References

Bernier, G. M., and Putnam, F. W. (1964), *Biochim. Biophys. Acta 86*, 295.

Dreyer, W. J., Gray, W. R., and Hood, L. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 353.

Easley, C. W., and Putnam, F. W. (1966), *J. Biol. Chem. 241*, 3671.

Ein, D., Kimura, S., Terry, W. D., Magnotta, J., and Glenner, J. D. (1972), *J. Biol. Chem. 247*, 5653.

Epstein, C. J. (1967), *Nature (London) 215*, 355.

Fitch, W. M., and Margoliash, E. (1967), *Science 155*, 279.

Gottlieb, P. D., Cunningham, B. A., Rutishauser, U., and Edelman, G. M. (1970), *Biochemistry 9*, 3155.

Gray, W. R. (1967), *Methods Enzymol. 2*, 469.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972a), *Biochemistry 11*, 4493.

Hermodson, M. A., Kuhn, R. W., Walsh, K. A., Neurath, H., Eriksen, N., and Benditt, E. P. (1972b), *Biochemistry 11*, 2934.

Hill, R. L. (1965), *Advan. Protein Chem. 20*, 37.

Hilschmann, N. (1967), *Hoppe Seyler's Z. Physiol. Chem. 348*, 1718.

Hilschmann, N. (1969), *Naturwissenschaften 56*, 195.

Köhler, H., Shimizu, A., Paul, C., and Putnam, F. W. (1970), *Science 169*, 56.

Levin, M., Franklin, E. C., Frangione, B., and Pras, M. (1973), *Biochemistry 12*, (in press).

Milstein, C. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 301.

Osserman, E. F., Takatsuki, K., and Talal, N. (1964), *Seminars Hematol. 1*, 3.

Pisano, J. J., and Bronzent, T. J. (1969), *J. Biol. Chem. 244*, 5597.

Putnam, F. W. (1969), *Science 163*, 633.

Putnam, F. W., Shimizu, A., Paul, C., Shinoda, T., and Köhler, H. (1971), *Ann. N. Y. Acad. Sci. 190*, 83.

Putnam, F. W., Titani, K., and Whitley, E., Jr. (1966), *Proc. Roy. Soc., Ser. B 166*, 124.

Shinoda, T., Titani, K., and Putnam, F. W. (1970), *J. Biol. Chem. 245*, 4463.

Terry, W. D., Page, D. L., Kimura, S., Isobe, T., Osserman, E. F., and Glenner, G. G. (1973), *J. Clin. Invest. 52*, 1276.

Titani, K., Shinoda, T., and Putnam, F. W. (1969), *J. Biol. Chem. 244*, 3550.

Titani, K., Wikler, M., Shinoda, T., and Putnam, F. W. (1970), *J. Biol. Chem. 245*, 2171.

Watanabe, S., and Hilschmann, N. (1970), *Hoppe Seyler's Z. Physiol. Chem. 351*, 1291.

Whitley, E. J., Jr. (1969), M.S. Thesis, Indiana University.

Whitley, E. J., Jr., Titani, K., and Putnam, F. W. (1969), *J. Biol. Chem. 244*, 3537.

Woods, K. R., and Wang, R. T. (1967), *Biochim. Biophys. Acta 133*, 369.

A Circular Dichroism Study of the Secondary Structure of Bradykinin†‡

John R. Cann,* John M. Stewart, and Gary R. Matsueda§

ABSTRACT: A systematic study has been made of the circular dichroism (CD) behavior of bradykinin, several of its analogs and peptide fragments, and model compounds. It is concluded that the secondary structure of bradykinin and its analogs is a time average of two interconverting structures—one disordered and one partially ordered due to a $3 \rightarrow 1$ type hydrogen bond bridging the Pro^7 residue. With increasing temperature the peptide spends a progressively greater fraction of its time intramolecularly hydrogen bonded until at 80° it spends all of its time in the partially ordered configuration. This transition is characterized by large positive values of $\Delta H°$ and $\Delta S°$, which is interpreted to mean that the structure of water plays a dominant role in determining the configuration of the peptide. Indeed, bradykinin possesses considerable conformational freedom and can assume different configurations in response to changes in solvent composition at constant temperature, *e.g.*, changing from water or buffer to 90% dioxane–water mixture. The nonadditivity of the CD spectra of peptide fragments and the comparative CD of bradykinin and its analogs in 90% dioxane are indicative of interactions between the amino acid residues in the bradykinin molecule.

The circular dichroism (CD) spectrum of bradykinin (the biologically active nonapeptide, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) in the wavelength interval 260–210 mμ, shows two weak bands, a negative one centered at 234 mμ and a positive one at 221 mμ. These spectral features suggested to Brady *et al.* (1971) that bradykinin has essentially a random-coil configuration in solution. More recently, however, the spectrum has been reinterpreted by one of us (Cann, 1972) as being indicative of a secondary structure having some order, most

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TABLE I: Properties of Peptides.

	CCD K[a]	Tlc[b]		Electrophoresis[c]		Amino Acid Composition (mol/mol of peptide)					
		R_FI	R_FF	$E_{lys}5$	$E_{lys}2.8$	Arg	Pro	Gly	Phe	Ser	Leu
Bradykinin (BK)	1.72[d]	0.54, 0.80[e]	0.32, 0.70[e]	0.64		2.12	2.70	1.00	1.97	0.98	
[Leu[5,8],Gly[6]]-BK	0.40[f]	0.58, 0.78[e]	0.50, 0.64[e]	0.69		1.87	2.92	2.00			2.28
[Leu[5]]-BK	0.57[d]	0.78	0.66	0.67		1.99	3.08	1.11	0.91	0.99	0.96
[Leu[8]]-BK	0.64[d]	0.74	0.64	0.73		2.29	2.82	1.00	1.04	0.97	1.03
Arg-Pro	0.10[g]	0.24	0.28	0.78		1.00	0.92				
Arg-Pro-Pro[k]		0.32	0.34	0.69		1.00	1.87				
Arg-Pro-Pro-Gly	*h*	0.34	0.38	0.64		1.00	1.98	1.02			
Arg-Pro-Pro-Gly-Phe	*i*	0.58	0.62	0.53		0.99	2.02	1.00	1.10		
Pro-Phe-Arg	0.60[d]	0.80	0.62	0.63		1.16	1.00		1.00		
Ser-Pro-Phe-Arg	0.38[d]	0.76	0.62	0.61	0.74	0.99	1.06		1.00	0.99	
Phe-Ser-Pro-Phe-Arg	2.23[d]	0.80	0.78	0.48		1.00	0.94		1.90	0.81	
Ser-Pro-Pro-Arg	0.09[d]	0.70	0.40	0.78		1.00	1.97			0.86	
Arg-Pro-NH_2	*j*	0.30	0.42	0.70							

[a] The observed partition coefficient after countercurrent distribution in the indicated system. [b] R_F's observed on cellulose tlc in systems I (1-BuOH–AcOH–H_2O, 4:1:1) and F (1-BuOH–AcOH–H_2O–pyridine, 15:3:12:10). [c] Mobilities relative to lysine at pH 5 and 2.8. [d] In 1-BuOH–1% trifluoroacetic acid (1:1). [e] Peptide trifluoroacetates frequently give two spots on tlc in acetic acid systems. The slower spot is characteristic of the peptide acetate, while the faster is the trifluoroacetic acid complex. [f] In 1-BuOH–0.67% trifluoroacetic acid (1:1). [g] In 1-BuOH–AcOH–H_2O (4:1:5). [h] Purified by Dowex 50 chromatography. [i] Purified by preparative electrophoresis. [j] Not purified. See text. [k] Purchased from Cyclo Chemical Co. Contained a trace of contaminant that appeared to be Arg-Pro.

probably due to an internally hydrogen-bonded proline residue. Thus, the negative band at 234 mμ was attributed to a configuration in which one of the proline residues is bridged by a 3→1 type hydrogen bond (Venkatachalam, 1968; Ramachandran *et al.*, 1966) to give a structure analogous to the intramolecularly hydrogen-bonded configuration of the sort shown by *N*-acetyl-L-proline *N'*-methylamide in nonpolar solvents (Tsuboi *et al.*, 1959; Madison and Schellman, 1970a, 1970b; Cann, 1972). As for the positive band at 221 mμ, it was noted that bradykinin contains two phenylalanine residues which in model compounds in aqueous solution have intense, positive bands centered at 217 mμ. In order to test these ideas we have made a systematic study of the CD characteristics of bradykinin, several of its analogs[1] and peptide fragments, and model compounds.

Materials and Methods

Bradykinin and the analogs and fragments used were synthesized by the solid-phase method (Stewart and Young, 1969), except for Arg-Pro-Pro, which was purchased from Cyclo Chemical Co. The peptides and their physical properties are given in Table I. Boc amino acids used were of the L configuration, and were purchased from Schwarz, Bachem, Fox, or Beckman. Boc-Arg(NO_2) and Boc-Ser(Bzl) were used. The C-terminal Boc amino acid was esterified to either 1 or 2% cross-linked chloromethylated polystyrene; the degree of substitution was 0.2–0.4 mmol/g. Syntheses were done on a 0.4-mmol scale on an improved version of the automatic instrument described earlier (Merrifield *et al.*, 1966) or on the Beckman 990 synthesizer. Removal of Boc groups was with either 4 M HCl–dioxane or trifluoroacetic acid–$CHCl_3$ (1:3, v/v); in each case a prewash of the deprotection reagent was used. The salt of the peptide-resin was neutralized by 5-min treatment with 10% Et_3N in $CHCl_3$ or CH_2Cl_2, following a prewash with the reagent. $CHCl_3$ was always used as neutralization solvent with peptide hydrochlorides. Coupling reactions were usually done for 2 hr with 2.5-fold amounts of Boc amino acids and dicyclohexylcarbodiimide in $CHCl_3$ or CH_2Cl_2. In some cases coupling times were shorter if ninhydrin monitoring (Kaiser *et al.*, 1970) indicated complete coupling. Boc-Arg(NO_2) was coupled in 30% DMF, due to its low solubility. After coupling the last amino acid, the Boc group was removed by a final deprotection step, and the resin was washed thoroughly with EtOH and CH_2Cl_2 and dried. Peptides were cleaved from the resin by treatment with anhydrous HF (10 ml/g of resin) in the presence of anisole (50 equiv/equiv of NO_2 or Bzl groups) for 30 min at 0°. Arg-Pro-NH_2 was synthesized on a benzhydrylamine resin (Pietta and Marshall, 1970) kindly supplied by Schwarz Bio-Research, which yielded the amide directly on treatment with HF. Boc-Pro was coupled to the benzhydrylamine resin with dicyclohexylcarbodiimide, and remaining amino groups were acetylated with acetic anhydride and triethylamine before deprotection of the Boc group. Peptides were purified as indicated in the footnotes to Table I by countercurrent distribution, by column chromatography on Dowex 50 in the pyridine–acetic acid system of Schroeder (1967), or by continuous flow electrophoresis on the Elphor instrument (Brinkmann). Homogeneity of the purified peptides was demonstrated by paper electrophoresis at pH 5 (pyridine–acetic acid, 0.1 M in pyridine) and/or pH 2.8 (1 M acetic acid) and by thin-layer chromatography on commercial cellulose plates. For amino acid analysis peptides were hydrolyzed in 6 N HCl in N_2-flushed sealed tubes; hydrolysates were analyzed on the Beckman 120 analyzer. Arg-Pro-NH_2 was used as the crude

[1] [Leu[5,8],Gly[6]]-bradykinin, Arg-Pro-Pro-Gly-Leu-Gly-Pro-Leu-Arg; [Leu[5]]-bradykinin, Arg-Pro-Pro-Gly-Leu-Ser-Pro-Phe-Arg; [Leu[8]]-bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-Arg.

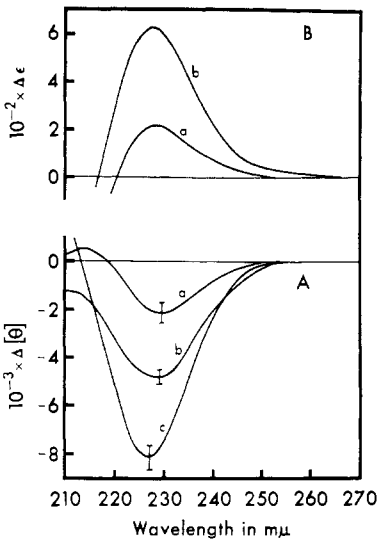


FIGURE 1: CD and ultraviolet absorption difference spectra of model proline compounds. A: curve a, molar CD difference spectrum of Gly-Pro-Gly-Gly produced by 90% dioxane–water mixture containing 8×10^{-4} M HCl referred to water at pH 4.5–5.8, band centered at 229.5 mμ; curve b, Gly-Pro-Gly-Gly produced by 96% dioxane containing 8×10^{-4} M HCl referred to water at pH 4.5–5.8, centered at 229 mμ; curve c, *N*-acetyl-L-proline *N'*-methylamide produced by 90% dioxane referred to water, 227 mμ. B: curve a, ultraviolet absorption difference spectrum of Gly-Pro-Gly-Gly produced by 96% dioxane containing 8×10^{-4} M HCl referred to water, λ_{max} 228.5 mμ; curve b, acetylproline methylamide produced by dioxane referred to water, λ_{max} 227.5 mμ. Error bars in this and following figures indicate mean derivations.

material, since all purification procedures tried caused complete cyclization to the diketopiperazine. The crude peptide was estimated to be 90% pure, and the contaminant appeared to be Arg-NH_2 as judged by electrophoresis and overall amino acid composition.

The peptide trifluoroacetates when dissolved in water to a concentration of about 0.15% gave about pH 4; the peptide acetates, about pH 5.

Acetylproline methylamide, acetylserine methylamide, acetylphenylalanine amide, Gly-Pro-Gly-Gly, and Gly-Phe-Gly were obtained from Cyclo Chemical Co. or Schwarz/Mann and were supplied with assay. Eastman *p*-dioxane was purified by passage through a column of activated aluminum oxide (Woelm basic, activity grade 1). The purified dioxane was stored over a small amount of aluminum oxide and under nitrogen in the dark, and was tested for absence of peroxide before use.

CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment, fitted with a thermostatable cell holder. The temperature of the solution was checked with a thermistor probe. Except where indicated, measurements were made at 27.0°. Slits were programmed to yield a 15-Å bandwidth at each wavelength. Concentration and path lengths were dictated by the absorbancy of the solution. In general, a 0.1-cm cell permitted reliable measurements down to a wavelength of 215 mμ with 0.15% solutions of the several peptides in 90% dioxane–water mixture. Molar ellipticities, $[\theta]$, and mean residue ellipticities, $[\theta]_{mrw}$, (deg cm²)/dmol, were calculated in the usual fashion. Each spectrum is the average of at least two determinations. The spectrum of bradykinin was checked from time to time over a period of 1.5 years and always found to agree within a small experimental error.

Ultraviolet absorption spectra and difference spectra were

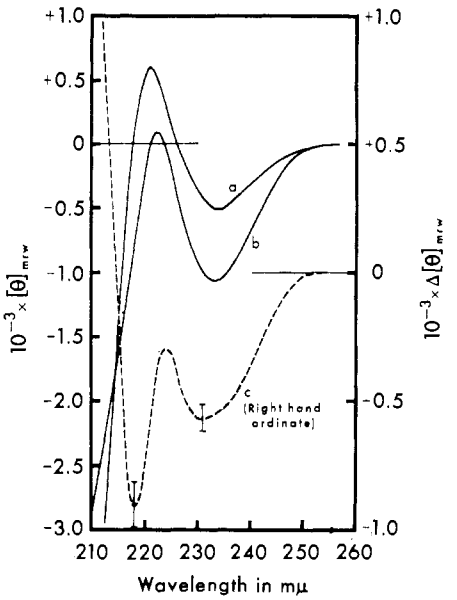


FIGURE 2: The effect of 90% dioxane on the CD of bradykinin: curve a, CD spectrum of bradykinin in H_2O (pH 4.12); curve b, CD spectrum in 90% dioxane–water mixture; curve c, CD difference spectrum produced by 90% dioxane–water referred to water. The CD spectra in 0.01 M sodium acetate buffer (pH 4.00) and 0.03 M potassium phosphate buffer (pH 7.18) are the same as curve a within experimental error.

recorded on a Cary Model 14 spectrophotometer through the courtesy of Dr. Oscar K. Reiss. Integral tandem absorption cells were employed for the difference spectra. $\Delta\epsilon$ is the difference molar extinction coefficient.

Results

Spectroscopic measurements have been made on the various peptides listed in Table I: bradykinin; three of its analogs; four of its N-terminal peptide fragments; three of its C-terminal fragments; and the peptide, Ser-Pro-Pro-Arg, which is an analog of the C-terminal fragment, Ser-Pro-Phe-Arg. The CD spectra of these peptides in water and in 90% dioxane–water mixture have been compared not only with each other but also with several model compounds. The effects of pH, concentration and type of supporting electrolyte, and temperature on the CD spectra have been examined for some of the peptides in water. Ultraviolet absorption measurements have also been made in certain instances. The results of these experiments are presented below in a format which first compares the spectra of members of the several forementioned groupings of peptides and then turns to the effect of temperature and salts.

Model Compounds. Experiments on model compounds are crucial to this investigation. The first hint that the secondary structure of bradykinin might have some order came from observations on the CD of the intramolecularly hydrogen-bonded configuration of acetylamino acid amides in dioxane. In particular, the intramolecularly hydrogen-bonded configuration of acetylamino acid amides is characterized by a well-developed n–π^* Cotton effect in the neighborhood of 220–230 mμ as contrasted to the poorly resolved n–π^* Cotton effect at about 212 mμ shown by the non-hydrogen-bonded configuration in water. Thus, acetylproline methylamide in water shows a strong negative CD band at 202 mμ with only a very weak shoulder in the vicinity of 212 mμ, while in dioxane it exhibits a strong negative band centered at 226 mμ. The CD

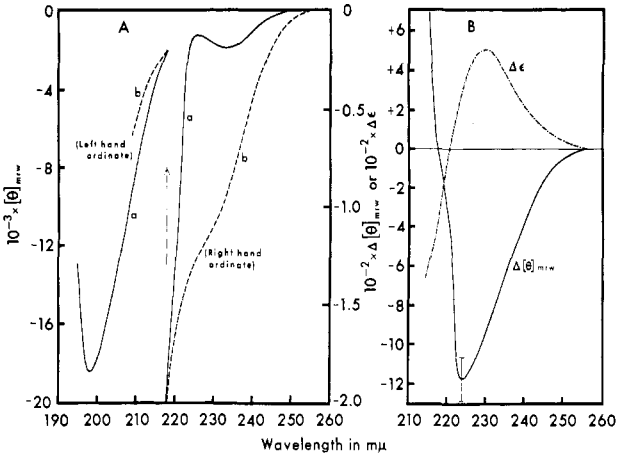


FIGURE 3: The effect of 90% dioxane on the CD and ultraviolet absorption of the bradykinin analog, [Leu[5,8],Gly[6]]-bradykinin. A: curve a, CD spectrum in water at pH 3.6–4.1; curve b, in 90% dioxane–water. B: $\Delta[\theta]_{mrw}$, CD difference spectrum produced by 90% dioxane–water referred to water; $\Delta\epsilon$, ultraviolet absorption difference spectrum produced by 90% dioxane–water referred to water.

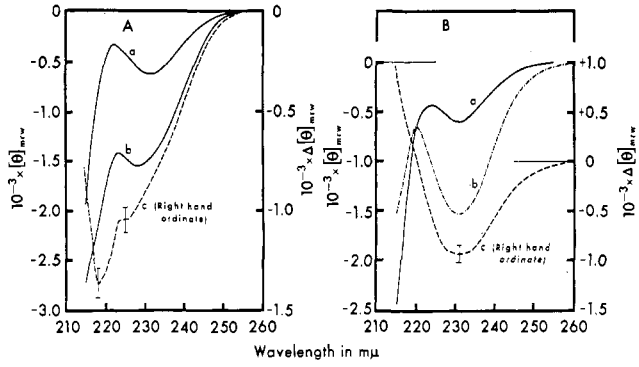


FIGURE 4: The effect of 90% dioxane on the CD of bradykinin analogs. A: [Leu[5]]-bradykinin; curve a, CD spectrum in water, pH 3.4–3.9; curve b, in 90% dioxane–water; curve c, CD difference spectrum produced by 90% dioxane referred to water. B: [Leu[8]]-bradykinin; curve a, in water at pH 3.6–4.1; curve b, in 90% dioxane; curve c, difference spectrum.

difference spectrum produced by dioxane relative to water shows a strong negative band centered at 227 mμ; and the ultraviolet absorption difference spectrum, a band with wavelength of maximum difference-absorption at 227.5 mμ. Similar, although weaker, difference spectra are produced by 90% dioxane–water mixture referred to water in the case of both acetylproline methylamide and Gly-Pro-Gly-Gly (Figure 1).

It is also pertinent to note that the intramolecularly hydrogen-bonded configuration of *N*-acetylproline in cyclohexane shows a Cotton effect at 226 mμ (Madison and Schellman, 1970a).

Other aliphatic amino acid amides such as acetylalanine methylamide and acetylserine methylamide in 90% dioxane give negative CD difference bands centered at 221 mμ. Acetylalanine methylamide and acetylleucine methylamide in pure dioxane give positive ultraviolet absorption difference bands referred to water which are centered at 225.5 mμ.

The CD difference spectra of acetylphenylalanine amide and Gly-Phe-Gly produced by 90% dioxane referred to water show a negative band centered at 216 mμ with a shoulder (or weak band in the case of the tripeptide) at 235 mμ. The difference spectra are similar to, although less intense than, the difference spectrum of acetylphenylalanine amide produced by pure dioxane and shown in Figure 3 of Cann (1972).

These various results suggested the idea of probing the secondary structure of bradykinin by examining the effect of 90% dioxane on its CD and the CD of related peptides.

Bradykinin. In Figure 2 the CD spectrum of bradykinin dissolved in 90% dioxane–water mixture (curve b) is compared to its spectrum in water or buffer (curve a). 90% dioxane causes[2] a twofold intensification of the negative band centered at 234 mμ and a strong diminution of the positive band at 221 mμ. These solvent-induced spectral charges are reversible. Thus, when a solution of bradykinin in 90% dioxane was diluted tenfold with water, the spectrum reverted to that shown in water by bradykinin never exposed to dioxane. The possibility that the effect of dioxane is due to molecular association of bradykinin or dissociation of aggregates seems to be eliminated by the following observations. (1) The CD in both water and 90% dioxane obeys Beer's law, within experimental error, over a twofold range of concentration. (2) 90% dioxane has only a small effect on the ultraviolet absorption spectrum of bradykinin in the 280–250-mμ wavelength range where the two phenylalanine residues absorb—the four bands are red shifted by about 0.5 mμ, and their molar extinction coefficients are increased by about 13%. (3) The molar absorption spectrum of bradykinin in water is essentially the same as the spectrum of 2 mol of Gly-Phe-Gly, except that the extinction of the former is about 15% greater than the latter. It is concluded, therefore, that as in the case of model compounds the configuration of bradykinin changes reversibly when the solvent is changed from water to 90% dioxane–water.

The CD difference spectrum produced by 90% dioxane referred to water (curve c in Figure 2) is characterized by two negative bands with extrema at 231 and 218 mμ. Comparison with the difference CD spectra of model compounds (*e.g.*, Figure 1A) indicates that 90% dioxane either induces formation of at least two 3→1 hydrogen bonds bridging Pro and Phe residues in the peptide and/or increases the degree of such intramolecular hydrogen bonding.

Bradykinin Analogs. The CD spectra in water of the three bradykinin analogs examined (Figures 3A and 4) show a common feature with the spectrum of bradykinin, namely, a weak negative band in the region 231–234 mμ. Moreover, the CD in this spectral region is greatly intensified by changing the solvent from water to 90% dioxane. The CD difference spectrum of [Leu[5,8],Gly[6]]-bradykinin produced by 90% dioxane (Figure 3B) shows a negative band at 224.5 mμ which appears to suggest involvement of an aliphatic amino acid. On the other hand, the ultraviolet absorption difference spectrum shows a positive band at 229.5 mμ, which suggests enhanced formation of a 3→1 hydrogen bond bridging a Pro residue. It may be that the CD difference band is a composite band and that the ultraviolet difference spectrum is the more diagnostic. In the case of [Leu[5]]-bradykinin (Figure 4A) the

[2] Brady *et al.* (1971) have reported that there are negligible effects of dioxane and temperature on the CD spectrum of bradykinin. Recently, however, they have reexamined their data and find that this is a matter of interpretation. Thus, dioxane at an unspecified concentration had an effect on the CD of Lys-Lys-bradykinin which is in the same direction as the effect we observe with 90% dioxane and bradykinin. Likewise, raising the temperature from 10 to 40° had qualitatively the same effect on the CD of bradykinin as observed by us.

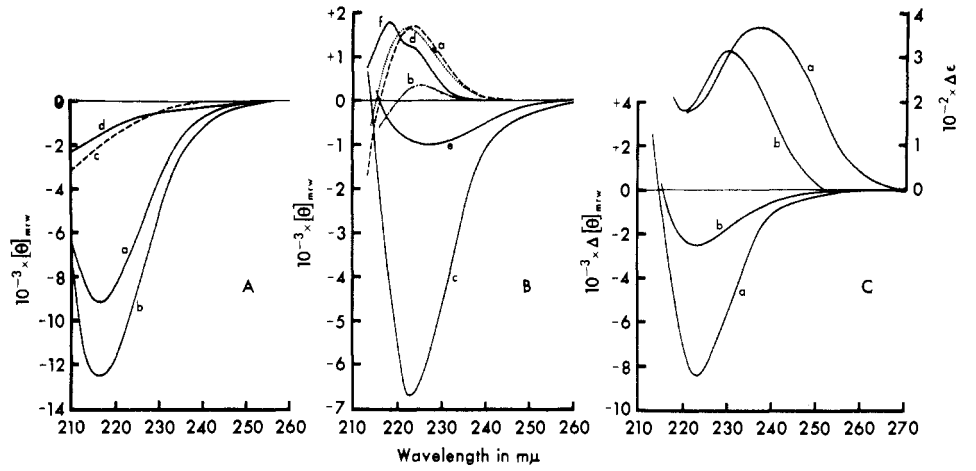


FIGURE 5: The CD and ultraviolet absorption behavior of the N-terminal peptide fragments of bradykinin. A: CD spectrum of Arg-Pro curve a, in water at pH 4.83; curve b, in 90% dioxane–water; curve c, in 0.1 M HCl; curve d, in 90% dioxane–water containing 0.1 M HCl B: curve a, Arg-Pro-Pro in water (pH 4.02); curve b, Arg-Pro-Pro in water (pH 4.01). 65.5°; curve c, Arg-Pro-Pro in 90% dioxane; curve d, Arg-Pro-Pro-Gly in water (pH 5.08); curve e, Arg-Pro-Pro-Gly in 90% dioxane; curve f, Arg-Pro-Pro-Gly-Phe in water (pH 4.04); the spectrum of Arg-Pro-Pro-Gly-Phe was not determined in 90% dioxane because of an insufficient supply of this peptide. C: CD and ultraviolet absorption difference spectra; curve a, Arg-Pro-Pro, produced by 90% dioxane referred to water at pH 4.02; curve b, Arg-Pro-Pro-Gly, produced by 90% dioxane referred to water at pH 5.08.

CD difference spectrum shows a negative band at 218 mμ with a strong shoulder at 225 mμ implicating both Phe and Pro residues. In contrast, [Leu^5]-bradykinin shows a single CD difference band centered at 231 mμ indicative of enhanced formation of 3→1 hydrogen bond(s) bridging a Pro residue(s).

These experiments along with those on bradykinin itself provide strong supporting evidence for our original contention that the secondary structure of bradykinin has some order. To define this structure as precisely as possible we made appeal to the CD behavior of the peptide fragments of bradykinin.

N-Terminal Fragments. The CD behavior of the N-terminal peptide fragments of bradykinin is summarized in Figure 5. The CD of the dipeptide, Arg-Pro, in either water or 90% dioxane, is rather indifferent (Figure 5A). On the other hand, Arg-Pro-Pro and Arg-Pro-Pro-Gly have an interesting CD behavior in that both exhibit a positive band at 222 and 222.5 mμ, respectively, in water but a negative one at 227.5 and 222.5 mμ, respectively, in 90% dioxane (Figure 5B). The CD difference spectra produced by 90% dioxane show a negative band centered at 223 mμ, and their ultraviolet absorption difference spectra, a positive band with wavelength of maximum absorption at 230.5 and 237 mμ, respectively (Figure 5C). Here too, the difference spectra are indicative of intramolecular hydrogen-bond formation in 90% dioxane; and we note their close similarity to the CD and absorption difference spectra of [Leu^{5,8},Gly^6]-bradykinin. The simplest picture is that in the case of Arg-Pro-Pro a hydrogen bond forms between C=O of the Pro^2-Pro^3 peptide bond and OH of the carboxyl group analogous to acetylproline in cyclohexane, while in Arg-Pro-Pro-Gly it is between C=O of Pro^2-Pro^3 and NH of Pro^3-Gly^4. This would be consistent with the red-shifted ultraviolet absorption difference spectrum of Arg-Pro-Pro relative to Arg-Pro-Pro-Gly. Although other configurations may be possible, *e.g.*, a 4→1 type hydrogen bond bridging Pro^2-Pro^3, nothing seems to be known about their CD. These results indicate clearly that the spectral behavior of [Leu^{5,8},Gly^6]-bradykinin involves a proline residue.

The CD of Arg-Pro-Pro-Gly-Phe in water (curve f in Figure 5B) is particularly revealing since the spectrum shows the positive 218-mμ band characteristic of Phe as well as a strong shoulder at 223 mμ associated with the Arg-Pro-Pro sequence.[3] This observation indicates that both the two Phe residues and the sequence Arg-Pro-Pro in the bradykinin molecule contribute to its positive 221-mμ CD band.

C-Terminal Fragments. Figure 6 summarizes the results of experiments on the C-terminal peptide fragments of bradykinin. It is immediately apparent that the CD characteristics of bradykinin reside to a large extent in the C-terminal portion of the molecule. Thus, in water Pro-Phe-Arg shows the positive 217-mμ band characteristic of phenylalanine (curve b in Figure 6A); Ser-Pro-Phe-Arg exhibits a negative band at 227 mμ characteristic of intramolecularly hydrogen-bonded proline compounds (curve a in Figure 6B); and Phe-Ser-Pro-Phe-Arg gives both a negative band at 231 mμ and a positive one at 219 mμ (curve c in Figure 6B). Moreover, the molar ellipticities of the Phe-Ser-Pro-Phe-Arg bands (−5700 and +8800°) compare favorably with those of bradykinin (−4700 and +5300°).

If the negative 227-mμ band of Ser-Pro-Phe-Arg is in fact indicative of a 3→1 hydrogen bond bridging Pro^2, then the CD spectrum of the analog, Ser-Pro-Pro-Arg, should not show the band since it is not possible to form a 3→1 hydrogen bond bridging Pro^2 in this molecule. As shown by curve d in Figure 6B, Ser-Pro-Pro-Arg does not show the band. This result gives twofold support to our thesis since it seems to eliminate the possibility that the 227-mμ band might have its origin in a type I β bend, *i.e.*, a 4→1 hydrogen bond bridging Pro-Phe (Venkatachalam, 1968). The occurrence of two Pro residues in a β bend would appear to be much more probable than Pro and Phe (Lewis *et al.*, 1971); and examination of a Corey–Pauling–Koltun model of Ser-Pro-Pro-Arg indicates that such a configuration is sterically possible. If it does happen to be present in Ser-Pro-Pro-Arg, its contribution to the CD must be below 215 mμ.

Changing the solvent from water to 90% dioxane has only a small effect on the CD of Pro-Phe-Arg (curve c in Figure 6A).

[3] That the positive, 223-mμ CD band of Arg-Pro-Pro and Arg-Pro-Pro-Gly is in fact associated with the Arg-Pro-Pro sequence *per se* derives from the observation that Pro-Pro (Figure 17 in Madison and Schellman, 1970a), Arg-Pro (our Figure 5A), and Arg-Pro-NH$_2$ (crude preparation) all show strongly negative CD down to 210 mμ.

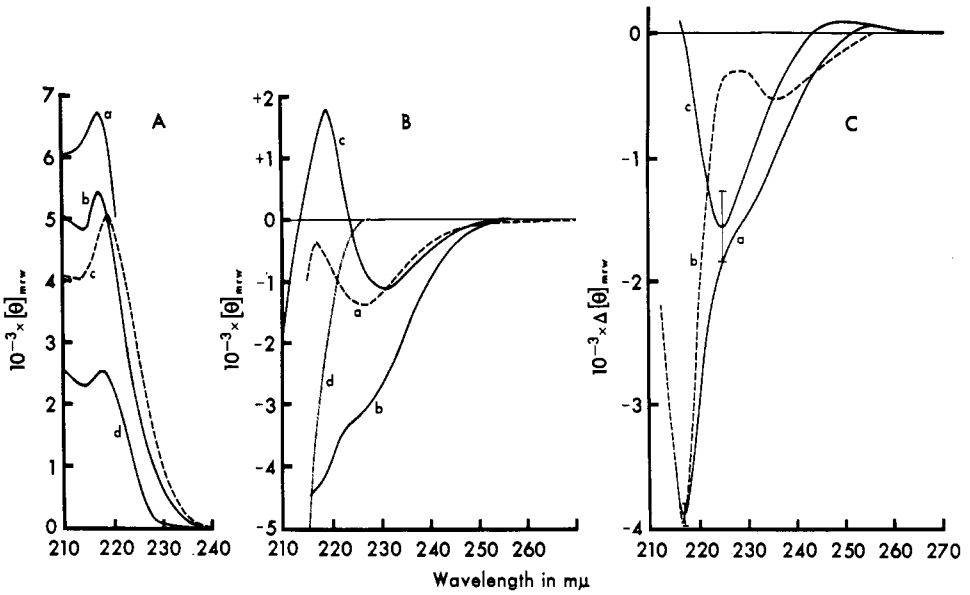


FIGURE 6: The CD behavior of the C-terminal peptide fragments of bradykinin. A: CD spectrum of Pro-Phe-Arg; curves a, b, and d in water at pH 3.95 and 10, 27, and 79°, respectively; curve c, in 90% dioxane–water. B: curve a, Ser-Pro-Phe-Arg in water (pH 4.02); curve b, Ser-Pro-Phe-Arg, in 90% dioxane–water; curve c, Phe-Ser-Pro-Phe-Arg in water (pH 4.12); curve d, Ser-Pro-Pro-Arg in water (pH 3.68 and 4.04). C: CD difference spectra: curve a, Ser-Pro-Phe-Arg, produced by 90% dioxane referred to water (pH 4.02); curve b, Gly-Phe-Gly, 90% dioxane–water mixture containing 4×10^{-4} M HCl referred to 2×10^{-2} M HCl (see footnote 2 in Cann, 1972) normalized such that the value of $\Delta[\theta]_{mrw}$ at 216 mμ is the same as shown in curve a; curve c, difference between curves a and b.

In contrast, it has a major effect on Ser-Pro-Phe-Arg whose spectrum in 90% dioxane (curve b in Figure 6B) is a composite of at least two bands. The difference spectrum (curve a in Figure 6C) shows a negative band at 217 mμ implicating the Phe residue, and a strong shoulder at about 228 mμ. Comparison with the normalized difference spectrum of Gly-Phe-Gly (curve b) or acetylphenylalanine amide suggests that the shoulder has its origin in the Pro residue. Thus, the rather large difference between the two spectra (curve c) is a negative band centered at 225 mμ. The conclusion seems justified that, whereas in water a 3→1 hydrogen bond bridges Pro[2] of Ser-Pro-Phe-Arg, in 90% dioxane the configuration of the peptide involves two 3→1 hydrogen bonds, one bridging Pro[2] and the other Phe[3] as in a 2_7 ribbon or one turn of a 2.2_7 helix (Dickerson and Geis, 1969). Finally, we note the similarities between the interpretations of the effect of dioxane on the CD of Ser-Pro-Phe-Arg, bradykinin, and [Leu[5]]-bradykinin.

Throughout the foregoing presentation of results stress has been placed on 3→1 hydrogen bonds. It is understood, however, that a residue need not spend all of its time internally hydrogen bonded. In other words, the secondary structure of a given peptide should be viewed as a time average of two or more rapidly interconverting structures—disordered and partially ordered. This view is underscored by the following description of the effect of temperature on the CD.

Effect of Temperature and Salts. Both bradykinin and Ser-Pro-Phe-Arg undergo a thermal transition in water or buffer as revealed by large and progressive changes in their CD spectra when the temperature is raised[2] from 10 to 80°. As illustrated in Figures 7A and 8A these spectral changes consist of a strong intensification accompanied by a small blue shift of the negative 234-mμ band (227 mμ in the case of Ser-Pro-Phe-Arg) and a concomitant diminution of the positive 221-mμ band (218 mμ in case of Ser-Pro-Phe-Arg and evidenced only at 10°). The transition is virtually complete at 80° and is reversible. Thus, for example, a bradykinin solution, which had been incubated for 15 min at 65.5°, then chilled to 0° and rewarmed to 27°, gave the same CD spectrum as bradykinin never exposed to either extreme of temperature.

Both sets of spectra exhibit an isosellipticity point. This fact permits quantitation of the transition in terms of two states of that part of the configuration of the peptide molecule which is responsible for the CD band. For the two-state transition, $A \rightleftharpoons B$, the equilibrium constant is given by

$$K = \frac{[\theta] - [\theta]_A}{[\theta]_B - [\theta]} \quad (1)$$

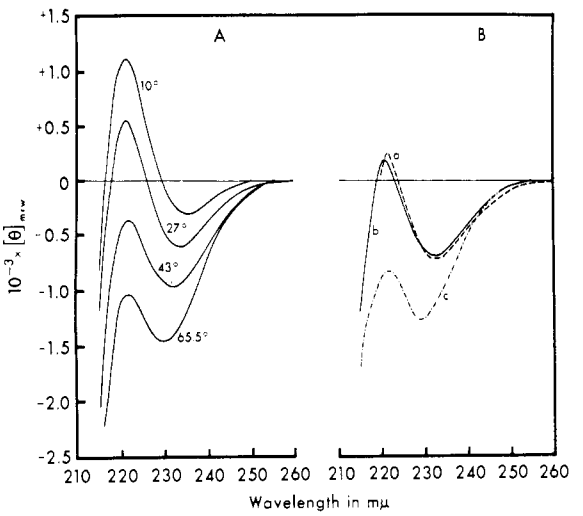


FIGURE 7: Effect of temperature and type of salt on the CD of bradykinin. A: CD spectra in water (pH 4.01) at indicated temperatures; isosellipticity point at 207.5 mμ, $[\theta]_{mrw}$ −6300°; the effect of temperature on the CD in 0.01 M sodium acetate buffer (pH 4.00) and in 0.03 M potassium phosphate buffer (pH 7.18) was the same within experimental error as in water (pH 4.01). B: CD spectra in 1 M $(NH_4)_2SO_4$ (curve a), 1 M NaCl (curve b), and 1 M $NaClO_4$ (curve c) (pH 4.03); the spectrum in 1 M NaBr is intermediate between NaCl and $NaClO_4$ but is not shown because this experiment was made at pH 7.4.

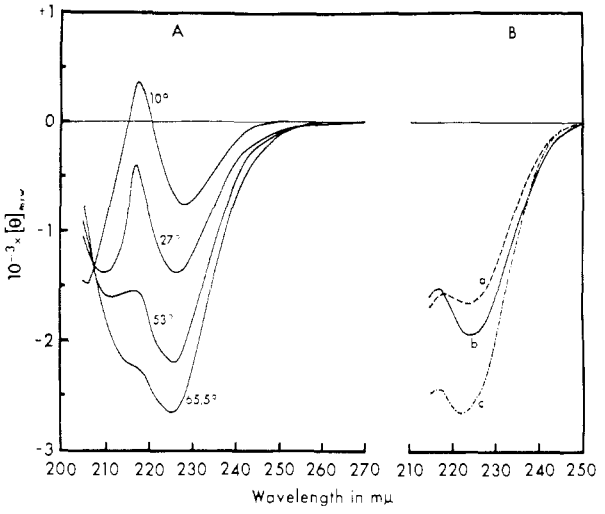


FIGURE 8: Effect of temperature and type of salt on the CD of Ser-Pro-Phe-Arg: A, CD spectra in water (pH 4.02) at indicated temperatures; B, CD spectra in 1 M $(NH_4)_2SO_4$ (curve a), 1 M NaCl (curve b), and 1 M $NaClO_4$ (curve c) (pH 4.04).

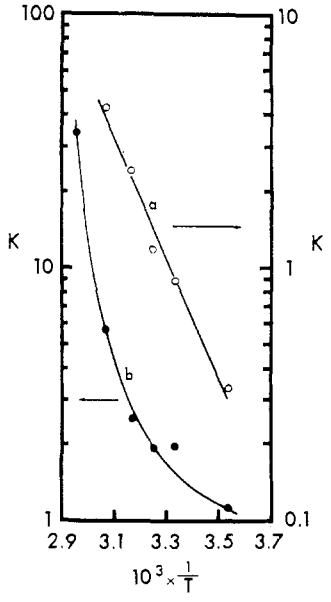


FIGURE 9: van't Hoff plot of logarithm of equilibrium constant (K) against reciprocal of the absolute temperature ($1/T$): a, bradykinin; b, Ser-Pro-Phe-Arg.

where $[\theta]$ is the observed ellipticity at 230 mμ in the case of bradykinin and 225 mμ for Ser-Pro-Phe-Arg; and the subscripts A and B designate initial and final states. The value of $[\theta]_B$ was taken as the value of $[\theta]$ at 80°. $[\theta]_A$ is the low temperature limit of the ellipticity which, unfortunately, could not be realized experimentally because spectral measurements below 10° are not technically possible with our instrumentation. Although $[\theta]$ for bradykinin at 10° is only slightly negative, $[\theta]_A$ cannot be taken to be zero because $[\theta]$ is still increasing algebraically at the rate of about 200 (deg cm²)/dmol for every 10° drop in temperature. Accordingly, $[\theta]_A$ must be positive; and, in fact, inspection of the spectra presented in Figure 7 indicates that at sufficiently low temperature the peptide would not show the 234-mμ band but would show the 221-mμ band to which both the two Phe residues and the N-terminal sequence, Arg-Pro-Pro, evidently contribute. In that event, the simplest procedure for assigning a value to $[\theta]_A$ might be to take the appropriately weighted sum of the ellipticities of the peptide fragments, Arg-Pro-Pro and Pro-Phe-Arg, neither of which exhibit the negative 234-mμ band. As shown in Figures 5B and 6A, the ellipticities of both fragments have a significant positive value at 230 mμ which is temperature dependent. This in itself is not particularly difficult to handle except for our limited knowledge as to the additivity of the spectra. As will become clear shortly, the latter is not a trivial consideration and dictated our final choice of $[\theta]_A$ as the weighted sum of the ellipticities at 230 mμ of the peptide fragments Arg-Pro-Pro-Gly-Phe (weight of 5/9) and Pro-Phe-Arg (weight of 3/9) at 27°. The resulting value[4] of $[\theta]_A$ is 400 (deg cm²)/dmol. Similar reasoning was applied to Ser-Pro-Phe-Arg for which $[\theta]_A$ was taken to be either (1) the value of $^3/_4$ $[\theta]_{mrw}$ for Pro-Phe-Arg at each temperature of interest; (2) its value at 27°, or (3) its value averaged over the entire temperature range. Each gave virtually the same results.[4]

The van't Hoff plots of log K against $1/T$ are presented in Figure 9. The plot for bradykinin is approximately linear and yields $\Delta H^\circ = 10.8$ kcal mol^{-1} and $\Delta S^\circ = 36$ eu at 27°. The plot for Ser-Pro-Phe-Arg is nonlinear and the large positive values of ΔH°, ΔS° and ΔC_p derived therefrom increase with increasing temperature: ΔH° increases from about 2.5 to 50 kcal mol^{-1} on going from 10 to 63°; ΔS°, from 8 to 150 eu; and ΔC_p, from 170 to almost 2000 cal deg^{-1} mol^{-1}. At 37°, for example, $\Delta H^\circ = 7$ kcal mol^{-1}; $\Delta S^\circ = 24$ eu; and $\Delta C_p = 400$ cal deg^{-1} mol^{-1}. The change in heat capacity is particularly striking in view of the fact that the spectra show an isosellipticity point. This seeming paradox is understood in statistical mechanical terms as follows: the heat capacity is determined by the redistribution of the occupancy of the many states of the system as a whole with changing temperature. However, all of the states of the system fall into only two classes insofar as the CD band is concerned—that is the meaning of the isosellipticity point. The relative occupancy of the two classes of states at any temperature depends on their relative distribution in energy. The change in heat capacity for the CD transition means that the density of the class of states showing the CD band increases more rapidly with increasing energy than does the density of the states of the other class.

The magnitude of the thermodynamic functions, which is reminiscent of the conformational transitions shown by proteins, is interpreted to mean that the structure of water plays a dominant role in determining the configuration of bradykinin and Ser-Pro-Phe-Arg. That being the case, one would predict salts to exert a lyotropic effect on the peptide configuration (von Hippel and Schleich, 1969) which, in turn, would be reflected in the CD. Thus, for example, at 27° a high concentration of $NaClO_4$, which is a breaker of water structure, should affect the CD spectrum in much the same way as raising the temperature. In contrast, the structure-maker, $(NH_4)_2SO_4$, should give an effect relative to NaCl analogous to lowering the temperature. These lyotropic effects will, of course, be modulated by possible binding of anions to the positively charged Arg residues. As shown in Figures 7B and 8B salts act

[4] This assignment of $[\theta]_A$ for bradykinin is a conservative one. Thus, if $[\theta]_A$ were to be incorrectly assigned the value of zero, the general conclusions would still be the same: the van't Hoff plot would be linear but of greater positive shape so that ΔH° and ΔS° would be overestimated by about 70%. The same holds for Ser-Pro-Phe-Arg, which gives a nonlinear van't Hoff plot for $[\theta]_A = 0$ (or even -300) as well as for the value of about 1100 (deg cm²)/dmol estimated from the ellipticity of Pro-Phe-Arg.

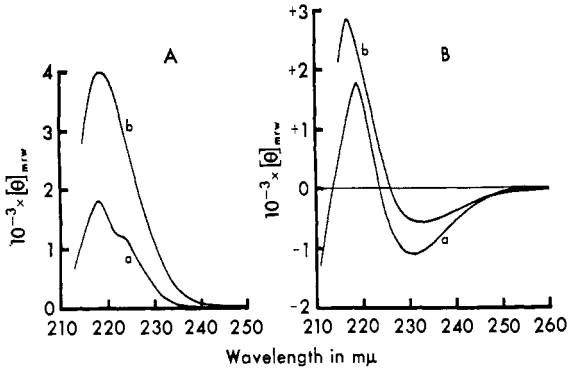


FIGURE 10: Nonadditivity of CD spectra. A: curve a, observed spectrum of Arg-Pro-Pro-Gly-Phe in water; curve b, weighted sum of the spectra of Arg-Pro-Pro-Gly and acetylphenylalanine amide. B: curve a, observed spectrum of Phe-Ser-Pro-Phe-Arg; curve b, weighted sum of the spectra of Ser-Pro-Phe-Arg and acetylphenylalanine amide.

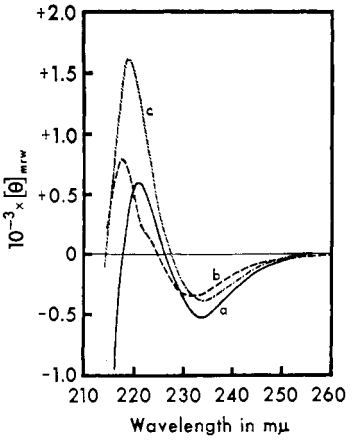


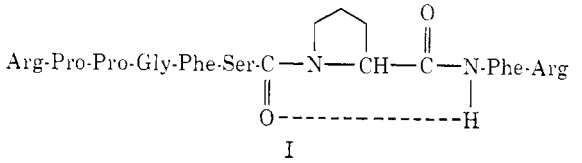
FIGURE 11: Attempted simulation of the CD spectrum of bradykinin in water: curve a, observed spectrum; curve b, weighted sum of the spectra of Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg; curve c, weighted sum of the spectra of Arg-Pro-Pro-Gly and Phe-Ser-Pro-Phe-Arg.

as anticipated, their order of increasing effect on the spectra being $(NH_4)_2SO \leqslant NaCl < NaBr < NaClO_4$ which is recognized as a Hofmeiseter series.

Finally, we note that the values of $\Delta H°$ and $\Delta S°$ are an order of magnitude greater than those (Torchia, 1972) for the trans $\rightleftharpoons$ cis interconversion of Gly-Pro peptide bonds in aqueous solutions of poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly). Also, the CD spectrum of the model compound, *N*-acetyl-L-proline *N'*,*N'*-diisopropylamide, which exists as 50% cis isomer in water, shows a negative band at 210 mμ (Madison and Schellman, 1970a) which is considerably further into the blue than the 227–234-mμ band of bradykinin and Ser-Pro-Phe-Arg. When taken together, these observations seem to eliminate the possibility that the 227–234-mμ band might be due to a cis Ser-Pro bond.

Discussion

It is evident from the results presented above that the secondary structure of bradykinin and its analogs in aqueous solution has some order due to an internally hydrogen-bonded Pro^7 residue



I

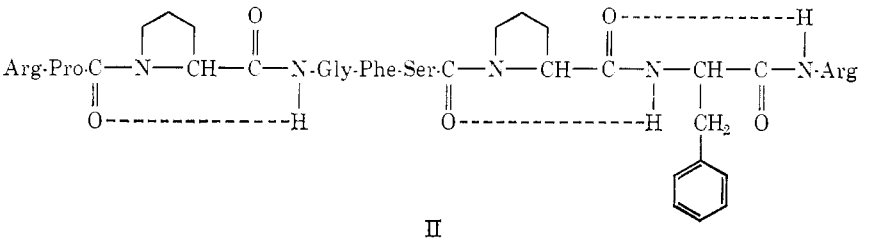
Conversely, the negative 234-mμ CD band of bradykinin is assigned to this hydrogen-bonded configuration. The positive 221-mμ band is evidently the composite of bands due to two chromophores, the 217-mμ band characteristic of the Phe residues overlying the 222-mμ band of the N-terminal sequence, Arg-Pro-Pro.

These assignments derive from comparison of the CD behavior of bradykinin and its analogs with that of the peptide fragments, but quantitative conclusions such as the relative contributions of Phe and Arg-Pro-Pro to the 221-mμ band are precluded by the nonadditivity of the spectra. Thus, for example, the spectrum of Arg-Pro-Pro-Gly-Phe cannot be simulated by the weighted addition of the spectra of Arg-Pro-Pro-Gly and acetylphenylalanine amide (Figure 10A); and the agreement between observed and calculated spectra is not greatly improved by using the 30% less-intense CD of phenylalanine at pH 2.5 and 10.1 (Beychok, 1967) instead of acetylphenylalanine amide. In some way the CD of Phe is strongly suppressed by residue interactions, as well as charge interactions, in the pentapeptide. A different situation obtains in the case of the C-terminal fragment, Phe-Ser-Pro-Phe-Arg, in which there seems to be some kind of interplay between Phe^1 and Pro^3 (Figure 10B). Strikingly similar effects thwart attempts at simulation of the spectrum of bradykinin itself by the weighted addition of the spectra of either Arg-Pro-Pro-Gly and Phe-Ser-Pro-Phe-Arg or Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg (Figure 11).

Returning to the secondary structure of bradykinin, we envision the peptide, which is quite hydrophobic in nature, as being encompassed by ordered water structures such that at 10°, for example, the molecule spends about 25% of its time in the intramolecularly hydrogen-bonded conformation I and the remainder of its time in a disordered conformation. In the disordered conformation the C=O and NH groups of the Ser^6-Pro^7 and Pro^7-Phe^8 peptide bonds, respectively, are hydrogen bonded to water. As the temperature is raised the ordered water structures are gradually disrupted ("melted"). Concomitantly, the peptide spends a progressively greater fraction of its time internally hydrogen bonded until at 80° it spends all of its time in this conformation. That is to say, as the temperature is increased the free energy of formation of the hydrogen bond bridging the Pro^7 residue (C=O · · · HN) becomes progressively more favorable than the free energy of formation of C=O · · · H_2O and H_2O · · · HN hydrogen bonds, the driving force being the entropy of "melting" of the ordered water structures. Similarly, breakers of water structure such as $NaClO_4$ favor the hydrogen-bonded conformation of bradykinin at constant temperature. The same considerations apply to the C-terminal fragment, Ser-Pro-Phe-Arg.

It might at first seem strange that an increase in the ordered conformation of the peptide occurs on heating. This is due, however, to the fact that there is competitive interaction between the water molecules themselves, the different parts of the peptide, and water and different parts of the peptide. Therefore, the outcome of this complex, competitive situation is not readily predictable when the temperature is changed.

An alternative explanation crosses one's mind, namely that



II

disruption of ordered water structure permits increased rotation of the aromatic side chain of Phe and/or internal rotation in the Arg-Pro-Pro sequence with concomitant decrease in their positive CD (Kauzmann *et al.*, 1940) and thus an apparent enhancement of the negative 234-mμ band which might be inherently temperature insensitive. The CD of both Pro-Phe-Arg and Arg-Pro-Pro is, in fact, temperature dependent (Figures 5B and 6A), but this is not an important effect in the region of 234 mμ, and in the case of bradykinin can account at most for only 20% of the enhanced CD over the temperature range, 10–80°. Moreover, this interpretation is inconsistent with the isosellipticity point shown by the spectra and difficult to reconcile with the effect of dioxane on the 234-mμ band.

Bradykinin and its analogs possess considerable conformational freedom and can assume different configurations in response to changes in the polarity of the solvent in much the same way as do acetylamino acid amides. We tentatively propose that in 90% dioxane–water mixture bradykinin has the hydrogen-bonded configuration II in which the sequence of two hydrogen bonds bridging Pro^7 and Phe^8 constitute a segment of a 2_7 ribbon or 2.2_7 helix.

Conformation II was arrived at by comparison of the CD of bradykinin, its analogs, and its fragments in 90% dioxane; and here too one finds evidence for residue interactions. Thus, the CD difference spectra of bradykinin and [Leu^5]-bradykinin produced by 90% dioxane referred to water are bimodal (Figures 2 and 4A). The two bands are interpreted in terms of internally hydrogen-bonded Phe and Pro residues which, in turn, is consistent with our interpretation of the difference spectrum of Ser-Pro-Phe-Arg, Figure 6C. In contrast, the difference spectrum of [Leu^8]-bradykinin shows a single band which is interpreted in terms of an internally hydrogen-bonded Pro. Evidently, in bradykinin a 3→1 hydrogen bond can form across a Phe only when that residue is located in the 8th position of the sequence where it follows a Pro. Such interactions have important implications not only for theoretical prediction of peptide configuration from first principles but also for understanding the relationship of structure to function.

Finally, the results of this investigation suggest that the adoption of a more highly ordered configuration of bradykinin in a hydrophobic environment may have implications for its interaction with cell membranes. It would be interesting to see if interaction of bradykinin with receptors would indeed produce spectral evidence of similar conformational changes.

References

Beychok, S. (1967), *in* Poly-α-Amino Acids, Peptide Models for Conformational Studies, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, Chapter 7.

Brady, A. H., Ryan, J. W., and Stewart, J. M. (1971), *Biochem. J. 121*, 179.

Cann, J. R. (1972), *Biochemistry 11*, 2654.

Dickerson, R. E., and Geis, I. (1969), The Structure and Action of Proteins, New York, N. Y., Harper & Row, Chapter 2.

Kaiser, E., Colecott, R. L., Bossinger, C. D., and Cook, P. I. (1970), *Anal. Biochem. 34*, 595.

Kauzmann, W. J., Walter, J. E., and Eyring, H. (1940), *Chem. Rev. 26*, 339.

Lewis, P. N., Momany, F. A., and Scheraga, H. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2293.

Madison, V., and Schellman, J. (1970a), *Biopolymers 9*, 511.

Madison, V., and Schellman, J. (1970b), *Biopolymers 9*, 569.

Merrifield, R. B., Stewart, J. M., and Jernberg, N. (1966), *Anal. Chem. 38*, 1905.

Pietta, P. G., and Marshall, G. R. (1970), *Chem. Commun.*, 650.

Ramachandran, G. N., Venkatachalam, D. M., and Krimm, S. (1966), *Biophys. J. 6*, 849.

Schroeder, W. A. (1967), *Methods Enzymol. 11*, 351.

Stewart, J. M., and Young, J. D. (1969), Solid-Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.

Torchia, D. A. (1972), *Biochemistry 11*, 1462.

Tsuboi, M., Shimanorechi, T., and Mizushima, S. (1959), *J. Amer. Chem. Soc. 81*, 1406.

Venkatachalam, C. M. (1968), *Biopolymers 6*, 1425.

von Hippel, P. H., and Schleich, T. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, Chapter 6.