TABLE VI

Compound	mμ (max., pH 14)	ε × 10⁻³ (pH 14)
Uridine	264.5	7.5
5-Fluorouridine	270	7.0
1-β-D-Ribofuranosylthymine[24]	268–269	7.6
2′-Deoxyuridine	263.5	7.86
5-Fluoro-2′-deoxyuridine	269–270	6.94
Thymine	268	7.51

ity by trace contamination by nucleosidase(s) in the extract preparation, was verified.

Spectrophotometric Studies.—Ultraviolet absorption data were determined with a Cary recording spectrophotometer, model 11, using buffers and techniques previously described.[30,32] The curves in the strongly acidic region up to *p*H 3 were measured in HCl solutions; acetate buffers were used for the *p*H range 4–6, phosphate buffers for range *p*H 6–9, glycine buffers *p*H 10–12, and sodium hydroxide solutions from *p*H 12–14. All benzoylated derivatives were dissolved in methylene chloride and aliquots delivered into absolute ethanol; all other compounds were dissolved in water and aliquots delivered into the proper buffer solution. The spectrophotometrically measured pK_a values were determined by methods previously described.[32,56]

Polarimetric Determinations.—Optical rotations were determined using equipment and techniques previously described.[24]

Electrophoretic Experiments.—All studies were made using an E.C. electrophoresis apparatus.[57] Whatman 3MM paper was employed; after completion of the run, the paper was air-dried and the products visualized under ultraviolet light.

Paper Chromatographic Determinations.—The R_f values for some 5-fluoropyrimidine nucleosides and related compounds are shown in Table V. The values shown were determined by the ascending method using Schleicher and Schuell #597 paper. The compounds were visualized on the paper chromatograms under ultraviolet light and were all found to be light absorbing with the exception of the thiated intermediates which showed a characteristic pink fluorescence.

Key to Figures: All the spectra listed were run in aqueous solutions at *p*H values indicated on the curves. The italicized letters refer to isosbestic points.[31]

(56) J. J. Fox and D. Shugar, *Bull. soc. chim. Belg.*, **61**, 44 (1952).

(57) Manufactured by E. C. Apparatus Co., Swarthmore, Pa.

[CONTRIBUTION FROM THE LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY OF THE DEPARTMENT OF BIOPHYSICS AND NUCLEAR MEDICINE, SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA AT LOS ANGELES*; INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORIES, WOODS HOLE+, AND THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION AND HARVARD MEDICAL SCHOOL, BOSTON 15, MASS.]

A Conformation-dependent Cotton Effect in α-Helical Polypeptides and Proteins[1,2]

BY N. S. SIMMONS*, C. COHEN, A. G. SZENT-GYORGYI+,[3a] D. B. WETLAUFER[3b] AND E. R. BLOUT

RECEIVED JUNE 8, 1961

A Cotton effect is described which is associated with the α-helical conformation of polypeptides and proteins. This Cotton effect appears to be negative, with an inflection point at about 225 mμ. It is suggested that the magnitude of the trough at 233 mμ may be used as an approximate measure of α-helix content. Transformation of the polypeptides to random coil conformations, or denaturation of the proteins, results in the loss of the 225 mμ Cotton effect.

Introduction

Anomalous rotatory dispersion, known as the Cotton effect, occurs in or near optically active absorption bands.[4] The chemical groupings making up polypeptide or protein chains give rise to at least three types of electronic absorption bands: the α-carbons of the constituent amino acids absorb in the far ultraviolet; the peptide chromophore absorbs strongly around 145 and 185 mμ[5]; and aromatic side chains absorb in the region below 300 mμ. These chromophores may exhibit Cotton effects when situated in a disymmetric environment. In particular, a helical conformation of the polypeptide chain,[6] or restricted rotation of side chains, would be expected to induce anomalous rotatory dispersion.

Some rotatory dispersion data have been reported for polypeptides and proteins in the spectral range 240–300 mμ. Examination of the α-helical forms of two synthetic polypeptides containing aromatic side chains, poly-γ-benzyl-L-glutamate and poly-β-benzyl-L-aspartate, did not reveal a Cotton effect in this region.[7] Recently, however, measurements on the protein subunits of tobacco mosaic virus showed the presence of a small inflection point at 293 mμ; this unusual rotatory dispersion might be due to a small Cotton effect from oriented aromatic amino acids.[8] Furthermore, the shape of the dispersion curve below 240 mμ indicated the possible presence of a large Cotton effect in this region. An investigation of model polypeptides and proteins was then undertaken to examine this effect.

In this communication we report the rotatory dispersion of certain polypeptides and α-proteins from 400 to about 220 mμ. In all cases, the α-helical conformation is characterized by what may be described as a large negative Cotton effect with a trough at 233 mμ and an inflection at about 225 mμ. When the helix is destroyed, the effect is lost; thus the rotatory dispersive behavior in this region is conformation-dependent.

Experimental

Materials. Polypeptides. Poly-γ-benzyl-L-glutamate (L-PBG).—This sample, RK-1262, was prepared by Mr. Roy

(1) This is Polypeptides. XXXVI. For the preceding paper in this series see E. R. Simons, G. D. Fasman and E. R. Blout, *J. Biol. Chem.*, **236**, Pc64 (1961).

(2) This work was supported in part by Contract No. AT(04-1)-GEN-12, between the Atomic Energy Commission and the University of California and by United States Public Health Service Grants A2633 to C. Cohen and A2558 to E. R. Blout.

(3) (a) Established investigator of the American Heart Association; (b) Department of Biochemistry, Indiana University Medical School, Indianapolis 7, Ind.

(4) See for example, W. Kuhn, *Ann. Rev. Phys. Chem.*, **9**, 417 (1958).

(5) D. L. Peterson and W. T. Simpson, *J. Am. Chem. Soc.*, **79**, 2375 (1957).

(6) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Natl. Acad. Sci., U. S.*, **37**, 205 (1951).

(7) R. H. Karlson, K. S. Norland, G. D. Fasman and E. R. Blout, *J. Am. Chem. Soc.*, **82**, 2268 (1960).

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

Karlson and had a reduced specific viscosity of 1.12 at a concentration of 0.2% in dichloroacetic acid from which a weight average molecular weight is estimated as 190,000. For rotatory dispersion measurements the sample was dissolved in freshly distilled dioxane at a concentration of 1.0%.

Poly-L-methionine (L-PMet).—This sample, SMB-405, was prepared by Dr. Stanley Bloom and had a reduced specific viscosity of 0.64 at a concentration of 0.2% in trifluoroacetic acid, from which a weight average molecular weight is estimated as 69,000. For rotatory dispersion measurements the sample was dissolved at a concentration of 1.4% in freshly distilled methylene chloride.

Poly-α,L-glutamic Acid (L-PGlu).—This sample, R-4273-112, was prepared according to the procedure of Idelson and Blout.[9] It had an intrinsic viscosity of 1.12 in 0.2 *M* NaCl at *p*H 7.3 from which a weight average molecular weight is estimated as 51,000. For rotatory dispersion measurements the sample was dialyzed against either acetate buffer (*p*H 4.75, μ = 0.20, 0.18 *M* NaCl) or phosphate buffer (*p*H 7.4, μ = 0.20, 0.18 *M* NaCl). Concentrations in the dialysates were determined by a biuret method previously calibrated with polyglutamic acid.

Proteins. **Paramyosin** was prepared from the adductor muscles of *Mercenaria mercenaria* as described previously.[10] The protein was stored as lyophilized powder. The nucleic acid contamination in the preparation was low, as indicated by the ratio of 2.1 in the extinctions at 278 and 260 mμ. The preparation chromatographed as a single sharp peak, using gradient elution against 1.0 *M* NaCl at *p*H 8.0 from diethylaminoethyl (DEAE)-substituted cellulose column, and the ratio of extinctions remained 2.1.

Tropomyosin was prepared from rabbit muscle following the procedure of Bailey[11] through stages 1–4. The protein was stored as a lyophilized powder. The preparation contained considerable amounts of nucleic acids which accounted for about half of the total extinction at 278 mμ. The nucleic acid was removed by passage through a DEAE column. The protein was applied to the column from a solution containing 0.02 *M* *p*H 7.0 phosphate buffer and eluted by gradient elution against a solution containing 0.02 *M* neutral phosphate buffer and 1.0 *M* NaCl. Tropomyosin came off at conductances of 0.015 mho/cm. as a sharp peak, the nucleic acids at conductances of 0.027 as a broader peak (conductance measured at 0°). The tropomyosin fraction was dialyzed against water, lyophilized and stored. The ratio of extinction at 278 and 260 mμ was 2.1.

Myosin was prepared according to Szent-Gyorgyi.[12] A twice-reprecipitated preparation was centrifuged for 3 hours at 40,000 r.p.m. and the lipid collected on the top was removed by filtering the solution through 8 layers of gauze.

Tropomyosin and paramyosin were dissolved in cold 0.6 *M* KCl, 0.1 *M* phosphate buffer *p*H 7, with gentle magnetic stirring for 24 hr. at 4°, and then centrifuged for 1 hour in the Servall at 7000 r.p.m. at 4°. Concentrations of these three protein solutions were determined from Kjeldahl nitrogen analyses and the biuret method. The stock solutions having concentrations around 1% were stored at 4° at all times until immediately before use or further dilution.

Denaturation.—The proteins were denatured by placing one volume of the stock solutions into 9 volumes of recrystallized urea solution which was saturated at 20° (9 *M*). Sealed tubes containing this mixture were then incubated for 2 hr. at 60° and cooled to room temperature. Control urea solutions similarly diluted with the protein solvent were treated in the same way and used as the blank for the rotatory dispersion measurements.

Methods.—Measurements of the optical rotation were made with the Rudolph automatic recording spectropolarimeter,[13] model 260/655/850/810/614 and with the Rudolph photoelectric spectropolarimeter, model 80Q3/200AS/650, using a General Electric AH-6 water-cooled high pressure arc as the light source. The automatic recording runs were made using a symmetrical angle of 15° and a slit width of 1.5 mm. Fused quartz cells of 0.1, 0.05 and 0.01 dm. length selected for minimal birefringence were used depending on the concentrations, materials and the solvents employed, in order to obtain maximum transmittance at the lowest possible wave lengths.

L-PBG was measured between 212 and 280 mμ in a 0.01-dm. cell at concentrations of 1% and 0.33%. L-PMet was measured between 222 and 280 mμ at a concentration of 0.28% in the 0.01-dm. cell. L-PGlu was measured at concentrations ranging from 1.35% in the visible to 0.016% in the region 226–240 mμ. Measurements where λ was less than 300 mμ were made in a 0.10-dm. cell.

The native proteins were measured at concentrations between 0.01 and 0.02% in 0.05-dm. cells between 225 and 280 mμ. Stock solutions of the denatured proteins were measured in the 0.05-dm. cell from 230 to 300 mμ.

It is important to use low concentrations and short path lengths since rotatory dispersions which may be termed "spurious Cotton effects" can be observed with solutions having high optical density (P. Urnes and P. Doty, private communication).

The data are expressed in terms of $[R']_\lambda$, defined as

$$[R']_\lambda = [\alpha]_\lambda \times \frac{\bar{M}}{100} \times \frac{3}{n^2 + 2}$$

where $[\alpha]\lambda = (100\ \alpha_\lambda)/(l \times c)$, and α is the observed rotation in degrees, l is the cell length (in decimeters), and c is the concentration of optically active solute in g./100 ml. Further, $\bar{M}$ is the mean amino acid residue weight and n is the refractive index of the solvent. For this work $\bar{M}$ was taken as 115 for all the proteins. Values of n at 546 mμ were used in calculations of the Moffitt equation in the wave length region 365 to 578 mμ. In the computations of $[R']_{233}$, values of n at 265 mμ were used, the shortest wave length for which data for all solvents were available. The n-values employed were: for 8 *M* urea, $n_{265} = 1.457$; for *p*-dioxane, $n_{265} = 1.470$; for dichloromethane, $n_{265} = 1.479$; for water, $n_{265} = 1.370$. We thank Professor John Schellman[14] for access to data of Foss, Yang and Schellman on dispersion of refractive index of several solvents.

The measurements of the near ultraviolet and visible rotatory dispersion were carried out with a 2-dm. cell in the model 200 polarimeter, at a symmetrical angle of 2°. The Moffitt equation has been discussed elsewhere (see for example, references 7, 15, 19); we used $\lambda_0 = 212$ mμ in our calculations.

Results and Discussion

The rotatory dispersion data from both the polypeptides and the proteins reveal the presence of a large trough at 233 mμ and an inflection point about 225 mμ which could be part of a negative Cotton effect. The rotatory dispersion of poly-γ-benzyl-L-glutamate (L-PBG) and poly-L-methionine (L-PMet) are shown in Fig. 1 for the wave length region 215–280 mμ. These synthetic polypeptides have been shown to exist in the α-helical conformation in the solid state and give evidence of existing in the same conformation in the organic solvents used for these measurements.[16] In Fig. 2 the rotatory dispersion of the helical and random forms of poly-α,L-glutamic acid (L-PGlu) are shown. In Fig. 3 there are shown the rotatory data for aqueous solutions of native paramyosin, tropomyosin and myosin, and the data for the same proteins in 8 *M* urea. These fibrous muscle proteins exist in the α-helical conformation in their native states.[17] As seen in Fig. 3, the α-proteins

(9) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958).

(10) W. H. Johnson, J. S. Kahn and A. G. Szent-Gyorgyi, *Science*, **130**, 160 (1959).

(11) K. Bailey, *Biochem. J.*, **43**, 271 (1948).

(12) A. Szent-Gyorgyi, "Chemistry of Muscular Contraction," 1st Ed., Academic Press, Inc., New York, N. Y., 1947.

(13) We thank Dr. Carl Djerassi for the use of this instrument.

(14) J. A. Schellman, private communication.

(15) E. R. Blout, chapter in "Optical Rotatory Dispersion," by C. Djerassi, McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p. 238.

(16) Recent unpublished data of Luzzati, *et al.* (*J. Mol. Biol.*, in press (1961)) indicate that L-PBG may exist in the 3_{10} rather than the 3.6_{13} (α) helix in low concentrations in organic solvents such as dimethylformamide. This important work implies that rotatory dispersion measurements may not distinguish between these two helical conformations.

(17) C. Cohen and A. G. Szent-Gyorgyi, *J. Am. Chem. Soc.*, **79**, 248 (1957).

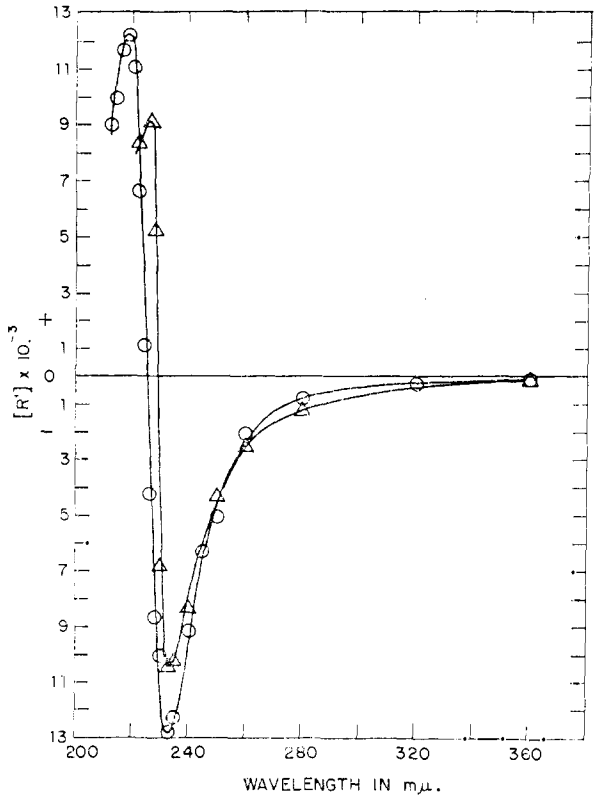

Fig. 1.—The ultraviolet rotatory dispersion of: —Δ—Δ—, poly-γ-benzyl-L-glutamate in dioxane solution; —O—O—, poly-L-methionine in methylene dichloride solution.

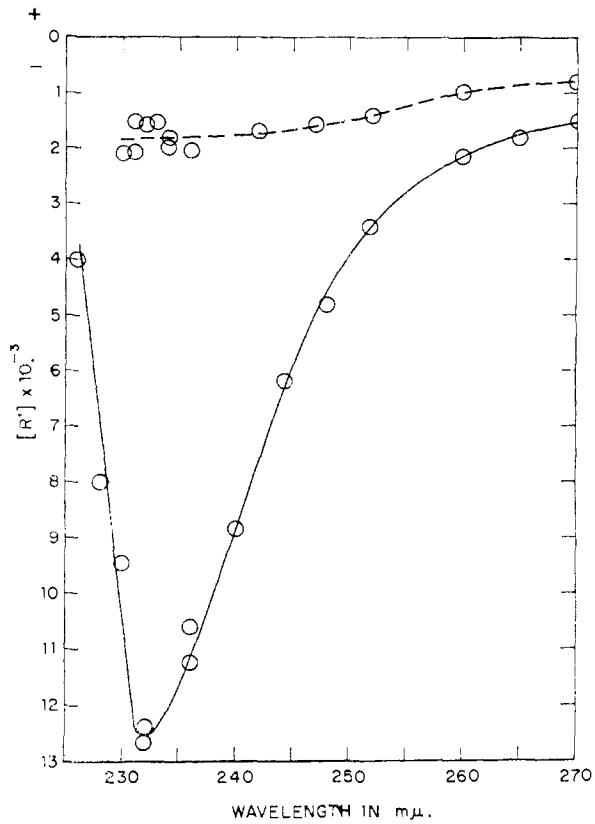

Fig. 2.—The ultraviolet rotatory dispersion of: ———, poly-L-glutamic acid at *p*H 4.5 in water; - - - -, poly-L-glutamic acid (sodium salt), *p*H 7.4 in water.

exhibit a trough similar to that observed with the synthetic polypeptides. In contrast, the random coil form of poly-γ-L-glutamic acid and the urea denatured tropomyosin and myosin do not show this effect.[18] Thus we may conclude that a Cotton effect with an inflection point at 225 mμ is associated with the α-helical form of polypeptides and proteins.

It appears that the magnitude of this trough at 233 mμ can be used as an approximate measure of α-helix content. We have calculated $[R']_{233}$ for the native and denatured or random coil forms of the polypeptides and proteins investigated (Table I). In the non-helical form, the observed rotation is due simply to the configurational contribution of the optically active amino acids. Our results indicate that there is a residue rotation difference at 233 mμ ($\Delta[R']_{233}$) of approximately 11,000° between the α-helical and random conformations of the polypeptide chains. Using this scale, we can then estimate the percentabe helix for native myosin as 63, and the percentage residual helix for paramyosin in 8 *M* urea as 28. We find substantial agreement between these values and data previously published using b_0-values calculated from the Moffitt equation[19] as an estimate of helix content. It is important to note that our scale based on $[R']_{233}$ is solvent independent. Hence these measurements differ from estimates made using visible wave length residue rotations which have large solvent dependence.

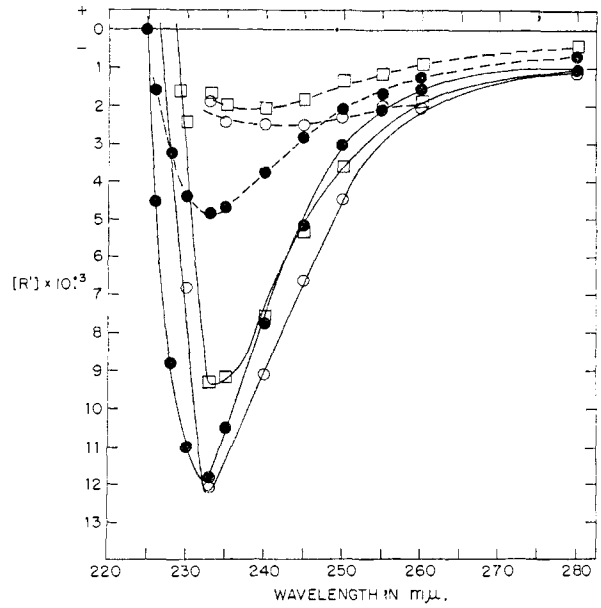

Fig. 3.—The ultraviolet rotatory dispersions of some fibrous α-proteins: ●, paramyosin; O, tropomyosin; □, myosin; ———, proteins in 0.6 *M* KCl; - - -, proteins in 8 *M* urea.

(18) It should be noted, however, that paramyosin from *Mercenaria mercenaria*, as previously reported,[17] is not completely unfolded in 8 *M* urea, and a portion of the effect remains (Fig. 3).

(19) W. Moffitt, (a) *J. Chem. Phys.*, **25**, 467 (1956); (b) *Proc. Natl. Acad. Sci., U. S.*, **42**, 736 (1956).

TABLE I

SOME ROTATORY PROPERTIES OF CERTAIN SYNTHETIC POLYPEPTIDES AND α-PROTEINS

Material	Helical				Random	
Polypeptides	b_0	% helix[a] calcd. from b_0	$[R']_{233}$	% helix[b] calcd. from $[R']$	b_0	$[R']_{233}$
Poly-L-glutamic acid	−600	95	−12,600	99	0	−1780
Poly-L-methionine	−630	100	−10,400	79	0	....
Poly-γ-benzyl-L-glutamate	−670	106	−12,800	101	0	....
Proteins	Native				Denatured	
Paramyosin	~−600	95	−11,600	90	−220	−4850
Tropomyosin	~−600	95	−12,100	95	0	−1920
Myosin	−380	60	− 8,700	63	0	−1670

[a] Assuming mean value of $b_0 = -630$ for 100% helix, $b_0 = 0$ for 0% helix, and a linear interpolation. [b] Assuming mean value of $[R']_{233} = -12{,}700$ for 100% helix, $[R']_{233} = -1800$ for 0% helix, and a linear interpolation.

It is not possible at this time to state unequivocally that the anomalous rotatory dispersion which we observe in the region 220–240 mμ is a negative Cotton effect. Instrumental limitations have prevented satisfactory measurements at shorter wave lengths than about 220 mμ. Therefore, the possibility must be considered that the change in sign of rotation and the trough at 233 mμ are the beginning of a *positive* Cotton effect with its inflection point at still shorter wave lengths. Further experiments, when better apparatus is available, will resolve this question. However, the shape of the 220 mμ peak of the rotatory dispersion of two synthetic polypeptides (Fig. 1) and the abruptness of the change in rotation, make it appear likely that the effect we have observed is a negative Cotton effect, with an inflection at 225 mμ.

The electronic origin of this effect is associated with a transition of the peptide chromophore. The effect therefore is relatively independent of side chain composition and solvent interaction, but depends on helix content. Although ester and carboxyl chromophores absorb in this spectral region, any conformational-induced disymmetry of these groups must be of minor significance in this phenomenon; poly-L-methionine exhibits the effect although lacking the COO^- chromophore.

A weak absorption band in proteins about 225 mμ has recently been reported[20] and, assuming the observed rotatory data represent a negative Cotton effect, we can relate this effect to the 225 mμ absorption band. Studies of model secondary amides indicate that the main absorption band of the amide group, due to the $N-V_1$ transition, lies at approximately 185 mμ. The formation of a simple exciton band in the α-helix would not be expected to displace this maximum to the observed 225 mμ. A more probable origin of the 225 mμ band in proteins and polypeptides is an $n-\pi^*$ electronic transition assigned in the spectra of secondary amides.[5]

Despite the weakness of the 225 mμ absorption, its rotational strength is large. Since this band is close to the visible, it will therefore make a large contribution to the optical rotation in the visible and near ultraviolet region where b_0 is normally evaluated. Whether the negative 225 mμ Cotton effect and another positive Cotton effect at shorter wave lengths are responsible for the observed anomalous rotatory dispersion of helical polypeptides and proteins, and hence their b_0-values, remains to be determined.[21]

The Cotton effect reported here appears to be a useful empirical characterization of polypeptides and proteins, independent of theoretical interpretations. In addition to providing a new parameter for estimates of helix content in simple proteins and polypeptides, this effect may provide structural information in more complex systems difficult to analyze by rotatory dispersion measurements at visible wave lengths. Examples are proteins containing absorbing chromophores (*e.g.*, hemoglobin), nucleoproteins, and polypeptides with aromatic amino acid residues. Moreover, this Cotton effect offers additional criteria for evaluating theoretical treatments of rotatory dispersion.

(20) A. N. Glazer and E. L. Smith, *J. Biol. Chem.*, **235**, PC 43 (1960).

(21) We thank Dr. John Schellman for clarifying this point and for helpful discussions.