# AN ANALYSIS OF THE OPTICAL ROTATORY DISPERSION OF POLYPEPTIDES AND PROTEINS*

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The characterization of many types of natural products by optical rotation has been a recognized procedure for about one hundred years. Recently optical rotatory dispersion has been shown to be a valuable technique, and the problem of correlating optical rotation and rotatory dispersion measurements with structure has challenged many investigators.[1, 2] For some time it has been known that native proteins, in general, show much less negative rotations than do denatured proteins; and in 1955 the suggestion was made[3] that such changes in rotation were associated with the loss of helical structures.

Following this proposal, Moffitt, in a brilliant series of papers,[4–6] suggested an equation to describe the optical rotatory dispersion of $\alpha$-helical polypeptides. This equation, which now bears Moffitt's name, has been used as the basis for the estimation of the $\alpha$-helix content of many types of synthetic polypeptides and many types of proteins.[7]

In this communication we present a new analysis of the visible and near-ultraviolet rotatory dispersions of synthetic polypeptides and proteins in aqueous solutions. This analysis makes use of, and is based on, the reeently discovered Cotton effects of synthetic polypeptides and proteins[8–12] which lie in the region 185–240 m$\mu$. The analysis not only allows the determination of $\alpha$-helix content of proteins in solution, but also provides a basis for the differentiation of $\alpha$-helix containing proteins from proteins involving other structures.

*Analysis of Optical Rotatory Dispersion Data.—The Drude equation:* It has long been suggested that the rotatory power of molecules at wavelengths removed from their absorption bands was a consequence of the anomalous rotatory dispersion, which is observed in optically active absorption bands.[1, 13] The general formulation of this relation was first stated by Drude[14] as:

$$[R'] = \sum_i K_i/(\lambda^2 - \lambda_i^2) \qquad (1)$$

where $\lambda_i$ is the wavelength of the $i$th transition, $K_i$ is a constant, and $[R']$ is the observed rotation corrected for the refractive index of the solvent. (In this and subsequent equations we use $[R']$ rather than $[\alpha']$ since in polypeptides and proteins we are concerned primarily with the rotation per peptide unit or amino acid residue.) From quantum mechanical considerations, Rosenfeld[15] was able to show that the Drude constant $K_i$ was equivalent to $A_i$ times $\lambda_i^2$ [equation (2)], where $A_i$ is related to the rotational strength $B_i$ as shown in equation (3):

$$[R'] = \sum_i \frac{A_i\lambda_i^2}{\lambda^2 - \lambda_i^2} \quad (2) \qquad B_i = \frac{hc}{96\pi N} A_i. \quad (3)$$

For the analysis of the rotatory dispersion of proteins, extensive use has been made of a one-term Drude equation,

$$[R'] = \frac{A_c\lambda_c^2}{\lambda^2 - \lambda_c^2} \quad (4)$$

which was suggested as an useful approximation[16] of the multiterm Drude equation (1). If two or more transitions contribute strongly to the rotation, then this simple equation no longer holds in spectral regions close to absorption bands, and in this situation at least one more term is needed. The necessity of a second term was recognized by several investigators.[17,18] Recently Yamaoka, working in our laboratory, found that a two-term Drude equation fits the rotatory dispersion data of two $\alpha$-helical synthetic polypeptides in several solvents in the wavelength range 275–700 m$\mu$.[19]

*Comment on Drude and Moffitt equations:* At this point it is worth while to consider the validity of the above expressions for the simple case of a polypeptide or protein existing in conformations which comprise only $\alpha$-helices and random forms. It is clear that a multiterm Drude equation, given sufficient parameters, will fit the rotatory dispersion data, from long wavelengths to wavelengths near the first optically active absorption band. It is not surprising therefore that a one-term Drude equation has been found to express the rotatory dispersion only under a limited range of conditions, namely, either when low $\alpha$-helix content is present or when the analysis only comprises wavelengths very far removed from the absorption band. Furthermore, even in these cases, for the most part, the values of $\lambda_c$ and $A_c$ obtained have no physical meaning in terms of molecular parameters. The Moffitt equation is related to the assumption that the important contributions to the rotation are due only to the 150 m$\mu$ (N $\rightarrow$ $V_2$) and the 190 m$\mu$ (N $\rightarrow$ $V_1$) amide absorption bands. It is now known that an important contributor to the rotatory dispersion of $\alpha$-helical peptide molecules is the relatively weak $n \rightarrow \pi^*$ transition in the region 215–225 m$\mu$ (specifically excluded from the Moffitt formulation), and we will show that the contributions of absorption bands below 185 m$\mu$ are neither important for, nor do they interfere with, the analysis proposed here.

*Experimental Observation of Optically Active Absorption Bands of Polypeptides and Proteins.*—During the past few years it has proved possible to measure the rotatory dispersion of synthetic polypeptides and proteins in the far-ultraviolet region (240–185 m$\mu$), where absorption due to the fundamental peptide sequence occurs. It has been found that associated with these absorption bands, Cotton effects are observed (Fig. 1, Table 1). The far ultraviolet rotatory dispersion curves of $\alpha$-helical polypeptides show a trough at 233 m$\mu$, a crossover point around 225 m$\mu$, a shoulder in the region 215–220 m$\mu$, a peak at 198 m$\mu$, and a second crossover point around 193 m$\mu$. It is quite certain from spectral, rotatory dispersion and circular dichroism data that for $\alpha$-helical polypeptides an optically active transition occurs around 193 m$\mu$. It was found that a two-term Drude equation with one term fixed at $\lambda_1$ = 193 m$\mu$ gives a very good fit of visible and near ultraviolet rotatory dispersion if $\lambda_2$ = 225 m$\mu$.[19] The second term includes the contributions of all the Cotton effects which influence the rotation in the visible and near ultraviolet region with the exception of the Cotton effect centered at 193 m$\mu$. If only two Cotton effects influence the rotation, then the second term involves the contribution of only one Cotton effect centered at 225 m$\mu$. If, on the other hand, more than two

Cotton effects are responsible for the rotation in the visible and near ultraviolet region, then the second term is the sum of the contributions from several Cotton effects, the major contribution to this second term probably being the $n \rightarrow \pi^*$ transition. It should be noted that the coincidence of the calculated wavelength of $\lambda_2$ and the observed crossover point ($\lambda = 225$ m$\mu$) may be fortuitous. Since the second term may include contributions from more than one Cotton effect, we will refer to it as a *virtual* Cotton effect. We shall show in a forthcoming communication that the knowledge of the precise physical significance of the 225 m$\mu$ term is not required to justify the following analysis.

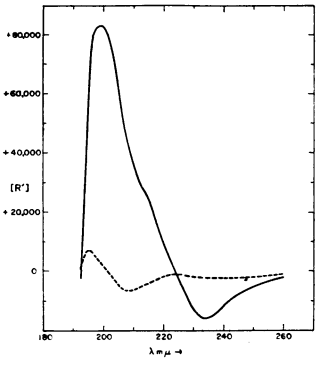


FIG. 1.—The ultraviolet rotatory dispersion of: ——, a synthetic polypeptide in an $\alpha$-helical conformation (poly-$\gamma$-methoxyethyl-L-glutamate in water: methanol 3:7 v/v); - - -, a synthetic polypeptide in a nearly random conformation (poly - $\gamma$ - morpholinylethyl - L-glutamamide in water).

The random conformation shows two Cotton effects: a negative Cotton effect, with its crossover point at 198 ± 3 m$\mu$; and a very weak Cotton effect, around 225 m$\mu$. By random conformation we mean a conformation with no periodic, but with possibly fixed, arrangement of peptide groups. Cotton effects associated with three other conformations have been observed (Table 1).

*A New Analysis of Rotatory Dispersion Data of Polypeptides and Proteins in $\alpha$-Helical and Random Conformations.—Mathematical analysis:* Using the Cotton effects described above, it is now possible to recast the fundamental Drude equation in terms of $\lambda_i$'s. For molecules that consist only of random and $\alpha$-helical segments, we take into account the Cotton effects at 225, 198, and 193 m$\mu$, and write

$$[R'] = \frac{A_{(\alpha)193}\lambda^2_{193}}{\lambda^2 - \lambda^2_{193}} + \frac{A_{(\alpha)225}\lambda^2_{225}}{\lambda^2 - \lambda^2_{225}} + \frac{A_{(\rho)198}\lambda^2_{198}}{\lambda^2 - \lambda^2_{198}} + \frac{A_{(\rho)225}\lambda^2_{225}}{\lambda^2 - \lambda^2_{225}}. \quad (5)$$

In this and subsequent equations we use the following notation: (1) The subscripts of the $\lambda$'s are the wavelengths in millimicrons used for $\lambda_i$. These wavelengths correspond to the crossover points. (2) Two subscripts are used with each $A$ constant. The first subscript in parentheses designates the conformations to which $A$ is related; $\alpha$ is used for the $\alpha$-helix, $\rho$ is used for a random conformation. The second subscript of $A$ is the wavelength of the crossover point. The second subscript is placed in parentheses when an approximate wavelength is used for two Cotton effects lying close together in wavelength.

TABLE 1

SUMMARY OF ROTATORY DISPERSION DATA OF L-POLYPEPTIDES IN VARIOUS CONFORMATIONS

| Structure | Crossover point[c] (m$\mu$) | Peak (m$\mu$) | Trough (m$\mu$) | Sign |
|---|---|---|---|---|
| $\alpha$-Helix[a] | 225 | | 233 | − |
| | 193 | 198 | 185[d] | + |
| Random[a] | | | 233 | − |
| | 198 | 190 | 204 | − |
| Poly-L-proline-II[a] | 203 | 194 | 216 | − |
| Poly-L-proline-I[b] | 210 | 218 | 202 | + |
| $\beta$ form[b] | 198 | 207 | 190[d] | + |

[a] In solution.
[b] In oriented solid state.
[c] See text for discussion of crossover points.
[d] Extrapolated.

In order to provide an easy graphical representation equation (5) should be transformed into a two-term equation. Term 3 on the right hand side of equation (5) can be written as

$$A_{(\rho)198}\frac{\lambda^2_{193}}{\lambda^2_{193}}\frac{\lambda^2-\lambda^2_{193}}{\lambda^2-\lambda^2_{198}}\frac{\lambda^2_{193}}{\lambda^2-\lambda^2_{193}}$$

where $\lambda^2_{198}/\lambda^2_{193}$ is a constant equal to 1.05 and $(\lambda^2-\lambda^2_{193})/(\lambda^2-\lambda^2_{198})$ varies from 1.01 to 1.06 when $\lambda$ varies from 600 to 260 m$\mu$. By taking a mean value of 1.08 for $(\lambda^2_{198}/\lambda^2_{193})[(\lambda^2-\lambda^2_{193})/(\lambda^2-\lambda^2_{198})]$ the maximum error compared to the four-term equation (5) is 2 per cent for $\lambda \geqslant 260$ m$\mu$. Thus equation (5) can be rewritten as

$$[\mathrm{R}'] = (A_{(\alpha)193} + 1.08\,A_{(\rho)198})\frac{\lambda^2_{193}}{\lambda^2-\lambda^2_{193}} + (A_{(\alpha)225} + A_{(\rho)225})\frac{\lambda^2_{225}}{\lambda^2-\lambda^2_{225}} \quad (6)$$

or using the above notation

$$[R'] = A_{(\alpha,\rho)(193)}\frac{\lambda^2_{193}}{\lambda^2-\lambda^2_{193}} + A_{(\alpha,\rho)225}\frac{\lambda^2_{225}}{\lambda^2-\lambda^2_{225}} \quad (7)$$

where $A_{(\alpha,\rho)(193)} = A_{(\alpha)193} + 1.08\,A_{(\rho)198}$ and $A_{(\alpha,\rho)225} = A_{(\alpha)225} + A_{(\rho)225}$.

A way of plotting a two-term Drude equation has been suggested.[19a] For this purpose equation (7) may be written as

$$[R']\frac{\lambda^2-\lambda^2_{193}}{\lambda^2_{193}} = A_{(\alpha,\rho)(193)} + A_{(\alpha,\rho)225}\frac{\lambda^2_{225}}{\lambda^2_{193}} + A_{(\alpha,\rho)225}\frac{\lambda^2_{225}-\lambda^2_{193}}{\lambda^2_{193}}\frac{\lambda^2_{225}}{\lambda^2-\lambda^2_{225}}. \quad (8)$$

Plotting $[R'](\lambda^2-\lambda^2_{193})/\lambda^2_{193}$, against $\lambda^2_{225}/(\lambda^2-\lambda^2_{225})$ should yield a straight line, where $A_{(\alpha,\rho)225}[(\lambda^2_{225}-\lambda^2_{193})/\lambda^2_{193}]$ is the slope and $A_{(\alpha,\rho)(193)} + A_{(\alpha,\rho)225}(\lambda^2_{225}/\lambda^2_{193})$ is the intercept.

Since $A_{(\alpha,\rho)(193)}$ includes the contributions of two Cotton effects lying at two different wavelengths and since the second term may include contributions of more than one Cotton effect, we call equation (7) a *modified two-term Drude equation*. It is now known that the Cotton effects in the region 185–240 m$\mu$ are conformation-dependent. Since $A_{(\alpha,\rho)(193)}$ is directly related to the rotational strength of the Cotton effect centered at 193 m$\mu$, its value should be related to helix content, and in cases of mixtures of only $\alpha$-helix and random conformations a linear relation should exist between $A_{(\alpha,\rho)(193)}$ and helix content. If a linear relation exists between $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$, then $A_{(\alpha,\rho)225}$ is also linearly related to helix content, irrespective of whether this quantity includes the contributions of more than one Cotton effect.

In this paper we apply this new analysis only to aqueous solutions of polypeptides and proteins. In forthcoming communications, after generalization of this analysis to polypeptides in organic solvents, we will show how the one-term Drude equation (in some cases) and the Moffitt equation are related to this new equation.

*Polypeptides and proteins in aqueous solution:* Using the analysis given above, we have calculated $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ for several water-soluble synthetic polypeptides and proteins in water at different pH's and temperatures and for various water-methanol mixtures. The data for polypeptides are shown in Figure 2.

TABLE 2

ROTATORY CONSTANTS OF POLYPEPTIDES IN AQUEOUS SOLUTION*

| Polypeptide of:[a] | pH | $A_{(\alpha,\rho)\,(193)}$ | $A_{(\alpha,\rho)225}$ | $H_{193}$ | $H_{225}$ |
|---|---|---|---|---|---|
| L-glutamic acid[b] | 4 | +2900 | −2050 | 100 | 100 |
| L-glutamic acid[c] | 7 | −750 | −60 | 0 | 0 |
| L-glutamic acid: L-lysine (1:1)[d] | 3 | +1400 | −1180 | 59 | 57 |
| | 8 | +240 | −590 | 27 | 27 |
| L-glutamic acid: L-lysine: L-alanine (1:1:3)[d] | 3 | +1800 | −1360 | 70 | 66 |
| | 8 | +1470 | −1210 | 61 | 58 |
| γ-morpholinylethyl L-glutamamide: L-alanine (7:3)[e] | 7 | +1520 | −1250 | 62 | 60 |
| γ-morpholinylethyl L-glutamamide[e] | 7 | −610 | −90 | 4 | 2 |
| γ-morpholinylethyl L-glutamamide[e, f] | 7 | +2020 | −1550 | 76 | 75 |
| γ-morpholinylethyl L-glutamamide[e, g] | 7 | +2500 | −1820 | 89 | 89 |
| γ-methoxyethyl L-glutamate[g, h] | 7 | +2835 | −1980 | 98 | 97 |
| L-serine[i] | 7 | +10 | −80 | 21 | 1 |
| L-proline-II[j] | 7 | −2680 | −590 | −53 | 27 |

* Measurements made using a Cary 60 recording spectropolarimeter at wavelengths of 300–600 mμ.
[a] At room temperature, in water solution, unless otherwise noted. [b] Assumed 100% helical. [c] Assumed 0% helical. [d] See ref. 25. [e] See ref. 26. [f] Methanol:water (5:5). [g] Methanol:water (7:3). [h] See ref. 27. [i] See ref. 22. [j] See ref. 23.

TABLE 3

ROTATORY CONSTANTS OF PROTEINS IN AQUEOUS SOLUTION*

| Number in Figure 3 | Proteins[a] | $A_{(\alpha,\rho)\,(193)}$ | $A_{(\alpha,\rho)225}$ | $H_{193}$ | $H_{225}$ |
|---|---|---|---|---|---|
| 1 | Paramyosin | +2780 | −1940 | 97 | 95 |
| 2 | Bovine serum albumin | +1250 | −1150 | 55 | 55 |
| 3 | Fibrinogen | +580 | −680 | 35 | 31 |
| 4 | Fibrinogen (8 *M* urea) | +200 | −500 | 26 | 22 |
| 5 | Fibrinogen (9 *M* urea) | +100 | −500 | 24 | 22 |
| 6 | Ribonuclease | +230 | −575 | 27 | 26 |
| 7 | Tropomyosin | +2410 | −1750 | 87 | 85 |
| 8 | Tropomyosin (pH 10) | +1325 | −1130 | 57 | 54 |
| 9 | Myosin | +1535 | −1200 | 63 | 58 |
| 10 | L. M. M.[b] | +2440 | −1770 | 88 | 86 |
| 11 | H. M. M.[c] | +1225 | −1020 | 54 | 49 |
| 12 | β-Lactoglobulin | +420 | −420 | 32 | 18 |
| 13 | Pepsinogen | +60 | −250 | 22 | 10 |

* Measurements made using a Rudolph 200S manual photoelectric spectropolarimeter at the wavelengths of the mercury lines between 313 and 578 mμ. The experimental data for proteins numbered 5 and 7–11 were obtained from Drs. Susan Lowey and Carolyn Cohen.
[a] At room temperature, pH 5–8 unless otherwise noted. [b] Light meromyosin, fraction I. [c] Heavy meromyosin.

Some of the data for polypeptides (at room temperature) are reported in Table 2. The data for proteins are shown in Table 3 and Figure 3. As may be seen from Figures 2 and 3, plotting $A_{(\alpha,\rho)\,(193)}$ as a function of $A_{(\alpha,\rho)225}$ a linear relation is obtained.

$$A_{(\alpha,\rho)225} = -0.55\,A_{(\alpha,\rho)\,(193)} - 430. \tag{9}$$

If we make the assumptions that (*a*) poly-α,L-glutamic acid is completely α-helical at pH 4 ($A_{(\alpha,\rho)\,(193)} = +2900$, $A_{(\alpha,\rho)225} = -2050$), (*b*) poly-α,L-glutamic acid has a completely random conformation at pH 7 ($A_{(\alpha,\rho)\,(193)} = -750$, $A_{(\alpha,\rho)225} = -60$), and (*c*) that linear relations exist between $A_{(\alpha,\rho)\,(193)}$ and helix content and therefore between $A_{(\alpha,\rho)225}$ and helix content, then one obtains the following relations

$$H_{193} = \frac{A_{(\alpha,\rho)\,(193)} + 750}{36.5} \tag{10}$$

$$H_{225} = -\frac{A_{(\alpha,\rho)225} + 60}{19.9} \tag{11}$$

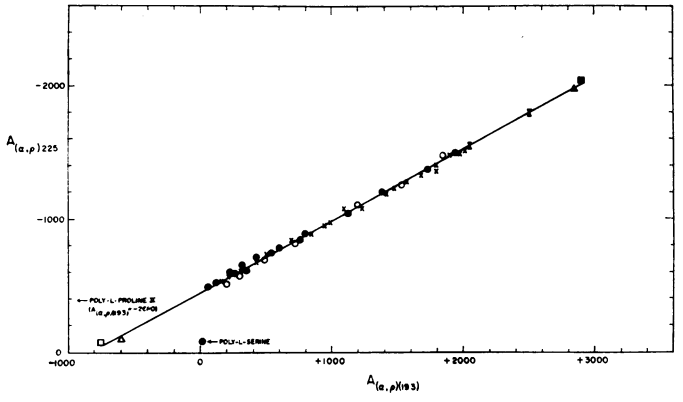


FIG. 2.—Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for polypeptides in solution. Polypeptides of L-glutamic acid: L-lysine (1:1) ●; L-glutamic acid: L-lysine:L-alanine (1:1:3) ×; γ-morpholinylethyl-L-glutamamide:L-alanine (7:3) ○; at different pH's and temperatures in water solution. Poly-L-glutamic acid at pH 4 ■; poly-L-glutamic acid at pH 7 □; poly-γ-morpholinylethyl-L-glutamamide Δ; in water solution. Poly-γ-morpholinylethyl-L-glutamamide ▼; poly-γ-methoxyethyl-L-glutamate ▲ in methanol:water solution.

where $H$ equals the per cent helix. The estimated helix contents obtained from the values of $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ are given in Tables 2 and 3. Since equations (10) and (11) derive from equation (9), it is obvious that for polypeptides and proteins which do not fit equation (9), the calculated helix contents from equations (10) and (11) will differ significantly (cf. last two entries in Tables 2 and 3). With the above assumptions, we estimate that the precision of this method is ±5 per cent helix content, with the main cause of this variation being the error in the experimental measurement of rotation and concentration. We will discuss this estimate in a forthcoming communication after generalizing this new analysis to polypeptides and proteins in organic solvents.

*Calculation of rotational strengths:* From equation (3) calculations have been made of the rotational strengths for the three Cotton effects lying at 225, 198, and 193 mμ, respectively. The data are given in Table 4 and compared with calculations of rotational strengths observed from circular dichroism data,[20] and from theoretical calculations.[21]

TABLE 4

ROTATIONAL STRENGTHS IN C.G.S. UNITS (×$10^{39}$) OF SOME PEPTIDE COTTON EFFECTS

| | Calculated from | | |
|---|---|---|---|
| Rotational strengths[a] | $A$'s | Circular dichroism measurements[b] | Theoretical analysis[c] |
| $B_{(\alpha)198}$ | +3.2 | +3.6 | |
| $B_{(\pi)225}$ | −2.2 | −4.1[d] | −1.7 |
| $B_{(\rho)198}$ | −0.9 | −1.4 | |
| $B_{(\rho)225}$ | −0.07 | | |

[a] For subscript explanation, see text. [b] See ref. 20. [c] See ref. 21. [d] The circular dichroism band shows a broad maximum at 216–220 mμ.

As can be seen, there is a good agreement between the rotational strength calculated from circular dichroism measurements and from $A_{(\alpha)193}$. Such agreement is to be expected if $A_{(\alpha)193}$ includes the contribution of a single Cotton effect. As noted above, the 225 mμ Cotton effect may be a virtual one, and if so, $A_{(\alpha)225}$ includes the contribution of Cotton effects outside the region 185–240 mμ. If these

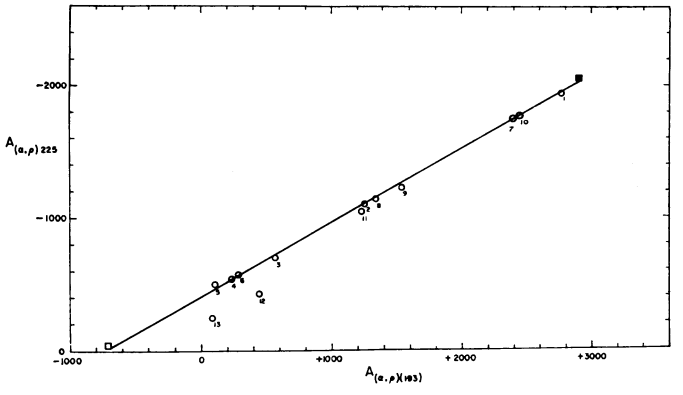


FIG 3.—Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for proteins in water solution; the numbers correspond to the entries in Table 3. Poly-L-glutamic acid pH 4 ■; poly-L-glutamic acid pH 7 □; in water solution.

contributions are important, a lack of agreement between the rotational strength calculated from $A_{(\alpha)225}$ and from the circular dichroism band around 216–220 mμ would be expected.

*Discussion.*—It has been shown above that the use of equations (10) and (11) allow the estimation of α-helix content of synthetic polypeptides and proteins in water or methanol:water solutions. This method is based on the premise that the visible and near-ultraviolet rotatory dispersion can be considered as due to only two Cotton effects characteristic of the α-helical conformation and the two Cotton effects characteristic of the random conformation. Logically, the question now arises as to whether all data for synthetic polypeptides and proteins fit the above analysis. We have calculated $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ for poly-L-serine and poly-L-proline II (Table 2), two synthetic polypeptides whose conformation has been shown to be non-α-helical.[22, 23] We find that the points so obtained do not fall on the curve of Figure 2. Similar calculations for two proteins suspected of being non-α-helical, β-lactoglobulin and pepsinogen (Table 3), also do not fit equation (9), and the points for the relation between $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ do not lie on the curve of Figure 3. We thus conclude that these substances do not consist of either α-helices, random conformations, or mixtures of these two conformations. This conclusion is supported by far-ultraviolet rotatory dispersion measurements of β-lactoglobulin and pepsinogen which indicate that other conformations are involved.[24]

Instruments capable of rotation measurements in the far ultraviolet are becoming available, and thus, in some cases, conformations and helix contents may be obtained by the direct measurements of far-ultraviolet Cotton effects. However, we believe that the above analysis will prove valuable in cases where rotation measurements in the far ultraviolet are not possible because of solvent or solute absorption. Moreover, even in cases where far-ultraviolet measurements are possible, the precision of the determination of helix contents may be better using this new analysis than by measurements of the magnitudes of the 233 mμ trough or 198 mμ peak.

Inasmuch as the above analysis depends only on the relative ratio of the magnitude of the 193, 198, and virtual 225 mμ Cotton effects, the presence of additional Cotton effects, known to contribute to the rotation of certain proteins, will not allow

the foregoing analysis. Examples of such Cotton effects may be intrinsic ones due to oriented absorbing side chains as in poly-L-tyrosine, or extrinsic ones due to chromophoric groups as in myoglobin and hemoglobin.

Since we have recently shown that the beta conformation of synthetic polypeptides as well as poly-L-proline *I* and poly-L-proline *II*, show characteristic Cotton effects in the far ultraviolet,[11, 12] it should be possible to develop similar types of mathematical analysis for the several molecular conformations of synthetic polypeptides and proteins. Such a development might allow the determination of the gross structural features of the conformation of proteins in solution.

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