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Internal Rotations of Side Chains and Backbone in Luteinizing Hormone-Releasing Hormone (LH-RH). Analysis of Carbon-13 Spin–Lattice Relaxation Times[1a]

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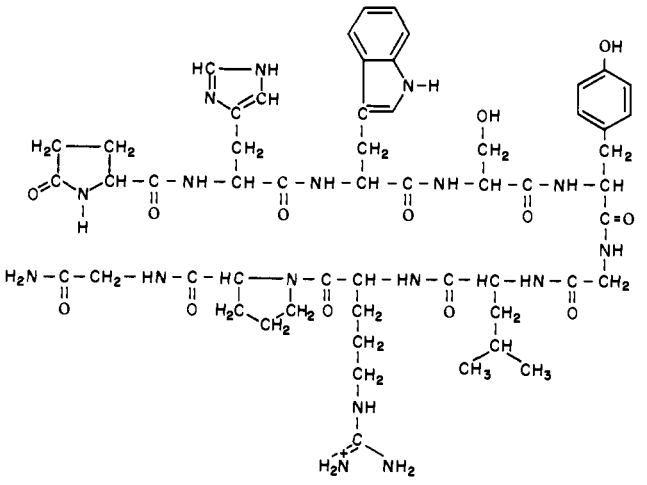
Abstract: We have analyzed the ^{13}C spin–lattice relaxation times obtained for LH-RH in aqueous solution in terms of contributions from both overall and internal motions of backbone and side chains. The method of analysis is based on a model of stochastic rotational diffusion about bonds. It assumes that these rotations about individual bonds are *independent* of and *uncorrelated* with rotations about all other bonds. We have calculated minimum and maximum dimensions for the LH-RH monomer and computed the corresponding T_1 values for *overall* molecular tumbling. For this T_1 range, corresponding to $2 \times 10^{-10} \leq \tau_{mol} \leq 2 \times 10^{-9}$, the *internal* motions of the backbone must be assumed slower than the overall molecular motion in order to simulate the observed near equality in NT_1 values for the α-carbons in positions 3–8 of LH-RH. For the side chains, the observed NT_1 values are good qualitative monitors of internal rotations about bonds whose relaxing carbons are more than one bond removed from C_α.

The purpose of this study is to gain qualitative insight into the types and rates of motion which can occur in linear peptides of intermediate molecular weight ($\simeq$1000) and which can produce the spin-lattice relaxation times (T_1) observed by carbon-13 nuclear magnetic resonance spectroscopy. T_1 values have been measured and can be analyzed in terms of both overall molecular and internal motion in both cyclic and linear molecules.[2-11]

The carbon-13 (^{13}C) spin-lattice relaxation times of luteinizing hormone-releasing hormone (LH-RH) (Figure 1) have been measured in aqueous solution and effective correlation times have been reported.[12] We now analyze the T_1 data in terms of rates of both overall and internal molecular motions. The latter comprise rotation about single bonds, both in the backbone and in side chains.

The method of analysis is essentially that of Levine and co-workers.[3,10,11] Because LH-RH is a linear and therefore potentially flexible peptide, it was necessary to explore the effect of both isotropic and anisotropic overall molecular motion on the calculated T_1 values of the backbone and side chains. We have used various methods to estimate the maximum and minimum dimensions plausible in LH-RH for fully extended and compact conformations and examined the effect of varying these size parameters on the calculated rates of internal motion for the side chains of the various residues.

Methods. Levine et al.[3,10,11] have presented a method which extends previous treatments[13-16] and permits the calculation of dipolar relaxation times when nuclei are reorienting in a magnetic field as a result of multiple internal motions in a molecule. The treatment applies to all values of rotational correlation times (τ). The relaxation times of nuclei in chains attached to bodies undergoing overall isotropic[10] and anisotropic[3] motion have been considered. The formulation assumes that the motions about each individual bond are *independent* and *uncorrelated* with the motions



LUTEINIZING-HORMONE RELEASING-HORMONE

Figure 1. Primary sequence of LH-RH.

about all other bonds. Furthermore, the calculations have been based on a model of stochastic rotational diffusion about the bonds. Threefold jump models of rotation[11,14,17] have also been considered, yielding qualitatively similar results.[3]

Levine et al.[3,10,11] dealt with linear hydrocarbons for which the angles between the bonds about which internal rotations occurred were identical (109.8°). In our study we found it necessary to include calculations of the matrix elements $d_{nm}^{(2)}(\beta_i)$ (Appendix) appropriate for peptides, where three different angles in the backbone must be considered.

The T_1 values arising from dipole-dipole interactions were calculated from the standard expression,[18a] which was derived for the extreme narrowing condition[18b]

$$1/T_1 = 1/T_1^{DD} = (N\gamma_H^2\gamma_C^2\hbar^2/10)\langle r^{-6}\rangle[J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)] \quad (1)$$

where the spectral density function $J(\omega)$, the Fourier transform of the angular autocorrelation function, is essentially that in Levine[3] but generalized to arbitrary angles β_i

$$J(\omega_j) = \sum_{m,r,s} a_{rm}a_{sm} \sum_{\alpha_1,\alpha_2,\ldots\alpha_N} [\tau^*/(1 + \omega_j^2\tau^{*2})] \times d^*_{r\alpha_1}(\beta_1)d_{s\alpha_1}(\beta_1)B_{\alpha_1\alpha_2}(\beta_2)B_{\alpha_2\alpha_3}(\beta_3)\ldots B_{\alpha_{N-1}\alpha_N}(\beta_N) \quad (2)$$

with

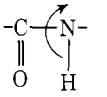
$$\tau^* = \left[E_m + \sum_{i=1}^{N} \alpha_i^2 D_i\right]^{-1} \quad (3)$$

$$B_{\alpha_i\alpha_j}(\beta_i) = |d_{\alpha_i\alpha_j}(\beta_j)|^2$$

a_{rm} is the coefficient of the rth eigenfunction of the spherical top rotor used in the expansion of the mth eigenfunction of the symmetric top rotor. The latter has an energy of rotation E_m, with $E_{|2|} = 2D_x + 4D_z$, $E_{|1|} = 5D_x + D_z$, $E_0 = 6D_x$. $D_x = D_y < D_z$ are the rotational diffusion coefficients about the molecular x, y, and z axes, D_i is the corresponding coefficient about the ith bond. The time-independent matrix elements $d_{mn}(\beta_i) \equiv d_{mn}^{(2)}(\beta_i)$ depend on the angle β_i between adjacent bonds about which internal rotations occur (see Appendix). ω_H and ω_C are the resonance frequencies of ^1H and ^{13}C, respectively, $\hbar$ is Planck's constant divided by 2π, N is the number of protons directly attached to the carbon under study, γ_H and γ_C are the magnetogyric ratios of proton and carbon, respectively, $\langle r^{-6}\rangle$ is the vibrationally averaged inverse sixth power of the ^{13}C-^1H internuclear distance.

We have used a number of analytical approaches for correlating the observed NT_1 values with the diffusion coefficients for rotation about each bond in the peptide backbone and side chains. For the side chains of individual residues, we have estimated the overall rate of molecular reorientation and then determined the rates of diffusion about each bond by varying the values of D_i separately until every observed T_1 value was fitted. Rates of overall molecular motion have been estimated from molecular dimensions and the rotational diffusion model. The minimum molecular dimensions were estimated from van der Waals radii, maximum dimensions of a monomer from measuring space-filling (CPK) models in fully extended conformations. Alternatively, the α-carbon of each side chain was assumed to be the "effective center of mass" for that side chain, and rates of internal motion (D_i) with respect to this point were calculated.

The general behavior of the observed NT_1 values was reproduced for the peptide backbone, using plausible values for the rate of overall molecular reorientation. This allowed us to simulate the effect of internal motions within the backbone itself. In one approach diffusion constants were evaluated for all the rotationally flexible bonds in a peptide link. Because of the partial double-bond nature of the peptide link it was assumed that rotation about the peptide



bond was not possible. An arbitrary, large value of $\tau = 1.0 \times 10^{10}$ s was used to simulate the rigidity of the peptide bond. Alternatively, diffusion was considered to occur about "pseudobonds" [19] which link adjacent α-carbons. The diffusion constants so obtained are only "effective" values and comprise contributions from rotations about both bonds in the peptide unit. Such an approach is justified since we measure only the T_1 values of the α-carbons and cannot monitor the rates of rotation involving C=O· and NH groups. There is little difference between the *true* motion of the C-H vector as ψ and ϕ of the peptide unit vary and its *effective* motion due to an equivalent rotation of angle θ about the pseudobond.[20] The exact relation between θ and (ψ,ϕ) is known.[21] The experimental uncertainties (15%) in T_1 measurements mask any error introduced by the pseudobond approximation. On the other hand, this approximation reduces by a factor of 3 the number of bonds that have to be considered to calculate diffusion constants that correspond to observable α-carbon T_1's. This leads to a ninefold decrease in calculation time/pseudobond. An additional twofold overall time saving results from using the single effective angle, (146°) characteristic of the pseudobond geometry, instead of the three different angles of the peptide unit (123, 114, 110°). Finally, the approximations of the model itself (independent *and* uncorrelated motion about bonds) are more likely to be valid for the pseudobonds than for the more strongly coupled bonds of the peptide unit; it is also less difficult to estimate a single effective diffusion constant for a pseudobond than to guess the two D_i needed in the peptide unit.

Results and Discussion

The T_1 values obtained at 67.9 MHz (sample concentration 200 mg/ml, temperature 308 K) are listed in Table I. The effective correlation time corresponding to each T_1 value is also given. This effective correlation time (τ_{eff}) is calculated from eq 1[22,23] with

$$J(\omega_j) = \tau_{eff}/(1 + \omega_j^2\tau_{eff}^2) \quad (4)$$

Table I. Spin-Lattice Relaxation Times[e] and Correlation Times of Carbons Bearing Protons in LH-RH

	Isotropic									Anisotropic,[d] τ_z = 0.9×10^{-9} s, τ_x = τ_y = 1.9×10^{-9} s, τ_{int} 10^{-10} s
	τ_{mol} chosen equal to τ_{eff} for C_α of each side chain[a]								τ_{mol} = 5.0×10^{-10} s,[b] τ_{int} 10^{-10} s	τ_{mol} = 1.0×10^{-9} s,[c] τ_{int} 10^{-10} s	
Residue	NT_1, ms	τ_{eff}, 10^{-10} s	τ_{int}, 10^{-10} s	NT_1 calcd	Residue	NT_1, ms	τ_{eff}, 10^{-10} s	τ_{int}, 10^{-10} s	NT_1 calcd			
<Glu α-CH	295	1.7	1.7	296	Gly α-CH_2	190	3.0	3.0	191			
β-CH_2	380	1.3	2.4	376								
γ-CH_2	460	1.0	1.3	492	Leu α-CH	175	3.4	3.4	175	5.0	2.1	1.8
					β-CH_2	240	2.2	3.0	234	2.1	1.8	1.7
His α-CH	210	2.6	2.6	210	γ-CH	260	1.9	3.9	260	4.2	4.3	4.3
β-CH_2	210	2.6	26.0	222	δ-CH_3	1545	0.31	0.04	1575	0.04	0.04	0.04
δ-CH	180	3.3	50.0	220	δ-CH_3	1440	0.32	0.05	1450	0.05	0.05	0.05
ϵ-CH	N.O.											
					Arg α-CH	185	3.2	3.2	185	3.8	1.9	1.6
Trp α-CH	160	4.1	4.1	160	β-CH_2	240	2.2	3.0	240	2.4	2.0	1.9
β-CH_2	200	2.8	4.0	200	γ-CH_2	330	1.5	1.5	336	1.6	1.6	1.6
C-2	200	2.8	8.0	206	δ-CH_2	350	1.4	3.5	358	3.7	3.7	3.7
C-4	160	4.1										
C-5	160	4.1			Pro α-CH	220	2.4	2.4	220	1.9	1.2	1.0
C-6	190	3.1			β-CH_2	470	1.0	2.4		0.5	0.5	0.5
C-7	185	3.2			γ-CH_2	480	1.0					
					δ-CH_2	260	2.0					
Ser α-CH	165	3.8	3.8	166								
β-CH_2	234	2.2	1.8	252	Gly α-CH_2	390	1.2	1.2	403	0.45	0.35	0.32
Tyr α-CH	165	3.8	3.8	166								
β-CH_2	200	2.8	5.0	200								
o-CH	230	2.3	5.0	214								
m-CH	210	2.6										

[a] Isotropic overall molecular motion, using correlation time of α-carbon of each amino acid as the effective correlation time for molecular motion in the calculations of internal motions of each side chain. The calculations assume rotation about individual bonds; in cyclic systems (i.e., <Glu, Pro), where only oscillations can occur, this treatment is not justified. For the aromatic moieties, the value of τ_{int} is that obtained for rotation about the C_β-C_γ bond. The rings were considered rigid. The calculated values of T_1 are shown in order to give an estimate of the fit obtained when calculating T_1 using our values of τ_{int} and τ_{mol} (τ_{mol} in this case is the correlation time of each α-carbon). [b] $\tau_{mol} = 5.0 \times 10^{-10}$ s corresponds to the value expected for a spherical particle 8 Å in radius undergoing rotational diffusion in a medium of viscosity equal to that of water. [c] $\tau_{mol} = 1.0 \times 10^{-10}$ s corresponds to the value expected for LH-RH in a compact conformation, as determined from CPK space-filling models. [d] τ values used in these calculations were obtained for the rotational diffusion of an ellipsoid of revolution having dimensions equal to those obtained from a CPK model of LH-RH in a fully extended conformation. The last three columns of the table correspond to Leu, Arg, Pro, Gly. [e] Observed at 67.9 MHz. Sample concentration 200 mg/ml, temperature 308 K. N.O., not observed. NT_1 values are given in milliseconds, N is the number of protons directly attached to the carbon under study. $\tau_{int} = 1/6D_{int}$.

In the case of flexible linear peptides both overall molecular tumbling and intramolecular motion of the peptide backbone and side chains can participate in the reorientation of the individual C-H bonds and thereby contribute to the relaxation process. In order to separate the various contributions to the observed relaxation time, we need an estimate of the rate of overall molecular motion. In some cases this can be obtained from the T_1 measurements if a given carbon is known not to have any freedom within the molecular framework. In LH-RH no carbon should be considered rigid within the molecular framework and we should estimate the rate of molecular reorientation by other means. However, because such estimates rely on a number of assumptions (vide infra), we can adopt an alternative approach in which we assume that in the extreme narrowing range, the shortest T_1 value in the peptide backbone is determined solely by the rate of overall molecular motion. One then calculates the *relative* rates of internal motion for the remaining carbons in the backbone and side chains with respect to the most "restricted" carbon. We have used both approaches in our calculations in order to determine the sensitivity of our results to the various assumptions used in the calculations.

A. Estimate of Rate of Overall Molecular Motion. (a) Isotropic Case. The correlation time for overall molecular reorientation (τ_{mol}) is often estimated from the rotational diffusion constant, D, which in turn can be related to molecular dimensions. In the case of isotropic motion (i.e., for spherically symmetric bodies)[24] a modified Stokes-Einstein relation gives

$$\tau_{mol} = 1/6D = \beta/6kT = 8\pi\eta r^3 f_r/6kT = V_m \eta f_r/kT \quad (5)$$

where k is Boltzmann's constant, T is the absolute temperature, V_m is the molecular volume, and β is the molecular friction constant

$$\beta = 8\pi r^3 f_r \eta \quad (6)$$

where r is the radius of a spherical solute, η is the viscosity of the medium, and f_r is the microviscosity factor which is ≤ 1, depending on the relative sizes of solute and solvent.[25,26]

(b) Molecular Dimensions. Several methods are available for estimating molecular volumes. We have calculated the volume for LH-RH using the method of atomic increments.[27] The method is based on the assumption that the distance of closest approach of two nuclei in different mole-

Table II. Rotational Diffusion Constants[a] and Corresponding Correlation Times[b] for Ellipsoids of Revolution of Various Models of LH-RH[c]

Model	Mol dimensions	Dimensions of ellipsoid of rev (semiaxes), Å	$D_\parallel$, 10^8 s^{-1}	$D_\perp$, 10^8 s^{-1}	$\tau_\parallel$, 10^{-9} s	$\tau_\perp$, 10^{-9} s	Calcd[d] T_1, s
α-Helix	20 × 20 × 20	10.0 × 10.0	1.65	1.66	1.00	1.00	0.118
		10.0 × 9.0	2.15	2.01	0.775	0.829	0.125
Compact	20 × 18 × 14	10.0 × 8.0	2.84	2.43	0.587	0.686	0.137
Type II β turn	24 × 19 × 16	12.0 × 10.0	1.49	1.32	1.119	1.263	0.113
		12.0 × 9.0	1.91	1.55	0.873	1.075	0.118
		12.0 × 11.0	1.19	1.13	1.401	1.475	0.110
β-Pleated sheet	27 × 21 × 10	14.0 × 10.0	1.35	1.05	1.234	1.587	0.110
		14.0 × 9.0	1.71	1.21	0.975	1.377	0.113
		14.0 × 11.0	1.08	0.91	1.543	1.831	0.108
Fully extended	36 × 16 × 11	18.0 × 8.0	1.85	0.86	0.901	1.938	0.111
		18.0 × 9.0	1.42	0.76	1.174	2.193	0.109
		18.0 × 7.0	2.48	0.97	0.672	1.718	0.115

[a] η = 0.01 P. T = 305 K. [b] $\tau = 1/6D$. [c] Measured on molecular space-filling models. [d] Assuming a rigid body, T_1 value is calculated for a CH group where the C-C bonds make an angle of 40° with the long axis. Rotation about C-C bonds is slow, $D_{int} = 1.0 \times 10^{10}$ s^{-1}. The calculations were performed for a magnetic field of 67.9×10^3 G.

cules is determined by their van der Waals radii. Each molecule is considered to be enclosed by a van der Waals surface which is spherical about each atom. The van der Waals volume of a molecule is obtained by summing the van der Waals increments for all the atoms in the molecule.[28,29] This method is accurate for small molecules (2-6-Å radius). However, for large flexible linear molecules which can fold upon themselves, the method will yield only a *lower bound* on the size. The reason is that folded peptides may trap solvent molecules and this might result in a larger effective rotational volume. For LH-RH, the method of atomic increments yields 6.3 Å for the radius r of the spherical particle. The effect of hydrogen bonding to solvent molecules has not been considered. For instance, if each carboxyl and (peptide) N-H group were strongly hydrogen bonded to one molecule of water, the minimum radius would increase to 7.2 Å.

Molecular space-filling models (CPK) of LH-RH yield dimensions of 20 × 18 × 14 Å for the axes when the peptide assumes its most compact shape. In a fully extended conformation values of 36 × 16 × 11 Å are obtained for the lengths of the axes of the ellipsoidal molecule. Table II contains the computed molecular dimensions when LH-RH is made to assume some of the classical conformations observed in peptides. These values are meant only as approximations in order to calculate possible (or plausible) values for the rates of overall molecular motion. In Table II we have used the two largest dimensions measured on each model to estimate the diffusion constants. This was done in order to obtain an *upper bound* for the molecular dimensions (the *lower bound* was obtained from the method of atomic increments). Table II also shows that the greatest ratio of axes is observed in the fully extended model and this ratio is never greater than 3.5:1. The actual value is undoubtedly less than this because it is unlikely that a linear peptide will at all times assume a fully extended conformation in solution. The above ratio does, however, provide a plausible upper limit for the monomer when calculating the effect of anisotropic motion on the observed T_1 values.

(c) Anisotropic Molecular Motion. If a molecule is nonspherical in shape, the overall molecular motion will not be isotropic and two or more correlation times will be needed to describe its rotational diffusion.[30] A rigid ellipsoid of revolution has two rotational diffusion constants:[24] one for rotation about the symmetry axis ($D_\parallel$) and another for rotation about any axis perpendicular to the symmetry axis ($D_\perp$). In Tables II and III diffusion constants for rotation about the symmetry axis as well as for rotation about an axis perpendicular to the symmetry axis in LH-RH are presented. The $D_\parallel$ and $D_\perp$ in Table II have been calculated from the molecular friction constants of an ellipsoid[30] tumbling in solution, assuming a viscosity (η) of 0.01 P, a temperature of 305 K, and dimensions as measured on the CPK models. It must be emphasized that this assumes that molecular reorientation is determined solely by molecular shape. The latter may be justified in cases where solute-solvent interactions do not produce anisotropic frictional forces. Table III shows the effect of varying the lengths of the semiaxes of an ellipsoid of constant volume (corresponding to the volume of a sphere 8 Å in radius) on the rotational diffusion coefficients about the different axes. Again η was 0.01 P and T = 305 K. The correlation time for reorientation of a given C-H internuclear vector (τ_{C-H}) held rigidly in the molecular framework depends on the angle between the C-H internuclear vector and the axis of symmetry of the ellipsoid.[24] When the molecular motion is not isotropic, different T_1, and consequently τ, values can be observed for different angles between the C-H vectors and the axis of symmetry of the ellipsoid. In Table II examples are given of T_1 values expected for CH groups which are held rigidly within the molecular framework. We assumed an ellipsoidal model for the peptide molecule, with an angle of 40° between the long axis and the C-C bond (as would be the case with the backbone extended along the symmetry axis of the ellipsoid). The angle between the C-C bond and the C-H bond is 109.8°. T_1 values in the range of 108-137 ms are calculated depending on the conformation chosen. These T_1 values are *lower bounds* because they correspond to the restricted (essentially rigid) groups in the models of maximum volumes.

Table III. Rotational Diffusion Constants and Correlation Times for Rotation about the Axes of Ellipsoids of Revolution of Volume Corresponding to a Sphere of 8-Å Radius[a]

Semiaxes, Å		Rotational diffusion coeff, 10^8 s^{-1}		Correlation times, 10^{-10} s	
		$D_\parallel$	$D_\perp$	$\tau_\parallel$	$\tau_\perp$
8.001	7.999	3.32	3.25	5.02	5.12
9.00	7.54	3.5	3.1	4.76	5.38
10.00	7.16	3.7	2.9	4.50	5.75
11.00	6.82	3.8	2.6	4.38	6.41
12.00	6.53	4.0	2.3	4.17	7.25
13.00	6.27	4.1	2.1	4.07	7.94
14.00	6.05	4.2	1.9	3.97	8.77
15.00	5.84	4.3	1.7	3.88	9.80

[a] Assuming η = 0.01 P. T = 305 K.

B. Effect of Internal Motion on T_1 Values of Side Chains. (a) Isotropic Overall Molecular Motion. We have already stated that in a linear peptide such as LH-RH, internal motion is possible in the peptide backbone. If such internal motion occurs at a rate comparable to or greater than that of overall molecular reorientation, the effective correlation time will comprise contributions from overall molecular motion and internal rotation. In the case of isotropic overall motion and one internal motion, the relation between the observed correlation time (τ_{eff}), the correlation time for internal motion ($\tau_{int} = 1/6D_{int}$), and that for overall motion (τ_{mol}) has been described.[13,16] Figure 2 shows the effect of internal motion on observed T_1 values (T_{1eff}) for various rates of overall molecular motion, assuming that the overall molecular motion is isotropic. We find that correlation times in the range between 5×10^{-9} and 2×10^{-10} s for overall molecular reorientation and greater than 7×10^{-11} s for internal motion will yield T_1 values in the range of those observed for the peptide backbone of LH-RH ($\simeq$200 ms). Figure 2 is mainly useful to set plausible limits for rates of overall as well as internal motion in a peptide backbone. It must be emphasized again that such calculations assume *independent* and *uncorrelated* motion about all bonds, an assumption which is probably not justified for the peptide backbone.

Table I shows the values of the correlation times obtained for internal motions about individual carbon-carbon bonds using various values for τ_{mol}. We have performed a number of calculations for the side chains. In one approach we assumed that the α-carbon of each residue was the effective center of mass for each side chain; i.e., the side chain was anchored at the α-carbon. The correlation time of the α-carbon was considered to be the effective correlation time for both overall molecular motion and internal motion of the peptide backbone. Furthermore, the overall motion was assumed isotropic. The τ values obtained are listed in Table I, column 1. The backbone α-carbons have the longest correlation time in the vicinity of the aromatic residues. The tryptophyl C_α has $\tau = 4.1 \times 10^{-10}$ s; the seryl and tyrosyl C_α's have an average τ value of 3.8×10^{-10} s. The correlation times are shorter for the backbone α-carbons near the N- and C-terminal ends, implying some segmental motion of the backbone. As noted previously[12] the glycine residue (no side chain) in position 6 appears to be conformationally more flexible than the residues on either side of it. The calculations for the cyclic residues pyroglutamate and proline are not entirely justified as these residues can undergo only torsional motion and not true rotation. In histidine, tryptophan, and tyrosine the internal motions of the side chains are slow compared to the motion of the α-carbon. The side chain of leucine shows a decrease in correlation time for rotation about the C_α-C_β bond compared to C_α, but an increase in correlation time for rotation about C_β-C_γ. The latter observation is likely to result from the presence of two CH_3 groups attached to C_γ. The two methyl groups of leucine have very short correlation times for rotation about the C_γ-C_δ bonds. The side chain of arginine shows a decrease in correlation time for rotations about bonds between carbons α and γ; however, a sharp increase in τ is noted about the C_γ-C_δ bond. Again this appears to be a consequence of the presence of a bulky group linked to C_γ.

In order to take into account internal motion of the peptide backbone we have carried out a series of calculations in which we fixed the value of the correlation time for overall molecular motion at either 5.0×10^{-10} or 1.0×10^{-9} s. The former value is expected for a spherical molecule 8 Å in radius dissolved in a medium of viscosity equivalent to that of water. Such a τ value has been observed for oxytocin, vasopressin, and angiotensin II (molecular weights 1000-1100)

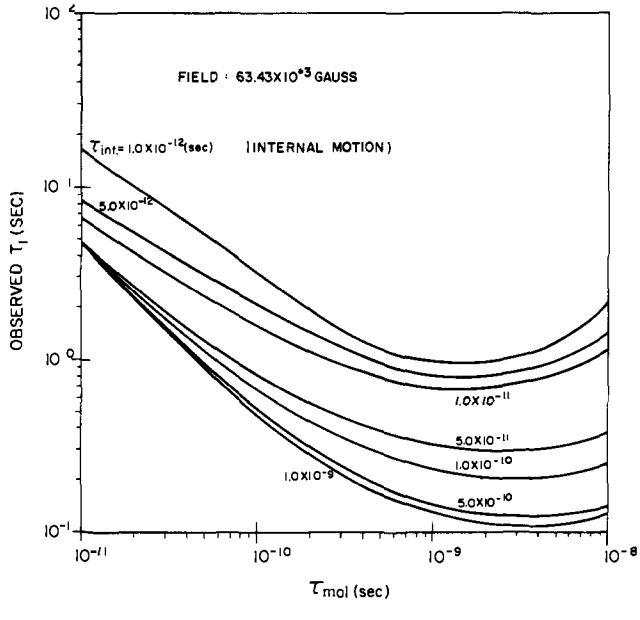


Figure 2. Effect of internal motion on observed T_1 values for various rates of overall motion assuming isotropic overall molecular motion and one internal motion. Applied magnetic field strength 63.43×10^3 G.

which have radii of $\simeq$8 Å as measured on space-filling models.[7,31] The value of 1.0×10^{-9} s is that obtained for a compact conformation of LH-RH (Table II). The above values are physically realistic; a τ value of 4.1×10^{-10} s is observed for the most restricted α-carbon of LH-RH. If the backbone of LH-RH were rigid, the T_1 values of the α-carbons would be determined by overall molecular reorientation and consequently the correlation time for this motion cannot be greater than 4.1×10^{-10} s. For a given T_1 value, if internal motion is possible, the correlation time for overall molecular motion will be longer because the internal motion of the backbone will contribute to the effective correlation time. Column 9 of Table I shows the values of τ_{int} obtained for rotation about all the C-C bonds in the leucyl and arginyl residues of LH-RH. For the α-carbons we have assumed that rotation occurs only about one bond, that which links C_α to the bulk of the molecule. As the overall motion is isotropic, the relaxation times have no angular dependence. Comparing the two sets of calculations indicates that changing the correlation time for overall molecular motion affects appreciably only the correlation time of the α-carbon. A small effect is observed on the calculated correlation time for rotation about the C_α-C_β bond. No effect is seen farther along the chain. We can compare these results, where we assume that overall molecular motion and internal motion of the peptide backbone both contribute to the T_1 values of the α-carbons, with those in which the α-carbon is used as the center of mass and considered effectively rigid. We again find that the values of the correlation times for rotation of C_α and for rotation about the C_α-C_β bonds are affected, but for C_β-C_γ the results differ by less than 10%. We can conclude that the values of the correlation times for internal motion in the side chains beyond C_γ are independent of the values chosen for overall molecular reorientation in the ranges which we consider physically significant.

The aliphatic side chains appear to be anchored at the α-carbons of the peptide backbone and undergo increased or segmental motion toward either end. A plot of T_1 vs. carbon number (for carbons in position 2 outward) would have a slope which is proportional to the rate of internal motion if

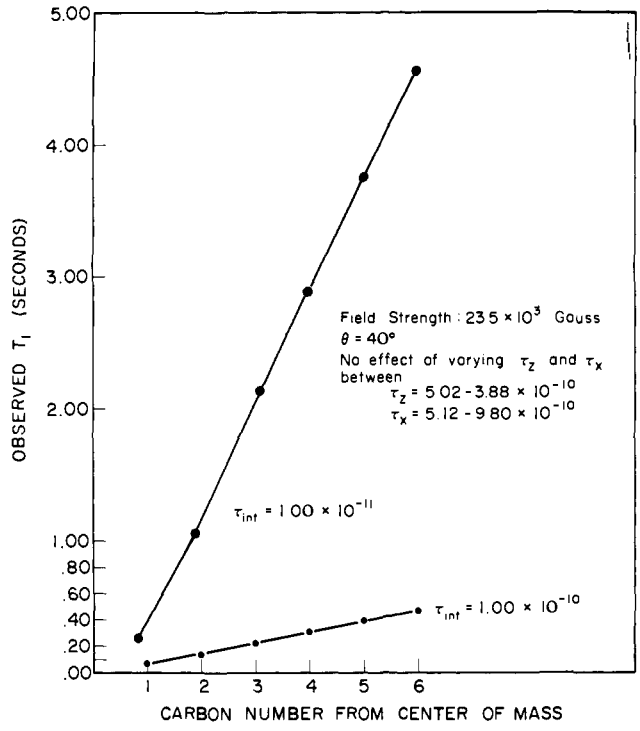


Figure 3. Effect of fast internal motions on observed T_1 values of CH_2 groups. Ellipsoids of revolution have a volume corresponding to that of a sphere of 8-Å radius. Both isotropic and anisotropic overall molecular motion were considered in physically realistic ranges for LH-RH.

all the rotational diffusion constants about each successive bond are equal. This is not observed for arginine, proline, and leucine. The side chains show gradations in the rates of internal motion. Furthermore, the gradation appears to be characteristic of the type of amino acid rather than of the framework of the peptide in which the side chain is embedded. The NT_1 values of the carbons in the amino acid side chains appear to be good qualitative monitors of internal motions, at least for carbons more than one bond removed from C_α.

(b) Anisotropic Overall Molecular Motion. In order to monitor the effect of anisotropic overall molecular motion on the T_1 values in linear peptide hormones we have carried out a set of calculations on a model system in which the volume of the molecule was kept constant and the ratio of semiaxes was varied. We chose a volume which was equal to that of a sphere of 8-Å radius. Rotational diffusion constants were calculated[30] as in Table II, assuming a viscosity of 0.01 P for the solution and a temperature of 305 K. The correlation times are given in Table III. Figure 3 shows the effect of anisotropic molecular motion on observed T_1 values in an alkane for different values of the angle between the first C-C bond and the long axis of the ellipsoid of revolution. The angle between each remaining C-C bond is 109.5° and the angle between the C-H internuclear vector and the axis of internal motion is also 109.5°. From Figure 3 we see that if internal motion is faster than overall molecular motion, then molecular shape has little effect. The observed T_1 values increase rapidly and linearly along the chain. If the internal motion is slower than the overall motion ($D_\perp$), then the molecular shape has a more pronounced influence. The observed T_1 values show a slight dip between carbons 1 and 2 but the increase in T_1 values along the rest of the chain appears linear, in accord with the results of Levine et al.[3]

In Table I, last column, we have calculated the correlation times for internal motion about the bonds in the side chains of leucine and arginine, where the overall molecular

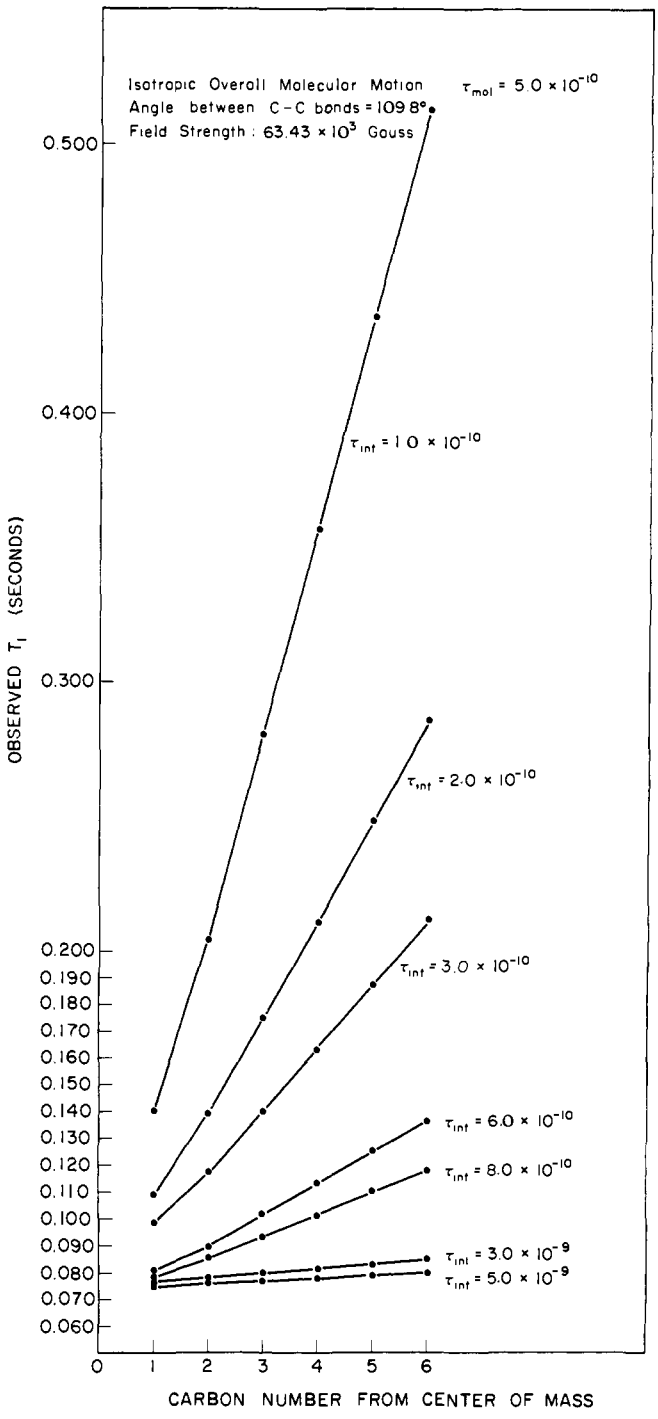


Figure 4. Simulation of backbone motion. Effect of varying τ_{int} on observed T_1 of CH_2 groups for a given value of τ_{mol}, assuming isotropic overall molecular motion. Center of mass is located at carbon 0.

shape of LH-RH was assumed to be an ellipsoid of rotation. We set τ_z, the correlation time for rotation about the z, or long axis of the ellipsoid, equal to 0.9×10^{-9} s. The correlation times for rotation about axes perpendicular to the long axis of the ellipsoid, τ_x and τ_y, were set to 1.9×10^{-9} s. These values were obtained from Table II and correspond to the values expected for LH-RH in a fully extended conformation. They can be compared with values obtained for the isotropic case (i.e., $\tau_x = \tau_y = \tau_z$) with $\tau_{mol} = 1.0 \times 10^{-9}$ s. The anisotropy of the motion has little effect beyond the β-carbon, and even there the effect is less than 5%. Similar conclusions have been reached by Levine et al.[3] In Table IV the calculated NT_1 values of leucyl carbons in the LH-RH are compared for the spherical and ellipsoidal

Table IV. Correlation Times for Internal Rotations in Leucine in LH-RH

Position	Obsd NT_1, ms	τ_{int},[a] 10^{-10} s	Calcd NT_1, ms	τ_{int},[b] 10^{-10} s	Calcd NT_1, ms $\theta = 0°$	$\theta = 40°$	$\theta = 90°$
α-CH	175	2.1	178	1.8	177	176	176
β-CH_2	240	1.8	242	1.7	240	240	242
γ-CH	260	4.3	260	4.3	258	260	260
δ-CH_3	1545	0.04	1540	0.04	1534	1538	1544
δ-CH_3	1440	0.05	1538	0.05	1434	1438	1444

[a] Isotropic overall molecular motion: $\tau_z = \tau_x = \tau_y = 1.0 \times 10^{-9}$ s. Calculated for a sphere of 10-Å radius (vol = 37 699 Å^3). [b] Anisotropic overall molecular motion: $\tau_z = 0.9 \times 10^{-9}$ s, $\tau_x = \tau_y = 1.9 \times 10^{-9}$ s. Calculation for an ellipsoid of revolution 18 × 8 Å (semiaxes) (vol = 43 429 Å^3). θ = angle between first α-carbon and long axis of the ellipsoid of revolution.

Table V. Simulation[a] of Backbone T_1 Values in LH-RH

Residue	Obsd NT_1, ms	Calcd τ_{int}, s	Calcd NT_1, ms
<Glu	295	1.6×10^{-10}	292
His	210	4.0×10^{-10}	210
Trp	160	4.5×10^{-9}	171
Ser	165	4.5×10^{-9}	168
Tyr	165	4.5×10^{-9}	165
Leu	175	1.2×10^{-9}	175
Arg	185	1.3×10^{-9}	185
Pro	220	4.3×10^{-10}	220
Gly-NH_2	390	7.6×10^{-11}	390

[a] Assuming isotropic overall molecular motion with $\tau_x = \tau_y = \tau_z = 4.1 \times 10^{-10}$ s. Calculation done separately for N-terminal and C-terminal halves of the molecule using the pseudobond approach. In the calculations the center of mass was assumed to be near position 6 and the possible internal motion at this position was neglected.

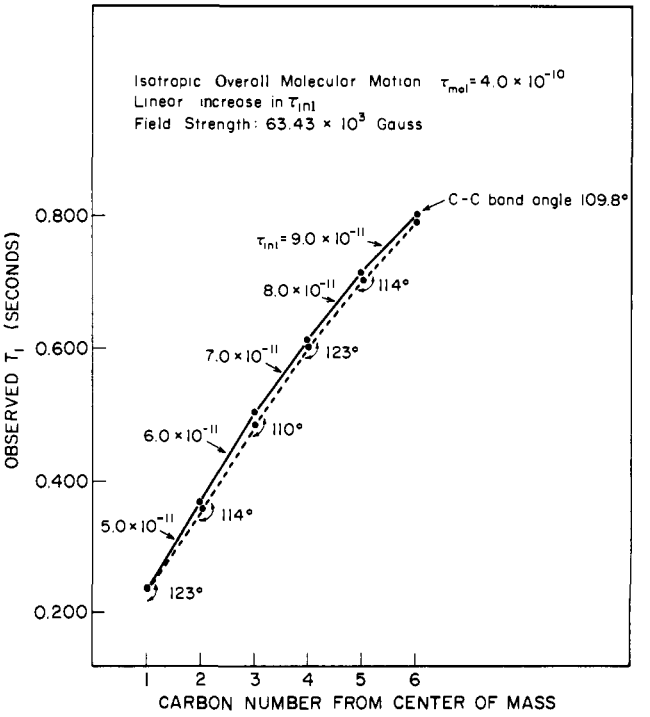


Figure 5. Effect of varying angle between C-C bonds. Isotropic overall molecular motion of polyglycine-type chain. $\tau_{mol} = 4.0 \times 10^{-10}$ s.

models of LH-RH. Table IV illustrates the insensitivity of the NT_1 values to the angle between the long axis of the ellipsoid of rotation and the C_α molecular framework bond.

C. Simulation of Motion of Backbone. In previous sections we have considered the motions of the side chains as units. The α-carbon of each side chain unit has been assumed either to be embedded in a rigid body which possesses the motional characteristics of the whole molecule or to be one bond removed from the molecular framework which rotates as a rigid body. We have not yet examined the motional characteristics of the peptide backbone as a unit. The NT_1 values of the backbone are fairly constant up to the penultimate positions at the N- and C-terminal ends, where an increase in NT_1 values is observed. This increase is more pronounced at the glycine-bearing C terminus.

Figure 4 shows the effect of different rates of internal motion on observed T_1 values for a chain undergoing isotropic overall molecular motion with correlation times of 5.0×10^{-10} and 4.0×10^{-10} s. In the model calculations an angle of 109.8° between each C-C bond was assumed. Only by having internal motions which are slow compared to the overall molecular motion can we obtain a fairly flat slope. This model, however, assumes that the center of mass is at the "carbon" designated as 0. Figure 5 shows the effect of using the correct dihedral angles for a peptide backbone compared with those for an alkane. The angular effect appears to be negligible for our conditions.

Table V contains the values obtained for internal motion in the backbone, assuming that the center of mass is located near carbon 6. In these calculations the "pseudobond" approach was used. This enabled us to consider a sufficient number of α-carbons.

Figure 6 shows the effect of very slow internal motions on the observed T_1 values in a linear chain which is undergoing isotropic overall motion. Such calculations simulate the effect of introducing the peptide link (no rotation, $\tau_{int} = 10^{10}$ s) in the linear chain.

We have performed calculations using all the bonds in a peptide for a segment which includes three α-carbons. We find that, to a good approximation, the correlation times assumed for rotations about -HC_α-CO- [$\tau_{int}(C_\alpha\text{-}C)$] and -HN-$C_\alpha$- [$\tau_{int}(N\text{-}C_\alpha)$] should be 2-3 times larger than the correlation time [τ_{int}(pseudo)] needed for the corresponding pseudobond. If τ_{mol}/τ_{int} (center of mass) is greater than 10, this factor is 3; otherwise it is 2. We considered $10^{-11} \leq \tau_{mol} \leq 10^{-9}$, assumed that τ_{int} (C_α-C) = τ_{int} (N-C_α), and that there is no rotation about the CO-NH bond.

In peptides, where the molecular framework is *not* rigid, motion of the backbone can lead to the observation of a "pseudoisotropic" behavior. This term describes the effect of time averaging a number of conformations in a flexible backbone; consequently, no point of the backbone remains fixed with respect to a point within the molecular framework. The *time averaged* molecular conformation then rotates in solution as if it were effectively isotropic.

In the case of LH-RH the similar NT_1 values of the backbone may be the result of "pseudoisotropic" motion. Internal motion in such a system would not produce a gradation of T_1 values and the results would resemble more those that were observed in cycloalkanes[5] than those found for acyclic *n*-alkanes.[2a]

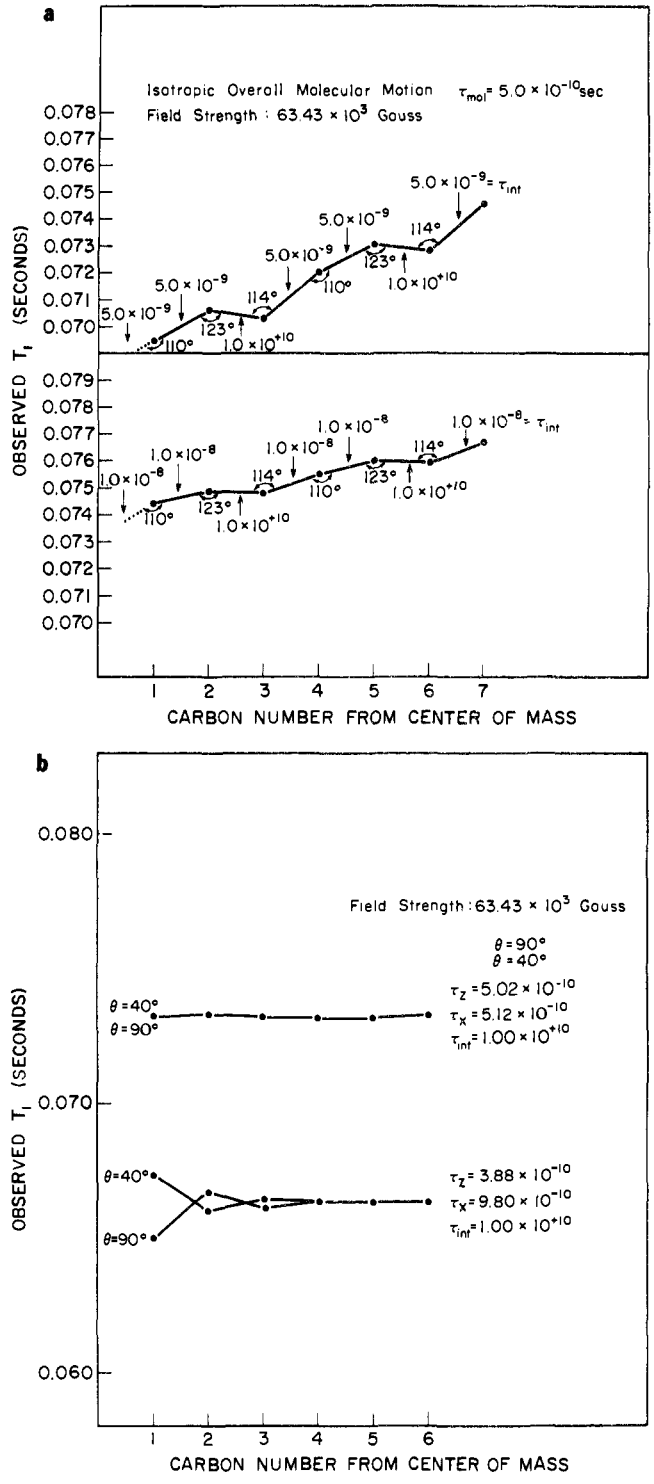


Figure 6. Effect of very slow internal motions on observed T_1 values of CH_2 groups. (a) Isotropic overall motion. Simulation of motions in a peptide backbone. (b) Isotropic and anisotropic overall molecular motion. Simulation of aliphatic side chain in which all internal motions are slow. θ is the angle between the first C-C bond and long axis of ellipsoid of revolution.

The α-carbons of residues **1, 2, 9,** and **10** show longer NT_1 values than residues **3–8**. This seems to be a consequence of increased motional freedom. Such residues can be considered as anchored at positions 3 and 8 with increasing flexibility of the chain toward the termini.

Conclusion

In analyzing the rates of internal motion of the side chain of LH-RH, we have considered both spherical and ellipsoidal models of overall molecular shape. In these models, the overall molecular motion of LH-RH was considered to be that of a rigid body rotating in solution about its principal moment of inertia axes with rates proportional to the relative values calculated from molecular friction coefficients.

We have found that the different models used to describe overall tumbling have no appreciable effect (<10%) on the calculated diffusion constants (and thus T_1 values) about individual bonds in side chains beyond the β-carbon. However, if the internal motion is much slower than the reorientation of the long axis of the molecule, nonisotropic motion can generate T_1 values which are shorter for the α-carbon than for the β-carbon of a given amino acid. Birdsall et al.[2b] reached the same conclusion in the case of hexane.

Monitoring the effect of the slight anisotropy in overall tumbling of peptides would necessitate very accurate T_1 measurements (uncertainty <3%). This greater accuracy should also permit a study of the relative amount of steric hindrance imposed on a given amino acid in a peptide by different adjacent residues. This may in turn help in elucidating some aspects of the structure-biological activity of peptide hormones.

An increased experimental accuracy will require correspondingly more sophisticated theoretical models for the T_1 data analysis. In particular, the stochastic rotational diffusion model currently used is unlikely to be equally valid or even reasonable for both overall and internal motions. Furthermore, assuming independent and uncorrelated rotations about bonds (a mathematical convenience) is clearly inappropriate when one wants to unravel the consequences of steric hindrance, H bonding, solvent effects, etc., on relaxation time results. Ultimately, one will need to calculate from and for a given conformation the corresponding correlation functions, spectral densities, etc., and *then* set up a model for the relaxation processes that reproduces the experimental results and mimics closely the "true" correlation functions, etc. Such a theoretical program is under development by one of us (R.L.S.).

Acknowledgment. We wish to thank Dr. Y. K. Levine for providing us with listings of the original programs used to calculate T_1 values. This gave us an invaluable starting point for the modifications we required. R.D. also wishes to thank Dr. Ian C. P. Smith for his support and encouragement during the course of this work.

Appendix

The calculations of spin-lattice relaxation times T_1 from the angular autocorrelation functions required the evaluation of the matrix elements $D_{mn}^{(2)}(0,\beta,\gamma) = d_{mn}^{(2)}(\beta)e^{-in\gamma(t)}$ ($m, n = -2, -1, 0, 1, 2$) of the 5 × 5 second-order Wigner rotation matrix.[10] In particular, the time-independent part, $d_{mn}^{(2)}(\beta)$, depends only on the angle β between adjacent bonds.

Our simple generalization of Levine's model allows us to include arbitrary angles β_i between any pair of bonds along the chain, as well as arbitrary diffusion constants D_i for the internal motions about the different bonds. However, in order to be able to deal efficiently with a larger number of bonds (>6), Levine's computer program had to be optimized considerably. This optimization is especially important when the β_i are all different because the computer time is particularly sensitive to how frequently the $d_{mn}^{(2)}(\beta_i)$ have to be reevaluated (for N bonds and anisotropic motion, the evaluation of $J(\omega_k)$ involves an $(N + 3)$-fold summation).

The matrix elements $d_{mn}^{(2)}(\beta)$ have the form[32]

$$d_{mn}^{(2)}(\beta) = C_{mn} \sum_{\alpha} \frac{(-1)^{\alpha}(\cos(\beta/2))^{4+n-m-2\alpha}(-\sin(\beta/2))^{m-n+2\alpha}}{(2-m-\alpha)!(2+n-\alpha)!(\alpha+m-n)!\alpha!} \quad \text{(A1)}$$

where

$$C_{mn} = [(2+n)!(2-n)!(2+m)!(2-m)!]^{1/2}$$

and the sum is over the values of the index $\alpha \geq 0$ for which the factorial arguments are ≥ 0.

Only the 15 matrix elements in the 5×3 block of $\mathbf{d}^{(2)}$ are needed for obtaining $J(\omega_k)$, and of these only nine are distinct

$$\mathbf{d}^{(2)}(2\beta) = \left|\begin{array}{ccc:cc} a & b & c & d & e \\ -b & f & g & h & d \\ c & -g & i & g & c \\ -d & h & -g & f & b \\ e & -d & c & -b & a \end{array}\right| \quad \text{(A2)}$$

where

$$\begin{aligned} a &\equiv d_{2,2}^{(2)}(2\beta) = \cos^4\beta \\ b &\equiv d_{1,2}^{(2)}(2\beta) = 2\sin\beta\cos^3\beta \\ c &\equiv d_{0,2}^{(2)}(2\beta) = \sqrt{6}\sin^2\beta\cos^2\beta \\ d &\equiv d_{-1,2}^{(2)}(2\beta) = 2\sin^3\beta\cos\beta \\ e &\equiv d_{-2,2}^{(2)}(2\beta) = \sin^4\beta \\ f &\equiv d_{1,1}^{(2)}(2\beta) = \cos^2\beta(\cos^2\beta - 3\sin^2\beta) \\ g &\equiv d_{0,1}^{(2)}(2\beta) = \sqrt{6}\sin\beta\cos\beta(\cos^2\beta - \sin^2\beta) \\ h &\equiv d_{-1,1}^{(2)}(2\beta) = \sin^2\beta(3\cos^2\beta - \sin^2\beta) \\ i &\equiv d_{0,0}^{(2)}(2\beta) = (\cos^2\beta - \sin^2\beta)^2 - 2\sin^2\beta\cos^2\beta \end{aligned} \quad \text{(A3)}$$

Their explicit evaluation via eq A3 requires the computation of only 1 sine, 1 cosine, 12 multiplications, and 8 additions-subtractions ($\sqrt{6}$ is brought in through COMMON). This takes less than $\frac{1}{60}$th of the time that the direct use of eq A1 would need.

Further time savings could be effected by building into the main subroutine all the checks required to minimize the number of evaluations of $\mathbf{d}^{(2)}$. Each new bond increases the total computation time t_N by a factor of 3. The program can now handle up to 12 bonds and its highly modular form allows for easy extension. In the most general case (all β_i different), we find

$$t_N = C3^N \text{ s} \quad \text{(A4)}$$

where $C = 5.625 \times 10^{-3}$ for an IBM 360/67, and N is the number of bonds. If all β_i are the same, the constant C in eq A4 reduces to 2.92×10^{-3}. For this case, the program could be optimized further by removing the checks and evaluating the $d_{mn}^{(2)}(\beta)$ only once in the main program.

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