Intramolecular Microdynamical and Conformational Parameters of Peptides from ^1H and ^{13}C NMR Spin-Lattice Relaxation. Tetragastrin†

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ABSTRACT: Measurements of ^1H and ^{13}C spin-lattice relaxation and ^{13}C nuclear Overhauser enhancement factors are reported for dimethyl sulfoxide solutions of tetragastrin, a pharmacologically active tetrapeptide. The use of the dipolar formalism for predicting ^1H and quaternary ^{13}C relaxation rates is discussed. Furthermore, the prospect is opened for the use of quaternary ^{13}C and ^1H relaxation times to obtain information on the peptide torsion angles ϕ, ψ, and χ in a way supplementing NMR coupling constant methods presently in use.

The microdynamical and conformational characteristics of peptide hormones in solution are of considerable importance in the elucidation of structure-function relationships of these biologically significant molecules. Although a variety of nuclei have been used as nuclear magnetic resonance (NMR) probes, the great majority of studies continue to involve either ^1H or ^{13}C. Moreover, NMR studies which stress relaxation parameters increasingly involve ^{13}C as the probe nucleus, to an extent that ^1H NMR relaxation studies in peptide systems are becoming somewhat of an endangered species. The reason for this development is well known and quite simple. ^{13}C NMR spectra under conditions of proton noise decoupling are relatively easy to interpret, as compared to ^1H spectra, and hence provide relaxation data from which a wealth of information may be extracted. This is illustrated by the recent work of many authors (Keim et al., 1974; Deslauriers et al., 1975; Oldfield et al., 1975; Torchia et al., 1975).

Interpretation of relaxation data, both ^{13}C and ^1H, rests primarily on the theoretical formalism describing the motionally modulated interaction between two magnetic dipoles. This formalism has been developed to such an extent (Solomon, 1955; Woessner, 1962; Woessner et al., 1969; Doddrell et al., 1972) that with care it may be applied profitably in a wide variety of situations. There is of course no a priori reason to expect nondipolar relaxation mechanisms to be absent. Fortunately, however, the extent to which a dipolar mechanism is contributing to ^{13}C relaxation often may be assessed by measuring the nuclear Overhauser enhancement (NOE) which results upon application of proton noise decoupling power (Kuhlmann et al., 1970). Other methods exist (Cutnell et al., 1975), but convenience will probably ensure that NOE measurements continue to provide the main experimental approach to this problem.

In this paper we take the position that, given the dipolar formalism, relaxation studies of protonated carbon resonances are more profitably viewed as providing a point of departure, rather than a self-contained but limited approach with which to investigate complicated motional phenomena. From this point of view such studies provide initial information with which analysis of ^1H and nonprotonated ^{13}C relaxation data may proceed. In discussing this approach in some detail we wish: (1) to illustrate the general utility of, and some of the difficulties associated with, the use of data on protonated carbons to predict the relaxation behavior of ^1H and nonprotonated ^{13}C nuclei, and (2) to show that ^1H and ^{13}C relaxation studies have the potential to provide information concerning oligopeptide conformation (torsion angles) in a form which supplements that available from measurements of CH-NH vicinal proton coupling constants (Barfield and Karplus, 1969). In addition, our analysis provides a microdynamical description of the molecular species studied.

We focus our attention on tetragastrin, the C-terminal tetrapeptide, Trp-Met-Asp-Phe-NH_2, of gastrin. Since tetragastrin has the same range of pharmacological activity as gastrin itself (Tracy and Gregory, 1964), it is a peptide of special interest in biophysical studies. Our investigation is carried out in Me_2SO-d_6[1] solution.

Experimental Section

Materials. Tetragastrin was synthesized according to published procedures (Davey et al., 1966) and isolated as the gastrin tetrapeptide amide trifluoroacetate. Our material was homogeneous to greater than 95% by thin-layer chromatography in two solvent systems, as determined before and after NMR experiments.

Sample Preparation. The gastrin tetrapeptide amide trifluoroacetate was dissolved in 100% Me_2SO-d_6 to yield a 0.3 M solution of tetragastrin (free amide mol wt = 596). This operation, as well as all sample transfers, was performed under dry nitrogen to minimize the amount of H_2O contaminating the solvent. Dissolved oxygen was removed via four freeze-pump-thaw cycles. The sample was transferred to 5- and 10-mm o.d. tubes for ^1H and ^{13}C studies, respectively, and sealed with pressure caps. For proton studies 0.015 and 0.03 M samples were prepared in a similar manner (Cutnell and Glasel, 1976b).

NMR—General. All spectra used in this study were obtained with a JEOL-PFT-100 Fourier transform spectrometer

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[1] Abbreviations used are: TFA, trifluoroacetic acid; Me_2SO-d_6, perdeuteriodimethyl sulfoxide; Me_4Si, tetramethylsilane; NOE, nuclear Overhauser effect; o.d., outside diameter; NMR, nuclear magnetic resonance.

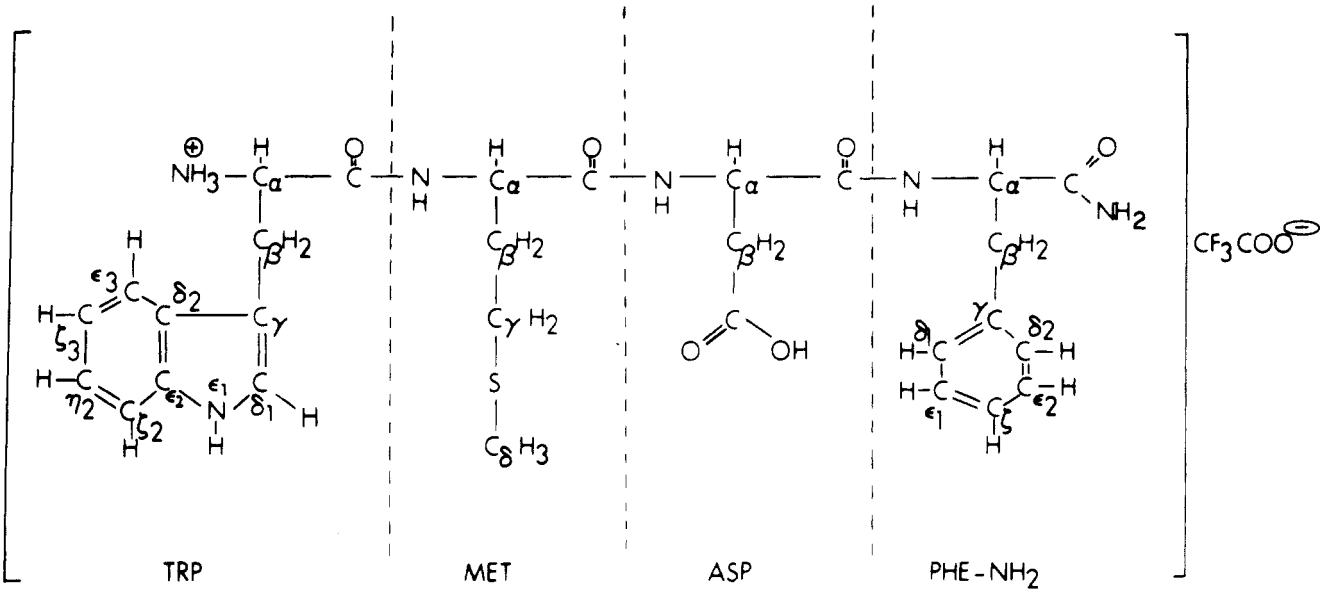

FIGURE 1: Primary sequence of amino acids in tetragastrin amide trifluoroacetate showing the nomenclature used for the four residues (IUPAC-IUB Commission, 1970).

using a disk storage system, employing an internal deuterium lock, and operating at 100.0 and 25.15 MHz for ^1H and ^{13}C, respectively. For all measurements of spin-lattice relaxation the standard 180°-τ-90°-t pulse sequence was used with the phase of the 90° pulse changed by 180° on every scan (Cutnell et al., 1976). Suppression of the water resonance, when necessary, was performed as described elsewhere (Bleich and Glasel, 1975). Temperature was controlled to ±1 °C via the standard JEOL temperature controller.

NMR—^{13}C. For ^{13}C spin-lattice relaxation measurements a bandwidth of 6.25 kHz was employed with 8192 data points. ^1H noise decoupling was used with a noise bandwidth of 2.5 kHz. The number of scans used per spectrum varied between 600 and 2284. With two exceptions the value of t used in the 180°-τ-90°-t pulse sequence was at least 3.5 times the measured spin-lattice relaxation time T_1 for all resonances. For the Phe γ and Trp δ_2 resonances the value of t was 3.3 and 2.8 times the measured T_1, respectively. T_1 values were determined via two parameter nonlinear regression analysis using a single exponential decay function (not its logarithm) to describe the approach of the magnetization to equilibrium. Magnetization was taken to be proportional to peak heights which were read manually from the recorded spectra.

Repeated 90° pulses were used to obtain the equilibrium spectra from which NOE factors were calculated. Such spectra were determined with a spectral bandwidth of 6.25 kHz and 8192 data points in the presence and absence of ^1H noise decoupling power (2.5-kHz noise bandwidth). That there was sufficient decoupling power was evident from studies we have carried out with much broader resonances (Cutnell and Glasel, 1976a). In the presence of noise decoupling power the time between 90° pulses was at least 3.4 times the longest T_1 characterizing the resonances. This value was increased to 4.8 times the longest T_1 in the absence of decoupling power in order to account for the nonexponential approach to equilibrium of the coupled ^1H-^{13}C spin system (Opella et al., 1976). The number of transients accumulated in determining these spectra was 8234. NOE factors were calculated from integrated intensities, these being determined by cutting out and weighing tracings (including noise) of the recorded spectra. This integration procedure was followed with at least two horizontal magnifications for each region of the spectrum integrated and the results were averaged after being weighted by the appropriate factor. This procedure was used to ensure that intensity was not being neglected in the wings of the resonances. Except for one deviation of 11% such multiple tracings yielded areas agreeing within ±9%. Since not all resonances are completely resolved even in the decoupled spectrum, individual resonances could not be separately integrated in the fully coupled spectrum. Only five separate regions of the spectrum could be separately integrated. These regions ranged from the most downfield (carbonyl) to the most upfield (methyl) resonances and gave five separate values of the nonenhanced integrated intensity per carbon atom. These values agreed to within ±6%, which indicates that our 90° pulse was sufficiently uniform throughout the spectrum to justify using an average of these five values as the nonenhanced intensity per carbon in NOE calculations. Where NOE values correspond to an average determined from the integrated intensity for a specific region of the enhanced spectrum, it is so indicated in our results, and reported values are accurate to ±10% (maximum deviation from the mean).

NMR—^1H. For ^1H spin-lattice relaxation measurements a bandwidth of 2.00 kHz was employed with 8192 data points. The number of transients accumulated per spectrum varied between 2 and 40. The value of t in the 180°-τ-90°-t pulse sequence was 17 s. Such a large value is necessary for the methyl protons of tetragastrin (Cutnell and Glasel, 1976b). With two exceptions, T_1 values were determined as described above for ^{13}C relaxation. For the unresolved α-^1H resonances of Met, Asp, and Phe it was necessary to use integrated intensities to ensure that a meaningful average T_1 was being determined. Secondly, for the methyl ^1H data it was necessary to employ a four-parameter nonlinear regression analysis using a weighted sum of two exponentials to describe the approach of the magnetization to equilibrium (Cutnell and Glasel, 1976b).

Results

^{13}C Assignments. The primary sequence of amino acids in tetragastrin is shown in Figure 1, together with the nomenclature (IUPAC-IUB Commission, 1970) used for the four

TABLE I: ^{13}C Chemical Shifts,[a] Spin-Lattice Relaxation Times,[b] and Nuclear Overhauser Enhancement Factors for Tetragastrin at 30 °C and 0.3 M in Me_2SO-d_6.

	Chemical Shift (ppm)	T_1 (s)	NOE
Trp α	52.5	0.0769 ± 0.0043	2.4[c]
β	27.3	0.0388 ± 0.0032	2.6[d]
γ	106.7	0.947 ± 0.069	2.1
δ_1	125.0	0.0864 ± 0.0031	2.7[e]
δ_2	127.0	1.87 ± 0.12	*f*
ϵ_2	137.7	1.34 ± 0.10	2.1[g]
ϵ_3, η_2	118.4	0.0856 ± 0.0040	2.7[e]
ζ_2	111.4	0.0874 ± 0.0071	2.5
ζ_3	121.0	0.0848 ± 0.0050	2.7[e]
Met α	51.9	0.0764 ± 0.0076	2.4[c]
β	32.2	0.0591 ± 0.0072	2.6[d]
γ	37.2	0.0562 ± 0.0077	*h*
CH_3	14.5	1.37 ± 0.09	2.0
Asp α	49.6	0.0735 ± 0.0060	2.4[c]
β	29.3	0.107 ± 0.009	2.6[d]
Phe α	53.9	0.0853 ± 0.0093	2.4[c]
β	35.9	0.0542 ± 0.0063	*h*
γ	136.2	1.60 ± 0.10	2.1[g]
δ_1, δ_2 (ϵ_1, ϵ_2)	129.0	0.248 ± 0.007	2.7[e]
ϵ_1, ϵ_2 (δ_1, δ_2)	128.0	0.246 ± 0.008	2.7[e]
ζ	126.1	0.125 ± 0.007	2.7[e]
C=O	172.6	1.13 ± 0.05	2.1[i]
C=O	171.8	1.30 ± 0.08	2.1[i]
C=O	170.4	0.875 ± 0.039	2.1[i]
C=O	170.0	1.09 ± 0.07	2.1[i]
C=O	168.4	0.900 ± 0.040	2.1[i]

[a] Measured ±0.06 ppm downfield from external tetramethylsilane. [b] (±) figures denote approximate 95% confidence limits (≈ two standard deviations). [c] Resonances integrated as a unit. [d] Resonances integrated as a unit. [e] Resonances integrated as a unit. [f] See results. [g] Resonances integrated as a unit. [h] Integration not possible due to proximity of solvent resonance. [i] Resonances integrated as a unit.

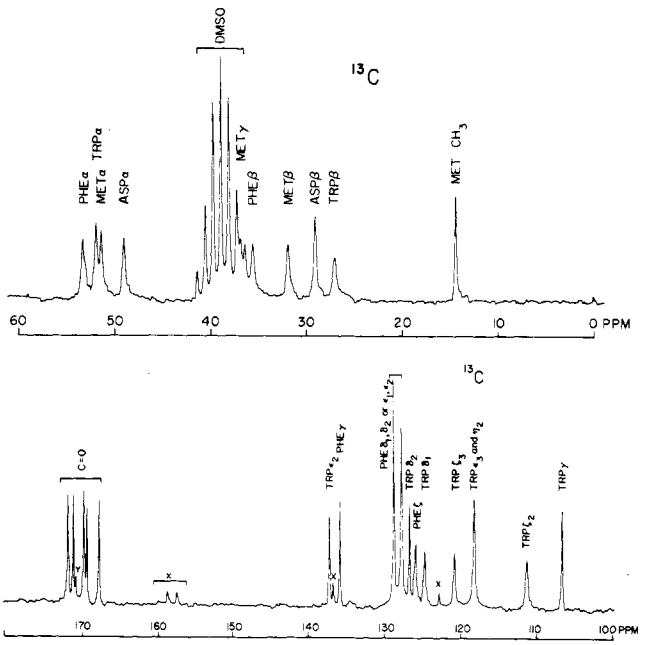

FIGURE 2: The 25.15 MHz ^{13}C spectrum of tetragastrin amide trifluoroacetate at 30 °C and 0.3 M in Me_2SO-d_6 (DMSO) (bandwidth = 6.25 kHz, number of transients = 1200, 90° pulse repetition = 5.0 s, number of data points = 8192). Chemical shifts are measured downfield with respect to external tetramethylsilane (TMS), whose resonance location was established using a separate sample containing a solution of Me_4Si in Me_2SO-d_6. X denotes resonances from trifluoroacetate. Y denotes unidentified resonance.

residues. The ^{13}C spectrum is shown in Figure 2, and the associated assignments are given in Table I.

With the exception of Trp β, the α and β resonances were assigned via selective heteronuclear spin decoupling using known proton assignments (see below). Since the corresponding proton resonances overlap too closely, it was not possible to use this procedure to distinguish between Trp β and Met γ. The Trp β resonance was therefore identified by using the known assignment for the free amino acid (Allerhand et al., 1973). This assignment places the resonance of Met γ at 37.2 ppm, which differs from the value of 29 ppm reported for model peptides in aqueous solution (Christl and Roberts, 1972). Our assignments, however, are supported by the observed relaxation times.

Since the five carbonyl carbon resonances are expected in the most downfield position, the four quaternary carbon resonances are immediately identifiable by their longer T_1 values and narrower line widths (Allerhand et al., 1973; Oldfield and Allerhand, 1975). Based on the assignments for the free amino acids (Christl and Roberts, 1972; Allerhand et al., 1973) and for *N*-acetylphenylalanine methyl ester (Johnson and Jankowski, 1972) the resonances at 106.7, 127.0, 136.2, and 137.7 ppm may be assigned to Trp γ, Trp δ_2, Phe γ, and Trp ϵ_2, respectively. We note that the T_1's of Trp γ and δ_2 are the smallest and largest tryptophan T_1's, respectively, in agreement with the results of Oldfield and Allerhand (1975). Data on appropriate model peptides conceivably could lead to interchanging the assignments of Phe γ and Trp ϵ_2. A resolution of the question is not possible without the aid of selectively deuterated tetragastrin analogues, but we note that our assignments lead to the result that the T_1 of Phe γ exceeds that of Trp ϵ_2, which is consistent with the observed (see Table I) larger T_1's for the protonated aromatic carbons of Phe as compared with protonated heterocyclic carbons of Trp.

Of the remaining resonances, that occurring at 111.4 ppm may be assigned unambiguously to Trp ζ_2 by comparison with the results for the free amino acid (Allerhand et al., 1973). Similarly, the resonance at 118.4 ppm may be assigned to Trp ϵ_3 and η_2 on the basis that its integrated intensity indicates the contribution of two carbons. In the free amino acid these resonances are resolved (Allerhand et al., 1973), with the resonance we measure falling (relative to Trp γ) between the two. The nearest unassigned resonance occurs 2.7 ppm further downfield and hence introduces no ambiguity in this assignment. The two resonances occurring at 128.0 and 129.0 ppm may be assigned to Phe δ_1, δ_2 and ϵ_1, ϵ_2 since the integrated intensities reveal the contribution of two carbons to each resonance. These assignments are confirmed by comparison with the results for the free amino acid (Christl and Roberts, 1972) and *N*-acetylphenylalanine methyl ester (Johnson and Jankowski, 1972). It is not possible to distinguish between Phe δ_1, δ_2 and ϵ_1, ϵ_2 on the basis of our data.

The remaining unassigned midfield resonances occur at 121.0, 125.0, and 126.1 ppm with a twofold ambiguity. Comparison with the free amino acid (Christl and Roberts, 1972) indicates that either of the two more downfield resonances may be assigned to Phe ζ. To eliminate this problem we note that the assigned protonated aromatic carbons of Phe have longer T_1's than the assigned protonated heterocyclic carbons of Trp (see Table I). Furthermore, all of the remaining protonated

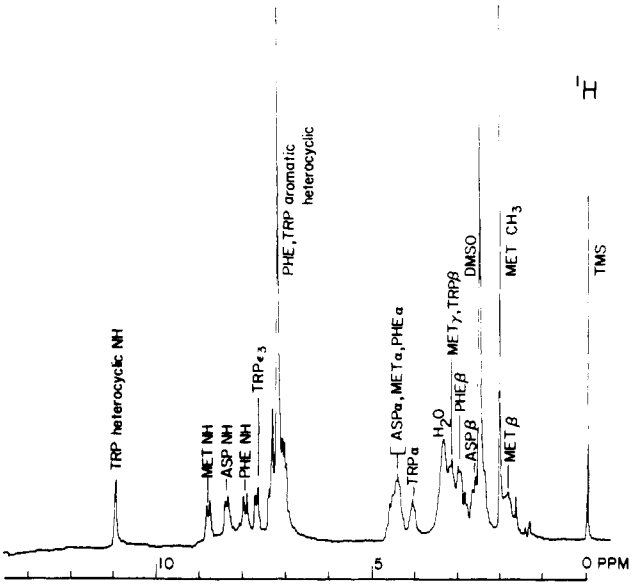

FIGURE 3: The 100.0 MHz ^{1}H spectrum of tetragastrin amide trifluoroacetate at 28 °C and 0.03 M in Me_2SO-d_6 (bandwidth = 2.00 kHz, number of transients = 30, 90° pulse repetition = 6.0 s, number of data points = 8192). Chemical shifts are measured downfield with respect to internal tetramethylsilane.

heterocyclic carbons have virtually identical T_1's of about 0.085 s (see Table I). On the basis of T_1 values, then, the resonance at 125.0 ppm may be assigned to either Trp δ_1 or Trp ζ_3, while that occurring at 126.1 ppm may be assigned to Phe ζ. Assignments of Trp δ_1 and Trp ζ_3 may be resolved by the following argument. Reference to the results for the free amino acid (Allerhand et al., 1973) indicates that resonances occur 14.5 and 17.6 ppm downfield from the Trp γ resonance and are assigned to Trp δ_1 and Trp ζ_3 with the ambiguity unresolved. Relative to Trp γ we find resonances downfield at 14.3 (121.0 ppm with respect to external Me_4Si) and 18.3 ppm (125.0 ppm with respect to external Me_4Si). The results for the free amino acid were obtained in aqueous solution, while our results are for a Me_2SO-d_6 solution. Because of the proximity of the indole NH, the Trp δ_1 resonance may be expected to exhibit a greater dependence upon solvent effects. With this point in mind the resonance found here to occur 14.3 ppm downfield from Trp γ may be assigned to Trp ζ_3, since it corresponds closely to the resonance found 14.5 ppm downfield from Trp γ in aqueous solution. On the other hand, the resonance found 18.3 ppm downfield from Trp γ may be assigned to Trp δ_1, since it exhibits a greater deviation from the resonance found 17.6 ppm downfield from Trp γ in aqueous solution.

We have not attempted a complete assignment of the carbonyl resonances. We do note, however, that the observed differences in T_1's (see Table I) provide some clues. The resonance at 171.8 ppm is characterized by a distinctly longer T_1 which is probably related to the greater side chain mobility of the Asp residue. Similarly, the remaining shorter and longer carbonyl T_1's may be possibly related to the two middle and terminal residues, respectively, on the basis that a greater degree of motional freedom is expected for the terminal residues. However, in view of the reduced NOE's observed for these carbons and in view of the absence of covalently bonded protons, the possibility of nondipolar relaxation mechanisms must be considered.

The two small peaks in Figure 2 near 122 and 137 ppm (X) are the central two resonances of the CF_3 carbon of trifluoroacetic acid (TFA). Similarly the two small peaks near 158 ppm (X) are the central two resonances of the TFA carbonyl carbon multiplet (Johnson and Jankowski, 1972). The small peak near 171 ppm is unidentified.

^{13}C Relaxation Times and NOE Factors. The T_1's determined for each resonance of tetragastrin are given in Table I. A single T_1 was found to be sufficient for characterizing the relaxation in each case. The (±) values shown in Table I are the approximate 95% confidence limits (corresponding to approximately two standard deviations), as determined by computer analysis of the raw data. The T_1's given reflect the results of nonlinear regression analysis using superimposed data from three different runs at the same temperature. It should be noted that it was possible to obtain T_1's for Met γ and Phe β even though the corresponding resonances are substantially obscured by the solvent resonances in Figure 2. This was because the relatively long T_1 of the Me_2SO-d_6 carbons resulted in the solvent resonances remaining inverted throughout the range of 180°-90° pulse intervals required to characterize the relaxation of Met γ and Phe β. Thus, the Met γ and Phe β resonances were easily identifiable in the partially relaxed spectra, although it is evident in Table I that the 95% confidence limits for the associated T_1's are somewhat larger on a percentage basis than for the other resonances.

The NOE factor for each resonance of tetragastrin is given in Table I, where we have been careful to indicate which NOE's were determined from integrating a particular region of the enhanced spectrum (containing several resonances). Attention should therefore be given to the footnotes appended to this table. It should be noted that NOE values are not given for the Trp δ_2, Met γ, and Phe β carbons. In the case of the Trp δ_2 resonance a separate integration could not be performed due to its proximity to other resonances in the region between 118.4 and 129.0 ppm. Since Trp δ_2 is a quaternary carbon, nondipolar interactions are expected to lower the NOE (see Table I, Trp γ, Trp ϵ_2, and Phe γ). Thus, the average NOE determined by integrating the above region of the enhanced spectrum (containing seven resonances) reflects primarily the NOE of the six protonated carbons. Considering the NOE's determined for the three other quaternary carbons in Trp and Phe, the error introduced by Trp δ_2 in the average NOE for these six protonated carbons is less than 3%. In the case of Met γ and Phe β, NOE values are not given in Table I because the proximity of these resonances to the solvent resonance (see Figure 2) prohibited accurate integration.

In determining the NOE factors we have ignored the effects of the small resonances from TFA and the small unidentified resonance since it is not possible to eliminate their contribution in the fully coupled spectrum. In any case it can be shown that they contribute an error which is less than the experimental error of ±10%.

^1H Assignments. The ^{1}H spectrum of tetragastrin is shown in Figure 3, and the associated assignments are given in Table II. These assignments were obtained via stepwise homonuclear spin decoupling of α-^{1}H resonances, using difference spectroscopy to aid in locating overlapping resonances. With the exception of that for the Met γ protons, the results given here agree with those of Feeney et al. (1972), who studied pentagastrin and several component peptides (excluding tetragastrin) in D_2O and Me_2SO-d_6. In the present study the Met γ protons resonate 0.6 ppm further upfield than those for the tripeptide Met-Asp-Phe-NH_2 in Me_2SO-d_6 (Feeney et al., 1972). The Met γ-^{1}H resonance was assigned by selective heteronuclear decoupling using the assigned Met γ-^{13}C resonance (see ^{13}C assignments). It should be noted that the

TABLE II: ^{1}H Chemical Shifts[a] and Spin-Lattice Relaxation Times[b] for Tetragastrin.

	Chemical Shift (ppm)	0.3 M T_1 (s)	0.03 M T_1 (s)
Trp α	4.06[c]		0.243 ± 0.022
β (Met γ)	3.1[d]		
Aromatic (Phe aro.)	7.22[c]	0.511 ± 0.012	0.724 ± 0.010
Indole NH	10.99[c]		
Met NH	8.82[c,e]		
α	4.4[d]		0.380 ± 0.025[g]
β	1.7[d]		
γ (Trp β)	3.1[d]		
CH_3	2.03[c]	0.741[f]	0.873[f]
Asp NH	8.39[c,e]		
α	4.5[d]		0.380 ± 0.025[g]
β	2.6[d]		
Phe NH	7.95[c,e]		
α	4.3[d]		0.380 ± 0.025[g]
β	3.0[d]		
Aromatic (Trp aro.)	7.22[c]	0.511 ± 0.012	0.724 ± 0.010

[a] Measured downfield from internal tetramethylsilane, on a 0.03 M sample at 28 °C. [b] Measured at 30 °C. (±) figures denote approximate 95% confidence limits (≈ two standard deviations). [c] ±0.01 ppm. [d] ±0.01 ppm. [e] Value given denotes center of doublet. [f] From initial slopes of nonexponential decay plots (Cutnell and Glasel, 1976b). [g] Determined from integrated intensities of these overlapping resonances (see Results).

chemical shifts given for the amide protons in Table II refer to the centers of the observed doublets, the NH splittings arising from interactions with the α-protons being 7.8, 7.3, and 7.8 Hz for Met, Asp, and Phe, respectively.

^1H Relaxation Times. The T_1's determined for various resonances of tetragastrin are given in Table II. With one exception a single T_1 was found to be sufficient for characterizing the relaxation of the resonances monitored. For the methyl resonance it was necessary to employ a weighted sum of two exponentials to characterize the relaxation in 0.015 and 0.03 M solutions in Me_2SO-d_6 (Cutnell and Glasel, 1976b). Such was also the case for the 0.3 M Me_2SO-d_6 solution, further confirming our earlier results. The methyl T_1's given in Table II are the average T_1's given by the *initial* slopes of the relaxation decay plots.

It should be noted that at 100 MHz it was not possible to determine the α-proton T_1's separately except for Trp α. In order to obtain a meaningful average T_1 from the overlapping Met, Asp, and Phe α resonances, integrated intensities were used in determining the relaxation decay plot. This was not done in the case of the aromatic resonance at 7.22 ppm even though it contains some contribution from both Trp and Phe since the broad resonances nearby prohibit a meaningful determination of integrated intensities. It should be noted further that the results in Table II for the 0.03 M solution do not reflect contributions from intermolecular interactions. Table II does indicate that such interactions come into play for concentrations between 0.03 and 0.3 M but intermolecular interactions are not important at 0.03 M since the results for a 0.015 and a 0.03 M solution are identical for the aromatic and -S-CH_3 T_1's (Cutnell and Glasel, 1976b).

Discussion

A. General. In this section we begin with a brief outline of the required dipolar formalism for nuclear spin relaxation. Following this outline, ^{1}H relaxation rates will be determined for various situations of interest. Similar calculations for nonprotonated ^{13}C nuclei will be then carried out. The uncertainty associated with the predicted relaxation times will reflect the experimental error in our initial ^{13}C data, propagated through the calculation to give the *maximum* error. During this discussion the question of peptide torsion angles will arise, but because of its importance it will be deferred and reconsidered separately in section F. As we proceed through this framework, a picture of the motional characteristics of tetragastrin will emerge, a summary of which will conclude our discussion. With respect to the main chain of tetragastrin we note that the overall molecular motion (of the peptide virtual bond structure) is isotropic. We are led to this conclusion by the near equality of the measured ^{13}C T_1's (after correcting for differences in number of bonded protons) for the α carbons and the protonated carbons of the Trp residue and the dipolar nature of the relaxation revealed by the measured NOE's (see Table I and section G). Since the various C-H vectors sample a wide variety of angles with respect to any potentially preferred axis for main-chain reorientation, anisotropic overall tumbling would have been apparent in sizable differences among the various T_1's, if it had been present. Thus, the dipolar relaxation formalism based on anisotropic overall tumbling will not be presented here (Woessner et al., 1969). We will point out, however, features of our data which indicate that analysis of certain side-chain motions potentially requires such a description of overall tumbling.

B. Theory. The dipolar formalism for nuclear spin relaxation, which is central to the interpretation of our data, is summarized below for both ^{13}C and ^{1}H. Equations 1-6 describe the relaxation parameters for the situation where the internuclear vector between two nuclei, the one relaxing the other, exhibits two kinds of motion. This vector reorients about an internal axis which in turn reorients by isotropic overall tumbling (Solomon, 1955; Woessner, 1962; Doddrell et al., 1972).

For both ^{13}C and ^{1}H:

$$A = (1/4)(3\cos^2\theta - 1)^2 \quad (1a)$$

$$B = 3\sin^2\theta\cos^2\theta \quad (1b)$$

$$C = (3/4)\sin^4\theta \quad (1c)$$

$$\tau_B^{-1} = \tau_R^{-1} + (6\tau_G)^{-1} \quad (1d)$$

TABLE III: Internuclear Separations[a] and Internal Rotation Angles[b] for the Aromatic Ring System of Phenylalanine.[c]

	H_β	$H_{\delta 1}$	$H_{\epsilon 1}$	H_ζ	$H_{\epsilon 2}$	$H_{\delta 2}$
C_γ	2.19	2.14 (86.2)	3.36 (39.3)	3.83 (0.00)	3.36 (39.3)	2.14 (86.2)
$H_{\delta 1}$			2.46 (0.00)	4.26 (30.0)	4.92 (60.0)	4.26 (90.0)
$H_{\epsilon 1}$		2.46 (0.00)		2.46 (60.0)	4.26 (90.0)	4.92 (60.0)
H_ζ		4.26 (30.0)	2.46 (60.0)		2.46 (60.0)	4.26 (30.0)
$H_{\epsilon 2}$		4.92 (60.0)	4.26 (90.0)	2.46 (60.0)		2.46 (0.00)
$H_{\delta 2}$		4.26 (90.0)	4.92 (60.0)	4.26 (30.0)	2.46 (0.00)	

[a] Given in Å by the value not in parentheses. [b] Given in degrees by the value in parentheses. [c] These values calculated by assuming a CH bond distance of 1.09 Å, an average C-C bond distance of 1.37 Å (Gurskaya, 1968), a planar and regular hexagonal phenyl ring, that the CH bond bisects the CCC angle, and that the $C_\gamma C_\beta H_\beta$ angle is 109.5°.

$$\tau_C^{-1} = \tau_R^{-1} + 2(3\tau_G)^{-1} \quad (1e)$$

For ^{13}C:

$$\chi_j = \frac{\tau_j}{1 + (\omega_H - \omega_C)^2\tau_j^2} + \frac{3\tau_j}{1 + \omega_C^2\tau_j^2} + \frac{6\tau_j}{1 + (\omega_H + \omega_C)^2\tau_j^2} \quad (2a)$$

$$\phi_j = \frac{6\tau_j}{1 + (\omega_H + \omega_C)^2\tau_j^2} - \frac{\tau_j}{1 + (\omega_H - \omega_C)^2\tau_j^2} \quad (2b)$$

$$T_1^{-1} = \left(\frac{\gamma_H^2\gamma_C^2\hbar^2N}{10r^6}\right)(A\chi_R + B\chi_B + C\chi_C) \quad (3)$$

$$\mathrm{NOE} = 1 + \left(\frac{\gamma_H}{\gamma_C}\right)\left(\frac{A\phi_R + B\phi_B + C\phi_C}{A\chi_R + B\chi_B + C\chi_C}\right)f_{dd} \quad (4)$$

For ^1H:

$$\chi_j = \frac{\tau_j}{1 + \omega_H^2\tau_j^2} + \frac{4\tau_j}{1 + 4\omega_H^2\tau_j^2} \quad (5)$$

$$T_1^{-1} = \left(\frac{3\gamma_H^4\hbar^2N}{10r^6}\right)(A\chi_R + B\chi_B + C\chi_C) \quad (6)$$

In eq 1-6 the symbols appearing are defined as follows: θ = angle between the internuclear vector and the axis of internal reorientation; τ_R = correlation time describing isotropic overall tumbling; τ_G = correlation time describing internal reorientation; ω_H, ω_C = Larmor frequencies of ^1H and ^{13}C, respectively; γ_H, γ_C = magnetogyric ratios of ^1H and ^{13}C, respectively; $\hbar$ = Planck's constant divided by 2π; r = internuclear separation; N = number of nuclei interacting with and situated at a distance r from the nucleus whose relaxation rates are given by eq 3 and 6; T_1 = spin-lattice relaxation time; NOE = ^{13}C nuclear Overhauser enhancement factor; f_{dd} = fraction of the total ^{13}C spin-lattice relaxation rate which is caused by dipolar interactions.

It should be noted that eq 1-6 employ the nomenclature which is in general use in ^{13}C studies (Doddrell et al., 1972) rather than that used in the original work (Woessner, 1962). It is also useful to note that ^{13}C-^{14}N *dipolar* interaction may also be described with eq 1-4 if ω_H and γ_H are replaced by ω_N and γ_N and eq 3 is multiplied by $^4/_{15}$.

In applying these equations it is useful to focus attention on the vectors between the nucleus of interest and the other nuclei which relax it. Thus, a given nucleus may be relaxed by several other nuclei in its molecular environment. The total relaxation rate in such a case is the sum of individual contributions, each calculated from eq 3 or 6, and each calculation potentially requiring a different value of r and θ. Furthermore, the effect of internal reorientation decreases as θ approaches zero and vanishes when the internuclear vector is parallel to the internal axis. Values of internuclear separations r and internal rotation angles θ, which will later be used in calculations applying eq 1-6 to the aromatic and heterocyclic ring systems of Phe and Trp, are summarized in Tables III and IV. Note that in these tables the angle θ is given between 0 and 90° since eq 1a-c are even trigonometric functions.

Equations 1-6 were derived under the simplifying assumption that only autocorrelation functions need be considered, which is valid for most relaxation mechanisms. However, it is known to be insufficient under some circumstances, most notably when methyl reorientation is involved (Cutnell and Glasel, 1976b). In this case the various internuclear vectors reorient as a unit, and thus cross-correlations must be considered. In general the influence of cross-correlations is always to retard the relaxation while leaving unaffected the relaxation rate determined from the initial slope of the nonexponential decay plot (Runnels, 1964). Thus, for example, the average methyl ^1H T_1's reported in Table II do not reflect the effects of cross-correlations. These effects will be considered separately in a forthcoming paper which deals with the measurement and interpretation of methyl ^1H and ^{13}C relaxation in peptides.

C. Calculation of the α-^1H Relaxation of Tetragastrin. The ability to calculate α-^1H relaxation rates has important implications for the determination of peptide tertiary structure, as will be apparent later in this publication. Our efforts are restricted here to the Phe residue because of the availability (Feeney et al., 1972) of useful information from proton-proton coupling constants concerning the conformations of the Phe side chain. Such information is necessary because, as we will show, the β protons provide a significant contribution to the

TABLE IV: Internuclear Separations[a] for the Heterocyclic Ring System of Tryptophan.

	H_β	$H_{\delta 1}$	$N_{\epsilon 1}$	$H_{\epsilon 1}$	$H_{\zeta 2}$	$H_{\eta 2}$	$H_{\zeta 3}$	$H_{\epsilon 3}$
C_γ	2.15	2.17	2.23	3.21	4.43	5.26	4.72	2.95
$C_{\delta 2}$	*b*	3.29	2.20	3.19	3.44	3.87	3.39	2.18
$C_{\epsilon 2}$	≥4.04	3.28	1.31	2.08	2.18	3.37	3.82	3.40

[a] Given in Å and calculated from known bond distances and angles (Gurskaya, 1968) by assuming a CH bond length of 1.09 Å, a NH bond length of 1.02 Å (Dickerson and Geis, 1969), and that CH or NH bonds bisect the angle opposite them. [b] See Discussion section F for treatment of $C_{\delta 2}$ . . . H_β interaction.

TABLE V: Dipolar Spin-Lattice Relaxation Times[a] for Tetragastrin.

	Experimental (Corrected for Nondipolar Contributions)	Partial Predicted[b]	Total Predicted[b]	Discussion Section Reference
^{1}H Phe α	0.380 ± 0.025[c]	0.519[e] ± 0.037	0.380 (ϕ = 48°)	C, F
^{1}H aromatic	0.724 ± 0.010[c]		0.602 ± 0.072	D
^{13}C Phe γ	2.81 ± 0.95[d]		2.24 ± 0.30	E
^{13}C Trp γ	1.55[g] ± 0.56[d]		1.53[g] ± 0.13	E
^{13}C Trp ϵ_2	2.19[g] ± 0.80[d]		2.06[g] ± 0.15	E
^{13}C Trp δ_2	3.06[g] ± 1.08[d]	3.95[f,g] ± 0.32	3.12[g] ($\chi^{(2)}$ = ±135°)	E, F

[a] In seconds. [b] (±) figures denote maximum propagated error. [c] (±) figures denote approximate 95% confidence limits in this measured quantity. [d] (±) figures denote maximum propagated error due to experimental error in both the measured T_1 and the NOE. [e] Calculation ignores effect of protons bonded to nearby nitrogens. [f] Calculation ignores effect of Trp β protons. [g] The experimental value does not reflect ^{13}C-^{14}N dipolar interaction since the measured NOE does not reflect a contribution from this interaction (Norton and Allerhand, 1976). Likewise the predicted values do not reflect ^{13}C-^{14}N dipolar interaction.

α-^{1}H relaxation. Similar information is also available (Feeney et al., 1972) for the Asp side-chain β protons, of which no use is made here for the following reason. Our ^{13}C data (see section G) indicate a large degree of internal reorientation about the Asp C_α-C_β bond which complicates the calculation of the α-^{1}H relaxation rate, as may be seen from the theory of Woessner (1965) which treats dipolar relaxation when the length of the internuclear separation varies. On the basis of this theory it may be seen that a simple weighting of the various r^{-6} terms is not sufficient.

From vicinal proton coupling constants derived from an ABX analysis of the Phe $C_\alpha H$-$C_\beta H_2$ fragment the fractional populations of the rotamers shown in Figure 4 have been determined (Feeney et al., 1972). These fractional populations were found to be nearly identical in the tripeptide Met-Asp-Phe-NH_2 and pentagastrin in aqueous solution. It is therefore likely that they provide a first approximation for the case of tetragastrin in aqueous solution. An equivalent analysis of the vicinal coupling constants in Me_2SO-d_6 solution is not possible because of interference from the solvent resonance, and therefore we assume that the rotamers and populations are the same as those in aqueous solution, as shown in Figure 4. The tenability of this assumption is supported by our ^{13}C relaxation data for the phenylalanine ring. These data indicate that the phenyl ring is reorienting internally about the $C_\gamma \ldots C_\zeta$ axis (see section D). Such reorientation is severely inhibited for rotamer III (but not for rotamers I and II) due to steric interactions, as may be seen from a space-filling model arranged according to energetically allowed values for the main-chain torsion angles (Feeney et al., 1972). Thus, the dominance of rotamers I and II reflected in Figure 4 is consistent with the internal mobility of the phenyl ring observed in our ^{13}C data. For each of the rotamers shown in Figure 4 the internuclear separations between the α proton and the β protons may be calculated from the known C_α-C_β bond distance (Gurskaya, 1968), a C-H bond distance of 1.09 Å, and the assumption of tetrahedral bond angles. The results of this calculation indicate that in rotamers I and II the two β protons are located 2.50 and 3.07 Å from the α proton, while in rotamer III both protons are equidistant from the α proton at 2.50 Å.

The ^{13}C results from Table I may be used to provide the requisite correlation time for the calculation of the α-^{1}H relaxation rate. The Phe α NT_1 of 0.0853 s and Phe β NT_1 of 0.108 s indicate a slight degree of internal rotation of β-CH_2. However, this difference is just barely beyond the 95% confidence limits and is therefore ignored as a first approximation. Thus, using an average of $NT_{1\alpha}$ and $NT_{1\beta}$, eq 3 may be employed with r = 1.09 Å and N = 1 to show that $\tau_R = 5.33 \times 10^{-10}$ s. Equation 3, as is well known, is a double-valued function of τ_R. However, tetragastrin is not sufficiently large a peptide that the larger value of τ_R to the low-temperature side of the T_1 minimum need be considered. The NOE values in Table I provide further support in this regard, a point which will later receive additional discussion (see section G). With a value for τ_R and the appropriate internuclear separations of 2.50 and 3.07 Å available, eq 6 may be used to calculate the contribution to the α-^{1}H relaxation from interactions with the β protons, according to $T_1^{-1} = 0.83T_1^{-1}$ (rotamer I or II) + $0.17T_1^{-1}$ (rotamer III). It is not surprising that the corresponding T_1 = 0.519 ± 0.037 s exceeds the measured value of 0.380 ± 0.025 s (see Table V), since we have ignored the effect on the α proton of nearby amide and amino protons. Because of the significance of this neglected contribution as a repository of valuable information about peptide conformation, it is discussed later in a separate section (see section F).

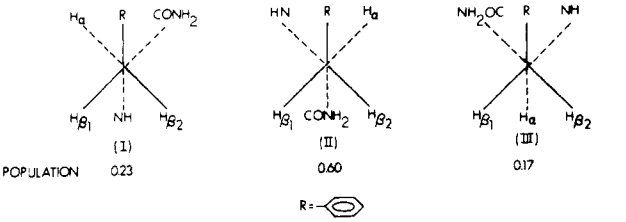

FIGURE 4: Rotamers and fractional populations assumed for the Phe residue of tetragastrin (Feeney et al., 1972).

D. Calculation of the Phe Aromatic ^{1}H Relaxation of Tetragastrin. The results in Table I reveal measurably different ^{13}C T_1's for Phe δ_1, δ_2, ϵ_1, ϵ_2, and ζ. Phe δ_1, δ_2 and Phe ϵ_1, ϵ_2 have virtually identical T_1's of 0.247 s, while Phe ζ exhibits a smaller value of 0.125 s. Furthermore, the Phe β NT_1 (0.108 s) is within experimental error of that for Phe ζ (0.125 s). These data provide strong indication that the phenyl ring is reorienting internally about an axis passing through C_γ and C_ζ. In addition, the equivalence of $NT_{1\beta}$ and $NT_{1\zeta}$ indicates that the $C_\beta H_2$-C_6H_5 unit may be treated as a rigid unit exhibiting overall tumbling. Using an average of $NT_{1\beta}$ and $NT_{1\zeta}$, eq 3 may be employed in the absence of internal reorientation with r = 1.09 Å to show that the overall reorientation of the $C_\beta H_2$-C_6H_5 unit is characterized by a correlation time $\tau_R = 4.26 \times 10^{-10}$ s. This τ_R indicates, via eq 4, that the expected NOE is 2.9, within experimental error of the measured value of 2.7. The correlation time describing the internal reorientation of the phenyl ring may now be obtained from eq 3 and the T_1 values measured for Phe δ_1, δ_2 and ϵ_1, ϵ_2. The values of A, B, and C to be used in eq 3 are obtained from eq 1a-c with

$\theta = 60°$, since all four C–H internuclear vectors are oriented at this angle with respect to the internal reorientation axis for purposes of calculation in eq 1a–c. With $r = 1.09$ Å and $\tau_R = 4.26 \times 10^{-10}$ s, eq 3 shows that the correlation time describing internal reorientation of the aromatic ring is $\tau_G = 6.01 \times 10^{-11}$ s. In performing this calculation we take account of the fact that in rotamer III (see Figure 4) internal reorientation of the phenyl ring is prohibited as determined from a steric model.

The above values for τ_R and τ_G may now be used in eq 6 to predict the ^1H relaxation rate. Within the aromatic ring any given proton interacts with four others, each internuclear vector characterized by its own length and angle with respect to the axis of internal reorientation, the appropriate angles and internuclear distances being given in Table III. It should be noted that the calculated relaxation rates are not identical for each proton, since for different θ's the internal reorientation does not affect each of the intra-phenyl interactions equivalently. Thus, in principle the relaxation of the aromatic resonance should be multiexponential. This effect was not observed, however (Cutnell and Glasel, 1976b), a result which is not surprising, since calculation of the relaxation curve from a weighted sum of differently decaying exponentials predicts a departure from exponentiality which is within the scatter of data. From the average of the calculated rates an effective T_1 of 0.602 ± 0.072 s may be determined and is to be compared with the value of 0.724 ± 0.010 s measured for the aromatic proton resonance of a 0.03 M solution (see Table V).

It is interesting to note that the predicted is less than the measured T_1 to an extent which cannot be accounted for completely by our experimental error. Nor can it be accounted for by effects of the Trp aromatic protons, the resonances for which overlap those of the Phe aromatic protons. The ^{13}C T_1's in Table I indicate that probably the Trp aromatic protons have T_1's which are slightly less than the aromatic protons of the Phe, since the internuclear distances involved are similar. The difference between predicted and measured values probably arises from our assumption that a single correlation time is sufficient to characterize the overall tumbling of the $C_\beta H_2$–C_6H_5 unit. Strictly speaking this is not true, since we observe a slight difference between the ^{13}C T_1's of Phe α and ζ (see Table I), and a formalism including the effect of anisotropic overall tumbling is therefore pertinent (Woessner et al., 1969). We do not include such a refinement, however, since our calculation is limited by our neglect of interactions between the Phe δ and Phe α and β protons. These interactions potentially can provide a contribution to the relaxation at least equal in size to the small discrepancy observed above between predicted and measured values. Accurate accounting of these interactions is not a straightforward matter in view of the nonconstant internuclear separations which are involved (Woessner, 1965), and a strictly applicable theory is not available. This issue of relaxation in the presence of a varying internuclear separation will recur later in other situations and constitutes a theoretical problem worth considerable attention in light of the potential of relaxation data to provide peptide torsion angles (see section F).

E. Calculation of Quaternary ^{13}C Relaxation Times for Tetragastrin. So far our efforts have been confined to calculating the ^1H relaxation behavior from information obtained from ^{13}C relaxation measurements on protonated carbons. However, from the same starting point it is also possible to calculate the ^{13}C relaxation rates of quaternary carbons. Such an analysis is equally important, since the relaxation rates of nonprotonated carbons, as well as those for α protons, may be used to monitor peptide conformations (i.e., torsion angles), as will be discussed in section F.

Phe γ illustrates the case where internal reorientation must be considered. Values for τ_R and τ_G were determined earlier (see section D) as $\tau_R = 4.26 \times 10^{-10}$ and $\tau_G = 6.01 \times 10^{-11}$ s, respectively, with the internal reorientation occurring about an axis between C_γ and C_ζ. Table III summarizes the internuclear distances and angles (relative to the $C_\gamma \ldots C_\zeta$ axis) required for internuclear vectors between C_γ and each aromatic proton. Table III also gives the internuclear separation between C_γ and each of the Phe β protons. Note that the β protons remain equidistant at constant r even though the phenyl ring exhibits internal reorientation and that no reorientation angle is needed for $C_\gamma \ldots H_\beta$ interactions since τ_R alone describes the reorientation of these internuclear vectors. Similarly, the internuclear vector for the $C_\gamma \ldots H_\alpha$ interaction reorients solely by overall molecular tumbling, and the internuclear separation may be calculated to be 2.84 Å in rotamers I and II and 3.54 Å in rotamer III (Gurskaya, 1968). The dipolar relaxation time of Phe γ arising from interaction with the Phe α, β and aromatic protons may now be determined from eq 3 to be 2.24 ± 0.30 s (see Table V). The experimental value for Phe γ may not directly be compared with this result, however, because the measured NOE of 2.1 indicates the presence of a contribution to the relaxation from nondipolar mechanisms. In order to determine the experimental dipolar T_1, eq 4 may be used to reveal that f_{dd} corresponds to very nearly 57% for each interaction. The measured value of 1.60 s may thus be corrected for the nondipolar contribution to show that experimentally $T_{1dd} = 2.81 \pm 0.95$ s (see Table V). The (±) figure reflects the maximum error resulting from experimental errors in the measured T_1 and, more important, in the NOE. The agreement between predicted and experimental quantities is within experimental error. Note, however, that the calculated is smaller than the experimental value, even though contributions from the Phe amide protons were not included in the above calculation. Inclusion of these contributions would reduce the calculated T_{1dd} even further. The direction of the discrepancy between theory and experiment thus has the same trend as noted previously in connection with the relaxation of the aromatic protons (see section D).

Calculations similar to those above may also be carried out for the three quaternary carbons of the Trp residue. Reference to Table I shows that the T_1's of the protonated carbons of the heterocyclic ring are practically identical. Furthermore, Trp α and β have T_1's (after accounting for $N_\beta = 2$) not significantly different from those for the protonated heterocyclic ring carbons. With these observations in mind note that the various CH internuclear vectors of the heterocyclic ring and the α and β carbons are oriented at quite different angles relative to any internal reorientation axis which might be drawn through the ring system. The relative equality of the various NT_1's for the protonated carbons is therefore a strong indication that the reorientation of the entire Trp residue may be described by a single correlation time. In the absence of internal reorientation the average of the NT_1's for the Trp protonated carbons yields a value of $\tau_R = 6.45 \times 10^{-10}$ s from eq 3. Corresponding to this value of τ_R, the expected NOE from eq 4 is 2.8, which is within experimental error of the measured values in Table I.

Each of the Trp quaternary carbons experiences a dipolar interaction within the ring system with six protons and the ^{14}N nucleus. In addition, interactions with the Trp β protons constitute a further source of relaxation. The requisite internuclear separations are given in Table IV. From the sum of contributions from the interactions (excluding ^{13}C–^{14}N, see footnote *g* of Table V) implied by Table IV, T_1 values of 1.53 ± 0.13,

2.06 ± 0.15, and 3.95 ± 0.32 s are calculated from eq 3 for Trp γ, ϵ_2, and δ_2, respectively, using $\tau_R = 6.45 \times 10^{-10}$ s. In the case of Trp ϵ_2 the contribution from the β protons was omitted, since the orientation about the C_β–C_γ bond is open to question. In any event this contribution is approximately only 1% of the total rate because of the internuclear separations involved. In the case of Trp δ_2 the contribution from the β protons was also omitted, but it will be considered separately in section F. In order to compare these predicted results with experimental quantities the presence of nondipolar relaxation mechanisms must be considered because of the reduced value of the measured NOE. From Table I the value of 2.1 may be used with τ_R in eq 4 to show that ^{13}C–^1H dipolar interactions constitute only 61% of the observed total relaxation rate for Trp γ and ϵ_2. Since it was not possible to determine a separate NOE for Trp δ_2, we assume the value of 2.1, which would seem reasonable in view of the fact that this value was measured separately for both the Trp γ and the combined Trp ϵ_2 and Phe γ resonances. In view of recent findings (Levy and Edlund, 1975) indicating appreciable chemical shift anisotropy in polycyclic systems the presence of roughtly 39% nondipolar interactions is not surprising. The measured T_1's of 0.947, 1.34, and 1.87 s thus correspond to ^{13}C–^1H dipolar T_1's of 1.55 ± 0.56, 2.19 ± 0.80, and 3.06 ± 1.08 s for Trp γ, ϵ_2, and δ_2, respectively. The ± figures reflect the same maximum error as above. The results of these calculations are summarized in Table V, where it can be seen that the predicted and experimental quantities agree within the propagated *maximum* error limits. We draw attention, however, to Trp δ_2 where several points should be kept in mind. Comparison of the results for the four quaternary carbons in Table V reveals that the discrepancy between predicted and experimental results is noticeably greater for Trp δ_2 than for Phe γ, Trp γ, and Trp ϵ_2. Note also that the predicted exceeds the experimental value for Trp δ_2, whereas for Phe γ, Trp γ, and Trp ϵ_2 the predicted is very nearly equal to or less than the experimental value. Finally we note that a similar discrepancy has also been observed in Trp residues in proteins (Oldfield et al., 1975). These considerations lead us to believe that the discrepancy between predicted and experimental dipolar T_1's for Trp δ_2 reflects in part our neglect of other important interactions, in particular those between Trp δ_2 and the Trp β protons. To illustrate the potential importance of quaternary ^{13}C T_1's in elucidating peptide conformation (i.e., torsion angles) we shall proceed in section F to analyze the contribution to the Trp δ_2 relaxation from the Trp β protons.

In concluding this section we call attention to the role played by NOE measurements in correctly calculating quaternary carbon T_1's. It is widely assumed that under noise decoupling conditions the NOE factor is independent of the internuclear separation r, as in eq 4. However, this is not strictly true for quaternary carbons which are relaxed by multiple interactions with noncovalently bonded protons. If the corresponding internuclear vectors do not all exhibit identical motional characteristics, if internal motions are not rapid compared to overall molecular tumbling, and if the motional narrowing limit does not apply, then the NOE expression used in place of eq 4 will have to take into account the additional dependence on r. This point will have to be kept in mind if quaternary carbon T_1's are used to extract values for torsion angles in larger polypeptides.

F. Determination of Peptide Torsion Angles from Spin–Lattice Relaxation Rates. Our earlier analysis of α-^1H and quaternary ^{13}C relaxation rates revealed certain discrepancies between the experimentally determined quantities and those calculated from relaxation data on protonated carbons. We now return to these discrepancies to show that α-^1H and quaternary ^{13}C T_1's constitute a potentially rich source of information about peptide conformation. In particular the α-^1H T_1's will be seen to relate to the main-chain torsion angles ϕ and ψ describing rotation about the N–C_α and C_α–C bonds, respectively. The quaternary ^{13}C T_1's, on the other hand, will be seen to relate to the torsion angles χ describing rotation about side-chain bonds.[2]

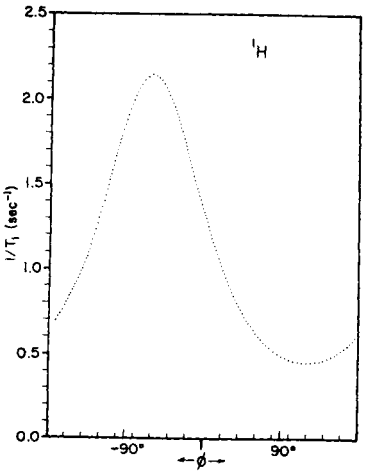

FIGURE 5: The dependence of the α-^1H spin–lattice relaxation rate on the main-chain torsion angle ϕ for the Phe residue of tetragastrin, this dependence arising from the interaction with the adjacent amide proton. See Discussion, section F.

Considering only contributions from the Phe β protons, the Phe α-^1H relaxation time was predicted in section C to be $T_1 = 0.519$ s, whereas the experimental value is $T_1 = 0.380$ s (see Table V). The discrepancy implies that a 0.705-s^{-1} contribution to the spin–lattice relaxation rate remains unaccounted for. As the next step in refining our earlier calculation, we now consider the contribution to the Phe α-^1H relaxation rate from the NH proton of the peptide bond. In order to determine this contribution the H_{NH} . . . H_α internuclear distance r is required in eq 6, and it is through r that the dependence of T_1^{-1} on the torsion angle ϕ arises. With the value of $\tau_R = 5.33 \times 10^{-10}$ s (see section C) eq 6 may be used in the absence of internal reorientation to reveal that the dependence of T_1^{-1} on ϕ is as shown in Figure 5. The required contribution of 0.705 s^{-1} corresponds to $\phi = +48°$ or $-169°$, but the latter value may be eliminated on the grounds that it is not energetically favorable (Feeney et al., 1972). We note for comparison that Feeney et al. (1972) report likely values of $\phi \approx -90°$ to $-100°$ and $\psi \approx +60°$ to $+90°$, on the basis of NH–C_αH coupling constants and potential energy calculations. However, with $\psi \approx +60°$ to $+90°$ a secondary minimum in the potential energy diagram exists at $\phi \approx 48°$ (Feeney et al., 1972), and this value of ϕ is also consistent with the measured NH–C_αH coupling constant (Gibbons et al., 1970). Thus, the additional information provided by our relaxation data implies the alternative ϕ value of 48°, which choice could not be made on the basis of NH coupling constants alone.

The above analysis concerning ϕ ignores contributions to the relaxation from sources other than the NH proton of the peptide bond. Reference to a space-filling model indicates that interaction of H_α with the Phe δ protons is possible depending on the orientation of the aromatic ring about the C_β–C_γ bond. In view of the internal reorientation of the phenyl ring and the

[2] For the conventions followed with respect to ϕ, ψ, and $\chi^{(2)}$ see IUPAC-IUB Commission (1970).

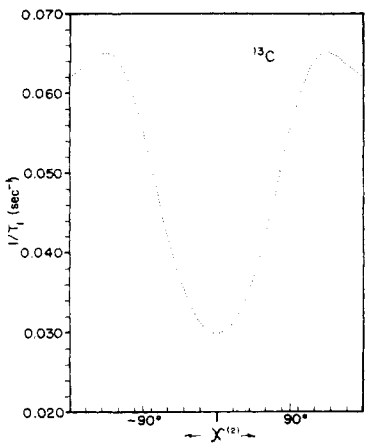

FIGURE 6: The dependence of the quaternary ^{13}C spin–lattice relaxation rate on the side-chain torsion angle $\chi^{(2)}$ for the Trp δ_2 carbon of tetragastrin, this dependence arising from interactions with the Trp β protons. See Discussion, section F.

resultant variation in $H_\alpha \cdots H_\delta$ internuclear separation, this contribution is not easily calculated (Woessner, 1965). Further experiments with the aromatic protons replaced by deuterium would clarify the extent of this contribution. In addition to $H_\alpha \cdots H_\delta$ interaction, it is also possible that interactions between H_α and the protons of the C terminal NH_2 group are important (see Figure 1). A calculation of the contribution from NH_2 protons is not a straightforward matter, however, because internal rotation is possible about the twofold symmetry axis of the NH_2 group and also about the C_α–C bond. Furthermore, these internal rotations result in nonconstant internuclear separations between H_α and the NH_2 protons (Woessner, 1965), and a strictly applicable theory is not available to describe the interactions. As a first approximation, and to evaluate the possibility of internal reorientations, we have calculated the contribution of the NH_2 protons via overall tumbling by assuming trigonal angles, the absence of either internal rotation, and a spatial average of r^{-6} terms over possible orientations of the NH_2 group about its twofold symmetry axis. This calculation yields a result for T_1^{-1} vs. ψ (which describes the orientation about the C_α–C bond) similar in character to that shown in Figure 5. On the basis of this result, the ψ values reported by Feeney et al. (1972) correspond to T_1^{-1} contributions exceeding 0.705 s^{-1}. Furthermore, on the basis of this same result the only ψ values consistent with our relaxation data and which correspond to energetically favorable conformations are associated with energetically unfavorable values of ϕ which are clearly inconsistent with the observed NH coupling constant. The implication is that internal rotation about the twofold NH_2 symmetry axis and about the C_α–C bond greatly reduce the efficiency of interactions between H_α and NH_2 protons as a relaxation mechanism. Further experiments are required to clarify the extent and nature of contributions from the NH_2 protons. ^{13}C and ^2H measurements on the analogue of tetragastrin where the labile protons have been replaced with deuterium would be useful.

An important feature of this approach to the elucidation of peptide main-chain conformation is that it rests on the foundation provided by the dipolar formalism for nuclear spin relaxation. This formalism has the advantage of being based on a through-space effect, well defined and extensively developed. In contrast, the use of NH–C_αH proton vicinal coupling constants for studying peptide main-chain conformation rests on the Karplus–Bystrov relationship (Bystrov et al., 1969; Barfield and Karplus, 1969) which, since it is basically a quantum chemical many-electron calculation, is not nearly as well defined and developed. Furthermore, the dipolar interaction as seen in Figure 5 is inherently less redundant than the Karplus–Bystrov relationship. A given T_1^{-1} value is related in Figure 5 to a pair of ϕ values, whereas a given coupling constant may correspond to as many as four values of ϕ (Barfield and Gearhart, 1973; Gibbons et al., 1970). The conclusion is clear that combined ^{13}C and α-^1H relaxation studies have the potential to provide a useful additional approach with which to investigate main-chain torsion angles in peptides. Such studies will probably be most useful when carried out at high magnetic fields where more accurate coupling constants may be derived from second-order multiplet patterns, and additional T_1's may be measured.

We now turn our attention to quaternary ^{13}C T_1 data as a potentially important source of information concerning side-chain torsion angles χ. In section E we calculated a value of T_1 = 3.95 s (see Table V) for Trp δ_2 by considering only interactions within the heterocyclic ring system. On the other hand, the experimental value for the dipolar T_1 was found to be 3.06 on the basis of NOE values and the measured T_1. This discrepancy (accounting for the effect of ^{13}C–^{14}N dipolar interaction) amounts to a contribution to T_1^{-1} of 0.071 s^{-1}, and to further refine our calculation of T_1 for this quaternary carbon we now consider the contribution from interaction with the Trp β protons. To calculate the contribution to the Trp δ_2 T_1^{-1} from the Trp β protons eq 3 must be used in the absence of internal reorientation with $\tau_R = 6.45 \times 10^{-10}$ s (see section E). The internuclear separation required in eq 3 depends on the orientation of the heterocyclic ring about the C_β–C_γ bond and is thus determined by the side-chain torsion angle $\chi^{(2)}$. The contribution to T_1^{-1} as a function of $\chi^{(2)}$ is shown in Figure 6. It should be noted that $\chi^{(2)} = 0°$ when $C_{\delta 2}$ is in the eclipsed position relative to C_α and that the positive sense for rotation of the ring is counterclockwise when sighting from C_β to C_γ. The maximum T_1^{-1} contribution of 0.065 s^{-1} occurs for $\chi^{(2)} = \pm 135°$, with the local minimum at $\chi^{(2)} = \pm 180°$ resulting from the presence of two β protons. The value of 0.065 s^{-1} corresponds closely to the 0.071-s^{-1} discrepancy in our earlier calculation of the Trp δ_2 dipolar T_1^{-1}. If other contributions to T_1^{-1} were negligible, the orientation of the Trp heterocyclic ring would thus be characterized approximately by $+135° \leq \chi^{(2)} \leq +180°$ and $-180° \leq \chi^{(2)} \leq -135°$, with $C_{\delta 1}$ thus roughly in the eclipsed position relative to C_α. We note that this position of $C_{\delta 1}$ with respect to C_α corresponds to a secondary minimum in the barrier for rotation about the C_γ–C_β bond in 3-ethylindole (Maigret et al., 1971). Further experiments are needed to clarify the extent of additional contributions to T_1^{-1}, however. Reference to a space-filling model indicates that the N-terminal NH_3^+ protons are the prime candidates for these other contributions, and experiments with these protons replaced by deuterium are necessary to resolve the question.

The exact nature of the dependence of a quaternary carbon T_1^{-1} on side-chain torsion angles will depend on the location of the carbon relative to the protons which relax it. In this respect we note that T_1 of Phe γ does not depend at all on rotation about the Phe C_β–C_γ bond because of its symmetric placement relative to the β protons. We note in addition the use of selective deuteration as a general technique for expanding the capability of ^{13}C relaxation studies to provide information about peptide main- and side-chain conformation. Any protonated carbon may be made to behave in its ^{13}C T_1 characteristics as a quasi-quaternary carbon if its bonded

TABLE VI: Overall and Internal Reorientation Correlation Times for Tetragastrin at 30 °C and 0.3 M in Me_2SO-d_6.

	τ_R (ns)	τ_G (ns)
1. Average main chain	0.709	
2. Trp side chain	Same as 1, fixed to main chain	
3. Asp β	0.709	0.0873
4. Phe aromatic ring	0.426	0.0601

proton(s) are replaced by deuterium. From this point of view the ideas which we have been stressing in this section offer exciting possibilities for experimental studies of peptide conformations.

G. Motional Characteristics of Tetragastrin. Throughout the preceding analysis a picture of the motional characteristics of tetragastrin has emerged from our relaxation time data on protonated carbons. To conclude our discussion we now summarize these characteristics, the correlation times for which are shown in Table VI.

As stated originally, the nearly constant T_1's observed for the α carbons (see Table I) imply that the various parts of the main chain are exhibiting very similar motions on the NMR time scale. This contrasts with observations on a series of pentapeptides where a definite lengthening of the T_1's and corresponding increased motional freedom was observed proceeding outward from the central residue toward each terminal residue (Keim et al., 1974). An average of the C_α T_1's reveals via eq 3 that $\tau_R = 7.09 \times 10^{-10}$ s for isotropic overall reorientation in the absence of internal motion. The correspondingly expected NOE from eq 4 is 2.7 and within experimental error of the measured values, thus confirming that the molecular motion of the main chain is to the high-temperature side of the T_1 minimum.

The motional characteristics of the side chains of tetragastrin vary considerably. Relative to the main chain the most rigid side chain is that of tryptophan, as may be seen in Table I. In contrast to the situation for the Trp side chain the data in Table I for the Met side chain reveal increasing motional freedom proceeding from C_α to the methyl carbon. On the basis of the nearly equal T_1's for C_β and C_γ the motional characteristics of these two carbons may be seen to be nearly the same. The somewhat larger NT_1's for C_β and C_γ, as compared to that for C_α, imply a slight degree of internal motion of C_β and C_γ relative to the main chain. However, the much longer NT_1 value for the methyl carbon (especially in view of the reduced NOE) implies a large degree of motional freedom of the S-CH_3 bond, considerably beyond that already present for C_β and C_γ. A more complete discussion of the methyl reorientation will be presented in a forthcoming publication, as mentioned above.

The β carbon of the aspartic acid residue exhibits considerable motional freedom relative to the main chain. This is apparent in Table I in that the NT_1 for Asp β appreciably exceeds that for Asp α. Using the main chain $\tau_R = 7.09 \times 10^{-10}$ s in eq 3 reveals a correlation time for internal reorientation (assumed to occur about the C_α-C_β bond) of $\tau_G = 8.73 \times 10^{-11}$ s, roughly eight times faster than the overall main-chain reorientation. The corresponding NOE from eq 4 is 2.9, agreeing with the experimental value to within experimental error.

In a manner similar to that for the methionine side chain, Phe β exhibits a slight degree of internal reorientation relative to Phe α. However, as indicated by the relative NT_1 values in Table I, the effect is less for Phe than for Met. The interesting feature of the Phe side chain is that the phenyl ring is reorienting internally about an axis between C_γ and C_ζ. Using the average of NT_1 for Phe β and Phe ζ, it was shown (see section D) that the overall reorientation of this axis was characterized by $\tau_R = 4.26 \times 10^{-10}$ s, while internal reorientation proceeded roughly seven times more rapidly with $\tau_G = 6.01 \times 10^{-11}$ s. Note also that the overall reorientation of the $C_\gamma \cdots C_\zeta$ axis ($\tau_R = 4.26 \times 10^{-10}$ s) is noticeably faster than the overall reorientation of the main chain ($\tau_R = 7.09 \times 10^{-10}$ s). This is apparent in Table I from the longer Phe ζ T_1 as compared to that for the α carbons.

Conclusion

Throughout this paper we have taken the position that ^{13}C relaxation measurements on protonated carbons are most profitably viewed as providing a starting point for detailed analysis of ^1H and quaternary ^{13}C relaxation data. We have illustrated how, from this point of departure, the basic and well-developed dipolar relaxation formalism may be used with ^{13}C data on protonated carbons to calculate the proton and quaternary carbon relaxation behavior in considerable detail. Because of this calculational ability ^1H and quaternary ^{13}C relaxation data have the potential, when obtained with suitably deuterated species, to provide information concerning the main- and side-chain torsion angles which characterize peptide conformations. Our analysis illustrates, however, that the dipolar relaxation formalism is deficient when interactions are important which involve internal reorientation and subsequently nonconstant internuclear separations. Considering its importance further theoretical and experimental work in this area is needed.

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Dual Labeling in Standard DNA·RNA Hybridization Studies Using ^{125}I-Labeled Nuclear RNA and ^{3}H-Labeled DNA†

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ABSTRACT: Standard DNA-RNA hybridization studies, using nucleic acids isolated from mammalian tissues, are frequently hindered by relatively low levels of radioactivity in pulse-labeled RNA and in an inability to reliably estimate the amount of DNA present in the hybrid. In the method described here nuclear RNA is labeled in vitro with ^{125}I to 400 000–800 000 cpm/μg and DNA is obtained from a rat glial tumor line grown in culture and labeled to specific activities of 42 000–79 000 cpm/μg. DNA-RNA hybridization is conducted in an all solution system at RNA:DNA ratios of 3.5:1 to 18:1. Assay background is controlled by pretreatment of the hybrid and free RNA at the conclusion of the annealing study with RNase, then isolation of the hybrid together with a small fraction of free RNA oligonucleotides on hydroxyapatite. The partially purified hybrids are then trapped on Millipore filters. Assay background is 0.004% of total counts present in the annealing reaction. Comparison of the annealing reactions of pulse-labeled liver nuclear RNA and in vitro ^{125}I-labeled nuclear RNA in saturation, kinetic, and competitive hybridization studies shows them to be essentially the same. Nuclear RNA labeled by either tritium or iodine shows a 10–20-fold greater concentration of the annealing sequences over that found in the microsomal RNA. Minor differences are noted between the nuclear RNAs in the initial rates of reaction and in the magnitude of the decrease in percent hybridization at low levels of unlabeled competitor RNA. This may be due to preferential labeling in pulse-labeled RNA of molecules which are present in lower concentrations or are transcribed from more frequently repeated DNA sequences than the average population of annealing RNA molecules. The technique has application in systems where the amount of tissue for RNA extraction is small or where the system does not permit the obtaining of pulse-labeled RNA, as in experimental rodent skin carcinogenesis or in dealing with RNA from the tissues of large mammals or humans.

Standard competitive hybridization studies measure only a small portion of RNA sequences, e.g., only those sequences transcribed from the reiterated portion of the genome (Holmes and Bonner, 1974a,b). In spite of this limitation, these studies have provided direct evidence for the possible importance of nuclear to cytoplasm transport mechanisms in maintaining the differentiated state in normal tissues and in the pathogenesis of neoplasia (Shearer and Smuckler, 1972; Garrett et al., 1973a–c; Shearer, 1974a,b).

Three factors control the sensitivity of the competition study, after such conditions as the assay incubation temperature, ionic strength, and duration of incubation have been determined. These are the ability to accurately determine the amount of RNA annealed, which is a function of the specific activity of the RNA, a means of accurately determining the DNA present in the hybrid, either chemically or by dual labeled testing, and a favorable net RNA counts annealed to assay background

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