General approach to measurements of pK_a differences by ^{13}C NMR spectroscopy

Tonis Pehk,*,[a] Ene Kiirend,[a] Endel Lippmaa[a] and Ulf Ragnarsson[b]

[a] *Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, Akadeemia tee 23, EE0026 Tallinn, Estonia*
[b] *Department of Biochemistry, University of Uppsala, Biomedical Center, PO Box 576, S-751 23 Uppsala, Sweden*

The method of measuring very small isotope effects on pK_a values using NMR spectroscopy has been adapted for wider use in comparative pK_a studies in mixtures of organic compounds. The method is illustrated initially on simple linear and branched monocarboxylic acids and on a mixture of methyladipic acids as examples of dicarboxylic acids. All measurements were performed by ^{13}C NMR spectroscopy. In the case of propionic and valeric acid with close pK_a values their relative order as determined by the new technique was found to depend on the composition of the mixture. The method does not require pH measurements and opens a whole array of new applications in studies of acid–base equilibria.

Introduction

The acid–base equilibrium is one of the fundamental concepts in chemistry. Acids and bases are characterized by pK_a values. Interpretation of pK_a variations due to structural changes within molecules ranging from simple ones to proteins has been the subject of endless speculation. Minor changes in pK_a values, which can also be induced by medium effects and even by isotopic perturbations, may modify mechanisms of reactions and shift chemical equilibria. For example, it has been demonstrated that the very small $^{14}N/^{15}N$ isotope effect on acid–base equilibria can be exploited for the separation of $^{14}N/^{15}N$ isotopomers by reverse phase liquid chromatography.[1] Dependence of pK_a values on solvent composition has also been demonstrated.[2] Therefore, among numerous methods for pK_a determination,[3] due attention should also be given to methods in which the comparative analysis of the acid–base equilibria is performed in mixtures in which all media effects are exactly the same. Although listed among 'other methods' in a classical manual, NMR spectroscopy is an attractive approach.[3] Normally, pK_a determination by NMR is based on the analysis of the variation of the degree of protonation of a molecule with pH. The degree of protonation is typically monitored by the changes of the chemical shifts close to the acidic (basic) site in the molecule. Owing to the low signal to noise ratio with other nuclei, the main efforts were originally focused on proton NMR studies. However, high sensitivity is not much use when the selectivity is too low to differentiate between individual sites in a polyfunctional compound or in a mixture of compounds. Two-dimensional Fourier-transform methods afford considerably greater selectivity and allow rather complicated compounds up to proteins to be studied.[4] The introduction of superconductive solenoids has considerably improved the signal to noise ratio with other nuclei, among them ^{13}C. By modern instrumentation a few milligrams of a typical organic compound can be successfully studied at ^{13}C natural abundance. Therefore, the very high selectivity of the ^{13}C nucleus to structural parameters makes it attractive for pK_a studies. pK_a determinations by ^{13}C NMR were first undertaken more than 25 years ago by INDOR experiments[5] and have since been used for studies of carboxylic acids,[6–12] amines,[7,8,13] heterocyclic compounds,[14] amino acids, peptides and proteins.[5,8,15–27] In all publications cited chemical shifts were plotted against pH (in ref. 9 also against added volume of NaOH) and from the graphs obtained pK_a values were calculated. Typical graphs of this kind are presented in Fig. 1 for [2-^{13}C,^{15}N]glycine in a mixture with unlabelled glycine, where two stages of protonation are clearly seen in all graphs. In addition to chemical shifts of carboxy and methylene carbons [Fig. 1(*a*) and (*b*)], methylene proton chemical shifts [Fig. 1(*c*)], and three types of spin–spin coupling constants [Fig. 1(*d*), (*e*) and (*f*)], connected with ^{13}C nuclei are also plotted against pH. Five of the six graphs are connected with ^{13}C parameters, demonstrating the multitude of possibilities for pK_a measurements by ^{13}C NMR spectroscopy. The method of choice depends on the actual variation of the NMR parameter during the experiment, the signal to noise ratio and the absence of interfering signals within the region of interest. From these points of view the coupling constants are generally ruled out, but should nevertheless be regarded as a possibility in specific cases. However, these graphs are not ideal for the presentation of data for compounds with close pK_a values [Fig. 1(*a*) and (*c*)]. A more conclusive presentation is obtained by plotting the degree of protonation as a function of pH for ordinate values around 50%. This approach is illustrated in Fig. 2 for the case of acetic, propionic, butyric and valeric acid. Recognizing that the degree of protonation n for an anion is equivalent to an ionization $(1 - n)$ for the corresponding acid, the relative strengths of the acids involved can be read directly. Larger differences in protonation are observed at pH $\simeq pK_a$ values, and the possibility of measuring these differences depends on the total change of the NMR parameter on protonation. For a typical carboxy carbon in acids *ca.* 5 ppm (630 Hz on an 11.7 T magnet) changes are observed. Reproduction of line position measurements under temperature control in ^{13}C NMR spectra is at least 0.1 Hz, and 0.1% changes in degrees of protonation can therefore be measured with no problem. This approach was proposed by Ellison and Robinson for the measurement of small isotope effects on acid–base equilibria.[2] They plotted the degree of protonation of a reference compound against the difference in NMR frequencies for the two compounds, and in this way they could eliminate any pH measurements, which often become the limiting factor in the determination of small pK_a differences. The proposed method is based on only the measurement of frequency differences, which can be done with much higher precision. Up to now it has been used by a couple of laboratories only for studies of isotope effects on pK_a values.[2,28–31]

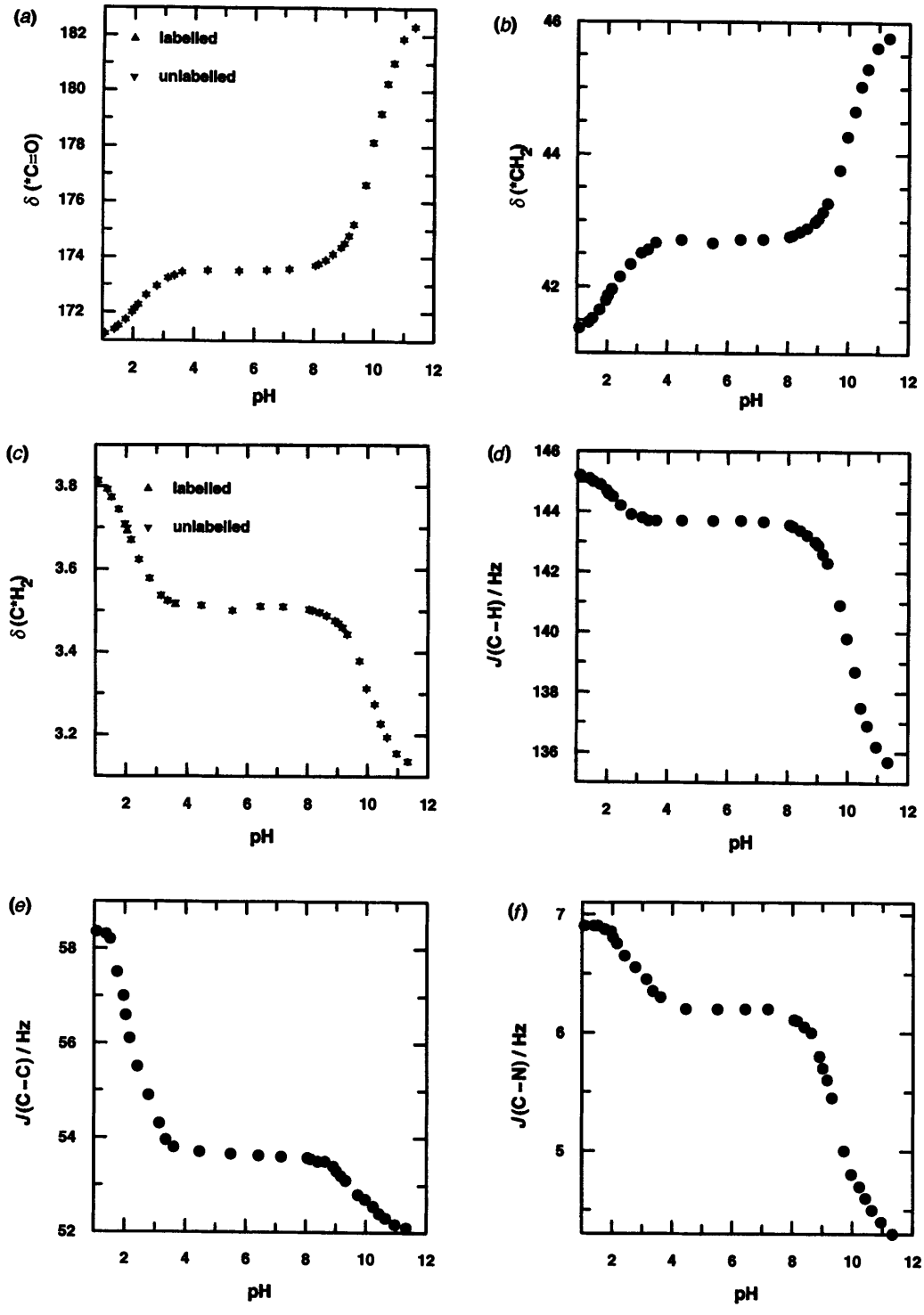

Fig. 1 Variation of different NMR parameters in the titration of a mixture of glycine and [2-^{13}C,^{15}N]glycine

In the present work the above-mentioned idea involving measurement of frequency differences is extended to determination of pK_a differences in closely related non-labelled compounds. Homologous carboxylic acids varying in chain length and branching and also a pair of substituted dicarboxylic acids were used as illustrations. Both quite big and very small pK_a differences are recorded.[32] Additional new results related to isotope effects on pK_a are presented and discussed elsewhere.[32]

Method

The ratio between the concentrations of deprotonated and protonated acid HA can be expressed *via* the NMR chemical shifts. If δ is the chemical shift for a partly protonated acid and δ_p and δ_d the corresponding chemical shifts under fully protonated and deprotonated conditions, eqn. (1) is valid.

$$[A^-]/[HA] = (\delta - \delta_p)/(\delta_d - \delta) \quad (1)$$

Furthermore, if only the ratio R between the dissociation constants for this acid and another one, HA^a, is needed, from the law of mass action one obtains eqn. (2).

$$R = K/K^a = (\delta - \delta_p)(\delta^a_d - \delta^a)/(\delta_d - \delta)(\delta^a - \delta^a_p) \quad (2)$$

Practically, the most convenient procedure is to measure the chemical shift δ for the reference, HA, and δ^a for the compound(s) under investigation, HA^a, and plot the difference

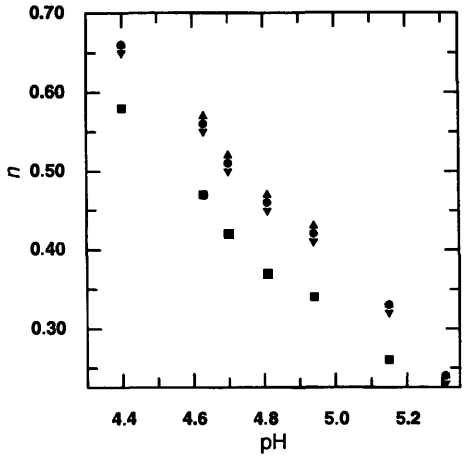

Fig. 2 Degrees of protonation in the mixture of carboxylic acids around their pK_a values as a function of pH: ■ acetic; ● propionic; ▼ butyric; ▲ valeric acid

$\delta - \delta^a$ against the degree of protonation, n, for HA, which can also be expressed in chemical shift parameters, eqn. (3).

$$n = (\delta_d - \delta)/(\delta_d - \delta_p) \quad (3)$$

Rearrangement of eqn. (3) gives eqn. (4). Insertion of eqn. (4) in

$$\delta = n\delta_p + (1 - n)\delta_d \quad (4)$$

eqn. (2) and rearrangement results in eqn. (5). Consequently,

$$\delta^a = [(1 - n)\delta^a{}_d + Rn\delta^a{}_p)]/(Rn + 1 - n) \quad (5)$$

eqn. (6) holds. It should be stressed that eqn. (6) is derived only

$$\delta - \delta^a = \delta_d - \delta^a{}_d - n(\delta_d - \delta_p) + Rn(\delta^a{}_d - \delta^a{}_p)/[1 + (R - 1)n] \quad (6)$$

from the law of mass action and two expressions relating NMR chemical shifts to the distribution of molecular populations during the exchange process without any additional restrictions.

In Fig. 3, eqn. (6) is illustrated for different values of $R > 1$, corresponding to pK_a(reference) < pK_a(measured compound). For values $R < 1$, corresponding to pK_a(reference) > pK_a(measured compound), one obtains concave curves instead of convex ones. The presented curves illustrate the sensitivity of the method to very small changes of R close to 1.0 [Fig. 3(*b*)], where the maxima of curves are around $n = 0.5$, and also for the bigger changes of R (from 1.5 to 10), where they are shifted towards lower fractions of protonated reference compound [Fig. 3(*a*)]. Equality of chemical shifts in fully protonated and deprotonated states, as assumed in Fig. 3, is generally rather exceptional even in the case of isotopic substitution.[32] Proper handling of experimental data allows improvement of the presentation of curves for the general case when the chemical shift differences in fully protonated and deprotonated forms are not equal, and thus to obtain, even without a curve-fitting procedure, information about the relative pK_a values of the measured compounds. These methods are most conveniently illustrated by practical examples, for which different mixtures of carboxylic acids as model compounds were studied, as described below.

Examples and discussion

Unbranched carboxylic acids

The feasibility to determine relative pK_a values of simple carboxylic acids was studied with a mixture of acetic, propionic, butyric and valeric acid (each *ca.* 0.2 M in H_2O). Acetic acid was used as a reference compound. Bell-shaped curves resulting from the plotting of ^{13}C carboxylic chemical shift differences against the degree of protonation of acetic acid are presented in four different ways in Fig. 4. Direct plotting [Fig. 4(*a*)] immediately shows that acetic acid has the lowest pK_a value in this series, but the fit between the experimental data and calculated curves is poorly presented, and no conclusion can be drawn about the relative acidities of the compounds. A better picture of the data is obtained by shifting all curves to a common value (0 Hz) either at $n = 0$ [Fig. 4(*b*)], or at $n = 1$ [Fig. 4(*c*)]. From these curves it is clear that the pK_a of butyric acid is closest to that of acetic acid. Skewed bell-shaped curves result from the differences in protonation shifts, as shown in Table 1. For the carboxy carbons they are quite close, within 0.32 ppm, but α-carbon shifts differ much more, 0.93 ppm. The experimental curves can be viewed as the sum of a typical bell-shaped curve (Fig. 3) and a linear dependence related to different protonation shifts. Even for small differences in protonation shifts, as between the propionic and valeric acid carboxy carbons (0.08 ppm, at 125.77 MHz 10.1 Hz), the resulting graph corresponding to an R value of about 1.02 is strongly influenced by both factors. The linear effect can be effectively eliminated by the additional introduction of the degree of protonation n^a [eqn. (3)] of the investigated compound by eqn. (7), closely related to

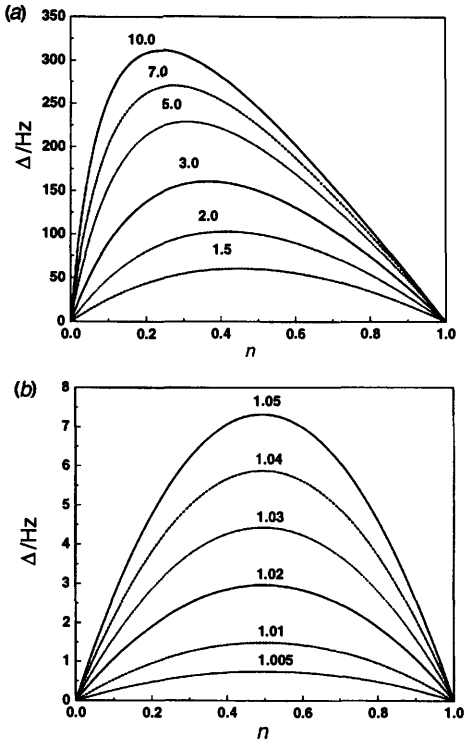

Fig. 3 Theoretical bell-shaped curves for different R values assuming a 600 Hz (4.8 ppm with 11.7 T magnet) protonation shift and the same chemical shifts in the deprotonated ($n = 0$) and protonated ($n = 1$) states

$$\delta^{a'} = n^a\delta_p + (1 - n^a)\delta_d \quad (7)$$

eqn. (4). Plotting $\delta - \delta^{a'}$ values against n gives undistorted bell-shaped curves [Fig. 4(*d*)], which characterize in the best way the pK_a changes in all compounds. All four ways of data presentation (Fig. 4) give the same R values by a curve-fitting program and they are only different visual representations of experimental NMR titration curves.

Measured data for linear carboxylic acids and the results of curve fitting are given in Table 1, where the relative acidities based on the chemical shifts of both the carboxy and α-carbons are presented. Close values were obtained from both sets of data. For comparison, literature values of the pK_a

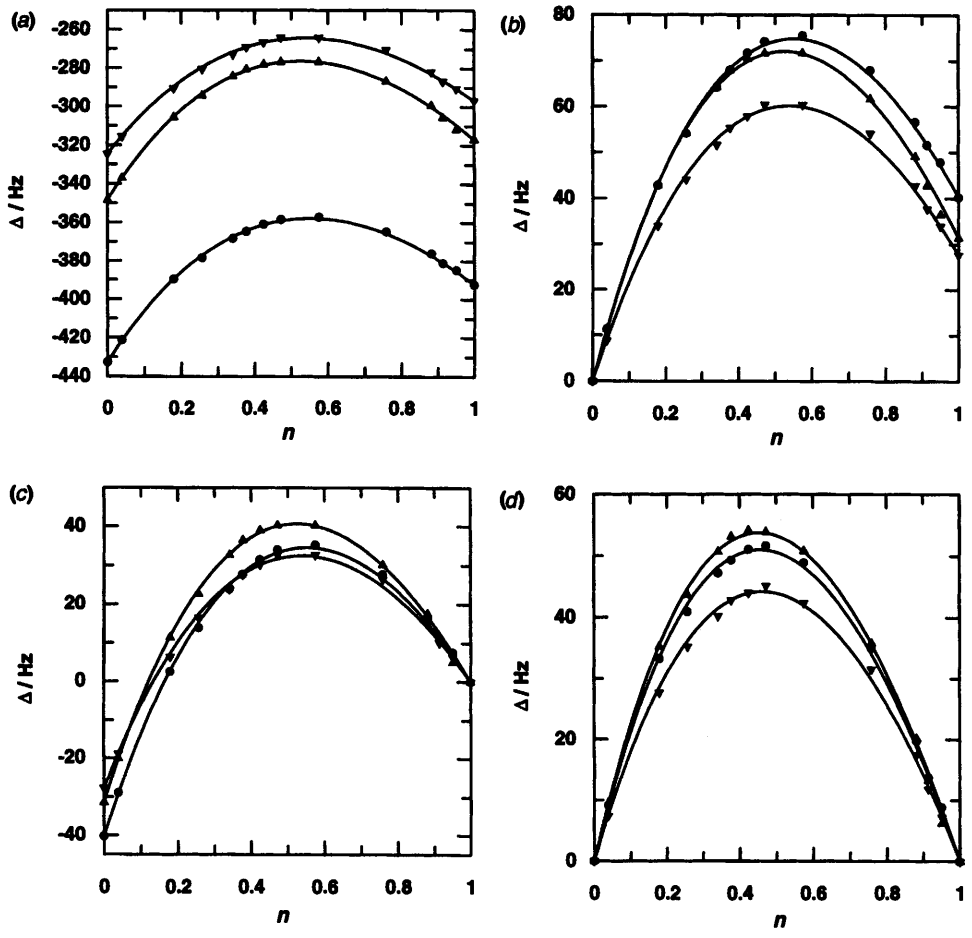

Fig. 4 Alternative presentations of relative K_a values for propionic (●), butyric (▼) and valeric (▲) acid (reference acetic acid): (*a*) by direct application of eqn. (6); (*b*) by equalizing chemical shifts at $n = 0$; (*c*) by equalizing chemical shifts at $n = 1$; (*d*) by equalizing chemical shifts at both extremes with the help of eqn. (7)

Table 1 Effect of alkyl chain length on the relative acidity in unbranched carboxylic acids RCOOH, measured from their composite mixture in water solution

	$\delta_d - \delta_p$, ppm		$K(CH_3COOH)/K(RCOOH)$		ΔpK_a values		
R	COO	CH_2 (CH_3)	from COO	from CH_2	COO	CH_2	Ref. [33]
CH_3	4.64	2.93	1	1	0	0	
CH_3CH_2	4.96	3.45	1.419 ± 0.003	1.407 ± 0.004	0.152	0.148	0.12
$CH_3CH_2CH_2$	4.86	3.86	1.355 ± 0.005	1.359 ± 0.004	0.132	0.133	0.06
$CH_3CH_2CH_2CH_2$	4.88	3.78	1.455 ± 0.005	1.453 ± 0.005	0.163	0.162	0.10

differences are also given. Both sets confirm that higher homologues have higher pK_a values than acetic acid, butyric being closest to acetic acid. However, the fit to generally accepted ΔpK_a values is rather poor, and even the order between propionic and valeric acid is reversed as compared to that obtained with classical methods on the individual acids. In order to study the effect of solution composition on the relative acidities, additional experiments with propionic and valeric acid mixtures were performed. Two binary mixtures (0.42 M propionic/0.14 M valeric and 0.05 M propionic/0.03 M valeric) were measured. In the last case, less than 1 mg of valeric acid is present in the active volume of the NMR coil. The results of a mutual comparison of these acids in all three experiments for carboxy and α-carbons are presented in Fig. 5, where the α-carbon chemical shift differences are equalized at $n = 1$ to the carboxy carbon differences. The differences in starting point of the three pairs of curves illustrate the dependence of the carboxy carbon shift differences on the composition of the mixtures. Only in the four-component mixture with a total concentration of carboxylic acids of 0.82 M were convex curves obtained. In dilute solution, the K_a ratio of propionic and valeric acid corresponds to valeric acid being stronger by 0.04 pK_a units, as commonly cited.[3] The same dilute binary mixture was also measured in 1 M KCl solution to check for a possible ionic strength effect. Only a marginal influence of salt solution on the relative dissociation constants was observed: $R = 0.927 \pm 0.002$, $\Delta pK_a = 0.033$ from carboxy carbons and $R = 0.930 \pm 0.001$, $\Delta pK_a = 0.032$ from α-carbons. Therefore the major factor in the determination of relative dissociation constants of carboxylic acids is not ionic strength, but the concentration of acids. Plotting of $K_{(propionic)}/K_{(valeric)}$ against total concentration of carboxlic acids gives a linear dependence with $r = 0.998$, intercept 0.919 ± 0.005 and slope 0.124 R units per 1 M total concentration of carboxylic acids, showing that in aqueous solution association processes play an important role and can be studied by the present method. The importance of media effects shows that utmost care has to be taken in using small differences in pK_a values for the discussion of practical problems of acid–base equilibria in solutions.

Table 2 Effects of branching on the relative acidity in simple carboxylic acids RCOOH, as measured from the mixture of five acids in water solution

	$\delta_d - \delta_p$, ppm		$K(CH_3COOH)/K(RCOOH)$		ΔpK_a		
R	COO	α	from COO	from α	COO	α	Ref. 3
H	5.51	—	0.11 ± 0.02	—	−0.96	—	−1.01
CH_3	4.94	3.21	1	1	0	0	0
CH_3CH_2	5.33	3.51	1.474 ± 0.007	1.49 ± 0.01	0.17	0.17	0.11
$(CH_3)_2CH$	5.50	3,09	1.70 ± 0.02	1.77 ± 0.02	0.23	0.25	0.08
$(CH_3)_3C$	5.30	1.53	2.88 ± 0.06	3.05 ± 0.09	0.45	0.48	0.27

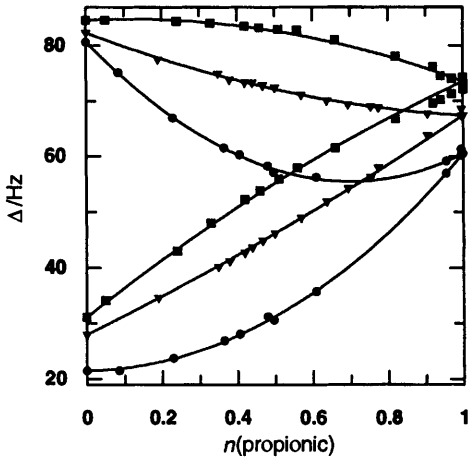

Fig. 5 Comparison of relative acidities of propionic and valeric acid for two- and four-component mixtures: ■ in 0.83 M four-component mixture $R(\alpha) = 1.023 \pm 0.003$, $R(COO) = 1.025 \pm 0.001$; ▼ in 0.56 M two-component mixture $R(\alpha) = 0.987 \pm 0.001$, $R(COO) = 0.982 \pm 0.001$; ●-in 0.08 M 2 component mixture $R(\alpha) = 0.920 \pm 0.001$, $R(COO) = 0.918 \pm 0.001$. In the three pairs of curves the upper ones belong to carboxy carbons.

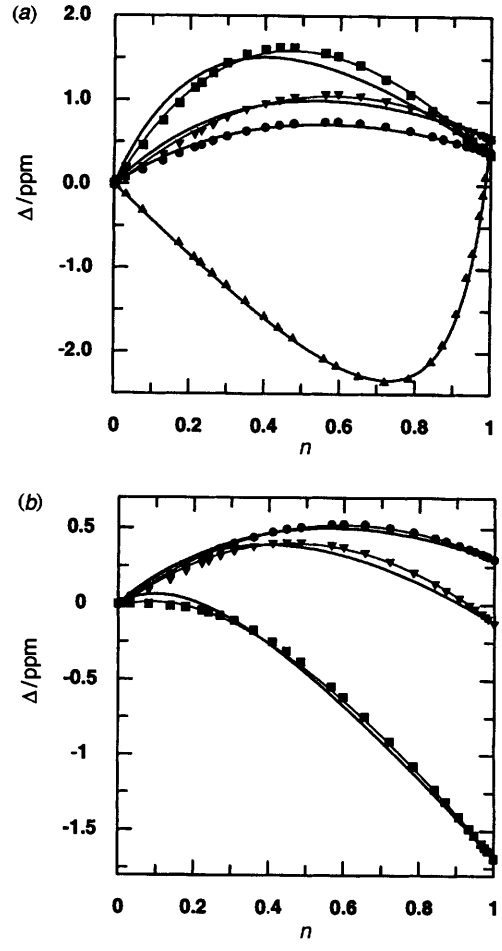

Fig. 6 Effects of alkyl branching on the relative acidities of carboxylic acids, referenced to acetic acid, measured from carboxy (*a*) and α (*b*) carbons: ▲ formic, ● propionic, ▼ isobutyric, ■ pivalic acid. Thick curves are calculated with the single variable (R), thin curves are obtained by multiparameter fit.

Branched carboxylic acids

The mixture of five carboxylic acids RCOOH, R = H (formic), CH_3 (acetic), CH_3CH_2 (propionic), $(CH_3)_2CH$ (isobutyric) and $(CH_3)_3C$ (pivalic) was studied (Table 2, Fig. 6). Formic acid was included in the mixture in order to check the method with respect to a relatively large difference in K_a values.

As seen from Table 2 and Fig. 6(*a*), differences in pK_a values at least up to 1 pK_a unit are quite well determined by the present method. R values calculated from the carboxy and α-carbon chemical shifts are practically the same. Only in the case of pivalic acid is the R value from the quaternary carbon somewhat different from that of the carboxy group. However, the protonation shift of the quaternary carbon atom of pivalic acid is about half of that in other acids (Table 2) and should be regarded as less accurate.

A comparison of the ΔpK_a values obtained for branched acids with literature data in relation to acetic acid again shows differences and even the order of dissociation constants of propionic and isobutyric acids is reversed. The present study indicates that addition of methyl groups in β-positions results in a progressive decrease of dissociation constants. As seen from Fig. 6(*a*) and 6(*b*), with increasing degree of branching systematic deviations appear between the calculated curve and experimental data. The reported R values were obtained by a single parameter (R) fit. The use of multiparameter fitting by varying chemical shift differences with reference at $n = 0$ and $n = 1$ does not improve the situation. Inclusion into the variables of the protonation shift of a reference (acetic acid) results in very good fits, as seen by additional thin-line curves in Fig. 6. But these good fits are obtained by unrealistic protonation shifts deviating up to 10 ppm from the experimental values and cannot therefore be accepted. Increase of branching induces some additional nonlinear effects, which may be caused, *e.g.* by nonlinear changes in chemical shifts of sterically crowded compounds during protonation. Considering the relatively small standard errors in the calculated R values with a single variable, the values obtained for the relative dissociation constants can still be used for the comparative analysis of these quite different types of acids. Additional experiments in more dilute solution will also give more accurate results for the branched acids.

Substituted dicarboxylic acids

In contrast to the situation with unsubstituted α,ω-dicarboxylic acids the comparative analysis of unsymmetrical dicarboxylic acids or unsymmetrically substituted α,ω-dicarboxylic acids is straightforward. A crude mixture of 2- and 3-methyladipic acids was chosen as an example, because in this case the interaction of the carboxylic groups, separated by 5 C–C bonds, is strongly attenuated and therefore the methyl group effects on pK_a values can be investigated simply. Our studies of carboxylic acids show that the carboxy group influence extends over quite long distances, more than 10 C–C

Table 3 Methyl group effects on pK_a values of carboxy groups in 2- and 3-methyladipic acids, as calculated relative C-6 of 2-methyladipic acid

COOH group	δ_p[a]	$\delta_d - \delta_p$, ppm	K(C-6 of 2-methyladipic)/K	ΔpK_a
C-1 of 2-methyladipic acid	181.79	5.17	0.905 ± 0.001	−0.043
C-1 of 3-methyladipic acid	178.10	4.78	0.885 ± 0.001	−0.053
C-6 of 3-methyladipic acid	178.70	4.90	0.965 ± 0.001	−0.015

[a]C-6 of 2-methyladipic acid at 178.52 ppm.

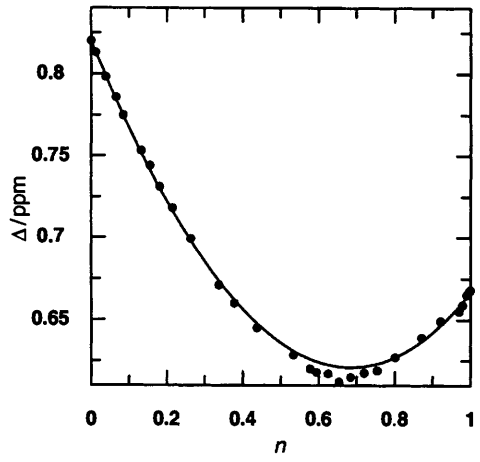

Fig. 7 Comparison of dissociation constants of C-1 and C-6 in 3-methyladipic acid: $K_{(C\text{-}6)}/K_{(C\text{-}1)} = 0.917 \pm 0.001$

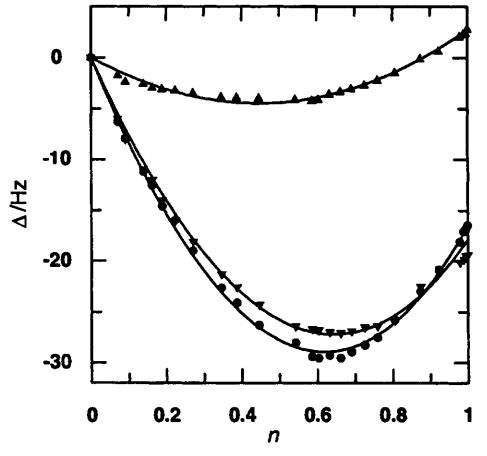

Fig. 8 Methyl group effects on pK_a values of carboxy groups in 2- and 3-methyladipic acids, internal reference C-6 of 2-methyladipic acid: ▲ C-6 of 3-methyladipic acid, ▼ C-1 of 2-methyladipic acid, ● C-1 of 3-methyladipic acid

bonds. However, these long range effects are rather small, in the ppm range, and special care has to be taken to prove their real existence. The five-bond effect in fatty acids is − 0.25 ppm. In methyladipic acids, protonation shifts are in the range of 4.78–5.17 ppm (Table 3). Comparison of these values with that of valeric acid (4.88 ppm) shows that for the calculation of degrees of protonation as a first approximation the mutual influence of carboxy groups can be ignored. Correct assignment of carboxy groups is needed for the starting point. This is a simple case due to the well known β-(to low field) and γ-(to high field) effects of the group. [34] With the use of methyl group substituent effects in 2-, 3- and 4-methylvaleric acid[35] the carboxy carbon chemical shifts in the methyladipic acids are predicted within 0.1 ppm. Further confirmation of correct assignment is given in the Experimental section. As the reference for the relative pK_a study, C-6 of 2-methyladipic acid was used, being the least influenced by the methyl group. Our results from NMR titration of methyladipic acids are presented in Figs. 7 and 8.

As anticipated, the pK_a of C-6 of 3-methyladipic acid has a value closest to that of 2-methyladipic acid, but somewhat unexpected is the methyl group effect to increase the dissociation constants of carboxylic groups both at close and remote positions. In addition, the 3-methyl effect on the C-1 dissociation constant is larger than that of the 2-methyl group. In the case of branched carboxylic acids the insertion of methyl groups resulted in weaker acids, which is explained nicely by their $+I$ effect. On the other hand there is no monotonic decrease of dissociation constants in homologous fatty acids and steric effects can modify inductive effects. The observed trends in dicarboxylic acids present a problem which needs further study. Unfortunately, we were not able to find literature data on dissociation constants of the reported methyladipic acids.

Experimental

All spectra were measured with a 5 mm C/H dual probehead at 20 °C on a Bruker AMX-500 spectrometer, equipped with a B-VT1000 temperature controller. The MLEV-16 pulse sequence was applied for effective low power proton decoupling. Chemical shifts were measured from internal dioxane: for the lock [$^2H_8$]dioxane was added. No dependence of the dioxane chemical shift or the difference between dioxane and [$^2H_8$]dioxane on pH (0 to 12) was observed. For data fitting a PC Microcal Origin 2.5 (Microcal. Inc.) and Grafit 2.07 (Erithacus Software Ltd.) packages running under Windows were used. Both programs gave identical results.

Titration of samples was performed in the NMR tube with the help of a MI-412 microelectrode (Microelectrodes, Inc.) by adding small amounts of 30% HCl (Suprapur, Merck) or concentrated aq. NaOH (Merck) by immersing a thin glass rod into the titrant and transferring small amounts of it from the rod surface to the NMR tube. In several cases the need to back-titrate from lower (higher) to higher (lower) pH values occurred. No additional deviations from correlation curves were observed due to these manipulations.

The mixture of 2- and 3-methyladipic acids was prepared from commercial methylcyclohexanol by HNO_3 oxidation. Their structures were confirmed by full assignment of ^1H and ^{13}C chemical shifts in CD_3OD solution with the help of 2D FT ^1H–^1H and ^1H–^{13}C COSY correlation diagrams. 2-Methyladipic acid: 181.77 (C-1); 39.05, 2.41 (CH-2); 32.31, 1.38/1.51 (CH_2-3); 21.95, 1.50 (CH_2-4); 33.57, 2.28 (CH_2-5); 178.49 (C-6); 16.26, 1.03 (CH_3). 3-Methyladipic acid: 178.05 (C-1); 41.02. 2.22/2.28 (CH_2-2); 29.42, 1.82 (CH-3); 30.86, 1.41/1.55 (CH_2-4); 31.43, 2.31 (CH_2-5); 178.72 (C-6); 18.40, 0.84 (CH_3). Unambiguous assignment of carboxy carbons follows from the single resonance spectra because different numbers of $^2J_{CH}$ and $^3J_{CH}$ coupling constants occur for the carboxy carbon atoms. In 3-methyladipic acid C-1 gives two two-bond couplings (6.8 Hz) and one 3-bond coupling (3.2 Hz); C-6 of this isomer has four long range coupling constants (2 × 7.2 and 2 × 3.6 Hz). In 2-methyladipic acid C-1 was assigned by selective decoupling from the methyl protons; the width of the broad unresolved multiplet decreased from 30 Hz (one two-bond coupling and five three-bond couplings) to 15 Hz by reducing the number of three-bond couplings to two.

All other compounds were commercial ones and were used without additional purification.

Conclusions

It is known that frequency is the parameter which can be measured with highest precision. Such measurements are the basis in the present determination of pK_a differences by ^{13}C NMR spectroscopy. Some applications have been demonstrated in relative pK_a studies within a series of linear and branched carboxylic acids and dicarboxylic acids. The ΔpK_a values obtained vary with the concentration. When accurate thermodynamic values are required, extrapolation to zero concentrations is therefore required. The following advantages of the method for measurements of pK_a differences by ^{13}C NMR spectroscopy should be stressed:

(*i*) No need for pH measurement, which is a limiting factor in nearly all pK_a studies.

(*ii*) Allows comparative studies of acid–base equilibria under truly identical conditions (solvents of various composition, temperature, ionic strength, *etc.*).

(*iii*) In contrast to most other methods for pK_a studies the requirement for purity of samples is quite low.

(*iv*) Affords very high selectivity: free energy differences corresponding to less than 4 $Jmol^{-1}$ can be measured.

(*v*) Multiple check possibilities from single measurement by monitoring of NMR signals at different molecular sites.

(*vi*) Mixture of series of compounds or homologous series can be studied in a single run.

(*vii*) Comparative analysis of different acidic or basic centres within the same molecule.

(*viii*) Measurements in non-aqueous solutions appear feasible, with potential applications therefore also in studies of super-acids and -bases.

(*ix*) Short and long range isotope effect studies. High enrichment is not needed. Overlap problems can be circumvented by the use of auxiliary isotopic substitution. Some new aspects of isotope effect studies are discussed elsewhere.[32]

The main limitations of the method are inherent to NMR and especially to ^{13}C NMR spectroscopy, *i.e.* relatively low sensitivity and the need to monitor the process of protonation by collecting a series of spectra. The unique information obtained justifies the time needed for the experiments. By the use of modern inverse detection methods the time needed can be considerably reduced.

Acknowledgements

This work was partially supported by the Royal Swedish Academy of Sciences and the Estonian Science Foundation.

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Paper 6/03087D
Received 5th *June* 1996
Accepted 17th *July* 1996