174. ^{17}O-NMR. of Enriched Acetic Acid, Glycine, Glutamic Acid and Aspartic Acid in Aqueous Solution. I. Chemical Shift Studies[1])

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Summary

The ^{17}O-NMR. chemical shifts of the enriched amino acids glycine, aspartic acid and glutamic acid were measured in aqueous solution as a function of pH. High magnetic fields are necessary to resolve the α,β- and α,γ-carboxyl resonances of aspartic acid and glutamic acid, respectively. The chemical shifts of acetic acid were measured for comparative reasons. Ionization constants and titration shifts were obtained by nonlinear least-squares fits to one-proton titration curves. The average excitation energy approximation is discussed in terms of the observed changes in ^{17}O-shielding on deprotonation. No intramolecular association between the α-amino group and the α-carboxyl group in the zwitterionic form is required to explain the high-frequency shift of the carboxylate ion. Also no indication of an intramolecular association between the α-amino group and the side-chain carboxyl groups of aspartic acid or glutamic acid was found.

1. Introduction. – One class of biological molecules of great importance are the amino acids and NMR. studies of various nuclei (^{1}H, ^{13}C, ^{15}N) have been reported [1–3]. However, despite the obvious importance of O-atoms in amino acid and peptide chemistry, ^{17}O-NMR. spectroscopy still remains a relatively unexploited technique with only four publications so far available [4–7]. The difficulties are due to the low natural abundance of ^{17}O (0.037%), the large quadrupolar coupling constants which lead to broad resonances and the strong rolling baseline distortions [8–11].

We have recently reported [7] the ^{17}O-NMR. spectra of five amino acids in aqueous solution at pH 6 under controlled conditions of concentration, ionic strength and temperature. Also important to the application of ^{17}O-NMR. spectroscopy to peptides is the knowledge of the behaviour of the ^{17}O-chemical shifts and line widths of the amino acids as a function of pH. We report here the results of an ^{17}O-NMR. study of the chemical-shift titration of monocarboxylic (glycine) and dicarboxylic (aspartic acid, glutamic acid) amino acids in aqueous solution.

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An attempt will be made to rationalize the contribution of each ionization state to the observed ^{17}O-chemical shifts. The results and information obtained from pH-dependent line-width studies are described in the following paper [12].

2. Experimental. – *Materials.* All amino acids *(Fluka AG)* were used without further purification. Enrichment with ^{17}O was obtained by isotopic exchange between the carboxyl function and $H_2{}^{17}O$ in the presence of a strong acid and at elevated temperatures [13]. In a typical experiment 3.0 ml of 5M HCl was added to a mixture of 3.0 g of glycine and 300 mg of H_2O enriched to 20 atom-% in ^{17}O (*Biogenzia Lemania*, Lausanne). The temperature was raised to 80° and after 5 h the solution was neutralized with 10M NaOH. Cooling gave a quantitative yield of glycine, approximately 1%-enriched in ^{17}O.

Sample preparation. The pH-titrations were performed at a sample concentration of 0.1M in 1M aq. NaCl. This minimizes the change in ionic strength and viscosity which could occur during the titration [7]. The pH was adjusted by the addition of either HCl- or NaOH-solution. The pH-values were measured directly in the 10-mm-NMR. tubes at 40°, before and after each experiment. Acetic acid was measured in H_2O and ^2H_2O, the pH-meter readings were used without correction for isotope effects [14]. To avoid back-exchange of ^{17}O [15] all samples were stored at neutral pH at 4°. Some natural abundance spectra of glycine have been measured in ^{17}O-depleted water (*Biogenzia Lemania*, ^{17}O-content *ca.* $1 \cdot 10^{-3}$%).

^{17}O-NMR. measurements. ^{17}O-NMR. spectra were obtained at 27.11 MHz using a *Bruker CXP-200* instrument with a high-resolution probe head. No field/frequency-locking system was used. The probe temperature was kept constant at $40 \pm 1°$. The chemical shifts were determined relative to the resonance position of 1,4-dioxane, measured in a separate experiment [16]. At 40° the chemical shift of dioxane relative to water is +0.2 ppm. The spectral parameters normally used were the following: spectral width = 20 kHz; 90° pulse angle = 50 μs (high-resolution probe); quadrature detection; acquisition time $T_{acq} = 5$–10 ms ($\simeq 4\ T_2$). A preacquisition delay $\Delta t = 130$–140 μs was used. Since the amino acid absorptions are 7 to 7.5 kHz away from the water resonance, Δt corresponds to the next maximum ($\Delta\nu \cdot \Delta t = 1$) of the *FID*-interferogram and hence no linear-phase correction is needed [17], which otherwise could introduce a sinusoidal baseline. Each FID was zero-filled up to 8 K before FT.

For single resonances a simple exponential function with line-broadening (LB.) = 50–75 Hz was applied to the FID in order to diminish the truncation of the water resonance. For strongly overlapping resonances such as those of glutamic and aspartic acids a *Gaussian*-exponential function was used to resolve the peaks with a minimum accumulation of noise [18]. This apodization-resolution enhancement function has the form $\exp(at - bt^2)$ where a and ab are adjustable parameters and are related to the *Aspect-2000* parameters (LB.) [in Hz, (LB.) < 0] and (GB.) [0 < (GB.) < 1] as follows: $a = -\pi$(LB.) and $b = a/(2(GB.) \cdot T_{acq})$.

pK_a-Calculations. Apparent pK_a-values for pH-dependent resonances were obtained nonlinear least-squares fits of the observed chemical shifts, δ(pH), to a one-proton titration curve

$$\delta(\mathrm{pH}) = \frac{\delta(\mathrm{AH}) + \delta(\mathrm{A}^-) \cdot 10^{(\mathrm{pH} - \mathrm{p}K_a)}}{1 + 10^{(\mathrm{pH} - \mathrm{p}K_a)}}$$

where δ(HA) and $\delta(A^-)$ are the chemical shifts for the protonated and the deprotonated species, respectively. *Equation 1* assumes that there is rapid exchange between the ionized species resulting in one average signal.

3. The case for ^{17}O-enrichment. – *Valentine et al.* [5] recently made some indication of the difficulties encountered in studying amino acids at natural abundance. The stringent requirements are the high concentrations and the extensive signal-averaging needed and the intense water peak which often contributes to memory overflow. Recording of the spectra can be facilitated by the use of ^{17}O-depleted water [16] [19–21] since O-exchange in amino acids is slow and important only at low pH-values [13] [15]. Our ^{17}O-NMR. investigation of amino acids in ^{17}O-depleted water and at elevated temperatures resulted in rela-

tively sharp resonances and appeared not too difficult. However, since we are interested in a future application of ^{17}O-NMR. to polypeptides we wanted to examine this technique at physiological temperatures. Unfortunately, despite fast pulsing and the application of a sensitivity enhancement function the spectra turned out to be very poor: *Figure 1A* shows the ^{17}O-natural-abundance-NMR. spectrum of glutamic acid, 0.1 M in ^{17}O-depleted water at 40°. It is obvious that the large number of transitions needed makes the recording very time-consuming and practically prohibitive for titration curve measurements. *Figure 1B* illustrates clearly the advantages of working with an ^{17}O-enriched sample of glutamic acid. Previously successful natural abundance studies of highly soluble phosphates [19] and monosaccharides [20] [21] have been performed. However on considering the low solubility of several amino acids and their relatively easy enrichment we decided to work with ^{17}O-enriched amino acids.

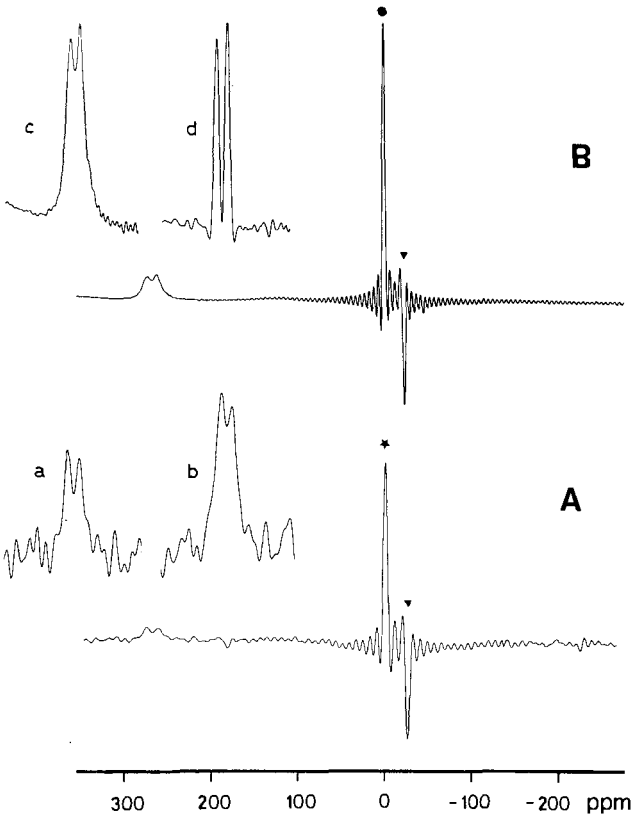

Fig. 1. *27.11 MHz-^{17}O-NMR. spectra of 0.1 M solutions of glutamic acid in H_2O containing 1 M NaCl* (Temperature = 40°; pH = 3.1). (A) Natural abundance spectrum in ^{17}O-depleted water (★). T_{acq} = 5 ms. NS = 3,000,000, total experimental time *ca.* 4.2 h. Glutamic acid resonances after *a)* vertical expansion (8×); *b)* exponential multiplication of the FID. (LB. = 100 Hz). (B) Spectrum of 1%-enriched glutamic acid in ordinary water (●). T_{acq} = 7.5 ms, NS = 150,000, total experimental time *ca.* 19 min. Glutamic acid resonances after *c)* vertical expansion (8×); *d)* multiplication of the FID. with a *Gaussian*-exponential function [18] (LB. = – 500 Hz, GB. = 0.45). ▼ is a transmitter residual spike.

4. Results. – In order to characterize the behaviour of the ^{17}O-chemical shift of an isolated carboxyl group we first turned our attention to the case of acetic acid. *Figure 2* shows the pH-dependence of the chemical shift of a 0.1 M solution of acetic acid in 1 M aqueous NaCl. Upon deprotonation of the carboxyl function we observe a shift to higher frequency with an inflection point at its pK_a. The least-squares titration shift of 23.8 ppm *(Table 2)* is in agreement with the value of 23.6 ppm reported by *Reuben* [22]. The pH-titration was also performed with deuteriated acetic acid, CH_3COO^2H, in a 1 M solution of NaCl in ^2H_2O in order to study the isotope effect *(Fig. 2)*. At high pH we observe a shift of 0.6 ppm to lower frequency for the deuteriated solution, and 1.1 ppm for the curve at low pH. This tendency is similar to that found for the ^{17}O-chemical shift of ^2H_2O relative to H_2O [23].

The titration curve of the amino acids can be divided into two distinct regions. Firstly, as observed for the case of acetic acid deprotonation of the carboxyl group in the cationic form results in a similar increase in the ^{17}O-chemical shift, showing an inflection point at the pK_a (*Figs. 3* and *4*). For aspartic acid overlapping of the two absorptions was observed just until both groups predominated as their anions (pH > 4), at higher pH two separate absorptions could be observed.

Secondly, the high pH-section of the titration curves consists of another inflection point due to the titration of the amino group resulting in a decrease in the chemical shift of ≈ 3 ppm for the α-carboxyl groups and ≈ 1 ppm for the β- and γ-carboxyl groups of aspartic and glutamic acid, respectively.

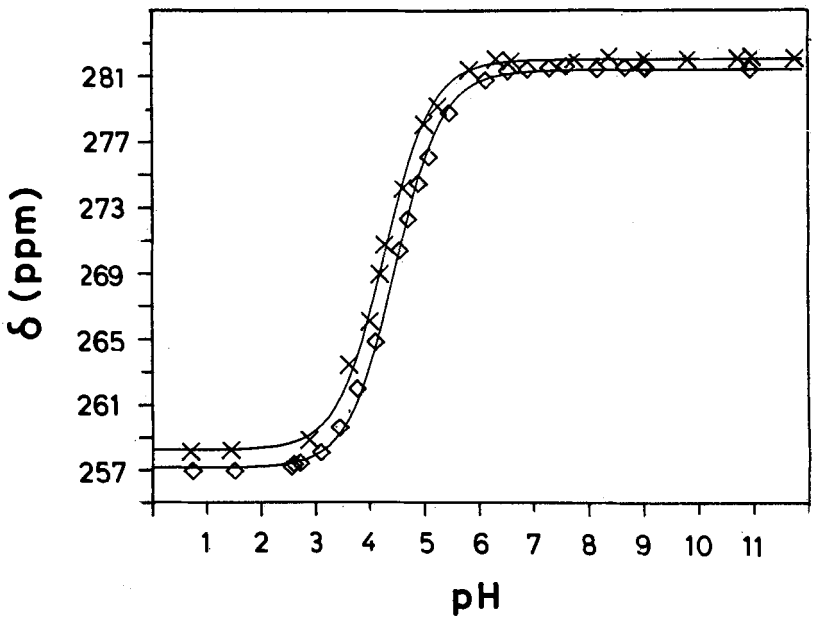

Fig. 2. *The ^{17}O-NMR. titration shifts of 0.1 M acetic acid at 40° measured in H_2O* (X) *and in ^2H_2O* (◇) *in the presence of 1 M NaCl* (The pH-meter readings in ^2H_2O were not corrected for isotope effects. The solid lines correspond to nonlinear least-squares fit of one-proton titration curves *(Eqn. 1)* to the experimental data. The titration parameters are listed in *Table 2*)

The titration curve of glycine is similar to that reported earlier [6]. However, we found that at pH-values > 11 the chemical shifts of all amino acids studied tend to a constant value (*Figs. 3* and *4*).

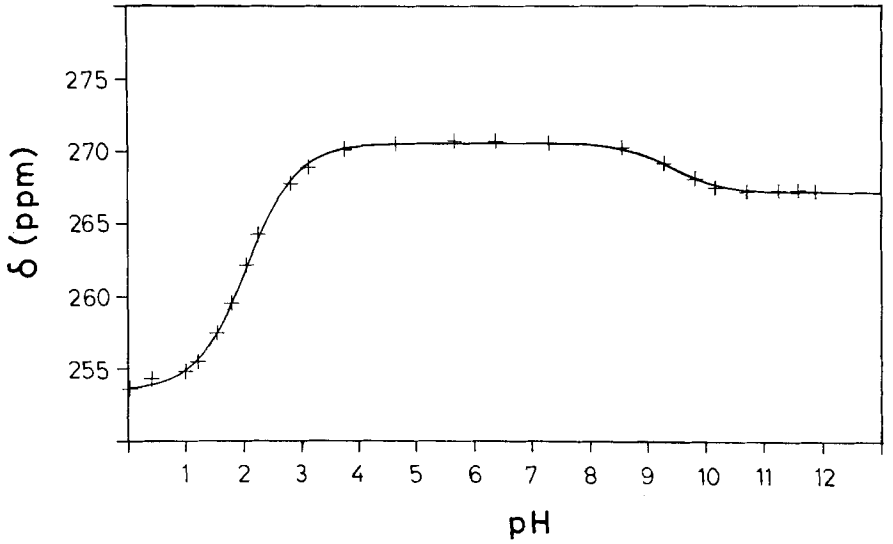

Fig. 3. *The ^{17}O-NMR. titration shifts of 0.1 M glycine at 40° measured in H_2O in the presence of 1 M NaCl* (The solid line corresponds to nonlinear least-squares fits of one-proton titration curves *(Eqn. 1)* to the experimental data. The titration parameters are listed in *Table 2*)

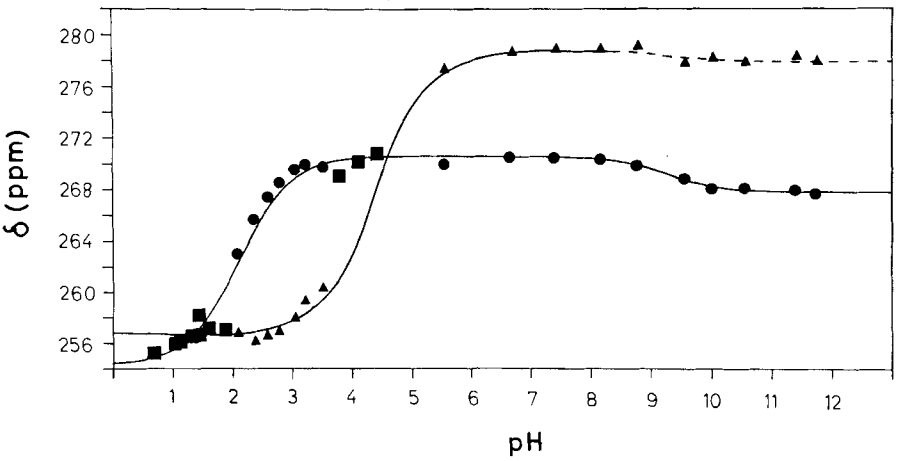

Fig. 4. *The ^{17}O-NMR. titration shifts of 0.1 M glutamic acid at 40° measured in H_2O in the presence of 1 M NaCl* (The solid lines correspond to nonlinear least-squares fits of one-proton titration curves *(Eqn. 1)* to the experimental data: (●) resonances from the α-carboxyl group, (▲) resonances from the γ-carboxyl group, (■) overlapping resonances. The titration curve for the γ-carboxyl group was calculated without the (■) points. The titration parameters are listed in *Table 2*. The dashed line was drawn to follow the experimental points)

The ^{17}O-chemical shifts corresponding to the three ionization states of the amino acids are presented in *Table 1*. The ^{17}O-titration shifts as well as pK_a-values obtained from least-squares fits to one-proton titration curves *(Eqn. 1)* are given in *Table 2*.

5. Discussion. – Amino acids can exist in three possible forms depending on their degree of protonation, the concentration of each species varies greatly with the pH;

$$^{+}NH_3CHRCOOH \rightleftarrows {}^{+}NH_3CHRCOO^{-} \rightleftarrows NH_2CHRCOO^{-}$$

At neutral pH the doubly charged zwitterion is dominant although there is a very small contribution from the uncharged form, $NH_2CHRCOOH$ [24]. The change in the relative population of the above species with pH will have a pronounced effect on the ^{17}O-chemical shifts and line widths of the amino acids. We shall now estimate the contribution of each ionization to the observed chemical shifts in the overall titration. The titration curves are compared with those obtained earlier by ^{13}C-NMR. [25] and ^{15}N-NMR. [26] [27] spectroscopy.

α-Carboxyl titration[3]). Only one O-line is observed in carboxylic acids since the (C=O)- and (C–OH)-resonance positions are averaged by rapid intermolecular proton transfer. From the ^{17}O-chemical shifts in methyl esters [28] (δ(C=O) $\simeq$ 350 ppm and δ(C–O–CH_3) $\simeq$ 130 ppm), and taking into account a methyl-shielding effect of $\Delta \simeq 30$ ppm, the chemical shift of the carboxyl group can be estimated as

$$\delta = \tfrac{1}{2}\{\delta(C{=}O) + \delta(C{-}O{-}CH_3) + \Delta\} \approx 255 \text{ ppm}$$

which is in very good agreement with the values observed for acetic acid and the amino acids investigated *(Table 1)*.

Table 1. *^{17}O-NMR. Chemical shifts of acetic acid and some amino acids*[a])

Compound	Resonance	Chemical shift (ppm)[b])		
		δ_1[c])	δ_2[d])	δ_3[e])
Acetic acid	α-COOH	258.2	282.0	
		257.1[f])	281.4[f])	
		257.9[g])	282.2[g])	
Glycine	α-COOH	253.5	270.5	267.4
Aspartic acid	α-COOH	(255)[h])	267.8	265.4
	β-COOH	(255)[h])	280.7	279.6
Glutamic acid	α-COOH	254.3	270.6	267.6
	γ-COOH	257.1	279.4	278.8

[a]) Measured in 0.1M solutions in H_2O which contained 1M NaCl; T = 40°. The chemical shifts were obtained from nonlinear least-squares fits of one-proton titration curves *(Eqn. 1)* to the experimental data. [b]) Chemical shifts were measured relative to 1,4-dioxane used as external reference, +0.2 ppm relative to water. The errors for the chemical shifts were ±0.3 ppm for acetic acid and glycine and ±0.5 ppm for glutamic acid and aspartic acid. [c]) δ_1 is the chemical shift of the protonated carboxyl O-atoms at acid pH. [d]) δ_2 is the chemical shift of the deprotonated carboxyl O-atoms at neutral pH. [e]) δ_3 is the chemical shift of the deprotonated carboxyl O-atoms after deprotonation of the α-amino group at basic pH. [f]) Chemical shifts measured in ^2H_2O. [g]) Chemical shifts measured in H_2O without added NaCl. [h]) Estimated values because of overlapping α- and β-carboxyl resonances.

[3]) α-Carboxyl= $-\overset{1}{C}OOH$, β-carboxyl= $-\overset{4}{C}OOH$.

It has been recently pointed out [29] that heteroatomic substitution at the C-atom β to an OH-group provokes shielding which is usually larger than that of CH_3 (−6 ppm): −11 ppm for OH; −12 ppm for $N(CH_3)_2$ and −8 ppm for Cl. This agrees well with the shielding found for the α-carboxyl group of the amino acids relative to acetic acid and that of the α-carboxyl group of glutamic acid relative to the γ-carboxyl group *(Table 1)*.

Additivity properties have similarly been used [22] to predict the chemical-shift tendency of the acetic acid anion. Since the chemical shift of the acetate ion relative to acetic acid is +24 ppm *(Table 2)*, the value per negatively charged O-atom is about +48 ppm and is of the same sign and order of magnitude as the chemical shift which has been roughly estimated [30] for the OH-ion (+70 ppm with respect to H_2O).

The chemical shift or the screening constant σ is usually discussed with respect to contributions from a diamagnetic (σ^d) and a paramagnetic (σ^p) term as defined by *Ramsey* [31]. It is usually assumed that the diamagnetic term is independent of change in the chemical environment of the O-atom [32] [33]. Thus, the shift differences are essentially induced by the paramagnetic term. The latter may be evaluated using several assumptions. Following the AEE. (average excitation energy)-approximation the paramagnetic term for a nucleus A bonded to other nuclei B may be written [34] [35]

$$\sigma_A^p = -(e^2h^2/2m^2c^2) \cdot \Delta E^{-1} \cdot \langle r^{-3} \rangle_{2p} \left\{ Q_{AA} + \sum_{B \neq A} Q_{AB} \right\} \qquad (2)$$

where ΔE is an AEE. usually taken as the magnitude of the lowest energy electronic transition, $\langle r^{-3} \rangle_{2p}$ is the so-called orbital expansion term, and Q_{AA} and Q_{AB} are defined in terms of the appropriate matrix elements used to calculate atomic charge densities and interatomic bond orders, respectively.

Table 2. *^{17}O-NMR. Titration shifts and pK_a-values of acetic acid and some amino acids*[a])

Compound	Resonance	α-Carboxyl titration		α-Amino titration		Side-chain titration	
		Δ[b])	pK_a	Δ	pK_a	Δ	pK_a
Acetic acid	α-COOH	23.8	4.26 ± 0.02				
		24.3[c])	4.47 ± 0.02[c])				
		24.3[d])	4.66 ± 0.02[d])				
Glycine	α-COOH	17.0	2.05 ± 0.02	−3.1	9.44 ± 0.06		
Aspartic acid	α-COOH	(12.8)[e])	[e])	−2.4	9.53 ± 0.06		
	β-COOH			−1.1[f])	[f])	(25.7)[e])	[e])
Glutamic acid	α-COOH	16.3	2.1 ± 0.1	−2.8	9.30 ± 0.06		
	γ-COOH			−0.6[f])	[f])	22.3	4.5 ± 0.1

[a]) Measured in 0.1 M solutions in H_2O which contained 1 M NaCl; T = 40°. The parameters were obtained from nonlinear least-squares fits of one-proton titration curves *(Eqn. 1)* to the experimental data. [b]) Δ-values are the chemical shift changes on deprotonation (in ppm). Positive values indicate deshielding. [c]) Parameters obtained in ^2H_2O. [d]) Parameters obtained in H_2O without added NaCl. [e]) No curve-fitting procedure was performed because of overlapping of the two carboxyl absorptions at pH values < 4. The titration shifts given in parentheses are estimated values. [f]) The computer program could not converge to an adequate fit. Approximate Δ-values were obtained by inspection.

Valentine et al. [6] recently applied this approach to amino acids. They pointed out that the sign of the ^{17}O-titration shift of the α-carboxyl groups is not consistent with *Equation 2* when only the reduced form $\sigma^p \sim \Delta E^{-1} \cdot \langle r^{-3} \rangle_{2p}$ is considered. Their argument was based on the facts that *a)* deprotonation of the carboxyl group should result in electron delocalization which expands the effective radius of the O-atom 2p-electrons and thus decreases $\langle r^{-3} \rangle$ and σ^p; *b)* the resonance forms of the ionized carboxyl group lower the ground electronic energy level relative to the covalently bonded carboxyl group. This will increase ΔE which for carboxyl as for carbonyl compounds is provided by the $(n \rightarrow \pi^*)$-transition [36], and σ^p will again decrease. In conclusion, the change in chemical shift of the carboxyl group resulting from deprotonation was expected towards low frequency, opposite to that observed experimentally. *Valentine et al.* [6] considered solvent effects as a possible reason for the sign reversal of the carboxyl titration shift. Assuming a strong intramolecular ionic association of the amino acids in the zwitterionic state, transition from the cation to the zwitterion would result in the breaking of intermolecular H-bonds between the solvent and the carboxyl groups accompanied by appreciable chemical shifts to high frequency [22]. Indication of an intramolecular H-bond between the amino group and the carboxyl group in the zwitterionic form was also obtained by ^{15}N-NMR. [27] since the resonance of the amino group was shifted to high frequency when the carboxyl group was deprotonated. We want to emphasize, however, that the AEE. model *(Eqn. 2)*; *a)* involves the additional terms Q_{AA} and Q_{AB} whose variation may play a significant role [11] [19]; *b)* appears valid only for carbonyl groups with low $(n \rightarrow \pi^*)$-transition energy [35] [37]. Indeed, the $(n \rightarrow \pi^*)$-electronic absorption shows a considerable hypsochromic shift on going from the carbonyl group to carboxyl and carboxylate groups (absorption maximum at 280 nm for acetone, compared to 204 nm for acetic acid [38], <197 nm for the acetate ion [38], and <213 nm for the various ionization states of amino acids [36]). Consequently, the other excitation energies $\sigma \rightarrow \pi^*$, $n \rightarrow \sigma^*$, $\pi \rightarrow \sigma^*$ and to a lesser extent the $\sigma \rightarrow \sigma^*$ are expected to play a significant role for the compounds considered here and the linear relationship between the O-chemical shifts and the inverse of the lowest excitation energy $(n \rightarrow \pi^*)$ appears unrealistic [37]. On the other hand, there is no *a priori* method for the selection of the additionally contributing transitions [35].

Deprotonation of the carboxyl group of acetic acid *(Fig. 2)* resulted in ^{17}O-deshielding similar to that in the amino acids. In addition, we have recently performed ^{17}O-NMR. studies on a large series of substituted carboxylic acids and their carboxylate ions [39]. Throughout, a net chemical shift to high frequency was observed on deprotonation. We conclude therefore that in order to understand the ^{17}O-titration shift of amino acids, no specific intramolecular association of the zwitterionic form is necessary. Furthermore, we observed [39] that electronegatively substituted acetic acids, *e.g.* $CH_2ClCOOH$, gave a smaller chemical shift difference between the acid and its ion which is in agreement with the tendency in amino acids ($\delta(CH_2ClCOOH) = 253.1$ ppm; $\delta(CH_2ClCOO^-) = 271.5$ ppm). In conclusion, the quantitative difference in the titration shift between acetic acid and glycine can be attributed to a simple inductive effect of the $\overset{+}{N}H_3$-group and not to a specific intramolecular association of the zwitterionic form.

β,γ-Carboxyl titration. The titration shifts of the β- and γ-carboxyl groups in aspartic and glutamic acid are very close to that of acetic acid *(Table 2)*. This indicates that neither of the side-chain carboxyls do undergo an efficient interaction with the positive charge of the α-amino group.

α-Amino titration. Figures 3 and *4* show that the ^{17}O-resonance signals are sensitive to the ionization state of the α-amino group. The titration shifts appear closely related to the number of bonds from the amino function. A shift to lower frequency has been observed on deprotonation, −2.4 to −3.1 ppm for the α-carboxyls, −1.1 ppm for the β-carboxyl of aspartic acid and −0.6 ppm for the γ-carboxyl of glutamic acid *(Table 2)*. The sign of the ^{17}O-titration shifts is opposite to that reported for those of ^{13}C [25] where titration of the amino group produces a surprisingly large effect on the α-carboxyl C-atom. ^{13}C-chemical shifts of amino acids and their variation with pH have been calculated by a modified CNDO/2 program [25]. However, only a poor agreement between predicted and observed shifts was obtained for C-atoms close to the site of ionization. This was attributed to the very delicate balance between the electron density and excitation energy terms. In conclusion, we believe that for a detailed explanation of either the ^{13}C- or ^{17}O-titration shifts the usual approximations in the calculation of the paramagnetic term have to be carefully examined. The calculation for both the C- and O-atoms of the carboxyl group and its comparison with experimental values is recommended.

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