[36] Stein, W. H., and S. Moore, in *Amino Acids and Proteins*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 14 (1949), p. 189.
[37] Fleissner, E., and E. Borek, these PROCEEDINGS, **48**, 1199 (1962).
[38] Gold, M., J. Hurwitz, and M. Anders, *Biochem. Biophys. Res. Commun.*, **11**, 107 (1963).
[39] Reid, E., *Nature*, **168**, 955 (1951).
[40] Nathans, D., and F. Lipmann, these PROCEEDINGS, **47**, 497 (1961).
[41] Biswas, B. B., and R. Abrams, *Biochim. et Biophys. Acta*, **55**, 827 (1962).
[42] Allfrey, V. G., and A. E. Mirsky, in *Nucleohistones*, ed. J. Bonner and P. O. P. Ts'o (San Francisco: Holden-Day, Inc., 1963).
[43] Mirsky, A. E., unpublished experiments.
[44] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

---

*AN ANALYSIS OF THE OPTICAL ROTATORY DISPERSION OF POLYPEPTIDES AND PROTEINS, II**

BY E. SHECHTER AND E. R. BLOUT

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL

Communicated by John T. Edsall, March 24, 1964

In a previous communication[1] we reported a new analysis of visible and near-ultraviolet rotatory dispersion data of aqueous solutions of polypeptides and proteins having α-helical or random conformations, or mixtures of both. The rotations were described by an expression designated a *modified two-term Drude equation:*[1]

$$[R'] = \frac{A_{(\alpha,\rho)\,(193)}\lambda^2_{193}}{\lambda^2 - \lambda^2_{193}} + \frac{A_{(\alpha,\rho)225}\lambda^2_{225}}{\lambda^2 - \lambda^2_{225}} \quad (1)$$

A linear relation was obtained by plotting $A_{(\alpha,\rho)\,(193)}$ versus $A_{(\alpha,\rho)225}$,

$$A_{(\alpha,\rho)225} = -0.55A_{(\alpha,\rho)\,(193)} - 430, \quad (2)$$

and it was found that the helix content could be expressed in terms of either one of the two parameters.

As a result of this analysis it was concluded that the rotatory dispersion parameters of polypeptides and proteins (in aqueous solutions) existing in α-helical or random conformations, or mixtures of the two, fit equation (2). A failure to fit this equation was taken as an indication of the presence of other structures.

In this communication we extend this analysis to the optical rotatory dispersions of polypeptides and proteins in a variety of organic solvents, and compare the results so obtained with the ones obtained in aqueous solutions.

Analysis of Some Optical Rotatory Dispersion Data of Synthetic Polypeptides and Proteins in Organic Solvents.—During the past ten years there have been a number of investigations into the effect of various organic solvents on the optical rotation and rotatory dispersion of several synthetic polypeptides and some proteins.[2] Much of this work arose from the fact that many high-molecular-weight polypeptides are soluble only in organic solvents. As a result of these investigations it was found that the type of organic solvent had a pronounced effect on the conformation of the solute. It is now recognized, for example, that highly polar

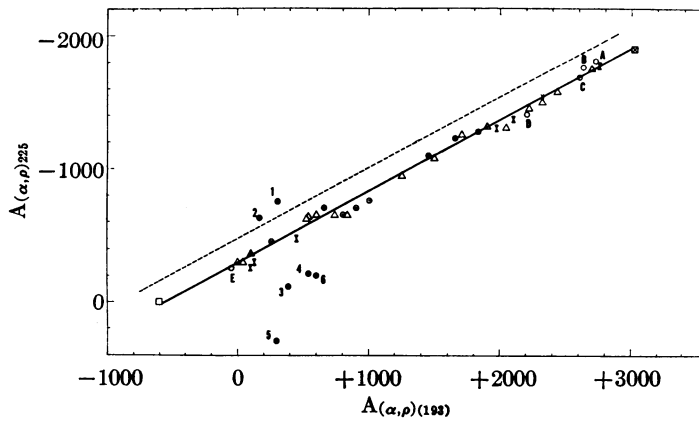

FIG. 1.—Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for polypeptides in organic solvents: poly-L-methionine ⊠ in chloroform; copoly-L-methyl-S-cysteine:L-methionine 1:1 □ in DCA:TFA 1:1; poly-γ-benzyl-L-glutamate ○ in dioxane (*A*), dioxane: DMF 1:1 (*B*), chloroform (*C*), pyridine (*D*), DCA (*E*); poly-γ-methoxyethyl-L-glutamate × in dioxane; poly-L-alanine ⊙, poly-L-leucine ⊗, in various mixtures of chloroform, DCA, TFA; poly-ε-carbobenzoxy-L-lysine ⧖ in various mixtures of chloroform, DCA; copoly-L-methyl-S-cysteine:L-methionine △ in various mixtures of chloroform, DCA, TFA at different ratios; copoly-L-valine:L-methionine 1:1 ▲ in DCA:TFA 2:8; poly-L-methyl-S-cysteine ● in chloroform:DCA 1:1 (*1*), DCA (*2*); poly-O-acetyl-L-serine ● in chloroform:DCA 2:8 (*3*), chloroform:DCA 8:2 (*4*), EDC: DCA 1:1 (*5*), DCA (*6*). ---- Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for polypeptides and proteins in aqueous solutions.

solvents such as dichloroacetic acid and trifluoroacetic acid often disrupt α-helical conformations in synthetic polypeptides, whereas less polar solvents such as chloroform, dioxane, and chloroethanol often promote helix formation in synthetic polypeptides and some proteins.

We have calculated $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ for several polypeptides in a variety of organic solvents. The data are reported in Figure 1; some of the data are recorded in Table 1. In these solvents, as in aqueous solutions, a linear relation between $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ has been found for the polypeptides whose conformations are α-helical, random, or mixtures of these forms. The plot of $A_{(\alpha,\rho)(193)}$ against $A_{(\alpha,\rho)225}$ for polypeptides dissolved in organic solvents shows greater scatter of the experimental points than that for the analogous plot of polypeptides dissolved in aqueous solutions. The scatter of the points in Figure 1 may be a result of two sources of error in the calculation of $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ which are not present with aqueous solutions: (1) The data for aqueous solutions were obtained on a recording spectropolarimeter, and many wavelengths were used which yielded more precise values of $A_{(\alpha,\rho)(193)}$ and $A_{(\alpha,\rho)225}$ than those for organic solvents where most of the data were obtained on a manual spectropolarimeter using fewer wavelengths. (2) There are refractive index corrections which are difficult to make, since indices of refraction for certain organic solvents are known only at visible wavelengths around the sodium D line. If such values of the indices of refraction are used over the entire wavelength range through which the rotatory dispersions were obtained, the absolute values of $A_{(\alpha,\rho)225}$ so obtained will always exceed the true values. These differences become significant at low helix content.

TABLE 1

ROTATORY CONSTANTS OF POLYPEPTIDES IN ORGANIC SOLVENTS

Polypeptide of	Solvent	$A_{(\alpha,\rho)\,(193)}$	$A_{(\alpha,\rho)225}$	Per cent helix calculated from equation (4)	(5)	(8)
L-methionine[a, b]	$CHCl_3$	+3020	−1900	100	100	100
L-methyl-S-cysteine: L-methionine(1:1)[b, c]	DCA:TFA(1:1)	−600	0	0	0	1
γ-benzyl-L-glutamate	Dioxane	+2710	−1810	92	96	93
	Dioxane:DMF(1:1)	+2630	−1770	90	93	91
	$CHCl_3$	+2600	−1680	89	89	89
	Pyridine	+2200	−1400	78	74	76
	DCA	−50	−250	15	13	15
γ-methoxyethyl-L-glutamate[d]	Dioxane	+2320	−1550	81	82	81
L-alanine	$CHCl_3$:TFA(1:9)	+245	−455	23	24	24
	DCA:TFA(1:1)	+800	−640	39	34	37
L-leucine	$CHCl_3$:DCA(9:1)	+1460	−1100	57	58	58
	DCA:TFA(1:1)	+700	−720	36	38	37
ε-carbobenzoxy-L-lysine[e]	$CHCl_3$	+2745	−1780	93	94	93
	$CHCl_3$:DCA(4:6)	+100	−270	19	14	18
L-methyl-S-cysteine L-methionine(1:9)[b]	$CHCl_3$:DCA(7:3)	+2320	−1500	81	79	80
	DCA	+735	−655	37	35	37
(2:8)	$CHCl_3$	+2700	−1750	92	92	92
	DCA	+520	−600	31	32	32
(3:7)	DCA	0	−310	17	16	17
L-valine:L-methionine (1:1)[b]	DCA:TFA(2:8)	+90	−370	19	20	19
5[h] O-acetyl-L-serine[f]	EDC:DCA(1:1)	+600	−210	33	11	
4[h] "	$CHCl_3$:DCA(7:3)	+530	−220	31	11	
3[h] "	$CHCl_3$:DCA(3:7)	+330	+340	26	−18	
6[h] "	DCA	+380	−125	27	7	
1[h] L-methyl-S-cysteine[b]	$CHCl_3$:DCA(1:1)	+300	−750	25	40	
2[h] "	DCA	+160	−630	21	32	
L-proline II[g]	Acetic acid	−2100	−750	−42	40	
L-proline I[g]	Propionic acid	−1460	+1180	−24	−62	

[a] Assumed 100% helical. [b] See ref. 3. [c] Assumed zero per cent helical. [d] See ref. 5. [e] See ref. 6. [f] See ref. 7. [g] See ref. 8. [h] The numbers correspond to the points plotted in Fig. 1 which do not fall on the line relating $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$.

There may be other causes of lack of complete linearity between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ in organic solvents which are not due to experimental errors. However, for our present purpose, which is principally the determination of helix contents, we may consider that in organic solvents a linear relation between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ exists for polypeptides in α-helical and random conformations as shown by the solid line in Figure 1. The points for the polypeptides which do not fall close to the line are shown by filled circles, are numbered, and shown in Table 1. Infrared measurements[3] indicate that these compounds exist in conformations other than α-helical or random. Therefore, as expected, these points lie well off the line relating $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$.

The relation between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ differs from the one obtained in aqueous solutions (eq. 2) and is given by

$$A_{(\alpha,\rho)225} = -0.55A_{(\alpha,\rho)\,(193)} - 280. \quad (3)$$

From equation (3) it is possible to derive two equations which allow the determination of helix content in organic solvents, making the assumptions that poly-L-methionine is completely α-helical in chloroform ($A_{(\alpha,\rho)\,(193)} = +3020$, $A_{(\alpha,\rho)225} =$

-1900) and that a 1:1 copoly L-methionine:L-methyl-S-cysteine exists in a completely random conformation in a 1:1 mixture of dichloroacetic:trifluoroacetic acid ($A_{(\alpha,\rho)\,(193)} = -600$, $A_{(\alpha,\rho)225} = 0$). The equations for helix content then become those shown in (4) and (5)

$$H_{193} = \frac{A_{(\alpha,\rho)\,(193)} + 600}{36.2} \quad (4) \qquad H_{225} = -\frac{A_{(\alpha,\rho)225}}{19.0} \quad (5)$$

where H is the per cent helix.

Since to a first approximation the linear relation between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ does not seem to depend on amino acid composition, organic solvent, or temperature as long as the conformation remains α-helical or random, we may assume at this point that in organic solvents these internal or external conditions do not affect in an appreciable way the values of $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ for a given α-helix content. We may therefore assume that the uncertainty in the estimation of helix content depends on the scatter of the experimental points defining the line in Figure 1, which is approximately ± 5 per cent helix content. Thus, in the case of aqueous solutions, where the experimental scatter is much smaller, the previous estimate of an accuracy better than ± 5 per cent is reasonable.

Since equations (4) and (5) derive from equation (3), rotatory dispersion parameters of polypeptides and proteins dissolved in organic solvents which do not fit equation (3) will give values of helix content from equations (4) and (5) which differ from each other (see last entries in Table 1). If the difference in calculated helix content from equations (4) and (5) is greater than the experimental error, this should, as in the case of the aqueous solutions data, point to the existence of structures in the solute other than α-helical and random. However, as we stated above, there can be reasons other than experimental errors which may allow for the lack of perfect linearity between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ in organic solvents. Therefore, the fact that polypeptides or proteins in organic solvents do not fit equation (3) exactly must at this time be regarded only as indicating the possibility that they contain other structures, except in those cases where the deviation from the relation is very great (Fig. 1).

The rotatory dispersion data for some proteins in organic solvents have been analyzed and plotted in the form described by equation (1). The values of $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ so obtained are plotted in Figure 2 and the values shown in Table 2. As can be seen, the points fall well within the experimental error referred to above.

The presence of Cotton effects other than those considered[1] (namely, the 193, 198, and virtual 225 mμ Cotton effects) will prevent a fit between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ in equation (3). In addition left-handed α-helices may not fit equation (3) with the signs of $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ reversed, unless the ratio of the magnitudes of the Cotton effects at 193, 198, and 225 mμ are the same as for right-handed α-helices.

Comparison of Rotatory Dispersion Parameters in Aqueous and Organic Solvents.—A comparison of the values of $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ for polypeptides of the same helix content in aqueous and organic solvents shows that the value of $A_{(\alpha,\rho)\,(193)}$ is larger in the organic media than in aqueous solutions, whereas $A_{(\alpha,\rho)225}$ is less negative in organic solvents. The observed differences between the two types of solvents are of the order of 150 degrees. Since the optical activity of the $n \rightarrow \pi^*$ transition has an electrostatic origin,[4] one may expect the dielectric constant to

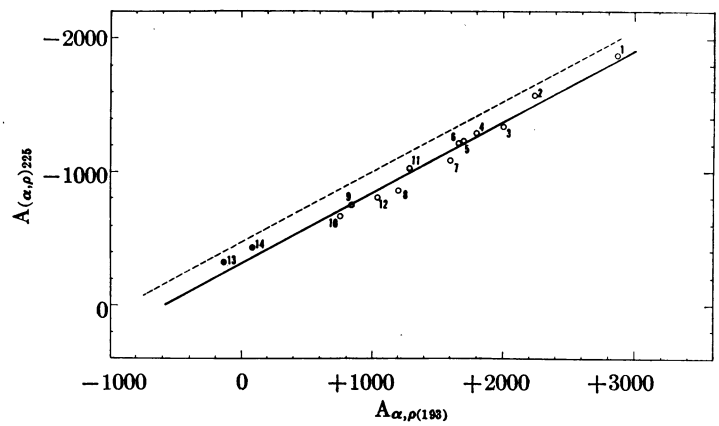

FIG. 2. —— Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for proteins in organic solvents. The numbers correspond to the entries in Table 2. ---- Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for polypeptides and proteins in aqueous solutions.

influence the values of $A_{(\alpha,\rho)\ (193)}$ and $A_{(\alpha,\rho)225}$. Although it may be premature to draw a definite conclusion, the smaller levorotation in organic solvents compared to aqueous solutions seems to have its origin in the differences in dielectric constant between these two classes of solvents.

The magnitude of the differences in dielectric constant among organic solvents (D = 2–20) is less than the difference in dielectric constant between water (D = 80) and organic solvents as a class. Thus, one may expect to observe differences in A's from aqueous to organic solvents, but smaller differences among A's from the various organic solvents. It can be seen from the data in Figures 1 and 2 that there is a linear relation between $A_{(\alpha,\rho)\ (193)}$ and $A_{(\alpha,\rho)225}$ which appears to be essentially independent of the organic solvent. Formic acid (D = 60) and methanol (D = 40) are, as expected, two exceptions among the organic solvents; the values of A's

TABLE 2

ROTATORY CONSTANTS OF PROTEINS IN ORGANIC SOLVENTS

Proteins	Solvent	$A_{(\alpha,\rho)}$ (193)	$A_{(\alpha,\rho)225}$	Per cent helix calculated from equation (4)	(5)	(8)
1. Tropomyosin[a]	Chloroethanol	+2870	−1880	96	99	97
2. Globin M	"	+2240	−1580	79	83	80
3. Ovalbumin	"	+2000	−1340	72	71	72
4. Globin H	"	+1800	−1290	67	68	67
5. Ribonuclease	"	+1700	−1230	64	65	64
6. Histone	"	+1670	−1210	63	64	63
7. Bovine serum albumin	"	+1600	−1095	61	58	60
8. Lysozyme	"	+1200	−850	50	45	48
9. Insulin	"	+845	−750	40	40	40
10. Pepsin	"	+750	−655	37	35	37
11. Bovine plasma albumin[b]	"	+1290	−1300	52	54	53
12. Oxidized bovine plasma albumin	"	+1060	−800	46	42	45
13. Bovine plasma albumin	Formic acid[c]	−140	−320	16	13	15
14. Oxidized bovine plasma albumin	"	+80	−420	23	18	20

[a] The values of a_0 and b_0 from the Moffitt equation given in ref. 2*b* were used to compute the specific rotations for the proteins numbered 1–10.
[b] The values of the specific rotations given in ref. 9 were used for the proteins numbered 11–14.
[c] Formic acid having a dielectric constant of 60, the values of helix content were calculated using the relations obtained for polypeptides and proteins in aqueous solutions.

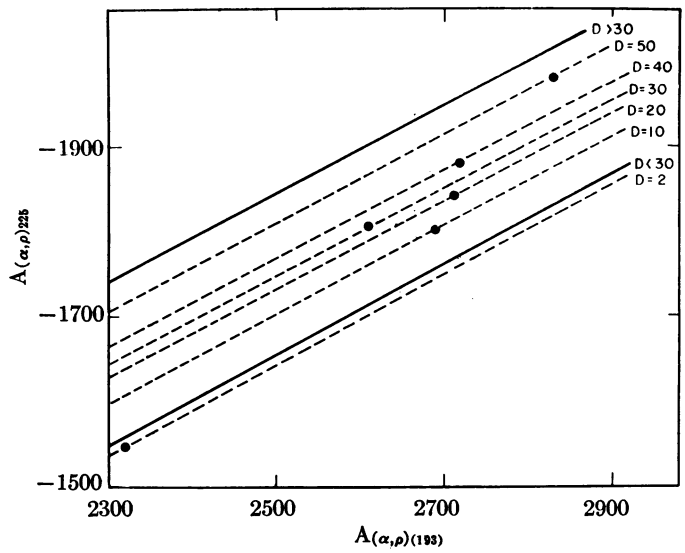

FIG. 3.—Plot of $A_{(\alpha,\rho)(193)}$ versus $A_{(\alpha,\rho)225}$ for poly-γ-methoxyethyl-L-glutamate in mixtures of solvents of different dielectric constant. The values of D shown are approximate values assuming contributions from each solvent component directly proportional to its volume fraction.

obtained in these solvents are closer to those obtained for aqueous solutions. To examine the variation of $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ with dielectric constant, we made careful measurements of rotatory dispersion for poly-γ-methoxyethyl-L-glutamate in various mixtures of dioxane ($D = 2$), methanol ($D = 40$), and water ($D = 80$). As can be seen in Figure 3, the plot of $A_{(\alpha,\rho)\,(193)}$ against $A_{(\alpha,\rho)225}$ gives a point approximately on the line obtained for organic solvents when $D = 2$, and the values gradually approach the line obtained for aqueous solutions as the dielectric constant increases. Since the variation of the rotatory parameters is small for an increase of 10 units of dielectric constant, we may arbitrarily divide solvents into two groups: those with high dielectric constant (water and some hydroxylic organic solvents), and those with low dielectric constant (most organic solvents).

Since two different relations exist between $A_{(\alpha,\rho)\,(193)}$ and $A_{(\alpha,\rho)225}$ in low and high dielectric constant solvents [eqs. (2) and (3)], neither $A_{(\alpha,\rho)\,(193)}$ nor $A_{(\alpha,\rho)225}$ can be regarded as a parameter of helix content which is independent of solvent. But since, as was noted above, a variation of $A_{(\alpha,\rho)\,(193)}$ is compensated by an opposite variation of $A_{(\alpha,\rho)225}$, then $A_{(\alpha,\rho)\,(193)} - A_{(\alpha,\rho)225}$ should be, to a good approximation, solvent-independent. In solvents of high dielectric constant the helix content is given by

$$H = \frac{A_{(\alpha,\rho)\,(193)} - A_{(\alpha,\rho)225} + 690}{56.4} \tag{6}$$

and in solvents of low dielectric constant by

$$H = \frac{A_{(\alpha,\rho)\,(193)} - A_{(\alpha,\rho)225} + 600}{55.2}. \tag{7}$$

These two equations are practically equivalent, and we may therefore use either of them to express the helix content, essentially independently of the solvent in which the material is dissolved. Helix contents calculated from an equation

$$H = \frac{A_{(\alpha,\rho)\ (193)} - A_{(\alpha,\rho)225} + 650}{55.8}, \tag{8}$$

which is the mean of equations (6) and (7), are shown in Tables 1 and 2.

Conclusions.—In this and the previous communication[1] we have shown that a new equation (eq. 1) describes the visible and near-ultraviolet rotatory dispersion data of α-helical and random polypeptides and proteins in organic solvents and aqueous solutions, respectively. This new equation is a modified two-term Drude equation. The two rotatory parameters $A_{(\alpha,\rho)\ (193)}$ and $A_{(\alpha,\rho)225}$ were shown to be α-helix parameters which are influenced appreciably only by the dielectric constant of the solvent. For a given α-helix content the variation of the rotatory parameters is small enough so that all solvents may be grouped in two categories: high and low dielectric constant solvents. In each of these classes the α-helix content can be expressed by two independent linearly related parameters [eqs. (2) and (3)].

It has been shown that if a polypeptide or protein gives values of $A_{(\alpha,\rho)\ (193)}$ and $A_{(\alpha,\rho)225}$ which do not fit equations (2) or (3), this indicates that other structures such as extended conformations, proline helices, or other types of helices are present. We infer that the fit of $A_{(\alpha,\rho)\ (193)}$ and $A_{(\alpha,\rho)225}$ to these equations indicates the presence in the polypeptides or the proteins of only α-helical or random conformations within the limits of experimental error described in this communication. Finally it was found that the quantity $A_{(\alpha,\rho)\ (193)} - A_{(\alpha,\rho)225}$ is independent of solvent to a first approximation and can therefore be used as an α-helix content parameter which is independent of solvent.

An advantage of this analysis over previous types of optical rotatory dispersion analyses is that the α-helix content can be obtained independently from two rotatory dispersion parameters, thus allowing an internal check on the α-helix content of the polypeptide or protein. Comparisons of previous types of optical rotatory dispersion analyses with the present one will be discussed in a forthcoming communication.

We are pleased to acknowledge the support of this work by U.S. Public Health Service grant AM-07300-01.

* This is "Polypeptides XLVII." For the preceding paper in this series and an explanation of the notations used, see ref. 1.

[1] Shechter, E., and E. R. Blout, these PROCEEDINGS, **51**, 695 (1964).

[2] For reviews see (*a*) chap. 17 by E. R. Blout in *Optical Rotatory Dispersion*, ed. C. Djerassi (New York: McGraw-Hill, 1960); (*b*) Urnes, P., and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1962).

[3] Bloom, S. M., G. D. Fasman, C. De Loze, and E. R. Blout, *J. Am. Chem. Soc.*, **84**, 458 (1962).

[4] Schellman, J. A., and P. Oriel, *J. Chem. Phys.*, **37**, 2114 (1962).

[5] Kulkarni, R. K., and E. R. Blout, in preparation.

[6] Fasman, G. D., M. Idelson, and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 709 (1961).

[7] Fasman, G. D., and E. R. Blout, *J. Am. Chem. Soc.*, **82**, 2262 (1960).

[8] Blout, E. R., and G. D. Fasman, in *Recent Advances in Gelatin and Glue Research* (London: Pergamon Press, 1957), p. 122.

[9] Marsh, M. M., *J. Am. Chem. Soc.*, **84**, 1896 (1962).