Interactions of acyl carnitines with model membranes: a ^{13}C-NMR study

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Abstract **Esterification of fatty acids with the small polar molecule carnitine is a required step for the regulated flow of fatty acids into mitochondrial inner matrix. We have studied the interactions of acyl carnitines (ACs) with model membranes [egg yolk phosphatidylcholine (PC) vesicles] by ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. Using AC with ^{13}C-enrichment of the carbonyl carbon of the acyl chain, we detected NMR signals from AC on the inside and outside leaflets of the bilayer of small unilamellar vesicles prepared by cosonication of PC and AC. However, when AC was added to the outside of pre-formed PC vesicles, only the signal for AC bound to the outer leaflet was observed, even after hours at equilibrium. The extremely slow transmembrane diffusion ("flip-flop") is consistent with the zwitterionic nature of the carnitine head group and the known requirement of transport proteins for movement of ACs through the mitochondrial membrane. The partitioning of ACs (8–18 carbons) between water and PC vesicles was studied by monitoring the [^{13}C]carbonyl chemical shift of ACs as a function of pH and concentration of vesicles. Significant partitioning into the water phase was detected for ACs with chain lengths of 12 carbons or less. The effect of ACs on the integrity of the bilayer was examined in vesicles with up to 25 mol% myristoyl carnitine; no gross disruption of the bilayer was observed. We hypothesize that the effects of high levels of long-chain AC (as found in ischemia or in certain diseases) on cell membranes result from molecular effects on membrane functions rather than from gross disruption of the lipid bilayer.**—Ho, J. K., R. I. Duclos, Jr., and J. A. Hamilton. **Interactions of acyl carnitines with model membranes: a ^{13}C-NMR study.** ***J. Lipid Res.*** **2002.** 43: **1429–1439.**

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Acyl carnitines (ACs) participate in a crucial step in the shuttling of fatty acids and breakdown products of carbohydrates into the inner mitochondrial matrix for oxidative phosphorylation. Carnitine, a small molecule with a nontitratable positive charge at the quaternary amine position and a titratable carboxyl group, is covalently linked to fatty acid to form ACs (**Fig. 1**). Therefore, ACs are zwitterionic in aqueous solution at neutral pH. Two AC transferases (CPT I and CPT II) and a carnitine-AC translocase located within the inner mitochondrial membrane control the conversion between AC and acyl-CoA and regulate the flux of AC into the inner matrix (1, 2). This transport is regulated by both fatty acid and carbohydrate metabolism. ACs are also formed in the peroxisome as products of partial β-oxidation of long- and very-long-chain fatty acids (3). The medium-chain ACs formed inside the peroxisome from partial degradation of very-long-chain saturated fatty acids and methyl-branched fatty acids are exported by a translocase. At the mitochondrion, these ACs bypass CPT I and go directly to a translocase (possibly a different translocase from that for long-chain unbranched fatty acids) in the inner membrane (4).

Concentrations of carnitine and AC in cells and serum are influenced by a host of normal physiological and pathophysiological processes. Increased levels of AC in the blood and urine are associated with most inborn errors of fatty acid oxidation (5). The concentration of short-chain ACs in cells increases and that of long-chain ACs decreases with exercise, although the ratio between total AC and acyl-CoA remains constant (6). Under ischemic and hypoxic conditions, levels of long-chain ACs may increase 8- to 10-fold in myocytes and may be elevated up to 100-fold in sarcolemma (7). Such levels of long-chain ACs elicit adverse pharmacological effects on cellular functions and make the heart more susceptible to arrhythmias and other dysfunction (8). These pathological effects may be caused by the binding of ACs to cell membranes and alteration of their functions. Limiting the formation of long-chain ACs by inhibiting CPT I might have protective effects against myocardial ischemia, although inhibiting CPT I did not reduce infarct size (7, 9).

Abbreviations: AC, acyl carnitine; CMC, critical micelle concentration; PC, phosphatidylcholine; SUV, small unilamellar vesicles.

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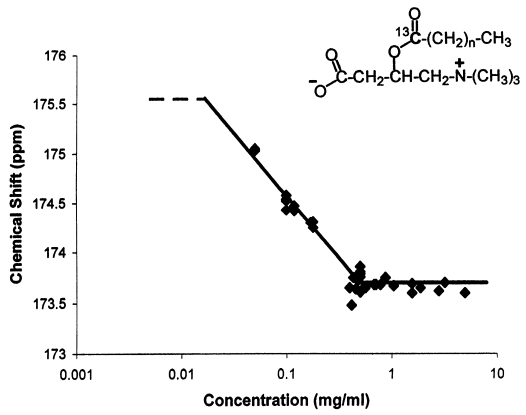

Fig. 1. Semi-log plot of the carbonyl chemical shift of myristoyl carnitine in water versus concentration. The constant chemical shift value at high concentrations (173.5 ppm) represents the micellar state, and the shift at low concentrations (175.5 ppm) represents monomeric acyl carnitine (AC), as measured for shorter-chain ACs. The critical micelle concentration (CMC) was determined from the concentration at which the straight line representing a mixture of monomers and micelles intersects the chemical shift of the monomer. The structural formula of ACs is shown above the plot. For the data illustrated in this figure (myristoyl carnitine), n = 12.

Malfunctions in the carnitine metabolic pathway can result in severe metabolic dysfunction, with poor clinical outcomes and early morbidity. Carnitine deficiency can cause a debilitating disease involving muscular and myocardial function by affecting fatty acid metabolism in cells (10). Inborn errors of fatty acid oxidation occur with one in 10,000 to 15,000 live births (11). The acyl-chain profile of ACs in fibroblasts of such patients is often sufficiently distinct to permit localization of the specific enzyme defect (12). Some of these infantile or juvenile onset myopathies, usually associated with mental retardation or regressions, are caused by defects in carnitine transport, carnitine-acylcarnitine translocase, and carnitine palmityl transferase (13). Deficiencies in serum AC sometimes are found in patients with chronic fatigue syndrome (14).

Despite the importance of ACs in human health and disease, physical studies of these common molecules have been sparse. The acyl chain alters the physical properties of carnitine and converts this water-soluble molecule into a molecule that, at a certain chain length, will partition favorably into the phospholipid bilayer of membranes. An understanding of how ACs interact with membranes is crucial for understanding the biophysical basis of the normal and pathological effects of these molecules. Previous studies have focused mainly on surfactant properties of ACs, such as their abilities to form micelles and to disrupt phospholipid bilayer structure. For example, surface-active properties of palmitoyl carnitine have been shown to lead to detergent effects at very high concentrations (e.g., 1 mol of AC per mol of phospholipid) (15, 16). Limited information is available about the molecular interactions of ACs with membranes at physiological concentrations (17, 18). The thermodynamics and kinetics of transfer of pyrene-labeled AC between model and natural membranes were studied by fluorescence spectroscopy (19). A model for medium-chain AC, (1-pyrenebutyryl) carnitine showed interesting properties of transmembrane movement: as assessed by its extraction from phospholipid bilayer vesicles, it moved from the outer to the inner leaflet rapidly but appeared to be trapped in the inner leaflet (20). Although definitive data about the transmembrane movement of natural ACs in model membranes are lacking, there is direct (21) and indirect (22) evidence that palmitoyl carnitine has a slow rate of transbilayer movement in erythrocyte membranes.

Our study focuses on the binding of ACs with different chain lengths (8–18 carbons) to model membranes composed of phospholipid bilayers. Labeling with ^{13}C permits the detection of low amounts of AC in model membranes and preserves the native structure of the lipid. We used ^{13}C-NMR spectroscopy of carbonyl-labeled AC to address the questions of *i*) whether ACs bind to both leaflets of a small unilamellar vesicle; *ii*) whether ACs cross the lipid bilayer spontaneously in either direction; *iii*) how the acyl-chain length affects partitioning into the membrane; and *iv*) whether low levels of AC are disruptive to the bilayer structure.

MATERIALS AND METHODS

Synthesis of ^{13}C-labeled ACs

A series of six [octanoyl (8:0), decanoyl (10:0), lauroyl (12:0), myristoyl (14:0), palmitoyl (16:0), and oleoyl (18:1)] *dl-O*-acylcarnitine-(*1′*-^{13}C) chloride analogs were prepared on scales of 100 to 300 mg from the corresponding carbonyl-labeled (99 atom% ^{13}C) fatty acid (Cambridge Isotope Laboratories, Woburn, MA) and thionyl chloride followed by treatment of the intermediate fatty acid chloride with *dl*-carnitine chloride in trichloroacetic acid as solvent according to the reported method (23). The saturated fatty acyl derivatives were each recrystallized three times from isopropanol/acetone. The unsaturated oleoyl carnitine chloride was purified by a slight modification of the reported procedure (24). The crude oleoyl carnitine chloride was washed with hexane followed by anhydrous diethyl ether and was then filtered through silica gel 60 (E. Merck), eluting with 9:1 chloroform-methanol (v/v). After these steps, three recrystallizations from methanol/anhydrous diethyl ether gave 143 mg (85% yield) of a white solid that melted/decomposed at 112°C to 114°C. The saturated AC chloride analogs melted/decomposed just above 150°C. The ^{13}C-labeled AC chlorides were homogeneous by analytical TLC on silica gel 60 (E. Merck), eluting with chloroform-methanol-water (60:30:4, v/v/v). ^{1}H-NMR showed the C3-H proton resonance of carnitine chloride starting material [δ 4.41 (DMSO-d_6) or δ 4.68 (D_2O)] shifted downfield to δ 5.44 (DMSO-d_6) or 5.61 (D_2O) for the ^{13}C-labeled acylcarnitine chloride products, which differed from the corresponding unlabeled analogs only by the additional ^{2}J-^{1}H-^{13}C = 7.3 Hz coupling observed for the [C2′-H_2]methylene observed at δ 2.32 in DMSO-d_6 and at δ 2.42 in D_2O. The ^{13}C-NMR spectra of the ^{13}C-labeled AC chlorides showed strong C1′ carbonyl signals, and the acyl group C2′ carbons appeared as doublets (^{1}J-^{13}C-^{13}C =57 Hz) at 34.4 ppm in D_2O-H_2O (1:3, v/v).

Small unilamellar vesicles

Small unilamellar vesicles (SUV) composed of egg yolk phosphatidylcholine (PC) were used as a model system for cell membranes.

Egg yolk PC in chloroform was purchased from Avanti Polar Lipid, Alabaster, AL (Lot #EPC-253, EPC-292). Its concentration was determined by evaporating a known amount of PC solution and measuring the dry weight on a Cahn C-31 microbalance. A measured volume of PC was transferred to a round-bottom Kimax centrifuge tube. Chloroform was removed by evaporation under a stream of nitrogen gas and lyophilization under vacuum (10 Torr) overnight. The dried PC sample, hydrated overnight in unbuffered 0.56% (75 mM) KCl solution for pH titration experiments or in 50 mM phosphate buffer solution (pH 7.4) for all other experiments to maintain a stable pH. D_2O (20%, v/v), was added to the aqueous sample to provide a lock signal for the NMR experiments. To make AC available to both leaflets of the vesicles, vesicles were prepared either by adding AC to the chloroform solution of PC before lyophilizing or by hydrating PC in an aqueous solution of AC. The hydrated sample (2.0 ml total) was transferred to a thick-walled polycarbonate centrifuge tube for sonication with a Branson Sonifier cell disrupter model 350 equipped with a titanium tip (power level 3; 30% duty cycle) under a stream of nitrogen gas for 60 min to achieve a characteristic slightly translucent, nonturbid suspension. The preparation was then centrifuged on a tabletop centrifuge for 30 min to remove any metallic fragments that had dislodged from the sonication probe. The phospholipid concentration of selected samples was determined by Bartlett analysis (25), and was typically ~90% of the concentration estimated from the initial weight measurement.

Aqueous solutions of AC were prepared by dissolving weighed amounts of crystalline AC in water. Comparisons with samples prepared by cosonication of PC as described above were made by adding dissolved AC at a known concentration to sonicated vesicles.

^{13}C-NMR spectroscopy

^{13}C-NMR studies were performed on *i*) a Bruker AMX-300 with the 10 mm QNP probe (^{13}C = 75 MHz), with data acquired and processed by Bruker software UXNMR, or *ii*) a Bruker Avance system DMX-500 (^{13}C = 125 MHz) with a 10-mm broadband probe, with data acquired and processed with UXNMR and XwinNMR. Continuous irradiation in the proton frequency provided decoupling and nuclear Overhauser enhancement. The spin lattice relaxation time (T_1) was measured at 75 MHz for the carbonyl signal using the inversion-recovery method (26). The chemical-shift reference was tetramethylsilane in $CDCl_3$ in a capillary tube. With such an external referencing configuration, the terminal methyl signal of phospholipids was seen at 13.9 ppm.

RESULTS

Aqueous AC

^{13}C-NMR spectra were obtained for [^{13}C]carbonyl-enriched ACs with different chain lengths (8–18 carbons) in water over a concentration range that was feasible with a time limit of 24 h for the most dilute samples (~0.05 mg/ml). A single, narrow (<10 Hz) carboxyl peak was seen for all aqueous samples. The chemical shift and T_1 of the carbonyl carbon of each AC were measured (**Table 1**). The chemical shift of the medium-chain ACs (octanoyl and decanoyl carnitine) was ~175.5 ppm, and the T_1 was ~7 s at ~1 mg/ml (<1 mM). In contrast, the chemical shifts of palmitoyl and oleoyl carnitine were significantly more upfield at ~173.5 ppm and the T_1 was shorter (2 s). For lauroyl and myristoyl carnitine, the chemical shift and the T_1 were concentration-dependent in the range of our study.

TABLE 1. The spin-lattice relaxation time (T_1) of the carbonyl carbon of ACs (C8:0–C18:1) in aqueous solution, measured at the stated value of chemical shift

Carnitine Acyl Chain	Chemical Shift	Spin Lattice Relaxation Time, T_1
	ppm ± 0.02 ppm	*s ± 10%*
Octanoyl	175.49	7.2
Decanoyl	175.55	10.0
Lauroyl	174.21	3.0
Myristoyl	173.70	2.2
Palmitoyl	173.50	2.0
Oleoyl	173.44	2.0

As shown below, these changes reflected the transition from monomer to micelle. For medium-chain ACs, the long T_1 and the carbonyl chemical shift of ~175.5 ppm suggest that these ACs are monomeric in aqueous solution under the conditions of these experiments. The longer T_1 of medium-chain AC reflects more rapid molecular motions compared with the long-chain ACs, and the downfield chemical shift reflects increased hydration of the carbonyl carbon (27, 28). The shorter T_1 of long-chain ACs and their carbonyl chemical shift of 173.5 ppm, which shows a less polar environment of the carbonyl group, imply a micellar state. This experimental finding is consistent with previous measurements by other methods (29).

Aqueous octanoyl carnitine and decanoyl carnitine have critical micelle concentration (CMC) values that are higher than the concentrations (1 mM) we used for these NMR studies. Therefore, the upper limit of the carbonyl chemical shift of 175.5 ppm for these ACs was presumed to represent the chemical shift of AC monomers in general. The chemical shift of long-chain ACs (>14 carbons), which are micellar at the concentrations in our study (see below), was 173.5 ppm. Because the [^{13}C]carbonyl is in the head group, we assumed that the length of the acyl chain would not affect its interaction with water in a given state (monomer or micelle) and that the limiting values obtained with shorter- and longer-chain ACs would be applicable to ACs with chain lengths of 12 (lauroyl) and 14 (myristoyl) carbons. At concentrations slightly above the CMC, monomers will be in fast exchange with the micelles and the carbonyl chemical shift is predicted to appear at values between 173.5 and 175.5 ppm. The concentration above which the carbonyl chemical shift first decreases below the value of 175.5 ppm signifies the CMC. Extrapolation of concentration-dependent chemical shift data for myristoyl carnitine (Fig. 1) yielded a value of 0.021 mg/ml, or 0.051 mM, a value somewhat lower than the previous value of 0.1 mM derived from light scattering measurements (29).

Lauroyl carnitine in aqueous solution showed similar concentration-dependent chemical shifts. Because the CMC is much higher than that for myristoyl carnitine, it was feasible to obtain data both below and above the CMC. Assuming that the monomer chemical shift is 175.5 and extrapolating from the chemical shift data (plotted as in Fig. 1), we estimated the CMC of lauroyl carnitine to be 4.3 mg/ml (1.15 mM), close to the published value of 1.2 mM (29).

Localization and transbilayer movement of ACs in vesicles

The use of [^{13}C]carbonyl-labeled AC permitted the detection of low amounts of AC in egg PC SUV (model membranes) by ^{13}C-NMR spectroscopy. The interactions of ACs with vesicles were studied as a function of acyl chain length of AC, concentration of AC, and the solution pH. The chemical shift of a lipid carbonyl group is highly sensitive to its local environment and its proximity to the aqueous-lipid interface, which determines its hydration (27, 28). The curvature difference between the outer and the inner leaflets in a small vesicle results in differences in the hydration of phospholipid carbonyls, allowing two [^{13}C]carbonyl signals to be observed for the phospholipid (27, 28). The natural-abundance ^{13}C-NMR spectrum shows phospholipids localized in the outer and inner leaflets by peaks at 173.6 and 173.4 ppm (**Fig. 2A**). The intensity of the phospholipid signal at 173.6 ppm (outer leaflet) is ~1.5 times that of the peak at 173.4 ppm (inner leaflet). The curvature effect can be exploited to locate other lipids in the two leaflets of vesicles (30–33).

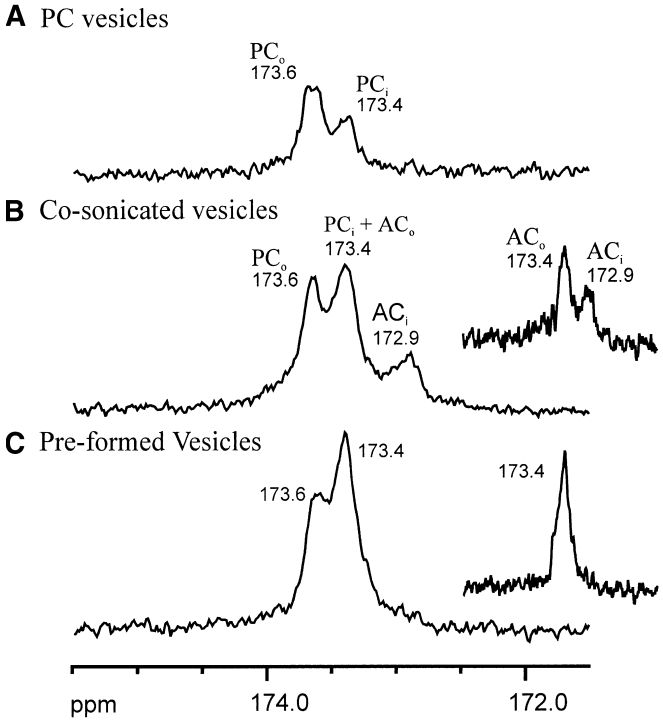

Fig. 2. ^{13}C-NMR spectrum (carbonyl region) of phosphatidylcholine (PC) vesicles without (A) and with (B,C) AC (lauroyl carnitine). The two signals (173.6 and 173.4 ppm) represent the carbonyls of the phospholipid on the outer and inner leaflet of the vesicles, respectively. B: Lauroyl carnitine (6 mol%) was incorporated into the vesicles prior to sonication. The increase in signal at 173.4 and the new peak at 172.9 ppm represent signals from the ^{13}C-carbonyl-enriched lauroyl carnitine. The difference spectrum (B-A; inset) clearly reveals two new signals from the lauroyl carnitine (173.4 and 172.9 ppm). C: Lauroyl carnitine (6 mol%) was added after preparation of the PC vesicles. The intensity of the signal at 173.4 ppm increased, but no new signal was observed. The difference spectrum (inset) shows only one new signal (173.4 ppm). These spectra were acquired at 75 MHz with a 3.0 s pulse interval and 1,800 spectral accumulations.

The general features of the ^{13}C-spectra (carbonyl region) of ACs in SUV are illustrated by the examples of decanoyl and lauroyl carnitine. For vesicles made by cosonication of lauroyl carnitine with PC, the intensity of the phospholipid peak at 173.4 ppm increased and a new signal was observed at 172.9 ppm (Fig. 2B). The two signals probably represent two distinct environments for the AC carbonyl group. In comparison, when lauroyl carnitine was added to preformed vesicles (Fig. 2C), the intensity of the signal at 173.4 ppm increased but the signal at 172.9 ppm was absent. The signals from the labeled lauroyl carnitine were visualized more clearly by computing a difference spectrum by subtracting the spectrum of SUV from that of SUV with added AC (Fig. 2, insets). The difference spectrum of the cosonicated preparation showed two distinct signals (Fig. 2B, inset), whereas that of the sample with AC added to preformed vesicles showed only one peak (Fig. 2C, inset). A signal at 173.4 ppm was observed in both preparations, whereas the signal at 172.9 ppm was observed only when lauroyl carnitine was added to preformed vesicles.

The carbonyl spectra of decanoyl carnitine in SUV were also different for the two types of sample preparation (**Fig. 3**). Both preparations showed a signal at 174.0 to 174.1 ppm, whereas only decanoyl carnitine cosonicated with PC showed a signal at 172.9 ppm. The signal of ACo is shifted downfield (174.0 ppm; Fig. 3A) relative to that of

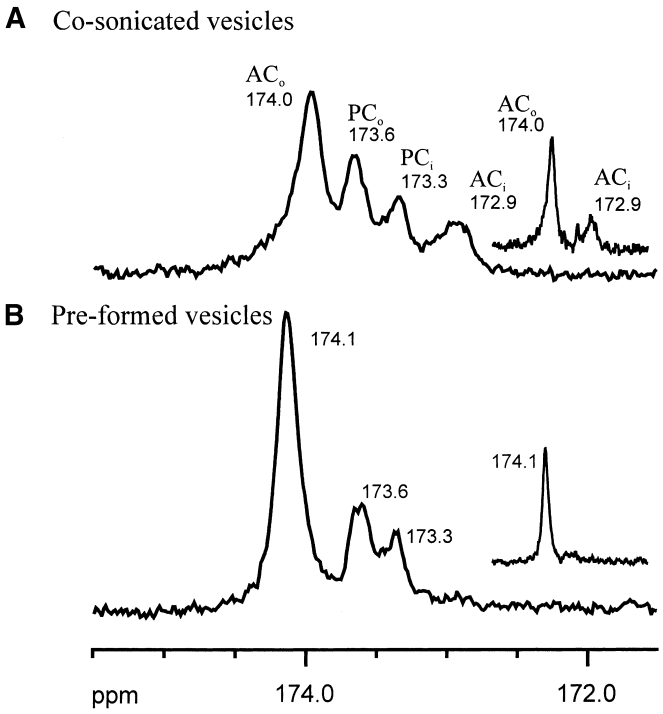

Fig. 3. ^{13}C-NMR spectrum (carbonyl region) of decanoyl carnitine in PC vesicles acquired under the same instrumental conditions as in Fig. 2. Difference spectra (insets) were obtained by subtracting the spectrum of PC vesicles containing no decanoyl carnitine from those containing decanoyl carnitine. A: Decanoyl carnitine (6 mol%) was added before sonication. The spectra reveal two new signals from the decanoyl carnitine (174.0 and 172.9 ppm). B: Decanoyl carnitine (10 mol%) was added after formation of vesicles. The spectra show only one new signal (174.1 ppm).

lauroyl carnitine (173.4 ppm; Fig. 2) because of partitioning into the aqueous phase, as discussed below. Figure 3 (insets) illustrates difference spectra of the experiments for decanoyl carnitine. The cosonicated preparation showed two distinct signals at 174.0 ppm and 172.9 ppm (Fig. 3A), whereas the sample for which AC was added to preformed vesicles showed only one peak at 174.1 ppm (Fig. 3B). The line widths of the carbonyl peaks were significantly larger than the line width of decanoyl carnitine in aqueous solution without PC. The ratio of the intensities from the decanoyl carnitine signals at 174.0 and 172.9 ppm was measured in the difference spectrum of Fig. 3. The ratio (3:1) is about twice as high as the ratio of the intensities of the phospholipid signals at 173.6 ppm (outer leaflet) and 173.4 ppm (inner leaflet) and shows a preference of AC for the outer leaflet.

The spectra of all ACs investigated showed two AC carbonyl signals for samples prepared by cosonication and one signal for samples prepared by adding AC to vesicles. These sites are assigned to AC in the outer (downfield peak) and inner (upfield peak) leaflet of the vesicle. The two signals are separated by 1.0 ppm (75 Hz), indicating that the exchange rate must be no greater than 34 s^{-1}. The ability of AC to move spontaneously from the outer to the inner leaflet of the bilayers on a slower time scale was assessed by obtaining ^{13}C-spectra as a function of time following the addition of AC to preformed vesicles. Even after 3 to 4 days of incubation of the sample at room temperature, no AC signal from the inner leaflet of the vesicles was observed. Spontaneous movement of AC from the outer to the inner leaflet would have resulted in the appearance of the AC carbonyl signal from the inner leaflet. The persistent absence of the signal from AC on the inner leaflet indicates that flip-flop is extremely slow.

pH studies

Because of the inductive effect from the titratable carnitine carboxyl group, the chemical shift of the nearby carbonyl carbon in the acyl chain (Fig. 1) is expected to be pH-sensitive. **Figure 4** shows pH-dependent spectra of decanoyl carnitine in cosonicated (A–C) and preformed (D–F) SUV in unbuffered 0.56% (75 mM) KCl solution. In both samples, the signals from AC in the outer leaflet of the vesicles shifted upfield by 1 ppm with the change from neutral to acidic pH. In the cosonicated sample, the signal from decanoyl carnitine on the inner leaflet of the vesicles (AC_o) also shifted upfield, but to a lesser extent (Fig. 4A–C). This effect is a result of a leak of protons across the bilayer, which occurs on the time scale of the collection of NMR data (min). The signals from the outer and inner leaflets of phospholipid bilayer represent nontitratable carbonyls, and their chemical shifts were constant at 173.6 and 173.3 ppm.

Lauroyl, myristoyl, and oleoyl carnitine also were studied at different pH values. The ^{13}C-spectra of these ACs were similar to those of palmitoyl carnitine for both cosonicated and preformed vesicle samples (data not shown). The ratios of the intensity of the two AC signals in cosonicated samples ranged from 3:1 to 4:1.

Even in acidic conditions under which the carboxyl on

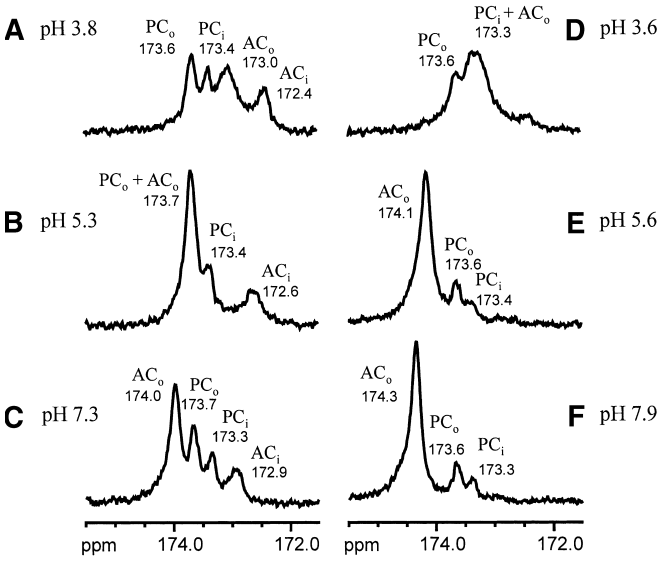

Fig. 4. ^{13}C-NMR spectrum (carbonyl region) of 6 mol% decanoyl carnitine in PC vesicles at selected pH values. Instrumental conditions were the same as in Figs. 2 and 3. A–C: Decanoyl carnitine was cosonicated with PC. The signals of the PC carbonyls are pH-insensitive and remain at 173.6 and 173.3 ppm, whereas the peak for AC_o (decanocyl carnitine in the outer leaflet of the vesicles) shifts upfield by 1.0 ppm when the pH is decreased from 7.3 (A) to 3.8 (C). The AC_i signal shifted to a smaller extent. D–F: Decanoyl carnitine was added after formation of vesicles. The AC_i was absent in these spectra.

the carnitine head group became protonated, the NMR spectra demonstrated that ACs did not move from the outer to inner leaflet across the bilayer, as evidenced by the absence of a peak representing AC on the inner leaflet in all samples prepared by adding AC to preformed vesicles (e.g, Fig. 4D–F). This behavior was expected because the positive charge on the ammonium group is unchanged with pH.

Effect of high AC levels in SUV

The effect of higher levels of AC on the gross structure of the vesicles was investigated systematically with myristoyl carnitine in small unilamellar vesicles (SUV). Disruption of the bilayer structure by formation of mixed micelles would be detected by the collapse of the two PC carbonyl peaks into a single peak representing the uniform environment of PC in micelles (32). Aggregation of vesicles would be seen as broadening of the lines of most peaks in the spectrum. We found that increasing the proportion of myristoyl carnitine from 0.5 mol% to 25 mol% with respect to phospholipid revealed no gross disruption of the phospholipid bilayers or aggregation of vesicles at any proportion of AC, as evidenced by the constancy of the phospholipid ^{13}C-spectrum. This result is consistent with the finding from infrared studies that palmitoyl carnitine mixes with dipalmitoyl PC without perturbing the gel-fluid phase transition even at high ratios (18).

Dilution studies

In the preceding results, we found that the carbonyl chemical shift of AC in the presence of SUV was dependent

on the acyl-chain length of the AC. This could reflect *i*) changes in the positioning of the AC in the bilayer or *ii*) partitioning of AC between the aqueous and lipid phases. The partitioning of AC between phospholipid bilayers and the aqueous buffer was studied by diluting vesicles (at fixed AC:PC ratios of 4–10 mol%) with buffer. For these experiments, samples were prepared and diluted in 50 mM phosphate buffer (pH 7.4) to prevent pH changes with dilution. Partitioning into the aqueous phase would be reflected in an increase in the carbonyl chemical shift toward that of the AC in buffer without vesicles (unbound AC).

Partitioning was studied by first characterizing, as reference points, the states in which ACs were completely membrane-bound and in which they were almost completely dissolved in solution. The reference point at which almost all ACs are bound to phospholipid bilayers was established by mixing long-chain ACs (palmitoyl and oleoyl) with phospholipids at high concentrations. The chemical shift of the carbonyl group of these ACs remained unchanged at 173.0 to 173.2 ppm even with 16-fold dilution of the suspension of PC vesicles. At the lowest concentrations studied (<0.1 mg/ml), the total concentration of AC in the sample was higher than the CMC of the AC in buffer alone, but the small amount of AC in the aqueous phase in the presence of PC would be in the monomer form. The chemical shift of the downfield AC peak (AC_o) was slightly upfield (~0.5 ppm) of the unbound AC_o in a micellar state (Table 1), signifying a small dehydration effect compared with the micellar state but a much more significant effect compared with the monomeric state (see Discussion). Because of the limited aqueous solubility of these long-chain ACs, we assumed that they were completely bound to PC and that their chemical shift is also representative of medium-chain ACs bound (completely) to vesicles. We established the reference point of the aqueous phase by mixing octanoyl carnitine in vesicles with a low concentration of phospholipid (8 mg/ml PC). The chemical shift of octanoyl carnitine in the presence of the PC (175.5 ppm) was the same as that for monomeric octanoyl carnitine in aqueous solution.

The most pronounced chemical-shift changes with respect to concentration of PC vesicles were observed in experiments with decanoyl carnitine (**Fig. 5**). The chemical shift of decanoyl carnitine in the outer leaflet (AC_o) was 173.9 ppm at a PC concentration of 50 mg/ml (Fig. 5, top panel). With dilution to a PC concentration of 25 mg/ml, the AC_o signal (174.3 ppm) became well resolved from the PC carbonyl and shifted further downfield with decreasing concentration. At a phospholipid concentration of 3.1 mg/ml (16-fold dilution from the original concentration), the chemical shift for AC_o (175.3 ppm) was the same as that of decanoyl carnitine in aqueous buffer at the same pH. Thus, nearly all of the decanoyl carnitine was partitioned from the membrane-bound phase to aqueous phase. The weak signal for AC_i showed no change with PC vesicle concentration, as expected, since dilution of the vesicles in buffer did not dilute the volume of the internal vesicle.

Spectra at different concentrations of lauroyl carnitine/vesicle preparations showed a small chemical shift change

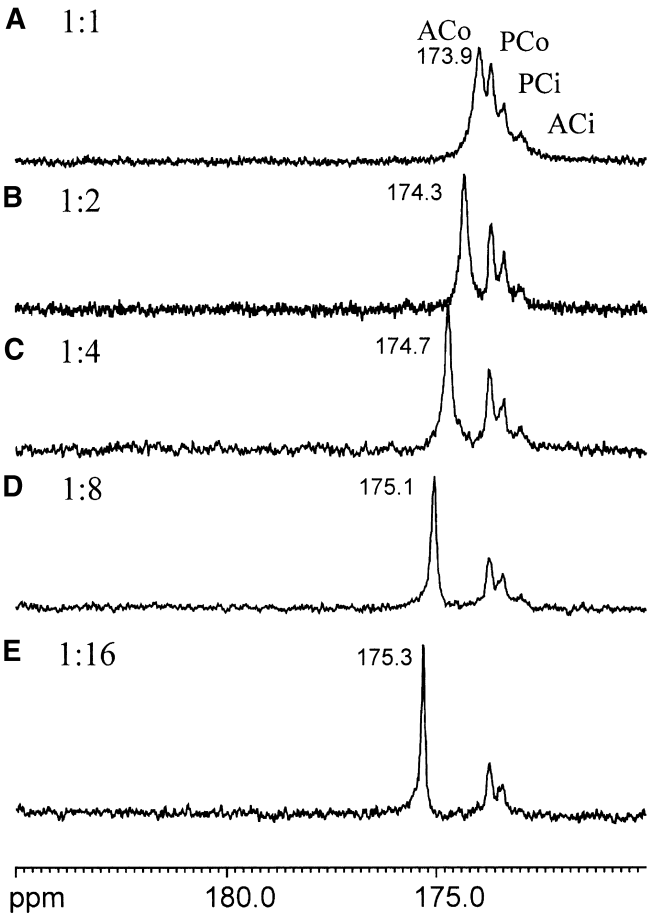

Fig. 5. ^{13}C-NMR spectrum (carbonyl region) of 6 mol% decanoyl carnitine cosonicated with PC vesicles. The small unilamellar vesicles (SUV) preparation (PC, 100 mg/ml) was diluted progressively in buffer. The peak for decanoyl carnitine in the outer leaflet shifted from 173.9 ppm at a PC concentration of 50 mg/ml (A) to 175.3 ppm at a PC concentration of 3.1 mg/ml (E). These spectra were acquired after 1,800 (A, B), 2,800 (C), 14,400 (D), and 28,800 (E) spectral accumulations with other conditions as in Figs. 2–4.

from 173.4 ppm at a PC concentration of 50 mg/ml to 173.8 ppm at a PC concentration of 12.5 mg/ml. These concentrations correspond to a concentration of lauroyl carnitine of <0.9 mg/ml, well below its CMC (4.3 mg/ml). The chemical shift values showed that lauroyl carnitine partitioned primarily to PC vesicles except at very low concentrations of phospholipid. For myristoyl carnitine, the only detectable change in the AC_o chemical shift was a slight shift at the lowest PC concentration studied (8 mg/ml). At the other extreme, results for octanoyl carnitine showed exclusive partitioning to the aqueous phase except at a very high concentration of PC (100 mg/ml). Thus, the chemical-shift data indicated a clear trend for the partition of ACs into the membrane as the acyl chain length of the AC increased.

The partition coefficient

These results for different chain lengths can be analyzed quantitatively for partitioning between the lipid and aqueous phase. Because the carnitine head group is

charged, it must be localized at the aqueous lipid interface when AC is bound to the phospholipid bilayer. The local environment of the labeled acyl carbonyl, which is adjacent to the head group, should be similar for all acyl chain lengths studied, and the chemical shift should remain constant when AC is membrane-bound. Long-chain ACs (palmitoyl and oleoyl) in the presence of vesicles showed a constant chemical shift for AC_o (173.2 ppm) that was unaffected by dilution. Myristoyl and lauroyl ACs showed the same chemical shift for AC_o (δ_b = 173.2 ppm) at the highest concentrations of vesicles. The chemical shift reference for unbound AC (δ_f = 175.5 ppm) is represented accurately by monomeric AC dissolved in aqueous buffer, as determined for the medium-chain AC (octanoyl and decanoyl). The most downfield chemical shift was observed at the highest dilution ratio of vesicles.

One narrow signal for AC_o was seen in all dilution experiments. The observed chemical shift varied between the two chemical shift extremes, indicating fast exchange of AC between the unbound and the lipid-bound phase (34). The observed chemical shift (δ_{obs}) is related to the population of the bound (p_b) and free state (p_f) by the following equation:

$$\delta_{obs} = p_b\delta_b + p_f\delta_f \qquad (Eq.\ 1)$$

Therefore, the membrane-bound fraction can be calculated as

$$p_b = \frac{\delta_{obs} - \delta_f}{\delta_b - \delta_f} \qquad (Eq.\ 2)$$

As the dilution ratio increased (the concentration of phospholipid decreased), the signal of AC on the outside of the vesicles shifted downfield, reflecting the partitioning of AC from the lipid phase into the aqueous phase. For decanoyl carnitine, most (67%) was bound to PC at the highest PC concentration and almost all partitioned into the aqueous phase for the most dilute sample (1:16 ratio). Similar experiments with ACs of different chain lengths clearly showed that increasing the length of the acyl chain favors the lipid-bound phase, as expected.

The partition coefficient for AC between the phospholipid-bound phase and the aqueous phase is defined by the equation

$$\frac{1}{K} = \frac{[AC_b]}{[AC_f][PC]} \qquad (Eq.\ 3)$$

The partition coefficient ($1/K$) was determined from the linear fitting of the ratio of bound over free AC vs. the concentration of PC, as illustrated for decanoyl carnitine in **Fig. 6**. The slope of this plot is the partition coefficient ($1/K$). The calculated partition coefficients for other ACs (up to 14 carbons) are given in **Table 2**. They range from 1.28 M^{-1} (octanoyl carnitine) to 1,050 M^{-1} (myristoyl carnitine). The semi-log plot in **Fig. 7** shows a linear relationship between the partition coefficient and the number of carbons in the acyl chain from 8 to 14 carbons. The calculated value for myristoyl carnitine has a much higher error because only small chemical shift changes were observed at the high dilution ratios. The chemical shift measurements can detect partitioning into the aqueous phase as low as 5% to 10% (corresponding to a chemical shift change of 0.1–0.2 ppm). Our approach did not give partition coefficients for long-chain ACs, but those for palmitoyl and oleoyl carnitine must be larger than that for myristoyl carnitine. Previously, the partition coefficient for palmitoyl carnitine was estimated as 34 M^{-1} (16), a value close to that extrapolated from our plot (not shown).

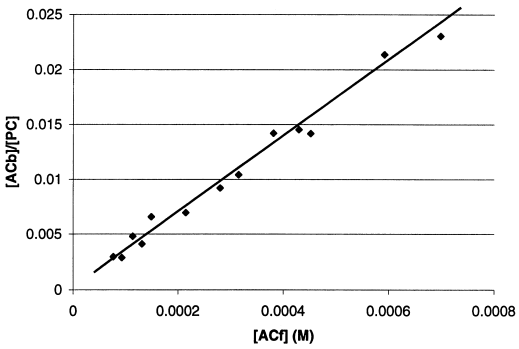

Fig. 6. The amount of decanoyl carnitine bound to SUV (mol AC/ mol PC) versus the concentration of decanoyl carnitine. The slope gives the reciprocal of the partition coefficient ($1/K$), which is 32 M^{-1}.

Compared with unesterified fatty acids, ACs show a more favorable partition into the aqueous phase in the presence of vesicles. For example, the partition coefficient for myristoyl carnitine measured in this study (~1,000 M^{-1}) is about 10-fold lower than that for myristic acid (~10,000 M^{-1}) (35). On the other hand, acyl-CoAs are more water-soluble than fatty acids or ACs with the same acyl chain, and the partition coefficient of myristoyl CoA is 194 M^{-1} (J. Boylan and J. A. Hamilton, unpublished data).

DISCUSSION

Localization of ACs in bilayers

Acyl carnitines, which are composed of a polar head group and a hydrophobic tail, are structurally similar to fatty acids. They presumably assume similar configurations in a phospholipid bilayer, with the head group positioned at the lipid-aqueous interface, a prediction that is validated in our studies. To study the interactions of ACs with model membranes, we focused on the carbonyl carbon in the acyl chain. The sensitivity of the carbonyl

TABLE 2. The partition coefficient ($1/K$) of ACs calculated by weight using units of mg/ml (column 2) and molar units (column 3)

Chain Length	Partition Coefficient ($1/K$)	Partition Coefficient ($1/K$) (1/M)
	ml/mg	
Octanoyl carnitine (C8:0)	0.00169	1.28
Decanoyl carnitine (C10:0)	0.0277	32.0
Lauroyl carnitine (C12:0)	0.306	233
Myristoyl carnitine (C14:0)	1.39	1050

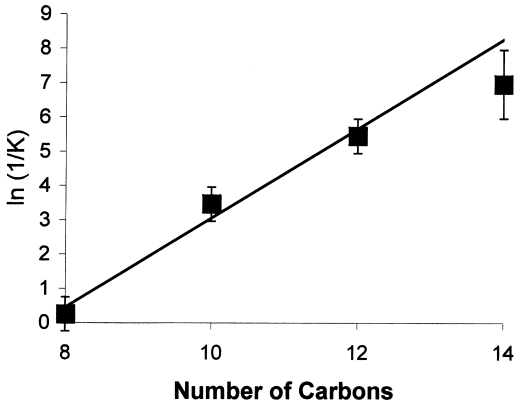

Fig. 7. The partition coefficient versus the number of carbons in the acyl chain of AC. The slope gives the free energy/CH_2 group, which is –714 cal/mol.

chemical shift to the local environment has been used to detect, without separation techniques, the binding of several types of acylated lipids to phospholipids, including cholesteryl esters (36–38), triglycerides (28, 36, 37, 39, 40), diglycerides (30, 36), acyl-CoA (32), and fatty acids (41, 42). Binding of ACs to vesicles was accompanied by an upfield shift relative to the micellar or monomeric form, as predicted by the behavior of the more polar lipids (acyl-CoA and fatty acids). This effect can be attributed to the partial dehydration of the carbonyl upon insertion of the acyl chain into the phospholipid monolayer (32).

The polar head region of ACs is localized at the aqueous interface of the phospholipid bilayer, analogous to the localization of other single-chain amphiphilic lipids, such as fatty acids, lysophospholipids, and acyl-CoA. Although these lipids can act as detergents, we have contended that this is probably not the primary mechanism by which they disrupt membrane processes when they accumulate at high concentrations in cells during ischemia and other disease states (32, 33, 43). The same argument can be made for ACs because they incorporate to high levels in model phospholipid membranes (up to 25 mol% in our experiments) without resulting in major structural reorganization of the bilayer structure. As discussed previously, many of the deleterious effects of ACs are not reproduced by other detergents (1). Thus, their unique effects in membranes are probably specific molecular effects that reflect the localization of their unique head group at the aqueous interface.

Absence of transmembrane movement in bilayers

Because of the curvature difference between the outer and inner leaflets of SUV, the two leaflets exhibit different hydration of carbonyl groups in the aqueous interface; these differences can result in a small difference in chemical shift of the carbonyl carbons. This property permitted two separate signals to be observed when ACs were cosonicated with phospholipid and distributed in both leaflets. The presence of the two narrow AC signals suggests a slow exchange between ACs between the inner and outer leaflets. Based on the chemical shift difference of 0.4 to 1.0 ppm at a ^{13}C-resonance frequency of 75 MHz, the exchange cannot be faster than 22.5 to 33.8 s^{-1} (35). This observation does not preclude spontaneous transmembrane movement (flip-flop) on a slower time scale. Therefore, additional experiments were designed to exclude slower flip-flop of ACs across the membranes. After adding AC to the outer leaflet of preformed vesicles, we observed only the signal characteristic of the AC in the outer leaflet, even after three to four days of incubation at room temperature or at 4°C and after various manipulations of these AC samples. The persistent absence of the AC signal from the inner leaflet of the vesicles argues against flip-flop of AC across the phospholipid bilayers. In contrast, fluorescence studies of 1-pyrenebutyryl carnitine concluded that its inward movement across the bilayer of SUV was rapid (complete within 30 s) but that outward movement was much slower (20). Our results also show that there was no breakdown (transient or permanent) of the vesicles, which would have permitted the transbilayer equilibration of AC. Previous studies found that much higher proportions of AC dissolved large unilamellar vesicles (15).

The observation that ACs cannot flip-flop across a protein-free phospholipid bilayer is consistent with the prediction that charged molecules or ions require a transporter or a channel for rapid transport across the lipid bilayer. The pKa of the carboxyl group of AC is 3.8 in aqueous solution, and the AC molecule is zwitterionic at neutral pH (29). The pKa of the carboxyl of lipid molecules can change significantly (up to 4 pH units in fatty acids), resulting in the presence of uncharged fatty acids in the bilayer (42) upon going from the unbound state to the bilayer interface. However, the positive charge on the quaternary amine on the choline group of AC would be unaffected by binding to SUV. This nontitratable positive charge on the head group would inhibit penetration of the molecule into the central hydrophobic core of the bilayer, irrespective of the ionization state of the carboxyl. It is interesting to note that there is strong evidence that palmitoyl carnitine can diffuse (flip-flop) across erythrocytes at a measurable rate ($t_{1/2}$ = hours). As discussed by Classen et al. (21), this finding is consistent with other observations that the rate of flip-flop of certain molecules can be enhanced in biomembranes relative to the rate in protein-free phospholipid bilayers as a result of membrane defects caused by proteins. This enhancement in erythrocytes was not the result of a specific transport protein, and the rates were too slow for effective metabolism. On the basis of this example, we can speculate that the leakage of ACs into the plasma in patients with disorders of fatty acid oxidation could occur by slow diffusion through the plasma membrane.

The absence of transmembrane movement of ACs in a simple phospholipid bilayer is in sharp contrast with the presence of such movement by unesterified fatty acids, which can diffuse rapidly ($t_{1/2} < 1$ s) through phospholipid bilayers via the equilibrium concentration of their un-ionized form (44, 45). Fatty acids also exhibit or modulate many physiological functions, such as cell signaling and ion channel activities (46). In some cases, the unrestricted and unregulated flow of fatty acids across mem-

branes into different compartments of the cell may not be desirable. According to our hypothesis of diffusion by flip-flop (46–48), unesterified fatty acids can diffuse into the inner mitochondrial matrix in an unregulated manner. Long-chain fatty acids cannot be activated in the inner mitochondrial compartment for entry into the β-oxidation cycle, but their presence in the inner mitochondrial membrane can uncouple the proton gradient that is used to generate ATP (49).

The charged head group of ACs, which prohibits spontaneous rapid movement through a lipid membrane, makes ACs suitable candidates for regulating the flow of fatty acyl chains into the mitochondria. Acyl-CoA is another molecular component in the transport of fatty acids. In the outer mitochondrial space, the fatty acyl group is transferred from coenzyme A to the carnitine group for transport through the mitochondrial membrane, after which the fatty acyl group of AC is transferred to give acyl-CoA. Why does this transport/metabolic mechanism involve both AC and acyl-CoA? Acyl-CoA cannot flip-flop across a lipid bilayer (32) and in principle could serve to regulate transport of the acyl chain. There are advantages to transporting AC rather than acyl-CoA, as the size of a carnitine head group is much smaller than that of a coenzyme A group (16), reducing the need for the protein transporter to build a large cleft within the membrane for the charged head group. In addition or alternatively, because acyl-CoA participates in and regulates many anabolic and catabolic pathways (50), its conversion to AC may provide an alternative mechanism not strictly dependent on the concentration of acyl-CoA to regulate the flow of fatty acyl chains into the inner mitochondrial matrix for β-oxidation.

Partitioning between aqueous and lipid phases

The partitioning of AC between the aqueous phase and phospholipid bilayers is an important determinant of both the structural and functional effects of ACs in membranes. In addition, the transport properties of ACs may be affected by partitioning. Whether the AC is largely membrane bound or dissolved in bulk water can have a potential effect on the rate of the translocase reaction if the translocase preferentially binds AC in the membrane or the aqueous phase.

Desorption of the AC molecule from the lipid phase requires energy to disrupt the hydrophobic interactions of the acyl chain of the AC with those of the phospholipids. With each additional carbon, hydrophobic interactions will be stronger (and aqueous solvation less favorable) and more energy will be required for desorption of the AC into the aqueous solution. The partition coefficient is related to Gibb's free energy as follows:

$$\frac{1}{K} = A\exp\left(-\frac{\Delta G}{RT}\right) \qquad \textit{(Eq. 4)}$$

$$\Delta G = RT\ln K + \text{constant}$$

$$\frac{\mathrm{d}(\Delta \mathrm{G})}{\mathrm{d}(\mathrm{CH_2})} = RT\,\frac{d(\ln K)}{d(CH_2)}$$

where K is the partition coefficient, R is the gas constant, and T is temperature in Kelvin.

The partition coefficient determined from our dilution studies (Figs. 5 and 6) permits calculation of the change of Gibb's free energy per methylene group, $\Delta(\Delta G)/CH_2$. From the linear relationship between the partition coefficient and the number of carbons in the acyl chain (Fig. 7), $\Delta(\Delta G)/CH_2$ (the slope) was calculated to be −714 cal/mol, or −3 kJ/mol. The negative sign denotes the release of Gibb's free energy when a methylene group enters the lipid bilayer from the aqueous environment. The result for ACs is quantitatively similar to reported values for fatty alcohols and fatty acids. The logarithm of the solubility of aliphatic acids, alcohols, and alkanes is linearly related to chain length (51). The free energy per methylene group ($\Delta(\Delta G)/CH_2$) of fatty acids has been reported to be 640 cal/mol (2.69 kJ/mol) and 850 cal/mol (3.57 kJ/mol) (52–54). Therefore, the membrane-binding properties of AC, as with other related lipids, are largely controlled by the hydrophobic effect.

The carnitine head group of ACs is quite polar and contributes to the Gibb's free energy, ΔG, of lipid-aqueous partitioning. Because of the carnitine head group, ACs partition more favorably than fatty acids into the aqueous phase (35). Moreover, the partitioning of an amphipathic lipid molecule also depends on the Gibb's free energy of the hydrophobic tail. The similarity of the $\Delta(\Delta G)/CH_2$ of ACs to that of other lipids signifies that, when bound to membranes, ACs assume a configuration similar to that of other amphipathic lipid molecules. Since $\Delta(\Delta G)/CH_2$ reflects only the energy required to move the hydrophobic acyl carbon between the lipid phase and the water phase, the acyl chain must penetrate into the hydrocarbon region of the bilayer.

Summary

Our results show that ACs with chain lengths longer than six carbons bind to phospholipid bilayers in the same manner as related amphipathic lipids, with the head group at the aqueous interface and the acyl chain intercalated between acyl chains of phospholipids. Increasing chain length favors partitioning into the membrane phase, and long-chain ACs (16 or 18 carbons) reside almost completely in the membrane phase. Extrapolation of our data indicates that short-chain ACs will be present mainly or exclusively in the aqueous phase of cells. The partitioning properties predict that short-chain ACs will access transport proteins and enzymes via, or in, the aqueous phase. The solubility of long-chain ACs in the lipid membrane phase relative to the aqueous phase is so high that long-chain ACs, if they are not passed directly between membrane-bound translocase and enzyme, most likely will reach their translocases and metabolic enzymes (e.g., CPT II) by lateral diffusion through the membrane (2). Thus, the long-chain AC has a rather specific orientation to present to membrane proteins as opposed to the random orientation of short-chain ACs, and this difference could govern or enhance selectivity of ACs with different chain lengths.

The extremely slow flip-flop of AC in phospholipid bilayers (SUV) strongly suggests that transport proteins are required for transmembrane movement of long-chain ACs at rates sufficient for effective metabolism of acyl chains in mitochondria. In our view, fatty acids can diffuse across the mitochondrial membranes, and do so in a largely unregulated manner. Long-chain fatty acids are not utilized for β-oxidation because only the soluble (and less abundant) medium-chain fatty acids are activated by the inner mitochondrial CoA synthase. The conversion of long-chain fatty acids to acyl-CoA and AC regulates the flux of fatty acid chains for β-oxidation.

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