- Levitzki, A., and Koshland, D. E., Jr. (1972), Biochemistry 11, 247.
- Main, A. R. (1969), J. Biol. Chem. 244, 829.
- Main, A. R., Tarkan, E., Aull, J. L., and Soucie, W. G. (1972), J. Biol. Chem. 247, 566.
- Mandelkern, L., and Flory, P. J. (1952), J. Chem. Phys. 20, 212.
- Noelken, M. E., and Timasheff, S. N. (1967), J. Biol. Chem. 242, 5080.
- Oki, Y., Oliver, W. T., and Funnell, H. S. (1965), Can. J. Physiol. Pharmacol. 43, 147.
- Reiner, E., Seyferth, W., and Hardegg, W. (1965), Nature (London) 205, 1110.
- Sanger, F. (1963), Proc. Chem. Soc. London, 76.
- Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
- Schachman, H. K. (1963), Cold Spring Harbor Symp. Quant. Biol. 28, 409.
- Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry* 5, 2681.

- Scheraga, H. A., and Mandelkern, L. (1953), J. Amer. Chem. Soc. 89, 179.
- Scott, E. M., and Powers, R. F. (1972), Nature (London), New Biol. 236, 83.
- Tanford, C. (1955), J. Phys. Chem. 59, 798.
- Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.
- Tanford, C., Kawahara, K., and Lapanje, S. (1967), J. Amer. Chem. Soc. 89, 729.
- Tomimatsu, Y., and Palmer, K. J. (1963), J. Phys. Chem. 67, 1720.
- Tucci, A. F., and Seifter, S. (1969), J. Biol. Chem. 244, 841.
- Van Holde, K. E. (1967), Fractions, 1, Palo Alto, Calif., Spinco Division of Beckman Instruments, Inc.
- Van Holde, K. E., and Baldwin, R. L. (1958), J. Phys. Chem. 62, 734.
- Waddell, W. J. (1956), J. Lab. Clin. Med. 48, 311.
- Yang, J. T. (1961), Advan. Protein Chem. 16, 323.
- Yphantis, D. A. (1964), *Biochemistry* 3, 297.
- Zech, R., and Engelhard, H. (1965), Biochem. Z. 343, 86.

Synthesis, Biological Activity, and ¹⁹F Nuclear Magnetic Resonance Spectra of Angiotensin II Analogs Containing Fluorine[†]

William H. Vine,[‡] David A. Brueckner,[§] Philip Needleman, [¶] and Garland R. Marshall^{*,}

ABSTRACT: [Asp¹,*p*-fluoro-L-phenylalanine⁴]-angiotensin II ([pFPhe⁴]-AII) and [Asp¹,*p*-fluoro-L-phenylalanine⁸]-angiotensin II ([pFPhe⁸]-AII) were synthesized by the solid-phase procedure in order to study their biological activity and ¹⁹F nuclear magnetic resonance (nmr) spectra. *p*-Fluoro-D,Lphenylalanine was resolved by enzymatic hydrolysis of *N*trifluoroacetyl-*p*-fluoro-D,L-phenylalanine with carboxypeptidase A. [pFPhe⁸]-AII is at least as potent as angiotensin II and [pFPhe⁴]-AII is an antagonist of angiotensin II in the

Т

Le replacement of hydrogen by fluorine has become a useful tool for pharmacological and structural analysis of organic compounds. The similarity of van der Waals' radii rat oxytocic, the rat blood pressure, and the prostaglandin release assay. The ¹⁹F nmr spectra of [pFPhe⁸]-AII show that the C-terminal carboxyl has a pK_a of 3.1, that above pH 7 two conformations exist, and that the rate of exchange between these two conformations is slow on the nmr time scale. A conformational change with a pK of 6.1 is the most likely cause of the chemical shift change seen in the ¹⁹F nmr spectra of [pFPhe⁴]-AII.

(H = 1.20 Å; F = 1.35 Å) and the dissimilarity of electronegativity (H = 2.1; F = 4.0) (Sheppard and Sharts, 1969) suggest that analogs with fluorine substituted for hydrogen might have interesting pharmacological activities. Fluorine replacement of hydrogen in corticosteroids, in pyrimidines (Goodman and Gilman, 1970), and in amino acids results in compounds with significantly altered properties. Fluorine amino acids can be either toxic or support the growth of bacterial auxotrophs (Loncrini, 1969). In particular pFPhe¹ is incorporated into *Escherichia coli* proteins and is bacterio-

[†] From the Departments of Physiology and Biophysics, of Biological Chemistry and of Pharmacology, Washington University Medical School, St. Louis, Missouri 63110. *Received October 31, 1972*. Supported by a grant-in-aid (AHA-70-672) from the American Heart Association and grants from the National Institutes of Health (HE 14397-02 and HE 14059-02). A preliminary report of this work was delivered at the 164th National Meeting of the American Chemical Society, New York, N. Y., Sept 1972.

[‡] Graduate Fellow, Medical Scientists training program (5-T05-GMO-2016-04).

[§] Present address: Institute for Psychosomatic and Psychiatric Research and Training, Michael Reese Hospital, Chicago, Ill. 60616.

[¶] Established Investigator, American Heart Association (AHA-68-115).

^{||} Established Investigator, American Heart Association (AHA-70-111).

¹ Abbreviations used are: pFPhe, *p*-fluoro-L-phenylalanine; [pFPhe⁴]-AII, [Asp¹,pFPhe⁴]-angiotensin II; [pFPhe⁸]-AII, [Asp¹,pFPhe⁸]angiotensin II; AII, [Asn¹,Val⁵]-angiotensin II; A, 1-butanol-acetic acid-water (4:1:1); B, 2% acetic acid-acetone; C, 1-butanol-pyridineacetic acid-water (15:10:3:2); D, 1-propanol-water (2:1); *t*-Boc, *tert*-butyloxycarbonyl.

static, even though the alkaline phosphatase from such strains retains normal activity (Richmond, 1963).

In addition to the interesting pharmacological activities of fluorine compounds ¹⁹F nuclear magnetic resonance (nmr) is superior in several aspects to ¹H nmr for conformational analysis. The ¹⁹F chemical shift spans a much wider range than ¹H and when ¹⁹F is substituted for ¹H in large molecules, it eliminates the difficult task of individual resonance assignment. The increased sensitivity of ¹⁹F to its environment has led to its use in the study of intramolecular conformational preferences of ethanes (Newmark and Sederholm, 1965), cyclohexanes (Cantacuzene and Jantzen, 1970), and glucopyranoses (Phillips and Wray, 1971) and the study of the intermolecular interactions due to hydrogen bonding of alcohols (Middleton and Lindsey, 1964) and phenols (Gurka and Taft, 1969). The study of the binding of N-acetyl-p-fluoro-D,-L-phenylalanine to α -chymotrypsin (Gammon et al., 1972) and the study of the interaction of trifluoroacetylated RNase S-peptide with RNase S-protein and of various inhibitors to the S-peptide-S-protein complex (Huestes and Raftery, 1971) exemplify the advantages of increased environmental sensitivity and of simplicity gained by the use of ¹⁹F nmr in the study of peptides and proteins.

To take full advantage of the similarity of van der Waals' radii of ¹⁹F and ¹H, it was decided to incorporate ¹⁹F into All analogs by solid-phase peptide synthesis (Marshall and Merrifield, 1972) using the appropriate fluorine-containing amino acid. For this study pFPhe was chosen since it has been incorporated into several peptide hormone analogs which have high biological activities. [pFPhe5]-bradykinin, in which pFPhe is substituted for phenylalanine, has 170 and 150% the activity of bradykinin in the bronchoconstriction and vasodilation assays, respectively (Nicolaides et al., 1963). Likewise, [pFPhe4]-gastrin tetrapeptide is fully active (Morely, 1968). The substitution of pFPhe for tyrosine in physalae- \min_{6-11} yields a peptide with approximately 100, 675, and 220% physalaemin's activity in the hypotensive, large intestine, and ileum assays, respectively (Bernardi et al., 1966). However, [pFPhe⁴]-angiotensin₃₋₈ was reported to be inactive (Bumpus and Smeby, 1968). Because fluorine analogs of biologically active compounds possess such diverse activities and because ¹⁹F nmr offers advantages over ¹H nmr, we undertook the synthesis of [pFPhe8]-AII and [pFPhe4]-AII in order to study their biological activity and ¹⁹F nmr spectra.

Experimental Section

Materials. AII was the generous gift of Ciba-Geigy, Inc. (Basle, Switzerland). Carboxypeptidase A was obtained from Sigma Chemical Co. (St. Louis, Mo.). Dimethylformamide was purified by distillation from KOH and benzene as suggested by Stuart and Young (1969). t-Boc-Phe-2% divinylbenzene-polystyrene resin was synthesized by the procedure of Marshall and Merrifield (1965).

Analytical Methods. Thin-layer chromatography was performed on 0.25-mm silica gel G plates obtained from Analtech, Inc., in 1-butanol-acetic acid-water (4:1:1), in 2% acetic acid-acetone, in 1-butanol-pyridine-acetic acid-water (15:10:3:12), or in propanol-H₂O (2:1) and developed by ninhydrin and Clorox-starch sprays (Nitecki and Goodman, 1966). Peptides were hydrolyzed in 12 N HCl-propionic acid (1:1) (Scotchler *et al.*, 1970) or 6 N HCl both containing 2% anisole for 2 or 24 hr, respectively. Amino acid analysis were performed on a Spinco 120C analyzer. pFPhe elutes after phenylalanine and has the same color constant. Optical rotations were determined on a Cary Model 60 spectropolarimeter. Melting points were determined on a capillary melting point apparatus and are uncorrected. Elemental analysis was obtained from PCR, Inc., Gainsville, Fla.

Nuclear Magnetic Resonance. Nuclear magnetic resonance spectra were obtained using 5-KHz side-band oscillation at 56.4 MHz on a Varian Associates T-60 and were referenced and locked to a saturated solution of tetrafluorodibromobenzene in benzene which had a chemical shift of approximately -958 Hz relative to a 1% solution of tetrafluorotetrachlorocyclobutane in benzene. Probe temperature was 35.5°. Timeaveraged spectra were obtained by means of an interface to a Digital Equipment Corp. PDP-12 computer. Peptides were dissolved in H₂O to give approximately 4.5% solutions and where necessary solutions were centrifuged in order to remove peptide precipitated between pH 6.0 and 9.3. pFPhe was dissolved in H₂O at a concentration of 1.3%. pH was raised by addition of NaOH and lowered by addition of HCl and measured on a Radiometer PHM 4b at room temperature.

N-*Trifluoroacetyl*-*p*-*fluoro*-D,L-*phenylalanine* (1). The method of Weygand and Geiger (1956) was adapted for the preparation of *N*-trifluoroacetyl-*p*-fluoro-D,L-phenylalanine. Racemic *p*-fluorophenylalanine (32.5 g) was dissolved in 160 ml of trifluoroacetic acid and cooled to 12° . Trifluoroacetic anhydride (67 g) was added over 10 min with stirring and allowed to react 1 hr at 10° . Distillation *in vacuo* of the solvent and excess reagent gave a white crystalline product which was recrystallized from benzene–hexane to give 37.2 g (75% yield) of *N*-trifluoroacetyl-*p*-fluoro-D,L-phenylalanine (mp 144–145°).

Anal. Calcd for $C_{11}H_8F_4NO_8$: C, 47.32, H, 3.25; F, 27.22; N, 5.02. Found: C, 46.73; H, 3.04; F, 26.46; N, 5.10.

p-Fluoro-L-phenylalanine (11). Compound I (22.2 g) was dissolved in 800 ml of water and the pH was adjusted to pH 7 carefully (to avoid alkaline hydrolysis) with 2 N LiOH. A suspension of 55.2 mg of carboxypeptidase A was added dropwise and the mixture was stirred for 4 hr at 37°. The pH was readjusted to 7 with 2 N LiOH and the enzyme was denatured by heating 30 min at 70° and removed by filtration. The mixture was acidified by addition of 8 ml of 12 N HCl and extracted nine times with a total of 2 l. of chloroform. The aqueous phase was evaporated and the resulting solid was recrystallized from 50% aqueous ethanol to give 6.36 g (87% yield) of *p*-fluoro-L-phenylalanine: mp 280–282° dec; $[\alpha]_{D}^{25}$ –23° (*c* 2.0, water), lit. $[\alpha]_{D}^{25}$ –23° (Bennett and Nieman, 1950).

Anal. Calcd for $C_8H_{10}FNO_2 \cdot 0.5H_2O$: C, 56.25; H, 5.24; F, 9.88; N, 7.29. Found: C, 56.51; H, 5.38; F, 9.45; N, 7.28.

N-*Trifluoroacetyl-p-fluoro-D-phenylalanine (III)*. The chloroform extracts from the preparation of II were evaporated to give 9.75 g (87% yield) of crystalline *N*-trifluoroacetyl-*p*fluoro-D-phenylalanine which was recrystallized from benzene-hexane: mp 134–135°, $[\alpha]_{D}^{25} - 10^{\circ} (c 2.0, methanol)$.

Anal. Calcd for $C_{11}H_{9}F_{4}NO_{3}$: C, 47.32; H, 3.25; F, 27.22; N, 5.02. Found C, 47.32; H, 3.12; F, 26.93; N, 5.06.

p-Fluoro-D-phenylalanine. Compound III (4.6 g) was deprotected by hydrolysis with 2 equiv of lithium hydroxide. Adjustment of the pH to 3.7 gave 2.92 g (97% yield) of white crystalline product which was recrystallized from 50% aqueous ethanol: mp 275–277° dec; $[\alpha]_D^{25} + 25^\circ$ (c 2.0, water), lit. $[\alpha]_D^{25} + 24^\circ$ (Bennett and Nieman, 1950).

Anal. Calcd for $C_8H_{10}FNO_2 \cdot 0.5H_2O$: C, 56.25; H, 5.24; F, 9.88; N, 7.29. Found: C, 57.25; H, 5.14; F, 9.64; N, 7.46.

tert-Butyloxycarbonyl-p-fluoro-L-phenylalanine (IV) was pre-



FIGURE 1: The response of rat blood pressure to $[pFPhe^{s}]$ -AII and AII. The same symbol represents the same animal, the dashed lines the response to $[pFPhe^{s}]$ -AII and the solid lines the response to AII. The response has been normalized such that 100% corresponds to the increase in blood pressure resulting from a dose of AII of 100-ng/kg rat body weight.

pared according to the procedure of Estrin et al. (1971), modified by the use of 16% citric acid in the extraction of IV into ether. To II (6.65 g), suspended with stirring in 110 ml of acetonitrile at room temperature, triethylamine (14 g) and tert-butyloxycarbonyl azide (5.45 g) were added. After 6 days the solvent was removed on a rotary evaporator in vacuo at 40°. The remaining yellow solid was dissolved in ether and 16% citric acid, the water layer was extracted three times with ether, and the extracts were combined and dried over anhydrous MgSO₄ overnight. The MgSO₄ was removed by filtration and the ether was evaporated on a rotary evaporator. The white solid was dissolved in ethyl acetate, a large excess of hexane was added, and crystals were allowed to form upon incubation at -20° for 1 week. The yield was 8.97 g (87%) of IV which gave one spot on thin-layer chromatography (tlc) in solvent systems A (R_F 0.86; R_F (t-Boc-Phe) 0.90) and B (R_F 0.71; R_F (t-Boc-Phe) 0.71): $[\alpha]_D^{25}$ +5.2° (c 0.9, ethyl acetate).

Anal. Calcd for $C_{14}H_{18}FNO_4$: C, 59.32; H, 6.36; N, 4.94. Found: C, 58.78; H, 6.27; N, 4.71.

t-Butyloxycarbonyl-p-fluoro-L-phenylalanyl-resin (V). Compound IV (2.1 g) was added to chloromethylated 2% divinylbenzene-polystyrene in 19 ml dimethyl sulfoxide and triethylamine (0.79 g) and stirred for 36 hr at 75°. The esterified resin was filtered, washed with dimethyl sulfoxide, ethanol, and dichloromethane, and dried. Amino acid analysis showed the resin was substituted with 0.48 mequiv/g of IV.

t-Boc- β -benzyl-L-aspartylnitro-L-arginyl-L-valyl-O-benzyltyrosyl-L-valyl-im-tosyl-L-histidyl-L-prolyl-p-fluoro-L-phenylalanyl-resin (VI). Amino acid-resin V (1.98 g) was placed in the reaction vessel, washed three times with dichloromethane, three times with *tert*-butyl alcohol, and then allowed to react with the appropriate amino acids by the previously described protocol (Hancock *et al.*, 1973) on a Schwarz synthesizer.

L-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-histidyl-L-prolyl-pfluoro-L-phenylalanine (VII). Compound VI was dried and then placed in a polypropylene reaction vessel of an HF apparatus (Pourchot and Johnson, 1969). Anisole (3 ml) and anhydrous HF (10 ml) were added and allowed to react for 45 min. The HF was removed by a stream of nitrogen and then *in vacuo*. The dry peptide and resin were washed with ethyl acetate and extracted with 1% acetic acid. The extract was lyophilized to give 743 mg of white, crude product of which 335 mg was purified by CCD in butanol-acetic acidwater (4:1:5) for 429 passages. Tubes 54–75, which gave two spots by tlc in solvent system D and one spot in the other systems, were combined, evaporated *in vacuo* to remove the 1butanol, and lyophilized to give 145 mg of VIIa. Compound VIIa (90 mg) was placed on a 49 \times 2.5 cm column of Sephadex G25-40 and eluted with 1% acetic acid. Fractions of 2 ml were collected and the contents of tubes 81–90 which gave one spot by tlc in solvent systems D and A were combined and lyophilized to give 60 mg of product, a yield of 26% based upon the initial pFPhe on the resin. Amino aid analysis showed: Asp, 1.10; Arg, 0.94; Val, 1.92; Tyr, 1.00; His, 1.02; Pro, 1.03; pFPhe, 0.98. One ninhydrin and Clorox-starchpositive spot was obtained by tlc in solvent systems A (R_F 0.090; R_F (AII) 0.065), C (R_F 0.55; R_F (AII) 0.57), and D (R_F 0.54; R_F (AII) 0.27): $[\alpha]_D^{25} - 42.6^{\circ}$ (c 0.22, 1 N HCl).

t-Boc- β -benzyl-L-aspartylnitro-L-arginyl-L-valyl-p-fluoro-Lphenylalanyl-L-valyl-im-tosyl-L-histidyl-L-prolyl-L-phenylalanyl-resin (VIII) was prepared from 4.50 g of t-Boc-L-phenylalanyl-resin substituted with 0.34 mequiv/g. The protocol used for VI was followed with substitutions of the appropriate amino acid derivatives.

L-Aspartyl-L-arginyl-L-valyl-p-fluoro-L-phenylalanyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine (IX). Compound VIII was dried, cleaved, and extracted from the resin as above except that 2.6 g of tryptophan was used as scavenger. White powder (1.48 g) was obtained by lyophilization. The crude product (487 mg) was purified by countercurrent distribution (CCD) in solvent system A (4:1:5) for 200 passages and tubes 19-50 were combined, the 1-butanol was evaporated in vacuo, and then the partially purified material was lyophilized to give 304 mg. This material was then further purified by CCD in 1-butanol-pyridine-acetic acid-water (8:2:1:9) for 200 passages. Tubes 45-75 were combined, partially evaporated in vacuo, and lyophilized to give 159 mg of white product, a yield of 35% based upon the phenylalanine initially on the resin. Amino acid analysis showed: Asp, 1.06; Arg, 0.94; Val, 1.89; pFPhe, 1.00; His, 0.93; Pro, 1.10; Phe, 1.09. Thin-layer chromatography gave one ninhydrin and Clorox-starch-positive spot in solvent systems A (R_F) 0.097; R_F (AII) 0.065) and C (R_F 0.56; (AII) 0.57): $[\alpha]_D^{25}$ -47.9° (c 0.36, 1 N HCl).

Bioassay. The oxytocic activity of the analogs was measured by the procedure of Marshall *et al.* (1970). All blood pressures were measured in pentobarbitol- (30 mg/kg, intraperitoneally) anesthetized rats (Holtzman) pretreated with phenoxybenzamine (30 mg/kg) and propranolol (15 mg/kg) by recording from the carotid artery (linear-core transducer, Physiograph). The agent (50 μ l) was injected into the cannulated jugular vein. An AII dose-response curve was determined before and after the administration of each analog so that each animal served as its own control. Antagonists were infused into the other cannulated jugular vein (20 μ l/min) for 15 min before and during the challenging doses of AII (Needleman *et al.*, 1972b).

An acute hypertensive model was prepared by clamping the left renal artery and vein with a hemostat (protected with polyethylene tubing) for 4.5 hr (Krieger *et al.*, 1971). Blood pressure was monitored before and after clamp removal and during infusion of the antagonist. These rats had a blood pressure after propranolol and phenoxybenzamine administration of 79 \pm 3 mm (n = 16) and after clamp removal of 172 \pm 4 mm (n = 16).

Prostaglandin release was measured from isolated rabbit kidney perfused with Krebs solution at 37° and at a constant flow of 8 ml/min. The venous effluent superfused over a series of isolated assay tissues chosen for their sensitivity to pros-



FIGURE 2: The agonistic activity of AII and [pFPhe⁸]-AII and the antagonistic activity of [pFPhe⁴]-AII on isolated rat uterus strips. The values are reported as the average (\pm SE). The concentration of the inhibitor, [pFPhe⁴]-AII, was 20 µg/ml.

taglandins (chick rectum and rat stomach strip) and to AII (rat stomach strip) as previously described (Needleman *et al.*, 1973).

Results

Biological Activitity. [pFPhe8]-AII is equipotent to AII in both the rat oxytocic (n = 8) and blood pressure assays (Figures 1 and 2) and is slightly more potent in the prostaglandin assay system. For release of a half-maximal amount of prostaglandin-like substance and also for a half-maximal contraction of rat stomach strip, a dose of $0.05 \,\mu g$ of [pFPhe⁸]-AII is required as compared with 0.15 μ g for AII. [pFPhe⁴]-AII is an antagonist of AII. (1) In the rat uterus assay it causes a 10-fold shift in the AII dose-response curve when present at 20 μ g/ml, and has a K_1^{*2} of 500 ng/ml relative to a $K_{\rm m}$ * for AII of 5 ng/ml (Figures 2 and 3). (2) The infusion of [pFPhe4]-AII at 100 ng/kg per min results in a 7-fold decrease in sensitivity to AII (n = 3) and in renal-clamped hypertensive rats a blood pressure decrease of $17 \pm 4 \text{ mm} (n = 4)$. (3) [pFPhe4]-AII neither releases prostaglandin-like substance or contracts rat stomach strips at a dose of 150 μ g and at this dose it completely blocks the response of this system to AII at doses as great as 500 ng.

Nuclear Magnetic Resonance. The ¹⁹F nmr spectrum of pFPhe at pH 5.06 appears as a AA'BB'X multiplet with a chemical shift of 890 Hz downfield from external tetrafluorodibromobenzene. The spectra of [pFPhe8]-AII and [pFPhe4]-AII are similar to those of pFPhe except for decreased resolution of the multiplet due to the larger size of the peptides (Figure 5). The titration curves of pFPhe, [pFPhe4]-AII, and [pFPhe8]-All are shown in Figure 4. The pK_a determined by inspection for pFPhe is 2.2 for the carboxyl and 9.1 for the amino group. The titration curve for [pFPhe4]-AII has a probably significant inflection of 2.5 Hz with an approximate pK_a of 6.1. The chemical shift of [pFPhe4]-AII does not change as the concentration is changed from 10 to 2.3% at pH 4.75. A shift of 30 Hz is observed for [pFPhe9]-AII as its C-terminal carboxyl group is titrated with an apparent pK_a of 3.1. The sensitivity of ¹⁹F nmr to through space charge effects is exemplified here in that the shift of the phenylalanine ring pro-



FIGURE 3: Reciprocal plot of uterine contractions vs. angiotensin concentration in the presence of different concentrations of [pFPhe⁴]-AII. Response is expressed as cm contraction (with 1 g of tension = 2 cm). Each curve represents the response obtained from three experiments. The number on each line represents the concentration of [pFPhe⁴]-AII in the bath.

tons as seen in the ¹H nmr of AII is 10 Hz at 220 MHz (Glickson *et al.*, 1972). A second peak appears in the ¹⁹F nmr spectra of [pFPhe⁸]-AII at a pH greater than 7 and is shown in Figure 5. This peak increases in size as the pH is raised from 7.08 to 10.15 and disappears when the pH is lowered from 10.15 to 5.23. The titration curve of this second peak is also shown in Figure 4.

Discussion

Resolution of p-Fluoro-D,L-phenylalanine. The original resolution (Bennett and Niemann, 1950) of *p*-fluorophenylalanine involved the papain-catalyzed synthesis of the phenylhydrazide of the N-acetyl-L-amino acid from the racemic mixture followed by acid hydrolysis and was rather laborious. A more reasonable scheme involved the hydrolysis of the Nchloroacetyl derivative by carboxypeptidase A to give the free L-amino acid and acid hydrolysis of the N-chloroacetyl-Damino acid as had been shown for phenylalanine (Gilbert et al., 1949). Since both of the enantiomers are useful for synthetic studies and acid hydrolysis has been shown to cause some racemization (Manning, 1970), we decided to use an acyl group which could be removed under milder conditions. We chose the trifluoroacetyl group which has been shown to be stereospecifically removed from phenylalanine by carboxypeptidase A at a rate much greater than any other N-acyl derivative (Fones and Lee, 1953). The N-trifluoroacetyl-pfluoro-D-phenylalanine could be converted to the free acid by mild alkaline hydrolysis or used directly in synthesis. An alternative approach which we did not investigate involves the removal of chloroacetyl groups by thiourea (Masaki et al., 1968). The convenience of this procedure and its freedom from potential racemization by acid hydrolysis would argue for its use in the resolution of other synthetic amino acids. We have used it successfully for the resoluton of other amino acids with aromatic and aliphatic side chains in accordance with the specificity of carboxypeptidase A.

Biological Activity. The activities of analogs of angiotensin substituted in positions 4 and 8 are summarized in Table I. That [pFPhe⁸]-AII is at least equipotent to AII is consistent with the activities of other AII analogs with modifications at position 8. The presence of a benzene ring in the β position of the eighth amino in such analogs as [Asp-NH₂-OH¹, *p*-bromo-L-Phe⁸]-AII (Bumpus and Smeby, 1968), [Asp¹, Tyr⁸]-

² $K_{\rm I}^*$ and $K_{\rm m}^*$ are constants determined from a reciprocal plot of uterine response vs. All concentration. These constants, analogous to the $K_{\rm I}$ and $K_{\rm m}$ of a Lineweaver-Burk plot, are intended to represent only relative binding potency of various All analogs.



FIGURE 4: Titration curves of pFPhe, [pFPhe⁴]-AII and [pFPhe⁸]-AII obtained from the ¹⁹F nmr spectra. Chemical shift is hertz downfield from tetrafluorodibromobenzene.

AII, and $[Asp^1,(OMe)Tyr^8]$ -AII (Chaturvedi *et al.*, 1970) results in pressor activities at least 33% of AII. Substitution of a variety of aliphatic amino acids in position 8 yields antagonists with varying potencies (Table I). Thus, a β -phenyl substitution is necessary for agonism but not for binding.

[pFPhe4]-AII is the first antagonist of AII with a modification solely at position 4. [Asp1,Phe4]-AII (Bumpus and

Analog	Activity		
	Pressor (%)	Uterus (%)	Ref
[Asp-NH ₂ /OH ¹ ,pBrPhe ⁸]-AII	50		а
[Asp ¹ ,Tyr ⁸]-AII	83	100	b
[Asn ¹ ,Tyr ⁸]-AII	10	3.7	а
[Asp ¹ ,(OMe)Tyr ⁸]-AII	33		Ь
[Asp ¹ ,Ile] ⁸ -AII	$K_{\rm I}*=5$ ng/ml		с
[Asp⊥,Ala⁴]-AII	0.31	0.06	а
[Asp ¹ ,Phe ⁴]-AII	10		а
[Asp ¹ ,(OMe)Tyr ⁴]-AII	0.4		b
[Asp ¹ ,Phe ⁴ ,Tyr ⁸]-AII	$K_{\rm I}^* = 240 \rm ng/ml$		с

^{*a*} Bumpus and Smeby (1968). ^{*b*} Chaturvedi *et al.* (1970). ^{*c*} Needleman *et al.* (1972b).



FIGURE 5: ¹⁹F nmr spectra of [pFPhe⁸]-AII recorded in the sequence b, c, d, e, f, and a: (a) 64 scans, (b) 8 scans, (c) 8 scans, (d) 8 scans, (e) 8 scans, and (f) 32 scans.

Smeby, 1968) has 10% and [Asp¹,(OMe)Tyr⁴]-AII has 0.9% (Chaturvedi *et al.*, 1970) the pressor activity of AII, but they have no antagonistic activity. Other analogs of AII with modifications solely at position 4 have varied agonistic activities. An analog of AII, in which Tyr and Phe are transposed, [Phe⁴,Tyr⁸]-AII is an antagonist of AII, $K_I^* = 240$ ng/ml (Marshall *et al.*, 1970). Since [Asp¹,Phe⁴]-AII and [Asp¹,Tyr⁸]-AII are purely agonistic, the antagonistic activity of [Phe⁴,Tyr⁸]-AII must be due to the fact that Tyr and Phe are transposed. The activities of the other position four analogs, then, do not suggest a rationale for the antagonistic activity of [pFPhe⁴]-AII and this activity must be specifically related to the substitution of fluorine for the hydroxyl of Tyr.

The activities of the two fluorine analogs of AII described in this paper are consistent with the diversity of activity displayed by fluorine analogs of other naturally occurring compounds. These results, the availability of synthetic routes to fluorine amino acids, the ability to resolve their racemic mixtures, and the ease of peptide synthesis by the solid-phase method, suggest that the incorporation of fluorine amino acids into analogs of peptide hormones would be a good route toward biologically active compounds—both agonists and antagonists.

Since [pFPhe⁸]-AII and [pFPhe⁴]-AII are biologically active, conformation restraints for AII can be determined from their ¹⁹F nmr spectra. Although a distortion of conformation because of the substitution of fluorine for hydrogen is unlikely on a steric basis, the difference in electronegativity and electronic structure of the two atoms could distort the conformation one wants to study. The fact that the conformations of [pFPhe[§]]-AII and AII are identical in solution and at the receptor is the simplest explanation for the equipotence of the two molecules. Thus, the ¹⁹F nmr studies of [pFPhe[§]]-AII are meaningful *vis-à-vis* the solution conformation of AII. On the other hand, the conformation of [pFPhe⁴]-AII must fulfill highly specific requirements in order to bind to the receptor but must be sufficiently different from AII so that [pFPhe⁴]-AII can be an antagonist. Thus, [pFPhe⁴]-AII and [pFPhe⁸]-AII are valid probes of AII receptor conformations and should provide in the future interesting data on hormonereceptor interactions.

Nuclear Magnetic Resonance Studies. The single most promising method for the determination of conformational restraints for peptide hormones in solution is nmr. The ability to resolve individual resonances, the general sensitivity of chemical shift to environment, the relationship between coupling constant and dihedral angle, and the ability to measure exchange rates combine to make nmr the best single technique for the determination of solution conformation of peptides.

In a proton nmr study of AII, Glickson *et al.* (1972) found NH-C_{α}H coupling constants incompatible with partial α -helix (Smeby *et al.*, 1962) or a γ -turn model (Printz *et al.*, 1972a) but compatible with the presence of partial random coil, antiparallel β structure (Fermandjian *et al.*, 1972) or a β -turn model (Printz *et al.*, 1972a). The β -turn model, however, assumes a conformation for Tyr⁴ which is not allowed by potential energy calculations for the i + I residue of a β turn (Venkatachalam, 1968). In addition, such an orientation for a non-glycine residue has not been observed in an analysis (Marshall *et al.*, 1972) of six protein structures determined by X-ray crystallography.

The presence of preferred solution conformation(s) and conformational changes in AII is suggested in the ¹H nmr study by resolution of the β (CH₃)₂ resonances of Val⁵ and Val³, the reciprocal effects of titration of His⁶ imidazole proton and Tyr⁴ hydroxyl proton on the chemical shifts of His⁶ and Tyr⁴ hydrogens, the change in chemical shift of the amide protons with a pK of 6.6 and of the Pro⁷ δ proton, the Pro⁷ β proton, and of a Val α proton near pH 6.6. The conformational change observed about pH 6.6 could be related to titration of His⁶ imidazole proton (pK_a = 6.3) or the Asn¹ amino proton (pK_a = 6.9) or both. However, the titration curves of AII obtained from ¹⁸C nmr studies showed firstorder chemical shift changes consistent with a random-coil structure (Zimmer *et al.*, 1972).

The ¹⁹F nmr spectra of [pFPhe⁴]-AII and [pFPhe⁸]-AII indicate that these two peptides also have preferred solution conformations. In the titration curve of [pFPhe4]-AII, a probably significant shift of 2.5 Hz is seen with an approximate pK of 6.1. This change in chemical shift could be due to a change effect of His⁶ which is known to have a pK_a of 6.3 in AII and may be held in proximity to pFPhe⁴ in the preferred conformations. It could also arise from the general conformational change seen in AII which has a pK of 6.6 or some combination of the two effects. In the titration curves for the Tyr⁴ ortho and meta protons, changes are also seen in the pH region near 6.1, but these are larger and of opposite direction to that seen in [pFPhe4]-AII. The differences seen in the ¹H nmr of AII and ¹⁹F nmr of [pFPhe⁴]-AII suggest that the conformation near the fourth residue is different. Further studies on the ¹H nmr of [pFPhe⁴]-AII should clarify this point. Nevertheless, the implication remains that both peptides have preferred solution conformations.

In the region below pH 7 the titration curve of [pFPhe8]-AH

is consistent with the existence of a single conformation. rapidly interconverting conformations, or a random-coil conformation. The pK_a of 3.1 determined for the C-terminal carboxyl group of [pFPhe8]-AII in H2O is in good agreement with the value of 3.07 determined for AII in H₂O (Glickson et al., 1972). However, above pH 7.0 a second peak appears in the ¹⁹F nmr spectrum at much higher field, increases in size as the pH is raised from 7.08 to 10.15 and disappears as the pH is lowered from 10.15 to 5.23. The gradual increase in the size and the reversibility of this second peak are compatible with a second conformation(s) of AII, more stable in alkaline solution which is in slow equilibrium with another conformation(s). The position of the second peak approximately 120 Hz upfield from that of fully titrated pFPhe suggests that the *p*-fluorine must be close to and strongly shielded by the His⁶ or Tyr⁴ rings or by a negative charge. The pK of this conformational change appears to be close to but greater than the pK of the conformational change seen in AII. Its apparent value would be lowered if the appearance of the second resonance is hidden by noise at lower pH values. Alternately, if the transition between the two sets of conformations is dependent upon the ionization state of the N-terminal amino group, the pK of the transition would be expected to be higher in [pFPhe8]-AII ([Asp1,pFPhe8]-AII) than in AII ([Asn1]-AII) since the N-terminal amino group of aspartyl peptides have higher pK_a 's than those of asparaginyl peptides. The possible existence of two conformations of AII in slow equilibrium was first suggested by Ferreira et al. (1969) but our nmr studies were not conducted at low ionic strength and it seems unlikely these observations are related. Slow equilibrium phenomena among different conformations of polypeptides have been seen in the 1H nmr studies of the denaturation of lysozyme (McDonald et al., 1971) and of the transformation of poly(γ -benzyl L-glutamate) (Ferretti and Ninham, 1970). However, slow equilibrium between conformations was not expected for a peptide the size of [pFPhe⁸]-AII; indeed, the question of whether or not a linear peptide like [pFPhe8]-AII would have a preferred solution conformation has been the subject of considerable debate.

Other Studies on Conformation. The solution conformation of AII has been studied by thin-film dialysis, ultracentrifugation, gel filtration, titration, hydrogen-deuterium or hydrogentitritium exchange, optical rotatory dispersion-circular dichroism (ORD-CD), and ultraviolet, infrared, laser Raman, and nmr spectroscopy. Each method has provided conformational restraints but even the same techniques have suggested contradictory ones. Analysis is further complicated by overinterpretation of data, by different experimental conditions, and by unjustified assumptions made in potential energy calculations.

Thin-film dialysis, ultracentrifugaton, and gel filtration measure the shape and size of a molecule and conformational changes affecting either of these properties may be detected. Ultracentrifugation studies show that AII in a concentration as great as 1.25% exists as a monomer over the pH range 2.5–8.6 (Paiva *et al.*, 1963) and ¹⁹F nmr spectra of [pFPhe⁴]-AII are not affected by changes in concentration from 2.3 to 10%. The initial thin-film dialysis study of Craig *et al.* (1964) suggested that AII exists in a coiled form which increases in size when the buffer is changed from 0.01 M acetic acid–0.15 M ammonia acetate (pH 5.58) to 0.001 M ammonia carbonate or 0.1 N ammonia hydroxide. A more recent study by Franze de Fernandez (1968) suggests this conformationl change occurs in [Asp¹,Val³]-AII and AII between pH 9.30 and 10.70. They believe this conformational change is related to the titration

of the Tyr⁴ hydroxyl which in turn causes the increased biological activity of AII at high pH. But the data of Needleman *et al.* (1972a) suggests that the pK of this activity increase is near 7.8 in rat uterus. Similar changes in activity are seen for *N*-acetyl-AII (P. Needleman and G. R. Marshall, unpublished data) and [pyrazolylalanine⁶]-AII (Needleman *et al.*, 1972a). The pH dependence of the activity implies that neither the Tyr⁴ hydroxyl, the amino-terminal group, nor the imidazole of His⁶ is involved. Thus, the increased activity must be due to a titratable group on the receptor. The results of another study of AII by thin-film dialysis are explained on the basis of two conformations of AII, one which exists only at low ionic strength, and which is partially separable by gel chromatography (Ferreira *et al.*, 1969).

Measurement of hydrogen exchange is another important experimental source of conformational restraint. For example, hydrogens involved in an intramolecular hydrogen bond are expected to be less labile and should be detectable by hydrogen-tritium or deuterium exchange, abnormal pK_a , or nmr spectroscopy. The titration curve of AII shows no abnormal pK_a 's (Paiva et al., 1963). These results are supported by ¹H and ¹⁹F nmr studies discussed previously. Paiva et al. (1963) also measured amide hydrogen-deuterium exchange rates and found the same rate of exchange for all hydrogens at each pH between 2.66 and 3.92. Both the titration and hydrogen-deuterium exchange data were interpreted in terms of a random coil structure. A recent hydrogen-tritium exchange study found different rates of exchange for amide hydrogens and Printz et al. (1972b) suggested that either one of perhaps two intramolecular amide hydrogen bonds existed in AII or that the amide hydrogens were well shielded from water. Evidence from ¹H nmr in H₂O suggests that only the Phe⁸ amide hydrogen undergoes exceptionally slow exchange because the C-terminal carboxyl group is ionized (Glickson et al., 1972). The advantage of nmr in exchange studies is that amide hydrogens can be seen and analyzed separately, whereas, in hydrogen-tritium exchange, complex curves must be resolved into individual curves which are assumed to represent similarly exchanging amide hydrogens.

The lack of resolution of absorption bands from individual chromophores prevents rigorous interpretation of results from most spectroscopic methods such as infrared and Raman spectroscopy or ORD-CD unless one is dealing with a regular structure. Fermandjian et al. (1972) have analyzed infrared and Raman spectra of AII in the solid state in D₂O and in H₂O. On this basis they found amide bands for AII in the solid state compatible with the existence of some antiparallel β structure. It must be pointed out that these amide bands could be compatible with many other structures of AII. The solution spectra generally resembled those in the solid state but were analyzed in far less detail. The CD spectra of AII in H₂O shows a gradual increase in ellipticity as the temperature is raised from 15 to 60° and Fermandjian et al. (1971) suggest a conformation change is taking place. Furthermore, the CD curve at 60° in H₂O is found to resemble part of the CD curve for AII in trifluoroethanol which in turn resembles the CD curve expected for a poly(α -amino acid) of antiparallel β structure. The structural change found in the CD studies as a function of temperature was not found in the ¹H nmr studies (Glickson et al., 1972). In general the CD, infrared, and laser Raman data suggest that AII does have one or more preferred solution conformations—one of which could be an antiparallel β structure—but do not constrain the conformation of AII in H₂O to any particular structure.

These experimental results suggest the existence of preferred solution conformations of angiotensin which are dependent on the conditions under which they are examined. This conclusion is consistent with the evidence seen in both ¹H and ¹⁹F nmr for preferred solution conformations of angiotensin II. In particular, minor modifications of the structure—*i.e.*, substitution of pFPhe for Tyr⁴ or a change in ionization of the imidazole and/or amino-terminal groups alter the predominate solution conformation. Current experimental results are not sufficient, however, to assign any particular set of conformations to angiotensin under a given experimental condition.

In summary, substitution of pFPhe for Tyr⁴ or Phe⁵ has resulted in two analogs of angiotensin II, the former a competitive antagonist and the latter an equipotent agonist. The use of fluorine substitution as an nmr probe of peptide conformation in these two analogs presents new evidence for preferred solution conformations of a linear octapeptide, angiotensin II.

References

- Bennett, E. L., and Nieman, C. (1950), J. Amer. Chem. Soc. 72, 1800.
- Bernardi, L., Bosesio, G., Chillini, F., deCara, C., de Castiglione, R., Ersparmer, V., and Goffrido, O. (1966), *Experentia* 22, 29.
- 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, pp 83–87.
- Cantacuzene, J., and Jantzen, R. (1970), Tetrahedron 26, 2429.
- Chaturvedi, N. C., Park, W. K., Smeby, R. R., and Bumpus, F. M. (1970), J. Med. Chem. 13, 177.
- Craig, L. C., Harfenest, E. J., and Paladini, A. C. (1964), Biochemistry 3, 764.
- Estrin, N. F., Alemany, A. A., and DeTar, D. F. (1971), Biochem. Prep. 13, 18.
- Fermandjian, S., Fromageot, P., Tistchenko, A., Leickman, J., and Leitz, M. (1972), *Eur. J. Biochem.* 28, 174.
- Fermandjian, S., Margot, J., and Fromageot, P. (1971), *Eur. J. Biochem.* 24, 252.
- Ferreira, A. T., Hampe, O. G., and Paiva, A. C. M. (1969), *Biochemistry* 8, 3483.
- Ferretti, J. A., and Ninham, B. W. (1970), *Macromolecules* 3, 30.
- Fones, W. S., and Lee, M. (1953), J. Biol. Chem. 201, 847.
- Franze de Fernandez, M. T., Delius, A. E., and Paladini, A. C. (1968), *Biochim. Biophys. Acta 154*, 223.
- Gammon, K. L., Smallcombe, S. H., and Richards, J. H. (1972), J. Amer. Chem. Soc. 90, 4573.
- Gilbert, J. B., Price, V. E., and Greenstein, J. P. (1949), J. Biol. Chem. 180, 473.
- 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, pp 563–569.
- Goodman, L. S., and Gilman, A. (1970), The Pharmacological Basis of Therapeutics, New York, N. Y., The Macmillan Co.
- Gurka, D., and Taft, R. W. (1969), J. Amer. Chem. Soc. 91, 4794.
- Hancock, W. S., Prescott, D. J., Vagelos, P. R., and Marshall, G. R. (1973), *J. Org. Chem.* 38, 774.
- Huestes, W. H., and Raftery, M. A. (1971), *Biochemistry 50*, 1181.

- Krieger, E. M., Salgado, H. C., Assan, C. J., Greene, L. J., and Ferreira, S. H. (1971), *Lancet 1*, 269.
- Loncrini, D. F. (1969), Advan. Fluorine Chem. 6, 43.
- Manning, J. M. (1970), J. Amer. Chem. Soc. 92, 7447.
- Marshall, G. R., Bosshard, H. E., Eilers, N. C., and Needleman, P. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, pp 571–578.
- Marshall, G. R., and Merrifield, R. B. (1965), *Biochemistry* 4, 2395.
- Marshall, G. R., and Merrifield, R. B. (1972), *in* Biochemical Aspects of Reactions on Solid Supports, Stark, G. R., Ed., New York, N. Y., Academic.
- Marshall, G. R., Vine, W., and Needleman, P. (1970), Proc. Nat. Acad. Sci. U. S. 67, 1624.
- Masaki, M., Kitahara, T., Kureta, H., and Ohta, M. (1968), J. Amer. Chem. Soc. 90, 4508.
- McDonald, C. C., Phillips, W. D., and Glickson, J. D. (1971), J. Amer. Chem. Soc. 93, 235.
- Middleton, W. J., and Lindsey, R. V. (1964), J. Amer. Chem. Soc. 86, 4948.
- Morely, J. S. (1968), Proc. Roy. Soc., Ser. B 170, 97.
- Needleman, P., Freer, R. J., and Marshall, G. R. (1972a), Arch. Int. Pharmacol. Therap. 200, 174.
- Needleman, P., Johnson, E. M., Vine, W., Flanigan, E., and Marshall, G. R. (1972b), *Circ. Res. 31*, 862.
- Needleman, P., Kaufman, A., Douglas, J., Johnson, E. M., and Marshall, G. R. (1973), *Amer. J. Phys.* (in press).

- Newmark, R. A., and Sederholm, C. H. (1965), J. Chem. Phys. 43, 602.
- Nicolaides, E. D., Croft, M. K., and De Wald, H. A. (1963), J. Med. Chem. 6, 524.
- Nitecki, D. E., and Goodman, J. W. (1966), Biochemistry 5, 665.
- Paiva, T. B., Paiva, A. C. M., and Scheraga, H. A. (1963), Biochemistry 2, 1327.
- Phillips, L., and Wray, V. (1971), J. Chem. Soc. B, 1618.
- Pourchot, L. M., and Johnson, J. J. (1969), Org. Prep. Proc. 1, 121.
- Printz, M. P., Nemethy, G., and Bleich, H. (1972a), *Nature* (*London*) 237, 135.
- Printz, M. P., Williams, H. P., and Craig, L. C. (1972b), Proc. Nat. Acad. Sci. U. S. 69, 378.
- Richmond, M. H. (1963), J. Mol. Biol. 6, 284.
- Scotchler, J., Lazier, R., and Robinson, A. B. (1970), J. Org. Chem. 35, 3151.
- Sheppard, W. A., and Sharts, C. M. (1969), Organic Fluorine Chemistry, New York, N. Y., W. A. Benjamin, Inc.
- Smeby, R. R., Arakawa, K., Bumpus, F. M., and Marsh, M. (1962), *Biochim. Biophys. Acta* 58, 550.
- Stuart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman & Co., p 31.
- Venkatachalam, C. M. (1968), *Biopolymers 6*, 1425. Weygand, F., and Geiger, R. (1956), *Chem. Ber. 84*, 647.
- Zimmer, S., Haar, W., Maner, W., Ruterjans, H., Fermandjian, S., and Fromageot, P. (1972), *Eur. J. Biochem.* 29, 80.

Intrinsic Optical Activity of Retinal Isomers. Implications for the Circular Dichroism Spectrum of Rhodopsin[†]

Barry Honig,*, ‡ Peter Kahn, and Thomas G. Ebrey

ABSTRACT: The source of the circular dichroism in the visible region of the rhodopsin absorption spectrum may be either an intrinsic chromophore asymmetry or one induced by its environment in rhodopsin. Since 11-*cis*-retinal is twisted about a number of bonds in its π electron system it is expected that separated conformers with opposite chirality will exhibit optical activity. Calculations of the extent of overlap of mirror image isomers show that the highly selective opsin binding site is probably incapable of binding two enantiomers of the

In recent years the optical activity of rhodopsin and other visual pigments has been the subject of numerous experimental investigations (Crescitelli *et al.*, 1966; Takezaki and Kito, 1967; Kito *et al.*, 1968; Mommaerts, 1969; Horwitz and Heller, 1971; Shichi, 1971; Waggoner and Stryer, 1971; Johnston and Zand, 1972). The interpretation of these experichromophore. In addition, the calculated magnitude of the rotational strength of 11-cis-retinal is comparable to that of rhodopsin. Thus, the optical activity of the visual pigment may be at least partially explained in terms of a selective binding of a particular conformer by the protein. The skewed ring-chain conformation of other retinal isomers may be the source of the optical activity of visual pigments whose chromophore is not 11-cis-retinal.

ments has been complicated by the variety of factors that may be responsible for the observed circular dichroism of the 500- (α) and 340- (β) nm bands of rhodopsin (Figure 1). Since these electronic transitions are due to the retinylidene chromophore, their optical activity must result from either an intrinsic molecular asymmetry due to twisting, induced twisting by the protein, or to an asymmetric environment in the region of the protein near the chromophore. Although the last mechanism has been generally accepted as the source of the optical activity of rhodopsin, we feel that the importance of the intrinsic optical activity of retinal isomers has not been fully recognized (see, however, the discussion by Mommaerts,

[†] From the Department of Biological Sciences, Columbia University, New York, New York 10027. *Received December 6, 1972.* Supported by Public Health Service Grant EY-00433.

[‡] Present address: Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel.