The values of $\Delta S^{\ddagger}$ are relatively small as compared to those reported for N-alkylated derivatives of formamide and acetamide.[2-5] The variation of $\Delta S^{\ddagger}$ obtained in different solvent systems suggests changes in solvation in proceeding from the ground state to the rotational transition states.[25]

From these studies, we conclude that the splittings observed in nmr for each set of protons are all due to cis–trans isomerism resulting from restricted rotation about the carbonyl–nitrogen amide bond. The results indicate that local minima associated with N–C_α and C_α–C bonds cannot explain the nmr splittings.[26] The coexistence of cis–trans isomers provides a reasonable explanation.

The cis–trans isomerization of the peptide bond has been discussed in detail by Madison and Schellman,[27] Ramachandran,[28] and Andrews[29] from the theoretical point of view. From their calculations, the ground-state energy difference between the cis and the trans isomers is estimated to be less than 2 kcal/mol. Our results agree with the calculated values. In spite of the low energy difference between the two isomers, solvent and intramolecular interactions unique to polypeptides could prevent the formation of the cis isomer in the long chain molecules.[29,30] Thus, it is reasonable to explain the all-trans conformation of poly(*N*-methyl-L-alanine) observed in helix-supporting solvents but with roughly equal cis–trans conformation in a helix-breaking solvent such as trifluoroacetic acid.[10]

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^{13}C Nuclear Magnetic Resonance Studies of 85% ^{13}C-Enriched Amino Acids. Chemical Shifts, Coupling Constants J_{C-C}, and Conformation

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Abstract: The ^{13}C chemical shifts and the coupling constants J_{C-C} of the amino acids: alanine, valine, leucine, and isoleucine, enriched to 85% in ^{13}C, were studied as a function of pH. The inductive effect on C_β is found to be slight during protonation of the COO^- group and negligible beyond C_γ in the pH range between 1 and 11. The spatial effect of the $\langle NH_3^+, COO^- \rangle$ zone is propagated as far as C_γ and probably further. The four amino acids studied fall into two categories: the first (alanine, leucine) characterized by a C_β carrying two or three protons and the second (valine, isoleucine) by a C_β with only one. The ^{13}C chemical shift variation with pH suggests that the single C_β proton of the second category lies outside the $\langle NH_3^+, COO^- \rangle$ zone, whereas in the first category one of the C_β protons is permanently inside it. Apart from $J_{C_0-C_\alpha}$, which is relatively high (53–60 Hz), the J_{C-C} values range from 31 to 36 Hz. Only $J_{C_0-C_\alpha}$ varies appreciably (by about 6 Hz) and reflects the COO^- group p*K*. On protonation of this group (pH 1–7) the chemical shifts of C_0 and C_α are linear functions of $J_{C_0-C_\alpha}$ and the total electron densities of these carbons vary in the same way for the four amino acids studied.

A large number of articles published in the last few years have stressed the advantages of the ^{13}C nuclear magnetic resonance (^{13}C nmr) technique as a means of studying biological molecules. In the amino acid field, the main parameters defining the chemical shifts of ^{13}C have been established by several authors,[1-4] often using the data of Grant, *et al.*[5] Unfortunately, very few results on the conformation of amino acids exist,[4] which information would lead to a better understanding of the behavior in solution of larger molecules such as peptides and proteins. However, some recent results based on chemical shifts,[6] spin–lattice relaxation times of different species,[7-9] and couplings of the type

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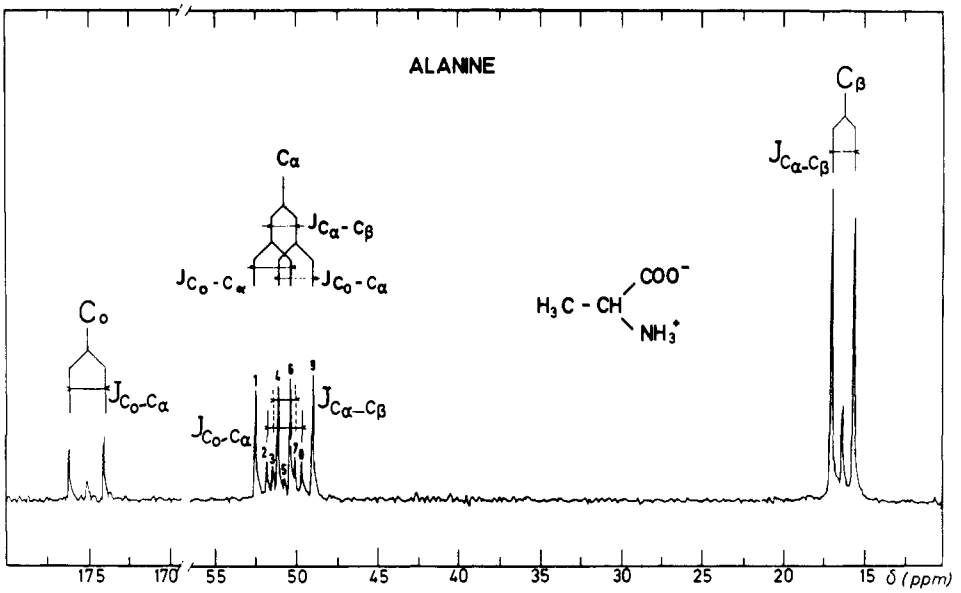

Figure 1. ^{13}C nmr spectrum of alanine (0.15 *M*, pH 7.6) enriched to 85% in ^{13}C. This spectrum was recorded on a Varian XL-100 nmr spectrometer operated in the Fourier transform mode (with complete proton decoupling).

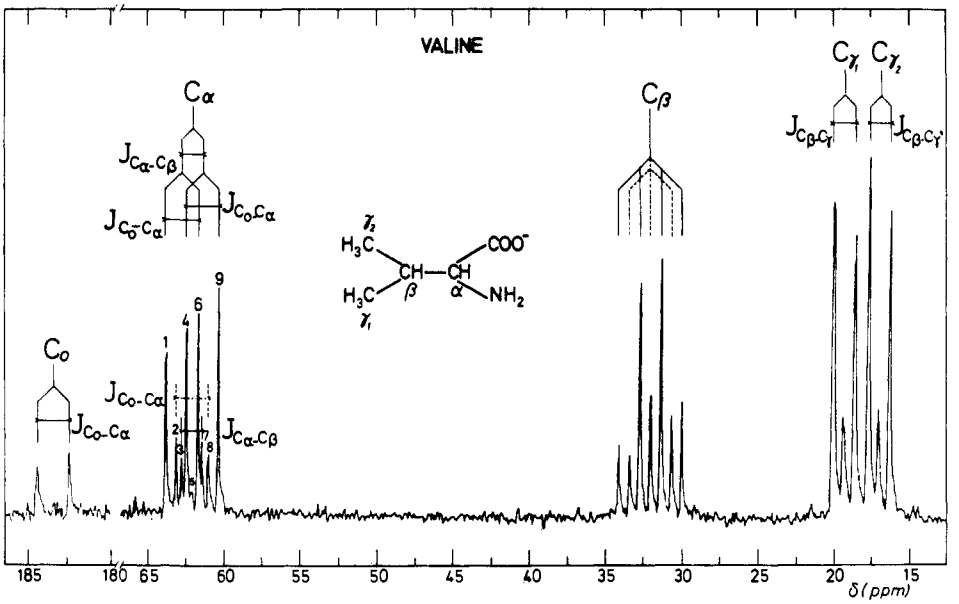

Figure 2. ^{13}C nmr spectrum of valine (0.1 *M*, pH 11) enriched to 85% in ^{13}C. The chemical shifts (in ppm) are measured from a TMS external reference.

^{13}C–H[10–12] and ^{13}C–^{13}C[13–15] show the utility of ^{13}C nmr in the conformational analysis of peptides. One result of the work on ^{13}C chemical shifts is the detection of the cis–trans isomerism of proline in a series of dipeptides.[16,17] A step forward in the understanding of these problems would be reached with the use of peptides containing one or more ^{13}C-labeled amino acids.[17] These molecules would not only be able to supply more detailed information on spatial structures but, above all, would throw light on the nature of interactions occurring in the presence of other molecules. However, such an approach implies a thorough knowledge of both the spectra of the ^{13}C-labeled amino acids and their new physicochemical parameters inside model peptides.

The preliminary results of a systematic study carried out with 85% ^{13}C-enriched amino acids are reported in this paper. The ^{13}C chemical shifts and the coupling constants J_{C-C} of the four amino acids, alanine, valine, leucine, and isoleucine, specially chosen for the aliphatic nature of their side chains, were followed as a function of pH. The conformational aspect of these

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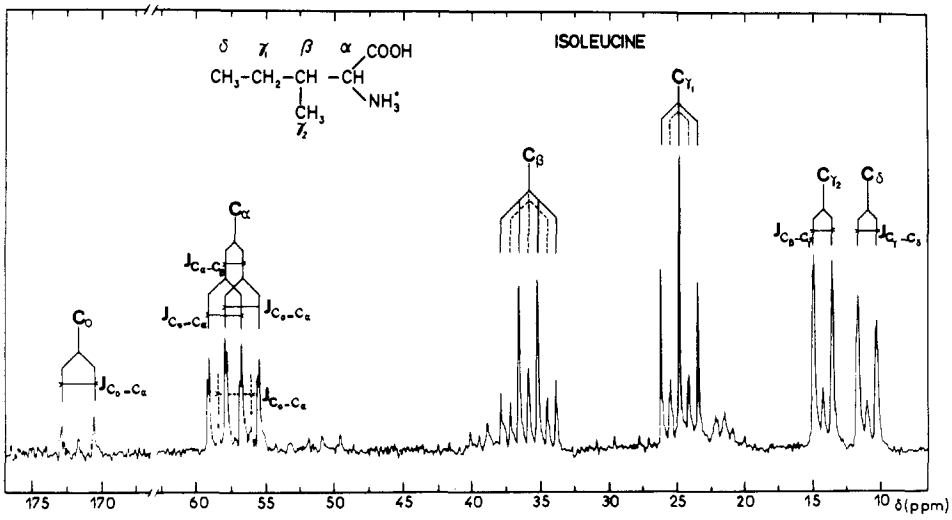

Figure 3. ^{13}C nmr spectrum (with complete proton decoupling) of isoleucine (≈ 0.1 *M*, pH 1.0). A small amount of leucine appears as an impurity.

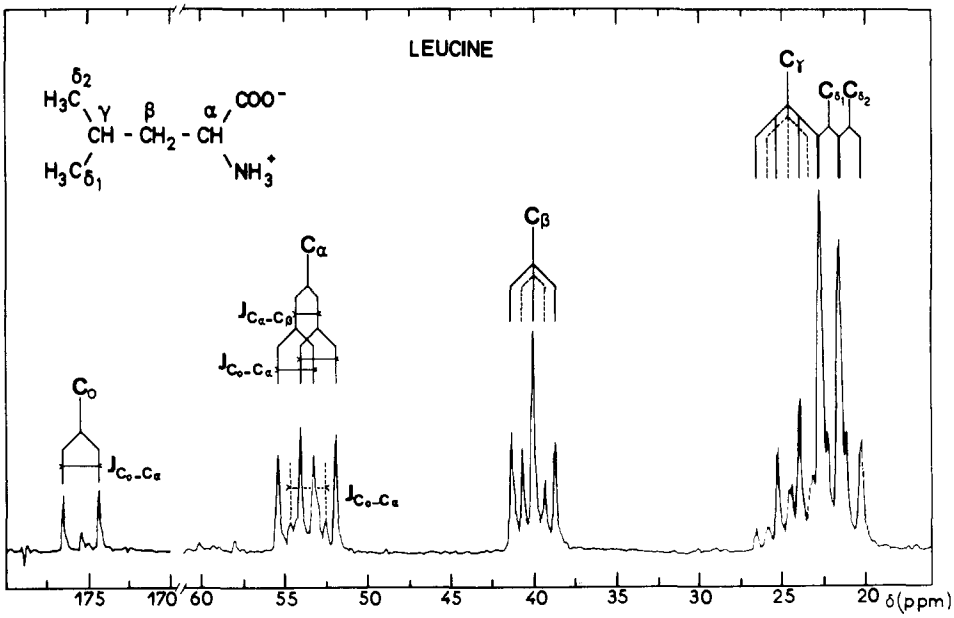

Figure 4. ^{13}C nmr spectrum of leucine (≈ 0.1 *M*, pH 7.8). Within the region 20–27 ppm, where C_γ, $C_{\delta 1}$, and $C_{\delta 2}$ appear, the spectrum is not first order. The bridges indicated above these carbons are simply symbolic.

molecules was studied in correlation with X-ray diffraction (XR) and proton nuclear magnetic resonance (^1H nmr) data derived from literature.

I. Experimental Section

Materials and Instrumentation. Uniformly carbon-13 labeled amino acids were prepared in our laboratory in large-scale biosynthesis from algae *Spirulina maxima*. Algae were grown in the presence of highly enriched $^{13}CO_3HNa$. Proteins extracted with trichloroacetic acid were hydrolyzed by pronase and then by H_2SO_4 (6 *N*) at 100°. Amino acids were finally separated by ion-exchange chromatography and the material collected was ^{13}C enriched to 85% on each carbon. The enriched amino acids dissolved in D_2O were run in the concentration range of 0.05–0.15 *M*. The pH was adjusted with concentrated solutions of DCl and NaOD and measured with a Tacussel pH meter. None of the measurements were corrected for pD.

The cmr spectra were recorded at 25.15 MHz on a Varian XL-100 12 WG spectrometer at about 30°. The apparatus operated in the pulsed-Fourier transform mode associated to a 16K 620 f computer which allowed an acquistion time of 0.8 sec for a 5000-Hz spectral width (resolution ~1.25 Hz). Proton decoupling was obtained with a Varian Gyrocode spin decoupler. The impulse generator, a V 4420 model, produced an amplification of 1 kW (τ 90° = 16 μsec). Neither sensitivity enhancement nor resolution enhancement was used in any of the spectra. Under these conditions the accumulation time was 10–15 mn in the case of ^{13}C-enriched samples and about 2–3 hr for normal amino acids.

For all the measurements D_2O used as solvent provided the deuterium lock signal; 10% TMS in $CHCl_3$, contained in a tube of 5 mm o.d. inside the 10-mm o.d. sample tube, was used as external reference.

II. Interpretation of ^{13}C-Labeled Amino Acid Spectra

Figures 1, 2, 3, and 4, respectively, show the spectra of alanine (pH 7.6), valine (pH 11), isoleucine (pH 1), and leucine (pH 7.8) at 30° in D_2O solution. At first sight, a larger number of signals than expected appears in each spectrum. In the C_α region in fact (between 45 and 65 ppm) nine signals are found instead of the four which should result from the coupling of $^{13}C_\alpha$ with $^{13}C_0$ and $^{13}C_\beta$. Comparison of these spectra with the

Table I. Standardized Probabilities and Relative Intensities of Combinations I, II, III, and IV[a]

Combination	C_0 C_α C_β[b]	Probability of combination[c]	Peaks obsd	Rel intensity of the peaks[d]
I	^{13}C ^{13}C ^{13}C	$P_1 = E^2$	| | | |	$I_1 = E^2/4$
II	^{13}C ^{13}C ^{12}C	$P_2 = (1 - E)E$	$\lvert J_{C_0-C_\alpha} \rvert$	$I_2 = (1 - E)E/2$
III	^{12}C ^{13}C ^{13}C	$P_3 = (1 - E)E$	$J_{C_\alpha-C_\beta}$ | |	$I_3 = (1 - E)E/2$
IV	^{12}C ^{13}C ^{12}C	$P_4 = (1 - E)^2$	|	$I_4 = (1 - E)^2$

[a] These calculations remain valid for any carbon C_b coupled with two carbons C_a and C_c where $J_{C_a-C_b} \neq J_{C_b-C_c}$. In the special case where $J_{C_a-C_b} = J_{C_b-C_c}$, combination I gives rise to a triplet with its central peak overlapping the single peak of combination IV, the latter defining the chemical shift of C_b. The resulting intensity of the central peak $I_1' = 2I_1 + I_4 = {}^3/_2E^2 - 2E + 1$. The doublets of combinations II and III overlap. The probability P_2' of the resulting doublet will be $P_2' = P_2 + P_3 = 2E(1 - E)$ and the corresponding relative intensity $I_2' = I_2 + I_3 = E(1 - E)$. The C_b multiplet thus consists of five peaks (example: leucine C_β, Figure 4; isoleucine $C_{\gamma 1}$, Figure 3). [b] In these combinations the C_α must be a ^{13}C to be detectable. [c] E = ^{13}C enrichment factor; $0 \leqslant E \leqslant 1$; the probabilities are standardized so that $\Sigma P_i = 1$. [d] In all cases the spectra are assumed to be first order.

theoretical spectra of LAOCOON III,[18] taking long-range couplings into account, does not give similar results. This means that these extra signals have another origin related to the partial enrichment. Since the sample is only 85% enriched in ^{13}C, the probability of a ^{13}C being adjacent to a ^{12}C or ^{13}C must be taken into consideration. The resulting spectrum then represents all possible combinations of different ^{13}C or ^{12}C sites, and the intensity of the lines is proportional to the probability of each of these combinations. Let us consider, for example, the simplest spectrum, that of alanine C_α (Figure 1). In the region between 48 and 53 ppm a nine-peak multiplet, resulting from the different combinations of C_α, C_0, and C_β, is observed. To be detectable the C_α must be a ^{13}C and, consequently, the combinations with C_0 and C_β are restricted to four (Table I). In the case of combination I, the spectrum consists of four lines which result from coupling with C_0 and C_β; E being the enrichment, the probability P_1 of the first combination will be equal to E^2, and the relative intensity of the corresponding peaks will be $E^2/4$. In the case of combinations II and III, the signal is made up of two lines which arise from coupling with either C_0 or C_β. The probabilities $P_2 = P_3$ are equal to $E(1 - E)$ and the relative intensities $I_2 = I_3 = E(1 - E)/2$. Combination IV gives a single line, the position of which defines the chemical shift of C_α. The probabilities and relative intensities of these combinations are given in Table I.

Figure 1 shows that the coupling constants $J_{C_0-C_\alpha}$ and $J_{C_\alpha-C_\beta}$ can be measured from the intervals 1–6, 4–9, 2–8 and 1–4, 6–9, 3–7, respectively. They can also be measured in the C_0 and C_β regions, as shown by bridges on the same figure. For all these coupling measurements an excellent agreement of 0.5 Hz was obtained.

In Figure 5 the combination probabilities and corresponding relative intensities as a function of enrichment are plotted. The results underline the importance of choosing a suitable enrichment factor, both when studying the isotope effect (for the different combinations) on the relaxation time and when observing the ^{13}C spectrum. In the former case the signal intensities corresponding to the combinations must be comparable, which requires an enrichment E of about 67% (solution of equations $I_1 = I_2 = I_3 = I_4$, Figure 5); in the latter case E must obviously be as high as possible. However, above 85% the intensity gain is small compared with the time loss and technical difficulties entailed by a high enrichment. Below 67% the observation of the main peaks (combination I) will be hindered by increased intensity of peaks due to a labeling fault.

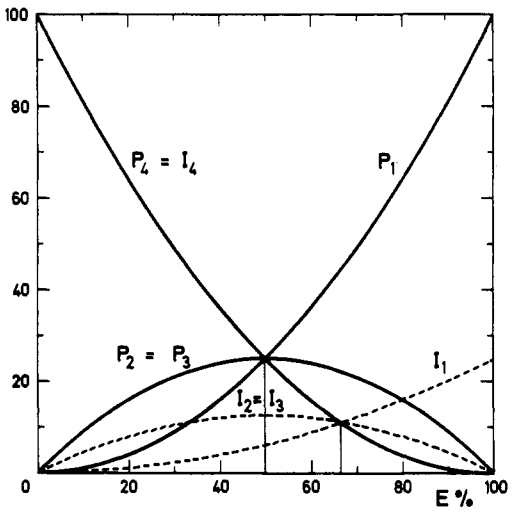

Figure 5. Standardized probabilities and relative intensities of different combinations as a function of enrichment factor (see Table I). All the probabilities are equal when E = 50% and the relative intensities of all four combinations are equivalent when E = 66.7%.

Multiplets due to ^{13}C–^{13}C coupling appear over a spectrum width varying from 60 to 110 Hz and in some cases this can interfere with the detection of other ^{13}C signals. As a result it is sometimes useful to choose a ^{13}C enrichment factor such that a single signal is obtained for each carbon (as is the case for naturally occurring products) but with an appreciable gain in sensitivity. In fact, the signal amplitude S of one carbon between two other carbons (example: C_α of an amino acid where $J_{C_0-C_\alpha} \neq J_{C_\alpha-C_\beta}$) will be proportional to the enrichment E multiplied by the probability P_4 (= I_4)

$$S = E(1 - E)^2$$

S, obtained as a function of E, is zero at E = 0 and 1 (0 and 100%) and maximum at E = $^1/_3$ (33%). At the latter enrichment the signal is 14 times, and at E = 20% 12 times, stronger than that of a natural occurring (~1%). The ratios of the signals due to combination II (or III) corresponding to E = 20% and E = 33% are respectively 1.5 and 3. The optimum enrichment for the purpose of improved sensitivity or simple and selective labeling of a product thus seems to lie between 15 and 20%, for the two following reasons: below E = 15% the sensitivity gain is small, and above 20% signals

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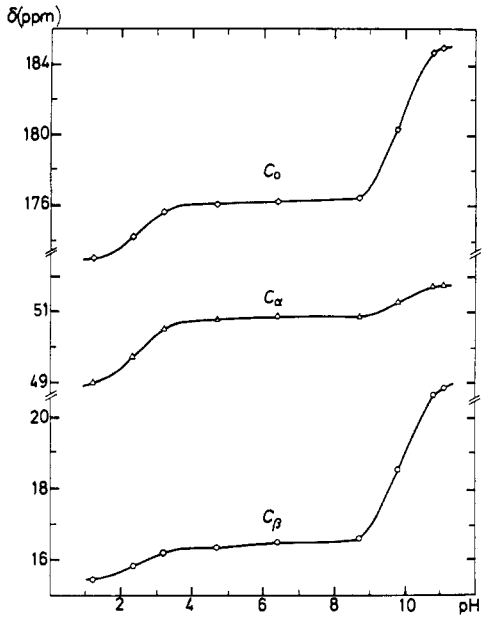

Figure 6. The ^{13}C chemical shifts (in ppm relative to TMS in $CHCl_3$ used as external reference) of alanine as a function of pH.

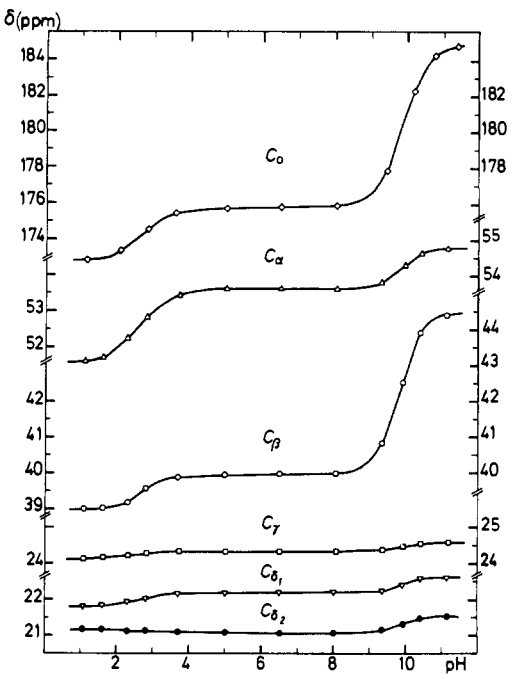

Figure 7. The ^{13}C chemical shifts of leucine as function of pH.

corresponding to other combinations appear, which fact, as mentioned above can interfere with the observation of neighboring ^{13}C signals.

Apart from the $J_{C_o-C_\alpha}$ value which is relatively high (53–60 Hz), the coupling constants ($J_{C_\alpha-C_\beta}$, $J_{C_\beta-C_\gamma}$, ...) of the saturated aliphatic chain are very similar and lie between 31 and 36 Hz (section IIIB). Consequently, any carbon (except C_α) bound to a number n carbons will possess a ^{13}C spectrum (with complete proton decoupling) consisting of a complex of $n + 1$ main peaks interlocked with submultiplet of n peaks. The $n + 1$ multiplet is due to ^{13}C coupling with n other ^{13}C while the submultiplet corresponds to the case where the ^{13}C is coupled with $n - 1$ ^{13}C, the last being a ^{12}C. The probability that this ^{13}C will see two ^{12}C's at same time is low when the enrichment is higher than 67%. The $n - 1$ peak submultiplet is, therefore, difficult to observe because of its low intensity, and if it did appear it would merge with the main $n + 1$ peak multiplet (first-order spectrum).

The chemical shift of the carbon investigated will be given by the central peak of the main $n + 1$ peak multiplet when n is even (isoleucine $C_{\gamma 1}$, Figure 3; leucine C_β, Figure 4) and by the position of the central peak of the n peak submultiplet when n is odd. This is true on condition that the isotope effect is nil or negligible, as verified in the case of the three amino acids alanine, valine, and isoleucine which have a nearly first-order spectrum. The central peak of the submultiplet (case where n is odd) is localized at the center of the main multiplet. In addition, if valine enriched to 85% in ^{13}C is mixed with nonenriched valine, the latter forming 97% of the total mixture, it is found that, within the limits of error, the ^{13}C signals of the nonenriched component carbons coincide with those which define the chemical shifts of the enriched component.

When the spectrum is not quite first order, the submultiplet will be slightly displaced inside the main $n + 1$ peak multiplet (leucine C_γ, $C_{\delta 1}$, $C_{\delta 2}$, Figure 4) in the direction of the signal of its coupled carbon.

To conclude, 85% ^{13}C enrichment appears to have several advantages: (1) the ^{13}C signal is obtained rapidly; (2) the measurement of the coupling constants and their attribution is more or less direct; (3) the submultiplet observed gives the chemical shift in some cases, allows the coupling constant values to be checked, and facilitates the spectral attribution.

III. Results and Discussion

A. Chemical Shifts of Amino Acid ^{13}C. The ^{13}C resonances of different carbons of the amino acids alanine, leucine, valine, and isoleucine were studied as a function of pH (Figures 6–9). In general, in the pH range explored (1–11) these resonances lie between 170 and 185 ppm for C_o, 48 and 62 ppm for C_α, 15 and 39 for C_β, 14 and 25 ppm for C_γ and 11 and 23 ppm for C_δ (Table II). Between acid and basic pH's the C_o signals shift toward low field by about 12–13 ppm, those of C_α by 2.7–3.8 ppm, and those of C_β by 2.8–5.8 ppm. These values agree well with those obtained by Gurd, *et al.*,[3] who examined valine and leucine at acid, neutral, and basic pH. The slight differences observed in the case of the $\Delta\delta C_{\gamma 1}$ of valine in neutral or basic pH can be attributed to the high concentrations used by these authors. During passage from the carboxyl (COOH) to the carboxylate form (COO^-), the $\Delta\delta C_o$ of the amino acids is relatively low (2.9 ± 0.4 ppm) in contrast to that of carboxylic acids ($\Delta\delta C_o$ = 4.7 ppm).[4] This difference is due to the presence of the NH_3^+ group which can interact ionically with the COO^- group and hence cause a partial neutralization of the charge carried by the latter. During passage from the zwitterion to the anion state, not only is the above mentioned ionic interaction abolished, the NH_3^+ group losing a proton, but, since the nitrogen hybridization changes, the result is a double variation of the inductive and of the spatial effect.[1,2,5] This explains the magnitude of the chemical shift shown by C_o in this pH range.

1. Conformation of Valine. The valine C_γ titration curves show (Figure 8) that each methyl group behaves

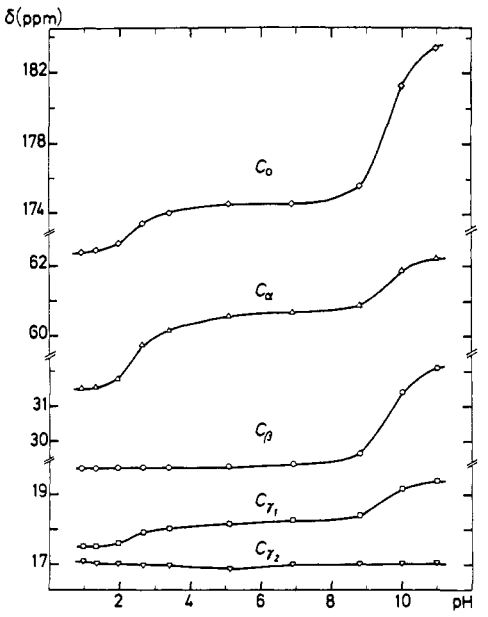

Figure 8. The ^{13}C chemical shifts of valine as a function of pH.

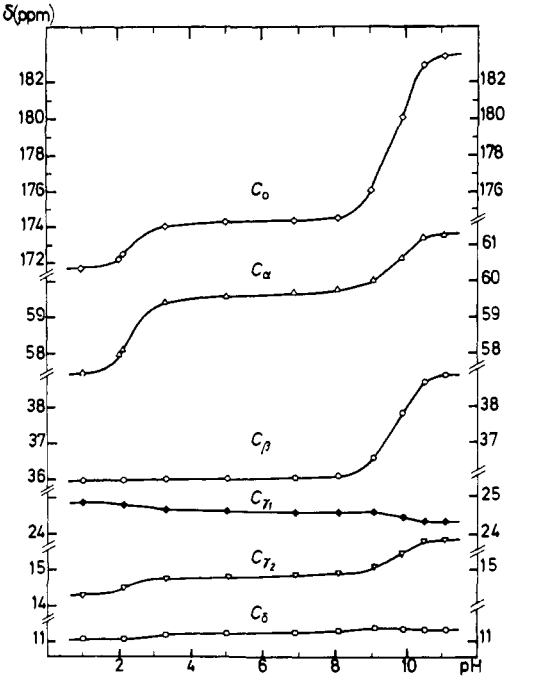

Figure 9. The ^{13}C chemical shifts of isoleucine as a function of pH.

in an individual way. Not only do these carbons possess distinct chemical shifts at a given pH, but the shapes of their curves differ. That of $C_{\gamma 1}$ reflects the two pK's (pK_1 of COO^- group and pK_2 of NH_3^+ group), while that of $C_{\gamma 2}$ remains constant. This shows that the inductive effect due to protonation of the COO^- and NH_2 groups is nil or negligible for the C_γ, their chemical shifts being influenced chiefly by the spatial effect. Consequently, this difference observed as a function of pH reveals a conformational situation in which $C_{\gamma 1}$ (which reflects the two pK's) must be localized in, and, hence, influenced by, the $\langle COO^-, NH_3^+\rangle$ zone. This is the situation illustrated by the rotamers I and II of Figure 10a in which the proton carried by the C_β lies outside the $\langle COO^-, NH_3^+\rangle$ zone. These in fact are the two conformations given by ^1H nmr[19-21] and by X-ray diffraction,[22-24] although the ^1H nmr results assign a small proportion (16%) to rotamer III, whereas the XR results exclude it entirely.

The fact that $\delta C_{\gamma 2}$ remains constant with varying pH means that the total proportions of rotamers I and II do not alter. This agrees with the ^1H nmr results which show that the relative proportions of the rotamers are not very dependent upon pH.[19-21]

The C_β titration curves show that the four amino acids fall into two categories. The first type, to which alanine and leucine belong, has a C_β carrying two or three protons, its curve reflecting the pK of both COO^- and NH_3^+ groups. The second includes valine and isoleucine and is distinguished by a C_β carrying a single proton, its curves reflecting only the pK_2 (NH_3^+). Moreover, the C_β curve amplitude for the first two amino acids between pH 1 and 11 is twice that observed in the corresponding case for valine (5.6 *vs.* 2.8 ppm); when the $C_{\gamma 1}$ curve of valine is added to its C_β curve, the

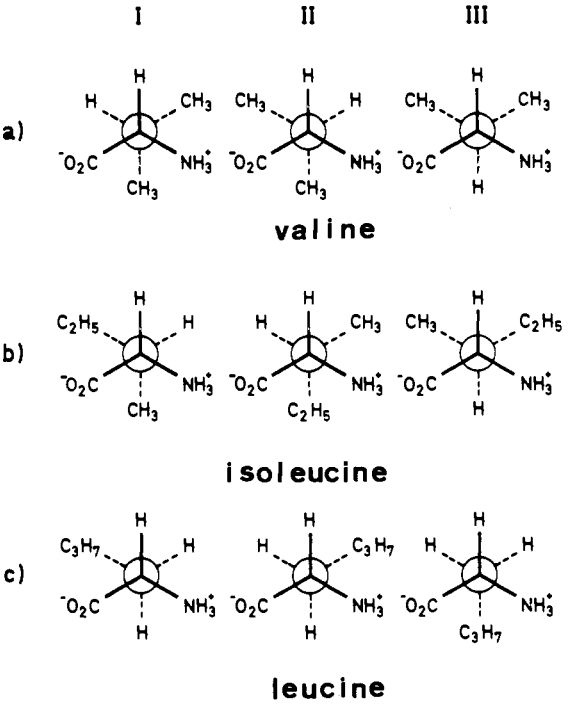

Figure 10. The three possible rotamers of the C_α–C_β bond of (a) valine, (b) isoleucine, and (c) leucine.

C_β curve of the first category of amino acids is obtained. The conclusions are as follows: at least for the pH range 1–7 it seems certain that the inductive effects on C_β caused by protonation of the carboxyl group is negligible, and the spatial effect of the $\langle COO^-, NH_3^+\rangle$ zone extends as far as C_γ and probably beyond.

2. Conformation of Isoleucine. The similarity between the C_β and $C_{\gamma 1}$ (or $C_{\gamma 2}$) titration curves of valine and isoleucine suggests that the isoleucine C_β proton lies outside and the $C_{\gamma 2}$ inside the $\langle COO^-, NH_3^+\rangle$ zone, as shown by rotamer I (Figure 10b). In fact, if the amplitude of the isoleucine $C_{\gamma 2}$ curve is compared with

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Table II. ^{13}C Chemical Shifts (in ppm Relative to TMS in $CHCl_3$, Used as External Reference) of Amino Acids Enriched to 85% in ^{13}C, Measured at Acid, Neutral, and Basic pH

Amino acids	pH	C_0	C_α	C_β	$C_{\gamma 1}$	$C_{\gamma 2}$	$C_{\delta 1}$	$C_{\delta 2}$
Alanine	1.2	172.98	48.97	15.45				
$CH_3-C(H)(NH_3^+)-COO^-$	6.4	176.25	50.87	16.49				
β α	11.1	184.98	51.75	20.83				
$\Delta\delta = f(\Delta pH)$	1.2 → 6.4	3.27	1.90	1.04				
	6.4 → 11.1	8.73	0.88	4.34				
Leucine	1.1	172.81	51.54	38.75	24.11		21.80	21.16
$(CH_3)_2CH-CH_2-C(H)(NH_3^+)-COO^-$	6.5	175.78	53.60	39.93	24.32		22.21	21.11
δ_1 γ β α δ_2	11.2	184.69	54.76	44.40	24.55		22.60	21.51
$\Delta\delta = f(\Delta pH)$	1.1 → 6.5	2.97	2.06	1.18	0.21		0.41	−0.05
	6.5 → 11.1	8.89	1.16	4.47	0.23		0.39	0.40
Valine	0.9	171.69	58.49	29.24	17.50	17.09		
$(CH_3)_2CH-C(H)(NH_3^+)-COO^-$	6.9	174.57	60.70	29.35	18.25	16.99		
γ_1 β α γ_2	11.1	183.43	62.20	32.09	19.38	17.06		
$\Delta\delta = f(\Delta pH)$	0.9 → 6.9	2.88	2.21	0.11	0.75	0.10		
	6.9 → 11.1	8.86	1.50	2.74	1.13	0.07		
Isoleucine	1.0	171.75	57.45	35.96	24.87	14.30	11.08	
$CH_3-CH_2-CH(CH_3)-C(H)(NH_3^+)-COO^-$	6.9	174.38	59.68	36.03	24.59	14.85	11.23	
δ γ_1 β α γ_2	11.1	183.42	61.26	38.85	24.27	15.74	11.23	
$\Delta\delta = f(\Delta pH)$	1.0 → 6.9	2.63	2.23	0.07	−0.28	0.55	0.15	
	6.9 → 11.1	9.04	1.58	2.82	−0.32	0.89	0.0	

that of valine, it is found that between pH 1 and 7 the $\Delta\delta C_{\gamma 2}$ (0.55 ppm) of the former amino acid is about $^3/_4$ that of the latter ($\Delta\delta C_{\gamma 1}$ = 0.75 ppm). This difference suggests that, in the case of isoleucine, other rotamers are present in equilibrium in the solution. Among the possibilities, however, rotamer III can be rejected since the isoleucine δC_β does not change during protonation of the COO^- group. Furthermore, if the sum of the proportions of valine rotamers I and II is assumed to be 100%, implying the permanent presence of a methyl group in the $\langle COO^-, NH_3^+\rangle$ zone, and if $\Delta\delta C_{\gamma 1}$ = 0.75 ppm is taken to reflect this situation, it is concluded that for isoleucine the respective proportions of rotamers I and II are 73 and 27%.

The above proportions can be also be calculated (78 and 22%, respectively) by comparison of the valine $\Delta\delta C_{\gamma 1}$ (1.13 ppm) with the isoleucine $\Delta\delta C_{\gamma 2}$ (0.89 ppm) between pH 7 and 11. These results show that the rotamer proportions do not vary greatly with pH. It may therefore be assumed that, as in the case of valine, the sum of the proportions of isoleucine rotamers I and II is 100%. This implies that the C_β proton lies entirely outside the $\langle COO^-, NH_3^+\rangle$ zone of influence and that, consequently, the amplitude of the C_β curve of the two amino acids must be the same for an identical pH variation. This following was observed: between pH 1 and 11, valine $\Delta\delta C_\beta$ = 2.85 ± 0.10 ppm and isoleucine $\Delta\delta C_\beta$ = 2.90 ± 0.10 ppm; and between pH 7 and 11, valine $\Delta\delta C_\beta$ = 2.74 ± 0.10 ppm and isoleucine $\Delta\delta C_\beta$ = 2.82 ± 0.10 ppm.

The difference between the proportions of the isoleucine rotamers I and II can be explained by the difference in the steric bulk of the methyl and ethyl groups. The existence of these two conformations was revealed by X-ray diffraction[25] which assigned conformation I to isoleucine crystallized in the form of monohydrate-HBr and conformation II to isoleucine monohydrate-HCl. With regard to the isoleucine $C_{\gamma 1}$, its titration curve faintly shows the two pK's (COO^- and NH_3^+) in accord with the presence of 25% rotamer II in the solution. However, the $C_{\gamma 1}$ chemical shifts observed vary inversely (toward high fields) with respect to the δ of the other carbons. Unfortunately, no adequate explanation can be found for this difference in behavior, which may correspond to the presence of a second asymmetric carbon (C_β) in the side chain of isoleucine.

3. Conformation of Leucine. In Figures 6 and 7 the titration curves of the alanine and leucine C_β are seen to be practically superimposable, suggesting that the presence of two C_β protons is sufficient for the maximum spatial effect to be observed. When the C_β has a single proton, as is the case for valine and for isoleucine,

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this proton was found to lie outside the $\langle COO^-, NH_3^+\rangle$ zone. In fact, the difference between the leucine and valine (or isoleucine) C_β titration curves is related to the fact that one of the two leucine C_β protons lies within the $\langle COO^-, NH_3^+\rangle$ zone, showing that leucine can only assume the conformations I and II (Figure 10c). Finally, it should be noted that the alanine C_β carries three protons but only one can be localized inside the $\langle COO^-, NH_3^+\rangle$ zone at a given time. This is why the curve amplitudes of the alanine and leucine C_β's observed as a function of pH are comparable. This means that rotamer III can be rejected for leucine in which no C_β proton lies inside the zone of influence; otherwise the amplitude of the leucine C_β curve would be smaller than that of alanine. In fact, steric factors also eliminate rotamer III in which the isopropyl group introduces a strong repulsion on the NH_3^+ and COO^- groups.

The leucine C_δ's also behave differently depending on pH (Figure 7, Table II). We find that while $\delta C_{\gamma 1}$ describes very faintly the pK_1 and pK_2, $\delta C_{\gamma 2}$ reflects only the pK_2 (NH_3^+), though with an amplitude equal to that of $C_{\gamma 1}$. It should be noted that the inductive effect due to protonation is negligible beyond C_β. As a result, the δC_γ variations are caused by the spatial effect of the $\langle COO^-, NH_3^+\rangle$ zone. This shows that between pH 1 and 7 the methyl groups are spatially nonequivalent, the $C_{\delta 1}$ group being influenced by the $\langle COO^-, NH_3^+\rangle$ zone and the $C_{\delta 2}$ group lying outside of it. At basic pH the two C_δ's are influenced in the same way, which fact seems to show a conformational change in the isopropyl group with respect to the $\langle COO^-, NH_3^+\rangle$ zone of influence.

B. ^{13}C–^{13}C Coupling Constants. Little information exists in this field since the natural abundance of ^{13}C (1.1%) is too small to allow the observation of coupling interactions between two ^{13}C nuclei, the probability for two ^{13}C carbons to be neighbors being practically nil (10^{-4}) (Table I, Figure 5). However, attempts were made in several laboratories to obtain labeled products (thus increasing the probability of ^{13}C–^{13}C coupling) in order to determine, for example, the state of hybridization of bound orbitals.[26,27] Frei and Bernstein[28] examining different hybridized carbon combinations showed that the coupling constant values depend roughly on the characters of the bound orbitals. In general the highest value was found in sp–sp systems and the lowest in sp^3–sp^3 systems. It was also shown that the nature of the bound carbon substituents can influence the value of $J_{C_{sp^3}-C_{sp^3}}$. This was observed in particular by Bartuska, *et al.*,[29] in a series of CH_3–CH_2–X-type compounds where a strong correlation exists between the coupling value J_{C-C} and the electronegativity of the group directly attached to methylene (J_{C-C} increases with the electronegativity). In the case of C_{sp^3}–C_{sp^2}-type couplings their order of magnitude generally varies between 38 and 59 Hz.[14] For the CH_3—C(X)=O type the coupling constants increase with the polarity of the α substituent.[15] Thus in acetic acid the coupling constant $J_{C_0-C_\alpha}$ obtained at acid pH is equal to 56.7 Hz whereas in acetate it is 51.6 Hz. The former value is 2.5 Hz lower than that observed in the alanine spectrum obtained at acid pH, the coupling constants of which are given in Table III with that of the other amino acids

Table III. Coupling Constants J_{C-C}, Obtained from Amino Acids Enriched to 85% in ^{13}C, Measured at Acid, Neutral, and Basic pH[a]

Amino acids	pH	$J_{C_0-C\alpha}$	$J_{C_0-C\beta}$	$J_{C\beta-C\gamma_1}$ ($J_{C\beta-C\gamma_2}$)	$J_{C\gamma-C\delta_1}$ ($J_{C\gamma-C\delta_2}$)
	1.2	59.2	34.1		
Alanine	6.4	54.1	34.9		
	11.1	52.7	35.2		
	1.1	59.5	32.9	31.7	31.2 (35.3)
Leucine	6.5	53.6	34.3	31.3	31.4 (34.9)
	11.2	52.6	34.6	31.7	31.3 (34.7)
	0.9	59.5	32.1	34.9 (34.6)	
Valine	6.9	53.5	33.2	33.2 (34.0)	
	11.1	53.1	33.6	35.1 (35.2)	
	1.0	59.0	32.5	34.5 (34.4)	34.2
Isoleucine	6.9	53.5	33.7	34.0 (34.9)	35.2
	11.1	53.4	34.0	34.1 (35.6)	35.1

[a] The maximum error is 0.5 Hz.

studied. Obviously the highest coupling constants are the $J_{C_0-C\alpha}$, their values measured at acid, neutral, and basic pH lying between 53 and 60 Hz. The other values, of the type $J_{C_{sp^3}-C_{sp^3}}$, fall between 31 and 36 Hz.

The effect of pH on the coupling constant values was examined (Table III). The largest variations are found for the constants $J_{C_0-C\alpha}$ (a mean decrease of 7 Hz having been observed between the cation and the anion state) although the greatest change is measured between acid and neutral pH (maximum $\simeq$6 Hz). This reduction in the $J_{C_0-C\alpha}$ value corresponds to the ionization of the CH group which amounts to decreasing the α polarity of C=O, in agreement with the observations of Gray, *et al.*,[15] who pointed out the effect of a polar group on the coupling constant $J_{C_0-C\alpha}$. In this way, for example, the passage from the CH_3COOH to the CH_3COO^- form is accompanied by a 5.1-Hz reduction of $J_{C_0-C\alpha}$. The passage from the zwitterion to the anion state hardly changes $J_{C_0-C\alpha}$: $\Delta J_{C_0-C\alpha} = 1.2 \pm 0.2$ Hz for alanine and leucine and 0.4 Hz for valine and isoleucine. Unlike the constant $J_{C_0-C\alpha}$, which decreases as the pH rises, the constant $J_{C\alpha-C\beta}$ increases slightly for each of the amino acids examined (1.3 ± 0.3 Hz). Although this latter value corresponds to the limit of experimental error, the measurements performed four times (section II) are reproducible to within 0.5 Hz.

C. Relationship between δ and J. As mentioned above, as the pH varies from 7 to 1, the shift of the signals of carbons C_0 and C_α toward high fields corresponds chiefly to the change in inductive effect caused by protonation of the carboxylate group. Together with these chemical shifts, the coupling constant $J_{C_0-C\alpha}$ was found to increase from 53 to 60 Hz. Both parameters (δ and J) vary continuously with pH and both reflect the pK of the terminal C group. At a given pH, each therefore depends on the proportions of the COO^- and COOH forms in equilibrium in the solution. Hence, the δ and J values observed correspond to mean values reflecting the simultaneous presence of zwitterion and cation forms in the medium. From eq 1 and 2 the observed δ and J can be determined,

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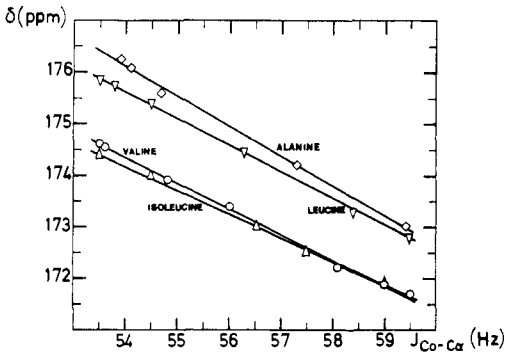

Figure 11. The C_0 chemical shifts of the four amino acids as a function of $J_{C_0-C_\alpha}$.

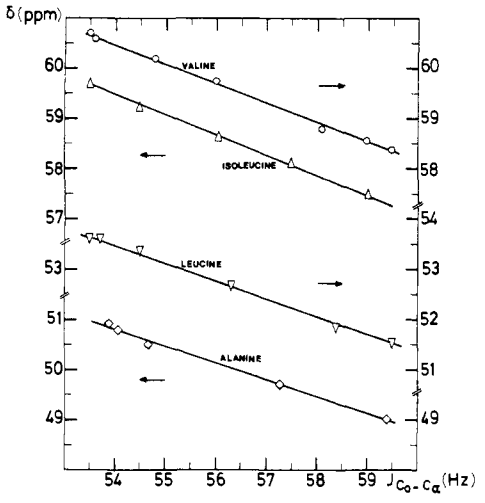

Figure 12. The C_0 chemical shifts of the four amino acids as a function of $J_{C_0-C_\alpha}$.

$$\delta_{obsd} = \delta_a[COOH] + \delta_b[COO^-] \quad (1)$$

$$J_{obsd} = J_a[COOH] + J_b[COO^-] \quad (2)$$

where δ_a and δ_b are the chemical shifts of the carbon C_0 (or C_α) in carboxyl and carboxylate form, respectively; J_a and J_b are the coupling constants $J_{C_0-C_\alpha}$ of the corresponding states. The stationary concentrations of the carboxyl COOH and carboxylate COO^- state are standardized as

$$[COOH] + [COO^-] = 1 \quad (3)$$

From eq 1-3 can be derived

$$\delta_{obsd} = kJ_{obsd} + C^{te} \quad (4)$$

where

$$k = \frac{\delta_b - \delta_a}{J_b - J_a} \quad (5)$$

and

$$C^{te} = \frac{\delta_a J_b - \delta_b J_a}{J_b - J_a} \quad (6)$$

k being expressed in ppm/Hz.

Since δ_{obsd} is a linear function of J_{obsd} one of these values can be determined from the other. Expression 4 is proved experimentally as shown by the plots of the chemical shifts of C_0 and C_α against $J_{C_0-C_\alpha}$ between pH 1 and 7 (Figures 11 and 12). It is found that $J_{C_0-C_\alpha}$

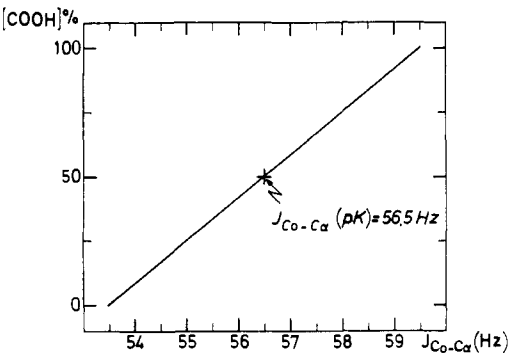

Figure 13. Theoretical COOH concentration of amino acids as a function of $J_{C_0-C_\alpha}$.

corresponding to either the carboxyl or the carboxylate state has much the same value for the four amino acids: $J_{C_0-C_\alpha}$ corresponding to COO^- = 53.5 ± 0.5 Hz and $J_{C_0-C_\alpha}$ corresponding to COOH = 59.5 ± 0.5 Hz.

As shown above, J_{obsd} (expression 2) represents an average coupling corresponding to the carboxyl and carboxylate states in equilibrium. From the J_{obsd} value can be determined the concentration of either COOH or COO^-. The curve in Figure 13 gives the variation of the theoretical COOH concentration with $J_{C_0-C_\alpha}$, which coupling constant is seen to equal 56.5 Hz at the pK_1. It is independent of the pK_1 value of each amino acid.

Figure 11 clearly reveals two types of $\delta C_0 = f(J_{C_0-C_\alpha})$ curve corresponding to the two amino acid categories mentioned: alanine and leucine on the one hand, valine and isoleucine on the other, the $^{13}C_0$ resonance fields of the latter category being lower than those of the former. It is observed that, within a category, for a given $J_{C_0-C_\alpha}$ value the δC_0 values are similar. This suggests that for a given state (zwitterion for instance) the position of the ^{13}C resonance signal of the C_0 of saturated amino acids depends chiefly on the nature of the β group. The relatively large difference between the chemical shift of the alanine C_0 and that of leucine (δC_0(Ala) − δC_0(Leu) = 0.47 ppm as compared with δC_0(Val) − δC_0(Ileu) = 0.19 ppm) is due to the fact that the C_β of the former has three protons whereas that of the latter has two. From these results, it may be inferred that the inductive effect of a substituent located beyond C_β on the chemical shifts of carbon C_0 is negligible.

Figure 12 also shows that the four amino acids can be divided into two categories although here the superposition of the $\delta C_\alpha = f(J_{C_0-C_\alpha})$ curves is not obtained within each category. This is due mainly to the inductive effects corresponding to the different side chains which distinguish the amino acids.[2] We note finally that whereas the resonance signals of the alanine and leucine C_0 (category 1) appear at lower field than those of category 2, the reverse is true for the C_α signals (Table II).

The k_{C_0} and k_{C_α} values can be determined from the slope of the $\delta C_0 = f(J_{C_0-C_\alpha})$ and $\delta C_\alpha = f(J_{C_0-C_\alpha})$ curves. They are listed in Table IV, bearing the (−) sign, δ and J varying inversely. An examination of these values raises two important comments: the absolute value of k_{C_0} higher than k_{C_α} shows that the electron density variation at the C_0 site is greater than at the C_α site during protonation of the COO^- group, and

Table IV

Amino acids	k_{C_0}[a]	$k_{C\alpha}$[a]	k_{C_0}[a] + $k_{C\alpha}$[a]	K_{C_0}	$\Delta\delta C_0/(\Delta\delta C_0 + \Delta\delta C\alpha)$	$K_{C\alpha}$	$\Delta\delta C_0/(\Delta\delta C_0 + \Delta\delta C\alpha)$
Alanine	−0.57	−0.34	−0.91	0.626	0.632	0.373	0.367
Leucine	−0.51	−0.35	−0.86	0.593	0.590	0.407	0.409
Valine	−0.49	−0.39	−0.88	0.556	0.565	0.443	0.434
Isoleucine	−0.51	−0.40	−0.91	0.560	0.541	0.439	0.459

[a] k_i being expressed in ppm/Hz.

the sum $k_{C_0} + k_{C\alpha}$ is appreciably the same for all the amino acids studied; $k_{C_0} + k_{C\alpha} = 0.88 \pm 0.03$ ppm/Hz.

The constants k_{C_0} and $k_{C\alpha}$ can thus be standardized by posing

$$K_{C_0} + K_{C\alpha} = 1 \tag{7}$$

where the standardized values K_{C_0} and $K_{C\alpha}$ are given by the equations

$$K_{C_0} = \frac{k_{C_0}}{k_{C_0} + k_{C\alpha}} \tag{8}$$

and

$$K_{C\alpha} = \frac{k_{C\alpha}}{k_{C_0} + k_{C\alpha}} \tag{9}$$

From expressions 4, 8, and 9 we can write

$$K_{C_0} = \frac{k_{C_0}}{k_{C_0} + k_{C\alpha}} = \frac{\Delta\delta C_0}{\Delta\delta C_0 + \Delta\delta C_\alpha} \tag{10}$$

$$K_{C\alpha} = \frac{k_{C\alpha}}{k_{C_0} + k_{C\alpha}} = \frac{\Delta\delta C_\alpha}{\Delta\delta C_0 + \Delta\delta C_\alpha} \tag{11}$$

where $\Delta\delta C_i$ is the difference between the chemical shifts of the carbon C_i corresponding to the COOH and COO^- states. It is observed in eq 10 and 11 that the standardized values K_{C_0} and $K_{C\alpha}$ are independent of $J_{C_0-C\alpha}$. The K_{C_0} and $K_{C\alpha}$ values for each of the amino acids are listed in Table IV. In principle they should be equal to those determined from the ratio $\Delta\delta C_i/(\Delta\delta C_0 + \Delta\delta C_\alpha)$ (Table IV), the slight differences being ascribable to the error on the estimation of $\Delta\delta C_i$ measured on the titration curve between acid and neutral pH.

From $J_{C_0-C\alpha}$ it is, therefore, possible to deduce δC_0 and δC_α on the one hand and the proportions of COOH and COO^- in equilibrium in the solution on the other. For ^{13}C products in natural abundance where the ^{13}C signal of C_0 is difficult to observe, the value $\Delta\delta C_0$ can be obtained by using that of $\Delta\delta C_\alpha$; knowing δC_α it is hence possible to calculate δC_0.

Direct Determination of Enantiomeric Compositions with Optically Active Nuclear Magnetic Resonance Lanthanide Shift Reagents[1a,2]

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Abstract: Tris(3-trifluoroacetyl-*d*-camphorato)europium(III), Eu(facam)$_3$, tris(3-heptafluorobutyryl-*d*-camphorato)-europium(III), Eu(hfbc)$_3$, and the corresponding praseodymium analogs, Pr(facam)$_3$ and Pr(hfbc)$_3$, are useful nmr shift reagents for direct determination of enantiomeric compositions. These optically active shift reagents are applicable to those classes of compounds that respond to conventional shift reagents such as Eu(dpm)$_3$ and Eu(fod)$_3$. In the presence of the above optically active lanthanide chelates, enantiomers have nonequivalent nmr spectra, and shift differences for enantiotopic protons of over 1 ppm have been observed. Generally, shift differences are large enough for complete separation of at least one set of enantiotopic signals, and enantiomeric compositions (optical purities) can be determined directly from relative peak areas. The hfbc chelates induce larger shifts than the facam chelates. However, shift differences for enantiomers are not consistently larger for a particular reagent. Also, magnitudes of nonequivalence vary with the shift-reagent-substrate ratio in unpredictable ways.

The use of optically active nmr lanthanide shift reagents for direct determination of enantiomeric compositions has been the subject of several recent communications.[3-6] We now complete our initial report[4] and present additional pertinent results of our work in this area.

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(2) Presented in part at the 165th National Meeting of the American Chemical Society, Dallas, Texas, April 1973.

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