Systematic Analysis of Chemical Shifts in the Nuclear Magnetic Resonance Spectra of Peptide Chains. II. Oligoglycines*

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ABSTRACT: Unequivocal assignments of glycine nuclear magnetic resonance peaks in oligoglycines are given, based on a comparative study of selectively deuterated oligoglycines. An erroneous earlier assignment (Mathur, R., and Martin, R. B. (1965), *J. Phys. Chem. 69*, 668) is thereby corrected. Two correction terms for the chemical shift additivity rules (Nakamura, A., and Jardetzky, O. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2212) are defined and their origin is traced to minor electron shifts in the oligopeptide chain. The nuclear magnetic resonance spectra also indicate that glycine oligomers in aqueous solution are *not* constrained to a small number of preferred conformations.

In the foregoing paper (Nakamura and Jardetzky, 1967) we have described certain general rules for the prediction of chemical shifts of glycine residues incorporated into a peptide chain, insofar as such rules become apparent from the study of dipeptides. In the present paper we have extended our inquiry into the physical nature of some of the more subtle effects and their dependence on the length of the peptide chain. For this purpose we have prepared a series of oligoglycines, selectively deuterated to avoid any ambiguity in the assignment. Our findings confirm the conclusions reached earlier on the additivity of chemical shifts resulting from different effects. They also show that the chemical shift of a given residue in a peptide chain is dependent on the state of ionization and, in some cases, on the structure of its nearest neighbor. In an extended peptide chain, the shift of a given amino acid is independent of its next nearest and further removed neighbors. These findings are of crucial importance for the interpretation of amino acid shifts in terms of the tertiary structure of proteins.

Experimental Section

Undeuterated glycylglycine (N, 21.1; triton B equiv, 125; perchloric acid equiv, 134), triglycine (N, 21.96; C, 37.98; H, 5.78), tetraglycine, and pentaglycine (N, 22.85) were purchased from Mann Research Laboratories, Inc., New York, and used without further purification.

For the preparation of the deuterated oligoglycines the *p*-nitrophenol active ester method was used. The *p*-nitrophenyl esters of CBZ-Gly (carbobenzoxyglycine), CBZ-Gly_2, and CBZ-Gly-Gly* (Gly* denotes glycine-CD_2) were synthesized by the condensation of CBZ-blocked compounds and *p*-nitrophenol with dicyclohexylcarbodiimide in methylene chloride or in dimethylformamide, according to the method reported by Rothe and Kuniz (1957) and Elliot and Russell (1957). The products were recrystallized from ethanol.

The coupling reactions were carried out following the procedure of Bodansky *et al.* (1957). The appropriate combination between a *p*-nitrophenyl ester of CBZ-Gly, CBZ-Gly_2, or CBZ-Gly-Gly*, and Gly*, Gly_2, Gly-Gly*, Gly_3, Gly-Gly*-Gly, or Gly-Gly*-Gly_2 (see Figure 1) was reacted in a mixed solvent of dioxane and water, triethylamine being used as a base. After the reaction was completed the mixture was cooled, filtered, and acidified by the addition of concentrated hydrochloric acid. Following filtration, the deuterated oligoglycine was recrystallized from hot water.

Unblocked deuterated oligoglycines were obtained by the catalytic hydrogenation of deuterated CBZ-oligoglycines. Palladium charcoal was used as a catalyst. All the procedures applied in the synthesis are listed in Figure 1, with yields and melting points of the products.

For taking nuclear magnetic resonance spectra, a solution containing 0.05 M concentration of a peptide was used whenever possible. Sample solutions were prepared as follows: 0.05 mmole of a peptide and approximately 0.004 mmole of DSS-H_2O[1] were dissolved in D_2O, 0.102 N DCl, or 0.102 N NaOD, yielding a zwitterionic, cationic, or anionic solution, and made up to a volume of 1 ml. As the solubility of the zwitterions of tetraglycine and pentaglycine, and their deuterated analogs, is very low, saturated solutions were used.

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[1] Abbreviations used: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate monohydrate.

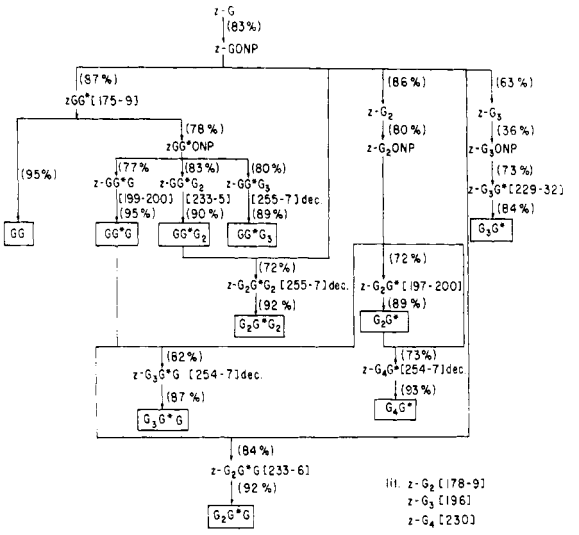

FIGURE 1: Flow diagram for the preparation of selectively deuterated oligoglycines.

The pD value of each sample solution of cations or zwitterions was measured by a glass electrode Beckmann pH meter Model G to ensure the ionic form of the solute. pD values of cationic solutions lay between 0.9 and 1.0, and those of zwitterionic solutions between 5.6 and 6.0. Estimating from the reported pK_a (COOH) values of 3.5–3.25 for these peptides (Cohn and Edsall, 1943), more than 99% of the solute molecules in these cationic solutions must exist as cations. From the reported isoelectric points of 5.4–5.6 (Cohn and Edsall, 1943) for the peptides, which is equivalent to 5.8–6.0 in deuterated systems, the observed pD's of 5.6–6.0 ensure that almost all the solute molecules were present as zwitterions. Approximate pD values of the anionic solutions were measured by pH test paper, which showed their pD's between 12 and 13. As the pK_a (NH_3^+) is 7.7–8.2 (Cohn and Edsall, 1943) for these peptides, the solute molecules must exist almost completely as anions.

The peptides are not very stable in alkaline solution. Accordingly the measurements of their spectra were completed within 2 hr after the preparation of the solutions. Sample solutions in trifluoroacetic acid were made by dissolving 0.05 mmole of a peptide in trifluoroacetic acid to a final volume of 1 ml.

All nuclear magnetic resonance measurements were made on a Varian Associates DP 60 high-resolution nuclear magnetic resonance spectrometer at room temperature. Chemical shifts were measured by the side-band technique, using a Hewlett Packard Model 200 CD wide-range oscillator and an HP 522B electronic counter. The chemical shifts are reported in cycles per second from DSS, which is used as an internal reference.

Results and Discussion

Sample spectra of deuterated tetraglycines are shown in Figure 2 and are compared to the spectrum of the fully protonated analog. The assignments are readily

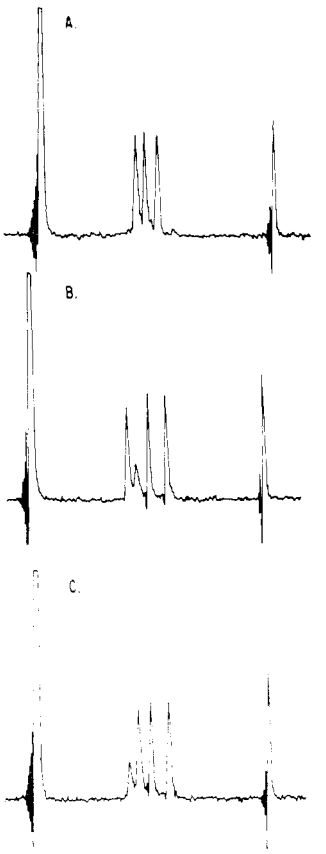

FIGURE 2: Nuclear magnetic resonance spectra of deuterated tetraglycines: (A) G-G-G-G*, (B) G-G-G*-G, and (C) G-G*-G-G.

made and generally confirm those reported previously on the basis of titration curves (Scheinblatt, 1966; Li *et al.*, 1962) but *not* those of Mathur and Martin (1965). Their advantage is that they completely eliminate the ambiguity which could exist even in the simple case of the glycylglycine dipolar ion (*cf.* Figure 3). The data on chemical shifts of all derivatives studied, in three ionic forms, are summarized in Table I and graphically represented in Figure 3.

The principal conclusions which emerge from a

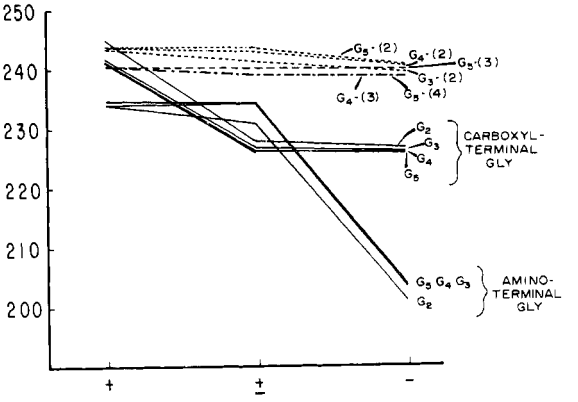

FIGURE 3: Titration shifts in glycine and oligoglycines. Chemical shifts of the glycyl CH_2 protons in Gly_2, Gly_3, Gly_4, and Gly_5. In cycles per second from DSS.

TABLE I: Chemical Shifts of the CH_2 Protons of Oligoglycines and Their Deuterated Analogs in D_2O (cycles per second from DSS, 60 Mcycles/sec, 30°).[a]

Ionic Forms	Cation (+)					Zwitterion (±)					Anion (−)				
Assignments	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Glycylglycine G_2	234.0	244.9				230.4	227.9				200.8	226.9			
GG*	233.9	—				231.3	—				201.2	—			
Triglycine G_3	233.9	243.1	241.7			234.4	242.1	227.0			203.6	239.6	226.3		
GG*G	234.1	(243.0)	241.8			233.8	—	226.6			203.5	(239.1)	226.2		
G_2G^*	234.5	243.5	—			234.0	241.7	—			203.4	239.7	—		
Tetraglycine G_4	235.0	244.2	241.7	241.7		233.7	242.6	238.6	226.1		203.3	240.6	238.6	226.1	
GG^*G_2	234.6	(243.5)	241.1	241.1		234.5	(243.3)	239.5	226.7		203.4	—	238.8	226.2	
G_2G^*G	234.6	243.6	—	241.4		234.4	243.4	(239.4)	226.6		203.4	240.6	(238.7)	226.0	
G_3G^*	234.9	243.4	240.7	—		234.1	243.1	239.1	—		203.8	240.7	238.6	—	
Pentaglycine G_5	234.7	243.8	240.9	240.9	240.9	235.8	243.6	240.0	240.0	226.4	203.6	240.7	240.7	239.1	226.3
GG^*G_3	234.5	(243.7)	240.5	240.5	241.3	233.6	—	240.4	238.9	226.0	203.1	—	240.0	238.8	226.0
$G_2G^*G_2$	234.8	244.1	(240.7)	240.7	241.6	233.9	244.1	—	239.2	226.3	203.7	240.9	—	238.9	226.2
G_3G^*G	234.9	244.0	240.9	(240.9)	241.6	243.4	243.4	240.1	—	226.1	203.5	240.4	240.4	(238.8)	225.9
G_4G^*	234.7	243.8	240.4	240.4	—	234.2	243.6	240.7	239.3	—	203.7	240.3	240.3	238.9	—

[a] G* denotes deuterated glycine residue; ( ) denotes that the intensity of the signal is very weak; — denotes that the intensity of the signal is too low to measure.

TABLE II: Proton Chemical Shifts of Oligoglycines in Trifluoroacetic Acid (cycles per sec from DSS, 60 Mcycles/sec, 30°).[a]

	CH_2				NH_3^+, NH			
Assignments	1	2	3	4	1	2	3	4
Glycylglycine	254.1 (q)	258.3 (d)			445.8 (b)	470.0 (t)		
Triglycine G_3	254.5 (q)	259.9 (d)	256.2 (d)		442.9 (b)	482.5 (t)	458.5 (t)	
G_2G^*	254.4 (q)	260.0 (d)	—		442.7 (b)	482.9 (t)	458.0 (s)	
Tetraglycine G_4	254.2 (q)	260.1 (d)	256.7 (d)	256.7 (d)	442.1 (b)	480.0 (t)	473.2 (t)	458.7 (t)
GG^*G_2	254.1 (q)	—	256.7 (d)	256.7 (d)	442.1 (b)	479.5 (s)	473.4 (t)	458.7 (t)
G_2G^*G	253.9 (q)	260.0 (d)	—	256.4 (d)	442.0 (b)	480.8 (t)	473.0 (s)	459.2 (t)
G_3G^*	254.4 (q)	260.4 (d)	257.7 (d)	—	442.8 (b)	480.8 (t)	474.1 (t)	459.3 (s)

[a] s, singlet; d, doublet; t, triplet; q, quartet; b, broad. (—) denotes intensity of signal too weak to measure.

TABLE III: The Effect of Temperature on the Chemical Shifts of CH_2 Protons of Zwitterionic Tetraglycine in D_2O (cycles per sec from DSS, 60 Mcycles/sec).

Assignment Temp (°C)	1	2	3	4
9.0	234.4	243.3	239.6	227.1
30.5	234.4	243.4	239.4	226.6
76.1	234.2	243.0	238.7	226.0
97.6	233.9	*a*	238.4	225.1

[a] Intensity too weak to measure.

scrutiny of these data may be summarized as follows. (1) The chemical shifts representing different effects are additive, confirming the conclusions drawn from the study of dipeptides (Nakamura and Jardetzky, 1967). Thus, for example, the chemical shifts of glycylglycine may be considered as the shift of the zwitterionic amino acid plus a sum of the appropriate N-terminal (or C-terminal) peptide and titration shifts along with N′-terminal or (C′-terminal) shifts reflecting the titration of a neighboring residue. To this sum must be added a small correction (*c*), $\Delta_{cN'}$ or $\Delta_{cC'}$ ($\sim$0.012 ppm), which seems to arise because a group affecting the electron density of its neighbor does so at the expense of its own electron density. Corresponding to the shifts $\Delta_{tC'}$ and $\Delta_{tN'}$, which modify the chemical shift of a given residue according to the ionization state of nearest neighbor residues, we thus have small correction factors, equal to $\Delta_{tC'}$ and $\Delta_{tN'}$ but opposite in sign, which are to be applied to the chemical shift of the neighboring residue. These correction terms are given by

$$\Delta_{cN'}(\mathrm{Y}) = -\Delta_{tN'}(\mathrm{X}) \tag{1a}$$

for an R–Y–X peptide, and

$$\Delta_{cC'}(\mathrm{Y}) = -\Delta_{tC'}(\mathrm{X}) \tag{1b}$$

for an X–Y–R peptide.

TABLE IV: Chemical Shifts of the Carbobenzoxy Derivatives of Oligoglycines.[a]

Assignment	Phenyl	CH_2 (benzyl)	Gly(1)	Gly(2)	Gly(3)	Gly(4)	Gly(5)
			CBZ-glycine				
CBZ-G-O$^-$	445.6	307.8	220.3				
			CBZ-glycylglycine				
CBZ-G_2-O$^-$	445.8	309.1	232.6	225.0			
CBZ-GG*-O$^-$		308.9	232.0	—			
Av	445.8	309.0	232.3	225.0			
			CBZ-triglycine				
CBZ-G_3-O$^-$	445.8	309.0	234.2	237.1	224.0		
CBZ-GG*G-O$^-$		308.7	234.2	—	224.3		
CBZ-G_2G*-O$^-$		308.9	233.9	237.0	—		
Av	445.8	308.9	234.1	237.1	224.1		
			CBZ-tetraglycine				
CBZ-G_4-O$^-$	445.3	308.9	234.2	238.1	236.5	225.3	
CBZ-GG*G_2-O$^-$		308.6	234.3	—	236.5	225.5	
CBZ-G_2G*-O$^-$		309.0	234.5	238.8	—	225.5	
CBZ-G_3G*-O$^-$		308.4	234.3	238.1	236.5	—	
Av	445.3	308.7	234.3	238.3	236.5	225.4	
			CBZ-pentaglycine				
CBZ-G_5-O$^-$	445.5	308.6	233.9	237.6			224.7
CBZ-GG*G_3O$^-$		308.5	234.0	237.5			224.7
CBZ-G_2G*G_2-O$^-$		308.5	234.0	237.6			224.7
CBZ-G_3G*G-O$^-$		308.4	233.9	238.1			224.8
CBZ-G_4G*-O$^-$		308.9	234.4	238.2			—
Av	445.5	308.6	234.0	237.8			224.7

[a] (—) denotes intensity of signal too weak to measure.

The existence of these correction terms is clearly apparent from a comparison of the titration shifts for diglycine with those of other oligoglycines (Figure 3). (2) With this proviso, the titration shifts of either N-terminal or C-terminal residues are found to be independent of chain length, as are the peptide shifts. (3) Owing to the neighbor titration effect (eq 1), the resonances of residues adjacent to the N-terminal residue are shifted to lower fields by about 0.01–0.02 ppm and those adjacent to the C-terminal residue to higher fields by 0.005–0.01 ppm by comparison with the shifts of residues in the interior of the chain. As a result, the second residue from the N terminal is invariably found at lowest fields, when the amino terminal is charged. (4) The chemical shift of residues removed by more than one residue from either end is unaffected by the state of ionization of the peptide. For a peptide without any secondary structure the chemical shift of a residue A is given to a very high degree of approximation by

$$\delta_{\text{calcd, coil}} = \delta(\text{A})^{\pm} + \Delta_{\text{pN}} - \Delta_{t\text{N}} + \Delta_{t\text{C}} \qquad (2)$$

where $\delta(\text{A})^{\pm}$ is the shift of the zwitterionic form of the free amino acid, Δ_{pN} is the N-terminal peptide-bond shift, and $\Delta_{t\text{N}}$ and $\Delta_{t\text{C}}$ are the N- and C-terminal titration shifts. In a heteropolymer additional small terms may have to be added to account for the effects of the nearest neighbor residue especially if such a neighbor has an aromatic side chain (Nakamura and Jardetzky, 1967). In view of the finding discussed in the foregoing paper that there is a conformational component in the chemical shift of some dipeptides, there might be some doubt as to whether the correction shift (eq 1) is electronic or conformational in nature. The temperature independence of the shifts of glycine residues in the interior of an oligoglycine chain shown in Table III, speaks strongly against the existence of conformational contributions. The view that the correction shift represents direct electron withdrawal or donation between amino acid residues is further supported by the data in Table II, which show a close parallel between the shifts of the CH_2 protons and of the amide and amino protons, measured in trifluoroacetic acid, in which exchange is sufficiently slow so that the latter can be observed. Needless to say, the possibility of attributing the shift to the Pople (1958) mechanism (Nakamura and Jardetzky, 1967) is out of the question, since there are no asymmetric carbons involved. This should be borne in mind when considering similar chemical shifts in the oligopeptides of other amino acids.

Of some interest in the present context are the chemical shifts of the carbobenzoxy (CBZ) derivatives of oligoglycines given in Table IV. In all of these the terminal amino group is blocked by the CBZ group. The most interesting feature of these data is that there exists a characteristic blocking shift, Δ_{CBZ}, which is in sign and magnitude almost equal to the N-terminal peptide shift, Δ_{pN}, and independent of chain length beyond the tripeptide. This suggests to us that the C–N linkage in the CBZ derivative contains a fair amount of double-bond character. A comparison of the chemical shifts for the N-terminal residues in the di- and longer CBZ peptides serves to further confirm the additivity rules.

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