may occur at any stage, it is not surprising that complete equilibrium is not observed. It is probable that the various fragmentation reactions occur from specific canonical forms, i.e., loss of NH_3 from e and loss of H_2O from f (and possibly d). However, even this model of equilibration involving only the labile hydrogens may be too simple. One would anticipate that the loss of ammonia and water from the MH^+ ion of 4-aminobutyric acid and 6-aminohexanoic acid should remove all the labile hydrogens along with the added proton. However, as summarized in Table VII, we find 21–27% deuterium retention in the fragment ions resulting from loss of ammonia and water from the MD^+ ion in the D_2 and CD_4 CI of these amino acids. This result indicates that there may be significant interchange of the labile hydrogens with hydrogens bonded to carbon prior to fragmentation of MH^+.

Several other deuterium retention results are of interest. In line with the results in Tables IV to VI, the fragment ion resulting from elimination of methyl mercaptan from MD^+ in methionine showed 20% deuterium retention in the CD_4 CI and 52% retention in the D_2 CI. This compares with the 75% calculated on the basis of equilibration. Obviously, fragmentation by this mode does not result solely from protonation at this site although there is a preference for loss of the added proton.

The *m/e* 74 ion ($H_2N^+{=}CHCOOH$) is particularly abundant in the H_2 CI of leucine, isoleucine, aspartic acid, and methionine. For leucine and isoleucine there was practically no deuterium incorporation in this fragment when D_2 was used as reagent gas, indicating that the ion does not originate by fragmentation of the MD^+ ion. The most likely origin is either by charge transfer, possibly involving excited states of H_3^+, or by decomposition of the $(M - H)^+$ ion. By contrast, in the D_2 CI *m/e* 74 showed 57% deuterium retention for aspartic acid and 60% retention for methionine. The *m/e* 74 ion also is observed in the CH_4 CI of aspartic acid and using CD_4 the deuterium retention was found to be 50%. These retention figures are consistent with extensive intramolecular hydrogen interchange and fragmentation by the mechanisms outlined in Schemes III and IV. In the same vein, the *m/e* 44 ion in the CI of 3-aminobutyric acid showed approximately 40% deuterium retention in both the D_2 and CD_4 CI. Again this is consistent with the mechanism of formation outlined in reaction 7.

Acknowledgment. The authors are indebted to the National Research Council of Canada for financial support. A.G.H. gratefully acknowledges the kind hospitality of Professor K. R. Jennings and the Department of Molecular Sciences, University of Warwick, where this manuscript was written.

References and Notes

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(5) A. G. Harrison and P.-H. Lin, *Can. J. Chem.*, **53**, 1314 (1975).
(6) *Natl. Stand. Ref. Data Ser., Natl. Bur. Stand.*, **No. 26** (1969).
(7) G. Junk and H. Svec, *J. Am. Chem. Soc.*, **85**, 839 (1963).
(8) A. G. Harrison, P.-H. Lin, and C. W. Tsang, *Int. J. Mass Spectrom. Ion Phys.*, **19**, 23 (1976).
(9) C. W. Tsang and A. G. Harrison, *J. Chem. Soc., Perkin Trans. 2*, 1718 (1975).
(10) Initial protonation at a specific site, followed by incomplete equilibration through proton transfer, would result in a preference for loss of the added proton (deuteron) in only one fragmentation reaction. Since such a unique preference is not observed we conclude that the initial protonation must occur at any one of several sites.

The Dependence of Geminal H–H Spin–Spin Coupling Constants on ϕ and ψ Angles of Peptides in Solution

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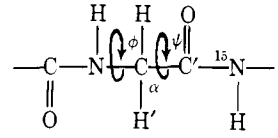
Abstract: A theoretical study is presented of the conformational dependence of geminal H–H coupling constants in compounds which provide models for the peptide structure. Calculated results for Fermi contact coupling in *N*-methylacetamide, N^{α}-acetylglycinamide, *cyclo*-(-Gly–Gly–), and a three-peptide fragment having a γ turn are based on the finite perturbation theory (FPT) formulation in the semiempirical approximation of intermediate neglect of differential overlap (INDO). It is shown that the effect of the amide carbonyl is to produce a shift in the value of the geminal coupling constant to more negative values, depending on the value of the dihedral angle ψ. However, the effect of the amide nitrogen is to shift the geminal coupling constants toward more positive values depending on the dihedral angle ϕ. Under the combined effects of the two groups, as in N^{α}-acetylglycinamide, the total variation of the coupling is calculated to be 8 Hz. Agreement of calculated and experimental values is quite satisfactory in those cases in which x-ray structural data for the molecules are known. Although polarity of the solvent is known to have an effect on geminal H–H coupling, the calculated results for the three-peptide fragment having a γ turn suggests that intramolecular hydrogen bonding may not be an important factor. Based on these results, it is concluded that geminal H–H coupling constants can complement other NMR parameters as a probe of peptide structure in solution.

Studies of the conformations of peptides in solution[1] have made extensive use of spin–spin coupling constants from nuclear magnetic resonance spectra. Especially important, in this regard, are the vicinal H–N–C–H coupling constants, which provide a measure of the dihedral angle ϕ measured about the N–C_{α} bond in the peptide backbone **1**. It has also been suggested that the vicinal ^{15}N–C′C_{α}–H coupling constants would provide a measure of the dihedral angle ψ measured about the C_{α}–C′ bond in the peptide backbone.[2-5] However, it appears that the difficulties of in-



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corporation of ^{15}N combined with the small magnitudes of the observed coupling constants[2] greatly limits the applicability of the technique.

In the present study it is shown that the geminal H–C–H′ coupling constants in the glycyl residues of the peptide backbone **1** depend on both dihedral angles ϕ and ψ. The form of this dependence is investigated by means of the finite perturbation theory (FPT) formulation for nuclear spin–spin coupling in the INDO (intermediate neglect of differential overlap) approximation of semiempirical molecular orbital (MO) theory.[6] The calculated results are found to be in reasonable correspondence with the experimental data. Any experimental criterion for estimating angles should be an improvement upon estimates from conformational energy calculations.[7]

Geminal H–H coupling constants in methylene groups exhibit unusually large substituent effects, ranging from +42 to −22 Hz. Following the original observations[8] and theoretical explanations[9-11] of the effects of adjacent unsaturated and electronegative substituents, a number of reviews on the subject have appeared,[12-16] and the conclusions may be summarized as follows.[9-11]

(i) Adjacent substituents bearing double bonds will produce shifts to more negative geminal coupling constants in reasonable correspondence with the original theoretical suggestion[9] of a dependence proportional to $\cos^2 \xi \cos^2 \xi'$ where ξ and ξ' are the dihedral angles between the planes formed respectively by C′–C_α–H and C′–C_α–H′ and the plane perpendicular to C_α—C′=O which includes the axis of the $2p_z$ atomic orbitals.

(ii) Adjacent electronegative substituents give more positive values of the geminal coupling, but there is no simple relationship between $^2J_{HH'}$ and substituent electronegativity.[12]

(iii) Adjacent substituents with lone pairs will also give more positive values of $^2J_{HH'}$ and might be expected to follow a dependence of the form $\cos \xi \cos \xi'$, where the dihedral angles ξ and ξ' are measured from the planes containing the N–C_α–H and N–C_α–H′ to a plane which includes the axis of the nitrogen lone pair.[10,17]

(iv) Substitution of an electronegative substituent at a position β to a methylene is expected to produce on the average slight negative shifts in the values of $^2J_{HH'}$.[11]

Geminal H–H coupling constants in the glycyl residues of the peptide backbone **1** will depend on all four of these substituent effects as well as the nature of the solvent[18] and conformational averaging. It is the purpose of this investigation to show that despite the extreme complexity of the physical situation, useful structural data may be obtainable from the $^2J_{HH'}$ in glycyl residues of peptides in solution, especially when complemented with other coupling constant data. In the following sections the INDO-FPT method will be applied to model systems and the calculated results compared with representative experimental data. Although the INDO method with the usual parameterization tends to underestimate the absolute values of the $^2J_{HH'}$,[19] the substituent and solvent dependencies appear to be adequately reproduced.

Molecular Orbital Description of Substituent Effects on $^2J_{HH'}$

1. Geminal H–H Coupling in the Methyl Adjacent to the Carbonyl in *N*-Methylacetamide.

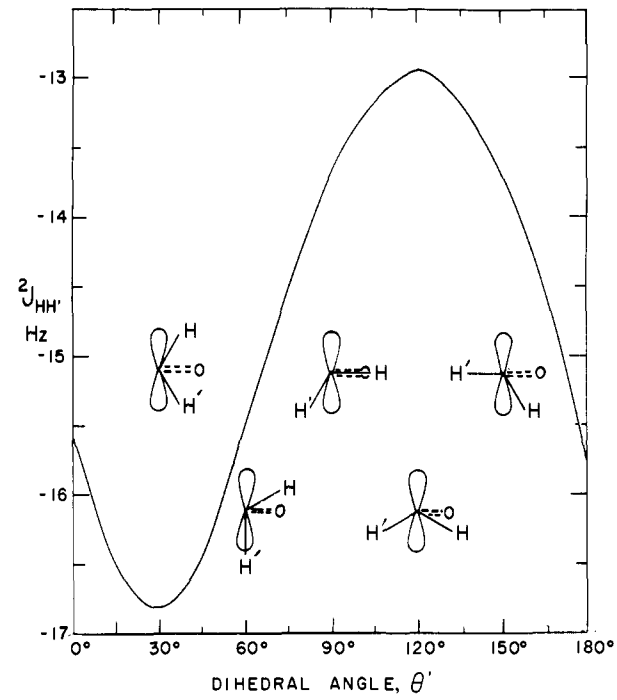
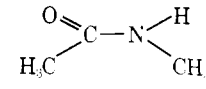


Figure 1. A plot of the calculated INDO-FPT results (shifted by −8.0 Hz) for geminal H–H coupling in the methyl group adjacent to the carbonyl of *N*-methylacetamide (**2**) as a function of the dihedral angle θ'. This dihedral angle is measured from the plane which includes the axis of the $2p_z$ atomic orbital of the carbonyl carbon. The situation is depicted for representative dihedral angles in the inset diagrams.

Table I. Calculated INDO-FPT Results for the Geminal H–H Coupling Constant of the Carbonyl Methyl of *N*-Methylacetamide (**2**) as a Function of the Dihedral Angle θ' and $\psi = 30° - \theta'$

θ', deg	ψ, deg	$^2J_{HH'}$, Hz	θ', deg	ψ, deg	$^2J_{HH'}$, Hz
0	30	−7.57	120	−90	−4.93
30	0	−8.82	150	−120	−5.66
60	−30	−7.57	180	−150	−7.73
90	−60	−5.64	210	−180	−9.03

Calculated INDO-FPT values of the geminal H–H coupling constants of the methyl group adjacent to the amide carbonyl of *N*-methylacetamide (**2**) were based on the Corey–Pauling geometrical



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model.[20] Calculated results are given in Table I at 30° intervals of the dihedral angle θ', and of ψ, which is related to θ' by the expression[21]

$$\psi = 30° - \theta' \qquad (1)$$

The dihedral angle θ' is depicted in the formulas inset in Figure 1.

The magnitudes of the calculated geminal H–H coupling constants in Table I are substantially smaller in magnitude than the experimental ones. Problems associated with accurate calculations of geminal H–H coupling constants have been discussed in detail,[10,13,19] and occur in both valence-bond and molecular-orbital formulations. Within the VB descriptions the difficulty has been ascribed[9,10] to cancellation between large (some empirically determined) integrals of opposite sign within the CH_2 moiety. The calculated ab initio MO result for methane is −1.27 Hz[22] in comparison

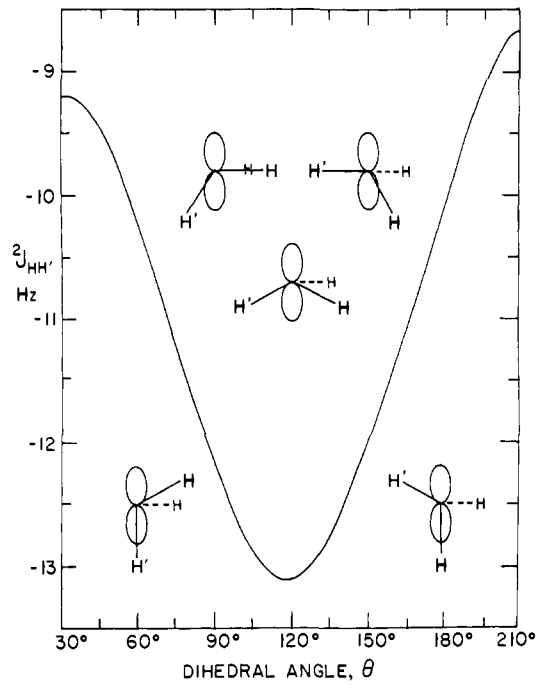


Figure 2. A plot of the calculated INDO-FPT results (shifted by −8.0 Hz) for geminal H–H coupling in the *N*-methyl of *N*-methylacetamide (**2**) as a function of the dihedral angle θ. This dihedral angle is measured between the N–C_α–H plane and a plane perpendicular to the C_α–N–H plane. This is depicted for representative dihedral angles in the inset diagrams.

with the experimental value of −12.4 Hz.[23] The inclusion of the effects of electron correlation in a third-order perturbation formulation gives the much more reasonable value of −15.0 Hz.[22] It would not be computationally feasible to perform such calculations for the compounds of this study which model the peptide structure. It is only possible to make use of the much simpler approximation of INDO wave functions because the substituent effects are adequately reproduced. For example, the range of values of $^2J_{HH'}$ in Table I is nearly four hertz depending on the dihedral angle θ'. This is close to the range found in previous semiempirical VB studies of the effects of adjacent unsaturated groups.[9] The form of the angular dependence of the substituent effects superimposed on a number close to the methane value is very well documented.[12-16]

The calculated results in Table I are reproduced to within about 0.1 Hz by the expression

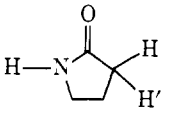
$$^2J_{HH'}(\theta') = -1.45\cos^2\theta'\cos^2(\theta' + 120°) - 3.26[\cos^2\theta' + \cos^2(\theta' + 120°)] + C \quad (2)$$

where $C = -3.20$ Hz. The conformational dependence of the substituent effect in eq 2 is just that expected for the case in which the interactions between the geminal C–H bonds and the adjacent $2p_z$ atomic orbitals are proportional to the product of the terms $A + B\cos^2\theta'$ and $A + B\cos^2(\theta' + 120°)$, which are of the mathematical form of the two-center VB exchange integrals between a carbon hybrid orbital and an adjacent $2p_z$ atomic orbital. In the previous theoretical study[9] the constant term A was neglected in comparison with B, thereby leading to a substituent dependence proportional to the first term in eq 2. The constant C in eq 2 was simply taken to be the methane value of −12.4 Hz.[9] Since the greatest inadequacy of the semiempirical descriptions seems to be associated with the constant term C either due to the integral parameterization and/or the neglect of the effects of electron correlation, the shift of all of the values by a constant amount is a reasonable procedure.

Table II. Calculated INDO-FPT Results for Geminal H–H Coupling in the *N*-Methyl Group of *N*-Methylacetamide (**2**) as a Function of the Dihedral Angle θ and $\phi = \theta - 30°$

θ, deg	ϕ, deg	$^2J_{HH'}$, Hz	θ, deg	ϕ, deg	$^2J_{HH'}$, Hz
30	0	−1.18	135	105	−4.79
45	15	−1.46	150	120	−4.01
60	30	−2.20	165	135	−2.93
75	45	−3.21	180	150	−1.82
90	60	−4.18	195	165	−0.98
105	75	−4.87	210	180	−0.68
120	90	−5.09			

Indeed, the interpretation of all geminal substituent effects has been based on the assumption that the substituents would not modify the parameters within the CH_2 moiety. The addition of −8.0 Hz gives a value of $C = -11.20$ Hz in eq 2, and leads to reasonable agreement with experimental values in representative systems.[12] For example, the value of −17.7 Hz was reported[12,24] for the cyclic lactam **3**, in comparison with a value of −16.8 Hz from eq 2.



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The calculated geminal H–H coupling constants from eq 2 are plotted in Figure 1 as a function of the dihedral angle θ'. The slight deviation of the calculated values in Table I from a periodicity of 180° can be attributed to the effects of inclusion of σ electrons as well as non-next-nearest interaction. The approximation of eq 2 restores the 180° periodicity of the curve in Figure 1. In fact, the dependence of $^2J_{HH'}$ on dihedral angle in the figure is quite similar to the original curve,[9] which was based on the valence-bond formulation. However, in the original curve there was a small "dip" near $\theta' = 120°$. This feature of the calculated results was almost certainly due to the overly simplified approximation of the physical situation by a model which contained only six atomic orbitals, and was not expected to occur in an all-valence electron calculation.

2. Geminal H–H Coupling in the *N*-Methyl of *N*-Methylacetamide. The calculated values of the geminal H–H coupling in the *N*-methyl group of **2**, which were based on the INDO-FPT method and the Corey–Pauling geometrical parameters, are entered in Table II at 15° intervals of the dihedral angle θ. The dihedral angle is measured from a plane perpendicular to the H–N–C_α plane to the H–C_α–N plane as depicted for several conformations in Figure 2. This dihedral angle is related to the usual ϕ angle by the relationship[21]

$$\phi = \theta - 30° \quad (3)$$

The data in Table II are satisfactorily represented by the equation

$$^2J_{HH'}(\theta) = 4.25\cos\theta\cos(\theta + 120°) + C \quad (4)$$

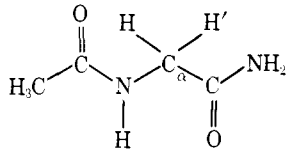
where $C = -4.1$ Hz. On shifting these results by −8.0 Hz, $C = -12.1$ Hz, and the resulting dependence of the $^2J_{HH'}$ on dihedral angle θ is depicted in Figure 2. It may be noted that the conformational dependence predicted from VB theory[10,17] is reproduced in the MO calculations, and that the effect of the lone pair electrons is to produce shifts in $^2J_{HH'}$ to more positive values than the methane value. Unfortunately, there is very little experimental data for geminal coupling constants in cyclic amides to be compared with the calculated results of this section.

Table III. Calculated INDO-FPT Results for $^2J_{HH'}$ in N^{α}-Acetylglycinamide (3) as a Function of the Dihedral Angles ϕ and ψ

ϕ,[a] deg	ψ,[b] deg	$^2J_{HH'}$ (ϕ, ψ), Hz	ϕ,[a] deg	ψ,[b] deg	$^2J_{HH'}$ (ϕ, ψ), Hz
0	0	[c]	120	−120	−3.69
0	−60	−0.64	120	180	−7.17
0	−120	−0.43	180	0	−4.75
0	180	[c]	180	−60	−0.08
60	0	−7.56	180	−120	0.19
60	−60	−3.74	180	180	−4.26
60	−120	−3.85	−120	0	−7.63
60	180	−7.31	−120	−60	−3.75
120	0	−7.63	−120	−120	−3.75
120	−60	−3.77	−120	180	7.17

[a] $\phi = \theta - 30°$. [b] $\psi = 30° - \theta'$. [c] Energy was too large due to strong steric interactions.

3. Geminal H-H Coupling in N^{α}-Acetylglycinamide. Geminal coupling constants in the methylene group of N^{α}-acetylglycinamide (**4**) were obtained from the INDO-FPT



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method with the Corey-Pauling geometry.[20] The results, obtained at 60° intervals of the dihedral angles ϕ and ψ, are entered in Table III. Although the magnitudes of the calculated coupling constants are still too small, the values range over almost 8 Hz due to differing orientations relative to the carbonyl and nitrogen of the two amide substituents.

For the (ϕ, ψ) = (0°, 0°) and (0°, 180°) conformations, steric interactions between the two groups lead to energies which are so large that it was concluded that the calculated values of $^2J_{HH'}$ must be spurious and were not included in the table. In fact, space-filling models suggest that conformations within 60° on either side of these would not be accessible. These inaccessible areas are shaded in Figure 3 to indicate that predictions must not be made for dihedral angles in these regions (see section 4).

Although the calculated values in Table III reflect the simultaneous effects of the adjacent carbonyl and amide functions, the lack of additivity of the two types of contributions, as inferred from the calculated results in the previous sections, is an important consideration. This lack of additivity, which ranges from 1 to 2.5 Hz, must be attributable to the simultaneous electronic perturbation of the CH_2 moiety by the two groups.

The data in Table III can be represented to generally better than 0.3 Hz by the equation

$$^2J_{HH'}(\theta, \theta') = -2.95[\cos^2\theta' + \cos^2(\theta' + 120°)] - 2.80\cos^2\theta'\cos^2(\theta' + 120°) - 4.65\cos\theta\cos(\theta + 120°) + C \quad (5)$$

where $C = -1.5$ Hz. From the experimental coupling constant value of −18.0 Hz (sign inferred) obtained[25] for several cyclic tetrapeptides, the constant C in eq 5 can be shifted by −9.6 Hz to give $C = -11.1$ Hz. Equation 5 can be put into a more symmetrical form in terms of ϕ and ψ,

$$^2J_{HH'}(\phi, \psi) = -13.91 - 1.55\cos^2\psi - 2.80\cos^4\psi + 4.65\cos^2\phi \quad (6)$$

The geminal coupling constants from eq 6 are plotted as a function of the two dihedral angles in Figure 3. Distinctive features of the plot in Figure 3 are as follows. (a) The absolute minima of −18.3 Hz at (ϕ, ψ) = (90°, 180°) and (90°, 0°). These arise for the symmetrical conformations in which a line connecting the coupled hydrogens on the C_{α} carbon is parallel to the axis of the $2p_z$ orbital of the adjacent carbonyl carbon (θ' = 30°, 210°) and parallel to the C_{α}-N-H plane (θ = 120°). Clearly, this corresponds to the two coupling constant minima in Figures 1 and 2. (b) A saddle point with coupling constant −13.9 Hz for (ϕ, ψ) = (90°, 90°), which arises from the cancellation between the θ' = 120° maximum (Figure 1) due to the interaction with the carbonyl and the θ = 120° minimum (Figure 2) due to the amide nitrogen. (c) The absolute maxima of −9.2 Hz for (ϕ, ψ) = (0°, 90°) and (180°, 90°) occur for those cases in which the substituent effects of the carbonyl (θ' = 120°) and nitrogen (θ = 30° and 210°) assume maximum values in Figures 1 and 2, respectively. (d) Saddle points with coupling constants −13.6 Hz at (ϕ, ψ) = (180°, 0°) and (180°, 180°) arising from the cancellation between the θ' = 30° minimum in the carbonyl effect and the θ = (30°, 210°) maxima associated with the nitrogen substituent effect. (e) Because of the symmetry implicit in eq 6, a value of −13.6 Hz is obtained for the points with (ϕ, ψ) = (0°, 0°) and (0°, 180°). However, for N^{α}-acetylglycinamide (**4**) these are conformationally inaccessible points. The first of these corresponds to a conformation analogous to that of *cyclo*-(-Gly-Gly-) which is to be discussed in the next section.

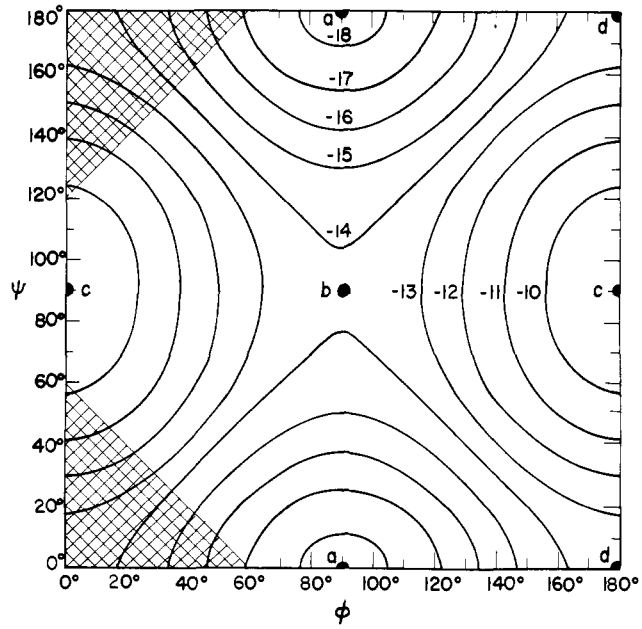


Figure 3. Contours of the calculated INDO-FPT results for geminal H-H coupling (shifted by −9.5 Hz) in the methylene group of N^{α}-acetylglycinamide (**4**) as a function of the dihedral angles ϕ and ψ. The contours are based on eq 6 and, therefore, include values near regions [(ϕ, ψ) = (0°, 0°) and (0°, 180°)] which are energetically inaccessible. The geminal coupling at the labeled points are as follows: (a) −18.3 Hz, (b) −13.9 Hz, (c) −9.2 Hz, (d) −13.6 Hz. The shaded regions provide a rough indication of the regions which would be energetically inaccessible for actual molecules because of strong steric interactions.

Calculated results from eq 6 are compared with available experimental values for cyclic peptides in Table IV. The criteria for inclusion in the table were the availability of structural information from x-ray data (items 1 and 2 in the table) or data in which structures have been based on symmetry, vicinal H-N-C-H coupling constants, and space-filling models. In the latter cases some of the calculated and experimental results do not conform so that several addi-

Table IV. Comparison between Representative Experimental Geminal Coupling Constant Data and Those Calculated Values from Eq 6 and Figure 3

Compd	ϕ, deg	ψ, deg	$^2J_{HH'}$(calcd), Hz	$^2J_{HH'}$(exptl), Hz
1. *cyclo*-(-Sar-)$_4$	121	−66	−13.0	−14.5[a]
2. Actinomycin	80	−170	−17.9	−17.5[b]
	73	179	−17.9	
3. *cyclo*-(-Gly-Pro-Gly-)$_2$	180 (set 1)	180	−13.6	−18.5[c,d]
	−165 (set 2)	150	−12.3	
	117 (set 3)	180	−17.3	
	103 (set 4)	180	−18.0	
	90 (set 1)	0	−18.3	−17.5[c]
	100 (set 2)	0	−18.1	
	100 (set 3)	10	−17.9	
	75 (set 4)	10	−17.7	
4. *cyclo*-(-Gly-Pro-)$_3$	180 (170)[f]	180 (−162)[f]	−13.6 (−13.1)	−16.3 (CD_2Cl_2)[e]
	120 (169)[f]	150 (−150)[f]	−15.5 (−12.2)	−15.2 (DMSO and NaSCN)[e]
	60	−150	−15.5	
5. *cyclo*-(-Pro-Ser-Gly-)$_2$	150	−120	−11.0	−16.5 (D_2O)[g,d]
				−15.1 (DMSO)[g,d]
	−150	180	−14.8	−16.6 (D_2O)[g]
				−15.1 (DMSO)[g]
6. *cyclo*-(-Ser-Pro-Gly-)$_2$	60	−90	−12.7	−15.1[h]

[a] Reference 25. Coupling constants measured in $CDCl_3$; x-ray: P. Groth, *Acta Chem. Scand.*, **24**, 780 (1970). [b] NMR measured in $CDCl_3$: B. H. Arison and K. Hoogsteen, *Biochemistry*, **9**, 3976 (1970); x-ray: H. M. Sobell and S. C. Jain, *J. Mol. Biol.*, **68**, 21 (1973). [c] NMR spectrum obtained in DMSO by L. G. Pease, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **95**, 260 (1973); R. Schwyzer, Ch. Grathwohl, J.-P. Meraldi, A. Tun-Kyi, R. Vogel, and K. Wütherich, *Helv. Chim. Acta*, **55**, 2545 (1972). [d] The glycyl residue in which the coupling occurred was involved in hydrogen bonding. [e] NMR spectra obtained by C. M. Deber, D. A. Torchia, S. C. K. Wong, and E. R. Blout, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1825 (1972). Values in parentheses are from V. Madison, M. Atryi, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 6725 (1974). [f] Energy map calculations given by V. Madison, *Biopolymers*, **12**, 1837 (1973), indicated accessible conformations with all-trans peptide bonds and three intramolecular hydrogen bonds. [g] Geminal coupling constant obtained from published spectra given by D. A. Torchia, A. di Corato, S. C. K. Wong, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **94**, 609 (1972). [h] Geminal coupling constant (in DMSO) inferred from published spectrum by D. A. Torchia, S. C. K. Wong, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, 94, 616 (1972).

tional factors were investigated as possible sources of the disparities.

Some of the results for which correspondence between calculated and experimental values in Table IV is not good are those in which the glycyl residues are expected to be involved in intramolecular hydrogen bonding. In section 5 an attempt is made to include this feature in the calculations. Another factor, which could partially explain some of the disparities, is due to the fact that many of the spectra were obtained with the peptides dissolved in quite polar solvents. Geminal coupling constants are known to be much more sensitive to solvent polarity than other types of coupling constants.[18] For the "normal" direction of the dipole moment vector, i.e., pointing away from the CH_2 moiety, the geminal coupling constants are expected to become more negative with increasing solvent polarity. However, exceptions are known.[18]

Even in the cyclic molecules in Table IV there will be contributions from other conformations of the molecules and the importance of including the effects of conformational averaging is being increasingly emphasized.[1,26-29] The effects of conformational averaging on geminal H–H coupling constants were based on the equation

$$\langle {}^2J_{HH'} \rangle = \frac{\int_0^{2\pi} \int_0^{2\pi} {}^2J_{HH'}(\phi, \psi) \exp[-E(\phi, \psi)/kT] \, d\phi \, d\psi}{\int_0^{2\pi} \int_0^{2\pi} \exp[-E(\phi, \psi)/kT] \, d\phi \, d\psi} \quad (7)$$

where $^2J_{HH'}(\phi, \psi)$ follows from eq 6 and the energies $E(\phi, \psi)$ were taken from the INDO-MO calculations leading to the calculated coupling constant values. Numerical integration of eq 7 gave a rather limited range of values of $\langle {}^2J_{HH'} \rangle$ from about −11.8 to −14.6 Hz, and does not at all help explain the apparent disagreement in Table IV. It is possible that potential functions from conformational energy calculations might give a better representation of the effects of conformational averaging on geminal H–H coupling constants.

It seems likely that a major source of disparity between the calculated and experimental results in Table IV is due to the uncertainties in the structures which were based on H–N–C–H coupling constants and molecular models. For example, two different groups investigated the NMR spectra of *cyclo*-(-Gly-Pro-Gly-)$_2$ (item 3, sets 1 and 2 in Table IV). Although estimates of the ϕ angles differed by only 10–15°, in at least one case the ψ angles differed by 30°. Furthermore, from the experimental vicinal coupling constant data in the two types of glycine residues it is possible to deduce at least two other sets of dihedral angles (item 3, sets 3 and 4 in Table IV), and from these construct a model for *cyclo*-(-Gly-Pro-Gly-)$_2$ that is reasonable and in conformity with the calculated geminal coupling constants. In this case it should be noted that the sets 3 and 4 correspond to conformations in which the hydrogen bonds are not planar in analogy to the situation deduced from the crystal structure of the cyclic hexapeptide *cyclo*-(-Gly-Gly-D-Ala-D-Ala-Gly-Gly-).[30] It is not our intention to suggest that the conformations for sets 3 and 4 are an improvement over the sets 1 and 2, but only to show that there are sufficient ambiguities in structures based on this type of model building to explain the gross disparities between the calculated and experimental results in Table IV.

Additional geminal coupling constant data are available for glycyl residues in a number of cyclic peptides.[31] Indeed, the incentive for this work stemmed from the wide range of experimental coupling constant data for a series of cyclic pentapeptides. Unfortunately, these molecules lacked symmetry, so that it was not possible to reduce the number of minima in the conformational energy calculations by the construction of models. As more structural data become available from NMR and other experimental techniques, the geminal coupling constants may become exceedingly useful in removing certain of these structural ambiguities.

4. Geminal H–H Coupling in *cyclo*-(-Gly-Gly-). It was

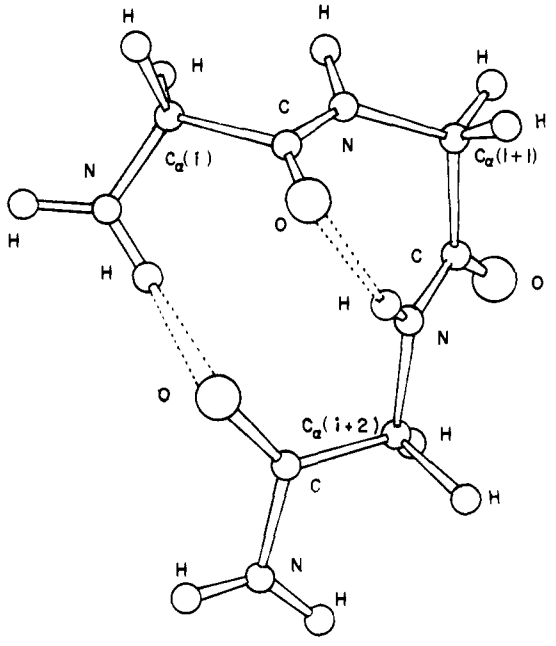
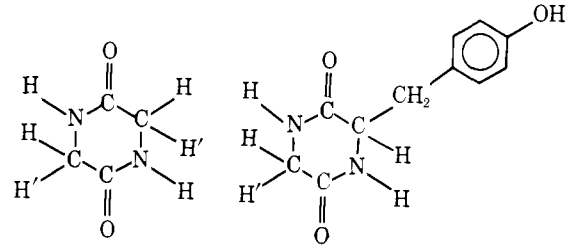


Figure 4. Schematic of the three peptide structure H-Gly-Gly-Gly-NH_2 exhibiting a γ turn. Hydrogen bonding is depicted by the dashed line. This figure is based on a similar one given in ref 34.

previously noted that eq 5 and 6 will give values for $^2J_{HH'}$ even in those conformations which are sterically inaccessible. An interesting example arises for the point $(\phi, \psi) = (0°, 0°)$ in Figure 3. This corresponds to the cis peptide linkage, so that the removal of N-H and C_α-H hydrogens of N^α-acetylglycinamide (**4**) corresponds on ring closure to *cyclo*-(-Gly-Gly-) (**5**). The calculated value of −13.1 Hz



5 **6**

from eq 6 is in the unacceptable region of Figure 3 and reflects contributions from mechanisms which would not arise in actual molecules. As a consequence, the value should not be compared with the experimental value of −17.0 Hz reported[32] for the related compound *cyclo*-(-Gly-Tyr-) (**6**) in DMSO-d_6. The INDO-FPT coupling constant results for compounds **5** and **6** with coordinates from the x-ray structural data[33] are $^2J_{HH'} = -16.5$ and −16.9 Hz, respectively (these values are shifted by the previously adopted value of −9.6 Hz). The geminal coupling constant for *cyclo*-(-Gly-Gly-) (**5**) seems not be have been reported because of the equivalence of the methylene protons, but would probably not be greatly different from **6**.

5. Geminal H-H Coupling in the Gly-Gly-Gly Sequence with a γ Turn. In an attempt to investigate the role of intramolecular hydrogen bonding on the value of the geminal coupling constant in a peptide sequence, an INDO-FPT calculation was performed for the relatively simple case of the tripeptide H-Gly-Gly-Gly-NH_2.[34] This was chosen because it exhibits a model for hydrogen bonding by means of the conformation which has a γ turn. A schematic representation for this is given in Figure 4. With conformational data from ref 34, the calculated geminal coupling constant in the $(i + 2)C_\alpha$ methylene was found to be −15.7 Hz (again the results were shifted by −9.6 Hz as in section 3). This is very close to the value of −15.6 Hz from eq 6. Since it is primarily the influence of the lone pairs and the nitrogen electronegativity which would be affected by the hydrogen bonding, it is not surprising that the effect appears to be unimportant. However, it should be realized that a different model for the hydrogen bond could lead to a different result.

Conclusions

Geminal H-H coupling constants depend on the ϕ and ψ angles in the peptide backbone **1**. In calculations on model compounds it is shown that this variation may be as much as 8 Hz. In cases where x-ray structural data are available and rigid molecules are expected, the agreement between calculated and experimental results is quite satisfactory and suggests that geminal coupling may complement other types of NMR parameters as a probe of peptide structure. In those cases in which the geometry is more uncertain because of inference from molecular models, it is more difficult to assess the significance of the disagreements which are found. Although it appears likely that geminal coupling constant data can provide useful structural information for peptides in solution, it would be foolhardy not to point out the dangers of placing too much confidence in a single curve.[35,36]

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Kinetics of Phase Equilibrium in a Binary Mixture of Phospholipids

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Abstract: A 50:50 mol % binary mixture of dipalmitoylphosphatidylcholine (DPPC) and dielaidoylphosphatidylcholine (DEPC) has been studied using ^{13}C nuclear magnetic resonance at 25.2 and 90.5 MHz. Each phospholipid was enriched in the choline methyl groups with ^{13}C. The line width of the ^{13}C resonance of the higher melting lipid (DPPC) in this binary mixture increases rapidly at temperatures below ~32 °C, the same temperature as determined earlier by spin label paramagnetic resonance and by freeze-fracture electron microscopy to mark the onset of a lateral phase separation in the plane of the membrane. The temperature dependence of the observed ^{13}C line widths differs quantitatively but not qualitatively from theoretically calculated line widths based on the previously reported phase diagram for this mixture of lipids. The discrepancy may be due to density and composition fluctuations (nucleation) in the fluid phase of the lipids. Such fluctuations are suspected to be of importance for the transport of certain molecules through cell membranes.

A number of membrane-related biological functions are strongly correlated with the physical properties of the membrane lipids. These functions include active transport,[1] enzymatic activity,[2] membrane assembly,[3] and complement-mediated immune attack.[4] Some of these correlations are relatively trivial; when all the membrane lipids are frozen or highly viscous, transport is inhibited or stopped. Other correlations are certainly not trivial, and their explanation should lead to a better understanding of the biophysical events associated with membrane function. A change in the uptake of β-galactosides and β-glucosides into cells of *Escherichia coli* associated with the *onset* of lateral phase separations in the plane of the membrane is one example, as discussed later.[5]

All of the membrane functions enumerated above are kinetic processes, and it is therefore of interest to investigate the kinetic properties of lipids, and lipid mixtures. In this paper we (a) describe the ^{13}C nuclear magnetic resonance spectra of a binary mixture of two phospholipids, dielaidoylphosphatidylcholine (DEPC) and dipalmitoylphosphatidylcholine (DPPC), in which the two lipids are enriched with ^{13}C in the choline methyl groups, (b) give a theoretical calculation of resonance line widths expected for a two-phase bilayer system in which the lipids undergo rapid lateral diffusion, and (c) show that the experimental and theoretical results indicate an unexpectedly rapid phase equilibration in this system, which may be due to rapid density and composition fluctuations (nucleation) in the fluid lipid phase. This study is relevant to the effects of phase separations on membrane functions, such as transport.[1]

Experimental Section

Reagents. The following materials were obtained from commercial sources and used without further purification: 2-aminoethanol (Sigma), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE) (Calbiochem), 90% ^{13}C-enriched methyl iodide (Merck), and elaidic acid (Analabs).

^{13}C Choline Enriched Dipalmitoylphosphatidylcholine (DPPC*). DPPE was methylated under basic conditions in freshly distilled methanol at 45 °C. The methylation was followed by thin-layer chromatography and the reaction usually stopped after 4 h. The various amines were separated by silica plate chromatography (Analtech) or silica gel column chromatography (Bio Sil-A, 200–325 mesh), eluted with a chloroform–methanol gradient. The product showed the same R_f as unlabeled DPPC on silica gel thin-layer chromatography, developed in chloroform:methanol:water 65:35:4, and stained with sulfuric acid. The concentration of the solution was measured by method of McClare.[6] The product was identified using ^1H and ^{13}C nuclear resonance, and thin-layer chromatography. An aqueous dispersion of the lipid showed the same transition temperature by the spin label TEMPO method (42 °C) as did the unlabeled material.

Dielaidoylphosphatidylcholine (DEPC). DEPC was prepared by the method of Cubero Robles and Van den Berg[7] from *o*-(*syn*-glycero-3-phosphoryl)choline (available as the $CdCl_2$ adduct from Sigma) and the sodium salt of elaidic acid. The product was purified by silicic acid column chromatography as for DPPC* above.

^{13}C Enriched Choline. This was prepared by direct methylation of ethanolamine under basic conditions, and was partially purified using an ion exchange column (Bio-Rad, AG-50W-x8, NH_4^+ form). Thin-layer chromatography was performed on cellulose plates (Analtech) and developed in propanol–NH_4OH(14.8 M)–H_2O, 6:3:1. The spots were visualized in an iodine chamber.

Phosphatidic Acid of DEPC. This phosphatidic acid was prepared by the action of phospholipase D, freshly extracted from cabbage according to Davidson and Long.[8] The purification followed the method of Kornberg and McConnell.[9]

^{13}C Enriched Dielaidoylphosphatidylcholine (DEPC*). The condensation of the phosphatidic acid with the ^{13}C enriched choline was carried out in freshly distilled pyridine and catalyzed by