Study of Nitrogen-15-Labeled Amino Acids and Peptides by Nuclear Magnetic Resonance Spectroscopy†

J. A. Sogn,‡ W. A. Gibbons,*‡ and E. W. Randall§

ABSTRACT: Several characteristics, uses, and advantages of studying amino acids and peptides enriched in ^{15}N are shown in this paper. ^{15}N substitution eliminates the objectionable effects of the ^{14}N electric quadrupole on proton spectra and offers a relatively simple, positive method of assigning amide signals in difficult circumstances. It was found that one-bond N–H coupling constants vary significantly with cis–trans isomerism of the peptide bond, suggesting their use in the study of proposed cis peptide linkages involving amino acids other than proline. Vicinal couplings to side-chain β protons from nitrogen have been confirmed to be sensitive indicators of the side-chain torsion angle χ, but the vicinal coupling from nitrogen through the peptide bond to the next α proton is too small to be similarly evaluated as yet with any confidence. The ^{15}N chemical shifts of a number of 95% enriched ^{15}N amino acids and one enriched peptide are reported and partially interpreted. Chemical shift parameters were found to resemble those found in ^{13}C nuclear magnetic resonance. Coupling and intensity relationships in the amino acid spectra are shown to be dominated by the pH-dependent proton chemical exchange.

Experience has shown that it is unreliable to base the determination of the total conformation of a peptide in solution on the data from any single chemical or physical technique, but at the present time nuclear magnetic resonance (nmr) is the most generally useful technique because of its high information content and the wide range of studies one can carry out with available instrumentation. But it is essential to take full advantage of all other available information including infrared (ir), circular dichroism–optical rotatory dispersion (CD–ORD), fluorescence, hydrodynamic data from rates of dialysis, T–H exchange, chemical reactivities, and theoretical studies as well (Bovey *et al.*, 1972; Gibbons *et al.*, 1970a). It is equally necessary to try to expand the methodology of nmr, achieve better interpretation of the data one can easily collect, and extract information now inaccessible. With this aim we have undertaken, as an extension of our nmr studies of peptide conformations (Gibbons *et al.*, 1970b, 1972a,b), a study of some of the possibilities opened up through use of the ^{15}N nucleus (Randall and Gillies, 1971). We have studied ^{15}N resonances directly and indirectly by observing the effects of ^{15}N enrichment on proton spectra.

Experimental Section

The 95% enriched amino acids were purchased from Merck Sharp and Dohme of Canada, Ltd., Prochem, Ltd., and the Norrell Chemical Co., and used without further purification. When necessary, the amino acids were resolved by acetylation followed by deacetylation of the l forms with hog kidney acylase I (Greenstein *et al.*, 1954), purchased from Sigma Chemical Co. These resolved forms were incorporated into dipeptides *via* the *N*-carboxyanhydride route (Hirschmann *et al.*, 1967). The dipeptides were cyclized to the corresponding diketopiperazines by the method of Kopple and Ghazarian (1968). Valinomycin (L-Val-^{15}N) was prepared by the method of Gisin *et al.* (1969).

^{15}N nmr magnitude spectra were recorded at 9.12 MHz on the Bruker HFX-90 spectrometer of the SRC at Queen Mary College, University of London, operated in the Fourier mode. Samples were spun in 10 mm diameter tubes with C_6F_6 as an external (^{19}F) lock contained in a 5 mm tube inside the sample tube. Samples ranged from 16 to 500 mg of amino acid dissolved in 1.5 ml of solvent and required between 128 and 4096 pulses with repitition every 0.4 sec and no delays (sweep width, 5 kHz). With 2048 output channels for the Fourier transform the resolution is 2.4 Hz or 0.27 ppm. All chemical shifts are based on at least two replicate spectra. Proton noise decoupling, where used, was performed without temperature control of the sample. The ambient probe temperature was 27°.

Proton spectra were obtained principally on the Consortium Varian HR-220 at Rockefeller University in 5 mm diameter tubes at an ambient probe temperature of 14°. They were checked on the Rockefeller Bruker HFX-90 in the continuous wave mode when a different field strength was required to make the assignment unambiguous. Coupling constants were measured to ±0.05 Hz with repeated spectra.

Results and Discussion

Proton Assignments and Elimination of Quadrupole Effects. The ^{14}N nucleus, with a spin of 1 and thus an electric quadrupole moment, exerts no observable effects on the proton spectra of amino acids and peptides, except for an undesirable broadening of the directly bonded amide proton. The broadening is a combination of unresolved coupling and quadrupole-induced relaxation effects. ^{15}N, with a spin of $^1/_2$, couples with nearby protons to give discrete narrow lines, with no broadening in the absence of exchange effects. The elimination of ^{14}N quadrupolar broadening effects from the amide signals gives better resolution in complicated spectra and makes

† From the Departments of Biochemistry, Rockefeller University, New York, New York 10021, and Queen Mary College, University of London, London, England. *Received January 3, 1973.* Supported in part by funds from National Institutes of Health Grant AM 02493-14 and National Science Foundation Grant GB-12278, and grants from the Research Corporation and Sloan Foundation to a consortium at the Rockefeller University for a 220-MHz nmr facility.

‡ Rockefeller University.

§ Queen Mary College.

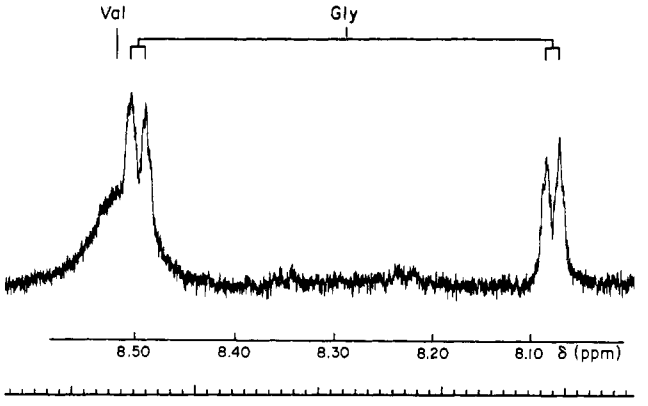

FIGURE 1: Amide region of c-L-Val-Gly-^{15}N in H_2O, pH 3.2 at 220 MHz.

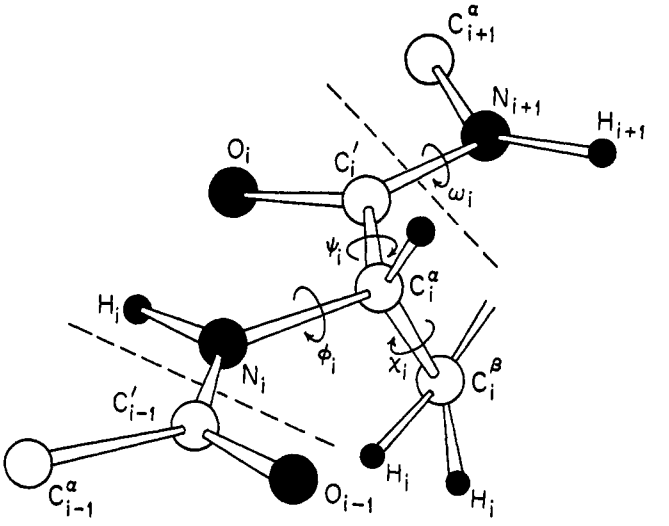

FIGURE 2: The peptide unit, showing the backbone torsion angles ϕ, ψ, and ω and the side-chain torsion angle χ.

possible easier and more accurate line-shape analyses for the determination of exchange relaxation times. Figure 1 shows the amide region of c-L-Val-Gly-^{15}N at pH 3.2. The Gly-^{15}N-amide is a doublet of doublets with $^1J_{NH}$ = 91.1 Hz and $^3J_{HH}$ = 3.0 Hz. The Val-^{14}N-amide is a broad singlet. Analysis of the Gly signal is straightforward, but the Val signal contains the effect of quadrupolar broadening as well as conformational averaging and proton chemical exchange.

Enrichment with ^{15}N can also be used as an aid to assigning amide proton signals in a peptide spectrum. No nmr study of a peptide is complete without an unambiguous assignment of all the amide resonances because they are the source of most conformational information. The assignment can usually be done only by tedious double resonance experiments and even these, in the cases where they are feasible, do not suffice to separately identify amides belonging to multiple residues of one amino acid, occupying different positions along the peptide chain. Partial or total substitution of ^{15}N for ^{14}N, by chemical or biological means, of specific known amino acid residues in peptides allows the trivial, nonperturbing assignment of amide resonances due to the effect of the large $^1J_{NH}$. This can again be seen in Figure 1. Ivanov *et al.* (1971) have used this technique, with low enrichment, to distinguish the amide protons of valinmycin.

^{15}N–H Coupling Constants and Peptide Conformational Parameters. In addition to these supportive roles in proton nmr, measurement of ^{15}N–H coupling constants from either the proton or ^{15}N spectra theoretically gives new conformationally sensitive data. Two-bond ^{15}N–H couplings are very small in these systems. They are not detectable at low pH although they increase to approximately 1 Hz above the amine pK, as has previously been found by Lichter and Roberts (1970a), but both one-bond and three-bond couplings yield interesting results.

Determination of ω. Figure 2 shows the three torsion angles ϕ, ψ, and ω, which define the peptide backbone and the first side-chain torsion angle χ (Kendrew *et al.*, 1970). Evidence relating to ω seems to be found in the value of $^1J_{NH}$ (Table I). All of the values in Table I fall within the known range for amides (Binsch *et al.*, 1964). The small variation in the values is correlated principally with cis–trans isomerism about the peptide bond. The normal trans (ω = 0°) peptides and the simple amide shown at the top of the table exhibit a range of 92.15–94.45 Hz with an average of 93.3 Hz, whereas the cis peptides of the cyclic dipeptide series at the bottom of the table show a range of 89.30–91.13 Hz with an average of 90.4 Hz. Other amides have previously been shown to display

TABLE I: ^{15}N–H Direct Coupling Constants.

Compound	Solvent	$^1J_{NH}$ (Hz)
N-Ac-DL-Ala-^{15}N	H_2O, pH 6	93.30
Valinomycin (L-Val-^{15}N)	CCl_4	93.55
L-Ala-L-Phe-^{15}N	H_2O, pH 2	92.15
L-Ala-L-Ala-^{15}N	H_2O, pH 2	93.24
L-Phe-Gly-^{15}N	H_2O, pH 2	93.15
L-Val-Gly-^{15}N	H_2O, pH 2	94.45
c-L-Ala-L-Ala-^{15}N	H_2O, pH 2	91.13
c-L-Val-Gly-^{15}N	H_2O, pH 2	91.07
c-L-Val-Gly-^{15}N	Me_2SO	90.00
c-L-Phe-Gly-^{15}N	Me_2SO	90.50
c-L-Ala-L-Phe-^{15}N	Me_2SO	89.30

similar behavior under a wide variety of solvent and concentration conditions: N-phenylformamide-^{15}N (Bourn *et al.*, 1964) and N-methylformamide-^{15}N, N-isopropylformamide-^{15}N, and acetamide-^{15}N (Bramwell, 1968; Bramwell and Randall, 1973[1]). The $^1J_{NH}$ values for the two amide protons of formamide-^{15}N show the same trend in the neat liquid and in acetone solution (Sunners *et al.*, 1960; Bourn and Randall, 1964) and also in water (Chuck *et al.*, 1969), despite earlier work (Sunners *et al.*, 1960). The majority of the results in Table I were obtained for aqueous solution, but the trend in the peptides was independent of solvent for the two solvents Me_2SO and water. There is a small but significant solvent effect, however, with the values for $^1J_{NH}$ for c-L-Val-Gly-^{15}N varying by 1.07 Hz between H_2O and Me_2SO. More studies will be necessary before all of the relevant variables affecting $^1J_{NH}$ are clear and their magnitudes predictable, but the data strongly indicate that in peptides the value of $^1J_{NH}$ is a reliable indicator of cis–trans isomerism about the peptide bond as suggested previously (Randall and Gillies, 1971).

Determination of χ and ψ. Vicinal coupling constants involving nuclei of first- and second-row elements of the periodic table seem generally to show a marked dependence on the di-

[1] Bramwell, M. R., and Randall, W. E. (1973), unpublished results.

TABLE II: ^{15}N–H_β Vicinal Coupling Constants.

Compound	Solvent	$^3J_{NH}$ (Hz)
Tyr-^{15}N	D_2O, pH 10	2.75, 2.95
Asp-^{15}N	D_2O, pH 10	2.80, 3.70
Phe-^{15}N	D_2O, pH 1	2.20, 2.35
Ala-^{15}N	D_2O, pH 1	3.10
N-Ac-DL-Ala-^{15}N	H_2O, pH 6	3.04
L-Ala-L-Ala-^{15}N	H_2O, pH 2	3.05
L-Ala-L-Phe-^{15}N	H_2O, pH 2	2.30, 3.40
c-L-Ala-L-Phe-^{15}N	Me_2SO	1.90, 3.20

TABLE III: ^{15}N–H_α Vicinal Coupling Constants.

Compound	Solvent	$^3J_{NH}$ (Hz)
Valinomycin (L-Val-^{15}N)	CCl_4	1.20
L-Ala-L-Ala-^{15}N	D_2O, pH 2	1.20
L-Val-Gly-^{15}N	D_2O, pH 2	<1
L-Ala-L-Phe-^{15}N	D_2O, pH 2	<1
c-L-Val-Gly-^{15}N	D_2O, pH 2	1.02
c-L-Ala-L-Phe-^{15}N	Me_2SO	1.85
c-L-Val-Gly-^{15}N	Me_2SO	<1
c-L-Phe-Gly-^{15}N	Me_2SO	<1

hedral angle (the so-called Karplus (1959) relationship). Provided that the significant limitations of this correlation are properly taken into account, the value of the coupling constants can be used to put strong restraints on the possible dihedral angle (Karplus, 1963). Limitation of possible values of the angle ϕ in the peptide backbone through measurement of $^3J_{NH-C\alpha H}$, with additional limitations coming from peptide conformational energy maps, is one of the keys to the study of peptide conformations by nmr (Gibbons *et al.*, 1970a; Bystrov *et al.*, 1969; Ramachandran *et al.*, 1971). Measurements of vicinal ^{15}N–H coupling constants in peptides could potentially be used in the determination of the side-chain torsion angle χ about the C–$C_{\alpha\beta}$ bond if the coupling $^3J_{N-H\beta}$ were measured or the torsion angle ψ if $^3J_{N-H\alpha}$ were measured.

Values measured for $^3J_{N-H\beta}$ can be related to conformation by performing a rotamer population analysis assuming three staggered minima in the potential energy curve for rotation about χ, using the values of $^3J_{H\alpha-H\beta}$ as input. This approach has been used by Lichter and Roberts (1970b) who determined $^3J_{N-H}$ for three ^{15}N-enriched amino acids at neutral pH. They analyzed for the three rotamer populations using Pachler's values (1963, 1964) of J_{trans} = 13.6 Hz and J_{gauche} = 2.6 Hz for the α–β H–H coupling constant. They then used the rotamer populations and $^3J_{N-H\beta}$ values to estimate J_{trans} and J_{gauche} for the ^{15}N–H_β system. We have applied this same type of analysis to a larger number of determinations (Table II) to assure its validity and determine the confidence limits of the correlation. Our results are in full agreement with those of Lichter and Roberts with J_{trans} = 4.8 ± 0.1 Hz and J_{gauche} = 1.8 ± 0.1 Hz. It should be noted that the actual values of these quantities, though not the strength of the correlation, are strongly dependent on the values of J_{trans} and J_{gauche} for $^3J_{H\alpha-H\beta}$. Thus the confidence limits are essentially the confidence limits for these quantities.

The other vicinal N–H coupling, $^3J_{N-H}$, would be of great practical value if conformational significance with respect to the torsion angle ψ could be attached to it. Assuming normal trans peptide bonds (ω = 0°) in a peptide of i residues, even if a single value could be determined for each angle ϕ_i, there are still i degrees of freedom left undefined because there is no experimental method for determining ψ_i. Because of this it has been possible so far to study with confidence only cyclic peptides, peptides of high symmetry, or otherwise highly constrained peptides. Eight measurements of $^3J_{N-H\alpha}$ have been made and these are shown in Table III. The principal drawback to studying these couplings is their small magnitude, necessitating very accurate determinations. These coupling constants are greatly different from the other vicinal coupling constants $^3J_{N-H\beta}$ because of variation in hybridization, bond lengths, bond angles, and substituent electronegativities between the two systems. All values of $^3J_{N-H\alpha}$ are in line with the prototype value of 1.3 Hz for acetamide ^{15}N (Binsch *et al.*, 1964). A very rough estimate of $^3J_{N-H\alpha}$ based on the value for acetamide and taking into account the more usual first-order effects on vicinal coupling constants (Karplus, 1963) leads us to predict a value for the totally rotationally averaged coupling constant of 0.9–1.0 Hz. Thus, the maximal value for the coupling constant, for trans orientation of the nitrogen and proton, is likely to be less than 3 Hz. The potential importance of the method led us to attempt these measurements despite the need for highly accurate measurement of very small couplings and the consequently poor prognosis for conformational studies. Line widths in peptide solutions are generally large enough to obscure the couplings directly, even with the elimination of quadrupolar broadening, but couplings on the order of 1 Hz or greater could be extracted with some confidence by line-shape analysis. The results are insufficient to permit any easy correlation of coupling constant with conformation as revealed by X-ray results (Webb and Lin, 1971) and the nmr studies of Kopple *et al.* on diketopiperazines (Kopple and Marr, 1967; Kopple and Ohnishi, 1969; Ziauddin and Kopple, 1970). The range of values is insufficient for even a qualitative interpretation. Thus any use of these coupling constants is unlikely unless more very precise values are obtained.

^{15}N Magnetic Resonance Studies. For many applications of ^{15}N to nmr, direct observation of the ^{15}N resonance would be preferable to observation of coupling effects in the proton spectrum. Overlap of signals is less of a problem in the ^{15}N spectrum because of the greater range of chemical shifts, and nitrogen signals in higher molecular weight compounds are less subject to dipole–dipole broadening than proton signals. In addition, enrichment in ^{15}N of only those residues of interest is a much easier procedure for the production of a simplified spectrum than the substitution of all undesired protons by deuterium. The drawbacks to routine ^{15}N nmr are the low 0.37% natural abundance and the poor sensitivity of ^{15}N, 10^{-3} that of protons. Instrumental advances, however, have now made it possible to measure ^{15}N spectra even at natural abundance with commercially available spectrometers, provided very high concentrations can be attained (Pregosin *et al.*, 1971). In addition, an increasing number of 95% enriched ^{15}N compounds are now available allowing a wider range of studies than is possible at natural abundance. We have accordingly measured the ^{15}N chemical shifts of a number of 95% enriched amino acids, and one peptide, in aqueous solution at varying pH, under both proton decoupling and nondecoupling conditions. Previous work by Pregosin *et al.* (1971) in-

TABLE IV: ^{15}N Chemical Shifts.

Compound	Solvent	Chem Shift[a] (+0.3 ppm)
Glycine	H_2O	0
Glycine	6 N HCl	−0.3
Glycine	6 N NaOH	−4.6
Alanine	H_2O	+12.8
Alanine	6 N HCl	+13.0
Valine	H_2O	+6.0
Valine	6 N HCl	+6.1
Ornithine (α)	H_2O	+11.0
Ornithine (α)	6 N HCl	+11.2
Phenylalanine	H_2O	+9.1
Aspartic acid	6 N HCl	+9.8
Leucine	6 N HCl	+11.4
Tyrosine	6 N HCl	+9.6
Glutamic acid	6 N HCl	+11.2
L-Val-Gly-^{15}N	H_2O, pH 2	+83.0

[a] Shifts in ppm relative to Gly in H_2O. Downfield shifts are positive.

TABLE V: ^{15}N and ^{13}C Substituent Parameters.[a]

	^{15}N (ppm)	^{13}C (ppm)
β	+12.9	+9.4
γ	−3.5	−2.5
δ	+1.2	+0.3

[a] Downfield shifts are positive.

vestigated the chemical shifts of a number of amino acid methyl ester hydrochlorides at natural abundance under proton-decoupled conditions.

Table IV shows the ^{15}N chemical shifts of the amino acids relative to Gly at its isoelectric point. All of the amino acids were studied in 6 N HCl to give a coherent set of data, but those with sufficient solubility were studied in water as well. In no case was any significant difference found between the chemical shifts of the fully protonated form and the zwitterion. In addition, these chemical shifts agreed very well in all but two cases with the chemical shifts published for the amino acid methyl ester hydrochlorides (Pregosin *et al.*, 1971). It is clear that the nitrogen shifts are very insensitive to the state of the carboxyl group. In the one amino acid studied at a pH above the amine pK, glycine, an upfield shift of 4.6 ppm is observed compared to an upfield shift of 24 ppm for anhydrous ammonia relative to ammonia chloride in water (Lambert *et al.*, 1964). As reported for the amino acid methyl ester hydrochlorides (Pregosin *et al.*, 1971), the chemical shifts can be semiempirically rationalized relative to glycine on the basis of a downfield β shift, an upfield γ shift, a downfield δ shift, and negligible longer range effects, the contributions of all of which are closely additive. The shift parameters obtained from an analysis of the data in Table IV are shown in Table V, where they are compared to the corresponding ^{13}C parameters from the literature (Grant and Paul, 1964). An α-substituent parameter could not be obtained for ^{15}N here because all of the amino acids have the same α-carbon structure. The close similarity in signs and relative magnitudes of the two sets of parameters confirms as one might expect that there are no large differences in the factors affecting ^{13}C and ^{15}N chemical shifts (Lichter and Roberts, 1972).

Spin coupling information was generally not obtained from the amino acid ^{15}N spectra. In all cases in water and in the only case in 6 N NaOH, only single peaks were observed because of the dominating effect of proton chemical exchange on the relaxation of the ^{15}N nucleus in these compounds at any pH above the acid range. In spite of the collapse to a singlet, proton noise decoupling in neutral solution still gave a significant signal enhancement. In 6 N NaOH the Gly signal diminished in intensity with proton noise decoupling as expected on the basis of studies of the nuclear Overhauser enhancement in the ^{15}N spectrum of the ammonium ion (Lichter and Roberts, 1971). Proton noise decoupling was necessary to obtain singlet spectra in 6 N HCl. It should be noted that phase information was lost in our Fourier transform experiments, which produced magnitude spectra, so no information on the sign of the nuclear Overhauser enhancement is available. One spectrum was taken in 6 N HCl without proton noise decoupling. This was glycine, and after 4096 pulses a broad quartet of poor signal-to-noise ratio was seen with a coupling constant of approximately 80 ± 5 Hz. A single peptide, L-Val-Gly-^{15}N, was run at pH 2 under coupled and proton noise decoupling conditions. The sharp singlet at +83.0 ppm relative to internal glycine observed with proton noise decoupling split into a doublet of 94.6 ± 0.6 Hz without irradiation of the protons. In this case longer range coupling could not be detected in the ^{15}N spectrum or in the proton spectrum.

Conclusion

Information obtained from the ^{15}N nucleus in the nmr of polypeptides complements information available from proton and ^{13}C nmr and from other techniques. Use of ^{15}N substitution to eliminate the effect of the ^{14}N electric quadrupole and to facilitate the assignment of amide proton signals in proton magnetic resonance has been demonstrated to be a useful tool in the nmr study of polypeptides amenable either to chemical synthesis or to selective biosynthetic incorporation of ^{15}N amino acids. Determination of the angle ω from $^1J_{^{15}NH}$ as shown in this paper should prove useful, particularly in studying small cyclic peptides where cis peptide bonds are common. Side-chain dihedral angles are obtainable both from proton and ^{15}N studies. In simple peptides where overlap is no significant problem $^3J_{H\alpha H\beta}$ is probably a better and easier determinant of the α–β dehedral angle χ than is $^3J_{^{15}NH\beta}$ since $^3J_{H\alpha H\beta}$ is larger and does not require special synthesis. However, in more complicated spectra the balance is considerably different. The α-proton region of the proton spectrum increases in complexity rapidly with the size of the peptide and the β region of many amino acids is difficult to analyze even for the free amino acid. Thus, $^3J_{H\alpha H\beta}$ may become difficult to measure. But at high enrichment it is extremely easy to determine $^3J_{^{15}NH\beta}$ from the ^{15}N nmr spectrum, especially as only one residue at a time need contain ^{15}N. With increasing complexity of the peptide ^{15}N nmr becomes less an adjunct to proton magnetic resonance and more a window clearly offering selected limited information no longer easily available in the proton spectrum.

The use of $^3J_{^{15}NH\alpha}$ for the determination of ψ as discussed here is potentially the most important use of ^{15}N in nmr of peptides in the sense that it offers hope of determining a param-

eter of peptide conformation not available by any technique previously. This idea was first presented by Gibbons *et al.* (1970a). After this manuscript was ready for submission an article appeared by Karplus and Karplus (1972) discussing the potential applicability of this method on a theoretical basis, with reference to several literature values of $^3J_{^{15}NH}$ and $^3J_{^{14}NH}$ for compounds other than peptides and one new determination, for acetylglycine-^{15}N. They used a valence bond formulation to obtain the equation

$$^3J_{^{15}NH}(\theta) = -1.514a^2(^3J_{HH'}(\theta))$$

where $^3J_{^{15}NH}$ and $^3J_{HH'}$ are coupling constants measured for fragments differing only in the replacement of ^{15}N in the first instance by a proton in the second instance and a^2 is the fractional s character of the nitrogen hybrid orbital. This predicts a similar dependence on the dihedral angle θ for $^3J_{^{15}NH}$ and $^3J_{HH'}$ provided only that a^2 is independent of θ. They compared the values calculated from this equation (and an analogous one for the ^{14}N case) with 12 literature values of $^3J_{^{15}NH}$ and $^3J_{^{14}NH}$ for compounds of varying hybridization with excellent agreement in some cases and fair agreement in others. The single new experimental value was from acetylglycine-^{15}N where $^3J_{^{15}NH} = (-)$ 1.5 Hz, in agreement with our observation of $^3J_{^{15}NH} = 1.4$ Hz for the acetyl methyl splitting in acetyl-DL-alanine and the literature value (Binsch *et al.*, 1964) of 1.3 Hz in acetamide-^{15}N, as well as with their calculated value of $^3J_{^{15}NH} = -1.44$ Hz. (Signs of these coupling constants in general have not been determined and are only tentatively assumed negative on the basis of theory.) Assuming three staggered rotamers of the methyl group in acetylglycine, $^3J_{^{15}NH} = {}^1/_3(J_{^{15}NH^t} + 2J_{^{15}NH^g})$ where t and g designate trans and gauche. If J^t, J^g, and $^3J_{^{15}NH} = -1.44$, then $J_{^{15}NH^t}$ is predicted to be -4.3 Hz, a value considerably higher than our predicted maximum of about 3.0 Hz. Our value was obtained by applying the admittedly approximate corrections of Karplus (1963) to the literature value of the acetamide-^{15}N coupling constant to try and correct for the small differences in bond lengths, bond angles, and substituents found between acetamide and a peptide. While the difference in the two estimates is quite small the practical significance is substantial, since in our experience coupling constants of less than 2.0 Hz in α-proton multiplets are only measurable with difficulty. Thus, the easy access range of coupling constants are those of $\geqslant 2$ Hz and thus the difference between 3.0 and 4.3 Hz is meaningful. We agree with the sentiments of Karplus and Karplus (1972) that neither method of estimating $J_{^{15}NH^t}$ is very rigorous and feel that at this time only further experiments can determine which is the more accurate. To this end we are currently attempting a more rigorous treatment of our data on $^3J_{^{15}NH\alpha}$, taking into account what is generally known about the conformation of the peptides studied, to establish quantitatively the shape and magnitude of the relationship between coupling constant and dihedral angle in this case. The dynamics of structure in solutions of the peptides we studied are not well known and we expect further studies of these and related compounds under various conditions, along with consideration of conformational energy restraints and conformational averaging deduced from theoretical studies, to yield more information on conformations and hopefully to allow experimental determination of the limiting value of the coupling constant.

When this has been done we propose to use the relationship between $^3J_{^{15}NH\alpha}$ and ψ, and the relationship between $^3J_{^{15}NH\beta}$ and χ as well, in conjunction with conformational energy calculations and maps, in a manner analogous to that proposed earlier (Gibbons *et al.*, 1970a) for the relationship between $^3J_{NH-C\alpha H}$ and ϕ, for the further definition of peptide conformation using the best features of nmr and conformational energy calculations.

Acknowledgment

The authors wish to acknowledge the help of Dr. P. S. Pregosin and Dr. A. I. White with the ^{15}N magnetic resonance experiments.

References

Binsch, G., Lambert, J. B., Roberts, B. W., and Roberts, J. D. (1964), *J. Amer. Chem. Soc. 86*, 5564.

Bourn, A. J. R., Gillies, D. G., and Randall, E. W. (1964), *Tetrahedron 20*, 1811.

Bourn, A. J. R., and Randall, E. W. (1964), *Mol. Phys. 8*, 567.

Bovey, F. A., Brewster, A. I., Patel, D. J., Tonelli, A. E., and Torchia, D. A. (1972), *Accounts Chem. Res. 5*, 193.

Bramwell, M. R. (1968), Ph.D. Thesis, London University.

Bystrov, V. F., Portnova, S. L., Tsetlin, V. I., Ivanov, V. T., and Ovchinnikov, Y. A. (1969), *Tetrahedron 25*, 493.

Chuck, R. J., Gillies, D. G., and Randall, E. W. (1969), *Mol. Phys. 16*, 121.

Gibbons, W. A., Nemethy, G., Stern, A., and Craig, L. C. (1970a), *Proc. Nat. Acad. Sci. U. S. 67*, 239.

Gibbons, W. A., Sogn, J. A., Craig, L. C., and Johnson, L. F. (1970b), *Nature (London) 227*, 840.

Gibbons, W. A., Alms, H., Sogn, J. A., and Wyssbrod, H. R. (1972a), *Proc. Nat. Acad. Sci. U. S. 62*, 1267.

Gibbons, W. A., Bockman, R. S., Alms, H., and Wyssbrod, H. R. (1972b), *Biochemistry 11*, 1721.

Gisin, B. F., Merrifield, R. B., and Tosteson, D. C. (1969), *J. Amer. Chem. Soc. 91*, 2691.

Grant, D. M., and Paul, E. G. (1964), *J. Amer. Chem. Soc. 86*, 2985.

Greenstein, J. P., Birnbaum, S. M., and Levintow, L. (1954), *Biochem. Prep. 3*, 84.

Hirschmann, R., Strachan, R. G., Schwam, H., Schoenewaldt, E. F., Joshua, H., Barkemeyer, B., Veber, D. F., Palaveda, W. J., Jr., Jacob, T. A., Beesley, T. E., and Denkewalter, R. G. (1967), *J. Org. Chem. 32*, 3415.

Ivanov, V. T., Laine, I. A., Abdulaev, N. D., Pletnev, V. Z., Lipkind, G. M., Arkhipova, C. F., Senyavina, L. B., Meshcheryakova, E. N., Popov, E. M., Bystrov, V. F., and Ovchinnikov, Y. A. (1971), *Khim. Prir. Soedin.*, 221.

Karplus, M. (1959), *J. Chem. Phys. 30*, 11.

Karplus, M. (1963), *J. Amer. Chem. Soc. 85*, 2870.

Karplus, S., and Karplus, M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 3204.

Kendrew, J. C., Klyne, W., Lifson, S., Miyazawa, T., Nemethy, G., Ramachandran, G. N., and Scheraga, H. A. (1970), *Biochemistry 9*, 3471.

Kopple, K. D., and Ghazarian, H. G. (1968), *J. Org. Chem. 33*, 862.

Kopple, K. D., and Marr, D. H. (1967), *J. Amer. Chem. Soc. 89*, 6193.

Kopple, K. D., and Ohnishi, M. (1969), *J. Amer. Chem. Soc. 91*, 962.

Lambert, J. B., Binsch, G., and Roberts, J. D. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 735.

Lichter, R. L., and Roberts, J. D. (1970a), *Spectrochim. Acta*,

Part A 26, 1813.
Lichter, R. L., and Roberts, J. D. (1970b), *J. Org. Chem. 35*, 2806.
Lichter, R. L., and Roberts, J. D. (1971), *J. Amer. Chem. Soc. 93*, 3200.
Lichter, R. L., and Roberts, J. D. (1972), *J. Amer. Chem. Soc. 94*, 2495.
Pachler, K. G. R. (1963), *Spectrochim. Acta 19*, 2085.
Pachler, K. G. R. (1964), *Spectrochim. Acta 20*, 581.
Pregosin, P. S., Randall, E. W., and White, A. I. (1971), *Chem. Commun.*, 1602.
Ramachandran, G. N., Chandrasekaran, R., and Kopple, K. D. (1971), *Biopolymers 10*, 2113.
Randall, E. W., and Gillies, D. G. (1971), *Progr. Nucl. Magn. Resonance Spectrosc. 6*, 119.
Sunners, B., Piette, L. H., and Schneider, W. G. (1960), *Can. J. Chem. 38*, 681.
Webb, L. E., and Lin, C. F. (1971), *J. Amer. Chem. Soc. 93*, 3818.
Ziauddin and Kopple, K. D. (1970), *J. Org. Chem. 35*, 253.

The Mechanism of Polymyxin B Action and Selectivity toward Biologic Membranes†

Chuen-Chin HsuChen and David S. Feingold*

ABSTRACT: The selectivity of action of the polymyxin antibiotics was studied using lipid spherules in aqueous suspension (liposomes) as a model system; the release of trapped glucose marker was the index of membrane damage. The polymyxin B induced glucose release was observed to be a specific process which differentiates among phospholipids with various "polar head" structures. Liposomes prepared from phosphatidylethanolamine were extremely sensitive to polymyxin B while none of those prepared from the *N*-methyl-substituted analogs (*N*-methylphosphatidylethanolamine, *N*,*N*-dimethylphosphatidylethanolamine, phosphatidylcholine) were sensitive to the antibiotic. The N-methylated analogs of phosphatidylethanolamine protected phosphatidylethanolamine-containing liposomes from polymyxin B. The efficiency of this protection was a function of the molar per cent of the analog in the membrane model and the antibiotic concentration. The pH optimum of the polymyxin B induced glucose leakage in liposomes with phosphatidylethanolamine as the major component was between 7.0 and 8.5 with a marked decrease in activity at extremes of pH. We conclude that the polymyxin susceptibility of biologic membranes requires both the presence of target molecules such as phosphatidylethanolamine, and a threshold density of these molecules on the membrane surface. On the basis of these data with the model membranes a mechanism to explain the selective toxicity of the polymyxin antibiotics is proposed. According to this mechanism proton transfer between the antibiotic and the membrane may be the initial step leading to the irreversible damage in membranes containing phosphatidylethanolamine as the dominant phospholipid.

The polymyxin family of peptide antibiotics has potent bactericidal activity against most Gram-negative bacilli. These antibiotics share several structural features including: (1) a cyclic heptapeptide; (2) a preponderance of the basic amino acid, α,γ-diaminobutyric acid, and (3) a side chain which terminates with a short-chain fatty acid such as methyloctanoic acid. Our present knowledge on the mode of action of the polymyxins began with the pioneer studies of Newton in the early 1950's (reviewed in Newton, 1956). He showed that the antibiotics interacted with cell surface components, probably phospholipids, and demonstrated the irreversible breakdown of the permeability barriers associated with the bactericidal action of polymyxin. Few (1955) demonstrated the interaction of polymyxin with monolayers of phospholipids and lipids of bacterial origin. The strict structural requirements for biological activity of the polymyxin molecule were recognized during the chemical synthesis of this compound (Vogler and Studer, 1966); the structural requirements of the phospholipid molecule determining antibiotic susceptibility have not been systematically studied. This is of special interest since the polymyxins are among the very few membrane-active antibiotics which show sufficient selective membrane toxicity to be of use in treating infections in man.

Previous work suggest that one can sharply differentiate two types of resistance to the polymyxins. *Proteus mirabilis*, and probably other bacteria, are resistant because the cell wall of the organism blocks access of the antibiotic to the susceptible cytoplasmic membrane (Teuber, 1969; Sud and Feingold, 1970; HsuChen and Feingold, 1972). Mammalian cells are also resistant but they lack cell wall and the membranes must be inherently resistant to destruction by the polymyxins. The studies reported herein were designed: (1) to define the nature of the inherent cytoplasmic membrane resistance to the antibiotic and (2) to demonstrate that phospholipids with different "polar head" structures respond to polymyxin differently. The model membrane system used was similar to that described by Haxby *et al.* (1968).

Experimental Section

Preparation of Lipids and Liposomes for Study. Lipids were extracted from *Escherichia coli 200P* (a K12 strain) as described by Kanfer and Kennedy (1963) followed by pre-

† From the Infectious Disease Unit, Beth Israel Hospital-Children's Hospital Medical Center and the Departments of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215. *Received June 12, 1972.* Supported in part by U. S. Public Health Service Grants AI-06313 and TO1-A1-00350 from the National Institute of Allergy and Infectious Diseases.