

must be important. The solvation part clearly enters through the  $a_{H_2O}$  term and has been adequately discussed above. Consideration of Figure 1 and the activity coefficients in eq 14 indicates that  $f_s/f_{ts}$  is decreasing with increasing acidity. We qualitatively observed greater solubility of our substrate in more acidic solutions in agreement with a marked decrease in  $f_s$ . Although salting in of a neutral organic molecule is not expected, it might be explained by our use of a mixed solvent; if water preferentially solvates protons, this leaves a greater effective concentration of dioxane for solvation of the neutral substrate. The acid-

catalyzed hydrolysis of a phosphinate soluble in water should be studied to resolve this question by elimination of the dioxane problem.

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## Analysis of the Optical Rotatory Dispersion of Polypeptides and Proteins. IV. A Digital Computer Analysis for the Region 190–600 $m\mu$ <sup>1,2</sup>

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**Abstract:** The lack of methods for resolving complex optical rotatory dispersion curves into their component Cotton effects has prevented an adequate comparison of experiment and theory. A nonlinear, least-squares curve fitting approach to the interpretation of optical rotatory dispersion data is shown to be effective in both resolving overlapping Cotton effects and revealing small Cotton effects obscured by larger ones adjacent to them. The application of this method to the optical rotatory dispersion data (from 600 to 190  $m\mu$ ) of various  $\alpha$ -helical polypeptides in solution yields results agreeing with the conclusions from the combination of circular dichroism and polarized ultraviolet absorption spectra which support the exciton (for  $\pi \rightarrow \pi^*$ ) and one-electron (for  $n \rightarrow \pi^*$ ) models. The method is also applied to the optical rotatory dispersion of the random form of poly- $\alpha$ -L-glutamic acid and the poly-L-proline II helix. For the former, three Cotton effects are found centered at 197.6, 216.6, and 235  $m\mu$  with rotational strengths  $-14.2 \times 10^{-40}$ ,  $1.9 \times 10^{-40}$ , and  $-0.13 \times 10^{-40}$  erg  $cm^3$ , respectively. For the poly-L-proline II helix, two Cotton effects are found centered at 206.9 and 221.0  $m\mu$  with rotational strengths  $-33 \times 10^{-40}$  and  $5 \times 10^{-40}$  erg  $cm^3$ , respectively. It is concluded that further theoretical work is needed before assignments can be made for the optically active transitions of the "random" polypeptide conformation and the poly-L-proline II helix.

In 1956, Moffitt<sup>3</sup> predicted that when the peptide chromophore is incorporated into an  $\alpha$  helix, the 185- $m\mu$   $\pi \rightarrow \pi^*$  monomer transition would be split into two perpendicularly polarized components separated by 2800  $cm^{-1}$  ( $\sim 10$   $m\mu$ ). In 1961, studies<sup>4</sup> on the far-ultraviolet polarized absorption spectra of oriented films of  $\alpha$ -helical polypeptides revealed two transitions near 190  $m\mu$  with the predicted polarization and approximately the predicted energy separation. Thus, it seemed that the validity of the application of the exciton model to polypeptides was established. Moffitt<sup>3</sup> also predicted that the two components of the split  $\pi \rightarrow \pi^*$  transition would have large rotational strengths of opposite sign but equal absolute magnitude. He assumed that the  $n \rightarrow \pi^*$  peptide transition near 220  $m\mu$  would make a negligible contribution to the optical activity. A year later Moffitt, Fitts, and Kirk-

wood<sup>5</sup> reported that certain terms in the original Moffitt treatment which would give rise to additional optical activity for the exciton band had been neglected; however, this additional contribution was not evaluated until 1964.<sup>6</sup> Therefore, even with these revisions, it was expected that all the optical activity above 170  $m\mu$  would arise from the 190- $m\mu$  exciton band, the perpendicular and parallel components of which were predicted to be separated by 10  $m\mu$ .

Thus, in 1960–1961, when the optical rotatory dispersion (ORD) of some proteins and  $\alpha$ -helical polypeptides was measured to 225  $m\mu$ ,<sup>7,8</sup> it was surprising to discover a sizeable trough at 233  $m\mu$ . This was thought to be due to a negative Cotton effect located near 225  $m\mu$  and was assigned to the  $n \rightarrow \pi^*$  transition. It was also pointed out at the time that an alternative ex-

(1) Polypeptides. LII. For the previous paper in this series, see *J. Am. Chem. Soc.*, **88**, 2041 (1966).

(2) We are pleased to acknowledge the support (in part) of this work by U. S. Public Health Service Grants AM-07300-01, -02, and -03.

(3) (a) W. Moffitt, *Proc. Natl. Acad. Sci. U. S.*, **42**, 735 (1956); (b) W. Moffitt, *J. Chem. Phys.*, **25**, 467 (1956).

(4) W. B. Gratzler, G. Holzwarth, and P. Doty, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1785 (1961).

(5) W. Moffitt, D. D. Fitts, and J. G. Kirkwood, *ibid.*, **43**, 723 (1957).

(6) (a) I. Tinoco, Jr., *J. Am. Chem. Soc.*, **86**, 297 (1964); (b) I. Tinoco, Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.*, **38**, 1317 (1963); (c) R. W. Woody, Dissertation, University of California, Berkeley, Calif., 1962, as quoted in G. Holzwarth, Dissertation, Harvard University, 1964.

(7) N. S. Simmons and E. R. Blout, *Biophys. J.*, **1**, 55 (1960).

(8) N. S. Simmons, C. Cohen, A. G. Szent-Gyorgyi, D. B. Wetlaufer, and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 4766 (1961).

planation of this minimum was that there was a positive Cotton effect at shorter wavelength superimposed on a steep, negative background. This interpretation would not require the  $n \rightarrow \pi^*$  transition to be optically active in agreement with Moffitt's assumption. However, support for the assignment of the 233-m $\mu$  minimum to a 225-m $\mu$   $n \rightarrow \pi^*$  Cotton effect came from calculations by Schellman and Oriol in 1962.<sup>9</sup> They demonstrated that a Cotton effect of approximately the observed magnitude was predicted when the effect of the static field of the helix on the borrowing between  $n \rightarrow \pi^*$  and  $\pi \rightarrow \pi^*$  transition dipole moments was considered.

Extension of the ORD measurements of  $\alpha$ -helical polypeptides even further into the ultraviolet<sup>10</sup> did not resolve this dilemma. Inspection of the ORD curve revealed the existence of at least two Cotton effects in the region 250–185 m $\mu$ , but it could not be stated unequivocally that a third was not also present. At the same time (1962) that the far-ultraviolet ORD of  $\alpha$ -helical polypeptides was reported, measurements of the circular dichroism (CD) over the same region were published.<sup>11</sup> Even with these data the question of whether two or three Cotton effects were present in the far ultraviolet could not be resolved.<sup>12</sup>

In 1963 the far-ultraviolet ORD of poly-L-proline II was reported.<sup>13</sup> The application of exciton theory to the poly-L-proline II helix predicts<sup>14</sup> a similar split in the 190-m $\mu$   $\pi \rightarrow \pi^*$  transition of the monomer. Yet, the far-ultraviolet ORD curve seemed to show only one Cotton effect.

Thus, at this point (1963), no satisfactory test had been made of the optical activity predictions of Moffitt's theory. It appeared likely that apparent discrepancies between theory and experiment were due in part to the inadequacies of the qualitative method of comparison. In order to compare experiment with theory quantitatively, it is necessary to extract from experimental data parameters which can be estimated from a theoretical model—in this case the position and rotational strengths of the optically active transitions. Thus, for the interpretation of complex ORD curves, some method had to be developed to resolve overlapping Cotton effects or to detect small Cotton effects buried by much larger neighbors.

Attempts to estimate the rotational strength and position of the far-ultraviolet optically active transitions of the  $\alpha$  helix have met with varying degrees of success. In trying to fit the far-ultraviolet ORD of poly- $\alpha$ -L-glutamic acid (PGA) (Na salt, pH 4.3) to Natanson's equation, Yamaoka<sup>15</sup> concluded that a third Cotton effect (possibly negative) was present in the region 185–250 m $\mu$  but he was unable to make any estimate of its size. Holzwarth<sup>16</sup> showed that an explanation consistent with the far-ultraviolet absorption and CD

of the  $\alpha$  helix was that three optically active transitions (190, 206, 222 m $\mu$ ) were present in the region 190–250 m $\mu$ . This explanation was based on his 1962 data<sup>11</sup> and on some assumptions regarding the degree of proportionality between the CD and absorption curves. Since the CD data have uncertainties of 10–20% over the 190–250-m $\mu$  range, and the calculations were not optimized, his parameter estimates are liable to quite large errors. Both the more recent CD data<sup>17, 18</sup> (which show better resolution than the 1962 data<sup>11</sup>) and the calculations presented below confirm the presence of three optically active transitions.

In this paper: (1) we describe a digital computer program for the nonlinear least-squares analysis of ORD data which allows more precise estimates of the Cotton effect parameters than have been available; (2) we report the results of the application of this program to (a) several  $\alpha$ -helical polypeptides, (b) a polypeptide having a different helical structure—poly-L-proline II, and (c) a polypeptide in the random conformation. With this approach we have been able to determine the positions, half-widths, and rotational strengths of the Cotton effects required to fit the observed curves.

Application of this method of analysis to ORD data from: (1) many different homopolypeptides each measured in the same solvent, and (2) one synthetic homopolypeptide measured in a variety of solvents, should prove useful in determining the precise quantitative nature of the effects of solvents and of side chains on the ORD of synthetic polypeptides. As is emphasized in the accompanying paper, a knowledge of such effects is essential for the valid interpretation of the ORD's of globular proteins in terms of structure.

## Methods

**A. The Moscovitz Method.** The pioneer effort at nonlinear, least-squares curve fitting for ORD data was the work of Moscovitz.<sup>19, 20</sup> His calculations were limited to fitting the single 290-m $\mu$  Cotton effect of the carbonyl group in various saturated ketones.<sup>20, 21</sup> The present work extends this approach to include more than one Cotton effect.

Moscovitz showed<sup>20, 22a</sup> that the Kronig-Kramers transform of a Gaussian circular dichroism band,  $[\theta']_{\lambda} = [\theta']_i \exp[-(\lambda - \lambda_i)^2/\Delta_i^2]$ , is

$$[R']_{\lambda} \cong \frac{2[\theta']_i}{\pi^{1/2}} \left\{ \exp\left[-\frac{(\lambda - \lambda_i)^2}{\Delta_i^2}\right] \int_0^{\frac{(\lambda - \lambda_i)}{\Delta_i}} \exp(y^2) dy - \frac{\Delta_i}{2(\lambda + \lambda_i)} \right\} \quad (1)$$

where  $[\theta']_{\lambda}$  = ellipticity in deg cm<sup>2</sup> decimole<sup>-1</sup>,  $\lambda$  = the wavelength of observation,  $\lambda_i$  = the wavelength of the extremum in the CD curve,  $[\theta']_i$  = the value of  $[\theta']_{\lambda}$  at the extremum,  $\Delta_i$  = the half-width of the CD band (half the separation in m $\mu$  between the wavelengths at which  $[\theta']_{\lambda}$  falls to  $(1/e)[\theta']_i$ ). The subscript  $i$  refers to the  $i$ th transition.

- (9) J. A. Schellman and P. Oriol, *J. Chem. Phys.*, **37**, 2114 (1962).  
 (10) E. R. Blout, I. Schmier, and N. S. Simmons, *J. Am. Chem. Soc.*, **84**, 3194 (1962).  
 (11) G. Holzwarth, W. B. Gratzner, and P. Doty, *ibid.*, **84**, 3194 (1962).  
 (12) G. Holzwarth, W. B. Gratzner, and P. Doty, *Biopolymers Symp.*, **1**, 389 (1964).  
 (13) E. R. Blout, J. P. Carver, and J. Gross, *J. Am. Chem. Soc.*, **85**, 644 (1963).  
 (14) W. G. Gratzner, W. Rhodes, and G. D. Fasman, *Biopolymers*, **1**, 319 (1963).  
 (15) K. Yamaoka, *ibid.*, **2**, 219 (1964).  
 (16) G. Holzwarth, Dissertation, Harvard University 1964.

- (17) M. Legrand and R. Viennet, *Compt. Rend.*, **259**, 4277 (1964).  
 (18) G. Holzwarth and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).  
 (19) A. Moscovitz, *Rev. Mod. Phys.*, **32**, 440 (1960).  
 (20) A. Moscovitz, "Optical Rotatory Dispersion," C. Djerassi, Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p 150.  
 (21) A. Moscovitz, *Tetrahedron*, **13**, 48 (1961).  
 (22) (a) A. Moscovitz, Dissertation, Harvard University, 1957; (b) for a discussion of the Lorentz correction see J. Cassim and E. W. Taylor, *Biophys. J.*, **5**, 553 (1965).

The right-hand side of eq 1 will be referred to as a Moscovitz term. If  $[\theta']_\lambda$  is in units of deg cm<sup>2</sup> decimole<sup>-1</sup> (the molecular weight being the mean residue weight) then  $[R']_\lambda$  is in the same units and is the residue rotation. The primes indicate that the Lorentz correction<sup>22b</sup> for the index of refraction of the solvent has been made.

The rotational strength,  $R_i$ , associated with a single CD band is

$$R_i = \frac{hc}{48\pi^2 N} \int \frac{[\theta']_\lambda d\lambda}{\lambda} \text{erg cm}^3 \quad (2)$$

where the integration is taken through the band. Hence, for a CD band Gaussian in wavelength

$$R_i = \frac{hc}{48\pi^2 N} \frac{\pi^{1/2} [\theta']_i \Delta_i}{\lambda_i} = 1.09 \times 10^{-42} \frac{2[\theta']_i \Delta_i}{\pi^{1/2} \lambda_i} \text{erg cm}^3 \quad (3)$$

In general we shall be dealing in terms of  $A_i$ , where

$$A_i \equiv \frac{2[\theta']_i \Delta_i}{\pi^{1/2} \lambda_i} = \frac{R_i}{1.09 \times 10^{-42}} \text{erg cm}^3 \text{ deg} \quad (4)$$

since the rotation can then be written as

$$[R']_\lambda = \frac{A_i \lambda_i}{\Delta_i} \left\{ \exp \left[ - \left( \frac{\lambda - \lambda_i}{\Delta_i} \right)^2 \right] \int_0^{\frac{(\lambda - \lambda_i)}{\Delta_i}} \exp(y^2) dy - \frac{\Delta_i}{2(\lambda + \lambda_i)} \right\} \text{deg cm}^2 \text{ dmole}^{-1} \quad (5)$$

It can be shown<sup>22a</sup> that

$$\lim_{x_i \rightarrow \infty} [\exp(-x_i^2) \int_0^{x_i} \exp(y^2) dy] = \frac{1}{2x_i} \quad (6)$$

hence, denoting  $(\lambda - \lambda_i)/\Delta_i$  by  $x_i$ , at large values of  $x_i$

$$[R']_\lambda \doteq \frac{A_i \lambda_i}{\Delta_i} \left[ \frac{1}{2x_i} - \frac{\Delta_i}{2(\lambda + \lambda_i)} \right] \equiv \frac{A_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \text{deg cm}^2 \text{ dmole}^{-1}$$

which is simply the Drude approximation.

A much better approximation can be obtained from an asymptotic expansion,<sup>23</sup> for  $x_i > 4$

$$\exp(-x_i^2) \int_0^{x_i} \exp(y^2) dy \approx \sum_{n=1}^{\infty} \left( \frac{4}{x_i} \right)^{2n-1} a_{2n-1} \quad (7)$$

where the coefficients  $a_{2n-1}$  can be evaluated, the first three being  $a_1 = 0.12499$ ,  $a_3 = 0.00392$ ,  $a_5 = 0.00031$ . A good estimate of the error involved in approximating a Moscovitz term by a Drude term is given by the second term in the expansion, *i.e.*

$$\text{error} \cong \left[ \frac{A_i \lambda_i}{\Delta_i} \right] \left( \frac{4}{x_i} \right)^3 a_3 \cong \frac{1}{4x_i^3} \frac{A_i \lambda_i}{\Delta_i} \equiv \frac{A_i \lambda_i \Delta_i^2}{4(\lambda - \lambda_i)^3}$$

In his treatment Moscovitz<sup>19</sup> used an abbreviated two-term Drude expression, with parameters  $B$ ,  $C$ , and  $Q$  to approximate the contribution of Cotton effects outside the region of concern (background). Under circumstances where it is known that a single Cotton effect, centered at a wavelength close to the

region of measurement, is contributing the major part of the background, the value of  $Q$  may be taken to be the center of that Cotton effect and  $B$  may be considered to be related to its rotational strength *via* eq 4. In addition, Moscovitz assumed that the term  $A_i \lambda_i / 2(\lambda + \lambda_i)$  on the right-hand side of eq 5 could be absorbed by the background and did not consider it explicitly. Thus, the relation he used had the form

$$[R']_\lambda = R_0 [\exp(-x_0^2) \int_0^{x_0} \exp(y^2) dy] + \frac{B}{\lambda^2 - Q^2} + \frac{C}{\lambda^2} \quad (8)$$

where  $x_0 = (\lambda - \lambda_0)/\Delta_0$ , and the subscript  $i$  has been replaced by 0, since only one Cotton effect is being considered.  $Q$  was held constant throughout a given calculation and estimates were obtained of  $R_0$ ,  $\lambda_0$ ,  $\Delta_0$  for the Cotton effect and  $B$  and  $C$  for the background. Some data gave an excellent fit to eq 8; others did not.<sup>20</sup> In his discussion of the calculations, Moscovitz pointed out that the most probable cause of unsatisfactory fit is that the CD curves are not well approximated as Gaussians.

An analysis of the type of Moscovitz's which assumes only one Cotton effect in the region of measurement cannot yield meaningful results with polypeptides and proteins, since the peptide Cotton effects overlap extensively. In fact, even if one limits oneself to the first extremum (data above 225  $\mu$ ), the other Cotton effects are too close to allow approximation as an abbreviated two-term Drude background.<sup>24</sup> It was considered worthwhile, therefore, to attempt an analysis of the ORD data along the lines of the Moscovitz calculations, but with more Cotton effects.

**B. The Present Method.** A new computer program was developed which differs from the Moscovitz program in two important respects: (1) more than one Cotton effect may be included during a given calculation; (2) weights are included in the nonlinear, least-squares analysis.<sup>25</sup> The use of weights compensates for variations in the absolute magnitude of the experimental error of observed rotations at different wavelengths. Thus, data from 600 to 185  $\mu$  can be used, which provide improved estimates of the parameters.

The terms used to approximate the contribution to the ORD of the optically active transitions outside the observable wavelength range were

$$\frac{BQ}{\lambda^2 - Q} + \frac{C}{\lambda^2} \quad (9)$$

rather than the form in eq 8. During a calculation using the Moscovitz program,<sup>19</sup>  $Q$  is fixed at some convenient value; however, in the program used for the calculations reported here,  $Q$  is included as a parameter in the nonlinear, least-squares analysis. Therefore, the  $Q$  was specifically included in the numerator in order to reduce the interdependence of  $B$  and  $Q$ .  $Q$  is used, instead of  $Q^2$ , in order to simplify the form of the first and second derivatives of the residual with respect to this parameter.

(24) G. R. Bird, private communication.

(25) For a description of the nonlinear, least-squares technique, see, for example: C. A. Bennett and N. L. Franklin, "Statistical Analysis in Chemistry and the Chemical Industry," John Wiley and Sons, Inc., New York, N. Y., 1954.

(23) For an introduction to this type of numerical approximation, see, for example: G. Stanton, "Numerical Methods for Science and Engineering," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1961.

In certain cases, the one Drude term, equivalent to the abbreviated two-term background (eq 9) of a given solution, is centered in the wavelength region of the observed values. Thus, if only one Drude term were being employed, the optimal solution would require the evaluation of the Drude term at or near  $\lambda = Q^{1/2}$ ; this would quite obviously prevent convergence. The same problem could arise with the abbreviated two-term Drude background (see eq 9). However, as will be seen from the results, there is usually a degeneracy in acceptable sets of background parameters ( $B$ ,  $C$ , and  $Q$ ). This degeneracy allows a value of  $Q^{1/2}$ , lying outside the wavelength range of the observed rotations, to be arbitrarily selected without loss of generality. Thus, the expression in eq 9 does not have to be evaluated near  $\lambda = Q^{1/2}$ , and we are essentially using the same procedure, with regard to background parameters, as Moscovitz. Evidently,  $B$ ,  $C$ , and  $Q$  have no immediate physical significance and cannot be related to the parameters of the Cotton effects in the vacuum ultraviolet which give rise to the background rotation.

The term  $\Delta_i/2(\lambda + \lambda_i)$  (see eq 1) was specifically included in the calculation of the contribution of each Cotton effect, since without it, the integral expression is not asymptotic to a Drude term. It has been shown<sup>22a</sup> that the

$$\lim_{x \rightarrow \infty (\lambda \gg \lambda_i)} [\exp(-x_i^2) \int_0^{x_i} \exp(y^2) dy]$$

is  $1/2x_i$  or ultimately  $\Delta_i/2\lambda$ . Thus, from eq 8, at  $\lambda \gg \lambda_i$  the expression Moscovitz used for  $[R']_\lambda$  approaches

$$\frac{R\Delta_i}{2\lambda} + \frac{B+C}{\lambda^2}$$

which, at large enough  $\lambda$ , has a  $1/\lambda$  dependence. By contrast, the correct asymptote is the form approached by the equivalent one-term Drude,  $A_i\lambda_i^2/(\lambda^2 - \lambda_i^2)$ , which is  $A_i\lambda_i^2/\lambda^2$ . Equation 5 has the latter asymptote.<sup>26</sup>

Thus, the expression fitted to the observed data in the calculations reported here is

$$[R']_\lambda = \sum_i \frac{A_i\lambda_i}{\Delta_i} \left\{ \exp \left[ - \left( \frac{\lambda - \lambda_i}{\Delta_i} \right)^2 \right] \int_0^{\frac{\lambda - \lambda_i}{\Delta_i}} \exp(y^2) dy - \frac{\Delta_i}{2(\lambda + \lambda_i)} \right\} + \frac{BQ}{\lambda^2 - Q} + \frac{C^2}{\lambda^2} \quad (10)$$

As a measure of fit, an estimate of the variance of an observation of unit weight is used. This is referred to as the weighted residual mean square, or the RESMS, and is defined as<sup>25</sup>

$$\text{RESMS} \equiv \sum_\lambda \frac{W_\lambda^2 ([R']_\lambda^{\text{obsd}} - [R']_\lambda^{\text{pred}})^2}{N - NP}$$

where  $W_\lambda$  = the weight of the observation at wavelength  $\lambda$  and is proportional to the inverse of the estimated (or calculated) standard deviation of the measurement at wavelength  $\lambda$  (the weights are scaled so that the smallest is unity);  $[R']_\lambda^{\text{obsd}}$  = observed rotation at wavelength  $\lambda$ ;  $[R']_\lambda^{\text{pred}}$  = the predicted rotation at wavelength  $\lambda$ ;  $N$  = the number of wavelengths at which optical rotation is recorded;  $NP$  = the number of parameters.

(26) Moscovitz was concerned with rotations in the immediate vicinity of a single Cotton effect and hence these remarks are not relevant to his case.

It is possible to estimate the expected RESMS for a solution which fits within the experimental error (*i.e.*, to within approximately one standard deviation at each wavelength), since such a RESMS is the variance of an observation of unit weight,<sup>25</sup> and this has already been estimated in determining the weights to be used. Such an estimated RESMS is referred to as the experimental RESMS.

A nonlinear, least-squares calculation is an iterative process. For the actual calculations initial guesses of the parameters ( $A_i$ ,  $\lambda_i$ ,  $\Delta_i$ ,  $B$ ,  $C$ ,  $Q$ ) are provided to the computer along with observed data (rotations, wavelengths, and weights). These guesses are then modified by the program at each iteration in such a manner as to reduce the RESMS. When the latter does not decrease by more than 1% in two consecutive iterations, the calculation is considered to have converged to a solution. It should be noted that the convergence of this type of calculation is very sensitive to the initial guesses; a poor estimate of one of the more critical parameters ( $\lambda_i$ ,  $\Delta_i$ ) can be sufficient to prevent convergence even though the estimates of all the remaining parameters are extremely good.<sup>27</sup>

It was found that for the data used in these calculations all three background parameters ( $B$ ,  $C$ , and  $Q$ ) were rarely simultaneously determinable. The most successful procedure was to determine the parameters  $A_i$ ,  $\lambda_i$ ,  $\Delta_i$ , and  $B$  for a series of pairs of values of  $C$  and  $Q$ . Then using the values from the best solution, allow  $C$  to vary in addition to  $A_i$ ,  $\lambda_i$ ,  $\Delta_i$ , and  $B$  for a series of values of  $Q$ . For almost all the cases considered, the values of  $A_i$ ,  $\lambda_i$ ,  $\Delta_i$  and the RESMS are essentially unchanged as  $Q^{1/2}$  varies from 170 to 100  $\mu$ . This result indicates that the various sets of values of  $B$ ,  $C$ , and  $Q$  correspond to background contributions (in the region of observation) which are, for each calculation, identical within experimental error. For this reason, the values of the individual parameters  $B$ ,  $C$ , and  $Q$  have no physical significance; only the *total* contribution of the terms involving these parameters to the rotation has meaning.<sup>28</sup> Therefore,  $B$ ,  $C$ , and  $Q$  cannot be used to locate transitions further into the ultraviolet.

**C. Experimental.** CD measurements were performed by Dr. S. Beychok of Columbia University on a modified Jouan dichrographe previously described.<sup>29</sup> All the ORD data used for the calculations were obtained

(27) It was initially found valuable to resort to a factorial analysis approach to search out good starting solutions. This is a method of obtaining a minimum of a well-behaved function of many variables. Each parameter is incremented by a preset amount to obtain a set of values over the probable range; the function is then evaluated at the set of points in "parameter space" obtained by taking all possible combinations of the parameter values calculated as described above. The process is repeated with a finer mesh, *i.e.*, smaller increments and over a range of parameter values centered on the point for which the function had a minimum value in the last calculation. This process is continued until the parameter values which minimize the function are determined to the desired accuracy. The method was practicable only when a small number of parameters ( $\leq 4$ ) were being used.

(28) Often, the background parameter values so evaluated differed by less than one or two of their respective standard errors from zero, and therefore were not considered significant. (See, for example, solutions 2 and 3, Table I.) It should be noted that convergent solutions can be obtained with *fixed* nonzero background parameters. The RESMS's in these cases are greater than those for the solutions in which  $B$ ,  $C$ , and  $Q$  vary, but may still be less than the experimental RESMS, provided that the values assigned to  $B$  and  $C$  are not excessive. Whether or not these solutions are significant depends on whether the weights have been chosen correctly, and will require further investigation.

(29) S. Beychok, *Proc. Natl. Acad. Sci. U. S.*, **53**, 999 (1965).

Table I. Two Cotton Effect Solutions for PAEMG in Methanol-Water (9:1)

	1	2	3
RESMS <sup>a</sup> × 10 <sup>-8</sup>	1.18	1.21	1.21
R × 10 <sup>40</sup> (ergs cm <sup>3</sup> )	34.7 ± 0.9	35 ± 2	34 ± 3
A × 10 <sup>-4</sup> (deg erg cm <sup>3</sup> )	0.318 ± 0.008	0.32 ± 0.02	0.31 ± 0.02
λ (mμ)	192.7 ± 0.4	192.7 ± 0.6	192.8 ± 0.5
Δ (mμ)	6.4 ± 0.4	6.4 ± 0.7	6.3 ± 0.5
R × 10 <sup>40</sup> (ergs cm <sup>3</sup> )	-27.4 ± 0.6	-27.2 ± 0.9	-27.5 ± 0.7
A × 10 <sup>-4</sup> (deg erg cm <sup>3</sup> )	-0.251 ± 0.005	-0.249 ± 0.008	-0.252 ± 0.007
λ (mμ)	220.0 ± 0.3	220.0 ± 0.3	220.0 ± 0.3
Δ (mμ)	13.9 ± 0.3	13.8 ± 0.3	14.0 ± 1.0
B × 10 <sup>-4</sup> (deg)	0.0 <sup>b</sup>	-0.02 ± 0.13	0.3 ± 1.0
C × 10 <sup>-4</sup> (deg mμ <sup>-2</sup> )	0.0 <sup>b</sup>	500 ± 2200	-3100 ± 9200
Q <sup>1/2</sup> (mμ)	0.0 <sup>b</sup>	173.2 <sup>b</sup>	100.0 <sup>b</sup>

<sup>a</sup> Experimental RESMS = 0.25 × 10<sup>8</sup>. For meaning of symbols, refer to text. <sup>b</sup> Indicates those parameters which were held constant.

using a Cary 60 recording spectropolarimeter (with sample compartment temperatures from 22 to 25°). The errors in observed rotations vary with wavelength for two reasons: (1) the increase in residue rotation with decreasing wavelength is less than the increase in extinction coefficient; hence, the observed rotation at a given optical density decreases with decreasing wavelength; (2) the instrumental noise increases with decreasing wavelength. The dependence of experimental error on wavelength will, therefore, vary somewhat between polypeptides because of differences in their absorption spectra. If differences in solubility exist, such that solutions cannot be obtained at sufficiently high concentrations for optimal use of the polarimeter, then the experimental error may be larger. To give some indication of the precision of the measurements, the standard deviation (expressed first in degrees, then as a percentage of the mean observed value at that wavelength) is given at a few wavelengths: 600 (1.6 deg, 5.8%), 400 (2 deg, 1.4%), 300 (19 deg, 2.4%), 250 (160 deg, 3%), and 195 mμ (5000 deg, 10%). These values were obtained from five separate measurements of a poly-γ-morpholinylethyl-L-glutamamide solution on different days. They, therefore, represent the extent of reproducibility of the measurements but are only a lower limit on the error of the data since they do not take into account errors in concentration or calibration of the instrument. The α-helix data were obtained using poly-γ-morpholinylethyl-L-glutamamide<sup>30</sup> (PAEMG)<sup>31</sup> in water-methanol (1:9) (c 0.2, path lengths 5 to 0.01 cm), poly-γ-methoxyethyl-L-glutamate<sup>32</sup> (PMEG) in water-methanol (3:7) (c 0.1, path lengths 5 to 0.05 cm), poly-α-L-glutamic acid (PGA) in water (pH 4.3) (c 0.05, path lengths 5 to 0.1 cm). The helix contents as estimated from the modified two-term Drude equation<sup>33</sup> were 90, 100, and 90%, respectively. The ORD of the random conformation was obtained using PGA in water at pH 7 (c 0.046, path lengths 5 to 0.05 cm). The ORD of poly-L-proline II was obtained with a high molecular weight sample (η<sub>sp</sub>/c (0.2% in DCA) = 1.13, mol wt = 38,000) lyophilized from glacial acetic acid and dissolved in water. The data used in the calculations were the means of three dispersions ob-

tained with a 0.23% solution using pathlengths from 5 to 0.01 cm.

### Results of the Calculations

**A. Rotatory Dispersion of the α Helix.** The ORD data for PAEMG are used to illustrate the steps in reaching a final solution; however, the PGA or PMEG data could have been used equally as well.

An initial attempt was made to fit the PAEMG data (from 600 to 190 mμ) to two Cotton effects in the region 190 to 240 mμ with zero background. The absolute values of the background parameters are less than their respective standard errors and, therefore, are not significantly different from zero (see Table I). The solution with the smallest RESMS is shown in Figure 1 as a dashed line. The fit is fair, but the RESMS is significantly above the experimental RESMS and the RESMS's for subsequent solutions with three Cotton effects. The most interesting features of the solution are: (1) the position of the negative Cotton effect (220 mμ), (2) the lack of fit at 233 mμ (Figure 1), and (3) the lack of fit at the shoulder on the long wavelength side of the 192-mμ Cotton effect (Figure 1). Therefore, a third Cotton effect was included in the calculation. Additional support for a third Cotton effect was inferred from the CD data of Holzwarth, *et al.*<sup>11</sup> These data show an asymmetric negative dichroism band, which looks as if it were composed of two bands. This asymmetric band is not present in the CD curve corresponding to the best two Cotton effect solution (see Figure 2).

The solutions with three Cotton effects are of two types: type I (columns 1-3 in Table II)—those with a positive Cotton effect around 192 mμ, a positive Cotton effect near 217 mμ, and a negative Cotton effect, also near 217 mμ; type II (columns 4-6 in Table II)—those with a positive Cotton effect around 192 mμ, a negative Cotton effect close to 209 mμ, and a negative Cotton effect close to 224 mμ. Both types have RESMS well below the experimental RESMS. These solutions are listed in Table II. The introduction of nonzero background parameters does not reduce the RESMS significantly. The zero background solutions were therefore considered representative.

A similar twofold degeneracy of the three Cotton effect solutions was found with the PGA (pH 4.3) and the PMEG data.

The CD curve calculated from the parameters of

(30) R. K. Kulkarni and E. R. Blout, *J. Am. Chem. Soc.*, **84**, 3971 (1962).

(31) Since it is derived from aminoethylmorpholine.

(32) R. K. Kulkarni and E. R. Blout, to be published.

(33) E. Shechter and E. R. Blout, *Proc. Natl. Acad. Sci. U. S.*, **51**, 695 (1964).

Table II. Three Cotton Effect Solutions for PAEMG in Methanol-Water (9:1)

	Type I			Type II		
	1	2	3	4	5	6
RESMS <sup>a</sup> × 10 <sup>-8</sup>	0.018	0.016	0.016	0.015	0.015	0.014
R × 10 <sup>40</sup>	40.0 ± 0.3	41.0 ± 0.70	40.5 ± 1.0	38.3 ± 0.4	38.9 ± 0.9	38 ± 1
A × 10 <sup>-4</sup>	0.367 ± 0.003	0.376 ± 0.006	0.371 ± 0.009	0.352 ± 0.004	0.357 ± 0.008	0.35 ± 0.01
λ	192.4 ± 0.1	192.3 ± 0.1	192.3 ± 0.1	192.2 ± 0.1	192.2 ± 0.1	192.2 ± 0.1
Δ	8.2 ± 0.1	8.5 ± 0.2	8.4 ± 0.2	7.9 ± 0.1	8.1 ± 0.2	8.0 ± 0.2
R × 10 <sup>40</sup>	150 ± 10	100 ± 20	100 ± 10	-12.1 ± 0.6	-12.5 ± 0.8	-12.4 ± 0.7
A × 10 <sup>-4</sup>	1.4 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	-0.111 ± 0.005	-0.115 ± 0.007	-0.114 ± 0.007
λ	216.9 ± 0.1	216.7 ± 0.2	216.7 ± 0.2	208.8 ± 0.2	208.7 ± 0.2	208.7 ± 0.2
Δ	11.1 ± 0.1	11.0 ± 0.1	11.0 ± 0.1	8.7 ± 0.3	8.9 ± 0.4	8.8 ± 0.4
R × 10 <sup>40</sup>	-180 ± 10	-130 ± 20	-130 ± 10	-18.4 ± 0.3	-18.4 ± 0.3	-18.5 ± 0.3
A × 10 <sup>-4</sup>	-1.7 ± 0.1	-1.2 ± 0.2	-1.2 ± 0.1	-0.169 ± 0.003	-0.169 ± 0.003	-0.169 ± 0.003
λ	216.9 ± 0.1	216.8 ± 0.2	216.8 ± 0.2	224.0 ± 0.1	224.0 ± 0.1	224.0 ± 0.1
Δ	12.0 ± 0.1	12.3 ± 0.2	12.3 ± 0.1	10.9 ± 0.1	10.9 ± 0.1	10.9 ± 0.1
B × 10 <sup>-4</sup>	0.0 <sup>b</sup>	0.08 ± 0.12	0.01 ± 0.01	0.0 <sup>b</sup>	0.1 ± 0.1	0.02 ± 0.02
C × 10 <sup>-4</sup>	0.0 <sup>b</sup>	900 ± 1100	500 ± 200	0.0 <sup>b</sup>	1000 ± 1000	500 ± 300
Q <sup>1/2</sup>	0.0 <sup>b</sup>	100.0 <sup>b</sup>	173.2 <sup>b</sup>	0.0 <sup>b</sup>	100.0 <sup>b</sup>	170.0 <sup>b</sup>

<sup>a</sup> Experimental RESMS = 0.25 × 10<sup>8</sup>. For units see Table I; for meaning of symbols, refer to text. <sup>b</sup> Indicates those parameters which were held constant.

solution 4, Table II, is shown in Figure 2. Solutions of either type I or type II give identical CD curves; therefore, the curve for only one type (type II) is given.

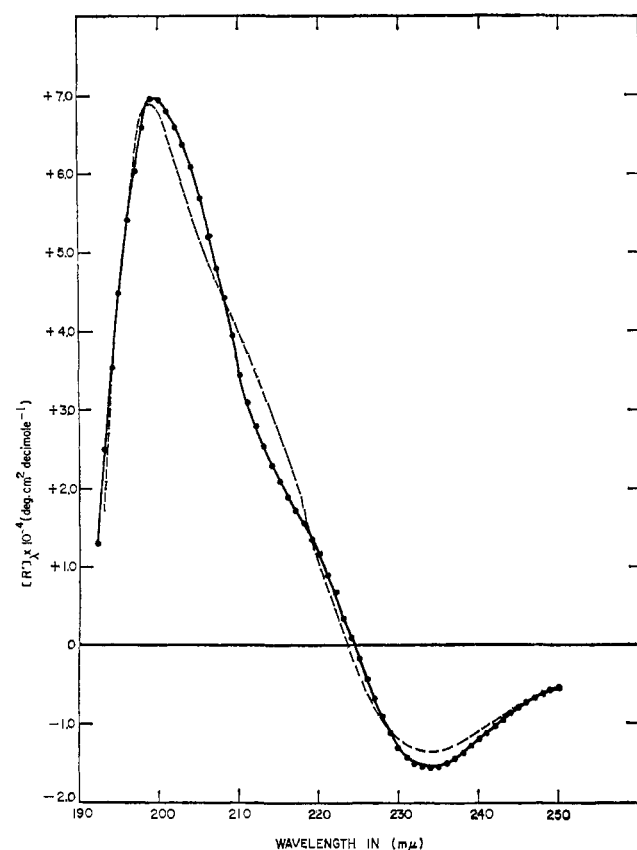


Figure 1. Solutions for the optical rotatory dispersion of poly- $\gamma$ -morpholinylethyl- $\alpha$ -L-glutamamide in methanol-water (9:1): - - -, two Cotton effect solution (solution 1, Table I); —, three Cotton effect solution (solution 4, Table II); —●—●—, observed values.

**B. Rotatory Dispersion of the Random Conformation.** The ORD of the random conformation was obtained using PGA in water at pH 7. Other polypeptide

systems are being investigated, but so far we have not obtained sufficiently precise data to warrant computer analysis. The parameter values giving an acceptable fit with two Cotton effects are listed in Table III. As

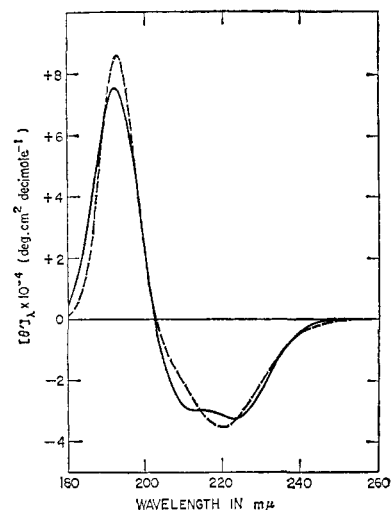


Figure 2. Circular dichroism calculated from parameter values for poly- $\gamma$ -morpholinylethyl-L-glutamamide in methanol-water (9:1): - - -, two Cotton effect solution (solution 1, Table I); —, three Cotton effect solution (solution 4, Table II).

might be expected from the standard error of  $B$  in solutions 2 and 3, it is possible to obtain acceptable solutions with  $B$  set equal to zero (solution 4), or with  $C$  set equal to zero and  $Q$  very small (solution 5). If a large value of  $Q$  is used with  $C$  set equal to zero, the RESMS obtained is significantly greater (solution 6). However, from these solutions it is clear that the Cotton effect parameters are not affected by this indeterminacy in background parameters, indicating once again that the total background contribution has been determined, but that the individual background parameter values have little significance.

A RESMS approximately one-third that of the best two Cotton effect solution can be obtained by including

Table III. Two Cotton Effect Solutions for PGA (pH 7.0) in Water

	1	2	3	4	5	6
RESMS <sup>a</sup> × 10 <sup>-8</sup>	0.0125	0.00435	0.00435	0.00430	0.00430	0.00618
R × 10 <sup>40</sup>	-12.5 ± 0.2	-14.0 ± 0.2	-14.0 ± 0.2	-14.0 ± 0.2	-14.0 ± 0.2	-14.3 ± 0.3
A × 10 <sup>-4</sup>	-0.114 ± 0.002	-0.128 ± 0.002	-0.128 ± 0.002	-0.128 ± 0.002	-0.128 ± 0.002	-0.131 ± 0.003
λ	197.7 ± 0.1	197.5 ± 0.1	197.5 ± 0.1	197.5 ± 0.1	197.5 ± 0.1	197.4 ± 0.1
Δ	7.8 ± 0.2	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.6 ± 0.2
R × 10 <sup>40</sup>	1.5 ± 0.1	1.74 ± 0.09	1.74 ± 0.09	1.74 ± 0.09	1.74 ± 0.09	1.7 ± 0.1
A × 10 <sup>-4</sup>	0.014 ± 0.001	0.0159 ± 0.0008	0.0159 ± 0.0008	0.0159 ± 0.0008	0.0159 ± 0.0008	0.016 ± 0.001
λ	217.6 ± 0.7	217.7 ± 0.4	217.7 ± 0.4	217.7 ± 0.4	217.7 ± 0.4	217.4 ± 0.5
Δ	8.6 ± 0.6	8.8 ± 0.3	8.8 ± 0.3	8.8 ± 0.3	8.8 ± 0.3	8.8 ± 0.4
B × 10 <sup>-4</sup>	0.0 <sup>b</sup>	-300 ± 2000	-0.0005 ± 0.0042	0.0 <sup>b</sup>	64 ± 5	0.022 ± 0.003
C × 10 <sup>-4</sup>	0.0	3000 ± 2000	700 ± 100	640 ± 50	0.0 <sup>b</sup>	0.0 <sup>b</sup>
Q <sup>1/2</sup>	0.0 <sup>b</sup>	3.2 <sup>b</sup>	173.2 <sup>b</sup>	0.0 <sup>b</sup>	3.2 <sup>b</sup>	173.2 <sup>b</sup>

<sup>a</sup> Experimental RESMS = 0.01 × 10<sup>8</sup>. For units see Table I; for meaning of symbols, refer to text. <sup>b</sup> Indicates those parameters which were held constant.

Table IV. Three Cotton Effect Solutions for PGA (pH 7.0) in Water

	1	2	3
RESMS <sup>a</sup> × 10 <sup>-8</sup>	0.00150	0.00144	0.00143
R × 10 <sup>40</sup>	-14.4 ± 0.2	14.4 ± 0.2	-14.2 ± 0.2
A × 10 <sup>-4</sup>	-0.129 ± 0.001	-0.132 ± 0.002	-0.130 ± 0.002
λ	197.6 ± 0.1	197.5 ± 0.1	197.6 ± 0.1
Δ	8.4 ± 0.1	8.5 ± 0.1	8.5 ± 0.1
R ± 10 <sup>40</sup>	1.9 ± 0.1	2.0 ± 0.5	1.9 ± 0.2
A × 10 <sup>-4</sup>	0.017 ± 0.001	0.019 ± 0.004	0.018 ± 0.002
λ	216.8 ± 0.4	216.7 ± 0.7	216.6 ± 0.4
Δ	10.1 ± 0.8	11 ± 1	10.3 ± 0.8
R × 10 <sup>40</sup>	-0.13 ± 0.05	-0.2 ± 0.4	-0.13 ± 0.05
A × 10 <sup>-4</sup>	-0.0012 ± 0.0005	-0.002 ± 0.003	-0.0012 ± 0.0005
λ	235 ± 3	230 ± 10	235 ± 3
Δ	10 ± 2	13 ± 6	10 ± 2
B × 10 <sup>-4</sup>	500 ± 200	0.15 ± 0.07	0.002 ± 0.001
C × 10 <sup>-4</sup>	50,000 ± 20,000	800 ± 600	660 ± 40
Q <sup>1/2</sup>	10.0 <sup>b</sup>	100.0 <sup>b</sup>	184.4 <sup>b</sup>

<sup>a</sup> Experimental RESMS = 0.01 × 10<sup>8</sup>. For units see Table I; for meaning of symbols, refer to text. <sup>b</sup> Indicates those parameters which were held constant.

a third Cotton effect. The three Cotton effect solutions are listed in Table IV. The ORD curve corresponding to solution 3, Table IV, is shown in Figure 3. The CD curves for solution 4 of Table III and solution 3 of Table IV are shown in Figure 4. Since both the two and the three Cotton effect solutions have RESMS values less than the experimental RESMS, independent evidence as to whether there are two or three Cotton effects is required. Because of the nature of the predicted CD curves, it should be much easier to choose between the two and three Cotton effect solutions for random PGA (pH 7) than between the type I and type II solutions for the  $\alpha$  helix. The CD of the two Cotton effect and three Cotton effect solutions predict, respectively, zero and nonzero values of  $[\theta']_{\lambda}$  in the region 245–235 m $\mu$ . Even though the predicted values are small, they should be distinguishable from zero and, hence, allow a choice between the two possible solutions to be made (*vide infra*).

**C. Rotatory Dispersion of the Poly-L-proline II Helix.** A one Cotton effect solution was initially obtained for the poly-L-proline II data (solution 2, Table V). The RESMS is well above the experimental RESMS; therefore, a two Cotton effect solution was sought and obtained (solution 1, Table V). Both one

Table V. Solutions for Poly-L-proline II in Water

	1	2
RESMS <sup>a</sup> × 10 <sup>-8</sup>	0.00431	0.532
R × 10 <sup>40</sup>	-33 ± 1	-26.8 ± 0.4
A × 10 <sup>-4</sup>	-0.30 ± 0.01	-0.246 ± 0.004
λ	206.9 ± 0.3	205.7 ± 0.3
Δ	13.4 ± 0.3	10.7 ± 0.3
R × 10 <sup>40</sup>	5 ± 1	...
A × 10 <sup>-4</sup>	0.049 ± 0.009	...
λ	221.0 ± 0.6	...
Δ	10.5 ± 0.3	...
B × 10 <sup>-4</sup>	-0.173 ± 0.008	-4.7 ± 0.3
C × 10 <sup>-4</sup>	2900 ± 200	2800 ± 2000
Q <sup>1/2</sup>	170 ± 17	80 ± 22

<sup>a</sup> Experimental RESMS = 0.023 × 10<sup>8</sup>. For units see Table I; for meaning of symbols, refer to text.

and two Cotton effect solutions require a nonzero background contribution in order to obtain the optimal fit. In addition, for both solutions the value of  $Q$  was sufficiently critical that a convergent solution could be obtained with all three background parameters ( $B$ ,  $C$ , and  $Q$ ) being varied in the calculation. The predicted

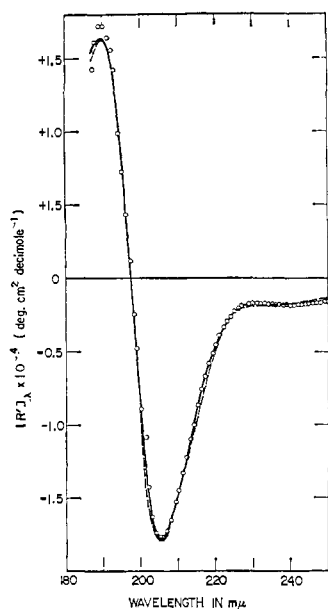


Figure 3. Solutions for the optical rotatory dispersion of poly- $\alpha$ -L-glutamic acid in water at pH 7: - - -, two Cotton effect solution (solution 5, Table III); —, three Cotton effect solution (solution 3, Table IV);  $\circ$ - $\circ$ - $\circ$ -, observed values.

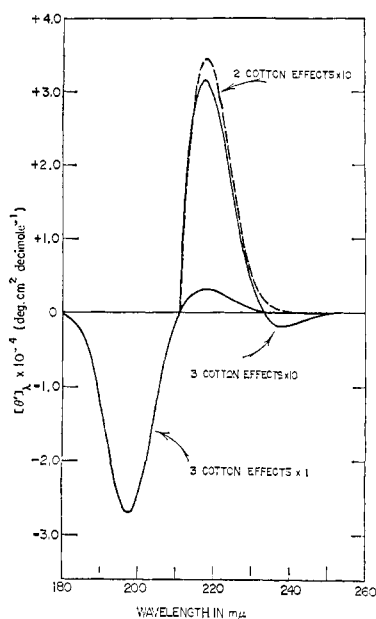


Figure 4. Circular dichroism calculated from parameter values for poly- $\alpha$ -L-glutamic acid in water at pH 7: - - -, two Cotton effect solution (solution 5, Table III); —, three Cotton effect solution (solution 3, Table IV). Below 210  $m\mu$ , the circular dichroism calculated from the two Cotton effect solution is identical with that calculated from the three Cotton effect solution.

and observed ORD curves are shown in Figure 5. The CD predicted from the two Cotton effect solution is shown in Figure 6; the CD observed in the region 260–220  $m\mu$  is shown as open circles in the same figure.

## Discussion

**A. The Criteria for an Acceptable Solution.** One important question must be considered; that is, the uniqueness of the decomposition of the ORD curve into a sum of the Kronig-Kramers transforms of Gaussian

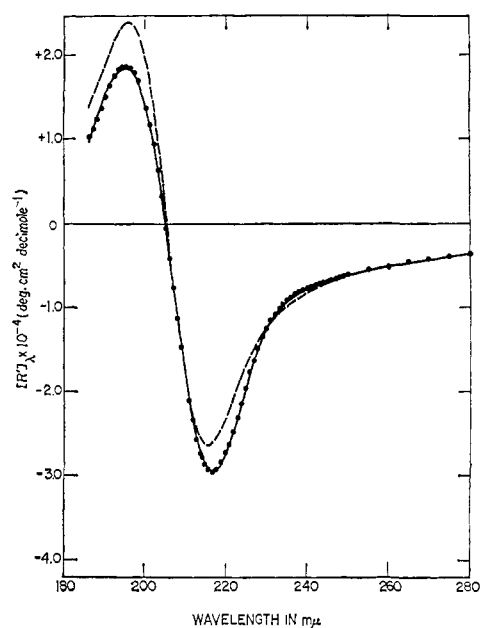


Figure 5. Solutions for the optical rotatory dispersion of the poly-L-proline II helix in water: - - -, one Cotton effect solution (solution 2, Table V); —, two Cotton effect solution (solution 1, Table V);  $\bullet$ - $\bullet$ - $\bullet$ -, observed values.

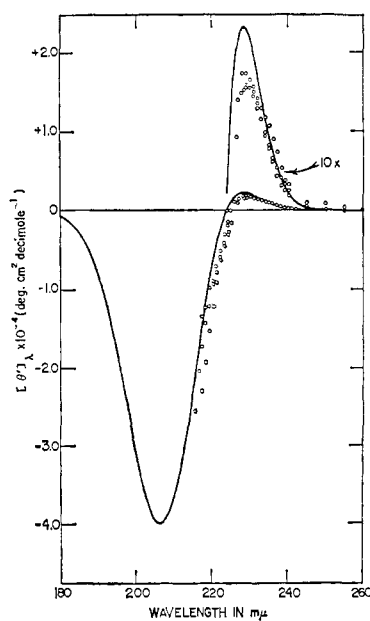


Figure 6. Circular dichroism calculated from parameter values for the poly-L-proline II helix in water: —, two Cotton effect solution (solution 1, Table V);  $\circ$ - $\circ$ - $\circ$ -, observed values.

CD bands. Unfortunately, the agreement between the calculated and observed CD curves (see below) is not in any way an indication that these are the only solutions. Any set of band shapes which can be adjusted so that they predict the observed CD curve, or so that the sum of their Kronig-Kramers transforms predicts the ORD curve, must of necessity in the one case predict the ORD, and in the other, the observed CD curves (within the error of the neglected backgrounds).

It is relevant, therefore, to consider in what manner actual CD bands may deviate from a Gaussian. An observed band may be asymmetric with respect to



Table VI. Calculated Rotatory Parameters for Different Helical Homopolypeptides

	1 PAEMG	2 PGA (pH 4.3)	3 PMEG
RESMS <sup>a</sup> × 10 <sup>-8</sup>	0.0153 <sup>c</sup>	0.0356 <sup>c</sup>	0.127 <sup>c</sup>
$R \times 10^{40}$	38.3 ± 0.4	41 ± 2	42.8 ± 0.8
$A \times 10^{-4}$	0.352 ± 0.004	0.37 ± 0.02	0.393 ± 0.007
$\lambda$	192.2 ± 0.1	191.8 ± 0.3	192.0 ± 0.1
$\Delta$	7.9 ± 0.1	8.7 ± 0.3	6.7 ± 0.3
$R \times 10^{40}$	-12.1 ± 0.6	-14 ± 2	-12.0 ± 0.8
$A \times 10^{-4}$	-0.111 ± 0.005	-0.13 ± 0.02	-0.110 ± 0.007
$\lambda$	208.8 ± 0.2	206.2 ± 0.7	207.9 ± 0.2
$\Delta$	8.7 ± 0.3	9.4 ± 0.8	7.2 ± 0.4
$R \times 10^{40}$	-18.4 ± 0.3	-17.5 ± 0.4	-20.5 ± 0.4
$A \times 10^{-4}$	-0.169 ± 0.003	-0.160 ± 0.004	-0.188 ± 0.004
$\lambda$	224.0 ± 0.1	223.5 ± 0.2	223.1 ± 0.2
$\Delta$	10.9 ± 0.1	10.8 ± 0.1	11.5 ± 0.2
$B \times 10^{-4}$	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>
$C \times 10^{-4}$	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>
$Q^{1/2}$	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>

<sup>a</sup> For units see Table I; for meaning of symbols, refer to text. <sup>b</sup> Indicates those parameters which were held constant. <sup>c</sup> Experimental RESMS for PAEMG, PGA (pH 4.3), and PMEG are  $0.25 \times 10^8$ ,  $0.25 \times 10^8$ , and  $0.36 \times 10^8$ , respectively.

wavelength and exhibit several relative extrema yet still be resolvable into physically meaningful Gaussian components (e.g., vibrational satellites or exciton band components). Such bands will not be considered to be "non-Gaussian" in the discussion to follow. What will be referred to as non-Gaussian are bands which have a single extremum, yet are not well approximated as a Gaussian (e.g., Lorentzians). Bands of this latter type will prevent a good fit using a number of Gaussian bands equal to the number of optically active transitions and will probably require the introduction of artificial bands, which do not correspond to optically active transitions, in order to obtain a fit. It follows that a set of data that is not well fitted by a certain number of Gaussians may be fitted by the same number of non-Gaussian bands, and that a set of data that is well represented by Gaussians cannot, in general, be fitted by the same number of non-Gaussians.

The aim of the type of calculation described in this paper is to represent the data in the most economic manner (with respect to parameters) which permits a physically meaningful interpretation.

**B. The  $\alpha$ -Helical Conformation. (1) Possibilities of Other Acceptable Solutions.** We have shown in the foregoing that the ORD data of  $\alpha$ -helical synthetic polypeptides are not adequately represented by two Gaussians, but that they are satisfactorily fitted by three Gaussians. In light of the discussion above, the only other possible solutions of interest would be either a two Cotton effect non-Gaussian solution, or other three Cotton effect Gaussian solutions. Recent CD data<sup>17,18</sup> confirm the double minimum predicted by our three Cotton effect solutions. The presence of these two extrema in the negative band precludes the possibility of a two Cotton effect non-Gaussian solution. Thus, the only question that remains is whether the degeneracy (type I vs. type II) among the three Cotton effect solutions is greater than two. This seems unlikely. The twofold degeneracy is introduced in attempting to resolve the two-component negative band. A similar problem does not seem to occur with the 192- and 208-m $\mu$  bands, since all sets of starting parameters which

we have tried yield only type I, type II, or divergent solutions.

To summarize, we have shown that the observed ORD of  $\alpha$ -helical polypeptides can be resolved into three Cotton effects, but that this resolution can be performed in two ways. The CD curves predicted by both three Cotton effect solutions are identical, and hence do not allow us to choose between the two solutions.

(2) **Type I vs. Type II.** As we indicated earlier, the far-ultraviolet absorption spectra of  $\alpha$ -helical polypeptide solutions<sup>34</sup> and solid films<sup>4</sup> have been interpreted as consisting of three absorption bands at 191, 205, and 210–225 m $\mu$ . Furthermore, these bands have generally been assigned to the perpendicular and parallel components of a split  $\pi \rightarrow \pi^*$  transition and to the  $n \rightarrow \pi^*$  transition of the peptide bond, respectively.

From a comparison of the ultraviolet absorption spectrum and CD of  $\alpha$ -helical polypeptides, Holzwarth<sup>16</sup> has concluded that the results from the two types of measurements are compatible if the CD curve is resolved into bands at 190, 206, and 222 m $\mu$ . This corresponds to solutions of type II.

Since no optically active absorption is obtained at 206 m $\mu$  with solutions of type I, such solutions cannot readily be reconciled with the observed ultraviolet absorption spectrum or theoretical calculations. In addition, the abnormally small energy separation between the two 217-m $\mu$  transitions (60 cm<sup>-1</sup>) is hard to explain. Thus, all the evidence supports the type II solutions, rather than the type I solutions. Table VI lists the zero background type II solutions for the three  $\alpha$ -helical polypeptides without background. The CD curves corresponding to solutions 1, 2, and 3 are shown in Figure 7. Note that there are significant differences between the rotational strengths, positions, and half-widths of corresponding Cotton effects for the three different polypeptides. However, for convenience in nomenclature, we denote the observed Cotton effects, for all three polypeptides, as the 224-, 208-, and 192-m $\mu$  Cotton effects. It should be borne in mind that, for a

(34) K. Rosenheck and P. Doty, *Proc. Natl. Acad. Sci. U. S. A.*, **47**, 1775 (1961).

Table VII. Comparison of Theory and Experiment<sup>a</sup>

Theory <sup>d</sup>		PMG <sup>b,c</sup>		PMEG		PAEMG			PGA (pH 4.3)		
$\lambda^a$	$R \times 10^{40}$	$\lambda$	$R \times 10^{40}$	$\lambda$	$R \times 10^{40}$	$\lambda$	$R \times 10^{40}$	$R^{100} \times 10^{40}$	$\lambda$	$R \times 10^{40}$	$R^{100} \times 10^{40}$
$\pi \rightarrow \pi^*$											
185	-115	...	...		~0		~0			~0	
191	242	190	66	192.0	42.8	192.2	38.3	42.5	191.8	40.7	45.3
195	-126	206	-23	207.9	-12.0	208.8	-12.1	-13.4	206.2	-14.0	-15.5
$n \rightarrow \pi^*$											
210-230	-3.4	222	-18	223.1	-20.5	224.0	-18.4	-20.4	223.5	-17.5	-19.4

<sup>a</sup> For units see Table I; for meaning of symbols, refer to text. <sup>b</sup> PMG = poly- $\gamma$ -methyl- $\alpha$ -L-glutamate, data from Holzwarth.<sup>16,18</sup> <sup>c</sup> Holzwarth's published values have been corrected for the index of refraction of the solvent, using an approximate value of 1.30 for  $n_D$ . <sup>d</sup> See ref 6 and 9.

given polypeptide-solvent system, the position of the actual Cotton effects may differ significantly from these nominal values.

**(3) Effect of Side Chain.** As would be expected from their respective parameter values, the calculated CD curves for PAEMG, PMEG, and PGA (Figure 7) have slightly different shapes in the region of negative dichroism. A similar variation is seen in Holzwarth's latest data<sup>18</sup> where the double minimum is resolved for poly- $\gamma$ -methyl- $\alpha$ -L-glutamate in trifluoroethanol, poly- $\alpha$ -L-lysine at pH 10.7 in 0.1 M NaF, copoly-L-Glu<sub>42</sub>-Lys<sub>28</sub>-Ala<sub>30</sub> in 0.1 M NaF at pH 3.0, and PGA at pH 4.5 in 0.1 M NaF. The relationship between  $[\theta']_i$  of an isolated band and its rotational strength is given by eq 4. It should be noted that the conversion factor involves  $\Delta_i/\lambda_i$ .  $[\theta']_\lambda$  at the extremum of an observed CD band cannot, therefore, be interpreted in terms of rotational strengths without knowing  $\Delta_i/\lambda_i$  for all the bands contributing to the ellipticity at that wavelength. Thus, the differences in the shape of the negative regions of the CD noted above cannot be directly interpreted to mean there exist differences in the relative magnitudes of the rotational strengths of the transitions involved. However, inspection of Table VI indicates that this is so for the polypeptide data reported here and leads to the suspicion that this will be so for the polymers measured by Holzwarth. In Table VII rotational strengths ( $R^{100}$ ) are given for 100% helix. These values are computed on the assumption that the helix content obtained from the modified two-term Drude equation is correct and that a linear extrapolation is valid.<sup>35</sup> From inspection of the  $R^{100}$  values it is evident that differences in the helix content of the polypeptides do not account for the differences in rotational strength. From these limited data, then, it would appear that the ratios of the rotational strengths of the far-ultraviolet peptide transitions are side chain dependent. This observation affects the estimation of

(35) Correcting,  $R_{\lambda_i}$  in this manner ignores the contribution of the small percentage of random conformation. However, the extent of the contribution of each Cotton effect of the random conformation to the observed rotational strengths is a matter of conjecture. From the estimated overlap of the Cotton effects of the random conformation with those of the individual helical polypeptides, one may make a plausible division of the contributions. If the helix content is chosen so that the rotational strengths of the 224-m $\mu$  Cotton effects agree for all three polypeptides, then the following values are obtained for  $R_{\lambda_i}^{100} \times 10^{40}$

PAEMG	PGA (pH 4.3)
42.9	48.1
-12.2	-15.0
-20.5	-20.5

and the per cent change in  $R_{208}$  is almost twice that in  $R_{192}$  so that the conclusion made above still stands.

helix content from these rotational strengths and will be discussed in the accompanying communication.<sup>36a</sup>

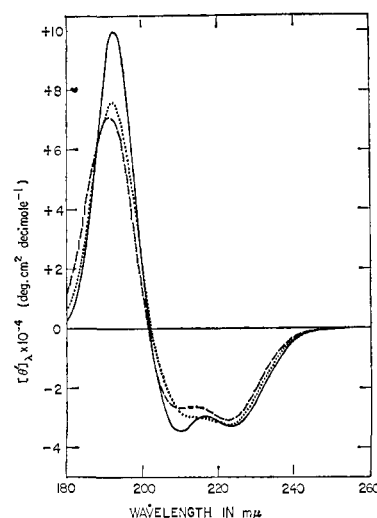


Figure 7. Circular dichroism calculated from the three Cotton effect solutions of the different  $\alpha$ -helical homopolypeptides: —, poly- $\gamma$ -methoxyethyl-L-glutamate in methanol-water (7:3) (solution 3, Table VI); ---, poly- $\alpha$ -L-glutamic acid in water at pH 4.3 (solution 2, Table VI); ····, poly- $\gamma$ -morpholinylethyl-L-glutamamide in methanol-water (9:1) (solution 1, Table VI).

**(4) Comparison of ORD and CD.** A direct comparison of the values of  $[\theta']_\lambda$  calculated from the observed ORD and those obtained from observed CD should give some idea of the degree of consistency between the experimentally determined ORD and CD. This comparison is most easily performed by consider-

(36) (a) J. P. Carver, E. Shechter, and E. R. Blout, *J. Am. Chem. Soc.*, **88**, 2562 (1966). (b) Subsequent to the completion of this work L. Velluz and M. Legrand, *Angew. Chem. Intern. Ed. Engl.*, **4**, 838 (1965), reported the results of a least-squares analysis of their CD data for PGA at pH 4.3. Their results show good agreement with ours except for the relative magnitude of the two negative bands.

Velluz and Legrand			Present work		
$\lambda$ (m $\mu$ )	$\Delta$ (m $\mu$ )	$R \times 10^{-40}$ (erg cm <sup>3</sup> )	$\lambda$ (m $\mu$ )	$\Delta$ (m $\mu$ )	$R \times 10^{-40}$ (erg cm <sup>3</sup> )
192	8.9	51	192	8.7	41
204	12.7	-29	206	9.4	-14
224	10	-15	224	10.8	-17.5

In the same paper they show a CD curve for PGA at pH 11 which, in contrast to their earlier results (see ref 17), shows a region of negative dichroism around 240 m $\mu$ . However, in the discussion, they indicate that only two bands are present—at 218 and 198 m $\mu$  (*vide supra*).

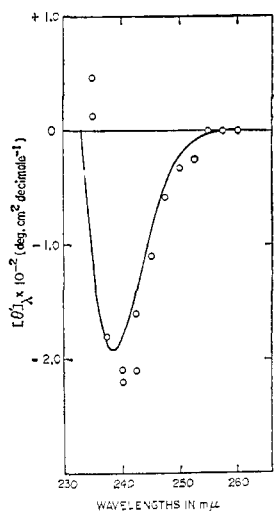


Figure 8. The 235-m $\mu$  optically active transition of poly- $\alpha$ -L-glutamic acid in water at pH 7: —, circular dichroism calculated from parameter values of three Cotton effect solution (solution 3, Table IV); -O-O-O-, observed circular dichroism.

ing the rotational strength estimates from the two sources. Unfortunately, the only rotational strength estimates available<sup>16,18</sup> for  $\alpha$ -helical polypeptides are for poly- $\gamma$ -methyl- $\alpha$ -L-glutamate, for which we do not have ORD data.<sup>18</sup> However,  $[\theta']_{\lambda}$  values in the far ultraviolet are available for  $\alpha$ -helical PGA from two sources.<sup>17,18</sup> The discrepancy between our predicted values and the observed values of Holzwarth and Doty<sup>18</sup> (once they are corrected for index of refraction) is about 10% at both 210 and 220 m $\mu$ . A comparison of our predicted value and a value of  $[\theta']_{220}$ , estimated from the published data of Legrand and Viennet,<sup>17</sup> shows the latter to be about 25% less negative.

**(5) Comparison of Theory and Experiment.** Theoretical predictions for the rotational strengths of the  $\alpha$  helix have been discussed recently.<sup>18</sup> Since the exciton theory calculations<sup>6</sup> were performed without regard for side chain, it is valid to compare the rotational strength estimates for any  $\alpha$ -helical polypeptide with the appropriate theoretically predicted quantity. Table VII summarizes the available rotational strength estimates from the calculations of Tinoco and co-workers<sup>6</sup> and Schellman and Oriol<sup>9</sup> and from experiment. As can be seen from the table, the results of the present study (columns 3–5) indicate a greater discrepancy between experiment and theory than do previous results<sup>18</sup> (column 2). However, there is qualitative agreement between the two sets of experimental results.

One unresolved problem is that current theory<sup>6</sup> predicts that three Cotton effects should be observed for the  $\pi \rightarrow \pi^*$  band; only two have been observed experimentally. The 192- and 208-m $\mu$  rotational strengths are of the same sign and relative magnitude as the values predicted for the two longer wavelength exciton Cotton effects of the  $\pi \rightarrow \pi^*$  transition. But this assignment would lead one to expect a negative Cotton effect near 185 m $\mu$ . The CD measurements<sup>16–18</sup> do not extend far enough to determine whether or not this band exists. However, the ORD data do not have to be extended into this region in order to detect the presence of a Cotton effect since, if such a Cotton effect existed, it would make a considerable contribution to

the observed rotations. Thus, one would expect that a large negative background would be required to obtain a fit of the ORD data. Although a slightly better fit is obtained using an abbreviated two-term Drude background with the Drude term at 170 m $\mu$ , the rotational strength associated with this term is positive. We have, therefore, indicated in Table VII that if the predicted 185-m $\mu$  exciton Cotton effect exists, the calculated value for the rotational strength is close to zero.

**(6) Overlap of 192- and 208-m $\mu$  CD Bands.** Another problem in interpreting the far-ultraviolet CD measurements of  $\alpha$ -helical polypeptides is to determine the extent of overlap between the positive and negative bands. This overlap can now be directly calculated. The rotational strengths listed in the tables are calculated using eq 4 and the appropriate values of  $A_i$ . From eq 2 it follows that the rotational strength of an isolated CD band can be obtained from the area under a plot of  $[\theta']_{\lambda}$  vs.  $\ln \lambda$ . Any discrepancy between the values obtained by these two methods must be due to overlap with adjacent bands. For the positive band at 192 m $\mu$  of PAEMG (solution 4, Table II), the values of  $R_{192}$  by these two methods are  $38.3 \times 10^{-40}$  and  $35.3 \times 10^{-40}$  erg cm<sup>3</sup>, respectively. The overlap, therefore, is approximately 8%.

**C. The Random Conformation. (1) Two Cotton Effects vs. Three Cotton Effects.** As was stated earlier, it was not possible to make the choice between the two Cotton effect and three Cotton effect solutions for random PGA (pH 7) on the basis of the observed ORD, but it might be possible to decide from the observed CD in the region 235–245 m $\mu$ . Figure 8 demonstrates that the choice should be in favor of the three Cotton effect solution. The discrepancies between observed and predicted CD data in the region 235–245 m $\mu$  (Figure 8) are within the errors of the estimates. The minimum appears to be at  $240 \pm 2$  m $\mu$ , whereas the predicted position is  $238 \pm 3$  m $\mu$ ; the observed value of  $[\theta']_{\min}$  is  $-230 \pm 20$  deg cm<sup>2</sup> dmole<sup>-1</sup>, whereas the predicted value is  $-190 \pm 80$  deg cm<sup>2</sup> dmole<sup>-1</sup>. It should be noted that although CD curves for random PGA have been published,<sup>16–18</sup> this band has not been previously reported.<sup>36b</sup>

Thus, the optical activity of random PGA (pH 7) in the far ultraviolet is due to three transitions at  $235 \pm 3$ ,  $216.6 \pm 0.4$ , and  $197.6 \pm 0.1$  m $\mu$  with half-widths  $8.5 \pm 0.1$ ,  $10.3 \pm 0.8$ , and  $10 \pm 2$  m $\mu$ , respectively, and rotational strengths  $-0.13 \pm 0.05 \times 10^{-40}$ ,  $1.9 \pm 0.2 \times 10^{-40}$ , and  $-14.2 \pm 0.2 \times 10^{-40}$  erg cm<sup>3</sup>, respectively.

**(2) Other Polypeptides in the Random Conformation.** To eliminate the possibility that the third Cotton effect in random PGA is unique to PGA, the CD of two other polypeptides in the random conformation—PAEMG in water and poly- $\alpha$ -L-lysine hydrochloride in water (pH 6)—were determined. For both polypeptides the longest wavelength CD band was negative; poly- $\alpha$ -L-lysine had a  $[\theta']_{\min}$  at approximately 240 m $\mu$ , PAEMG showed a  $[\theta']_{\min}$  around 228 m $\mu$ . Thus, it would appear that this third Cotton effect in PGA (pH 7) is not associated with the  $\gamma$ -carboxyl or arising from a unique conformation adopted by PGA due to  $\gamma$ -carboxyl interactions; on the contrary, it seems to be a general phenomenon associated with the random conformation of polypeptides.

(3) **Significance of the Third Cotton Effect.** The presence of three Cotton effects for the random conformation is entirely unexpected. It has been noted<sup>37</sup> that the optical activity of the random conformation is an order of magnitude larger than that anticipated for a truly random polymer with an inherently symmetric chromophore. The observation that a "random" polypeptide is really limited to a small number of the possible conformations of a mathematically random chain has been put forward as a possible explanation.<sup>37</sup> Thus, to point out that the 198-m $\mu$  Cotton effect in random PGA has a rotational strength equal to that of the 208-m $\mu$  Cotton effect of the  $\alpha$  helix ( $-14 \times 10^{-40}$  erg cm<sup>3</sup>), serves only to emphasize an existing problem. However, to note that PGA and other "random" polypeptides show three Cotton effects suggests a need to completely reconsider the origin of the optical activity of the "random" conformation.

**D. The Poly-L-proline II Helix.** In order to fit the ORD of poly-L-proline II in aqueous solution, a quite large positive Cotton effect at 221 m $\mu$  is required in addition to the large negative Cotton effect at 207 m $\mu$ . The observation that a second Cotton effect is needed to fit the curve, which appears to be a single Cotton effect, emphasizes the limitation of the "by inspection" approach to the analysis of ORD data. The 221-m $\mu$  Cotton effect is so located that its negative limb is superimposed on the negative limb of the 207-m $\mu$  Cotton effect and the positive limb of the 221-m $\mu$  Cotton effect occurs at the long wavelength shoulder of the 207-m $\mu$  Cotton effect. Thus, the only evidence of the presence of this second Cotton effect is a slight change of the 207-m $\mu$  Cotton effect long wavelength shoulder.

At present, the resolution of the observed ORD for poly-L-proline II into two Cotton effects is not readily interpretable on theoretical grounds. In the absorption spectra of models for the prolyl residue a  $\pi \rightarrow \pi^*$  transition is observed in the region 195–200 m $\mu$  and a very weak  $n \rightarrow \pi^*$  around 225–235 m $\mu$ .<sup>14, 38</sup> Using the exciton theory as developed for the  $\alpha$  helix<sup>2, 6</sup> and assuming that the transition moment for the  $\pi \rightarrow \pi^*$  band of the prolyl residue is identical with that observed<sup>39</sup> for the amide model (myristamide), it is predicted<sup>14, 40</sup> that the  $\pi \rightarrow \pi^*$  transition of the monomer will be split into two components upon incorporation into the poly-L-proline II helix. The calculated separations between these two components are 3400 ( $\sim 12$  m $\mu$ ) or 4700 cm<sup>-1</sup> ( $\sim 17$  m $\mu$ ) depending on whether a transition monopole<sup>40</sup> or point dipole<sup>14</sup> interaction is assumed. Presumably calculations along the lines of the recent work of Tinoco for the  $\pi \rightarrow \pi^*$  transition in the  $\alpha$  helix<sup>6a</sup> would predict an additional pair of optically active bands centered on the shorter wavelength perpendicularly polarized exciton band. If the relative separations of the two pairs of bands are similar to those for the  $\alpha$  helix, then the total system for the  $\pi \rightarrow \pi^*$  transition would be equivalent to three bands—the two longer wavelength components having the

separation predicted from Moffitt theory. No rotational strength estimates have been made for either the exciton components of the  $\pi \rightarrow \pi^*$  or of the  $n \rightarrow \pi^*$ . However, Schellman<sup>37</sup> indicates that the poly-L-proline II  $n \rightarrow \pi^*$  rotational strength should be much less than that calculated for the  $\alpha$  helix.

There are two possible assignments which do not contradict the limited theoretical predictions: (1) the rotational strength of the  $n \rightarrow \pi^*$  is negligible and the 221- and 207-m $\mu$  Cotton effects are the two longer wavelength components of a  $\pi \rightarrow \pi^*$  exciton band; (2) the 221-m $\mu$  Cotton effect arises from an  $n \rightarrow \pi^*$  transition and the 207-m $\mu$  Cotton effect is the longest wavelength component of a  $\pi \rightarrow \pi^*$  exciton band. The third possible assignment, which ignores the predictions of exciton theory as applied to the poly-L-proline II helix, is (3) the 221-m $\mu$  Cotton effect arises from an  $n \rightarrow \pi^*$  transition and the  $\pi \rightarrow \pi^*$  transition of the monomer in the helix does not show exciton splitting, and hence gives rise to only one Cotton effect (the 207-m $\mu$  Cotton effect).

## Conclusion

The application of the nonlinear, least-squares curve-fitting approach to the interpretation of the ORD curves for  $\alpha$ -helical polypeptides allows the resolution of the observed, strong, overlapping, ultraviolet Cotton effects. For the ORD of random PGA this method predicts the existence of a very weak Cotton effect ( $R_{235} = -0.13 \times 10^{-40}$  erg cm<sup>3</sup>) which has not been detected previously. The presence of this Cotton effect was confirmed by CD measurements. Finally, the ORD of the poly-L-proline II helix was shown to contain a quite large positive Cotton effect in addition to the larger negative one previously noted.<sup>11</sup>

The results of the calculations for the  $\alpha$ -helical polypeptides agree qualitatively with the prediction from exciton (for  $\pi \rightarrow \pi^*$ ) and one-electron (for  $n \rightarrow \pi^*$ ) theory. However, the calculations for random PGA and for the poly-L-proline II helix indicate a need for a reconsideration and extension of the present theoretical treatments. In the case of the poly-L-proline II helix, the apparent existence of only one Cotton effect in the far-ultraviolet ORD had suggested that the exciton model was not applicable to this structure. The present results, however, indicate that this could be a fruitful direction for further investigation.

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