Binding of ethyl oleate to low density lipoprotein, phospholipid vesicles, and albumin: a ^{13}C NMR study

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Abstract Fatty acid ethyl esters (FAEE), esterification products of ethanol and fatty acids, have been implicated as mediators of ethanol-induced organ damage. After ethanol ingestion in humans, FAEE circulate in blood, bound to lipoproteins and albumin. We have analyzed the binding of ethyl (1-^{13}C, 99%) oleate (EO) to small unilamellar phospholipid vesicles (SUV), human low density lipoprotein (LDL), and bovine serum albumin (BSA) by ^{13}C-NMR spectroscopy. Binding of ≤25 mol% EO to SUV yielded a single EO carbonyl peak (172.6–172.9 ppm) downfield from that of EO oil (171.9 ppm). Thus, the carbonyl forms hydrogen bonds with water in the SUV aqueous interface. At 30 mol% EO in SUV, a second EO carbonyl peak appeared, indicating a limit in FAEE solubility in SUV. Addition of EO to isolated human LDL yielded a peak at 171.9 ppm, suggesting that the EO exists in an unhydrated environment, most likely the core of the lipoprotein. This binding was also observed using high levels of EO added to human serum. The addition of EO dissolved in ethanol or as an oil to a solution of BSA yielded no visible EO peak, whereas addition of (1-^{13}C, 99%) oleic acid resulted in several narrow peaks, demonstrating a much greater affinity of BSA for oleic acid than for EO. Bidirectional transfer of EO between LDL and SUV was observed and was 85% complete within 30 min. There was no measurable transfer of EO from LDL or SUV to albumin. The weak binding of EO to albumin will result in increased transport of EO by lipoproteins as plasma levels of EO increase.—**Bird, D. A., M. Laposata, and J. A. Hamilton.** Binding of ethyl oleate to low density lipoprotein, phospholipid vesicles, and albumin: a ^{13}C NMR study. *J. Lipid Res.* 1996. **37:** 1449–1458.

Supplementary key words plasma • bilayer • alcoholism • ethyl ester

Fatty acid ethyl esters, esterification products of ethanol and fatty acids, have been implicated as mediators of ethanol-induced organ damage. In a study of individuals who were acutely intoxicated at the time of death, fatty acid ethyl esters (FAEE) and the enzyme responsible for their synthesis known as FAEE synthase were found selectively in the organs damaged by ethanol abuse (1). It was implied by this study that FAEE may mediate ethanol-induced organ damage because it is known that ethanol itself is unable to produce organ damage. In addition, the oxidative products of ethanol metabolism such as acetaldehyde are not found in all the organs damaged by ethanol abuse, particularly the pancreas, which is commonly affected. Recently, it has been demonstrated that FAEE reconstituted in low density lipoprotein (LDL) particles can be incorporated into HepG2 cells and subsequently decrease their rate of cell proliferation and protein synthesis (2). This was the first direct demonstration that FAEE could be incorporated and produce a detrimental effect in intact cells.

FAEE are found circulating in the blood after ethanol ingestion (3). Moreover, in the blood, they are bound to lipid carriers, consistent with their neutral, hydrophobic structure. Approximately 30% of the FAEE were bound to lipoproteins, and the remainder were bound to a protein in the fraction with a density >1.21 g/ml in ultracentrifugation preparations, which was shown to have a molecular weight corresponding to that of albumin (3). It is not known whether the FAEE found in the blood are generated in the liver and/or the pancreas and secreted into the blood or whether they are made by white blood cells, which have been shown to have a small amount of FAEE synthase activity (4).

The goals of the present investigation were *i)* to determine molecular interactions of FAEE with lipid carriers in the circulation and model membranes, and *ii)* to measure their partitioning among various environments. We have utilized a biophysical approach, ^{13}C

Abbreviations: FAEE, fatty acid ethyl esters; EO, ethyl oleate; SUV, small unilamellar phospholipid vesicles; LDL, low density lipoprotein; BSA, bovine serum albumin; PC, phosphatidylcholine.

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NMR spectroscopy, to assess binding of FAEE to albumin, LDL, and lipid bilayers in the form of small unilamellar vesicles. ^{13}C NMR approaches permit assessment of binding of lipids to model membranes and proteins in both simple and complex mixtures (5–13). The chemical shift of the carbonyl carbon was the major focus because it is a sensitive indicator of the molecular environment of the polar region of carbonyl-containing lipids, such as triglycerides (5, 9), diglycerides (11), cholesteryl esters (7, 12), and fatty acids (13). In the present study we demonstrated binding of FAEE to lipoproteins and phospholipid bilayers. We also demonstrated that fatty acids have a much greater affinity than FAEE for albumin. In studies of the transfer of FAEE between lipid carriers, bidirectional transfer between phospholipid bilayers and LDL was found, but minimal FAEE transfer to albumin was observed. FAEE partitioning between LDL and phospholipid membranes depended on the ratio of LDL to phospholipid. These data indicate that albumin has a much lower affinity for FAEE than for fatty acids, although its much greater concentration than lipoprotein particles in plasma could result in significant binding of FAEE in the circulation.

MATERIALS AND METHODS

FAEE synthesis

^{13}C-enriched ethyl palmitate and ethyl oleate were synthesized from (1,1,1-$^{13}C_3$, 99%) tripalmitin and (1,1,1-$^{13}C_3$, 99%) triolein (Cambridge Isotope Laboratories, Woburn, MA), respectively, as described by Turk et al. (14) using ethanol in the place of methanol. Briefly, the triglycerides, dissolved in dichloromethane, were incubated with 0.5 M KOH in ethanol for 45 min at room temperature. Upon termination of the reaction with 6 M HCl, the lipids were extracted into dichloromethane and the FAEE were purified by thin-layer chromatography (TLC) on a silica gel 60 plate (E. Merck, Darmstadt, Germany) developed in petroleum ether–diethyl ether 75:5 (v/v). After the ^{13}C-enriched FAEE were eluted with acetone, the solvent was evaporated under nitrogen and the FAEE were resuspended and stored in chloroform. The concentration of FAEE for binding studies was determined by measuring the dry weights.

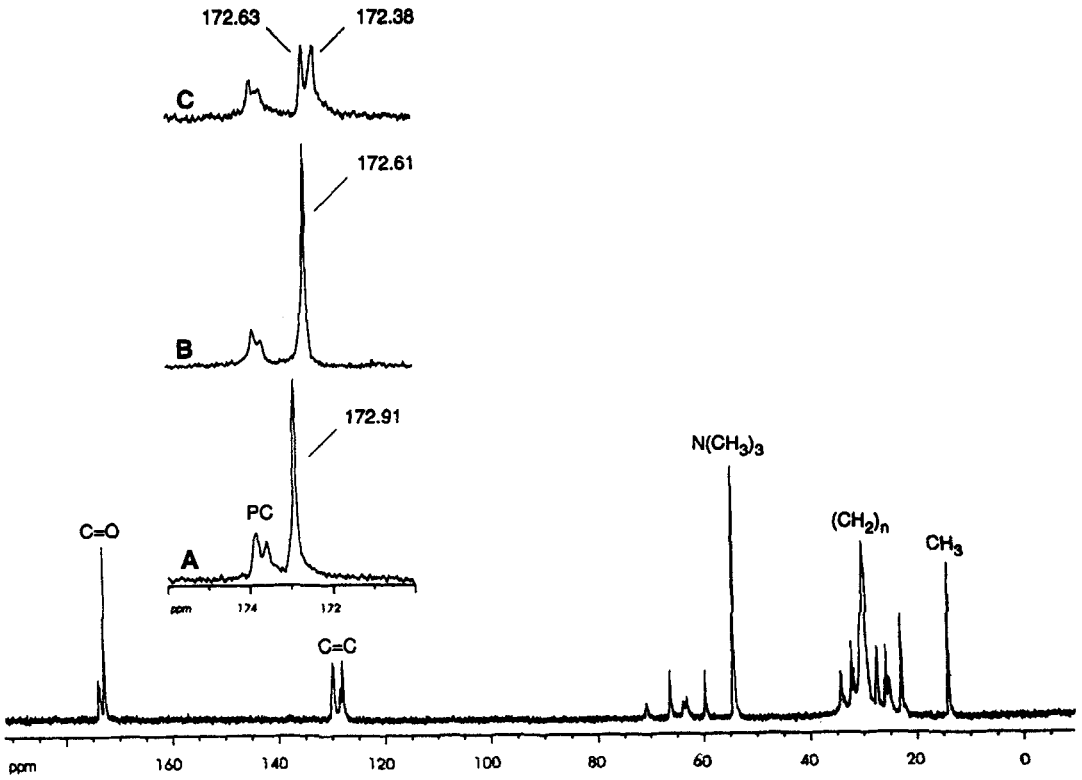

Fig. 1. *Solubility limit of ethyl oleate in phospholipid bilayers* (egg PC SUV) suspended in 0.56% KCl. NMR spectra were obtained on a Bruker AMX 300 NMR spectrometer operating at 75 MHz. Spectra shown are the result of 1,000 accumulations with 16 K time domain points, a spectral width of 15,150 Hz and a pulse interval of 8.0 s. The entire NMR spectrum of 5 mol% ethyl [1-13C]oleate in SUV is shown with the carbonyl (C=O), olefinic (C=C), choline ($NCH_3)_3$, methylene (CH_2), and methyl (CH_3) groups labeled. The three insets are expansions of the carbonyl region at (A) 5 mol%, (B) 25 mol%, and (C) 30 mol% with the chemical shift of the carbonyl of ethyl oleate indicated in ppm.

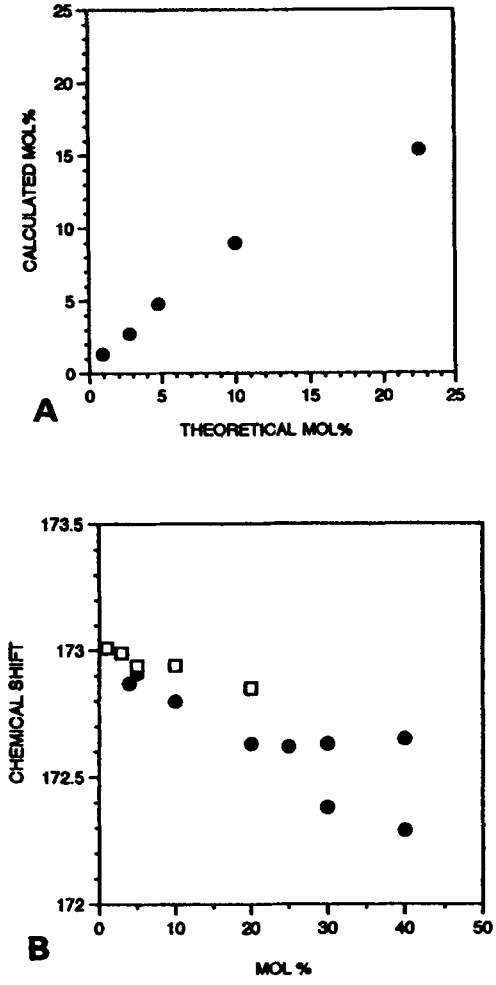

Fig. 2. The ^{13}C-carbonyl chemical shift and peak intensity of FAEE in lipid bilayers as a function of FAEE content. SUV were prepared with egg phosphatidylcholine and increasing amounts of ethyl palmitate or ethyl oleate. (A) Integration of the carbonyl peaks of the ethyl palmitate in SUV spectra was used to calculate the mole percent of FAEE incorporated into vesicles and compared to the mole% in the sonication mixture ("theoretical mole percent.") (B) The chemical shift in ppm of the carbonyl peak of ethyl palmitate (open squares) and ethyl oleate (closed circles) as a function of the mole percent of the FAEE in SUV.

Preparation of small unilamellar vesicles (SUV)

SUV were prepared by mixing 100 mg of egg phosphatidylcholine (Avanti Polar Lipids, Pelham, AL) with 0.38 mg, 1.16 mg, 1.97 mg, 4.15 mg, and 9.34 mg of ethyl palmitate to make 1, 3, 5, 10, and 20 mole percent FAEE, respectively. Similar studies were performed with 2.14 mg, 12.30 mg, 14.30 mg, 19.60 mg, and 29.30 mg of ethyl oleate to make 20, 25, 30, and 40 mole percent of FAEE, respectively. The solvent was evaporated under nitrogen and the lipid film was dried under a vacuum overnight. The dried lipids were resuspended in 1.8 ml of 75 mM (0.56%) KCl, vortexed, and sonicated for 50 min. The SUV preparation was then centrifuged for 30 min in a counter-top centrifuge to remove metal particles from the sonicator tip. Except for samples with high amounts of FAEE (>30 mole percent), SUV samples were translucent.

Albumin binding

An aqueous solution of bovine serum albumin (BSA) was prepared by dissolving fatty acid-free BSA (Sigma, St. Louis, MO) in 75 mM KCl. The solution was vortexed gently and filtered through a 0.2-μm sterile filter connected to a syringe. The concentration of the BSA solution was determined by absorbance at 279 nm (6).

Ethyl [^{13}C]oleate (2.36 mg) was evaporated to dryness under nitrogen and resuspended in 20 μl of ethanol. The ethanol was added to a solution (2.0 ml) containing 100 mg BSA (pH = 7.4) and the mixture incubated for 5 min at 37°C. For comparison, this procedure was followed with 2.15 mg of [^{13}C]oleic acid and 2.25 mg of methyl [^{13}C]oleate. The same molar ratio of lipid to protein (5/1) was used in all these cases.

LDL binding

Human blood was collected into 2.7-ml vacuum tubes containing 0.3 ml of 0.129 M (3.8%) sodium citrate. Tubes were centrifuged at 130 *g* for 15 min at room temperature. Plasma was collected, transferred into dialysis tubing with a molecular weight cut off of 3,500 Daltons (Spectrum Medical Industries, Houston, TX), and dialyzed against 2 mM EDTA in 0.9% sodium chloride for 24 h. The buffer solution was changed three times at approximately 8-h intervals. After dialysis, 10 μl of 1% thimerosal was added per each ml of plasma as an antioxidant. LDL was isolated by ultracentrifugation according to the method of Havel, Eder, and Bragdon (15) followed by dialysis against 0.2 M phosphate buffer (pH = 7.4) containing 2 mM EDTA, 50 mM KCl, and 15.4 mM sodium azide at 4°C for 24 h. The isolated LDL was filtered through a 0.2-μm sterile filter connected to a syringe and concentrated by centrifugation in CF50A membrane cones (Amicon, Beverly, MA) following the procedure described by the manufacturer. LDL protein was measured by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) and the values were multiplied by 0.75 to correct for phospholipid interference (16). To study binding of FAEE to LDL, ethyl [^{13}C]oleate (1.74 mg) was dissolved in 20 μl of ethanol and added to the LDL preparation.

Transfer experiments

SUV were prepared containing 4 mol% ethyl [^{13}C]oleate in the phosphate buffer described above for LDL. These SUV (50.75 mg of phospholipid) were

mixed with isolated LDL (7.60 mg of LDL protein) and incubated at 30°C. NMR spectra were taken at 0.5 h, 1.0 h, 1.5 h, 2.0 h, and 19.0 h.

Serum binding

Human serum was collected from coagulated blood after centrifugation at 2300 *g* for 15 min at room temperature. The serum was then concentrated 5-fold by centrifugation in CF50A membrane cones as described above. Ethyl [^{13}C]oleate (1.0 mg) was dissolved in ethanol and added to 2 ml of serum.

NMR methods

^{13}C NMR spectra were obtained at 30–35°C on a Bruker AMX 300 spectrometer operating at 75 MHz. In samples containing phospholipid vesicles or LDL, the terminal methyl resonance at 14.10 ppm was used as an internal reference (17). In samples containing albumin, the protein peak at 39.54 ppm was used as a chemical shift reference (6). The chosen internal chemical shift references are insensitive to the types of perturbations in the present experiments. A line broadening of 2 Hz was used to improve signal-to-noise ratios for all spectra. Internal D_2O was used as a lock and shim signal.

RESULTS

The first series of experiments assessed the conformation of FAEE in phospholipid bilayers and determined the maximal amount of ethyl oleate that could be incorporated into small unilamellar phospholipid vesicles (SUV). SUV were prepared by co-sonication of FAEE and egg phosphatidylcholine. **Figure 1A** shows the spectrum of phospholipid vesicles with 5 mol% ethyl oleate. The main spectrum reflects the natural abundance signals of the host phospholipid. The ^{13}C-enriched FAEE produced a narrow peak in the carbonyl region (inset) well resolved from the two phospholipid carbonyl peaks at 173.6 and 173.8 ppm characteristic of SUV (5). The chemical shift for the carbonyl peak of both ethyl palmitate and ethyl oleate in SUV was 172.9 ppm. This peak was shifted downfield 1.0 ppm compared to the carbonyl resonance for the pure ethyl palmitate or ethyl oleate oils (171.9 ppm), which indicates increased hydrogen bonding of the carbonyl in the bilayer phase relative to the pure oil phase. Thus, ethyl palmitate and ethyl oleate are incorporated into the phospholipid bilayer with the carbonyl oriented at the aqueous interface.

To determine the maximal amount of FAEE that could be incorporated into the phospholipid lamellar structure, samples were prepared with increasing mole percentages of FAEE. Figure 1 shows spectra in which the amount of ^{13}C-enriched ethyl oleate was held constant at 5 mol% (2.14 mg) and supplemented with unlabeled (unenriched) ethyl oleate to achieve the desired mole percent of ethyl oleate (25 mol% for Fig. 1B and 30 mol% for Fig. 1C). Supplementation with unenriched ethyl oleate prevented the signal of the ethyl oleate carbonyl peak in the NMR spectrum from obscuring the signals for the phospholipid carbonyls. At 25 mol% (Fig. 1B), a single peak representing ethyl oleate was observed at a chemical shift slightly upfield from that of a comparable peak in the spectrum of the 5 mol% sample (Fig. 1A). The natural abundance phospholipid peaks remained characteristic of phospholipids in SUV. At 30 mole percent (Fig. 1C), a second peak at 172.4 ppm was observed for the FAEE, reflecting a second pool of FAEE in slow exchange with the bilayer-solubilized pool. The results suggest that ethyl oleate is completely soluble in the lamellar structure of the bi-

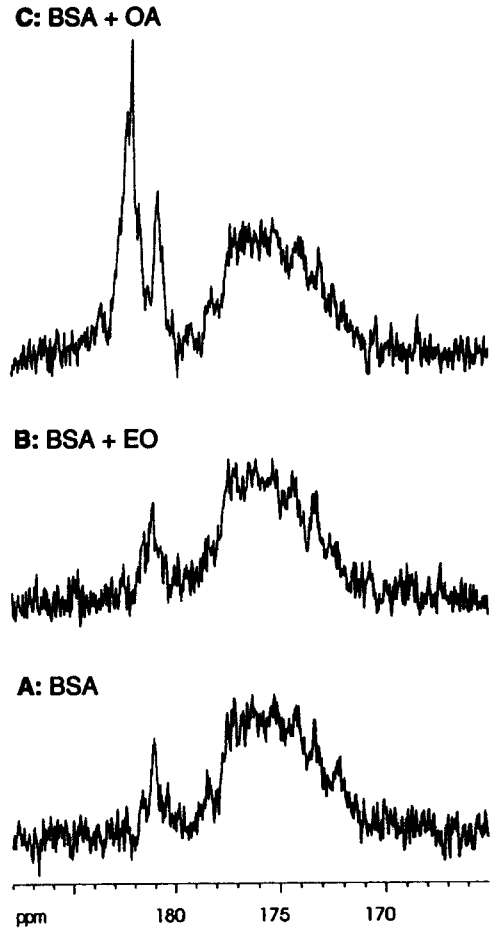

Fig. 3. Binding of ethyl oleate (EO) and oleic acid (OA) to albumin. Ethyl [1-^{13}C]oleate and [1-^{13}C]oleic acid were dissolved in ethanol and added to a solution of BSA (lipid/protein equal to 5/1). NMR spectra of BSA alone (A), BSA with ethyl oleate (B), and BSA with oleic acid (C) were obtained as in Fig. 1 except for the pulse interval (7 s) and number of accumulations (2000).

layer between 25 and 30 mol% before forming a separate phase. **Figure 2B** summarizes chemical shift data for ethyl oleate and ethyl palmitate. The data show a decrease in chemical shift between 5 and 20 mol% for the vesicle-incorporated FAEE. This chemical shift trend suggests a progressively decreasing average hydration of the FAEE carbonyl groups with increasing proportions of FAEE in the bilayer. The ethyl palmitate in SUV underwent a similar shift as the ethyl oleate in SUV. At ≥30 mol% ethyl oleate, two peaks are observed as in Fig. 1C.

A more quantitative measurement of the incorporation of FAEE into phospholipid bilayers can be made by integration of the signal intensity of the FAEE relative to phospholipids (Fig. 2A). The pulse interval of 8.0 s will give equilibrium peak intensities for carbonyl signals (5, 7, 9, 11, 13). Analysis of the data for ethyl palmitate shows a linear correlation between the amount of FAEE added and the percent incorporated up to 10 mol%. However, the sample prepared with 20 mol% FAEE showed incorporation of only 15 mol%. The smaller fraction that was not incorporated may have floated to the top of the NMR tube outside the detection coils.

^{13}C NMR experiments were next performed to compare the binding capacity of albumin for FAEE compared to free fatty acids (**Fig. 3**). In these studies, small aliquots of concentrated solutions of ^{13}C-enriched oleic acid or ethyl oleate in ethanol were added to an aqueous solution of albumin (50 mg/ml) at a lipid/protein ratio of 5/1. Mixtures were incubated for 5 min at 37°C and then analyzed by ^{13}C NMR. Some samples were incubated in the NMR probe at 37°C for 12 h during analysis. The spectrum of the oleic acid/BSA mixture (Fig. 3A) was similar to that reported previously for samples prepared by addition of potassium oleate to aqueous albumin (6). The [^{13}C]oleic acid produced three major signals reflecting different binding sites on the albumin molecule. Because of the ^{13}C-enrichment, the oleic acid signals are quite strong relative to the protein carbonyls, which appear as a broad envelope of resonances from 170 to 178 ppm. The spectrum of the FAEE/BSA mixture (Fig. 3B) showed a weak, narrow signal at 181 ppm which has previously been identified as originating from glutamate residues of BSA (6) and is thus seen in the spectrum of BSA without the addition of fatty acid or FAEE (Fig. 3A).

From these data, it can be argued that either very little FAEE (<0.5 mol/mol BSA) were bound to BSA or that the resonance lines were too broad to detect. The latter possibility could result either from highly restricted molecular motions of the carbonyl head group or intermediate exchange of FAEE between different binding sites. However, for complexes of fatty acids with albumin, we have observed NMR signals both in cases of high affinity binding with slow exchange (e.g., oleic acid; ref. 6) and lower affinity binding with slow, intermediate, or

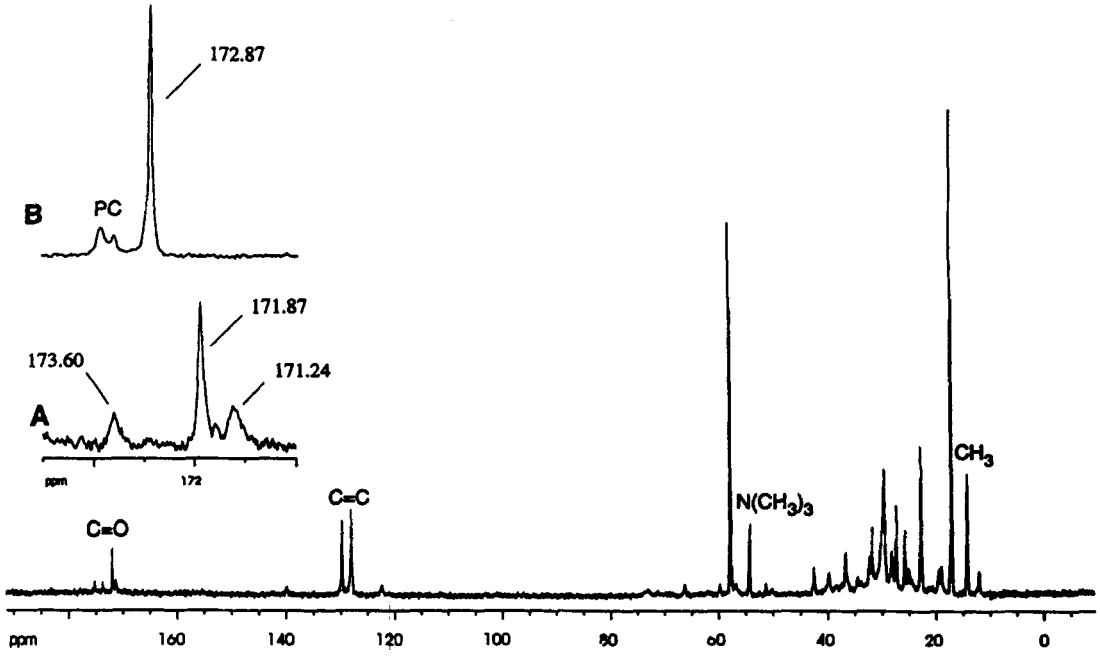

Fig. 4. Comparison of ethyl oleate binding to LDL and SUV. Ethyl [1-^{13}C]oleate in ethanol was added to isolated LDL (15.2 mg of LDL protein). The complete NMR spectrum after 6000 accumulations is shown with an expansion of the carbonyl region (A). Other spectrometer conditions are as in Fig. 1. An expansion of the carbonyl region of 4 mol% ethyl oleate in SUV is shown for comparison (B).

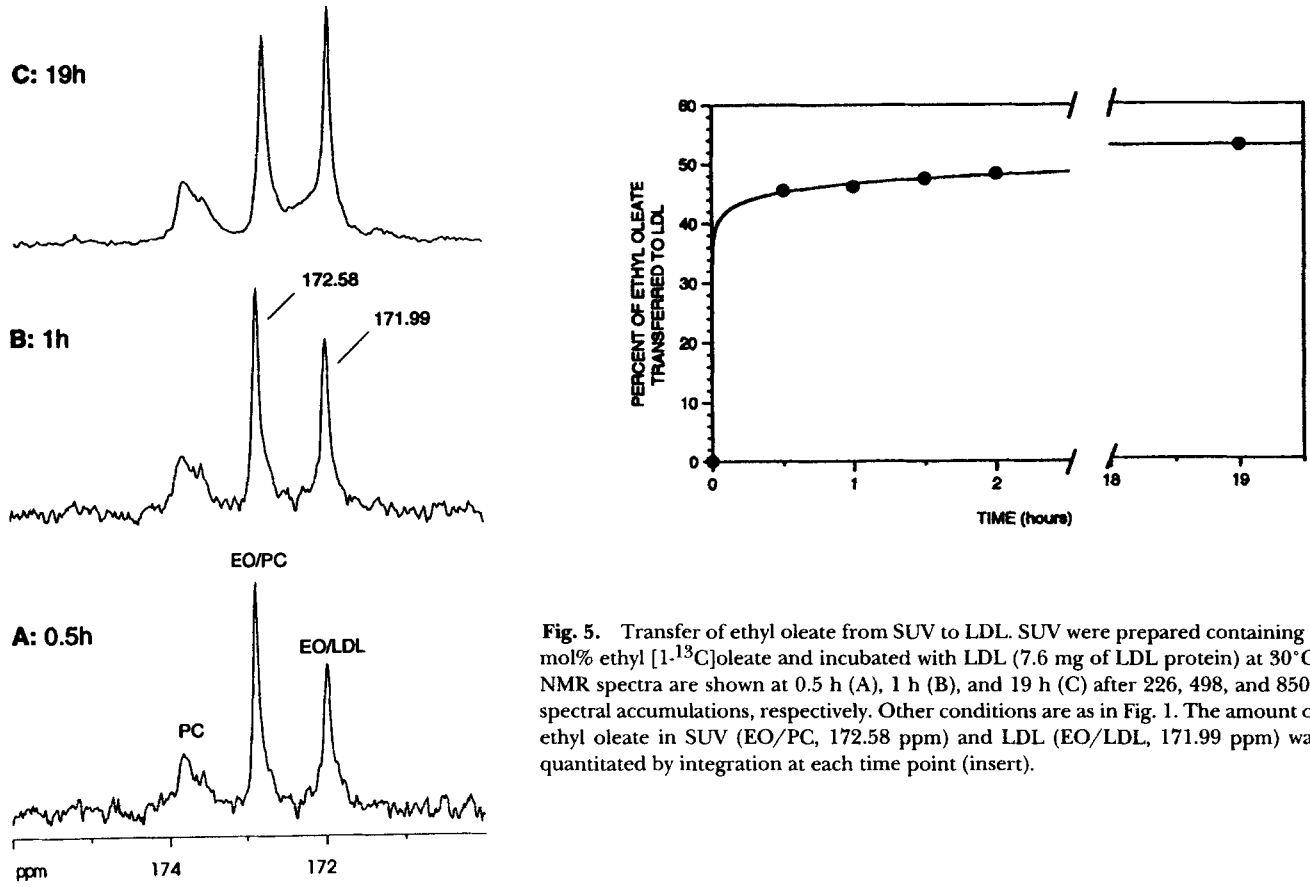

Fig. 5. Transfer of ethyl oleate from SUV to LDL. SUV were prepared containing 4 mol% ethyl [1-^{13}C]oleate and incubated with LDL (7.6 mg of LDL protein) at 30°C. NMR spectra are shown at 0.5 h (A), 1 h (B), and 19 h (C) after 226, 498, and 8501 spectral accumulations, respectively. Other conditions are as in Fig. 1. The amount of ethyl oleate in SUV (EO/PC, 172.58 ppm) and LDL (EO/LDL, 171.99 ppm) was quantitated by integration at each time point (insert).

fast exchange (e.g., octanoic or decanoic acid; ref. 18). Moreover, oil droplets were visible floating on the top of the NMR samples with ethyl oleate, and signals from this pool would not be detected. Therefore, the most likely explanation for the absence of observable resonances is a very low level of binding of FAEE to BSA. Independent measurements have shown that albumin has a higher affinity and capacity for fatty acids than for FAEE (19). The data in Fig. 3 support the conclusion that albumin has a much greater capacity for fatty acids than for FAEE. Based on the signal to noise ratios in our spectra, we could detect binding of as little as 0.5 mol of FAEE bound per mol BSA if the FAEE were present in a single binding site.

Because FAEE also bind to lipoproteins in human plasma (3), we studied the binding of ethyl oleate to isolated human LDL, which was chosen because it is the most abundant lipoprotein in fasting plasma. ^{13}C-labeled ethyl oleate, in ethanol, was added to aqueous LDL and the ethyl oleate/LDL complex was analyzed by ^{13}C NMR (**Fig. 4**). The carbonyl region of the spectrum showed a new signal at 171.87 ppm, which is similar to the shift of pure ethyl oleate oil. The carbonyl region also contained signals from phospholipids and cholesteryl esters in LDL (peaks labeled at 173.60 ppm and 171.24 ppm, respectively). As the chemical shift of ethyl oleate in LDL is distinct from that of ethyl oleate in the SUV, where the carbonyl is at the interface, and resonates at an upfield position characteristic of the unhydrated oil, ethyl oleate added to LDL appears to partition mainly into the core of the LDL particle. This was expected because other neutral lipids such as triglycerides and cholesteryl esters, as well as un-ionized fatty acids, also exist primarily in the core of lipoproteins (20).

As a model for the transfer of FAEE between plasma lipoproteins and membranes, the transfer of FAEE between phospholipid vesicles and isolated human LDL was studied. Phospholipid vesicles containing 4 mol% ethyl oleate were added to aqueous human LDL, and ^{13}C NMR spectra were obtained as a function of time. The data in **Fig. 5** indicate that ethyl oleate transferred rapidly from vesicles to LDL. In the spectrum representing the first 30 min after mixing, an intense signal characteristic of ethyl oleate bound to isolated LDL (Fig. 4) was observed, in addition to the signal from the donor vesicles. The spectra after an additional 1 (Fig. 5B) and 19 (Fig. 5C) hours showed additional FAEE transfer

from vesicles to LDL, though small in comparison to the transfer in the initial 30 min. Within 30 min, 40% of the ethyl oleate was transferred from the phospholipid vesicles to LDL (inset in Fig. 5). At 19 h, an equilibrium distribution was reached with 45% of the ethyl oleate bound to vesicles and 55% bound to LDL. Experiments to assess transfer of FAEE in the opposite direction, from LDL to SUV, were performed by first incorporating the ethyl oleate into LDL and adding SUV as acceptors. Transfer rates of FAEE from LDL to SUV were similar to those from SUV to LDL (data not shown). Experiments were also carried out to investigate the transfer of ethyl oleate from LDL and SUV to albumin using mass ratios of 0.87 for phospholipid to BSA (as in studies with fatty acids; refs. 8, 10, 21) and 0.16 for LDL protein to BSA. In each case, ^{13}C-NMR spectra revealed only signals for ethyl oleate bound to the donor species, the intensities of which did not decrease. Thus, no transfer of ethyl oleate from LDL or from SUV to BSA was detected (data not shown). Important to note is that the physiologic mass ratio of LDL protein to BSA is approximately 0.02. Therefore, there was a much greater proportion of LDL in our experiments as compared to the in vivo situation.

The final series of experiments investigated the binding of ethyl oleate in whole serum. Ethyl [^{13}C]oleate was added to concentrated normal human serum and the mixture was analyzed by NMR. Even with ^{13}C enrichment, the FAEE in plasma after ethanol injection would be difficult to detect by ^{13}C NMR. Therefore, higher levels of FAEE were used. The natural abundance ^{13}C NMR spectrum of plasma is complex and reflects primarily contributions of albumin and lipoproteins. The spectral region of interest for this study, the carbonyl region, is shown in the expansion A. The predominantly broad signals resemble those of isolated albumin (Fig. 3A) and represent unresolved protein carbonyl signals (170–180 ppm) and amino acid carboxylates (181 ppm). The addition of FAEE resulted in only one new signal, which occurred in the carbonyl region at 171.97 ppm (**Fig. 6,** inset B). This peak matches the chemical shift for ethyl oleate bound to isolated LDL and likely represents ethyl oleate bound to LDL or possibly other lipoproteins. Therefore, FAEE added at these concentrations to serum preferentially bind to lipoproteins.

DISCUSSION

In cells, FAEE are expected to interact primarily with membranes because of their very low aqueous solubility. We detected interactions of FAEE with phospholipid bilayers (SUV) by a chemical shift change in the carbonyl resonance. FAEE incorporated at low levels in SUV showed a downfield shift of 1.0 ppm relative to the oil phase, in which the carbonyl groups are not hydrogen-bonded. Very similar shifts have been observed for triglycerides and cholesteryl esters incorporated into SUV or multilamellar vesicles (5, 7, 9, 13, 22). It has been argued that the downfield shift represents partial hydration and hydrogen bonding of the carbonyl group. Therefore, the polar head group must be oriented at the aqueous interface (5, 13, 22). The same argument can be applied to FAEE, which appear to have a similar

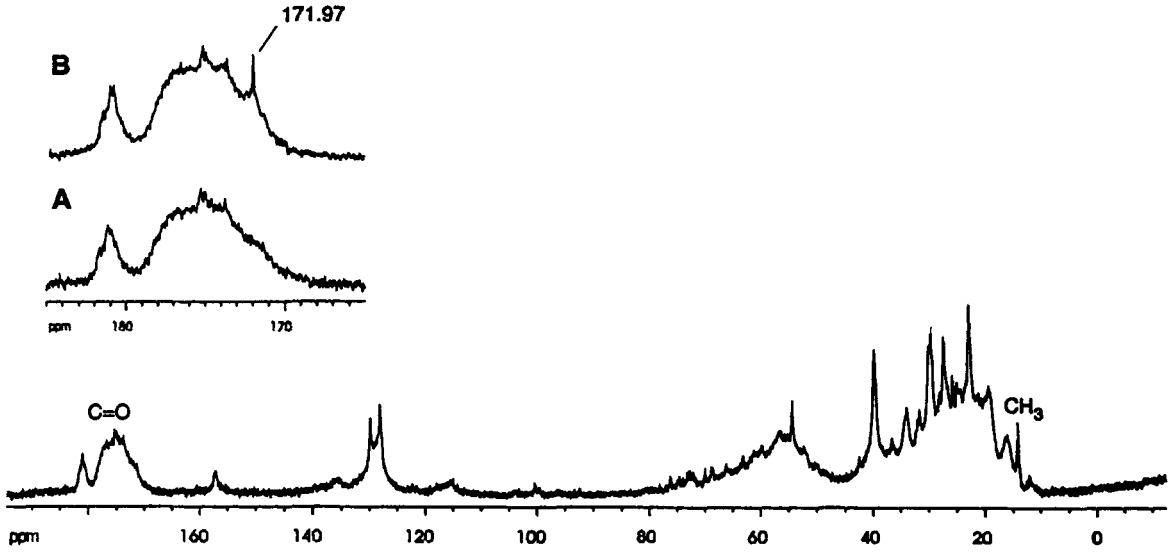

Fig. 6. Ethyl oleate binding in human serum. Human serum was isolated, concentrated, and analyzed by NMR using a pulse interval of 5.0 s and 3103 scans (spectrum and expansion A). Ethyl [1-^{13}C]oleate, dissolved in ethanol, was added to 2.0 ml of serum followed by NMR analysis with 10,000 scans (B). Other spectrometer conditions are as in Fig. 1.

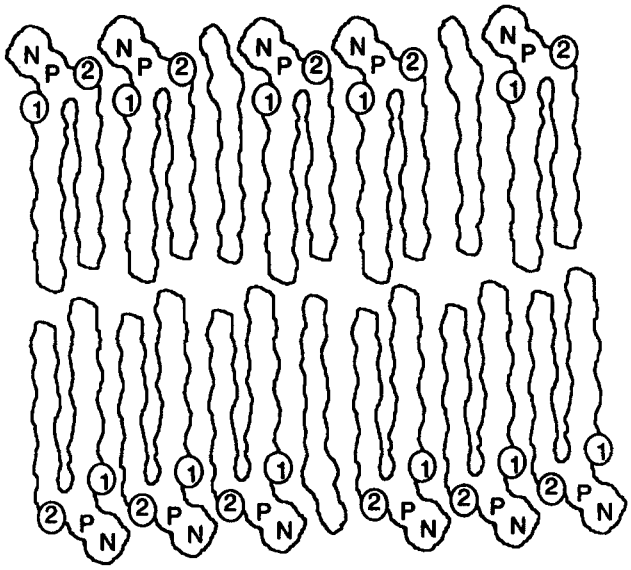

Fig. 7. Schematic diagram representing binding of FAEE to a phospholipid bilayer. N represents the choline group, P the phosphate group, and 1 and 2 the *sn*-1 and *sn*-2 carbonyl groups.Our results demonstrate that the carbonyls of ethyl oleate and ethyl palmitate in phospholipid vesicles are exposed to the aqueous interface. Therefore, FAEE most likely align parallel to the fatty acid moieties of the phospholipids. This scheme is meant to represent low proportions of FAEE in vesicles (≤5 mol%). As discussed in the text, at higher levels the average position of the headgroup may lie slightly deeper in the bilayer or a second population of FAEE that is not in the interface may be present.

hydration as other interfacial lipids. **Figure 7** shows a schematic diagram reflecting this general conclusion. This orientation, which is also the same as that for fatty acids in a phospholipid bilayer (21), places the carbonyl group in a position favorable for hydrolysis by enzymes acting at a lipid–water interface. With increasing FAEE content in SUV, the carbonyl chemical shift of the FAEE moved progressively upfield toward the position of oil-phase FAEE, which suggests a decreasing average hydration of the FAEE carbonyl in the vesicle environment. This result can be explained by an increasing population of FAEE that is not at the interface but is in fast exchange with molecules in the same position in the interface as FAEE in low proportions. An alternative explanation (and not distinguishable from the first, based on present data) is that the average position of the headgroup lies deeper in the bilayer with higher FAEE concentrations.

FAEE incorporated to relatively high levels in vesicles (25–30 mol%) before formation of a separate oil phase was detected. Our experiments thus establish a high interfacial solubility of FAEE. ^{13}C spectra (data not shown) showed that oleic acid also incorporated to a similar extent in egg PC vesicles without phase separation or disruption of the PC bilayer. In contrast, less polar molecules such as triglycerides and cholesteryl esters incorporate to the extent of only about 3 mol% into PC bilayers (5, 7, 9, 13, 22). The ^{13}C NMR spectrum of the phospholipid revealed no changes in the vesicle structure with less than saturating amounts of FAEE, indicating that small amounts of FAEE can incorporate non-disruptively into a membrane. Studies of ethyl oleate incorporation in piglet intestinal mucosal cells showed no effect on the plasma membrane under conditions where oleic acid showed significant damaging effects (23).

In the spectra of vesicles with FAEE incorporated at less than maximal amounts, there is a single carbonyl resonance from the FAEE. In contrast, these spectra show two resonances for PC carbonyls, one (downfield) reflecting PC on the outer leaflet and the other reflecting PC on the inner leaflet. These signals are observed not only because the local interfacial environments are different on the two leaflets, but because the exchange is slow on the NMR timescale ($< <20$ sec^{-1}). Because of the very fast flip-flop rate of un-ionized fatty acids in SUV ($t_{1/2} <10$ msec) (24), it can be assumed that the uncharged, slightly less polar FAEE molecule also flips rapidly and that single signal for FAEE in SUV reflects the average environment of FAEE rapidly exchanging between leaflets. FAEE that are transported to cells from the plasma compartment should, therefore, rapidly equilibrate across the plasma membrane and be quickly available for intracellular processes.

We attempted to measure binding of FAEE to serum albumin by a ^{13}C NMR approach used to detect binding of fatty acids to individual sites on albumin and to determine the maximal capacity of binding (8–10 mol of long chain fatty acids/mol albumin) (6). Binding of FAEE was not observed under NMR experimental conditions that would have detected binding of as little as 0.5 mol of FAEE/mol of BSA. With samples of low amounts of oleic acid, binding was readily observed by NMR (Fig. 3). We also incorporated FAEE into vesicles for potential delivery to BSA under conditions where fatty acids would partition almost entirely to albumin (8). However, we observed no binding to albumin. This demonstrates a much higher affinity of PC bilayers for FAEE compared to albumin. In cell studies, it has been shown that fatty acid methyl esters readily partition into cells from albumin (25), consistent with our results.

Our conclusion that FAEE bind to BSA to a lesser extent than fatty acids is qualitatively similar to conclusions reached with human serum albumin (26). However, we conclude that maximal binding of FAEE (<0.5 mol/mol albumin) is much lower than previously estimated for methyl palmitate by partitioning experiments (2.4 mol/mol albumin). Our result illustrates the importance of the negatively charged carboxyl moiety in sta-

bilization of the binding interactions of fatty acids with albumin. Previous NMR studies showed that the ionized form of fatty acid is bound to albumin and that titration to the un-ionized form is correlated with desportion of fatty acid from the binding sites (6). On the other hand, measurement of the distribution of low levels of FAEE among plasma components in vivo suggested some binding of FAEE to albumin [<0.01 mol FAEE/mol albumin (3)], and our results are not inconsistent with this finding.

Binding of ethyl oleate to plasma LDL was characterized by a single carbonyl signal at the chemical shift of ethyl oleate in a pure (unhydrated) oil phase. This result shows that the FAEE partitions into the nonaqueous core of LDL, which consists mainly of cholesteryl esters and triglycerides. Because of the amphipathic nature of FAEE and its demonstrated orientation in phospholipid bilayers, it is also expected that a small fraction of FAEE in LDL is present in the lipoprotein surface with an interfacial orientation of the carbonyl group. As exchange between surface and core pools is most likely very rapid, a single signal would be observed, and the chemical shift will reflect the predominant pool in the core. The finding that most of the FAEE is partitioned into the LDL core thus does not imply that FAEE cannot leave the particle by desorption from the surface.

It was possible to study transfer of ethyl oleate between SUV and LDL because of the different chemical shifts of the ethyl oleate carbonyl signal in each environment. Bi-directional transfer was observed with a $t_{1/2}$ <30 min (Fig. 5, inset). Because of the long time interval (30 min) required for obtaining spectra, it was not possible to measure t½ accurately. If it is assumed that the kinetic process of Fig. 5 is described by a single exponential and the process is 80% complete at 30 min, the calculated $t_{1/2}$ is approximately 13 min. This is about 10^2 times slower than that of oleic acid from SUV and comparable to the rate of dissociation of a saturated 22-carbon fatty acid from SUV (J. A. Hamilton, unpublished observation). A slow rate of transfer of FAEE is consistent with a process that is limited by the dissociation rate from the interface and the lower aqueous solubility at physiological pH of FAEE compared with a fatty acid with the same unsaturation and chain length. Thus, exchange of FAEE between SUV and LDL most likely occurs by a first order rate process consisting of desorption of the FAEE from the vesicle or lipoprotein interface and diffusion of monomers to the acceptor particles, analogous to the mechanism of transfer of fatty acids from SUV (27).

The time scale of transfer of FAEE in our experiments with LDL and model membranes is similar to that for desorption of fatty acid methyl esters from cells. In measurements of release of radioactive methyl esters from Ehrlich tumor cells into media containing albumin, it was found that 53% of methyl palmitate and 25% of methyl oleate were released from cells after 1 min of incubation (25). Furthermore, the time course of Fig. 5 is fast enough to be consistent with the time course (minutes) of the methyl oleate uptake and utilization in Ehrlich tumor cells (25).

Our experiments determining the distribution at equilibrium (partitioning) of ethyl esters between model membranes, LDL, and albumin also have physiological significance for transport and utilization of FAEE. At the high levels of FAEE used, FAEE were bound to both LDL and SUV, whereas no binding to BSA was detected. In human serum, binding was detected in a nonaqueous lipoprotein environment, similar to that in isolated LDL. This result suggests that as the concentration of plasma FAEE increases, plasma lipoproteins could take on an increasing role in binding and transport of FAEE. In fact, recent experiments have demonstrated a positive correlation between serum FAEE concentration and the percent of FAEE bound to lipoproteins. (D. Bird and M. Laposata, unpublished observations) Transport by LDL could result in a different metabolic fate of FAEE, as some of the FAEE would enter cells by receptor-mediated endocytosis of LDL. The present results from NMR assays of binding contrast with, but do not contradict, previous assays of FAEE binding in human plasma, which found that the majority of FAEE was bound to albumin and about 30% to lipoproteins (3). The NMR studies were performed with higher levels of FAEE than observed physiologically, which can greatly affect the partitioning of FAEE between lipoproteins and albumin if the capacity of albumin for FAEE is very low. In the latter study, the low levels of FAEE corresponded to FAEE/albumin mole ratios of <0.01. This low level of binding clearly is not excluded by our NMR studies. Furthermore, this level of FAEE bound to albumin will not interfere with fatty acid binding, which has a stoichiometry of 0.5 to 1.5 mol/mol of albumin in plasma of normal fasting patients (28).

The binding of FAEE to carriers in the blood is a particularly important issue if the pathogenesis of ethanol-induced organ damage involves the delivery of FAEE to target organs. FAEE-induced toxicity may be produced by FAEE synthesized endogenously with release into the circulation and uptake into cells. If it is demonstrated in subsequent experiments that circulating FAEE account for a portion of ethanol-induced toxicity, the possibility exists that the toxicity of ethanol-induced organ damage could be limited if the FAEE is removed from the blood. Plasmapheresis removes lipid components as well as albumin from the plasma. Patients with FAEE in the plasma as a result of high levels of ethanol intake could be treated in a similar fashion to decrease

any potential organ damage. Although most ethanol-induced organ damage does not occur after a single bout of high ethanol intake, it is known that extremely high amounts of ethanol can be lethal with few therapeutic options to inhibit this outcome. If FAEE are important in this setting, it is possible that FAEE-induced lethal toxicity can be prevented by removal of the FAEE from the blood.

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