Conformational Studies of Angiotensin Peptides in Aqueous Solution by Proton Magnetic Resonance†

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ABSTRACT: Observations of the proton magnetic resonance spectrum of [Asn^1,Val^5]angiotensin II during hydrogen–deuterium exchange and spin decoupling permit assignment of two slowly exchanging amides to Val^3 and Val^5. Spin decoupling during hydrogen–deuterium exchange and pH and temperature studies allow the identification of the remaining amide resonances. Comparative experiments have been performed with [Asn^1,Val^5]angiotensin I and with the C-terminal hexapeptide fragment of angiotensin II. A third amide hydrogen, assigned to Tyr^4, becomes more shielded from the solvent as the length of the peptide chain is increased from the hexapeptide to angiotensin I.

Angiotensin II is a naturally occurring linear octapeptide. It has been the subject of numerous studies on the relationship of its primary structure to its biological activities, which include reversible blood pressure elevation and smooth muscle contraction (Bumpus and Smeby, 1968). However, an understanding of the relevance of the tertiary structure to these biological functions has been limited not only by our lack of knowledge of the nature of specific receptor sites but also by the available physical techniques able to establish the details of the conformation of the peptide. The quest for the conformation of angiotensin II has therefore reflected the refinements in biophysical instrumentation and in our understanding of the interactions of peptides with their solvent environment. Spectroscopic investigations of angiotensin II have ranged from optical rotatory dispersion (Smeby *et al.*, 1962) to infrared and Raman studies (Fermandjian *et al.*, 1972a). Thin-film dialysis experiments (Craig *et al.*, 1964; Franze de Fernandez *et al.*, 1968) provided early evidence in support of a compact conformation and for its variation with pH. Ultracentrifuge measurements have indicated that angiotensin II is not associated in aqueous solution down to pH 2.5 and for concentrations up to 1.25% w/v (Paiva *et al.*, 1963; Craig *et al.*, 1964).

The present study by proton nuclear magnetic resonance (nmr) spectroscopy is an extension of earlier tritium–hydrogen-exchange experiments on angiotensin II in aqueous solution (Printz *et al.*, 1972b). The tritium–hydrogen-exchange data were interpreted in terms of classes of amide hydrogens having distinctly different rates of exchange with the solvent. The evidence for two slowly exchanging amide hydrogens suggested the presence of one if not two hydrogen bonds in angiotensin II. In conjunction with known structure–activity relationships two possible hydrogen-bonded conformations were proposed (Printz *et al.*, 1972a): a β-turn model requiring hydrogen bonds from Val^3 N—H to His^6 C=O and from His^6 N—H to Val^3 C=O and a novel γ-turn model with hydrogen bonds from Val^3 N—H to Val^5 C=O and from Val^5 N—H to Val^3 C=O. Based on electron paramagnetic resonance (epr) studies of free-radical analogs and on nmr studies, Weinkam and Jorgensen (1971) proposed a conformation for the C-terminal end of the peptide with an ion–dipole bond from the C-terminal carboxylate anion to the imidazole ring of His^6 and a hydrogen bond from Phe^8 N—H to His^6 C=O. Fermandjian *et al.* (1972a–c) proposed a conformation which has the features of the β-turn model at its N-terminal end and the features of the model of Weinkam and Jorgensen at its C-terminal end. The previous models are in sharp contrast to the helical model for [Asp^1,Ile^5]angiotensin II of Smeby *et al.* (1962) with three hydrogen bonds: Ile^5 N—H to Asp^1 C=O, His^6 N–H to Arg^2 C=O, and Phe^8 N—H to His^6 C=O. A unique assignment of the slowly exchanging amide hydrogens to specific residues should therefore provide a basis for a better discrimination among these various models. Our study has been guided by the knowledge of the pH dependence of the exchange rates from tritium–hydrogen exchange (Printz *et al.*, 1972b) and by a study by Molday *et al.* (1972) on the influence of neighboring side chains on the rates of exchange when hydrogen bonding can be excluded. To facilitate spectroscopic assignments we have studied not only [Asn^1,Val^5]angiotensin II but also its precursor [Asn^1,Val^5]-angiotensin I which has two additional residues, His^9 and Leu^{10}, at its C-terminal end and the hexapeptide lacking the N-terminal Asn^1 and Arg^2 of angiotensin II.

Materials and Methods

[Asn^1,Val^5]Angiotensin II, [Asn^1,Val^5]angiotensin I, and the angiotensin II C-terminal hexapeptide were synthesized by the solid phase method (Merrifield, 1963) using N^{α}-*t*-Boc-amino acids which were coupled to the peptide resin with dicyclohexylcarbodiimide except for Boc-Asn which was coupled *via* its *p*-nitrophenyl ester. Trifunctional amino acids were coupled as Boc-His(Ts), Boc-Tyr(Bzl), and Boc-Arg(Ts) or Boc-Arg(NO_2). Deprotection was achieved with 25% CF_3-$COOH$ in CH_2Cl_2 and cleavage from the resin was with anhydrous HF.

Purification of the crude angiotensin II from the synthesis using Boc-Arg(NO_2) gave a 38% yield of product based on the attachment of the first amino acid to the resin. Yields of the other pure peptides were 50% for angiotensin II using Boc-Arg(Ts), 45% for angiotensin I using Boc-Arg(Ts), and 26% for the C-terminal hexapeptide of angiotensin II. All of these purified peptides were found to be homogeneous

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by high-voltage electrophoresis at pH 1.7 and 5.6 and by paper chromatography in three solvent systems. All of these peptides gave the correct amino acid analyses (including ammonia).

An additional sample of [Asn[1],Val[5]]angiotensin II was purified from Ciba [Val[5]]hypertensin II amide by countercurrent distribution in the system 1-butanol–acetic acid–water (4:1:5). Purified Ciba angiotensin II was identical with the two synthesized angiotensin II peptides by electrophoresis and paper chromatography. Details of the syntheses and purifications will be reported elsewhere. All of these angiotensin II peptides gave identical nmr spectra. In the following the results for Ciba hypertensin II and synthesized angiotensin II will be described together but the data obtained from each of them will be identified.

The nmr spectra were recorded on a Varian HR-220 spectrometer operated by a consortium at the Rockefeller University. The temperature of the nmr probe was measured by the nmr line separation of methanol or ethylene glycol. The pH was measured with indicator paper in order to avoid contamination by an excess of salt. Calibration of several samples at 22° with an electrode confirmed the results within 0.15 pH unit. The pH was adjusted by the addition of small amounts of CF_3COOH or ammonia. The chemical shifts of the amide resonances were measured with respect to the aromatic resonances of Tyr[4] and Phe[8] and these resonances were in turn calibrated with respect to an internal standard of sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$. The samples approximated a concentration of 6% w/v.

In order to perform the hydrogen–deuterium-exchange experiments close to the desired pH, use was made of the following method. Two identical samples were dissolved in H_2O and brought to the desired pH by the addition of CF_3COOH. The samples were then lyophilized. One of the samples, an empty nmr tube, and 0.7 ml of D_2O were kept in ice. The other sample was dissolved in H_2O and used for preadjustment of the spectrometer. The cold sample was then dissolved in the D_2O and the exchange was recorded by repeatedly scanning the amide region of the spectrum. The half-lives for exchange were obtained from a least-square fit of a semilogarithmic plot of the resonance amplitudes *vs.* time. The uncertainties of these half-lives varied from 5 to 15% (Bleich *et al.*, 1973). In order to identify the resonances of the α-proton adjacent to a slowly exchanging amide, the amide region and the α-proton region of the nmr spectrum were sequentially scanned during the exchange. Spin decoupling during hydrogen–deuterium exchange was performed by irradiating a particular amide resonance and recording spectral changes in the α-proton region. This allows spin decoupling to be performed until the amide is deuterated to 80%. The alternate method of irradiating an α-proton resonance and recording spectral changes in the amide region limits the experiment to one half-life for exchange due to loss in signal intensity. The spin-decoupling experiments were performed with a Hewlett Packard oscillator 4204A. A variable-temperature controller was used for the exchange experiments at 30° but not during the hydrogen–deuterium-exchange experiments at 17°. We have recently observed that the temperature of the nmr probe varies over the time period of an experiment. This problem could be eliminated in the future by using a variable-temperature controller in all experiments.

Results

In the following the hexapeptide was treated as the C-terminal fragment of angiotensin II and its residues were numbered accordingly from 3 to 8. Figures 1, 2, and 3 show the spectra of the three peptides in aqueous solution and they illustrate the difficulty of recording proton nmr spectra in H_2O. The lower spectrum in each case, taken in D_2O of 99.96% isotopic purity, contains a large resonance near 5 ppm due to the residual HOD. This resonance is overwhelmingly large in H_2O and blanks out a part of the spectrum. Fortunately, the region below 6.5 ppm can be observed in H_2O and this allows the study of the amide resonances which are absent in D_2O due to exchange with the solvent. The labeling of amide resonances is changed from peptide to peptide in order to avoid misinterpretation.

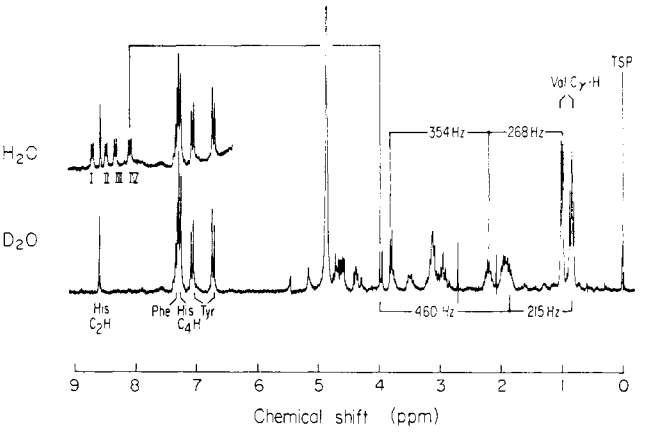

FIGURE 1: The 220-MHz proton nmr spectrum of the C-terminal hexapeptide analog of [Val[5]]angiotensin II (Val-Tyr-Val-His-Pro-Phe) in aqueous solution at pH 2.5 and 17°.

The peptides were examined as their trifluoroacetate salts by lyophilization after titration to pH 2 with CF_3COOH. In each spectrum the sharp resonance near 2.1 ppm is due to residual acetate. The spectrum of the hexapeptide contains a sharp resonance at 2.7 ppm which is due to an impurity of Me_2SO in this particular nmr tube. The region of the spectrum from 3.5 to 5 ppm contains the α-proton resonances. In the case of angiotensin II one additional resonance is hidden under the HOD resonance. It can be resolved at lower temperature and has been assigned to His[6] by Glickson *et al.* (1972). The spectra of angiotensin II and angiotensin I in H_2O show a resonance near 7.75 ppm which is due to one of the primary amides of Asn[1]. Temperature studies show the other resonance to be located under the resonance of Tyr[4] at 7.1 ppm. These amides were observed to exchange rapidly in all hydrogen–deuterium-exchange experiments.

pH Studies. The solid curves in Figure 4 show the pH dependence of the four amide resonances of the hexapeptide at 17°. A conformational change seems to occur above pH 5 which also changes the chemical shifts of the aromatic proton resonances as is indicated by the dashed lines in Figure 4. Below pH 5 only the amide resonance of the C-terminal Phe[8] shows a pH profile. The conformational change occurring above pH 5 prevents the identification of the amide resonance of His[6] by its pH profile. As the pH is raised the amide resonances broaden and vanish since the base-catalyzed exchange rate with the solvent increases. By following the analysis of Molday *et al.* (1972) some of the amide protons can be classified according to the contributions of their neighboring side chains to the exchange rate. Resonance III, already assigned to the amide proton of Phe[8] by its pH profile, is characterized by its weak base-catalyzed exchange rate due to its C-terminal position; it is still visible at pH 8. Resonance I is

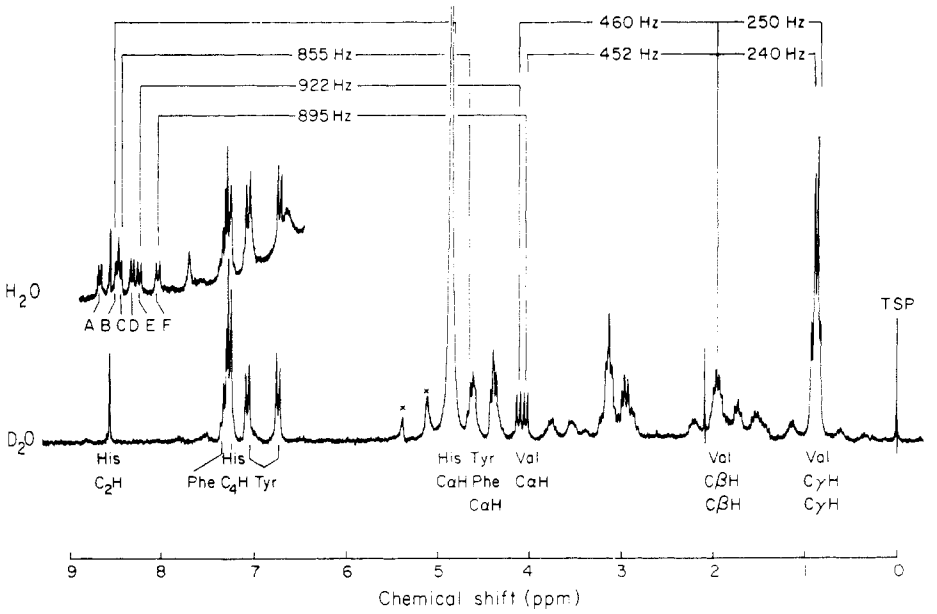

FIGURE 2: The 220-MHz proton nmr spectrum of [Asn^1,Val^5]angiotensin II (Asn-Arg-Val-Tyr-Val-His-Pro-Phe) in aqueous solution at pH 2.3 and 17°.

strongly base catalyzed and it therefore can be assigned to the amide proton of Tyr^4.

In order to resolve some of the amide resonances from the C_2-proton resonance of His^6 the titration experiments for angiotensin II were performed at various temperatures. Figure 5 shows the pH dependence at 20° of the chemical shifts of the six amide resonances of Ciba hypertensin II. The pH profile of resonance D assigns it to the amide resonance of Phe^8. Angiotensin II becomes less soluble above pH 5.7 and only a partial pH profile of resonance B reveals it as being due to the amide proton of His^6. Resonance A broadens between pH 4.4 and 5.5 due to its strong base-catalyzed exchange rate and it eventually disappears and can be assigned to the amide of Arg^2.

Figure 6 shows the pH dependence of the chemical shifts of the amide resonances of angiotensin I at 17°. Resonance 8 is assigned to the amide proton of Leu^{10} and resonance 6 to the amide proton of its neighbor His^9. Owing to the complexity of the amide resonances and to the loss of solubility above pH 5.5 the amide resonance of His^6 has not yet been identified.

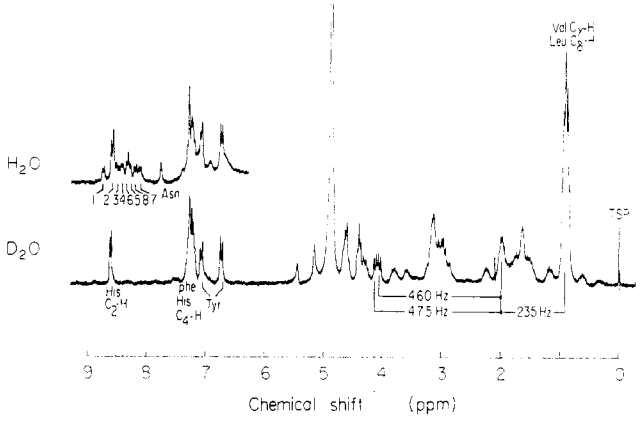

FIGURE 3: The 220-MHz proton nmr spectrum of [Asn^1,Val^5]-angiotensin I (Asn-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu) in aqueous solution at pH 3.5 and 17°.

Resonance 1 is again assigned to the amide of Arg^2 due to its strongly base-catalyzed exchange rate.

Temperature Studies. In order to investigate the conformational stability of the various peptides under a variety of conditions the temperature dependence of the chemical shifts of the amide resonances was studied at two or three different pH values. The experimental results are summarized in Figure 7. The upper left shows the temperature profiles of the four amides of the hexapeptide at pH 3.0 and the upper right at pH 5.3. In both cases resonance IV has a slightly smaller temperature coefficient. At pH 5.3 (Figure 7, upper right) resonance I broadens and disappears at higher temperature due to its strong base-catalyzed exchange with the solvent. The center left and right of Figure 7 show the temperature profiles of Ciba hypertensin II at pH 1.9 and 4.2, respectively. Not shown is the temperature study of synthetic angiotensin II at pH 3.8 which was also performed. The general behavior is very

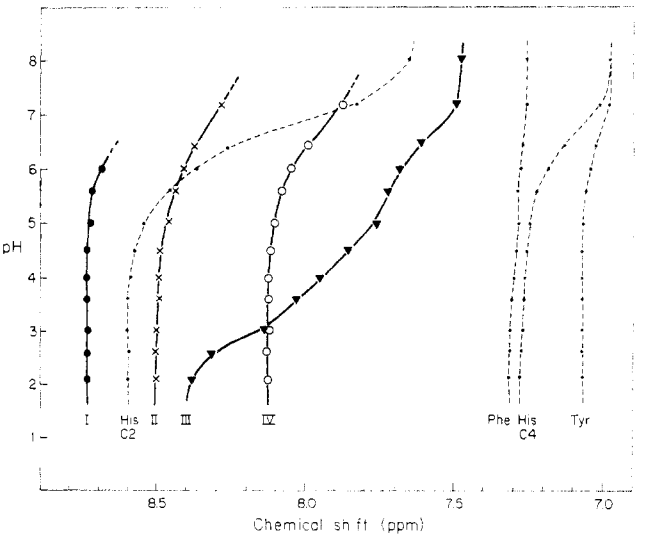

FIGURE 4: The pH dependence at 17° of the chemical shifts of the amide resonances (solid curves) and of the aromatic resonances (dashed curves) of the hexapeptide.

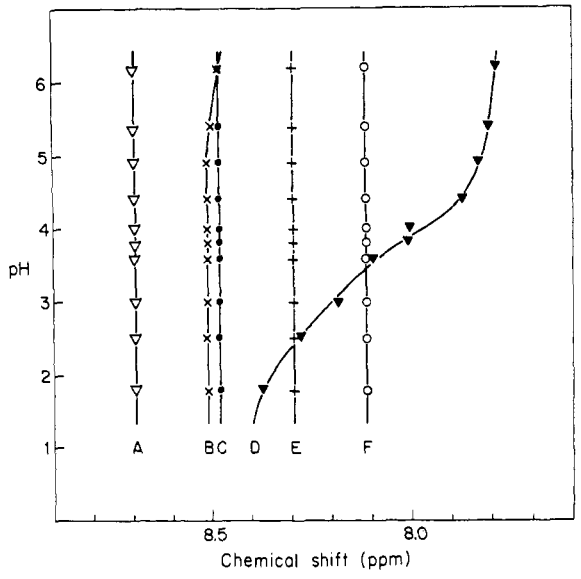

FIGURE 5: The pH dependence at 20° of the amide resonances of Ciba [Asn[1],Val[5]]hypertensin II.

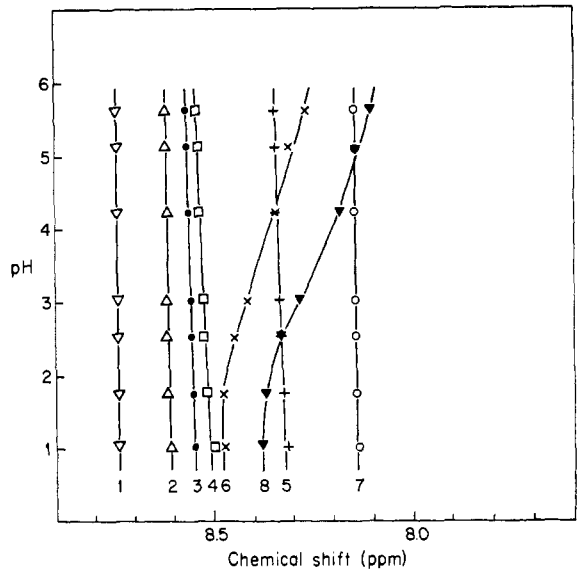

FIGURE 6: The pH dependence at 17° of the amide resonances of [Asn[1],Val[5]]angiotensin I.

similar for all resonances, with an increase in the inflection of the curves with increasing pH. As indicated in the center right of Figure 7, the exchange with the solvent of the amide of resonance A is strongly base catalyzed and it broadens and disappears with increasing temperature. The bottom left and right of Figure 7 give the temperature profiles of the amide resonances of angiotensin I at pH 2.7 and pH 5.3. The strongly base-catalyzed exchange rate of resonance 1 assigns it to the amide proton of Arg[2]. At pH 5.3 (Figure 7, bottom right) resonance 7 shows a smaller temperature coefficient than the remaining amide resonances. The pH dependence of the chemical shifts has assigned this resonance to the amide proton of the C-terminal Leu[10]. At pH 2.7 the resonance of the amide proton of its neighboring His[9], resonance 6, shows also a reduced temperature coefficient, but not as pronounced as for the amide resonance of Leu[10]. The inflections in the temperature profiles of resonances A and 1 might be overestimated because these resonances are not well resolved from the C_2 proton resonances of the histidine residues.

Hydrogen–Deuterium Exchange. The hydrogen–deuterium-exchange experiments are summarized in Tables I, II, and III. An exchange is described as being "fast" if its resonance is no longer observed in the first spectrum after initiation of the exchange (usually 3.5–10 min). Table I summarizes all exchange experiments performed on the hexapeptide. Resonance I is strongly base catalyzed but it is weakly acid catalyzed and at pH 1.6 its exchange rate is smaller than the exchange rates of resonances II and III. In all experiments it is found that resonance IV has an exchange rate that is markedly smaller than the other three amide resonances.

TABLE I: Half-Lives (minutes) for Hydrogen–Deuterium Exchange of the Amides of the Hexapeptide.

Temperature (°C)	17	17	17	17	17
pH	1.6	1.9	2.4	2.5	3.0
I (Tyr[4])	6.1	2	Fast	Fast	Fast
II (His[6])	2	6	7.0	11	3.5
III (Phe[8])	2	5	6.2	9	5
IV (Val[5])	10	37	40	33	17.5

The first column of Table II refers to an exchange of Ciba hypertensin II at pH 2.5 and 18°. The other columns give the results for our synthetic angiotensin II. To simplify the discussion the various amides can be classified as fast (A), intermediate (B, D), and slow (E, F). This classification does not include resonance C which is located between the amides with intermediate and those with slow exchange. As the data for the hexapeptide show, such a classification can only apply at a given pH, near the pH minimum for exchange in this case, but it allows comparison with the tritium–hydrogen-exchange experiments of Printz *et al.* (1972b).

Table III summarizes the various exchange experiments performed on angiotensin I. Resonance 2 is excluded because it is unfortunately hidden under the C_2 proton resonances of His[6] and His[9]. Using the fact that the amide resonances in aqueous solution have generally a larger temperature coefficient than aromatic proton resonances, this resonance could be resolved at 30°. But at this temperature it is observed to exchange very rapidly. At 18° it can therefore be expected to exchange with a rate that is either fast or intermediate. The amides can be classified into fast (1), intermediate (4, 6, 8), and slow (3, 5, 7). Amide resonances 6 and 8, which have a reduced temperature coefficient and which are assigned to His[9] and Leu[10] from pH studies, are among the amide resonances with intermediate exchange rates.

Resonance Assignments. Some of the amide resonances of the three peptides have been assigned on the basis of their behavior under variations in pH and in temperature. The

TABLE II: Half-Lives (minutes) for Hydrogen–Deuterium Exchange of the Amides of Angiotensin II.

Temperature (°C)	18	17	17
pH	2.5	2.3	2.7
A (Arg[2])	Fast	Fast	Fast
B (His[6])	8	5	7.5
C (Tyr[4])	32	16	24
D (Phe[8])	10	6	6.5
E (Val[5])	40	29	32
F (Val[5])	64	30	28

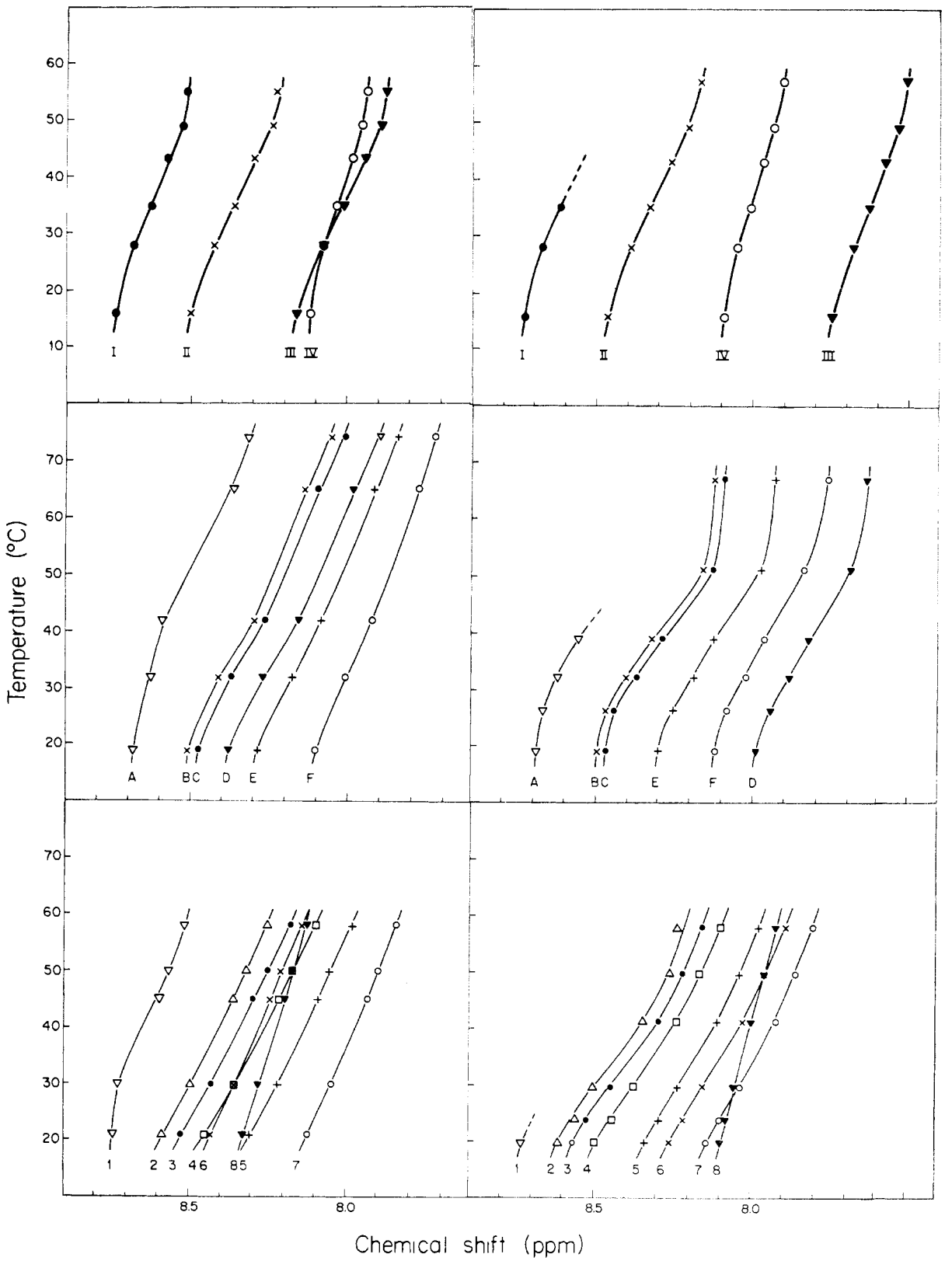

FIGURE 7: The temperature dependence of the chemical shifts of the amide resonances. Upper left and right: hexapeptide at pH 3.0 and 5.3; center left and right: Ciba hypertensin II at pH 1.9 and 4.2; lower left and right: angiotensin I at pH 2.7 and 5.3.

hydrogen–deuterium-exchange experiments have revealed amides with half-lives for exchange which would suggest their involvement in hydrogen bonding or a steric hindrance for access to the solvent. Spin decoupling in H_2O is difficult to perform (Dadok *et al.*, 1972) and we have made use of the long half-lives for exchange to assign these amides to specific residues. At the beginning of a hydrogen–deuterium-exchange experiment all amides are protonated and the α-proton resonances will show coupling to both the neighboring amide proton and to the neighboring β protons. At the end of an exchange experiment all amides are deuterated and the α-proton resonances show coupling to the β protons alone.

TABLE III: Half-Lives (minutes) for Hydrogen–Deuterium Exchange of the Amides of Angiotensin I.

Temperature (°C)	17	18	18	30
pH	2.5	2.8	3.2	2.8
I (Arg^2)	Fast	Fast	Fast	Fast
2 (His^6/Phe^8)	Hidden	Hidden	Hidden	Fast
3 (Tyr^4)	27	40	35	10
4 (Phe^8/His^6)	10	16	Fast	Fast
5 (Val^3)	52	50	34	11
6 (His^9)	9	15	Fast	
7 (Val^5)	42	42	32	12
8 (Leu^{10})	14	12	Fast	Fast

Alternate scanning of the amide region and of the α-proton region of the nmr spectrum during exchange thus allows identification of at least some of the α protons adjacent to the slowly exchanging amide protons. Figure 8 illustrates such an experiment performed on the hexapeptide at pH 2.4 and 17°. The amide region and the α-proton region are sequentially scanned and the time of the beginning of each scan is given on the left-hand side. As the amide resonance IV near 8.1 ppm slowly disappears, the α-proton resonance at 4.0 ppm evolves into a doublet reflecting the coupling to the neighboring β proton alone. Similar experiments have been performed on angiotensin II and angiotensin I. Further spin decoupling in D_2O provides the assignment of these α-proton resonances to specific residues. The results of the spin-decoupling experiments are indicated in Figures 1, 2, and 3 by the frequency separation of coupled resonances.

In the case of the hexapeptide the hydrogen–deuterium-exchange experiment and spin decoupling permit assignment of the slowly exchanging amide, resonance IV, to Val^5. Spin decoupling also shows that the doublet at 3.8 ppm is due to the α proton of the N-terminal Val^3. The pH studies have assigned resonance III to the amide proton of Phe^8 due to its pH profile. The behavior of resonance I under conditions of acid- and base-catalyzed exchange permits its assignment to the N-terminal amide of Tyr^4. By elimination resonance II can be assigned to the amide proton of His^6.

Hydrogen–deuterium exchange and spin decoupling of angiotensin II permit assignment of the two slowly exchanging amide protons to Val^3 and Val^5. Comparison of the exchange rates and of the chemical shifts with those of the hexapeptide suggests that resonance F can be assigned to Val^5 and therefore resonance E to Val^3. The pH profile of resonance D indicates that it is due to the C-terminal Phe^8 and a partial pH profile indicates that resonance B arises from the amide proton of His^6. The behavior of resonance A under variations of pH and temperature shows that its exchange is strongly base catalyzed and it is therefore assigned to the N-terminal amide proton of Arg^2. By elimination resonance C is assigned to Tyr^4.

The assignment of resonance C of angiotensin II to the amide of Tyr^4 has also been determined by spin decoupling during a hydrogen–deuterium-exchange experiment. It shows resonance C to be coupled to an α-proton resonance near 4.65 ppm. Glickson *et al.* (1972) have assigned this position to the α-proton resonances of Phe^8 and Tyr^4. Since resonance D has been assigned to the amide of Phe^8 by the pH profile of its chemical shift, amide resonance C is therefore assigned to Tyr^4. The assignments of the slowly exchanging amides, resonances E and F, to the two valine residues were also confirmed by spin decoupling during hydrogen–deuterium exchange. A similar experiment at 0° allowed assignment of resonance B to the amide of His^6. The low temperature decreases the exchange rate of this amide and also shifts the resonance of the residual HOD sufficiently to reveal the α-proton resonance of His^6 located under the solvent peak (Glickson *et al.*, 1972).

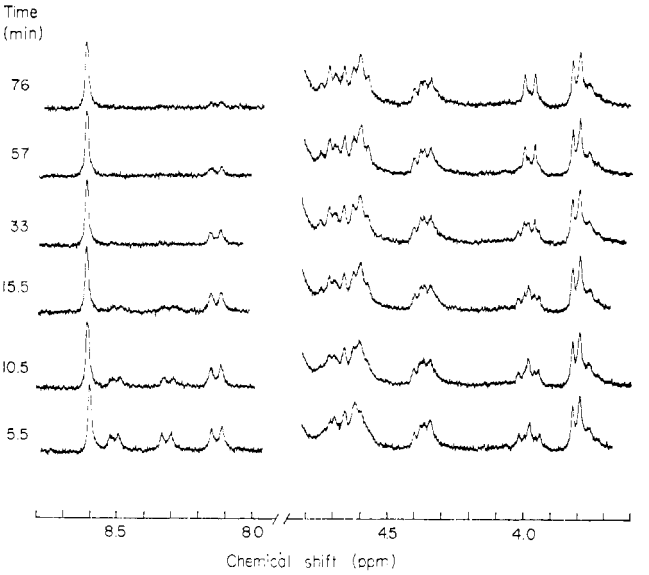

FIGURE 8: The amide region and the α-proton region of the nmr spectrum of the hexapeptide during hydrogen–deuterium exchange at pH 2.4 and 17°. The α-proton resonance at 3.98 ppm evolves into a doublet as the amide resonance IV at 8.13 ppm disappears.

In angiotensin I the pH profile of resonances 6 and 8 permits assignment of them to the C-terminal amide protons of His^9 and Leu^{10}. The strong base-catalyzed exchange rate of resonance 1 indicates it to arise from the N-terminal amide proton of Arg^2. Hydrogen–deuterium exchange and spin decoupling allow assignment of two of the three slowly exchanging amide resonances—3, 5, and 7—to the two valine residues (Figure 3). Comparison of the exchange rates and of the chemical shifts of the amide resonances with those of angiotensin II would suggest assigning resonance 3 to Tyr^4, resonance 5 to Val^3, and resonance 7 to Val^5. The remaining amide resonances of angiotensin I have not yet been assigned to specific residues.

Discussion

The observation of two slowly exchanging amide protons in angiotensin II confirms the tritium–hydrogen-exchange experiments of Printz *et al.* (1972b). However, these observations are not in agreement with those of Glickson *et al.* (1972). This discrepancy may arise from pH differences. But the findings that these amide protons are those of the two valine residues are in agreement with the proposed γ-turn model for angiotensin II in aqueous solution (Printz *et al.*, 1972a). Table IV gives the measured coupling constants of the amide resonances and also summarizes the resonance assignments made at the present time. While most of the coupling constants measured for angiotensin II are in fair agreement with the coupling constants calculated from the proposed torsional angles (Printz *et al.*, 1972a), the coupling constants measured for the amide resonances of Val^3 and Val^5 are 7.5 and 8.5 Hz instead of 2.3 and 9.7 Hz as predicted for the γ turn (Nemethy and Printz, 1972). Matthews (1972) has reported the observa-

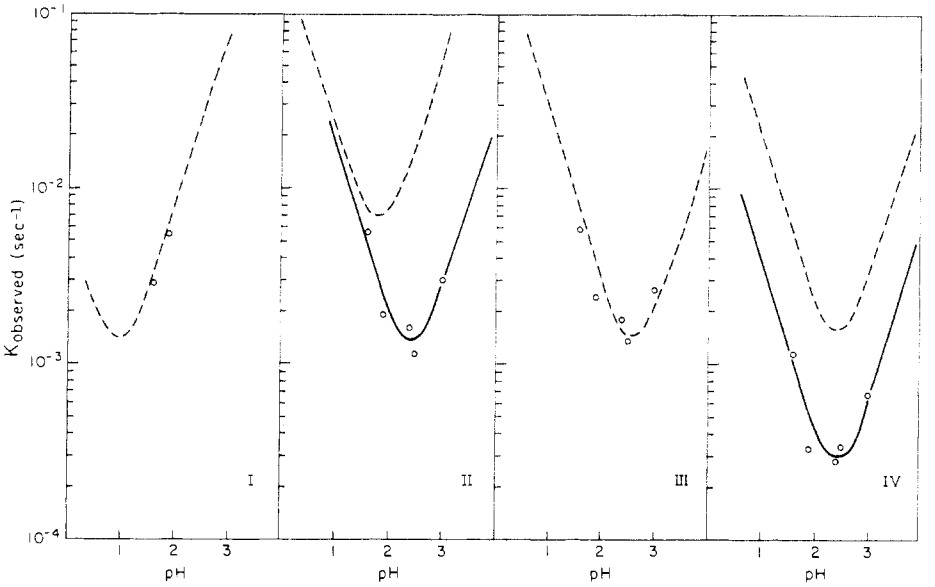

FIGURE 9: The pH profiles for hydrogen–deuterium exchange for the hexapeptide at 17°. The dashed curves represent the expected exchange profiles. The amides labeled I, II, III, and IV are assigned to Tyr^4, His^6, Phe^8, and Val^5, respectively.

tion from X-ray data of a γ turn in thermolysin. The torsional angle ϕ for the first residue in the turn (Val^3 in the present case) is reported to be $-148°$ instead of the predicted 172°. Such a torsional angle can be expected to give a coupling constant of 7.5 Hz (Gibbons *et al.*, 1970) in agreement with the observed coupling constant for the amide of Val^3 in anigotensin II.

The existence of one amide proton, indirectly assigned to Tyr^4 in angiotensin I, whose half-life for exchange increases as the length of the peptide chain is increased from the hexapeptide to angiotensin I could imply shielding by the C-terminal end of the peptide in a conformation differing from the proposed γ-turn model at its C-terminal end. With the exception of the slowly exchanging amide protons and the amide protons of the histidine residues the exchange data are in fair agreement with the half-lives calculated from the data of Molday *et al.* (1972) for other peptides and from the rates for poly(D,L-alanine) measured by Englander and Poulsen (1969). The analysis of the data made use of the following assumptions: all rates in D_2O are 2.5 times faster than in H_2O and the temperature dependence of the logarithm of the rates is 0.05 deg^{-1} (Englander and Poulsen, 1969), pD = pH reading + 0.4 (Glasoe and Long, 1960), and pK (D_2O, 17°) = 15.004 (Kirshenbaum, 1951). Figure 9 shows the predicted pH profile (dashed curves) and the measured pH profile (solid curves) for exchange of the hexapeptide. The data for the N-terminal amide of Tyr^4 (curve 1) and for the amide of Phe^8 (curve III) fall close to the predicted curves. The base-catalyzed exchange rate of the amide of His^6 (curve II) is decreased by a factor of 20 with respect to the data of Molday *et al.* (1972). No complete pH profile has been determined for angiotensin II and angiotensin I but the data available indicate a similar behavior for His^5 in angiotensin II and for His^9 in angiotensin I. Further studies with other histidine containing peptides should explain this effect.

TABLE IV: Coupling Constants and Assignments for the Amides of the Angiotensin Peptides.

	Resonance	J_{NH-CH} (Hz)	Assignment	Evidence
Hexapeptide	I	7.0 ± 0.3	Tyr^4	Exchange rate
	II	6.4 ± 0.3	His^6	
	III	7.5 ± 0.3	Phe^8	pH profile
	IV	8.5 ± 0.3	Val^5	Spin decoupling
Angiotensin II	A	6.7 ± 0.3	Arg^2	Exchange rate
	B	7.0 ± 0.3	His^6	pH profile, decoupling
	C	8.0 ± 0.3	Tyr^4	Spin decoupling
	D	7.5 ± 0.3	Phe^8	pH profile
	E	7.6 ± 0.3	Val^3	Spin decoupling
	F	8.5 ± 0.3	Val^5	Spin decoupling
Angiotensin I	1	6.8 ± 0.3	Arg^2	Exchange rate
	2	7.5 ± 0.3		
	3	8.0 ± 0.3	Tyr^4	Angiotensin II
	4	6.8 ± 0.3		
	5	8.0 ± 0.3	Val^3	Spin decoupling
	6	7.7 ± 0.3	His^9	pH profile
	7	8.5 ± 0.3	Val^5	Spin decoupling
	8	7.6 ± 0.3	Leu^{10}	pH profile

The pH profile for the amide of Val^5 in the hexapeptide (curve IV of Figure 9) suggests a pH independent unfolding of the peptide with subsequent unhindered hydrogen exchange with the solvent. The following mechanism for exchange has been proposed by Linderstrøm-Lang (1955)

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange}$$

where the exchange in sterically hindered in the closed form N and where the exchange proceeds unhindered in the open form I. The observed rate of exchange can be given for the case $k_2 \gg k_3$ and $k_2 \gg k_1$ (Hvidt and Nielsen, 1966): $k_{obsd} = (k_1/k_2)k_3 = Kk_3$. The data of Figure 9 for the amide of Val^5 gives $K = 0.2$ which compares with $K = 0.11$ found for the slowly exchanging amide in bacitracin A (Galardy *et al.*, 1971). It must be noted that the smallest rates for exchange in the angiotensin peptides and in bacitracin A are ten times larger than those observed for the cyclic decapeptide grami-

cidin S-A (Stern *et al.*, 1968; Laiken *et al.*, 1969) which reflects the greater conformational flexibility of the former peptides.

The present study illustrates the unreliability of the interpretation of the temperature dependence of amide resonance which has also been noted by Pitner and Urry (1972) in the case of amides and by Glickson (1972) for indoles. While the slowly exchanging amide proton of Val^5 in the hexapeptide has a reduced temperature coefficient, no such effect is observed for the slowly exchanging amides in angiotensin II and angiotensin I. On the other hand, the amide resonances of His^9 and Leu^{10} in angiotensin I have a reduced temperature coefficient but they exchange with rates that are intermediate. While caution might be required in the interpretation of temperature studies, the general behavior of the temperature curves of the amide resonances of angiotensin II must be noted (Figure 7): the inflection of the curves becomes more pronounced as the pH increases.

Some effects observed in this study warrant further experiments. The conformational change observed for the hexapeptide at pH 6.5 can be explained either by the decreased lifetime of the amide proton involved in hydrogen bonding or by the ionization of the imidazole of His^6 and of the amino group. A similar evidence for a conformational change has been observed for angiotensin II by Glickson *et al.* (1972). The partial shielding of the amide of Tyr^4 in angiotensin II would not be expected from the γ-turn model (Printz *et al.*, 1972a). The existence of the γ-turn conformation remains uncertain due to the lack of any evidence for the peptide carbonyls involved in the hydrogen bonding, a difficulty encountered in other conformational models as well. Our present nmr data disagree with the interpretation of the nmr findings of Fermandjian *et al.* (1972b,d) which postulated a hydrogen bond from Phe^8 N—H to His^6 C=O. Since these studies were performed in Me_2SO this discrepancy could reflect the effects of the different solvent on the conformation of angiotensin II. Circular dichroism studies by Fermandjian *et al.* (1971) failed to indicate an ordered conformation for the hexapeptide. However, these studies were done at pH 6.0 and the present pH studies have shown that the hexapeptide seems to undergo a conformational change between pH 5 and 8 which could account for this discrepancy.

In summary, this nmr study in aqueous solution provides a measure of evidence consistent with a conformation of the hexapeptide similar to that of the proposed γ-turn model, with a slowly exchanging Val^5 N—H, but it does not prove it. The conformation is stabilized in angiotensin II and in angiotensin I by a hydrogen bond involving Val^3 N—H. From the hexapeptide to angiotensin I, one additional amide proton assigned to Tyr^4 becomes increasingly shielded from the solvent, probably by a clustering of the terminal residues about the hydrophobic side chains of Val^3, Tyr^4, and Val^5.

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References

Bleich, H. E., Galardy, R. E., and Printz, M. P. (1973), *J. Amer. Chem. Soc. 95*, 2041.

Bumpus, F. M., and Smeby, R. R. (1968), *in* Renal Hypertension, Page, I. H., and McCubbin, J. W., Ed., Chicago, Ill., Year Book Medical Publishers, p 83.

Craig, L. C., Harfenist, E. J., and Paladini, A. C. (1964), *Biochemistry 3*, 764.

Dadok, J., Von Dreele, P. H., and Scheraga, H. A. (1972), *J. Chem. Soc., Chem. Commun.*, 1055.

Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7*, 379.

Fermandjian, S., Fromageot, P., Tistchenko, A. M., Leiknam, J. P., and Lutz, M. (1972a), *Eur. J. Biochem. 26*, 174.

Fermandjian, S., Greff, D., and Fromageot, P. (1972b), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 545.

Fermandjian, S., Morgat, J. L., and Fromageot, P. (1971), *Eur. J. Biochem. 24*, 252.

Fermandjian, S., Morgat, J. L., Fromagoet, P., Lutz, M., and Leicknam, J. P. (1972c), *in* Structure-Activity Relationships of Proteins and Peptide Hormones, Margoulies, M., and Greenwood, F. C., Ed., New York, N. Y., Excerpta Medica, p 460.

Fermandjian, S., Pradelles, P., Fromageot, P., and Dunand, J. J. (1972d), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28*, 156.

Franze de Fernandez, M. T., Delius, A. E., and Paladini, A. C. (1968), *Biochim. Biophys. Acta 154*, 223.

Galardy, R. E., Printz, M. P., and Craig, L. C. (1971), *Biochemistry 10*, 2429.

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

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Glickson, J. D. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 124.

Glickson, J. D., Cunningham, W. D., and Marshall, G. R. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 563.

Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.

Kirshenbaum, I. (1951), Physical Properties and Analysis of Heavy Water, New York, N. Y., McGraw Hill, p 36.

Laiken, S. L., Printz, M. P., and Craig, L. C. (1969), *Biochemistry 8*, 519.

Linderstrøm-Lang, K. (1955), *in* Symposium on Peptide Chemistry, Special Publication 2, London, The Chemical Society, p 1.

Matthews, B. W. (1972), *Macromolecules 5*, 818.

Merrifield, R. B. (1963), *J. Amer. Chem. Soc. 85*, 2149.

Molday, R. S., Englander, S. W., and Kallen, R. G. (1972), *Biochemistry 11*, 150.

Nemethy, G., and Printz, M. P. (1972), *Macromolecules 5*, 755.

Paiva, T. B., Paiva, A. C. M., and Scheraga, H. A. (1963), *Biochemistry 2*, 1327.

Pitner, T. P., and Urry, D. W. (1972), *Biochemistry 11*, 4132.

Printz, M. P., Nemethy, G., and Bleich, H. E. (1972a), *Nature (London), New Biol. 237*, 135.

Printz, M. P., Williams, H. P., and Craig, L. C. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 378.

Smeby, R. R., Arakawa, K., Bumpus, F. M., and Marsh, M. M. (1962), *Biochim. Biophys. Acta 58*, 550.

Stern, A., Gibbons, W. A., and Craig, L. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 734.

Weinkam, R. J., and Jorgensen, E. C. (1971), *J. Amer. Chem. Soc. 93*, 7038.