC and 3 mg of [24-$^{13}$C]bile salt were mixed in chloroform-methanol 2:1 and the solvent was evaporated under N$_2$. The sample was hydrated, adjusted to pH 10, and ultrasonically irradiated using a Branson W-350 Sonifier in a pulsed mode, 35% duty cycle, and 3.5 output level. The sonication vial was suspended in an ice bath to keep the sample temperature at 25-30°C. Sonication was carried out for 45 min, titanium was removed by low speed centrifugation, and the translucent supernatant (pH 10) was transferred to an NMR tube. For the second method, 97 mg of PC was hydrated and sonicated as before; then 3 mg of [24-$^{13}$C]NaC (pH 10) was added to the vesicle preparation and mixed by vortexing for 1 min. The concentration of egg PC in the NMR tube was 6.06 g/dl (78 mM). The mol ratio of PC to bile salt was ~20:1. The size of the vesicles was monitored by negative stain electron microscopy using 1% phosphotungstic acid on a 1/50 dilution of 78 mM (5% NaCl-97% PC) preparation on carbon-coated Formvar grids at 97,875 × magnification.
Titration

Samples were titrated from high to low pH with 1.0 N HCl, except for 0.2 mM NaC which was titrated with 0.1 N HCl. The pH was monitored using a Beckman model 3560 digital pH meter equipped with a Markson microtip probe to allow measurement in the NMR tube. At selected pH values, the amount of HCl added was recorded, the sample appearance was noted, and \(^{13}\)C NMR spectra were obtained. The pH measured before and after each NMR run was the same (± 0.1 pH units). The C24 chemical shift was plotted versus pH, and the

Fig. 1. \(^{13}\)C NMR spectra at 50.3 MHz of aqueous bile salts; 5% w/w, pH 9.3. Chemical shifts are relative to \((\text{CH}_3)_2\text{Si}\) and selected assignments of individual resonances indicated are based on Barnes and Geckle (16) and Leibfritz and Roberts (17). Note that assignments of the hydroxyl carbons (3, 7, 12) are according to Leibfritz and Roberts. A, NaC; B, NaDC; C, NaCDC. Inserts are expanded hydrocarbon region of the bile salts from 11 ppm to 60 ppm.
apparent pKa was defined as the pH corresponding to the chemical shift at one-half the maximum chemical shift difference (12).

RESULTS

Peak assignment

Natural abundance $^{13}$C NMR spectra of 5% aqueous solutions of NaC (116 mM), NaDC (120 mM), and NaCDC (120 mM) at pH 9 are shown in Fig. 1. All 24 carbons yielded resolved resonances; selected assignments indicated are based on those of Barnes and Geckle (18) for aqueous micellar cholate and on those of Leibfritz and Roberts (19) for the acids in methanol. Concentrations of NaC and NaDC were adjusted from 5 mM to 116 or 120 mM, and the pH was maintained at 9.0 throughout. The chemical shift of numerous peaks showed a concentration dependence, the magnitudes and directions of which were in agreement with those of Conte et al. (21) and Murata et al. (22) for NaDC. The concentration-dependent chemical shift change for the carboxyl resonance of NaC and NaDC, -0.5 ppm, was small compared with those produced by titration (4.5-5.3 ppm).

Chemical shift of monomeric cholic acid as a function of pH

A dilute solution of [24-$^{13}$C]NaC (0.2 mM) below its CMC (9 mM in H$_2$O at 20°C) and below the solubility limit of CA (9) was titrated from pH 10 to pH 2 and gave no visible precipitate during titration. The NMR titration curve (chemical shift vs pH) given in Fig. 2 shows that the chemical shift decreased from 184.7 ppm at high pH to 180.0 at pH 2. The apparent pKa was 4.6. At the low concentration used, an adequate signal to noise ratio (S/N) was obtained for the $^{13}$C-enriched carboxyl carbon only after 14-20 hr (25,000-35,000 accumulations). The S/N of this peak was not sufficient for measuring an accurate linewidth, although the resonance was narrow (<10 Hz) at all pH values. Resonances from the carbons at natural abundance were not detected. A micellar solution (116 mM) of NaC at pH 10 was also titrated. Natural abundance $^{13}$C spectra taken at pH 10.0-7.0 gave an invariant C24 chemical shift of 184.30 ppm (Fig. 2). However, at pH 6.5 the acid precipitated and no NMR signal was detected from the solution containing the precipitated CA.

Chemical shift of micellar bile acids as a function of pH

Natural abundance $^{13}$C spectra of mixed micelle systems containing a mol ratio of one unconjugated to seven taurine-conjugated bile salts (NaC/NaTC, NaDC/NaTCDC, and NaCDC/NaTCDC) were obtained as a function of pH. The height of the unconjugated carboxyl resonance, ~0.1 that of its taurine conjugate carboxyl resonance, was proportional to its concentration in the mixed micelle. A single, narrow (<10 Hz) carboxyl carbon peak was observed at each pH, but the S/N was not sufficient to measure an accurate linewidth. The pKa values (5.3, 6.1, and 6.3) for CA, DCA, and CDC, respectively, were in good agreement with potentiometrically determined values for simple micelles of each acid (8-11). The titration curve for CA in mixed micelles with NaTC is shown in Fig. 2. The mixed micelles were back-titrated from low pH to two intermediate pH values and to pH 9. The measured carboxyl chemical shifts fell on the titration curves.

Chemical shift of bile acids bound to BSA as a function of pH

$^{13}$C NMR spectra of the bile salt/BSA complexes (2:1 mol/mol, respectively) gave a single peak from the $^{13}$C-enriched carboxyl carbon for each bile salt at pH 9. Fig. 3 shows spectra for [24-$^{13}$C]CDCA bound to BSA at three pH values. The CDCA carboxyl peak was well resolved from the protein resonances except for the small glutamate carboxyl resonance (see Fig. 3A). In addition to chemical shift changes with pH, there were marked changes in the linewidth of the carboxyl peak. As illustrated in the CDCA/BSA spectrum, the C24 peak was slightly asymmetric at pH 7.4 (Fig. 3A). It showed a greater asymmetry and broadened to 53 Hz at pH 3.9 (Fig. 3B). After complete protonation of the bile acid (Fig. 3C), the carboxyl peak became very narrow (4 Hz) and
Fig. 3. $^{13}$C NMR spectra at 50.3 MHz of [24-$^{13}$C]CDCA in complexes with BSA (2.1 mol/mol; 3.2 x 10^{-3} mM CDCA and 1.6 x 10^{-4} mM BSA in 1.5 ml), pulse interval 2.0 s, 3.0 Hz line-broadening used in processing. Chemical shifts relative to the narrow BSA peak at 35.84 ppm (17). A, pH 7.4; B, pH 3.9; C, pH 2.0. Inserts show expanded carboxyl/carboxyl region (170 ppm-184 ppm) for the indicated pH values. BSA and Glu indicate the carbonyl and glutamatic carboxyl peaks from BSA, respectively. Note: these spectra and those in Fig. 4 were not obtained under spectrometer conditions that allow peak intensities to be directly related to the number of carbons contributing to that peak.

symmetrical. In the absence of BSA, CDCA would have precipitated and no peak would be observed below pH ~6.5. Therefore, CDCA was bound to the BSA even at low pH. Linewidth changes similar to those for CDCA occurred for CA/BSA and DCA/BSA. The apparent pKa's of CA, DCA, and CDCA when bound to BSA (4.5, 4.9, and 4.2, respectively) did not differ significantly. The titration curve for CA bound to BSA is shown in Fig. 2. After titration of each bile salt/BSA sample to low pH, the sample was back titrated to two intermediate pH points, and the measured chemical shifts fell on the titration curve. $^{13}$C NMR spectra of DCA/BSA complexes were also obtained. A single carboxyl peak at 182.7 ppm was observed for DCA/BSA mol ratios of 2:1, 3:1, 4:1, and 6:1.

Chemical shift of bile acid in phospholipid membranes as a function of pH

Vesicles were prepared by cosonication of CA, DCA, or CDCA with egg PC (~1:20 mol/mol) and were stable at all pH values investigated (pH 11-3), showing only a slight increase in turbidity by visual inspection at low pH values. Spectra obtained for the same sample up to 48 hr apart were similar. Spectra of PC vesicles with DCA or CDCA gave a single carboxyl peak throughout the titration (Fig. 4 for CDCA). The carboxyl peaks of both DCA and CDCA broadened slightly at and below the pKa. The apparent pKa's for DCA (6.5) and CDCA (6.6) were not significantly different. Spectra of the CA/PC cosonicated vesicle samples gave two carboxyl peaks, a major peak and a minor peak (varying from <3% to 10% of the major peak at high pH, depending on the particular sample preparation). Fig. 5 shows spectra of the CA/PC cosonicated vesicles at selected pH values (pH 10.2, 7.0, and 3.4). In addition to chemical shift changes, the CA peaks exhibited linewidth

Because of the greater linewidth and asymmetric lineshape, the uncertainty in measuring chemical shift in the spectra of bile acid-BSA complexes was much greater than that for the other systems reported herein.
changes during titration. At high and low pH the CA carboxyl resonances were narrow; as the pKa was approached the peaks broadened. The two CA carboxyl peaks gave titration curves with differing apparent pKa's (Fig. 2). The major peak had an apparent pKa (6.8) similar to CDCA and DCA. The minor CA peak had a significantly higher pKa of 7.3. After complete protonation of CA, the pH was immediately raised to pH 10, where only the major peak was seen at 183.2 ppm (spectrum not shown). Since [24-13C]CA below its CMC would not be detected in the number of accumulations (~500) used to obtain the vesicle spectra and would have a very different chemical shift if present (~184.7 ppm) and since the relative concentrations of CA and PC were such that CA micelles would not be present (9), we reasoned that the two CA peaks in the vesicle spectrum resulted from CA in two different magnetic environments within the PC bilayer. Negative stain electron micrographs of BA/PC (3% NaC-97% PC) samples showed a homogeneous population of unilamellar vesicles with a diameter of 229 ± 54 Å (mean ± SD, 199 particles counted). Vesicle diameter was the same at low and high pH.

A second method for NaC/PC vesicle preparation was used to help identify the two CA environments. In this method PC vesicles were prepared and [24-13C]NaC was added at pH 9.3. At this pH, CA in the absence of vesicles would be completely ionized and would have a chemical shift of ~184.7 ppm (see Fig. 2). However, the 13C spectrum showed a single carboxyl resonance at 183.3 ppm (Fig. 6A), the same chemical shift as the major peak in the spectrum of PC cosonicated with CA at high pH (Fig. 5). Thus at this pH, CA was incorporated into the vesicle and was completely ionized. After 24 hr of incubation, a single peak at the same chemical shift and intensity was seen. After decreasing the pH to 3.1, two peaks were seen immediately at 177.36 ppm and 177.97 ppm (Fig. 6B), identical to the chemical shifts of the minor and major peaks of the cosonicated system at the same pH. Titration of this sample from pH 3.1 to pH 7.2 (Fig. 6C-E) with 1.0 N NaOH yielded two titration curves. The titration points for the outer CA peak fell on the titration curve of the major peak in the cosonicated system at all pH values (Fig. 2). The minor peak, identified as CA in the inner monolayer (see Discussion), gave a titration curve from pH 3.1 to 7.2 before broadening beyond detectability at pH 7.8 (Fig. 6F). The chemical shifts above pH 5 were lower than those of the minor peak for the cosonicated vesicles (Fig. 2). In addition, the minor peak broadened and decreased in intensity as the pH was raised; the area ratio of the major peak to minor peak increased from ~2:1 at pH 3.1 to >10:1 at pH 7.8. When 3 mg of [24-13C]NaC was added to vesicles at pH 10 and then titrated to pH 3, two titration curves identical to those found when titrating from pH 3 to 9 were obtained. This reversibility suggests that the partial titration curve for the CA minor peak represented the equilibrium ionization behavior of CA in the inner monolayer of small unilamellar phospholipid vesicles.

**DISCUSSION**

The apparent pKa of a carboxyl group in a simple or complex environment can be determined from pH-dependent measurements of the carboxyl 13C chemical shift (12, 23). Table 1 summarizes the apparent pKa values of CA, DCA, and CDCA in several molecular...
environments as measured by $^{13}$C NMR spectroscopy. Aqueous CA below its CMC and solubility limit had an apparent pKa of 4.6, a significantly lower value than previously reported from potentiometric titration of 3 mM CA (pKa 4.98; ref. 11) and from extrapolation of apparent pKa values for CA in methanol-water solutions (pKa 5.06; ref. 24). Our value is only slightly lower than the values for dilute aqueous carboxylic acids (pKa 4.7–4.9; ref. 12). In that case, the pKa value measured by NMR corresponded precisely to the value determined potentiometrically on the same sample (12). Our lower pKa may reflect the lower concentration used to prevent precipitation of the acid form. The very low water solubilities of DCA and CDCA prevented determination of the monomeric pKa values of these acids by NMR.

Micellar solutions of unconjugated bile acids precipitate at intermediate pH (pH 6–7) on formation of the acid in amounts above what the micelle can solubilize (8, 9). In contrast, the taurine conjugates have low pKa’s (pKa <2) and remain soluble at all pH values (9). Incorporation of 10 wt% of the unconjugated bile acid into micelles of the taurine conjugate will prevent precipitation of the free acid (8, 9), allowing determination of the pKa of the unconjugated acid in the taurine-conjugate micelle. The pKa's determined from the complete NMR titration curves for CA, DCA, and CDCA in the mixed micelles were quite similar to those determined potentiometrically for micelles of the unconjugated bile acids alone (9–11). The apparent pKa of CA in the mixed micelle was significantly higher than that for monomeric CA. Thus, comparison of pKa values in these systems suggests that the local environment of the carboxyl group was altered to a greater extent on going from monomer to micelle than from simple micelle to mixed (taurine conjugate) micelle.

The apparent pKa values for the three bile acids bound to BSA are similar to those determined for long-chain fatty acids bound to BSA in titratable sites (14, 15). $^{13}$C NMR has detected one titrating site and several non-titrating sites for fatty acids bound to BSA. In contrast,
only a titrating site was present for the bile acids. In egg phosphatidylcholine vesicles, the apparent pKa values of CA, DCA, and CDCA were increased relative to all other systems. The pKa's of DCA, CDCA, and the CA major peak were not greatly different. However, they were significantly lower than the pKa for small concentrations of oleic acid in PC vesicles (pKa 7.4; ref. 15, 23).

Details of the molecular environments and motions of the bile acid carboxyl carbon can also be obtained from the 13C NMR results. In monomeric and micellar systems, the bile acid carboxyl resonance was narrow, indicative of a relatively mobile carboxyl group and a relatively homogenous environment. The carboxyl peak for bile acids complexed to BSA was quite broad and somewhat asymmetric except at low pH. These results suggest that the bile acids bound to BSA might be present in slightly different magnetic environments in slow exchange. In addition, the rotational motion of the carboxyl may be inhibited by interaction with amino acid groups. In contrast, long-chain fatty acids showed multiple narrow resonances when complexed with BSA (13, 14). Our results demonstrated binding of DCA to BSA at molar ratios up to 6:1 DCA/BSA, but even at the higher DCA/BSA molar ratios, multiple environments such as occurred with oleic acid (13) or myristic acid (14) were not clearly seen. Equilibrium binding studies have shown a single high affinity site and several low affinity sites for DCA binding (7). The intense, narrow bile acid C24 resonance at low pH, where the bile acid is fully protonated, shows that the bile acid is bound to albumin and that its local magnetic environment was more homogenous and/or its rotational motion was more rapid. Thus, both hydrophobic and hydrophilic interactions appear to be important in the binding of bile acids to BSA.

In the CA/PC vesicle system, two environments in slow exchange on the NMR time scale were detected. The relative population of the two environments changed with pH, showing the greatest disparity at high pH. From the chemical shift (see below) and pH-dependent intensity changes, these environments were identified as the outer and inner monolayers of the bilayer. When the vesicles were prepared by cosonication of CA and PC at pH 11 (to allow CA to distribute to both inside and outside monolayers of the vesicles) two peaks were seen—a peak at 183.2 ppm and a small peak at 182.1 ppm. However, when NaC was added to preformed vesicles as NaC monomers, only the large peak at 183.3 ppm was seen. In this case NaC was present only in one magnetic environment, presumably the outer monolayer of the vesicle. These spectra were stable for >12 hr indicating very slow exchange of cholate between the outer and inner monolayers. Reducing the pH from 9.3 to 3.1 caused immediate protonation of the external CA, which could then flip to the inner monolayer of the vesicle and establish an equilibrium ratio (~2/1), similar to the ratio of PC in the outer and inner monolayers of a small vesicle (25). The exchange between the two monolayers was much less than 30 exchanges/sec but faster than the time needed to accumulate the spectra (~40 min). At pH 5.5, when CA in the outer monolayer began to titrate, the resonances

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1The upper limit for the exchange rate may be determined from the chemical shift difference or the linewidth, whichever is more stringent.
TABLE 1. Apparent pKa's of bile acids in different molecular environments

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cholic</th>
<th>Deoxycholic</th>
<th>Cheno-deoxycholic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below CMC and solubility limit*</td>
<td>4.6</td>
<td>ND†</td>
<td>ND†</td>
</tr>
<tr>
<td>Bile acid/BSA complex (2:1 mol/mol)†</td>
<td>4.5</td>
<td>4.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Mixed micelles (1:9 w/w) bile acid/taurine conjugate‡</td>
<td>5.3</td>
<td>6.1</td>
<td>6.3</td>
</tr>
<tr>
<td>Bile acid/egg PC vesicles (3:97 w/w, ~1:21 mol/mol)§</td>
<td>6.8 major peak</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>7.3 minor peak, cosonicated</td>
<td></td>
<td></td>
</tr>
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</table>

*Apparent pKa ± 0.2.
†ND, not determined.
‡Apparent pKa ± 0.3.

became clearly separated. Between pH 6 and 8, CA in both environments titrated, showing that the proton flux through the vesicle was relatively rapid. However, the relative peak area of the CA in the inner monolayer decreased markedly in this pH range, indicating a net flux of CA to the outer monolayer. As the outer monolayer began to become ionized, the amount of CA in the inner monolayer diminished. This would occur if the protonated form of the bile acid exhibited rapid flip-flop relative to the ionized form. Thus, the titration to the ionized form on the outside depleted the protonated CA on the inner monolayer, since once the protonated form flipped out it became ionized and remained in the outer monolayer. The reversibility of this titration series and the inability to observe the CA minor peak above pH 7.8, except when CA and PC are cosonicated at high pH (Figs. 5 and 6), suggest that adding NaC to preformed vesicles represented the equilibrium location and ionization state of CA in the outer and inner monolayers of small sonicated vesicles. We were not able to identify the reason for the differences in chemical shift of the CA minor peak from pH 5.5–7.2 between the two methods of vesicle preparation. It is possible, however, that cosonication of CA with PC forced a small amount of NaC into the inner monolayer of the vesicle (which resulted in the minor peak in the cosonicated spectra at high pH) and that this "trapped" NaC altered the titration behavior of cholic acid in the inner monolayer. Our results may be relevant for the transport of CA across physiologic membranes because small changes in pH near 7.4 could significantly increase or decrease the net rate of bile acid movement across the bilayer.

Table 2 summarizes the maximum, minimum, range of chemical shifts, and the chemical shift at the pKa for the CA carboxyl resonance in the systems studied. At a fixed ionization state the chemical shift of the carboxyl carbon will be directly proportional to the net H-bonding of the carboxyl group, and as the environment of the carboxyl carbon becomes more hydrophobic, the carboxyl resonance will shift upfield (12, 26, 27). The observed chemical shifts for CA suggest that the monomeric environment is the most hydrophilic and the PC inner monolayer is the most hydrophobic. This general result would be expected since maximum hydration will occur for monomers, and since the phospholipid bilayer will provide a strongly hydrophobic environment for the bile acid causing partial dehydration of the carboxyl group. The carboxyl groups of PC molecules on the outer monolayer have more extensive H-bonding interactions with H2O (greater degree of hydration) and these resonances are therefore shifted downfield relative to resonances for carbonyl groups on the inner monolayer (28). A close

Table 2. Chemical shift ranges (ppm) for CA

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximum Chemical Shift</th>
<th>Minimum Chemical Shift</th>
<th>Chemical Shift Range</th>
<th>Chemical Shift at pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM</td>
<td>184.68</td>
<td>180.00</td>
<td>4.68</td>
<td>182.34</td>
</tr>
<tr>
<td>5% w/v (116 mM)</td>
<td>184.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA/NaTC micelles (1:9 w/w)</td>
<td>184.12</td>
<td>178.59</td>
<td>5.58</td>
<td>181.38</td>
</tr>
<tr>
<td>CA/BSA complexes (2:1 mol/mol)</td>
<td>183.52</td>
<td>178.65</td>
<td>4.87</td>
<td>181.08</td>
</tr>
<tr>
<td>CA/egg PC vesicles (3:97 w/w)</td>
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<td></td>
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<td>177.31</td>
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342 Journal of Lipid Research Volume 27, 1986
examination of the NMR chemical shift data suggests that for ionized CA, the order of increasing hydrophobicity is monomer < micelle < mixed micelles < BSA-bound < PC outer monolayer < PC inner monolayer cosol- ciated System. The hydrophobicity of protonated CA increases in the order; monomer < BSA-bound < mixed micelle < PC outer monolayer < PC inner monolayer. It is also known that, in the absence of complicating factors, the apparent pKa of a carboxyl group increases with increasing hydrophobicity (29). Monomeric CA was found to have the lowest pKa and CA in the inner mono- layer of PC vesicles had the highest. With the exception of CA/BSA complexes, an upfield shift for the CA carboxyl peak correlated with an increase in the apparent pKa. Thus the relative hydrophobicity estimated from the apparent pKa corresponded to that estimated from the chemical shift values. 4

We gratefully acknowledge Donald Gantz for performing the electron microscopy and thank Anne Gibbons and Irene Miller for preparation of the manuscript. This work was supported by grants HL-26335, HL-07291, and HL-07429 from the United States Public Health Service.

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REFERENCES


Natural occurring triacylglycerols in both animals and plants are comprised of three fatty acids esterified to glycerol. Most naturally occurring triacylglycerols are asymmetric since the fatty acids esterified at the 1- and 3-positions are different (Kukis, 1978). The final step in the synthesis of triacylglycerols involves the enzyme diacylglycerol acyltransferase that utilizes activated fatty acid to esterify the sn-3 (or sn-1) carbon of the glycerol. The preferred substrate is 1,2-diacyl-sn-glycerol (O'Doherty, 1978). The fatty acid in the 3-acyl position in most fats and oils is usually a long-chain fatty acid. However, certain plant oils and especially milk triacylglycerols from ruminants are highly enriched with short-chain fatty acids in the 3-position, that is, fatty acids containing eight carbons or less (Breckenridge, 1978; Breckenridge & Kukis, 1968). In bovine milk triacylglycerols at least 50% of the sn-3 positions are esterified with short-chain fatty acids (Breckenridge, 1978).

The utilization of short-chain fatty acids probably arises from the production of many short-chain fatty acids in the rumen which are absorbed, activated, and utilized by the diacylglycerol acyltransferase in the mammary gland. In fact, when linoleic-rich seed oils are coated by formalized casein that is resistant to rumen enzymes and are fed to sheep, the unsaturated seed oil fatty acids are absorbed without being catalyzed to the various smaller fatty acids. The bovine milk fat derived from such animals has a large portion of sn-3 position preferentially esterified to linoleic acid rather than to short-chain fatty acids (Mills et al., 1976). Even in some nonruminants such as the rat the sn-3 position is greatly enriched in short- and medium-chain fatty acids (Staggers et al., 1981; Aw & Grigor, 1980). These shorter chain fatty acids are more easily hydrolyzed by pancreatic lipase (Jensen et al., 1964) and lingual lipase (Staggers et al., 1981; Fernandes-

Warnakulasuriya et al., 1981; Paltauf et al., 1974).

In an attempt to understand the mechanisms of this preferential hydrolysis of short- and medium-chain fatty acids at the sn-3 position, we have synthesized a homologous series of 1,2-dipalmitoyl-3-acyl-sn-glycerols with acyl chains varying from two to eight carbons (Kodali et al., 1984). The bulk properties of this series has been examined (Kodali et al., 1984), but because triacylglycerols are hydrolyzed at a triacylglycerol–water interface (Entressangles & Desnuelle, 1968), we have examined the interfacial characteristics of these triacylglycerols. We found them to be a particularly interesting group of molecules in that their physical properties at the interface are dictated by the length of the fatty acyl chain at the sn-3 position. The acetyl derivative prefers the aqueous side of the interface and allows the two palmitate chains to solidify at a very low pressure. On the other hand, if the sn-3 chain contains eight carbons, the octanoate cannot be forced into water and thus must lie side by side in the monolayer with the two palmitate chains. The presence of the octanoate in the film prevents the crystallization of the palmitate residues and thus fluidizes the film to very low temperatures. If the sn-3 acyl group is intermediate in length, having from 3 to 6 carbons in the fatty acid moiety, it can be pushed into the aqueous phase, but this requires increasing energy the longer the chain length. At surface pressures believed to occur in emulsions in stomach or intestinal contents, we suggest that the short-chain fatty acids actually protrude into the aqueous phase, making them more available to enzymatic hydrolysis.

MATERIALS AND METHODS

Stereospecific 1,2-dipalmitoyl-sn-glycerol and a series of 1,2-dipalmitoyl-3-acyl-sn-glycerols with even carbon saturated fatty acyl chains of two through eight carbons in length were synthesized (Kodali et al., 1984). The triacylglycerols with 3-acyl chains of three and five carbons in length were also

\[
\text{ABSTRACT:} \text{ Stereospecific 1,2-dipalmitoyl-sn-glycerol and a series of 1,2-dipalmitoyl-3-acyl-sn-glycerols (TGs) with 3-acyl chains of two through six and eight carbons in length were synthesized. Pressure–area isotherms at 27 °C, surface melting temperatures (T_m), and equilibrium spreading pressures (ESP) measured at the bulk melting temperature (T_3) were obtained for each TG and for dipalmitin. Whereas dipalmitin and the 3-acytethyl-TG condense directly to an expanded mesomorphic state (30–33 Å²/palmitoyl chain at the vapor pressure, \( \pi_v \)), the 3-propionyl- through 3-octanoyl-TGs show an area per molecule in the liquid at \( \pi_v \) that increases linearly from 105 to 130 Å²/molecule (slope = 5 Å²/CH₂ group). This slope suggests that the 3-acyl chains are lying flat on the water at the end of the gas–liquid transition. Before solidification at 42–47 Å²/molecule, the 3-propionyl- through 3-hexanoyl-TGs show a transition corresponding to the immersion of the 3-acyl chain. The pressure at this transition, \( \pi_{cr} \), vs. 3-acyl carbon number is linear and indicates a chain immersion energy of 497 cal mol⁻¹ per CH₂. In contrast, the 3-octanoyl chain is not forced into the water but rather is pushed into the monolayer to lie parallel to the palmitoyl chains. As the sn-3 chain is lengthened, \( T_m \) decreases from 68 to 25 °C, but the 3-octanoyl monolayer does not solidify even at 5 °C because the short upright octanoyl chains fluidize the palmitoyl chains. The esp (at \( T_3 \)) drops from 31.7 mN m⁻¹ for dipalmitin to 20.6 mN m⁻¹ for the 3-acytethyl-TG. The esp for the 3-octanoyl-TG is 14.8 mN m⁻¹. In summary, increasing the length of the shorter 3-acyl chain of these diacyl TGs decreases the \( T_m \) and the esp. In monolayers, the shorter 3-acyl chains, lying flat on the surface at \( \pi_v \), either submerge into the aqueous phase if \( \pi_{cr} \) is less than esp or stand up and fluidize the monolayer if \( \pi_{cr} \) is greater than esp.}
\]
1,2-DIPALMITOYL-3-ACYL-SN-GLYCEROLS

synthesized. Approximately 5 mg of each lipid was measured to the nearest 0.01 mg and dissolved in 25 mL of high-pressure liquid chromatography (HPLC) grade heptane in a chronic acid rinsed volumetric flask. Care was taken to mark the fluid level on the volumetric flask so that solvent evaporation during storage could be detected.

Pressure–Area Isotherms. The Langmuir film balance consisted of a Teflon-coated trough 14 cm wide by 70 cm long by 1.6 cm deep. All isotherms were obtained by continuous compression at room temperature (27 ± 0.5 °C). Surface pressures were measured with a 1-cm² platinum Wilhelmy plate suspended from a Cahn electrobalance. Isotherms were directly recorded by a Hewlett-Packard X-Y recorder. A Hamilton syringe was used to spread 84 nmol of solute on the surface, and at least 10 min were allowed for the solvent to evaporate before lowering the Wilhelmy plate to the surface and starting compression. Each isotherm was obtained with a freshly prepared film that was compressed at a rate of 53.6 cm²/min (8.9 × 10⁻² A²/s) (3.5 A²/(chain-min)). Three isotherms obtained under identical conditions were superimposable to within 1.5 A²/molecule. Merck extraclean talc baked at 700 °C was dusted on a portion of the monolayer in order to monitor viscosity by observing talc movement in response to a light jet of air. Solidification of the monolayer indicated by nonmovement of the talc was reproducibly observed to within 1.5 A²/molecule. A test for hysteresis and compression rate dependence was carried out for one of the isotherms (PP5). A difference in compression and expansion isotherms at the transition of 0.6 mN m⁻¹ and 3 A²/molecule was observed. The pressure in the two-phase region decreased by 0.2 mN m⁻¹ when the compression was stopped for 5 min. These values indicate that the deviation from equilibrium is small enough to not affect the conclusions of the study.

Surface Melting Temperature and Equilibrium Spreading Pressure. Chromic acid rinsed glass jars 4.5 cm in diameter by 2.5 cm tall were half filled with double-distilled water and equipped with a clean glass thermometer and a magnetic stirring bar. Enough lipid was spread on the surface to make the surface concentration about 1.5 times the amount necessary to cover the entire surface as a monolayer. This assured that excess lipid was present but not enough to form a thick multilayered crystal on the surface. Talc dusted on the solid film in order to monitor viscosity as the entire system was heated and the subphase stirred by a combination magnetic stirrer and hot plate. The temperature at which the surface became fluid was recorded as the surface melting temperatures, Tₛ. The accuracy was ±1 °C. The equilibrium spreading pressures (esp) were measured at just above (2–4 °C) the bulk melting temperature of each compound. By use of the same apparatus used in the measurement of Tₛ, several small crystals were placed on the surface and the subphase heated until the crystals melted. Approximately 1 min later the surface tension was measured and then used to calculate the esp. An uncertainty in the esp of ±1.0 mN m⁻¹ mainly due to temperature fluctuation was estimated. The effect of

<table>
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<th>3-substituent</th>
<th>Tₛ² (°C)</th>
<th>Tₛ¹ (°C)</th>
<th>Tₛ¹ - Tₛ (°C)</th>
<th>esp (mN m⁻¹)</th>
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<td>3.0</td>
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<td>1.0</td>
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<td>46</td>
</tr>
<tr>
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<td>40.0</td>
<td>&lt;5</td>
<td>&gt;39</td>
<td>14.8</td>
<td>47</td>
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</tbody>
</table>

*Stable bulk melting temperatures. From Kodali et al. (1984). Note the depressed values for the two odd members. *Surface melting temperatures. The octanoyl derivative remained as a fluid monolayer down to 5 °C. *Melting temperatures of the α-phase. From Kodali et al. (1984). *Temperature at which esp was measured; 2–4 °C above Tₛ in all cases. *Error is estimated as ±1.0 °C for Tₛ and ±1.0 mN m⁻¹ for esp.

temperature on the esp for the 3-octanoyl compound was measured between 30 and 52 °C by attaching a thermocouple, with its junction placed just below the water surface, directly to the X-Y recorder. After calibration, a continuous graph of surface tension vs. temperature was produced by gradually changing the temperature.

RESULTS

Surface Melting Temperature and Equilibrium Spreading Pressure. Values of the stable bulk melting temperature, Tₛ, the surface melting temperature, Tₛ, and Tₛ¹ - Tₛ for each compound are given in Table I. The metastable α-form melting temperatures (Tₛ) (Kodali et al., 1984) are given for comparison. The Tₛ values are depressed for the two odd members of the series. A surface melting temperature for the 3-octanoyl compound was not obtained since the monolayer stayed fluid down to 5 °C.

The esp values measured at just above Tₛ for each compound are also given in Table I. It was shown that spreading pressure rises nonlinearly as the temperature is increased for the 3-octanoyl compound. The spreading pressures were 14.1 mN m⁻¹ at 30 °C, 14.3 mN m⁻¹ at 40 °C, 14.6 mN m⁻¹ at 45 °C, and 15.2 mN m⁻¹ at 50 °C.

Pressure–Area Isotherms. Pressure–area isotherms for 1,2-dipalmitoyl-sn-glycerol (PPOH) and the 3-acyetyl (PP2), 3-propionyl (PP3), 3-butyryl (PP4), 3-pentanoyl (PP5), 3-hexanoyl (PP6) and 3-octanoyl (PP8) compounds are shown in Figure I. The area per molecule of the liquid phase (Av) in equilibrium with its gas (i.e., at the vapor pressure, pᵥ), can be read from the isotherm as the area per molecule at the end of the gas to liquid transition (i.e., at the departure from the base line indicated by the horizontal bars in Figure I). In all cases Av was estimated as a range because there was not a sharp break from the base line. PPOH showed a base-line surface pressure of 0.4 mN m⁻¹, Av was 58–68 A²/molecule, and the isotherm rose sharply after 45 A²/molecule. Solidification occurred at 44.3 A²/molecule at a pressure of 2.3 mN m⁻¹. PP2 showed a base-line surface pressure of 0.45 mN m⁻¹, Av was 65–70 A²/molecule, and the isotherm rose abruptly after 47 A²/molecule. Solidification occurred at 45 A²/molecule at a pressure of 3.6 mN m⁻¹. PP3 showed a base-line surface pressure of 0.65 mN m⁻¹, Av was 102–106 A²/molecule. The pressure then increased with a discontinuity in the slope at 95 A²/molecule at a pressure of 1.3 mN m⁻¹. Solidification occurred at 45 A²/molecule at a pressure of 3.8 mN m⁻¹. The isotherm then rose abruptly. PP4 showed a base-line surface pressure of 0.45 mN m⁻¹, Av was 110–113 A²/molecule. The pressure then increased with a discontinuity in the slope at 84.5 A²/molecule at a pressure of 5.8 mN m⁻¹.
Solidification occurred at 46.8 Å²/molecule at a pressure of 7.6 mN m⁻¹. PP5 showed a base-line surface pressure of 0.6 mN m⁻¹. πₐ was 116–119 Å²/molecule. The pressure then increased with a discontinuity in the slope at 77.8 Å²/molecule at a pressure of 10.9 mN m⁻¹. Solidification occurred at 47.2 Å²/molecule at a pressure of 11.6 mN m⁻¹. PP6 showed a base-line surface pressure of 0.55 mN m⁻¹. πₐ was 120–123 Å²/molecule. The pressure then increased with a discontinuity in the slope at 72.2 Å²/molecule at a pressure of 16.0 mN m⁻¹. Solidification occurred at 42.2 Å²/molecule at a pressure of 16.8 mN m⁻¹. PP8 showed a base-line surface pressure of 0.3 mN m⁻¹. πₐ was 125–130 Å²/molecule. The pressure then increased with a sharp break at 79.5 Å²/molecule at a pressure of 15.5 mN m⁻¹. Further compression produced a perfectly horizontal isotherm down to 39 Å²/molecule with solidification at 30.4 Å²/molecule.

DISCUSSION

Pressure–Area Isotherms. PPOH shows a horizontal low-pressure isotherm down to approximately 60 Å²/molecule with solidification at 44.3 Å²/molecule. At solidification there are 22 Å² per acyl chain, indicating close chain packing. PP2 shows a similar isotherm to that of PPOH with solidification at 45 Å²/molecule. At solidification there is enough area per molecule for only two acyl chains so that the 3-acyl chain must be submerged in the water.

Dervichian has classified fatty chain monolayer states according to the area per chain, πₐ, at the vapor pressure, π, (Dervichian, 1954). Briefly, they are liquid (38 Å²/chain), expanded mesomorphic (24–33 Å²/chain), mesomorphic (fluid, 23.5 Å²/chain), and solid (rigid, 20.5–22 Å²/chain). For example, tripalmitin at its π, is liquid above 45 °C, expanded mesomorphic from 40 to 45 °C, and solid below 35 °C. At 27 °C PPOH and PP2 probably condense directly to an expanded mesomorphic state since πₐ is 61 (30.5 Å²/chain) and 67 Å²/molecule (33.5 Å²/chain), respectively. However, PP3, with an πₐ of 102–106 Å²/molecule, probably condenses into the liquid state with an area per palmitoyl chain of at least 38 Å². This amounts to at least 76 Å² for the two palmitoyl chains leaving at most 26–30 Å² for the 3-propionyl chain. This area is too large for propionic acid lying flat on the water surface. If the 3-acyl chain is truly lying flat, then the area occupied would be equivalent to the chain length times its width or about 4.5 Å (1.25 Å + 1.25 Å + 1.5 Å) = 18 Å². However, if the 3-carbon of the glycerol and the ester group were also in the surface then the area would total about 6.7 Å × 4.5 Å = 30 Å² (Figure 2).

Assuming that the 3-acyl chains of PP4 through PP8 are also lying flat on the surface in the liquid phase at πₐ, then πₐ should increase by ~5 Å² for each additional carbon in the sn-3 chain. In Figure 3a, πₐ is plotted vs. 3-acyl carbon number. A line which accommodates ranges of πₐ has a slope of ~5 Å² per carbon and an intercept at zero carbons of 92 Å². This slope is consistent with the suggestion that the sn-3 chains of three to eight carbons are lying flat on the surface at the end of the gas–liquid transition.

In contrast to PP8 which solidifies as a bilayer at 30.4 Å²/molecule, PP3 through PP6 solidify between 42 and 47 Å²/molecule, indicating that only two and not three chains are solidifying as a monolayer. The shorter sn-3 chains of PP3 through PP6 must be submerged in the subphase at solidification. Since the sn-3 chains are not submerged in the liquid state at πₐ, they must be forced into the water at some point during compression of the liquid. PP3 through PP6 have discontinuities in their isotherms at 95.0, 84.5, 77.8, and 72.2 Å²/molecule, respectively (at asterisk in Figure 1), indicating phase transitions. One phase consists of the triacylglycerol with all three chains out of the water and the other phase consists of the triacylglycerol with the shorter sn-3 chain and the sn-3 glycerol carbon submerged in the water. As the film is compressed in this two-phase region, the sn-3 chains are pushed into the water. After all the sn-3 chains are pushed into the water, further compression of the palmitoyl chains solidifies them at 42–47 Å²/molecule. The pressures at the start of the two-phase regions (discontinuities) vs. 3-acyl carbon number is linear with a slope of 4.9 mN m⁻¹ per carbon (Figure 3b). Note that the PP8 discontinuity pressure does not fall on this line, suggesting that monolayer collapse rather than sn-3 chain immersion is occurring.

Dividing the surface pressure by the surface concentration at the two-phase initiation area (at asterisk in Figure 1) yields the energy per mole of monolayer at the transition. A plot of this energy vs. 3-acyl carbon number (Figure 3c) is linear with a slope of 497 cal mol⁻¹ per carbon and a y intercept of 1298 cal. The 497 cal mol⁻¹ per carbon slope represents the surface energy (μₐwater - μₐsurface) necessary for chain immersion per additional carbon in the sn-3 chain. This energy is only about 56% of the 884 cal mol⁻¹ per carbon for transfer of a hydrocarbon chain from a hydrocarbon environment to a water.
to be 14 mN m\(^{-1}\) at 27 °C (from the temperature dependence studies), monolayer collapse must occur well before reaching the pressure necessary to immerse the 3-octanoyl chain. The horizontal portion of the PP8 isotherm corresponds to the transition of a monolayer to a bilayer with solidification of the bilayer at 30.4 Å\(^2\)/molecule (20 Å\(^2\)/chain).

**Surface Melting Temperature and Equilibrium Spreading Pressure.** Changes in the value of \(T_1 - T_2\), from one compound to the next, must depend on differences in the amount and stability of chain–chain interactions in the solid bulk phase and in the solid monolayer. General packing properties in the bulk state for this series are known (Kodali et al., 1984). Briefly, the stable bulk phase packing arrangements consist of the double-layer dicacylglycerol type for PPOH, PP2, and PP4 and a trilayer with the shorter sn-3 chains segregated from the palmitoyl chains for PP6 and PP8. The general packing properties of the solid monolayer deduced from the isotherms consist of solidified palmitoyl chains for PPOH and 3-acyl chains immersed in the water with solidified palmitoyl chains on the surface for PP2 through PP6. PP8 does not solidify as a monolayer even at 5 °C because the 3-octanoyl chain cannot be immersed and must mix with and therefore fluidize the palmitoyl chains.

PPOH has nearly identical surface and bulk melting temperatures (Table 1) because the extent of palmitoyl chain–chain interaction is similar in the double layer bulk phase and in the monolayer. The hydroxyl group may experience similar hydrogen bonding in the bulk (with neighboring carbonyls) and in the monolayer (with water). PPOH has a high esp of 31.7 mN m\(^{-1}\) probably due to the high affinity of the hydroxyl group for the water. In contrast, PP2 has an esp of only 20.6 mN m\(^{-1}\). The added acetyl group causes a larger drop in the esp whereas each additional carbon in the sn-3 chain causes a much smaller reduction. The loss of the hydroxyl has little or no effect on the stability of the solid palmitoyl chains since \(T_1 - T_2\) for PP2 is near zero. In the PP2 through PP6 series increasing sn-3 chain length coincides with a smoothly decreasing value of \(T_1 - T_2\). Since the sn-3 chains are in the water, this chain is lengthened there is increasing palmitoyl chain destabilization and a decreasing \(T_2\). Note that \(T_1\) is depressed for PP3 and PP5 and that for PP3 \(T_1\) is actually greater than \(T_2\). The fact that \(T_1\) and \(T_2\) are much higher than the melting temperature of hexagonally packed chains (\(T_m\)) may indicate a specific chain packing on the surface monolayer like that in the stable bulk phase.

In summary three categories of these triacylglycerols defined by the behavior of the shorter acyl chains during compression of the triacylglycerol monolayers are schematized in Figure 4. Dipalmitin and triacylglycerols with 3-acyl chains of two or less carbons are always hydrophilic at the sn-3 position (Figure 4, top). 3-Acyl chains of three to six carbons in length lie flat on the surface of the water at low surface pressure but can be completely hydrated at the sn-3 position when the triacylglycerol monolayer is compressed (Figure 4, middle). If the 3-acyl chain contains eight carbons (and probably seven, nine, and ten), the chain cannot be submerged at monolayer surface pressures and is pushed up into the palmitoyl chain phase preventing monolayer solidification (Figure 4, bottom).

Triacylglycerols with short sn-3 chains are found in nature with bovine milk butterfat containing a large percentage of butyric acid in the 3-position (Breckenridge & Kuksis, 1968; Pitas et al., 1967). In rats, lingual lipase preferentially hydrolyzes the shorter sn-3 chain from rat milk triacylglycerols (Staegs et al., 1981). Pancreatic lipase is specific for hydrolysis of the 3-acyl ester.
The efficiency and specificity of enzymes like lingual lipase and pancreatic lipase may be related to the availability of the 3-acyl chain to the aqueous side of an oil/water interface.

Fluidization of a lipid bilayer is caused by chain unsaturation, cholesterol, or other fluidizing components. The concept that large differences in chain length can also fluidize a monolayer (or bilayer) has been demonstrated in this study.

ACKNOWLEDGMENTS

We thank Dharma Kodali for synthesizing the triacylglycerols, John Steiner for technical assistance, and Anne M. Gibbons and Irene Miller for preparation of the manuscript.

Registry No. PPOH, 30334-71-5; PP2, 92734-29-7; PP3, 102871-10-3; PP4, 92841-82-2; PP5, 102871-11-4; PP6, 92841-83-3; PP8, 92734-30-0.

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Plasma Lipoproteins

Editor

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Preface

In an earlier volume of New Comprehensive Biochemistry, Dr. Paul Miller and I contributed a chapter on the current status of the metabolism of the plasma lipoproteins [1]. In this rapidly evolving field of research, an enormous amount of new knowledge and understanding of lipoprotein structure, function and metabolism has emerged. Since the last volume was published, Michael S. Brown and Joseph L. Goldstein received the Nobel Prize in medicine and physiology in 1985 for their pioneering work on the LDL receptor. Their fundamental investigations have had a great impact not only on lipoprotein metabolism but on other areas of biology and medicine as well. Their work on the LDL receptor helped clarify several aspects of lipoprotein metabolism as they relate to LDL. Recently, the complete structure of apoB-100, the apolipoprotein of LDL, has been elucidated. The determination of the structure of this protein had been the subject of intensive study for many years in various laboratories, but until recently, relatively little progress had been made. The application of methods of molecular biology enabled the determination of the structure of cDNA to be determined and a great deal of the protein structure has been completed as well. This work is reviewed in detail in the present volume by Yang and Chan.

The volume begins with chapters on structure, then proceeds to analyses of lipid and lipoprotein dynamics, metabolism, function, genetics, and molecular biology. Doctor Breslow covers the subject of lipoprotein genetics in molecular biology in his review in the present volume; Dr. Nestel discusses overall regulation and metabolism of the plasma lipoproteins; Drs. Gianterco and Bradley, the role of lipoprotein receptors; and Dr. Fogelman, the role of cellular regulation of cholesterol metabolism. The chapter by Dr. Patsch describes the latest developments and views on the metabolism of HDL.

The metabolism of the plasma lipoproteins is dependent on their structure and on the activities of various enzymes; the former being covered by Drs. Pownall, Sparrow, Massey and Small, and the latter by Drs. Tall, Jonas and Schotz in this volume. Doctors Morrisett and Guyton review Lp(a), a topic that has been underrepresented in volumes on lipoproteins, but one that has begun attracting the attention of more investigators.

We expect that this volume would be mainly of interest to researchers who are interested in lipid and lipoprotein structure and metabolism. The subjects covered are technical and biochemical in places but have great implications for clinical medicine and biology in general.

Antonio M. Gotta, Jr.
References

Contents

Preface ................................................................. v
Acknowledgement .................................................... vii

Chapter 1
Structure of triglyceride-rich lipoproteins: an analysis of core and surface phases
Kurt W. Miller and Donald M. Small (Boston, MA, USA) ............. 1

1. Introduction .......................................................... 1
2. Chylomicron and VLDL metabolism .................................. 2
   (a) Synthesis of nascent chylomicrons and VLDL ..................... 3
   (b) Metabolic transformation of chylomicrons and VLDL ............. 6
3. Structural features of triglyceride-rich lipoproteins and physical-chemical properties of their components ............... 8
   (a) Chemical compositions of chylomicron and VLDL subfractions ... 8
   (b) Early attempts to isolate surface and core lipids ................. 10
   (c) Physical properties and phase solubilities of triglyceride-rich lipoprotein lipids: lecithin, cholesterol, triglyceride and cholesterol ester 10
4. Emulsions: structural models of triglyceride-rich lipoproteins .......... 18
   (a) Basic emulsion properties ....................................... 19
   (b) Triolein-lecithin-water emulsions and triolein-cholesterol-lecithin-water emulsions ... 19
   (c) Triolein-cholesterol oleate-cholesterol-lecithin-water emulsions ... 27
5. Phase equilibria of chylomicron and VLDL lipids ................. 32
   (a) Compositions of surface and core lipids ......................... 32
   (b) Equilibration of lipids between lipoprotein subfractions .......... 36
   (c) Phase compositions obtained by other methods ................... 42
6. Phase diagram analysis of triglyceride-rich lipoprotein metabolism 44
   (a) The interpretation of changes in relative lipid composition as plotted on the phase diagram ................. 44
   (b) Increase in cholesterol content ................................ 46
   (c) Lipid transfer/exchange reactions ................................ 50
   (d) Triglyceride hydrolysis and remnant formation ................... 53
   (e) Abnormal chylomicrons and VLDL ................................ 63
7. Concluding remarks ................................................ 67

References .......................................................... 67

Appendix: computer program for analysis of triglyceride-rich lipoprotein structure .......................... 72
CHAPTER 1

Structure of triglyceride-rich lipoproteins: an analysis of core and surface phases

KURT W. MILLER* and DONALD M. SMALL

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1. Introduction

Intestinal chylomicrons and hepatic very low density lipoproteins (VLDL) serve as the major transport vehicles of triglyceride within the circulation. These lipoproteins are collectively designated the 'triglyceride-rich' lipoproteins since under normal conditions of diet and time of residence in the plasma triglyceride is their major component. Mammalian chylomicrons typically consist of 1–2% protein and 98–99% lipid, of which 90% is triglyceride, 1–2% cholesterol ester, 1% cholesterol, and 5–8% phospholipid**. VLDL contain appreciably more protein, ~7–10%, and of their lipids, 65% is triglyceride, 12% cholesterol ester, 5% cholesterol, and 18% phospholipid. Since they consist predominantly of lipid, chylomicrons and VLDL have buoyant densities less than plasma and can be isolated from other blood components by centrifugation. VLDL and chylomicron size and density distributions overlap, and thus, to obtain VLDL largely of hepatic origin, patients or animals must be fasted for sufficient time to allow dietary chylomicrons to be cleared from their plasma. VLDL obtained from fasted individuals range in diameters from 350–750 Å. If intestinal lymph VLDL are included in the category of intestinal chylomicrons, the range of lymph chylomicron particle sizes measured prior to their entry into the bloodstream range from 350 to > 2 000 Å, with a diameter of 1 200 Å being an average value after the ingestion of a meal containing fat.

Since the content of triglyceride-rich lipoprotein lipids greatly exceeds that of the apoproteins, a reasonable working hypothesis is that the arrangement of the lipids is key to governing the overall structure of the lipoproteins. The lipids are held

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** Unless otherwise indicated, all composition data are presented in weight percent units.
together solely by noncovalent forces, and are organized to lessen the unfavorable free energy of contact between hydrophobic lipid moieties and the surrounding water in which they are suspended. Apoproteins are bound to the surface of the lipoproteins, and participate in stabilizing the lipid-water interface. Since most of the apoproteins have several domains of amphiphilic α helices [1], the hydrophobic part of the helix may form part of the surface by either directly acting with the core surface and essentially displacing surface phospholipid, or by adsorbing to surface lipids. From this surface position in the particle, certain exposed hydrophilic regions may act as receptor ligands (apolipoprotein (apo)B, apoE), or serve as cofactors (apoCII, for lipoprotein lipase, the enzyme responsible for the cleavage of chylomicron and VLDL triglyceride). Certainly the structure of the lipid domains at the surface of the lipoprotein influences the binding conformation and catalytic properties of apoproteins and enzymes which adsorb to its surface. Since the compositions of the lipid and apoprotein components change in some cases dramatically during metabolism of the lipoprotein particle, it becomes important to determine how lipid and protein compositional changes are interrelated.

We will attempt to summarize what is presently known about the structural organization of chylomicron and VLDL lipids. Since the arrangement of lipids within these lipoproteins is analogous to that of simple emulsion particles, it will be useful to discuss the properties of emulsion systems to acquire insight into the properties of the more complex lipoproteins. After summarizing features of their structural organization, it will be possible to look in greater detail at their metabolism and address areas such as mechanisms of lipoprotein assembly, hydrolysis of triglyceride by lipoprotein lipase and formation of remnants, transfer of cholesterol ester and triglyceride between lipoproteins, and transfer of cholesterol into nascent triglyceride-rich lipoproteins after they enter the circulation. Thus, one of the goals of this review is to discuss the compositional and structural changes which take place during the metabolism of chylomicrons and VLDL.

2. Chylomicron and VLDL metabolism

The metabolism of triglyceride-rich lipoproteins has been extensively reviewed in the recent literature. The reader is referred to reviews of lipoprotein and apolipoprotein synthesis and metabolism [2–14], action of lipoprotein lipase [15–18], and related areas such as fat absorption [19–21] and lipid metabolism [22, 23]. We will discuss the metabolism of chylomicrons and VLDL in parallel since many steps of their synthesis and transformation occur by common pathways. Where possible, we will try to indicate how a thorough description of triglyceride-rich lipoprotein particle structure would facilitate the interpretation of metabolic data.
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structural aspect of lipids because it will be into the process of their metabolism as is of cholesterol into nascent lipoprotein of the goals that take place for the lipoprotein, and related e will discuss of their synthesis, we will try to elucidate the structural and functional aspects of lipoproteins.

(a) Synthesis of nascent chylomicrons and VLDL

The synthesis of triglyceride-rich lipoproteins occurs within the intracellular membrane compartments of intestinal enterocytes and liver hepatocytes. The fatty acid and 2-monooacylglycerol precursors of chylomicron triglycerides are taken up by the enterocyte after being transported to the cells in bile salt micelles [19, 20, 24]. Apparently, the monoglycerides subsequently are re-esterified to triglycerides and therefore most of the synthesis of triglyceride occurs independently of the glyceraldehyde 3-phosphate pathway, the predominant pathway for synthesis of triglyceride in the liver [22]. Since little or no de novo synthesis of fatty acids occurs during the absorption of fat, the fatty acid profile of the chylomicron triglycerides closely resembles that of the dietary fat [25, 26]. Thus, chylomicron triglycerides have relatively high melting points if derived from ingested cream or butter fat or have low melting points if derived from most vegetable oils, such as corn or safflower oil [26, 27]. The fatty acid composition of chylomicron phospholipids is relatively independent of that of the dietary fat [26, 28], and a high percentage of the phospholipid species has saturated fatty acids at the sn-1 position and polyunsaturated fatty acids at the sn-2 position of the glycerol backbone. A small percent of the dietary cholesterol is in the form of cholesterol esters and must be hydrolyzed before absorption [21]. Within the enterocyte a fraction of the cholesterol is esterified to fatty acids by acyl CoA:cholesterol acyltransferase (ACAT) to reform cholesterol esters [29–32]. Cholesteryl oleate and cholesteryl linoleate are common species of cholesterol esters found in nascent chylomicrons and VLDL.

The fatty acids which are incorporated into VLDL lipids in the hepatocyte are derived from multiple sources, namely de novo synthesis from acetyl-CoA units produced by carbohydrate utilization, free fatty acids taken up into the cells from plasma albumin, and from the hydrolysis of lipids transported to the liver in plasma lipoprotein such as chylomicron remnants [33–39]. Furthermore, cholesterol can be supplied by de novo synthesis, or by uptake from the plasma [40]. Most, if not all, of the synthetic machinery for triglyceride-rich lipoprotein lipid synthesis is present on the cytoplasmic side of the endoplasmic reticulum (ER) membranes [23]. The synthesized lipids are then segregated into the luminal aspects of the ER during the remainder of their transit through the cell. It is clear that the cholesterol content of newly secreted, or nascent, chylomicrons and VLDL is significantly less than that of their plasma counterparts [41]. The difference probably arises simply because the sites of nascent lipoprotein assembly are located at the minimum of a cholesterol concentration gradient which is lowest in the intracellular membranes [42], and highest in the circulatory system. However, the level of intracellular cholesterol in the hepatocyte can be increased by prolonged feeding of cholesterol, and under these conditions, nascent VLDL become relatively enriched in their cholesterol contents [43–45].

The composition of apoproteins in chylomicrons and nascent hepatic VLDL are
similar. Both contain apoB, a high molecular weight, extremely hydrophobic glycoprotein which contributes 10–30% to the total chylomicron and VLDL apoprotein mass in mammalian species [11, 46], and up to 50% in avian species [47, 48]. Intestinal cells secrete only the small apoB of about 250 000 daltons, while hepatocytes produce large apoB which has a molecular weight of 350 000–400 000 [49]. Also present on lymph chylomicrons (nascent triglyceride-rich particles)* are apoAII (Mr 8–12 000) of which apoCII (Mr 9 500) serves as the cofactor for lipoprotein lipase [50]. Many of the apoC peptides present on lymph chylomicrons probably have been acquired by the chylomicrons upon their entry into the lymph [11, 51]. The intestine secretes significant amounts of de novo synthesized apoAII and apoAIV (Mr 46 000) on chylomicrons [52]. However, it does not secrete significant levels of chylomicron-associated apoE (Mr 32–35 000). In contrast, a small amount of apoE is probably secreted on nascent hepatic VLDL [53]. As will be discussed below, the percentages of specific apoproteins bound to the lipoproteins change dramatically after nascent particles first enter the circulation, and then change continuously during their time of residence in the circulation.

The secretion of lipoprotein lipids is contingent upon the synthesis and secretion of apoproteins, as demonstrated by studies which show a complete block of lipid secretion after administration of cycloheximide, an inhibitor of protein synthesis [54]. Study of patients with the disease abetalipoproteinemia has documented the importance of apoB synthesis and secretion in the process of chylomicron and VLDL production [6, 55, 56]. These patients have no chylomicron or VLDL particles in their plasma, and also lack LDL, the metabolic end-product of catabolized VLDL. Thus, their plasma triglyceride levels are extremely low, and do not rise after the ingestion of a fatty meal. Rather, the digested fat is esterified to triglyceride within their enterocytes and accumulates in intracellular fat droplets. Apparently the secretion of HDL apoproteins is not markedly affected by the block in chylomicron and VLDL secretion, since plasma apoAII and apoC levels are fairly normal. Since intracellular apoB cannot be detected in enterocytes by immunological procedures [57], it seems possible that a highly truncated and immunologically unrecognizable apoB molecule, or no apoB at all, is synthesized by these patients. In another genetic abetalipoproteinemia, the synthesis of hepatic apoB is impaired while that of intestinal apoB is normal [56]. These patients can absorb and transport dietary fat but cannot produce hepatic VLDL.

Early studies of hepatocytes in the process of VLDL synthesis suggest that apoB and presumably other apoproteins are combined with VLDL lipids, synthesized in the smooth ER, at or near specialized elements of the rough ER which have smooth-

* We will use ‘nascent triglyceride-rich particles’ to mean particles which have been secreted and collected from intestinal lymph or hepatic perfusion in the absence of blood cells or plasma. These particles are not truly nascent as they have been exposed to intestinal lymph or VLDL lipoprotein fluid.
surfaced ends [58]. Subsequently, VLDL-sized lipoprotein particles are observed within the Golgi apparatus. These presumably represent the initial VLDL assembly products. Smaller HDL-like particles are often seen to be intermixed with the larger VLDL within the same elements of the Golgi. In view of the avid lipid-binding properties of the apoproteins, it is likely that they are associated with at least a subset (perhaps phospholipids) of VLDL lipids at all stages of their transport through the secretory pathway. The majority of their lipids appear to become associated with them after their entry into the Golgi. Furthermore, a fraction of VLDL phospholipids may be added late in the secretory pathway, just prior to secretion, by some intracellular organelle transporting the nearly completed lipoproteins [59]. Based upon ultrastructural study of chylomicron formation in intestinal cells, chylomicrons follow a similar pathway of export, except that they are discharged into the lymph ducts and not directly into the plasma, as in the case of most of the hepatic VLDL.

Not much is known about the precise molecular events which lead to the assembly of triglyceride-rich lipoproteins. One aspect of assembly requires knowledge about the limits of solubility of the relatively nonpolar lipids, triglyceride and cholesterol esters, within the membrane phospholipid bilayer. As discussed below, the solubilities of these lipids in phospholipid are quite low, and once they attain levels exceeding the limits of their solubility in the bilayer, they would be expected to form an oily phase which in time may become a lipoprotein core. Thus, the assembly of the lipid particle may occur spontaneously. However, it is possible that the intervention of apoproteins, and/or an intracellular assembly ‘apparatus’ is required to direct the departure of the nascent lipoprotein particle into the lumen of the ER instead of into the cytoplasm [8]. However, intact apoB does not appear necessary for this process since patients with abetalipoproteinemia form nascent-like particles which appear in (secretory) vesicles. They are not secreted; thus intact apoB is required for secretion.

Under conditions of cholesterol feeding, cholesterol ester rich VLDL are secreted from hepatocytes. In these VLDL the cholesterol ester/triglyceride weight ratio may exceed 1/1 [60], whereas in normal nascent VLDL the ratio is typically < 1/4. These abnormal VLDL are also enriched in cholesterol, apoB, and apoE. Due to the relative enrichment in apoE and depletion of other small molecular weight apoproteins, these VLDL exhibit altered electrophoretic mobility and are designated β-migrating, or β-VLDL. Presently the relationship between cholesterol feeding and increased synthesis and secretion of apoE is not well understood. Perhaps the synthesis of apoE facilitates the assembly or secretion of cholesterol ester enriched VLDL. In this regard, it has been demonstrated that apoE is secreted along with phospholipid and cholesterol ester from several cell types [61, 62].
(b) Metabolic transformation of chylomicrons and VLDL

Nascent chylomicrons and VLDL undergo several major compositional changes after entering the plasma. Both apoproteins and lipids are exchanged between triglyceride-rich lipoproteins and plasma elements such as erythrocytes and other classes of lipoproteins. Red blood cells contain an enormous reservoir of unesterified cholesterol which potentially can be transferred to nascent triglyceride-rich lipoproteins. Assuming a hematocrit of 40% blood volume, red blood cells, which have a cholesterol/phospholipid molar ratio of approximately 1/1 [42], contribute about 60–70 mg/dl cholesterol to the total blood cholesterol concentration. In humans another 50–75 mg/dl of unesterified cholesterol is present in circulating lipoprotein pools. During the peak phase of chylomicron entry into the plasma, plasma triglyceride levels may approach 500 mg/dl. Since the cholesterol content of chylomicrons is < 1% of their total weight, the addition of < 5 mg/dl chylomicron cholesterol to plasma only expands the cholesterol pool slightly. Since the nascent lipoproteins contain little cholesterol, it would be anticipated that they are not initially in equilibrium with the other blood cholesterol carriers with respect to cholesterol distribution.

Several in vitro studies have shown that chylomicrons and VLDL do in fact acquire cholesterol when incubated with plasma or erythrocytes. Zilversmit showed that cholesterol was transferred into dog lymph chylomicrons and phospholipid was lost from the lymph lipoproteins when they were incubated with dog serum [63]. The transfer of lipid components was dependent upon both the length of time of incubation and the ratio of chylomicrons to serum in the incubation mixtures. Faergerman and Havel demonstrated that rat plasma VLDL experienced a doubling of their cholesterol percentage when incubated with rat erythrocytes for prolonged periods of time (6 hours) [64]. It should be noted that the residence time of nascent VLDL in rat plasma is normally only 5–10 min and thus the extent of uptake of cholesterol may be considerably less in vivo. Nevertheless, these and other [65] experiments suggested that cholesterol not only exchanges between triglyceride-rich lipoproteins and both plasma lipoproteins and red blood cells but that also net amounts of cholesterol transfer from blood to nascent particles in vivo.

The mechanism of cholesterol transfer probably involves the spontaneous movement of cholesterol molecules between donor and acceptor particles [66, 67] independent of protein carriers. While other lipids can transfer to a limited extent without protein carriers, their potential to do so is much less than that of cholesterol because their movement through the aqueous phase requires overcoming a higher energy barrier of transfer from nonpolar to aqueous phases. Therefore, it is likely that a major fraction of the phospholipid which is transferred between lipoproteins, is carried by apoproteins and transfer proteins which shuttle between triglyceride-rich lipoproteins and high density fractions of the plasma. For example, apoA1 and apoAIV transfer off VLDL and chylomicrons and enter the HDL or \( p > 1.21 \) g/ml
fractions when nascent lipoproteins are incubated with plasma [68]. ApoC peptides undergo transfer from HDL to triglyceride-rich lipoproteins in a process by which chylomicrons and VLDL are activated for subsequent lipolysis in the peripheral circulation by the binding of apoCII [69]. These apoprotein and lipid transfer reactions occur independently of any catalytic action of lipoprotein lipase. Since apoprotein transfer reactions are quite rapid [48, 69, 70], they presumably can occur to completion during the short plasma residence times of chylomicrons and some species of VLDL.

When activated triglyceride-rich lipoproteins enter the peripheral circulation, they attach to the capillary walls and undergo degradation by lipoprotein lipase which is bound to cell surface glycosaminoglycans [71] and can be released from its binding sites by heparin [15]. ApoCII is absolutely required for the action of lipoprotein lipase. Patients with Type I hypertriglyceridemia, who lack the cofactor but have lipoprotein lipase, have extraordinarily high levels of circulating chylomicron and VLDL triglyceride (> 1 g/dl) [72]. As a consequence of lipase action, a large percentage (> 75%) of the particle triglyceride is degraded to free fatty acids and monoglycerides which eventually enter muscle or fat cells or are bound to albumin. A small fraction of the lipoprotein phospholipids are also cleaved to fatty acids and lysophospholipids by lipoprotein lipase [73]. The net result of lipase action is the production of a lipoprotein core 'remnant' which is reduced in size and has an altered lipid and apoprotein composition [74, 75]. ApoC peptides are largely removed from the degraded particle and enter the HDL fraction [76]. As a result, the lipoprotein becomes a poor substrate for continued lipoprotein lipase action. During this process apoB remains with the lipoprotein particle that ultimately is taken up by the liver [77]. Hepatic remnant uptake is mediated by apoE which acts as a ligand for the hepatic remnant receptor (apoE receptor) protein [78, 79]. Possibly the enrichment of the remnant with unesterified and esterified cholesterol may promote the transfer of apoE from HDL to the remnant lipoprotein [80].

In all species examined, chylomicrons are efficiently cleared from the circulation within 5-10 minutes of their entry into the plasma. However, the rate of clearance of VLDL varies greatly between species. While the half-life of rat VLDL is short, isolated human plasma VLDL have a plasma half-life of almost 6 hours [81]. In humans large VLDL are cleared like chylomicrons while small VLDL are converted to IDL and LDL [49]. During the process apoB remains bound to the particle on which it initially was secreted. The transformation of VLDL to LDL may occur entirely within the plasma compartment and probably involves the concerted action of lecithin:cholesterol acyltransferase (LCAT), cholesterol ester and triglyceride exchange proteins (CEEP and TGEP) and lipoprotein lipase [5]. That is, cholesterol ester molecules contained in LDL or formed in HDL by LCAT are transferred to the IDL particle by CEEP in exchange for residual triglyceride molecules. Residual triglyceride is hydrolyzed and subsequently removed by lipases. Thus, cholesterol ester gradually accumulates and triglyceride is lost from the lipoprotein. Because
human VLDL have a relatively long half-life they probably can equilibrate with plasma cholesterol pools to a greater extent than chylomicrons.

Presently, biochemical studies of lipolysis or lipid exchange have not been able to determine the exact location(s) where lipoprotein or hepatic lipase, and CEEP and TGEP encounter their substrate molecules. Assuming that the classical emulsion droplet-like structural model of lipoprotein organization [82] is basically correct, then these catalytic proteins conceivably could act at the lipoprotein surface if their substrates are soluble in this region. Alternatively, they may penetrate into the lipoprotein core and encounter cholesterol ester and triglyceride molecules there. Similarly the exact location of cholesterol molecules taken up by the particle cannot be determined without knowledge of the phase solubilities of the lipids in triglyceride-rich lipoproteins.

3. Structural features of triglyceride-rich lipoproteins and physical-chemical properties of their components

In their review of the structure and metabolism of chylomicrons and VLDL, Dole and Hamlin presented a model for the structure of triglyceride-rich lipoproteins that was based upon available knowledge of the gross physical properties of their lipid and apoprotein components [82]. According to their model phospholipid, cholesterol and apoproteins reside in a surface emulsifier layer around an apolar core of triglyceride and cholesterol ester molecules. These basic features of organization have been discussed in other review articles [8, 83]. The emulsion droplet-like model predicts a structure which satisfies the thermodynamic requirement for low energy dispersion of the nonpolar lipids, triglyceride and cholesterol ester, in the polar aqueous environment. In this section we present data which led to refinements in this simple model and a better description of the detailed aspects of chylomicron and VLDL structural organization.

(a) Chemical compositions of chylomicon and VLDL subfractions

Chylomicrons and VLDL can be separated by gel filtration or centrifugation into subfractions which vary in size and buoyant density. When the chemical compositions of subfractionated lipoproteins are determined, several consistent features are observed for both chylomicrons and VLDL. In all cases the percentage of triglyceride declines, whereas percentages of phospholipid, cholesterol, cholesterol ester, and apoprotein increase as particle sizes decrease [84 – 86]. While the percentage of cholesterol ester increases, its increase is not sufficient to counterbalance the decrease experienced in the particle triglyceride content. Thus, the combined percentages of triglyceride and cholesterol ester are depleted in smaller-sized particles. These size relationships are to be expected if the majority of polar phospholipid,
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osphopholid,  
cholsterol and apoprotein components are present in the surface and triglyceride  
and cholesterol ester are in the core, since the surface area to volume ratio of a  
spherical lipoprotein particle will increase as its diameter declines. Fraser [87]  
verified this relationship with rabbit lymph chylomicrons sized by centrifugation  
when he determined that the particle volume/surface area ratios correlated positive-  
ly with the particle triglyceride/phospholipid ratios.  

Sata et al. calculated that the phospholipid, cholesterol, and apoprotein compo- 
ents of subfractionated human VLDL could be fitted into a 21.5 Å thick  
monolayer at the surface of lipoprotein particles independent of their diameter [86].  
The thickness of the surface region corresponds approximately to the expected  
length of the acyl chains of phospholipid if they are radially oriented at the surface  
of the lipoprotein. These investigators also noted that the cholesterol/phospholipid  
ratios of the subfractionated lipoproteins decreased as particle sizes declined. This  
led them to speculate that some of the unesterified cholesterol molecules may be  
located within the cores of large VLDL since the values of the particle  
cholesterol/phospholipid ratios in some cases exceeded 1/1 and were, therefore,  
higher than the maximum ratio which could be obtained in single-phase dispersions  
of cholesterol and phospholipid in water [88–91].  

Within subfractions of chylomicrons and VLDL, considerable variation also ex- 
ists in the relative proportions of apoB and low molecular weight apoproteins, most  
notably the apoC peptides. Eisenberg et al. showed that the ratio of apoB to low  
molecular weight apoproteins increased in smaller particles [92]. This observation  
contributed to speculation that there may be a fixed number of apoB molecules per  
triglyceride-rich lipoprotein. Subsequent experiments have supported this point,  
although there is still some controversy over the exact number of apoB molecules  
per particle, e.g., one versus two copies per lipoprotein [74, 93]. In fact, it has been  
suggested that, since the mass of apoB per particle does not change during the  
transformation of chylomicrons or VLDL to their remnants, apoB never leaves the  
particle during its metabolism [74]. This idea is consistent with the marked hydro-  
phobicity of apoB. Conversely, the apoC peptides readily transfer between donor  
and acceptor lipoprotein particles and will readily adsorb from solution to  
phospholipid vesicles or phospholipid-triglyceride emulsions [94]. Thus, the size-  
dependence of the apoB/apoC ratio may be partly explained by the reduction of the  
amount of surface area unoccupied by apoB in small particles. As a consequence  
of the apoprotein and lipid heterogeneity of differently sized particles, metabolic  
variability within subfractionated lipoproteins would be expected. However, the  
study of this aspect of metabolism is hampered by the cross-contamination of  
metabolically different lipoproteins within the subfractions owing to the intrinsic  
polydispersity of triglyceride-rich lipoproteins.
(b) Early attempts to isolate surface and core lipids

Several techniques have been applied to isolate the putative surface ‘membrane’ and oil core lipids of triglyceride-rich lipoproteins. Procedures such as solvent extraction, i.e. particle delipidation [85], freeze-thawing cycles [26], and rotary evaporation of water [65, 95] have been used to disrupt the native lipoproteins. Each of these methods has potential for altering the true composition of lipids in a given region of the particle, or altering the distribution of lipids between the surface and core of the lipoprotein. For example, partial extraction of nonpolar lipids from VLDL with heptane [85] yielded a phospholipid-apoprotein residue which originated from the lipoprotein surface but probably lacked some cholesterol and nonpolar lipids that might have been present in it since these lipids are soluble in heptane. The technique of freeze-thawing or rotary evaporation of water to induce coalescence [26, 95] may have had less tendency to separate cholesterol ester and triglyceride which could be trapped in the aggregated surface (membrane) fraction. For instance, after disruption, low density oil and high density membrane lipid fractions were obtained by centrifugation [26, 65]. The oil lipids of human, dog and rat chylomicrons contained > 99% triglyceride and < 1% cholesterol ester and cholesterol. The membrane lipids consisted mostly of phospholipid, 5–8% cholesterol, no cholesterol ester, and highly variable levels of triglyceride (5–40% of total membrane lipids). The accumulation of large but variable amounts of triglyceride in the membrane phospholipid were assumed to result from sedimentation of crystalline triglyceride produced by freezing out of saturated triglyceride species during freeze-thaw cycles [26]. In this regard, somewhat less but still variable amounts of triglyceride were present in the membrane fraction when the chylomicrons were coalesced by rotary evaporation at 24–37°C [65]. Because freeze-thawing crystallizes some triglyceride and allows it to precipitate into the membrane fraction and also may alter cholesterol partitioning into phospholipid, this technique is inappropriate. Furthermore, rotary evaporation alters the water content of phospholipids and thus will change the distribution of cholesterol and nonpolar lipids into phospholipids. For instance when water is absent, cholesterol ester can be quite soluble in phospholipid, and vice versa [96, 97]. Thus this technique is also perturbative. Although no reliable estimate of surface triglyceride content could be obtained, the results did suggest that cholesterol may partition between the lipoprotein surface and core.

(c) Physical properties and phase solubilities of triglyceride-rich lipoprotein lipids: lecithin, cholesterol, triglyceride and cholesterol ester

Another way of obtaining information about the phase compositions of chylomicrons and VLDL is by studying simple lipid systems which model the structure of one or both phases of the lipoproteins.
Studies on the principal surface components

Air-water lipid monolayers exhibit many properties of the surface monolayer region of lipoproteins, since lipids with the best interfacial activities are present in both. Phospholipids spread at an air-water interface can form monolayers in which the lipids are oriented roughly perpendicular to the plane of the water surface. In this configuration their polar and/or charged headgroups interact with substrate water molecules and their nonpolar acyl chains extend up into the air above the water surface. If spread at sufficient surface density, the acyl chains will be in contact with one another, and the monolayer is said to be 'condensed' [98–101]. Egg lecithin is reasonably representative of the phospholipids found in lipoproteins [28, 102]. It contains a high percentage of unsaturated fatty acids at the sn-2 carbon of its glycerol backbone and principally palmitic acid at the sn-1 carbon. Monolayers of egg lecithin exhibit the properties of compressibility and elasticity, owing to the presence of these unsaturated acyl chains. Unsaturated acyl chains do not pack well when the lateral pressure on the monolayer is increased, and they tend to maintain the separation between the phospholipid headgroups. Monolayers of egg lecithin are quite stable to lateral pressure and can exist up to a surface pressure, \( \pi \), of \(-43 \) dyne/cm before the monolayer collapses. At the collapse point the area/molecule reaches its limiting value of \( 62 \, \text{\AA}^2 \) [91, 99]. Thus, even at maximum compression the area per acyl chain, \( 31 \, \text{\AA}^2/\text{chain} \), is much greater than the area (18.5–20 \( \text{\AA}^2/\text{chain} \)) of a saturated hydrocarbon chain packed in a crystalline lattice [103]. For comparison, the surface area/lecithin molecule in a maximally hydrated multilamellar vesicle has been reported as low as \( 66 \, \text{\AA}^2 \) and as high as \( 72 \, \text{\AA}^2 \) [91, 104]. Such areas are obtained in monolayers between \(-30–22 \) dynes/cm [91]. Thus, bilayer lecithin molecules exist in a relatively expanded state. Other things being equal, this means that the surface pressure could be increased up to \(-43 \) dynes/cm before phospholipid would buckle from the surface. The contraction in surface area resulting from this would be only \( 4–10 \, \text{\AA}^2/\text{molecule} \), or about a decrease in \( 6–14\% \). Thus, at an egg lecithin-triolein emulsion surface a potential space of \( 6–14\% \) exists at the core-surface interface which could be realized if compression of the surface occurred. Such compression could be produced by external lipids (e.g. cholesterol), lipolytic products or apoproteins entering the surface. It is likely that this potential ability of unsaturated species of phospholipid to be compressed or expanded may be important to the stabilization of the lipoprotein surface as it undergoes apoprotein and lipid adsorption/desorption or lipid hydrolytic reactions during its metabolism.

Cholesterol also spreads at an air-water interface and forms monolayers in which the polar hydroxyl group of the molecule is hydrogen-bonded to water molecules and the steroid nucleus projects up into the air [98]. Although the monolayer remains fluid up to its collapse, the monolayers are less compressible than those formed by egg lecithin because the steroid nucleus is rigid. Furthermore, the cross-sectional area of the steroid nucleus (36–38 \( \text{\AA}^2 \)) is greater than that of the aliphatic
isoctyl tail (31 Å²) of the molecule, and therefore the tail probably does not contribute significantly to the surface area measured in the monolayer. Monolayers of cholesterol are stable to ~38 dynes/cm. They can be compressed to 42–44 dynes/cm, at which point they will collapse. The limiting area per cholesterol molecule is 37–39 Å, or about twice that of an all-trans-saturated hydrocarbon chain.

Mixtures of unsaturated lecithin, such as egg yolk lecithin and cholesterol, form monolayers which exhibit nonideal properties. That is, at a given surface pressure, the area/molecule is less than that calculated from the mole fractions of lecithin and cholesterol in the monolayer at the same pressure, assuming a priori that they should form an ideal mixture [98, 105]. Typically an area reduction of ~15% occurs at 25 mole % cholesterol and 20 dynes/cm. Two possible explanations for this behavior have been offered. In one, the reduction is thought to occur because cholesterol binds to the lecithin acyl chains and reduces their tendency to spread laterally [98]. The other possible explanation views the apparent area reduction as arising from the localization of the cholesterol molecules to the region of the monolayer near the lecithin headgroups where the lecithin molecules are held apart by the contacts between the kinked acyl chains [106]. In either case, the incorporation of cholesterol into the phospholipid is critically dependent upon the hydration of the lecithin headgroups. The bound water molecules probably reduce the cohesive forces between adjacent lecithin molecules and allow cholesterol molecules to incorporate [89, 91].

The maximum solubility of cholesterol in egg lecithin bilayers has been measured by a number of physical techniques [88–91, 107]. The equilibrium value is 33 wt. % or 50 mole % cholesterol at 22–37°C. The addition of cholesterol stiffens the acyl chains of the phospholipid, increases their average length, and further separates the headgroups, allowing water to penetrate deeper into the headgroup region [91]. Cholesterol-supersaturated lecithin bilayers can be prepared which contain >33 wt.% cholesterol. With time the excess cholesterol molecules will eventually precipitate as cholesterol monohydrate crystals [107, 108]. These crystals melt at a much higher temperature than body temperature, the first of the polymorphic crystalline phase transitions occurring above 85°C [109]. Since the cholesterol/phospholipid ratio of large triglyceride-rich lipoproteins may exceed 1/1, it becomes important to determine if (a) a separate phase of crystalline cholesterol is present in the surface of these lipoproteins, (b) the surface is supersaturated with cholesterol and thus metastable, or (c) cholesterol also partitions into the lipoprotein core, as suggested from the presence of some cholesterol in isolated chylobinon oil lipids [26, 95].

Although triglyceride is much less polar than phospholipid, it has sufficient polar character at its glycerol backbone region to allow it to spread on water [98, 110, 111]. However, monolayers of triglycerides containing unsaturated fatty acids are much less stable than those formed by phospholipid and cholesterol and at room
temperature collapse at $\pi = 12 - 15$ dynes/cm [111]. The instability of the monolayer to lateral pressure may be attributable to the relatively weak interactions of the ester groups with water and interrelated factors arising from poor potential for the acyl chains to pack perpendicular to the water surface. The ability of triglyceride to localize at the air-water interface is promoted when it is mixed with phospholipid, as studies of mixed phospholipid-triglyceride monolayers have revealed [110, 112]. The percentage of triglyceride in the mixed monolayer is high at low pressures but decreases to about 5% (mole fraction = 0.04) when $\pi = \sim 43 - 45$ dynes/cm – a pressure at which pure triglyceride could only exist as a bulkphase of oil on the water surface. While these results suggest that triglyceride may be present to a limited extent in the surface monolayers of triglyceride-rich lipoproteins, the exact percentage of triglyceride actually present in the surface cannot be predicted, since the lateral surface pressure at the lipoprotein interface is difficult to measure directly. Therefore, more suitable models for the surface region of chylomicrons and VLDL must be studied.

Phospholipid vesicles are an example of one such structural analog of the lipoprotein interface. The solubility of triolein in egg lecithin unilamellar vesicles has been measured by chemical and $^{13}$C NMR spectroscopic methods [113, 114]. At 24 - 37°C, a maximum of 3 wt.% of triolein can be incorporated into the vesicle. Using triolein labeled with $^{13}$C at all three acyl carbonyl groups, it was demonstrated that these groups are probably hydrogen-bonded to water molecules present at the vesicle surface, since the chemical shifts of the residues were deshielded compared to the chemical shifts arising from triolein carbonyl groups present in an oil phase. Further the $\beta$ carbonyl was less deshielded than the $\alpha$ carbonyls, indicating that the $\beta$ position is in a more hydrophobic region (see Fig. 1). The same techniques were used to demonstrate that cholesteryl oleate was slightly less soluble (2 wt.%) in egg lecithin vesicles [115]. $^{13}$C NMR indicated that cholesterol esters assume a hairpin-like conformation with acyl chain and steroid groups lying side by side parallel to the lecithin bilayer chains and the ester group exposed to the aqueous phase (Fig. 1). Previous studies, in which polarized light microscopy, X-ray diffraction and differential scanning calorimetry (DSC) had been employed to monitor the presence of cholesterol esters in hydrated multilamellar egg lecithin bilayers, had demonstrated that the maximum solubility of cholesteryl linolenate in phospholipid was 2 wt.% [116]. Furthermore, triolein and cholesteryl oleate were found to be cosoluble in egg lecithin vesicles [114]. These mixtures were prepared by adding a slight net excess of these lipids to egg lecithin before sonication. The ratio of triolein/cholesteryl oleate in the vesicular fraction, i.e., the surface phase, slightly favored triolein but was very close to that in the starting mixture. The combined solubility of the two lipids in the bilayer was always limited to 4 mole %, suggesting that phospholipid interfaces have a maximum solubility for these two lipids which is independent of their relative proportions.
Studies on the principal core components

Most, but not all, biological triglycerides are liquids at > 20°C and form an immiscible oil phase when in contact with water [100]. The low melting points of triglycerides obtained from vegetable oils, such as corn or safflower oil, is a function of their high contents of esterified mono- and polyunsaturated fatty acids [117]. Liquid triglyceride oils are good solvents for cholesterol esters. The solubility of a given cholesterol ester is dependent upon the temperature of the mixture and the melting point ($T_m$) of the ester. For example, triolein ($T_m = 4°C$) can incorporate ~ 12% cholesteryl oleate ($T_m = 51°C$) at 24°C and ~ 25% cholesteryl oleate at 37°C [118]. At temperatures greater than 50°C, the two components are miscible in all

Fig. 1. Conformation of triglyceride and cholesterol ester in egg phosphatidylcholine surfaces. The maximum mole % of cholesteryl oleate (CO) and triolein (TO) and mixtures of both which can be incorporated into egg phosphatidylcholine (PC) bilayers is shown by the black points outlining the stippled zone in the diagram. 2.8 mole % of either may be maximally incorporated. To the left excess oil triglyceride and/or cholesterol ester are prominent as a second phase. Line A – C would indicate complete competition of triolein for cholesteryl oleate or vice versa, whereas boundary ABC would indicate complete additivity. Some competition exists as the observed line is less than complete additivity. The $^{13}$C NMR experiments indicate that the conformation of triolein is as shown below. The $\alpha$ carbonyls (sn-1, 3) protrude more into the aqueous environment than the $\beta$ (sn-2) carbonyls. The NMR experiments indicate that the cholesteryl oleate molecule is bent at the carbonyl group which protrudes slightly into the aqueous compartment. The conformation of these molecules makes them available in the surface for enzymatic reactions (e.g., lipolysis), or for transfer reactions. (Data from [114])
proportions. While the precise temperature-composition phase diagrams of triolein and cholesteryl linolenate \( T_m \sim 32^\circ C \) and cholesteryl arachidonate \( T_m \sim 19^\circ C \) have not been determined, they should both be completely miscible with melted triglycerides at body temperature since they are both liquids at 37°C [118]. Cholesteryl linoleate, an ester found in triglyceride-rich lipoproteins, is largely soluble in liquid triglyceride at body temperature since its melting point (42°C) is close to 37°C.

Study of the thermal properties of the lipids in human plasma VLDL has provided insight into the physical state of the core of this lipoprotein [119a]. VLDL typically contain a 4/1 ratio of triglyceride to cholesterol ester. Of the ester fraction, 70% is cholesteryl olate, cholesteryl linoleate, cholesteryl linolenate, and cholesteryl arachidonate. When samples of VLDL are heated and cooled in a calorimeter, no thermal transitions are observed in the range of 10–50°C in which the VLDL remain undenatured. Thus, although the average sized VLDL in the population contains a greater number of cholesterol ester molecules than does LDL — a lipoprotein which exhibits liquid-to-liquid crystalline phase transitions in this temperature range [119] — the cholesterol ester molecules are dissolved in the triglyceride core. The cholesterol ester transition occurs in the normal LDL core just below body temperature [119–122]. The melting point of the esters is influenced by their overall fatty acid composition [118, 123] and by the few percent of triglyceride which is dissolved in them [120, 124]. Thus, in lipoproteins which contain a high ratio of polyunsaturated cholesterol esters or a large amount of triglyceride such as normal LDL and VLDL, the core lipids most often exist in a liquid state at body temperature.

The phase transitions in LDL are reversible liquid crystal-liquid transitions and occur at about the same temperature regardless of the direction of heating or cooling [119, 120]. In contrast, triglycerides do not undergo liquid crystal transitions but these complex molecules can undergo several polymorphic transitions before melting. Once melted, triglycerides undercool \( \sim 20–30^\circ C \) and crystallize to an \( \alpha \) form before reverting to more stable forms with time. For an in-depth review of triglyceride physical properties see [111]. In general, the greater the percentage of long chain saturated fatty acids in a triglyceride mixture the higher the melting point \( T_m \) and crystallization temperature \( T_p \). In humans and other omnivores and in carnivores, increased saturated fatty acids in the diet lead to increased saturated fatty acids absorbed and esterified to triglyceride in chylomicrons. However, since saturated triglycerides have such high melting points, intestinal absorption is limited and, thus, limits on the saturation of chylomicron triglycerides are present. However, in ruminants the rumen saturates many plant fatty acids and the gut appears to be presented with a very saturated chyme. These animals absorb and esterify the saturated fatty acids into chylomicron triglycerides so that up to 80–90% of the fatty acids may be saturated [125].

When monkeys are fed high-saturated fat diets containing 40% of their caloric
intake as butterfat, their chylomicron triglycerides undergo crystallization abruptly at 14–17°C [126] when cooled. When heated, the crystalline triglyceride fraction does not completely melt until ~45°C. Rats fed palmitate-rich diets produce palmitate-rich lymph chylomicrons and VLDL which begin to crystallize at 26°C and do not melt completely until 58°C [127, 128]. The chylomicrons produced in ruminants [125] crystallize at ~30°C and are not completely melted until 60°C [129]. The intestinal lipoproteins produced by these ruminants or by saturated fat-fed animals actually contain metastable, undercooled liquid triglyceride cores which remain liquid at 37°C [126, 127, 129]. However, care must be taken in the collection and storage of these lipoproteins so as not to induce triglyceride crystallization, particularly when the lipoproteins are to be used subsequently for metabolic studies. We have found that triglyceride-rich lipoproteins having more than ~50% palmitic acid (16:0) + stearic acid (18:0) circulate as undercooled metastable particles. If the particles are cooled to their crystallization point (Tc) some of the triglycerides crystallize. Since the Tc is ~20–30°C below Tm, a fraction of the triglyceride remains crystalline at body temperature. If the particles are reheated such particles have abnormal metabolism. Although several correlations were tested between Tc and lipoprotein fatty acid composition, none were highly correlated. The best correlation (r = 0.79) was for Tc vs. % (16:0 + 18:0) (see Fig. 2).

While cholesterol has considerable air-water and lipid-water interfacial activity, it is also soluble in triglyceride and cholesterol ester oils [27, 118, 123, 130–133]. The solubility of cholesterol in nonpolar solvents can be attributed to the large

Fig. 2. Temperature of crystallization of native triglyceride-rich lipoproteins vs. percentage of stearic and palmitic acids in triglyceride. Between 50 and 84%, 16:0 + 18:0 the correlation is $T_c = 5 + 0.21 \times (\% \text{16:0 + 18:0})$ °C; $r = 0.79$. The rat, monkey and bovine data are from [127], [126] and [129], respectively. The VLDL, IDL and chylomicrons (CM) are all intestinal particles named by density.
hydrophobic portion of the molecule. At 37°C, the solubility of cholesterol in triolein is 4.3%, and at 21°C, its solubility is 2.8%. The addition of water to the oil reduces the solubility of cholesterol to 3.2% at 37°C and 1.9% at 21°C [132]. Similarly, addition of water to anhydrous cholesteryl linoleate-cholesterol mixtures decreases the solubility of cholesterol from 5.0% to 3.8% at 37°C [132, 133]. The addition of a water phase promotes the migration of cholesterol molecules from the interiors of the oil droplets to their interfaces where they reduce the interfacial tension by the hydrogen-bonding of their 3-hydroxyl groups to water molecules. The hydration of the hydroxyl group apparently makes the cholesterol molecule less soluble in the oil, and the excess cholesterol molecules precipitate as cholesterol monohydrate crystals. The addition of water to triolein-cholesteryl oleate oil mixtures had no effect on the solubility of cholesteryl oleate in the oil, because cholesteryl oleate displays little interfacial activity.

Before moving on to the discussion of the phase behavior of emulsified mixtures of triglyceride-rich lipoprotein lipids, some comment should be made concerning the studies on the equilibrium distribution of cholesterol between the surface and core of cholesterol ester rich systems such as LDL. Unfortunately, the precise distribution of cholesterol between core (oil) and surface phases has not been systematically studied. Loomis [96] established the phase boundaries for the cholesteryl linoleate-cholesterol-lecithin-H₂O system at 37°C at 4% cholesterol, 96% cholesteryl linoleate for the oil phase and ~32% cholesterol, 1% cholesteryl linoleate, 67% lecithin for the surface phase. Thus the distribution ratio of cholesterol between the surface and core, \( K_{C/s/o} = 32/4 = 8 \). However, when he made an emulsion of 10.9% cholesterol in a 50 cholesteryl linoleate:50 lecithin mixture, and separated the

### TABLE 1

<table>
<thead>
<tr>
<th>CE* Emulsion system</th>
<th>%TG</th>
<th>%C in surface</th>
<th>%C in oil</th>
<th>( K_{C/s/o} )</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CL-C-L-H₂O (37°C)</td>
<td>0</td>
<td>16</td>
<td>3.4</td>
<td>-4.7</td>
<td>[96]</td>
</tr>
<tr>
<td>2 Tangier spleen CE-rich droplets (39°C)</td>
<td>1%</td>
<td>17</td>
<td>3</td>
<td>-5.7</td>
<td>[134]</td>
</tr>
<tr>
<td>3 LDL lipids (35°C)</td>
<td>3%</td>
<td>24.5</td>
<td>3</td>
<td>-8.1</td>
<td>[120]</td>
</tr>
</tbody>
</table>

*CE, cholesterol ester; TG, triglyceride; CL, cholesteryl linoleate; C, cholesterol; L, lecithin.

* \( K_{C/s/o} \) % cholesterol in surface: % cholesterol in core (oil) phase.
oil and surface in the ultracentrifuge, he found that the $K_{C_{250}}$ was $16/3.4 = 4.7$ (Table 1). Isolated and fractionated cholesterol ester droplets from the spleen of a Tangier disease patient [134] gave a similar $K_{C_{250}}$ of 5.7. However, when the purified surface and core lipids of LDL were prepared by centrifuging emulsified LDL lipids in the ultracentrifuge at 37°C [120], the oil phase contained 92.6% cholesterol ester, 4.5% triglyceride, and 3% cholesterol, while the surface lipid fraction contained 72.9% phospholipid, 2.5% cholesterol ester, 24.5% cholesterol, giving a $K_{C_{250}}$ of 24.5/3 = 8.1. The reasons for the apparent variability of $K_{C_{250}}$ in these different systems is not known, but it may be related to their different phospholipids and cholesterol esters. As a rough approximation, $K_{C_{250}}$ in cholesterol ester rich systems appears to be about 5–8, and this is quite different from very triglyceride-rich systems, as will be discussed later.

In any event, these results confirm predictions that cholesterol may partition between core and surface lipids of emulsions of lipids and probably between phases of intact LDL. Similar evidence for partitioning of cholesterol between the surface and core of HDL has been obtained by nonperturbative methods, e.g. NMR spectroscopy [135]. Based upon the strict relationship of the surface cholesterol ester/triglyceride ratio to that of the total system that was observed in model systems [114], the lack of a detectable mass of triglyceride in the surface of LDL is consistent with the concept that triglyceride and cholesterol ester partially compete with one another for orientation at the phospholipid interface. Since the triglyceride content of LDL is low, the minute amount of triglyceride in the surface was less than could be detected.

4. Emulsions: structural models of triglyceride-rich lipoproteins

As discussed in the preceding sections, considerable information concerning the structural organization of chylomicron and VLDL lipids has been obtained by study of the physical properties of native triglyceride-rich lipoproteins and simple lipid mixtures modeling their structure. These studies support the predicted general model or organization of lipids but also suggest that several of the lipids may partition between the surface and core regions as in LDL. Since chylomicrons and VLDL are considerably larger than LDL and, therefore, have much greater proportions of core mass relative to surface mass, it is possible that large amounts of cholesterol may be in their cores. In order to predict the fraction of the total particle cholesterol present in the core and the equilibrium phase compositions of lipoproteins, model systems in which both phases are present and at equilibrium must be examined. In this section we will describe our studies with simple triglyceride-rich emulsions and demonstrate methods of preparation of purified surface and core lipid regions. It should be noted that these techniques, which will be applied to the study of lipoprotein lipid emulsions, are also applicable to the study of the phase compositions of intracellular fat droplets, emulsified intestinal fat, milk globules, etc.
(a) Basic emulsion properties

In addition to the similarities which exist in the organization of lipids within the two types of particles, emulsion systems have two other features in common with native triglyceride-rich lipoproteins. First, they are of low density and can be floated in the ultracentrifuge. Second, they are polydisperse, and size subfractions can be obtained by centrifugation. These features permit accurate structural modeling if the compositions of the starting lipid mixtures are adjusted to resemble the overall composition of the lipoprotein, e.g., low in cholesterol in the case of chylomicrons, or relatively higher in cholesterol and cholesterol ester in the case of VLDL. However, emulsions differ from lipoproteins in a technically important way. Due to their lack of protein (and due to the presence of extremely large particles within coarse emulsions), they are much less stable to coalescence when centrifuged. This feature allows one to separate and isolate emulsion core and surface phases on the basis of density.

(b) Triolein-lecithin-water emulsions and triolein-cholesterol-lecithin-water emulsions

The simplest system which can be used as a relatively crude model of chylomicron structure is composed of triolein, egg lecithin, and water. To prepare a coarse, that is, highly polydisperse emulsion from these lipids, 80 mg of triolein and 20 mg of egg lecithin in organic solvents are dried under vacuum to remove all traces of solvent. In the dry solvent-free system the lecithin is dissolved in the liquid triolein. To the 100 mg of lipid, 0.9 ml of water is added to give a 10% lipid, 90% water system. The vial is sealed under nitrogen and agitated. Initially the lecithin hydrates and the oil swells [104]. Lecithin and associated triolein oil droplets are sheared off the walls of the tube. A polydisperse population of particles is generated by the process, and particle sizes eventually attain a limiting range of values which depend upon the relative proportions of surface and core lipids in the mixture and the intensity and duration of agitation. For the coarse emulsions prepared this way, particles of > 10 μm down to ~ 300 Å are present in the system. It is important to realize that although the chemical composition of the droplets depends upon their size according to the ratio of surface to core mass in each particle, the composition of the surface and core phases, respectively, of each droplet in the system are the same once chemical equilibrium is attained. This is because all droplets in the system interact with one another and are subject to disruptive and fusive forces and lipid transfer reactions which tend to make the system homogeneous.

When samples of emulsions are centrifuged inside narrow diameter capillary tubes at 50 000 × g for 12 – 16 h, the emulsion is broken and the floating triglyceride oil and sedimented surface phospholipid phases can be recovered [120, 136]. The oil is obtained in pure form after one centrifugation but to remove a minor amount of
### TABLE 2

Chemical compositions of coarse emulsions and their phases

<table>
<thead>
<tr>
<th>Group 1 emulsions</th>
<th>Emulsion</th>
<th>Oil$^d$</th>
<th>Surface$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TO</td>
<td>CO</td>
<td>C</td>
</tr>
<tr>
<td><strong>Low cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. no CO</td>
<td>79.1$^a$</td>
<td>2.0</td>
<td>18.9</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.05</td>
<td>0.7</td>
</tr>
<tr>
<td>B. low CO</td>
<td>76.8</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>79.1$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. moderate CO</td>
<td>64.4</td>
<td>13.7</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.2</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>78.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II emulsions</td>
<td>Emulsion</td>
<td>Oil^d</td>
<td>Surface^d</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>High cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. no CO</td>
<td>80.9^a</td>
<td>4.69</td>
<td>99.3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>25.7</td>
<td>72.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>E. low CO</td>
<td>74.8</td>
<td>2.2</td>
<td>95.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.05</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.6</td>
<td>73.8</td>
</tr>
<tr>
<td>F. moderate CO</td>
<td>64.6</td>
<td>13.7</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.1</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>1.79</td>
</tr>
<tr>
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<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.10</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.28</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.6</td>
<td>71.4</td>
</tr>
</tbody>
</table>

^a Values represent the percentage, by weight, ± 1 SD from the mean (n = 3). SD is positioned just below the mean percentage.

^b The sum of triolein (TO) + cholesteryl oleate (CO), the nonpolar lipids, (N). Other abbreviations as in Table 1. (From [137]).

^c The surface samples for emulsion C were pooled (n = 3) for chemical analysis.

^d Surface and core (oil) compositions obtained for emulsions subjected to centrifugation to isolate phases.
oil droplets trapped in the surface phase it must be resuspended in water and recenterfuged. Control studies were performed to show that after one resuspension and recentrifugation of the surface lipids, a limiting level of triolein was obtained [136]. Furthermore, the speed of centrifugation, and hence the pressure exerted on the lipids, had no effect on the composition. These controls were also performed for emulsions containing cholesterol (see below). The results of the chemical analysis of the lipids showed that the oil was composed of pure triolein and no detectable phospholipid. The surface lipids consisted of 3% triolein and 97% lecithin. Thus, the results obtained by this technique are identical to those obtained from direct measurement of vesicular triolein content by $^{13}$C NMR spectroscopy or by chemical measurement of the compositions of lecithin-triolein vesicles from which all emulsion particles had been removed by centrifugation (see Fig. 1) [114].

Emulsions composed of triolein, cholesterol, and egg lecithin were then studied. The isolation of the phases of these emulsions allowed us to measure the equilibrium distribution of cholesterol between the surface and core regions. Since cholesterol is much more soluble in phospholipid than in triolein, we anticipated that cholesterol would partition preferentially into the surface phase, and this prediction was confirmed by the analyses. For example, the compositions of two typical emulsions (emulsions A and D) and the oil and surface phases isolated by centrifugation are given in Table 2 [137]. The data show that cholesterol was present in the core phases even though the amount of cholesterol in these emulsions was below the maximum that could be incorporated into the emulsion droplets (see below). Furthermore, the amount of cholesterol in the phases was dependent upon its level in the starting mixture. The incorporation of cholesterol into the emulsions did not prevent a small amount of triolein from partitioning to the surface lipids.

The combined phase composition data for a number of emulsions in which the cholesterol level was increased to and above its maximum solubility were compiled and used to construct the phase diagram for this system [136]. Although the system contains four components, the data are best analyzed on a triangular coordinate phase diagram which shows only the three lipid components. This triangular coordinate diagram is actually a slice taken at 90% water content of the larger tetrahedral phase diagram in which the content of water in the systems is included (Fig. 3). When using the three-component diagrams it must be remembered that the water phase is also present in the system, and that water can markedly influence some of the properties of the lipids, such as the solubility of cholesterol in the triolein oil [132] and the swelling of phospholipids [104]. However, for the sake of all other graphical manipulations of data that will be performed with these diagrams, the presence of the water phase can be ignored.

The method of plotting composition data points on the diagram is as follows (see Fig. 3). Each apex of the diagram represents the location of a pure (100%, by weight) single component system of one lipid (and of course water). Along the edges of the triangle, the compositions of mixtures of two lipid components are plotted.
Systems with all three lipid components plot within the edges of the triangle. We have chosen to place cholesterol at the top apex of the figure to emphasize the partitioning of cholesterol between the triolein oil and egg lecithin surface phases and to remain consistent with the graphs of cholesterol ester, cholesterol, and lecithin systems [120, 134, 138].

Fig. 4 shows the triolein-cholesterol-egg yolk lecithin phase diagram. The compositions of two representative emulsions (E), and their oil (O), and surface (S) lipid compositions are also shown in Fig. 4. Lines have been drawn on the figure through the oil and surface phases and their parent emulsion compositions (lines OES). These lines are called tie lines and join the compositions of all points on the figure which are in chemical equilibrium [139]. The line ab which intersects the triolein-

Fig. 3. The method of representing triglyceride-cholesterol-lecithin composition on triangular coordinates. The true system is triglyceride-lecithin-cholesterol-water and would be represented by a regular tetrahedron, upper left. However, by fixing the water content at 90%, the lipid system can be expressed as the 3-component system triglyceride-lecithin-cholesterol at constant water. The percentage total weight of triglyceride (TG), lecithin (L) and cholesterol (C) constituted by each of these components are shown on the scales along the sides of the triangle. Since the sum of triglyceride, lecithin and cholesterol equals 100%, the composition of any mixture containing these components can be represented as a single point within triangular coordinates. Thus, a mixture containing 80% triglyceride, 15% lecithin and 5% cholesterol is represented by a single point (P) formed at the intersection of the dashed lines extended from the 80% level on the triglyceride scale at the base of the triangle, the 15% level on the lecithin scale at the right of the triangle, and the 5% level on the cholesterol scale at the left of the triangle. We will generally plot weight %, although mole % can also be used.
lecithin edge of the figure at 3% triolein and 97% lecithin (point a) delineates the surface phase boundary and was drawn as the best fit line through the compositions of several isolated surface phases. The ratio of triolein to lecithin along the line (0.036) is approximately constant. (Recent NMR studies suggest that at high cholesterol:phospholipid ratios (~1:1 mole:mole), triglycerides and cholesterol esters are squeezed out of the surface.) The phase diagram consists of five zones or regions which differ with respect to the number and compositions of their phases. Zone I represents the surface phase which can incorporate from 2–4% triolein and 0–32% cholesterol. At fixed H₂O composition (90%) and at constant temperature

Fig. 4. The phase diagram of triolein (TO), cholesterol (C) and egg yolk phosphatidylcholine (L) in excess water, pH 7, at 22–24°C. Five regions (I–V) have been designated. I, the emulsion surface phase (Labd) which contains 2.3–4.0% TO and varying amounts of C and L. Points a and b represent the surface phase compositions in the absence of C and in the presence of the maximum amount of incorporated C, respectively. II, the emulsion oil phase (line TOe) which contains only TO and C, and can incorporate a maximum of 2.0% C (point e). III (abeTO), a 2-phase region in which emulsions (E) are composed of oil (O) and surface (S) phases whose compositions lie at the intersections of the tie lines (dashed lines) with the phase boundaries of the oil (TOe) and surface (ab) phases. IV (Cbe), a 3-phase region which is separated from Region III by the bold dashed line (be). Systems such as shown by point g in Region IV are saturated with C and are composed of oil (point e), surface (point b), and C monohydrate crystals (point C). V, a 2-phase region consisting of a surface phase saturated with C (line bd) and C monohydrate crystals (point C). The data points for the oil, surface, and emulsion compositions are plotted on the figure. (Data taken from [136] and [137])
and pressure, the system may be treated as a three-component system, triolein, cholesterol and lecithin. The phase rule [139] states that the degrees of freedom, $F$, are equal to the number of components ($C$) minus the number of phases ($P$), $F = C - P$. Thus, in Zone I since $C = 3$ and $P = 1$, $F = 2$. That is, the composition of two of the components must be fixed to define the system. Zone II gives the range of possible oil phase compositions for the emulsions. Up to 2% cholesterol was found to be soluble in the triolein oil. Lecithin is not measurably soluble in the oil phase. Zone III shows the range of possible emulsion compositions which have less than saturating levels of cholesterol. Two phases are present: surface and oil, and $F = 1$. Tie lines in this region connect the compositions of the equilibrated oil and surface phases. Zone IV is a three-phase zone where $F = 0$. Mixtures having compositions in this region consist of an oil phase saturated with cholesterol, point e, a surface phase saturated with cholesterol, point b, and an additional phase of cholesterol monohydrate crystals, point C. The lower limit of this region is line eb. At equilibrium, emulsion compositions falling on eb would be saturated with cholesterol. Finally, Zone V is the region in which a surface phase saturated with cholesterol, and cholesterol monohydrate crystals are both present. No oil phase is present in this zone.

Considerable information about the properties of emulsions which plot in Zone III can be gathered by phase diagram data analysis. From graphical inspection, the relative proportion of oil in an emulsion increases the closer it plots to the triolein apex of the figure. The actual fraction of the total lipid mass present in either phase of the emulsion is calculated using the tie line on which it plots. For the emulsion (E) the ratio of surface to oil phase masses, $M_s/M_o$, is obtained by measurement of the tie line segments OE and ES and the relation

$$\frac{M_s}{M_o} = \frac{OE}{ES}. \tag{1}$$

Similarly the fraction of surface lipids, for example, in the total system mass, $M_t$, is given by

$$\frac{M_s}{M_t} = \frac{OE}{OS}. \tag{2}$$

Since the respective phase compositions of all particles within the parent emulsion system should be the same at equilibrium, the compositions of subfractions of the total system will plot on the same tie line as demonstrated for hypothetical particles A, B and C in Fig. 5. These particles differ only in size and in the relative proportions of their surface and core phases, and therefore have different OE/ES ratios. The weight average sum of their compositions determine the value of point E.

To calculate the fractions of the total particle cholesterol, for example, present in the two phases of each emulsion droplet, one additional parameter must be introduced. This parameter, the phase distribution ratio for cholesterol, $K_C (K_{CS/o}$,
Fig. 5. Illustration of triangular coordinate phase diagrams used for the study of the phase behavior of triglyceride-rich emulsions and lipoproteins. Data points for a coarse emulsion (E) and its two lipid phases, the oil (O) and surface (S), form tie lines OES in the 2-phase region of the triangular coordinate diagram. The coarse emulsion (E) consists of polydisperse (variable sized) particles such as A, B, C. Because particles A, B and C are in equilibrium, they have identical weight fractions of cholesterol in their respective surface (S) and oil (O) phases. Particle diameters decrease from left to right along the tie line. Apex symbols: N, nonpolar lipids; P, polar lipids; C, cholesterol. (From [140])

above), is defined as the ratio of the weight fraction of cholesterol in the surface lipids, \( x_{cs} \), to the weight fraction of cholesterol in the oil lipids, \( x_{co} \).

\[
K_C = \frac{x_{cs}}{x_{co}}.
\] (3)

For the range of emulsions with 0–1% C in the oil phase and 0–28% C in the surface lipids the mean value \( \pm \) SD of \( K_C \) is 18 \( \pm \) 5 \( (n = 16) \) at 24°C [136]. At 37°C the mean \( K_C \) was estimated to be 22. Using \( K_C \), the ratio of the amount of the total particle cholesterol in the surface phase to that in the oil core is obtained using

\[
K_C \left( \frac{M_s}{M_o} \right) = \frac{x_{cs}}{x_{co}}
\] (4)

in which \( x_{cs} \) and \( x_{co} \) represent the fractions of the total emulsion cholesterol pre-
sent in the surface and oil regions, respectively. Then the percentage of the total emulsion cholesterol in the surface phase, \( \%C_s \), is given by

\[
\%C_s = \frac{X_{cs}}{X_{co} / (1 + X_{cs}/X_{co})} \times 100
\]  

(5)

and the percentage of the total emulsion cholesterol in the oil, \( \%C_o \), is given by

\[
\%C_o = 100 - \%C_s.
\]  

(6)

Thus, no measurement of the sizes of a subfractionated system of droplets need be made to calculate these parameters for each particle in the system. The same set of equations can be used to calculate all of the above values for each lipid in the emulsion, e.g., triolein, etc. This simple triolein-cholesterol-lecithin-water system (Fig. 4) serves as the basic model system upon which the study of more complicated emulsion and lipoprotein phase behavior has been built.

(c) Triolein-cholesterol oleate-cholesterol-lecithin-water emulsions

When cholesteryl oleate is added to the mixtures of lipids described in the last section, better models of triglyceride-rich lipoproteins are obtained. Pure cholesteryl oleate melts from a crystal at \(-50.5^\circ C\) and undergoes two metastable liquid crystal transitions: isotropic liquid-cholesteric liquid crystal at \(47^\circ C\) and cholesteric to smectic liquid crystal at \(42^\circ C\) [118, 123]. Cholesteryl oleate is reasonably soluble in triolein at \(24^\circ C\) [118]. While an exhaustive study of systems having a wide range of triolein/cholesterol oleate ratios has not yet been completed, two model systems with triglyceride/cholesterol ester ratios similar to lymph chylomicrons and plasma VLDL have been studied. Using these systems, the effect of incorporating cholesteryl oleate on the phase distribution of cholesterol in the emulsion was examined. Furthermore, they were also used to study the equilibration of lipids between individual particles within an emulsion or lipoprotein system.

To compare the phase behavior of lipids in emulsions containing variable levels of cholesteryl oleate, mixtures of roughly 80% triolein-cholesterol oleate and 20% cholesterol + egg lecithin were prepared [137]. They differed in their relative amounts of cholesteryl oleate as shown in Table 2. The values for the triolein/cholesterol oleate ratios of the emulsions were: (A and D), no cholesteryl oleate present; (B and E), triolein/cholesterol oleate = 33/1; and (C and F), triolein/cholesterol oleate = 5/1. These values were set at approximately the limits of the range of triglyceride/cholesterol ester ratios commonly found in normal triglyceride-rich lipoproteins. In emulsions A–C the cholesterol content was low (2–2.8%) to model lymph chylomicrons, and in emulsions D–F it was higher (4.7–5.8%) to model plasma VLDL.

As shown in Table 2, cholesteryl oleate was present to a very limited extent in the
surface region and was much more soluble in the triolein oil phase. In these experiments the oil to surface distribution ratio $K_{o/s}$ for cholesteryl oleate was about 60 ($K_{s/o} = 0.016$, Table 3). The level of cholesteryl oleate in the surface was strongly influenced by the amount of cholesteryl oleate in the emulsion, but for emulsions with 14% cholesteryl oleate, the percentage of cholesteryl oleate in the surface only attained a level of about 0.28 - 0.35%. This is reasonable, assuming that the amount in the surface is roughly proportional to the amount in the total composition (see above). For instance, if the maximum solubility of cholesteryl oleate in the surface is 2.8% [114, 115] in a pure cholesteryl oleate-lecithin system then 14% of 2.8 is about 0.39%. Incorporation of this relatively small amount of cholesteryl oleate into the emulsion did not significantly shift the amount of triolein in the surface phase from the range (2 - 4%) observed in emulsions without cholesterol ester. On the other hand, incorporation of cholesteryl oleate into the emulsions markedly influenced the solubility of cholesterol in the oil phase. The solubility of cholesterol in the emulsion oil phase was increased by 3 - 4-fold by increasing the percentage of cholesteryl oleate in the oil to 19 - 20%. Consequently,

**TABLE 3**

Surface: oil phase distribution ratios and standard free energies of phase transfer for the coarse emulsion lipids

<table>
<thead>
<tr>
<th>Emulsion group</th>
<th>$K_{TO}^a$</th>
<th>$K_{CO}$</th>
<th>$K_{C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Low cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. no CO</td>
<td>0.044$^b$</td>
<td>0.0001</td>
<td>27.8 (+ 1.8)$^c$</td>
</tr>
<tr>
<td></td>
<td>0.048</td>
<td>0.019 (− 2.5)$^c$</td>
<td>18.2 (+ 1.6)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.003 (− 2.5)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.065</td>
<td>0.018 (− 2.5)</td>
<td>7.5 (+ 1.1)</td>
</tr>
<tr>
<td><strong>II. High cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. no CO</td>
<td>0.019</td>
<td>0.002</td>
<td>38.5 (+ 2.0)</td>
</tr>
<tr>
<td></td>
<td>0.018</td>
<td>0.015</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.004</td>
<td>26.1 (+ 1.7)</td>
</tr>
<tr>
<td></td>
<td>0.039</td>
<td>0.015</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.001</td>
<td>14.1 (+ 1.4)</td>
</tr>
</tbody>
</table>

*The weight fraction phase distribution ratios $K_{TO}$, $K_{CO}$, and $K_{C}$ were calculated by using the equation: $K_i = x_i^s / x_i^o$ where $x_i^s$ and $x_i^o$ are the weight fractions of component $i$ in surface and oil phases.

$^b$ The mean ± 1 SD ($n = 3$).

$^c$ The standard free energy change for transfer of $i$ between surface and oil phases in Kcal/mole: $\Delta G_{surface to oil} = RT \ln (x_i^s / x_i^o) = \mu_i^o - \mu_i^s$ where $x_i^s / x_i^o$ is the ratio of mole fractions of $i$ in surface and oil and $\mu_i^o$ and $\mu_i^s$ is the standard chemical potential of each.
(From [137])
the values of $K_C$ decreased 3-4 fold (Table 3). It is possible that even more cholesterol would be shifted into the oil phase if the level of cholesteryl oleate in the oil were increased (see earlier discussion and Table 1). Furthermore, phase equilibrium studies should be carried out using lower melting cholesteryl esters which are more soluble in the oil phase [123]. Information of this sort would be useful for prediction of the behavior of cholesterol in cholesterol ester-rich β-VLDL, IDL and LDL.

The compositions of emulsions containing cholesteryl oleate can also be plotted on triangular coordinate phase diagrams after making a few alterations in the way components are treated. Furthermore, the combined solubility of triolein and cholesteryl oleate in the lecithin surface is similar to the maximum solubility of either component alone in lecithin. Therefore, for the purpose of simplifying graphical analysis of these mixtures, we combined the percentages of triolein and cholesteryl oleate and designated them as the nonpolar lipid component, $N$. Component $N$ is then assigned to the left axis of the diagram that formerly was assigned to triolein (see Fig. 4). The percentages of triolein and cholesteryl oleate in each data point were summed for two emulsions, B and F, which are listed in Table 2. Using

![Initial text]

Fig. 6. Plot of compositions of representative emulsions in Table 4. (Inset) oil phase compositions. (a) Total emulsion with isolated oil (O) and surface (S) phases. (b) Plot of individual fractions produced by sonication of emulsions B (B) and F (F) superimposed on Fig. 6a. Fractions 1 - 4 are the creams isolated by sequential centrifugation steps. Fraction 5 is the combined infranatant and resuspended pellet (vesicle) fraction. Symbols: N, TO + CO, L, Lecithin, C, cholesterol; E, emulsion compositions; O, oil and S, surface phase compositions. (From [137])
the values for \(N\), the plots of systems B and F have been made in Fig. 6a. As in the case of triolein-cholesterol-lecithin-water systems, the compositions of the emulsions (E) plot on tie lines which join the compositions of their surface (S) and oil (O) phases.

To illustrate the equilibrium distribution of lipids between individual particles within the emulsion systems, two emulsions with compositions similar to emulsions B and F were sonicated, and the compositions of five different size subfractions prepared by centrifugation were measured (Table 4, emulsions \(B_s\) and \(F_s\)). When the compositions of the subfractions were plotted on a triangular coordinate diagram they were found to lie on the appropriate tie lines (Fig. 6b). This result showed that the theoretical predictions of the way subfractions should plot on phase diagrams (Fig. 5) can in fact be experimentally verified. Note that the subfractionated emulsion droplets were in equilibrium with respect to their core content of triolein and cholesteryl olate since the triolein/cholesterol olate ratios of the subfractions were identical (Table 4) (and nearly all of the particle triolein and cholesteryl olate are in the oil cores). Furthermore because the composition of the subfractionated particles in each specific system (i.e., \(B_s\) or \(F_s\)) fell on the appropriate tie line all the particles of a specific system were also in equilibrium with respect to the surface-to-core and interparticle distribution of cholesterol molecules. Thus all particles in the system had the same respective surface and oil compositions. In contrast, note that the total cholesterol/phospholipid ratios of the subfractions vary (Table 4). As the particles get larger, the C:L ratio increases because the ratio of core to surface phases in the particle increases and the core carries proportionately more of the total cholesterol (Table 4).

The phase diagram can be used to calculate the mean size of the weight average particle in each of the subfractions. Two assumptions must be made to perform these calculations. First, since values for the densities of the lipid components are required in the calculations, we have assumed that the values of the bulk phase densities can be applied to these mixtures*. Second, the thickness of the surface monolayer is assumed to be 20 Å, the approximate length of extended phospholipid acyl chains. Using these assumptions emulsion particle diameters can be calculated as follows. First, the weight fraction densities of the oil and surface phases, \(P_o\) and \(P_s\), are calculated from

\[
P_o = x_{TO_o}P_{TO} + x_{CO_o}P_{CO} + x_{C_o}P_{C}
\]

\[
P_s = x_{TO_s}P_{TO} + x_{CO_s}P_{CO} + x_{C_s}P_{C} + x_LP_L
\]

* Lipid densities (\(p\)) are listed for 23°C from the following sources: \(p\) triolein = 0.913 g/ml [140]; \(p\) cholesteryl olate = 0.96 g/ml [141] \(p\) cholesterol = 1.045 g/ml [142] and \(p\) lecithin = 1.016 g/ml [104].
### TABLE 4

Sonicated emulsion $B_1$ and $F_5$ subfraction compositions and calculated structural parameters

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Sample</th>
<th>TO$^a$</th>
<th>CO</th>
<th>N$^b$</th>
<th>C</th>
<th>L</th>
<th>TO: CO$^c$</th>
<th>C:L$^c$</th>
<th>C:L molar</th>
<th>$M_s/M_o$ $^d$</th>
<th>D$^h$</th>
<th>%C$_s$ $^i$</th>
<th>%C$_c$ $^i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_1$, low C, low CO</td>
<td>E$^e$</td>
<td>75.6</td>
<td>2.3</td>
<td>77.9</td>
<td>2.1</td>
<td>20.0</td>
<td>32.9</td>
<td>0.11</td>
<td>0.22</td>
<td>0.3</td>
<td>530</td>
<td>79.0</td>
<td>21.0</td>
</tr>
<tr>
<td>1$^f$</td>
<td>86.0</td>
<td>2.6</td>
<td>88.6</td>
<td>1.4</td>
<td>9.9</td>
<td>33.1</td>
<td>0.14</td>
<td>0.28</td>
<td>0.12</td>
<td>0.14</td>
<td>1150</td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td>2$^f$</td>
<td>82.9</td>
<td>2.5</td>
<td>85.4</td>
<td>1.7</td>
<td>13.0</td>
<td>33.2</td>
<td>0.13</td>
<td>0.26</td>
<td>0.18</td>
<td>0.18</td>
<td>840</td>
<td>69.2</td>
<td>30.8</td>
</tr>
<tr>
<td>3$^f$</td>
<td>77.4</td>
<td>2.3</td>
<td>79.7</td>
<td>2.0</td>
<td>18.3</td>
<td>33.7</td>
<td>0.11</td>
<td>0.22</td>
<td>0.26</td>
<td>0.26</td>
<td>590</td>
<td>76.5</td>
<td>23.5</td>
</tr>
<tr>
<td>4$^f$</td>
<td>65.7</td>
<td>2.0</td>
<td>67.7</td>
<td>2.7</td>
<td>29.6</td>
<td>32.9</td>
<td>0.11</td>
<td>0.18</td>
<td>0.26</td>
<td>0.26</td>
<td>340</td>
<td>86.2</td>
<td>13.8</td>
</tr>
<tr>
<td>$F_5$, high C, moderate CO</td>
<td>E$^e$</td>
<td>63.5</td>
<td>13.8</td>
<td>77.3</td>
<td>5.7</td>
<td>17.0</td>
<td>4.6</td>
<td>0.34</td>
<td>0.68</td>
<td>0.28</td>
<td>560</td>
<td>75.2</td>
<td>24.8</td>
</tr>
<tr>
<td>1$^f$</td>
<td>71.4</td>
<td>15.9</td>
<td>87.3</td>
<td>4.2</td>
<td>8.6</td>
<td>4.5</td>
<td>0.49</td>
<td>0.98</td>
<td>0.12</td>
<td>0.12</td>
<td>1150</td>
<td>56.5</td>
<td>43.5</td>
</tr>
<tr>
<td>2$^f$</td>
<td>68.3</td>
<td>14.7</td>
<td>83.0</td>
<td>4.8</td>
<td>12.2</td>
<td>4.6</td>
<td>0.39</td>
<td>0.78</td>
<td>0.19</td>
<td>0.19</td>
<td>800</td>
<td>67.3</td>
<td>32.7</td>
</tr>
<tr>
<td>3$^f$</td>
<td>64.5</td>
<td>13.8</td>
<td>78.3</td>
<td>5.7</td>
<td>16.0</td>
<td>4.7</td>
<td>0.36</td>
<td>0.72</td>
<td>0.26</td>
<td>0.26</td>
<td>590</td>
<td>73.8</td>
<td>26.2</td>
</tr>
<tr>
<td>4$^f$</td>
<td>55.0</td>
<td>11.4</td>
<td>66.4</td>
<td>7.5</td>
<td>26.2</td>
<td>4.8</td>
<td>0.29</td>
<td>0.58</td>
<td>0.50</td>
<td>0.50</td>
<td>340</td>
<td>84.4</td>
<td>15.6</td>
</tr>
<tr>
<td>$5^f$</td>
<td>13.5</td>
<td>2.9</td>
<td>16.4</td>
<td>16.9</td>
<td>66.7</td>
<td>4.7</td>
<td>0.25</td>
<td>0.50</td>
<td>6.11</td>
<td>(&lt;100)</td>
<td>98.5</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Weight percent values are listed.

$^b$ Nonpolar lipids, $N = TO + CO$.

$^c$ Lipid weight ratios.

$^d$ Unfractionated emulsion (E) compositions.

$^e$ Subfractions 1–4, isolated by sequential ultracentrifugation steps.

$^f$ The combined infranatant and pelletted vesicle fraction obtained after the final centrifugation, fraction 5.

$^g$ Ratio of surface to core masses.

$^h$ Calculated diameter in Å.

$^i$ Phase distributions of cholesterol.

$^j$ The calculated diameter of fraction 5 is erroneous since these fractions contain a mixture of microemulsion particles and vesicles.

(Compiled from [137])
where $x_{TO_o}$, etc., are the weight fractions of the lipids in the phases, and $p_{TO_o}$, etc., are the densities of each component. The surface/oil volume ratio, $V_s/V_o$, is obtained using knowledge of $P_o$, $P_s$, and $M_s/M_o$ in the equation

$$V_s/V_o = (M_s/M_o) (P_o/P_s).$$

(9)

Then the values of the surface radius, $r_s$, and the oil-surface boundary radius, $r_o$, can be assigned using the equation

$$V_s/V_o = (r_s^3 - r_o^3) (r_o^{-3}).$$

(10)

The second assumption above sets $r_o = r_s - 20 \text{ Å}$. These equations were employed to calculate the sizes of the particles in the subfractions listed in Table 4. The diameters calculated from the phase diagram demonstrate that particles in the range of sizes commonly exhibited by triglyceride-rich lipoproteins can be obtained by sonication.

Finally, the percentages of the total particle cholesterol molecules carried in the two phases of the emulsion droplets were calculated using Eqs. (5) and (6) and have been listed in Table 4. For the large particles, > 40% of their total cholesterol molecules are present in their cores, and this value declines considerably in smaller particles. Across the entire range of particle sizes, the percentages of the total particle triolein and cholesteryl oleate molecules in the surface phase never exceeds 2% of the total for triolein, and 1% of the total for cholesteryl oleate [137].

5. Phase equilibria of chylomicron and VLDL lipids

(a) Compositions of surface and core lipids

Native chylomicrons and particularly native VLDL are inherently stable to coalescence during centrifugation, and their surface and core lipids cannot be separated by the ultracentrifugal forces which are attained during routine preparative centrifugation steps. We therefore extracted the total lipids from these lipoproteins, prepared emulsions from the extracted lipids and broke them in the centrifuge using the conditions described in the previous section. Of course, it is possible that the compositions of the phases obtained using this technique may be slightly different from intact lipoproteins, but the data will show that this is probably not the case.

Human plasma VLDL and monkey lymph chylomicrons were chosen as examples of triglyceride-rich lipoproteins [143]. Samples obtained by centrifugation at $p = 1.006 \text{ g/ml}$ were washed free of adsorbed plasma albumin, and the lipids were extracted by the Folch procedure [144]. The dried lipids were emulsified in water by
overnight agitation at 24°C. Subsequently, the equilibrated surface and core lipids were isolated by centrifuging the samples in capillary tubes as described in Section 4(b), above. The gross physical properties of the emulsions were identical to those of the model systems, i.e., they lacked any evidence of myelin figures or of cholesterol or cholesterol ester crystals.

The compositions of the isolated phases are presented in Table 5. The surface phases consisted mostly of phospholipid and cholesterol, but significant levels of triglyceride (2–4%) and measurable levels of cholesterol ester (0.2–0.4%) were present in them. The relative amounts of cholesterol in the two classes of emulsions differed greatly. The human plasma VLDL surface phase contained on the average 23% cholesterol, while the monkey lymph chylomicron surface contained only 5% cholesterol. Likewise the percentage of cholesterol in the oil lipids was much higher in human VLDL (1–2%) than in the monkey chylomicrons (0.3%). The lymph chylomicron samples were obtained from an animal fed a high fat diet containing safflower oil, and under these conditions the triglyceride fraction contained

| TABLE 5 |
| Chemical compositions of human plasma lipoprotein and safflower oil-fed monkey lymph \( \lambda_p > 20 \) lipoprotein lipid emulsions and their phases |

<table>
<thead>
<tr>
<th>Sample</th>
<th>TG(^a)</th>
<th>CE</th>
<th>N(^b)</th>
<th>FA</th>
<th>DG</th>
<th>C</th>
<th>PL(^c)</th>
<th>P(^d)</th>
<th>C:PL(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>emulsion (E)</td>
<td>64.9</td>
<td>9.0</td>
<td>73.9</td>
<td>-</td>
<td>-</td>
<td>6.4</td>
<td>19.7</td>
<td>19.7</td>
<td>0.32</td>
</tr>
<tr>
<td>oil (O)</td>
<td>85.2</td>
<td>13.3</td>
<td>98.5</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface (S)</td>
<td>4.5</td>
<td>0.4</td>
<td>4.9</td>
<td>-</td>
<td>-</td>
<td>21.1</td>
<td>74.0</td>
<td>74.0</td>
<td>0.28</td>
</tr>
<tr>
<td>Monkey chylomicrons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>emulsion (E)</td>
<td>88.2</td>
<td>1.6</td>
<td>90.3</td>
<td>2.3</td>
<td>0.5</td>
<td>0.9</td>
<td>6.5</td>
<td>8.8</td>
<td>0.14</td>
</tr>
<tr>
<td>oil (O)</td>
<td>95.8</td>
<td>2.6</td>
<td>99.7</td>
<td>1.3</td>
<td>Trace</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface (S)</td>
<td>2.1</td>
<td>0.2</td>
<td>2.8</td>
<td>6.3</td>
<td>0.5</td>
<td>4.7</td>
<td>86.2</td>
<td>92.5</td>
<td>0.05</td>
</tr>
<tr>
<td>( E_c )</td>
<td>51.6</td>
<td>1.3</td>
<td>52.9</td>
<td></td>
<td></td>
<td>29.9</td>
<td>17.1</td>
<td>17.1</td>
<td>1.75</td>
</tr>
<tr>
<td>( O_c )</td>
<td>94.9</td>
<td>2.4</td>
<td>97.3</td>
<td></td>
<td></td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FA, fatty acids; DG, diglycerides; E, emulsion; O, oil; S, surface; \( E_c \), C-saturated emulsion; \( O_c \), C-saturated oil phase isolated from \( E_c \).

\(^a\) Weight percentage values.
\(^b\) Nonpolar lipids: \( N = TG + CE + DG \), for \( E \) and \( S \); \( N = TG + CE + FA \), for \( O \); and \( N = TG + CE \) for \( E_c \) and \( O_c \).
\(^c\) Total percentage PL.
\(^d\) Polar lipids \( P = PL + FA \), for \( E \) and \( S \); and \( P = PL \), for \( E_c \).
\(^e\) Lipid weight ratios.

(From [143])
predominantly mono-unsaturated (12%) and poly-unsaturated (75%) fatty acids, of which the major component was linoleic acid [145]. As shown in Table 5, the small amount of total free fatty acids partitioned into both oil and surface phases of the monkey chylomicron lipids. After adding an excess of cholesterol to the lipids we measured the maximum solubility of cholesterol in emulsions prepared from the extracted monkey chylomicron lipids. A maximum of 2.7% cholesterol was soluble in the oil at 24°C. Thus, the monkey chylomicron oil was a better solvent for cholesterol than was the triolein oil at 24°C. The increased capacity (2.7% vs. 1.9%) of the oil to solubilize cholesterol might be attributable to its content of free fatty acids [27, 131], the triglyceride acyl chain composition, the cholesterol ester content (see above), or cholesterol ester acyl chain composition [123]. The relative importance of each of these parameters has not yet been thoroughly evaluated.

The method of plotting lipoprotein composition data is analogous to that used to plot the compositions of the cholesteryl olate-containing emulsions in Section 4 (see Fig. 6). We grouped triglyceride and cholesterol ester together in the category designated the nonpolar lipids, N. Free fatty acids found in the oil phase and both oil and surface diglyceride were treated as members of the nonpolar lipid class. Fatty acids found in the surface are probably partially ionized [83] and were treated as polar lipids. They were combined along with all classes of phospholipids, e.g., phosphatidylycholine, sphingomyelin, and lysophosphatidylycholine, in the polar lipid category, P. In the literature data presented below in Section 6, for the few cases where free fatty acids or diglycerides were listed in the compositions of triglyceride-rich lipoproteins, fatty acids were arbitrarily included in the polar lipid

![Fig. 7. Composition of emulsions made from human VLDL and monkey chylomicron (CM) lipids. E, emulsion; O, oil; S, surface; N, nonpolar lipids. Note that the VLDL system is much richer in free cholesterol than the CM system. (From [143])]
category and diglycerides were included with the nonpolar lipids. These assignments are generally consistent with the gross surface and bulk properties of these lipids [100] but in fact they probably distribute into both phases (see Table 5). Using these assumptions, the compositions of the emulsified lipoprotein lipids and their isolated oil and surface phases were plotted in Fig. 7. The compositions of the data points for each emulsion determine tie lines as in the case of the simple lipid systems. Again all points on the tie lines are in equilibrium (see Section 5(b) for discussion of native lipoprotein phase behavior). It is obvious that the graphical method facilitates comparison of the cholesterol contents of the phases. The relatively cholesterol-rich plasma VLDL emulsion plots on a tie line well above the tie line for the chylomicron

| TABLE 6 |
| Chemical compositions of human plasma $p < 1.006^\text{g/ml}$ and monkey lymph $S_f > 20$ lipoproteins and their subfractions |

<table>
<thead>
<tr>
<th>Fraction$^a$</th>
<th>Protein</th>
<th>Lipid</th>
<th>TG$^b$</th>
<th>CE</th>
<th>N$^c$</th>
<th>C</th>
<th>PL</th>
<th>TG:CE$^d$</th>
<th>C:PL$^e$</th>
<th>C:PL molar</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human plasma</strong>, $p &lt; 1.006$ (T)$^f$</td>
<td>10.1</td>
<td>89.9</td>
<td>66.8</td>
<td>9.5</td>
<td>76.3</td>
<td>5.8</td>
<td>17.9</td>
<td>7.0</td>
<td>0.32</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Subfractions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.7</td>
<td>97.3</td>
<td>87.3</td>
<td>3.1</td>
<td>90.4</td>
<td>3.5</td>
<td>6.2</td>
<td>28.2</td>
<td>0.56</td>
<td>1.12</td>
</tr>
<tr>
<td>II</td>
<td>7.9</td>
<td>92.1</td>
<td>75.9</td>
<td>5.2</td>
<td>81.1</td>
<td>4.9</td>
<td>14.1</td>
<td>14.6</td>
<td>0.35</td>
<td>0.70</td>
</tr>
<tr>
<td>III</td>
<td>10.1</td>
<td>89.9</td>
<td>67.4</td>
<td>8.4</td>
<td>75.8</td>
<td>5.7</td>
<td>18.5</td>
<td>8.0</td>
<td>0.31</td>
<td>0.62</td>
</tr>
<tr>
<td>IV</td>
<td>10.0</td>
<td>90.0</td>
<td>61.9</td>
<td>11.3</td>
<td>73.2</td>
<td>6.3</td>
<td>20.6</td>
<td>5.5</td>
<td>0.31</td>
<td>0.62</td>
</tr>
<tr>
<td>V</td>
<td>13.1</td>
<td>86.9</td>
<td>58.5</td>
<td>13.1</td>
<td>71.6</td>
<td>6.7</td>
<td>21.8</td>
<td>4.5</td>
<td>0.31</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Monkey lymph</strong>, $S_f &gt; 20$ (T)</td>
<td>92.8</td>
<td>1.8</td>
<td>94.6</td>
<td>0.9</td>
<td>4.5</td>
<td>51.6</td>
<td>0.20</td>
<td>0.40</td>
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<td></td>
</tr>
<tr>
<td><strong>Subfractions</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_f &gt; 3500$ (I)</td>
<td>95.3</td>
<td>1.8</td>
<td>97.1</td>
<td>0.8</td>
<td>2.2</td>
<td>52.9</td>
<td>0.26</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_f = 3500 - 3500$ (II)</td>
<td>87.2</td>
<td>2.1</td>
<td>89.3</td>
<td>1.1</td>
<td>9.5</td>
<td>41.5</td>
<td>0.12</td>
<td>0.24</td>
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</tr>
<tr>
<td>$S_f = 400 - 1500$ (III)</td>
<td>85.5</td>
<td>2.2</td>
<td>87.7</td>
<td>1.1</td>
<td>11.2</td>
<td>38.9</td>
<td>0.10</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_f = 20 - 400$ (IV)</td>
<td>85.2</td>
<td>2.2</td>
<td>87.4</td>
<td>1.2</td>
<td>11.4</td>
<td>38.7</td>
<td>0.10</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Subfractions obtained by gel filtration (human plasma VLDL) and centrifugation (monkey lymph chylomicrons).

$^b$ Percentage, by weight, of the total lipid.

$^c$ Nonpolar lipids; $N = TG + CE$.

$^d$ Lipid weight ratios.

$^e$ $T$, total triglyceride-rich lipoproteins.

(From [143])
sample. When the data in Fig. 7 and Fig. 6 are compared, the behavior of the model systems is shown to closely resemble that of the lipoprotein lipid emulsions.

(b) Equilibration of lipids between lipoprotein subfractions

To assess the state of equilibrium of cholesterol between lipoproteins in the total lipoprotein systems, the compositions of native triglyceride-rich lipoprotein subfractions, obtained by gel filtration of human plasma VLDL and by centrifugation of monkey lymph chylomicrons, were analyzed using phase diagram plots. The lipids

---

**Fig. 8.** Phase diagram analysis of human plasma VLDL and nascent monkey chylomicrons. (a) Human plasma $p < 1.006$ g/ml lipoprotein lipid emulsions and subfractionated intact lipoproteins, (b) monkey intestinal lymph $S_p = 20$ lipoprotein lipid emulsions and subfractionated intact lipoproteins. Points O (oil), E (emulsion), and S (surface) designate the compositions of the emulsified lipids and their isolated phases. Points I–V represent the compositions of native lymph chylomicron and plasma VLDL subfractions of the total (T) triglyceride-rich parent lipoproteins (Table 6). N, nonpolar lipids; P, polar lipids; C, cholesterol. (Taken from [143])
from these same lipoprotein samples were used to make emulsions and to generate
the oil and surface compositions shown in Fig. 7. The composition data are sum-
murized in Table 6. In the Table, the particle sizes of the lipoproteins decrease from
fractions I – V. The lipoprotein triglyceride/cholesterol ester ratios of the fractions
also undergo a marked decline across the fraction numbers, while lipoprotein
cholesterol/phospholipid ratios show a more modest decline. Notice that, in con-
trast to model emulsions containing triolein and cholesteryl oleate in which the par-
ticle triglyceride/cholesterol ester ratios remain constant (Table 4), the smaller
lipoprotein subfractions are richer in cholesterol ester. Thus, the native lipoprotein
fractions are not in equilibrium with respect to the distribution of triglyceride and
cholesterol ester between individual lipoproteins. This further implies that the sur-
face triglyceride/cholesterol ester ratios will decline in smaller particles [114].
However, as indicated from the plots of the native lipoprotein subfraction com-
positions on the diagram in Fig. 8, the subfractions are in a state of surface-to-core and
interparticle equilibrium with respect to the distribution of cholesterol molecules.
We arrived at this conclusion because the native lipoprotein subfraction composi-
tion points fall on tie lines generated by their very emulsion phase compositions (line
OES, Figs. 7, 8). Furthermore, the compositions of the phases obtained from the
lipid emulsions are representative of the phases present in the parent intact lipopro-
teins or the data for the native lipoprotein subfractions would not have plotted on the
the tie lines. In summary, the native lipoprotein subfractions from a given sample
all have the same surface lipid composition (given by point S) and core composition
(given by point 0) on the phase diagram. The total particle cholesterol/phospholipid
ratios vary because the ratio of core/surface masses in the subfractionated lipopro-
teins and the percentage of the total particle cholesterol in each phase are size-
dependent. Note that the protein content in the VLDL fractions varies from
2.7 – 13.1%. Assuming a protein density of 1.34 g/ml and that it is all in the surface,
it must occupy about 20 – 25% of the surface volume. However, it appears that the
protein content of these particles does not significantly alter the surface lipid com-
position. If protein either bound cholesterol or pushed it out of the surface, the
compositions (at equilibrium) would be respectively above or below the tie line in
Fig. 8.

Since the publication of our original paper [143] we have analyzed the composi-
tions of subfractionated lipoproteins obtained from other literature sources. The
data points were plotted on phase diagrams (Fig. 9) and best-fit 'tie' lines were
drawn through the compositions to see if they behaved similarly to the samples just
described. If the line was straight a rough $K_C$ was calculated from the intercepts on
the phase diagram. For the samples of nascent triglyceride-rich lipoproteins (Fig.
9a – c) and the monkey chylomicrons in Fig. 8b, an average $K_C$ of 25 was obtained.
For the plasma lipoproteins plotted in Fig. 9d – f and Fig. 8a, the average $K_C$ was
17. All of the particles were separated and analyzed at room temperature or cooler
temperatures. From the study of cholesterol ester-containing emulsions, the value
Fig. 9. Demonstration of the equilibration of cholesterol between triglyceride-rich lipoprotein subfractions. The composition of lipoprotein fractions are plotted on the left and estimated surface compositions on the right. (a) Human chylomic urine $p < 1.006$ chylomicrons (CM) and VLDL [70]. (b) Dog lymph chylomicrons from corn oil (•) and cream-fed (o) dogs [84]. Particle sizes: 1, > 200 nm; 2, 200–140 nm; 3, < 140 nm. (c) Rat liver perfusate $p < 1.006$ g/ml (total), $p = 1.006–1.02$ g/ml, and $p = 1.02–1.063$ g/ml lipoproteins [53]. (d) Rat plasma VLDL from fructose-fed rats [64]. Total VLDL fraction and 1–4 subfractions. (e) Human plasma $S_p > 20$ lipoproteins [146]. Total $p < 1.006$ g/ml fraction and 1–5 subfractions. (f) Human VLDL subfractions [86]. Note: different scales on figure edges; for explanation, see text and Table 7.

of $K_C$ was shown to change depending on the cholesterol ester content of the particle (Table 3). It decreases abruptly with the addition of a few percent cholesterol ester and then probably decreases very gradually on further increase of cholesterol ester content. It may also change slightly with different triglyceride or cholesterol esters. Finally $K_C$ decreases of course with increasing temperature, since this increases oil solubility disproportionately. Our best estimate at present is that in lipoproteins with < 3% cholesterol ester, the value for the cholesterol phase distribution ratio at 37°C should be approximately 22, i.e., $K_C = 22$. For lipoproteins with 3–25% cholesterol ester the value is probably more like $K_C = 11$. As noted in Table 1, the $K_C$ for very cholesterol ester-rich particles is as low as 6–9.
For some of the literature data the percentage of cholesterol ester in a given set of fractionated lipoproteins was < 3% in large particles but > 3% in small particles. To avoid possible inconsistency in the calculation of the phase compositions, a single value of $K_C$ was assumed for all subfractions that was most representative of the greater number of subfractions, i.e., if only the largest subfraction had < 3% cholesterol ester, the value of $K_C = 11$ was chosen since the majority of the subfractions had > 3% cholesterol ester. Only a few cases were encountered for which this adjustment had to be made. In fact, the finding that subfractions generally plotted on the same tie line even though the subfractions varied in their cholesterol ester contents, indicates that the application of a single $K_C$ to all of the subfractions is justified. Having determined the values of $K_C$ by the graphical analysis of the data in Figs. 8 and 9, and then after adjusting the $K_C$ value for the systems at body temperature, i.e., 37°C, we used the $K_C$ values of 11 and 22 in a computer program (see Appendix) to calculate the compositions of the lipoprotein surface and core regions.

The compositions of subfractions of human chyluric urine $p < 1.006$ g/ml lipoproteins [70] and dog intestinal lymph chylomicrons [84] are plotted in Fig. 9a and b. The human intestinal triglyceride-rich lipoproteins were obtained from the urine of chyluric patients fed a corn oil meal. Lymphatic chylomicrons enter the bladders of these patients through mesenteric lymphatic-urinary fistulae which formed as a result of filarial infection. As shown on the diagrams, the compositions of the intestinal lipoproteins plot on a tie line indicating that they are in equilibrium with respect to cholesterol distribution. The surface and core cholesterol contents were calculated for both examples using $K_C = 22$. In the case of the human lymph VLDL sample, the cholesterol ester content exceeded 3%, but since chylomicrons contributed the bulk of the lipid mass in the total system, the value of $K_C = 22$ was taken to be a more representative value for calculation of the weight fractions of cholesterol in the lipoprotein phases. With this adjustment, the calculated surface cholesterol content was 8 - 10% and the oil cholesterol was 0.36 - 0.46% (Table 7). The dog lymph chylomicrons had average surface cholesterol contents of 9% (cream-fed) and 7% (corn oil fed), and oil cholesterol contents of 0.4% and 0.3%, respectively (Table 7). We noted earlier [143] that monkey chylomicrons obtained from butter fat fed monkeys appeared to have slightly greater total cholesterol content (0.5%) than those from safflower oil fed monkeys (0.4%). Presently not enough data have been collected to determine whether diet-related differences are actually significant. It is intriguing to speculate that cholesterol absorption and hence chylomicron cholesterol content are influenced by the degree of saturation of the dietary fatty acids since cholesterol is more soluble in saturated than polyunsaturated oils [27, 130]. However, the role that this difference may play in cholesterol absorption is controversial.

Several other examples of subfractionated lipoproteins were examined using phase diagrams (Fig. 9c-f) and the weight fraction solubilities of cholesterol in
TABLE 7
Phase behavior of subfractionated plasma and lymph triglyceride-rich lipoproteins

<table>
<thead>
<tr>
<th>Source</th>
<th>Ref.</th>
<th>$K_C$</th>
<th>Sub-</th>
<th>$x_{cs}$</th>
<th>$x_{co}$</th>
<th>%$C_o$</th>
<th>%$C_s$</th>
</tr>
</thead>
<tbody>
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<td>a. Human urine CM</td>
<td>[70]</td>
<td>22</td>
<td>1</td>
<td>0.079</td>
<td>0.0036</td>
<td>38</td>
<td>62</td>
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<td></td>
<td></td>
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<td>2</td>
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<td>0.0046</td>
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<td>85</td>
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<td>b. Dog lymph CM (cream)</td>
<td>[84]</td>
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<td>1</td>
<td>0.075</td>
<td>0.0034</td>
<td>51</td>
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<td>3</td>
<td>0.107</td>
<td>0.0049</td>
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<tr>
<td>Dog lymph CM (corn oil)</td>
<td>[84]</td>
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<td>0.003</td>
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<td>0.071</td>
<td>0.0032</td>
<td>27</td>
<td>73</td>
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<td>c. Rat nascent VLDL</td>
<td>[53]</td>
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<td>total</td>
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<td>0.0018</td>
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<td>95</td>
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<tr>
<td></td>
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<td></td>
<td>1.06 - 1.02</td>
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<td>96</td>
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<td>1.02 - 1.063</td>
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<td>98</td>
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<td>d. Rat plasma VLDL</td>
<td>[64]</td>
<td>11</td>
<td>total</td>
<td>0.147</td>
<td>0.013</td>
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<td>4</td>
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<td>72</td>
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<td>e. Human plasma $S_p &gt; 20$</td>
<td>[146]</td>
<td>11</td>
<td>total</td>
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<td>0.021</td>
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<td>5</td>
<td>0.196</td>
<td>0.018</td>
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<td>72</td>
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<tr>
<td>f. Human plasma VLDL</td>
<td>[86]</td>
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<td>0.187</td>
<td>0.017</td>
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<td>57</td>
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<tr>
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<td></td>
<td></td>
<td>4</td>
<td>0.197</td>
<td>0.018</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>5</td>
<td>0.204</td>
<td>0.018</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>0.207</td>
<td>0.019</td>
<td>18</td>
<td>82</td>
</tr>
</tbody>
</table>

$a$ CM, chylomicrons.

$b$ $K_C$, phase distribution ratio for cholesterol, Eq. (3).

c Total, parent triglyceride-rich lipoprotein, and 1 - 6, size subfractions decreasing in size from 1 - 6.

d $x_{cs}$, weight fraction of cholesterol in the surface lipids.

e $x_{co}$, weight fraction of cholesterol in the oil lipids.

f $\%C_o$, percentage of the total particle cholesterol molecules in the core.

$eC_s$, percentage of the total particle cholesterol molecules in the surface.
their surface, \( x_{35} \), and oil, \( x_{co} \), phases are listed in Table 7. Examination of the plots in Figs. 8 and 9 reveals that surface-to-core and interparticle equilibration of cholesterol appears to occur in all of the subfractionated VLDL and chylomicron samples. In addition, each lipoprotein in the system has approximately the same weight fraction of cholesterol in its respective surface and core phases. Presently it is unknown whether the equilibration of cholesterol between subfractions occurred while the lipoproteins were in the circulation or whether it occurred during and after isolation procedures. However, it will be shown that plasma lipoprotein and red blood cell membrane lipid pools probably are not completely equilibrated at the time of lipoprotein isolation.

As in the case of model emulsion systems, the percentage phase distribution of cholesterol and other lipids between the lipoprotein phases can be calculated from the phase diagram plots. Since the weight fraction of nonpolar lipids in the surface lipids was 0.03 – 0.04 for the four VLDL and chylomicron lipid emulsions that were examined [143] and also was consistently in this range for the model systems, we have assumed that this value is observed for the majority of triglyceride-rich lipoproteins. It is necessary to make this assumption so that a surface phase boundary line can be drawn on the lipoprotein phase diagram plots. The position of this line is required for calculation of tie line segment lengths (Eq. 1) in the series of equations used to calculate \( \% C_o \) and \( \% C_o \) values (Section 4).

The values for the \( \% C_o \) and \( \% C_o \) in the examples plotted in Fig. 9 are listed in Table 7. For large chylomicrons, > 50% of the cholesterol is carried in their cores. For small chylomicrons and VLDL size ranges, 10 – 30% of the total particle cholesterol is in the core. The extremely small rat hepatic nascent VLDL [53] have very little cholesterol in their cores. In summary, the core phases in some cases play a major role in the transport of cholesterol in triglyceride-rich lipoproteins, particularly since large particles contain the bulk of the lipid mass in the total lipoprotein system. For comparison, most of the cholesterol in LDL is carried in the surface phase (\( \% C_o < 15\% \)) [120].

Lipoprotein particle diameters can also be calculated from the phase diagrams. The method is similar to that used to calculate emulsion particle diameters. First, the percentages of each component (protein included) in the total particle are expressed in weight fraction units, \( x_{i,t} \), of the total mass. Then the weight fraction units are multiplied by the percentages of the total particle components in the surface and oil phases, \( \% i_s \) and \( \% i_o \), to obtain the weight fractions of the total particle mass (for each component) in the surface, \( w_{is} \), and oil, \( w_{io} \), phases

\[
\begin{align*}
  w_{is} & = x_{i,t} (\% i_s /100) \\
  w_{io} & = x_{i,t} (\% i_o /100).
\end{align*}
\]
All of the protein and phospholipid molecules are assumed to be located in the surface region, and thus

\[ w_{\text{PROTS}} = x_{\text{PROTT}} \]
\[ w_{\text{PLs}} = x_{\text{PLt}} \]

The values for the total particle weight fraction of each component in a given phase are multiplied by their respective partial specific volumes, \( \nu_i \), and are summed for each phase to obtain the unit phase volumes, \( \nu_{\text{ot}} \) and \( \nu_{\text{st}} \)

\[ \nu_{\text{ot}} = \sum w_{i\text{ot}} \nu_i \]
\[ \nu_{\text{st}} = \sum w_{i\text{st}} \nu_i \]

Then Eq. (10) is used to calculate \( r_i \) and particle diameters, again assuming the surface coat thickness is 20 Å. This method of diameter calculation [143] is more rigorous than that in which protein, phospholipid and cholesterol are assumed to be in the surface and triglyceride and cholesterol are assumed to be in the core [76]. However, the two methods give essentially the same diameters for small lipoproteins because most of the cholesterol molecules in small particles are in the surface. In large particles, the two methods give values which may differ by 5–15%, since a large percentage of the total cholesterol molecules are present in the cores of these lipoproteins.

Once the value of the fractional molecular volume of a given component in a phase is calculated, e.g., \( \nu_{i\text{ot}} = w_{i\text{ot}} \nu_i \), the number of molecules of that component in each region of the lipoprotein can be calculated from the particle diameter assuming molecular volumes of triglyceride = 1610 Å³, cholesterol ester = 1126 Å³, cholesterol = 642 Å³ and phospholipid = 1260 Å³ [86, 111, 123, 143]. Thus the total molecular composition of a lipoprotein can be estimated using phase diagram plots and a few assumptions. As more information becomes available concerning the molecular volumes of components in mixtures and the precise locations of apoproteins within the surface, it will be possible to refine the above calculations.

(c) Phase compositions obtained by other methods

Before moving on to discussion of the lipid composition changes which occur during metabolism, we thought it of interest to present a comparison of our results for the measurement of phase compositions with those appearing in earlier publications. The compositions of dog lymph chylomicron surface 'membrane' and oil core phases obtained by freeze-thaw and dehydration techniques [26, 65] are listed in Table 8. The data that have been analyzed represent samples of the results obtained
when subjects or animals were fed corn oil fat loads. The surface lipids contained from 5 - 43% triglyceride depending on the sample and the method of isolation. It is clear that the technique of freeze-thawing augmented the content of the putative surface triglyceride above the level obtained using rotary evaporation at 24°C. Furthermore, freezing increased the relative fraction of saturated triglyceride species in the membrane by 3 - 4-fold [26]. Otherwise the older data are quite consistent with data obtained by recovery of lipid phases from centrifuged lipid emulsions (Table 5). Notably the cholesterol/phospholipid ratios of the surface phases obtained by the freeze-thaw or rotary evaporation techniques (0.04 - 0.12) compare with that of the surface of monkey chylomicrons (0.05) (Table 5). Thus, these early papers indicated that the surface cholesterol/phospholipid ratios are typically less than those for the total lipoprotein particle (compare also C/PL ratios in Table 5 with subfrac-

### Table 8

Compositions of oil and surface phases obtained by freeze-thaw or dehydration of chylomicrons

<table>
<thead>
<tr>
<th>Source</th>
<th>Ref.</th>
<th>Technique</th>
<th>Sample</th>
<th>TG (mg/g)</th>
<th>CE (%)</th>
<th>C (mg/g)</th>
<th>PL (mg/g)</th>
<th>C/PL</th>
<th>C/PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>[27]</td>
<td>F-T</td>
<td>CM</td>
<td>96.5</td>
<td>0.1</td>
<td>0.5</td>
<td>2.8</td>
<td>0.18</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>43.0</td>
<td>4.8</td>
<td>52.8</td>
<td>0.09</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Dog No. 9</td>
<td>[26]</td>
<td>F-T</td>
<td>CM</td>
<td>94.7</td>
<td>0.2</td>
<td>0.6</td>
<td>4.3</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O</td>
<td>99.3</td>
<td>0.25</td>
<td>0.26</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>25.2</td>
<td></td>
<td>6.9</td>
<td>67.9</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>Dog No. 24</td>
<td>[26]</td>
<td>F-T</td>
<td>CM</td>
<td>95.4</td>
<td>0.2</td>
<td>0.66</td>
<td>3.5</td>
<td>0.19</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O</td>
<td>99.3</td>
<td>0.3</td>
<td>0.19</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>16.1</td>
<td>8.7</td>
<td>75.3</td>
<td>0.12</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>[26]</td>
<td>F-T</td>
<td>CM</td>
<td>95.1</td>
<td>0.06</td>
<td>0.25</td>
<td>4.6</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>10.5</td>
<td>3.0</td>
<td>86.5</td>
<td>0.03</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Dog No. 46</td>
<td>[65]</td>
<td>F-T</td>
<td>M</td>
<td>19</td>
<td></td>
<td>7.0</td>
<td>74</td>
<td>0.09</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RE (25°C)</td>
<td>M</td>
<td>5</td>
<td>3.4</td>
<td>92</td>
<td>0.04</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Dog No. 50</td>
<td>[65]</td>
<td>F-T</td>
<td>M</td>
<td>16.8</td>
<td>4.9</td>
<td>78</td>
<td>0.06</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RE (25°C)</td>
<td>M</td>
<td>11.8</td>
<td>3.0</td>
<td>85</td>
<td>0.04</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

*a F-T, freeze-thaw denaturation; RE, denaturation by rotary evaporation of water.
*b CM, parent chylomicrons; O, oil; M, membrane (surface phase).
*c Compositions are given in weight percent values.
*d Cholesterol/phospholipid weight ratio.
*e Mole ratio.
tion C/PL ratios in Table 6). Furthermore, the weight fractions of cholesterol which were detected in the core lipids (0.002 - 0.003) are also similar to that observed in monkey oil lipids (Table 5). The authors concluded that 25 - 35% of the unesterified cholesterol was in the core of chylomicrons [26, 65]. While these values are too small (40 - 60% would be a more accurate estimate) and large variations in surface triglyceride were found, the qualitative conclusions of these early studies were correct.

6. Phase diagram analysis of triglyceride-rich lipoprotein metabolism

(a) The interpretation of changes in relative lipid composition as plotted on the phase diagram

When nascent triglyceride-rich particles enter lymph or plasma and are subsequently acted on by lipoprotein lipase, hepatic lipase and the different lipid transfer proteins, their composition changes. These changes may be illustrated graphically on triangular coordinate diagrams. However, to interpret these changes some understanding of triangular coordinates is necessary. Using the nonpolar lipid (N) (triglyceride + cholesterol ester)-phospholipid (P)-cholesterol (C) phase diagram with a designated distribution ratio for cholesterol $K_C = 11$, consider a hypothetical lipoprotein having a composition given by the point A (Fig. 10). The changes in the relative composition which occur while the lipoprotein particle undergoes various physical and enzymatically catalyzed lipid changes can be described by three lines extending through the particle composition to the three apices, NAX, PAY and CAZ. Any compositions falling on NAX will have varying amounts of nonpolar lipids (N) and the same fixed ratio of phospholipid and cholesterol (point X). Likewise any composition falling on CAZ will have varying C but the same N/P ratio (point Z). Now if the composition of the lipoprotein changes in such a way that the resulting new composition moves away from the nonpolar lipid apex (N) along the line AX, then the interpretation is that there has been a relative decrease in the nonpolar lipid (e.g. triglyceride) content of the particle without changing the ratio of cholesterol and phospholipid. This could occur as a result of: (1) net removal of triglyceride, (2) the net addition of the exact ratio of cholesterol and phospholipid (point X) to the lipoprotein. Singular triglyceride hydrolysis leading to a net decrease in triglyceride without changing the cholesterol-phospholipid composition would follow line AX. Furthermore, the composition could change by moving either towards or away from the phospholipid apex along PAY. The implications of this are similar, if it moves towards the phospholipid apex there is a relative increase in phospholipid caused either by a net increase of phospholipid or a net decrease in the exact ratio of cholesterol and nonpolar lipid indicated by the point Y. Conversely, if the composition moves away from
phospholipid there is either a net loss of phospholipid from the particle or the addition of cholesterol and neutral lipid of an exact composition given by point Y. Finally, if the composition moves towards the free cholesterol apex along line AC, there is a relative increase in free cholesterol, brought about either by net addition of cholesterol or net subtraction of nonpolar lipid and phospholipid with a ratio given by point Z. In general, the most logical interpretation of these lines is that a specific molecule has either been added or removed rather than a specific combination indicated by points X, Y, Z added or subtracted. We should emphasize here that these plots only show the relative changes in the particle and do not give net changes. Net

![Figure 10](image)

Fig. 10. Interpretation of compositional changes on triangular coordinates. The fine lines in the lower part of the diagram represent tie lines running from the composition of the oil (near the N apex) to the composition of the surface (at the right hand side). These lines are drawn for a surface to core distribution ratio for cholesterol \( C_{11} \) equal to 11. Consider a point with the composition A at about 80% nonpolar lipid, 5% cholesterol, and 15% phospholipid. The three lines drawn through A (NAX, PAY, and CAZ) represent the direction the composition would take if a single component of the system, for instance N or P or C was added or subtracted from A. For instance, if the composition at A changes along line NAX towards X, the direction of the arrow, then N is being removed from composition A but the composition of the other two components remains constant at point X. If A moves towards C along line ZAC then C is being added to A and the ratio of N to P (point Z) remains constant. In a similar fashion if P is removed from composition A along line PAY then the composition moves from A towards Y. If a compositional change involves more than one of these processes, for instance if the composition moves from point A to point B, then the compositional change may be interpreted as the sum of two vectors one along AX and the other along AC as shown by thin dashed lines in the Fig. Such vectorial changes can be quantitated geometrically. See text for further explanation.
changes must be made by measuring the total mass changes within a given mass of lipoprotein. Note, if only nonpolar lipid, e.g., triglyceride, is removed from our hypothetical particle, as the composition moves away from the apex along AX it crosses tie lines such that the particle and its surface and core phases become relatively enriched in free cholesterol.

If a lipoprotein at point A acquires a new composition (e.g., B, Fig. 10) which does not lie on one of the three lines just discussed, then the new line connecting the starting and final compositions may be considered as a vector sum of two or more processes. For instance, line AB (bold dashed line) is the vector sum of segments lying on AX and AC (dotted lines). As we will discuss later, the formation of remnants from chylomicrons results from several physical and enzymatic alterations which can be described vectorally. With this introduction in mind, we will now use the phase diagram to analyze the metabolism of triglyceride-rich lipoproteins.

(b) Increase in cholesterol content

Comparison of the phase compositions of nascent lymph chylomicrons and plasma VLDL revealed that VLDL contain much greater weight fractions of cholesterol in their core and surface phases (Section 5). We wished to determine if this difference held for a much broader group of examples, so compositions of nascent and plasma triglyceride-rich lipoproteins from a number of different animal and human sources were compared by plotting the compositional data on phase diagrams. Data were

Fig. 11. (a) Phase composition of nascent triglyceride-rich lipoproteins from several species. The points correspond to the lipoproteins listed in Table 9. The best fit line through these compositions gives a surface composition of 8% cholesterol, 2% nonpolar lipid, and 88% phospholipid. Points from [143]. For further explanation see text and Table 9. (b) Composition of intracellular membranes which are thought to be involved in the synthesis of nascent lipoproteins (Mic, microsomes; GM, Golgi membranes; GC, Golgi complex, taken from [42]). The compositions of these membranes are rather similar, at least in cholesterol content to the surface of the nascent lipoprotein (see point S in a above).
also analyzed by computer to calculate phase compositions (see Appendix). Calculations took into account the cholesterol ester content of the lipoproteins and thus $K_C$ was assigned at either 22 (CE < 3%) or 11 (CE > 3%).

As shown in Fig. 11a and Table 9 all examples of chylomicrons and nascent VLDL from animals fed normal diets and isolated under conditions which did not expose them to cholesterol-rich sources such as plasma or erythrocytes had low levels of cholesterol in their phases. The average cholesterol content of nascent

<table>
<thead>
<tr>
<th>Data point</th>
<th>Source</th>
<th>$K_C$</th>
<th>$x_{co}$</th>
<th>$x_{cs}$</th>
<th>Surf C/PL</th>
<th>Mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>dog, intestinal lymph CM [26]</td>
<td>22</td>
<td>0.003</td>
<td>0.07</td>
<td>0.080</td>
<td>0.16</td>
</tr>
<tr>
<td>2.</td>
<td>monkey, Table 6 [143] total CM fraction</td>
<td>22</td>
<td>0.003</td>
<td>0.05</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>3.</td>
<td>rat, intestinal lymph CM, fat-fed [68]</td>
<td>22</td>
<td>0.002</td>
<td>0.04</td>
<td>0.043</td>
<td>0.09</td>
</tr>
<tr>
<td>4.</td>
<td>human, chylous urinary CM [70]</td>
<td>22</td>
<td>0.004</td>
<td>0.08</td>
<td>0.088</td>
<td>0.18</td>
</tr>
<tr>
<td>5.</td>
<td>human, intestinal lymph CM [147]</td>
<td>22</td>
<td>0.006</td>
<td>0.12</td>
<td>0.143</td>
<td>0.29</td>
</tr>
<tr>
<td>6.</td>
<td>rat, intestinal lymph CM [76]</td>
<td>22</td>
<td>0.003</td>
<td>0.07</td>
<td>0.073</td>
<td>0.15</td>
</tr>
<tr>
<td>7.</td>
<td>rabbit, intestinal lymph CM [87]</td>
<td>22</td>
<td>0.004</td>
<td>0.09</td>
<td>0.106</td>
<td>0.21</td>
</tr>
<tr>
<td>8.</td>
<td>human, chylous urinary VLDL [70]</td>
<td>11</td>
<td>0.008</td>
<td>0.09</td>
<td>0.102</td>
<td>0.20</td>
</tr>
<tr>
<td>9.</td>
<td>rat, intestinal lymph CM, glucose-fed [68]</td>
<td>11</td>
<td>0.006</td>
<td>0.07</td>
<td>0.076</td>
<td>0.15</td>
</tr>
<tr>
<td>10.</td>
<td>rat, liver Golgi VLDL [45]</td>
<td>11</td>
<td>0.005</td>
<td>0.06</td>
<td>0.061</td>
<td>0.12</td>
</tr>
<tr>
<td>11.</td>
<td>rat liver Golgi VLDL [41]</td>
<td>11</td>
<td>0.009</td>
<td>0.10</td>
<td>0.113</td>
<td>0.23</td>
</tr>
<tr>
<td>12.</td>
<td>rat, liver perfusate VLDL [53]</td>
<td>22</td>
<td>0.002</td>
<td>0.04</td>
<td>0.043</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Refer to Fig. 11a.
* Value of $K_C$ used in computer calculations.
* $x_{co}$, weight fraction of cholesterol in the oil.
* $x_{cs}$, weight fraction of cholesterol in the surface.
* Surf C/PL, surface cholesterol/phospholipid weight ratio.
* Mole ratio, surface cholesterol/phospholipid molar ratio.
lipoprotein surface regions was 8%, and values fell within a range of 4–12%. The content of cholesterol in the oil was only 0.2–0.9%. Graphical analysis reveals strikingly that particles with widely different total unesterified cholesterol contents e.g. dog chylomicrons, 0.2%, and rat perfusate VLDL, 5%, essentially plot on the same tie line. We wish to emphasize that this method of analysis clearly shows the similarity of the surface lipid compositions of these lipoproteins, a fact which is not always apparent from consideration of only their total particle compositions.

We have also plotted (Fig. 11b) the compositions of several cellular membrane fractions through which nascent lipoproteins transit on their way to the extracellular environment [42]. These compositions lie close to the $P$ axis of the diagrams since they contain minor amounts of nonpolar lipids and cholesterol. For comparison, the tie line on which the compositions of nascent triglyceride-rich lipoproteins plot has been drawn to show where it intersects the right-hand side of the phase diagram. The cholesterol content of the intracellular membranes are all quite low and fall near the intersection of the nascent lipoprotein tie line with the surface phase boundary. The cholesterol/phospholipid ratios of the ER membrane (0.05) and Golgi membrane (0.07) are nearly identical to the surface cholesterol/phospholipid ratio of nascent chylomicrons and VLDL (Table 9). In contrast, the cholesterol content of liver membranes, which are partially exposed to the bloodstream, are greater (cholesterol/phospholipid = 0.16, not shown) than those of intracellular membranes. Thus, we suggest that nascent lipoprotein particles are in equilibrium with the intracellular membranes from which they are formed and secreted. This could be anticipated a priori since the lipoproteins are probably assembled from the same pool of lipids which are channeled into ER membrane synthesis. Thus triglyceride-rich lipoproteins have no intrinsic ability to collect cholesterol at the time of their synthesis, and their phase compositions are more or less dictated by the supply of lipids in the internal cellular membranes. In this regard, comparison of the

Fig. 12. Composition of plasma VLDL from several species. The composition of plasma VLDL is plotted in points 1–12. The source, species, and specific surface and core compositions are given in Table 10. The best fit line for the human VLDL is given by the line OS. For comparison, the phospholipid-cholesterol compositions of human and rat red blood cells (rbc) (taken from [42]) are also plotted.
phospholipid species of nascent lipoproteins with circulating lipoproteins [63] reveals that the nascent lipoprotein phospholipids are significantly enriched in non-
choline phospholipid species, which are major components of cellular membranes
[108]. Furthermore, the intracellular membranes that are involved in synthesis of
nonpolar lipids should contain up to a maximum of 3–4% triglyceride and
cholesterol ester. Detectable amounts of these lipids are in fact in some intracellular
membranes [42, 148].

The compositions of ‘normal’ plasma VLDL from eight species are plotted in Fig.
12 and the weight fractions of cholesterol in their phases are listed in Table 10. The
phase compositions of plasma VLDL exhibited much more variation than did those
of nascent lipoproteins. For example, as little as 5% cholesterol was present in the
surface of swine VLDL and 23% cholesterol was in the surface of one human VLDL
sample. Human VLDL consistently exhibited the highest phase concentrations of
cholesterol of any species examined (see Table 10). In humans, apparently a large
amount of cholesterol is transferred from the relatively large plasma lipoprotein and
erthrocyte membrane pools into nascent VLDL during its long residence in the
plasma. However, it would be useful to analyze data for human hepatic nascent
VLDL to confirm that they have surface cholesterol contents which are similar to
other nascent lipoproteins plotted in Fig. 11.

In an attempt to relate the phase compositions of VLDL to that of the

### TABLE 10

Cholesterol content of plasma VLDL core and surface lipids

<table>
<thead>
<tr>
<th>Data point</th>
<th>Source</th>
<th>( K_c )</th>
<th>( x_{co} )</th>
<th>( x_{cs} )</th>
<th>Surf C/PL</th>
<th>C/PL mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. rat (64)</td>
<td>11</td>
<td>0.012</td>
<td>0.14</td>
<td>0.164</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2. human (146)</td>
<td>11</td>
<td>0.021</td>
<td>0.23</td>
<td>0.296</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>3. dog (3)</td>
<td>11</td>
<td>0.012</td>
<td>0.13</td>
<td>0.154</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>4. human (149)</td>
<td>11</td>
<td>0.016</td>
<td>0.18</td>
<td>0.226</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>5. rabbit (3)</td>
<td>11</td>
<td>0.017</td>
<td>0.19</td>
<td>0.238</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>6. human (143)</td>
<td>11</td>
<td>0.016</td>
<td>0.18</td>
<td>0.219</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>7. chicken (3)</td>
<td>11</td>
<td>0.017</td>
<td>0.19</td>
<td>0.232</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>8. human (150)</td>
<td>11</td>
<td>0.017</td>
<td>0.19</td>
<td>0.238</td>
<td>0.48</td>
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</tr>
<tr>
<td>9. swine (151)</td>
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<td>0.05</td>
<td>0.054</td>
<td>0.11</td>
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<tr>
<td>10. turkey (3)</td>
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<td>11. rat (41)</td>
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<td>0.207</td>
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<tr>
<td>12. bovine (3)</td>
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<td>0.013</td>
<td>0.14</td>
<td>0.170</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

* Refer to Fig. 12.
| Same as in Table 9. |
erythrocyte, we have also plotted the compositions of human and rat erythrocyte membranes [42] on the phase diagram in Fig. 12. Presently, we cannot directly compare data for VLDL and erythrocytes from the same source for more than a few cases, but suspect that the following conclusions would be supported. As shown by the graph, the cholesterol contents of the erythrocyte membranes are consistently higher than surface lipids of nascent lipoproteins (Fig. 11) but only marginally higher than the surface lipids of plasma VLDL of humans. Since plasma VLDL surface cholesterol levels are intermediate between those of nascent lipoproteins and erythrocytes, it appears that VLDL begins to equilibrate with erythrocytes after entering the circulation but perhaps does not achieve complete equilibration before isolation. Thus, in disease conditions, such as Type III or V hyperlipidemia, in which the clearance of triglyceride-rich lipoproteins is impaired, IDL, VLDL and chylomicrons may equilibrate to greater extents with the blood components and may have measurably higher contents of cholesterol in their phases (but see Section 6d).

(c) Lipid transfer/exchange reactions

In vitro studies of transfer of cholesterol between triglyceride-rich lipoproteins and plasma lipoproteins [63, 152] or red blood cells [64] have been analyzed on phase diagrams to obtain information about the mechanisms of cholesterol transfer. Samples of dog lymph chylomicrons from dogs fed cream were incubated with varying proportions of dog serum at 37°C [63]. It may be important to note that the chylomicrons were stored at 4°C before use in the incubations. During the time period of incubation, chylomicrons showed progressive increases in their unesterified cholesterol content and losses of surface phospholipids. The extent of change in the chylomicron compositions were related to the relative proportions of the incubated serum and chylomicrons, i.e., when a small mass of chylomicrons was incubated with serum, the extents of transfer of cholesterol into and phospholipid out of chylomicrons were greater than when a large mass of chylomicrons were present in the incubation mixtures.

Since no total chylomicron composition was reported in the original paper [63], we have selected as representative of the incubated chylomicrons, the composition of the dog chylomicrons given in a later paper from the same laboratory [84]. The dogs used in the two studies were fed similar loads of cream following 12–16 hour fasts. The data for the relative masses of chylomicron subfractions [84] were used to calculate the weight average lipid composition of the total lipoprotein fraction — 93.8% triglyceride, 0.6% cholesterol ester, 0.7% cholesterol, and 5.0% phospholipid. Assuming this total composition, the compositions of the particle during the time course of incubation were calculated and are plotted in Fig. 13a. No triglyceride was lost or gained during the incubations.

The data analysis in the original paper indicated that chylomicrons rapidly lost phospholipids and gained cholesterol slowly during the incubation. This result is
TABLE 11

Calculated change in phase cholesterol contents of dog chylomicrons incubated with serum

<table>
<thead>
<tr>
<th>Timea (min)</th>
<th>(10/90)b</th>
<th>(30/70)</th>
<th>(50/50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$x_{cs}$</td>
<td>$x_{co}$</td>
<td>$x_{cs}$</td>
</tr>
<tr>
<td>0</td>
<td>0.08</td>
<td>0.0038</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
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<td>0.0057</td>
<td>0.10</td>
</tr>
<tr>
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<td>0.14</td>
<td>0.0064</td>
<td>0.12</td>
</tr>
<tr>
<td>60</td>
<td>0.14</td>
<td>0.0064</td>
<td>0.12</td>
</tr>
<tr>
<td>120</td>
<td>0.18</td>
<td>0.0081</td>
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<tr>
<td>180</td>
<td>0.21</td>
<td>0.0096</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Fold change, 180 2.5 2.0 1.7

a Ratio of chylomicron to serum lipid mass in the incubation.

b Total elapsed time of incubation in minutes.

c $x_{so}$ and $x_{co}$ are the weight fractions of cholesterol in the surface and oil phases calculated using $K_C = 22$ in all cases.

(From [61] and [84])

readily apparent from the examination of the graphs. The compositions initially moved markedly away from the polar lipid apex of the figure and then more slowly towards the cholesterol apex in a series of steps. Probably phospholipid transfer occurs rapidly when apoproteins are transferred between chylomicrons and plasma lipoproteins. The majority of cholesterol influx into the nascent particle occurs after the rapid phase of phospholipid transfer is complete.

As the compositions of the chylomicrons change, they move across tie lines. Consequently the phase contents of cholesterol change. The values for the weight fractions of cholesterol in the surface and oil have been summarized in Table 11. The phases are enriched by 2.5-fold in cholesterol during a 3-h incubation of chylomicrons and serum at a 10/90 ratio at 37°C. The magnitude of the phase composition changes was less when the relative amount of serum in the incubations was reduced. Although the percentage compositions of the lipoproteins do not change dramatically, very large numbers of lipids were transferred since the particles are quite large. By the end of the incubations, the weight fractions of cholesterol in the chylomicron phases are comparable to those of dog plasma VLDL and other types of plasma VLDL (Table 10). Thus, if given sufficient length of exposure to serum, chylomicrons can equilibrate with endogenous plasma VLDL. This conclusion could already be drawn on the basis of the data in Fig. 8a and Fig. 9e which show that the large particles (Fraction I) of exogenous origin present in the total $p < 1.006$ g/ml fractions of plasma also plot on the tie lines for endogenous VLDL. In
another study in which chylomicrons were equilibrated with plasma, the data indicated similar losses of chylomicron phospholipid and gains of cholesterol (Fig. 13b).

Faegeman and Havel [64] found that rat VLDL incubated for 6 h at 37°C with red blood cells to remove radiolabeled 3H-cholesterol gained cholesterol mass during the incubation. These investigators reported the starting and final compositions of the VLDL which are shown in Fig. 13c. During the incubation we estimated that

---

**Fig. 13.** The effects of incubation of nascent lipoproteins with serum or red blood cells. (a) Dog chylomicrons incubated with dog serum. The open circles represent the incubation of 10 parts of chylomicrons to 90 parts of serum, and the closed circles represent incubation of equal parts of both. The starting composition is given as the 0 time point and the change in compositions of the isolated particles are given as a function of minutes after 0 time. Note that the early change from 0 to 15 min moved the composition away from the phospholipid apex as phospholipid is being removed from the particle. The later time points show that compositional change moves in a direction towards the cholesterol apex as cholesterol is added from plasma to the particle. Changes are greater when the proportion of plasma to nascent chylomicrons is greater (data from [63] and [84]). (b) Rat lymph chylomicrons added to serum (data from [152]). Point 1 control rat chylomicrons, point 2, chylomicrons incubated 1 h at 37°C, and point 3, incubated 7 h at 37°C. (c) Rat plasma VLDL incubated with rat red blood cells at 37°C for 6 h (data from [64]).
the surface cholesterol increased from 14% to 23%. The core lipids likewise experienced an 1.6-fold increase in cholesterol content. The fact that rat plasma VLDL gain cholesterol when incubated with erythrocytes confirms the conclusion made above, that as isolated from the plasma, VLDL are not quite in equilibrium with red blood cells with respect to their cholesterol contents. When injected back into rats, the cholesterol-enriched VLDL were more rapidly cleared from the circulation than controls. Whether this was a consequence of their increase in free cholesterol content, apoprotein changes or their acquisition of a foreign lipid or protein component from the erythrocytes is not known.

In summary, the core lipids of triglyceride-rich lipoproteins play an important role in the uptake of cholesterol from red blood cells and plasma lipoproteins because cholesterol is soluble in the core and the core contributes the bulk of the particle mass. Since chylomicron remnants ultimately are cleared from the circulation by the liver, the transport of cholesterol in chylomicrons and their remnants plays a significant role in the cholesterol homeostasis of the organism.

(d) Triglyceride hydrolysis and remnant formation

The lipolysis of triglyceride-rich lipoprotein triglyceride, and to a lesser extent phospholipid, produce much more marked alterations in lipoprotein composition than do lipid transfer reactions (Section 6C). Often 70–90% of the triglyceride is removed from the lipoprotein in 1 h of in vivo or in vitro incubation. When cholesterol-carrying elements (e.g. plasma, rbc) are present during the course of the reaction, the lipoproteins can still participate in lipid transfer reactions while undergoing degradation. It is possible that these secondary reactions, which may be facilitated by apoprotein transfer, may contribute significantly to the overall changes in particle composition. In this section we will examine the process of lipolysis from the standpoint of monitoring the changes in particle surface and core regions.

In 1970, Fielding demonstrated that the ability of post-heparin plasma lipoprotein lipase to hydrolyze in vitro a sonicated triolein emulsion activated with VLDL apoproteins was sensitive to the cholesterol content of the emulsion [153]. Emulsions composed of triolein, cholesterol, and egg lecithin, with or without added cholesteryl oleate were studied. The addition of high levels of cholesterol to either type of emulsion completely blocked the activity of lipoprotein lipase. The compositions of the phases for the series of emulsions can now be obtained since the phase behavior of these systems has been defined. Assuming $K_C = 22$ for emulsions lacking cholesteryl oleate and $K_C = 11$ for those containing cholesteryl oleate, the compositions of the phases were calculated and tie lines for the systems are plotted in Fig. 14. The analysis shows that the activity of the lipase decreases as the surface cholesterol content increases and eventually ceases when the level of cholesterol in the emulsion reaches a maximum value, that is, when its phases are saturated with
Fig. 14. In vitro lipolytic degradation of emulsions. Below are shown the estimated compositions of the starting emulsions used by Fielding [153]. The calculated surface compositions based on a partition coefficient of 22 are plotted on the right hand side of the triangle. Point 5 would have been supersaturated with cholesterol and no estimate of surface composition is given. Above are plotted the lipolysis rate in micromoles of fatty acid released per milliliter of assay per hour [153] against the calculated surface cholesterol concentration. Lipolysis was completely inhibited in emulsion 5. Clearly the surface composition of cholesterol has a strong inhibitory effect on the lipolysis in vitro. Similar results have been obtained in vivo by injecting similar emulsions into animals. Lipolysis is completely inhibited at surface composition of 34% cholesterol [154, 155].

cholesterol. For this series of emulsions without cholesteryl oleate, the relative hydrolysis of triolein decreased progressively as the phase compositions of the particles were enriched with cholesterol. However, emulsions containing cholesteryl oleate were maximally active when the weight fraction of cholesterol in the surface phase was 0.07. As discussed above, this is the approximate level of cholesterol in the surface of nascent lipoproteins (see Fig. 11) – particles which are excellent substrates for lipoprotein lipase. The reason that the addition of cholesteryl oleate to the emulsions shifted the activity maximum from 0% surface cholesterol to 7% surface cholesterol is not clear. However, the results point to the cholesterol content of the phases as being a critical parameter which influences the susceptibility of a triglyceride-containing particle to lipolysis.

Recently, our laboratory has shown that in vivo lipolysis of emulsion particles was
totally blocked by high particle cholesterol [154, 155]. Particles composed of triolein, cholesteryl oleate, egg yolk lecithin and cholesterol were prepared according to [137] so that the surface composition ranged from 10–40% cholesterol. Particles were injected into rats and the rate of lipolysis was followed for 10 min. Lipolysis was inversely proportional to cholesterol content and was completely inhibited at a surface composition of about 34%, that is a 1:1 mole ratio of cholesterol to phospholipid.

The in vitro hydrolysis of human plasma VLDL by bovine milk lipoprotein lipase [150, 156] and rat plasma VLDL by lipoprotein lipase in post-heparin plasma [157] have been extensively studied. Within 1 h of incubation at 37°C in the presence of albumin, as much as 97% of the particle triglyceride can be cleaved to fatty acids milk lipoprotein lipase and removed from the ‘remnant’ [156].

The VLDL remnant is enriched in cholesterol ester and cholesterol but depleted in phospholipid and triglyceride. The phospholipid which is not degraded by the lipase is removed along with small molecular weight apoproteins and is recovered in the HDL density range. The remnant, which now contains only apoB, is recovered in the LDL density range. Similar transformations occurred for rat plasma VLDL incubated with plasma obtained from animals administered heparin [157].

The compositions of the starting intact VLDL and their remnants were plotted on the phase diagrams shown in Fig. 15. As expected the particle compositions shift away from the N apex of the diagrams since triglyceride is removed from the nonpolar lipid fraction. The compositions also shift towards the C apex of the figure indicating that the remnants are enriched in their total percentages of cholesterol.

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**Fig. 15.** VLDL lipolysis in vitro. Data points are from: Eisenberg and Rachmilewitz [157] (■), for plasma equilibrated rat plasma VLDL before (point 1) and after incubation with post-heparin plasma; Deckelbaum et al. [156] (o) for point 1 – control human VLDL, point 2 – native human LDL, and point 3 – in vitro ‘LDL’ produced by incubation with milk lipoprotein lipase; Patsch et al. [150] (*), for point 1 – control human VLDL, point 2 – VLDL incubated with HDL, and point 3 – VLDL + HDL incubated with milk lipase. The starting VLDL composition is connected to the remnant composition by arrows. Note that in most cases the starting compositions have a lower surface cholesterol content than their remnants. Furthermore, most of the remnants have a rather similar surface composition of about 23–26% cholesterol. These are given at the right of the figure.
For the human VLDL, the particle compositions move across tie lines indicating that the weight fractions of cholesterol in their phases are raised (from 18–19% to 25–26% cholesterol) by the removal of triglyceride. In one of these examples [156], no other source of cholesterol besides that contained in the VLDL was present in the incubation and the enrichment in cholesterol resulted only from the removal of the other lipids. Thus in the absence of other sources of lipids, loss of triglyceride is equivalent to loss of core solvent for cholesterol, and as the core concentration of cholesterol increases some distribute to the surface increasing the weight fraction of cholesterol in the surface. For the other experiment in which human VLDL was incubated with bovine milk lipoprotein lipase, HDL was also present in the incubation mixture [150]. While some of the cholesterol enrichment could have resulted from the transfer of cholesterol from HDL, this seems unlikely since incubation of HDL with VLDL without lipolysis had little effect on the composition of VLDL (see Fig. 15). The rat VLDL degradation studies [157] were conducted using postheparin plasma (40%). No increase in the relative contents of cholesterol compared to the control were noted. This probably indicates that the control also gained cholesterol during its incubation with normal plasma. In this regard, the other examples of rat plasma VLDL described above (see Fig. 12 and Table 10) had less cholesterol in their phases than the control in [157].

Fig. 16. Chylomicron degradation during perfusion through the isolated rat heart. The original starting composition of the rat chylomicrons are given in the left hand side at time point 0. The changes in composition as a function of minutes of circulation through the perfused heart are shown at 30, 45 and 60 min. The calculated surface compositions using a $K_C = 11$ are given in the right hand side. The original surface composition was approximately 8% cholesterol and did not change appreciably in the first 30 min of recirculation, even though lipolysis occurred. However, between 30 and 60 min the composition of the particle became enriched in cholesterol and the resulting surface composition increased from 8% to 16%. (Plotted from data of Fielding [159])
Lipoprotein lipase-mediated degradation of chylomicrons and VLDL has also been studied using the perfused rat heart system as a model of in vivo degradation [158, 159] (Fig. 16). In these studies the compositions of the remnants were monitored over the 60–90 min of their recirculation through the perfused heart. As a consequence of degradation by heart lipoprotein lipase the triglyceride contents of chylomicrons were reduced to 6–20% of their initial values, and up to 75% of the VLDL triglyceride was also hydrolyzed. Heart perfusion fluids always contained

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Fig. 17. Chemical compositions (a) of triglyceride-rich lipoprotein remnants and comparison (b) of remnant and starting intact lipoprotein compositions. In (b) initial and final lipoprotein compositions are joined by dashed lines. (c) Difference between in vitro and in vivo formation of remnants. For remnants produced in vitro, compositions change by moving away from the $N$ apex along vector $\vec{a}$ (point 12 to 13) indicating that only triglyceride is removed from particles. Remnants formed in vivo (points 1 to 2) can be described by two vectors: ($\vec{b}$) occurs as triglyceride is lost and ($\vec{c}$) occurs as C is transferred into the particle. (1 and 2) Mjöls et al. [74], rat, large chylomicron (CM) (1) and CM remnants (CMR) (2); (3 and 4) Pattniak and Zilversmit [160]. C-fed rabbit, CM (3) and CMR (4); (5 and 6) Redgrave and Small [76], rat, CM (5) and CMR (6); (7 and 8) Mjöls et al. [74], rat, small CM (7) and CMR (8); (9 and 10) Mjöls et al. [74], rat, VLDL (9) and VLDL remnants (VLDLR) (10); (11) Szara et al. [86], human Type III β-VLDL; (12 and 13) Deckelbaum et al. [156], human, VLDL (12) and VLDLR (13); (14 and 15) Patsch et al. [150], human VLDL (14) and VLDLR (15); (16) Deckelbaum et al. [120], human LDL.
$p > 1.006 \text{ g/ml or } p > 1.063 \text{ g/ml plasma but lacked red blood cells. The compositions of the partially degraded chylomicrons from [159] were plotted in Fig. 16 to determine how their phase compositions changed during lipolysis. By 30 min, note that even though lipase is reducing the triglyceride content the surface cholesterol changes little. However, between 30 and 60 min the surface content doubled from \(-8\% -16\%\).}

The final model of remnant formation that was analyzed was the technique of preparation of triglyceride-rich lipoprotein remnants in hepatotomized rats (Fig. 17). In this animal model, the partially degraded remnants that are produced in the peripheral circulation cannot be removed by the liver. Therefore, lipolysis and transfer reactions may be somewhat exaggerated due to the prolonged time period of circulation. After lipoproteins were in the circulation for one hour the compositions of VLDL remnants (Fig. 17a, point 10) and chylomicron remnants (points 2, 4, 6, 8) were found to plot on a single tie line. The estimated weight fractions of cholesterol in the phases of the remnants were 0.023 in the core and 0.24 - 0.28 in the surface. The compositions of the human VLDL remnants produced in vitro by the action of milk lipoprotein lipase (points 13, 15 [150, 156]), human LDL (point 16) and VLDL isolated from a patient with Type III hyperlipoproteinemia (point 11) [86] also plotted roughly on this same tie line. For comparison, the starting compositions of the undegraded lipoproteins have been plotted in Fig. 17b. Clearly the lipoproteins become greatly enriched in cholesterol content during circulation in the hepatotomized rat. Since the remnant tie line intersects the phase boundary approximately at the position where the red blood cell membrane composition also plots, the remnants are probably nearly in equilibrium with the red blood cells with respect to cholesterol distribution. On the basis of the lipoprotein metabolic and structural data, and the results of study of the phase compositions of model systems, we will now present what we think is a reasonable description of some of the events which occur during lipolysis of the lipoprotein.

Upon entering the circulation from the intracellular secretory pathway, nascent chylomicrons and VLDL undergo apoprotein and lipid transfer/exchange reactions with circulating lipoproteins, erythrocytes, white blood cells, and perhaps endothelial cells. The nascent particles rapidly lose apoAI, apoAIV, and phospholipids, and acquire apoC and apoE peptides. However, at least in the absence of lipolytic events they experience a slower enrichment in unesterified cholesterol (63) and Fig. 13). After binding of apoCII to their surfaces, the lipoproteins interact with lipoprotein lipase situated on peripheral endothelial cells. Although other forms of hydrolysis cannot be ruled out, we favor the model that lipoprotein lipase cleaves surface-oriented triglyceride.

The hydrolysis of surface as opposed to core located triglyceride molecules seems likely for the following reasons. The most direct reason comes from the study of lipoprotein emulsions and phospholipid vesicles cited above which showed that triglyceride is soluble in the lipoprotein surface lipids. Furthermore, $^{13}$C NMR
studies have demonstrated that the two most actively cleaved acyl groups, sn-1 and sn-3, are probably hydrogen-bonded to interfacial water molecules [113]. Thus, surface triglyceride is ideally situated to be acted on by an enzyme which ultimately requires water for its catalytic action. In addition, studies of the action of pancreatic lipase on mixed lecithin-triglyceride monolayers [161] and lipoprotein lipase on pure triglyceride monolayers [162] show directly that a bulk oil phase need not be present for the enzyme to encounter and cleave its substrate. While it may be argued that the action of pancreatic lipase differs from that of lipoprotein lipase, it should be noted that their substrates are nonetheless both present in emulsified form [21].

The hydrolysis of surface triglyceride would rapidly deplete the surface triglyceride concentration and reduce the rate of triglyceride cleavage unless a mechanism were operating to maintain the surface triglyceride concentration at a more or less constant level. This mechanism may simply be the partitioning of triglyceride molecules to the surface to restore the equilibrium solubility of triglyceride in the surface lipids. Recent $^{13}$C NMR experiments on microemulsions (J.A. Hamilton, G.S. Ginsburg, D.M. Small, unpublished results) indicate that the rate of exchange of core molecules (cholesteryl oleate) with the surface is rapid ($\leq$ msec). Since both the cholesterol ester and triglyceride should be able to partition between the surface and core, the relative amounts of triglyceride vs. cholesterol ester which transfer to the surface would be governed, in part, by their ratio within the core [114]. If core-to-surface transfer of triglyceride ultimately supplies lipoprotein lipase with substrate then it would be interesting to know if the rate of phase transfer can become rate-limiting to the overall turnover rate of the enzyme. Possibly, under conditions of decreased temperature or increased saturation of the triglyceride acyl chains, a situation may be encountered where partitioning may be a slower event than the actual cleavage of the triglyceride by the enzyme, once triglyceride is bound to its active site.

Eventually, lipolysis leads to the formation of partial catabolic remnants which are relatively poorer substrates for lipoprotein lipase than their nascent predecessors. Several interrelated factors may be involved in the decreased susceptibility of the remnant towards further hydrolysis. Among these are the roles played by the cleavage products, fatty acids, diglycerides, and monoglycerides, produced by the catalytic reaction. If the end products of cleavage are not removed from the surface by albumin, etc., as fast as they are produced by the enzyme, they will accumulate and may alter the surface lipid structure and/or phase solubility of triglyceride in either a positive or negative way. They may decrease the enzyme turnover rate via end-product inhibition. Since an acyl-enzyme complex may be an intermediate, a high local concentration of fatty acid might lead to formation of a long-lived inhibitory intermediate. As discussed above, the surface of the remnants become enriched in unesterified cholesterol due to depletion of core triglyceride and net movement of a fraction of the core cholesterol molecules into the surface. Also cholesterol may be transferred into the remnant from outside sources. $^{13}$C NMR
studies with model systems have shown that cholesterol enrichment proportionally reduces the solubility of triglyceride in the surface phase from 3 - 4% to 0.15% of the surface lipid mass at 33% surface cholesterol (Spooner and Small, unpublished data, 1986). The surface triglyceride concentration may be further reduced by partitioning of cholesterol ester to the surface since cholesterol ester competes with triglyceride for surface orientation. Note that the core remnant becomes enriched in cholesterol ester by triglyceride removal. However, since in most remnants (Fig. 17) the final triglyceride/cholesterol ester ratio usually remains ≥ 4/1, there would be only a small reduction in surface triglyceride concentration due to substitution of cholesterol ester for surface triglyceride. Thus, the ~50% depletion of the surface triglyceride content alone seems not to be sufficient to reduce the triglyceride cleavage to the extent observed in model systems (Fig. 14 and [153]).

One reason for the loss of activity in the remnant is the depletion of apoCII occurring as a result of the departure of surface lipids. Factors such as a change in the particle’s surface curvature or an increase in lateral surface pressure may contribute to loss of apoC peptides. Since apoB stays with the core remnant, the surface area to which other apoprotein can bind is reduced in the shrunken remnant. If apoE binds with higher affinity than apoCII to the cholesterol-enriched surface, then further reduction in apoCII content will result. Ultimately, due to the acquisition of apoE the chylomicron remnant is recognized by the liver and removed from the circulation [78, 79]. Whether cholesterol enrichment acts primarily by reducing apoC’s or directly stimulates the binding of apoE to the chylomicron remnant or exerts a negative effect on the catalytic properties of lipoprotein lipase is unknown.

The overall transformation of a nascent particle by lipolytic and lipid transfer reactions to its remnant can be depicted graphically as in Fig. 18, using the data for in vivo production of remnants in [76]. The composition of the nascent chylomicron (point W) and its remnant (point Z) lie on two different tie lines. The enrichment of the lipoprotein phases accompanying lipolysis results from the combined processes of triglyceride removal via lipolysis, phospholipid removal via departure of surface phospholipid and/or phospholipid degradation, and cholesterol enrichment via transfer into the remnant from external sources (refer to Fig. 10).

Removal of 85 - 90% of the particle triglyceride would change the composition from 90% N, 1% C, 9% P (point W) to 50% N, 5% C, 45% P (point X). This remnant contains more surface phospholipid than is required to cover the remaining core and may have an extension of bilayer phospholipids (refer to [143]). The composition of this particle lies on a tie line slightly above that of the original chylomicron since triglyceride removal along line WX shifts the particle composition across tie lines. For the particle to move to point Z, the combined processes of loss of the redundant phospholipid region and gain of cholesterol from external sources must occur. The line joining the composition of the remnant (point X) its original surface (point Y), and the final remnant (point Z) in Fig. 7 [143] represents this final transformation, but the transformation does not occur directly as implied
Fig. 18. The use of triangular coordinates to study the catabolism of triglyceride-rich particles. Above, the compositional changes occurring during in vivo catabolism of chylomicrons, and below expanded lower section of the diagram to show details of the changes. The composition of starting chylomicrons (W) and the remnants produced in vivo (Z) are taken from [76]. Similar changes are shown in Fig. 17b and 17c. Chylomicrons in this study were approximately 1300 Å in diameter and their remnants were considerably smaller. However, the total mass of free cholesterol in the two fractions was approximately the same or slightly greater in the remnant fraction [76]. The catabolism may be described in three relatively simultaneous processes which will be described in sequence: (1) the removal of triglyceride from the particle, (2) the separation of surface and core remnants, and (3) the transfer of free cholesterol into the core remnant. As triglyceride is hydrolyzed from the chylomicon the composition would move along an extension of the line NW. This extension is shown as the solid line WX. When the composition of point X is reached, 85-90% of the initial triglyceride has been hydrolyzed and removed. The system would consist of residual triglyceride and redundant surface present as a bilayer. The original surface composition of the chylomicron W would lie on the tie-line OWY and have a surface composition at Y. Note that as lipolysis occurs and the composition moves along WX, the composition moves away from tie-line OWY to a new tie-line describing the surface and core composition of point X. This tie-line is O’XS and is now richer in free cholesterol in both core and surface, albeit a small change. When the particle at point X separates its redundant surface this surface remnant will have a composition of S and generate a core particle at composition X’ which also lies on the tie-line O’XS. The final change in composition to Z is effected by the addition of free cholesterol to the core particle along line X’ZC to the position Z. Thus, three vectors are summed to describe the change from W to Z: vector $\overrightarrow{a}$ (triglyceride hydrolysis), vector $\overrightarrow{b}$ (separation of core and surface remnants) and vector $\overrightarrow{c}$ (addition of free cholesterol to the core remnant). Since the original chylomicron fraction and its remnant fraction have approximately the same mass of free cholesterol and since the surface remnant must contain free cholesterol, then net movement of cholesterol from other sources into the chylomicron system must occur during catabolism. In short, cholesterol moves from other sources (other lipoproteins, membranes, etc.) into the chylomicron surface remnant to ultimately enter higher density lipoprotein fractions. (Modified from [143])
in the original publication. Rather the core and surface regions of the remnant with the excess surface would be in equilibrium via surface-to-core lipid transfer reactions. Thus the composition of the core particle remaining after departure of the redundant surface lies at point \(X'\), on a line drawn through the compositions of the surface which actually departs (point S) and the remnant with excess surface (point X). Finally for the composition of the remnant to end up at point \(Z\), a transfer of cholesterol into the remnant at point \(X'\) along a line towards the C apex must occur. Thus although the final remnant (point \(Z\)) and the starting chylomicron (point \(W\)) have the same number of cholesterol molecules [76], a net flux of cholesterol into the chylomicron during its transformation must have occurred to replace the cholesterol molecules which departed to HDL in the surface remnant.

While the graphical analysis in Fig. 18 necessarily depicts the process as occurring in discrete steps, the transformation appears to occur continuously by a simultaneous combination of the three distinct steps, as indicated by the data of Fielding et al. [159] in Fig. 16. Thus the vectorial treatment of remnant formation is useful to understand the relative magnitudes of the processes of hydrolysis, surface departure, and cholesterol enrichment. Since triglyceride-rich lipoproteins accept cholesterol from blood constituents, contribute cholesterol to the HDL fraction, and cholesterol esters from HDL and ultimately carry cholesterol to the liver.

### TABLE 12

Effect of cholesterol feeding on guinea pig hepatic perfusate VLDL composition

<table>
<thead>
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<th>Days fed cholesterol</th>
<th>0</th>
<th>10</th>
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<td></td>
</tr>
<tr>
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<td>71.1</td>
<td>57.6</td>
<td>45.8</td>
<td>36.3</td>
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<td>CE</td>
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<td>13.3</td>
<td>23.9</td>
<td>32.2</td>
</tr>
<tr>
<td>C</td>
<td>3.2</td>
<td>7.0</td>
<td>9.0</td>
<td>13.8</td>
</tr>
<tr>
<td>PL</td>
<td>16.3</td>
<td>13.2</td>
<td>12.8</td>
<td>11.6</td>
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<tr>
<td>Protein</td>
<td>8.9</td>
<td>8.8</td>
<td>8.5</td>
<td>6.0</td>
</tr>
<tr>
<td>(x_{cs})</td>
<td>0.14</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x_{co})</td>
<td>0.006</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_C)</td>
<td>22</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values given as percent by weight.
* \(x_{cs}\), weight fraction of cholesterol in the surface; \(x_{co}\), weight fraction of cholesterol in the oil.
* \(K_C\), surface/oil cholesterol distribution ratio.

(Compiled from [163])
in the remnant, triglyceride-rich lipoproteins play an important role in cholesterol homeostasis. The lipoprotein core region participates in this overall process.

(e) Abnormal chylomicrons and VLDL

The examples of abnormal triglyceride-rich lipoproteins which have been chosen for analysis are β-VLDL from cholesterol-fed animals and a human Type III hyperlipoproteinemic patient. Also, the triglyceride-rich lipoproteins from a hypertriglyceridemic patient have been examined. The results will show that under conditions of cholesterol feeding, the concentration of cholesterol in the phases of VLDL and chylomicrons can reach extraordinarily high levels. Study of these lipoproteins has provided some information on the values of \( K_C \) under conditions of elevated core cholesterol ester content.

As a result of feeding cholesterol, guinea pigs synthesize a cholesterol ester- and cholesterol-enriched hepatic VLDL which exhibits β-mobility by agarose gel electrophoresis [163]. Over the course of 12 weeks of cholesterol feeding, the VLDL cholesterol ester and cholesterol contents gradually rise (Table 12). The increase in unesterified cholesterol shifts the particle composition towards the cholesterol apex of the phase diagram (Fig. 19) and hence leads to an increase in the phase cholesterol contents. Using the computer and assigned \( K_C \) values of \( K_C = 22 \) for zero days of cholesterol feeding and \( K_C = 11 \) for 10 days on the diet, the surface and core cholesterol contents were calculated (Table 12). The results show a 2-fold increase in the concentration of cholesterol in the surface lipids and a 4-fold increase in the core lipids. For the data collected at 28 and 84 days no analysis of the tie line position of these β-VLDL was undertaken since we are uncertain what \( K_C \) to assign for

---

Fig. 19. Composition of guinea pig liver perfusate VLDL in response to cholesterol feeding. Plotted from the data in [163] and Table 12. Note that the perfusate VLDL at day 0 has a relatively low surface and core composition of cholesterol given by S and O. As cholesterol feeding continues the perfusate VLDL contains higher and higher surface cholesterol levels. By day 10 it contains nearly 30% surface cholesterol and by days 28 and 84 the compositions are actually supersaturated with cholesterol. Thus, the liver appears to be able to incorporate supersaturating amounts of cholesterol into its nascent lipoproteins.
these samples containing even higher levels of cholesterol. In the case of the day 84 sample, the cholesterol content is so high that this β-VLDL may be a metastable particle or a particle containing cholesterol monohydrate in a microcrystalline phase.

Examples of plasma β-VLDL from four species of cholesterol-fed animals have been analyzed on the phase diagram (Fig. 20). We have drawn a tie line on the figure to approximate the phase compositions of these samples. We estimate that the surface lipids are close to or saturated with cholesterol, e.g., $x_{cs} \geq 0.33$. The oils contain from $3-5\%$ cholesterol and in the case of the pig sample (point 2, Fig. 20) perhaps an even greater level of oil cholesterol. The enrichment of VLDL cholesterol in response to diet can occur at the level of synthesis of VLDL by the liver [44, 45] (see Fig. 19) and/or by retardation of the clearance of nascent triglyceride-rich lipoproteins from cholesterol-rich blood [167, 168]. Cholesterol feeding may also depress hepatic B/E receptors. In any case, the relationship between the increased surface (and core) cholesterol content and the altered apoprotein content of these lipoproteins has not yet been adequately explained.

In humans, Type III hyperlipoproteinemia or dysbetalipoproteinemia, is characterized by the increase of plasma β-migrating VLDL [167]. These VLDL are thought to accumulate due to defective hepatic receptor recognition of their bound apoE which has an abnormal ligand site [169]. Partly as a consequence of their retarded rate of clearance, the VLDL become enriched in unesterified and esterified cholesterol. To determine the phase contents of cholesterol in this lipoprotein, we plotted the data for subfractionated human type III β-VLDL from [86] (Fig. 21). By plotting the subfraction compositions, we can estimate the surface and core compositions of these VLDL by the logic presented in Fig. 8. Since the subfractions plot on a single line, they are in equilibrium with respect to interparticle cholesterol distribution. The tie line intersects the oil phase boundary at $\sim 4\%$ oil cholesterol and intersects the surface boundary at $\sim 24\%$ surface cholesterol. Thus, a calculated $K_C$ from the graph is $K_C = 24/4 = 6$. It should be noted that this value is similar

![Fig. 20. Chemical compositions of plasma β-VLDL. (1) Sata et al. [86], human Type III β-VLDL; (2) Mahley et al. [164], swine; (3) Goldstein et al. [60], dog; (4) Mahley and Holcombe [165], rat; (5) Mahley et al. [166], monkey. Note that β-VLDL is approximately saturated with cholesterol.](image-url)
to that of the $K_C$ for human LDL (Table 1). Furthermore, the surface cholesterol content is similar to that of remnants (Fig. 17a) and LDL, the ultimate VLDL remnant. In this particular example it is less than the estimated surface of $\beta$-VLDL from other species (compare Fig. 20).

The phase compositions of human $\beta$-VLDL can be compared to those of VLDL from a hypertriglyceridemic patient (Fig. 21, [86]). The comparison shows that $\beta$-VLDL are more enriched in both core and surface cholesterol concentrations. However, the hypertriglyceridemic VLDL, which generally exhibit prolonged circulation within the plasma, is quite similar in surface cholesterol ($x_{cs} = 0.18$) and core cholesterol ($x_{cc} = -0.02$) compared to controls (see Fig. 9f, and Table 7, sample f). The best fit line for these VLDL is $K_C = 9$ which is significantly different from $\beta$-VLDL. This difference may be explained by the increased cholesterol ester content of $\beta$-VLDL.

Finally, we have compared the phase behavior of triglyceride-rich lipoproteins from cholesterol-fed normal and diabetic rabbits [170]. Rabbits rapidly develop atherosclerotic lesions in response to cholesterol feeding. However, in the diabetic state they are more resistant to development of atherosclerosis. When the compositions of subfractions of the two classes of lipoproteins are plotted on a phase

![Fig. 21. Human triglyceride-rich lipoprotein fractions taken from hypertriglyceridemic individuals and patients with dysbetalipoproteinemia. Plotted from the data of Sata et al. [86]. The size distribution was approximately the same although the dysbetalipoproteinemia particles are slightly smaller in size. The best fit lines for the points give a $K_C = 9$ for the hypertriglyceridemias which is quite similar to that calculated for normals (see Figs. 7, 9). The calculated $K_C$ is only 6 for dysbetalipoproteinemias indicating a greater partitioning of cholesterol into the core. The surface compositions calculated on the basis of these two distribution ratios indicate that the hypertriglyceridemic surface contains about 18% cholesterol which is quite normal whereas the dysbetalipoproteinemic lipoprotein surface contains 24% cholesterol. Core compositions are also different. In the dysbetalipoproteinemic patients the core contains almost 4% cholesterol. Thus, both core and surface compositions of dysbetalipoproteinemic triglyceride-rich fractions are distinctly abnormal and contain more core and surface cholesterol. Presumably such particles are resistant to lipolysis (see Fig. 14).]
diagram (Fig. 22) the effect of substituting cholesterol ester for triglyceride in the core becomes apparent. The cholesterol-fed control rabbits have cholesterol ester/triglyceride ratios > 10. In contrast, the cholesterol-fed diabetic rabbits have more typical amounts of triglyceride and cholesterol ester and the cholesterol ester/triglyceride ratio is 1/2. Apparently the increase in core cholesterol ester in the control contributes to the overall greater solubility of cholesterol in the core, since the two categories of VLDL have different oil phase compositions. For the control rabbits the value of $x_{co} = 0.04$ whereas for diabetic rabbits $x_{co} = 0.02$. The two classes of lipoproteins nevertheless maintained similar surface cholesterol concentrations ($x_{cs} = 0.25$, by tie line extrapolation). Thus cholesterol-fed controls exhibited $K_C$ values of $K_C = 0.25/0.04 = -6$ and diabetics exhibited $K_C$ values of $K_C = 0.25/0.02 = -12$. Presently it is unknown why the two types of VLDL differed in their capacity to be taken up by macrophages, i.e., control cholesterol-fed VLDL was more rapidly taken up than diabetic cholesterol-fed VLDL. In this

![Diagram](image)

**Fig. 22.** The composition of lipoprotein subfractions of $S_t > 20$ from cholesterol-fed control and cholesterol-fed diabetic rabbits. The top of the figure is an expansion of the lower left-hand corner. The lipoprotein compositions of fractions from diabetic cholesterol fed animals (●) and cholesterol-fed controls (▲) are shown. The dotted line is the boundary of cholesterol saturation. Fractions 1, 2 and 3 in both groups fall on lines, indicating that the fractions within a given group are in equilibrium and differ only in size. The largest lipoproteins are on the left-hand side and the smallest on the right. The intersection of these lines with the phase boundaries on the left and right sides of the diagrams indicate the core and surface compositions of these lipoprotein particles, respectively. Thus, the surface compositions of both groups are similar (~73% phospholipid, 25% cholesterol), 2% (cholesterol ester plus triglyceride) (A), whereas the core compositions are different. The diabetic cholesterol-fed animals have only ~2% cholesterol in the core (B), while the cholesterol-fed controls contain ~4% of cholesterol (C). (From [170])
in the sterol s have sterol in the, since control ate two molecules ex-ues of LDL differ in this regard, the apoE/apoC ratio in the diabetic VLDL was lower than that in the control. This finding suggests that the surface triglyceride and cholesterol ester contents of the two particles may also influence apoprotein binding to the surface, since the overall triglyceride and cholesterol ester contents of the lipoproteins influences the surface triglyceride and cholesterol ester concentrations [114].

7. Concluding remarks

Important concepts concerning the fine structure of triglyceride-rich lipoprotein lipid organization have been developed by study of the phase solubility properties of lipid model systems and native lipoproteins in conjunction with phase diagram analysis. The data analysis has begun to reveal the relationships between lipoprotein phase compositions and lipoprotein metabolism. As model systems and phase diagram analysis are applied to study the influence of lipid structure and phase solubility properties on the binding and function of triglyceride-rich lipoprotein apoproteins and enzymes, a greater understanding of lipoprotein metabolism in normal and disease states should be forthcoming.

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Appendix: computer program for analysis of triglyceride-rich lipoprotein structure

The program entitled ‘Lipoprotein Phase Diagram Analysis’ presented herein is written in ‘Apple Basic’ language and is ready to run on personal computers such as the Apple II Plus. However, it can be readily modified for use with any computer. The program permits graphical analysis of triglyceride-rich lipoprotein composition data using triangular coordinate phase diagrams. It calculates the values of all of the parameters discussed in Eqs. (1 – 16) of the text and lists many of these values in printout form. The authors will gladly provide a step-by-step description of the program lines upon request.

The major aspects of the program will now be explained. First, the lipoprotein composition data are entered as percentages of the total particle weight, including protein (program lines 400 – 600). The value of $K_C$ (the phase distribution ratio for cholesterol, Section 4b, Eq. (3) can be entered, or a default value of $K_C = 22$ (for $< 3\%$ cholesterol ester) or $K_C = 11$ (for $> 3\%$ cholesterol ester) will be set by the computer for subsequent calculations (lines 301 – 302). Second, the composition data point is plotted on an appropriate phase diagram (taking into account the value of $K_C$) that shows the surface phase boundary and the phase boundary between the two- and three-phase regions (lines 1501 – 2300, and refer to Figs. 3 and 4). Third, the tie line is drawn on the graph, and the Zone (Fig. 4) in which the composition lies is indicated (lines 2450 – 4850). Fourth, using the position of the tie line and the graphical method presented in detail in [136], the surface and core lipid compositions are calculated (lines 4650 – 4700 and 5000 – 5150). Fifth, the particle diameter is calculated using the total composition of the lipoprotein and the value of $M_s : M_o$ (Eq. (1)) that is measured graphically from the lengths of the tie line segments (lines 5200 – 7800). Six, the molecular compositions of the surface and core phases (i.e., the number of lipid molecules in each phase) are calculated as described in Section 5b (lines 7850 – 9000).

This program has also been written in Fortran. For specific details please contact D.M. Small.
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This program has also been written in Fortran. For specific details please contact D.M. Small.
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100 HIMEM: 38191
150 REM PROGRAM TITLE:
LIPIDPROTEIN PHASE DIAGRAM
ANALYSIS: WRITTEN BY KURT W.
MILLER
200 HOME
210 VTOY 4
250 INPUT "SOURCE OF DATA = ";IS
300 INPUT "DATA POINT (HUMAN PLA
SMA VLDL, ETC.) = ";JS
301 PRINT "DO YOU WISH TO ENTER
A VALUE FOR THE PHASE DIS
TRIBUTION RATIO OF CHOLESTEROL
GLYCO (KC) ?"
302 INPUT "IF SO, ENTER THE VALU
E OF KC. IF NOT, ENTER ZERO
0. KC = ";KC
350 REM ENTER THE LIPOPROTEIN C
OMPOSITION DATA
400 INPUT "ENTER THE VALUE FOR T
G = ";TG
450 INPUT "ENTER THE VALUE FOR C
P = ";CE
500 INPUT "ENTER THE VALUE FOR C
L = ";CL
550 INPUT "ENTER THE VALUE FOR P
E = ";PE
600 INPUT "ENTER THE VALUE FOR P
O = ";PO
650 FOR PAUSE = 1 TO 3000: NEXT
PAUSE
700 HGR
750 HPL 45.152 TO 45.159
800 HPL 51.152 TO 51.159
850 HPL 45.152 TO 51.159
900 HPL 149.2 TO 154.2
950 HPL 149.2 TO 149.8
1000 HPL 149.8 TO 154.8
1050 HPL 226.152 TO 226.159
1100 HPL 226.152 TO 231.152
1150 HPL 231.152 TO 231.156
1200 HPL 231.156 TO 226.156
1250 HPL 233.152 TO 233.159
1300 HPL 233.159 TO 238.159
1350 HPL 238.0 TO 220.159
1400 HPL 220.159 TO 60.159
1450 HPL 60.159 TO 140.0
1501 IF (KC = 0) THEN GOTO 1550
1502 CL = 33 / KC
1503 NL = 100 - CL
1504 PY = (100 - CI) * 1.59
1505 PX = (((100 - NL) * (1.6) + 6
0) / (0.8 * CL)
1506 HPL 193.1,106.53 TO FX,PY
1507 GOTO 1951
1550 IF (CE < 3) THEN GOTO 1
800
1600 KC = 11
1650 FX = 52.4
1700 FY = 154.23
1750 GOTO 1950
1800 KC = 22
1850 FX = 61.2
1900 PY = 154.615
1950 HPL 193.6,106.53 TO FX,PY
1951 MS = 2.1157
1952 MB = MS * -140
1953 KG = (FY - 106.53) / (FX - 1)
1954 KB = FX - (KG * PX)
1955 EX = -(MB - EX) / (MS - MS)
1956 EY = (EX * MS) - MS
1957 HPL 215.2,159 TO EX,EY
2000 LD = TY + CE + C + PL
2050 N2 = (TG + CE) * 100 / LD
2100 C2 = (C * 100) / LD
2150 CE = (PL * 100) / LD
2200 EX = (100 - C2) * 1.59
2250 EX = ((100 - N2) * (1.6) + 6
0) / (0.8 * C)
2300 HPL 1500: NEXT
2350 FOR PAUSE = 1 TO 1500: NEXT
PAUSE
2400 HM = MS
2450 HM = (MS - EX) + EY
2500 HN = 0
2550 HN = 159
2600 HN = - (BM - BE) / (MM - ME)
2650 MM = 0
2700 MU = 159
2750 HX = -(BM - BE) / (MM - ME)
2800 MU = (KX + MM) + BM
2850 HX = -(BM - BN) / (MM - ME)
2900 KX = (KX + MM) + BM
2950 KX = (KX - EX) * 2 + (KY -

3000 JE = (LX - EX) * 2 + (LY -
3050 JF = JF / JK
3100 AD = 58.03569
3150 Z = R + AB
3200 IP (Z < AB) THEN GOTO 4
3250 HPL 140.0 TO 140.0
3300 HPL 140.0 TO FX,PY
3350 HPL 140.0 TO TX,EY
3400 FOR PAUSE = 1 TO 2000: NEXT
PAUSE
3450 TEXT
3500 JX = 33 / KC
3550 JX = 100 - JJ
3600 GOTO 3950
3650 FM 1
3700 PRINT "OIL N = ";JJ
3750 PRINT "OIL C = ";JJ
3800 PRINT
3850 PRINT "SURFACE PHASE LIPID
COMPOSITION (B)"
4100 PRINT 7000
4150 PRINT "SURFACE N = 2" 7050 AJ = (AH) / (AG + AH)
4200 PRINT "SURFACE C = 33" 7100 VN = AI * VM + AJ * VE
4250 PRINT "SURFACE PL = 65" 7150 AX = NA * VN
4300 PRINT 7200 AL = CB * VC
4350 PRINT "THE LIPOPROTEIN COM- 7250 AM = NB * VN
POSITION LIES IN THE THREE PHASE 7300 SV = AS + AD + AK + AC
REGION" 7350 CV = AM + AL
4400 PR = 0 7400 VR = SV / CV
4450 END 7450 SR = 40
4500 X = 0.42329 * Z 7500 SR = SR + 10
4550 SX = 215.2 - X 7550 HO = SR - 20
4600 SY = (WS * SX) - 296.29664 7600 VS = 4.18879 * (SR - 3 - HO - 3)
4650 CS = 100 - (SV / 1.59) 7650 VO = 4.18879 * (HO - 3)
4700 CO = CS / KC 7700 Q = VS / VO
4750 C = (100 - CO) * 1.59 7750 IF (Q > VR) THEN GOTO 7500
4800 OK = (CO * 1.6 + 60) - 0.8 * CO
4850 HPLD = SX, SY TO OK, OK 7800 D = 2 * SR
4900 FOR PAGE = 1 TO 4500: NEXT 7850 PA = (AZ / SV) * VS
PAGE 7900 PB = (AO / SV) * VS
4950 TEXT 7950 PC = (AK / SV) * VS
5000 NS = 137.5 - (0.625 * SX) - 8000 PD = (AC / SV) * VS
(0.5 * CS) 8050 QA = (AM / CV) * VO
5050 M0 = 137.5 - (0.625 * OK) - 8100 QB = (AL / CV) * VO
(0.5 * CO) 8150 NW = (AZ * TV) + (AJ * EV)
5100 PS = 100 - NS - CS 8200 S = PB / PV
5150 PO = 0 8250 T = FC / NV
5200 PV = 1260 8300 U = PO / CV
5250 CV = 642 8350 V = QA / NV
5300 EV = 1126 8400 W = QB / CV
5350 TV = 1610 8450 AA = T * AI
5400 VP = 0.970 8500 BB = T * AJ
5450 VC = 0.968 8550 CC = V * AI
5500 VE = 1.044 8600 DD = V * AJ
5550 VT = 1.093 8650 S = INT (S)
5600 VR = 0.705 8700 O = INT (O)
5700 MR = ((OC - EX) 8750 U = INT (U)
* 2 + (CY - EX)) / 2 + (EX - SY) / 2 + (CY - SY) * 0.5 8800 W = INT (W)
5750 AS = (BE) * (NS / NO) 8850 AA = INT (AA)
5800 BS = (BE) * (CS / CO) 8900 CC = INT (CC)
5850 SR = 100 8950 BB = INT (BB)
5900 SP = 100 9000 DD = INT (DD)
5950 SN = (AS) / (1 + AS) * 100 9050 PR = 1
6000 NN = 100 - SN 9100 PRINT "REFERENCE = ";RS
6050 SC = (BS) / (1 + BS) * 100 9150 PRINT "DATA POINT = ";DS
6100 OC = 100 - SC 9200 PRINT
6150 P = P / 100 9250 PRINT "WEIGHT FRACTION COMP- OF THE TOTAL PARTICI-
6200 PL = PL / 100 PATION E";
6250 TS = TG / 100 9300 PRINT
6300 CE = CE / 100 9350 PRINT "TG = ";TG
6350 C = C / 100 9400 PRINT "CE = ";CE
6400 N = TG + CE 9450 PRINT "C = ";C
6450 NA = N * (SN / 100) 9500 PRINT "PL = ";PL
6500 NS = N * (NN / 100) 9550 PRINT "PROT = ";P
6550 CA = C * (SC / 100) 9600 PRINT
6600 CB = C * (CC / 100) 9650 PRINT "VALUE OF NC (37'C) = "
6650 AS = P * VR 9700 PRINT
6700 NC = CA * VC 9750 PRINT "OF THE TOTAL LIPID S";
6750 AD = PL * VP 9800 AE = (TG) / (TG + CE)
6800 AE = (TG) / (TG + CE) 9850 AP = (CE) / (TG + CE)
6900 AQ = AE / 885.4 9900 AH = AF / 651.1
9800 PRINT 11200 PRINT "PHASE DISTRIBUTION
9850 PRINT "N = "":N2 OF CHOLESTEROL, C"
9900 PRINT "C = "":C2
9950 PRINT "PL = "":PL
10000 PRINT 11300 PRINT "ICO = "":ICO
10050 PRINT "OIL PHASE COMPOSITION (\%)
11000 PRINT "ICS = "":ICS"
10100 PRINT 11350 PRINT
10150 PRA 0 11400 PRINT "LIPROPROTEIN DIAMETER A (A) = "":A"
10200 IF (Z < = AB) THEN GOTO
11450 PRINT "R (A) = "":D"
10300 10500 PRINT
10250 GOTO 3850 11500 PRINT
10300 PRI 1 11550 PRINT "NUMBER OF LIPID MOL
10350 PRINT "OIL N = "":NO ECULES IN THE SURFACE, OIL,
10400 PRINT "OIL C = "":CO AND TOTAL LIPROPROTEIN PARTIC
10450 PRINT "OIL PL = "":PL LE"
10500 PRINT 11600 PRINT
10550 PRINT "SURFACE PHASE COMPO-
11650 PRINT "SURFACE PL = "":S
SITION (\%)
11700 PRINT "OIL PL = "":O
10600 PRINT 11750 PRINT "TOTAL PL = "":S + O
10650 PRINT "SURFACE N = "":NS
11800 PRINT
10700 PRINT "SURFACE C = "":CS
11850 PRINT "SURFACE C = "":U
10750 PRINT "SURFACE PL = "":PS
11900 PRINT "OIL C = "":N
10800 PRINT 11950 PRINT "TOTAL C = "":U + W
10850 PRINT "SURFACE/OIL LIPID M
12000 PRINT
ASS RATIO (MS/MO) = "":MB
12050 PRINT "SURFACE TG = "":AA
10950 PRINT "PHASE DISTRIBUTION
12100 PRINT "OIL TG = "":CC
OF NONPORAL LIPIDS, N"
12150 PRINT "TOTAL TG = "":AA + C
11000 PRINT 12200 PRINT
11050 PRINT "NO = "":AN
12250 PRINT "SURFACE CE = "":BE
11100 PRINT "N = "":SN
12300 PRINT "OIL CE = "":DO
11150 PRINT 12350 PRINT "TOTAL CE = "":FB + D
12400 PI 0
Carbon 13 NMR Studies of Saturated Fatty Acids Bound to Bovine Serum Albumin

I. THE FILLING OF INDIVIDUAL FATTY ACID BINDING SITES*

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$^{13}$C NMR chemical shift and intensity results for a series of carboxyl $^{13}$C-enriched saturated fatty acids (8–18 carbons) bound to bovine serum albumin (BSA) are presented as a function of increasing fatty acid (FA)/BSA mole ratio. Spectra for long-chain (≥12 carbons) FA–BSA complexes exhibited up to five FA carboxyl resonances, designated $a$, $b$, $b'$, $c$, and $d$. Only three resonances (peaks $b$, $b'$, and $d$) were observed below 3:1 FA–BSA mole ratio, and at ≥3:1 mole ratio, two additional resonances were observed (peaks $c$ and $a$). In a spectrum of 5:1 stearic acid–BSA complexes, peaks $b$, $b'$, and $d$ each represented approximately one-fifth, and peak $c$ approximately two-fifths, of the total FA carboxyl intensity. Plots of total carboxyl/carboxyl intensity ratio as a function of FA–BSA mole ratio were linear up to 7–9 mole ratio. Deviation from linearity at mole ratios ≥7 was accompanied by the detection of crystalline unbound FA (as 1:1 acid/soap) by x-ray diffraction. In contrast to long-chain FA–BSA complexes, $^{13}$C NMR spectra of octanoic acid–BSA complexes yielded only one FA carboxyl resonance (peak $c$) at FA–BSA mole ratios between 1 and 20. We conclude: (i) peaks $b$, $b'$, and $d$ represent FA bound to three individual high affinity (primary) long-chain FA binding sites on BSA; (ii) peak $c$ represents FA bound to several secondary long-chain (or primary short-chain) FA binding sites on BSA; (iii) peak $a$ represents long-chain FA bound to an additional lower affinity binding site. We present a model that correlates the observed $^{13}$C NMR resonances with individual binding site locations predicted by a recent three-dimensional model of BSA.

Utilization of circulating FFA$^1$ by tissues is influenced not only by the avidity of FA binding to albumin in the circulation (Scow and Chernick, 1970; Spector and Fletcher, 1978). In normal human subjects, this ratio is variable and is elevated under certain metabolic or environmental conditions such as fasting (Frederickson and Gordon, 1958) and/or prolonged exercise (Havel et al., 1967). Under certain pathological conditions, FFA/albumin ratios may be transiently or consistently elevated secondary to increased FFA mobilization (diabetic ketoacidosis, myocardial infarction, acute anxiety) or decreased circulating albumin (nephrotic syndrome, liver disease, familial hypoalbuminemia). It is conceivable that increased FFA production and/or decreased circulating albumin could result in abnormal partitioning of FFA into other components of the circulation (lipoproteins, blood cell membranes, endothelial cell membranes; Spector and Fletcher, 1978). This abnormal FFA partitioning might result in detrimental structural and/or functional alterations such as decreased neutrophil phagocytic and bacteriocidal activity (Hawley and Gordon, 1976), platelet aggregation (Hoak et al., 1970), and endothelial cell damage (Zilversmit, 1976).

As one approach to predicting FFA/albumin interactions at different mole ratios in vivo, the FA binding properties of albumin have been extensively examined in vitro using several approaches (for a review, see Spector, 1975). Binding data obtained from equilibrium partitioning methods have been analyzed by the Scatchard model (Scatchard, 1949) or the stepwise association model (Klotz et al., 1946; Spector et al., 1971). The Scatchard analyses for long-chain FA bound to human (Goodman, 1958) and bovine (Spector et al., 1969) albumin have yielded the concept of three classes of FA binding sites with respect to relative affinities. In contrast, the stepwise association analyses assumed no grouping of binding constants into classes and suggested that this grouping is somewhat arbitrary (Spector et al., 1971; Ashbrook et al., 1975). Second, mapping studies using peptide fragments (King and Spencer, 1970; King, 1973; Reed et al., 1975), chemical modifications (Koh and Means, 1979), or affinity labeling (Lee and McMammy, 1980) have aided in the localization of ligand binding sites to general regions on the polypeptide sequence. However, pitfalls include possible disruptive changes in protein conformation and binding properties following fragmentation, inconsistencies in affinity labeling, and a lack of specificity with chemical modification (Brown and Schockley, 1982). Third, spectroscopic studies using fluorescence (Sklar et al., 1977; Berde et al., 1979), ESR (Kuznetsov et al., 1975; Morris et al., 1975; Rehfild et al., 1978; Perkins et al., 1982), and NMR (Müller and Mead, 1973; Inoue et al., 1979) spectroscopy have yielded information concerning the physicochemical interactions of ligands and
albumin. However, pitfalls include the need for fatty acids containing structure-perturbing spin-label probes (ESR) or conjugated double bonds (fluorescence). Also, $^{13}$C NMR at natural abundance has been hampered by a lack of sensitivity (Kragh-Hansen and Rissom, 1976).

As an alternative approach, we have utilized $^{13}$C NMR spectroscopy with $^{13}$C-enriched fatty acids to investigate the interactions of biologically important FA with bovine albumin. Carbon 13 enrichment greatly enhances spectral sensitivity and permits investigation of FA/albumin interactions in the range of physiologically relevant FA/albumin mole ratios. Using this approach, we have shown that the carboxyl chemical shift of oleic acid bound to BSA is highly sensitive to the FA binding environment on albumin; spectra revealed multiple FA carboxyl resonances corresponding to multiple FA binding environments (Parks et al., 1988). In addition, we have utilized fatty acids with $^{13}$C enrichment in hydrocarbon chain carbons (C-3, C-14) to probe the interactions of myristic acid with BSA (Hamilton et al., 1984).

This paper presents $^{13}$C NMR results for a series of carboxyl $^{13}$C-enriched saturated fatty acids (8-18 carbons) bound to BSA as a function of increasing FA/BSA mole ratio. The results provide direct physicochemical information regarding the order of filling and saturation of individual FA binding sites with increasing FA/BSA ratio as well as the relative occupation of individual sites at a given mole ratio. Second, the results delineate differences between shorter chain and longer chain FA with regard to the number and type of FA binding sites. Third, accompanying powder x-ray diffraction results provide information about the physical state of unbound FA at high mole ratios. Finally, the results permit direct correlation of observed NMR resonances with the FA binding sites predicted by recent models of BSA structure (Brown and Shockley, 1982) and provide insights into the binding locations of FA at different FA/albumin ratios in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Essentially FA-free crystallized lyophilized BSA was purchased from Sigma (A-7511, lot 22F-9340). The supplier used method IV of Cohn et al. (1947) to recrystallize fraction V albumin and the charcoal defatting procedure of Chen (1967) to remove bound fatty acid. The content of bound fatty acid, as determined by gas-liquid chromatography, was <0.02 mol of FA/mol of BSA prior to the addition of FA. The protein content of the BSA sample was assigned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the gels were overloaded with sample in order to search for minor impurities. In addition to the major albumin band at 66,000 daltons, minor bands were observed at ~120,000 (~5%), 55,000 (~1%), and 160,000 (~1%). These minor bands most likely corresponded to BSA dimers (Friedl and Kietler, 1970; Foster, 1977), e$, antitrypsin (Leurell and Jeppson, 1975), and immunoglobulins, respectively (Putnam, 1975; Peters, 1975). Although apoprotein A-I is often present as a contaminant in commercial albumin preparations (Fainaru and Deckelbaum, 1979), no bands were observed at the appropriate molecular weight (28,000).

The dimer/polymer content of Sigma A-7511 BSA (with added FA), as determined by column chromatography (Parks et al., 1985), was 25%. To determine whether NMR results were affected by the presence of disulfide-linked dimers and polymers, Sigma BSA was further fractionated by gel filtration chromatography. A 2.0 ml aliquot of hydrated BSA (100 mg/ml) was applied to a column of Sephadex G-150 (90 x 2.6 cm) equilibrated with 20 mM KCl, 0.1% NaN$_3$ at 4°C. 6 ml fractions were collected at a flow rate of 7 ml/h. The protein concentration of each fraction was determined by absorbance at 279 nm. The elution profile was essentially identical to one previously published (Morisset et al., 1975). Fractions containing monomeric BSA were pooled and concentrated by ultrafiltration using Amicon (Danvers, MA) PM-10 filters. The final monomeric BSA sample contained 1.5 ml of 60 mg/ml BSA. No reformation of dimers/oligomers occurred in the concentrated monomeric fraction, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. $^{13}$C NMR spectra for C$_{16}$:0-BSA complexes using monomer or unfractiected BSA (obtained under identical conditions) were indistinguishable. This result is consistent with previous observations for C$_{18}$:1-BSA complexes (Parks et al., 1985). Therefore, unfractiected BSA was used throughout this study.

$^{13}$C carboxyl-enriched (90%) fatty acids were purchased from KOR Isotopes (Cambridge, MA) (C$_{6}$:0, C$_{8}$:0, C$_{10}$:0, C$_{12}$:0) and Merck Sharp and Dohme Isotopes (St. Louis, MO) (C$_{14}$:0). Sample purity as determined by thin layer chromatography (hexanes:ethyl ether, acetic acid, 90:9:1) was >98%. In addition, no impurities were visualized by H NMR for $^{13}$C-enriched FA samples dissolved in deuterated chloroform.

**Sample Preparation**—BSA solutions (7%, w/v) were prepared using doubly distilled deionized water. After adjusting the pH to 7.4, the solutions were centrifuged at 16,000 rpm for 30 min to remove trace amphoters. The protein concentration was determined from the absorbance at 279 nm of 1:100 dilutions using an extinction coefficient of 6.67 for a 1% sample (Janota et al., 1968). Crystalline of $^{13}$C-enriched FA were dissolved in 2:1 chloroform/methanol, and the concentrations were determined by measuring dry weights on an electrobalance (Cahn model 25, Cerritos, CA). Stoichiometric amounts of fatty acids in solvent were added to 10-mm NMR tubes, and the solvent was evaporated under N$_2$. D$_2$O (200 µl) and 1.2 eq of 1 N KOH were added, and samples were thoroughly mixed until all BSA crystals dissolved to form an optically clear micellar soap solution. Hydrated BSA samples (1.8 ml, pH 7.4) were added to the soap solutions (0.2 ml) with continuous vortexing for several minutes, and samples were equilibrated and intermittently vortexed for 30 min. Potassium stearate solutions formed a gel phase at room tempature and had to be gently and briefly heated before BSA solutions were added. Sample pH was adjusted from pH 7.5-7.6 (following mixing) to 7.4 and samples were equilibrated at room temperature (25°C) for 8-12 h prior to $^{13}$C NMR experiments. The NMR results were independent of equilibration time.

All pH measurements were made directly in the NMR tube using a pH meter (Beckman 3560, Fullerton, CA) equipped with a 29 cm x 4 mm glass combination electrode (Markson MiraMark, Phoenix, AZ). Values measured before and after obtaining NMR spectra agreed within 0.1 pH unit.

The final FA-BSA samples used for NMR contained from 0.5 to 20 mol of FA/mol of BSA and 7% w/v protein. As reported elsewhere (Cistola, 1985), $^{13}$C NMR spectra (FA as well as protein resonances) for C$_{16}$:0-BSA complexes at 3.8, 7.5, and 11.4% w/v BSA (all at 51 mol ratio, pH 7.4, 35°C) were essentially identical. Hence, it is unlikely that noncovalent protein aggregation occurred over this concentration range. The salt concentrations (as determined by flame photometry) of 7% hydrated samples with 1.5% Na$_2$SO$_4$, sodium, and potassium. No salt was added to FA-BSA samples in this study. Addition of KCl to FA-BSA samples up to a final concentration of 0.1 M does not change $^{13}$NMR results provided that the sample temperature is kept below 38°C (Cistola, 1985).

**$^{13}$C NMR Spectroscopy**—$^{13}$C NMR spectra were obtained on a Bruker WP-200 NMR spectrometer (Billerica, MA) as described elsewhere (Hamilton and Small, 1981; Cistola et al., 1982). Internal D$_2$O was used as a lock and shim signal. Chemical shift values were measured digitally with an estimated uncertainty of ±0.1 ppm. The chemical shift (δ = 39.57 ppm) of the narrow resonance from protein ε-Lys/ε-Leu carbons (Gurd and Reim, 1973) was used as an internal reference after calibrating this resonance against external tetramethylsilane. To enhance spectral resolution in selected cases, the convolution difference method was used (Campbell et al., 1975). FA carboxyl/BSA carboxyl intensity ratios were measured using the integration routine provided in the Bruker DISNMR program. NMR sample temperatures were controlled to 34 °C and measured as described previously (Cistola et al., 1982). Spin-lattice relaxation times (T$\text{L}_1$) were measured using a fast inversion recovery technique (Canet et al., 1975) and calculated using a three-parameter fitting routine (Sass and Ziesow, 1977). Nuclear Overhauser enhancements (NOE) were determined from comparisons of peak heights from spectra accumulated with broad-band and inverse-gated decoupling (Opella and Allara, 1976). For all spectral accumulations, pulse intervals were equal to the $T_1$ value of the largest FA carboxyl peak in the spectrum, and 90°C pulses (15 µs) were used.

NOTE: In our previous study, a 90 x 2.5-cm column was used for preparative fractionation of monomeric BSA, rather than a 90 x 1.5-cm column (incorrectly noted in Fig. 1 caption of Parks et al., 1985).
13C NMR of Fatty Acid–Albumin Interactions

X-ray Diffraction—For samples containing suspended crystalline material (>7:1 FA : BSA), the material was pelleted by centrifugation at 10,000 rpm for 30 min at 30 °C. The pellet was transferred to 1-mm quartz capillary tubes (Charles Supper Co., Natick, MA) and the capillaries were placed in a sample holder kept at constant temperature (30 °C) by a circulating antifreeze/water bath. Nickel-filtered CuKα x-radiation (λ = 1.5418 Å) from a microfocus x-ray generator (Jarrell-Ash, Waltham, MA) was focused by a single nickel-coated mirror and further collimated by a Luzzati-Baro camera with slit optics. Low-angle powder x-ray diffraction patterns were recorded with a position-sensitive detector (Tennelec PSD-1100, Oak Ridge, TN) and a computer-based analysis system (Tracer Northern TN-1710, Middleton, WI).

RESULTS

13C NMR spectra at various C14:0 BSA mole ratios (at fixed pH, BSA concentration, ionic strength, and temperature) are shown in Fig. 1, A–E. The broad envelope centered at ~176 ppm represents carbonyl carbons of glutamine, asparagine, and the peptide backbone (Gurd and Keim, 1973) as well as aspartate carbonyl carbons (Shindo and Cohen, 1976) of BSA. The narrower resonances falling between 179 and 184 ppm primarily represent carbonyl carbons of 13C-carboxyl-enriched FA bound to BSA (Parks et al., 1983; Cistola et al., 1983; Hamilton et al., 1984), except for the resonance at 180.9–181.1 ppm, which represents protein glutamate carbonyl carbons (Shindo and Cohen, 1976). Protein-free saturated FA (>10 carbons) exist as crystalline 1:1 acid/soap compounds at pH 7.4 and 35 °C (Cistola et al., 1986), and crystalline phases do not give rise to high resolution NMR resonances. Furthermore, none of the observed carbonyl resonances had chemical shifts coincident with those of soluble short-chain FA in water (without protein) under the same conditions (Cistola et al., 1988; Cistola, 1985). Hence, the observed carbonyl resonances do not represent unbound FA. In addition, none of these resonances represented BSA peaks that shifted into the carbonyl region upon FA binding, since spectra of FA-BSA complexes using C14:0 with no 13C enrichment were identical to FA-free BSA samples (Fig. 1A). In order to compare FA carbonyl peaks in different FA-BSA spectra, we have named analogous FA carbonyl peaks, based on their chemical shifts at pH 7.4 (and their ionization behavior; Cistola et al., 1987), as follows: peak a (183.7–184.1 ppm), peak b (182.5 ppm), peak b' (182.3–182.4 ppm), peak c (181.8–182.1 ppm), and peak d (180.4–180.7 ppm). In addition, we have named the glutamate carbonyl resonance as peak pr (180.9–181.1 ppm). It is notable that peak b' was not observed for C14:0-BSA complexes (Parks et al., 1983) either because peak b' was not present or because it was not resolved from peaks b and c.

For most of the FA-BSA spectra presented in this study, the intensities of individual FA carbonyl peaks could not be quantitatively measured as peak areas or peak heights because of closely overlapping FA or protein carbonyl resonances. Therefore, increases in FA carbonyl peak intensity with increasing mole ratio are represented qualitatively in the form of difference spectra. Digital subtraction of the upper spectra from the lower spectra yielded the difference spectra shown in the right column of Fig. 1 (F–I). The difference spectra contained only those FA carbonyl peaks which increased or decreased in intensity or changed chemical shifts between the two different mole ratios. The intensities resulting from unperturbed carbonyl or carbonyl resonances of BSA were subtracted out by this procedure.

At 1:1 mole ratio (Fig. 1B), peaks b and b' were clearly visible, but peak d was difficult to distinguish because of closely overlapping protein glutamate resonance(s). However, subtraction of FA-free BSA (Fig. 1A) from 1:1 C14:0-BSA (Fig. 1B) revealed that peak d was present at 1:1 mole ratio (Fig.

Fig. 1. Carbonyl/carboxyl region of 13C NMR spectra (A–E) and difference spectra (F–I) for C14:0-BSA complexes with different C14:0 BSA mole ratios at pH 7.4 and 34 °C. Difference spectra were obtained by digitally subtracting a spectrum at a given mole ratio from one at a higher mole ratio. This method removes BSA resonances from spectra and shows which FA carbonyl resonances increased between two corresponding FA-BSA mole ratios. The pairs of spectra which were subtracted are indicated by the dashed lines in the middle of the figure. The lower case letters above each peak indicate specific FA carbonyl resonances with characteristic chemical shifts (see "Results"). For all samples, the BSA concentration was 7% (w/v). All spectra were recorded after 6,000 accumulations with a pulse interval of 2.0 s, 16,384 time domain points, and a spectral width of 10,000 Hz. Line broadening (3 Hz) was used in all spectral processing: A, FA-free BSA spectrum; B, 1:1 C14:0-BSA spectrum; C, 3:1 C14:0-BSA spectrum; D, 5:1 C14:0-BSA spectrum; E, 7:1 C14:0-BSA spectrum; F, difference spectrum, 1:1 C14:0 BSA minus FA-free BSA; G, difference spectrum, 3:1 C14:0-BSA minus 1:1 C14:0 BSA; H, difference spectrum, 5:1 C14:0-BSA minus 3:1 C14:0-BSA; I, difference spectrum, 7:1 C14:0-BSA minus 5:1 C14:0 BSA.

1F). Between 1:1 and 3:1 mole ratio (Fig. 1, B, C, and G), peaks b, b', and d increased, and peaks c and a appeared above 2:1 mole ratio (2:1 spectrum not shown). Between 3:1 and 5:1 mole ratio (Fig. 1, C, D, and H), intensity increases occurred in all five FA carbonyl peaks. Between 5:1 and 7:1 mole ratio (and up to 13:1 mole ratio), only peak c increased in intensity (Fig. 1, D, E, and I). Peak d decreased in intensity between 5:1 and 7:1 mole ratio (Fig. 1I).

A plot of total carbonyl/carboxyl area ratio as a function of C14:0-BSA mole ratio is shown in Fig. 2 (circles). The plot is linear up to 8–9:1 mole ratio, above which point the sample became increasingly turbid (suspended crystals; downward pointing arrow in Fig. 2). The samples were centrifuged and yielded a transparent supernatant and a crystalline pellet.
Examination of the pellet by powder x-ray diffraction showed first order long spacings (41.0 ± 0.9 Å) characteristic for crystalline 1:1 potassium (or sodium) hydrogen dimyristate, a 1:1 acid/soap compound (Piper, 1929; Cistola et al., 1986). Therefore C16:0-BSA samples at high molar ratio contained unbound FA in the form of crystalline 1:1 acid/soap. In general, long-chain fatty acids in water (<micromolar concentrations) form 1:1 acid/soap crystals or fatty acid/soap lamellar liquid crystals between pH 7 and 8 (Small, 1986).

$^{13}$C NMR spectra and difference spectra at various C18:0-BSA mole ratios are shown in Fig. 3, A–I. At 1:1 mole ratio, peaks b, b', and d were present (Fig. 3, B and F), although peak d was barely detectable. Peak d was much more clearly seen at 2:1 (spectrum not shown) and 3:1 (Fig. 3, C and G). Between 1:1 and 3:1 mole ratio, peaks b, b', and d increased in intensity (Fig. 3, B, C, and G). Between 3:1 and 5:1 mole ratio, peaks c and a became visible and all five FA carbonyl peaks increased in intensity (Fig. 3, C, D, and H), and between 5:1 and 7:1 (spectrum not shown), peaks c, b/b', and a increased in intensity (Fig. 3, D, E, and F).

A plot of total carboxyl/carboxyl area ratio as a function of C18:0-BSA mole ratio (not shown) was linear up to about 7:1 mole ratio. Deviation from linearity was accompanied by the visual appearance of sample turbidity (suspended crystals) at and above 7:1 mole ratio. As with C14:0-BSA (see above), these crystals most likely represent unbound C18:0 in the form of crystalline 1:1 acid/soap.

$^{13}$C NMR spectra (but not difference spectra) for C12:0-BSA complexes at four mole ratios are shown in Fig. 4. These spectra were very similar to corresponding spectra for C14:0-BSA complexes. At 1:1 mole ratio, peaks b/b' and pr/d were visualized; at 3:1, three FA peaks were visualized (b, b', d). At 5:1 and 7:1 mole ratios, all five FA carbonyl peaks were distinguishable. Convolution difference spectra (not shown) permitted greater resolution of peaks b and b'.

A plot of total carboxyl/carboxyl area ratio as a function of C12:0-BSA mole ratio (not shown) was essentially identical to that for C14:0-BSA (Fig. 2, circles). Deviation from linearity and the appearance of sample turbidity (crystal formation) occurred above 8:9:1 mole ratio. Centrifugation and examination of crystals by powder x-ray diffraction revealed first order long spacings (35.4 ± 0.7 Å) characteristic for crystalline 1:1 potassium (or sodium) hydrogen laurate, an acid/soap compound (Cistola et al., 1986).

$^{13}$C NMR spectra for C16:0-BSA complexes as a function of mole ratio (Cistola, 1985) are not shown here. Cistola et al. (1983) show that for C16:0-BSA complexes. A plot of total carboxyl/carboxyl area ratio as a function of C16:0-BSA mole ratio is shown in Fig. 2 (triangles). Deviation from linearity and sample turbidity (crystals) appeared at and above 7:1 mole ratio. The crystals most likely represented crystalline 1:1 acid/soap, as was demonstrated by x-ray diffraction, for C12:0-BSA and C14:0-BSA samples.

Although peaks b, b', and c closely overlapped in many FA-BSA spectra, peaks d and a were nearly completely resolved from the b/b'/c envelope, and their relative intensities (areas) could be quantitatively estimated (Table I). To determine the relative area of peak d, the area from the glutamate carboxyl resonance (peak pr) had to be subtracted out (see legend to Table I). The results indicate that the relative intensities of peak d increased and peak a decreased, with increasing FA chain length. However, the relative intensity of the b/b'/c

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TABLE I
Relative $^{13}$C NMR intensities of peaks a and d

The areas of all resonances were measured by integration (see "Experimental Procedures"). The areas of peak d and the total FA carboxyl region were determined by subtracting out the contribution of BSA glutamate carboxyl resonances. This glutamate contribution (glutamate/carboxyl ratio) was determined from spectra of FA-free BSA accumulated under the same conditions as FA/BSA spectra. All three samples reported in this table contained 5:1 mole ratio of FA/BSA. $C_{14,0}$-BSA and $C_{16,0}$-BSA results were derived from spectra shown in Figs. 1D and 3D, respectively, and $C_{18,0}$-BSA results were derived from a spectrum not shown here (Cistola, 1985). The estimated uncertainty is ±10%.

<table>
<thead>
<tr>
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<tr>
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<tr>
<td>$C_{14,0}$-BSA</td>
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</tr>
<tr>
<td>$C_{16,0}$-BSA</td>
<td>0.7</td>
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<tr>
<td>$C_{18,0}$-BSA</td>
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Fig. 5. Carboxyl/carboxyl region of $^{13}$C NMR spectra for 1-$^{13}$C C$_{18,0}$-BSA with increasing C$_{18,0}$-BSA mole ratio at pH 7.4 and 34 °C. The numbers at the right of each spectrum indicate the mole ratio of C$_{18,0}$-BSA. Spectra were recorded after 4000 accumulations with a pulse interval of 2.8 s. All other spectral conditions are as described in the legend to Fig. 1. The BSA concentration was 7.5% (w/v).

chemical shift (ppm)

182.1 ppm; this value suggested that this FA resonance was analogous to peak c in the other FA-BSA spectra. This correlation was also supported by NMR titration results which demonstrated that peak c was the only one of the five observed FA carboxyl peaks to exhibit a titration shift between pH 7.4 and 3.0 (Cistola et al., 1987). The chemical shift of peak c in C$_{18,0}$-BSA spectra increased with increasing mole ratio from 182.0 ppm (1:1 and 3:1 mole ratio) to 182.1 ppm (5:1), 182.2 ppm (7:1), 182.3 ppm (9:1), and 182.9 ppm (20:1). The line widths of this resonance remained narrow (<10 Hz) at all mole ratios studied.

$^4$Unbound C$_{18,0}$ under these conditions would have existed as monomers in solution, rather than crystalline or liquid-crystalline aggregates or micelles. For C$_{18,0}$ BSA samples at >3:1 mole ratio, a progressive increase in the chemical shift of peak c (see "Results") toward the chemical shift of monomeric aqueous C$_{18,0}$ (184.2 ppm) provided evidence that monomeric unbound C$_{18,0}$ was present at concentrations >1 µM and in rapid exchange (>100 exchanges/s) with protein-bound C$_{18,0}$.  

envelope exhibited little or no change with increasing FA chain length. (Results for C$_{12,0}$-BSA were not included because peak d was not well resolved from the b-b'-c complex; Fig. 4.)

$^{13}$C NMR spectra for C$_{18,0}$-BSA complexes (Fig. 5), unlike those for all other FA-BSA complexes studied, showed only one FA carboxyl peak at all mole ratios (up to 20:1). At 5:1 mole ratio (pH 7.4), the chemical shift of this resonance was
13C NMR spectroscopy using carboxyl 13C-enriched saturated FA permitted resolution of FA carboxyl resonances from all BSA resonances (except glutamate carboxyl carbons) and provided good signal-to-noise ratios at physiologically relevant mole ratios (as low as 0.5 mol of FA/mol of BSA). Except for C16:0-BSA, all FA-BSA complexes studies exhibited multiple carboxyl resonances, each corresponding to FA bound in a different binding environment. 13C NMR spectra and difference spectra for FA-BSA samples with increasing FA/mole ratio provided a method for observing which binding sites were occupied at a given mole ratio and which binding sites filled with increasing mole ratio. The results for C16:0-BSA and C18:0-BSA, shown in Figs. 1 and 3, respectively, demonstrated that at low FA-BSA mole ratios (≤1:1), only three FA carboxyl peaks were observed (b, b', and d). At or above 1:1 mole ratio, two additional FA carboxyl peaks (c and a) were observed for C16:0-BSA and C18:0-BSA samples. Peak c demonstrated the largest intensity increase between 3 and 7 mole ratio; peak a also increased between 3 and 7 mole ratio but remained relatively small. For samples containing ≥7 mole

4 Strictly speaking, each observed NMR peak could represent the weighted average of FA in two or more binding environments in fast exchange. However, it is likely that the exchange rates between all long-chain FA binding environments on BSA were slow since the rates of hydration of long-chain FA at 37 °C are slow (k = 0.1-0.01 s⁻¹; Daniels et al., 1985). Therefore, we have assumed that each FA carboxyl peak represents only bound FA in slow exchange between different binding environments.

5 Throughout this manuscript, long-chain FA are arbitrarily defined as FA containing ≥12 carbon atoms. Similarly, short-chain FA are defined as FA containing <12 carbon atoms. This nomenclature is used in order to be congruent with a pertinent review article (Brown and Schockley, 1982).
spectrum (data not presented). Therefore, peaks $b$, $b'$, and $d$ each represent about one molecule of FA/molecule of BSA, at least in $C_{20}$-BSA spectra. According to Brown and Schockley's model, binding sites 1-C, 2-C, and 2-C can each accommodate one FA molecule. (iii) According to NMR titration results at low pH (Cistola et al., 1987) the FA carboxyl groups represented by peaks $b$, $b'$, and $d$ (but not peak $c$) were involved in electrostatic interactions with positively charged residues on BSA. In the model, binding sites 1-C, 2-C, and 3-C each contain clusters of positively charged residues at the mouths of the binding channels (Fig. 6). (iv) According to NMR titration results at high pH (Cistola et al., 1987), the FA carboxyl groups represented by peak $b'$ (but not peaks $b$ and $d$) are directly adjacent to the lysine residue(s). Since only one of the three high affinity FA binding sites contains lysine residues (site 3-C), we proposed that peak $b'$ represents FA bound to site 3-C (Cistola et al., 1987) and, for reasons mentioned below, peak $d$ represents FA bound to site 2-C. By the process of elimination, peak $b$ represents FA bound to site 1-C.

The evidence for assigning peak $c$ to binding sites 1-AB, 2-AB, and 3-AB (Fig. 6, Scheme A) is as follows. (i) In $C_{20}$-BSA spectra (Fig. 5), peak $c$ was the only FA carboxyl peak observed at low mole ratios. Therefore, peak $c$ represents FA bound to primary short-chain FA binding site(s). Since peak $c$ was also present in spectra of long-chain FA-BSA complexes at $>3:1$ mole ratio, peak $c$ may also represent FA bound to secondary (lower affinity) long-chain FA binding sites. According to the model, the primary short-chain FA (or secondary long-chain FA) binding sites are sites 2-AB, 3-AB, and perhaps 1-AB (Brown and Schockley, 1982). (ii) The intensity of peak $c$ increased with increasing $C_{20}$-BSA mole ratio above 1:1 (Fig. 5). Therefore, peak $c$ must represent more than 1 mol of $C_{20}$-BSA at mole ratios $>1$. Similarly, the intensities of peak $c$ represent $>1$ mol of FA at long-chain FA-BSA mole ratios $>5$. Although peak $c$ may represent several molecules of FA (per molecule of BSA) bound at several different locations on BSA, the local molecular environments experienced by each FA carboxyl group must be sufficiently similar, or the exchange rates between different sites must be sufficiently rapid, to yield only one narrow peak (one chemical shift). (iii) According to NMR titration data (Cistola et al., 1987), the FA carboxyl groups represented by peak $c$ are not involved in strong electrostatic interactions with BSA and are accessible to the aqueous milieu. In addition, the length of the narrow hydrophobic binding channel running through the middle of each of the three subdomains is 30 Å (Fig. 6), a distance not long enough to accommodate two noninterdigitating $C_{20}$-BSA molecules (19–20 Å each). Therefore, FA molecules represented by peak $c$ are probably bound hydrophobically (not electrostatically) with their carboxyl groups and several methylene carbons protruding from the channel (Fig. 6, Scheme A).
The intensity data also showed albumin can bind >6 mol of FA/mol of albumin. This additional intensity fell entirely under peak c. The fact that FA bound in these additional locations gave rise to chemical shifts identical to those for FA bound to the AB sites could be because the FA carboxyl environment is the same (i.e. exposed to solvent) or, less likely, because these bound FA are in rapid exchange with FA bound to the AB sites.

The evidence for assigning peak a to binding site 2-AB (Fig. 6, Scheme B) is as follows. (i) Peak a was observed only at mole ratios ≥3; therefore, peak a represents FA bound to lower affinity binding sites (i.e. the AB sites). (ii) According to NMR titration data at high pH (Cistola et al., 1987), FA carboxyl groups represented by peak a are directly adjacent to lysine residues. The lower affinity sites 2-AB and 3-AB (but not 1-AB) contain several lysine residues near the opening of the binding channel (Brown and Schockley, 1982). (iii) As shown in Table II, the relative intensities of peaks a and d changed reciprocally; as peak d increased, peak a decreased and vice versa. These results suggested a structural interaction between bound FA molecules represented by NMR peaks a and d. Since peak a cannot represent FA bound in site 1-AB (see above) and peak d cannot represent FA bound in site 3-C (Cistola et al., 1987), we propose that the structural interaction between two FA molecules occurs through the 30 Å binding channel in domain 2. Hence, an FA molecule bound to site 2-AB with its carboxyl group interacting with nearby lysines would give rise to NMR peak a (Fig. 6, Scheme B). In the same BSA molecule, FA bound to site 2-C would be unable to interact electrostatically with amino acids at the mouth of the channel because of constraints in the length of the binding channel, and this FA molecule would give rise to NMR peak c (Fig. 6, Scheme B). Alternatively, an FA molecule bound to site 2-C in a manner allowing electrostatic interactions (Fig. 6, Scheme A) would give rise to peak d, and an FA molecule bound to site 2-AB would be unable to interact electrostatically with amino acids at the mouth of the channel because of constraints in the length of the binding channel. Hence, the latter would give rise to NMR peak c. Each binding arrangement (Scheme A or B) would result in the same relative intensity of peak c if all six binding sites were filled. According to this binding arrangement (Fig. 6), FA molecules bound to domain 2 of a given BSA molecule could give rise to either NMR peak d or peak a, but not both.

Since each 13C NMR spectrum of FA-BSA complexes represents FA binding arrangements averaged over a population of BSA molecules, we propose that in a given FA-BSA sample, some individual BSA molecules bind FA in an arrangement depicted by Scheme A, and other molecules, by Scheme B. As FA chain length increases, Scheme A is favored over Scheme B.

In conclusion, 13C NMR spectra of FA-BSA complexes as a function of mole ratio have yielded physicochemical information regarding the order of filling and relative occupation of individual FA binding sites on BSA. In lieu of three-dimensional crystal structures obtained from x-ray crystallography, this 13C NMR approach may provide valuable information regarding FA interactions with individual binding sites on other proteins (e.g. cytosolic fatty acid binding proteins, α-fetoprotein, human serum albumin).

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