Chemical Shift Nonequivalence in Proton Magnetic Resonance Spectra of Glycyl Peptides[1]

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Abstract: Glycyl methylene splitting due to chemical shift nonequivalence occurs in the proton magnetic resonance spectra of a dozen amino acid–glycyl dipeptides. The magnitude of the splitting does not appear to be correlated with any single structural element and is reduced by the addition of salt or urea and by an increase in temperature. The chemical shift of the high-field glycyl methylene hydrogen in phenylalanylglycine actually moves downfield upon loss of a proton from the ammonium group. Glycyl methylene splitting also occurs in the central glycyl residue of aminoacylglycylglycines, but not in aminoacylglycinamides. Despite the almost exclusive appearance of chemical shift nonequivalence in the dipolar ion form of di- and tripeptides, it is argued that head-to-tail folding is not implied but rather that the oppositely charged termini of the molcules provide a field gradient which acts upon nonequivalent atoms or groups of atoms. Nonbonded interactions of the amino acid side chains with atoms of planar, *trans* amide bonds restrict the number of allowed rotamers about single bonds in the peptide backbone, reducing the averaging effect and resulting in possible augmentation of field gradients at nonequivalent atoms. Examples of chemical shift nonequivalence of methyl group resonances in some derivatives of valine are also reported.

A recent survey of proton magnetic resonance spectra of amino acids and peptides reported splitting of the methylene hydrogens in leucylglycine and in the middle residue of leucylglycylglycine.[2] This splitting was ascribed to preferences for certain unspecified rotamers. Because of the importance of this conclusion for protein conformation and the efficacy of proton magnetic resonance spectroscopy in describing these apparent rotamers, we undertook a pmr study of amino acid–glycine dipeptides for 19 of the 20 (all except asparaginyl) amino acid residues commonly occurring in proteins and for others as well. We find methylene splitting in at least 12 dipeptides and also confirm its existence in tripeptides.

Experimental Section

The peptides used in this study were the best grade commercially available from Cyclo Chemical Corp., Mann Research Laboratories, and Calbiochem Corp. N-Benzylbutyramide was synthesized from *n*-butyric anhydride and benzylamine following the standard procedure, mp 49–49.5°, lit.[3] 41–44°. The infrared and pmr spectra are consistent for the product. D-Phenylalanyl-L-valine was synthesized from N-carbobenzoxy-D-phenylalanine and L-valine methyl ester hydrochloride, purchased from Cyclo Chemical Corp. The synthesis was carried out in the usual way with N,N-dicyclohexylcarbodiimide.[4] N-Carbobenzoxy-D-phenylalanyl-L-valine methyl ester was prepared in methylene chloride,[5] followed by basic hydrolysis[6] at room temperature and hydrogenolysis in a Parr hydrogenation apparatus. The infrared and pmr spectra are consistent with the products of each step. Carbon, hydrogen, and nitrogen analyses are in excellent agreement with the intermediate and final products.

Proton magnetic resonance spectra were recorded on a Varian A-60 nmr spectrometer equipped with variable-temperature probe. All spectra were run at room temperature (about 25°) except where noted for leucylglycine. Chemical shifts were generally calculated from the spectra recorded on the 50-cycle sweep width. Sweep widths were frequently calibrated with the Model 200 CD wide-range oscillator and 5512 A electronic counter manufactured by the Hewlett-Packard Co.

In general, 0.3 to 0.5 mmole of the compound was dissolved in 0.5 ml of D_2O. In cases where the compound failed to dissolve completely, NaOD was added dropwise until the sample was completely dissolved. The pH was measured with a Beckman Model G pH meter equipped with a Beckman No. 40316 one-drop glass electrode. Observed pH meter readings are reported. After the spectrum was recorded at this pH, either NaOD or DCl, depending on the initial pH, was added. The pH was again measured and the spectrum recorded. In all dipeptides, spectra were recorded at high and low pH and at or near to the isoelectric pH. Where possible excess acid or base was added to suppress ionizations. Some peptide spectra were recorded at intermediate pH values. The pH dependence of the chemical shifts of phenylalanylglycine was determined by titrating a 0.9 *M*, pH 0.5 solution with 3.2 *M* NaOD and a 0.8 *M*, pH 11.5 solution with 3.3 *M* DCl using a Manostat microburet. All chemical shifts are reported in ppm downfield from sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) as an internal standard.

Results

In acidic or basic solutions, the glycyl methylene absorption appears as a single resonance line for most of the aminoacylglycines studied in this research. Frequently this resonance peak is broadened or split into an AB quartet in the dipolar ion form of the dipeptide. In the last case a $\Delta\nu$ and J_{AB} may be calculated from the spectrum. For these dipeptides the high-field, ν_B, and low-field, ν_A, chemical shifts in ppm downfield from DSS as an internal standard are recorded in Table I. The dipeptides are listed in approximate order of increasing $\Delta\nu$. Values of J_{AB} vary only slightly from 17.0 to 17.6 cps with a tendency for the greater values to appear in more acidic solutions. The pH values for the solutions recorded in Table I were carefully regulated so that a single charged species predominated. Charges are indicated by beginning with the N terminal ammonium group and ending with the carboxylic acid charge of the glycyl residue. For example, the three charge signs for α-glutamylglycine refer successively to the N terminal ammonium group,

(1) This research was supported by grants from the National Science Foundation and the National Institutes of Health. Similar results have been obtained by O. Jardetzky and A. Nakamura, private communication.

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the γ-carboxylic acid group, and the glycyl carboxylic acid group. In the leucylglycine spectra of the earlier study,[2] one of the peaks designated as an impurity resonance is actually the highest field component of an AB quartet, while the lowest field component occurs at the same field strength as the CH triplet.

Table I. Chemical Shifts (ppm) of Glycyl Methylene Protons in L-Aminoacylglycines[a]

L-Amino-acylglycine		ν_A		ν_B
Glutaminyl	+0		4.10	
	+−	3.85		3.76
	0−		3.78	
α-Glutamyl	+00		3.99[b]	
	+−−	3.88		3.73
	0−−		3.76	
α-Aspartyl	+00		4.08	
	+−−	3.84		3.71
	0−−		3.78	
Histidyl	++0		4.09	
	++−	3.98		3.83
	+0−		3.82[c]	
	00−		3.73	
Norvalyl	+0		4.04	
	+−	3.88		3.71
	0−		3.75	
Valyl	+0		4.08	
	+−	3.90		3.71
	0−		3.76	
Norleucyl	+0		3.99[b]	
	+−	3.88		3.71
	0−		3.75	
Leucyl	+0		4.06	
	+−	3.89		3.70
	0−		3.74	
Isoleucyl	+0		4.07	
	+−	3.90		3.70
	0−		3.76	
Tyrosyl	+00		4.02	
	+0−	3.95		3.75
	00−	3.82		3.71
	0−−		3.70	
Phenylalanyl	+0		4.00	
	+−	3.89		3.60
	0−	3.80		3.67
Tryptophanyl	+0		4.00	
	+−	3.97		3.64
	0−		3.79	

[a] In ppm downfield from DSS as internal standard in D_2O. [b] Solution insufficiently acid so that this value is a minimum one. [c] Indication of splitting.

Using a peak width at a half-height of 2 cps or less on the 50-sweep width as the criterion, as well as no indication of the low-intensity outer components of the AB pattern, we observed no splitting of methylene hydrogens in neutral solutions of the following dipeptides and related compounds: glycylglycine, D,L-alanylglycine, β-alanylglycine, L-serylglycine, L-threonylglycine, L-prolylglycine, hydroxy-L-prolylglycine, L-arginylglycine, L-methionylglycine, L-cystinyl-bisglycine, glutathione, acetylglycine, benzoylglycine (basic solution), N-benzylbutyramide (in $CDCl_3$ where it exhibited a *cis–trans* mixture), sarcosylglycine, glycylsarcosine (exhibits a *cis–trans* mixture), glycyl-L-tyrosine, glycyl-L-leucine, L-valylglycinamide, and L-tyrosylglycinamide. L-Lysylglycine exhibited a peak width of 2.5 cps which was unresolvable. Chemical shift differences of less than about 0.1 ppm would be detected only with difficulty on our instrument. Consideration of these results and those reported in Table I indicates that no simple single factor, such as electronegativity or the number of substituents on the β-carbon atom, appears to account for the magnitude of splitting.

The magnitude of the methylene splitting in dipolar ion leucylglycine was observed under a variety of solution conditions. The increment of splitting with a decrease in concentration is about 0.017 ppm/mole. Solutions of this dipeptide in 8 *M* urea or 2.5 *M* KCl exhibited a decrease in $\Delta\nu$ of about 0.04 ppm or 20%. Increasing concentrations of $MgCl_2$ are more effective on a molar basis than KCl in causing decreases in $\Delta\nu$. A solution of dipolar ion leucylglycine in D_2O at 100 or 120° exhibits a decrease in $\Delta\nu$ of 0.04 ppm compared to the same solution at room temperature.

Several miscellaneous features of the spectra of the dipeptides recorded in Table I are worth mentioning. Splitting of the glycyl methylene hydrogens in tryptophanylglycine persists into quite acid solutions but finally becomes unanalyzable on our instrument with a peak width at half-height of 4 cps near zero pH. The phenylalanyl part of phenylalanylglycine exhibits an ABX spectrum in basic solutions with $J_{AX} = 5.1$, $J_{BX} = 8.4$ cps, and $\Delta\nu_{AB} = 0.125$ ppm. In neutral and acid solutions only an A_2X type spectrum is obtained. The results are similar to those reported for phenylalanine[7] and other amino acids.[8,9] Upon ionization of the phenolic group in tyrosylglycine, the high-field hydrogens of the A_2B_2 system of the aromatic ring undergo an upfield shift greater than do the low-field hydrogens, confirming the assignment of the high-field pair as those *ortho* to the hydroxyl group.[10]

The interesting case of phenylalanylglycine, where the methylene resonance is split even in basic solutions, was carefully studied over a range of pH. Individual variations of the low- and high-field proton resonances, A and B, respectively, are shown in Figure 1. A sufficient number of pH values were studied in order to establish that the curves in Figure 1 do not cross. Plateaus occur where one kind of charged species predominates. Upon removal of protons from the carboxylic acid or ammonium groups, the resonance frequency changes in a manner typical of a titration curve. The midpoint of the change in basic solution where the ammonium group is undergoing deprotonation occurs at an observed pH in D_2O of 7.8 for both A and B hydrogens. A similar analysis is not possible for the region of carboxylic acid ionization because the spectra collapse into a single average line before the extreme acid limit frequencies of the A and B protons may be established. The average value of the chemical shifts of A and B protons moves upfield markedly with deprotonation of the carboxylic acid group and slightly with deprotonation of the ammonium group. These average shifts are similar to those observed for the other dipeptides and for the single peak of the C terminal residue of glycylglycine[11] upon

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equivalent ionizations. In all dipeptides where splitting is observed, the greatest value of $\Delta\nu$ occurs in solutions of the dipolar ion.

Unequal chemical shift changes are exhibited by the A and B protons as the observed pH is varied in Figure 1. Even more noteworthy is the downfield shift of the high-field B hydrogen on loss of a proton from the ammonium group leaving a negatively charged molecule. This downfield shift of the B hydrogen is just compensated by an upfield shift of the A hydrogen so that the average shift of both hydrogens taken together is slightly upfield by 0.01 ppm on ammonium ionization.

The pronounced difference in behavior between the A and B protons of phenylalanylglycine upon ammonium ionization, along with their continued separate resonances in basic solutions, suggested that a comparison of the resonances of L-phenylalanyl-L-valine with its diastereomeric L,D or D,L dipeptide would be informative. Valine was chosen for the carboxyl terminal residue because there is only one asymmetric carbon atom, and its α-CH hydrogen resonance is only a doublet and is not split more extensively. Unfortunately after synthesizing the D,L compound, we found the dipolar ion form insufficiently soluble in water. The results are presented in Table II.

Table II. Valyl Resonances of Phenylalanylvalines[a]

	L,L		D,L	
Charge	α-CH	Methyls	α-CH	Methyls
+0	4.23	0.97, 0.88	4.18	0.78, 0.67
+−	4.05	0.97, 0.95, 0.86, 0.84	Insoluble	
0−	4.03	0.91, 0.79	3.97	0.71, 0.59

[a] In ppm downfield from DSS as an internal standard in D_2O.

In the cationic and anionic forms, the valyl methyl groups of phenylalanylvaline appear as a doublet split by the β-CH hydrogen with coupling constants of about 6.8 cps. In the dipolar ion form four peaks appear in the methyl region: β-CH splitting yields a similar coupling constant, and there is a second splitting of 0.018 ppm due to chemical shift nonequivalence. The double doublet for methyl resonances also occurs in all three ionic forms of the amino acid valine.[9] The splittings due to chemical shift nonequivalence are 0.017, 0.053, and 0.072 ppm for the cationic, dipolar ion, and anionic forms, respectively, with the high-field methyl group undergoing the largest shift with pH. With no β-CH hydrogen present, the appearance of two peaks in the methyl region of penicillamine (β-mercaptovaline) must be due to chemical shift nonequivalence. The chemical shift differences are 0.06, 0.08, 0.25, and 0.24 ppm for the +1, 0, −1, and −2 charged forms, respectively. Again the high-field methyl peak undergoes the greater shift with pH. Unlike the dipeptides, where the greatest chemical shift nonequivalence for either methyl group or hydrogen splitting occurs in the dipolar ion form, the splitting due to this cause is greater in the most negatively charged species of amino acids.[8] The tripeptide valylglycylglycine exhibits a double doublet for the methyl groups only in basic solution with a splitting due to chemical shift nonequivalence of about 0.02 ppm.

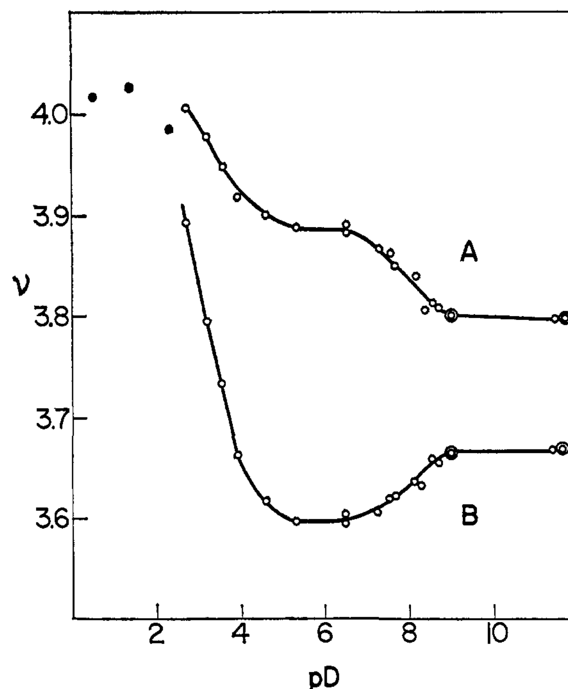

Figure 1. Chemical shift in ppm downfield from DSS as internal standard *vs.* observed pH meter readings for glycyl methylene protons A and B of 0.75 *M* phenylalanylglycine in D_2O. Bullseyes represent two points. Solid points in most acid solutions are average values for unresolved peaks.

A select sample of aminoacylglycylglycine tripeptides was also examined for glycyl methylene splitting in neutral solutions. Consistent with the behavior of the corresponding dipeptides, no splitting was observed in alanylglycylglycine and prolylglycylglycine. A small splitting in the central glycyl residue was observed in valylglycylglycine. Sufficient splitting of the methylene hydrogens in the central glycyl residue to permit analysis was observed in leucylglycylglycine and phenylalanylglycylglycine. Though no splitting of the terminal glycine appeared, the methylene hydrogens of the central glycyl residue exhibited an AB quartet in leucylglycylglycine (ν_A = 4.06, ν_B = 3.98 ppm) and phenylalanylglycylglycine (ν_A = 3.98, ν_B = 3.88 ppm). No splitting was observed in either glycyl residue of glycyl-L-leucylglycine.

Discussion

In order to exhibit chemical shift nonequivalence in nuclear magnetic resonance spectra, two conditions must be met. To be chemical shift nonequivalent, atoms or groups may not be related by a symmetry operation taking into consideration internal motions that are rapid compared to the time scale of an nmr experiment. Secondly, there must exist a sufficient field gradient so that the nonequivalent atoms or groups exhibit chemical shifts resolvable on the instrument employed.

For the L-amino acid–glycine dipeptides of this study, no symmetry element exists along the $N-CH_2$ bond axis. Even if rotation about the planar and *trans* amide bond were rapid, the methylene hydrogens would still be nonequivalent in these optically active dipeptides and their racemic mixtures. Only if inversion at the asymmetric carbon atom were rapid compared to the nmr

chemical shift would a plane of symmetry include the $N-CH_2$ bond axis. This explanation for chemical shift nonequivalence arising from molecular dissymmetry does not invoke rotamer preferences about the $N-CH_2$ or C–CO bonds. If such favored rotamers do exist, they might serve to enhance dissymmetry and hence nonequivalence. The main question posed by the results reported here is, "To what extent are the observed chemical shift differences in glycyl methylene hydrogens augmented or due solely to rotamer preferences?"

Since rotations about all single bonds except the C–N bond in the peptide backbone are rapid, proton magnetic resonance spectra represent weighted average spectra over all rotamers present. Each kind of rotamer gives rise in general to unique shielding at each hydrogen or other atom. If rotamers are equally represented about the whole 360°, then parts of their individual contributions probably cancel and observed chemical shifts represent highly averaged values. In this case contributions to chemical shifts from rotamer preferences are considered insignificant, and any splitting is ascribed to field effects operating on an asymmetric environment.

One of the problems in assessing the importance of rotamer preferences is that potential barriers about C_α–N and C_α–CO single bonds in the peptide backbone are not well defined, but appear to be very low, with the result that large numbers of rotamers may be significant. Primarily because of the *trans* conformation of the planar amide group, rotations about single bonds within a given amino acid residue are nearly independent of rotations about neighboring residues in the peptide chain.[12] However, nonbonded interactions of the amino acid side chains with atoms of *trans*, planar amide bonds restrict the number of allowed rotamers even in the case of glycine and markedly more so for larger side chains.[13] In the aminoacylglycines certain rotamers are disadvantaged because of nonbonded interactions, but there remains a considerable arc over which rotamers can occur, especially about the $N-CH_2$ single bond. There does not seem to be a good correlation between the extent of methylene splitting and the permissible arc over which rotamers can occur. Though large groups with restricted rotamers show chemical shift nonequivalence and appear in Table I, side-chain groups with equally restricted rotamers appear in the list where any splitting was too small to be observed.

Several studies of the physical properties of di-, tri-, tetra-, and pentapeptides have been interpreted to indicate a lesser end-to-end distance in those peptides that contain some D amino acid residues than in those that contain only L residues. This conclusion has been drawn from comparisons of acid ionization constants,[14] transition metal ion interactions,[15] dielectric increments,[16,17] rates of ring closure,[17,18] and proton magnetic resonance spectra.[19] Not all of these experiments were performed on the dipolar ion form of the peptide; in the last two kinds of measurements the carboxylic acid groups were uncharged.

Examination of nonbonded interactions by use of Courtauld's molecular models indicates that the amino and carboxylic acid groups are brought closer together in L,D than in L,L dipeptides. This result is independent of pH or charge on the terminal groups; the conclusion is forced by consideration of nonbonded interactions alone. Thus the results referred to in the previous paragraph receive a natural explanation so that it is not necessary or desirable to invoke interactions between opposite charges inducing preferred rotamers. Nearly the same rotamer preferences exist in the presence and absence of the dipolar ion charge distribution. No special folded conformation is produced by attraction of oppositely charged ends of the molecule. In an independent study, the chemical shifts produced upon proton ionization in glycine, alanine, and their di- and tripeptides are correlated with the separation along the chain of the measured hydrogen from the protonic center, indicating that head-to-tail folding does not occur to an observable extent in these compounds.[20]

The same nonbonded interactions that force the amino and carboxylate groups to be closer together in the D,L dipeptide also force the side chains to be closer together than in the L,L diastereomer. When one of the side chains is aromatic, the other appears in its shielding cone in the D,L dipeptide with the result that all valyl resonances in Table II for phenylalanylvalines appear at higher field in this diastereomer than in the L,L compound. An identical explanation accounts for the observed higher field resonances in the nonaromatic side chain of the D,L diastereomers of leucyltyrosine[19] and alanyltyrosine[21] compared to the L,L dipeptides.

Counting separately individual charged forms, nearly 100 compounds were examined in this study of chemical shift nonequivalence, yet methylene hydrogen or methyl group nonequivalence was observed only in dipolar ion forms or with aromatic amino acid residues. The lack of correlation between the magnitude of nonequivalence and the allowed rotamers as derived from peptide maps, along with the dependence of rotamer preferences on nonbonded interactions and their independence of charge distribution, suggests that rotamer preferences are not the primary cause for nonequivalence in the di- and tripeptides of this study. Rather the asymmetric carbon provides the potential for chemical shift nonequivalence which is realized by the field gradients produced in dipolar ion forms and by aromatic groups. Any rotamer preferences that occur as a result of nonbonded interactions might serve to heighten nonequivalence.

Methylene splitting also occurs in the central glycyl residues of the dipolar ion forms of the tripeptides leucylglycylglycine and phenylalanylglycylglycine where the charge separation is almost 50% greater than in

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dipeptides. The splitting in these two tripeptides is about 40% of that observed in the corresponding dipeptides. Lack of observed methylene splitting in the terminal glycyl residues of tripeptides and either glycyl residue in glycyl-L-leucylglycine is evidently due to their being out of a region of sufficient field gradient.

Temperature dependence of the extent of methylene splitting may in principle distinguish field from rotamer effects since all rotamers should be nearly equally populated at high temperatures, leaving only the averaged field effect to contribute to chemical shift nonequivalence. In the case of leucylglycine an increase in temperature from 25 to 120° reduced the methylene splitting by about 20%. Not even this degree of splitting may be ascribed to rotamer preferences about C–C single bonds because collapse of amide spectra has been observed at less than 100° owing to the onset of rapid rotation about the C–N bond. Because of the small energy differences between potential minima, a very wide temperature range would have to be studied in order to assess quantitatively the importance of rotamers about C–C single bonds. It does not seem possible to separate rotamer from field effects on the basis of the ionic strength dependence of $\Delta\nu$. We are unable to determine precisely the extent to which rotamer preferences due to nonbonded interactions contribute to chemical shift nonequivalence, which is due primarily to field effects in the compounds reported in this study.

Methylene or methyl group splitting has also been observed in many compounds that contain no charged groups. Chemical shift nonequivalence has been observed in the nitrogen substituents of uncharged amides with an asymmetric center in the carbonyl substituent.[22]

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The presence of an AB quartet of the methylene hydrogens of neopentyl O-methylmandelate[23] demonstrates that nonequivalence may occur across ester as well as amide bonds. Methyl group nonequivalence has been observed in several isopropyl esters.[24] In these compounds polar and aromatic groups may produce field gradients and nonequivalence as do charged and aromatic groups in peptides. The important role of solvent in contributing to the magnitude of chemical shift nonequivalence of solutes has been stressed. In cases where rotamer preferences appear unable to account for the results, greater magnitudes of methylene splitting are observed in media of lower dielectric constants.[25]

Two arguments suggest that the high-field resonance in the aminoacylglycine dipeptides is the one that would remain if the glycyl residue were stereoselectively deuterated so that the deuterium atom appears in the position of the side chain in an L,D or D,L dipeptide. Table II shows that the high-field α-CH hydrogen resonance of phenylalanylvalines occurs in the D,L diastereomer. In addition, selective deuteration of the methylene group in neopentyl O-methylmandelate such that the product might be considered analogous to a D,L compound results in retention of the high-field resonance.[23] Since both of these examples contain phenyl groups, the conclusion is valid for the compounds with nonaromatic side chains in Table I only if the additional effect of an aromatic group is simply to augment and not to reverse the sign of the chemical shift difference found with aliphatic groups.

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A New Evaluation of Platt's Model for Diatomic Hydrides[1]

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Abstract: Platt's model for diatomic hydrides has been reevaluated using Clementi's Roothaan–Hartree–Fock atomic wave functions. The diatomic hydrides from H_2 through HBr have been treated. The calculated equilibrium internuclear distances are in excellent agreement with experimental values for the united-atom model, $(Z + 1)^0 \rightarrow (ZH)^0$, but are too large for the separated-ion model, $(Z)^-, H^+ \rightarrow (ZH)^0$. The calculated force constants are also in better agreement with experiment for the united-atom model.

The united-atom model for diatomic hydrides has been the subject of a considerable literature since the initial formulation by Platt in 1950.[3–10] In the model one begins with a neutral atom of atomic number $Z + 1$. The wave function is assumed to have averaged spherical symmetry. A proton is removed from the nucleus and allowed to move out through the fixed

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