The interactions of the carboxyl group of oleic acid with bovine serum albumin (BSA) were studied by $^{13}$C NMR spectroscopy at 50.3 MHz using 90% isotopically substituted [1-13C]oleic acid. $^{13}$C NMR spectra were obtained as a function of the mole ratio of oleic acid to BSA (from 0.5–10.0) and, for selected mole ratios, as a function of $pH$ (between $pH$ 3.0 and 10.6) and temperature (between 15 and 55°C and thermally denatured at 95°C). Except for spectra of highly acidic ($pH \leq 3.9$) and denatured samples, spectra of oleic acid/BSA complexes showed multiple narrow resonances from the oleic acid carboxyl carbon in a region (179–184 ppm) downfield from protein carboxyl and carboxyl carbon resonances. At low oleic acid/BSA ratios (0.5 and 1.0), at least two oleic acid carboxyl carbon peaks were observed; at high ratios ($\geq 3.0$), at least four peaks were present. The intensities of individual peaks, but not their chemical shifts, varied with the oleic acid/BSA ratio. The chemical shift of individual oleic acid peaks was invariant between $pH$ 6.0 and 10.6; below $pH$ 6.0, one of the oleic acid resonances exhibited an NMR titration curve with an apparent $pK_a$ of $\sim 4$. Thus, BSA binding sites for oleic acid are heterogeneous as monitored by the magnetic microenvironment of the oleic acid carboxyl carbon. The number of different oleic acid environments and the relative population of oleic acid molecules in these environments is dependent on the mole ratio of oleic acid/BSA. Our results suggested that the anionic form of oleic acid is bound to BSA at physiological pH and that the multiplicity of NMR peaks for [1-13C]oleic acid resulted from, at least in part, different electrostatic and hydrogen bonding interactions between the oleic acid carboxyl group and specific amino acid residues of BSA.

Albumin is one of few proteins which can bind unesterified fatty acids and is the major vehicle for transport of fatty acid in plasma (1, 2). In human plasma, the mole ratio of fatty acid to albumin normally varies between 0.5 and 1.5 (1, 3) but is much higher under certain conditions, such as heparin administration (4) or extreme exercise (5).

In spite of the obvious importance of fatty acid/albumin interactions, studies by physical techniques have often been hampered by technical problems. The low water solubility of long chain fatty acids has made it difficult to obtain accurate binding constants for fatty acid/albumin complexes (1). Spectroscopic studies at physiological mole ratios of fatty acid to albumin using the fatty acid as a probe are either impractical or impossible because of the simplicity of the fatty acid molecule (e.g. lack of intrinsic chromophoric or fluorescent properties) or the relatively low abundance, compared to the background abundance from albumin, of spectroscopic signals (e.g. proton or $^{13}$C NMR signals). Consequently, fatty acids containing nitroxide groups or conjugated double bonds have been used in electron spin resonance (6–10) and fluorescence (11, 12) spectroscopic studies, respectively. Natural abundance $^{13}$C NMR spectroscopy has been used to study interactions of long chain alkyl sulfates at very high molar ratios of surfactant to albumin (13, 14).

Bovine serum albumin and human serum albumin have similar structures, based on the amino acid sequences and on the proposed location of disulfide linkages (15–17). Each protein molecule has three homologous cylindrical domains and a total of nine loops (2, 15–17). Recently this model of structural organization has been utilized for interpretation of fluorescence and ESR results. Thus, it has been suggested that the first two fatty acids bind to a single specific domain (domain III) (11) and that each of the remaining two domains can bind two fatty acid molecules (11, 18).

This study demonstrates the feasibility of using $^{13}$C NMR spectroscopy to probe interactions between albumin and a biological fatty acid, oleic acid. Of the numerous fatty acids found in association with albumin, oleic acid is the most abundant in both human serum albumin (19) and BSA (20) and also appears to be the most tightly bound (1, 21). To achieve adequate spectral sensitivity and to focus specifically on the oleic acid carboxyl carbon, 90% isotopically substituted [1-13C]oleic acid was used. The use of the $^{13}$C nucleus as a nonperturbing probe should avoid some ambiguities encountered with perturbing probes, as discussed by Perkins et al. (10) for the case of nitroxide spin-labeled fatty acids. The NMR chemical shift (δ) of the carboxyl carbon is highly sensitive to ionization and hydrogen bonding of the carboxyl group (22–25). We have studied the effect on the oleic acid carboxyl $^{13}$C NMR signal of (a) varying the molar ratio of oleic acid to BSA, (b) varying the temperature, and (c) varying the $pH$.

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EXPERIMENTAL PROCEDURES

Materials—Fatty acid-free BSA was obtained from Sigma (Lot 10F-9707). It had $<0.02$ mol of fatty acid bound/mol of BSA by thin layer chromatography (hexane:dichloroacetic acid, 70:30:1) of a 2:1 chloroform:methanol extract of a 12% BSA solution. Oleic acid was purchased from KOR Isotopes, Cambridge, MA (90% $^{1-13}$C) and New England Nuclear (9,10-$^3$H). Both isotopes were $>96\%$ pure by thin layer (26) and gas-liquid (27) chromatography.

Sample Preparation—BSA solutions were made with doubly distilled, deionized water which had been boiled for 30 min to decrease dissolved CO$_2$ content. The concentration of BSA solutions was determined by measuring the extinction coefficient ($E_{1%}$) at 270 nm (Ref 28) of an appropriate dilution. Unless noted otherwise, BSA concentrations were 12% w/v. The oleic acid-BSA mixtures were made by two methods, one using neat oleic acid and the other an aqueous solution of potassium oleate. In the first method, a known amount of oleic acid in CHCl$_3$ was added to an NMR tube, the solvent evaporated under nitrogen atmosphere, and a known amount of aqueous (unbuffered) BSA solution was added. In the second method, 1.2 eq of base (6 N KOH) were added to a known amount of neat oleic acid to make a soluble potassium oleate solution and the BSA solution then was added to the aqueous potassium oleate. The BSA-oleic acid mixtures were allowed to equilibrate at room temperature at least 18 h and the BSA-aqueous potassium oleate mixtures for $\approx$ 1 h prior to NMR analysis. The resulting mixtures were adjusted to appropriate pH values by adding small amounts of 6 N KOH or $\text{HCl}$ directly into the NMR tube using a microsyringe. The pH was measured in the NMR tube with a Beckman model 3560 pH meter equipped with a microelectrode (5-mm diameter) and standardized with pH 4.0, 7.0, and 10.0 buffers. Values for pH reported in the text were measured immediately after the NMR spectra were obtained. These values differed by $<0.2$ pH unit from values obtained before NMR runs.

Column Chromatography—To analyze the monomer-polymer content of BSA with bound fatty acid, mixtures containing $^{1-13}$C oleic acid and BSA were prepared as above and were applied (100 $\mu$l containing 10.0 mg of BSA at pH 7.0) to a Sephadex G-150 column (0.9 x 30 cm). The column was eluted with doubly distilled, deionized water adjusted to pH 7 with KOH. The flow rate was $13$ ml/h and the eluent was monitored for absorbance at 280 nm. Individual tubes (2.2 ml) were monitored for radioactivity by mixing 0.2 ml of eluent with 10 ml of Aquasol (New England Nuclear). Fractions were counted in a liquid scintillation counter (Beckman LS-250) to a 2-$\sigma$ error of $<3\%$.

For preparative separation of monomeric BSA, the following procedure was used. Mixtures of 90% $^{1-13}$C oleic acid and BSA were made as described above. An aliquot of the mixture (2.0 ml containing 240 mg of BSA at pH 7.0) was applied to a Sephadex G-150 column (90 x 2.5 cm) and eluted as described above. The flow rate of the column was 36 ml/h and 6-ml fractions were collected. The monomeric fraction was pooled and concentrated by vacuum dialysis. The

![Fig. 1. Column chromatography of 5 mol of 90% $^{1-13}$C oleic acid/mol of BSA at pH 7. The sample was prepared by adding BSA (420 mg in 3.5 ml of H$_2$O) to 8.8 mg of potassium oleate. The pH was adjusted to 7.0 and a 2-ml aliquot (240 mg of BSA; 5 mg of fatty acid) was applied to a Sephadex G-150 column (90 x 1.3 cm). Column elution is described under "Experimental Procedures." Fractions indicated by the bar were pooled and concentrated for NMR analysis. The void and total volumes of the column are designated by $V_0$ and $V_t$, respectively. The major fraction of albumin came out before the total volume as monomeric albumin ($M_t$ = $\approx$66,000).

![Fig. 2. Proton-decoupled Fourier transform $^{13}$C NMR spectrum of fatty acid-free BSA (A) and of 5 mol of 90% $^{1-13}$C oleic acid/mol of BSA before (B) and after (C) column chromatographic separation. Spectra were recorded at 50.3 MHz after 18,000 spectral accumulations with a pulse interval of 2.0 s, 16,384 time domain points, and a spectral width of 10,000 Hz. A line broadening of 2 Hz was used to improve signal-to-noise ratios. Other details of NMR methodology are given under "Experimental Procedures." pH of the pooled, concentrated solution (6.7) was adjusted to 7.4 prior to NMR analysis. $^{13}$C NMR—Mixtures of 90% $^{1-13}$C oleic acid-BSA (1.2 ml of 12% solution) plus 100 $\mu$l of D$_2$O were used for NMR analysis. Spectra were obtained on a Bruker WP-200 NMR spectrometer at 50.3 MHz as described previously (29). Internal D$_2$O was used as a lock and shim signal. Chemical shift values were measured digitally to an estimated uncertainty of $\pm 0.10$ ppm, except as noted. The chemical shift of the narrow resonance in the aliphatic region from protein $\varepsilon$-lysine and $\beta$-leucine carbons (23) was measured as a function of pH using tetramethylsilane as an external reference for a sample containing 4 mol of oleic acid/mol of BSA. The chemical shift was independent of pH between pH 3 and 10 ($\delta$ = 39.54 ppm) and shifted to 39.88 ppm at pH $\geq$ 10.6. In other experiments with BSA, this peak was used as an internal reference and the appropriate corrections were made for spectra obtained at pH 10.6. In spectra of aqueous potassium oleate, the terminal CH$_3$ resonance of D$_2$O was measured at 14.10 ppm or the terminal CH$_3$ resonance of the BSA solution at pH 7.0 was used as an internal reference. Spin lattice relaxation times were measured using a fast inversion recovery technique (30) and calculated using a three-parameter exponential fitting routine (31) provided in the Bruker DISNMR program. On selected spectra with good signal-to-noise ratios, broad spectral components were removed by the convolution difference procedure (32) using the calculation contained in the Bruker DISNMR program with a digital line broadening of 2.0 Hz.

Nuclear Overhauser enhancement was estimated from peak heights with broad band decoupling and with inverse-gated decoupling by the method of Opella et al. (33). Sample temperature was controlled (± 1°C) with the Bruker B-VT-1000 variable temperature unit and...
Fig. 3. Carboxyl and carbonyl region of the proton-decoupled Fourier transform $^{13}$C NMR spectra of 0.5–7 mol of fatty acid (FA)/BSA at pH 7.4. BSA was added to neat $[^{13}$C]$^1$oleic acid and the pH was adjusted to 7.4. Spectra accumulation conditions were the same as in Fig. 2 except 4000 spectral accumulations were obtained for each spectrum in the bottom and top rows. Spectra shown in the middle row are from samples with mole ratios indicated in the bottom line of the table. The spectra (middle row) for 0.5, 2, and 3 mol of oleic acid/mol of BSA were obtained following 20,000, 24,000, and 31,227 accumulations, respectively, and are printed with higher vertical gain. The spectrum (middle row) for 1 mol of oleic acid/mol of BSA is a 2-fold vertical expansion of the bottom spectrum (4,000 accumulations). Major oleic acid carbonyl peaks are labeled as a–d. The broad signal is primarily from protein backbone carbonyl groups.

RESULTS

Fig. 1 shows the elution profile from a Sephadex G-150 column of a solution containing 5 mol of [1,13]$^1$C$^1$oleic acid/mol of BSA. The majority (74%) of the protein eluted in the monomeric size range ($M_r = 66,000$) just before the total volume of the column. There was no distinguishable dimeric peak, but small amounts of polymeric species of BSA were present. Using analytical columns with [9,10-2H]$^1$oleic acid/BSA complexes (1–6 mol of oleic acid/mol of BSA), it was found that 65–74% of the radiolabeled oleic acid was bound to monomeric BSA and the remainder was bound to polymeric forms. Free oleic acid was not recovered in the total volume, indicating that oleic acid remained bound to BSA and was not liberated as free un-ionized oleic acid or as potassium oleate. Recovery of radiolabeled fatty acid from the column was 105 ± 12% of that added.

The $^{13}$C NMR spectra of fatty acid-free BSA and a preparation of 5 mol of oleic acid/mol of BSA before and after fractionation are shown in Fig. 2. Both 5:1 complexes (unfractionated material, Fig. 2B, and pooled, concentrated material from the monomeric peak of the preparative G-150 column, Fig 2C) gave essentially identical spectra. Compared to the spectrum of fatty acid-free BSA (Fig. 2A), the spectra of $^{13}$C$^1$oleic acid/BSA complexes had at least four additional resonances between 180.6 and 183.8 ppm. The relatively weak resonance at 180.9 ppm in the spectrum of fatty acid-free BSA (Fig. 2A) is probably from glutamic acid carboxyl groups in the protein peptide backbone carbonyl groups and some side chain carbonyl groups (23, 34). The peak at ~180 ppm is from certain protein aromatic groups, the broad signal at ~40 ppm from α-carbons in the protein backbone, and the 14–55 ppm region from methylene and methyl groups of amino acid side chains (23). The relatively narrow resonance at 39.54 ppm is from the ε-carbon and the β-carbon of lysine and leucine (23), respectively, both of which are abundant amino acid species in BSA (15).

Studies at Differing Oleic Acid/BSA Mole Ratios—The region of the $^{13}$C NMR spectrum containing protein carbonyl and carboxyl and oleic acid carboxyl resonances (170–190 ppm) is shown in Fig. 3 for oleic acid/BSA mole ratios varying from 0.5–7.0 at constant pH (7.4) and constant BSA concentration. At low oleic acid/BSA ratios, narrow peaks ($\nu_{1/2} \approx 10$ Hz) were present at 180.9 ppm and 182.4 ppm (0.5 mol of oleic acid) and at 180.8 ppm and 182.4 ppm (1.0 mol of oleic acid). In these spectra, the peak at 180.8 may include an appreciable contribution from protein carbonyl groups (Fig. 2A). At higher oleic acid/BSA ratios, the intensity of the oleic acid carboxyl peaks increased, and at least four resonances between 180.5 ppm and 184.0 ppm were detected in spectra of samples containing >2 mol of oleic acid/mol of BSA. There were no detectable differences in the 170–180 ppm region between the fatty acid-free BSA spectrum and spectra at any oleic acid/BSA ratio. For convenience, four oleic acid peaks which showed little or no chemical shift change as a function of the oleic acid/BSA ratio were designated a–d, as indicated in Figs. 3 and 4. Peak a was not present in spectra of <3 mol of oleic acid/BSA (Fig. 3) and peak d could not be detected in a spectrum of 10 mol of oleic acid/mol of BSA (spectrum not shown).

The peak heights of the oleic acid carboxyl resonances increased relative to protein resonances (e.g. the protein carboxyl envelope; Fig. 3) with increasing oleic acid/BSA mole ratios. The increase was disproportionate, with a large increase for peak c and a small increase for peak a, as shown in plots of peak heights versus the oleic acid/BSA mole ratio (Fig. 5). The peak height was used as a relative measure of peak area because individual peaks were not sufficiently resolved for area measurements. The comparison of peak intensities using peak heights is not intended to be rigorously quantitative because there appear to be differences in peak widths (e.g. between peaks b and c) and there were some detectable changes in line widths at different mole ratios, as in the spectrum of 4 mol of oleic acid/mol of BSA, in which peak d showed an anomalous line broadening and consequently a smaller peak height.
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FIG. 4. Carboxyl chemical shift as a function of mol of 90% [1-13C]oleic acid/mol of BSA at pH 7.4. Small letters are used to identify peaks shown in Fig. 3. Chemical shift values were obtained from samples where BSA was added to neat 90% [1-13C]oleic acid (squares) or potassium oleate (circles). The former values (squares) were obtained from the spectra in Fig. 3 (except 10 mol of fatty acid/mol of BSA; spectrum not shown).

FIG. 5. Carboxyl peak height as a function of mol of 90% [1-13C]oleic acid/mol of BSA at pH 7.4. Lettering is as in Figs. 3 and 4. Peak heights were obtained from spectra shown in Fig. 3 with the same number of accumulations (4,000) and processing conditions. Inset shows a plot of the total peak height (a + b + c + d) for each oleic acid/BSA ratio.

of the individual peaks increased linearly with increasing oleic acid/BSA, as shown in the inset in Fig. 5. In addition, the plot of the total oleic acid carboxyl peak area (integral of the 180.5–184.0 ppm region) normalized against the BSA carbonyl peak area (integral of the 170–180 ppm region) was linear (r = 0.95; p < 0.01) with respect to moles of oleic acid/BSA (data not shown).

The resolution of peaks b and c was significantly increased by the convolution difference technique, as exemplified in Fig. 6 for three different oleic acid/BSA mole ratios (2.0, 3.0, and 5.0). Peak b appeared to be somewhat broader and possibly less homogeneous than peaks c and d. An additional small peak at 181.7 ppm was present in the convolution difference spectra of 3 and 5 mol of oleic acid/mol of BSA (and possibly in the 2-mol spectrum). This peak was seen as a shoulder in spectra at several mole ratios (Fig. 6) and probably represents an additional microenvironment of the oleic acid carboxyl groups. 

T1 and NOE values for oleic acid peaks were measured for a sample of 5 mol of oleic acid/mol of BSA. The T1 values for peaks b, c, and d were between 1.8 and 2.1 s and the NOE values for peaks c and d were approximately 1.8. Peak a was too weak to measure either T1, or NOE. Thus, T1 and NOE values were not significantly different for the major oleic acid resonances and differences in oleic acid peak intensity in the spectrum of BSA with 5 mol of added oleic acid cannot be attributed to differences in T1 and NOE values of the individual resonances. Furthermore, it is reasonable to assume, particularly since the total peak height and area are linearly related to the oleic acid/BSA ratio, that the peak intensity increases (Figs. 3 and 5) are not a result of T1 and NOE changes, but reflect the increasing amounts of oleic acid bound to BSA.

pH Studies—13C NMR spectra were obtained as a function of pH at three different oleic acid/BSA mole ratios (4.0, 5.0, and 6.0). The spectra in general, and the oleic acid carboxyl
region in particular, were unaffected by pH changes between pH 6.5 and 10, except for small changes in resolution which made it difficult to distinguish between resonances b and c at some pH values. At high pH (pH 10.4 and 10.6), peaks b and c were not distinguishable and appeared as a single broadened resonance at the chemical shift corresponding to peak b; in addition, the lysine c-carbon peak shifted downfield slightly (see "Experimental Procedures"). At low pH (<6), significant changes in the oleic acid carboxyl region were observed, as illustrated by the selected spectra shown in Fig. 7. Qualitatively, the carboxyl region first showed a loss of resolution, changes in relative intensities, and an apparent line broadening of the major resonances with decreasing pH (e.g. pH 5.0 and 4.4; in Fig. 7); at pH 4.4, a peak at 179.5 ppm (a chemical shift value different from those for peaks a-d) first appeared and the intensity of the new peak increased while all other peak intensities decreased to yield a single narrow peak at pH < 4.0 (e.g. pH 3.9 in Fig. 7).

The carboxyl chemical shift values are plotted as a function of pH in Fig. 8A. At pH > 6.0, four resonances were detectable (corresponding to a, b, c, and d of Figs. 3 and 4) which showed little or no chemical shift change between pH 6.0 and 10. Since peaks b and c were in general poorly resolved above pH 7.4, the chemical shift values are connected by a dotted line; even if crossover of the two peaks occurred, the chemical shift change would be small (<0.5 ppm). Note that similar chemical shift values were obtained whether oleic acid was added to BSA as un-ionized oleic acid (circles) or ionized potassium oleate (squares). In addition, the NMR results were identical when the pH was decreased from 10.6 to 3.9 (open circles) and when pH was increased from 3.9 to 10.6 (closed circles). Significant decreases in chemical shift values occurred in the pH range (pH < 5.8) in which qualitative spectral changes and gross sample changes (turbidity) were observed (see below). In Fig. 8A, the chemical shift values for the most intense peak (corresponding to peak c at pH > 5.5) were connected. It thus appeared that the major oleic acid peak had a sigmoidal behavior of chemical shift versus pH. The smaller resonances a, b, and d either coalesced into the single intense resonance or broadened beyond detection at low pH.

To study the low pH region in greater detail, additional titrations of oleic acid/BSA complexes were carried out by adding BSA to oleic acid and titrating from pH 6.0 to pH 3.4. Fig. 8B shows the plot of carboxyl chemical shift values as a function of added equivalents (or moles) of HCl for a representative experiment (5 mol of oleic acid/mol of BSA). The chemical shift values of the most intense peak showed a linear dependence on the number of added HCl equivalents, with two break points. The general form of the curve resembled that for the \( ^{13} \text{C} \) NMR titration of aqueous carboxylic acids (25); the linearly decreasing chemical shift values with added HCl were consistent with protonation of the carboxyl group and the onset of protonation occurred at ~20 \( \mu \)l of HCl (Fig. 8B). The total chemical shift change (\( \Delta \delta_{\text{max}} - \delta_{\text{min}} = 2.5 \) ppm)
was smaller than \( \delta_{\text{max}} - \delta_{\text{min}} \) for aqueous carboxylic acids (4.7 ppm, Ref. 25).

Turbidity measurements (optical density at 700 nm) also were made on several titration samples as a function of pH. At pH 3.9, samples were very turbid but cleared with increasing pH, so that there was no detectable turbidity at neutral pH and above. Sample turbidity was not dependent on the method of addition of oleic acid to BSA and turbidity changes as a function of pH were completely reversible. Samples of BSA with no added fatty acid were optically clear at all pH values between 4.5 and 10.6.

**Temperature Studies**—The effect of temperature variations between 15 and 55 °C and of thermal denaturation on oleic acid/BSA complexes also was investigated by \(^{13}\)C NMR. Spectra were obtained at 5 different temperatures (15, 25, 36, 44, and 55 °C) on a sample of 4 mol of oleic acid/mol of BSA at pH 7.4 (data not shown). The resolution of oleic acid carboxyl peaks was temperature-independent between 15 and 37 °C.

At the higher temperatures, resonances were slightly broader and resolution somewhat poorer; in these spectra, peaks \( b \) and \( c \) appeared as one broad peak. A sample with 5 mol of oleic acid/mol of BSA at pH 7.4 was studied before and after heat denaturation at 35 °C for 5 min (35). After heat denaturation, the sample remained clear but was noticeably more viscous. The spectrum of the 5 mol of oleic acid/mol of BSA sample before heat denaturation was identical with that shown in Fig. 3. However, the NMR spectrum of the heat-denatured sample had only one broad peak in the fatty acid carboxyl region with a chemical shift of ~181.1 ppm at pH 7.4 (data not shown); in addition, the protein resonances were much broader in the heat-denatured spectrum.

**Aqueous Potassium Oleate**—An aqueous solution of potassium oleate at pH 11.2 was prepared from \([1-^{13}\text{C}]\)oleic acid at the concentration of oleic acid corresponding to that for samples with 4 mol of oleic acid/mol of BSA (0.19% w/v). The sample was titrated with HCl and NMR spectra were obtained at pH values of 11.2, 10.6, 9.7, 9.1, and 8.5. At high pH values, samples were optically clear and the \(^{13}\)C spectra had a narrow, intense resonance (~4 Hz) at 183.50 ppm (pH 11.2) and 183.39 ppm (pH 10.5) from the \(^{13}\)C-enriched carboxyl group. As the pH was decreased below 10, samples became progressively more turbid. The carboxyl region of \(^{13}\)C spectra had a very broad resonance (~50–100 Hz) centered at ~182.0 ppm (pH 9.7), ~181.0 (pH 9.1), and ~180.4 (pH 8.5).

**DISCUSSION**

The binding of fatty acid to albumin is mediated through nonpolar and polar interactions between the protein and fatty acid, but the polar interactions are thought to play a relatively minor role (1). For example, hydrocarbons can bind to albumin (36) and the longer chain fatty acids have a greater affinity for albumin than medium chain fatty acids (21, 37). However, data on binding of native fatty acids to albumin are insufficient to describe in molecular detail the albumin binding sites and mechanisms of fatty acid binding. This study provides insight into the microenvironments experienced by the carboxyl carbon of oleic acid in the presence of BSA.

The \(^{13}\)C NMR spectra of oleic acid/BSA mixtures showed multiple resonances from the carboxyl carbon of the \([1-^{13}\text{C}]\)oleic acid moiety. The \(^{13}\)C NMR spectrum was not altered by removal of a small proportion of aggregated protein. It is unlikely that uncomplexed oleic acid can account for any of the observed resonances for several reasons: (i) the concentrations of free oleic acid in the aqueous medium would be extremely low (1); (ii) such oleic acid, if present, would not yield a narrow NMR resonance, except at pH extremes (see below); and (iii) the oleic acid/BSA spectrum was unaffected by fractionation procedures which would remove uncomplexed oleic acid. Therefore, the observation of multiple oleic acid carboxyl resonances resulted from the heterogeneity of oleic acid binding sites on individual BSA molecules. The different \(^{13}\)C resonances from the single carbon nucleus of the oleic acid were the result of different magnetic microenvironments of the carboxyl carbon in BSA binding sites. These carboxyl groups were in sufficiently slow exchange to produce distinguishable resonances (the exchange rate must be less than the chemical shift difference, in hertz, between the different peaks). In contrast to the albumin results, \(^{13}\)C NMR spectra of phosphatidylcholine vesicles containing 4–7 weight % of \([1-^{13}\text{C}]\)oleic acid showed a single narrow resonance (<10 Hz) between pH 3.0 and 11.0, and similar results have been reported for myristic acid incorporated into phospholipid vesicles (24).

The minimum number of different oleic acid microenvironments, obtained from the number of distinguishable resonances, and the relative populations of oleic acid in the different microenvironments, estimated from peak heights, varied as a function of oleic acid content at constant pH (7.4). At least two microenvironments were observed at low mole ratios of oleic acid/BSA (0.5 and 1.0) and at least three at a mole ratio of 2.0. At higher mole ratios (>3.0), at least four different microenvironments were observed. The relative population of oleic acid in different microenvironments appeared to be approximately equal at 1.0–3.0 mol of oleic acid/mol of BSA. At high oleic acid/BSA ratios (>3), the disproportionate increase in the peak heights of the different resonances showed that different oleic acid microenvironments contained grossly different numbers of molecules. A new oleic acid microenvironment (peak \( a \)) was detected at oleic acid/BSA ratios of >3.0 and contained the smallest proportion of oleic acid at all oleic acid/BSA ratios. At high oleic acid/BSA ratios, the microenvironment corresponding to peak \( c \) accounted for the largest proportion of oleic acid.

It has previously been suggested from ESR (18) and fluorescence (12) results that the first two fatty acid molecules bind to albumin in a single domain in an antiparallel manner, i.e. with a head-to-tail arrangement. Assuming that a given carboxyl resonance represents at least one physically distinct binding site on BSA, our results show at least two binding sites with 0.5 and 1.0 mol of oleic acid/mol of BSA, which could result from the binding of pairs of oleic acid molecules in nonequivalent sites on the same BSA molecule, as in the antiparallel arrangement, or from the binding of oleic acid on different binding sites on different BSA molecules. However, the fact that (at least) three binding sites were discernable by NMR at a 2.0 mol ratio of oleic acid/BSA shows that two molecules of oleic acid cannot be homogeneously distributed in two binding sites in the population of BSA molecules. From the spectra in Fig. 2 and the corresponding intensity data, no discrete stepwise pattern was seen for the addition of higher mole ratios of oleic acid/BSA, as might be suggested from the pairing of fatty acid molecules in three binding sites (18), and one binding site corresponding to resonance \( a \) appeared to have, on the average, fewer than one molecule of oleic acid. In addition, although a maximum of four peaks has been noted in general, additional peaks may be present in the \( b, c \) region (as suggested in the convolution difference spectra) and a splitting of peak \( a \) was observed in some spectra (e.g.

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Fig. 2, B and C). It is interesting to note that our results contrast with results of fluorine NMR spectroscopy, which could not detect any magnetic inequivalence in the first 14–15 binding sites of long chain trifluoroalkylsulfate ions on BSA (38).

One remarkable feature of the oleic acid/BSA interactions was the insensitivity of the oleic acid carboxyl function to changes in bulk pH (Fig. 6, a and b) between pH 6.0 and 10.6, as indicated by the constancy of the chemical shifts of the individual oleic acid carboxyl peaks. This result shows that the oleic acid carboxyl groups (bound to BSA) did not titrate in this pH range, in marked contrast to aqueous oleic acid in the absence of albumin (apparent pK = 7.2, Ref. 39) and to oleic acid in egg phosphatidylcholine vesicles (apparent pK = 7.6, Footnote 3). This result is physiologically significant because small changes in bulk pH near pH 7.4 result in changes in the ionization state and physical state of protein-free aqueous oleic acid (39).

An NMR titration curve was obtained for the major oleic acid carboxyl peak (peak c) at low pH (Fig. 8, A and B). This titration curve may be incomplete, based on the smaller total chemical shift change (δmax - δmin) compared with aqueous short chain carboxylic acids (25). The apparent pK values from the NMR titration curve (pK ~ 4) was somewhat lower than pK values for carboxylic acids (25). Since BSA undergoes a conformational change which expands the structure in the same pH range (40), it is not clear whether the protonation of oleic acid molecules occurred as a result of the disruption of specific lipid-protein interactions and exposure of oleic acid carboxyl groups or whether the protonation occurred independent of protein changes. The chemical shifts of oleic acid carboxyl peaks a, b, and d were more resistant to pH changes than peak c, suggesting that oleic acid molecules in binding sites corresponding to peaks a, b, and d were more protected by specific lipid-protein interactions and/or steric hindrances. Highly acidic (pH ≤ 4.0) conditions removed specific oleic acid carboxyl interactions with BSA and resulted in a more homogeneous population of oleic acid molecules.

The relatively minor changes in tertiary structure induced by temperature changes below the denaturation temperature (35) produced minor changes in the oleic acid carboxyl spectrum. The loss of resolution and apparent broadening of peaks b and c at higher temperatures may have been caused by protein conformational changes or by an increased rate of exchange of oleic acid between these magnetically similar sites. Following gross structural changes induced by thermal denaturation, the multiple oleic acid peaks collapsed to a broad peak centered at 181.0 ppm. Thus, the magnetic environment of all peaks except c changed markedly. The upfield shift of peaks a, b, and c could indicate a change in the net ionisation state of these populations of oleic acid carboxyl groups as a result of increased exposure of these groups to solvent molecules.

Our results provide evidence that the ionized form of oleic acid is bound to BSA at pH 7.4. This conclusion can be inferred from the pH dependence of the oleic acid carboxyl chemical shift and is consistent with the chemical shift changes following protein denaturation. The observation of several distinct resonances for the oleic acid carboxyl carbon may have been a result of differences in electrostatic and hydrogen bonding interactions between the oleic acid carboxyl group and specific amino acid groups. However, in addition to the reported large effects of ionization and hydrogen bonding on fatty acid carboxyl carbon chemical shifts in lipid systems (22–25), other extrinsic factors may affect the chemical shift of oleic acid in the presence of protein, so that a precise interpretation of chemical shift values cannot be made at present. Several previous studies have suggested that basic amino acid residues, including arginine (41), lysine and arginine (42), and tyrosine (43), are located at or near fatty acid binding sites on albumin. The ionization of tyrosine residues was hindered by the addition of fatty acid (43), suggesting close and specific interactions between the fatty acid carboxyl group and tyrosines. In addition, hydrogen bonding interactions between ligands and binding sites have been proposed from studies of steroid (44) and alcohol (45) binding to BSA. 13C NMR spectra of oleic acid/BSA complex at pH 7.4 contained oleic acid carboxyl resonances with line width values as low as 10 Hz, in contrast to spectra of aqueous oleic acid (at the same oleic acid concentration but with no BSA) at pH values near physiological pH. In the latter case, the resonances were severely broadened, probably as a result of slow reorientation of lamellar liquid crystalline structures and/or highly restricted internal molecular motions of the oleic acid carboxyl group. The narrower oleic acid carboxyl resonances in the oleic acid/BSA complex may be a result of rapid internal motions or overall reorientation of the complex or both. Although the relaxation mechanism(s) of the fatty acid carboxyl carbon is not known and a detailed analysis of molecular motions cannot be made, resonances of nonprotonated carbons relaxing either by a dipolar mechanism or chemical shift anisotropy mechanism (46, 47) will be much narrower than resonances for protonated carbons with the same molecular motions. In fact, using the longer correlation times for anisotropic overall reorientation of BSA, it is clear that narrow (<10 Hz) carboxyl resonances could be observed even with rigid attachment of the carboxyl group to BSA binding sites. Studies of the relaxation behavior of fatty acid α-CH 2 or β-CH 2 carbons should provide an understanding of whether the head group region is rigidly attached or whether motions which are significantly more rapid than the overall reorientation of the fatty acid/BSA complexes are present.

The present study showed that carboxyl carbons of 90% [1-13C]oleic acid yielded observable NMR resonances at physiological mole ratios of oleic acid/BSA. The oleic acid carboxyl resonances were in a region that was nearly devoid of protein resonances, a result which allowed quantitation of NMR features of the oleic acid resonances. The observation of multiple oleic acid carboxyl peaks suggested that specific ionic and/or hydrogen-bonding interactions may be an essential aspect of fatty acid binding to albumin.

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Interactions of Oleic Acid with Albumin

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