hydrophobic bonding and hydrogen bonding operate in a concerted manner is supported by the evidence presented herein. However it appears to be essential that the intramolecular hydrogen bonds be situated in a region of the molecule which is shielded from the solvent water molecules. The studies of ϵ-caprolactam association in dioxane, tetrahydrofuran, and 1,2-dimethoxyethane, Table II, and of *N*-methylacetamide association in carbon tetrachloride, dioxane, and water (Klotz and Franzen, 1962) demonstrate that in the presence of effective hydrogen acceptors or donors interamide hydrogen bonds are readily broken. Moreover, the presence of dipoles in the immediate vicinity of the interamide hydrogen bond can lower its stability both by competitive effects and by decreasing the electrostatic energy of the hydrogen bond. Hydrogen bonds existing within hydrophobic regions, on the other hand, would have considerable stability due to the absence of competing groups and polar surroundings. This, in fact, implies that interamide hydrogen bonds exist only in regions where shielding by hydrocarbon groups occurs.

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REFERENCES

Amaya, K. (1961), *Bull. Chem. Soc. Japan 34*, 1278.
Applequist, J., and Doty, P. (1962), *in* Polyamino Acids, Polypeptides, and Proteins, Stahman, M. A., ed., Madison, University of Wisconsin Press, pp. 161–176.
Arnett, E. M., and Wu, C. Y. (1962), *J. Am. Chem. Soc. 84*, 1684.
Bender, P. (1946), *J. Chem. Ed. 23*, 179.
Carpenter, G. B., and Donohue, J. (1950), *J. Am. Chem. Soc. 72*, 2315.
Corey, R. B. (1938), *J. Am. Chem. Soc., 60*, 1598.
Daniels, F., Mathews, J. H., Williams, J. W., Bender, P., and Alberty, A. (1956), Experimental Physical Chemistry, New York, McGraw-Hill, p. 205.
Davies, M., and Thomas, D. K. (1956), *J. Phys. Chem. 60*, 763.
Hermans, J., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82*, 5156.
Katz, J. L. (1957), *Dissertation Abstr. 17*, 2039.
Klotz, I. M. (1960), *Brookhaven Symp. Biol. 13*, 25.
Klotz, I. M., and Franzen, J. S. (1962), *J. Am. Chem. Soc. 84*, 3461.
Lord, R. C., and Porro, T. J. (1960), *Z. Elektrochem. 64*, 672.
Mizushima, S., Simanouti, T., Nagakura, S., Kuratani, K., Tsuboi, M., Baba, H., and Fujioka, O. (1950), *J. Am. Chem. Soc. 72*, 3491.
Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Pimentel, G. C., and McClellan, A. L. (1960), The Hydrogen Bond, San Francisco, Freeman.
Riddiford, L. M., and Scheraga, H. A. (1962), *Biochemistry 1*, 108.
Rosenheck, K., and Doty, P. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1775.
Schroeder, R., and Lippincott, E. R. (1957), *J. Phys. Chem. 61*, 921.
Tanford, C. (1962), *J. Am. Chem. Soc. 84*, 4240.
Wesson, L. G. (1948), Tables of Electric Dipole Moments, Boston, Massachusetts Institute of Technology Press, p. 14.

The Conformation of Angiotensin II in Aqueous Solution*

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Some physicochemical properties of angiotensin octapeptides were studied. The molecular weight of Asp(NH_2)^1-Val^5-angiotensin II (angiotensinamide) was determined in the *p*H range 2.5–8.6 using the Archibald method of approach to sedimentation equilibrium, and was found to be 1000 ± 100 in agreement with the theoretical value for the monomer. Direct and spectrophotometric titrations showed that the *pK* values and enthalpies of ionization of the carboxyl, amino, imidazole, and phenolic groups of angiotensinamide are normal. The optical rotatory dispersions of Val^5-angiotensin II and angiotensinamide were normal, and the b_0 values obtained from Moffitt plots were 38 and 32, respectively. The optical rotatory dispersion of angiotensinamide was not significantly affected by *p*H, temperature, or the presence of urea or nonpolar solvents. The kinetics of hydrogen-deuterium exchange of angiotensinamide were also studied and the first-order rate constants obtained were of the same order of magnitude and had similar *p*H-dependence as those for poly-D,L-alanine and simple peptides. It is concluded that Val^5-angiotensin II and angiotensinamide exist in a random conformation in aqueous solutions.

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The angiotensins are peptides produced by the action of the proteolytic enzyme renin on a protein substrate contained in the α_2-globulin fraction of mammalian blood plasma (Braun-Menendez *et al.*, 1946). They are the most potent vasopressor substances known and are characterized, among other pharmacological properties, by a powerful stimulating activity on smooth muscles (Braun-Menendez, 1956). The decapeptides Ileu^5-angiotensin I (horse) and Val^5-angiotensin I (ox), and the octapeptide Ileu^5-angiotensin II (horse) have been isolated from *in vitro* renin digests of plasma

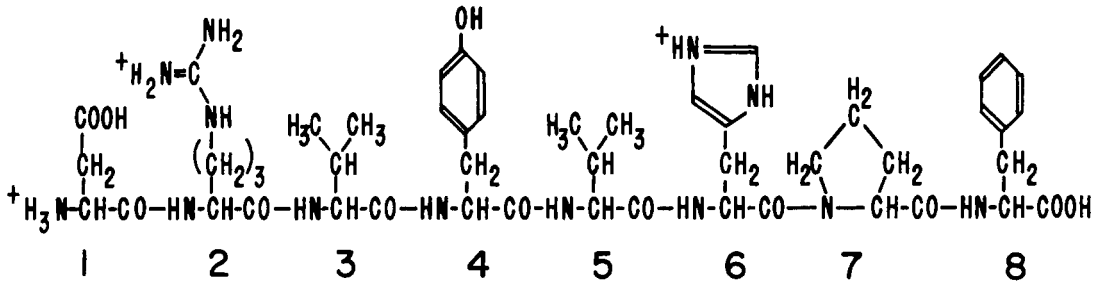

FIG. 1.—Valyl[5]-angiotensin II. All the amino acid residues of the L configuration. The state of ionization is that of maximum proton binding. In angiotensinamide the β-carboxyl of residue 1 is amidated.

globulins (Skeggs, 1960; Elliott, 1960). Although Val[5]-angiotensin II (Fig. 1) has not been isolated from such renin digests, it is probably the active ox angiotensin, since angiotensin I exerts its biological activity on being converted into angiotensin II by proteolytic enzymes contained in blood plasma (Skeggs *et al.*, 1956) and other tissues (Page and Bumpus, 1961). The synthesis of the four naturally occurring angiotensins, as well as that of several analogs, has permitted a thorough study of the relationships between the covalent structure of angiotensin II and its activity (Schwyzer and Turrian, 1960; Page and Bumpus, 1961; Paiva and Paiva, 1960b, 1961; Seu *et al.*, 1962). A relatively high degree of structural specificity has been observed, since most of the chemical modifications of the molecule resulted in a reduction of activity. Thus, the removal of either the C-terminal phenylalanine residue or the N-terminal aspartyl-arginyl portion of the molecule yields an inactive product. Also very important for activity are: (1) a free C-terminal carboxyl group; (2) the pyrrolidine ring of the proline in position 7 of the peptide chain; (3) the imidazole ring of the histidyl side chain in position 6; (4) the phenolic hydroxyl in position 4; (5) the guanidino group in the arginyl side chain in position 2. The N-terminal portion of the molecule, though not essential for activity, is of some importance, since significant loss of activity resulted from the blocking or removal of the N-terminal amino group (Deodhar, 1960; Arakawa *et al.*, 1962) or the removal of the aspartyl residue (Paiva and Paiva, 1960a; Schwyzer and Turrian, 1960).

Although the main object in studying the structure-activity relationships of angiotensin was to acquire insight on the nature of its receptor in the muscle cell, it is obvious that observations on the importance of the different groups of the molecule for biologic activity do not necessarily reflect their importance for the interaction with the receptor site. Even the isolated muscle preparations used for the activity assays are complex biological structures and different biodynamical steps might become rate limiting for the different angiotensin analogs studied. Furthermore, the possibility must be considered that some of the chemical groups of the angiotensin molecule might owe their importance for biological activity to a role in maintaining an active conformation of the molecule. It is also possible that the active species of angiotensin might be an aggregate of two or more molecules, and that some of the groups important for activity would be involved in bridging the associated monomers.

A study of the conformation and association of angiotensin in solution would be important for a better understanding of its mode of action and also, when compared to similar studies made with other myotropic peptides, might reveal structural similarities that are not apparent from an examination of the primary structures. This paper describes the results of such a study of synthetic angiotensin II, in which the possibility of association was investigated by molecular weight determinations in the ultracentrifuge, and the possibility of a stable conformation in aqueous solutions was examined through a study of optical rotatory properties, titration curves, and hydrogen-deuterium exchange behavior.

Because of the difficulty in obtaining large enough amounts of the naturally occurring angiotensin octapeptides, most of the experiments to be described were performed with synthetic angiotensinamide,[1] which is either as active (Schwyzer and Turrian, 1960) or almost as active (Paiva and Paiva, 1960a) as Val[5]-angiotensin II. Some of the optical rotatory dispersion experiments were also made with synthetic Val[5]-angiotensin II.

EXPERIMENTAL

Materials.—The synthetic angiotensin peptides used in these experiments were Val[5]-angiotensin II and angiotensinamide, kindly supplied by Dr. R. Schwyzer of Ciba Limited, Basel. These peptides had been purified by countercurrent distribution as free peptides, after the last stage of the synthesis, and the lyophilized products contained from one to three molecules of water and from one to two molecules of acetic acid per molecule of peptide. Before the titration experiments the acetic acid was removed by prolonged lyophilization from 0.1 M HCl. The *p*H of the solutions used for optical rotation and ultracentrifuge measurements was adjusted by addition of dilute HCl or KOH solutions. The concentration of the angiotensin solutions used in all the experiments was determined from the optical density of neutral solutions at 275 mμ, using the value 1379 for the molar extinction coefficient with 1-cm cells.

The standard buffers used for calibration of the *p*H-meter were 0.05 M sodium tetroxalate, 0.05 M potassium acid phthalate, 0.025 M phosphate, and 0.01 M sodium borate solutions prepared according to Bates (1954). Carbonate-free potassium hydroxide was prepared by the method of Kolthoff (1922) and standardized against potassium acid phthalate. The HCl solution was prepared from the azeotropic mixture and standardized against the KOH solution.

Synthetic poly-D,L-alanine, $n = 30$, was a gift from Dr. Joseph Kurtz of the Weizmann Institute, Rehovoth, Israel. All chemicals were reagent grade, and urea solutions were prepared fresh from recently recrystallized material.

Ultracentrifuge Measurements.—A Spinco Model E ultracentrifuge equipped with a phase plate and an RTIC temperature unit was employed. Angiotensinamide solutions were made in 0.1 M KCl and the desired

[1] In this paper the abbreviation angiotensinamide is used for Asp(NH$_2$)[1]-Val[5]-angiotensin II.

*p*H was obtained by addition of dilute HCl or KOH. All runs were made at 20.0°. The Archibald method of approach to sedimentation equilibrium (Schachman, 1959) was used for the determinations of molecular weight. The approach-to-equilibrium runs were made at 42,040 rpm in conventional 12-mm cells with 4° sector aluminum centerpieces. No silicone fluid was added to obtain an artificial bottom. The synthetic boundary runs were performed at approximately 7,000 rpm, with a Kel-F double-sector interference type synthetic-boundary centerpiece. Photographs were taken with schlieren phase plate angles near 70°, and five or more frames were recorded in each run. Plate measurements were made with a Gaertner two-dimensional microcomparator having a sensitivity of 0.001 mm. Since no artificial bottom was used, the molecular weights were calculated only from the meniscus measurements. The partial specific volume of angiotensinamide was estimated to be 0.734 from the amino acid composition and the apparent specific volumes of the amino acid residues (Cohn and Edsall, 1943).

Direct Titration.—The titration experiments were done on a Radiometer Model 4 *p*H meter with a G222B type glass electrode and a K100 saturated calomel electrode. The solutions were placed in a jacketed glass chamber equipped with a magnetic stirrer and shielded from electrostatic disturbances by a Faraday cage. The temperature desired inside the glass chamber was maintained to within ±0.05° by the circulation, through the jacket, of water from a thermostated bath. To assure temperature equilibration, the electrodes were kept at the proper temperature for 20–24 hours before use. Both before and after each titration the *p*H meter was calibrated with phosphate buffer and the *p*H of the tetroxalate, phthalate, and borax buffers was checked. A maximum variation of 0.01 *p*H unit during the time of titration was accepted. During the standardization of the *p*H meter and the titrations, the solutions were flushed by a stream of nitrogen that had passed through a column of soda lime and bubbled through water at the proper temperature.

Five-ml samples of 8–10 mM angiotensinamide in 0.1 M KCl solution were titrated, by addition of 0.999 N HCl, down to about *p*H 2, then of 0.995 N KOH up to about *p*H 12, followed by 0.999 N HCl again back to *p*H 2. The acid and base solutions were added from calibrated Agla syringes driven by Shardlow micrometers graduated in 0.01 mm, so as to change the *p*H by about 0.05 to 0.1 unit after each addition. Solvent blanks were titrated in exactly the same manner at each temperature, and the calculation of the experimental titration curve was done by using the apparent activity coefficients calculated from the blank titrations according to the equations:

$$p\mathrm{H} = -\log [\mathrm{H}^+] - \log \gamma'_{\mathrm{H}}$$

$$pK_w - p\mathrm{H} = p\mathrm{OH} = -\log [\mathrm{OH}^-] - \log \gamma'_{\mathrm{OH}}$$

The apparent activity coefficients, γ'_{H} and γ'_{OH}, include any errors due to the glass electrode response and liquid junction potential (Tanford, 1950; Bates, 1954).

Spectrophotometric Titrations.—Spectrophotometric titrations were performed by a point-by-point technique. To aliquots of angiotensinamide solution were added the appropriate amounts of 0.973 M KOH, 1 M KCl, and water to obtain 10^{-4} M solutions of angiotensinamide of 0.1 ionic strength and *p*H varying from 7 to 11.5. The *p*H of each solution was measured at 21.0° and 31.0°, and the differential absorbance at 2950 A was also measured at the same temperatures, using a neutral solution as a reference blank. A Beckman Model DU spectrophotometer with photomultiplier attachment and thermostated cell compartment was used for the absorbance measurements.

Optical Rotation.—Optical rotatory dispersion measurements were made on a Rudolph photoelectric polarimeter, Model 200, equipped with a Beckman DU quartz monochromator and an oscillating polarizer. The light sources were a General Electric AH-6 water-cooled high pressure mercury arc and a zirconium lamp. The symmetrical angle was always 5°. Most of the dispersions were obtained in the wavelength range 2967–6500 A using 0.5-dm jacketed polarimeter tubes with fused quartz windows and 7-mm bore. The temperature in the polarimeter tubes was maintained by the circulation, through the jacket, of water from baths at constant temperature. In some of the dispersion measurements the wavelength range was extended down to 2400 A by the use of more dilute solutions and of nonjacketed fused silica cells of 0.1-dm path-length. The average of the readings obtained with a blank solution of the whole wavelength range was subtracted from the readings obtained at each wavelength with the peptide solution. For each set of conditions at least two dispersion measurements were obtained using different solutions, and the average values were taken. The specific rotations were obtained with a reproducibility of ±2%. The *p*H of the angiotensin solutions used for the optical rotation measurements was adjusted by addition of dilute HCl or KOH.

The optical rotatory dispersion data were plotted according to a one-term Drude equation, $[\alpha] = k/(\lambda^2 - \lambda_c^2)$, and the parameters k and λ_c were derived from the intercept and slope, respectively, of the least-square straight line relating $[\alpha]\lambda^2$ to $[\alpha]$ (Yang and Doty, 1957).

The dispersion data were also plotted according to the Moffitt equation (Moffitt, 1956):

$$[m'] = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2$$

where $[m']$ is the reduced mean residue rotation. In most cases the value of λ_0 was taken to be 2120 A (Moffitt and Yang, 1956) and values for b_0 and a_0 were derived from the slope and intercept of the least-square straight line fitted to a plot of $[m'](\lambda^2 - \lambda_0^2)/\lambda_0^2$ versus $\lambda_0^2/(\lambda^2 - \lambda_0^2)$. For the dispersion of angiotensinamide at 20°, the most probable value for the parameter λ_0 to fit the experimental data was evaluated by the statistical method described by Sogami *et al.* (1963).

Deuterium-Hydrogen Exchange.—Angiotensinamide and poly-D,L-alanine solutions in citrate buffers of *p*H ranging from 2.2 to 4.0 were lyophilized for 24 hours over $CaCl_2$ and KOH pellets. The kinetics of deuterium-hydrogen exchange was followed by dissolving the lyophilized material in D_2O and observing the decrease in absorption at 1550 cm^{-1} in a Perkin-Elmer Model 21 double-beam spectrophotometer with a sodium prism. The amount of D_2O necessary to yield a 1% solution of the peptide in 0.1 M citrate was added from a syringe, and the same syringe was used for rapid mixing and transference of the solution to a 0.1-mm calcium fluoride cell with thermospacers. Solvent compensation was attained by placing, on the reference beam's path, a variable space CaF wedge cell (Connecticut Instrument Co.) containing 0.1 M citrate buffer in D_2O. The first reading was usually made 60 seconds after the addition of D_2O to the lyophilized peptide. Water at 25° ± 0.1° was circulated through the cell thermospacers.

Results

Ultracentrifuge Experiments.—Table I summarizes

TABLE I
ARCHIBALD MOLECULAR WEIGHT OF ANGIOTENSINAMIDE IN 0.1 M KCl

*p*H	Concentration (%)	Molecular Weight
2.5	0.99	900
3.5	0.42	1010
	0.80	870
	1.25	1000
6.3	0.61	1050
	0.95	1070
8.6	0.88	1140

the results of thirteen Archibald runs carried out with angiotensinamide solutions ranging in *p*H from 2.5 to 8.6 and in concentration from 0.42 to 1.25%. Within these limits no significant dependence of molecular weight on *p*H or concentration was observed, and the average value was found to be 1000 with a standard deviation of 100.

Titration.—The curve obtained for the direct titration of angiotensinamide between *p*H 2 and 12 at 25° is shown in Figure 2. Since the titration was reversible, with no time dependence in the *p*H of the solutions observed up to 1 hour, the titration data were interpreted according to the equation (Linderstrøm-Lang, 1924) $pH - \log x_i/(1 - x_i) = pK_i' - 0.868\, wZ$, where x_i represents the dissociated fraction of the *i*th group (in angiotensinamide there is only one group of each kind) at the given *p*H, pK_i' is the negative logarithm of the intrinsic dissociation constant at the ionic strength used, and w is an empirical parameter which corrects for any electrostatic interaction between the proton and the molecule of net charge Z.

TABLE II
APPARENT pK VALUES AND ENTHALPIES OF IONIZATION OF ANGIOTENSINAMIDE IN 0.1 M KCl[a]

Groups	pK' 10.0°	pK' 25.0°	pK' 35.0°	ΔH (kcal/mole)
α-Carboxyl	3.38	3.34	3.29	1.4
Imidazole	6.50	6.30	6.15	5.4
α-Amino	7.46	6.92	6.59	14.4
Phenolic	10.30	10.04	9.92	6.1

[a] Direct titration.

The titrations of the carboxyl and phenolic groups did not overlap those of the other groups. Assuming no binding of ions other than H^+, the pK values for these two groups were obtained by plotting $pH - \log [x_i/(1 - x_i)]$ vs. Z (Tanford, 1950) for the regions below *p*H 4.3 (carboxyl group) and above 9 (phenolic group). Figure 3 shows that in both cases straight lines parallel to the abscissae were obtained ($w = 0$) and, at 25°, pK values of 3.34 and 10.04 were found for the carboxyl and phenolic groups, respectively. The pK' value found for the phenolic group agreed with the previously reported one obtained from spectrophotometric titrations (Paiva and Paiva, 1962). By assuming that $w = 0$ for the whole titration curve,

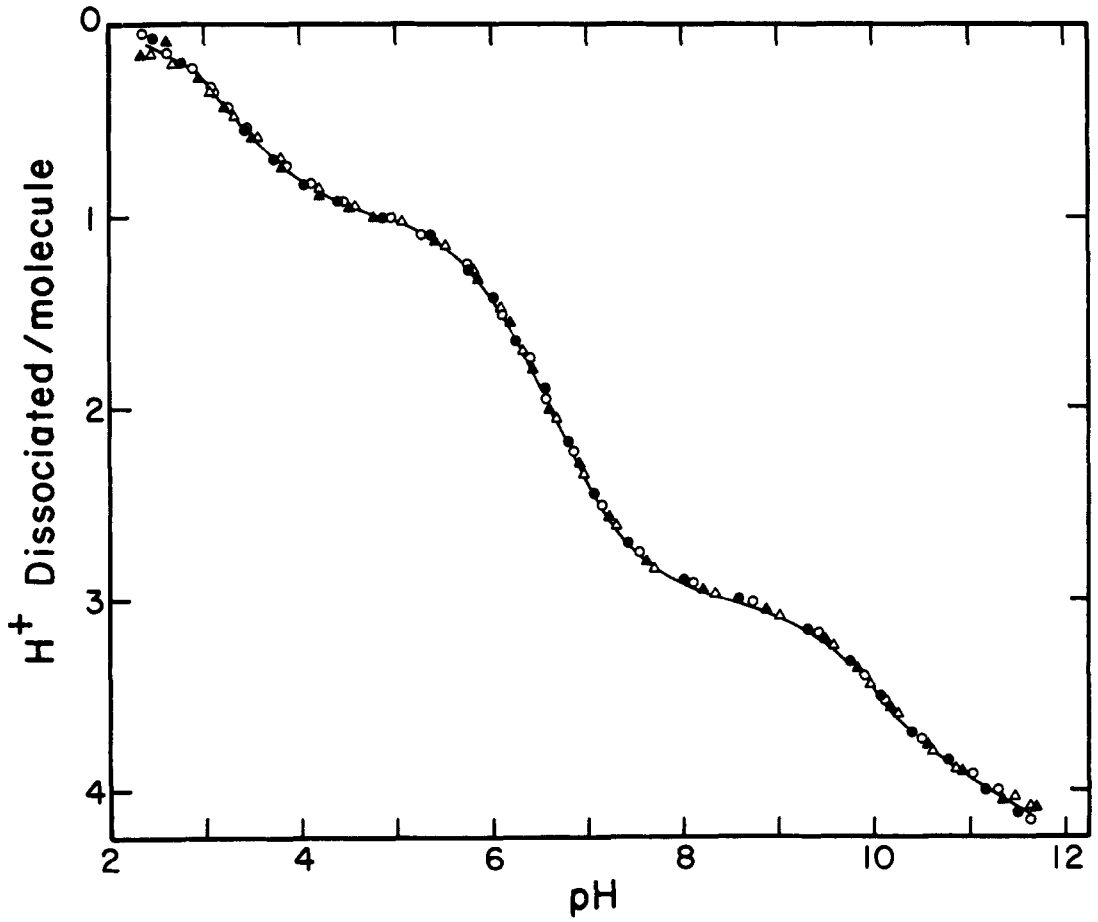

FIG. 2.—Titration curve of angiotensinamide in 0.1 M KCl at 25°. The circles and triangles denote two separate experiments. Open symbols represent titration with HCl and filled ones represent titration with KOH. The curve is a theoretical one calculated with the pK' values 3.34, 6.30, 6.92, 10.04, and 12.40.

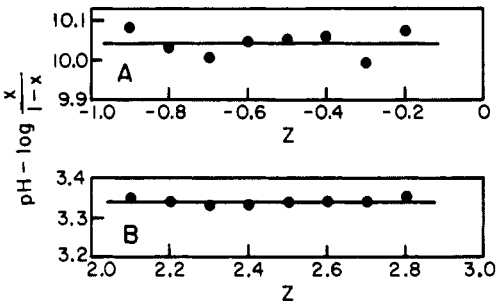

FIG. 3.—Plot of the titration data for the phenolic (A) and carboxyl (B) groups of angiotensinamide in 0.1 M KCl according to the equation $pH - \log [x/(1 - x)] = pK' - 0.868\ wZ$.

a theoretical curve was calculated that fitted the experimental data when the values 6.30, 6.92, and 12.40 were assigned to the pK' values of the imidazole, amino, and guanidino groups, respectively (Fig. 2). The data obtained at 10° and 35° were also treated in the same way, and the results are shown in Table II. The enthalpies of ionization, calculated from the linear plots of pK' vs. $1/T$, are also shown in Table II. The parameters for the guanidino group are not shown because, due to large errors in the measurements above pH 12, the titrations were not carried out beyond that pH, where only a fraction of the guanidino group is titrated.

The pK' and enthalpy of ionization of the phenolic group were also calculated from spectrophotometric titrations performed at 21° and 31° (Fig. 4). The pK' values obtained were 10.19 at 21° and 10.06 at 31°. The enthalpy of ionization was calculated using the equation $\Delta H = 2.303\ R\ [\Delta pH/\Delta(1/T)]$. This form of the vant'Hoff equation is valid when the difference in pH at the two temperatures, ΔpH, is taken at the same degree of ionization, calculated as the ratio of the optical density at that pH to the optical density at pH 12.5. The results of such calculations (Table III) confirm those obtained by direct titrations.

Optical Rotatory Dispersion.—The optical rotatory dispersion of aqueous solutions of angiotensinamide at 20° obeyed a one-term Drude equation with $\lambda_c = 203$, $k = -2.12 \times 10^9$ and a specific rotation of −1250 at 2420 A. A typical plot of $[\alpha]\lambda^2$ vs. $[\alpha]$ between 2420 A and 6500 A is shown in Figure 5. When the data were plotted according to the Moffitt equation, assuming $\lambda_0 = 2120$ A, a linear relationship was also obtained, with $b_0 = 32$ and $a_0 = -463$. However, when the parameter λ_0 was allowed to vary between 203 and 230 mμ (Sogami *et al.*, 1963), the best fit to the Moffitt equation was obtained when $\lambda_0 = 218$ mμ. Figure 6 shows the Moffitt plot for angiotensinamide in the range 2420–6500 A using the λ_0 value of 2180 A. From this plot, the values 38 and −430 were obtained for b_0 and a_0, respectively.

TABLE III
ENTHALPY OF PHENOLIC IONIZATION FROM SPECTROPHOTOMETRIC TITRATION

Per Cent Ionization	pH 21°	pH 31°	ΔH (kcal/mole)
30	9.85	9.67	7.5
40	10.03	9.89	5.8
50	10.19	10.06	5.4
60	10.35	10.22	5.4
70	10.52	10.38	5.8
Average:			6.0

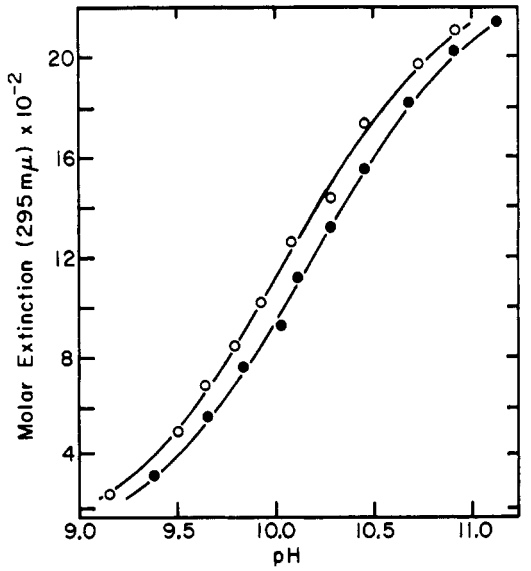

FIG. 4.—Spectrophotometric titration curves of angiotensinamide in 0.1 M KCl at 21° (filled circles) and 31° (open circles). The curves are theoretical ones assuming $w = 0$ and pK values 10.19 (21°) and 10.06 (31°).

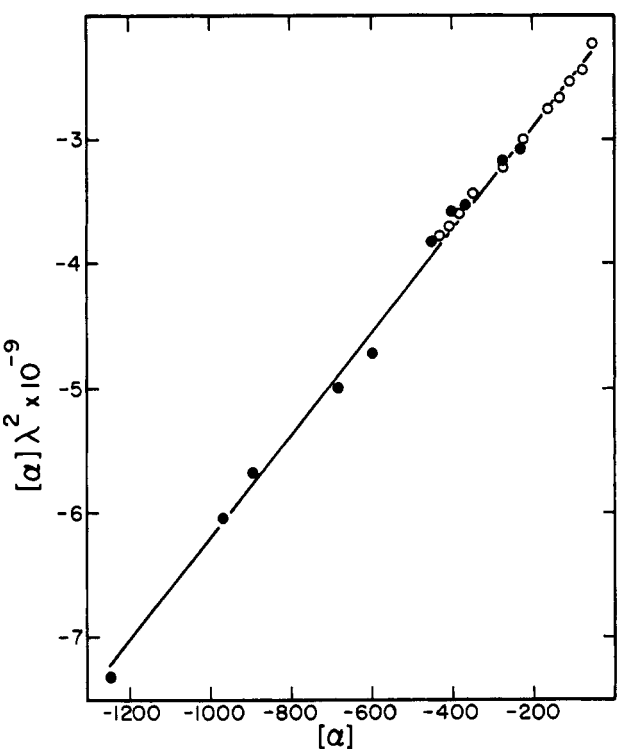

FIG. 5.—A modified Drude plot of the optical rotatory dispersion of angiotensinamide at pH 6.0, 20°. Open circles, $c = 0.534\%$, filled circles, $c = 0.040\%$. λ in angstroms.

Val⁵-angiotensin II also had a simple dispersion, with $\lambda_c = 202$, $k = -1.78 \times 10^9$, and a specific rotation of −1050 at 2420 A; when the dispersion was plotted according to the Moffitt equation, assuming $\lambda_0 = 2120$ A, values of 38 and −426 were found for b_0 and a_0, respectively. The Drude plot for Val⁵-angiotensin II, as well as for angiotensinamide, was independent of concentration in over a 10-fold range of concentration.

The optical rotatory dispersion of angiotensinamide was not dependent on temperature, between 10° and 80°, or on pH, between 2.5 and 9.7 (Table IV). The dispersion was also not significantly influenced by the

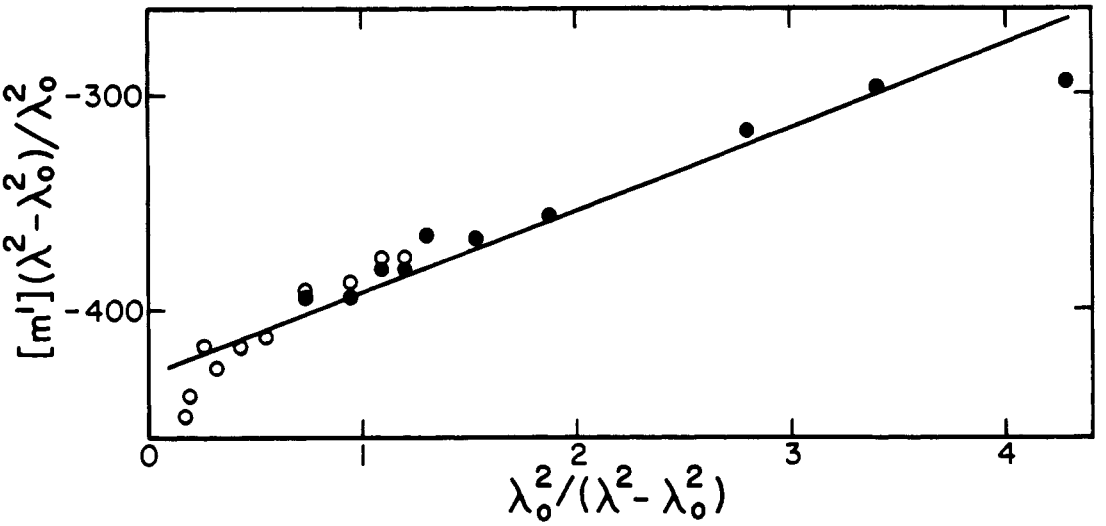

FIG. 6.—A Moffitt plot of the rotatory dispersion of angiotensinamide at *p*H 6.0, 20°. Open circles represent averages from five dispersion measurements between 2967 A and 6500 A using solutions varying in concentration from 0.425% to 0.727%. Filled circles are averages of two experiments between 2420 A and 3342 A with 0.040% and 0.056% solutions. λ in angstroms, $[m']$ in degrees cm²/decimole; λ_0 = 2180 A.

presence of 5 M urea or high concentrations of "helix-forming" solvents. Because of the insolubility of angiotensinamide in the common nonpolar solvents, th only solvents that were used, besides water, were 70% dioxane-water and dichloroethane containing 13% acetic acid. Table IV shows that the dispersion was normal also in these solvents.

Hydrogen-Deuterium Exchange.—A comparison was made between the kinetics of H-D exchange of angiotensinamide and of poly-D,L-alanine. Very similar results were obtained with both peptides. In both cases the recorded absorbancy at 1550 cm^{-1} rapidly decreased after dissolution of the peptide in D_2O, and approached asymptotically a constant value A_∞ within 10–15 minutes. When log $(A_t - A_\infty)$ was plotted against time, linear plots were obtained up to about 80% conversion (Fig. 7). From the slope of such plots, the first-order rate constants were calculated and are shown in Table V. Our results with poly-D,L-alanine were in good agreement with those of Bryan and Nielsen (1960) not only in the magnitude of the rate constants, but also in its *p*D dependence, with a minimum at *p*D 3.6. The first-order rate constants found for angiotensinamide were of the same order of

TABLE IV
EFFECT OF *p*H, TEMPERATURE, SOLVENT, AND UREA ON THE OPTICAL ROTATORY DISPERSION OF ANGIOTENSINAMIDE

Solvent	*p*H	Temperature	λ_c (mμ)	$k \times 10^{-9}$	b_0	a_0
Water	6.0	10.0	208	−1.95	45	−425
		20.0	203	−2.12	32	−463
		40.0	206	−1.98	43	−482
		80.0	202	−2.08	37	−451
	2.5	10.0	200	−2.15	50	−430
		20.0	196	−2.17	77	−480
		40.0	198	−2.15	59	−473
		60.0	189	−2.09	43	−458
	8.5	20.0	202	−2.16	48	−497
5 M Urea	2.0	20.0	196	−2.25	49	−503
	6.6	20.0	199	−2.33	78	−496
70% Dioxane		20.0	203	−2.06	37	−372
DCE-AA[a]		20.0	210	−2.48	57	−552

[a] Acetic acid (13%) in dichloroethane.

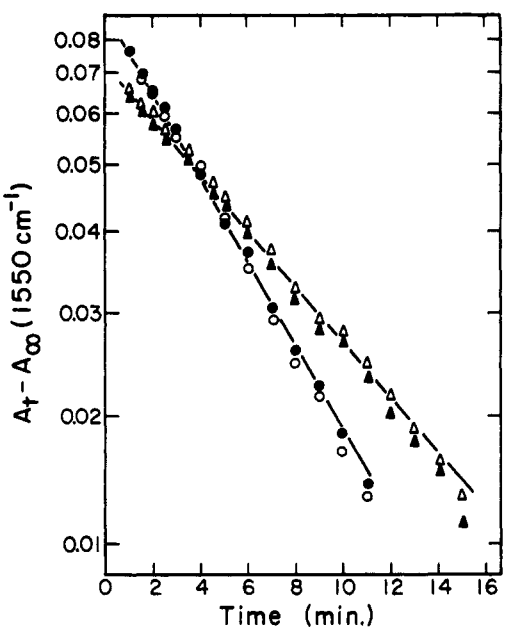

FIG. 7.—Semilog plot of the decrease in absorbancy at 1550 cm^{-1} during hydrogen-deuterium exchange of angiotensinamide at *p*D 3.14 (triangles) and poly-D,L-alanine at *p*D 3.60 (circles). Filled and empty symbols represent separate experiments. 1% peptide solutions in 0.1 M citrate buffer were used.

TABLE V
APPARENT FIRST-ORDER RATE CONSTANTS FOR THE H-D EXCHANGE OF ANGIOTENSINAMIDE AND POLY-D,L-ALANINE AT 25°

Angiotensinamide		Poly-D,L-alanine	
*p*D	k (min^{-1})	*p*D	k (min^{-1})
2.66	0.19	2.91	0.33
2.92	0.14	3.14	0.28
3.14	0.10	3.39	0.26
3.42	0.13	3.60	0.16
3.64	0.16	3.81	0.18
3.92	0.39	4.04	0.25
		4.26	0.39

magnitude as those found for poly-D,L-alanine, but the minimum rate was observed at pD 3.14.

Discussion

Our determinations of the molecular weight of angiotensinamide in aqueous solution indicated that it is a monomer, with no signs of association between pH 2.5 and 8.6, and at concentrations up to 1.25%. The value of 1000 with a standard deviation of 100, found with the Archibald method, is in agreement with the value of 1032 from the amino acid composition. This indicates that in the very low concentrations in which angiotensin exerts its physiological actions the monomer is probably the species that interacts with the cellular receptor.

Knowing that angiotensinamide in aqueous solution is monomeric, the question of whether the molecule exists in a random or in a folded conformation is an important one for the study of activity-structure relationships. Thus, although so many of the chemical groups of angiotensinamide are important for its biological activities, it is conceivable that only two or three of those groups are directly involved in the drug-receptor interaction, while the importance of the other groups would be in maintaining the molecule in an active conformation through side-chain interactions. The titration experiments were made in order to find whether any of the charged groups of angiotensinamide are involved in such side-chain interactions. Of these charged groups, the carboxyl, imidazole, phenolic, and guanidino groups are very important for activity, and the amino group is of less importance, though still necessary for full biological activity. With the exception of the guanidino group, which was not titrated in our experiments, all other groups had normal pK values and enthalpies of ionization, indicating that these groups are "exposed" and not involved in side-chain interactions. The pK' values around 3.3, found for the carboxyl group, are low when compared with those for normal side-chain carboxyls in proteins, but they are within the normal range for C-terminal carboxyl groups in peptides (Ellenbogen, 1952). The experimental titration data could be fitted with a theoretical curve without correction for electrostatic interactions ($w = 0$).

To obtain further information on the conformation of angiotensin octapeptides in solution, optical rotatory dispersion studies were perfomed on two peptides, Val^5-angiotensin II and angiotensinamide. The dispersion of the optical rotation of aqueous solutions of both peptides was found to be simple, following a one-term Drude equation. The reduced mean residue rotations of Val^5-angiotensin II and angiotensinamide at 2420 A were -1030 and -1278, respectively. These values are much smaller than what would be expected if the peptides were in an α-helical conformation. According to Woody's (1962) calculations, the mean residue rotation of α-helical octapeptides should be around $-10{,}000$ at the minimum that occurs in the optical rotatory dispersion around 2330 A. Experimentally, random-coiled polypeptides have mean residue rotation values around $-2{,}000$ while the helical forms are associated with values around $-15{,}000$ at about 2400 A (Blout *et al.*, 1962; Holzwarth *et al.*, 1962; Jirgensons, 1962). The dispersion of angiotensinamide was normal even in the presence of high proportions of low-hydrogen-bonding solvents such as dioxane and dichloroethane. Unfortunately, the low solubility of angiotensinamide in these solvents did not permit a study of solutions in pure nonpolar solvents. The dispersion data for angiotensinamide were not significantly altered in the presence of 5 M urea or by varying the temperature between 10° and 80° or the pH between 2.5 and 8.5. This indicates that no conformational transitions take place within those limits of pH and temperature.

When the dispersion data were plotted according to the Moffitt equation, b_0 values ranging from 29 to 78 were observed. These small positive values of b_0 probably have no significance regarding helical content, since polypeptides known to be in random conformation frequently give nonzero b_0 values up to ± 100 (Urnes and Doty, 1961). According to Woody's (1962) calculations, b_0 values for α-helical octapeptides should be around ± 400.

Our results are in contrast with those of Smeby *et al.* (1962), who proposed a helical conformation for Ileu^5-angiotensin II, based on optical rotatory studies. These authors proposed a conformation in which there are three hydrogen bonds between the first and fifth, the second and sixth, and the third and eighth residues of the angiotensin octapeptide. A perfect α-helix, with bonds between the third and seventh and fourth and eighth residues, would not be possible because of the presence of a prolyl residue in position 7 of the chain. The main support for the above model of angiotensin was the finding of a b_0 of -133 (with $\lambda_0 = 210$ mμ), which was changed to $+40$ in the presence of 0.8 M urea (Smeby *et al.*, 1962). However, the significance of their relatively high negative value for b_0 may be open to question, since the same authors found a b_0 of $+204$ for the tetrapeptide L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, obviously not capable of forming a helix. We also have not observed an effect of urea on the optical rotatory dispersion of angiotensinamide, even when 5 M urea was used. The examination of molecular models does not suggest that the discrepancy between our results and those of Smeby *et al.* (1962) may be due to the differences between the covalent structures of Ileu^5-angiotensin II and Val^5-angiotensin II.

More evidence against the helical model for angiotensinamide was obtained in the hydrogen-deuterium exchange experiments. Apparently, all the hydrogens exchanged at the same rate, and the first-order rate constants were of the same order of magnitude as those for simple amides and peptides (Nielsen *et al.*, 1960) and of random coiled poly-D,L-alanine (Bryan and Nielsen, 1960). A slower rate of exchange would be expected for half the NH groups of angiotensinamide if three intrachain hydrogen bonds were present, as proposed by Smeby *et al.* (1962).

The helical conformation of the angiotensin octapeptides is also not favored by thermodynamic considerations, unless it is stabilized by other interactions besides the intra-chain hydrogen bonds. Examination of the helical model proposed by Smeby *et al.* (1962) suggests that the only possible stabilizing interactions between the side chains would be a tyrosyl–carboxylate hydrogen bond, or hydrophobic bonds between the tyrosyl and phenylalanine side chains, or between the valyl residue in position 3 and the phenylalanine side chain. The tyrosyl–carboxylate hydrogen bond can be ruled out in view of the normal behavior of the carboxyl and phenolic groups on titration. On the other hand, examination of molecular models shows that the two possible hydrophobic bonds mentioned above can only be of minimal strength (Nemethy and Scheraga, 1962). A rough estimate of the free energy of unfolding of the helical angiotensin model, including the stabilization from the hydrophobic interactions, was made by using the equation $\Delta F^0_{unf} = 3\,\Delta H^0_{res} - 7\,T\,\Delta S^0_{res} + \Delta F^0_{H\phi}$ (Scheraga, 1961; Scheraga *et al.*,

1962), using the values for the enthalpy (ΔH^0_{res}) and entropy (ΔS^0_{res}) of unfolding proposed by Schellman (1955) and the parameters calculated by Nemethy and Scheraga (1962) for the free energy of breaking hydrophobic bonds ($\Delta F^0_{H\phi}$). Even assuming that the hydrophobic bonds between phenylalanine and tyrosyl and valyl side chains were of maximum strength, a negative free energy of unfolding of about 2 kcal at 25° is found. Therefore, such a helical structure would be unstable.

We conclude that angiotensin octapeptides in aqueous solutions are predominantly in a random conformation, and that a predetermined ordered spatial arrangement of the molecule is not necessary for biologic activity. Whether or not the molecule is stabilized in a definite conformation by interaction with its cellular receptor is a question that cannot be answered until further investigation on the characterization and isolation of the receptor is carried out.

References

Arakawa, K., Smeby, R. R., and Bumpus, F. M. (1962), *J. Am. Chem. Soc. 84*, 1424.

Bates, R. G. (1954), Electrometric *p*H Determinations, New York, Wiley, p. 118.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Braun-Menendez, E. (1956), *Pharmacol. Rev. 8*, 25.

Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Munoz, J. M., and Taquini, A. C. (1946), Renal Hypertension, Springfield, Charles C. Thomas.

Bryan, W. P., and Nielsen, S. O. (1960), *Biochim. Biophys. Acta 42*, 552.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides, New York, Reinhold.

Deodhar, S. D. (1960), *J. Exp. Med. 111*, 419.

Ellenbogen, E. (1952), *J. Am. Chem. Soc. 74*, 5198.

Elliott, D. F. (1960), *in* Polypeptides Which Affect Smooth Muscles and Blood Vessels, Schacter, M., ed., New York, Pergamon, p. 99.

Holzwarth, G., Gratzer, W. B., and Doty, P. (1962), *J. Am. Chem. Soc. 84*, 3194.

Jirgensons, B. (1962), *Biochemistry 1*, 917.

Kolthoff, I. M. (1922), *Z. Anal. Chem. 61*, 48.

Linderstrøm-Lang, K. (1924), *Compt. Rend. Trav. Lab. Carlsberg 15*, no. 7.

Moffitt, W. (1956), *J. Chem. Phys. 25*, 467.

Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 596.

Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.

Nielsen, S. O., Bryan, W. P., and Mikkelsen, K. (1960), *Biochim. Biophys. Acta 42*, 550.

Page, I. H., and Bumpus, F. M. (1961), *Physiol. Rev. 41*, 331.

Paiva, A. C. M., and Paiva, T. B. (1960a), *Biochem. Pharmacol. 5*, 187.

Paiva, T. B., and Paiva, A. C. M. (1960b), *Brit. J. Pharmacol. 15*, 557.

Paiva, A. C. M., and Paiva, T. B. (1961), *Biochim. Biophys. Acta 48*, 412.

Paiva, A. C. M., and Paiva, T. B. (1962), *Biochim. Biophys. Acta 56*, 339.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, Academic, p. 182.

Schellman, J. A. (1955), *Compt. Rend. Trav. Lab. Carlsberg (Ser. Chim.) 29*, 223, 230.

Scheraga, H. A. (1961), Protein Structure, New York, Academic, p. 81.

Scheraga, H. A., Nemethy, G., and Steinberg, I. Z. (1962), *J. Biol. Chem. 237*, 2506.

Schwyzer, R., and Turrian, H. (1960), *Vitamins Hormones, 18*, 237.

Seu, J. H., Smeby, R. R., and Bumpus, F. M. (1962), *J. Am. Chem. Soc. 84*, 3883.

Skeggs, L. T. (1960), *in* Polypeptides Which Affect Smooth Muscles and Blood Vessels, Schacter, M., ed., New York, Pergamon, p. 106.

Skeggs, L. T., Kahn, J. R., and Shumway, N. P. (1956), *J. Exp. Med. 103*, 295.

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

Sogami, M. W., Leonard, W. J., Jr., and Foster, J. F. (1963), *Arch. Biochem. Biophys. 100*, 260.

Tanford, C. (1950), *J. Am. Chem. Soc. 72*, 441.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Woody, R. W. (1962), Ph.D. dissertation, University of California, Berkeley.

Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.