papers and notes on methodology

Ionization behavior of aqueous short-chain carboxylic acids: a carbon-13 NMR study

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Abstract The ^{13}C chemical shift of each carbon of aqueous acetic, propionic, and butyric acids has been measured as a function of pH or of added equivalents of base. A plot of chemical shifts for the carboxyl, α, and β carbons as a function of pH is sigmoidal and yields pK_a values that agree closely with values obtained by potentiometric titration. In contrast, a plot of chemical shift as a function of added equivalents of base is linear and has a sharp break at the equivalence point. Based on this result, we propose that the local (microscopic) ionization state of the carboxyl group can be determined directly by NMR without need for pH or pK determinations. In addition to titration curves, the effects of concentration, ionic strength, and temperature upon fatty acid chemical shifts are reported. For aqueous acids, changes in ionic strength and temperature have no effect on chemical shifts. However, changes in concentration do affect chemical shifts, probably as a result of changes in the relative degree of acid-acid and acid-water hydrogen bonding. Our results provide necessary background data for ^{13}C NMR studies of higher fatty acids in lipid-lipid and lipid-protein systems. **Cistola, D. P., D. M. Small, and J. A. Hamilton.** Ionization behavior of aqueous short-chain carboxylic acids: a carbon-13 NMR study. *J. Lipid Res.* 1982. **23:** 795–799.

Supplementary key words ^{13}C chemical shift • fatty acids • pH • ionic strength concentration • NMR titration curves

NMR studies have demonstrated that the ionization state of the carboxyl group has a significant influence upon the chemical shifts of ^{13}C resonances of carboxylic acids (1, 2). The chemical shift difference between the fully ionized and un-ionized group allows an estimation of its pK_a in a variety of carboxylic acids, amino acids, and peptides (3–6). Although NMR titration curves (chemical shift vs. pH) have been determined for many amino acids and have been applied extensively to ionization studies of peptides (1, 7, 8), there are no such curves for aqueous fatty acids other than acetic acid (1). In addition, the effect of other variables such as concentration, ionic strength, and temperature on ^{13}C chemical shifts has not been systematically investigated for these acids.

We report ^{13}C NMR titration curves for three water-miscible short-chain acids: acetic, propionic, and butyric acids. These acids are not complicated by the limited water solubility and complex phase behavior characteristic of the higher fatty acids (9). The titration curves, as well as the dependence of ^{13}C chemical shifts upon concentration, temperature, and ionic strength reported herein, provide a detailed background necessary for interpretation of ^{13}C chemical shifts of longer-chain fatty acids in water and in lipid-lipid and lipid-protein systems such as phospholipid bilayers (10, 11), membranes, lipoproteins, and serum albumin, and will aid in the description of the complex phase behavior of fatty acids in these systems.

EXPERIMENTAL

Materials

Glacial acetic acid (reagent A.C.S., Fisher), propionic acid (certified, Fisher), and butyric acid (reagent, Fisher) were diluted to appropriate concentrations with de-ionized, doubly-distilled water; concentrations are given as %v/v unless otherwise noted. The neat propionic acid used for temperature studies was obtained from Nu-Chek Prep, Elysian, MN. There were no impurity peaks in any of the ^{13}C NMR spectra of the samples used. The pH of samples for concentration studies was adjusted with either 10 N NaOH, 10 N HCl, or solid NaOH.

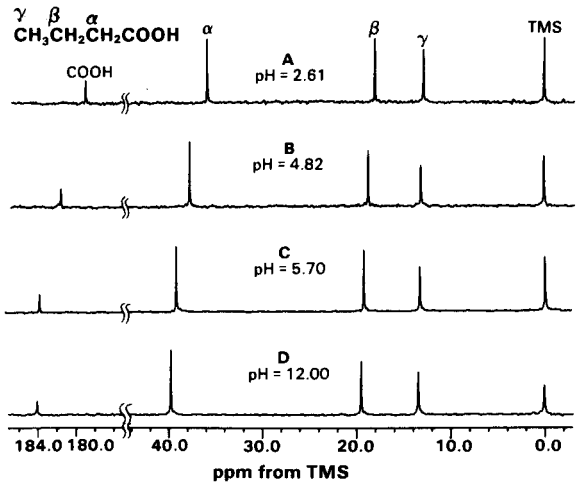

Fig. 1. Natural abundance proton-decoupled Fourier transform ^{13}C NMR spectra of 4% (0.045 M) butyric acid recorded at 50.3 MHz with a pulse interval of 14.8 sec, a spectral width of 200 ppm, and 16,384 time domain points. 200, 300, 1000, and 1600 scans were accumulated for spectra A, B, C, and D, respectively; signal-to-noise ratios decreased with added titrant (12), requiring more spectral accumulations for the more ionized samples. The region from 44 to 176 ppm contains no peaks and has been deleted from the figure.

Solid NaCl (certified A.C.S., Fisher), was added to samples for ionic strength studies, and pH values were adjusted to 2.5. All sample pH values were measured with a Beckman model 3560 pH meter equipped with an Altex model 531167 5mm combination electrode; the pH meter was standardized to pH 4, 7, and 10 buffers depending on the pH range of measurement. The estimated uncertainty in pH values is ±0.05.

Titration

Ten-ml samples of dilute acid in a scintillation vial were titrated in a nitrogen atmosphere with 10 N NaOH or HCl using a 50 μl Hamilton syringe; sample dilution during titration was insignificant. At appropriate pH values, 2-ml aliquots were transferred to a 10-mm NMR tube. Sample pH values checked before and after each NMR run agreed within ±0.03 pH units.

NMR

Proton-decoupled Fourier transform ^{13}C NMR spectra were obtained on a Bruker WP 200 spectrometer operating at 47 kGauss (50.3 MHz for ^{13}C). The system was equipped with an Aspect 2000 data system with 32K data memory. Deuterochloroform and tetramethylsilane (TMS) in a coaxial insert were used as external lock and reference, respectively. The ^{1}H irradiation for proton decoupling (1.0 watt) was centered at 3.2 ppm downfield from the ^{1}H resonance of TMS. Chemical shifts were

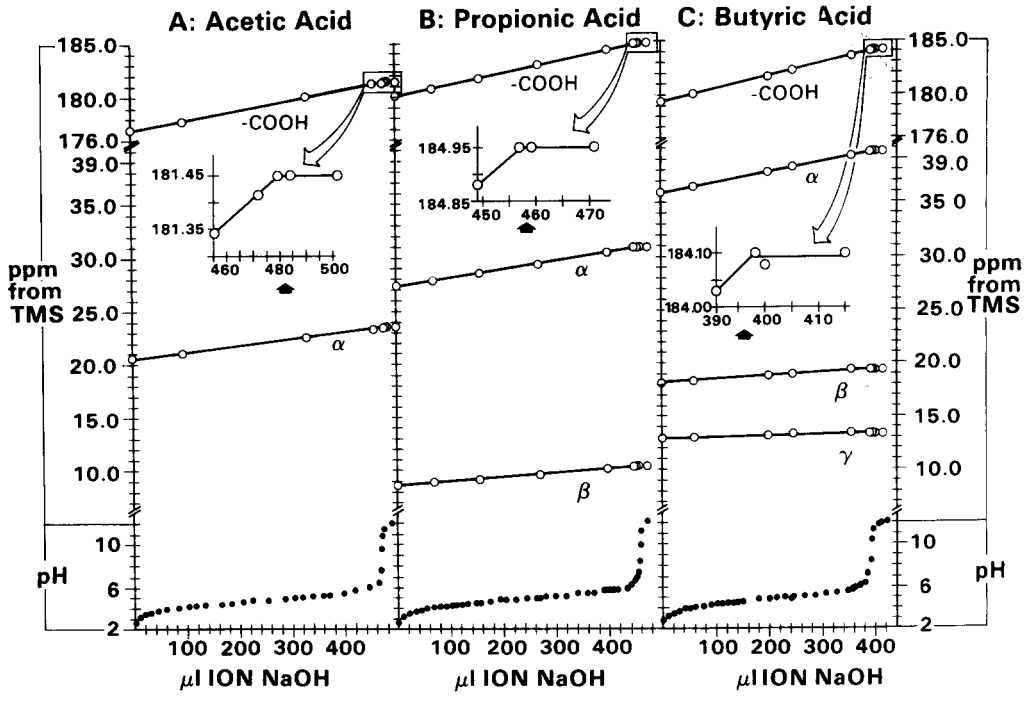

Fig. 2. Chemical shift and pH as a function of added 10 N NaOH for 3% acetic (A), 4% propionic (B), and 4% butyric (C) acids. The lowermost curves were determined by potentiometric titration. The equivalence points determined from these curves were 481 μl (acetic), 458 μl (propionic), and 396 μl (butyric). Above these curves, the ^{13}C chemical shifts for each resonance of each acid are plotted. Plots of carboxyl chemical shifts in the region of the equivalence point are shown on an expanded scale (insets) to demonstrate that the break in each plot corresponds to the equivalence point as determined by potentiometric titration (solid arrows). Sample temperatures varied from approximately 30°C to 45°C because of decoupler heating (13), although these changes did not alter chemical shift values (see Results and Discussion).

measured digitally, and their estimated uncertainty was ±0.05 ppm. Probe temperature was regulated with a Bruker B-VT-1000 variable temperature unit; sample temperature was measured using a thin, copper-constantan thermocouple and an Omega model 400A digital readout unit. Temperatures were recorded at 20-sec intervals following removal of sample from magnet and extrapolated back to 0 sec to obtain reported values with ±1°C uncertainty.

RESULTS

Representative spectra from the titration studies are shown in **Fig. 1** for 4% butyric acid at pH values corresponding to protonated (spectrum A), deprotonated (spectrum D), and two intermediate states (spectra B and C). Linewidths of all resonances were narrow (<4 Hz) for butyric acid spectra, as well as for all acetic and propionic acid spectra.

Conventional titration curves are shown at the bottom of **Fig. 2A** (acetic), **Fig. 2B** (propionic), and **Fig. 2C** (butyric). The equivalence point corresponds to the steepest portion of the curves. Chemical shifts for each carbon resonance are plotted above the titration curve in each figure. The chemical shift for each carbon resonance increases linearly with added equivalents of base up to the equivalance point, after which the chemical shift remains constant, as shown in the insets of Figs. 2A–C. Thus, the equivalence point may be determined directly from these NMR plots, employing the carboxyl as well as the aliphatic (up to the β carbon) shift data.

Plots of chemical shift as a function of pH for each carbon resonance of acetic, propionic, and butyric acids are shown in **Figs. 3A, B,** and **C,** respectively. These curves are similar to those published for related compounds (1, 7, 8). The magnitude of the chemical shift difference between the ionized and un-ionized forms of each acid (titration shift) is largest for the carboxyl group

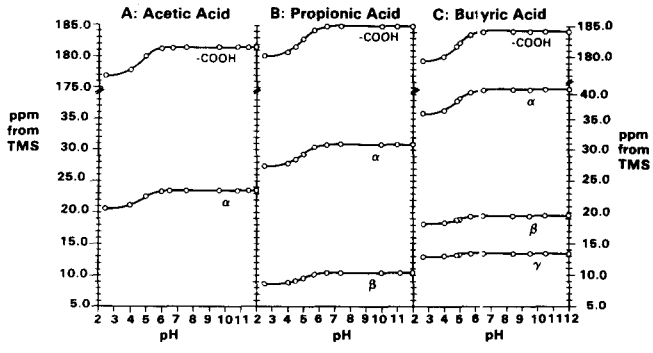

Fig. 3. Chemical shift of indicated resonances as a function of pH for 3% acetic (A), 4% propionic (B), and 4% butyric (C) acids. The chemical shift values plotted here correspond to values plotted in Fig. 2.

TABLE 1. Summary of carboxyl chemical shifts and pK_a values

	δ_{max} COO^-	δ_{min} COOH	pK_a (A)	pK_a (B)	pK_a (C)
Acetic acid	181.45	176.80	4.7	4.6	4.75
Propionic acid	184.95	180.02	4.9	4.8	4.87
Butyric acid	184.10	179.22	4.8	4.8	4.82

Summary of carboxyl chemical shifts (ppm from external tetramethylsilane) for each acid in its fully deprotonated (δ_{max}) and fully protonated (δ_{min}) state and of pK_a values derived from (A) NMR titration, (B) potentiometric titration, and (C) the literature (14). pK_a (A) represents the pH values that correspond to ½ ($\delta_{max} - \delta_{min}$). pK_a (B) values were determined from the potentiometric titration curves in Fig. 2. To ensure that δ_{min} was reached, samples were back-titrated with "10 N" HCl until chemical shifts reached a constant value. A plot of carboxyl chemical shift as a function of added μl of "10 N" HCl showed a sharp change in slope at δ_{min} (data not shown) similar to that observed at δ_{max} (Fig. 2).

and decreases for each successive carbon away from the carboxyl group. The pK_a values can be determined from data for all carbon resonances except the γ carbon of butyric acid. **Table 1** summarizes carboxyl titration shifts and corresponding pK_a values; pK_a values obtained by NMR agree with those obtained by potentiometric titration.

The effects of ionic strength and temperature on chemical shift were studied independently by *a*) changing ionic strength by adding NaCl at fixed pH, concentration, and sample temperature; and *b*) changing the NMR probe temperature at fixed pH, concentration, and ionic strength. For aqueous acids (10%, pH 2.5), large changes in ionic strength (up to $\mu_i = 2$) and temperature (T_{sample} = 30–55°C) had negligible effects upon chemical shifts (< 0.1 ppm). However, for neat propionic acid, variation of sample temperature from 8°C to 50°C produced a significant linear decrease in the carboxyl chemical shift (0.46 ppm) and smaller linear increases in aliphatic chemical shifts.

The concentration dependence of chemical shifts for each acid at pH 2.5 is shown in **Fig. 4.** The carboxyl chemical shift exhibited the strongest concentration dependence, while the α carbon exhibited the weakest concentration dependence for each acid. The sample temperature was controlled, but ionic strength increased (up to $\mu_i \simeq 2$) with increasing concentration, since NaOH was added to maintain constant pH. For acetic acid (Fig. 4A), the carboxyl chemical shift decreased from 176.8 ppm at 0.032 mole fraction (10%) to a minimum at about 0.4 mole fraction (60%) and then increased to 177.9 ppm for undiluted glacial acetic acid; the methyl resonance was concentration independent, except at high concentrations (glacial acetic acid). Propionic acid (Fig. 4B) exhibited qualitatively similar results, with a minimum at approximately 0.3 mole fraction for the carboxyl shift. However, a larger chemical shift change occurred with the carboxyl carbon of propionic acid as compared with

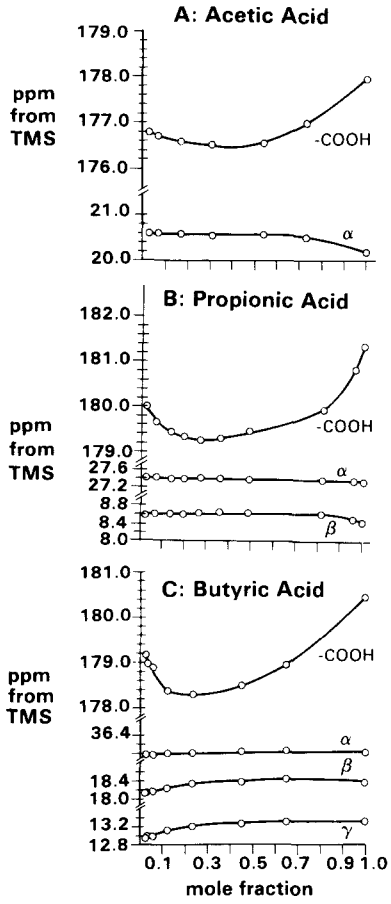

Fig. 4. Chemical shift as a function of concentration for acetic (A), propionic (B), and butyric acids (C), all at pH = 2.5. Sample temperatures were 35 ± 2°C (acetic), 29 ± 2°C (propionic), and 33 ± 2°C (butyric).

acetic acid. Butyric acid (Fig. 4C) showed a minimum carboxyl shift at about 0.2 mole fraction, no significant change for the α carbon resonance, and a larger linear increase for the γ carbon resonance.

DISCUSSION

The ionization state of an acid in solution is often determined by using the Henderson-Hasselbach equation (15) to compare pH and pK_a values. Determinations of both pH and pK_a involve measurement of hydrogen ion activities; thus, pH and pK_a are bulk (macroscopic) properties of the solution. However, the local (microscopic) ionization state of a given chemical group is not always reflected by bulk measurements such as pH (7, 10, 11, 16). The resulting deviation of pKs from expected values in some systems has led to use of the terms "apparent pK" (11), "effective pK" (10), or "microscopic pK" (16). The widespread reliance upon apparent pK values to describe microscopic ionization is surprising in light of the macroscopic nature of pK determinations.

Our results as plotted in Fig. 2 suggest a simple, direct method for obtaining microscopic ionization states without need for pH or pK_a determinations. Since the carboxyl carbon gives rise to only one ^{13}C resonance because of fast chemical exchange between protonated and unprotonated species, and since its ^{13}C chemical shift increases linearly with added base up to the equivalence point, the ionization state of the carboxyl group can be described by the following simple equation:

$$[(\delta - \delta_{min})/(\delta_{max} - \delta_{min})] \times 100\% = \% \text{ ionization} \quad \text{Eq. 1}$$

where δ_{max} and δ_{min} are the carboxyl chemical shifts of the fully ionized and un-ionized acids, respectively, at a given concentration (e.g., 4%), and δ is the measured chemical shift for a given sample. Thus, a plot of chemical shift as a function of added titrant yields a direct measure of the ionization state of a specific chemical group.[1] To our knowledge, this straightforward analysis has not been previously published; studies of similar compounds have always related ^{13}C chemical shifts to pH (1, 3, 6, 7, 10, 11) and have assumed a linear relationship between ionization and chemical shift without actually demonstrating it (7, 11).

In our concentration studies, the carboxyl carbon showed the greatest chemical shift changes with concentration. A probable explanation is that concentration alters the relative degree of solute-solute and solute-solvent hydrogen bonding (17). At the lowest acid concentrations, hydrogen bonding between acid and water molecules is maximized, and at the highest acid concentrations, hydrogen bonding between acid molecules is maximized (17); our results show that the carboxyl resonance appears more downfield at concentrations where hydrogen bonding is maximized. Previous results for acetic acid in water are qualitatively similar to ours and also show a minimum chemical shift at approximately 0.3 mole fraction (Fig. 4); however, quantitative differences probably reflect the lack of pH control in the study of Maciel and Traficante (17). In another study, Hagen and Roberts (2) concluded that ^{13}C chemical shifts of carboxylic acids are rather insensitive to concentration; however, they considered only a narrow concentration range (<0.1 mole fraction). It is clear from our results that carboxyl chemical shifts change significantly with concentration at values <0.3 mole fraction (Fig. 4). In addition, the concentration dependence of all resonances (except the α carbon) is greatest for the longest

[1] This result also replies to resonances α and β to the carboxyl. This result assumes that concentration changes are minimal during titration and that each molecule has only one ionizable group. If more than one group is present, computer curve fitting can be used (7, 16).

acid (butyric), in agreement with previous findings over a much smaller concentration range (2).

In contrast to the marked effects of ionization and concentration upon chemical shifts, large changes in ionic strength and temperature have no significant effect upon chemical shifts for aqueous acids when other variables are controlled. This insensitivity of chemical shift to ionic strength and temperature is important for two reasons. First of all, addition of NaOH during our titrations resulted in unavoidable changes in ionic strength and consequential changes in sample temperature (13). The above findings for ionic strength and temperature demonstrate that these changes do not affect the chemical shifts obtained from our titration studies. Secondly, in contrast to our results, others have found chemical shift changes with ionic strength for aqueous sodium acetate (18), aqueous sodium octanoate (18), and (aqueous) phospholipid/fatty acid vesicles (11). Our results suggest that these changes reflect either a lack of pH control (sodium acetate) (18), a difference in the ionization behavior of longer-chain fatty acids (18), or a difference in the molecular environment near the carboxyl group (11).

Free fatty acids are a major product of triglyceride lipolysis in the intestine, bloodstream, and tissues. In the bloodstream, released free fatty acid can remain in association with serum albumin and lipoproteins, or can cross the capillary endothelium to enter the underlying tissue. One way to probe the molecular environment of fatty acids in these different locations is by NMR chemical shift measurements. However, in order to properly interpret chemical shifts in these complex systems, it is necessary to understand the simplest model systems. The data presented in this paper will help to distinguish the effects of hydrogen bonding and ionization on chemical shifts in complex fatty acid-lipid and fatty acid-protein systems.

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