Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry 5*, 2681.

Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.

Traube, J. (1899), *Samml. Chem. Chem. Tech. Vorträge 4*, 255.

Development of Ordered Structures in Sequential Copolypeptides Containing L-Proline and γ-Hydroxy-L-proline*

Wayne L. Mattice† and Leo Mandelkern‡

ABSTRACT: The conformational properties of poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Pro-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) have been investigated by measuring the circular dichroism in water, ethylene glycol–water (2:1, v/v), and trifluoroethanol as a function of temperature and by determining the intrinsic viscosity in water over the range 5–70°.

It has been possible to demonstrate the formation of ordered structures for poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) under suitable conditions. Poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) are not ordered under the conditions studied. The ordered structures for poly(Pro-Ala) and poly(Hyp-Gly) are suggested to be similar to poly-L-proline form II. In poly(Pro-Ala) this conformation results from severe steric restraints to rotation about the dihedral angles ϕ and ψ. Puckering of the pyrrolidine ring allows intrachain hydrogen bonding between the hydroxyl group and the carbonyl oxygen on the second preceding residue to stabilize a similar conformation in poly(Hyp-Gly). A model for the ordered state of poly(Gly-Gly-Hyp-Gly) is not as clear but could involve an associated structure with some similarities to the known ordered state of poly(Pro-Gly-Gly).

The pyrrolidine ring in L-proline and γ-hydroxy-L-proline limits the nitrogen–α-carbon rotational angle, ϕ (Edsall *et al.*, 1966a–c), to about 102–122° (Donohue and Trueblood, 1952; Mathieson and Welsh, 1952; Cowan and McGavin, 1955; Leung and Marsh, 1958; Sasisekharan, 1959a,b), restricts the rotational freedom of the preceding residue in the chain (DeSantis *et al.*, 1965; Schimmel and Flory, 1967, 1968; Hopfinger and Walton, 1969; Holzwarth and Chandrasekaran, 1969; Madison and Schellman, 1970a,b), and does not allow the peptide bond to function as a proton donor in hydrogen-bond formation. In the solid state poly-L-proline form I exists as a right-handed helix with cis peptide bonds (Traub and Shmueli, 1963), and poly-L-proline form II forms a left-handed helix with trans peptide bonds (Cowan and McGavin, 1955; Sasisekharan, 1959a). In poly(γ-hydroxy-L-proline) A the chain conformation is similar to that of poly-L-proline form II, but in addition all possible intermolecular hydrogen bonds are formed between the hydroxyl groups and carbonyl oxygen atoms (Sasisekharan, 1959b). Polyglycine can form an ordered structure closely related to poly-L-proline form II and poly(γ-hydroxy-L-proline) A, but in polyglycine form II right- and left-handed helices are equally probable and all possible intermolecular hydrogen bonds are formed between the peptide units.

The conformational properties of a large number of sequential copolypeptides containing glycine and either L-proline or γ-hydroxy-L-proline have been reviewed (Harrington *et al.*, 1966; Andreeva *et al.*, 1967; Carver and Blout, 1967; Ramachandran, 1967; Venkatachalam and Ramachandran, 1969). The major emphasis has been on sequential copolypeptides containing glycine at every third residue since this periodicity occurs throughout large portions of the collagen chain (Schroeder *et al.*, 1954; Kang *et al.*, 1967; Bensusan, 1969; Butler, 1970; Kang and Gross, 1970). The proposed structures for the ordered portion of collagen also require that every third residue be glycine (Ramachandran and Kartha, 1955; Rich and Crick, 1955, 1961; Ramachandran and Sasisekharan, 1965; Ramachandran *et al.*, 1968). It has been shown that in the solid state poly(Pro-Gly-Pro) forms a collagen II type structure (Traub and Yonath, 1966; Yonath and Traub, 1969) and that several copolyhexapeptides with glycine at every third residue have a similar conformation (Segal *et al.*, 1969). Poly(Pro-Gly-Gly), however, forms a double-layered sheet in which each chain has a conformation similar to that of poly-L-proline form II and where there are two interchain hydrogen bonds per tripeptide unit (Traub, 1969). In solution it has been possible to demonstrate a heat-induced cooperative structural transition in poly(Pro-Gly-Pro) (Engel *et al.*, 1966). Subsequently it was found that the formation of the disordered form in monodisperse poly(Pro-Pro-Gly) is accompanied by a threefold reduction in molecular weight (Kobayashi *et al.*, 1970). Ordered conformations in solution at or near room temperature have also been detected in poly(Gly-Pro-Gly) (Oriel and Blout, 1966) and in several polyhexapeptides containing glycine at every third residue (Segal, 1969). Although poly(Gly-Pro-Ala) adopts a statistical conformation at room temperature, an ordered conformation is developed at low temperatures in ethylene glycol–water (2:1, v/v) (Brown *et al.*, 1969).

In the present work the conformational properties, in dilute solution, of five sequential copolypeptides containing L-proline or γ-hydroxy-L-proline are reported. Although four of

* From the Department of Chemistry and Institute of Molecular Biophysics, The Florida State University, Tallahassee, Florida 32306. *Received September 30, 1970.* This work was supported by a contract with the Division of Biology and Medicine, Atomic Energy Commission.

† Recipient of Public Health Service Postdoctoral Fellowship 5-F02-GM-41,377-02 from the National Institute of General Medical Sciences.

‡ To whom to address correspondence.

TABLE I: Average Number of Amino Acid Residues in the Sequential Copolypeptides.[a]

Sequential Copolypeptide	No. Av	Wt Av	Wt Av/ No. Av
Poly(Pro-Gly)	104 ± 5	171 ± 4	1.66 ± 0.12
Poly(Hyp-Gly)	102 ± 4	120 ± 4	1.18 ± 0.08
Poly(Gly-Gly-Pro-Gly)	73 ± 8	100 ± 3	1.39 ± 0.19
Poly(Gly-Gly-Hyp-Gly)	132 ± 13	229 ± 6	1.75 ± 0.21
Poly(Pro-Ala)	30 ± 4	37 ± 1	1.26 ± 0.19

[a] Calculated from the molecular weights reported by Mattice and Mandelkern (1971b).

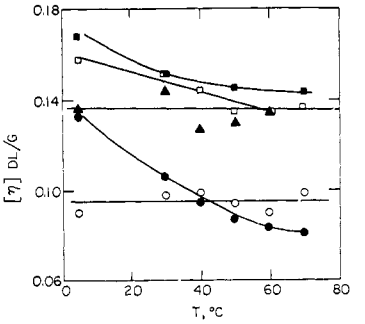

FIGURE 1: Intrinsic viscosity of poly(Pro-Ala) (●), poly(Pro-Gly) (□), poly(Hyp-Gly) (■), poly(Gly-Gly-Pro-Gly) (○), and poly(Gly-Gly-Hyp-Gly) (▲) in water.

these copolymers contain glycine, this unit is not located at every third residue so that the collagen triple helix is not an expected structure. In addition, we also have the opportunity of assessing and comparing the influence of the two pyrrolidine residues in chain molecules of otherwise identical composition. A preliminary report of this work has been presented (Mattice and Mandelkern, 1970c).

Materials and Methods

Sequential Copolypeptides. Poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), poly(Gly-Gly-Pro-Gly), and poly(Pro-Ala) were kindly supplied by Professor D. F. DeTar. They were synthesized by the *p*-nitrophenyl ester method (DeTar and Vajda, 1967; DeTar *et al.*, 1967a,b) which avoids racemization (DeTar and Estrin, 1966). The polymerization was carried out with the *p*-nitrophenyl ester of the indicated unit except for poly(Pro-Ala), which was prepared from the *p*-nitrophenyl ester of L-prolyl-L-alanyl-L-prolyl-L-alanine. The sequential copolypeptides were purified by exhaustive dialysis. Solutions were prepared by weight using copolypeptides which had been dried under vacuum using a Dry Ice–butoxyethanol trap.

Viscosity. Flow times were measured in Cannon-Ubbelohde semimicro dilutions viscometers, in baths controlled to ±0.03°. In order to prevent erroneous results due to possible evaporation at high temperature or condensation at low temperature, the flow times were first measured at 30°, then at one or two other temperatures, and then at 30° again. Within the experimental error, the viscosities were found to be unchanged.

Circular dichroism was measured using a Durrum-Jasco recording spectropolarimeter, Model ORD/UV-5, equipped with a circular dichroism attachment and utilizing an expanded scale. The instrument had been calibrated with 10-camphorsulfonic acid as described by DeTar (1969). Light paths varied from 0.1 to 10 mm. Temperature control above 0° was attained by circulating water from a constant-temperature bath. The relationship between the temperature in the bath and the cell was determined by measuring the latter with a thermocouple. Temperature control down to −25° was attained by circulating coolant from a thermostated ethylene glycol–water bath. Lower temperatures were attained by circulating alcohol cooled by an unthermostated Dry Ice–alcohol bath. The temperature in the cell was measured immediately after the completion of a spectrum using a thermocouple at temperatures below 0°. Resolution of the circular dichroism spectra into Gaussian bands was accomplished using computer program CDORD, written by Professor D. F. DeTar, in the manner previously described (Mattice and Mandelkern, 1970a). Resolution was accomplished using wave numbers. The spectra were not corrected for the refractive index of the solvent.

The circular dichroism for all copolypeptides, including those which contain glycine, is reported as the average $\Delta\epsilon$ per peptide bond.

Results

Molecular Weights. A detailed description of the determination of the weight- and number-average molecular weights of the samples studied is presented in the following paper (Mattice and Mandelkern, 1971b). For present purposes the average numbers of amino acid residues of each copolymer are listed in Table I. The glycine-containing sequential copolypeptides all contain at least 100 amino acid residues on the weight-average basis. Poly(Pro-Ala) has a much lower degree of polymerization. The molecular weight distributions are all narrower than the most probable distribution.

Intrinsic Viscosities. The intrinsic viscosities of the sequential copolypeptides in water are shown as a function of temperature in the range 5–70° in Figure 1. The intrinsic viscosities of poly(Gly-Gly-Pro-Gly) and poly(Gly-Gly-Hyp-Gly) show no discernible trend with temperature in this range. Both poly(Pro-Gly) and poly(Hyp-Gly) exhibit a slight increase in intrinsic viscosity on cooling, with $d \ln [\eta]/dT = -0.002$ deg^{-1} in each case. At 70° poly(Pro-Ala) exhibits the lowest intrinsic viscosity of all of the sequential copolypeptides. However, on cooling the intrinsic viscosity of this polymer increases markedly, the major increase occurring below 40°. At 5° it is greater than that of poly(Gly-Gly-Pro-Gly) and comparable to the result obtained with poly(Gly-Gly-Hyp-Gly), even though poly(Pro-Ala) contains by far the smaller number of peptide bonds.

At 30°, with trifluoroethanol as solvent, there is an increase of 40–50% in the intrinsic viscosity of poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly). The intrinsic viscosity of poly-L-proline approximately doubles with this change in solvent (Mattice and Mandelkern, 1971a). In contrast, the intrinsic viscosity of poly(Gly-Gly-Hyp-Gly) decreases by 10% on going from water to trifluoroethanol at 30°.

Circular Dichroism. The circular dichroism of the five sequential copolypeptides was determined at various temperatures in water, ethylene glycol–water (2:1, v/v), and trifluoroethanol. At comparable temperatures the spectra were

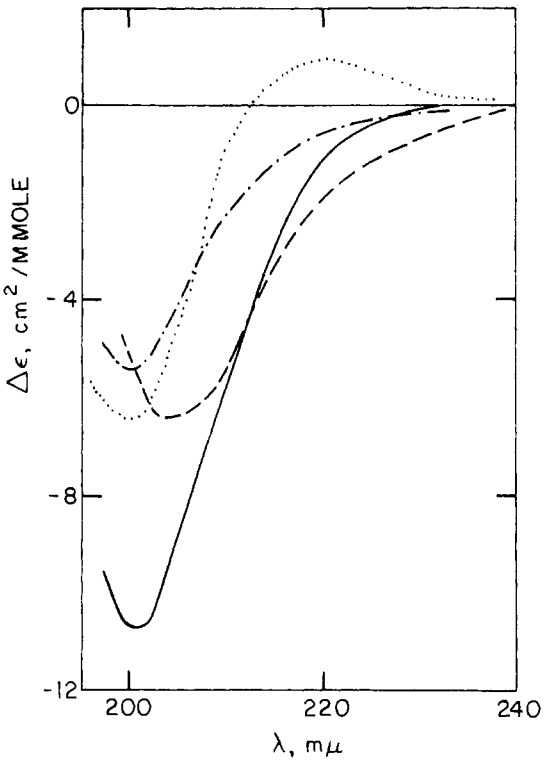

FIGURE 2: Circular dichroism of poly(Pro-Gly) at 50° (----) and −48° (——) and of poly(Hyp-Gly) at 50° (- · -) and −48° (. . . . .) in ethylene glycol–water (2:1, v/v).

usually only slightly altered when the solvent was changed from water to ethylene glycol–water (2:1, v/v). Figure 2 illustrated the effect of temperature on the circular dichroism of poly(Pro-Gly) and poly(Hyp-Gly) in ethylene glycol–water (2:1, v/v). At high temperature both copolypeptides exhibit only negative circular dichroism over the observable range, and the single minimum occurs at 200–204 mμ with a Δε of −5 to −6 cm² per mmole. These spectra are similar to that of heat-denatured collagen, which exhibits only negative circular dichroism with a single minimum at 195–198 mμ with Δε = −4 cm²/mmole (Tiffany and Krimm, 1969). Cooling increases the strength of the minimum in the circular dichroism of poly(Pro-Gly) but no new band is apparent down to temperatures as low as −48°. In contrast, cooling of poly-

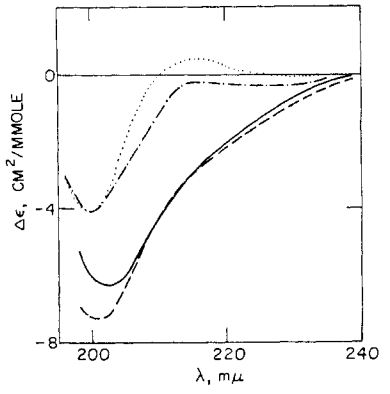

FIGURE 3: Circular dichroism of poly(Pro-Gly) at 30° (----) and −25° (——) and of poly(Hyp-Gly) at 25° (- · -) and −25° (. . . . .) in trifluoroethanol.

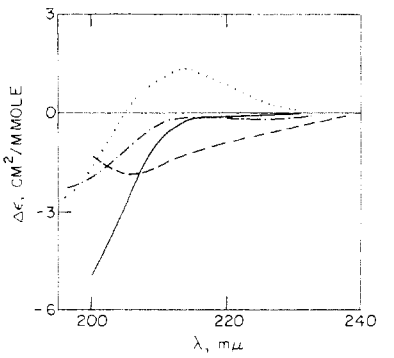

FIGURE 4: Circular dichroism of poly(Gly-Gly-Pro-Gly) at 75° (----) and −45° (——) and of poly(Gly-Gly-Hyp-Gly) at 75° (- · - · -) and −45° (. . . .) in ethylene glycol–water (2:1, v/v).

(Hyp-Gly) results in the development of a positive band which has a maximum at 220 mμ with Δε = +0.9 cm²/mmole at −48°. Poly(Hyp-Gly) exhibits a positive circular dichroism below about 10° in either water or ethylene glycol–water (2:1, v/v).

The circular dichroism of the same two copolypeptides in trifluoroethanol is given in Figure 3. Poly(Hyp-Gly) displays qualitatively similar behavior as in ethylene glycol–water (2:1, v/v). However, the positive peak at −25° is blue shifted, occurring at 216 mμ. A small positive circular dichroism has previously been observed with poly(Hyp-Gly) in trifluoroethanol (D. F. DeTar, personal communication). There is relatively little temperature effect on the circular dichroism of poly(Pro-Gly) in trifluoroethanol.

The circular dichroism of poly(Gly-Gly-Pro-Gly) and poly(Gly-Gly-Hyp-Gly) in ethylene glycol–water (2:1, v/v) is shown in Figure 4. Poly(Gly-Gly-Pro-Gly) exhibits only negative circular dichroism over the accessible spectral range at −45 to +75°. At 75° the minimum is near 204 mμ as was the case with poly(Pro-Gly), but is weaker. In water the minimum occurs at 200 mμ. Poly(Gly-Gly-Hyp-Gly) also exhibits only negative circular dichroism at 75° in ethylene glycol–water (2:1, v/v). As was the case with poly(Hyp-Gly), cooling gives rise to a positive circular dichroism. At −45° the maximum has a magnitude of +1.3 cm²/mmole at 214 mμ with poly(Gly-Gly-Hyp-Gly). Positive circular dichroism is observed below about 50° in ethylene glycol–water (2:1, v/v) and below 25° in water. The minimum in the poly(Gly-Gly-Hyp-Gly) circular dichroism is at about 195 mμ.

Somewhat different results are obtained with these two copolypeptides in trifluoroethanol, as is shown in Figure 5. Poly(Gly-Gly-Hyp-Gly) shows a positive circular dichroism with a maximum of +1.3 cm²/mmole at 212 mμ at −25°. The maximum is reduced only slightly by heating, attaining a value of +0.9 cm²/mmole at 25° and +0.8 cm²/mmole at 50°. Measurement of the spectrum in this region at the boiling point of trifluoroethanol, 74° (Timmermans, 1950), reveals that the circular dichroism is still positive, but a quantitative result cannot be obtained. In contrast to the results obtained in water and ethylene glycol–water (2:1, v/v), poly(Gly-Gly-Hyp-Gly) always exhibits a positive circular dichroism in trifluoroethanol. The positive circular dichroism band exhibited by poly(Gly-Gly-Hyp-Gly) in trifluoroethanol has been previously observed (D. F. DeTar, personal communication). Poly(Gly-Gly-Pro-Gly) exhibits only negative circular dichroism in trifluoroethanol. At −25° there is a local maximum at about 216 mμ with Δε = −0.2 cm²/mmole. It cannot

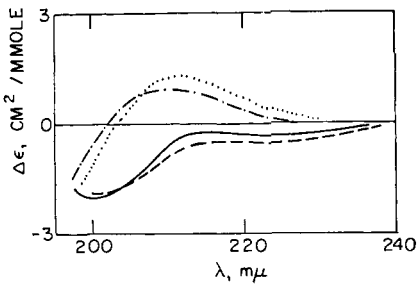

FIGURE 5: Circular dichroism of poly(Gly-Gly-Pro-Gly) at 30° (----) and −25° (——) and of poly(Gly-Gly-Hyp-Gly) at 25° (–·–·) and at −25° (. . . . .) in trifluoroethanol.

be stated whether this is due to the appearance of a positive component in the spectrum or whether it merely signifies the separation of two negative bands.

The circular dichroism of poly(Pro-Ala) is shown in Figure 6. At +75° in ethylene glycol–water (2:1, v/v) only negative circular dichroism is observed, and the single minimum occurs at 205 mμ with $\Delta\epsilon = -5$ cm²/mmole. Cooling results in an intensification and blue shift of the minimum and the appearance of a positive circular dichroism at higher wavelengths. At −42° the maximum is at 224 mμ with $\Delta\epsilon = +2.1$ cm²/mmole. A positive circular dichroism is observed below about 10° in either water or ethylene glycol–water (2:1, v/v). In contrast to the results in water and ethylene glycol–water mixtures, the circular dichroism of poly(Pro-Ala) in trifluoroethanol is negative throughout the entire observable region at both +25 and −25°.

For comparison with the results for the sequential copolypeptides, the circular dichroism of poly-L-proline is only slightly affected by changes in temperature. In trifluoroethanol, poly-L-proline (M_n = 53,000, M_w = 99,000) has a minimum at 206 mμ and a maximum at 227–228 mμ. Changing the temperature from +30 to −25° causes no change in the minimum ($\Delta\epsilon_{min}$ = 20 cm²/mmole) and results in only a very slight increase in the maximum from 1.2 cm²/mmole at 30° to 1.3 cm²/mmole at −25°. Very large changes in temperature have been reported to have little effect on the circular dichroism of poly-L-proline in ethylene glycol–water (2:1, v/v) (Brown *et al.*, 1969). The circular dichroism of guinea pig skin collagen in ethylene glycol–water (2:1, v/v) is also little affected by changes in temperature (Brown *et al.*, 1969).

On the basis of these results, the circular dichroism spectra of the sequential copolypeptides can be divided into two classes. One of these, exemplified by poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly), exhibits only negative circular dichroism under all conditions. On the other hand, poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) develop a positive circular dichroism at 214–224 mμ at low temperatures in water and ethylene glycol–water (2:1, v/v). However, changing the solvent from ethylene glycol–water (2:1, v/v) to trifluoroethanol yields quite different results for these three polymers. There is an increase in the temperature at which positive circular dichroism is observed with poly(Gly-Gly-Hyp-Gly), while there is little change with poly(Hyp-Gly). With poly(Pro-Ala), however, positive circular dichroism is never observed in trifluoroethanol.

The features observed at the lowest temperature studied in ethylene glycol–water (2:1, v/v) and trifluoroethanol are listed in Table II. Spectra reported for poly-L-proline, poly(γ-hydroxy-L-proline), and various collagens are also tabulated. The λ_{max} and $\Delta\epsilon_{max}$ for poly(Pro-Ala) and poly(Hyp-Gly) fall in the same range as found for the collagens and homopolypeptides, while λ_{max} for poly(Gly-Gly-Hyp-Gly) is shifted to the blue. The minima observed with the copolypeptides are less intense than those of the collagens and homopolypeptides.

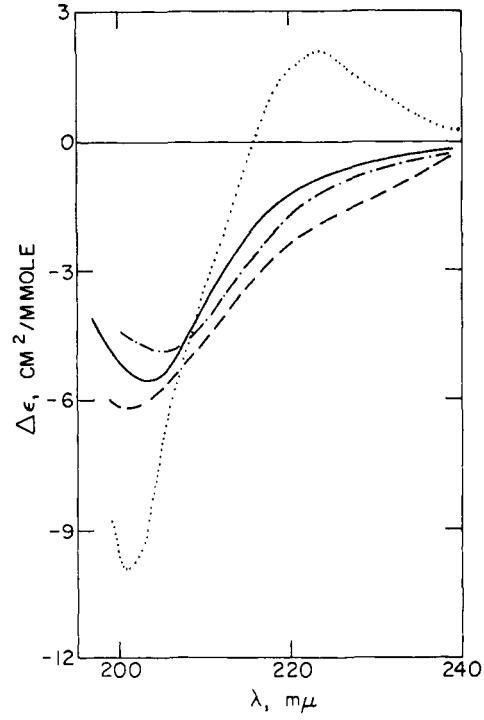

FIGURE 6: Circular dichroism of poly(Pro-Ala) at 25° (----) and at −25° (——) in trifluoroethanol and at 75° (–· –·) and −42° (. . . . .) in ethylene glycol–water (2:1, v/v).

Since the positive band in poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) is overlapped by the adjacent negative band, the magnitude of the circular dichroism at the maximum will not necessarily be a linear function of the observed strength of the positive circular dichroism band. Therefore the spectra were resolved into a positive and negative Gaussian band. This procedure may be an oversimplification for the copolypeptides since the monomer transition energies for the constituent amino acids may differ (Madison and Schellman, 1970b). The theoretical treatment of the circular dichroism of poly-L-proline form II originally used only two bands (Pysh, 1967). More recent theoretical work on a dipeptide model for poly-L-proline has suggested that more than two bands may be present (Madison and Schellman, 1970a,c). While the circular dichroism of this model could be reproduced using two Gaussian bands, it was also possible to obtain fits using three or four Gaussian bands (Madison and Schellman, 1970a,c). The presence of a third band in poly-L-proline has also been suggested experimentally by linear dichroism measurements (Rosenheck *et al.*, 1969). The positive Gaussian band obtained by the resolution procedure may therefore contain contributions from more than one spectral band.

The area under the positive Gaussian band in poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) in water and in ethylene glycol–water (2:1, v/v) is shown as a function of the temperature in Figure 7. Even at the highest temperature studied the spectra cannot be fitted without the use of a small positive Gaussian band. With poly(Pro-Ala) the area

TABLE II: Features in the Circular Dichroism in Order of Decreasing λ_{max}.[a]

Polymer	Solvent	Temp (°C)	λ_{max}	$\Delta\epsilon_{max}$	λ_{crs}	λ_{min}	$\Delta\epsilon_{min}$
Poly-L-proline[b]	Water	30	228	0.5	224	206	−13
Poly-L-proline[c]	EGW	24	228	1.4	222	206	−15
Poly-L-proline	TFE	30	228	1.3	222	206	−20
Poly(γ-hydroxy-L-proline)[d]	Water	30	225	2.2	219	205	−14
Poly(Pro-Ala)	EGW	−42	224	2.1	216	201	−10
Guinea pig skin collagen[e]	EGW	24	220	3.0	212	197	−17
Poly(Hyp-Gly)	EGW	−48	220	0.9	213	201	−6
Ichthyocol collagen[e,f]	0.2 M $MgSO_4$ (pH 7)	15	220	1.8	212	198	−14
Rat-tail collagen[g]	*h*	*h*	219	1.3	214	197	−12
Poly(Hyp-Gly)	TFE	−25	216	0.5	210	200	−4
Poly(Gly-Gly-Hyp-Gly)	EGW	−45	214	1.3	205	~195	−3
Poly(Gly-Gly-Hyp-Gly)	TFE	−25	212	1.3	203	<198	<−2
Poly(Pro-Ala)	TFE	−25				204	−5 to −6
Poly(Pro-Gly)	TFE	−25				202–203	−6
Poly(Pro-Gly)	EGW	−48				200–201	−11
Poly(Gly-Gly-Pro-Gly)	EGW	−45				<200	<−5
Poly(Gly-Gly-Pro-Gly)[i]	TFE	−25				200	−2

[a] The subscripts max, crs, and min refer to the observed maximum, crossover, and minimum, respectively, $\Delta\epsilon$ is the average circular dichroism per peptide bond, λ is the wavelength in millimicrons, EGW is ethylene glycol–water (2:1, v/v), and TFE is trifluoroethanol. [b] Mattice and Mandelkern (1970a). [c] Brown *et al.* (1969). [d] Mattice and Mandelkern (1970b). [e] Madison and Schellman (1970a). [f] Corrected for the refractive index of the solvent. [g] Tiffany and Krimm (1969). [h] Not specified. [i] Another minimum occurs at about 224 mμ with $\Delta\epsilon = -0.3$ cm²/mmole, and there is a local maximum at 216 mμ with $\Delta\epsilon = -0.2$ cm²/mmole.

under the positive Gaussian band, located at 218–220 mμ, begins to increase below about 40°. At −48° in ethylene glycol–water (2:1, v/v) the area has attained a value of 7000 ± 1000 cm/mmole, which is comparable to the area of 5950 ± 1350 and 5250 ± 900 cm per mmole found by the resolution of the circular dichroism spectra obtained with poly-L-proline (Mattice and Mandelkern, 1970a) and poly(γ-hydroxy-L-proline) (Mattice and Mandelkern, 1970b), respectively, in water at 30°.

The area under the positive Gaussian band in poly(Hyp-Gly), located at 214–216 mμ, does not rise appreciably until very low temperatures, attaining a value of 4000 ± 1000 cm/mmole at −48°. The intrinsic viscosity in water changes only slightly with temperature. With poly(Gly-Gly-Hyp-Gly) there is a substantial uncertainty in the area under the positive Gaussian band, located at 218–220 mμ, because the negative peak is not as well defined for this copolypeptide as it is for poly(Pro-Ala) and poly(Hyp-Gly). However, it is apparent that the positive band in poly(Gly-Gly-Hyp-Gly) increases appreciably in area upon cooling, reaching 8500 ± 2500 cm/mmole at −45°. At low temperatures it is larger than that found for poly(Hyp-Gly), and comparable to that of poly(Pro-Ala).

The spectra for poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) cannot be reproduced by a single negative Gaussian band. Several resolutions can be obtained using two Gaussian bands. In each case a large negative Gaussian band is required at about 200 mμ. The second band can be chosen in three ways: (a) a negative band at about 200 mμ with a very large bandwidth, (b) a positive band slightly to the red of the large negative band, or (c) a small negative band located at wavelengths corresponding to the location of the positive band in poly(Hyp-Gly) and poly(Gly-Gly-Hyp-Gly).

The resolved circular dichroism spectra of poly(Pro-Ala) at +25 and −25° in trifluoroethanol confirm the solvent effect suggested by the unresolved spectra. Resolution requires a large negative Gaussian band at 200–203 mμ and a small negative one at 218–220 mμ. The latter is located at about the same position as the positive band in water and ethylene glycol–water (2:1, v/v). At 25°, the circular dichroism of poly(Hyp-Gly) in trifluoroethanol requires a positive band with an area of about 6000 cm/mmole at 212 mμ, despite the fact that no positive circular dichroism is apparent in the directly observed spectra. The resolution of the spectrum for this system at −25° is not as definite. The best fit suggests that the bandwidths are small and that the positive band has an area of only 500 cm/mmole. However, it is also possible to obtain a fit, which yields results only slightly less satisfactory

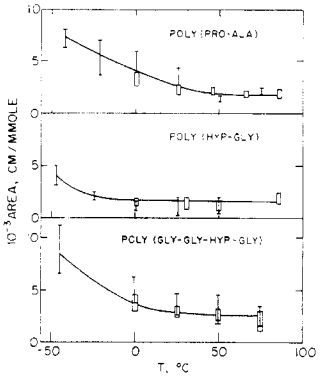

FIGURE 7: Area under the resolved Gaussian band in water (□) and in ethylene glycol-water (2:1, v/v) (I).

with experiment, wherein the band widths are similar to those found at 25°. The positive band has an area of 6000 cm/mmole and is located at 210 mμ. The result indicates the possibility that the positive band found in trifluoroethanol at both −25 and +25° is larger than that found in ethylene glycol–water (2:1, v/v) under comparable conditions. The observed maxima are not more intense because of the greater overlap of the positive and negative bands. The spectra obtained with poly(Gly-Gly-Hyp-Gly) in trifluoroethanol could not be resolved in any meaningful way because of the inability to locate the minimum experimentally (see Figure 5).

Discussion

The observed circular dichroism and the resolved spectra are similar in water and ethylene glycol–water (2:1, v/v) at those temperatures where both solvent systems were studied. The influence of trifluoroethanol as a solvent will be discussed subsequently. Although there are minor changes in the circular dichroism of poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) in ethylene glycol–water (2:1, v/v) and in water upon changing temperature, a positive circular dichroism is not observed under any circumstances. We conclude from these spectral results and the lack of any significant change in the intrinsic viscosity–temperature relations that statistical (random) conformations are maintained for these two copolymers. In contrast the magnitude of the resolved positive band of poly(Pro-Ala) increases below about 40° and correlates with the 50% increase in the intrinsic viscosity observed on cooling from 40 to 5°. These results give strong evidence for the development of an ordered conformation below 40° for this copolypeptide.

With poly(Hyp-Gly) and poly(Gly-Gly-Hyp-Gly) the increase in the magnitude of the resolved positive band also occurs abruptly on cooling. However, for these cases the increase occurs below 0° so that a correlation with the intrinsic viscosity cannot be made. The abruptness with which the spectral changes occur and the similarity of the magnitude of the positive bands with that of poly-L-proline lead to the conclusion that ordered conformations are being developed for these two copolypeptides at low temperatures. We note the major difference in structure and spectral properties that results when γ-hydroxy-L-proline is substituted for L-proline. The magnitude of the resolved positive band for the three copolypeptides which develop ordered structure is similar to that of poly-L-proline. However, it should be made clear that measurements of this type cannot establish the extent of conformational ordering that occurs in a molecule.

In attempting to understand the basis for the difference in the conformational properties of these copolypeptides, it is extremely helpful to make reference to the appropriate conformational energy maps of Brant *et al.* (1967) and Schimmel and Flory (1968). The major features of these calculations have been shown to be correct for they adequately predict the characteristic ratios of these sequential copolypeptides (Mattice and Mandelkern, 1971b).

From these maps we find that the L-proline residue followed by L-alanine has ϕ restricted to about 120° while ψ, the α-carbon–carbonyl carbon bond, may attain values near 125 or 325° (Schimmel and Flory, 1968). The L-proline residue also restricts the rotational freedom of the preceding L-alanine residue, and at room temperature nearly all of the L-alanine residues will have $\phi = 65 + 65°$ and $\psi = 300 \pm 40°$ (Schimmel and Flory, 1968). Cooling will tend to order the rotational angles at the energy minimum. Two possible ordered structures could result, depending upon which minimum was occupied by the ψ of the L-proline residue. The solvent could conceivably play a role in dictating which ψ was chosen by the L-proline residue. Using the minima in the conformational maps of Schimmel and Flory (1968), one structure would have $\phi_{Pro}\psi_{Pro}\phi_{Ala}\psi_{Ala} \cong 120, 125, 100, 300°$ and the other would have $\phi_{Pro}\psi_{Pro}\phi_{Ala}\psi_{Ala} \cong 120, 325, 100, 300°$. The latter combination is very close to $\phi, \psi = 102–104°, 325–326°$ observed for poly-L-proline form II in the solid state (Cowan and McGavin, 1955; Sasisekharan, 1959a), and development of this conformation should lead to optical properties similar to those exhibited by poly-L-proline form II. We observe that the circular dichroism ofpoly (Pro-Ala) becomes similar to that of poly-L-proline upon cooling in water or in ethylene glycol–water (2:1, v/v). As can be seen in Table II, the primary difference is a blue shift of all of the features in the circular dichroism of poly(Pro-Ala). This may be caused by the monomer transitions of the alanine residue occurring at somewhat higher energies than those of the proline residue (Madison and Schellman, 1970b).

The conformational map for glycine followed by L-proline differs from that for L-alanine followed by L-proline in two important ways. The region of low conformational energy covers a much larger area and the conformational energy for any given ϕ, ψ is the same as that for $-\phi, -\psi$ (Schimmel and Flory, 1968). Due to the flexibility and symmetry of the glycine residue, it is thus expected to be more difficult to order poly(Pro-Gly) in comparison with poly(Pro-Ala) if only nearest neighbor interactions are involved. This analysis is consistent with the experimental inability to observe a positive circular dichroism with poly(Pro-Gly) under conditions which yield a positive circular dichroism for poly(Pro-Ala).

In contrast to the above the dipeptide conformational maps do not explain the difference in properties that are observed between poly(Pro-Gly) and poly(Hyp-Gly). It thus becomes necessary to search for other interactions in poly(Hyp-Gly). Since the hydroxyl group participates in intermolecular hydrogen bonds in the crystal structures of γ-hydroxy-L-proline (Donahue and Trueblood, 1952) and poly(γ-hydroxy-L-proline) A (Sasisekharan, 1959b), intermolecular hydrogen bonding *via* the hydroxyl group in solution is one possible mechanism to account for the difference between poly(Pro-Gly) and poly(Hyp-Gly).

It is also possible that the hydroxyl group could be involved in short-range intramolecular hydrogen-bond formation. Inspection of Corey–Pauling–Koltun molecular models shows that the hydroxy group in γ-hydroxy-L-proline cannot participate in interactions with adjacent peptide groups if the pyrrolidine ring is planar. However, the pyrrolidine ring is known to undergo puckering at the γ carbon (Donohue and Trueblood, 1952; Mathieson and Welsh, 1952; Leung and Marsh, 1958). In the crystal structure of γ-hydroxy-L-proline, the γ carbon is puckered by about 0.4 Å out of the plane specified by the other four atoms in the pyrrolidine ring. The puckering occurs in the direction away from the carboxyl group (Donohue and Trueblood, 1952). If the ring is puckered in this direction in poly(Hyp-Gly), and if the ϕ and ψ of the glycine residue preceding the γ-hydroxy-L-proline residue are rotated to about 120 and 300°, respectively, a hydrogen bond forms between the hydroxyl group and the carbonyl oxygen of the preceding γ-hydroxy-L-proline residue. The conformation $\phi, \psi = 120°, 300°$ has an energy which is only 1 kcal above the minimum in the conformational map for glycine followed by L-proline (Schimmel and Flory, 1968). Thus, this conformation can be made plausible on steric

grounds. Hydrogen-bond formation cannot occur when the ϕ and ψ of the glycine residue are rotated to -120 and $-300°$, respectively. Thus the symmetry in the conformational map for glycine is destroyed if this interaction manifests itself. Depending upon which energy minimum is adopted by ψ of the γ-hydroxy-L-proline residue, this could lead to ordering either to $\phi_{Hyp}\psi_{Hyp}\phi_{Gly}\psi_{Gly}$ = 120, 125, 120, 300° or at $\phi_{Hyp}\psi_{Hyp}\phi_{Gly}\psi_{Gly}$ = 120, 325, 120, 300°. The latter is a left-handed helix related to poly-L-proline form II. The formation of this structure at low temperatures is consistent with the circular dichroism pattern at −48° in ethylene glycol–water (2:1, v/v). The blue shift of the spectrum of poly(Hyp-Gly) compared to poly-L-proline and poly(Pro-Ala) might arise because of a higher monomer transition energy for glycine than for L-proline or L-alanine (Madison and Schellman, 1970b).

It is more difficult to propose possible ordered structures for poly(Gly-Gly-Hyp-Gly). Intermolecular hydrogen-bonding interactions are still possible in poly(Gly-Gly-Hyp-Gly) leading to aggregated ordered structures. In addition a short range intramolecular hydrogen bond of the type proposed for poly(Hyp-Gly) is also possible. This bond only fixes the ϕ and ψ for the glycine residue immediately preceding the γ-hydroxy-L-proline. The other two glycine residues would still have identical energies for any ϕ, ψ and $-\phi$, $-\psi$ (Brant *et al.*, 1967). It can be noted that by fixing the ϕ and ψ of the glycine preceding the γ-hydroxy-L-proline at 120 and 300° a polymer somewhat analogous to poly(Gly-Pro-Gly) is created. For both poly(Gly-Pro-Gly) and poly(Gly-Gly-Hyp-Gly) two glycine residues per repeating unit possess considerable rotation about ϕ and ψ. The remaining rotational freedom for poly(Gly-Pro-Gly) is in ψ_{Pro}, while for poly(Gly-Gly-Hyp-Gly) it is in ψ_{Hyp}, if the hydrogen bond between the hydroxyl group and the second preceding carbonyl oxygen atom has been formed. Poly(Gly-Pro-Gly) has been shown to develop an ordered conformation in solution which has been attributed to aggregation (Oriel and Blout, 1966). Poly(Pro-Gly-Gly), which is equivalent to poly(Gly-Pro-Gly), forms a sheet-like ordered structure in the solid state involving interchain hydrogen bonds (Traub, 1969). Although analogies can be seen for the ordered chain structure observed in poly(Gly-Gly-Hyp-Gly) a definitive conclusion in regard to this structure either in solution or the solid state cannot be made.

The circular dichroism spectra show quite different effects of the conformational properties of the copolypeptides when trifluoroethanol is the solvent. The ordered form of poly(Pro-Ala) is no longer observed while the stability of the ordered form of poly(Gly-Gly-Hyp-Gly) is apparently enhanced. These results are consistent with the postulate that the ordering of these two copolymers occurs for different reasons. Ordering of poly(Pro-Ala) occurs because of extreme steric restrictions to rotation. If trifluoroethanol changes the relative energies of the minima at 125° and 325° in the proline conformational map (Schimmel and Flory, 1968) in a manner which favors the former minimum, it would be more difficult for the polymer to attain a conformation similar to poly-L-proline form *II* in trifluoroethanol. Specific solvent effects, including trifluoroethanol and water, on polypeptide rotational potential functions have been demonstrated for poly-L-proline (Mattice and Mandelkern, 1971a). A similar influence of the solvent could be expected for poly(Pro-Ala). Ordering of poly(Gly-Gly-Hyp-Gly) apparently depends on interactions such as hydrogen bonding, and these interactions are favored in trifluoroethanol. The same type of solvent effect has been observed with poly-N^5-(ω-hydroxyethyl)-L-glutamine, which exists at room temperature as a random coil in water and as an α helix in trifluoroethanol (Lotan *et al.*, 1966).

Consistent with these ideas, the intrinsic viscosity of poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) increase about 50% as the solvent is changed from water to trifluoroethanol at 30°. In contrast, under similar conditions the intrinsic viscosity of poly(Gly-Gly-Hyp-Gly) decreases about 10%.

Hydrogenbonding of side chain hydroxyl groups to the peptide backbone has been suggested to occur previously. This type of interaction was invoked to explain the lack of α-helix formation in poly-L-serine (Blout *et al.*, 1960; Bohak and Katchalski, 1963). It has also been suggested that poly-L-serine might form a left-handed α helix stabilized by side-chain backbone hydrogen bonds (Sarathy and Ramachandran, 1968).

We have recently found that poly(Pro-Ala), poly(Hyp-Gly), and poly(Gly-Gly-Hyp-Gly) yield wide-angle X-ray diffraction patterns in the solid state typical of ordered structure (W. L. Mattice, P. Scanlan, and L. Mandelkern, to be published).

References

Andreeva, N. S., Esipova, N. G., Millionova, M. I., Rogulenkova, V. N., and Shibnev, V. A. (1967), *in* Conformation of Biopolymers, Ramachandran, G. N., Ed., Vol. 2, New York, N. Y., Academic Press, p 469.

Bensusan, H. B. (1969), *Biochemistry 8*, 4716.

Blout, E. R., DeLoze, C., Bloom, S. M., and Fasman, G. D. (1960), *J. Amer. Chem. Soc. 82*, 3787.

Bohak, Z., and Katchalski, E. (1963), *Biochemistry 2*, 228.

Brant, D. A., Miller, W. G., and Flory, P. J. (1967), *J. Mol. Biol. 23*, 47.

Brown, F. R., III, Carver, J. P., and Blout, E. R. (1969), *J. Mol. Biol. 39*, 307.

Butler, W. T. (1970), *Biochemistry 9*, 44.

Carver, J. P., and Blout, E. R. (1967), *in* Treatise on Collagen, Ramachandran, G. N., Ed., Vol. 1, New York, N. Y., Academic Press, p 441.

Cowan, P. M., and McGavin, S. (1955), *Nature (London) 176*, 501.

De Santis, P., Giglio, E., Liquori, A. M., and Ripamonti, A. (1965), *Nature (London) 206*, 456.

DeTar, D. F. (1969), *Anal. Chem. 41*, 1406.

DeTar, D. F., and Estrin, N. F. (1966), *Tetrahedron Lett. 48*, 5985.

DeTar, D. F., Gouge, M., Honsberg, W., and Honsberg, U. (1967a), *J. Amer. Chem. Soc. 89*, 988.

DeTar, D. F., Rogers, F. F., Jr., and Bach, H. (1967b), *J. Amer. Chem. Soc. 89*, 3039.

DeTar, D. F., and Vajda, T. (1967), *J. Amer. Chem. Soc. 89*, 998.

Donohue, J., and Trueblood, K. N. (1952), *Acta Crystallogr., Sect. A, 5*, 419.

Edsall, J. T., Flory, P. J., Kendrew, J. C., Liquori, A. M., Nemethy, G., Ramachandran, G. N., and Scheraga, H. A. (1966a), *Biopolymers 4*, 130.

Edsall, J. T., Flory, P. J., Kendrew, J. C., Liquori, A. M., Nemethy, G., Ramachandran, G. N., and Scheraga, H. A. (1966b), *J. Biol. Chem. 241*, 1004.

Edsall, J. T., Flory, P. J., Kendrew, J. C., Liquori, A. M., Nemethy, G., Ramachandran, G. N., and Scheraga, H. A. (1966c), *J. Mol. Biol. 15*, 339.

Engel, J., Kurtz, J., Katchalski, E., and Berger, A. (1966), *J. Mol. Biol. 17*, 255.

Harrington, W. F., Josephs, R., and Segal, D. M. (1966), *Annu. Rev. Biochem. 35*, 599.
Holzwarth, G., and Chandrasekaran, R. (1969), *Macromolecules 2*, 245.
Hopfinger, A. J., and Walton, A. G. (1969), *J. Macromol. Sci.-Phys. B3*, 171.
Kang, A. H., Bornstein, P., and Piez, K. A. (1967), *Biochemistry 6*, 788.
Kang, A. H., and Gross, J. (1970), *Biochemistry 9*, 796.
Kobayashi, Y., Sakai, R., Kakiushi, C., and Isemura, T. (1970), *Biopolymers 9*, 415.
Leung, Y. C., and Marsh, R. E. (1958), *Acta Crystallogr., Sect. A, 11*, 17.
Lotan, N., Yaron, A., and Berger, A. (1966), *Biopolymers 4*, 365.
Madison, V., and Schellman, J. (1970a), *Biopolymers 9*, 65.
Madison, V., and Schellman, J. (1970b), *Biopolymers 9*, 511.
Madison, V., and Schellman, J. (1970c), *Biopolymers 9*, 569.
Mathieson, A. M., and Welsh, H. K. (1952), *Acta Crystallogr., Sect. A, 5*, 599.
Mattice, W. L., and Mandelkern, L. (1970a), *Biochemistry 9*, 1049.
Mattice, W. L., and Mandelkern, L. (1970b), *Macromolecules 3*, 199.
Mattice, W. L., and Mandelkern, L. (1970c), *J. Amer. Chem. Soc. 92*, 5285.
Mattice, W. L., and Mandelkern, L. (1971a), *J. Amer. Chem. Soc. 93*, 1769.
Mattice, W. L., and Mandelkern, L. (1971b), *Biochemistry 10*, 1934.
Oriel, P. J., and Blout, E. R. (1966), *J. Amer. Chem. Soc. 88*, 2041.
Pysh, E. S. (1967), *J. Mol. Biol. 23*, 587.
Ramachandran, G. N. (1967), *in* Treatise on Collagen, Ramachandran, G. N., Ed., Vol. 1, New York, N. Y., Academic Press, p 103.
Ramachandran, G. N., Doyle, B. B., and Blout, E. R. (1968), *Biopolymers 6*, 1771.
Ramachandran, G. N., and Kartha, G. (1955), *Nature (London) 176*, 593.
Ramachandran, G. N., and Sasisekharan, V. (1965), *Biochim. Biophys. Acta 109*, 314.
Rich, A., and Crick, F. H. C. (1955), *Nature (London) 176*, 915.
Rich, A., and Crick, F. H. C. (1961), *J. Mol. Biol. 3*, 483.
Rosenheck, K., Miller, H., and Zakaria, A. (1969), *Biopolymers 7*, 614.
Sarathy, K. P., and Ramachandran, G. N. (1968), *Biopolymers 6*, 461.
Sasisekharan, V. (1959a), *Acta Crystallogr., Sect. A, 12*, 897.
Sasisekharan, V. (1959b), *Acta Crystallogr., Sect. A, 12*, 903.
Schimmel, P. R., and Flory, P. J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 52.
Schimmel, P. R., and Flory, P. J. (1968), *J. Mol. Biol. 34*, 105.
Schroeder, W. A., Kay, L. M., Legget, J., Honnen, L., and Green, F. C. (1954), *J. Amer. Chem. Soc. 76*, 3556.
Segal, D. M. (1969), *J. Mol. Biol. 43*, 497.
Segal, D. M., Traub, W., and Yonath, A. (1969), *J. Mol. Biol. 43*, 519.
Tiffany, M. L., and Krimm, S. (1969), *Biopolymers 8*, 347.
Timmermans, J. (1950), Physico-Chemical Constants of Pure Organic Compounds, New York, N. Y., Elsevier, p 508.
Traub, W. (1969), *J. Mol. Biol. 43*, 479.
Traub, W., and Shmueli, U. (1963), *Nature (London) 198*, 1165.
Traub, W., and Yonath, A. (1966), *J. Mol. Biol. 16*, 404.
Venkatachalam, C. M., and Ramachandran, G. N. (1969), *Annu. Rev. Biochem. 38*, 45.
Yonath, A., and Traub, W. (1969), *J. Mol. Biol. 43*, 461.