Fatty Acid Distribution in Systems Modeling the Normal and Diabetic Human Circulation

A ^{13}C Nuclear Magnetic Resonance Study

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Abstract

A nonperturbing ^{13}C nuclear magnetic resonance (NMR) method was used to monitor the equilibrium distribution of carboxyl ^{13}C-enriched fatty acids (FA) between distinct binding sites on human serum albumin, native human lipoproteins, and/or phospholipid model membranes, under conditions that mimic the normal and diabetic human circulation. Two variables pertinent to the diabetic circulation were examined: FA/albumin mole ratio (as elevated in insulin deficiency and/or nephrosis) and pH (as decreased in acidosis). ^{13}C NMR spectra for samples containing carboxyl ^{13}C-enriched palmitate, human serum albumin, and phospholipid vesicles or native lipoproteins (all samples at pH 7.4, 37°C) exhibited up to six carboxyl NMR resonances corresponding to FA bound to distinct binding sites on albumin and nonalbumin components. When the sample FA/albumin mole ratio was 1, three FA carboxyl resonances were observed (182.2, 181.8, and 181.6 ppm; designated peaks β, γ, and β', respectively). These resonances corresponded to FA bound to three distinct high-affinity binding sites on human serum albumin. When the sample mole ratio value exceeded 1, additional carboxyl resonances corresponding to FA bound to phospholipid vesicles (179.0 ppm, peak ϕ), lipoproteins (180.7 ppm, peak σ), and lower affinity sites on albumin (183.8 ppm, peak α; 181.9 ppm, peak γ'), were observed. The intensity of peaks ϕ and σ increased with increasing mole ratio or decreasing pH. Using Lorentzian lineshape analysis, the relative mole quantities of FA bound to albumin and nonalbumin binding sites were determined. Plots of the fraction of FA associated with nonalbumin components as a function of FA/albumin mole ratio were linear and extrapolated to the abscissa at a mole ratio value of 1. This pattern of FA distribution was observed regardless of the type of nonalbumin acceptor used (phospholipid vesicles, human high- or low-density lipoproteins) or the type of FA used (palmitate, oleate, or stearate), and provided evidence for negative cooperativity for human serum albumin upon binding of 1 mol of FA per mole albumin. These in vitro NMR results suggest that the threshold FA/albumin mole ratio value for alterations in FA distributions in the human circulation may be 1, rather than 3, as previously held. The pathophysiological implications of these findings are discussed. (*J. Clin. Invest.* 1991. 87:1431–1441.) Key words: diabetes mellitus • fatty acids • lipoproteins • model membrane • nuclear magnetic resonance • serum albumin

Introduction

In humans, ~ 200 g of fatty acids are mobilized from adipose tissue each day and transported in the circulation at concentrations ~ 100–1,000-fold higher than their monomer solubility limit.[1] Solubilization and transport is mediated primarily by serum albumin, a three-domain protein with at least six high- and medium-affinity binding sites for long-chain fatty acids (1–4). Albumin prevents the self-association of fatty acids into liquid-crystalline or crystalline aggregates at neutral pH (5, 6) and provides tissues with a readily available source of fatty acids for energy production and lipid synthesis (7).

Under normal conditions in humans, it is thought that > 99% of the circulating fatty acids are bound to serum albumin (7–9). However, plasma lipoproteins and cellular membranes also have a high affinity for fatty acids (8, 10) and may, under certain normal or abnormal conditions, compete with albumin for fatty acid binding. Such conditions could include elevated circulating fatty acid levels (secondary to uncontrolled diabetes mellitus, myocardial infarction, hyperthyroidism, or sepsis), decreased albumin levels (secondary to nephrotic syndrome, liver diseases, genetic defects), and/or altered albumin binding properties (secondary to acidosis, drug administration, nonenzymatic glycosylation, or hyperbilirubinemia). The most severe abnormalities in fatty acid transport are expected in diabetic ketoacidosis complicated by nephrosis, since large elevations in circulating fatty acids are superimposed with decreased albumin levels and acidosis.

In vitro, fatty acids in blood cells, endothelial cells, and lipoproteins have been shown to induce a variety of detrimental functional effects (reviewed in reference 7) that may contribute to the pathogenesis of vascular complications in diabetic subjects. However, the in vivo significance of these effects remains unknown, largely because the distribution of fatty acids in the human circulation is not easily determined or predicted. A number of variables, such as those mentioned above, may affect the distribution of fatty acids between albumin and nonalbumin components, but the quantitative contribution of each variable is not known. In addition, the molecular basis for

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1. The term "fatty acid," rather than "nonesterified fatty acid" or "free fatty acid," is used throughout this manuscript. Use of the term "fatty acid" is not meant to imply any information regarding the ionization state of the carboxyl group.

fatty acid interactions with binding sites on human serum albumin, cell membranes, and lipoproteins is only partially understood. Moreover, nonperturbing techniques for quantitating fatty acid distributions between albumin and nonalbumin binding sites under physiological conditions have not been available.

In the present study, a new ^{13}C nuclear magnetic resonance (NMR)[2] model system approach was used to monitor the equilibrium distribution of carboxyl ^{13}C-enriched fatty acids in reconstituted and whole human plasma and blood under conditions that mimic steady-state conditions in the normal and diabetic human circulation. This NMR approach, based on earlier work (11), is nonperturbing and provides physicochemical information regarding the distribution of fatty acids between individual albumin and nonalbumin binding sites and the mechanisms that govern this distribution. The effects of two variables pertinent to the diabetic circulation were examined: fatty acid/albumin mole ratio (increased during insulin deficiency and/or nephrotic syndrome), and pH (decreased during ketoacidosis). The influence of these variables on fatty acid distributions have previously been examined using biochemical approaches (1, 7, 9). In the present study, the ^{13}C NMR approach has been applied to reexamine the quantitative contributions of these variables and to investigate the molecular mechanisms governing fatty acid transport in the normal and abnormal human circulation.

Methods

Materials. Palmitic acid [1-^{13}C, 90% enriched] was purchased from KOR Stable Isotopes, Cambridge, MA (Lot DM-I-89), and was > 99% pure fatty acid by thin-layer chromatography and > 95% pure palmitic acid by gas-liquid chromatography. Egg yolk phosphatidylcholine was purchased from Lipid Products, Nutley, England, and was > 99% pure by thin-layer chromatography.

Crystallized, lyophilized fatty acid-free human serum albumin was purchased from Sigma Chemical Co. St. Louis, MO (A-3782, Lot 76F-9335), and analyzed as follows. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of human albumin using 3–24% gradient gels (25 μg human serum albumin per lane) demonstrated a major band at 66 kD and several minor bands (totaling ~ 5%) with molecular masses corresponding to albumin oligomers. Previous studies indicated that the presence of covalently linked albumin oligomers did not affect ^{13}C NMR spectra of fatty acid/albumin complexes (3). In addition, ^{13}C NMR carboxyl resonances for fatty acids bound to human serum albumin from lyophilized preparations were identical to those observed in spectra of whole human plasma freshly isolated from individual healthy donors. No impurity bands were observed at a molecular mass corresponding to apoprotein A-I. "Fatty acid-free" albumin was analyzed for residual fatty acid content using gas-liquid chromatography. Fatty acids were extracted twice using a benzene/chloroform/methanol procedure (12) and transesterified using borontriflouride-methanol. Gas-liquid chromatographic analysis of the methyl ester derivatives, using heptadecanoic acid as a quantitative internal standard, indicated that the albumin preparation contained < 0.01 mol fatty acid per mole albumin.

Native human HDL (d = 1.081–1.21) and LDL (d = 1.025–1.050) fractions were purified by ultracentrifugation from one unit of plasma after a 12-h fast. Samples of heparinized whole human plasma used for NMR analysis were drawn from healthy donors after a 12-h fast, and levels of albumin, total protein, total cholesterol, HDL cholesterol, total bilirubin, triglycerides, and nonesterified fatty acids were measured in each plasma sample.

The buffer solution used throughout this study contained 135 mM NaCl, 4 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM NaN_3, 0.1 mM ascorbic acid, and 40 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid buffer, at pH 7.4.

Preparation of NMR samples containing fatty acid, human albumin, and model membranes. The general approach for equilibrating potassium salts of fatty acids with albumin (3) and the physical-chemical basis for that approach (5, 6) have been discussed in detail elsewhere. In short, stock solutions of ^{13}C-enriched fatty acids in chloroform were prepared and their concentrations were determined by measuring dry weights on a microbalance (Cahn Instruments, Inc., Cerritos, CA). An appropriate aliquot (typically 400 μg) was pipetted directly into a 10-mm NMR tube and the solvent was evaporated under a stream of nitrogen. A 100-μl aliquot of H_2O or D_2O (for NMR lock signal) and 1.2 equivalents of 1 N KOH were added to the NMR tube, and the sample was mixed and equilibrated at 35–45°C until all crystalline- or oil-phase fatty acid was completely converted to the potassium salt and dissolved in the aqueous phase. The resulting sample consisted of an optically clear micellar solution (~ 15 mM). For potassium palmitate, the samples underwent a reversible gel to micellar phase transition at ~ 30°C and were warmed in a 37°C water bath before mixing with albumin, vesicle, lipoprotein, or plasma samples.

Unilamellar phospholipid vesicles (100 mg/ml in buffer) were prepared by sonication (50 min, 25 watt, 35% duty cycle) under N_2 in an ice-water bath using a Sonifier (Branson Sonic Power Co., Inc., Danbury, CT) equipped with a microprobe tip. The phospholipid preparations contained no detectable fatty acids or lysolecithin by thin-layer chromatography (100 μg of phospholipid spotted), either before or after sonication. Aliquots of phospholipid vesicles (0.8 ml) and albumin (0.8 ml, 100 mg/ml) were combined in a 10-mm NMR tube, and a small quantity of D_2O (100 μl, for NMR lock signal) was added. The sample pH was adjusted to 7.4, and a ^{13}C NMR spectrum was acquired. Then the sample was transferred to a second NMR tube containing carboxyl ^{13}C-enriched potassium palmitate in 100 μl of H_2O, equilibrated for five minutes, and the pH was adjusted to 7.4. This sample contained a fatty acid/albumin mole ratio of 1:1, an albumin/phospholipid weight ratio of 1:1, and an albumin concentration of 47 mg/ml. An NMR spectrum was acquired, and additional ^{13}C-enriched palmitate was added using the procedure noted above to yield total palmitate/albumin mole ratio values of 2:1 and 3:1. In a similar manner, a separate sample was prepared with starting mole ratio of 4:1, and palmitate was added to yield samples containing 5:1 and 6:1 fatty acid/albumin mole ratio values. NMR spectra were acquired at each mole ratio increment. Negative-stain electron micrographs of the 6:1 mole ratio sample after NMR analysis were essentially identical to those for the vesicle preparation with no added albumin or palmitate and revealed a relatively homogeneous population of unilamellar vesicles with a mean diameter of ~ 30 nm.

In a similar manner, otherwise identical samples containing less phospholipid (final concentrations, 24 and 11 mg/ml) were prepared.

Preparation of NMR samples containing fatty acid, albumin, and native human lipoproteins. HDL and LDL fractions were pressure dialyzed against three volumes of sample buffer using a 10-ml Amicon ultrafiltration apparatus (Amicon Corp., Danvers, MA) equipped with a XM300 filter. HDL- and LDL-protein concentrations were determined by a modified Lowry method (13). Human serum albumin and HDL (0.8 ml each) were combined in a 10-mm NMR tube so that their final protein concentrations were 47 and 24 mg/ml, respectively. A ^{13}C NMR spectrum was acquired at pH 7.4 before the addition of fatty acid. The sample was subsequently transferred to a second 10-mm NMR tube containing 0.1 ml of aqueous ^{13}C-enriched potassium palmitate to yield a total fatty acid/albumin mole ratio of 2:1. Samples with mole ratio values of 4:1 and 6:1 were prepared in a similar man-

2. *Abbreviations used in this paper:* NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; T_1, spin-lattice relaxation time.

ner. In addition, separate samples containing a different concentration of HDL (final HDL protein concentration, 9 mg/ml) or containing LDL (final LDL protein concentration, 11 mg/ml) were prepared using identical procedures.

Gas-liquid chromatographic analyses of HDL and LDL fractions before addition of fatty acid or albumin contained 0.39 and 0.32 mol of natural abundance (no ^{13}C enrichment) fatty acid, as expressed per mole of albumin in the final NMR samples. The reported fatty acid/albumin mole ratio values include the endogenous unenriched plus the added ^{13}C-enriched fatty acids. Negative-stain electron micrographs of the samples containing the highest fatty acid/albumin mole ratio were essentially identical to those for isolated HDL or LDL with no added fatty acid or albumin and showed a relatively homogeneous population of round particles with a mean diameters of 11 and 22 nm, respectively.

^{13}C NMR spectroscopy. ^{13}C NMR spectra were recorded on a model WP-200 NMR spectrometer (Bruker Instruments, Inc., Billerica, MA; 50.3 MHz for ^{13}C) as described elsewhere (14) and a home-built 360 MHz NMR spectrometer (90.58 MHz for ^{13}C) at the Molecular Biophysics Laboratory, Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology. NMR internal sample temperatures were controlled to 37±1°C. The chemical shift of the narrow resonance from albumin ϵ-Lys/β-Leu carbons (3) was used as an internal reference after calibrating this resonance (39.52 ppm) against external tetramethylsilane. The estimated uncertainties in chemical shift values were ±0.1 ppm. In some cases, relative peak intensities were measured using the integration routines provided within the Bruker DISNMR and M.I.T. RNMR software packages. In order to deconvolute overlapping resonances and measure individual peak intensities, Lorentzian spectral simulations were generated using the Bruker GLINFIT program. Ninety-degree pulses were used throughout and spectral pulse intervals (4.82 s; ≥ 4–5 × spin-lattice relaxation time [T_1]) were sufficiently long to obtain full relaxation and equilibrium NMR intensities for palmitate and oleate carboxyl resonances. Spin-lattice relaxation and nuclear Overhauser enhancement (NOE) values were measured as previously described (3). The minimum time between sample mixing and the beginning of NMR data collection was 30 min. NMR results were independent of equilibration time for times ≥ 30 min.

pH measurements. All sample pH measurements were made directly in the NMR tube using a thin glass combination electrode (Microelectrodes, Inc., Londonderry, NH). Measurements made at room temperature (21–24°C) were corrected to values at 37°C using the following conversion factor: $\Delta pH/\Delta T = -0.0146$ (15). All reported pH values in this manuscript represent values corrected to 37°C.

Results

To examine the effect of increasing fatty acid/albumin mole ratio on the equilibrium distribution of fatty acids between binding sites on human serum albumin and model membranes, ^{13}C NMR spectra were obtained for samples containing albumin, phospholipid vesicles, and increasing amounts of carboxyl ^{13}C-enriched fatty acid, all at pH 7.4 and 37°C. Sonicated phospholipid vesicles were chosen as model systems to mimic the fatty acid binding properties of cell membranes and lipoprotein surfaces for the following reasons. First, phospholipid model membrane systems have affinities for fatty acids similar to those of red blood cells and platelets (10). Secondly, the ^{13}C carboxyl resonances for fatty acids bound to phospholipid vesicles are narrow, unlike those for fatty acids bound to red blood cells. Finally, carboxyl resonances for fatty acids bound to vesicles, which occur at ~ 179 ppm at pH 7.4, are well resolved from those for fatty acids bound to human serum albumin (181.6–183.8 ppm). The biological relevance of vesicles as a model fatty acid acceptor was assessed by comparing the results using vesicles as acceptors to those obtained using native acceptors (isolated human lipoprotein fractions and whole human plasma).

The carboxyl/carbonyl region of ^{13}C NMR spectra for samples containing human serum albumin, phospholipid vesicles, and increasing amounts of carboxyl ^{13}C-enriched palmitate are shown in Fig. 1. With no added palmitate (Fig. 1 *A*), several resonances were observed in addition to the broad carbonyl fringe centered at 175 ppm. The latter represents natural abundance resonances from the carbonyl and carboxyl carbons of the protein polypeptide backbone and aspartate side-chain residues (16, 17). The resonance at 181.0 ppm, designated *g*, represents glutamate side-chain carboxyl carbons of albumin (3), and the narrow doublet at 173.6 and 173.3 ppm, the carbonyl carbons of phosphatidyl-choline molecules on the outer and inner monolayer leaflets, respectively, of phospholipid vesicles (18).

When the fatty acid/albumin mole ratio in the sample was 1:1 (Fig. 1 *B*), a partially resolved triplet was observed at 182.14, 181.82, and 181.58 ppm, in addition to the peaks noted above. These three resonances (designated peaks β, γ, and β', respectively) were also seen in samples containing palmitate and human albumin but no phospholipid. They were assigned to the carboxyl carbons of palmitate bound to the three highest-affinity fatty acid binding sites on human serum albumin based on independent results presented elsewhere (D. P. Cistola and J. A. Hamilton, manuscript submitted for publication). Resonances β, γ, and β' are analogous to peaks b, d, and b′ that represent fatty acids bound to the three highest affinity binding sites on bovine serum albumin (3, 4). With an increase in sample fatty acid/albumin mole ratio to 2/1 (Fig. 1 *C*), peaks β, γ, and β' increased in intensity, and an additional resonance was marginally detected at 179.1 ppm (designated peak ϕ). At higher mole ratio values (Fig. 1, *D–F*), peak ϕ was unequivocally observed and increased in intensity with increasing mole ratio. The chemical shift of peak ϕ was identical to a peak observed in samples containing palmitate and phospholipid vesicles, but no albumin, and was assigned to the carboxyl carbons of palmitate associated with the phospholipid bilayer (see Fig. 2 and reference 11).

To determine whether peak ϕ was reproducible at 2:1 mole ratio and to improve its signal-to-noise ratio, an NMR spectrum was acquired for a sample prepared in an identical manner to that shown in Fig. 1 *C*, except that four times more NMR transients were acquired. A small, but clearly defined resonance at 179.0±0.1 ppm was observed (spectrum not shown). In addition, an NMR spectrum for a third, identically prepared sample also exhibited a resonance corresponding to peak ϕ at 179.1±0.1 ppm.

To determine the ionization state of fatty acids corresponding to peak ϕ, an NMR titration curve was obtained for palmitate associated with phospholipid vesicles under conditions identical to those used in Fig. 1, except that no albumin was present. This titration curve and a selected NMR spectrum are shown in Fig. 2. The carboxyl chemical shift of palmitic acid/palmitate bound to phospholipid vesicles increased from 175.9 ppm at pH 3.3 to 181.2 at pH 10.5 and exhibited a sigmoidal titration curve with an apparent pK_a value of ~ 7.3. The chemical shift at pH 7.4 was 179.0 ppm, essentially identical to that for peak ϕ in Fig. 1. This result indicated that, in samples containing both albumin and vesicles, ~ 58% of the fatty acid molecules bound to phospholipid vesicles were ionized at pH 7.4. This is in contrast to the fatty acids bound to serum albu-

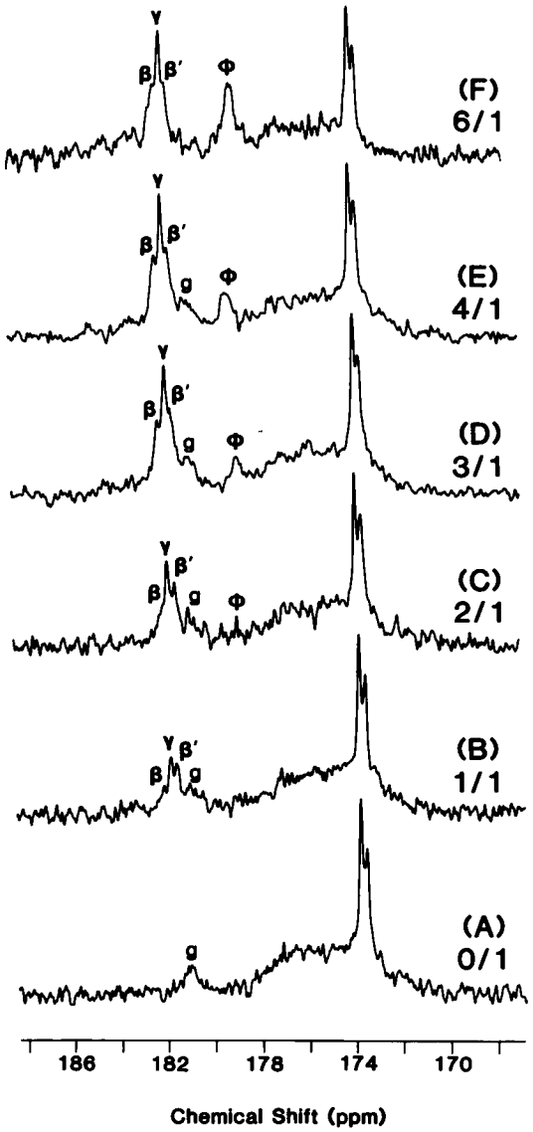

Figure 1. Carboxyl/carbonyl region of 50.3-MHz ^{13}C NMR spectra for samples containing human serum albumin, sonicated phospholipid vesicles, and increasing quantities of carboxyl ^{13}C-enriched palmitate, all at pH 7.4 and 37°C. The numerical ratios above the right edge of each spectrum indicate the total mole ratio of palmitate to human serum albumin in each sample. The letter *g* denotes natural abundance glutamate carboxyl resonance from human serum albumin (see Results). The letters β, γ, and β' denote carboxyl resonances of ^{13}C-enriched palmitate bound to the three high-affinity binding sites on human serum albumin. At higher mole ratio values, peak γ may also contain a contribution from an overlapping resonance representing fatty acid bound to medium-affinity binding sites (peak γ'; D. P. Cistola and J. A. Hamilton, manuscript submitted for publication). The resonance labelled ϕ represents palmitate associated with phospholipid vesicles. The doublet at 173.6/173.3 ppm corresponds to phospholipid carbonyl carbons in the outer and inner monolayer leaflets, respectively, of phospholipid vesicles. NMR spectra were recorded after 2,000 accumulations using 90° pulses and a pulse interval of 4.82 s. The T_1 values for peaks $\beta/\gamma/\beta'$ and ϕ were 1.1±0.1 and 0.9±0.1 s, respectively, and the NOE values for all of these peaks was identical (1.4±0.1). A line broadening factor of 3 Hz was used in spectral processing. Spectra shown in *D* and *E* were recorded after 4,000 accumulations. The concentrations of human serum albumin and phosphatidylcholine were 47 mg/ml, each.

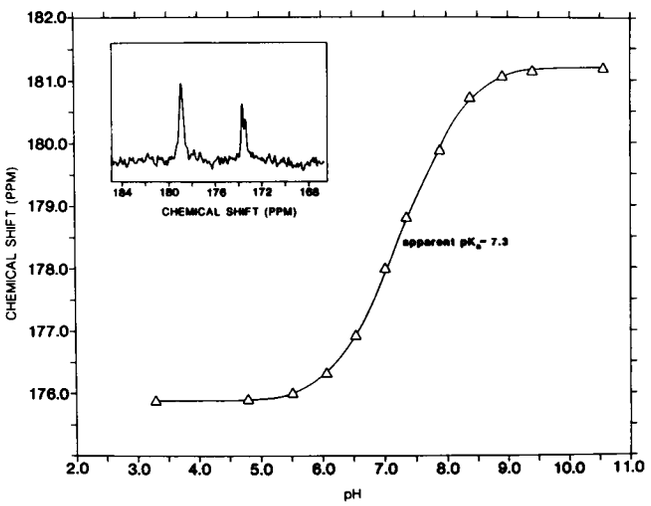

Figure 2. NMR titration curve for sonicated phospholipid vesicles containing 5 mol% ^{13}C-enriched palmitate at 37°C, and a selected ^{13}C NMR spectrum at pH 7.3 (*inset*). Corresponding spectra were recorded after 400–1,600 accumulations, and the spectrum shown in the *inset*, after 1,200 accumulations. Reported sample pH values were corrected to values at 37°C as described in Methods. Each data point was derived from a single NMR spectrum.

min binding sites in the same sample, which, at equilibrium, were fully ionized at pH 7.4 (D. P. Cistola and J. A. Hamilton, manuscript submitted for publication).

To estimate the exchange rates of palmitate between human serum albumin and phospholipid vesicles and between individual binding sites on human albumin, NMR spectra in Fig. 1 were compared with spectra for samples containing palmitate and human serum albumin (but no vesicles) and palmitate and vesicles (but no albumin). The corresponding carboxyl resonances had essentially identical chemical shifts and linewidths in all cases. Therefore, palmitate appeared to be in slow exchange, on the NMR chemical shift time scale, between binding sites on human albumin and phospholipid vesicles ($\ll$ 140 exchanges per second) and between individual sites on human albumin ($\ll$ 10 exchanges per second). No detectable changes were observed in these exchange rate upper limits with increasing fatty acid/albumin mole ratio.

To examine the effect of small decreases in pH on the distribution of fatty acids, ^{13}C NMR spectra were obtained for samples containing human serum albumin, phospholipid vesicles, and palmitate at pH values of 7.4, 7.2, 7.0, and 6.8 (Fig. 3). The fatty acid/albumin mole ratio in the sample was 4:1. This mole ratio value was chosen because the ratio of plasma free fatty acids to albumin in patients with diabetic ketoacidosis averages ~ 4 (19–21). With decreasing pH, the relative intensity of peak ϕ increased, indicative of an increasing fraction of palmitate associated with phospholipid vesicles. In addition, the chemical shift of peak ϕ decreased from 179.1 ppm at pH 7.4 to 177.3 ppm at pH 6.8, consistent with a change in the ionization state of palmitate bound to phospholipid vesicles (refer to Fig. 2). The percentage of palmitate bound to phospholipid vesicles that was ionized (anionic) was 58%, 46%, 37%, and 27% at pH 7.4, 7.2, 7.0, and 6.8, respectively. In contrast, the chemical shifts of peaks β, γ, and β' did not change between pH 7.4 and 6.8. Therefore, essentially 100% of the palmitate bound to human serum albumin at equilibrium was anionic at all four pH

values, consistent with our previous studies with bovine and human albumins (reference 4 and D. P. Cistola and J. A. Hamilton, manuscript submitted for publication).

To quantitate the percentage of fatty acids associated with non-albumin components, the intensities (areas) of individual carboxyl resonances were obtained by Lorentzian spectral simulation and deconvolution (see Methods). Since NMR pulse intervals were chosen to be $> 4 \times T_1$ for palmitate carboxyl resonances (longest T_1 = 1.1 s), and since nuclear Overhauser enhancement values for the observed fatty acid carboxyl resonances were equal (NOE = 1.4), the relative intensities of palmitate carboxyl resonances could be directly converted into relative mole quantities of palmitate bound to albumin binding sites and phospholipid vesicles.[3] A plot of the fraction of total fatty acids associated with phospholipid vesicles as a function of total sample fatty acid/albumin mole ratio and pH is shown in Fig. 4. The fraction of palmitate associated with phospholipid vesicles increased from 0.07 at 2:1 mole ratio to 0.34 at 6:1 mole ratio, all at pH 7.4 (Fig. 4, ●). A least squares fit of these points yielded a straight line with a slope of 0.068, a correlation coefficient of 0.99, and an x-intercept of 1.0. Data for similar systems containing oleate (Fig. 4, ■) or stearate (Fig. 4, ◇) yielded results qualitatively and quantitatively similar to those of palmitate (Fig. 4, ●). In addition, the percentage of palmitate associated with phospholipid vesicles increased from 0.22 at

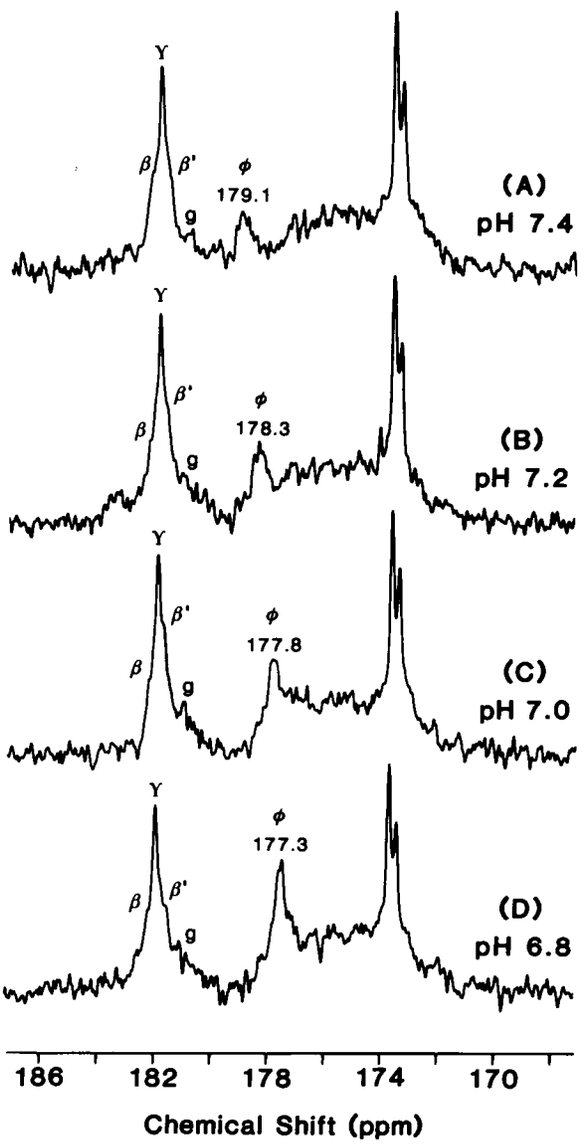

Figure 3. Carboxyl/carbonyl region of 50.3-MHz ^{13}C NMR spectra for samples containing human serum albumin, sonicated phospholipid vesicles, and ^{13}C-enriched palmitate at four different pH values. All spectra were accumulated at 37°C for a single sample with a palmitate/albumin mole ratio of 4/1. Spectra were recorded after 2,000 accumulations and processed with 3-Hz linebroadening. Chemical shift values in ppm are noted directly above peak ϕ in each spectrum. All other nomenclature as in Fig. 1 legend.

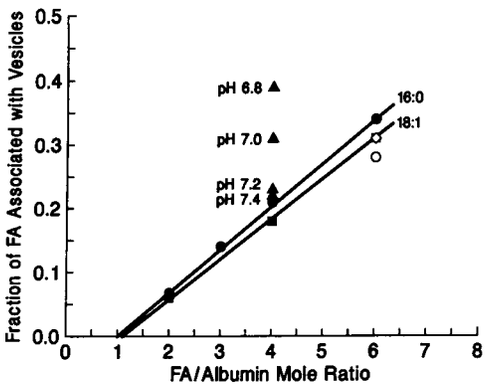

Figure 4. Plots of the fraction of fatty acid (FA) molecules associated with phospholipid vesicles as a function of the total sample fatty acid/albumin mole ratio and pH. The fractions of fatty acid molecules associated with phospholipid vesicles were calculated from the relative intensities of ^{13}C NMR carboxyl resonances corresponding to fatty acids bound to binding sites on vesicles and albumin. Peak intensities were quantitated using Lorentzian lineshape analyses of spectra shown in Figs. 1 and 3 and other spectra not shown. Each data point was derived from a single NMR spectrum. The data for palmitate at 2/1 mole ratio was not obtained from the spectrum shown in Fig. 1 *C*, but from a similar spectrum containing 8,000 transients and a twofold greater signal-to-noise ratio. No T_1 or NOE corrections were required to convert relative peak intensities into relative mole quantities of fatty acids. All samples contained human serum albumin (47 mg/ml), phospholipid vesicles (47 mg/ml), and different types and amounts of ^{13}C-enriched fatty acids. All NMR spectra were obtained at 37°C and pH 7.4, except where noted otherwise. (●) Palmitate, (■) oleate, (◇) stearate, (▲) palmitate at four different pH values. (○) Sample containing palmitate and a lower concentration of phospholipid vesicles (24 mg/ml). The line designated 16:0 represents a linear least squares fit of the solid circles, with a slope of 0.068, a correlation coefficient of 0.99, and an x-intercept of 0.97. The line designated 18:1 represents a linear least squares fit of the solid squares, with a slope of 0.063, an correlation coefficient of 0.99, and an x-intercept of 1.1. The estimated uncertainties in x and y values are ±0.1 and ±0.02, respectively.

3. In the present study, no attempt was made to quantitate the intensities of individual carboxyl resonances corresponding to fatty acids bound to distinct binding sites on human serum albumin, since significant uncertanties were associated with individual measurements under these conditions. Rather, Lorentzian lineshape analysis was performed to quantitate the total carboxyl intensity associated with albumin vs. nonalbumin binding sites. The occupation of individual binding sites on human serum albumin was assessed qualitatively by inspection of NMR spectra.

pH 7.4 to 0.39 at pH 6.8, all at 4/1 mole ratio (Fig. 4, ▲). For a similar sample containing one-half the concentration of phospholipid (24 mg/ml) and a palmitate/albumin mole ratio of 6.0, the fraction of palmitate associated with the vesicles was 0.28 at pH 7.4 (Fig. 4, ○).

To determine whether the distribution of fatty acids was affected by the type of non-albumin fatty acid acceptor used, ^{13}C NMR spectra were accumulated for samples containing human serum albumin and native human HDL with increasing amounts of carboxyl ^{13}C-enriched palmitate, all at pH 7.4 and 37°C. The carboxyl/carbonyl region of these spectra, shown in Fig. 5, were similar to those shown in Fig. 1, except for the following. First, the narrow resonances between 171.2 and 173.9 ppm represent carbonyl carbons of triglycerides, cholesteryl esters, and phospholipids in HDL (22). Secondly,

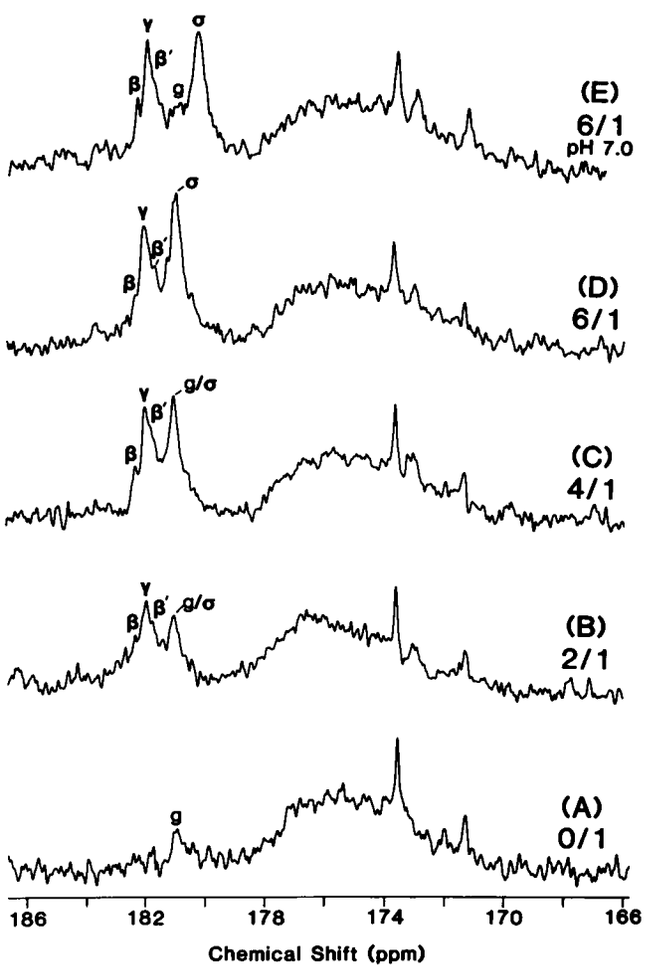

Figure 5. Carboxyl/carbonyl region of 50.3 MHz ^{13}C NMR spectra for samples containing human serum albumin (47 mg/ml), native human HDL (24 mg protein/ml), and increasing quantities of ^{13}C-enriched palmitate at pH 7.4 (*A–D*) and pH 7.0 (*E*). Peak σ represents palmitate associated with lipoproteins. Where peaks g and σ overlapped, the composite peak is noted as g/σ. Unlike bilayered vesicles (Figs. 1–3), phospholipids in HDL exhibited only one carbonyl carbon resonance because HDL has a monomer surface. All other nomenclature as in Fig. 1 legend. NMR spectra were recorded after 2,000 accumulations using 90° pulses and a pulse interval of 4.82 s. The T_1 values for peaks $\beta/\gamma/\beta'$ and g/σ were 1.1 and 1.0 s, respectively, for the sample shown in *D*.

peak g represents glutamate carboxyl carbons from both human albumin and the apoproteins of HDL. Finally, peak σ, which overlaps with peak g at pH 7.4, represents palmitate bound to the monolayer surface of HDL. This assignment is based on spectra not shown for otherwise identical samples containing palmitate and HDL but no albumin; these spectra exhibit a major fatty acid carboxyl resonance at 180.8 ppm at pH 7.4.

For samples containing added ^{13}C-enriched palmitate, peaks β, γ, and β' were observed (Fig. 5). As discussed above, peaks β, γ, and β' represent palmitate bound to the three high affinity sites on human serum albumin. The intensity of peak g/σ increased with increasing fatty acid/albumin mole ratio, as shown in Fig. 5, *B–E*, indicating an increase in the amount of palmitate associated with HDL. A decrease in sample pH from 7.4 (Fig. 5 *D*) to 7.0 (Fig. 5 *E*) resulted in a decrease in the chemical shift of peak σ from 180.8 to 180.1 ppm. Since peak g remained unchanged at 180.9 ppm, peaks g and σ were better resolved at pH 7.0 than at 7.4.

Qualitatively and quantitatively similar results were obtained for samples containing human LDL, rather than HDL, as a nonalbumin fatty acid acceptor (Fig. 6). The intensity of the carboxyl resonance corresponding to palmitate bound to LDL (peak σ) increased with increasing mole ratio and its chemical shift decreased from 180.6 ppm at pH 7.4 to 179.5 ppm at pH 6.8. Peak σ was assigned based on independent spectra for samples containing fatty acid and LDL, but no albumin (J. A. Hamilton, personal communication).

As observed for samples containing human serum albumin and phospholipid vesicles, the exchange rates of palmitate between albumin and native human lipoproteins ($\ll 35\ s^{-1}$) and between individual binding sites on albumin ($\ll 10\ s^{-1}$) were slow on the NMR chemical shift time scale. In addition, changes in fatty acid/albumin mole ratio and sample pH resulted in no detectable changes in the upper limits for these rates.

Plots of the fraction of total fatty acids bound to lipoproteins (HDL or LDL) as a function of total sample fatty acid/albumin mole ratio, are shown in Fig. 7. Plots for samples containing HDL at 24 mg/ml (●) and 9 mg/ml (■) were linear (r = 0.98 and 0.99, respectively) and extrapolated to the x-axis at a mole ratio value of 1.1. These plots were very similar to those obtained for samples containing phospholipid vesicles, rather than HDL, as nonalbumin fatty acid acceptors (cf. Fig. 4). For a sample containing LDL, the fraction of palmitate associated with LDL was 0.19 at pH 7.4 (◇) and 0.28 at pH 6.8 (□), all at a 6:1 mole ratio.

To assess their spectral resolution and appearance in the carboxyl/carbonyl region, samples of whole human plasma and whole blood with added ^{13}C-enriched palmitate were prepared and examined by ^{13}C NMR (Fig. 8, *A–C*). Carboxyl resonances corresponding to fatty acids bound to human serum albumin (peaks β, γ, β', α) and lipoproteins (peak σ) were observed and the degree of spectral resolution was similar to that observed for the reconstituted systems shown above. As previously observed for samples containing fractionated HDL or LDL, resolution of peaks σ and g was improved by lowering the pH from 7.4 to 6.8. A spectrum for a sample of whole blood (Fig. 8 *D*) was similar to that of plasma, except for the greater intensity of natural abundance carbonyl resonances resulting from hemoglobin in red blood cells. Carboxyl resonances for

fatty acids bound to red blood cell membranes are extremely broad and not observable under these conditions.

To quantitate the distribution of added ^{13}C-enriched palmitate under conditions that mimic the fatty acid binding properties of the intact human circulation, a sample was prepared containing whole human plasma, phospholipid vesicles, and ^{13}C-enriched palmitate. This sample was prepared so that the ratio of phospholipid vesicle surface area per mole of human serum albumin was comparable to the ratio of blood and endothelial cell membrane surface area per mole of albumin found in the normal human circulation.[4] ^{13}C NMR spectra for this sample (not shown) were similar to those shown in Figs. 8, *A–C* and exhibited carboxyl resonances corresponding to peaks β, γ, β', α, σ, and ϕ. At a pH value of 7.4 and a mole ratio value of 5.9, the total fraction of palmitate bound to nonalbumin components (lipoproteins and membranes) was 0.31 (Fig. 7, ▽). In the same plasma sample containing no added phospholipid vesicles, the total fraction of palmitate associated with non-albumin components (lipoproteins only) was 0.13 (Fig. 7, △).

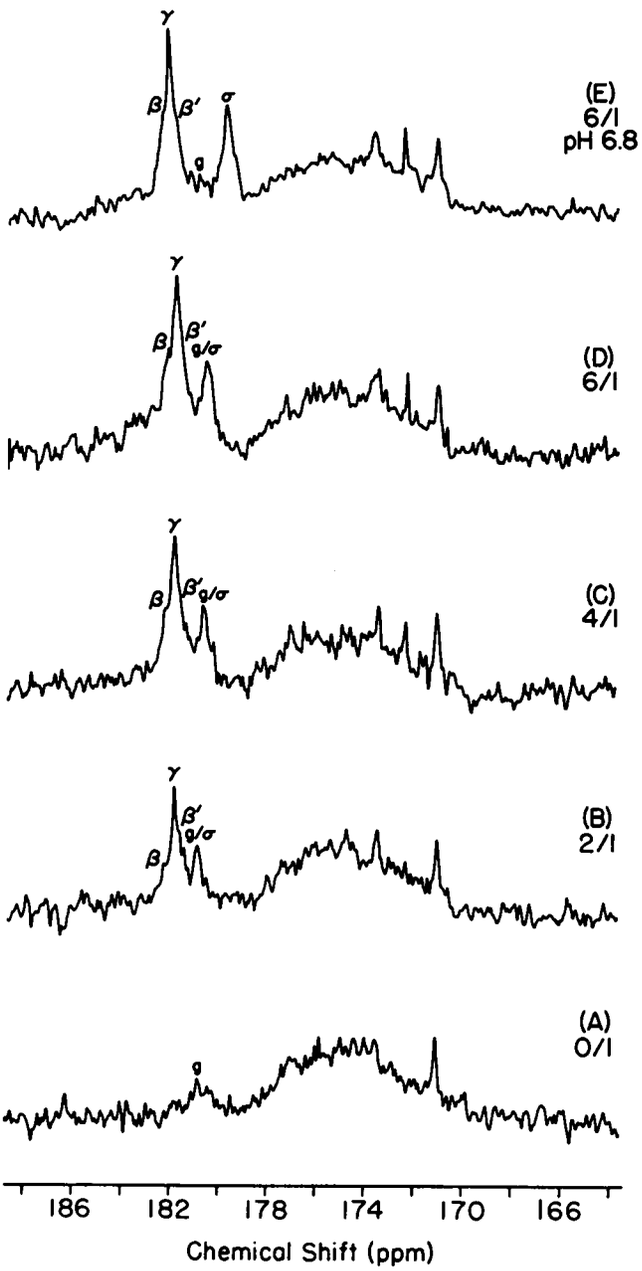

Figure 6. Carboxyl/carbonyl region of 50.3 MHz ^{13}C NMR spectra for samples containing human serum albumin (42 mg/ml), native human LDL (12 mg protein/ml) and increasing amounts of ^{13}C-enriched palmitate, at pH 7.4 (*A–D*) and 6.8 (*E*). All peak nomenclature, sample, and spectral conditions as in Fig. 1 and 5 legends. Spectra for samples obtained at 90.6 MHz (not shown) were qualitatively and quantitatively similar.

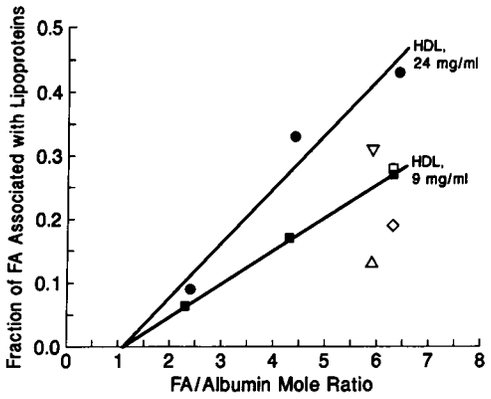

Figure 7. Plots of the fraction of fatty acids (FA) associated with human plasma lipoproteins as a function of the total sample fatty acid/albumin mole ratio. The fractions of fatty acids associated with lipoproteins were calculated from the relative intensities of ^{13}C NMR carboxyl resonances as described in the Fig. 4 legend and in the text. Each data point was derived from a single NMR spectrum. (●) Samples containing HDL at a concentration of 24 mg protein/ml and correspond to values obtained from spectra shown in Fig. 5, *B–D*. (■) Samples containing HDL at a concentration of 9 mg protein/ml and correspond to NMR spectra not shown. (◇,□) A sample containing LDL at a concentration of 12 mg protein/ml, and spectra shown in Fig. 6, *D* and *E*, respectively. (△) Data from an NMR spectrum of whole plasma from a healthy, nondiabetic human donor containing added ^{13}C-enriched palmitate and accumulated at pH 7.4 and 37°C. This spectrum was similar to that shown in Fig. 8 *B*. (▽) Data from an NMR spectrum of the same plasma sample with added ^{13}C-enriched palmitate, except that it was accumulated at pH 6.8. This spectrum was similar to that shown in Fig. 8 *C*. The plasma fatty acid/albumin mole ratio before the addition of ^{13}C-enriched fatty acid was 0.5:1. Other laboratory values were as follows: total cholesterol, 275 mg/dl; HDL cholesterol, 49 mg/dl; triglycerides, 156 mg/dl; albumin, 5.2 g/dl.

Discussion

We have used a novel ^{13}C NMR approach to determined the equilibrium distribution of ^{13}C-enriched fatty acids in reconstituted and whole human plasma and blood under conditions

4. The ratio of phospholipid vesicle surface area per mole albumin in these samples ranged between 0.23 and 0.45 × 10^7 m^2/mol. The ratio of blood and endothelial cell surface area per mol albumin in healthy human subjects is ~ 0.3 × 10^7 m^2/mol. These calculations assumed the following: mean vesicle diameter, 250Å; normal plasma albumin concentration, 4.2 g/dl; erythrocyte surface area, 163 μm^2 (reference 15, p. 69); mean white blood cell diameter, 12 μm; mean platelet diameter, 3 μm; total endothelial surface area, 1.1 × 10^3 m^2 (see reference 52).

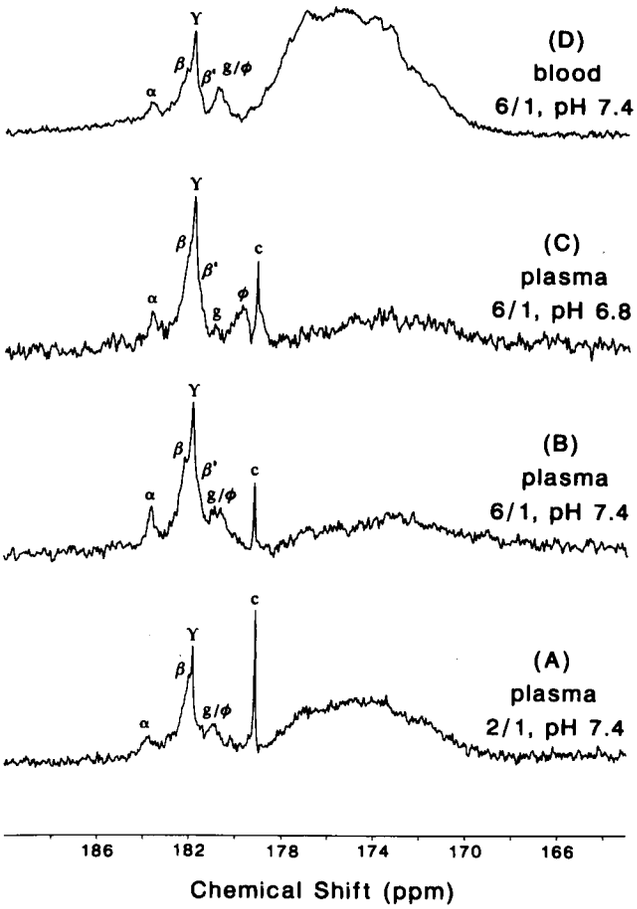

Figure 8. Carboxyl/carbonyl region of 90.6-MHz ^{13}C NMR spectra of whole human plasma (*A–C*) and whole human blood (*D*) with added ^{13}C-enriched palmitate, all at 37°C. The total fatty acid/albumin mole ratio and sample pH are shown above the right edge of each spectrum. The resonance labeled *c* represents carboxyl carbons of citrate, the anticoagulant used in this sample. The plasma and blood samples were obtained from a healthy, nondiabetic human donor after a 14-h fast. The plasma fatty acid/albumin mole ratio prior to the addition of ^{13}C-enriched palmitate was 1.0. Other laboratory values were as follows: albumin, 4.7 g/dl; total cholesterol, 164 mg/dl; HDL cholesterol, 39 mg/dl; triglycerides, 65 mg/dl; total bilirubin, 0.5 mg/dl; total protein 6.7 g/dl.

that mimic steady-state conditions in the normal and diabetic human circulation. The effect of two variables relevant to the diabetic circulation were examined: fatty acid/albumin mole ratio (as increased in insulin deficiency and/or nephrosis) and pH (as decreased in acidosis).

^{13}C NMR has several advantages over other methods for monitoring fatty acid distributions (11). First, distributions can be quantitated under nonperturbing conditions, without need for separation of components by centrifugation or chromatography. Earlier studies utilizing ultracentrifugation (23–26) produced artifactual results, in part, because of the high salt concentrations utilized to separate lipoproteins. Even using "milder" conditions, it was not certain that the separation of components did not alter the equilibrium distribution of fatty acids (8). Secondly, physiological conditions of temperature, salt composition, salt concentration, and albumin concentration can be utilized, including the use of whole human plasma. Some studies have been performed at non-physiological temperatures (8, 27), or in the absence of calcium (24–29). Decreases in calcium concentration below physiological levels alter the conformation of albumin near physiological pH (30) and may affect its binding properties. Thirdly, structural and kinetic information can be obtained from NMR spectra, such as the relative occupation of individual binding sites on albumin, lipoproteins, and model membranes, the ionization states of fatty acids bound to each site, and estimates of the exchange rates of fatty acids between albumin and nonalbumin binding sites (3, 4, 11).

A limitation of this ^{13}C NMR approach is that fatty acids associated with red blood cells cannot be quantitated. Because of their large diameter, red blood cells tumble slowly in solution, resulting in broad unobservable carboxyl NMR resonances for bound fatty acids. To circumvent this problem, small unilamellar phospholipid vesicles (diameter ~ 30 nm) were used as a model system to simulate the fatty acid binding properties of cell membranes (see Results).

Molecular mechanisms governing fatty acid distribution. The equilibrium distribution of fatty acids between human serum albumin, model phospholipid membranes, and/or native human lipoproteins as a function of total fatty acid/albumin mole ratio exhibited the following general pattern. At a mole ratio value of 1, essentially all of the fatty acid was associated with albumin and distributed among its three highest affinity binding sites (Figs. 1 *B*, 5 *B*, and 6 *B*). At mole ratio values > 1, fatty acids associated with both albumin and nonalbumin components, and the fraction associated with nonalbumin components increased linearly with increasing mole ratio (Figs. 4 and 7). At the highest mole ratio examined, a large fraction of the total fatty acid was associated with nonalbumin components. The same general pattern of fatty acid distribution was found regardless of the type of fatty acid acceptor used (phospholipid vesicles, native human HDL, or LDL) or the type of fatty acid used (palmitate, oleate, or stearate). Therefore, this distribution pattern appeared to be governed primarily by the binding properties of human serum albumin, rather than the properties of a particular nonalbumin acceptor or fatty acid.

Two mathematical models have been often used to describe the fatty acid binding properties of albumin and to analyse binding data. The Scatchard model assumes that fatty acid binding sites fall into distinct classes with respect to binding affinities. For palmitate binding to human serum albumin, the highest affinity class contains two or three binding sites, the medium affinity class, three to five, and the lowest affinity class, > 20 (reviewed in reference 7). Each class is described by an average affinity constant. The Scatchard model is oversimplified in that it does not account for possible cooperativity or configurational adaptability, and the validity of grouping individual binding sites into classes has been questioned (7, 9). A second model, the stepwise association model, is more general and makes no assumptions about the groupings of sites into classes (7, 9). Association constants for each mole of bound ligand are obtained, rather than average constants for a class of sites. For palmitate binding to human serum albumin, the stepwise association constants decrease, but show no abrupt changes, with increasing fatty acid/albumin mole ratio (7).

The NMR results support certain features of both models while providing new evidence for negative cooperativity in the interactions of fatty acids with human serum albumin. At a mole ratio value of 1, palmitate was distributed among three structurally distinct binding sites in the population of albumin molecules (peaks β, γ, and β' in Figs. 1 *B*, 5 *B*, and 6 *B*). Hence, the NMR results provided independent physicochemical rationale for the grouping of sites into a high affinity class in accordance with the Scatchard analysis, and also indicated that there are three, rather than two, high-affinity sites for human serum albumin (7, 31, 32). However, at a mole ratio value of 2, before the high affinity sites were saturated, fatty acid was also associated with nonalbumin components, indicating that the affinity of human serum albumin for the second mole of fatty acid per mol albumin was lower than that of the first. Moreover, plots of fatty acid distribution as a function of mole ratio (Figs. 4 and 7) intersected the *x*-axis at 1, rather than at 0 or 3, and indicated a discontinuous decrease in the affinity of albumin for fatty acid following the binding of 1 mol fatty acid per mole HSA. These findings provide evidence for negative cooperativity in the interactions of long-chain fatty acids with human serum albumin.

Two possible explanations have been proposed for the curvilinear Scatchard plots obtained for fatty acid binding data (7): (*a*) fatty acid binding sites are heterogeneous, and/or (*b*) fatty acid binding is associated with negative cooperativity. These two explanations could not be distinguished using previous equilibrium binding data. The present NMR results indicate that, at least for *human* serum albumin, both explanations are valid.

The uptake of fatty acids as a function of increasing fatty acid/albumin mole ratio has been monitored in previous studies using bovine serum albumin and Erlich ascites tumor cells (9, 33, 34). The observed patterns were similar in some respects to the distribution patterns in the present study, except that negative cooperativity at 1:1 mole ratio was not readily apparent. However, a reexamination of this earlier work in light of the present results seems to indicate the possibility of a biphasic pattern, with most of the data points extrapolating in a linear fashion to 1:1 mole ratio. (See Fig. 4 of reference 33).

Small decreases in pH analogous to those seen in moderate to severe acidosis resulted in an additional shift in the distribution of fatty acids from albumin to nonalbumin components. For example, at a fatty acid/albumin mole ratio of 4, approximately 23%, 31%, and 39% of the total fatty acid was associated with phospholipid vesicles at pH 7.2, 7.0, and 6.8, respectively (Figs. 3 and 4). Concurrently, the percentage of fatty acids bound to phospholipid vesicles that were ionized (anionic) decreased from ~ 50% at pH 7.4 to 46%, 37%, and 25% at pH 7.2, 7.0, and 6.8, respectively. In contrast, the fatty acids bound to albumin binding sites at equilibrium were essentially 100% ionized at all pH values between pH 7.4 and 6.8. Based on these distribution and ionization state values, a mechanism that involves a much higher affinity of protonated fatty acids for phospholipid vesicles relative to human serum albumin seems likely. Analysis of these and other related results using mathematical models will help to further evaluate this proposed mechanism. As has been suggested, small decreases in pH in the extracellular fluids in certain tissues may promote the uptake of fatty acids by cells by altering the distribution of fatty acids between albumin and cell membranes (18, 34).

Implications for fatty acid transport and uptake in vivo. It has often been assumed that > 99% of the circulating fatty acids are bound to albumin under normal conditions in humans (7, 8). The present NMR results support this assumption as long as fatty acid/albumin mole ratio values remain ≤ 1. It has also been suggested, based on the concept of three high affinity fatty acid binding sites, that fatty acid/albumin mole ratio values must exceed three or four before lipoproteins and cell membranes can effectively compete for fatty acid binding (1, 7, 9). However, the present NMR results indicate that lipoproteins and membranes can compete for fatty acid binding when the fatty acid/albumin mole ratio value exceeds 1, before the three "high-affinity" binding sites are completely filled. Therefore, the threshold mole ratio value for alterations in fatty acid distributions in the human circulation appears to be 1, rather than 3.

This finding has important implications for both normal and abnormal transport of fatty acids in the circulation. Under most physiological conditions, steady-state ratios of plasma fatty acids to albumin are < 1 (7, 35, 36). Although the distribution of fatty acids (governed by thermodynamic processes) is heavily weighted toward albumin binding, efficient removal of fatty acids from the circulation occurs because of rapid kinetic processes, that is, rapid dissociation from albumin and clearance from the circulation (7, 9). However, the ratios of fatty acids to albumin in plasma can transiently exceed 1 in healthy individuals, particularly following prolonged exercise and/or fasting (19, 37, 38). In this case, there may be an additional thermodynamic contribution to fatty acid clearance. This contribution, mediated by the negative cooperativity property of human serum albumin, would transiently shift the distribution of fatty acids from albumin toward cell membranes and facilitate their clearance from the circulation under conditions where fatty acids are being rapidly metabolized by tissues. Therefore, the negative cooperativity property of albumin provides a fine tuning capability that allows adjustment of its affinities for fatty acids to the present metabolic needs. Such a negative cooperativity and fine tuning capability is analogous to that observed for oxygen interactions with hemoglobin.

In several abnormal conditions, steady-state fatty acid/albumin ratios in plasma become acutely or chronically elevated above 1.[5] Chronic elevations to values typically between 1 and 2 occur in obesity (21, 35, 36, 39), hypertriglyceridemia (40), and insulin-treated diabetes mellitus (21, 36). Acute elevations occur in uncontrolled diabetes mellitus (19–21, 36), gram-negative infection and sepsis (41), nephrotic syndrome (24), hyperthyroidism (35), myocardial infarction (42, 43), and acute emotional or psychological stress (44–46). The highest mole ratio values are observed in uncontrolled diabetes, where values often exceed 2 and occasionally reach 6 (19–21, 36). In patients with diabetic ketoacidosis complicated by nephrosis, mole ratio values may exceed 6, and pH values below 7.2 are not uncommon.

5. The plasma fatty acid/albumin mole ratio values reported here are based on an assumed normal plasma albumin of 4.2 g/dl. Since plasma fatty acid measurements neglect the potential fatty acid fraction associated with blood and endothelial cell membranes, it is likely that plasma fatty acid/albumin mole ratio values underestimate the total mole ratio values in the circulation when the former exceed 1.

Extrapolating from the present in vitro NMR results, we predict that a substantial fraction (up to 50%) of the total circulating fatty acid pool may associate with plasma lipoproteins and the membranes of blood cells and vascular endothelial cells during uncontrolled diabetes in humans. The degree of fatty acid association with nonalbumin components depends on the value of the fatty acid/albumin mole ratio and the presence and severity of acidosis, in accordance with previous results (7). However, we also predict that significant alterations in fatty acid distributions may occur in less extreme conditions such as obesity, nephrotic syndrome, and diabetes controlled with conventional insulin therapy.

Possible consequences of abnormal fatty acid distributions. Fatty acids are known to exert a variety of effects on cells in vitro. For example, fatty acids inhibit neutrophil chemotaxis (47), enhance platelet aggregation (48, 49), and increase the transfer of macromolecules across endothelial cells in culture (50). In addition, fatty acids have been recently shown to inhibit the binding of LDL to human fibroblast LDL receptors (51). Interestingly, the inhibition was observed at fatty acid-albumin mole ratios at and above 2, which corresponds to the values where abnormal distributions were observed in the present study. Such a fatty acid-mediated inhibition of LDL uptake could have important pathological consequences in abnormal states such as diabetes mellitus.

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