preciably longer than for the ^{13}C satellite. This leads to better resolution of the main line(s) than of the ^{13}C satellite lines (see Figure 3). Theory suggests[13] and our data support the view that differential saturation should be greatest in large radicals with a highly localized electron.[16] The phenomenon should also be more pronounced in more viscous solvents,[13] and this too was observed.

Although our analysis illustrates the general nature of differential saturation, it is not capable of providing a quantitative account of the measurements. These were, of necessity, made under conditions of partial saturation and to improve signal intensity the lines were generally overmodulated, the modulation frequency being 100 kHz. Moreover, in some cases there was partially resolved hyperfine structure, which means that the lines were not homogeneously broadened. There are no available treatments of line shape which include simultaneously the effect of saturation, modulation amplitude, modulation frequency, and inhomogeneous broadening.[17] The effects of modulation amplitude (2.5 and 5.0 G) and frequency (10 and 100 kHz) on the saturation behavior of the main lines in the spectrum of **3** are illustrated in Figure 4.

Finally, we note that magnetic nuclei in quite remote positions can also produce differential saturation effects of sufficient magnitude to make radical identification difficult, e.g., $^{13}C_{ortho}$ and $^{13}C_{meta}$ in **1** (Figures 2 and 3) and the $^{29}Si\beta$ in **3** (see Table I).

References and Notes

(1) Issued as N. R. C. C. No. 16706.
(2) N. R. C. C. Research Associate, 1974–1976.
(3) N. R. C. C. Research Associate, 1975–1977.
(4) That is, when dynamic line broadening effects[5] are absent.
(5) See, e.g., J. E. Wertz and J. R. Bolton, "Electron Spin Resonance", McGraw-Hill, New York, N.Y., 1972, Chapter 9.
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(7) G. Brunton, D. Griller, L. R. C. Barclay, and K. U. Ingold, *J. Am. Chem. Soc.*, **98**, 6803 (1976).
(8) G. Brunton, J. F. Taylor, and K. U. Ingold, *J. Am. Chem. Soc.*, **98**, 4879 (1976).
(9) G. Brunton, K. U. Ingold, B. P. Roberts, A. L. J. Beckwith, and P. J. Krusic, *J. Am. Chem. Soc.*, **99**, 3177 (1977).
(10) The $^{13}C_{\alpha}/^{12}C_{\alpha}$ ratio of peak heights for undeuterated **1** also increased with increasing microwave power, but since this is a less persistent radical[6] quantitative measurements could not be made.
(11) A. M. Portis, *Phys. Rev.*, **91**, 1071 (1953).
(12) E. R. Andrew, "Nuclear Magnetic Resonance", Cambridge University Press, New York, New York, N.Y., 1955, Equations 2.54 and 3.15. See also ref 3, Equation D-3.
(13) The relaxation time is[14] $1/T_1 = (3/10\hbar^2)b^2M_I^2 2\tau_c(1 + \omega_0\tau_c)^{-1}$, the rotational correlation time is[14] $\tau_c = 4\pi\eta a^3/3kT$, and the microwave magnetic field is[15] $H_1^2 = 2 \times 10^{-3}$ *QP*. The anisotropic hyperfine interaction for ^{13}C ($M_I = 1/2$) is $b \approx 30$ G, the microwave frequency $\omega_0 = 2\pi \times 9 \times 10^9$ s^{-1}, viscosity $\eta = 0.3$ cP at a temperature $T = 300$ K, the cavity $Q = 2000$, and we take $T_1 = T_2$ and the radius $a = 5$ Å.
(14) G. E. Pake and T. S. Estle, "The Physical Principles of Electron Paramagnetic Resonance", 2nd ed, W. A. Benjamin, New York, N.Y., 1962.
(15) C. P. Poole, "Electron Spin Resonance", Interscience, New York, N.Y., 1967.
(16) That is, in radicals having large values for a and b.[13]
(17) The problem of the line shape of an inhomogeneously broadened line under saturation has been treated recently,[18] but only for slow passage and small modulation amplitudes.
(18) M. K. Bowman, H. Hase, and L. Kevan, *J. Magn. Reson.*, **22**, 23 (1976).
(19) Under similar conditions but with power = 10 mW the ratio, to the ^{12}C lines, of the normalized maximum peak heights are $^{13}C_{meta} \sim 6$, $^{13}C_{ortho} \sim 6.7$, $^{13}C_{\alpha} = 10.6$, and the fine structure is still observed, while with power = 170 mW the ratios are $^{13}C_{meta}$, not measurable; $^{13}C_{ortho} \sim 4$; $^{13}C_{\alpha} \sim 15$, and all lines are now broad singlets.

Studies of the pH Dependence of ^{13}C Shifts and Carbon–Carbon Coupling Constants of [U-^{13}C]Aspartic and -Glutamic Acids

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Abstract: ^{13}C NMR studies of the chemical shifts and carbon–carbon spin–spin coupling constants of 90% [U-^{13}C]aspartic and -glutamic acids are reported. Effects of titration of the two carboxyl groups are separated computationally and the results compared with those for asparagine and glutamine, aspartate and glutamate containing peptides, and a series of amino-*n*-butyric acids. The results indicate that the carboxyl carbon shift resulting from titration of the carboxyl group is strongly dependent on its distance (number of bonds) from an amino group. Alternatively, remote methyl groups exhibit a much smaller titration induced shift than carboxyl groups in the corresponding position. Significant remote effects of pH titration on the one-bond carbon–carbon coupling are also observed, particularly for couplings involving the side-chain carboxyl carbons. These results are discussed in terms of polarization of the C–O bonds in response to titration of a remote carboxyl group. Values of $^3J_{CC}$ in asparate and glutamate indicate a strong conformational dependence. Rotamer populations predicted on the basis of the observed couplings and theoretical INDO calculations are in good agreement with values based on an analysis of the $^3J_{HH}$ and $^3J_{CH}$ couplings. For a given conformation of glutamic acid, it is found that $^3J_{14}$ is considerably smaller than $^3J_{25}$. This result is consistent with observations on a number of other ^{13}C-labeled amino acids.

Introduction

^{13}C-Labeled amino acids have been used as nonperturbing probes of proteins[1-11] and peptides[14-22] into which they have been incorporated. Several of these studies have utilized uniformly labeled amino acids, the information content of which is dependent on the degree of isotropic labeling. At low levels ($\lesssim$20%) the labeling results in enhanced intensity, although small ^{13}C satellites due to $^1J_{CC}$ coupling can frequently be observed. At intermediate labeling levels the spectra become particularly complex owing to the different isotopic isomers which are present. Thus resonances corresponding to the singly, doubly, triply, etc., labeled amino acids can be observed.[23] At higher enrichments the spectrum is simplified since the fully labeled compound becomes the dominant isotopic isomer.[24-27] It then becomes possible to obtain values for the long-range carbon–carbon coupling constants which can be used to study molecular conformation. Owing to the complexity of the

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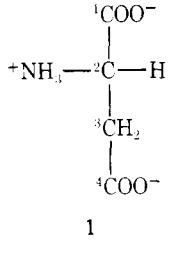
spectra obtained, the use of highly labeled amino acids must, in general, be restricted to synthetic peptides in which at most several of the residues are uniformly labeled.

Although several previous studies on uniformly labeled 85 atom % ^{13}C amino acids have been reported, there has been no systematic study of the pH dependence of the ^{13}C–^{13}C couplings in the dicarboxylic acids. Aspartic acid (**1**) and glutamatic acid (**2**) would be expected to show complex titration



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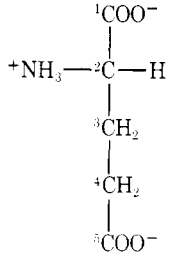

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behavior both because of the close proximity of the carboxyls and the similarity of their pKs. The present studies of uniformly labeled 90 atom % ^{13}C asparate and -glutamate ([U-^{13}C]asparate and -glutamate) have been undertaken in order to provide background information for studies with peptides containing uniformly labeled amino acids. Asparate is a particularly useful choice for determining the conformational dependence of $^3J_{CC}$ coupling since extensive conformational data based on $^3J_{HH}$ couplings[28-34] and $^3J_{CH}$ couplings[35-38] exist and because the conformation is strongly pH dependent.

In addition to the use of $^3J_{CC}$ as a conformational probe, the information available from chemical shift and $^1J_{CC}$ data has also been evaluated. In contrast to the studies done on the aliphatic amino acids,[24] determination of the effects of titration of the two carboxyl groups in asparate and glutamate cannot be deduced directly from the spectrum but must be accomplished computationally. We have used an approach similar to that of Cohen and co-workers.[39-41] In addition to long-range effects on chemical shifts which have been found for the aliphatic amino acids, long-range effects on $^1J_{CC}$ can be observed in asparate. In general, the studies show that useful information can be obtained from the carbon–carbon coupling in situations where the use of a uniformly enriched amino acid is feasible.

Materials and Methods

L-Asparagine, L-glutamine, DL-α-amino-n-butyric acid, DL-β-amino-n-butyric acid, and γ-amino-n-butyric acid were obtained from Sigma. The labeled amino acids used in these studies were obtained from the alga *Chlorella pyrenoidosa* (strain 71105). The algae were grown on ^{13}C-enriched (ca. 90%) carbon dioxide using described techniques.[42] The lyophilized cells were disrupted with trichloroacetic acid and the lipids extracted with chloroform–methanol, 3:1 (v/v). The dried cell debris was hydrolyzed with hydrochloric acid and the resulting amino acid mixture was desalted using a Dowex-50W cation exchange column. The crude mixture was placed on a Dowex-50W column (5 × 200 cm) and the amino acids were serially eluted with a series of pyridinium formate and pyridinium acetate buffers.[43,44] This chromatographic procedure provided pure fractions of various amino acids, among which were those used in these experiments. The pure fractions of glutamate and asparate were concentrated to dryness in vacuo and crystallized. Unresolved or partially resolved amino acids were separated using two additional chromatographic procedures; these procedures will be described in more detail elsewhere.[45] Purity was established by column chromatography, ^{13}C NMR, and infrared spectroscopy.

Pulse ^{13}C NMR spectra were obtained with a Varian XL-100-15 spectrometer (25.2 MHz) interfaced to a Nova 1210 computer. All spectra were obtained for ca. 0.1 M aqueous solutions at 25 °C and the spectrometer was locked to the resonance of D_2O contained in a capillary. The spectra for the carboxyl carbons were recorded with a spectral width of 500 Hz and 2K spectral points at an acquisition time of 4 s with no pulse delay and a 30° pulse. The upfield carbon resonances were recorded with a spectral width of 800 Hz for aspartate and 1000 Hz for glutamate with 2K spectral points. Acquisition times were 2.5 and 2 s, respectively, with no pulse delay and a 90° pulse. All chemical shifts are given relative to external neat tetramethylsilane using a D_2O capillary for a lock and are accurate to within ±0.1 ppm. No exponential multiplication of the free induction decay was performed. Peak positions were determined by computer examination of the spectrum using a program developed at Los Alamos which interpolates between data points and allows resolution of peaks to within ±0.1 Hz. The amino acid titrations were performed directly in the NMR tube using ~1 M KOH and HCl. Quirt et al.[41] found that the effect of changes in solute or salt concentrations were small, and we have assumed such effects to be less than the experimental error. The pK values determined either from the shift data or the coupling data were in close agreement with literature values.[46]

As the pH of the solutions approached the pK of the amino group, the α-carboxyl and the α-amino group broadened, owing to traces of metal ions either added during the titration or inherent in the sample. Above the amino pK, the resonances again sharpened, presumably owing to competition for the metal ions by hydroxyl ions. Paramagnetic broadening of amino acid resonances appears to be worst near the pK of the amino group and is particularly severe for ^{15}N NMR.[47] Treatment of the amino acid solutions with Chelex 100 ion exchange resin and addition of ca. *1-mg* quantities of EDTA produced very sharp lines throughout the pH range observed.

Residual broadening of the carbonyl resonances is probably due to unresolved $^2J_{CC}$ coupling. This effect made a determination of $^3J_{CC}$ difficult in many cases and is responsible for some of the scatter observed. In particular, C-4 of aspartate is coupled both to C-1 ($^3J_{14}$) and C-2 ($^2J_{24}$) to produce a doublet of doublets. Aspartate C-1 appeared as a doublet ($^3J_{14}$); however, the lines were broad reflecting unresolved $^2J_{13}$ coupling. This problem is aggravated by an apparent pH dependence of the aspartate $^2J_{24}$ and $^2J_{13}$ coupling which, if the resolution is insufficient, results only in broadening, leading to incorrect values for $^3J_{14}$. Glutamate C-1 appears as a broad resonance, but at higher resolution (0.25 Hz) it appears as a poorly resolved quartet. Coupling to C-4 ($^3J_{14}$) is evident from the small doublets seen for C-4; however, an additional smaller coupling to C-3 ($^2J_{13}$) would explain the shape of the C-1 resonance. Glutamate C-5 shows long-range coupling only to C-2 ($^3J_{25}$), although the upfield doublet was consistently larger than the downfield doublet, presumably owing to some type of higher order effect.

The finite perturbation theory INDO calculations of spin–spin coupling constants were performed on a CDC 7600 computer using a program of Seidman and Maciel.[48] Calculations based on the L-aspartic acid crystal structure of Derissen et al.[49] agreed closely with those using standard bond lengths of 1.53 Å for C–C, 1.09 Å for C–H, 1.25 Å for C–O, and 1.48 Å for C–N bonds and assuming tetrahedral bond angles in all cases. The sensitivity of the calculated $^3J_{CC}$ couplings to all conformational parameters other than the dihedral angle subtended by the coupled atoms was found to be quite small. Numerical results given in the paper correspond to the planes defined by the two carboxyl groups perpendicular to the all anti carbon skeleton. Calculated coupling constants, par-

Table I. Carboxyl Titration Induced Shifts of Aspartate, Glutamate, and Several Analogues

Carbon	Δ_A	Δ_B	I. Aspartate and Analogues Asn	Gly-Gly-Asp-Gly-Gly[54]	Asp-Phe-NH_2[56]
C-1	2.14	1.20	2.1	1.1	1.1
C-2	1.60	1.45	1.7	1.3	1.2
C-3	0.94	2.03	0.6	2.7	2.2
C-4	1.24	3.26	1.0	3.2	3.6

Carbon	Δ_A	Δ_B	II. Glutamate and Analogues Gln	Gly-Gly-Glu-Gly-Gly[54]	Ser-Glu-Gly[55]
C-1	2.41	0.61	2.4	0.6	0.4
C-2	1.99	0.71	1.8	1.1	0.7
C-3	0.60	1.50	0.7	1.2	1.6
C-4	0.45	3.56	0.2	3.3	3.6
C-5	0.57	4.33	0.4	4.4	4.9

Carbon	III. Butyric Acids α-Amino-*n*-butyric[a]	β-Amino-*n*-butyric[b]	γ-Amino-*n*-butyric[c]	*n*-Butyric[57]
C-1	2.6	3.8	4.4	4.7
C-2	1.7	2.8	3.5	3.9
C-3	0.4	0.9	1.5	1.4
C-4	−0.1	0.0	0.3	0.5

[a] Model for Δ_A of aspartate and glutamate. [b] Model for Δ_B of aspartate. [c] Model for Δ_B of glutamate.

ticularly $^1J_{CC}$ involving the carboxyl carbons, were found to be extremely sensitive to the state of protonation of the carboxyl groups, probably reflecting the neglect of the hydrogen bonding interactions with the solvent in the calculation.[50] As in similar studies,[51,52] we have calculated the conformational effects for the fully protonated species only. Changes in the electronic structure resulting from deprotonation will clearly dominate the one-bond carboxyl carbon couplings, but appear to be less significant for the $^3J_{CC}$ couplings discussed in the text.

Results and Discussion

A. Chemical Shift Data. The chemical shift titration curves for aspartate and glutamate are characteristic of a titration corresponding to a single p*K* at high pH but exhibit more complex behavior at low pH. This behavior reflects sensitivity of the observed shifts to the titration of both carboxyl groups. Contributions corresponding to the titration of C-1 (Δ_A) and the side chain carboxyls of aspartate or glutamate (Δ_B) can be separated computationally for each of the carbons (Table I) using a method essentially equivalent to that of Quirt et al.[41] No such separation has been reported for aspartate which shows the most pronounced dependence on the titration of both carboxyl groups. The glutamate values are in good agreement with those of ref 41. Owing to the closer proximity of the two carboxyl groups in aspartate there is a considerably greater dependence of all chemical shifts on *both* titrations than is the case for glutamate.

A question suggested by the computational separation of shifts noted above is whether the separate contributions Δ_A and Δ_B can be observed in systems with either of the carboxyl groups derivatized. A comparison with data obtained for glutamine (for which our results are identical with a previous study)[53] and asparagine on the one hand, and several previously studied peptides on the other,[54-56] indicates that Δ_A or Δ_B shifts are indeed observed (Table I). We can, therefore, conclude that the titration shifts produced by C-1 or the side chain carboxyls are relatively independent of whether the remote carboxyl and/or amino groups are derivatized to amides.

In contrast to the above observations, all carboxyl shifts were found to be significantly smaller than the shifts observed in aliphatic carboxylic acid analogues. In order to further understand these deviations carboxyl titration shifts for the series α-, β-, and γ-aminobutyric acids were measured and the results summarized in Table I. The strong dependence of the titration shift of the carboxyl carbon on the distance from the amino group is apparent and suggests that the presence of the amino group produces the primary perturbation of the carboxyl carbon shifts relative to an aliphatic carboxylic acid such as butyric acid (titration shift = 4.7 ppm).[57] This same dependence on distance is also observed for the titration shifts of the dicarboxylic amino acids. Thus, the Δ_A values for C-1 of aspartate and glutamate are fairly close to the C-1 shift in α-aminobutyric acid; the Δ_B value for C-4 of aspartate is similar to the C-1 shift in β-aminobutyric acid, and the Δ_B value for C-5 of glutamate is close to that for C-1 of γ-aminobutyric acid. The discrepancy between the Δ_A or Δ_B values and the titration shifts for the corresponding carboxyl carbons of the aminobutyric acid models reflects the difference between a remote carboxyl compared to a hydrogen or a methyl group.

Although the aminobutyric acids appear to serve as reasonable models for the shifts of the carboxyls being titrated, they are very poor models for the remote titration shifts observed in aspartate and glutamate. Thus, for example, titration of aspartate C-1 results in a 1.24-ppm shift of C-4 whereas titration of α-aminobutyric C-1 results in a negligible shift for C-4. The titration shift for the carboxyl carbon of β-aminobutyric acid is similar to that of aspartate C-4; however, the carbon three bonds from the carboxyl is hardly shifted in the former case while in the latter the shift is substantial (Table I). These discrepancies may reflect the greater polarizability of the remote carboxyl group in the dicarboxylic amino acids compared with a remote hydrogen or methyl group in the analogues as has been discussed by Batchelor et al.[60] If such an effect is assumed to dominate the observed shifts of the carboxyl carbons, it can be concluded that the carboxyl groups in the dicarboxylic amino acids are polarized in two steps corresponding to the two p*K*s.

B. Effects of pH on $^1J_{CC}$ Values. In general, the pH sensitivity of the one-bond coupling constants $^1J_{CC}$ is expected to depend on the ionization state of the carboxyl carbon involved but to be relatively insensitive to remote titration effects. Although this analysis turns out to be sufficient for most of the cases studied, several one-bond couplings are sensitive to remote titrations, notably those not involving carboxyl carbons. In order to obtain quantitative estimates of the long-range

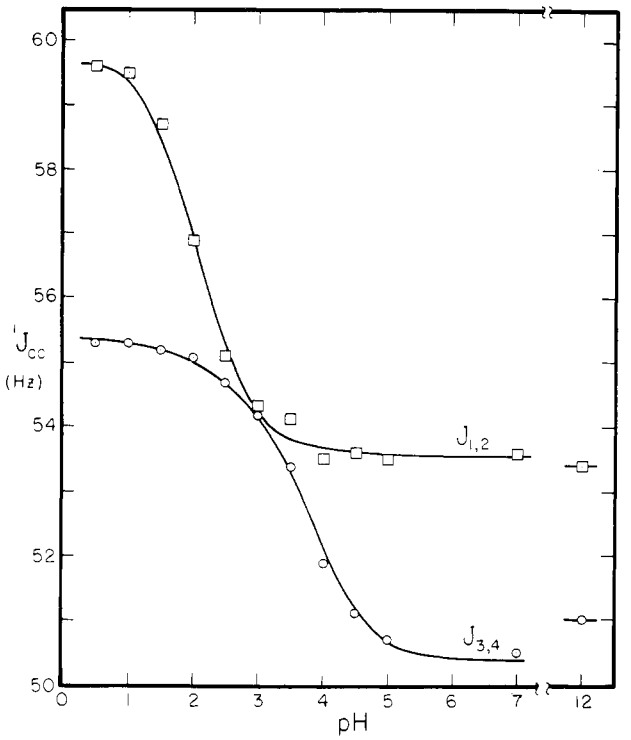


Figure 1. Dependence of the one-bond carbon-carbon coupling constant $^1J_{12}$ (□) and $^1J_{34}$ (⊙) in aspartate on pH. Curves were calculated using the parameters in Table II as described in the text.

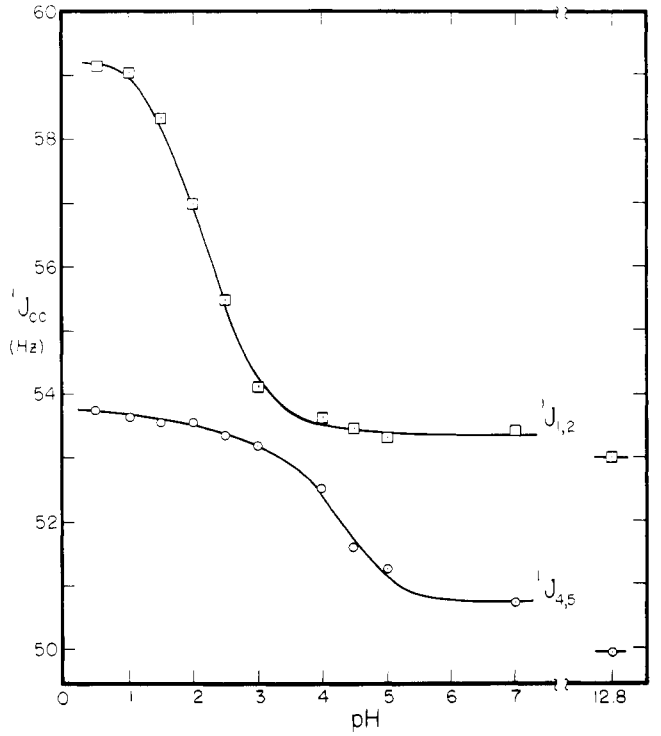


Figure 2. Dependence of the one-bond carbon-carbon coupling constant $^1J_{12}$ (□) and $^1J_{45}$ (⊙) in glutamate on pH. Curves were calculated using the parameters in Table II as described in the text.

effects, we have calculated ΔJ_A and ΔJ_B values (Table II) using a method equivalent to that used for the shifts. Some of the data obtained are presented in Figures 1-3.

As expected, the one-bond coupling constants involving the carboxyl groups show a substantial pH dependence while the $^1J_{23}$ exhibits changes of 1 Hz or less. A particularly surprising result (Figures 2 and 3 and Table II) is that although the $^1J_{12}$ couplings in both glutamate and aspartate are sensitive only

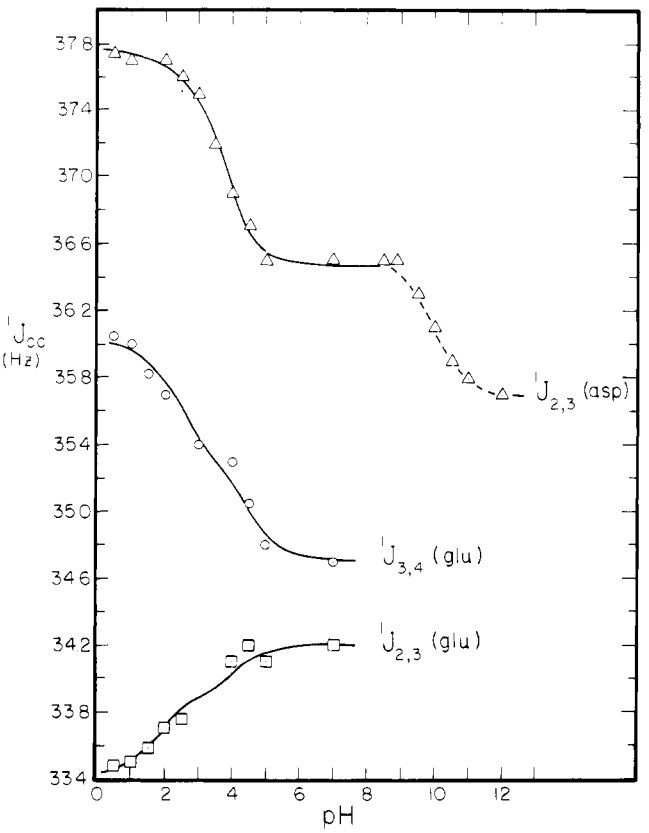


Figure 3. Dependence of the one-bond carbon-carbon coupling constant $^1J_{23}$ (△) (aspartate), $^1J_{23}$ (□) (glutamate), and $^1J_{34}$ (⊙) (glutamate) on pH. Curves were calculated using the parameters in Table II as described in the text.

Table II. Computed Changes in $^1J_{CC}$ Values Corresponding to Titration of αa-Carboxyl Group (ΔJ_A) and Side Chain Carboxyl Group (ΔJ_B)

Amino acid	Coupling constant	J, Hz (pH 7.0)	ΔJ_A, Hz[a]	ΔJ_B, Hz[a]
Aspartate	J_{12}	53.6	6.2	0.1
	J_{23}	36.5	0.2	1.1
	J_{34}	50.4	0.8	4.2
Glutamate	J_{12}	53.4	5.8	0.1
	J_{23}	34.2	−0.5	−0.2
	J_{34}	34.7	0.6	0.7
	J_{45}	50.7	0.4	2.6

[a] Positive signs for all but Glu J_{23} indicate an increased coupling at lower pH.

to titration of C-1 ($\Delta J_B \sim 0$), the $^1J_{34}$ coupling in aspartate and, to a lesser extent, the $^1J_{45}$ coupling in glutamate are sensitive to both titrations. As noted for the ^{13}C shifts, in both cases the effect of the C-1 titration on the carboxyl coupling is in the same direction as that produced by the direct titration of the side-chain carboxyl group. The glutamate $^1J_{23}$ value increases by ~0.7 Hz as the carboxyl groups are deprotonated. This behavior is similar to that of the aliphatic amino acids in which $^1J_{23}$ increases by ~1.1 Hz over a similar pH range.[24] On the other hand, the aspartate $^1J_{23}$ decreases by 1.2 Hz and a computer analysis indicates that this decrease corresponds almost entirely to ΔJ_B. Thus, the expected dependence of $^1J_{23}$ on the titration of C-1 is completely overwhelmed in aspartic acid by an effect involving the side-chain carboxyl. Since deprotonation of C-1 perturbs the $^1J_{34}$ coupling in aspartate while at the same time failing to produce the expected effect on the $^1J_{23}$ coupling, this indicates the existence of a perturbation of the electronic structure of the molecule which is significant for all of the carbons.

Table III. Calculated INDO-FPT Coupling Constants for the Carboxyl Carbons of Aspartic and Glutamic Acids as a Function of Dihedral Angle[a]

Dihedral angle, deg	$^1J_{C_1C_2}$, Hz	$^2J_{C_1C_3}$, Hz	$^3J_{C_1C_4}$, Hz	$^3J_{C_1H_B}$, Hz	$^3J_{C_1H_A}$, Hz
		Aspartic Acid			
0	79.36	−8.78	3.97	4.21	2.43
30	79.66	−8.49	2.43	7.70	0.43
60	78.45	−8.25	0.99	8.17	2.35
90	77.11	−8.47	0.82	5.29	5.49
120	77.44	−8.71	2.08	1.56	6.82
150	79.10	−8.35	3.85	0.44	4.72
180	80.16	−7.98	4.46	2.67	1.61
210	79.88	−8.31	3.24	5.54	0.88
240	78.49	−8.76	1.30	6.18	3.39
270	177.58	−8.49	0.61	3.93	7.16
300	77.20	−7.92	1.48	1.26	8.76
330	77.90	−8.12	2.99	1.14	6.65
		Glutamic Acid			
60	80.99	−7.35	0.09	8.02	2.08
180	76.72	−7.16	2.98	1.98	1.11
300	81.34	−7.42	1.05	1.28	7.97
	$^1J_{C_5C_4}$	$^2J_{C_5C_3}$	$^3J_{C_5C_2}$		
60	83.09	−9.64	1.69		
180	78.66	−8.50	5.15		
300	81.83	−8.25	0.98		

[a] For couplings involving C_1, the dihedral angle is that subtended by C_1 and C_4; for couplings involving glutamic acid C_5, the dihedral angle is that subtended by C_5 and C_2. [b] H_A and H_B are defined so that when C_1 is anti relative to C_4, H_A is anti relative to the nitrogen.

The $^1J_{CC}$ values are relatively insensitive to deprotonation of the amino group and are particularly difficult to obtain for glutamate owing to the existence of higher order effects involving C-3 and C-4 and the overlap of C-1 and C-2 resonances which occur at high pH. Even in this pH range, the aspartic acid couplings appear to differ significantly from those observed in the aliphatic amino acids with $^1J_{23}$ decreasing by 0.8 Hz in the former and increasing by ~0.3 Hz in the latter.

These one-bond couplings are not readily interpreted at present since the INDO calculations do not give very good agreement for the one-bond couplings (Table III), perhaps reflecting the neglect of hydrogen-bonding interactions of the carboxyl groups with the water.[50] In all cases observed, however, one-bond couplings directly involving carboxyl carbons decrease upon deprotonation of the carboxyl group and the long-range dependence of the one-bond couplings on titration of the remote carboxyl groups is in the same direction. This result suggests the possibility that titration of C-1 leads to a small polarization of the side-chain carboxyl groups as discussed for the shifts.

C. Effect of pH on $^3J_{CC}$ Values. Experimental studies on three-bond ^{13}C–^{13}C scalar coupling constants involving a series of aliphatic and alicyclic carboxylic acids indicate that such couplings exhibit a pronounced dihedral angle dependence.[58] Consistent with these observations, no resolvable coupling has been observed between the gauche C-1 and C-4 carbons of a series of ^{13}C-1 labeled carbohydrates.[59] However, the $^3J_{COCC}$ couplings between the anti C-1 and C-6 carbons are apparent in all cases and were determined to be 4.3 and 3.2 Hz for β- and α-D-glucopyranose, respectively. Finally, theoretical finite perturbation theory calculations in the INDO approximation of butane, 2-butanol, and butanoic acid indicate a strong dihedral angle dependence for the $^3J_{CC}$ couplings.[51,52] However, the observation of different values of $^3J_{COCC}$ for the α and β anomers of D-glycopyranose suggests that additional factors may be operative.[59]

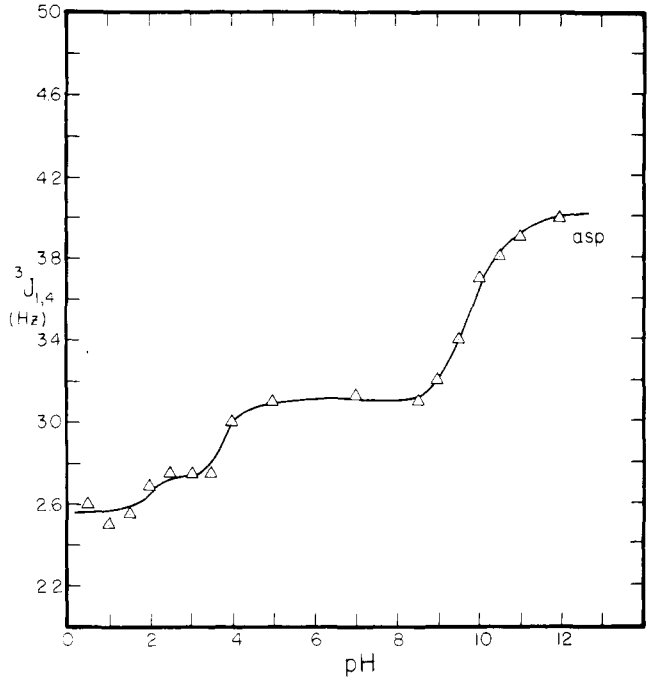
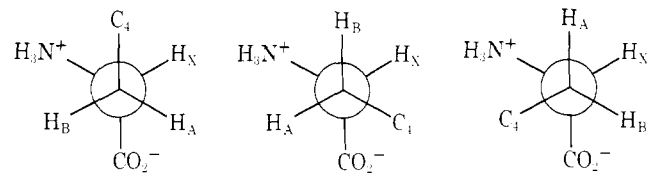


Figure 4. Dependence of the three-bond carbon-carbon coupling constant $^3J_{14}$ of aspartate on pH.

In considering the conformational dependence of $^3J_{CC}$ we restrict the discussion to a determination of the relative probabilities of the anti and the sum of the two gauche orientations. The validity of a Karplus-type relationship for the $^3J_{14}$ coupling observed in aspartate is consistent with the increase in coupling which occurs on increasing the pH (Figure 4), since the fraction of anti isomer is known to increase with increasing pH.[28-38] In order to make a more quantitative evaluation of the validity of a Karplus-type equation, it is first necessary to extract values for the anti and gauche coupling constants from the data. The most straightforward method is to utilize the aspartate rotamer populations deduced from $^3J_{HH}$ and $^3J_{CH}$ studies at various pH values. Unfortunately, there is a rather wide spread in the reported results with values for the anti population at high pH, ranging from 62 to 83%.[28-38] In several cases, attempts to fit the data in this way have resulted in large negative gauche coupling constants which are theoretically unlikely.



We have, therefore, attempted to analyze the data on the basis of theoretical coupling constants obtained from a finite perturbation theory calculation in the INDO approximation.[48] Using this approach, very poor results for all coupling constants are obtained if the carboxyl groups are deprotonated. Presumably, this reflects the failure to include hydrogen-bonding interactions between the solvent and the carboxyl oxygens.[50] For this reason, all calculations have utilized the different rotamer populations for the fully protonated species. The results obtained for aspartate and glutamate are summarized in Table III. $^3J_{C_1H_A}$ and $^3J_{C_1H_B}$ values are included since they are useful in studies of amino acid conformation.[35-38] The $^1J_{CC}$ and $^2J_{CC}$ values are similar to those which have been calculated

Table IV. Aspartate and Glutamate $^3J_{CC}$ Values and Computed Rotamer Populations

Amino acid	Ionization	Theoretical coupling[c] Gauche[b]	Anti	Exptl coupling	Calcd anti rotamer probability	Lit. value	Ref
		I. Analysis of $^3J_{14}$ Couplings					
Aspartate	2⁻	1.2	4.5	4.0	0.85	0.83	37
						0.71	38
	1⁻	1.2	4.5	3.1	0.58	0.61	38
						0.65	*a*
	1⁺	1.2	4.5	2.4	0.36	0.38	36
						0.39	*a*
Glutamate	1⁻	0.6	3.0	1.5	0.38		
Glutamate γ-Methyl ester	0	0.6	3.0			0.46	36
		II. Analysis of $^3J_{25}$ Coupling					
Glutamate	1⁻	1.34	5.15	3.0	0.44		
	2⁻	1.34	5.15	4.4	0.80		

[a] Data of ref 34 analyzed using equations of ref 37. [b] Average of the two gauche conformations. [c] As noted in the text, all theoretical couplings correspond to the fully protonated amino acids. The use of such couplings to obtain conformational information is based on the assumption that changes in $^3J_{CC}$ are dominated by conformational effects rather than electronic perturbations due to carboxyl group deprotonation.

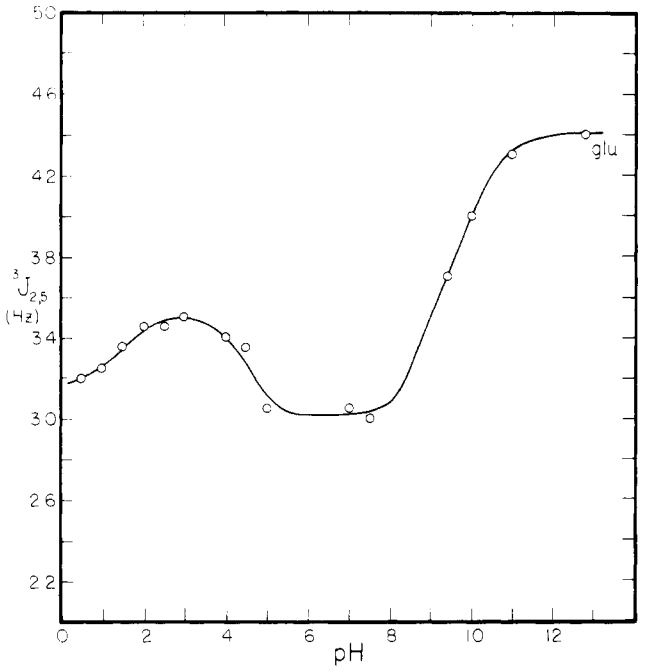


Figure 5. Dependence of the three-bond carbon–carbon coupling constant $^3J_{25}$ of glutamate on pH.

for butanoic acid[52] but are substantially larger than the measured values. However, the $^3J_{CC}$ values are quite reasonable and predict rotamer populations in substantial agreement with those available from the literature. Average gauche and anti coupling constants and deduced rotamer populations for aspartate and glutamate are given in Table IV.

At high pH, the anti probability deduced from the ^{13}C data is in close agreement with the result of Feeney.[37] At lower pH values the agreement with values reported by Espersen and Martin,[38] Kainosho and Ajisaka,[34] and Hansen et al.[36] is similarly good, as is the agreement with the value obtained by analyzing the ^1H–^1H couplings obtained by Kainosho and Ajisaka using the semiempirical formulas deduced by Feeney.[37] The agreement throughout the pH range suggests that the electronic perturbations resulting from deprotonation of the carboxyl groups probably exert minimal effects on the $^3J_{CC}$ values compared with changes in conformation.

In addition to providing a good quantitative fit for the aspartate data, the theoretical $^3J_{CC}$ values obtained for glutamate reproduce a trend which appears to hold for all of the amino acids for which data can be obtained: $^3J_{25}$, $^3J_{36}$, etc. > $^3J_{14}$.[25] Thus, the anti coupling calculated for glutamate, $^3J_{25}$ = 5.2 Hz, is significantly greater than the anti $^3J_{14}$ = 3.0 Hz. This suggests that differences observed in the amino acids have an electronic origin which does not reflect conformational differences between the dihedral angles defined by C-1 and C-4, and by C-2 and C-5. The $^3J_{14}$ coupling in glutamate was found to be sufficiently small so that in many cases it was not resolvable, perhaps owing to the existence of a small, unresolvable $^2J_{13}$ coupling. Thus, the $^3J_{14}$ couplings may be too small in general to be of practical use in obtaining conformational data. Clearly, aspartic acid represents an exception to this rule. On the other hand, the $^3J_{25}$ coupling in glutamate is easily observed and exhibits a dramatic pH dependence (Figure 5). Using the calculated gauche and anti couplings, the corresponding rotamer populations are included in Table IV. The fact that $^3J_{25}$ appears to drop above pH 3 may reflect a conformational change; however, the possibility that this is an electronic effect produced by deprotonation of C-5 cannot be ruled out. In particular, deprotonation of a carboxyl typically causes a decrease in the one-bond carbon–carbon coupling and may similarly produce a decrease in the three-bond coupling. The large effect corresponding to amino deprotonation clearly corresponds to an increase in the probability of the conformation with C-2 and C-5 anti, similar to the effect observed in aspartate.

The results obtained illustrate the primary difference which exists in the interpretation of $^3J_{CC}$ couplings and $^3J_{HH}$ couplings: the large substituent effects which clearly must be taken into account before conformational predictions can be made on the basis of $^3J_{CC}$ couplings. Thus, a set of $^3J_{CC}$ gauche and anti coupling constants generally applicable to the amino acids cannot be deduced. However, the predictions of the theoretical finite perturbation theory calculations appear to be particularly useful since the deduced rotamer populations are in good agreement with literature values. It thus appears that the approximations inherent in keeping only the Fermi contact term and in making the INDO approximation may be most serious for the $^2J_{CC}$ values and least serious for the $^3J_{CC}$ calculations. The conformational data obtained from the analysis of the $^3J_{CC}$ values suggest that this technique may be useful for analyzing the conformation of labeled amino acids incorporated into peptides. Indeed, attempts along this line have already been made.[17]

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