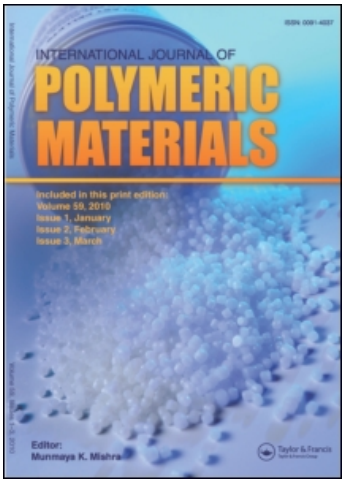

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Optical Activity Related to Ordered Aggregation in Some Biological Molecules: I. Poly(L-proline) and Collagen

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Optical Activity Related to Ordered Aggregation in Some Biological Molecules

1. Poly(L-proline) and Collagen†

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Optical activity, due to differential light scattering, is shown to produce large effects on the circular dichroism (CD) spectra of several biopolymers. It is suggested that helical association on aggregation is the causative factor. Thus, under the salt conditions for formation of native fibrils of collagen from solution, new CD bands are found to appear and intensify with time. Similarly, in the case of polyproline I, $M = 13{,}500$ (thought to associate in a side-to-side manner), large CD bands are observed throughout the visible and UV spectrum in addition to the normal CD bands of the unassociated form. These bands can be related to scattering phenomena. The new bands appear to be overtones of a fundamental frequency, similar, in some respects, to Bragg X-ray diffraction from crystals, and not to be confused with the ordinary Duysens absorption flattening effect on the CD spectra that is theoretically calculable from Mie scattering of spherical aggregates. It does, however, correspond to the optical activity observed in liquid crystals.

1 INTRODUCTION

Understanding the organization of biological systems is of vital importance to interpreting structure-function relationships.

Many attempts have been made recently to use circular dichroism (CD) and optical rotatory dispersion (ORD) of particulate systems to obtain meaningful information. Usually researchers have tried to correct for the effects of scattering. Thus, Gordon,[1] using Mie scattering theory, has calculated the effects on CD and ORD of scattering in the forward direction for

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optically active spherical and spherical shell particles which were taken to have the optical properties of a synthetic helical polypeptide. The calculated spectra were very similar to the observed results on aggregated poly(L-glutamic acid).[2] This type of scattering is seen quite often and should be viewed as an artifact distorting the informational content at the molecular level. Maestre and coworkers,[3] on the other hand, suggested that the scattered light deviated from the forward direction can contain information about ordered asymmetry at the scattering center. However, the theory of light scattered by an asymmetric particle in terms of its influence on CD and ORD has not been attempted.

A very different approach to understand ordered systems may be to look for similarities in structure to ordered liquid crystals. Very high optical activity was found in derivatives of cholesterol as early as 1888[4] and a theory[5] has been proposed built upon the supposition that ordered molecules exist in layers which stack with a helical twist one upon the other. Many cholesteric liquid crystals show anomalous rotatory dispersion and give a CD band due, not to absorption, but to selective reflection.[6] Robinson[7] has compared solutions of poly(γ-benzyl-L-glutamate) (PBLG) in organic solvents to cholesteric liquid crystals in many of their properties, but no CD bands seem to have been recorded.

It should be noted that very large ellipticity ($-$ 300,000 deg cm^2/d mol) at 197 nm has been found for the hexamer of BOC(Ileu)$_n$OMe in trifluoroethanol,[8] and high optical rotation measured at 597 nm for the pentamer of methyl-L-glutamate in dioxane.[9] There is some evidence that association is the causative factor.

2 EXPERIMENTAL

CD spectra were recorded on a Jasco 5 instrument, both before and after modification by Spoul Co. A Cary 15 spectrograph was used to take absorption spectra.

2.1 Poly(L-proline)

Poly(L-proline) (PP) was obtained from many sources, but one particular (125 B-1700) sample, of stated molecular weight 13,500 purchased from Sigma Chem. Co., was used in the experiments because it was only sparingly soluble in water in form I. Samples of molecular weight 1550 dissolved in water in form I,[10] and higher molecular weight samples were not soluble in form I in water. There are many indications in the literature[11,12,13] that poly(L-proline) in form I occurs in an aggregated state. It was surprising, however, to find the cloudy solutions to have extraordinary optical activity as represented by CD

bands in the visible region of the spectrum where there is no indication of any corresponding absorption bands (Figure 1). As the sample dissolved and became clear the anomalous CD bands disappeared. Also, if a solvent was used in which solution was immediately possible, such as trifluorethanol, there was no indication of these bands.

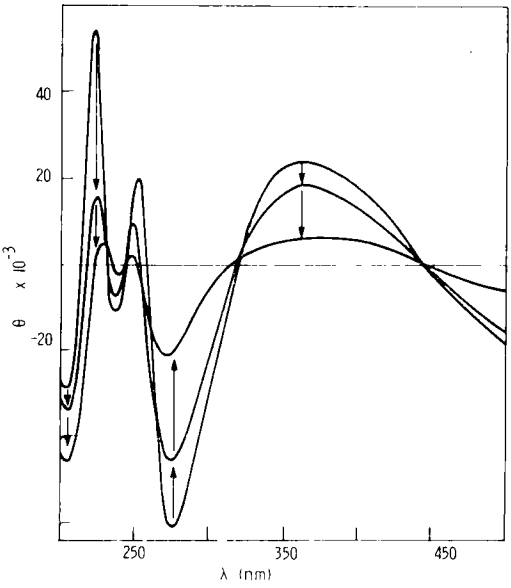

FIGURE 1 CD spectra of poly(L-proline) I, M = 13,500, in water. Arrows indicate changes in spectra with time over a period of a few hours. (Bands at 230, 215, and 200 nm are typical of form I of same sample dissolved in trifluoroethanol. These are replaced by bands at 227 nm and 208 nm as sample changes to form II.) The additional bands slowly disappear as the sample dissolves.

Many factors were shown to influence the intensity and position of the bands.

1) The anomalous bands occurred only when aggregated samples were noted. Thus micropore filtering reduced, but did not necessarily eliminate, the intensity of the bands.

2) The position of the sample holder in the optical track influenced the recorded CD spectrum (Figure 2).

3) Since the samples in water slowly dissolved, the length of time in solution is a factor. It was found that the bands decreased in intensity and shifted to shorter wavelengths over a period of several hours.

4) Temperature is known to be an important control of the structure of liquid crystals. We found that reducing the temperature to 5° shifted our bands

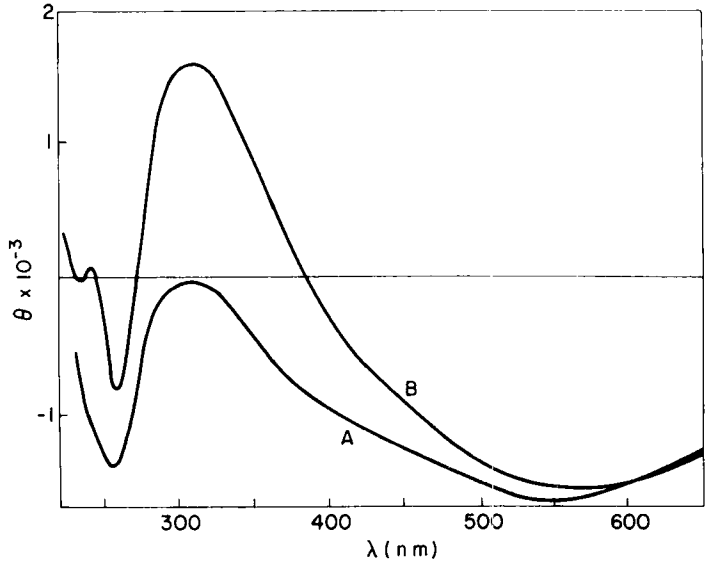

FIGURE 2 CD spectra of poly(L-proline) I, M = 13,500, in trimethyl phosphate: (A) sample close to the detector; (B) sample moved 10 cm toward light source. (Sample concentration 10^{-3} g/cm^3; 1 mm cell.)

of PP to shorter wavelengths. On raising the temperature back to 25° the bands returned to their initial position.

5) Many salts were found to influence the position and intensity of the bands. Immediately on addition ($CaCl_2$ > LiBr > $NaClO_4$ = $LiClO_4$ ≫ NaCl; see Table I), there was a shift to shorter wavelengths. By diluting out the salt the bands returned to longer wavelengths. 6 M NaH^4PO^2 was found to stabilize the aggregate and the CD spectrum remained constant with time over a period of days.

6) Some solvents also changed the position of the bands. Thus the positive band occurring at 350 nm in water was found at 300 nm in trimethyl phosphate, a solvent in which the sample had limited solubility. No change in the CD spectrum was found over a period of several days.

7) Both urea and guanidine hydrochloride were effective agents in shifting the bands to shorter wavelengths. In 4.8 M guanidine hydrochloride the samples were almost clear (Figure 3).

8) Scattering from the samples was evident. On attempting to record an absorption spectrum it was noted that scattering was most intense at the long wavelength region. A large band, apparently due to scattering, was recorded using a Cary 15 spectrograph. Many minor maxima were superimposed on the large band in the regions of the recorded CD bands.

TABLE I

Initially recorded position of CD spectra anomalous bands of poly(L-proline), with the sign of the bands as indicated

Solvent	Wave length (nm) and sign of bands			
water	250 (+)	280 (−)	370 (+)	650 (−)
4 *M* NaCl	247 (+)	280 (−)	365 (+)	650 (−)
4 *M* $LiClO_4$	247 (+)	270 (−)	340 (+)	
4 *M* $NaClO_4$	248 (+)	270 (−)	350 (+)	
5 *M* LiBr	230 (+)	245 (−)	290 (+)	
5 *M* $CaCl_2$	230 (+)	245 (−)	275 (+)	
5 *M* urea		245 (−)	295 (+)	350 (−)
10 *M* urea		230 (−)	255 (+)	350 (−)

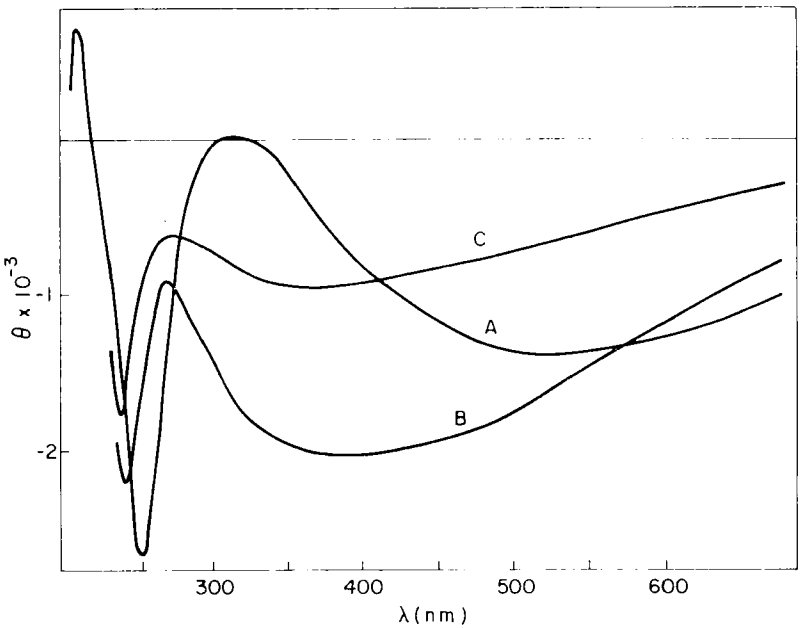

FIGURE 3 CD spectra of poly(L-proline) I, M ~ 13,500 with sample close to detector: (A) in water; (B) in 3M guanidine hydrochloride; (C) in 4.8M guanidine hydrochloride. (Sample concentration 2 × 10^{-4} g/cm^3; 5 mm cell.)

2.2 Collagen

Collagen was obtained by dissolving rat tail tendons in 2% acetic acid. It was purified by repeated precipitation with concentrated NaCl solutions. Neutral salt soluble rat skin collagen was donated by Dr. Barbara Doyle. It had been highly purified.

CD spectra were recorded with and without the addition of salts that were hoped to produce native type collagen fibers.[14]

Room temperature conditions were found to be right for the slow formation of fibers in 0.5 to 0.9 *M* NH_4F or NaF. New bands appeared in the CD spectra. Over the period of an hour the bands could be seen to grow in intensity (Figures 4 and 5). The metastable condition of the samples was evident since simple shaking caused the new bands to disappear. On observing these samples further, the bands could be found to reform. If the temperature was kept at 30° the bands formed more rapidly than at 25°. Samples from both sources acted similar.

Other types of distortion of the CD spectrum were found to be produced by concentrated solutions of NH_4F and KF with no new bands appearing.

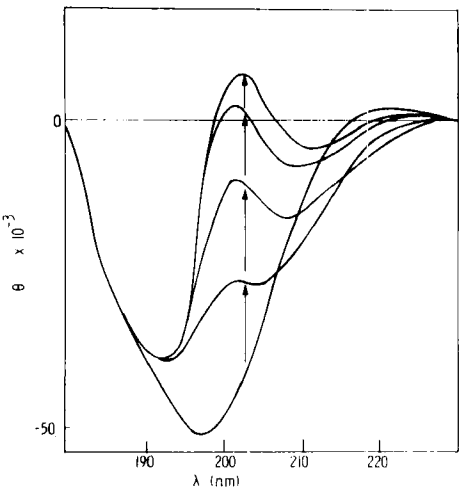

FIGURE 4 CD spectra of collagen in 0.9*M* NaF showing formation of optically active aggregate. The time between curves was approximately 20 min. (Sample concentration 2.5×10^{-5} g/cm^3; 5 mm cell.)

3 DISCUSSION

It is difficult to discuss results that seem to have no obvious relationship to recorded phenomena. Bands were appearing "out of the blue". In particular

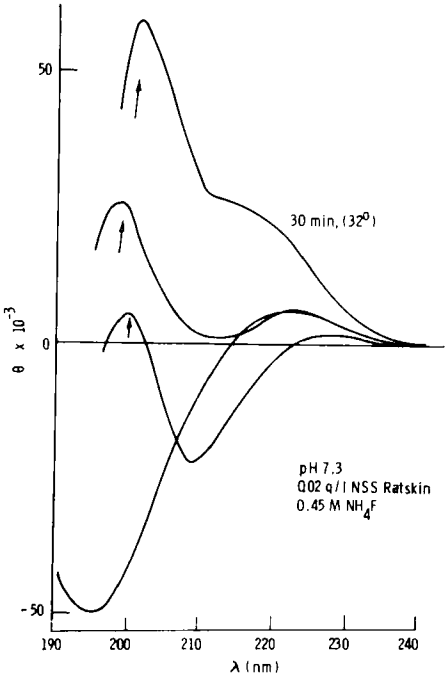

FIGURE 5 CD spectra of neutral salt soluble collagen in 0.45*M* NH_4F. Thirty minutes total elapsed time indicated by the arrows.

in the case of aggregated poly(L-proline) I, four new CD bands, some positive, some negative, were evident in addition to the normal CD bands present in low molecular weight samples in the same media, or when a solvent, such as trifluroethanol was used which gave complete solubilization of all samples. Instrumental artifacts are ruled out since in many other systems where aggregation and precipitation were evidently taking place, such as near the isoelectric point of many proteins, no new bands were produced and any distortion observed was similar to the results discussed by Gordon.[1] A relationship to differential scattering of circularly polarized light is suggested.

That scattering is a factor in the production of the bands follows from the differential effect in the recorded spectra dependent upon the distance of the sample from the detector. Thus, in the presence of scattering, if the sample is far from the detector, some of the incident beam may have deviated from the beam axis. Such scattered light will appear to have been absorbed and produce anomalous high optical densities, but only in the case of differential scattering of left *vs.* right circularly polarized light will this produce a change in the shape of a recorded CD spectra[3] as observed in Figure 2.

The helical array of oriented molecules occurring in layers in liquid crystals is known to produce a CD band at the wave length of maximum reflection.[6] It seems plausible that any helical array of optically active molecules should also produce a CD effect. The molecules may well be staggered in helical array in cylindrical bundles as suggested for the structure of native collagen in order to interpret the electron micrographs.[14]

In the case of poly(L-proline), with four bands present related to scattering, we find they may be ordered in frequency as the second, third, fourth, and fifth overtones of a fundamental that may be occurring in the infra-red out of range of detection. It is puzzling that bands of both signs seem to be present, if there is some relationship to the helix-hand of the suggested arrangement. It may be an artifact due to the simple method of recording. Thus, if we take the band appearing in the 500 nm region as the fundamental and assume distortion of the base line produces the next apparent band toward the blue as being positive when in effect it is on the base line as in Figure 4, only negative bands occur and can be ordered as fundamental and first and second overtones. In relation to this, Robinson[7] found that in the case of poly(γ-benzyl-L-glutamate) the sign of the optical rotation in the visible region of the spectrum depended on the solvent and concluded that the "twist" of the macrostructure had no simple relationship to the sense of the α helix conformation of the molecule. We would like to point out that the sign of the optical rotation would change dependent on which wavelength side from the proposed, but unobserved CD band, was being monitored.

The bands of poly(L-proline) that are related to scattering were found to shift to shorter wavelengths under conditions of disruption of the aggregate by lyotropic salts, urea, and guanidine hydrochloride. Lowering the temperature produced a similar effect which may be related to increased solubility also known to occur. Simple change of aggregate size, however, could hardly produce wavelength changes observed in the CD spectra, and an explanation of the experimental results will not be attempted at this time.

Perhaps more interesting from a biological point of view are the results recorded on collagen. Here we may have a prototype for many of the ordered systems occurring in nature. The effects observed were dramatic and reproducible with samples from different sources. Thus, under conditions that were expected to lead to native fiber type aggregation, we found new CD bands to grow in intensity as fibril formation was suggested to be taking place. It is most likely that they are related to differential scattering due to an ordered array of collagen molecules. Under conditions where aggregation took place too fast for the molecules to align themselves no such bands were observed. This could correspond to non-banded microfibrils recorded by Schmitt and coworkers.[15]

It is hoped that further study will show that differential scattered bands may be used as a tool to understand and detect superstructure existing in other biological systems.

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DISCUSSION

Prof. P. L. Luisi (*Swiss Federal Institute of Technology, Zurich*): Let me first give an example of the same kind of feature in the case of low molecular weight compounds. We have been investigating the spectroscopic properties of peptides containing two aromatic residues separated one from the other by an increasing distance, for instance, Gly-Trp-$(Gly)_n$-Trp-Gly with n = 0.1 and 2. In the case of n = 1 and n = 2, the CD spectrum at 290 nm shows a very weak negative band. The peptide with n = 0 shows in the same region a large positive band, which however, by dilution, decreases in intensity and eventually turns into the small negative band characteristic of the other two peptides. We attributed this effect to intermolecular interactions which for some reason are effective when n = 0 and not in the other cases. We think that this effect in the CD spectrum reflects a change in the aromatic chromophores upon interaction, whereas you seem in general to emphasize the scattering effect over the electronic perturbations. My question is to what extent in the polymeric systems you discussed, this discrimination between electronic effects and scattering effects has some meaning and can be operatively done?

Prof. M. L. Tiffany: If bands appear in the circular dichroism spectra in regions where there is no chromophore absorption the bands are suspect of being due to differential scattering. The experimental fact that band shape changes with amount of scattered light picked up by the detector ensures that differential scattering is present. Whereas aggregation and the possibility of optically active array of molecular units can increase with concentration, exciton interaction of two chromophores can also occur and is, of course, not a scattering phenomenon. One should be aware, also, that band inversion can occur in concentrated solutions due to overloading of the amplifier. This effect can be eliminated by using thinner cells.

Prof. H. Sund (*University of Constance, Constance*): Some of your results were obtained with clear solutions, some others with solutions containing undissolved material. Can some of the differences in the results be explained by effects of light scattering?

Prof. M. L. Tiffany: Differential scattering is dependent on scattering taking place. Many solutions can look clear and pass through a microporous filter and still contain particulate matter.