Unperturbed Dimensions of Sequential Copolypeptides Containing Glycine, L-Alanine, L-Proline, and γ-Hydroxy-L-proline*

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ABSTRACT: The characteristic ratios of poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Pro-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) have been determined in water. The results confirm the main features of the theoretical conformational maps derived by Flory and coworkers for glycine followed by either L-proline or a nonproline residue. Small adjustments, well within the uncertainty described by Schimmel and Flory, are suggested in the conformational map for L-proline followed by glycine. The constants for the Lennard-Jones functions of Scheraga and coworkers, as used by Madison and Schellman, produce a conformational map for L-proline followed by a nonproline residue which is in somewhat poorer agreement with experiment. The two sets of modified constants introduced by Madison and Schellman fail to predict the conformational properties of these sequential copolypeptides. L-Proline and γ-hydroxy-L-proline have the same effect on the unperturbed dimensions of the glycine-containing sequential copolypeptides. The characteristic ratios of the glycine-containing sequential copolypeptides are relatively insensitive to the details of the L-proline conformational map, so this does not prove that the conformational maps for L-proline and γ-hydroxy-L-proline are identical. The validity of the conformational map for L-alanine followed by L-proline remains uncertain.

Theoretical consideration of various types of interactions in different amino acid residues has led to maps which predict the conformational energy as a function of the rotational angles ϕ, ψ, and ω (Edsall *et al.*, 1966a–c) for polypeptides. The most elementary map divides the ϕ–ψ space into allowed, disallowed, and partially allowed areas solely on the basis of steric interactions (Ramachandran *et al.*, 1963, 1965; Nemethy *et al.*, 1966). More detailed conformational maps were subsequently obtained by computing the conformation energy arising from sources such as London, van der Waals, torsional, electrostatic, and hydrogen-bonding interactions (Brant and Flory, 1965b; Ramachandran *et al.*, 1966; Scott and Scheraga, 1966; Ramachandran and Sasisekharan, 1968; Flory, 1969a). It has been found that the conformational map for a particular amino acid residue is very sensitive to the occurrance of L-proline as the succeeding residue (Schimmel and Flory, 1967, 1968; Madison and Schellman, 1970a,b). The assumption has been that γ-hydroxy-L-proline can be treated as L-proline (Flory, 1969b).

The most rudimentary test of the validity of a conformational map is the prediction of minima in the conformational energy for rotational angles which correspond to observed ordered structures in the solid state. This procedure can at best, however, only verify the location of the energy minimum. If packing energies are large, it is also possible that ϕ and ψ deduced by X-ray studies of the solid state need not coincide exactly with the energy minimum in the conformational map. Of more general interest, however, is an assessment of the complete conformational map. This can be obtained from a determination of the characteristic ratio, which is directly calculable from the dependence of the conformational energy on the dihedral angles (Brant and Flory, 1965b; Flory, 1969c). The characteristic ratio is defined as $\langle r^2\rangle_0/n_p l_p^2$, where $\langle r^2\rangle_0$ is the unperturbed mean-square end-to-end distance, n_p is the number of peptide bonds, and l_p is the distance between adjacent α-carbon atoms, which is 3.8 Å for a planar trans peptide unit (Brant and Flory, 1965b).

Flory and coworkers have calculated conformational maps designated as Gly(Y) (Brant *et al.*, 1967), Gly(P) (Schimmel and Flory, 1968), Ala(Y) (Brant and Flory, 1965b; Brant *et al.*, 1967), Ala(P) (Schimmel and Flory, 1968), Pro(Y) (Schimmel and Flory, 1968), and Pro(P) (Schimmel and Flory, 1967), where (P) indicates that the given residue is followed by glycine or L-proline and (Y) indicates that it is followed by glycine or L-alanine. The Ala(Y) conformational map is presumed to apply to any amino acid with a CH_2R side chain (Brant and Flory, 1965b). It satisfactorily predicts the experimental characteristic ratios of 4 homopolypeptides with CH_2R side chains (Brant and Flory, 1965a) and the dipole moments of 14 small alanine peptides (Flory and Schimmel, 1967). Lack of a suitable solvent has prevented the determination of a characteristic ratio for polyglycine. However, the dipole moments of small glycine peptides are in agreement with the Gly(Y) conformational map (Flory and Schimmel, 1967), and the characteristic ratios of "random" copolypeptides of glycine and L-glutamic acid imply that the characteristic ratio of polyglycine would be low (Miller *et al.*, 1967). Conformational maps corresponding to Pro(P) have been derived by several groups (De Santis *et al.*, 1965; Schimme land Flory, 1967; Hopfinger and Walton, 1969; Holzwarth and Chandrasekaran, 1969; Madison and Schellman, 1970a,b). The experimental characteristic ratio of poly-L-proline at 30° (Mattice and Mandelkern, 1971a) is predicted adequately only by the conformational map of Hopfinger and Walton (1969). However, this conformational map is incorrect in predicting the temperature dependence of the characteristic ratio (Mattice and Mandelkern, 1971a). Experimental analysis of the valid-

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† Recipient of Public Health Service Postdoctoral Fellowship 5-F02-GM-31,377-02 from the National Institute of General Medical Sciences.

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TABLE I: Molecular Weights.

Copolypeptide	$10^{-3}\ M_n$	$10^{-3}\ M_w$	M_w/M_n	$10^{-3}\ M_v$[a]	$(n_p)_v$[b]
Poly(Pro-Gly)	8.0 ± 0.4	13.2 ± 0.3	1.66 ± 0.12	11.5 ± 0.4	148 ± 5
Poly(Hyp-Gly)	8.7 ± 0.3	10.2 ± 0.3	1.18 ± 0.08	9.7 ± 0.3	113 ± 4
Poly(Gly-Gly-Pro-Gly)	4.9 ± 0.5	6.7 ± 0.2	1.39 ± 0.19	6.1 ± 0.3	90 ± 4
Poly(Gly-Gly-Hyp-Gly)	9.4 ± 0.9	16.3 ± 0.4	1.75 ± 0.21	14.0 ± 0.6	196 + 4
Poly(Pro-Ala)	2.5 ± 0.3	3.1 ± 0.1	1.26 ± 0.19	2.9 ± 0.2	34 ± 2

[a] Estimated as $(2M_w + M_n)/3$; see text. [b] Number of peptide bonds corresponding to M_v.

ity of the conformational maps Gly(P), Ala(P), and Pro(Y) requires the study of copolypeptides which contain substantial amounts of L-proline and in which the amino acid sequence is precisely known. Given a conformational map and the sequence distribution for a copolypeptide, the characteristic ratio can be calculated exactly (Miller *et al.*, 1967; Flory, 1969c).

The characteristic ratios determined experimentally in water for five sequential copolypeptides containing L-proline and γ-hydroxy-L-proline are reported here. The results are compared to the predictions from the conformational maps Gly(P), Gly(Y), Ala(P), and Pro(Y) of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968) and to three conformational maps for *N*-acetyl-L-proline-*N*-methylamide with a trans peptide bond (Madison and Schellman, 1970b), the proline of which is analogus to Pro(Y) of Schimmel and Flory (1968).

Materials and Methods

Sequential Copolypeptides. The samples of poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Pro-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) are the same as those described in the previous paper (Mattice and Mandelkern, 1971b).

Molecular Weights. Weight-average molecular weights were obtained in water by low-speed sedimentation equilibrium using a Beckman Model E analytical ultracentrifuge equipped with interference optics. The partial specific volumes of the glycyl, L-alanyl, and L-prolyl residues were taken from Schachman (1957), and that of the γ-hydroxy-L-proline residue was taken from Sasisekharan (1959). Plates were read using a Nikon comparator. The apparent weight-average molecular weight, $M_{w,app}$, was evaluated for each initial concentration from the slope of the logarithm of the fringe number *vs.* the square of the displacement from the center of rotation. The true weight-average molecular weight, Mw, was obtained by extrapolation to infinite dilution according to eq 1, where

$$\frac{1}{M_{w,app}} = \frac{1}{M_w} + A_2(c_t + c_b) \qquad (1)$$

A_2 is the second virial coefficient and c_t and c_b are the equilibrium concentrations at the top and bottom of the cell, respectively (Williams *et al.*, 1958). This treatment is justified here due to the low molecular weights and relatively narrow molecular weight distributions of the sequential copolypeptides (Mandelkern *et al.*, 1957).

Number-average molecular weights were measured in water using a Mechrolab 503 high-speed membrane osmometer and an S & S B-20 membrane. The accuracy in the determination of M_n was limited by permeation, particularly for poly(Pro-Ala) and poly(Gly-Gly-Pro-Gly). The ratio M_w/M_n for poly(Pro-Ala) and poly(Gly-Gly-Pro-Gly) in Table I is within the range determined for the other sequential copolypeptides.

Virial Coefficients. The second virial coefficient, A_2, could not be determined to the accuracy required for the subsequent analysis by membrane osmometry. Therefore A_2 was obtained from the concentration dependence of $M_{w,app}$ using eq 1.

Viscosity. The intrinsic viscosities of the sequential copolypeptides from 5 to 70° in water have been reported elsewhere (Mattice and Mandelkern, 1970, 1971b).

Computations were carried out using a Control Data Corp. 6400 computer. The evaluation of an averaged transformation matrix from a conformational energy map was carried out using a program designed to follow the procedure presented by Flory (1969d). Characteristic ratios were computed from the appropriate averaged transformation matrices using a program written to carry out this process as required for sequential copolypeptides (Flory, 1969e).

Results and Discussion

The hydrodynamic and optical properties of poly(Pro-Gly), poly(Hyp-Gly), and poly(Gly-Gly-Pro-Gly) show that the sequential copolypeptides are not ordered in water (Mattice and Mandelkern, 1970, 1971b), so that these sequential copolypeptides were studied at 25°. Poly(Pro-Ala) and poly(Gly-Gly-Hyp-Gly) present evidence of conformational ordering at low temperatures in water, but are not so at 40° (Mattice and Mandelkern, 1970, 1971b). Consequently, these copolypeptides were studied at the elevated temperature.

Typical plots of the logarithm of the fringe number *vs.* the square of the displacement from the center of rotation are shown for poly(Pro-Ala), poly(Pro-Gly), and poly(Gly-Gly-Hyp-Gly) in Figure 1. Figure 2 shows $1/M_{w,app}$ as a function of $c_t + c_b$ for all five sequential copolypeptides. The A_2 values, determined from the slope of each, are presented in Table II. They are within the range previously obtained for polypeptides in good solvents (Doty *et al.*, 1956; Brant and Flory, 1965a; Miller *et al.*, 1967; Lapanje and Tanford, 1967; Mandelkern, 1971b). The $[n]$ are also listed in Table II. There is very little temperature effect on $[n]$ for poly(Pro-Gly), poly(Hyp-Gly), and poly(Gly-Gly-Pro-Gly) at 25–30° in water (Mattice and Mandelkern, 1970, 1971b). Therefore, the slight difference in temperature at which A_2 and $[n]$ were determined for these sequential copolypeptides is not important for present purposes.

The characteristic ratio can be calculated from eq 2 (Brant and Flory, 1965a). The intrinsic viscosity in a θ solvent is $[n]_\theta$, ϕ is a universal constant, M_v is the viscosity-average molecu-

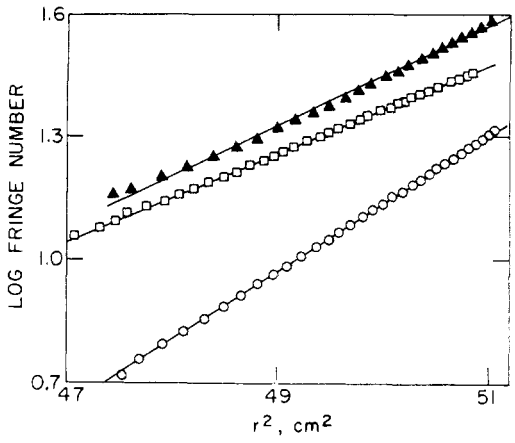

FIGURE 1: Log fringe number *vs.* r^2 for poly(Pro-Gly) at 2,000 rpm, 25°, c_0 = 2.78 mg/ml (○), poly(Pro-Ala) at 40,000 rpm, 40°, c_0 = 4.86 mg/ml (□), and poly(Gly-Gly-Hyp-Gly) at 17,000 rpm, 40°, c_0 = 4.90 mg/ml (▲).

$$\frac{\langle r^2\rangle_0}{n_p l_p} = \left(\frac{[n]_\theta}{\phi M_v^{1/2}}\right)^{2/3} \frac{M_0}{l_p^2} \qquad (2)$$

lar weight, and M_0 is the average residue weight. The characteristic ratio depends only on the cube root of M_v, so small errors in the estimation of this quantity are not consequential. Under θ conditions, the conditions of relevance here, the sequential copolypeptides as originally prepared should have $M_n:M_v:M_w$ = 1.00:1.67:2.00 (Flory, 1953a). The molecular weight distribution will be narrowed by the purification process. As shown in Table I, the actual molecular weight distributions are considerably narrower than the most probable distribution. Under θ conditions $M_v = (2M_w + M_n)/3$ for both the most probable distribution and for a monodisperse sample (Flory, 1953a), and M_v was estimated from this relationship. The uncertainties to be presented below for each tabulated characteristic ratio and for the expansion coefficients are due to the probable errors in the measured quantities and in the derived M_v.

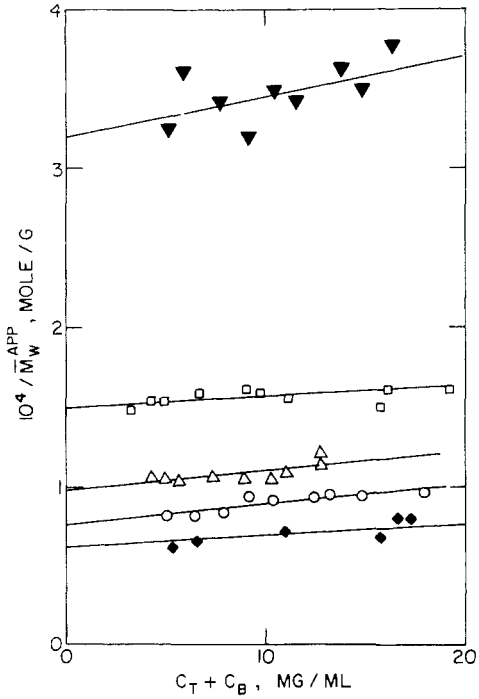

FIGURE 2: Concentration dependence of the apparent molecular weight calculated from sedimentation equilibrium in water for poly(Pro-Gly) (○), poly(Hyp-Gly) (△), and poly(Gly-Gly-Pro-Gly) (□) at 25°, and poly(Gly-Gly-Hyp-Gly) (◆) and poly(Pro-Ala) (▼) at 40°.

TABLE II: Second Virial Coefficients and Intrinsic Viscosities.

Copolypeptide	$10^4 A_2$ ((cm³ mole)/g²)	$[n]$ (dl/g)[a]
Poly(Pro-Gly)	12.5 ± 1.5[b]	0.151 ± 0.005[c]
Poly(Hyp-Gly)	11 ± 2[b]	0.151 ± 0.006[c]
Poly(Gly-Gly-Pro-Gly)	5.5 ± 2.5[b]	0.098 ± 0.004[c]
Poly(Gly-Gly-Hyp-Gly)	8 ± 2[d]	0.127 ± 0.006[d]
Poly(Pro-Ala)	25 ± 10[d]	0.094 ± 0.003[d]

[a] From Mattice and Mandelkern (1970) and Mattice and Mandelkern (1971b). [b] 25°. [c] 30°. [d] 40°.

While eq 2 is exact, it has only been possible so far to experimentally establish the θ condition in a mixed solvent system for certain polypeptides (Takahashi *et al.*, 1969). Consequently, in general, statistically coiling polypeptides must be studied in good solvents (Doty *et al.*, 1956; Doty and Nishihara, 1957; Brant and Flory, 1965a; Miller *et al.*, 1967; Lapanje and Tanford, 1967; Mattice and Mandelkern, 1971b). The intrinsic viscosity in a good solvent can be corrected to the corresponding value for the θ condition by use of the expansion coefficient, α, according to eq 3 and 4 (Flory, 1949; Orofino and Flory, 1957). Characteristic ratios of polypeptides have previously been determined from measurements in good solvents using eq 2–4 (Brant and Flory, 1965a; Miller *et al.*, 1967; Mattice and Mandelkern, 1971b). In their analysis of the expansion coefficients of proteins in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol, Lapanje and Tanford (1967) used an empirical expression obtained from light-scattering studies of polystyrene (Berry, 1966). This expression, given as eq 5, requires a preliminary estimate of the characteristic ratio before the expansion coefficient can be calculated.

$$[n] = \alpha^3 [n]_\theta \qquad (3)$$

$$\frac{A_2 M}{[n]} = \frac{2^{5/2}\pi N_0}{3^3 \phi} \ln\left[1 + \frac{\pi^{1/2}}{2}(\alpha^2 - 1)\right] \qquad (4)$$

$$A_2 M^{1/2} = \frac{420 N_0}{134}\left(\frac{\pi l_p^2}{6 M_0 n_p l_p^2}\langle r^2\rangle_0\right)^{3/2}(\alpha^2 - 1) \qquad (5)$$

When dimensions are expressed in ångströms and $[n]$ in deciliters per gram, the theoretical value for ϕ under θ conditions is 0.00266 (Pyun and Fixman, 1966). Experimentally, for other chain molecules ϕ is found to be about 0.0025 in θ solvents (Krigbaum and Carpenter, 1955; McIntyre *et al.*, 1962; Berry, 1967). The ϕ appropriate for polymers in good solvents is found to be slightly lower (Krigbaum and Carpenter, 1955; McIntyre *et al.*, 1962). Previously ϕ = 0.0021 has been used in the determination of the characteristic ratios of polypep-

TABLE III: Expansion Coefficients in Water.

Copolypeptide	Eq 4 ϕ = 0.0021	Eq 4 ϕ = 0.0025	Eq 5
Poly(Pro-Gly)	1.22 ± 0.05	1.27 ± 0.06	1.28 ± 0.04
Poly(Hyp-Gly)	1.25 ± 0.06	1.30 ± 0.08	1.29 ± 0.05
Poly(Gly-Gly-Pro-Gly)	1.09 ± 0.05	1.11 ± 0.06	1.09 ± 0.04
Poly(Gly-Gly-Hyp-Gly)	1.20 ± 0.08	1.24 ± 0.10	1.18 ± 0.05
Poly(Pro-Ala)	1.24 ± 0.13	1.29 ± 0.16	1.09 ± 0.04

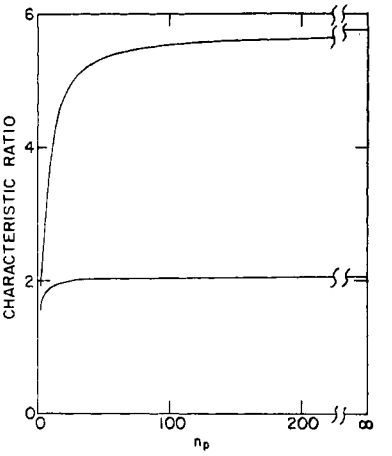

FIGURE 3: Theoretical characteristic ratios, calculated from the transformation matrices of Brant *et al.* (1967) and Schimmel and Flory (1968), as a function of the number of peptide bonds for poly(Pro-Ala) (upper curve) and poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Pro-Gly), and poly(Gly-Gly-Hyp-Gly) (lower curve).

tides from measurements in good solvents (Brant and Flory, 1965a; Miller *et al.*, 1967; Lapanje and Tanford, 1967; Mattice and Mandelkern, 1971b). However, in a recent study of poly(L-lactic acid), the ester analog of poly-L-alanine, ϕ = 0.0025 was used even though the polymer was studied in a good solvent, where α = 1.5 (Tonelli and Flory, 1969). In order to allow for this uncertainty, results obtained utilizing ϕ = 0.0021 and 0.0025 will be presented.

The expansion coefficients calculated using eq 4 and 5 are presented in Table III. The preliminary estimate of the characteristic ratio, required in the use of eq 5, was obtained from the appropriate conformational maps of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968). The values of α obtained with eq 5 are similar to those obtained with eq 4 for the glycine-containing sequential copolypeptides. For poly(Pro-Ala) a somewhat lower α is obtained using eq 5.

The characteristic ratios deduced from experiment using eq 2–4, and eq 5 for poly(Pro-Ala), are given in Table IV. The results are lower than the characteristic ratios of 9.0 ± 0.5 found with homopolypeptides with CH_2R side chains (Brant and Flory, 1965a), and much lower than the characteristic ratios of 14 and 18–20 found for the homopolymer poly-L-proline in water and organic solvents, respectively (Mattice and Mandelkern, 1971b). The characteristic ratio of poly(Pro-Ala) is similar to that of denatured proteins (Lapanje and Tanford, 1967), while the results with the glycine-containing sequential polypeptides are lower. As had been predicted (Flory, 1979b), the characteristic ratios are little affected by the substitution of γ-hydroxy-L-proline for L-proline.

The experimental characteristic ratios in Table IV are based on the assumption, inherent in eq 2, that the polymer can be adequately represented by a Gaussian distribution of chain elements (Flory, 1953b). Under these circumstances the characteristic ratio will be independent of molecular weight. The anticipated characteristic ratio, calculated from the appropriate conformational maps of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968), is shown as a function of the number of peptide bonds in Figure 3. The viscosity-average number of peptide bonds, given in the last column of Table I, is in the asymptotic region for the glycine-containing sequential copolypeptides. However, for poly(Pro-Ala) it corresponds to a characteristic ratio which is only about 90% of that at infinite degree of polymerization. The assumption of Gaussian statistics is thus not strictly correct in the case of poly(Pro-Ala).

The characteristic ratios predicted by theory are also presented in Table IV. Column 4 shows the characteristic ratios derived from the appropriate conformational maps of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968). The glycine-containing sequential copolypeptides are all predicted to have a characteristic ratio of 2.1. The predictions for these copolypeptides are in very good agreement with the experimental results, particularly those obtained with higher values for ϕ. The characteristic ratio for poly(Pro-Ala) is predicted to be higher than that of the glycine-containing copolypeptides, which is in qualitative accord with the experimental results. For poly(Pro-Ala) the best agreement is obtained with the lower value of ϕ and using eq 5 for α.

The last three columns in Table IV present the characteristic ratios that are predicted by substituting rotational potential functions for *N*-acetyl-L-proline-*N*-methylamide with a trans peptide bond (Madison and Schellman, 1970b) for the Pro(Y) of Schimmel and Flory (1968). Madison and Schellman (1970b) used three sets of constants for the Lennard-Jones 6–12 functions in their calculations of the conformational energy. The constants of Ooi *et al.* (1967) gave results NBE1, which are similar to the predictions of Flory and coworkers for the glycine-containing sequential copolypeptides. However, the characteristic ratio for poly(Pro-Ala) is predicted to be twice as large as predicted by Flory and coworkers, and is also much larger than that observed. The constants of Ooi *et al.* (1967) were modified in NBE2 to allow the formation of a hydrogen bond between the peptide hydrogen following a prolyl residue and the carbonyl oxygen preceding that prolyl residue. The occurrence of this hydrogen bond was postulated in order to explain a large negative circular dichroism band observed in some organic solvents (Madison and Schellman, 1970b,c). In NBE3 the constants of Ooi *et al.* (1967) were further modified to reduce repulsions between neighboring residues. Madison and Schellman (1970b,c) were able to explain several features in the circular dichroism observed with several small derivatives of L-proline if the conformations were determined by the constants designated NBE2 or NBE3. Agreement between their predicted and observed circular dichroism was poor if the conformations were determined by NBE1. We find, however, that NBE1 predicts reasonably well the conformational properties of the glycine-containing copolypeptides. The modifications used to produce NBE2 and NBE3 cause a marked deterioration in the ability to predict

TABLE IV: Observed and Predicted Characteristic Ratios.

Copolypeptide	Experimental[a]		Predicted from Theory[b]			
	ϕ = 0.0021	ϕ = 0.0025	Flory[c]	NBE1[d]	NBE2[e]	NBE3[d]
Poly(Pro-Gly)	2.77 ± 0.32	2.28 ± 0.29	2.1	1.9	1.3	1.2
Poly(Hyp-Gly)	3.09 ± 0.41	2.53 ± 0.38	2.1	1.9	1.3	1.2
Poly(Gly-Gly-Pro-Gly)	2.80 ± 0.38	2.41 ± 0.36	2.1	2.1	1.8	1.7
Poly(Gly-Gly-Hyp-Gly)	2.23 ± 0.40	1.87 ± 0.38	2.1	2.1	1.8	1.7
Poly(Pro-Ala)	3.51 ± 0.89	2.94 ± 0.84	5.7	10.5	2.6	1.5
Pro(-Pro-Ala)[e]	4.37 ± 0.51	3.88 ± 0.46				

[a] Using α from eq 4 unless otherwise noted. [b] Limiting value at high molecular weight. [c] Calculated from the appropriate transformation matrices of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968). [d] The transformation matrix calculated from the indicated conformational map for *N*-acetyl-L-proline-*N*-methylamide with a trans peptide bond (Madison and Schellman, 1970b) was substituted for Pro(Y) of Schimmel and Flory (1968). NBE1, NBE2, and NBE3 identify the curves in Figure 5 of Madison and Schellman (1970b). [e] Using α calculated from eq 5.

the characteristic ratios, These two sets of constants are thus not supported by the results reported here.

Pro(Y) of Schimmel and Flory (1968) exhibits minima at $\psi \cong 125$ and 325° which are of nearly equal energy within the reliability of the calculation, which cannot distinguish differences of about 2 kcal (Schimmel and Flory, 1968). NBE1 of Madison and Schellman (1970b) shows minima at $\psi \cong 140°$ and 280° with the latter being more stable by about 3 kcal (Madison and Schellman, 1970b). The differences in Pro(Y) and NBE1 could arise from sources other than the different constants used in evaluating the nonbonded energy. Both groups state that they took their proline coordinates from the crystal structure of L-leucyl-L-prolylglycine (Leung and Marsh, 1958). According to Leung and Marsh (1958), "a twist of about 120° from the fully extended configuration occurs at C_4–N_2 bond," which sets ϕ at about 120°. Schimmel and Flory (1968) state that their ϕ is 120–122°, in agreement with Leung and Marsh (1958), but Madison and Schellman (1970c) refer to a "standard value" of 112° for ϕ. Therefore, the possibility exists that different coordinates for the proline residue were used by Schimmel and Flory (1968) and by Madison and Schellman (1970b). The precise geometry assumed for the proline unit is crucial in correctly predicting the characteristic ratio of poly-L-proline (Mattice and Mandelkern, 1971b), and could be responsible for at least some of the difference in the results predicted from Pro(Y) and NBE1. In addition, Madison and Schellman (1970b), but not Schimmel and Flory (1968), included the electrostatic interaction between peptide units in their calculations. If the electrostatic term is similar to that found in homopolypeptides with CH_2R side chains, as has been suggested (Holzwarth and Backman, 1969; Yan *et al.*, 1970), it would stabilize the minimum at $\psi = 325°$ by as much as 1 kcal relative to the minimum at 125° (Schimmel and Flory, 1968). The inclusion of electrostatic interactions by Madison and Schellman (1970b) accounts for part of the differences in the energies at the minima at 140 and 280° in their rotational potential function NBE1 for *N*-acetyl-L-proline-*N*-methylamide with a trans peptide bond.

It has been shown that small amounts of glycine cause a large reduction in the theoretical characteristic ratios predicted for random copolypeptides (Schimmel and Flory, 1968). The effect of glycine is also pronounced in determining the characteristic ratios of sequential copolypeptides. To illustrate this point, the characteristic ratios anticipated for all possible sequential polypeptides formed from glycine, L-alanine, and L-proline in which there is a repeating unit of three, four, or five residues, have been computed. The Gly(Y), Gly(P), Ala(Y), Ala(P), and Pro(Y) transformation matrices were taken from Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968). The choice of a transformation matrix corresponding to Pro(P) is more difficult, since none of the theoretical treatments adequately account for the conformational properties of poly-L-proline (Mattice and Mandelkern, 1971a). Fortunately, it can readily be shown that the characteristic ratios predicted for sequential copolypeptides are generally insensitive to the details of the Pro(P) conformational map. Calculations were performed using two transformation matrices for L-proline followed by L-proline: (a) the transformation matrix given by Schimmel and Flory (1967), which predicts a characteristic ratio over 100 for poly-L-proline, and (b) the transformation matrix calculated from a square well rotational potential function located at $\phi = 102°$ and $\psi = 310 \pm 55°$, which predicts a characteristic ratio of 20.6 for poly-L-proline. The latter is close to the experimental characteristic ratio of 18–20 observed in three organic solvents at 30° (Mattice and Mandelkern, 1971a). The ratio of the results predicted with (b) to those predicted with (a) for the sequential copolypeptides which have at least one L-prolyl-L-prolyl sequence is shown as a function of the mole fraction of peptide bonds bounded on both sides by pyrrolidine rings in Figure 4. With the exception of poly(Pro-Pro-Ala), this ratio is greater than 0.8, and for the majority of the sequential copolypeptides it is greater than 0.9. The uncertainty in the rotational potential function for poly-L-proline is therefore of comparatively minor importance in predicting the characteristic ratios of sequential copolypeptides containing L-proline.

The anticipated characteristic ratio for the sequential copolypeptides, calculated using the square well for Pro(P), is shown as a function of the mole fraction of glycine in Figure 5. All of the sequential copolypeptides containing only L-proline and L-alanine are predicted to have a characteristic ratio greater than 5.7, while all of the sequential copolypeptides containing glycine are predicted to have characteristic ratios between 2.0 and 4.7. Comparison of these calculations to the work of Miller *et al.* (1967) and Schimmel and Flory (1968) shows that the influence of glycine is slightly more pronounced in sequential than in random copolypeptides.

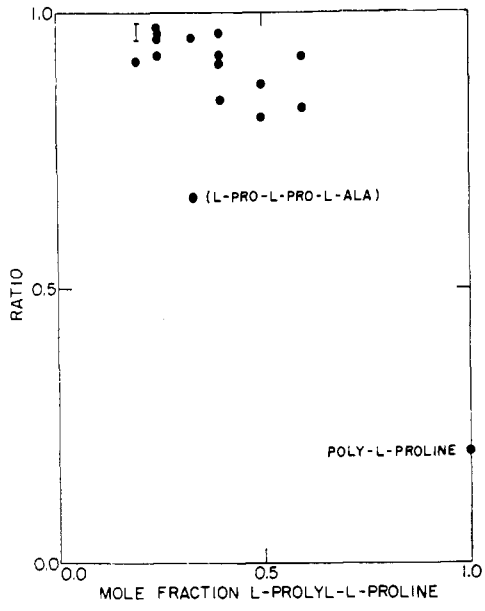

FIGURE 4: Ratio of the predicted characteristic ratios calculated using (a) to those using (b) for all sequential copolypeptides of glycine, L-alanine, and L-proline which have a repeating sequence of five or fewer residues and at least one Pro-Pro unit. (a) is the result obtained with the Pro(P) transformation matrix of Schimmel and Flory (1967), and (b) is the result obtained with a square-well rotational potential function at $\phi = 102°$ and $\psi = 310 \pm 55°$. Results for eleven sequential copolypeptides fall within the range marked I.

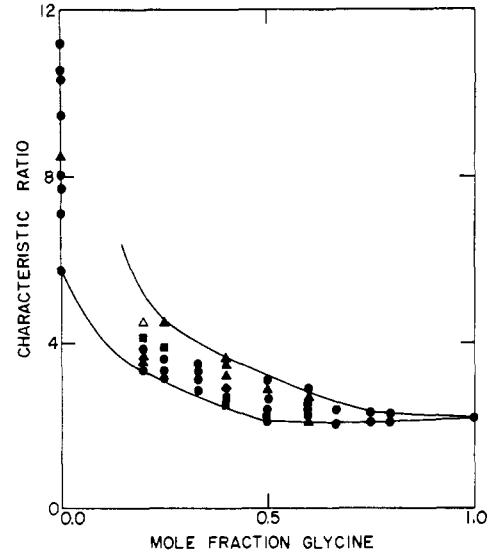

FIGURE 5: Anticipated characteristic ratios of all possible sequential copolypeptides of L-alanine, L-proline, and glycine, in which the repeating unit is five or fewer residues, plotted against the mole fraction of glycine. Characteristic ratios were calculated from the conformational maps of Brant *et al.* (1967), Schimmel and Flory (1968), and a square well at $\phi = 102°$ and $\psi = 310 \pm 55°$ for Pro-(P). Th symbols (●), (▲), (■), (◆), and (△) represent essentially identical predicted characteristic ratios for one, two, three, four, and six sequential copolypeptides, respectively. Poly-L-proline and poly (Pro-Pro-Ala) are predicted to have characteristic ratios greater than 12 and are not plotted.

With reference to poly(Pro-Gly), Figure 5 predicts that a sequential copolypeptide containing at least 50% glycine should have a characteristic ratio of 2.0–3.2 regardless of whether the remaining 50% is L-alanine, L-proline, or glycine and independent of the sequence. This can be contrasted with Figure 6, in which the same data are plotted against the mole fraction of L-proline. If a sequential copolypeptide is at least 50% L-proline, the characteristic ratio may vary from 2 to 21, depending both upon the composition of the remaining 50% of the sequential copolypeptide and upon the sequence. Thus the characteristic ratio of poly(Pro-Gly) is determined primarily by the conformational map Gly(P) and the glycine content.

Schimmel and Flory (1968) also showed that the predicted characteristic ratios of copolypeptides containing L-proline were sensitive to the difference in energy between the minima at $\psi = 125$ and 325° in the Pro(Y) conformational map providing the glycine content was not large. The relative energies of these two minima also have a perceptible effect on the predicted unperturbed dimensions of random coil proteins, even though L-proline is only a minor constituent of these polypeptides (Miller and Goebel, 1968). The effect of this uncertainty on the predicted characteristic ratio of poly(Pro-Gly) is shown in Figure 7. Here the predicted characteristic ratio calculated from Gly(P) and Pro(Y) with the minimum at $\psi = 125°$ increased in energy by an amount Δ over that given by Schimmel and Flory (1968), but with its shape unchanged, is plotted against Δ. The characteristic ratio is only slightly sensitive to Δ, being within 20% of 2.0 for $\Delta = \pm 3$ kcal.

The experimental characteristic ratio of poly(Pro-Gly) is predicted quite well by using the Gly(P) and Pro(Y) conformational maps. The agreement could be improved slightly by postulating that Δ is positive. This effect could be caused by electrostatic interactions or uncertainties in the theoretical calculations. Electrostatic interactions were also ignored in computing the Gly(P) conformational map. In order to estimate how large an effect might be involved, the energies in the conformational map for the peptide electrostatic interaction in the monopole approximation (Brant *et al.*, 1967) were added to the Gly(P) conformational map. This process is approximate because it requires estimating the conformational energies between contour lines in both maps. Characteristic ratios

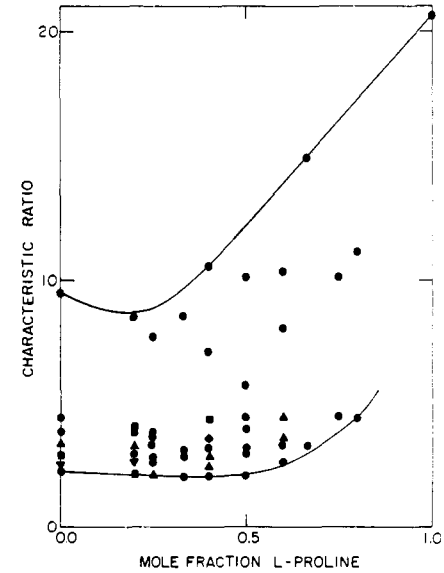

FIGURE 6: Results in Figure 5 plotted against the mole fraction of L-proline. The symbols are the same as in Figure 5.

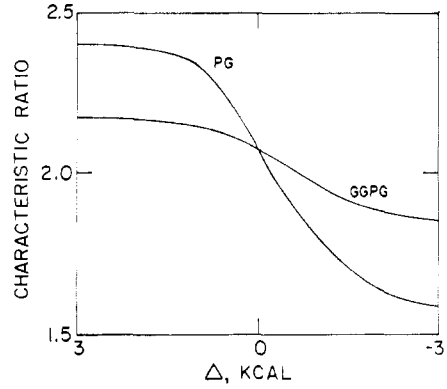

FIGURE 7: Effect of changes in Δ on the characteristic ratio for poly(Pro-Gly) (PG) and poly(Gly-Gly-Pro-Gly) (GGPG) at 30°. Δ is the energy added to the minimum at $\psi = 125°$ in the conformational map for Pro(Y) (Schimmel and Flory, 1968).

were calculated for poly(Pro-Gly) using this new conformational map for Gly(P). The change in the predicted characteristic ratio was less than 10%.

For the reasons discussed in connection with poly(Pro-Gly), the characteristic ratio of poly(Gly-Gly-Pro-Gly) should be determined almost entirely by its glycine content and the conformational maps Gly(Y) and Gly(P). Reference to Figure 5 shows that a sequential copolypeptide containing at least 75% glycine is predicted to have a characteristic ratio of 2.0–2.4, and Figure 7 shows that the characteristic ratio of poly(Gly-Gly-Pro-Gly) varies only 10% from two for $\Delta = \pm 3$ kcal. As was the case with poly(Pro-Gly), the agreement between the experimental and predicted characteristic ratios of poly(Gly-Gly-Pro-Gly) can be improved slightly by postulating that Δ is positive.

The experimentally determined characteristic ratios are similar within experimental error for poly(Pro-Gly) and poly(Hyp-Gly) and for poly(Gly-Gly-Pro-Gly) and poly(Gly-Gly-Hyp-Gly). This is consistent with the assumption that γ-hydroxy-L-proline and L-proline have the same effect on the unperturbed dimensions of polypeptides (Flory, 1969b). However, due to the relative insensitivity of the

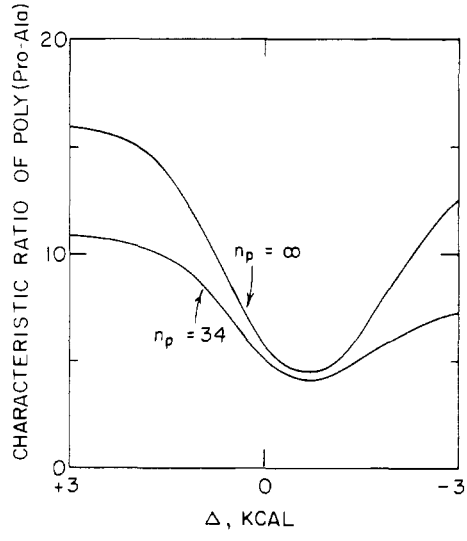

FIGURE 8: Effect of changes in Δ on the predicted characteristic ratio of poly(Pro-Ala) at $n_p = 34$ and $n_p = \infty$.

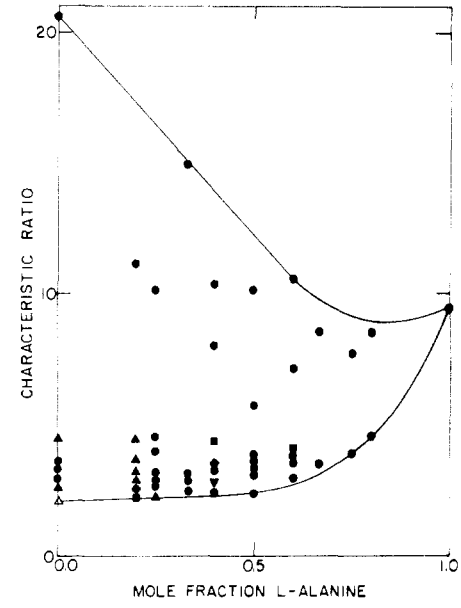

FIGURE 9: Results of Figure 5 plotted against the mole fraction of L-alanine. Symbols are the same as in Figure 5.

characteristic ratios of the glycine-containing sequential copolypeptides to the details of the Pro(Y) conformational map, this result does not uniquely establish the fact that the conformational maps for L-proline and γ-hydroxy-L-proline are precisely identical.

While an individual collagen chain is not a compositionally exact sequential copolypeptide, glycine does occur as every third residue throughout large portions of the molecule (Schroeder *et al.*, 1954; Kang *et al.*, 1967; Bensusan, 1969; Butler, 1970; Kang and Gross, 1970). Hence based on the predicted characteristic ratios in Figure 5 for poly(Gly-Ala-Ala), poly(Gly-Ala-Pro), poly(Gly-Pro-Ala), and poly(Gly-Pro-Pro), denatured collagen should have a characteristic ratio in the range 2.8–3.5. Flory (1960) calculated the ratio $\langle r^2\rangle_0^{1/2}/\langle r^2\rangle_{0,f}^{1/2}$, where $\langle r^2\rangle_{0,f}$ is the unperturbed mean-square end-to-end distance assuming free rotation about ϕ and ψ for denatured calf skin collagen from the data obtained by Doty and Nishihara (1957) in dilute citrate buffer.[1] The result corresponds to $\langle r^2\rangle_0/n_p l_p^2$ of about 2.5, which is in reasonably good agreement with the results predicted for a sequential copolypeptide containing glycine at every third residue. McBride and Harrington (1967) estimated a characteristic ratio of five for reduced carboxymethylated Ascaris collagen. This collagen contains only 26–28% glycine (Josse and Harrington, 1964; McBride and Harrington, 1967), which may account for its higher characteristic ratio.

The stability of the ordered conformation of collagen is correlated with the content of L-proline plus γ-hydroxy-L-proline (Burge and Hynes, 1959; Piez and Gross, 1960). It has been postulated that the pyrrolidine ring decreases the conformational entropy of the denatured collagen, thereby increasing the melting temperature (Garrett, 1960; Mandelkern, 1964; Josse and Harrington, 1964; Harrington

[1] Flory (1960) also presents results for the denatured forms of two other collagens in concentrated salt solutions. The characteristic ratio for denatured ichthyocol collagen is 3.3. The other sample has a characteristic ratio of 1.5–1.7, but these results were obtained under extremely nonideal conditions ($\alpha = 1.6$–1.7) so that the conclusion could be somewhat in error for this reason.

and Rao, 1967; Carver and Blout, 1967). We thus realize a rather unique situation in this case where the decrease in conformational entropy of the disordered chain has little effect on the characteristic ratio. This results because the location of glycine at every third residue maintains the characteristic ratio in the range of 2.8–3.5.

In contrast to the glycine-containing copolypeptides, the characteristic ratio of poly(Pro-Ala) is very sensitive to Δ, as is shown in Figure 8. The experimental results are in agreement with the predictions from the Ala(P) and Pro(Y) conformational maps if Δ is zero or slightly negative. They can be improved by using the lower value of ϕ and eq 5 for the estimation of α. This conclusion is in contrast to the results with the glycine-containing sequential copolypeptides, which suggest that Δ is positive.

The possibility exists that the experimental characteristic ratio of poly(Pro-Ala) is lower than that anticipated with a positive Δ because of small inaccuracies in the Ala(P) conformational map. Figures 6 and 9 show that the characteristic ratio of a sequential copolypeptide which is 50% L-proline (or 50% L-alanine) is sensitive to the remainder of the copolypeptide. The Ala(P) conformational map is similar to that of Pro(P) in that it is dominated by one minimum and a large portion of the ϕ–ψ surface is of high energy. In the case of poly-L-proline, relatively small changes in the conformational map have an enormous effect on the experimental characteristic ratio (Schimmel and Flory, 1967; Mattice and Mandelkern, 1971a). Puckering of the pyrrolidine ring (Mathieson and Welsh, 1952; Donohue and Trueblood, 1952; Leung and Marsh, 1958; Hopfinger and Walton, 1969) could increase the size of the area with low energy in the Ala(P) conformational map. The possibility exists that the characteristic ratio predicted by Pro(Y) with a positive Δ and Ala(P), taking account of pyrrolidine ring puckering, would be in still better agreement with experiment. It is also possible that interaction with the methyl group of the L-alanine residue causes the conformation of the pyrrolidine ring to change slightly so that Δ is somewhat dependent upon whether the residue preceding the L-proline residue does or does not contain a side chain.

The experimental characteristic ratios reported here confirm in general the deductions from the theoretical conformational maps. Particularly good quantitative agreement is obtained from the conformational maps Gly(P), Gly(Y), and Pro(Y) of Flory and coworkers (Brant *et al.*, 1967; Schimmel and Flory, 1968). The status of Ala(P) is a little less certain due to the larger experimental error in the determination of the characteristic ratio of poly(Pro-Ala) and the unknown effects of pyrrolidine ring puckering. The characteristic ratios of sequential copolypeptides containing glycine are determined primarily by the glycine content and are unaffected by the substitution of γ-hydroxy-L-proline.

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Identification of Phospholipase A_1 and A_2 in the Soluble Fraction of Rat Liver Lysosomes*

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ABSTRACT: Rat liver lysosomes were isolated by sucrose density gradient centrifugation from rats previously injected with Triton WR-1339. As measured by acid phosphatase activity, the lysosomes were purified 32-fold over the homogenate with an average yield of 6.0%. Mitochondrial, microsomal, and peroxisomal contaminations were each less than 0.05% of the total activity of the homogenate. When the lysosomes were incubated at pH 4.0 with 1.0 mM EDTA, [^{14}C]linoleic acid and [^{14}C]monoacylglycerophosphorylethanolamine were produced from 1-acyl-2-[^{14}C]linoleyl-3-glycerophosphorylethanolamine. After osmotic rupture of purified lysosomes the phospholipases (A_1 and A_2) were in the soluble fraction entirely. The two phospholipases were not inhibited to the same extent by increasing concentrations of Ca^{2+} or EDTA. Phospholipases A_1 and A_2 were separated by gel filtration on Sephadex G-200.

Mellors and Tappel (1967) reported the first lysosomal phospholipase from rat liver. This enzyme had an acid pH optimum, hydrolyzed both the C-1 and C-2 fatty acid ester linkages of phosphatidylcholine and phosphatidylethanolamine, and was found in both soluble and particulate fractions of lysosomes. Stoffel and Greten (1967) and Mellors *et al.* (1967) confirmed the presence of a phospholipase A with optimal activity in the acid pH range. These reports were contradictory, however, with regard to pH optima and Ca^{2+} requirement.

On the basis of selective inhibition studies, Stoffel and Trabert (1969) suggested the presence of two soluble phospholipases with acid pH optima. Waite *et al.* (1969) confirmed the presence of lysosomal phospholipase(s) A active at pH 4.5 and reported the presence of a phospholipase A in the lysosomal preparation which was stimulated by Ca^{2+} ions and was active in the neutral pH range. The localization of the Ca^{2+}-stimulated enzyme was uncertain since the distribution of this activity did not parallel the major activity found in either the lysosomes or the mitochondria. Recently Rahman *et al.* (1970) reported a Ca^{2+}-stimulated phospholipase A_2 from the particulate fraction of rat liver lysosomes. Nachbaur and Vignais (1968) and Waite (1969) described a Ca^{2+}-stimulated phospholipase A_2 in the outer membrane of mitochondria which is similar to the particulate lysosomal enzyme reported by Rahman *et al.* (1970). This observation suggests that mitochondrial contamination could account for the activity found by Rahman *et al.*

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