Circular Dichroism of Polypeptide and Protein Conformations. Film Studies*

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ABSTRACT: Films of poly-L-glutamic acid, sodium poly-L-glutamate, and poly[N^5-2(hydroxyethyl)-L-glutamine] were examined by infrared absorption in the amide I band region and by ultraviolet circular dichroism under various relative humidity conditions at which these polypeptides assume the α, β, and random conformations. It was shown that the circular dichroism spectra of polypeptide films in the random conformation differ from those observed in solution. In films, these spectra are characterized by a negative band at 200–205 mμ and a negative shoulder at 215–230 mμ. Similar circular dichroism spectra were obtained with denatured, but not totally unfolded, proteins. The difference between the circular dichroism spectra of randomly coiled polypeptides in solution and of unordered polypeptide films and denatured proteins are interpreted in terms of steric constraints imposed on the latter two systems.

The use of ultraviolet circular dichroism has been widely applied for the investigation of the solution structure of proteins and polypeptides (Timasheff and Gorbunoff, 1967; Timasheff *et al.*, 1967; Beychok, 1967, 1968). The relevance of the optical rotatory dispersion and circular dichroism spectra of poly-α-L-amino acids to that of proteins has been frequently questioned, especially with relation to the effects of environment and particle size (*e.g.*, membranes) (Urry *et al.*, 1967; Yang, 1967a,b; Urry and Ji, 1968; Ji and Urry, 1969). Recent optical rotatory dispersion and circular dichroism studies on films of polypeptides which exist in the β conformation (Davidson *et al.*, 1967; Fasman and Potter, 1967; Stevens *et al.*, 1968) have demonstrated that β structure in these models may give rise to two different types of spectra. Furthermore, the circular dichroism spectrum of a film of poly-L-lysine in the unordered conformation was found to differ considerably from that found in solution and to be similar in character to that reported for several denatured proteins (Stevens *et al.*, 1968). To investigate further the possibility that the circular dichroism solution properties of the various poly-α-amino acids might differ from those of proteins, due to solvent effects (which might be absent in the interior of proteins), neighboring group effects, and light scattering (Urry and Ji, 1968), our previous work on films was extended, with the intention of examining in greater detail the various conformations.

In an infrared study, Lenormant *et al.* (1958) had shown that the conformation of films of PGA-Na[1] could be altered by controlling the relative humidity, so that both the α and β conformations could be obtained. In the present paper, the film circular dichroism of PGA-Na in the α, β, and unordered structures was examined under the appropriate conditions of relative humidity (and checked by infrared studies); similar measurements were carried out on PHEG and PGA, as well as on aqueous solutions of several proteins in the unordered conformation.

Experimental Section

Materials. PGA-Na, degree of polymerization 610, synthesized by the method of Idelson and Blout (1958), was purchased from Pilot Chemicals, lot G-32.

PHEG was synthesized by the method of Lupu-Lotan *et al.* (1965) and had a specific viscosity of 0.59 (0.2% in 0.2 M NaCl).

Poly-L-lysine·HCl was synthesized as previously described (Fasman *et al.*, 1961) and had an intrinsic viscosity of 0.67 in 1 M NaCl, pH 4.0 (estimated mol wt 55,000).

Poly-L-glutamic acid was obtained from PGA-Na by dialysis against 0.01 M HCl which caused a flocculent precipitate. The suspension was lyophilized.

The proteins used were β-lactoglobulin A, which was prepared by the method of Aschaffenburg and Drewry (1957) from the milk of a homozygous cow; S-sulfonated β-lactoglobulin A, which was prepared from this material by the method of Pechère *et al.* (1958); α_{s1}-casein B, which was prepared by the method of Thompson and Kiddy (1964) from the milk of a homozygous cow; oxidized bovine pancreatic ribonuclease, which was purchased from Mann, Lot T4319; rabbit muscle aldolase, which was purchased from Calbiochem, lot 53669.

Methods. INFRARED SPECTROSCOPY. A Perkin-Elmer double-beam grating spectrophotometer No. 621 was used. The polarized infrared radiation was obtained using a Perkin-Elmer common beam wire grid polarizer no. 186-0187. The instrument was calibrated with a polystyrene film of 0.05 mm thickness.

NONORIENTED FILMS. The films were cast on AgCl plates from a concentrated polypeptide aqueous solution. For

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[1] Abbreviations used are: PGA-Na, sodium poly-α-L-glutamate; PHEG, poly[N^5-2(hydroxyethyl)-L-glutamine]; PGA, poly-α-L-glutamic acid.

TABLE I: Infrared Spectra Bands of Polypeptides.

Spectrum Number	% Relative Humidity	Amide I[a]		Amide II[a]	COO^{-}[a]	COO^{-}[a]	Conformation
			A. Oriented Films of PGA-Na				
179	70	1690 (∥)	1612 (⊥)	1530 (∥)	1653	1405	β
199	35	1690 (∥)	1615 (⊥)	1525 (∥)	1562	1405	β
201	84		1630–1640 (∥)		1550	1400	α
201	92		1637 (∥)		1548	1402	α
			B. Unoriented Films of PGA-Na				
132	Dried at room temperature		1652	1520	1570	1404	Unordered
159	35		1655	1520	1575	1405	Unordered
142	64	1690	1620	1520	1570	1404	β
153	84		1648	1555	1565	1405	α
160	90		1647	1555	1565	1405	α
161	97		1648	1555	1565	1405	α
			C. Other Polypeptides				
					O ‖ C | OH		
Poly-L-glutamic acid, from dioxane–H_2O (4:1)							
144	Unoriented		1652	1549	1712		
176	Oriented		1652 (∥)	1549 (⊥)	1710		
Poly[N^5(2-hydroxyethyl)-L-glutamine] from H_2O							
162	Unoriented		1651	1547			
185	Oriented		1650 (∥)	1546 (⊥)			
Poly-L-lysine, in β form[b] from aqueous solution, pH 11.2, heated							
		1690	1625	1538			

[a] In cm^{-1}; the dichroism is noted in parentheses. [b] Dried with a hair dryer to convert into β.

PGA-Na samples, the films were dried at ambient relative humidity at room temperature, or in a desiccator containing KOH under vacuum. When the film was dry, it was put in a desiccator of the desired relative humidity and left for 2–3 days. There was no difference between the spectra of the films dried either at ambient relative humidity or under vacuum. The PGA was dissolved in a 4:1 mixture of dioxane and H_2O, for casting. Poly-L-lysine·HCl was dissolved in water, the pH was raised to 11.2, and the film was then cast on the disk and dried with a hair dryer.

The desired relative humidity was obtained by having a saturated salt solution in the desiccator at 25°, as listed below; it was further checked by measurement on an Airguide No. 605 hydrometer, the values of which are recorded throughout. To maintain a constant humidity while the sample was being measured, the film was covered by a second AgCl plate, with a rubber gasket wetted with Nujol between the two plates, which were firmly held together in a standard cell assembly.

Salts used to maintain the desired relative humidity were $CaSO_4$, 98%; Na_2SO_4, 93%, KBr, 84%; $NaClO_3$, 75%; $NaNO_2$, 66%; $Ca(NO_3)_2$, 56%; $CaCl_2$, 32%.

ORIENTED FILMS. The films were cast in a glove bag maintained at a relative humidity between 65 and 70%, from aqueous solutions of the polypeptides and were oriented by unidirectional stroking with a spatula, the orientation being continued until the film was completely dry. The film was then placed in a desiccator of the desired relative humidity and left for 2–3 days. Films which were oriented and placed at either 84 or 92% relative humidity, causing conversion into α structure and then brought back to 65% relative humidity, converted into the β conformation, but lost their orientation.

CIRCULAR DICHROISM. Films were cast on a quartz disk from concentrated solutions of the polypeptides. The solution was smeared out on the disks with a finger wrapped in parafilm. The film was dried at room temperature or, in some cases, in a vacuum desiccator over KOH. The films of PGA-Na were placed in a desiccator of the desired relative humidity (see above for salts used) and maintained for 2–3 days to equilibrate. To maintain the same relative humidity during measurement, the cell holder, etc., was also kept in the desiccator and assembled before removal from the desiccator. The cell consisted of two quartz disks as cell windows, the film being cast on the inside of one window. A rubber spacer was placed between the plates. The disks and rubber ring were mounted in a demountable cell holder, consisting of two metal rings which screwed into each other.

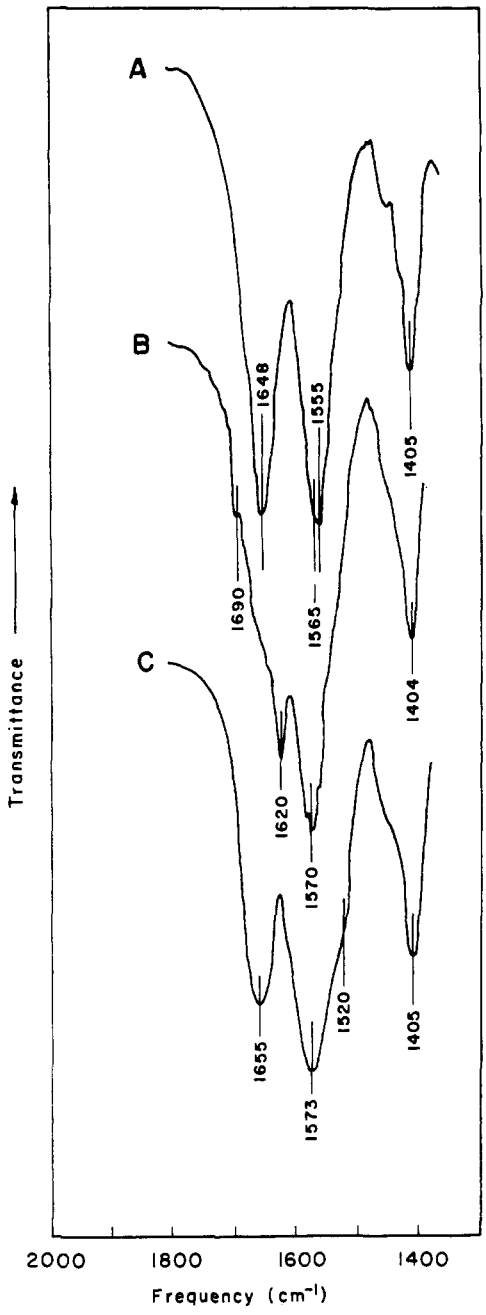

FIGURE 1: The infrared spectra of nonoriented films of PGA-NA: (A) α-helical form, film dried at prevailing room relative humidity at room temperature and kept for 2 days at relative humidity of 84%; (B) β form, film dried at prevailing room relative humidity and room temperature and kept 4 days at 64% relative humidity; (C) unordered form, film dried under vacuum over KOH and kept 3 days at relative humidity of 35%.

Films prepared by drying at ambient humidity at room temperature or under vacuum over KOH or under vacuum over P_2O_5 at 80° yielded similar spectra.

The gel of PGA-Na was prepared by smearing a concentrated aqueous solution of PGA-Na on the quartz disk, and the solution was allowed to evaporate until a gel formed.

Circular dichroism data were obtained with a Cary Model 60 recording spectropolarimeter equipped with a 6001 circular dichroism accessory, and set for a half-band width of 15 Å. The circular dichroism results are reported in terms of θ observed in deg $\times 10^{-3}$ for films and of $[\theta]$ observed in (deg cm^2/dmole) $\times 10^{-3}$ for proteins.

Results

Conformation of Polypeptides as Determined by Infrared Studies. As it has been shown that the conformation of polypeptide films can be assigned by their infrared band positions and dichroism (Miyazawa, 1960, 1967; Miyazawa and Blout, 1961), the films used in this study were first examined by that technique to evaluate their conformation. Following the procedure of Lenormant *et al.* (1958) for obtaining the α-helical and β conformations of PGA-Na, films were cast from water and oriented at 70% relative humidity, and then placed in desiccators of the appropriate relative humidity to obtain the desired conformation (α at >70%; β <70%).

The infrared spectral data for oriented samples of PGA-Na is shown in Table I. Films maintained at 70 or 35% relative humidity were found to exist in the antiparallel β pleated sheet [amide I 1690 cm^{-1} ($\|$), 1615 cm^{-1} ($\perp$)], while films produced at 84 or 92% relative humidity were found to be in the α-helical conformation [amide I 1637 cm^{-1} ($\|$) (Miyazawa and Blout, 1961)]. Thus, from the dichroism and positions of the amide I and II bands, it was found that these results agreed with the conditions reported by Lenormant *et al.* (1958) for the generation of α and antiparallel β conformations. As oriented films are undesirable for circular dichroism work, due to higher birefringence and light scattering, unoriented films were cast on AgCl plates, dried at room temperature, and then placed in desiccators of the desired relative humidities. The band positions of PGA-Na under various conditions of relative humidities are listed in Table I and the spectra are shown in Figure 1. The samples dried at room temperature and at 35% relative humidity have amide I bands at 1652–1655 cm^{-1} and amide II bands at 1520 cm^{-1}. This amide I band is at a frequency higher than those reported here and previously (Lenormant *et al.*, 1958) for the oriented α-helical form (1637 cm^{-1}) and exhibits the highest amide I position of all the unoriented films reported here. In their calculations, Miyazawa and Blout (1961) assigned the random coil amide I band to a frequency higher than those of the antiparallel β and the α-helical conformations (random coil, 1658; α, 1650; β, 1632 cm^{-1}). Thus, these spectra of PGA-Na with an amide I band at 1652–1655 cm^{-1} can be assigned to a random coil, in agreement with the position of 1657 cm^{-1} of the amide I band of PGA-Na film, found by Susi *et al.* (1967). It should be noted that the film maintained at 35% relative humidity remained in the unordered conformation, rather than converting into the β form as was the case with the oriented films. The unoriented film produced at 64% relative humidity is assigned to the β conformation, due to the presence of the 1690-cm^{-1} band and the lowest position of an amide I band, namely, 1620 cm^{-1} (Miyazawa and Blout, 1961). Thus, at 64% relative humidity, both the oriented and unoriented films of PGA-Na have the same conformation, antiparallel β. Films maintained at relative humidities of 84, 90, and 97% with amide I bands at 1648 cm^{-1} are assigned the α structure, in agreement with the oriented samples. Both the α and β forms in the unoriented films have amide I bands which lie at higher frequencies than those in oriented films. On examination of the oriented film spectra of Lenormant

et al. (1958), however, it can be seen that the summation of the two polarized spectra would tend to shift the bands to slightly longer frequencies, as has been observed here.

In Table I are also reported the infrared spectra of two polypeptides known to assume the α conformation, PGA (Blout and Idelson, 1958) and PHEG (Lupu-Lotan *et al.*, 1966; Adler *et al.*, 1968), both in their oriented and unoriented forms. The values of the band positions for both forms, oriented and unoriented, are seen to be the same. The oriented sample of un-ionized PGA gave band positions and dichroism as previously reported (Blout and Idelson, 1956) for the α-helical conformation. The fact that both oriented and unoriented samples give similar band positions suggests that, upon drying the PGA sample becomes oriented. Another explanation might lie in the fact that both polarized infrared spectra, *i.e.*, the parallel and perpendicular components are at the same frequencies as reported by Miyazawa and Blout (1961), and therefore, little band shift might be expected on summation. The spectrum of oriented PHEG has the amide I band at 1650 cm^{-1} and gives parallel dichroism, as expected for the α-helical conformation. The unoriented sample spectrum yields similar band positions. To correlate the infrared spectrum of the unoriented β structure, poly-L-lysine at pH 11.2 was cast and dried at an elevated temperature (Davidson and Fasman, 1967; Susi *et al.*, 1967; Stevens *et al.*, 1968); the amide I bands found at 1690 and 1625 cm^{-1} define the film as being antiparallel β, in agreement with the values reported by Susi *et al.* (1967). These values agree reasonably with that found for PGA-Na at 64% relative humidity, and further establish that the structure is β at this relative humidity.

In this manner, the conformations of unoriented films of PGA-Na produced at several relative humidities have been defined, as well as the helical conformations of PGA and PHEG, and these preparations could be studied by circular dichroism to define their rotatory properties.

Circular Dichroism Studies. The conformation of PGA-Na at pH 7.0 is known to be random (for reviews, see Yang, 1967b, Beychok, 1967, and Fasman, 1967). The circular dichroism spectral properties of this conformation have been reported by Holzwarth and Doty (1965), Velluz and Legrand (1965), Carver *et al.* (1966), Yang (1967a), and Adler *et al.* (1968). Recent circular dichroism studies in the 190- to 260-mμ region for both PGA and PHEG have shown that a prominent positive ellipticity band appears at 217 mμ and negative bands at 238 and 197 mμ; the ellipticity values for PGA are $[\theta]_{238}$ -180, $[\theta]_{217}$ $+4700$, and $[\theta]_{197}$ $-27{,}000$. These are representative of the unordered form (Timasheff *et al.*, 1967). The circular dichroism spectrum of the gel–film of PGA-Na (Figure 2) has the characteristics of the unordered structure, *i.e.*, a weak negative band at 234, a positive band at 217, and a negative band at 205 mμ. The latter band is red shifted relative to the solution spectrum. When the relative humidity is lowered to 97% and 92%, the circular dichroism spectra change, with a decrease in the peak at 217 mμ and a slight red shift; concomitantly the intensity of the negative peak at 235 mμ is markedly increased and the maximum is shifted to 230 mμ. Although the gel has the typical characteristics of the unordered form, the spectra of the 92 and 97% relative humidity films are more difficult to define. However, based on the infrared data discussed above, where the spectra had been defined as predominantly those of an α helix, it can be assumed that at these relative humidities, the polypeptide is shifting

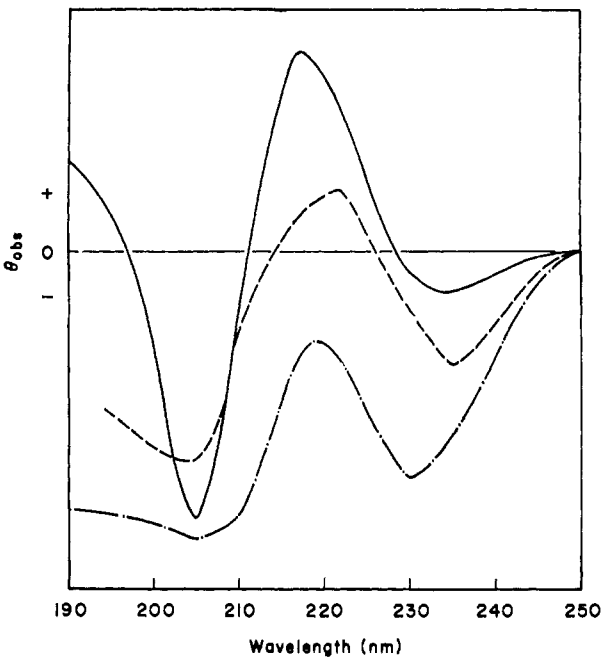

FIGURE 2: Circular dichroism of PGA-Na films: (———) gel; (– – –) film at relative humidity of 97%; (—·—·—·) film at relative humidity of 92%.

from the disordered to the α-helical structure, and still contains a significant fraction of the disordered form.

In Figure 3 are shown the circular dichroism spectra of two films completely in the α-helical form, PGA and PHEG, and of two forms of PGA-Na, at relative humidities of 75 and 84%, which, by infrared criteria, are also α helical. The PHEG sample has negative dichroic bands at 223 and 208 and a peak at 194 mμ. (In solution, these are found at 221, 209, and 193 mμ.) The relative intensities of the 208- and 223-mμ bands differ from those found in methanol solution where the 208-mμ extremum was larger than that at 223 mμ (Adler *et al.*, 1968). The band positions, however, have been only slightly altered (from 209 to 208 mμ). The PGA spectrum has negative dichroic bands at 223 and 209 mμ, with a positive band at 191 mμ (in solution, the positions are 222, 209, and 191 mμ). The 209-mμ band is of lesser magnitude than that at 223 mμ. Thus, these two helical spectra show variations in the relative magnitude of the 209- and 223-mμ bands. The spectra for the PGA-Na films at relative humidities of 75 and 84% have ellipticity bands at 223, 210, and 197 mμ. The 210-mμ band has decreased relative to the 223-mμ band, while the lowest wavelength band decreases in height relative to the other bands with increasing relative humidity.

When the relative humidity is further decreased to 59 or 64%, where the infrared spectra are characteristic of the β structure, the circular dichroism spectra (Figure 4) display a negative ellipticity band at 219–220 mμ and a positive band at 200–203 mμ, similar to the positions reported for the β structure of class I (Stevens *et al.*, 1968). The circular dichroism spectra of PGA-Na at low relative humidity are shown in Figure 5. The infrared spectra of these films indicate a random conformation. These circular dichroism spectra, however, are strikingly different from those of the gel (Figure 2) or of polypeptide solutions in the random conformation. A broad

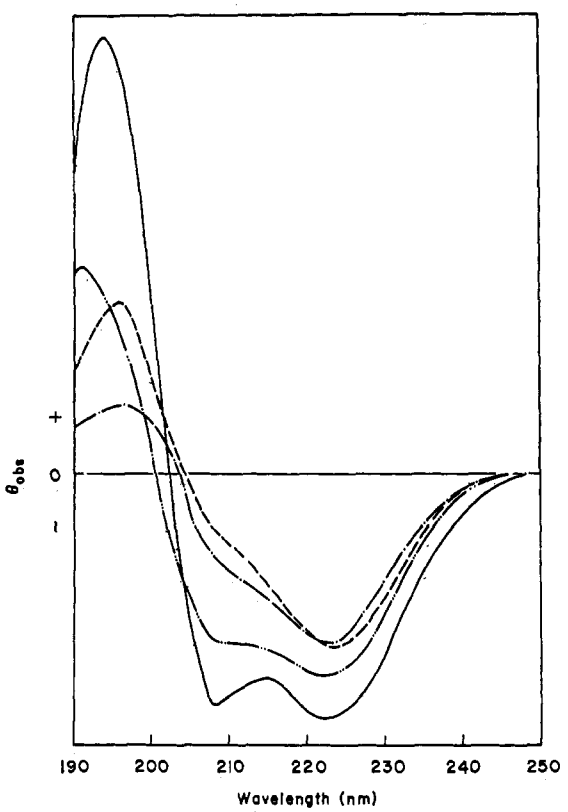

FIGURE 3: Circular dichroism of films of PHEG, PGA, and PGA-Na: (———) PHEG cast from H_2O; (—·—·—) PGA cast from mixture of dioxane–H_2O (4:1); (—·—·—·—) PGA-Na, film at 84% relative humidity; (– – –) PGA-Na film at 75% relative humidity.

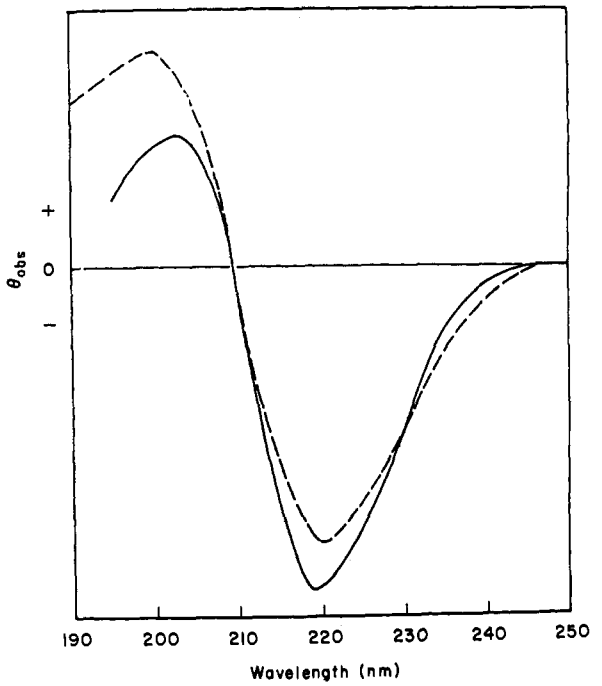

FIGURE 4: Circular dichroism of PGA-Na films in the β conformation: (– – –) relative humidity of 64%; (———) relative humidity of 59%.

negative shoulder is found in the 220- to 230-mμ region, and a larger negative peak at 203 mμ. The negative band observed in solution at 230–235 has been shifted and the positive band at 217–222 mμ has completely vanished. Thus, the unordered form found in low humidity films exhibits quite different circular dichroisms characteristics than those found in solution.

Unordered Proteins. The circular dichroism spectra of several proteins considered to have no structures with long-range order within them are shown on Figure 6, along with the circular dichroism spectra of unordered poly-L-lysine in solution and in a dry film. It is striking that none of the protein spectra are similar to those of random poly-L-lysine in solution nor of PGA-Na in gel (Figure 2). Instead, the spectra of the unordered proteins are strikingly similar in shape to those of unordered poly-L-lysine in film form and of the unordered PGA-Na dry films (Figure 5). The circular dichroism spectra of the proteins are characterized by negative bands between 200 and 204 mμ and negative shoulders of varying intensity between 215 and 230 mμ. The poly-L-lysine film circular dichroism spectrum has a negative extremum at 202 mμ and a negative shoulder between 215 and 230 mμ.

The circular dichroism spectra of aldolase in the native, two denatured, and one partly renatured forms are shown on Figure 7. The spectrum of the native protein is characterized by negative bands at 210 and 221 mμ and positive rotation below 202 mμ, suggesting the presence of α-helical regions. In the near-ultraviolet region, the spectrum is positive between 263 and 292 mμ, with maxima at 280 and 289 mμ; a negative rotation, maximal at 295 mμ, appears between 292 and 310 mμ. This portion of the spectrum reflects the asymmetry of aromatic residues and points to a constrained tertiary structure (Strickland *et al.*, 1969). Exposure of this enzyme to either pH 12 or pH 2 is known to denature it with normalization of the spectral properties of previously buried aromatic side chains (Donovan, 1964; Stellwagen and Schachman, 1962) and a marked increase in viscosity (Stellwagen and Schachman, 1962), which indicates unfolding of the native globular structure and the onset of predominantly unordered (or random) conformation (Tanford, 1968). The circular dichroism spectral changes brought about by such treatment are shown on Figure 7. In both denatured states the circular dichroism spectrum is characterized by a negative band, maximal at 202 mμ for the acid-denatured protein and at 199 mμ for the product of alkaline denaturation; both spectra have negative shoulders between 215 and 230 mμ in similar fashion to other denatured proteins and to polypeptide films in unordered conformation. Simultaneously, the circular dichroism spectrum above 250 mμ vanishes, indicating randomization of aromatic side-chain conformations, which must occur when the protein unfolds. Dialysis of the alkali-denatured protein back to pH 8.5 resulted in partial reversal of the spectral changes: in the far ultraviolet, extrema at 210 and 220 mμ reappeared, with about 65% of their original intensity, but no circular dichroism absorption could be observed above 250 mμ. Thus, as has been proposed by Donovan (1964), this procedure regenerates order in the protein without restoring the original native structure. It is striking, however, that even such partial refolding of the protein causes the circular dichroism spectrum to change drastically in the far-ultraviolet region.

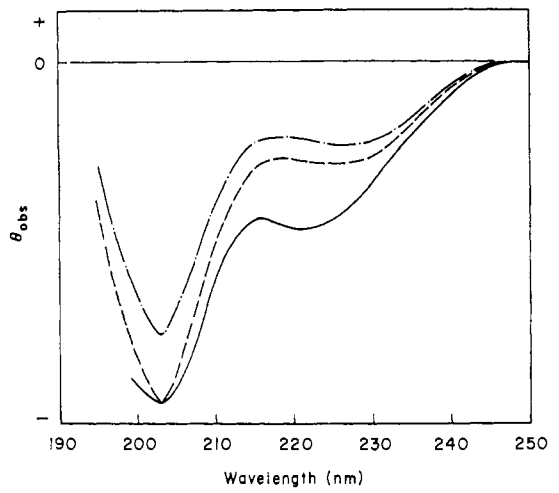

FIGURE 5: Circular dichroism of PGA-Na films in the unordered conformation: (———) film dried under vacuum over KOH and kept 3 days at 35% relative humidty; (– – –) film dried under vacuum over P_2O_5 at 80°; (— · — · —) film dried at room temperature.

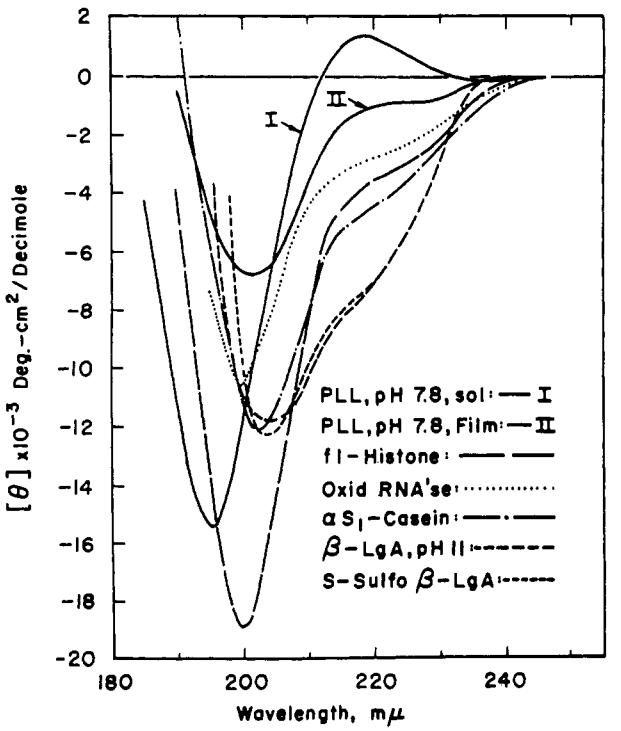

FIGURE 6: Circular dichroism of unordered poly-L-lysine and of several proteins in unordered conformation.

Discussion

The circular dichroism spectra of the various conformations assumed by PGA-Na at different relative humidities indicate that considerable variation is exhibited in both the α-helical and the random conformations. When compared to the α-helical spectrum of PGA and PHEG, the sodium salt of PGA exhibits a reduced ellipticity in the 208- to 210-mμ band relative to the 222-mμ band, as well as a reduced value in the positive peak below 200 mμ.

Urry and coworkers (Urry and Ji, 1968; Ji and Urry, 1969) have proposed that changes in circular dichroism spectra may be the result of absorption flattening and dispersion distortion due to light scattering, causing damping of ellipticity near 208 mμ in the helical conformations. Also slight shifts to longer wavelengths have been observed with changes in the dielectric constant of the solvent (from 222 to 225 mμ). Yang (1967a) has shown that aggregation of PGA at low pH also causes slight shifts to longer wavelengths (222–223 mμ) and increases the ellipticity for the helical conformation. An additional explanation for the decreased values of the 208-mμ ellipticity band in helical polymers has been offered by Urry (1968a) who attributed it to the ionization of vicinal carboxyl groups in the PGA helix.

Previous studies have shown that the $[\theta]_{208}/[\theta]_{222}$ ratio seems to be altered when the solvent becomes less polar as the 208-mμ band increases with decreasing dielectric constant (*e.g.*, $[\theta]_{208}/[\theta]_{222}$ PHEG in MeOH (Adler *et al.*, 1968), poly-α-methyl-L-glutamate in trifluoroethanol (Urry, 1968b), poly-L-alanine (Quadrifoglio and Urry, 1968). These differences have been attributed to vicinal effects for n–π^* transitions, due to changes in the conformation and charge of the side chain (Urry, 1968a). However, it may be due to changes in the frequencies and the intensities of the transitions which may affect the interactions between transitions of chromophoric groups caused by dielectric constant changes of the medium (Woody, 1968).

On examination of the circular dichroism of α-helical films, it is seen that in all cases $[\theta]_{208} < [\theta]_{222}$. With increasing relative humidity, the $[\theta]_{208}$ decreases further, as does $[\theta]_{197}$. Thus, addition of water leads to considerable changes in these ratios. Such changes cannot be attributed solely to changes in vicinal charge or of dielectric constant.

An even more striking difference is observed in films of the random, or unordered conformation. The gel–film, shown in Figure 2, displays the ellipticity bands found previously in solution work (Adler *et al.*, 1968), with position shifts from 197 to 205 mμ and from 238 to 234 mμ.

Films at 97 and 92% relative humidity which show predominantly helical infrared spectra have circular dichroism spectra intermediate between those of random and helical structures. The circular dichroism spectra of dried unordered forms (Figures 5 and 6) deviate considerably from those of the random polypeptides in solution or in the gel–film. In the dried films, the circular dichroism bands at 238 and 217 mμ have disappeared, being replaced by a negative shoulder at 220–230 mμ, and a large negative band at 205 mμ has replaced the corresponding solution band at 195 mμ. It might be tempting to assign the 220- to 230-mμ shoulder to the presence of a small amount of helix (Myer, 1969); however, such circular dichroism curves do not have minima at 205 mμ, and thus, cannot be responsible for the observed curves.

A possible explanation for the observed differences between the unordered dry film circular dichroism spectra and those in solution may be found in the following considerations. In aqueous solution, a polypeptide chain, when devoid of ordered structure, has a high degree of freedom of motion. Thus, it is capable of undergoing the random flight structural fluctuations characteristic of a random coil in solution (Flory and Miller, 1966; Miller *et al.*, 1967). A similar situation prevails in the gel, where the water contents are sufficiently high

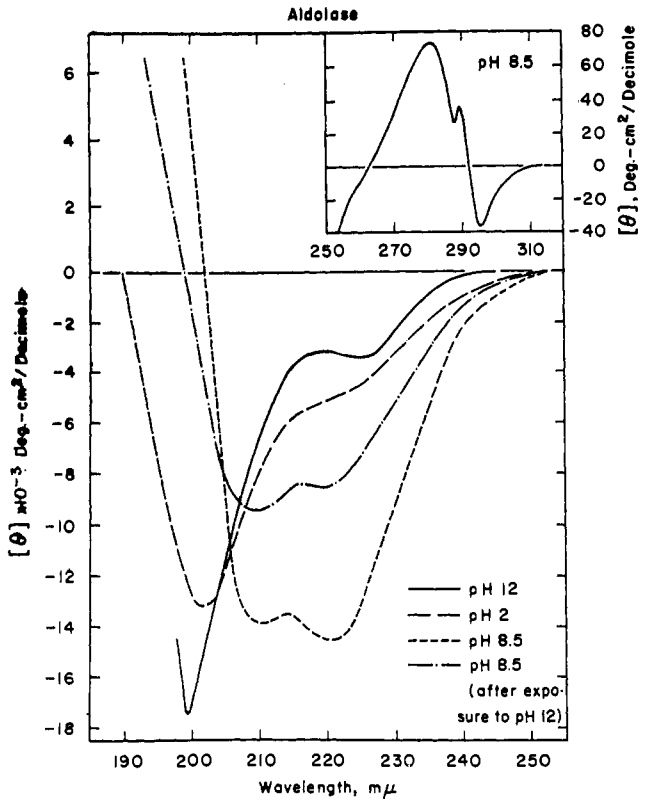

FIGURE 7: Circular dichroism of aldolase in the native state, acid denatured, alkali denatured, and "renatured."

to afford a sufficient free volume for the flexible chain to undergo extensive thermal fluctuations; in fact, while highly viscous, the gel flows. Drying the polypeptide film, however, removes the interchain water, *i.e.*, the medium in which the segments could flow; this greatly reduces the degree of freedom of motion of the chain segments so that the chain is no longer capable of the random flight motions of a true statistical coil. It is true that, even in the dry state, the polypeptide is still undergoing thermal motion. Due to the rigidity of the structure, however, these fluctuations cannot be of a long range, with the result that the polypeptide film assumes a state similar to that of an amorphous polymer glass (Birshtein and Ptitsyn, 1966), and it is quite likely that it becomes frozen in a state which favors particular short-range conformations over others. These may be dictated by discrete side-chain interactions and the steric requirements of the mutual packing of individual chains within a film, in which the concentration per unit volume of polypeptides is much higher than in solution and, therefore, interchain contacts are much more prevalent.

Similar reasoning may be applied to the denatured proteins. Of the proteins shown in Figures 6 and 7, β-lactoglobulin at pH 11 is known to be irreversibly denatured, but constrained by S–S bonds (Timasheff *et al.*, 1966); S-sulfonated β-lactoglobulin at pH 7.8 has the S–S bridges cleaved, but at that pH, it should not be fully unfolded (Aune *et al.*, 1967); the same is true of oxidized ribonuclease at pH 4.0; α_s-casein is known to belong to the unordered conformational type, even though its chain is folded into a structure considerably more compact than a random coil (Swaisgood and Timasheff, 1968). Histone in 0.14 M NaF at pH 7 is essentially a structureless polyelectrolyte (Jirgensons, 1969). Aldolase, denatured in acid or alkali, loses its native long-range ordered structure (Donovan, 1964), but should not unfold to a random coiled state. Thus, the denatured proteins examined in this study have unordered structures, which are, nevertheless, highly constrained by side-chain interactions and disulfide bridges, and are, therefore, not truly random. It is, therefore, not surprising that their circular dichroism spectra should resemble those of unordered polypeptides in the film form rather than those of randomly coiled chains in solution.

Similar circular dichroism spectra have been observed with other unstructured proteins. Phosvitin (Timasheff *et al.*, 1967) at pH 3.4 has a circular dichroism spectrum with a negative extremum at 199 mμ and a shoulder between 215 and 230 mμ. Change of pH to 6.6, where this protein carries a high positive charge, results in a shift of the circular dichroism spectrum to one characteristic of a randomly coiled polypeptide in solution, with negative bands at 198 and 232 and a positive extremum at 219 mμ. Viscosity data on this protein indicate that it becomes a random coil at the high pH (Jirgensons, 1958). Recently, Tiffany and Krimm (1969) (see also Krimm *et al.*, 1969) reported the circular dichroism spectra of some denatured proteins including myoglobin in 4.8 M $CaCl_2$ and ribonuclease in 0.24 M sodium dodecyl sulfate. The reported spectra were of a type similar to those presented in this paper for unordered proteins and dry polypeptide films. Tiffany and Krimm (1969), however, have assigned these spectra to random coils, for which no evidence is offered. They were also able to generate similar spectra in polypeptides (PGA and poly-L-lysine) under very special conditions, such as at high salt concentration (PGA in 4.5 M $LiClO_4$ and poly-L-lysine in 4 M $CaCl_2$). At these conditions of high salt concentration, PGA is known to collapse into a compact structure which cannot be termed a statistical coil (Iizuka and Yang, 1965). This is brought about by a decrease in hydration and possible charge neutralization of the side-chain carboxyls. Furthermore, specific interaction of the cations with the peptide carbonyl (Bello and Bello, 1962) can easily bring about shifts in the transition moments. The contention (Tiffany and Krimm, 1969) that PGA at neutral pH in dilute salt forms an extended helical structure due to charge–charge repulsion is not supported by other experimental evidence as well as theoretical analyses (Brandt and Flory, 1965a,b; Flory and Miller, 1966; Miller *et al.*, 1967), which have clearly demonstrated its existence in a randomly coiled state. It is true, however, that a highly kinked chain containing very short segments of regular structure would behave hydrodynamically as a coil. It would seem, therefore, that there is no reason to doubt the earlier conclusion that PGA in solution at neutral pH is in the form of a randomly coiled chain (Fasman, 1967) and not an extended structure. Furthermore, Adler *et al.* (1968) have shown that the nonionic polypeptide PHEG gives a random form circular dichroism spectrum similar to that of PGA at neutral pH; in this case, there can be no interside-chain residue electrostatic interactions, as would be expected from the assignment of Tiffany and Krimm.

It seems desirable at this point to examine the question whether the circular dichroism spectra of unordered proteins and dry polypeptide films could not result from the incipient

formation of ordered regions, such as short α helices. Such a situation seems highly unlikely. Greenfield and Fasman (1969a) have computed theoretical circular dichroism spectra for various combinations of α-helical, I-β, and random coil conformations based on solution circular dichroism spectra of poly-L-lysine and PGA. None of the combinations of these three conformations result in spectra similar to those of the constrained unordered structures described in this paper. It appears, therefore, that this type of spectrum with a negative shoulder between 215 and 230 mμ and a negative band between 200 and 205 mμ is truly characteristic of an unordered constrained polypeptide chain. Due to the constraints, a tightly folded unordered chain should possess a high level of local interactions and possibly even some local order. In this respect, it resembles the situation found in the unordered portions of the three-dimensional structures of globular proteins. In these structural regions, the chain is folded in a definite pattern, characterized by the high degree of local order and interactions, but totally devoid of long-range order, *i.e.*, the order is such that knowledge of the space coordinates of one residue does not permit an assignment to be made to the coordinates of adjacent residues. Such structural regions may quite possibly have circular dichroism spectra similar to those of constrained unordered structures rather than those of randomly coiled polypeptides.

One further point seems worthy of consideration. It is known that amide groups in different asymmetric environments have different characteristic optical activities. Optical rotatory dispersion and circular dichroism studies on a number of cyclic amides (Litman and Schellman, 1965; Balasubramanian and Wetlaufer, 1967; Greenfield and Fasman, 1969b) have revealed that these may have a large variety of optical rotatory spectra, some of them similar to those of polypeptides in solution. Furthermore, these are highly sensitive to the environment. For example, the circular dichroism spectrum of L-alanylglycyl anhydride changes drastically with a change in solvent. In trifluoroethanol, its circular dichroism spectrum is similar to that of unordered polypeptides in film form, with a negative band at 201 mμ and a negative shoulder at 215 mμ. In 2-propanol, its circular dichroism spectrum at first sight may be confused for that of an α helix; it has a negative doublet with apeces at 221 and 204 mμ. In acetonitile, it almost mimics the circular dichroism spectrum of randomly coiled poly-L-lysine in solution. Thus, in the interpretation of protein spectra, one must constantly be cognizant of exact band positions, relative intensities, overlaps, etc. A striking example of possible misinterpretation has been pointed out recently by Laiken *et al.* (1969) for gramicidin S-A. The circular dichroism spectrum of this cyclic peptide displays two negative maxima at 207 and 215 mμ and, at first glance, might be confused with that of an α helix. However, the band positions are incorrect for that conformation and must represent the result of the addition of a number of bands, each characteristic of the various conformations assumed by the individual residues of this molecule. It is interesting to note that the circular dichroism spectrum of ribonuclease, which is similar, has been analyzed successfully in terms of 9–11% α helices, 33% I-β, and 40–60% random conformations (Greenfield and Fasman, 1969a; Pflumm and Beychok, 1969).

The present observations that the circular dichroism spectra of unordered polypeptide chains can vary strongly in band position, intensity, and sign, similarly to the earlier observation on β-structure spectra, suggest once again that a quantitative analysis of protein conformation in solution is at present a very risky task. The limited amount of success attained at present (Timasheff *et al.*, 1967; Greenfield and Fasman, 1969a; Pflumm and Beychok, 1969) must be regarded with a great deal of caution. In none of the cases was it possible to fit over the entire spectral range the experimental circular dichroism pattern with a combination of spectra obtained from polypeptides in solution; in all cases, reasonable explanations could be offered for the lack of fit; basically such explanations may be reduced to considerations of spectral shifts due to side-chain interactions, variation in the local polarity of the chain environment in a folded structure in the local polarity of the chain environment in a folded structure, and contribution of aromatic and disulfide chromophores. It seems equally plausible that the observed lack of fit was due to a contribution made by the presence of a variety of conformations other than the fundamental three employed, each with its own characteristic circular dichroism spectrum. Such conformations may be, for example, the II′-β structure or the constrained unordered structure described in the present paper.

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