The Conformation of Angiotensin II in Aqueous Solution. II. Dialysis and Gel Filtration Behavior of [Asn^1-Val^5]-Angiotensin II*

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ABSTRACT: In a study of the kinetics of dialysis of [Asn^1-Val^5]-angiotensin II through cellophane membranes, at low ionic strength, it was possible to detect two components with different first-order rate constants of dialysis. The two components were present in approximately equal amounts in the pH range 2–9, but at pH 1.1 and 11.0 the component that dialyzed faster was present in a significantly lower proportion. At ionic strength 0.1 only the slow-dialyzing component was found in the pH range 1.1–11.0. A slow conversion from the fast- into the slow-dialyzing form was observed when the ionic strength of an aqueous solution of the peptide was raised to 0.1 by the addition of KCl. Chromatography on Sephadex G-25 columns also showed the presence of two components at low ionic strength.

The effect of salt on the behavior of angiotensinamide toward Sephadex chromatography indicated that the component that dialyzed faster was more retained by Sephadex than the slower dialyzing one. These results are interpreted as evidence for the possibility of two conformations of angiotensin II in aqueous solutions.

The study of the structural requirements for activity of angiotensin peptides has led to the belief that a better understanding of that problem will depend upon the knowledge of the conformation of these peptides in aqueous solution (Bumpus *et al.*, 1961). Smeby *et al.* (1962) have suggested that, in physiological conditions, the octapeptide angiotensin II may assume a helical conformation that might be responsible for some of the observed structure–activity relationships. Paiva *et al.* (1963) presented experimental evidence against such a conformation and argued that it would not be stable in aqueous solution. Craig *et al.* (1964), however, interpreted the behavior of angiotensin peptides on dialysis through cellophane as consistent with the helical model. They measured the 50% escape time of angiotensin II and compared it with the values obtained with other substances, concluding that its conformation must be near that of a tightly packed sphere. They also observed that increasing the ionic strength from 0.01 to 0.16 resulted in very little effect on the 50% escape rate of angiotensin peptides as compared with other peptides.

A more detailed study, however, shows that the dialysis of angiotensin solutions at low ionic strength is not a simple first-order process and that this behavior is affected by the presence of salt. The results of such a study, complemented by evidence from chromatography on Sephadex, are presented in this paper.

Materials and Methods

Angiotensinamide[1] was a synthetic [Asn^1-Val^5]-angiotensin II prepared by Ciba Ltd., Basel, and kindly supplied by Drs. R. Schwyzer and B. Riniker. It was purified by countercurrent distribution as the free peptide, after the last stage of the synthesis, and the lyophilized product contained from one to three molecules of water and two molecules of acetic acid per molecule of peptide. Chromatographic analysis of this product showed some contamination by [Val^5]-angiotensin II (less than 1%) but no other peptides or free amino acids were detected.

The dialysis chamber consisted of a piece of polyethylene tubing, 1 cm long and with 0.4-cm i.d., closed at one end with a stretched piece of cellophane. The cellophane, as purchased from Arthur H. Thomas Co., is said to have an average pore diameter of 4.8 mμ, but before its use it was stretched and submitted to different degrees of acetylation, as described by Craig and Konigsberg (1961); 0.050 ml of angiotensinamide solution was placed inside the dialysis chamber, which was inserted and tightly fitted to the top of a square photometer cell, of 1-cm light path, with fused silica windows. This cell contained 2.2 ml of the same solvent used for the angiotensin solution placed inside the chamber, and its contents were stirred by means of a small magnetic bar. The liquid was kept at the same level inside and outside the chamber. At regular time intervals the magnetic stirring was interrupted for 3–5 sec and the absorbance of the solution in the outer cell was read against a solvent blank in a Beckman DB double-beam spectrophotometer at 230 mμ. All the dialysis experiments were done at room temperature (18–23°).

The kinetics of dialysis was analyzed as a reversible first-order process in which the absorbance (A_t) in the outside

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[1] Abbreviation used is: angiotensinamide, [Asn^1-Val^5]-angiotensin II.

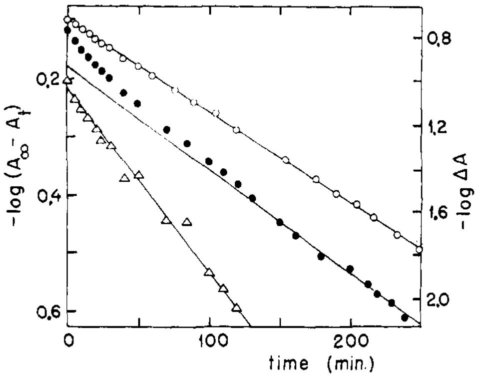

FIGURE 1: Kinetics of dialysis of angiotensinamide: (●) in 0.01 M HCl, pH 2.1; (○) in 0.01 M HCl containing 0.09 M KCl, pH 2.1. The ordinate scale for these two plots is on the left. The kinetics observed at the lower ionic strength is decomposed into a straight line through the last nine points (slow component) and a semilogarithmic plot (△) of the difference between the absorbance readings in the first 2 hr and the corresponding values from the extrapolated straight line (fast component). The ordinate scale for the latter plot is on the right. The initial concentration of angiotensinamide inside the dialysis chamber was 0.11 M.

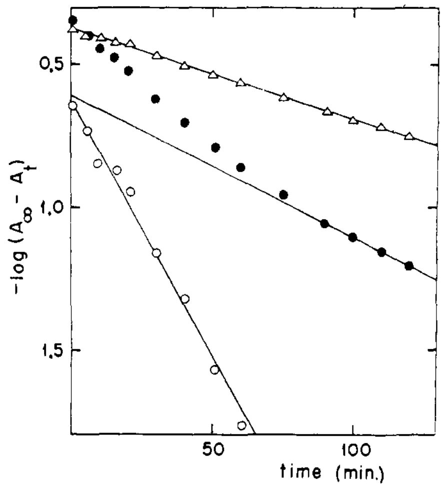

FIGURE 2: Kinetics of dialysis of angiotensinamide: (●) in 0.015 M acetic acid, pH 3.2, initial peptide concentration inside the dialysis chamber: 0.055 M; the kinetics was resolved into 52% of a slow component, with $k_1 = 2.4 \times 10^{-4}$ min^{-1}, and 48% of a fast component (○), with $k_1 = 9.3 \times 10^{-4}$ min^{-1}. (△) Dialysis of the same solution, with the same membrane, initiated 24 hr after the salt concentration was brought to 0.1 M by addition of solid KCl ($k_1 = 1.6 \times 10^{-4}$ min^{-1}).

chamber at time t is given by

$$\log (A_\infty - A_t) = \log A_\infty - 0.434ak_1t \qquad (1)$$

where A_∞ is the absorbance at equilibrium, a is the ratio of the volumes outside and inside the dialysis chamber, and k_1 is the first-order rate constant. The latter is assumed to be the same for the dialysis in both directions through the membrane.

For the gel filtration experiments, angiotensinamide was dissolved either in 0.015 M acetic acid or in 0.015 M acetic acid containing 0.1 M KCl; 1 ml of the solution was applied to a 48 × 1.15 cm column of Sephadex G-25 previously equilibrated with the solvent. The elution was made with the same solvent and the eluates were collected in fractions of 0.7 ml with a G. M. E. fraction collector. Each fraction was diluted with 0.1 M KCl for the determination of the absorbance at 230 mμ, done on a Beckman DB double-beam spectrophotometer.

All pH measurements were made on a Radiometer Model 4 pH meter. Molecular models of angiotensin were made with "Courtauld" atomic models purchased from the Ealing Corp., Cambridge, Mass.

Results

When the lyophilized angiotensinamide powder was dissolved in a solvent of ionic strength 0.1 or higher, the solution behaved on dialysis as predicted by eq 1. This behavior was observed in the pH range of 1.1–11.0 with all the membranes that were used and is illustrated by the plot of log $(A_\infty - A_t)$ against time, at pH 2.1, represented by the open circles in Figure 1. Solutions of low salt concentration, however, behaved anomalously on dialysis, as shown by the filled circles in Figure 1. A deviation from linearity was evident for the points obtained in the first 2 hr of dialysis, while the remaining points could be fitted with a straight line by the method of least squares. The deviations, ΔA, between the absorbance readings during the first 2 hr and the corresponding values obtained from the extrapolation of the straight line fitted to the latter points were also plotted semilogarithmically in Figure 1. These points fell into a straight line from which there were no systematic deviations.

The resolution of the kinetics of dialysis into two first-order components was observed with solutions of low ionic strength at all the pH values that were studied, but only when membranes of appropriate permeability were employed. The importance of membrane permeability is illustrated in Table I by the results of the dialyses of a solution of pH 5.5 and ionic strength <0.001 through three membranes of different degrees of acetylation: with membrane D the peptide dialyzed as a single component but with the less permeable membranes C and E the resolution into two components was evident. The values found for the rate constants varied with the degree of acetylation of the membranes, but the relative proportions of the two components did not depend significantly upon membrane permeability. This is also shown by the data for pH 5.5 in Table I. For the sake of clarity, similar observations made at other pH values were left out of Table I, which summarizes the results obtained with five different membranes, chosen by their selectivity for the two components at low ionic strength. At pH 1.1 it was not possible to have solutions of ionic strength below 0.1 and the dialysis followed a course according to eq 1. At the higher pH values observations made at lower ionic strengths revealed the presence of the two components in approximately equivalent amounts, except at pH 2.1 and 11.0, where the proportion of the slow component was significantly higher than that of the fast one.

With regard to the effect of pH on the dialysis rate, there was a tendency for the k_1 values for both components to decrease with the increase of pH, especially between pH 2.1 and 7.2. This effect is illustrated in Table I by the results obtained with membranes C and D and was probably due to a change in membrane permeability rather than changes in the effective diameter of solute. Membrane D, for example, was too

TABLE I: Effect of pH and Ionic Strength on the Dialysis Behavior of Angiotensinamide.

pH	Membrane[a]	Ionic Strength[b]	Fast Components %	Fast Components $k_1 \times 10^4$ min	Slow Component %	Slow Component $k_1 \times 10^4$ min
1.1	A	0.1	0		100	0.8
2.1	B	0.01	15	4.4	85	0.9
	B	0.1	0		100	0.8
3.3	C	0.001	45	6.4	55	3.6
	C	0.1	0		100	4.2
5.5	D	0.001		($k_1 = 5.3 \times 10^{-4}$ min^{-1})[c]		
	E	0.001	46	3.9	54	2.6
	C	0.001	50	2.6	50	0.3
	C	0.1	0		100	0.4
7.2	D	0.01	45	2.8	55	1.1
	D	0.1	0		100	0.6
9.0	D	0.001	58	2.2	42	0.4
	D	0.1	0		100	0.3
11.0	D	0.001	27	1.8	73	0.4
	D	0.1	0		100	0.4

[a] The observations were made with five membranes, of different degrees of acetylation, designated by A, B, C, D, and E. [b] The ionic strength was calculated from the amount of acid or base added to adjust the pH. When necessary, the ionic strength was brought to 0.1 by addition of KCl. [c] At pH 5.5 the two components present at low ionic strength were not resolved by membrane D, and the observed k_1 value cannot be attributed to either component.

permeable at pH 5.5 to resolve the two components revealed by membranes C and E, but at higher pH values its permeability became low enough for that resolution.

There was no significant differences between the results of dialysis experiments carried out with the same solutions and the same membrane at different times, from the moment the solution was made up to 20 days later. This was observed whether the solution was made with a solvent of low salt concentration or with ionic strength 0.1 or higher. However, when solid KCl was added to solutions of angiotensinamide, previously prepared with solvents of low salt concentration, a slow change in the dialysis behavior occurred. Figure 2 shows an example of this, in which KCl was added to an aqueous solution of angiotensinamide to bring its ionic strength to 0.1. The kinetics of dialysis, run before the addition of salt, was typical of solutions of low salt concentration, with 48% of a fast component and 52% of a slow component. In subsequent dialyses of the same solution it was found that the proportion of the slow component increased and that of the fast one decreased; 24 hr after the addition of salt the dialysis behavior (Figure 2) was similar to that observed with angiotensinamide dissolved in 0.1 M KCl. That behavior did not alter significantly in the following 9 days, during which the solution was kept in the refrigerator (4–6°).

Chromatography of angiotensinamide solutions on Sephadex G-25 also indicated the presence of at least two components in solutions of low ionic strength. Figure 3 shows that the elution profile of a solution of angiotensinamide in 0.015 M acetic acid–0.1 M in KCl was approximately symmetrical, but when the solvent was 0.015 M acetic acid, in the absence of KCl, a partial separation of two peaks was observed. This is better seen in a graph of the first derivative of the elution curve (Figure 4).

Discussion

The behavior of angiotensinamide on dialysis indicates that this peptide may assume at least two forms in aqueous solutions of low ionic strength. To simplify the following discussion the form that dialyzes more slowly will be designated by A and the faster dialyzing one will be called the B form. The presence of KCl favored the A form, and an increase in the salt concentration produced a slow conversion of the peptide from the B into the A form. Since angiotensin-

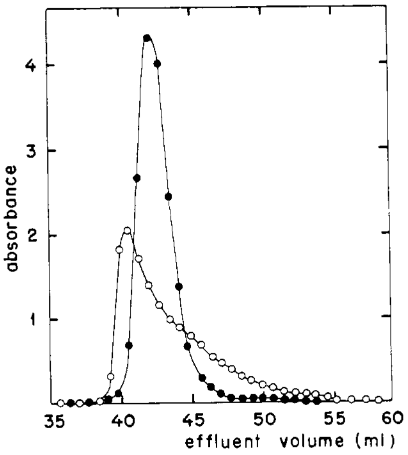

FIGURE 3: Chromatography of angiotensinamide on Sephadex G-25, monitored at 230 mμ: (●) 7.5 mg of peptide eluted with 0.015 M acetic acid–0.1 M in KCl, pH 3.2; (○) 8.8 mg of peptide, eluted with 0.015 M acetic acid, pH 3.2. The recovery of angiotensinamide in the eluates was 100 ± 5% in both chromatographies.

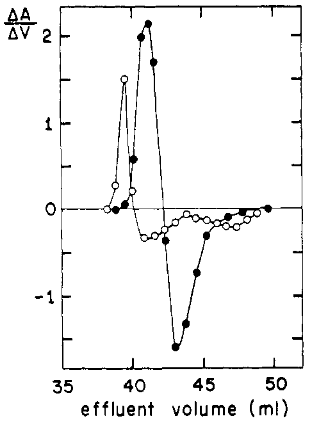

FIGURE 4: First derivative curves of the elution profiles of angiotensinamide on Sephadex G-25 shown in Figure 3. (●) Eluted with 0.015 M acetic acid–0.1 M in KCl and (○) eluted with 0.015 M acetic acid.

amide has been shown to be monomeric in the conditions of these studies (Paiva *et al.*, 1963; Paladini *et al.*, 1963), it may be concluded that two types of conformation may have some stability in aqueous solution. This was corroborated by the results of Sephadex chromatography in which the elution profiles of angiotensinamide, at low salt concentration, were characteristic of interconverting systems (Winzor and Scheraga, 1963). The effect of salt on the behavior of angiotensinamide toward Sephadex chromatography indicates that the species that was eluted first from the Sephadex column corresponds to the A form.

Craig and Ansevin (1963) have demonstrated that the rate of dialysis through cellophane membranes is a true measure of the effective diffusional volume, except where adsorption is found. If angiotensinamide is not an exception, we might conclude that the A form corresponds to a more expanded conformation and the B form to a more compact one. On the other hand, it is known that aromatic amino acids are adsorbed on cellophane, resulting in a faster rate of dialysis (Craig and Ansevin, 1963), and are retarded by adsorption on Sephadex columns (Porath, 1960; Gelotte, 1960). The presence of aromatic side chains in positions 4 and 8 of angiotensinamide makes it possible that the A form represent a conformation in which the aromatic residues are placed favorably for adsorption to the membrane and to Sephadex. In this case it is not possible to say which form of angiotensinamide is the more expanded one.

The favoring of form B by the increase of the salt concentration suggests that electrostatic interactions may play a role in stabilizing the corresponding conformation. However, Table I shows that the two forms of angiotensinamide are about equally present in a wide range of pH values, the proportion of fast component being significantly lower only at pH 2.1 and 11.0. Confrontation of this result with the titration curves of angiotensinamide (Paiva *et al.*, 1963) indicates that the protonated arginyl residue and the ionized C-terminal carboxyl are the only ionizable groups of that peptide whose electrostatic interaction might play a role in stabilizing the B conformation. This would not be enough to maintain the octapeptide in a stable compact conformation and the possibility of contributions from other types of interaction must be considered. The evidence at hand (Paiva and Paiva, 1962; Paiva *et al.*, 1963) points to the inexistence of hydrogen bonding in angiotensinamide in aqueous solutions. Hydrophobic bonding between side chains are possible, but by examination of molecular models it was not possible to postulate enough interactions to obtain a conformation that would be stable in aqueous solution. A rough calculation of the free energy of unfolding of these models, based on the parameters for hydrophobic bonds proposed by Nemethy and Scheraga (1962), and including the above-mentioned electrostatic interaction, yielded relatively, large negative values.

The addition of salt to an aqueous solution of angiotensinamide produced a very slow conversion from the B into the A conformation, while only the B form was evident in solutions prepared directly with 0.1 M KCl, even when the dialysis was performed immediately after the solution was made. This may be interpreted as an indication that the B species is closer to the conformation of the peptide in the solid phase, and that the activation energy for the observed conformational transition is unusually high. In view of these considerations, the model proposed by Warner (1961, 1964) for other peptides might be appropriate for the B conformation of angiotensinamide. In this model the oxygen atoms of the carbonyl groups belonging to the six first amino acid residues are placed in the corners of an hexagon with 4.8-Å side length. The electrostatic repulsion caused by the proximity between these oxygen atoms is a disrupting factor for this structure which might be counteracted by the presence of salt. Another feature of the Warner model is that four of the peptide bonds would be in a *cis* configuration. If the A form corresponds to a random conformation in which the peptide bonds are in a *trans* configuration, this model would explain the slow speed of the conversion from the A into the B form, involving the high-activation energy for rotation around the amide bond. Further evidence is necessary, however, before this model can be accepted with some confidence.

Although the results presented in this paper do not allow a definitive proposal concerning the types of conformation involved, they are evidence that two forms of angiotensinamide may be stable in aqueous solution. Craig *et al.* (1964) did not observe two components in their study of the dialysis behavior of angiotensinamide. We believe that the apparent discrepancy between their results and those reported here may be due to differences in membrane selectivity. There is reason to believe that our membranes differed from the ones employed by those authors, since we have observed an effect of pH on permeability. It seems probable that the selectivity toward the two forms of angiotensinamide was not simply related to the membrane's "pore size," but that other factors, such as adsorption and electrostatic interactions, may have played an important role in our experiments.

Fernandez *et al.* (1968) found that the half escape time of angiotensinamide did not vary significantly between pH 5 and 9, rising sharply on passing from pH 9.3 to 10.7. It is difficult to compare their results with ours in view of possible differences in membrane properties and lack of information about the ionic strength of their solutions, but it appears that the results presented in Table I are not in agreement with the findings of those authors. The conformational transition associated with the ionization of the phenolic group of the tyrosyl side chain, postulated by the same authors on the basis their observations, are also not supported by the previously reported titration studies of angiotensinamide (Paiva and Paiva, 1962; Paiva *et al.*, 1963).

Acknowledgments

We are grateful to Professors R. Schwyzer and B. Riniker, and to Ciba Ltd., Basel, for the generous gift of synthetic angiotensinamide.

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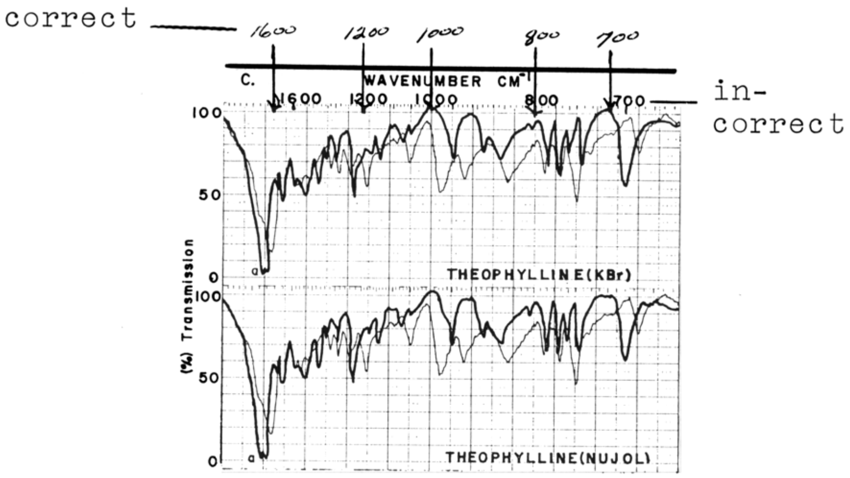
CORRECTIONS

In the paper "Interaction of Copper Ion with Guanosine and Related Compounds," by Anthony T. Tu and Christian G. Friederich, Volume 7, December 1968, p 4367, the C portion of Figure 4, which deals with the infrared spectra of theophylline, is incorrect; the wavenumber scale (cm^{-1}) should be corrected in the following manner.



In the paper "Minimization of Polypeptide Energy. IV. Further Studies of Gramicidin S," by F. A. Momany, G. Vanderkooi, R. W. Tuttle, and H. A. Scheraga, Volume 8, February 1969, p 744, the following addition should be noted: in the legend of Figure 1, the document number for the ASIS Publication Service is NAPS-00203.

In the paper "The Role of Sulfhydryl Groups in the Catalytic Function of Isocitrate Dehydrogenase. I. Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid)," by Roberta F. Colman, Volume 8, March 1969, p 888, the following change should be made.

In Table I (p 891) the line referring to the ninth rate constant should read

Substrates or coenzymes alone

8×10^{-5} M *threo*-D_s-isocitrate 38.3

note 38.3 instead of 8.33.

In the paper "Studies on the Mechanism of Inhibition of Glutamine Synthetase by Methionine Sulfoximine," by Robert