O-benzyltyrosylisoleucylleucylasparaginyl-S-benzylcysteinylprolylleucylglycyl-nitrated resin.

Dry ammonia was bubbled into a stirred suspension of the protected polypeptide-nitrated resin in 100 ml of anhydrous methanol at 0° until the solution was saturated with ammonia. The reaction mixture was then stirred overnight at 2°, and the solvent and ammonia were carefully removed by evaporation *in vacuo*.

The resulting dry powder was stirred with 150 ml of dimethylformamide at 110° for 20 min. The resin was then filtered off and washed with two 10-ml portions of dimethylformamide. The combined filtrate and washings were evaporated to dryness *in vacuo*, and the resulting oil was triturated with water. The product was filtered off and washed with methanol to give a pale tan powder (0.55 g). Precipitation from a dimethylformamide solution with ethanol gave 0.49 g of the protected polypeptide amide as a white powder, mp 256–258° (dec), $[\alpha]^{24}D$ −42.7° (*c* 0.5, dimethylformamide).

Anal. Calcd for $C_{65}H_{88}N_{10}O_{11}S_2$: C, 62.5; H, 7.10; N, 11.2. Found: C, 62.1; H, 7.09; N, 11.0.

[1-Deamino,4-isoleucine]-oxytocin was prepared from the preceding compound (250 mg) by the same procedures used for the preparation of this analog from the protected polypeptide that had been synthesized by the *p*-nitrophenyl ester method. The analog was obtained as a white powder; yield, 50.1 mg, $[\alpha]^{24}D$ −88.2° (*c* 0.5, 1 *N* acetic acid).

Anal. Calcd for $C_{44}H_{68}N_{10}O_{11}S_2$: C, 54.1; H, 7.01; N, 14.3. Found: C, 54.0; H, 7.02; N, 13.9.

Acknowledgments. The authors thank Mr. Joseph Albert for the elemental analyses, Mr. Roger Sebbane for the amino acid analyses, and Miss Margitta Wahrenburg and Mrs. Jessie Lawrence for the bioassays, under the direction of Dr. W. Y. Chan.

Carbon-13 Magnetic Resonance Studies of Amino Acids and Peptides. II

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Abstract: Carbon-13 (C-13) nuclear magnetic resonance studies of C-13 enriched (15%) amino acids are reported. The amino acids were isolated from C-13 enriched algae grown on $^{13}CO_2$. The spectrometer used operates either in the continuous wave or pulse mode. In the latter mode, the Fourier transform of the C-13 resonances is obtained. The C-13 nuclei were generally noise decoupled from the protons. The observed chemical shifts are compared with those predicted on the basis of empirical rules. The carbon chemical shifts in parts per million are more than an order of magnitude larger than the corresponding proton shifts of the amino acids.

A number of proton magnetic resonance (pmr) studies of enzyme conformation and enzyme-inhibitor interactions have recently been reported.[2–7] Interest in the pmr technique has been stimulated by new instrumental developments, particularly the availability of commercial high-resolution superconducting magnets operating at *ca.* 52,000 G. Proton resonance spectra of proteins are difficult to interpret. The number of protons per enzyme is very large, while the intrinsic line widths are generally broad, exceeding 10 Hz, and the chemical shift spread is relatively small. In an effort to circumvent these difficulties, Jardetzky, *et al.*,[7] and Katz, *et al.*,[8] have prepared deuterated enzymes which contain one or more amino acids in the normal proton form. This approach gives significantly simplified pmr spectra and permits studies not otherwise possible.

It would appear that C-13 labeling of proteins offers an alternate or complementary approach. One expects large chemical shifts and narrow line widths relative to the pmr case. C-13 labeling is a positive approach as contrasted with deuterium labeling[7,8] in the sense that its own resonance is observed. Also relatively small quantities of isotopic material are required. The range of systems which can be potentially C-13 labeled is large, and include such diverse systems as tissue cultures and bacteria.[9]

In this paper we present magnetic resonance data of most of the naturally occurring amino acids using direct C-13 detection at 15 MHz. This work is an extension of an earlier indirect, *i.e.*, INDOR study of the C-13 resonances.[10] Measurements were done mainly in D_2O under high-resolution conditions using noise[11] or coherent decoupling of the protons from the carbons. No carbon–proton coupling constants are reported. Both continuous and pulse techniques were employed. The results are contrasted with the pmr measurements at *ca.* 52,000 G.[12]

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Experimental Section

A. **Isolation and Characterization of C-13 Amino Acids.** *Chlorella vulgaris* was grown on C-13 enriched (*ca.* 15%) CO_2 as the sole carbon source. Lyophilized algae (10.7 g) were hydrolyzed to a protein fraction, and then to an amino acid mixture (4.9 g) by the TCA-6 *N* HCl method previously developed.[13] The mixture was dissolved in 2 *N* HCl (30 ml) and absorbed onto a column (6 ft × 2 in.) of Bio-Rad AG 50 W×4 (−400 mesh) resin and separated using volatile pyridine buffers.[14] A Technicon amino acid autoanalyzer system was used to monitor the amino acids in the effluent. The separation was carried out as previously described.[13] Samples of 20-μl volume were used to determine the amino acid content of a single fraction using thin layer chromatography (tlc) on silica gel. Fractions were pooled to give 3.1 g of separated amino acids as shown in Table I. Materials were worked up by concentration to small volume under reduced pressure followed by precipitation with alcohol–ether mixtures, except that tyrosine was filtered directly from the aqueous concentrate. All products were characterized by mixed chromatograms on tlc, and trimethylsilyl derivatives were prepared and analyzed by combined gas–liquid partition chromatography–mass spectrometry. The carbon-13 content obtained was 14.6 ± 1.0%. Details will be reported elsewhere.[15]

Table I. Yields of C-13 Enriched Amino Acids

Amino acid	Fraction	Weight,[a] mg	Amino acid purity, %
Asp	145–158		100
Asp, Pro	159–163		
Pro, Asp	164–175	24	95, 5
Asp, Pro	210–218	26	60, 40
Asp, Pro	219–230		
Asp, Pro, Thr	231–243	223	11, 70, 19
Thr, Asp	244–251	136	70, 30
Thr, Ser, Asp	252–259	92	71, 6, 23
Ser	260–280	150	100
Glu	300–334	336	100
Ala	355–395	453	95
Gly, Ala	396–403	121	75, 25
Ala, Gly, Val	404–408	69	2, 49, 49
Gly, Val	409–419	114	75, 25
Val	620–650	223	100
Met, Ileu	535–545	89	50, 50
Ileu, Leu	546–555	200	69, 31
Leu	556–566	270	100
Phe	656–665	248	100
Tyr	676–688	146	100
Lys	720–759	200	100
Lys, His	760–765	264	90, 10
His	766–771	48	100

[a] Total yield 3.2 g (70%).

The separation was generally complete except in one instance where methionine was not separated from isoleucine. This presented little or no difficulty in the measurements. No tryptophan, crystein, or cystine were isolated because the algae proteins had relatively few such residues. No glutamine or asparagine were obtained because these were hydrolyzed to glutamic and aspartic acid, respectively. The C-13 resonances of these five amino acids are not reported.

The C-13 enriched amino acids were further treated by passing aqueous solutions through a Chelex 100 (Calbiochem) resin bed to remove paramagnetic ion impurities. Several of the solutions were weakly colored, generally light brown, because of the presence of variable small amounts of nonparamagnetic[16] organic impurities, presumably pyridine by-products, introduced earlier during the chromatographic separation. No effort was made to eliminate these colored impurities as such efforts would have resulted in a reduced yield of amino acids. The magnetic resonance measurements were generally done at neutral pD and at 25° on saturated solutions in D_2O, when sufficient material was available, using 5-mm sample tubes containing 0.3–0.5 ml of liquid. All pD adjustments were done on a Beckman Zeromatic pH meter. The usual reading corrections were made for the D_2O solutions [*i.e.*, pD = pH(meter reading) + 0.4].

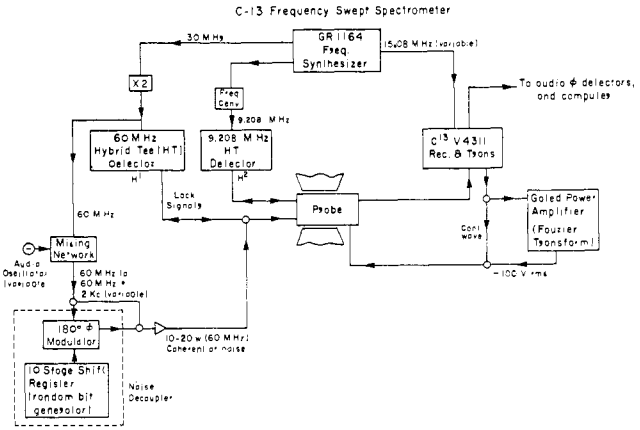

Figure 1. The experimental setup for direct observation of the carbon-13 magnetic resonances at *ca.* 14,000 G.

B. **Equipment and Operational Details. Continuous Mode.** The C-13 spectrometer was built in this laboratory. The experimental setup is illustrated in Figure 1.[17] The operation of the spectrometer in the continuous mode is briefly as follows. A General Radio frequency synthesizer, GR1164, was used in conjunction with several frequency divide, multiply, and sum networks to generate all the frequencies required. The magnetic field was stabilized by locking to an internal deuterium resonance, *i.e.*, to D_2O in this study. The lock detection system made use of a Relcom hybrid H_2 Tee detector[18] as a balanced bridge. A 2.5-KHz audiofrequency was used to modulate the magnetic field. The upper first sideband resonance of D_2O at 9.208000 MHz was used as the lock signal. The D_2O line width was *ca.* 0.7 Hz at room temperature. The instrument has the capability of stabilizing the field by locking either on a proton, fluorine, or phosphorus resonance, if required. The deuterium lock, however, was the most convenient and provided excellent stabilization.

The Varian 15-MHz V4311 unit was modified so that the transmitter output was a variable frequency derived from the synthesizer. This ratio frequency sweep mode of operation was very convenient, allowing easy manipulation of scan rates and widths with either manual or computer control. The frequency was measured with a Monsanto counter, Model 1120. A small field modulation[19] β factor was used and the C-13 resonances in the lower first side band were detected using a PAR H8 audio phase detector. The longitudinal relaxation times, T_1, were longer than the transverse relaxation times, T_2. This is often the case in C-13 nmr of small molecules. Intermediate passage conditions[20] were therefore used to observe the resonances so as to increase the detection sensitivity. The frequency was swept in both directions, and the average of the resonance positions was taken. The line widths were passage broadened by 1–3 Hz. The amino acid lower side band resonances varied from 15.084850 to 15.087500 MHz in this study and the resonance positions are believed to be accurate to at least ±2 Hz. The spectra were accumulated in a Varian C1024 computer of average transients.

A 60-MHz noise generator decoupled all protons simultaneously from the carbons. This was done to simplify the C-13 spectra and to improve the signal-to-noise ratio.[11,21] We found it necessary to wind a separate set of coils in a special configuration[22] in order to

(13) J. S. Cohen, I. Putter, and O. Jardetzky, in preparation.
(14) J. Liebster, K. Kopoldova, and M. Dobiasova, *Nature*, **191**, 1198 (1961).
(15) W. VandenHeuvel and J. S. Cohen, in preparation.
(16) This was ascertained by electron spin resonance measurements on the samples.
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(18) M. Klein and D. Phelps, *Rev. Sci. Instru.*, **38**, 1545 (1968).
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(21) R. Ernst, *J. Chem. Phys.*, **45**, 3845 (1966).
(22) R. H. Lyddane and A. E. Ruark, *Rev. Sci. Instr.*, **10**, 253 (1939).

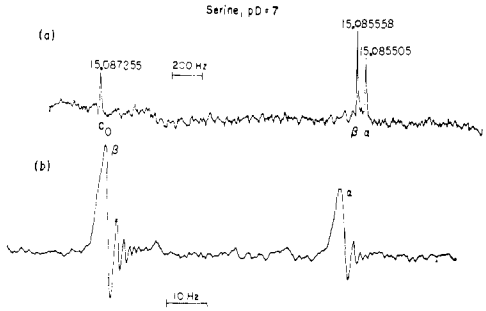

Figure 2. The accumulated noise-decoupled continuous mode spectra of 0.5 *M* (15% C-13 enriched) serine, neutral pD. (a) A 2000-Hz scan showing all three carbon resonances (*ca.* 100 accumulations, 30 min, 0.03-sec filter time constant); (b) a 120-Hz scan showing the α- and β- carbon resonances on an expanded scale (27 accumulations, 4 min, 0.03-sec filter time constant).

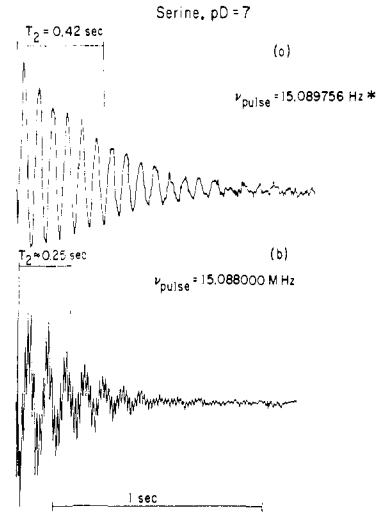

Figure 3. (a) The pulse mode spectra of the C_0 region of the same serine sample. The $\pi/2$ pulse was 14 Hz downfield of the C_0 resonances as indicated by the free induction decay pattern. (b) The α and β resonances of serine (100 accumulations, 8 min, 0.01-sec filter time costant). The $\pi/2$ pulse was 10 and 68 Hz upfield of the α and β resonances, respectively, as indicated by the free induction decay pattern. * Unit should be MHz.

produce a large, highly uniform decoupling field across the sample. With these coils we were able to generate a 2-G radiofrequency field at the sample when using a 10-W coherent 60-MHz source. The noise generator was essentially a ten-stage shift register constructed from ten Motorola MC 790P Dual J–K Flip-Flops with a module 2 adder, and was similar to the design outlined by Ernst.[21] The output consisted of a pseudorandom set of signals of the type ···010010···, where "0" and "1" denote, respectively, the absence or presence of a pulse which had a duration determined by a clock. (This clock frequency determined the proton range which could be simultaneously decoupled from the carbons.) We used the pseudorandom generator in conjunction with an HP 1054A mixer to phase modulate a 60-MHz carrier. (The "1" resulted in a 180° phase shift, whereas "0" left the original phase unchanged.) We could achieve complete and simultaneous decoupling of all protons with 10 W of noise into the proton center band, and a clock frequency of 1.3 KHz. A 60.000900-MHz carrier, which was *ca.* 5 ppm downfield of DSS [3-(trimethylsilyl)-1-propane sulfonate], was used. The decoupled C-13 resonances experienced a residual line broadening that was less than 0.3 Hz. The sample was air cooled to keep the temperature constant at *ca.* 25°.

Pulse Mode. Often it was more convenient to use a pulse mode of operation rather than to sweep with a continuous C-13 frequency. This was the case when the resonances were particularly weak. In this mode a pulsed frequency was used to rotate the C-13 magnetization $\pi/2$ radians from the equilibrium orientation along the magnetic field. The resulting free induction decay was accumulated in a Varian C1024. The frequency chosen to be pulsed depended on the region to be examined. For technical reasons, the C-13 center band, rather than the side band, was pulsed and the *unmodulated* centerband was detected, amplified, and accumulated. The pulsed frequency was derived from a gated power amplifier capable of putting out 300 Vrms of 15 MHz into the Varian transmitter coils. The pulse width was *ca.* 30–40 μsec, the period was 5–10 sec, depending on the T_1 relaxation times.

The free induction decay is the Fourier transform in time of the frequency signal.[20,23,24] We could use the pulsed spectrometer as described to detect simultaneously all resonances within several hundred hertz of each other in a time approximately equal to T_1 (*i.e.*, 5–10 sec). No loss of sensitivity relative to the continuous mode was experienced. In addition accurate line widths were obtained since passage broadening was eliminated.[20] Furthermore, the free induction decay of a weak signal could be more readily recognized than the corresponding absorption spectrum even though the two methods might give very comparable signal to noise after identical accumulation times. The lock and decoupling procedure described earlier was also used in the pulse mode.

C. Chemical Shifts. In paper I[10] we used a double resonance technique to determine that the α carbon of a saturated solution of glycine at room temperature and neutral pD had a chemical shift 86.5 ± 0.1 ppm above benzene. In this paper we report all chemical shifts relative to the glycine α carbon. The chemical shifts were determined by the following procedure. The (lower first side band) resonance frequency, ν_{cg} = 15.085286 MHz, of the glycine α-carbon reference solution was directly measured as described in the above section. The magnetic field was locked to the D_2O resonance. The glycine sample was replaced by the amino acid sample of interest, and the field was relocked to D_2O. The carbon resonance frequencies, ν_c, of the new sample were measured. The relative shift in parts per million is given by $10^6(\nu_{cg} - \nu_c)/(15.085$ MHz) plus a small correction which was never more than ±0.1 ppm. This small correction had to be made because the D_2O resonance shifted[25] with sample.

Results

The continuous and pulse mode, noise-decoupled spectra of a *ca.* 0.5 *M* neutral pD solution of serine, one of the more soluble amino acids, are shown as examples in Figures 2 and 3. The continuous mode spectrum (Figure 2b) and pulse mode spectrum (Figure 3b) were accumulated in approximately the same time and cover approximately the same frequency range. Intermediate passage[20] was used in the continuous mode case, and the spectra (Figure 2) show passage broadening and shifts of the order of a few hertz. Attempts to obtain accurate line widths, *i.e.*, slow-passage line widths, generally resulted in an appreciable signal-to-noise reduction. In the pulse mode (Figure 3) the free induction decay signal is a superposition of exponential decaying sinusoidal frequencies. The sinusoidal frequencies observed are equal to the frequencies of the resonances relative to the 30 μsec $\pi/2$ pulse frequency. The exponential decay times, T_2, determine the line widths, $\Delta\nu$, at half-height [*i.e.*, $\Delta\nu = (\pi T_2)^{-1}$]. The pulse mode line widths are observed to be narrower than

(23) A. Abragam, "Principles of Magnetic Resonance," Oxford University Press, London, England, 1961, pp 114.

(24) R. Ernst and W. A. Anderson, *Rev. Sci. Instr.*, 37, 93 (1966).

(25) The D_2O shifts were determined by the following procedure. DSS was added to each sample, and the pmr spectrum was measured on a Varian T-60. The chemical shift between the residual HDO in the sample and the methyl protons of DSS was recorded for each sample. The HDO resonances in all samples differed by less than ±0.1 ppm from the HDO resonance (τ 4.90) of the reference glycine solution. The D_2O resonances were assumed to undergo the same parts per million shift relative to the D_2O of the glycine reference as was observed for the HDO protons. It is known from the deuterium work reported by P. Diehl and T. Leipert [*Helv. Chim. Acta*, 47, 545 (1964)] that within a ±0.06-ppm experimental error the relative shift in parts per million between proton resonances are the same as the corresponding shifts of the deuterium isotope.

the continuous mode since, as we stated previously, passage broadening is eliminated.

The C-13 resonance positions of the 15 amino acids measured at neutral pD are summarized in Table II. The calculated values are in parentheses. The positions are displayed in Figure 4a, and can be contrasted with the corresponding proton resonances as reported by McDonald and Phillips[12] (Figure 4b). The C-13 chemical shifts in parts per million are observed to be more than an order of magnitude larger than the corresponding proton shifts. Thus, the C-13 chemical shift frequencies in hertz at 14,000 G are comparable to or generally larger than the proton shifts at 52,000 G.

The carboxyl resonances were observed to fall in a small region extending from −140 to −130 ppm below the α carbon of glycine. Generally, the amino acids which have a β carbon were observed to have very similar carboxyl resonances. The γ and δ carboxyls ($C_{0,\gamma}$, $C_{0,\delta}$) of aspartic and glutamic acid, respectively, occur −136.3 and −139.7 ppm below the α carbon in glycine. The resonances of the carboxyl group were measured as a function of pD. The resonances shift downfield with increasing pD, similar to previous observations for the α-carbon.[10] The total shift of the carboxyl is approximately 6 ppm over the pD range 1–9.

The C_β and C_δ carbons of phenylalanine and histidine, respectively, were not observed, possibly because of the very long T_1 relaxation times since these carbons have no bonded protons which can act as dipolar relaxers[26a] of the C-13 spin. The C_2 and C_4 carbons of the imidazole ring of histidine were also difficult to detect. This may be an indirect consequence of the fact that the N-14 nuclei bonded to these carbons have a quadrupole moment. (It is known that protons bonded to N-14 are often broadened).[26b] It is also possible that these ring carbons have long T_1 relaxation times. At this point, it is worth pointing out that no unusual difficulty was experienced in measuring either the carboxyls or the α carbons, even though the former have no protons bonded to them and the latter are bonded to N-14. The α carbons, however, were generally broader than the other carbons (Figure 2).

Discussion

The C-13 resonance positions of the amino acids can be estimated with considerable accuracy. In fact, the carbon resonances were identified and assigned in the majority of cases by comparing the observed resonances with the predicted resonances. The spread in chemical shifts were sufficiently large for this procedure to be practical and unambiguous. In some cases, however, coherent decoupling was found useful in confirming the assignment. The observed and calculated shifts are presented in Table II and are seen to be generally in good agreement. The resonance positions of five amino acids were not measured for reasons discussed earlier. However, reliable predictions as to their resonance positions can be made.[27]

(26) J. Pople, W. Schneider, and H. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, (a) p 307; (b) p 227.

(27) The chemical shifts of glutamine and asparagine are expected to be the same, within a few parts per million, as the shifts of glutamic and aspartic acid, respectively. The resonance positions of cysteine and cystine can be estimated using Table III and treating the sulfur atom as a carbon. The estimated tyrosine resonances are given in Table II.

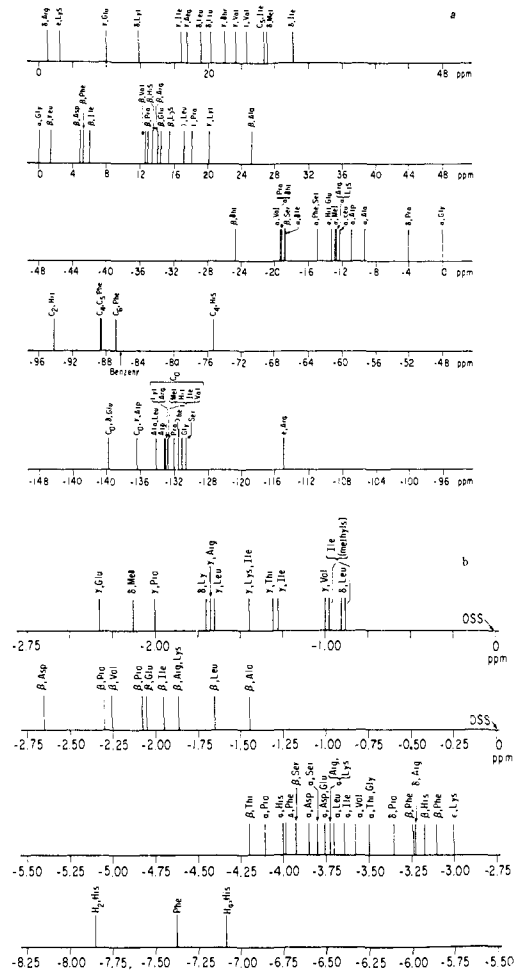

Figure 4. (a) The observed C-13 shifts of the amino acids, neutral pD, relative to the observed glycine α carbon (86.5 ppm upfield of benzene). (b) The observed proton chemical shifts[12] of the amino acids at neutral pD. Proton spin–spin couplings are not included.

The expected C-13 resonances were calculated as follows. Grant and coworkers[28] showed that if one uses a suitable set of chemical shift parameters then (1) the resonances of many substituted alkanes can be predicted from the parent hydrocarbons; (2) the parent hydrocarbon resonances in turn can be derived as substituted methanes. For example, in the case of the alkanes, if a proton is replaced by a methyl group, one measures a 9 ppm downfield shift in the α carbon, a 9 ppm downfield shift in the β carbon, an ~2.5 ppm downfield shift for the γ carbon, and small downfield shifts for the δ and ϵ carbons. The methyl shift parameters are reproduced in Table II, and suffice for purposes of predicting the positions of a primary carbon bonded to a secondary or tertiary carbon, or a secondary carbon bonded to a secondary or tertiary carbon, or a secondary carbon bonded to a primary or secondary carbon. However, if one wishes to predict the resonances of (i) a secondary carbon bonded to a tertiary or quaternary carbon [denoted by 2° (3°) and 2° (4°), respectively]; or (ii) a tertiary bonded to a secondary or tertiary [denoted by 3°(2°) and 3°(3°), respectively], refinement corrective terms must be added.[28] These are indicated in Table III.

(28) D. M. Grant and E. G. Paul, *J. Am. Chem. Soc.*, 86, 2984 (1964).

Table II. Resonance Positions of the Amino Acids in Parts per Million Relative to the α Carbon of Glycine (Saturated Solution) in Neutral D_2O at 25°

Compound	C_0	α	β C_2[a]	γ C_3[a]	δ C_4[a]	ε C_5[a]	C_6[a]
Leucine ($NC_\alpha C_\beta C_\gamma C_\delta$)	−134.1	−12.2 (−10.0)	1.5 (1.7)	17.1 (18.6)	19.3, 20.4[b] (19.2)		
Isoleucine ($NC_\alpha C_\beta C_\gamma C_\delta$)	−132.7	−18.4 (−15.9)	5.4 (10.0)	16.8 (17.1)	30.2 (32.2)	26.6 27.4	
Valine ($NC_\alpha C_\beta C_\gamma$)	−132.8	−19.1 (−18.4)	12.3 (15.7)	23.4, 24.6[b] (24.9)			
Alanine ($NC_\alpha C_\beta$)	−134.3	−9.1 (−9.1)	25.2 (25.7)				
Threonine ($NC_\alpha C_\beta C_\gamma$)	−131.5	−19.0 (−19.0)	−24.6 (−23.6)	22.0 (24.3)			
Glycine (NC_α)	−131.0	0.0 [−86.5 ppm relative to benzene]					
Lysine ($NC_\alpha C_\beta C_\gamma C_\delta C_\epsilon N$)	−132.9	−12.8 (−12.6)	15.3 (10.4)	20.1 (19.2)	11.8 (10.8)	2.5 (2.2)	
Arginine ($NC_\alpha C_\beta C_\gamma C_\delta NC_\epsilon N$)[c]	−132.7	−12.6 (−12.9)	14.0 (13.1)	17.6 (15.1)	1.0 (−4)	−115.0	
Serine ($NC_\alpha C_\beta OH$)	−130.6	−14.9 (−15.4)	−18.8 (−21.5)				
Proline	−132.1	−19.1 (−20.0)	12.8 (12.7)	18.1 (18.0)	−4.0 (−3.0)		
Glutamic acid ($NC_\alpha C_\beta C_\gamma C_{0,\delta}$)	−133.1, −139.8 ($C_{0,\delta}$)	−13.2 (−12.3)	14.4 (15.0)	8.0 (8.6)			
Aspartic acid ($NC_\alpha C_\delta C_{0,\delta}$)	−133.0, −136.3 ($C_{0,\gamma}$)	−10.7 (−9.0)	4.9 (2.5)				
Methionine ($NC_\alpha C_\beta C_\gamma SC_\delta$)[d]	−132.8	−12.7 (−12.7)	11.5,[e] (10.9)	12.4[e] (15.0)	27.3 (28.2)		
Histidine	−132.4	−13.3	13.5, −94.7		−75.7	N.O.[f]	
Phenylalanine	−132.5	−14.8	5.0	N.O.[f]	−88.6 to −88.2		−87
Tyrosine	(−132.5)	(−14.8)	(5.0)	?	(−88)	(−75)	(−114)[h]

[a] With one exception (Isoleucine) the C_1, C_2... C_6 labeling is generally reserved for carbons on aromatic rings. [b] The methyl carbons are nonequivalent. [c] The $-NHC_6N_2$ was treated as a $-NHC(O)OH$ in calculating substituent shifts. This would seem justifiable since the C_ϵ is near that of the carboxyl. [d] The sulfur was treated as a carbon in calculating substituent effects. To a first approximation the sulfur atom has about the same electronegativity as carbon, and similar shift effects might be expected. This assumption appears to be correct. [e] The assignment is uncertain. [f] Not observed. [g] The calculated values are estimated from the observed phenylalanine (above) and phenol resonances [P. C. Lauterbur, *J. Am. Chem. Soc.*, **83**, 1846 (1961)]. [h] The calculated values are in parentheses.

The shift parameters for $-NH_2$, $-NH_3^+$, $-OH$, $-COOH$, and $-COO^-$ substitutions on hydrocarbons are reproduced in Table III. One suspects that refinement corrections analogous to the 2°(3°), 3°(2°), etc. corrections for the methyls should be made for these groups also. Unfortunately sufficiently detailed studies that

Table III. Chemical Shift Parameters

Substituent group	Shift parameters (ppm) for carbon positions α	β	γ	δ	Refinement corrections,[a] ppm 1°(3°)	2°(3°)	3°(2°)	3°(3°)
Methyl[a]	−9.1 ± 0.1	−9.4 ± 0.1	2.5 ± 0.1	−0.3 ± 0.1	1.2 ± 0.2	2.52 ± 0.2	3.7 ± 0.2	9.5
Alcohols[b]	−48.5	−10	5.7	−0.5				
Amines								
($-NH_2$)[b]	−29	−11.4	4.6	−0.6				
(NH_3^+)[c]	−26 ± 0.5	−7.5	~4.6					
Carboxylic acid								
(−COOH)[c]	−21.5	−2	2.5	−0.6				
($-COO^-$)[c]	−24.5	−3.5	~2.5					

[a] D. Grant and E. G. Paul, *J. Am. Chem. Soc.*, 86, 2984 (1964). [b] T. D. Brown, Ph.D. Thesis, University of Utah, Salt Lake City, Utah, 1967, pp 34. [c] W. Horsley and H. Sternlicht, *J. Am. Chem. Soc.*, 90, 3738 (1968).

would permit one to assign statistically valid corrections, as was done in the methyl case,[28] have not been reported in the literature.

We have found in the case of the amino acids that (i) the shift parameters for different substituent groups are to a good approximation additive; (ii) the refinement corrections deduced by Grant[28] for hydrocarbons also apply to the amino acids. Amino and carboxyl group bond linkages have to be counted for purposes of deciding whether a carbon is a primary, secondary, or tertiary carbon. For example, in the case of amino acids having β carbons, the α carbon must be regarded as a tertiary carbon. Thus in the case of leucine, a 3.7 ppm [3°(2°)] upfield correction must be made to the (unrefined) predicted α carbon resonance. Similarly, a 2.5 ppm [2°(3°)] upfield correction must be made to the (unrefined) predicted β-carbon resonance in order to obtain agreement between the observed and predicted resonances. In the case of valine, the α carbon must be treated as a tertiary carbon bonded to a tertiary β carbon, and the appropriate 3°(3°) correction made. These observations were general.[29] (iii) In the case of the α carbons, best agreement between the observed and predicted shifts are obtained if the predicted shifts are taken relative to the *predicted* glycine α-carbon position rather than relative to the observed α-carbon glycine position (predicted: 80.5 ppm above benzene assuming additivity of the chemical shift parameters;[10] observed: 86.5 ppm). For the other carbons one can use the shift parameters and refinement corrections directly to predict the resonance positions relative to the observed glycine α-carbon position. (iv) In the case of proline, which has a cyclic residue, best agreement was obtained by the following procedure. A particular carbon on the cyclic residue might have a carbon or nitrogen β to it when counting counterclockwise, and γ to it when counting clockwise. This carbon or nitrogen must be considered as a *β and γ* substituent. Thus, if the atom was a carbon rather than a nitrogen a −9.4 + 2.5 = 6.9 ppm shift contribution would be expected to a first approximation. The usual refinement corrections were then made. (v) In the case of the aromatic residues, as in histidine and phenylalanine, the observed imidazole[30] and toluene resonances[31] were used to assign the ring carbon resonances. (vi) The reasons for the observed differences among the carboxyl resonances are unclear. Studies by Stothers and Lauterbur,[32] and Maciel[33] indicate that to a first approximation the chemical shifts of the carboxyls correlate with electronic charge density changes at the carbon.

It is interesting to compare the proton and carbon resonances (Figure 4a, b). It is apparent that while the proton chemical shifts in parts per million an order of magnitude smaller than the carbon shifts, the pattern of the proton resonances is very similar to the carbon resonances. This suggests that, to a first approximation at least, it should be possible to predict the proton resonances using a set of parameters analogous to Table III. The usefulness of such a table, if one could be constructed, is expected to be less in the proton case than in the carbon case. The pmr chemical shifts are small, and the proton shift parameters would be a few tenths of 1 ppm or less. Accurate predictions based on such a set of shift parameters would be difficult since perturbations arising from pD changes, electric fields, neighboring ring current effects, and proton spin–spin couplings, can be relatively significant. Pullman, *et al.*,[34] have attempted to correlate the pmr of the amino acids to the charge density at the protons.

The present study of amino acid C-13 resonances was undertaken to provide a framework for further studies on C-13 enriched enzymes.[9] The measurements reported here were generally done at neutral pD where the amino acids exist as zwitterions. Nevertheless, aside from the obvious case of the carbonyl (carboxyl) carbons, we expect that the shifts reported here should approximate the shifts for a random-coil polypeptide in aqueous solution. Sequence effects on the shifts will most likely be less than a few parts per million. This is reasonable in terms of the relatively small δ and ε shift parameters (Table III). In addition, replacing the terminal carboxyl and amino groups by amide groups apparently does not significantly affect the α,β,... carbon resonances. For example, in triglycine, the α carbon of the middle unit was within 0.2 ppm of that observed[10] for the α carbon of glycine at neutral pD. The α carbons of the terminal units in triglycine are different by several parts per million presumably be-

(29) These refinement corrections were neglected for the α carbon in paper I. Consequently some of the predicted values in Table I of that study are in error.

(30) R. J. Pugmire and D. M. Grant, *J. Am. Chem. Soc.*, 90, 4232 (1968).

(31) P. C. Lauterbur, *ibid.*, 83, 1838 (1961).

(32) J. B. Stothers and P. C. Lauterbur, *Can. J. Chem.*, 42, 1563 (1964).

(33) G. Maciel, *J. Chem. Phys.*, 42, 2746 (1965).

(34) G. Del Re, B. Pullman, and T. Yonezawa, *Biochem. Biophys. Acta*, 75, 153 (1963). It is interesting to note that if one plots the observed C-13 resonances against the Pullman-Del Re carbon charge densities one obtains a straight line fit with a slope of 240 ± 40 ppm/electronic charge. This is very close to the value 150–200/electronic charge one expects both theoretically [J. Pople. *Mol. Phys.*, 7, 301 (1963–1964)] and empirically [H. Spiesecke and W. G. Schneider, *Tetrahedron Lett.*, No. 14, 468 (1961)] if the carbon resonances correlate with charge density.

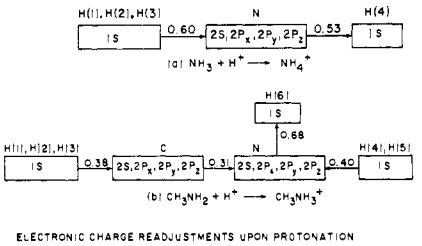

Figure 5. The electronic charge density readjustments upon protonation of the amino groups of ammonia and methylamine as calculated using Pople's CNDO/2 procedure.[38] The protonating hydrogens are denoted as H(4) and H(6) in a and b, respectively.

cause of charge effects.[10] In the case of the alanine residue in alanyl glycine at neutral pD, the shifts were within 1 ppm of that observed[10] for alanine at neutral pD. At this point it is perhaps worth emphasizing that while the sequence-induced shifts are expected to be small relative to the spread in the C-13 resonances of the residues, they most certainly would be large enough to be used for nondestructive sequencing of small peptides as has been done with pmr.[35]

It may eventually be possible to observe carbonyl group interactions in proteins, *e.g.*, helix or pleated sheet structure, using C-13 nmr. Maciel and Natterstad[36] have carried out a study of solvent effects on the $^{13}C{=}O$ resonances of ketones and esters. They reported large downfield shifts in the carbonyl resonances of ketones dissolved in hydrogen donating solvents. Smaller shifts were observed for the esters. It is pertinent to note that a 6- and 3-ppm downfield shift occurs in the carboxyl and α-carbon resonances, respectively, upon ionization of the carboxyl group.

The behavior of the α carbon[10] and the carboxyl carbon shifts upon ionization of the carboxyl is also interesting from the point of view of electronic structure. The α-shift parameters correlate with the electronic charge density[10] at the α carbon. The relatively small changes in the α carbon and carboxyl carbon upon ionization suggest that the carbon charge densities remain essentially constant. In paper I we also observed a small α carbon shift upon protonation of the amino group. The shift behavior of the α carbon upon protonating the amino group was analogous to that observed upon protonating methylamine to form methylammonium ion. We proposed that (1) transmission of electronic charge from the hydrogens of the amino acid to the incoming proton occurs through the carbon–nitrogen skeleton; (2) the electronic charge density at the carbons remains essentially constant (rearrangements of electrons in different orbitals can occur, but the overall carbon charge densities remain constant). This proposal was based on Clementi's observation[37] that an ab initio calculation of the charge densities of ammonia and ammonium ion gave almost identical electronic charge densities at the nitrogen.

We have repeated Clementi's calculation for ammonia and the ammonium ion using Pople's CNDO/2 procedure,[38] and have also calculated the charge densities in methylamine and methylammonium ion (Figure 5). The results for the ammonia–ammonium ion system are in good agreement with Clementi's.[37] Furthermore, the calculation supports our earlier proposal in paper I. The charge densities on the carbon remain essentially constant in the methylamine–methylammonium system. The methylamine protons transmit electronic charge through the carbon–nitrogen framework to the incoming proton. On this basis it would seem reasonable that a similar effect is occurring in the amino acids upon protonation as the C-13 measurements suggest.

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(35) M. Sheinblatt, *J. Am. Chem. Soc.*, **88**, 2123, 2845 (1966).
(36) G. Maciel and J. Natterstad, *J. Chem. Phys.*, **42**, 2752 (1965).
(37) E. Clementi, *ibid.*, **47**, 2323 (1967).
(38) J. A. Pople and G. A. Segal, *ibid.*, **44**, 3289 (1966).