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Transfer of oleic acid between albumin and phospholipid vesicles

(¹³C NMR/fatty acids/phospholipid bilayers)

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ABSTRACT **The net transfer of oleic acid between egg phosphatidylcholine unilamellar vesicles and bovine serum albumin has been monitored by ^{13}C NMR spectroscopy and 90% isotopically substituted [1-^{13}C]oleic acid. The carboxyl chemical shifts of oleic acid bound to albumin were different from those for oleic acid in phospholipid vesicles. Therefore, in mixtures of donor particles (vesicles or albumin with oleic acid) and acceptor particles (fatty acid-free albumin or vesicles), the equilibrium distribution of oleic acid was determined from chemical shift and peak intensity data without separation of donor and acceptor particles. In a system containing equal masses of albumin and phospholipid and a stoichiometry of 4–5 mol of oleic acid per mol of albumin, the oleic acid distribution was pH dependent, with ≥80% of the oleic acid associated with albumin at pH 7.4; association was ≥90% at pH 8.0. Decreasing the pH below 7.4 markedly decreased the proportion of fatty acid bound to albumin; at pH 5.4, ≤10% of the oleic acid was bound to albumin and >90% was associated with vesicles. The distribution was reversible with pH and was independent of whether vesicles or albumin acted as a donor. These data suggest that pH may strongly influence the partitioning of fatty acid between cellular membranes and albumin. The ^{13}C NMR method is also advantageous because it provides information about the structural environments of oleic acid bound to albumin or phospholipid, the ionization state of oleic acid in each environment, and the structural integrity of the vesicles. In addition, minimum and maximum limits for the exchange rates of oleic acid among different environments were obtained from the NMR data.**

It is well known that fatty acids (FA) bind with high affinity to serum albumin and that albumin is the major transport system for unesterified FA in the bloodstream (1, 2). FA also bind to phospholipid bilayers (3–5) and to cell membranes (6, 7). As the interface between the plasma and the cytoplasm, cellular plasma membranes are a critical part of the transport system for FA. The mechanism(s) of FA transfer and the factors regulating the transfer of FA between albumin and membranes are largely unknown.

We have recently undertaken systematic studies of the interactions of FA with albumin (8–11) and with protein-free model membranes in the form of unilamellar phospholipid vesicles (11). We found that ^{13}C NMR spectroscopy provides a straightforward way of examining partitioning of FA between albumin and phospholipid vesicles (11). To monitor the small, but physiologically relevant, amounts of FA bound to albumin or to phospholipid (1), we used oleic acid with 90% ^{13}C isotopic substitution in the carboxyl carbon. Oleic acid is the most abundant FA found in association with albumin (12) and it is the FA with the highest binding affinity to albumin (13).

Using ^{13}C NMR spectroscopy, we have (*i*) determined the equilibrium distribution of oleic acid between bovine serum albumin (BSA) and egg phosphatidylcholine (PtdCho) vesicles; (*ii*) demonstrated a marked effect of pH on partitioning; and (*iii*) assessed kinetic factors, such as the rate of oleic acid transfer between protein and lipid and flip-flop of oleic acid in the PtdCho bilayer.

MATERIALS AND METHODS

Materials. Egg yolk PtdCho was obtained from Lipid Products (Nutley, England) and was >99% pure by thin-layer chromatography (TLC); 90% [1-^{13}C]oleic acid was purchased from KOR Isotopes (Cambridge, MA) and was >96% pure by TLC and gas/liquid chromatography (8). All oleic acid used in these studies was [^{13}C]carboxyl carbon enriched. Essentially FA-free (crystalline, lyophilized) BSA from Sigma contained <0.02 mol of FA bound per mol of BSA by TLC (8) and ≤5% protein impurities as measured by 10% $NaDodSO_4$/PAGE (9).

Sample Preparation. PtdCho vesicles with oleic acid were prepared from the co-dried lipids essentially as described (14), except the long ultracentrifugation step was omitted. After low-speed centrifugation to remove Ti fragments and a small amount of undispersed lipid (14), the pH of the aqueous medium (0.56% KCl) was adjusted to 7.4. Selected samples were analyzed for lyso-PtdCho and FA content by TLC. No lyso-PtdCho was detected. The FA/PtdCho ratio, based on TLC quantitation of FA and quantitation of PtdCho by a modified Bartlett method (15), was that expected from the calculated composition. A solution of BSA in 0.56% KCl was prepared and the concentration (10%, wt/vol) was determined from the absorbance at 279 nm (16). Oleic acid–BSA complexes were made from aqueous potassium oleate as described (8). In some cases, 5 mM EDTA was added to the 0.56% KCl solution. For transfer studies, PtdCho vesicles with oleic acid were mixed with FA-free BSA, or oleic acid–BSA complexes were mixed with PtdCho vesicles containing no added FA. Samples were incubated in an NMR tube for 30 min at 35°C with frequent mixing prior to NMR analysis. Samples were initially slightly turbid but showed no significant changes within the time course of transfer studies (2–10 hr).

NMR Methods. ^{13}C NMR spectra were obtained on a Bruker WP-200 spectrometer at 50.3 MHz as described (8, 9). Internal ^2H_2O was used as a lock and shim signal. Chemical shifts were measured (±0.1 ppm) with the PtdCho fatty acyl methyl resonance at 14.10 ppm as an internal reference (14). In previous studies of FA–BSA complexes, the protein peak at 39.54 ppm (relative to external tetramethylsilane) was used as a chemical shift reference (8, 9). In spectra of mixtures of PtdCho and BSA, this protein peak appeared at 39.85 ppm relative to the methyl at 14.10 ppm; therefore, 0.3 ppm must be added to previous chemical shift values for the carboxyl peaks from oleic acid bound to BSA (8) in order to make a direct quantitative comparison with values reported in the

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Abbreviations: FA, fatty acid(s); BSA, bovine serum albumin; PtdCho, phosphatidylcholine; S/N, signal-to-noise ratio.

present study. After 15–20 min of spectral accumulation time, signal-to-noise ratios (S/N) were often sufficient to ascertain the chemical shifts, relative peak intensities, and line shapes of the carboxyl peaks. In all cases, these features were essentially the same as those seen after longer accumulation times (1–2 hr).

RESULTS

The ^{13}C NMR spectrum of PtdCho vesicles with [1-^{13}C]oleic acid contained a single resonance for the ^{13}C-enriched carboxyl carbon; no other oleic acid resonances were detected. PtdCho resonances (chemical shifts, line widths, and relative intensities) were unaffected by the incorporation of small amounts (2–20 mol %) of oleic acid. With increasing mol ratio of FA, a single FA carboxyl resonance of progressively increasing intensity was observed. With changes in pH, there were significant spectral changes of the oleic acid carboxyl peak. Most important, there was a large change in chemical shift, as shown for the carboxyl region of three selected spectra in Fig. 1. At all pH values there was a single FA carboxyl peak that exhibited modest line width changes with changes in pH, as shown. Fig. 1 also shows that there were two peaks for the PtdCho carbonyl carbons, corresponding to carbonyl groups in the outer leaflet (the more intense peak at 173.9 ppm) and the inner leaflet (173.6 ppm), as expected (17). There were no significant pH-dependent changes in S/N or line width of any PtdCho peak, as illustrated by the carbonyl peaks (Fig. 1). In contrast to the case for PtdCho, a single peak was detected from the FA carboxyl carbon. There could be three different explanations for this result: (*i*) FA is distributed on the inner and outer monolayer leaflets, but the carboxyl environment is the same in both leaflets; (*ii*) FA is distributed in both monolayers in different environments, but the chemical shift differences are averaged by rapid exchange; or (*iii*) FA is only present in one leaflet. The last explanation is considered unlikely when vesicles are prepared by co-sonication of FA and PtdCho (see *Materials and Methods*).

A complete NMR titration curve (chemical shift vs. pH) for 5 mol % oleic acid in vesicles is shown in Fig. 2 (lower curve). An apparent pK_a of 7.6 was determined as the pH at ½ the difference between the maximum and minimum chemical shift (4, 18).

The interactions of FA with BSA, as assessed by ^{13}C NMR, are more complex than those of FA with PC bilayers. Previous studies of oleic acid–BSA (8) and myristic acid–BSA complexes (9) revealed multiple FA peaks at all mol ratios studied (0.5–10.0 mol of FA per mol of BSA). Oleic acid showed more than one peak at all pH values except low pH (pH < 4; ref. 8); at pH 7.4, the number of peaks and the relative intensities of the different peaks depended on the oleic acid–BSA mol ratio. Only one peak, the major peak seen with ≥3 mol FA per mol of BSA, showed chemical shift changes with pH (8). The partial titration curve for this peak obtained for a complex containing 5 mol of oleic acid per mol of BSA (8) is shown in Fig. 2. Although the titration curve is incomplete, the NMR data suggest an apparent pK_a of ≈4.2 for oleic acid bound to BSA in the environment monitored by this peak. Fig. 2 shows the large difference between the pK_a values of oleic acid bound to BSA and to PtdCho. It also shows that oleic acid bound to PtdCho and to the major binding environment on BSA has a significantly different chemical shift at all pH values. Thus, in mixtures of albumin and vesicles with oleic acid, it should be possible to determine whether the oleic acid is associated with albumin or phospholipid by the carboxyl chemical shift, provided that

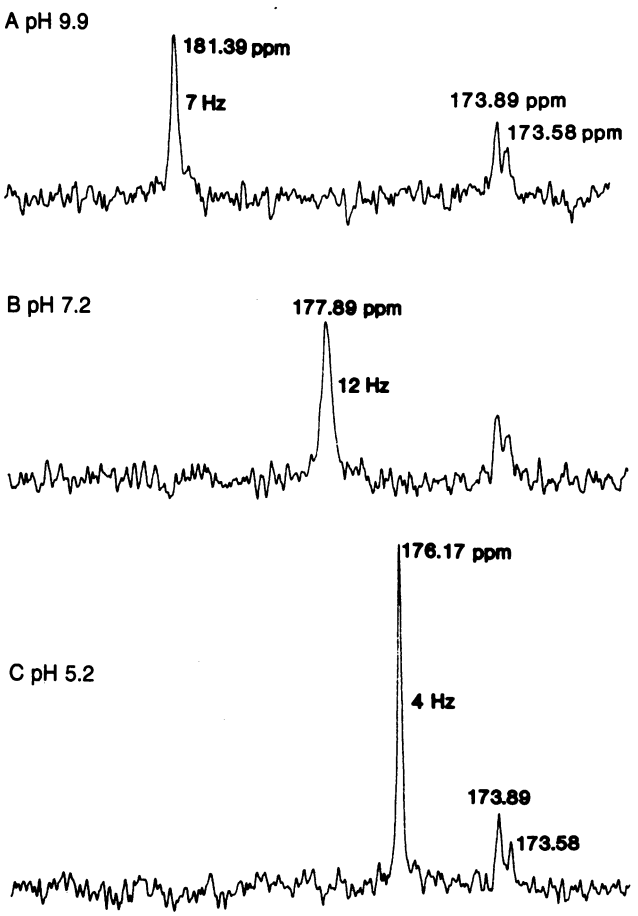

FIG. 1. The carboxyl and carbonyl region of the ^{13}C NMR spectrum at 35°C of egg PtdCho vesicles with 5 mol % of 90% [1-^{13}C]oleic acid at selected pH values as indicated: (*A*) pH 9.9; (*B*) pH 7.2; (*C*) pH 5.2. Spectra were recorded after 2000 accumulations and a pulse interval of 1.8 sec. Chemical shifts are indicated in ppm and linewidth values are in Hz.

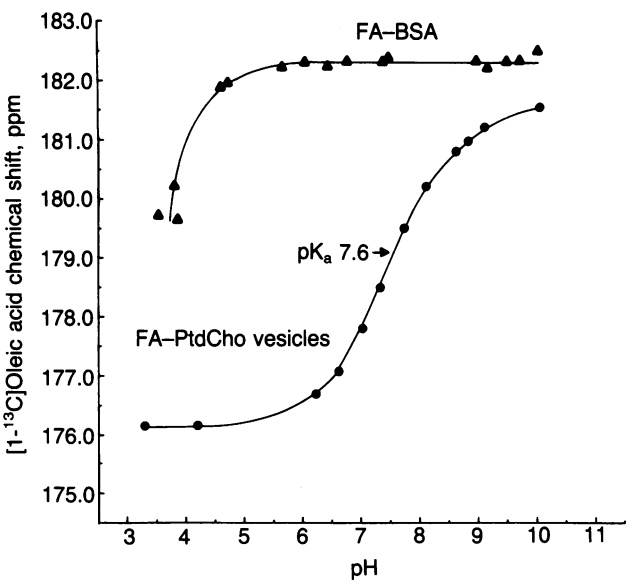

FIG. 2. Plot of the carboxyl ^{13}C chemical shift of 90% [1-^{13}C]oleic acid in egg PtdCho vesicles (lower curve) and of the major peak of 90% [1-^{13}C]oleic acid bound to BSA. The concentration of oleic acid in the vesicles was 5 mol % (2 wt %); the mol ratio of oleic acid to BSA was 5:1. The oleic acid–BSA titration data are from ref. 8; the chemical shifts are corrected as described in *Materials and Methods*.

the rate of exchange of FA between the lipid and protein is not fast on the NMR time scale.

To investigate the partitioning of oleic acid between the two systems, we mixed either vesicles containing oleic acid with FA-free BSA or oleic acid bound to BSA with PtdCho vesicles containing no FA. The final results were independent of which system acted as donor. After equilibration at pH ≈7.4 for 30 min, ^{13}C spectra were obtained at 35°C (≈1 hr per spectrum).

Fig. 3 shows the results of a transfer experiment starting with 6 mol % oleic acid in vesicles (100 mg of PtdCho per ml) to which an equal volume of aqueous FA-free BSA (100 mg/ml) was added to yield a stoichiometry of 5 mol of oleic acid per mol of BSA. Fig. 3*A* shows the carboxyl region of the ^{13}C NMR spectrum at pH 7.4 of oleic acid incorporated into vesicles prior to addition of BSA. A single peak for the [^{13}C]carboxyl carbon of oleic acid was present at the expected chemical shift (Fig. 2). Fig. 3*B* shows the same spectral region after addition of BSA and adjustment of the pH to 7.0. Most of the oleic acid transferred to BSA, as shown by appearance of peaks (a–d) that have the same chemical shift as found for oleic acid bound to BSA (8). A small oleic acid peak occurred at the chemical shift (177.4 ppm) obtained for oleic acid in PtdCho vesicles at pH 7.0 in the absence of BSA (Fig. 2). All the peaks for oleic acid bound to BSA were well separated from the peak for oleic acid in PtdCho. By comparison with previous spectra obtained under the same spectrometer conditions (8), the relative intensities of peaks a–d indicated that between 3 and 4 mol of oleic acid was bound per mol of BSA. Since ≈33% of the total oleic acid was bound to PtdCho (as approximated by the area of the peak at 177.4 ppm relative to total area of peaks a–d), essentially all the oleic acid in the incubation mixture yielded observable ^{13}C resonances. In addition, spectra obtained at later times for the same sample were identical, showing that equilibration occurred within ≈60 min. Since the oleic acid peaks had the same chemical shift and line width as in the individual systems (FA/PtdCho or FA/BSA), the exchange of oleic acid between lipid and protein environments was slow on the NMR time scale. After adjustment of the pH of this mixture from 7.0 to 8.0, the area of peaks a–d (FA bound to BSA) increased, while the peak representing FA in vesicles shifted to 179.8 ppm (as predicted; Fig. 2) and decreased in intensity (spectrum not shown).

Fig. 4 shows the pH dependence in the acid range (pH 5.5–7.3) of the transfer of oleic acid between BSA and

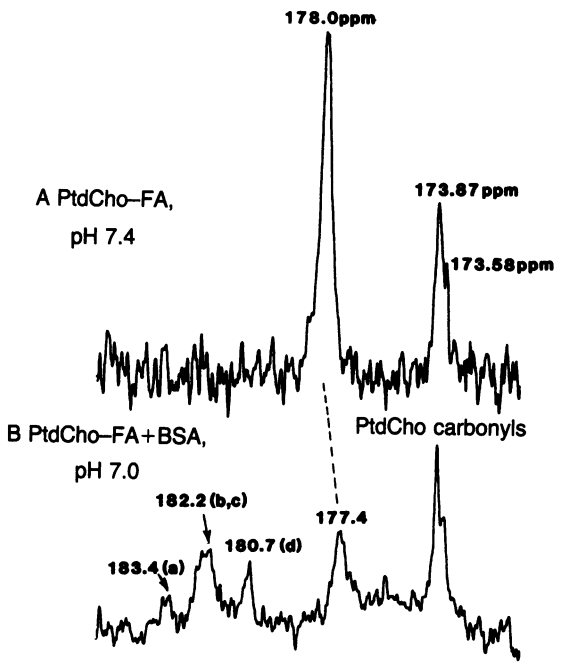

FIG. 3. The carboxyl and carbonyl region of the ^{13}C NMR spectrum at 40°C of (*A*) egg PtdCho vesicles (50 mg/ml) with 6 mol % of 90% [1-^{13}C]oleic acid at pH 7.4 after 1000 spectral accumulations and a pulse interval of 1.8 sec; and (*B*) a 50:50 (vol/vol) mixture of PtdCho/oleic acid vesicles (final concentration, 50 mg/ml) and 10% (wt/vol) BSA [final concentration, 5% (wt/vol)] at pH 7.0 after 4000 spectral accumulations and a pulse interval of 1.8 sec. In *B*, the number given above each peak is the chemical shift in ppm. Peaks from oleic acid bound to BSA are designated by letters in parentheses, according to ref. 7. The change in chemical shift of the peak from oleic acid in PtdCho (dashed line) is a titration shift (Figs. 1 and 2).

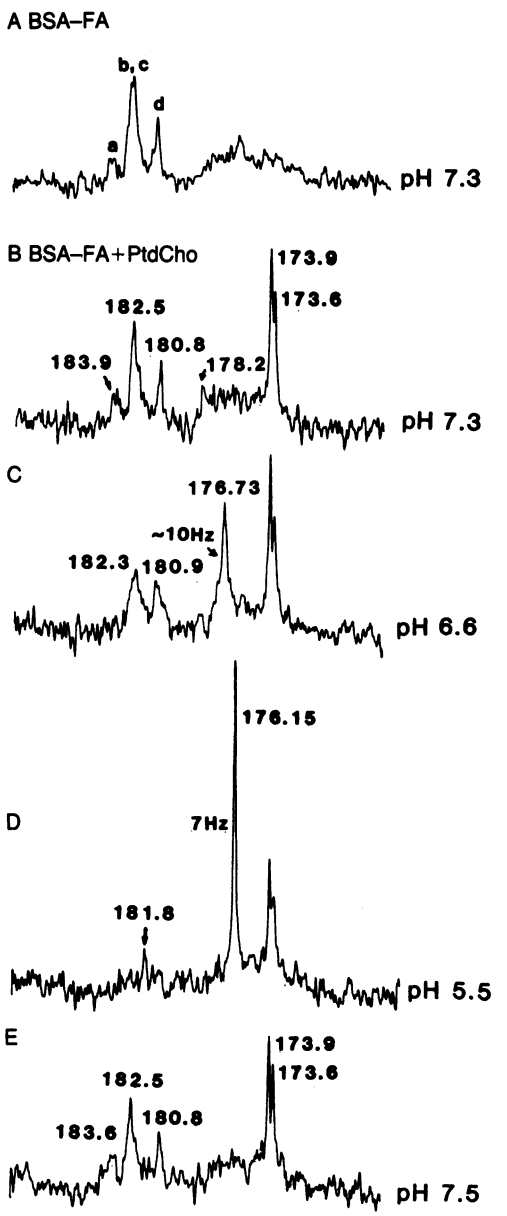

FIG. 4. The carboxyl and carbonyl region of the ^{13}C NMR spectrum at 35°C of 4 mol of 90% [1-^{13}C]oleic acid per mol of BSA at pH 7.3 (*A*), a 50:50 (vol/vol) mixture of the BSA–FA complex from (*A*) and egg PtdCho vesicles containing no oleic acid at pH 7.3 (*B*), and the same mixture at pH 6.6 (*C*), pH 5.5 (*D*), and pH 7.5 (*E*). All spectra were recorded after 2000 accumulations at a pulse interval of 1.8 sec. The letter designations in *A* are the same as those in Fig. 3 and ref. 8. The number given above each peak is the chemical shift in ppm; line width values of selected peaks in *C* and *D* are indicated in Hz.

PtdCho vesicles, as monitored by changes in the [^{13}C]carboxyl carbon spectrum. In this experiment, PtdCho vesicles were added to the donor (4 mol of oleic acid per mol of BSA) and the effect of decreasing pH was investigated. The spectrum of the donor before addition of PtdCho (Fig. 4*A*) is similar to spectra previously obtained at the same mol ratio (8). Fig. 4*B* shows that most (>90%) of the oleic acid was bound to BSA at pH 7.3, because the envelope of peaks at 180.0–184.0 ppm closely resembled that of Fig. 4*A*. Thus, the presence of PtdCho vesicles did not perturb the FA binding properties of BSA with respect to the local magnetic environments of the FA carboxyl carbon and the relative occupancy of these different environments. A weak resonance at 178.2 ppm (Fig. 4*B*) indicates that a small amount of FA may have transferred to PtdCho at pH 7.3. However, a major redistribution of oleic acid occurred on decreasing the pH to 6.6. At this pH, the oleic acid was distributed approximately equally between BSA and PtdCho vesicles (Fig. 4*C*). (Since the mixture contained equal masses of BSA and PtdCho, a partition coefficient based on grams could be calculated from relative peak intensities. However, in the absence of spin–lattice relaxation time and nuclear Overhauser enhancement measurements, peak areas cannot be related directly to the amounts of oleic acid contributing to the different resonances.) At pH 5.5, nearly all (>90%) the oleic acid was associated with PtdCho vesicles, as indicated by the intense narrow (7 Hz) resonance at the predicted (Fig. 2) chemical shift (176.15 ppm) and by the very weak peak (181.8 ppm) in the chemical shift region for FA bound to BSA. After adjusting the pH from 5.5 to 7.5, the mixture yielded a spectrum (Fig. 4*D*) nearly identical to the initial spectrum at pH 7.3 (Fig. 4*B*), showing the reversibility of FA transfer. In addition, the observations that the PtdCho carbonyl peaks were narrow, exhibited no decreases in S/N, and had the same relative intensities (within experimental error) in all spectra, provided evidence that the small unilamellar vesicles remained intact for the duration of the experiments.

Spectra as a function of pH were also obtained starting with (*i*) a mixture of 6 mol % oleic acid in vesicles (donor) and FA-free BSA (acceptor) and decreasing the pH from 8.4 to 5.4; and (*ii*) a mixture of the same composition starting at pH 5.4 and increasing to pH 7.3. The results were similar to those illustrated in Fig. 4. Additional transfer experiments at pH ≈ 7.4 with FA/BSA or FA/PtdCho ratios as described above consistently showed that most (>80%) of the oleic acid partitioned to BSA at pH ≥ 7.2.

DISCUSSION

The carboxyl carbon chemical shift is dependent on environmental factors such as ionization state (4, 18) and H bonding (18, 19) of the carboxyl group. Because of the high sensitivity of the carboxyl ^{13}C chemical shift to environmental factors, heterogeneity of FA binding sites on BSA has been detected by ^{13}C NMR spectroscopy (8–10). In this study, we have shown that the carboxyl ^{13}C chemical shift can be used to distinguish oleic acid bound to PtdCho from oleic acid bound to BSA in aqueous mixtures containing oleic acid, BSA, and PtdCho vesicles. It was thus possible to monitor net transfer of FA between BSA and vesicles without separation of donor and acceptor complexes. Previous studies of FA transfer between protein and phospholipid have relied on separation of FA–protein and FA–phospholipid complexes by centrifugation (5) or column chromatography (20), procedures that could alter the FA binding.

While the most important advantage of ^{13}C NMR over previous approaches to examine FA transfer may be the capability of determining FA binding to different species under equilibrium conditions, there are several other features of the present NMR method that make it an attractive tool for FA transfer studies. First, native FA are used, and the ^{13}C enrichment of the carboxyl carbon is an essentially nonperturbing modification. Thus, the use of FA labeled with a perturbing (e.g., fluorescent; see ref. 20) probe is circumvented. Second, binding of FA to protein or phospholipid is demonstrated directly by the narrow ^{13}C peaks (Figs. 3 and 4). Long-chain FA in aqueous solution in the absence of albumin or PtdCho at pH ≈ 7 form lamellar liquid crystalline or crystalline structures (21) and have extremely broad carboxyl resonances (8, 9). Third, structural information is provided by the ^{13}C spectrum—e.g., the observation of multiple binding environments on BSA. Fourth, the ionization state of oleic acid bound to PtdCho or to BSA can be determined from the carboxyl chemical shift. Fifth, the integrity of PtdCho vesicles can be evaluated from the ^{13}C spectrum of PtdCho. Since the S/N and line width of PtdCho peaks did not change with time or with pH alterations when BSA was present, FA transfer took place without gross disruption of the vesicles.

The chief limitation of ^{13}C NMR in the present application was the relatively long time required to obtain spectra with good S/N. Additional time-consuming measurements of spin–lattice relaxation time and nuclear Overhauser enhancement of the carboxyl resonances at different pH values are required before accurate partition coefficients can be calculated. Nevertheless, the approximate ratios of FA bound to BSA or to PtdCho and the marked change in the FA partition coefficient with pH can be reliably extracted from our results. Another consequence of the long spectral accumulation times (≈1 hr) was that the kinetics of transfer could not be studied in detail. However, some kinetic features can be inferred from the NMR data. First, net transfer was complete and equilibrium was reached within 60 min. This upper limit is consistent with previous results with FA transfer between albumin and PtdCho multilayers (5) and fluorescent-labeled FA transfer between PtdCho vesicles and FA binding proteins (20). Neither of these methods, however, allowed quantitation of FA binding without perturbing the equilibrium. Second, in the equilibrated mixtures of BSA and PtdCho, the exchange rate of FA among different binding environments was slow on the NMR time scale (≤30 per sec), since narrow (≤10 Hz) resonances were observed in the mixtures at the same chemical shift as the isolated systems. These results place the FA exchange rate between PtdCho and BSA on the time scale of seconds or minutes, consistent with a half time of seconds for dissociation of long-chain FA from albumin (22). In addition, the half-time for transfer of the fluorescent fatty acid, 9-(3-pyrenyl) nonanoic acid, between phospholipid vesicles was in the range of 50 msec to 4 sec, the rate-limiting step being desorption from the vesicle (23). Third, the rate of flip-flop of oleic acid from the inner to outer PtdCho monolayer must be on the time scale of minutes, or faster. Since oleic acid is presumably distributed on both bilayer halves and since essentially all of the FA transferred to BSA at pH 7.2–8.0 without disruption of the vesicle, the flip-flop of oleic acid from the inner to the outer leaflet occurred (at pH 7.6 ± 0.4) within the time of mixing and NMR analysis (≈1 hr). [It is possible that the flip rates of un-ionized and ionized FA are different; presumably, the un-ionized form would flip faster. In the oleic acid–PtdCho vesicles at pH 7.6, 50% of the oleic acid was un-ionized. This large proportion of un-ionized FA may promote rapid reequilibration of FA between bilayer halves when the equilibrium is disturbed—e.g., by removal to BSA. Furthermore, since the percentage of un-ionized FA increases greatly on decreasing the pH from 7.6 to 5.5 (Fig. 2), the net flip rate may be faster at pH < 7.6.] To our knowledge, the rate of transbilayer movement of FA in phospholipid bilayers has not been measured for native FA.

The detailed mechanism of transfer of FA between membranes and albumin is not known. Theories range from a highly specific receptor-mediated mechanism (24) to simple diffusion (25). For example, liver cells may contain receptors for albumin that facilitate uptake of FA (24). A kinetic analysis of dissociation of the dye rose bengal from albumin in perfused rat livers led to the suggestion that albumin binds to specific sites on the hepatocyte membrane (26). Studies with whole cell systems have led to suggestions that pH gradients promote release of FA from albumin and that the unassociated FA diffuses and binds to membranes (25). For example, FA transfer from albumin to isolated mammalian cells and to rat liver and heart slices was enhanced by small decreases in pH of the incubation medium below 7.4 (25). These physiological systems all contain membrane proteins that could bind FA, and the binding of FA to the phospholipid bilayer could not be studied independently. *In vivo*, a variety of mechanisms may operate. However, because of the complexity of cell and organ systems, it is difficult to derive a unique interpretation of experimental results. Our results show that FA transfer to phospholipid bilayers can occur without specific receptor or membrane carrier proteins and that pH could be an important factor in regulating FA uptake from albumin. The partitioning of oleic acid to PtdCho vesicles increased markedly with small decreases in pH below pH 7.4, and almost all of the FA was bound to PtdCho at pH 5.5. A similar pH dependence was recently found for the partitioning of oleic acid between PtdCho multilayers and BSA (5). A comparison of the titration curves for oleic acid bound to BSA and to PtdCho vesicles (Fig. 2) may provide additional insight into the mechanism of FA transfer. At pH 7.4, oleic acid bound to BSA is fully ionized (8), while oleic acid in the PtdCho vesicle is ≈50% un-ionized. The pool of albumin-bound oleic acid that titrates (pK_a, ≈4.2) will begin to become protonated at pH values just below 7.4. For example, at pH 7.2, 0.1% of this pool of oleic acid will be protonated if the Henderson–Hasselbalch relationship (27) is observed. Un-ionized FA have altered interactions with BSA and bind with lower affinity to BSA (5, 25) but are readily accommodated in the more hydrophobic environment of the PtdCho bilayer. Thus, slightly acidic pH gradients (25) could provide a driving force for removal of FA from albumin and uptake into a phospholipid membrane.

In summary, we have shown that partitioning of FA between two complex systems can be studied noninvasively without separating the systems and thereby potentially altering the equilibrium. The information in the ^{13}C NMR spectrum permitted (*i*) distinction of bound from unbound FA, (*ii*) distinction of BSA-bound from PtdCho-bound FA, (*iii*) determination of FA ionization state, (*iv*) assessment of vesicle integrity, and (*v*) estimation of the kinetics of transfer and exchange. The ^{13}C NMR method will be valuable in evaluating factors other than pH, such as phospholipid composition and physical state (3), the presence of other chemical components in bilayers, and the FA/BSA mol ratio (1, 25), all of which may influence FA equilibration between albumin and membranes.

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