

[27] Circular Dichroism and Optical Rotatory Dispersion of Proteins and Polypeptides

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Introduction

Considerable information concerning the structure of proteins in solution can be obtained from measurement of their optical activity. The great asymmetry of protein molecules is responsible for the large signals they display in the interrelated methods of optical rotatory dispersion (ORD) and circular dichroism (CD). ORD is the measurement, as a function of wavelength, of a molecule's ability to rotate the plane of linearly polarized light; CD is similar data evaluating the molecule's unequal absorption of right- and left-handed circularly polarized light. Although all the amino acids except glycine contain at least one asymmetric carbon atom (the L or D configuration), most amino acids display only small ORD and CD bands.² It is the conformation of the protein, that is, the asymmetric and periodic arrangement of peptide units in space, which gives rise to their characteristic ORD and CD spectra.

In recent years X-ray diffraction analysis has lead to the complete mapping of the peptide backbone and side-chain positions of lysozyme,^{3,4} several other enzymes,⁵ and quite a few other proteins^{6,7} in the solid state. Newer techniques such as neutron diffraction (for an example, see Schoenborn⁸) and high resolution nuclear magnetic resonance (for a

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² C. Toniolo, *J. Phys. Chem.* **74**, 1390 (1970).

³ C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature (London)* **206**, 757 (1965).

⁴ D. C. Phillips, *Sci. Amer.* **215**, 78 (1966); *Proc. Nat. Acad. Sci. U.S.* **57**, 484 (1967).

⁵ D. M. Blow and T. A. Steitz, *Annu. Rev. Biochem.* **39**, 63 (1970).

⁶ D. R. Davies, *Annu. Rev. Biochem.* **36**, 321 (1967).

⁷ R. E. Dickerson and I. Geis, "The Structure and Action of Proteins." Harper, New York, 1969.

⁸ B. P. Schoenborn, *Nature (London)* **224**, 143 (1969).

review see Roberts and Jardetzky⁹) have yielded information about the hydrogen atoms of proteins; this is beyond the resolution of X-ray analysis. ORD and CD are techniques which lack the capacity for exact structural determination possible with the methods mentioned above; however, they have the advantage that one can rapidly approximate the percentages of the conformations present in dilute solutions of proteins. Because of the small volume and low concentration required, a protein sample of less than 0.1 mg is often sufficient for a CD and/or ORD determination.

For studies of the structure of synthetic polypeptides, macromolecules that often exhibit regular repeating conformations, ORD and CD may be unsurpassed. Moreover, ORD and CD are very sensitive spectral tools and thus may be of use in studying any reactions involving changes in optical activity. Thus they are excellent for measuring protein denaturation and helix-coil transitions of polypeptides. They can also be used in measurements of enzyme interactions with substrates, inhibitors, or coenzymes and of the binding of metal ions and dyes to proteins and polypeptides.

In 1963 a chapter on the ORD of proteins was written by Fasman¹⁰ for this series. At that time, because of instrumental limitations, most studies of protein ORD involved measurements in the wavelength range above 240 nm and data analysis by means of the Drude¹¹ or Moffitt¹²⁻¹⁴ equations. Examination of the Cotton effects derived directly from asymmetric peptide chromophore transitions (the 233 nm ORD trough)¹⁵ was just beginning. In the intervening years two types of improvements in recording spectropolarimeters have revolutionized measurements of protein rotatory properties, causing a proliferation of polypeptide and protein investigations, and more than justifying a new review in "Methods in Enzymology." The first advance was development of commercial instruments permitting routine ORD measurements in the spectral range 185-600 nm, thus including Cotton effects arising from peptide chromophores. The second, even more useful, development was that of circular dichroism spectrophotometers capable of operating in this same spectral range. The advantages of CD over ORD for protein conformational studies are that (1) each optically active electronic transition gives

⁹G. C. K. Roberts and O. Jardetzky, *Advan. Protein Chem.* **24**, 448 (1970).

¹⁰G. D. Fasman, see Vol. 6 [126] p. 928.

¹¹P. Drude, "Lehrbuch der Optik," 2nd ed. Hirzel, Leipzig, 1906.

¹²W. Moffitt, *J. Chem. Phys.* **25**, 467 (1956).

¹³W. Moffitt, *Proc. Nat. Acad. Sci. U.S.* **42**, 736 (1956).

¹⁴W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci. U.S.* **42**, 596 (1956).

¹⁵N. S. Simmons and E. R. Blout, *Biophys. J.* **1**, 55 (1960).

rise to only one CD band instead of both positive and negative ORD signals (see Fig. 1), and the bands are thus more easily resolved and assigned, and that (2) CD, unlike ORD, bands are of finite width and, therefore, CD spectra contain no contribution from transitions outside of the measured spectral range. For these reasons most investigations of proteins utilized CD instead of ORD beginning about 1968, and the present chapter will concentrate on CD.

Another important advance was the discovery of the water-soluble β conformation of heated poly-L-lysine.¹⁶⁻¹⁹ Therefore, the contribution of β forms, as well as α -helical and random structures, to protein conformation in solution could be considered. This made it possible to analyze protein ORD²⁰ and CD²¹ spectra for mixtures of these three structures by using the synthetic polypeptide, poly-L-lysine, as a model. These methods seem to be a useful approximation for the conformation of highly structured proteins. However, unresolved problems in interpretation arise from the choice of model structures, the contribution of optically active nonpeptide chromophores, and the effects of light scattering.

Thus, although excellent CD and ORD data on proteins can now be obtained, the interpretation of these data must be approached with caution. The purpose of this article is to bring up to date the methodology of CD and ORD data gathering and analysis in the rapidly changing field of protein structure, making use of synthetic polypeptide studies when necessary. A systematic review of the theory of optical activity²²⁻³⁴ or the history of rotational analysis of proteins and other bipolymers will not be attempted, as these can be found elsewhere.^{10,26,30,32,33,35-46} Nor will the structure of collagen and polyproline,^{37(b,d)47-49} proteins with prosthetic groups like heme, or the new fields of infrared ORD⁵⁰ and magnetic CD⁵¹⁻⁵³ be discussed.

¹⁶ B. Davidson, N. M. Tooney, and G. D. Fasman, *Biochem. Biophys. Res. Commun.* **23**, 156 (1966).

¹⁷ R. Townend, T. F. Kumosinski, S. N. Timasheff, G. D. Fasman, and B. Davidson, *Biochem. Biophys. Res. Commun.* **23**, 163 (1966).

¹⁸ P. Sarkar and P. Doty, *Proc. Nat. Acad. Sci. U.S.* **55**, 981 (1966).

¹⁹ B. Davidson and G. D. Fasman, *Biochemistry* **6**, 1616 (1967).

²⁰ N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967).

²¹ N. J. Greenfield and G. D. Fasman, *Biochemistry* **8**, 4108 (1969).

²² T. M. Lowry, "Optical Rotatory Power." Longmans, Green, New York, 1935.

²³ E. U. Condon, *Rev. Mod. Phys.* **9**, 432 (1937).

²⁴ W. Kauzmann, J. E. Walter, and H. Eyring, *Chem. Rev.* **26**, 339 (1940).

²⁵ W. Kuhn, *Annu. Rev. Phys. Chem.* **9**, 417 (1958).

²⁶ C. Djerassi, "Optical Rotatory Dispersion." McGraw-Hill, New York, 1960; (a) Chapter by A. Moscowitz, p. 150.

²⁷ I. Tinoco, Jr., *Advan. Chem. Phys.* **4**, 67 (1962).

²⁸ S. F. Mason, *Quart. Rev.* **17**, 20 (1963).

The Phenomena of CD and ORD

A beam of linearly polarized light of wavelength λ can be considered as the sum of two components: beams of right- and left-circularly polarized light, with electric vectors E_R and E_L , respectively. When such light interacts with an asymmetric molecule (such as most biological macromolecules) two phenomena, CD and ORD, are observed, and the molecule

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- ²⁹ L. Velluz, M. Legrand, and M. Grossjean, "Optical Circular Dichroism." Academic Press, New York, 1965.
- ³⁰ N. J. Greenfield and G. D. Fasman, in "Encyclopedia of Polymer Science and Technology." Vol. 15, p. 410. Wiley, New York, 1971.
- ³¹ W. F. H. M. Mommaerts, see Vol. 12B, p. 302.
- ³² D. W. Urry, *Annu. Rev. Phys. Chem.* **19**, 477 (1968).
- ³³ S. Beychok, *Science* **154**, 1288 (1966).
- ³⁴ H. Eyring, H.-C. Liu, and D. Caldwell, *Chem. Rev.* **68**, 525 (1968).
- ³⁵ S. Beychok, *Annu. Rev. Biochem.* **37**, 437 (1968).
- ³⁶ W. B. Gratzer and D. A. Cowburn, *Nature (London)* **222**, 426 (1969).
- ³⁷ G. D. Fasman, ed., "Poly- α -Amino Acids: Protein Models for Conformational Studies." Marcel Dekker, New York, 1967. See in particular, chapters by (a) W. B. Gratzer, p. 177; (b) A. Elliot, p. 1; (c) S. Beychok, p. 293; (d) L. Mandelkern, p. 675.
- ³⁸ P. J. Urnes and P. Doty, *Advan. Protein Chem.* **16**, 401 (1961).
- ³⁹ J. A. Schellman and C. G. Schellman, in "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. 2. Academic Press, New York, 1964.
- ⁴⁰ S. N. Timasheff and M. J. Gorbunoff, *Annu. Rev. Biochem.* **36**, 13 (1967).
- ⁴¹ J. T. Yang, in "A Laboratory Manual of Analytical Methods of Protein Chemistry" (P. Alexander and H. L. Lundgreen, eds.), Vol. 5, p. 23. Pergamon, Oxford, 1969.
- ⁴² G. N. Ramachandran, "Conformation of Biopolymers." Academic Press, New York, 1967.
- ⁴³ C. W. Deutsche, D. A. Lightner, R. W. Woody, and A. Moscowitz, *Annu. Rev. Phys. Chem.* **20**, 407 (1969).
- ⁴⁴ I. Tinoco, Jr. and C. R. Cantor, in "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 18, p. 81. Wiley, New York, 1970.
- ⁴⁵ B. Jirgensons, "Optical Rotatory Dispersion of Proteins and Other Macromolecules." Springer-Verlag, Berlin and New York, 1969.
- ⁴⁶ I. Tinoco, Jr., in "Molecular Biophysics" (B. Pullman and M. Weissbluth, eds.), p. 269. Academic Press, New York, 1965.
- ⁴⁷ F. A. Bovey and F. P. Hood, *J. Amer. Chem. Soc.* **88**, 2326 (1966).
- ⁴⁸ F. A. Bovey and F. P. Hood, *Biopolymers* **5**, 325 (1967).
- ⁴⁹ W. L. Mattice and L. Mandelkern, *Biochemistry* **10**, 1926 (1971).
- ⁵⁰ Y. N. Chirgadze, S. Y. Venyaminov, and V. M. Lobachev, *Biopolymers* **10**, 809 (1971).
- ⁵¹ G. Barth, R. Records, E. Bunnenberg, C. Djerassi, and W. Voelter, *J. Amer. Chem. Soc.* **93**, 2545 (1971); E. Bayer, A. Bacher, P. Krauss, W. Voelter, G. Barth, E. Bunnenberg, and C. Djerassi, *Eur. J. Biochem.* **22**, 580 (1971).
- ⁵² D. D. Ulmer, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 1179 (1971).
- ⁵³ B. Holmquist, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 1179 (1971).

is said to be optically active. These phenomena arise from the following events:

1. E_R and E_L travel at different speeds through the molecule. This difference in refractive index leads to optical rotation, the rotation of the plane of polarization, measured in degrees of rotation, α_λ . ORD is the dependence of this rotation upon wavelength. In a region where the molecule does not absorb light, the rotation plotted against wavelength yields a plain curve. In the region of light absorption, however, the dispersion is anomalous. The rotation first increases sharply in one direction, falls to zero at the absorption maximum, and then rises sharply in the opposite direction. This anomalous dispersion is called a Cotton effect.

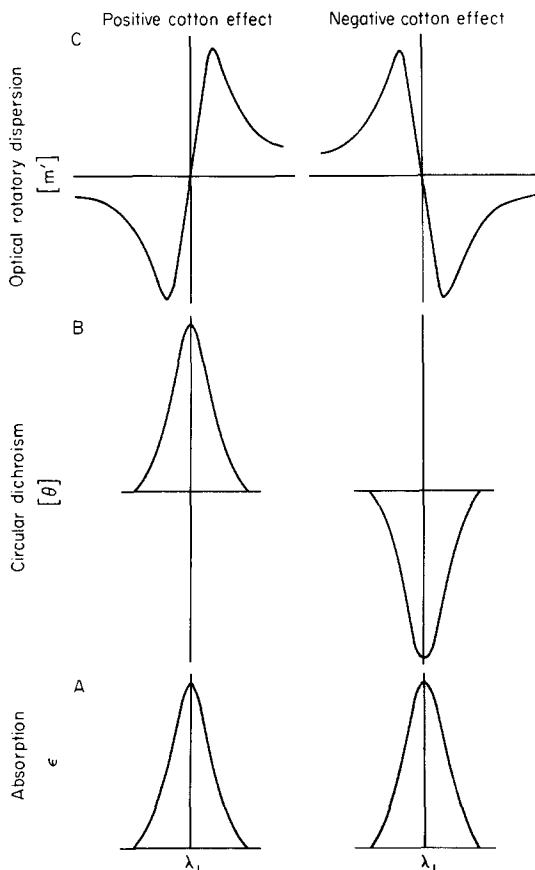


FIG. 1. A typical electronic absorption band (A) with its associated circular dichroism (B) and optical rotatory dispersion (C) curves.

2. In the region of its Cotton effect, an asymmetric molecule which exhibits ORD will also show unequal absorption of left- and right-handed circularly polarized light; this difference in extinction coefficient, $(\epsilon_L - \epsilon_R)$ is known as circular dichroism, and can be measured directly in some instruments as a differential absorbance. When CD occurs the emerging light beam is no longer linearly polarized, but instead is elliptically polarized. Thus, the ellipticity of the resulting light, θ_λ , is another measure of CD, and is proportional to $(\epsilon_L - \epsilon_R)$. A typical absorption band with its associated ORD and CD Cotton effects is shown in Fig. 1. Both dispersive and absorption phenomena are caused by the same charge displacements in a molecule; therefore, ORD and CD are closely related to each other. By means of the Kronig-Kramers transform equations developed by Moscovitz,^{26(a)} ORD curves can theoretically be computed from CD data and vice versa. This calculation is sometimes useful for evaluation of CD bands at very low wavelength.

Experimental Methods

General Considerations in Measurement

The practical considerations involved in recording CD or ORD data in a modern circular dichrometer or polarimeter are basically the same as those already indicated^{31,54} with respect to nucleic acids. Briefly, sufficient light must pass through the optically active sample and strike the photomultiplier to allow a meaningful rotation or CD measurement with tolerably high signal-to-noise ratio. This requirement becomes important to consider (1) below 225 nm (in the interesting spectral region of backbone peptide chromophores) where light sources begin to lose intensity, and where some light is absorbed by the optics and by solvents, and (2) in the region of aromatic side-chain absorption where molecular optical activity is relatively small. In such cases the parameters of protein concentration, cell path length, and solvent must be chosen with care. Under conditions of high absorbance and low CD or ORD signal, it is desirable to reduce path length and/or concentration.⁵⁴

Our previous article⁵⁴ in this series should be consulted for considerations affecting choice of spectral band width, scan speed, and instrumental time constant or pen period, and details affecting resolution and measurement, including tests for rotational artifacts. For tests of CD artifacts *d*-10-camphorsulfonic acid (or any compound or polymer displaying a CD band in the wavelength region of interest) should be used instead of sucrose, which is utilized in ORD. In a given instrument, the maximum allowable scan speed for CD and for ORD might not be

⁵⁴ A. J. Adler and G. D. Fasman, see Vol. 12B, p. 268.

identical. For example, in a Cary 60 spectropolarimeter with 6001 CD attachment, a time constant of 3 seconds in the CD mode corresponds to a pen period of 10 seconds in ORD operation, in that both would require a maximum scan speed of 4 nm per minute and a maximum photomultiplier dynode voltage of 500 V if the spectral band width is 1.5 nm (see Table I of the previous article⁵⁴).

Instruments

At present three commercial instruments, the Cary, the Jasco, and the Jouan, are capable of yielding high resolution CD and/or ORD spectra with relatively low noise levels down to about 185 nm, and are suitable for protein studies.

1. Cary 60 recording spectropolarimeter with 6001 CD accessory, and Cary 61 circular dichrometer.⁵⁵ The Cary instruments utilize a double-prism monochromator, a Faraday cell for rotational modulation of the light beam, Rochon prisms for polarizer and analyzer, and a Pockels cell as CD modulator and quarter-wave plate. A description of the polarimeter in the ORD mode has been given⁵⁴; operation and precision of the Cary 60 or 61 as a CD instrument are very similar. Data are reproducible to within 0.001° of rotation (ORD) or ellipticity (CD), and optical densities of ≤ 2 can usually be tolerated. The noise level is 0.0005° rotation or ellipticity under conditions of small absorbance at $\lambda > 220$ nm, and increases to about 0.005° for a protein sample of absorbance ~ 2 at 190 nm. Spectra are recorded on a continuous roll of chart paper.

2. Durrum-Jasco Model J-20 CD (ORD optional).⁵⁶ The Jasco J-10 (CD and spectrophotometer) and J-5 (ORD and spectrophotometer) instruments also are similar to the J-20 in specifications. All these instruments have been considerably improved over the Jasco Model 5,⁵⁴ and performance is now similar to that of the Cary 60/61. One improvement is that the baseline can now be multipotted. A possible disadvantage is the preprinted chart. The Jasco instruments differ from the Cary in not utilizing the Faraday effect, and in measuring CD directly in differential dichroic absorbance ($A_L - A_R$) rather than in ellipticity.

3. Roussel-Jouan Dichrograph CD 185 Model II.⁵⁷ This instrument is similar to the Jouan previously described,^{29,31} but has recently been

⁵⁵ Manufactured by Varian Instrument Division, 611 Hansen Way, Palo Alto, California 94303.

⁵⁶ Distributed by Durrum Instrument Corp., 3950 Fabian Way, Palo Alto California 94303.

⁵⁷ Manufactured by Jouan Société, 113, Bd. St. Germain, Paris 6^e; distributed in the United States by Bio-cal Instrument Co., 2400 Wright Ave., Richmond, California 94804.

modified to measure CD spectra, as $(A_L - A_R)$, down to 185 nm. It has not yet been extensively employed for proteins or polypeptides. However, data taken on Jouan instruments in some European laboratories⁵⁸ are of good quality with respect to noise level.

4. Bendix-Ericsson Polarimatic 62 Recording Spectropolarimeter.⁵⁹ The Bendix is capable of precise ORD measurement only at wavelengths above about 220 nm and is, therefore, not useful for peptide chromophores.

Calibration

The absolute accuracy of any quantitative measurement depends upon the standardization of the method. It is easy to check the calibration of ORD instruments: specific rotation values (at several wavelengths from 250 to 589 nm) of a 0.25% solution of National Bureau of Standards sucrose can be compared to literature values.⁶⁰ If necessary the polarimeter can be recalibrated.

However, there is at present no CD standard available with the consistent purity of N.B.S. sucrose. The compound commonly used for calibration of circular dichrometers is *d*-10-camphorsulfonic acid in 0.1% aqueous solution, which displays a large ellipticity band at 290 nm. But 10-camphorsulfonic acid forms a hydrate containing about 7% water under normal laboratory conditions,^{61,62} so that weight may not be an accurate measure of concentration. Furthermore, yellow impurities were found⁶³ in some batches of reagent grade (Eastman Kodak) 10-camphorsulfonic acid. The acid may be purified by recrystallization from acetic acid,⁶¹ followed by vacuum sublimation, drying at 80° under vacuum, and storage in a desiccator.⁶³ It is then suitable as a CD standard by means of which the signal gain adjustment controlling the magnitude of the observed CD signal on an instrument can be manipulated.

The exact value of the peak molecular ellipticity, $[\theta]_{290}$, of 10-camphorsulfonic acid is not known with certainty, partially because of impurity problems. Fortunately, the $[\theta]_{290}$ value can be calculated,^{61,62,64}

⁵⁸ See footnotes 219–221, 242, and 262 for articles giving examples on data taken on Jouan dichrographs.

⁵⁹ Manufactured by Bendix-Ericsson U.K. Ltd., and distributed in the U.S.A. by Bendix Corp., Cincinnati, Ohio.

⁶¹ E. Brand, E. Washburn, B. F. Erlanger, E. Ellenbogen, J. Daniel, F. Lippmann, and M. Scheu, *J. Amer. Chem. Soc.* **76**, 3037 (1954).

⁶² D. F. DeTar, *Anal. Chem.* **41**, 1406 (1969).

⁶³ W. C. Krueger and L. M. Pschigoda, *Anal. Chem.* **43**, 675 (1971).

⁶⁴ G. D. Fasman and P. Lituri, unpublished data.

⁶⁵ J. Y. Cassim and J. T. Yang, *Biochemistry* **8**, 1947 (1969).

by means of the Kronig-Kramers transform, from accurate ORD data on the same 10-camphorsulfonic acid sample obtained on a well-calibrated polarimeter. For this calculation to be valid the sample must not contain optically active impurities, although small amounts of water are tolerable. A simple way to obtain an absolute CD value for a rotationally pure aqueous solution of 10-camphorsulfonic acid standard is to use Cassim and Yang's⁶⁴ calculated ratios of peak molecular ellipticity to peak and trough molecular rotations: $[\theta]_{290}/[M]_{306} = 1.76$ and $[\theta]_{290}/[M]_{270} = -1.37$. For example, one dry, purified sample of 10-camphorsulfonic acid⁶³ yielded measured rotations of $[M]_{306} = +4480$ and $[M]_{270} = -5700$, from which $[\theta]_{290}$ equals the average of $4480 \times 1.76 = 7880$ and $-5700 \times -1.37 = 7800$, or $[\theta]_{290} = 7840$ (corresponding to $\Delta\epsilon_{L-R} = 2.37$). The resulting $[\theta]_{290}$ magnitude can then be used to calibrate the circular dichrometer, even though the 10-camphorsulfonic acid sample may contain some water. Note that the calibration setting recommended in the Cary 60 operating manual corresponds to $[\theta]_{290} = 7150$, and should not be used with 10-camphorsulfonic acid standards of unknown purity and dryness. The value of 7840 agrees well with that for another⁶² pure sample of 10-camphorsulfonic acid, but not with a third⁶¹ value, possibly because of different handling of the Kronig-Kramers relationships.

Solutions

The conditions of solvent, protein concentration and cell path length must be chosen so that the solution remains relatively transparent in the wavelength region of interest (optical density below 2 in most cases), but so that enough solute is present to register an easily measurable rotation or ellipticity with high signal-to-noise ratio.

The first consideration is to use a solvent which transmits sufficient light. Water is the usual solvent for protein and polypeptide spectral studies. Tris and acetate buffers, in moderate concentration, may be used at $\lambda \geq 200$ nm, but farther in the ultraviolet there is no buffer of suitably low absorbance. Fluoride and perchlorate salts may be employed down to the spectral limit of instrumentation to maintain desired ionic strengths, but most common salts, including chlorides, hydroxides, and phosphates, are to be avoided in the far ultraviolet.^{37(a)} Many organic solvents in which some proteins and polypeptides are soluble, such as dimethyl formamide and dimethyl sulfoxide, are not suitable for rotatory studies at $\lambda < 250$ nm unless cells of extremely thin path length are used.⁶⁵ There are a number of protein solvents of sufficient transparency,

⁶⁵ J. Engel, E. Liehl, and C. Sorg, *Eur. J. Biochem.* **21**, 22 (1971).

for example, trifluoroethanol, hexafluoroisopropanol, trimethyl phosphate, and methanesulfonic acid. Addition of any organic solvent, as well as any change of pH or temperature, may affect the protein's conformation, and must be used with caution.

The next consideration is that of sample concentration. For protein and polypeptide studies in the 185–240 nm spectral range, solution concentrations of 0.01–0.1%, with CD or ORD measured in cells of 0.1–1 mm path length, usually result in data showing a good compromise between sufficient signal and adequate light intensity. An absorbance of about 0.7 is usually optimal. Near-ultraviolet experiments ($\lambda > 240$ nm) usually require larger concentrations and/or longer cells. It may be necessary to vary the concentration and/or path length during the experiment in order to measure accurately each CD or ORD band of interest. In circumstances of low rotatory strength or low light intensity, or if aggregation is suspected, it is a good practice to take measurements at different concentrations (or path lengths), as a test for artifacts.

The solutions to be measured should be filtered⁶⁴ (Millipore filters⁶⁵ are useful) or centrifuged to remove suspended material.

The concentrations of samples must be known accurately in order to calculate molar or residue ellipticities or rotations. The measured weight of the sample is not a sufficient concentration determination, since most proteins and polypeptides retain some water even after drying under vacuum at moderate temperatures. Therefore, the concentration of the solution used for CD or ORD must be assayed (after filtration). For many proteins, accurate values for aromatic side-chain extinction coefficients are known^{67,68}; in these cases concentration can be obtained by a simple optical density determination. For all proteins and polypeptides, the Nessler micro-Kjeldahl analysis⁶⁹ for total nitrogen can be employed; however, in order to obtain concentration in terms of peptide residues per liter the amino acid composition must be known. In addition biuret,⁷⁰ Lowry,⁷¹ or ninhydrin⁷² colorimetric assays can be used, but for accurate results each method should be standardized with the protein to be measured.

⁶⁴ Manufactured by Millipore Filter Corp., Bedford, Massachusetts.

⁶⁵ D. M. Kirschenbaum, *Int. J. Protein Res.* **3**, 109, 157, 237 (1971).

⁶⁶ D. M. Kirschenbaum, in "The Handbook of Biochemistry," (H. Sober, ed.), 2nd ed. Chem. Rubber Publ. Co., Cleveland, Ohio, 1970.

⁶⁷ C. A. Lang, *Anal. Chem.* **30**, 1692 (1958).

⁶⁸ S. Zamenhof, see Vol. 3, p. 696.

⁶⁹ O. H. Lowry, N. J. Rosebrough, A. G. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

⁷⁰ S. Moore and W. H. Stein, *J. Biol. Chem.* **211**, 893 (1954).

Cells

Only fused quartz, circular cells should be used for ORD and CD. Such cells are available⁷³ in a great variety of path lengths and special adaptations (such as water jacketed), and should be tested for birefringence.⁵⁴ For path lengths of 1 mm or less, face-filling cells and double-necked cells are recommended because they are relatively easy to fill and clean. In any case, syringes fitted with thin Teflon tubing will be needed for filling thin cells. The exact path length of thin cells (1 mm or less) may be measured by counting interference fringes in the near infrared⁷⁴; this procedure is recommended for cells thinner than 1 mm.

Cell holders should be designed so that cells can be positioned reproducibly and firmly; V-blocks are not usually suitable. A cell holder assembly for ORD measurements in the Cary 60 has been described.⁵⁴ A very similar assembly can be machined for use in the Cary CD compartment. However, because of space limitations, the brass block with the groove (part A, Fig. 3, of reference cited in footnote 54) must be removable; it can be equipped with two holes on its underside which fit onto pins on a small brass plate permanently fixed to the floor of the sample compartment. Similar assemblies can be designed for other instruments.

Measurements

Most polarimeters and circular dichrometers now in use can be adjusted to yield a flat baseline (for a given solvent in a given cell). The previous discussion of ORD data collection⁵⁴ (the need to take frequent air blanks, etc.) applies also to CD. In CD measurements the sample signal should coincide with the solvent baseline in spectral regions where there is no absorption; if it does not, then the cell may not be properly positioned. (There is no such simple test for ORD data.)

Some instruments can be equipped with modified sample cell holders to permit direct measurement of difference ORD^{75,76} and difference CD.⁷⁶ These methods involve compensation for artifacts, but they may be useful for analysis of small changes in protein conformation, such as may occur upon binding of substrates, inhibitors, or coenzymes. (The same information can be obtained indirectly, for example, by subtraction of independently determined enzyme and inhibitor CD spectra from the

⁷³ Manufactured by Opticell Co., 10792 Tucker St., Beltsville, Maryland 20705.

⁷⁴ W. J. Potts, "Chemical Infrared Spectroscopy," Vol. I, p. 119. Wiley, New York, 1963.

⁷⁵ B. J. Adkins and J. T. Yang, *Biochemistry* **7**, 266 (1968).

⁷⁶ J. T. Yang and K. H. Chan, this volume [28].

measured CD of enzyme-inhibitor complex under the same conditions, e.g., see Simons⁷⁷).

Most instruments are thermostated so that measurements can be performed at a constant temperature (usually near 25°). Sample temperatures between about -20° to 100° can be obtained by means of circulating thermostat baths⁷⁸ connected to jacketed sample cells or to hollow cell holders. Electrical heating and cooling may also be employed.^{44,79} It is necessary to monitor the sample temperature during heating or cooling experiments.⁸⁰ At present extensive apparatus modification is necessary to achieve very low sample temperatures.⁸¹

Calculations

Treatment of Raw Data

The recorder chart tracing of any CD or ORD spectrum will contain significant noise, which must usually be averaged by eye. If the spectrum has been scanned slowly enough, this presents no problem. Alternatively, if digitizing accessories are available,⁴⁴ the ORD or CD curve (a single spectral scan) can be smoothed by computer. This technique improves data precision by reducing noise. In addition, a computer of average transients (CAT) can be utilized to increase resolution through repetitive scans.^{44,82,83} This method is particularly useful in cases of small rotational strength, for example, with L-tryptophan.⁸³

The noise-averaged baseline (for the same cell, solvent, and sensitivity range) then has to be subtracted from the sample spectrum at each wavelength of interest, and the chart difference multiplied by the instrumental sensitivity. The result for ORD is the observed rotation, α_{obs} , in degrees; for CD, depending upon the instrument, it is either the observed ellipticity, θ_{obs} , in degrees, or the differential circular dichroic absorbance,

⁷⁷ E. R. Simons, *Biochim. Biophys. Acta* **251**, 126 (1971).

⁷⁸ Circulating thermostat baths with built-in heating and cooling units, suitable for measurements in the -10 or -20 to 100° range, are manufactured by Tamson, Lauda, and Haake (among others). They are distributed, respectively, by Neslab Instruments (Durham, New Hampshire), Brinkmann Instruments (Westbury, New York), and Greiner Scientific (22 N. Moore St., New York, New York). Mixtures of ethylene glycol and water can be used as the circulating fluid.

⁷⁹ C. Formoso and I. Tinoco, Jr., *Biopolymers* **10**, 531 (1971).

⁸⁰ The plastic tip of a YSI thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) can be inserted into the cell jacket and plugged into a YSI Telethermometer. A small flat, YSI surface temperature probe may be clipped to the Pockels cell of a Cary 60 to monitor its temperature during a heating experiment.

⁸¹ E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry* **8**, 3205 (1969).

⁸² J. Horwitz, E. H. Strickland, and E. Kay, *Anal. Biochem.* **23**, 363 (1968).

⁸³ Y. B. Myer and L. H. MacDonald, *J. Amer. Chem. Soc.* **89**, 7142 (1967).

$(A_L - A_R)$, in absorbance units. The calculations which follow convert these data into quantitative measures of rotation or CD, and are amenable to computerization with most desk-top calculators.

Optical Rotatory Dispersion

Optical activity is usually reported in terms of molar rotation $[M]$ (for low molecular weight substances such as amino acid derivatives) and of residue rotation $[m]$ (for macromolecules). The residue rotation gives an indication of the optical activity of a single chromophoric peptide unit in a polypeptide or protein chain. The specific rotation at fixed wavelength, $[\alpha]_\lambda$, is of use mainly as a criterion of purity, for comparison to the older literature, and for calculation of Drude plots.

1. *Specific Rotation.* The optical activity is defined in terms of the specific rotation, $[\alpha]_\lambda$:

$$[\alpha]_\lambda^T = \frac{\alpha_{\text{obs}} \times 100}{l \times c}$$

where T = temperature; λ = wavelength; α_{obs} = observed rotation in degrees (solution minus solvent blank); l = path length of cell in decimeters; c = concentration in grams per 100 ml. For films, c = concentration in grams/cm², and l is unnecessary.

2. *Molar Rotation.* For low molecular weight substances $[M]_\lambda$ is defined as:

$$[M]_\lambda = \frac{\text{MW}}{100} [\alpha]_\lambda$$

where MW = molecular weight of the solute.

3. *Residue Rotation.* For comparison of rotations of proteins, oligopeptides and polypeptides, which differ greatly in molecular weights, a more meaningful unit, the mean residue rotation, is used:

$$[m] = \frac{\text{MRW}}{100} [\alpha]_\lambda$$

where MRW = mean residue weight of the repeating unit. The physical units for $[m]$ (as well as for $[M]$ and for these quantities reduced to vacuum) are degree centimeters² per decimole. The repeating molecular unit for proteins is the amino acid residue, so that the residue weight is the sum of the atomic weights in the unit—[C(=O)—CHR—NH]—. The MRW's of synthetic polypeptides and of proteins with known compositions can be calculated. The MRW for a large group of proteins is approximately 115, and this value can be used for comparative purposes when the exact amino acid composition is not known.

A convenient equation for calculation of $[m]$ when the molar residue concentration, c' (moles peptide residue/liter), is known is:

$$[m] = \frac{\alpha_{\text{obs}} \times 10}{l \times c'}$$

where the other symbols have been defined above.

4. *Reduced Molar and Residue Rotations.* The optical rotatory power is dependent upon the refractive index of the medium. Therefore, to compare observed rotations in a variety of solvents, the rotations are reduced to the value they would have in a vacuum, by means of the Lorentz correction factor, $3/(n^2 + 2)$, where n is the refractive index of the solvent at wavelength λ . Values of n and of $3/(n^2 + 2)$ for many solvents have been tabulated,^{10,54} and methods for measuring n have been given.⁵⁴ For example, $3/(n^2 + 2)$ values for water are 0.77 at 250 nm, 0.76 at 220 nm, and 0.74 at 195 nm. The reduced mean residue rotation, $[m']$, at wavelength λ incorporates the refractive index correction in the following manner:

$$[m'] = [m] \frac{3}{(n^2 + 2)} = \frac{\text{MRW}}{100} \times \frac{3}{(n^2 + 2)} [\alpha]_{\lambda}$$

A similar expression can be written for $[M']$, the reduced molar rotation, by substitution of molecular weight, MW, for residue weight.

5. *Drude and Moffitt Equations.* The most informative method of ORD data analysis in recent studies is to present dispersion plots ($[M']$ or $[m']$ vs. λ), and to obtain the characteristic parameters (positions and magnitudes) for the Cotton effects resulting from peptide and side-chain absorption in the ultraviolet. However, before ultraviolet ORD studies became feasible, much use was made of the Drude¹¹ and, especially, the Moffitt¹²⁻¹⁴ equations for protein ORD analysis. The application of these equations to protein studies, and the graphical methods employed for data reduction (including examples of calculation) have been extensively discussed.¹⁰ The Moffitt calculation is still performed occasionally.

Very briefly, the Drude equation, which describes the ORD in spectral regions far from optically active absorption bands, is:

$$[\alpha]_{\lambda} = \frac{k}{\lambda^2 - \lambda_c^2}$$

where k is a constant, and λ_c represents the mean wavelength of optically active electronic transitions.

The Moffitt equation, developed for synthetic polypeptides, is:

$$[m']_{\lambda} = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

where a_0 , b_0 , and λ_0 are constants. When λ_0 is taken to be 212 nm, b_0 is about -630 for polypeptides in a totally right-handed α -helical conformation and zero for random coils; thus b_0 for a protein can be used as a measure of its helical content, if it is devoid of any beta structure.

Circular Dichroism

Circular dichroism data are reported either as $[\theta]$, the molar or residue ellipticity, or as $(\epsilon_L - \epsilon_R)$, the differential molar circular dichroic extinction coefficient. The two measurements are proportional: $[\theta] = 3300(\epsilon_L - \epsilon_R)$. The rotational strength, R_K , of each optically active absorption band is sometimes calculated, provided that the experimental CD bands can be resolved; a du Pont 310 Curve Resolver⁸⁴ is useful for this purpose. There is no method of phenomenological CD analysis comparable to the Drude or Moffitt equations.

1. *Molar or Residue Ellipticity*, $[\theta]$. The molar ellipticity (for small molecules) or mean residue ellipticity (for proteins and polypeptides) is defined as:

$$[\theta]_\lambda = \frac{\theta_{\text{obs}} \times \text{MW (or MRW)}}{10 \times d \times c''}$$

where λ = wavelength; θ_{obs} = observed ellipticity, in degrees; MW = molecular weight; MRW = mean residue weight (see ORD); c'' = concentration in grams per milliliter; d = path length in centimeters. If the molar concentration of peptide residues, c' , is known directly, then $[\theta]$ may be calculated from:

$$[\theta] = \theta_{\text{obs}} \times \frac{10}{l \times c'}$$

where l = path length in decimeters c' = concentration in moles residue/liter. The units for $[\theta]$ are degrees per square centimeter per decimole.

2. *Reduced Molar or Residue Ellipticity*, $[\theta']$. The Lorentz refractive index correction (see ORD) is not usually applied to CD data. However, this correction is occasionally useful for literature comparisons:

$$[\theta'] = [\theta] \times \frac{3}{n^2 + 2}$$

3. *Differential Molar CD Extinction Coefficient*. In some instruments the difference in absorbance between left- and right-handed circularly polarized light, $(A_L - A_R)$ is measured directly. In such cases, the molar

⁸⁴ Manufactured by du Pont Instrument Products Division, Wilmington, Delaware 19898.

circular dichroic extinction coefficient (also called the molar dichroic absorption), $\epsilon_L - \epsilon_R$, is obtained from:

$$(\epsilon_L - \epsilon_R) = \frac{(A_L - A_R)}{c' \times d}$$

where d = path length in centimeters; c' = concentration in moles of residue per liter; $(\epsilon_L - \epsilon_R)$ has units of liters per mole centimeter.

4. *Relation between Ellipticity and Differential Absorption.* The proportionality, $[\theta] = 3300(\epsilon_L - \epsilon_R)$, has already been given. Another useful relationship, for comparison of raw CD data, is: $\theta_{\text{obs}} = 33(A_L - A_R)$. Thus, an observed ellipticity of 0.001 degree corresponds to an observed differential dichroic absorbance of 3×10^{-5} absorbance units.

5. *Rotational Strength.* The rotational strength R_K of the K th optically active absorption band is defined by an integral which can be found elsewhere.^{26(a),29,31} If the CD band is nearly Gaussian in shape, then:

$$R_K \approx 1.23 \times 10^{-42} [\theta_{\text{max}}]_K \frac{\Delta}{\lambda_{\text{max}}}$$

where λ_{max} = wavelength of the K th transition; $[\theta_{\text{max}}]_K = [\theta]$ at λ_{max} ; Δ = half-width of the band.

Relations between CD and ORD

CD and optical rotation are related, for the K th optically active transition (Cotton effect), by the Kronig-Kramers relations.^{85,86} By means of these integral transforms^{26(a),44} information contained in a complete ORD spectrum may be deduced, in principle, from a CD curve, and vice versa. Computer programs are available for calculation.^{61,62,64} Various methods of data manipulation can be compared in studies^{61,62,64} on *d*-10-camphorsulfonic acid. The transforms can be used to search for optically active transitions beyond the observable UV range.⁸⁷ A generalization useful for qualitative calculation of band magnitude is that, if a CD band is approximately Gaussian in shape then:

$$([M]_{\text{peak}} - [M]_{\text{trough}})_{\text{ORD}} \approx 1.2([\theta]_{\text{maximum}})_{\text{CD}}$$

Analysis of Data

The interpretation of protein CD and ORD spectra in terms of backbone conformation, side-chain interactions or active site geometry, is not a routine or straightforward matter, even with precise data collected for pure material under the best of experimental conditions. The fault

⁸⁵ R. de L. Kronig, *J. Opt. Soc. Amer.* **12**, 547 (1926).

⁸⁶ H. A. Kramers, *Atti Congr. Int. Fis. Como* **2**, 545 (1927).

⁸⁷ J. Y. Cassim and J. T. Yang, *Biopolymers* **9**, 1475 (1970).

lies in the methods of data analysis currently available; these are usually based upon synthetic polypeptides as models for protein structure, and cannot cope with the complexities of structure found in natural proteins. The model polypeptides can assume only three fundamental conformations in solution: α -helix, extended β structure, and random coil. Even so, the CD and ORD spectra for each standard conformational form vary with the polypeptide chosen (see Figs. 4-6 and 9-11). Furthermore, the unique tertiary structure of any protein in solution may contain distorted or extremely short segments of α or β chains (which have no counterpart in synthetic polypeptides), portions which are "random" in the sense that there is no regular, repeated structure (but which may have rotational properties very different from ionized poly-L-glutamic acid or poly-L-lysine), side-chain interactions which may contribute to the CD and ORD, and optically active disulfide and aromatic groups (which may cause rotational bands in the far- as well as the near-ultra-violet region). Additional complications in the CD and ORD of real protein systems may be caused by aggregation and light scattering. It is at present impossible to adequately calculate the rotational effects of all these complicating factors, although increasingly sophisticated and successful attempts are being made. A recent paper on insulin⁸⁸ demonstrates some of the problems involved.

Nevertheless, as shown below in the table, fair to excellent approximations to real protein structures determined by X-ray diffraction can be obtained from chiroptical data, especially if the protein contains large amounts of conventional secondary structure. It should be emphasized that CD and ORD techniques are now applied to proteins mainly to yield information on gross secondary structure; however, these methods are beginning to be used for study of individual peptide residues in proteins.⁸⁹⁻⁹² Considerable information concerning enzyme action can be deduced by measuring the small conformational changes that occur upon complex formation with inhibitors^{77,90-95} or coenzymes.^{96,97}

⁸⁸ M. J. Ettinger and S. N. Timasheff, *Biochemistry* **10**, 824 (1971).

⁸⁹ M. Z. Atassi, M. T. Perlstein, and A. F. S. A. Habeeb, *J. Biol. Chem.* **246**, 3291 (1971).

⁹⁰ V. I. Teichberg, C. M. Kay, and N. Sharon, *Eur. J. Biochem.* **16**, 55 (1970).

⁹¹ W. D. McCubbin, K. Oikawa, and C. M. Kay, *Biochem. Biophys. Res. Commun.* **43**, 666 (1971).

⁹² J. P. Halper, N. Latovitzki, H. Bernstein, and S. Beychok, *Proc. Nat. Acad. Sci. U.S.* **68**, 517 (1971).

⁹³ L. Fretto and E. H. Strickland, *Biochim. Biophys. Acta* **235**, 489 (1971).

⁹⁴ E. Breslow, *Proc. Nat. Acad. Sci. U.S.* **67**, 493 (1970).

⁹⁵ P. Cuatrecasas and C. A. Anfinsen, *Proc. Nat. Acad. Sci. U.S.* **64**, 923 (1969).

⁹⁶ D. D. Ulmer and B. L. Vallee, *Advan. Enzymol.* **27**, 37 (1965).

⁹⁷ R. Koberstein and H. Sund, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **19**, 149 (1971).

The use of ORD and CD for the evaluation of polypeptide and protein structure has been the subject of several extensive reviews.^{10,32-46} Since the 1950's great progress has been made in the elucidation of such structure, largely through X-ray diffraction analysis. Pauling and Corey⁹⁸ showed that polypeptide chains could assume only a limited number of stable structures, among them the α -helix (with 3.6 peptides per turn) and the interchain hydrogen-bonded β -structure (a fully extended parallel or antiparallel arrangement of peptide chains). These conformations were then found in various synthetic polypeptides by means of X-ray studies (see A. Elliot^{37(b)} for a review).

As the various conformations of polypeptides were elucidated they were correlated with the optical activity of the polymers, and the application of CD and ORD spectra to characterize polypeptide and protein structure was initiated. At first only ORD in the spectral region $\lambda > 250$ nm was available; much use was made of the Drude and (especially) the Moffitt equations. The early work has been reviewed.^{10,38} Then, as instrumentation improved, Cotton effects in the far-ultraviolet peptide region (185–240 nm) could be examined directly in both ORD and CD. The great majority of studies since about 1963 have utilized these Cotton effects; the near-ultraviolet region above 240 nm is now examined primarily for side-chain chromophore Cotton effects, not for backbone conformation.

Evaluation of Protein and Polypeptide Conformation from ORD in the Visible and Near-Ultraviolet Region

In 1955 Cohen suggested that the change in optical rotation that was noted upon protein denaturation may be due to a helix to random coil conformational transition.⁹⁹ The first measurements of optical activity of polypeptides and proteins were performed in the visible and near-ultraviolet regions ($\lambda > 250$ nm). Empirical equations were found to correlate ORD with polypeptide structure. The Drude equation (see calculations) could be satisfied for polypeptides of low helical content and for some proteins, but the ORD data of completely helical polypeptides and many other proteins^{12,13,100} could not be fitted. For polypeptides λ_c was found to be proportional to the percentage of helical content¹⁰⁰ up to 40% helix.

The Moffitt equation^{12,13} (see calculations) describes the rotation of helical polypeptides (such as un-ionized poly-L-glutamic acid) in the visible spectral region. The constant, b_0 , is proportional to backbone

⁹⁸ L. Pauling and R. B. Corey, *Proc. Nat. Acad. Sci. U.S.* **37**, 729 (1951); *ibid.* **39**, 247, 253 (1953).

⁹⁹ C. Cohen, *Nature (London)* **175**, 129 (1955).

¹⁰⁰ J. T. Yang and P. Doty, *J. Amer. Chem. Soc.* **79**, 761 (1957).

helical content and independent of side chain and environment, while a_0 is a function of side-chain and solvent. The ORD of many fully helical polypeptides yielded $b_0 = -630$, while fully random polypeptides gave a value of $b_0 = 0$. Polypeptides of mixed conformation gave a b_0 value which was proportional to helicity. Thus the value of b_0 was used as a measure of helical content, $b_0/-630 = \% \alpha$ -helix.^{14,101-103} However, the contribution of any β structure was ignored. The use of the Moffitt equation has been reviewed.^{10,38} Moffitt's equation was originally derived theoretically. However, it was later shown to have neglected some important terms.^{104,105} The equation, nonetheless, gave reasonable results for proteins and peptides whose helical content was then known, e.g., myoglobin.^{106,107}

Evaluation of Protein Structure from ORD in the Far Ultraviolet:

Use of Cotton Effects

In 1960 Simmons and Blout¹⁵ were the first to obtain ORD measurements in the absorption region of the polypeptide backbone. They detected the trough of a Cotton effect at 233 nm for tobacco mosaic virus. Following this, instrumentation rapidly improved and measurements of the entire ORD and CD spectra from 185 to 300 nm were soon achieved.

The first attempts to utilize ORD Cotton effects as a measure of helical content involved correlation of the magnitude of the 233 nm trough of proteins and polypeptides with helicity.¹⁰⁸ Later Yamaoka¹⁰⁹ and Blout and co-workers¹¹⁰⁻¹¹⁴ proposed a modified four-term Drude equation to account for the near ultraviolet and visible ORD. This Schechter-Blout equation is essentially a summation of the dispersion

¹⁰¹ C. Cohen and A. Szent-Györgyi, *J. Amer. Chem. Soc.* **79**, 248 (1957).

¹⁰² K. Imahori, E. Klemperer, and P. Doty, *Abstr. 131 Meet. Amer. Chem. Soc.*, Miami, April, 1957.

¹⁰³ J. T. Yang, *Tetrahedron* **13**, 143 (1961).

¹⁰⁴ W. Moffitt, D. D. Fitts, and J. G. Kirkwood, *Proc. Nat. Acad. Sci. U.S.* **43**, 723 (1957).

¹⁰⁵ J. A. Schellman and P. Oriel, *J. Chem. Phys.* **37**, 2114 (1962).

¹⁰⁶ S. Beychok and E. R. Blout, *J. Mol. Biol.* **3**, 769 (1961).

¹⁰⁷ P. J. Urnes, K. Imahori, and P. Doty, *Proc. Nat. Acad. Sci. U.S.* **47**, 1637 (1961).

¹⁰⁸ N. S. Simmons, C. Cohen, A. G. Szent-Györgyi, D. B. Wetlaufer, and E. R. Blout, *J. Amer. Chem. Soc.* **83**, 4766 (1961).

¹⁰⁹ K. K. Yamaoka, *Biopolymers* **2**, 219 (1964).

¹¹⁰ E. Shechter and E. R. Blout, *Proc. Nat. Acad. Sci. U.S.* **51**, 695 (1964).

¹¹¹ E. Shechter and E. R. Blout, *Proc. Nat. Acad. Sci. U.S.* **51**, 794 (1964).

¹¹² E. Shechter, J. P. Carver, and E. R. Blout, *Proc. Nat. Acad. Sci. U.S.* **51**, 1029 (1964).

¹¹³ J. P. Carver, E. Shechter, and E. R. Blout, *J. Amer. Chem. Soc.* **88**, 2550 (1966).

¹¹⁴ J. P. Carver, E. Shechter, and E. R. Blout, *J. Amer. Chem. Soc.* **88**, 2562 (1966).

of the α -helical and random coil forms of polypeptides, and attempts to account for the rotation due to the Cotton effect of each electronic transition of both polypeptide forms. The Shechter-Blout equation actually does not take into account all the terms present and combines many terms. Thus it is empirical, like the Moffitt equation, can be put into the same form as the Moffitt equation,¹¹⁵ and thus gives similar results for the estimation of helical content as the Moffitt equation.

The methods of helical content estimation considered so far involved measurements of optical rotation in the visible and near UV ($\lambda > 220$ nm) region. They generally ignored regular polypeptide conformations other than α -helix and random, although other conformations were known to exist in proteins. In particular, the β -form (that is the pleated sheet interchain hydrogen bonded form) was neglected. The β -form had not been studied extensively because of the difficulty of obtaining water-soluble model polypeptides in this conformation. Nevertheless, some attempts were made to include the β -form in the estimation of protein structure by means of the Moffitt equation.¹¹⁶⁻¹¹⁹ This extension was difficult because precise values for the contribution of the β structure to a_0 and b_0 were hard to determine due to uncertainties in the conformation of model polypeptides. Estimates of the Moffitt parameters for β polymers have resulted in a wide range of values for a_0 and b_0 .^{16,18,116,120-128}

In 1966 two groups¹⁸⁻¹⁹ obtained good ORD (and CD) measurements of the β form of poly-L-lysine in aqueous solution. (Similar data were obtained for the β form of silk fibroin in organic solvents.^{42,129}) ORD curves for the three fundamental conformations of poly-L-lysine (α -

¹¹⁵ J. T. Yang, *Proc. Nat. Acad. Sci. U.S.* **53**, 438 (1965).

¹¹⁶ A. Wada, M. Tsuboi, and E. Konishi, *J. Phys. Chem.* **65**, 1119 (1961).

¹¹⁷ J. A. Schellman and C. G. Schellman, *J. Polym. Sci.* **49**, 129 (1961).

¹¹⁸ G. V. Troitski, *Biofizika* **10**, 895 (1965).

¹¹⁹ S. N. Timasheff, R. Townend, and L. Mescanti, *J. Biol. Chem.* **241**, 1863 (1966).

¹²⁰ K. Imahori, *Biochim. Biophys. Acta* **37**, 336 (1960).

¹²¹ G. D. Fasman and E. R. Blout, *J. Amer. Chem. Soc.* **82**, 2262 (1960).

¹²² E. M. Bradbury, A. Elliott, and W. E. Hanby, *J. Mol. Biol.* **5**, 487 (1962).

¹²³ B. S. Farrap and I. W. Stapleton, *Biochim. Biophys. Acta* **75**, 31 (1963).

¹²⁴ S. Ikeda, H. Maeda, and T. Isemura, *J. Mol. Biol.* **10**, 223 (1964).

¹²⁵ K. Imahori and J. Yahara, *Biopolym. Symp.* **1**, 421 (1964).

¹²⁶ E. V. Anufrieva, I. A. Bolotina, B. Z. Volchek, N. G. Illarionova, V. I. Kalikhevich, O. Z. Korotkina, Y. V. Mitin, O. B. Ptitsyn, A. V. Purkina, and V. E. Eskin, *Biofizika* **10**, 918 (1965).

¹²⁷ E. V. Anufrieva, N. G. Illarionova, V. I. Kalikhevich, O. Z. Korotkina, Y. V. Mitin, O. B. Ptitsyn, A. V. Purkina, and B. Z. Volchek, *Biofizika* **10**, 346 (1965).

¹²⁸ L. Velluz and M. Legrand, *Angew. Chem. Int. Ed. Engl.* **4**, 838 (1965).

¹²⁹ E. Iizuka and J. T. Yang, *Biochemistry* **7**, 2218 (1968).

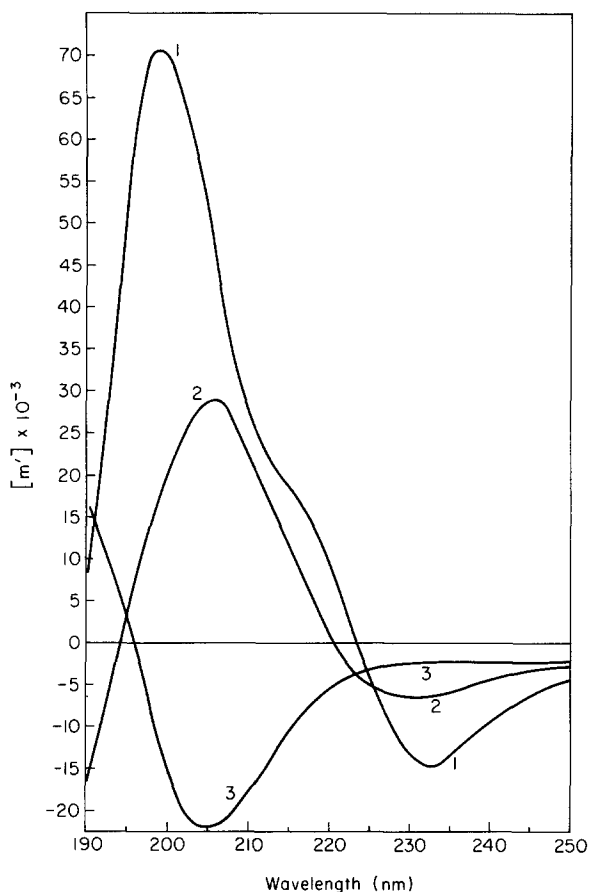


FIG. 2. The optical rotatory dispersion of poly-L-lysine in the α -helical (curve 1), β (curve 2), and random (curve 3) conformations. [From N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967).]

helix, β , and random) are shown in Fig. 2. At this point in time, realistic calculations of protein conformation, based upon polypeptide ORD Cotton effects, could be attempted. Greenfield *et al.*²⁰ used these data for poly-L-lysine to calculate ORD curves expected for various mixtures of the three basic conformations, and compared these calculated curves with measured ORD spectra for several proteins. (The proteins chosen for this study had conformations determined by X-ray diffraction.) The ORD comparison for myoglobin is shown in Fig. 3. The estimation of conformation for both myoglobin and lysozyme tended to overestimate the amount of β structure and to underestimate the amount of α -helix and random coil as found by X-ray diffraction studies. The

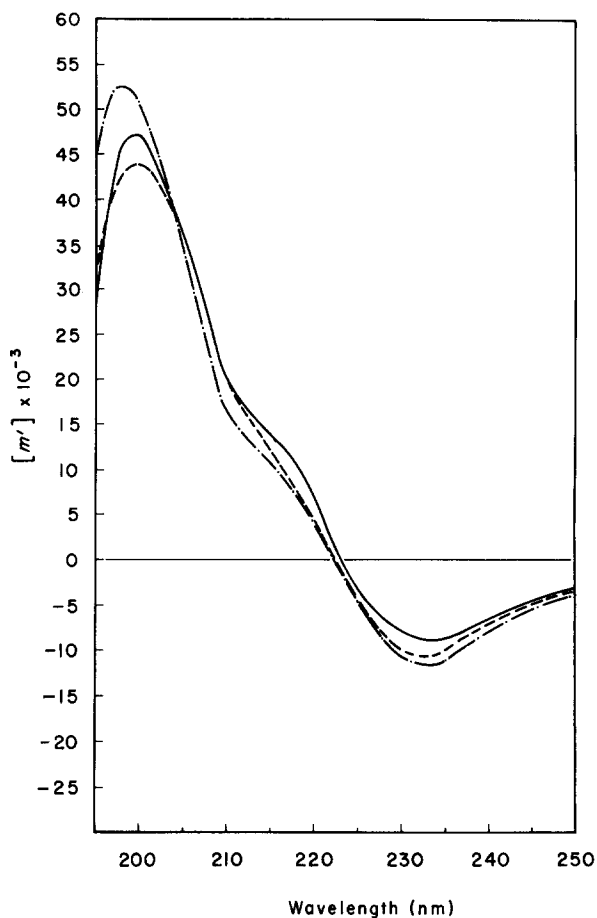


FIG. 3. The measured optical rotatory dispersion of sperm whale myoglobin (—) [from S. C. Harrison and E. R. Blout, *J. Biol. Chem.* **240**, 294 (1965)] compared with the calculated optical rotatory dispersion for 77% α -helix and 23% random coil (-·-·-) and for 54% α -helix, 36% β structure, and 10% random coil (-----) [from N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967)].

differences between the calculated and X-ray determined structures were attributed to aromatic side chain chromophores, disulfide bridge contributions, prosthetic groups contributions, and possible contributions from conformations of the amide groups other than those in the three reference conformations. It can also be seen from Figs. 4-6 that the absolute rotatory values for the three reference conformations are in doubt, since they vary with polypeptide side chain and solvent, adding further

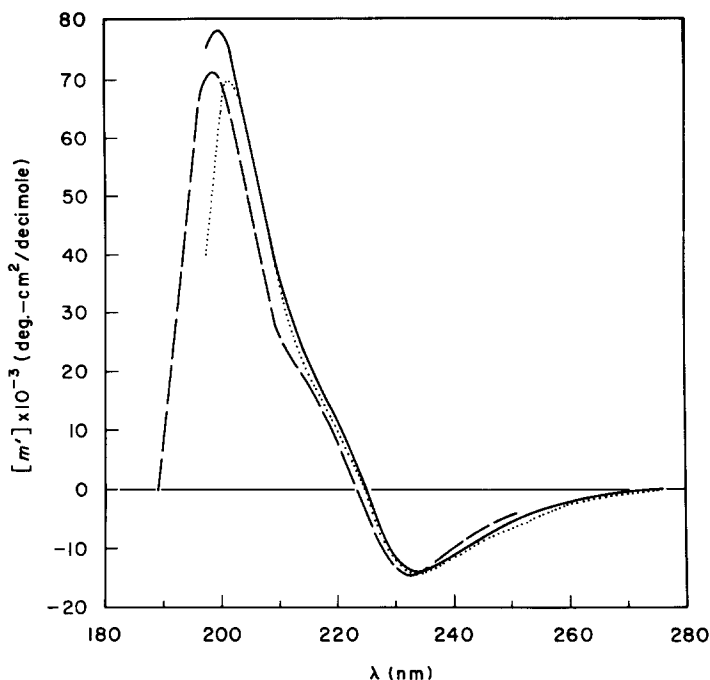


FIG. 4. The optical rotatory dispersion of the α -helix in various polypeptides: Poly-L-glutamic acid in water:dioxane, 1:1, — [from E. Iizuka and J. T. Yang, *Biochemistry* **4**, 1249 (1965)]. Poly-L-lysine in water, pH 11.0, - - - - - [from N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967)]. Poly-[N^{ϵ} -(2-hydroxyethyl)-L-glutamine] in methanol:water, 8:2, · · · · · [from A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.* **90**, 4736 (1968).]

difficulty in estimating protein conformation. Magar¹³⁰ performed similar calculations using a more precise method of minimizing the variance between the calculated and experimental ORD curves, but he reached essentially the same conclusions as Greenfield *et al.*²⁰

Evaluation of Protein Conformation from CD Measurements in the Ultraviolet

The preceding methods of estimating protein structure all used optical rotatory dispersion because instrumentation was not available to study circular dichroism in the far ultraviolet. The first measurements of the circular dichroism of polypeptides were made by Holzwarth

¹³⁰ M. E. Magar, *Biochemistry* **7**, 617 (1968).

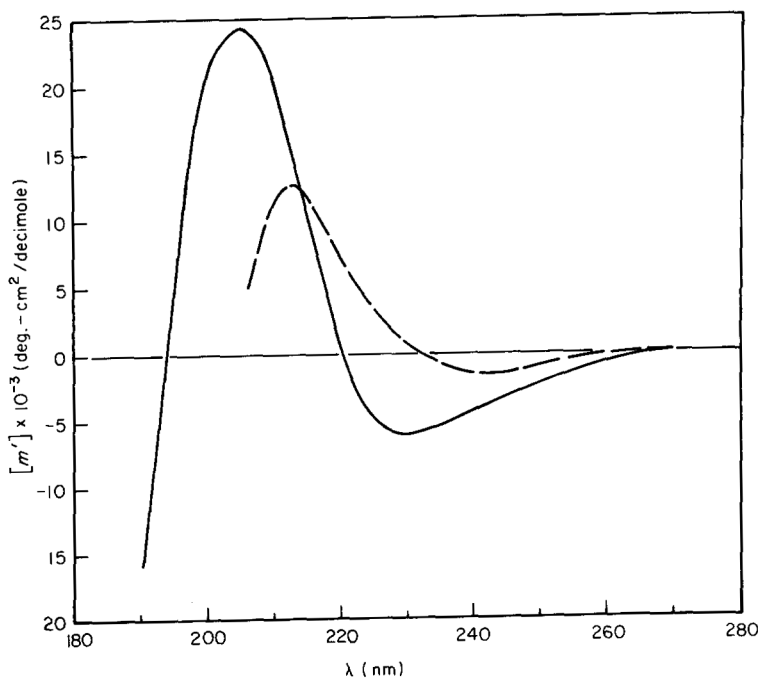


FIG. 5. The optical rotatory dispersion of the β form of various polypeptides: Poly-L-lysine in water, pH 11, — [from N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967)]. Poly-S-carboxymethyl-L-cysteine in water, pH 4.25, - - - - [from S. Ikeda and G. D. Fasman, *J. Mol. Biol.* **30**, 491 (1967)].

et al.,^{131,132} who examined the Cotton effects associated with peptide electronic transitions in α -helical and randomly coiled synthetic polypeptides and in myoglobin (which is largely helical). For helical polymers they assigned the negative CD band at 222 nm to the $n \rightarrow \pi^*$ amide transition, and the negative 208-nm band and positive 190-nm band to the parallel and perpendicularly polarized, respectively, $\pi \rightarrow \pi^*$ amide transitions. (This exciton splitting had been predicted by Moffitt.^{12,13}) They also made tentative assignments of the random coil CD bands, and showed (by means of the Kronig-Kramers transform) that the ORD helical and random-coil spectra were consistent with the CD bands.

After this major breakthrough, improved CD instruments became

¹³¹ G. M. Holzwarth, W. B. Gratzer, and P. Doty, *J. Amer. Chem. Soc.*, **84**, 3194 (1962).

¹³² G. M. Holzwarth and P. Doty, *J. Amer. Chem. Soc.* **87**, 218 (1965).

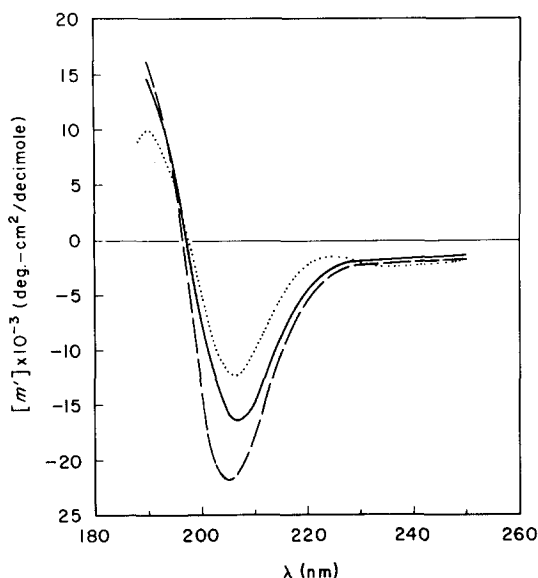


FIG. 6. The optical rotatory dispersion of the random coil form of various polypeptides: Poly-L-glutamic acid in water, pH 7.3, — [from E. Iizuka and J. T. Yang, *Biochemistry* **4**, 1249 (1965)]. Poly-L-lysine in water, pH 4.7–5.0, ---- [from N. J. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967)]. Poly-[*N*²-(2-hydroxyethyl)-L-glutamine] in water, ····· [from A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.* **90**, 4736 (1968)].

widely available, CD spectra for β -form poly-L-lysine were measured,^{16–19} and it became feasible to interpret CD data on proteins in terms of polypeptide conformations, as was done previously for ORD.²⁰ CD is now being used extensively to study the conformation of polypeptides and proteins in solution. Circular dichroism has an advantage over ORD in that there is less overlap between optically active transitions, and usually one can separate transitions due to the polypeptide backbone from other chromophores.

CD spectra for the three reference conformations of poly-L-lysine (α -helix, β -form, and random coil) are shown in Fig. 7. (Note that the β structure exhibits only one negative CD band in the 220-nm region.) Greenfield and Fasman²¹ utilized these curves for poly-L-lysine to calculate CD spectra expected for various mixtures of the three fundamental conformations. They used these calculated CD curves (in a manner analogous to their ORD study²⁰) to compare with experimental CD spectra of several proteins of known conformation (from X-ray studies). The results of this analysis, based on data from 208 to 250 nm,

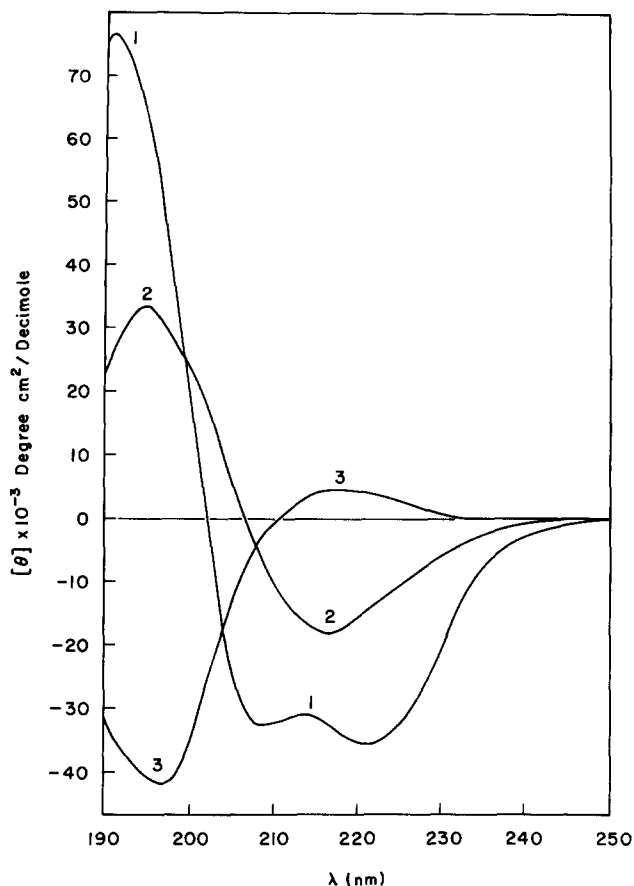


Fig. 7. Circular dichroism spectra of poly-L-lysine in the 100% α -helical (curve 1), β (curve 2), and random coil (curve 3) conformations. [From N. J. Greenfield and G. D. Fasman, *Biochemistry* 8, 4108 (1969).]

are shown in Fig. 8 and in the table. Greenfield and Fasman²¹ found, essentially, that when a protein is highly ordered, with either α -helix or β structure predominating, the results are within 5% of the X-ray data. Thus the estimates obtained from myoglobin, lysozyme, and RNase are quite good. When the proteins studied lacked a large amount of regularity, the deviations were much larger. In the latter case the fit of the estimated structure to the actual conformation obtained was not as good; however, these results did give an approximate idea of the secondary structure of the proteins and were more informative than all previous ORD methods of estimating protein conformation based on such param-

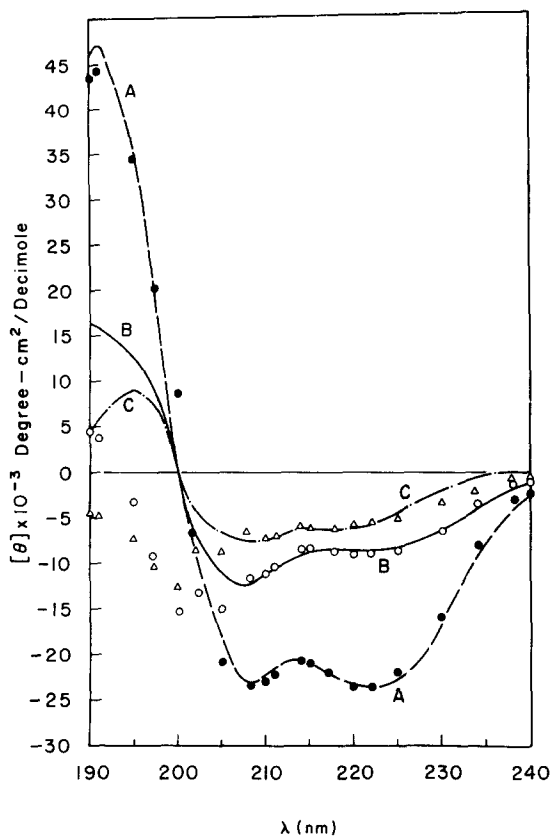


FIG. 8. The circular dichroism of (A) myoglobin (experimental curve) and 68.3% α -helix, 4.7% β structure, and 27.0% random coil, calculated from poly-L-lysine reference spectra in water (●●●●●). (B) Lysozyme (curve) and 28.5% α -helix, 11.1% β structure, and 60.4% random coil (○○○), calculated as in part A. (C) RNase (curve) and 9.3% α -helix, 32.6% β structure, and 58.1% random coil, calculated as for A ($\Delta \Delta \Delta$). [From N. J. Greenfield and G. D. Fasman, *Biochemistry* 8, 4108 (1969).]

eters as the Moffitt equation, the Shechter-Blout equation or the magnitude of the 233 nm trough of the ORD Cotton effect.

Refinements of the Analysis of Cotton Effects

As in the case of the ORD analysis,²⁰ the differences between calculated and experimental protein CD curves²¹ can be attributed largely to the imperfect nature of synthetic polypeptides as CD standards for protein chain conformations. The problems involved will be discussed in detail later. It can be mentioned here that (1) CD band parameters for α , β , and random-coil polypeptides vary with side chain and solvent (Figs. 9-11),

COMPARISON OF CONFORMATION OBTAINED BY CIRCULAR DICHROISM (CD) AND X-RAY^{a,b}

Protein	X-Ray structure: fraction			Refer- ence	CD calculated structure: fraction ^c							
	α	β	Random coil		α			β			Random coil	
					b	c		b	c		b	c
Myoglobin	65-72, 77 ^d	0	32-23	^h	68.3	68.2		4.7	7.9	27.0	23.9	
Lysozyme	28-42 ^d	10	62-48	^{i, j}	28.5	29.9		11.1	9.3	60.4	61.0	
RNase	6-18 ^d	36	58-46	^k	9.3	12.0		32.6	43.4	58.1	44.5	
RNase S	15 ^e	31	54	^l								
Carboxypeptidase A	23-30 ^d	18	59-52	^m	13.0	15.9		30.6	39.9	56.4	44.2	
α -Chymotrypsin ^f	3 ^g	—	—	ⁿ	11.8	13.4		22.8	31.9	65.5	54.8	
Chymotrypsinogen					13.8	17.3		25.2	28.9	60.9	53.8	

^a From N. J. Greenfield and G. D. Fasman, *Biochemistry* **8**, 4108 (1969).^b The best fit to the experimental CD curve was found by minimizing the variance between the experimental CD curve and a linear combination of CD curves for the α -helix, β structure, and random coil from 208 to 240 nm.^c Columns b: Values used in calculations are for poly-L-lysine in H₂O. Columns c: Values used in calculations same as for columns b with data for β -poly-L-lysine in SDS substituted for β poly-L-lysine in H₂O.^d Lower value represents true regular α -helix; upper value, total helix including 3_{10} and distorted helices.^e Helical type not distinguished by authors.^f α -Tosyl chymotrypsin used for X-ray work.^g Only one short-chain section α -helix included; isolated helical turns and β structure not reported, although they may be present.^h J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Shore, *Nature (London)* **185**, 422 (1960).ⁱ C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature (London)* **206**, 757 (1965).^j D. C. Phillips, *Sci. Amer.* **215**, 78 (1966); D. C. Phillips, *Proc. Nat. Acad. Sci. U.S.* **57**, 484 (1967).^k G. Kartha, J. Bello, and D. Harker, *Nature (London)* **213**, 862 (1967).^l H. W. Wyckoff, K. D. Hardman, N. M. Allenwell, T. Inagami, L. N. Johnson, and M. Richards, *J. Biol. Chem.* **242**, 3984 (1967).^m G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, J. A. Steitz, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.* **58**, 2220 (1967).ⁿ P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.* **35**, 143 (1968).

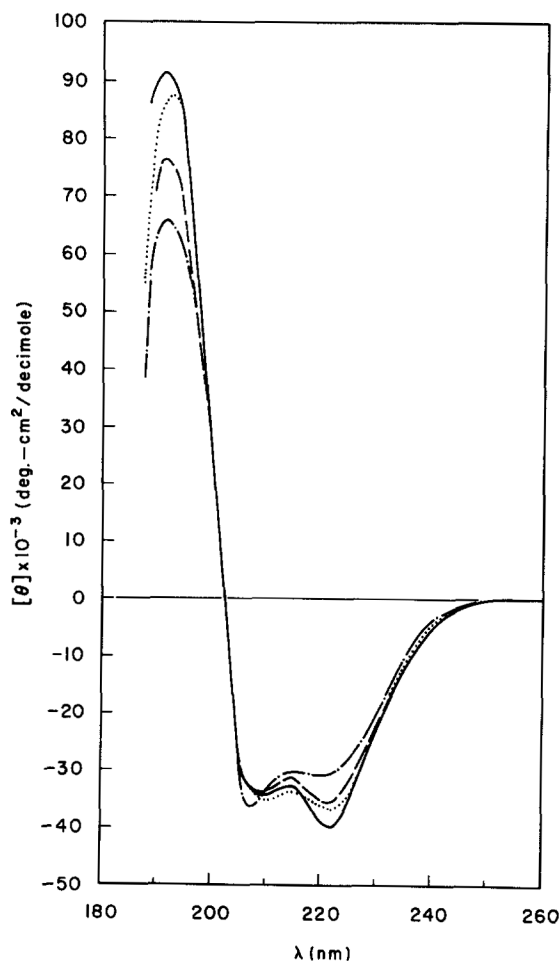


FIG. 9. The circular dichroism of the α -helix in various polypeptides: Poly-L-glutamic acid in water, pH 4.4— [from G. M. Holzwarth and P. Doty, *J. Amer. Chem. Soc.* **87**, 218 (1965)]. Poly-L-lysine in water, pH 11.0, - - - - - [from R. Townend, T. F. Kumosinski, S. N. Timasheff, G. D. Fasman, and B. Davidson, *Biochem. Biophys. Res. Commun.* **23**, 163 (1966)]. Poly-[N^5 -(2-hydroxyethyl)-L-glutamine] in methanol:water, 8:2, \cdots (G. D. Fasman, unpublished data). Poly-L-alanine in trifluoroethanol:trifluoroacetic acid, 98.5:1.5, - · - · - [from F. Quadrioglio and D. W. Urry, *J. Amer. Chem. Soc.* **90**, 2755 (1968).]

(2) CD curves of unordered proteins bear only a qualitative relation to those for random-coil poly-L-lysine or poly-L-glutamic acid, (3) other regular conformations (such as 3_{10} -helix) and very short or distorted segments of the three standard forms undoubtedly contribute to protein CD in a different manner, and (4) optically active aromatic, cystine disulfide,

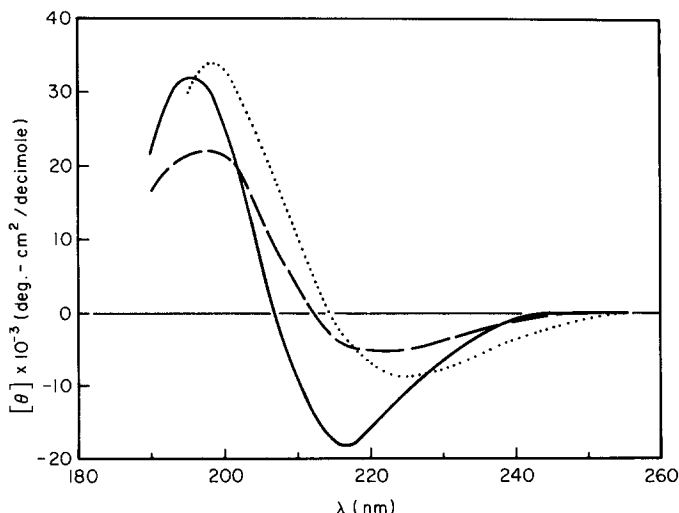


FIG. 10. The circular dichroism of the β form of various polypeptides: Poly-L-lysine in water, pH 11, — [from N. J. Greenfield and G. D. Fasman, *Biochemistry* **8**, 4108 (1969)]. Poly-L-serine in water, ---- [from F. Quadrifoglio and D. W. Urry, *J. Amer. Chem. Soc.* **90**, 2760 (1968)]. Poly-S-carboxymethyl-L-cysteine in water, pH 4.3, (G. D. Fasman, unpublished data).

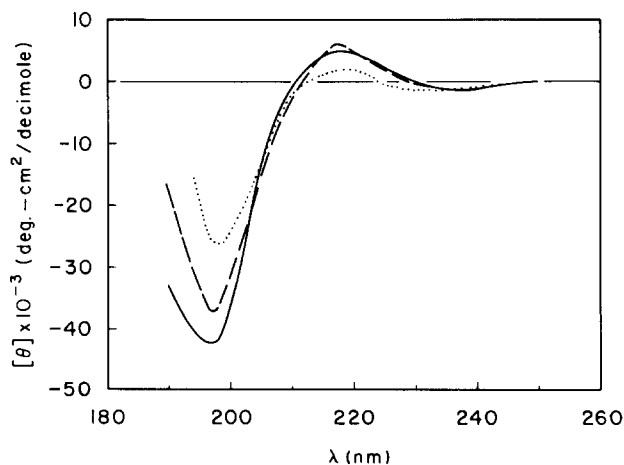


FIG. 11. The circular dichroism of the random coil form of various polypeptides: Poly-L-glutamic acid in water pH 7.5, — [from A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.* **90**, 4736 (1968)]. Poly-L-lysine in water, pH 5.7, ---- [from N. J. Greenfield and G. D. Fasman, *Biochemistry* **8**, 4108 (1969)]. Poly-[N^5 -(2-hydroxyethyl)-L-glutamine] in water, [from A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.* **90**, 4736 (1968)].

and prosthetic group chromophores may well influence protein CD spectra in the far ultraviolet. Although many data have been collected on these subjects, there has been little attempt as yet to apply this mass of information to protein studies.

Several recent papers represent variations and refinements of the methods of CD²¹ and ORD²⁰ analysis presented above. Straus *et al.*¹³³ made similar calculations of percentage of α , β , and random forms for several proteins, utilizing various polypeptides (not only poly-L-lysine) as standards, and allowing CD band parameters to vary for the calculation. Myer¹³⁴ utilized isodichroic points at 198, 204, and 208 nm for poly-L-lysine in its various conformations for analysis of CD spectra of myoglobin, lysozyme, RNase, and cytochrome *c*. Rosenkranz and Scholtan¹³⁵ used poly-L-serine at high salt concentration as their reference for the unordered form and calculated CD curves for myoglobin, lysozyme, and RNase. They obtained a better fit to the experimental data at $\lambda = 195\text{--}208$ nm; but a worse fit at $\lambda > 208$ nm than did Greenfield and Fasman.²¹

Saxena and Wetlaufer¹³⁶ avoided the problems inherent in polypeptide standards; they used native proteins of known conformation to obtain reference CD curves for α , β , and unordered conformations. These standard spectra, obtained from crystallographic and CD data for myoglobin, RNase, and lysozyme [and considerably different from those for poly-L-lysine (Fig. 7), especially in the random form], were then used for computation of CD curves for other proteins. Saxena and Wetlaufer¹³⁶ obtained very good agreement (better than did Greenfield and Fasman²¹) for carboxypeptidase, but only a fair fit for α -chymotrypsin. Thus, even total avoidance of polypeptide standards does not ensure a perfect method of CD conformational analysis of proteins. Each protein probably contains regions of unique structure which cannot be analyzed by any set of α , β , and random standard curves; furthermore, side-chain chromophore CD contributions are likely to be different for each protein. However, this type of CD interpretation,¹³⁶ based on protein data, is a step in the right direction. Chen and Yang¹³⁷ made use of crystallographically determined proteins to obtain b_0 , $[m]_{233}$ and $[\theta]_{222}$ parameters for α -helix and random-coil forms. An additional study bearing on CD analysis¹³⁸ shows that ORD data in the visible and near UV region may

¹³³ J. H. Straus, A. S. Gordon, and D. F. H. Wallach, *Eur. J. Biochem.* **11**, 201 (1969).

¹³⁴ Y. P. Myer, *Res. Commun. Chem. Pathol. Pharm.* **1**, 607 (1970).

¹³⁵ H. Rosenkranz and W. Scholtan, *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 896 (1971).

¹³⁶ V. P. Saxena and D. B. Wetlaufer, *Proc. Nat. Acad. Sci. U.S.* **68**, 969 (1971).

¹³⁷ Y.-H. Chen and J. T. Yang, *Biochem. Biophys. Res. Commun.* **44**, 1285 (1971).

¹³⁸ S. Sugai, K. Nitta, and M. Ishikawa, *Biophysik* **7**, 8 (1970).

be used to calculate CD and ORD peptide Cotton effects for polypeptides. There are many investigations utilizing CD or ORD of polypeptides (for example, the references cited in footnotes 139–143) whose main concern is stability or thermodynamics of the polymers, and which will not be discussed here.

Critique of the Analysis: Polypeptide Backbone

Long-chain synthetic polypeptides are an imperfect choice as models for protein ORD and CD structure determination. Conformational reference parameters for α , β , and random forms vary with the polypeptide, and a variety of possible protein structures are excluded in these simple polymers. The use of synthetic polypeptides as standards for the protein backbone, and the effects of chain length and of aggregation will be discussed. This section will also include a consideration of light scattering (which is particularly applicable to membrane studies), and the final section will discuss aromatic and cystine side-chain chromophore contributions.

α -Helix

Long-chain acidic poly-L-glutamic acid and alkaline poly-L-lysine have long been used as the canonical models for the α -helix in proteins. Although the chiroptical spectra are qualitatively similar for polypeptides with different side chains in various solvents, as illustrated in Figs. 4 (ORD) and 9 (CD), the peak values vary considerably. For example, the CD data for α -helical poly-L-glutamic acid,¹³² poly-L-lysine,^{17,20} poly-[N⁵-(2-hydroxyethyl)-L-glutamine],¹⁴⁴ poly-L-alanine,¹⁴⁵ poly- γ -methyl-L-glutamate,^{87,146} poly-L-methionine,¹⁴⁶ and poly-L-homoserine¹⁴⁶ (in various solvents) are far from coincident. This change in α -helix parameters is not due only to solvent effects.^{87,146} Calculations¹⁴⁷ have predicted that nonaromatic side-chain effects will contribute to the rotatory strength of α -helical peptide bands, due to changes in the geom-

¹³⁹ H. A. Scheraga, *Chem. Rev.* **71**, 195 (1971).

¹⁴⁰ M. Goodman, A. S. Verdini, N. S. Choi, and Y. Masuda, in "Topics in Stereochemistry" (E. L. Eliel and N. L. Allinger, eds.), Vol. 5, p. 69. Wiley, New York, 1970.

¹⁴¹ G. Conio, E. Patrone, and S. Brighetti, *J. Biol. Chem.* **245**, 3335 (1970).

¹⁴² J. Steigman and A. Cosani, *Biopolymers* **10**, 357 (1971).

¹⁴³ D. Puett and A. Ciferri, *Biopolymers* **10**, 547 (1971).

¹⁴⁴ A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.* **90**, 4736 (1968).

¹⁴⁵ F. Quadrifoglio and D. W. Urry, *J. Amer. Chem. Soc.* **90**, 2755 (1968).

¹⁴⁶ J. R. Parrish and E. R. Blout, *Biopolymers* **10**, 1491 (1971).

¹⁴⁷ J. N. Vournakis, J. F. Yan, and H. A. Scheraga, *Biopolymers* **6**, 1531 (1968).

etry of the helical backbone. This change in rotatory strength has been shown experimentally with copolymers of L-leucine and L-lysine in aqueous solution,¹⁴⁸ for which $[\theta]_{208} = -34,000$ remains constant, but $[\theta]_{222}$ varies with the leucine content. Furthermore, the CD spectrum of the α -helix constructed from data on proteins¹³⁶ differs from those based on synthetic polypeptides. Aggregation is known to change the ORD of helical poly-L-glutamic acid,¹⁴⁹ and care should be taken to avoid comparable situations in proteins.

Another respect in which long-chain polypeptides may not be a good model is that of chain length. Segments of α -helix in proteins are short, ranging from 3 to about 20 peptide units. ORD data¹⁵⁰ show that $[m']_{233}$ values for oligo-L-lysines, even at $n = 22$, do not approach that for poly-L-lysine because of incomplete helix formation. No helical models for short peptide chains are available. Theoretical calculations indicate that the rotatory strength of the $\pi - \pi^*$ transition of the α -helix 208 and 191 nm CD bands should be greatly dependent upon chain length,^{151,152} but vary in predictions about the $n - \pi^*$ transition (222 nm CD band).^{147,153,154}

β -Forms

Poly-L-lysine at high pH, after heating and recooling, is usually used as a reference for the β form. However, it has been shown that the β form of poly-L-lysine produced at neutral pH with sodium dodecyl sulfate has slightly different ORD¹⁸ and CD¹⁵⁵ spectra than those found in water alone at pH 11. The magnitude of the CD ellipticity band at 218 nm for this β form has only one-half the magnitude of the β form produced by heating poly-L-lysine in water at pH 11, as found by Townend *et al.*¹⁷ and by Sarkar and Doty.¹⁸ Li and Spector¹⁵⁵ stated that β poly-L-lysine in water alone may form an intermolecular "infinite" pleated sheet and may not be a good model for the short sections of β structure found in proteins. Furthermore, depending upon the concentration and chain length, poly-L-lysine is able to form either inter- or intramolecular β pleated sheets (or, in some cases, mixtures).¹⁵⁶

¹⁴⁸ C. R. Snell and G. D. Fasman, *Biopolymers* **11**, 1723 (1972).

¹⁴⁹ J. Y. Cassim and J. T. Yang, *Biochem. Biophys. Res. Commun.* **26**, 58 (1967).

¹⁵⁰ A. Yaron, G. D. Fasman, E. Katchalski, H. A. Sober, and A. Berger, *Biopolymers* **10**, 1170 (1971).

¹⁵¹ R. W. Woody and I. Tinoco, Jr., *J. Chem. Phys.* **46**, 4927 (1967).

¹⁵² R. W. Woody, *J. Chem. Phys.* **49**, 4797 (1968).

¹⁵³ I. Tinoco, Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.* **38**, 1317 (1963).

¹⁵⁴ D. W. Urry, *Proc. Nat. Acad. Sci. U.S.* **60**, 394 (1968).

¹⁵⁵ L. K. Li and A. Spector, *J. Amer. Chem. Soc.* **91**, 220 (1969).

¹⁵⁶ S.-Y. C. Wooley and G. Holzwarth, *Biochemistry* **9**, 3604 (1970).

Different poly- α -amino acids in the β form display different CD (Fig. 10) and ORD (Fig. 5) spectra depending on side-chain and solvent.¹⁵⁷⁻¹⁶¹ For example, the CD curves for poly-L-lysine,²¹ poly-L-serine,¹⁵⁷ and poly-S-carboxyethyl-L-cysteine¹⁶¹ in aqueous solution bear little more than qualitative relationship to one another or to a β -form CD curve constructed for globular proteins.¹³⁶ Fasman and Potter¹⁵⁸ have examined films cast from several β -forming polypeptides, and have grouped their ORD spectra into two classes; these films have also shown two classes of CD spectra.¹⁶⁰ Poly-L-lysine (Figs. 5 and 10) and poly-L-valine are found in form I, while poly-L-serine (Fig. 10) is found in form II.^{158,160}

Another difficulty is that there are three types of β structure possible in polypeptides⁹⁸: intermolecular parallel and antiparallel hydrogen-bonded sheets, and intramolecular antiparallel cross- β structure. It has been impossible to assign CD or ORD spectra unequivocally to one of these forms or another; this problem has been discussed experimentally^{156,158,160}, and theoretically.¹⁶²⁻¹⁶⁴ An exception is poly-[L-al α -L-glu(OEt)-gly],¹⁶⁵ which appears to be in a cross- β conformation¹⁶⁶ and has the optical properties of form I- β .

Urry¹⁶⁷ calculate an extremely large theoretical chain-length dependence for the $n - \pi^*$ transition in antiparallel pleated β sheets. However, Goodman *et al.*¹⁶⁸ found that oligo-isoleucines, $n = 7$ and 8, in organic solvents had CD spectra similar to those of long-chain β polypeptides.

Random Coil

The use of synthetic polypeptides, usually fully charged poly-L-lysine·HCl, sodium poly-L-glutamate, as models for "unordered" seg-

¹⁵⁷ F. Quadrifoglio and D. W. Urry, *J. Amer. Chem. Soc.* **90**, 2760 (1968).

¹⁵⁸ G. D. Fasman and J. Potter, *Biochem. Biophys. Res. Commun.* **27**, 209 (1967).

¹⁵⁹ N. M. Tooney and G. D. Fasman, *J. Mol. Biol.* **36**, 355 (1968).

¹⁶⁰ L. Stevens, R. Townend, S. N. Timasheff, G. D. Fasman, and J. Potter, *Biochemistry* **7**, 3717 (1968).

¹⁶¹ H. Maeda and S. Ikeda, *Biopolymers* **10**, 1635 (1971).

¹⁶² E. S. Pysh, *Proc. Nat. Acad. Sci. U.S.* **56**, 825 (1966).

¹⁶³ K. Rosenheck and B. Sommer, *J. Chem. Phys.* **46**, 532 (1967).

¹⁶⁴ R. W. Woody, *Biopolymers* **8**, 669 (1969).

¹⁶⁵ J. M. Anderson, W. B. Rippon, and A. G. Walton, *Biochem. Biophys. Res. Commun.* **39**, 802 (1970).

¹⁶⁶ W. B. Rippon, J. M. Anderson, and A. G. Walton, *J. Mol. Biol.* **56**, 507 (1971); J. C. Andries and A. G. Walton, *J. Mol. Biol.* **56**, 515 (1971).

¹⁶⁷ D. W. Urry, *Proc. Nat. Acad. Sci. U.S.* **60**, 114 (1968).

¹⁶⁸ M. Goodman, F. Naider, and C. Toniolo, *Biopolymers* **10**, 1719 (1971).

ments of proteins (which have well defined although not regularly repeating asymmetric structures) is open to criticism. First, use of such polyelectrolytes has been questioned by Krimm and co-workers,¹⁶⁹⁻¹⁷² who stated that in water at low salt concentration charged sodium poly-L-glutamate forms an extended helical structure due to charge repulsion. (Such a structure exists in the solid state.¹⁷³) Because of this, the suggestion has been made that polypeptides (sodium poly-L-glutamate,¹⁷⁰ poly-L-proline,¹⁷⁰ or poly-L-serine¹³⁵) in very concentrated salt solutions may be better standards for unordered protein chains. However, this suggestion is disputed by Fasman *et al.*,¹⁷⁴ who cite work¹⁷⁵ showing that in high salt it is possible that PGA collapses into a compact structure which cannot be termed a true statistical coil. Moreover, it was found¹⁷⁶ that salt may interact with the peptide carbonyl groups and consequently may shift transition moments. There is evidence against any extended helical form for protonated poly-L-lysine at low salt concentration.¹⁷⁷ Furthermore, the random coil ORD (Fig. 6) and CD (Fig. 11) curves for uncharged poly-[N⁵-(2-hydroxyethyl)-L-glutamine]¹⁴⁴ in water and for several un-ionized polyamino acids in various solvents¹⁷⁸ are qualitatively similar to those of charged polypeptides at low ionic strength; all exhibit negative CD bands at about 198 nm and positive bands at about 218 nm, although the band magnitudes are greater for the polyelectrolytes. It may be mentioned here that strong sulfuric acid (sometimes employed to produce random coil forms) may protonate the peptide backbone.^{179,180} The origin of the small 238-nm CD band is uncertain,^{40,113,181} and the assignment of other bands has been theoretically discussed.¹⁸²

A second, more serious, criticism of the use of synthetic polypeptides

¹⁶⁹ M. L. Tiffany and S. Krimm, *Biopolymers* **6**, 1379 (1968).

¹⁷⁰ M. L. Tiffany and S. Krimm, *Biopolymers* **8**, 347 (1969).

¹⁷¹ S. Krimm, J. E. Mark, and M. L. Tiffany, *Biopolymers* **8**, 695 (1969).

¹⁷² S. Krimm and J. E. Mark, *Proc. Nat. Acad. Sci. U.S.* **60**, 1122 (1968).

¹⁷³ H. D. Keith, *Biopolymers* **10**, 1099 (1971).

¹⁷⁴ G. D. Fasman, H. Hoving, and S. N. Timasheff, *Biochemistry* **9**, 3316 (1970).

¹⁷⁵ E. Iizuka and J. T. Yang, *Biochemistry* **4**, 1249 (1965).

¹⁷⁶ J. Bello and H. R. Bello, *Nature (London)* **194**, 681 (1962).

¹⁷⁷ D. G. Dearborn and D. B. Wetlaufer, *Biochem. Biophys. Res. Commun.* **39**, 314 (1970).

¹⁷⁸ D. Balasubramanian and R. S. Roche, *Chem. Commun.* **1970**, 862 (1970).

¹⁷⁹ E. Peggion, A. Cosani, M. Terbojevich, and A. S. Verdini, *Macromolecules* **3**, 318 (1970).

¹⁸⁰ K. Rosenheck, in "Molecular Associations in Biology" (B. Pullman, ed.), p. 517. Academic Press (1968).

¹⁸¹ Y. P. Myer, *Macromolecules* **2**, 624 (1969).

¹⁸² D. Aebersold and E. S. Pysh, *J. Chem. Phys.* **53**, 2156 (1970).

as model random coils is that their CD patterns in solution are quite different from those of denatured (presumably unordered) proteins. These proteins, denatured by a variety of methods, may still have polypeptide chains subject to some conformational constraints; they exhibit CD spectra characterized by a negative shoulder at about 220 nm and a negative band at about 200 nm.^{170,174,177} This type of CD curve is also shown by films of unordered synthetic polypeptides,¹⁷⁴ in which the peptide chains are presumably restricted although they do not have definite regular, repeating asymmetry. Thus, synthetic polypeptides in solution can be seriously questioned as suitable models for unordered proteins.

Other Backbone Conformations

The applicability of long-chain polypeptides as models for α -helix, β -sheet, and random-coil segments in proteins is even more tenuous if these segments are distorted or very short. In addition, backbone structures other than α , β , and unordered are known to occur in proteins, and each of these structures may contribute its own chiroptical properties to the ORD or CD data. For example, calculations^{151,152} indicate that the rotatory strength of the 3_{10} -helix, found in proteins,³ differs from that of the α -helix and displays its own chain-length dependence.

The situation may occur where an arbitrary backbone structure may mimic the CD of one of the reference conformations (α , β , random) and thus seriously interfere with interpretation of CD data. A good example of this is gramicidin S. This molecule has ten amino acids arranged in a cross- β -like structure.¹⁸³ The ORD and CD patterns obtained¹⁸⁴⁻¹⁸⁷ are somewhat similar to those of an α -helix. The ORD curve^{184,186,187} is particularly misleading,¹⁸⁷ containing a trough, $[m]_{233} = -18,000$. CD data^{185,187} include negative bands at 208 and 217 (not 222) nm of $-30,000$ to $-40,000$, and a positive band,¹⁸⁷ $[\theta]_{186} = 28,000$ (not $[\theta]_{192} = 80,000$ which is the value for an α -helix). (Another CD study¹⁸⁶ found only one broad negative band.) Therefore, the similarity of gramicidin CD spectra to α -helix is only superficial. Pysh¹⁸⁸ calculated that the gramicidin S structure could indeed yield optical parameters

¹⁸³ A. Stern, W. Gibbons, and L. C. Craig, *Proc. Nat. Acad. Sci. U.S.* **61**, 734 (1968); W. A. Gibbons, J. A. Sogn, A. Stern, L. C. Craig, and L. F. Johnson, *Nature (London)* **227**, 840 (1970).

¹⁸⁴ L. C. Craig, *Proc. Nat. Acad. Sci. U.S.* **61**, 152 (1968).

¹⁸⁵ S. Laiken, M. Printz, and L. C. Craig, *J. Biol. Chem.* **244**, 4454 (1969).

¹⁸⁶ D. Balasubramanian, *J. Amer. Chem. Soc.* **89**, 5445 (1967).

¹⁸⁷ F. Quadrifoglio and D. W. Urry, *Biochem. Biophys. Res. Commun.* **29**, 785 (1967).

¹⁸⁸ E. Pysh, *Science* **167**, 290 (1970).

resembling those of the α -helix, the resemblance being coincidental. Another example is the simple amide, L-5-methylpyrrolid-2-one, whose CD curve in cyclohexane¹⁸⁹ is similar to that of a right-handed α -helix (although the bands are shifted and are much smaller in magnitude).

The variety of CD and ORD spectra obtained for other small model compounds containing one,^{190,191} two,^{191,192} and six¹⁹³ amide groups illustrates the great variability in rotatory properties conceivable for peptide residues in regular conformations. In addition, there is evidence¹⁹⁴ that some polypeptides can change conformation upon aggregation with other polypeptides; this finding may have relevance to proteins under conditions of inter- or intramolecular aggregation.

Light Scattering

A final factor that can distort the CD and ORD spectra of proteins is light scattering. There have recently been several studies¹⁹⁵⁻²⁰⁶ showing that rotational bands can be red-shifted and reduced in magnitude for turbid suspensions (for example, for proteins in membrane preparations). Urry and Ji¹⁹⁵ were the first to attempt to correlate the known distortions in α -helical CD bands of membrane proteins with calculations of expected differential light scattering and absorption flattening in particulate systems. Such calculations have since been refined.¹⁹⁶⁻¹⁹⁹ CD data have been obtained for suspensions^{195,200} and scattering films²⁰¹ of polyamino acids and for membrane preparations^{198,199,202-206}. The tentative conclusion (based largely on studies of

¹⁸⁹ D. W. Urry, *J. Phys. Chem.* **72**, 3035 (1968).

¹⁹⁰ N. J. Greenfield and G. D. Fasman, *J. Amer. Chem. Soc.* **92**, 177 (1970).

¹⁹¹ N. J. Greenfield and G. D. Fasman, *Biopolymers* **7**, 595 (1969).

¹⁹² E. B. Nielsen and J. A. Schellman, *Biopolymers* **10**, 1559 (1971).

¹⁹³ S. M. Ziegler and C. A. Bush, *Biochemistry* **10**, 1330 (1971).

¹⁹⁴ G. G. Hammes and S. E. Schullery, *Biochemistry* **7**, 3882 (1968).

¹⁹⁵ D. W. Urry and T. H. Ji, *Arch. Biochem. Biophys.* **128**, 802 (1968).

¹⁹⁶ C. A. Ottaway and D. B. Wetlaufer, *Arch. Biochem. Biophys.* **139**, 257 (1970).

¹⁹⁷ D. J. Gordon and G. Holzwarth, *Arch. Biochem. Biophys.* **142**, 481 (1971).

¹⁹⁸ D. J. Gordon and G. Holzwarth, *Proc. Nat. Acad. Sci. U.S.* **68**, 2365 (1971).

¹⁹⁹ D. W. Urry, L. Masotti, and J. R. Krivacic, *Biochim. Biophys. Acta* **241**, 600 (1971).

²⁰⁰ D. W. Urry, T. A. Hinners, and L. Masotti, *Arch. Biochem. Biophys.* **137**, 214 (1970).

²⁰¹ D. W. Urry, T. A. Hinners, and J. Krivacic, *Anal. Biochem.* **37**, 85 (1970).

²⁰² T. H. Ji and D. W. Urry, *Biochem. Biophys. Res. Commun.* **34**, 404 (1969).

²⁰³ A. S. Schneider, M.-J. T. Schneider, and K. Rosenheck, *Proc. Nat. Acad. Sci. U.S.* **66**, 793 (1970).

²⁰⁴ M. Glaser and S. J. Singer, *Biochemistry* **10**, 1780 (1971).

²⁰⁵ G. L. Choules and R. F. Bjorklund, *Biochemistry* **9**, 4759 (1970).

²⁰⁶ J. C. Reinert and J. L. Davis, *Biochim. Biophys. Acta* **241**, 921 (1971).

fragmented, nonscattering red blood cell membranes^{198,203,204} and of scattering suspensions of helical proteins²⁰³) is that particulate distortion effects (mainly absorption flattening^{196,197}) and not special protein conformations, are responsible for the characteristic CD spectra of membranes. Because of this distortion there is considerable ambiguity in interpretation of CD spectra of particulate systems: films of poly-L-alanine known to be α -helical from IR spectroscopy, display β -type CD spectra when scattering is present,²⁰¹ and analysis shows that *Mycoplasma* membrane proteins are largely in the β conformation, although their CD curve appears similar to that of an α -helix with scattering distortion.²⁰⁵ Therefore, great caution should be exercised in the interpretation of CD spectra of turbid systems, and independent physical methods of conformation determination should be used whenever possible. Recently a cell has been devised which can correct scattering artifacts and shows great promise.^{206a}

Contribution of Nonamide Chromophores

General Comments

Two types of nonbackbone chromophores, aromatic amino acid residues and disulfide groups, may cause complications in the interpretation of CD data. The absorption spectra²⁰⁷ of the aromatics (phenylalanine, tyrosine, and tryptophan) and cystine include bands in the near-UV region (240–300 nm). In addition, these amino acids and histidine have absorption bands²⁰⁷ in the 185–240 nm “peptide” region. Any of these bands may be optically active in a protein, especially if the side chain is held in an asymmetric environment. The near-UV Cotton effects, when properly assigned to specific side chains,^{89,92} can be helpful in determining conformational interactions in proteins, for example, the tertiary structure of active sites. On the other hand, the lower wavelength side-chain CD and ORD bands can interfere with estimation of secondary structure from analysis of peptide Cotton effects. Studies of absorption and chiroptical properties of model compounds (amino acid derivatives and polypeptides) and of proteins have shown that in the near-UV range although the wavelengths of Cotton effects due to a given side-chain chromophore are relatively constant, the amplitude and even the sign of these bands can vary with the composition and geometry of the molecule. In the far-UV region ($\lambda < 240$) interaction between side-chain and peptide chromophores can even cause the position of CD and

^{206a} B. P. Dorman, J. E. Hearst, and M. F. Maestre, this volume [30].

²⁰⁷ D. B. Wetlaufer, *Advan. Protein Chem.* **17**, 303 (1962).

ORD bands to be variable. The purpose of this section is to summarize methods of utilizing side-chain Cotton effects to gain structural information, as well as to point out the dangers inherent in trying to interpret CD or ORD spectra containing overlapping peptide and side-chain bands.

Goodman and co-workers have written two very useful review articles on aromatic Cotton effects in proteins (and model compounds)²⁰⁸ and in polyamino acids,²⁰⁹ which cover the important literature up to 1968. An interesting series of articles from Strickland's laboratory^{81,82,93,210-217} deals with the resolution and analysis of near-UV aromatic CD bands in proteins (and models), usually at 77° K where characteristic fine structure becomes apparent. Another method often used for classification of side-chain bands is the red-shift upon ionization (at high pH) of tyrosine absorption and Cotton effects (see Fig. 13 for an example).

Many studies have been concerned with aromatic poly- α -amino acids as models for these chromophores locked into fixed orientations such as may exist in proteins. Two problems are common in these studies: overlapping peptide and aromatic bands, and low solubility of the polypeptides in water. To overcome the second problem, extensive use is made of nonaqueous solvents,^{65,218-221} and of copolymers (random or block) with water-soluble residues^{218,221-227} and modified side chains.^{209,228-232}

- ²⁰⁸ M. Goodman and C. Toniolo, *Biopolymers* **6**, 1673 (1968).
²⁰⁹ M. Goodman, G. W. Davis, and E. Benedetti, *Accounts Chem. Res.* **1**, 275 (1968).
²¹⁰ J. Horwitz, E. H. Strickland, and C. Billups, *J. Amer. Chem. Soc.* **91**, 184 (1969).
²¹¹ E. H. Strickland, M. Wilchek, J. Horwitz, and C. Billups, *J. Biol. Chem.* **245**, 4168 (1970).
²¹² J. Horwitz, E. H. Strickland, and C. Billups, *J. Amer. Chem. Soc.* **92**, 2119 (1970).
²¹³ E. H. Strickland, J. Horwitz, E. Kay, L. M. Shannon, M. Wilchek, and C. Billups, *Biochemistry* **10**, 2631 (1971).
²¹⁴ E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry* **9**, 4914 (1970).
²¹⁵ E. H. Strickland, E. Kay, and L. M. Shannon, *J. Biol. Chem.* **245**, 1233 (1970).
²¹⁶ J. Horwitz and E. H. Strickland, *J. Biol. Chem.* **246**, 3749 (1971).
²¹⁷ L. Fretto and E. H. Strickland, *Biochim. Biophys. Acta* **235**, 473 (1971).
²¹⁸ E. Peggion, A. S. Verdini, A. Cosani, and E. Scoffone, *Macromolecules* **2**, 170 (1969).
²¹⁹ E. Peggion, L. Strasorier, and A. Cosani, *J. Amer. Chem. Soc.* **92**, 381 (1970).
²²⁰ V. N. Damle, *Biopolymers* **9**, 937 (1970).
²²¹ A. Cosani, E. Peggion, A. S. Verdini, and M. Terbojevich, *Biopolymers* **6**, 963 (1968).
²²² H. J. Sage and G. D. Fasman, *Biochemistry* **5**, 286 (1966).
²²³ H. E. Auer and P. Doty, *Biochemistry* **5**, 1708 (1966).
²²⁴ G. D. Fasman, M. Landsberg, and M. Buchwald, *Can. J. Chem.* **43**, 1588 (1965).
²²⁵ A. Ohnishi, K. Hayashi, and J. Noguchi, *Bull. Chem. Soc. Jap.* **42**, 1113 (1969).
²²⁶ B. Shechter, I. Shechter, J. Ramachandran, A. Conway-Jacobs, and M. Sela, *Eur. J. Biochem.* **20**, 301 (1971).

The optical activity of cystine residues has been discussed by Beychok.^{33,37(c)} Examples of proteins displaying near-UV CD bands, at 250–280 nm, attributed to S-S bridges are insulin,²³³ ribonuclease,²³⁴ and the neurophysins⁹⁴

In general, the presence of aromatic residues may result in too low an estimate for the α -helical (or β -form) content of a protein. This conclusion is based upon many studies of far-UV CD and ORD for synthetic aromatic containing polypeptides in ordered conformations. (Little can be predicted from smaller aromatic amino acid derivatives about the sign and magnitude of Cotton effects in proteins.) Positive side-chain Cotton effects partially (or completely) cancel the negative peptide Cotton effects in the range $\lambda = 200\text{--}240$ nm for polymers and copolymers of L-phenylalanine,²¹⁹ L-tyrosine,^{65,220,226,227,235–241} L-tryptophan,^{241,242} and L-histidine.²⁴³ This may be true under a variety of solvent conditions, including many where the first three polymers are thought to be α -helical, and where poly-L-histidine may be in an α or a β structure. Thus, overlap of peptide with aromatic CD bands may lead to apparently weak negative ellipticity values for the peptide wavelength region in structured proteins, and result in an underestimation of the amount of secondary structure actually present. For example, one study on poly-L-tyrosine⁶⁵ notes that “in unfavorable cases one tyrosine side chain may compensate for the contribution of about one peptide bond in an α -helical or β conformation.” A striking case of far-UV aromatic bands in proteins is

²²⁷ J. Ramachandran, A. Berger, and E. Katchalski, *Biopolymers* **10**, 1829 (1971).

²²⁸ M. Goodman, A. M. Felix, C. M. Deber, A. R. Brouse, and G. Schwartz, *Biopolymers* **1**, 371 (1963).

²²⁹ M. Goodman, A. M. Felix, C. M. Deber, and A. R. Brouse, *Biopolym. Symp.* **1**, 409 (1964).

²³⁰ C. Toniolo, M. L. Falxa, and M. Goodman, *Biopolymers* **6**, 1579 (1968).

²³¹ M. Goodman, C. Toniolo, and E. Peggion, *Biopolymers* **6**, 1691 (1968).

²³² M. Goodman and E. Peggion, *Biochemistry* **6**, 1533 (1967).

²³³ S. Beychok, *Proc. Nat. Acad. Sci. U.S.* **53**, 999 (1965).

²³⁴ M. N. Pflumm and S. Beychok, *J. Biol. Chem.* **244**, 3982 (1969).

²³⁵ G. D. Fasman, E. Bodenheimer, and C. Lindblow, *Biochemistry* **3**, 1665 (1964).

²³⁶ S. Beychok and G. D. Fasman, *Biochemistry* **3**, 1675 (1964).

²³⁷ E. Patrone, G. Conio, and S. Brighetti, *Biopolymers* **9**, 897 (1970).

²³⁸ F. Quadrifoglio, A. Ius, and V. Crescenzi, *Makromol. Chem.* **136**, 241 (1970).

²³⁹ S. Friedman and P. O. P. Ts'o, *Biochem. Biophys. Res. Commun.* **42**, 510 (1971).

²⁴⁰ M. Shiraki and K. Imahori, *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo* **16**, 215 (1966).

²⁴¹ G. D. Fasman, R. McKinnon, and R. Hoving, unpublished results (1969).

²⁴² E. Peggion, A. Cosani, A. S. Verdini, A. Del Pra, and M. Mammi, *Biopolymers* **6**, 1477 (1968).

²⁴³ Y. P. Myer and E. A. Barnard, *Arch. Biochem. Biophys.* **143**, 116 (1971).

that of avidin,²⁴⁴ whose CD spectrum contains a positive tryptophan band at 228 nm; this protein has an ORD peak near 233 nm instead of the trough at this wavelength characteristic of the α -helix.

There is little information on the contribution of asymmetric disulfide chromophores to the CD of proteins or polypeptides in the wavelength region below 240 nm. However, the presence of CD bands below 240 nm for cystine itself (in mulls²⁴⁵ and in KBr disks^{246,247}), and the existence of a positive CD band at 230 nm, for gramicidin S, attributed (at least partially) to an S-S transition²⁴⁸ may serve as cautionary notes. Several other amino acids (cysteine, methionine, asparagine, and glutamic and aspartic acids and amides) also absorb light in the 185–240 nm region,²⁰⁷ although they do not have absorption maxima in this region. Their contribution to protein rotatory properties has not been extensively investigated, but it is not expected to be large. The remainder of this section will summarize the CD and ORD properties of individual aromatic and S-S chromophores in model compounds and in proteins.

Amino Acid Derivatives and Poly-L-Amino Acids

(i) *Phenylalanine*. Phenylalanine can be taken as typical of the aromatic chromophores, although somewhat fewer studies have been performed with it than with tyrosine or tryptophan. All aromatic studies have in common the problems of choice of model compounds and resolution of aromatic rotatory bands from peptide bands; the same reviews^{208,209} can be consulted for all aromatic residues. The ultraviolet absorption spectrum²⁰⁷ of phenylalanine is characteristic of a monosubstituted benzene ring.²⁰⁸ The weak band, with vibrational fine structure, in the 260-nm region gives rise to several weak Cotton effects; not all of the transitions are optically active in any given compound. In addition, there are strong, optically active, electronic absorption bands further in the UV.

ORD studies of L-phenylalanine,^{249–252} its small peptides,²⁵¹ and N-acetyl-L-phenylalanine amide²⁵³ (a more realistic model than the free amino acid for the side-chain chromophore in proteins) showed very

²⁴⁴ N. M. Green and M. D. Melamed, *Biochem. J.* **100**, 614 (1966).

²⁴⁵ P. C. Kahn and S. Beychok, *J. Amer. Chem. Soc.* **90**, 4168 (1968).

²⁴⁶ A. Imanishi and T. Isemura, *J. Biochem. (Tokyo)* **65**, 309 (1969).

²⁴⁷ N. Ito and T. Takagi, *Biochim. Biophys. Acta* **211**, 430 (1970).

²⁴⁸ U. Ludescher and R. Schwyzer, *Helv. Chim. Acta* **54**, 1637 (1971).

²⁴⁹ A. Moscovitz, A. Rosenberg, and A. E. Hansen, *J. Amer. Chem. Soc.* **87**, 1813 (1965).

²⁵⁰ A. Rosenberg, *J. Biol. Chem.* **241**, 5119 (1966).

²⁵¹ E. W. Gill, *Biochim. Biophys. Acta* **133**, 381 (1967).

²⁵² N. Sakota, K. Okita, and Y. Matsui, *Bull. Chem. Soc. Jap.* **43**, 1138 (1970).

²⁵³ M. Shiraki, *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo* **19**, 151 (1969).

weak multiple Cotton effects near 260 nm superimposed on a large rotation at 220–230 nm due to the amide (or carboxyl) chromophore. The development of CD instrumentation permitted much better resolution of the optically active transitions for L-phenylalanine,^{210,252,254,255} N-acetyl-L-phenylalanine amide,^{201,253,256} N-acetyl-L-phenylalanine esters,^{208,210} and peptides (linear²⁵⁷ and cyclic^{258,259}). Simmons *et al.*²⁵⁶ were the first to achieve good resolution of the near-UV CD bands (all negative in the case of N-acetyl-L-phenylalanine amide) and to measure the positive CD bands at 195 and 217 nm; they attributed a small, negative, band at 240 nm to the primary amide group (by analogy to N-acetyl-L-alanine amide). The CD spectra of N-acetyl-L-phenylalanine amide (and the analogous L-tyrosine and L-tryptophan derivatives) in water are shown in Fig. 12.

As pointed out by Horwitz *et al.*,²¹⁰ the CD vibrational fine structure varies with the compound: in L-phenylalanine itself only the prominent absorption bands at 264 and 258 nm are dichroic²⁵⁴ (giving rise to positive Cotton effects), but, on the other hand, in the amide²⁵⁶ and in proteins²⁶⁰ only the weak absorption bands of the phenylalanine chromophore at 268 and 262 nm are optically active. The resolution of the near-UV CD bands can be increased by working at 77° K²¹⁰; band assignments useful in characterizing phenylalanine in proteins can be made. Organic solvents can greatly affect the entire CD spectrum of N-acetyl-L-phenylalanine amide (as well as those of the other aromatics).²⁵³

Studies of the phenylalanine chromophore in polypeptides are complicated by the insolubility of poly-L-phenylalanine in water (necessitating the use of copolymers or organic solvents) and by the overlapping of side-chain and peptide bands (making conformational determination very difficult). Early ORD studies in aqueous solution of block copolymers of L-phenylalanine with DL-glutamic acid^{222,223} revealed the presence of near-UV aromatic bands and of a weak 228-nm trough, not typical of normal polypeptide conformations, but suggestive of an α -helix. Chiroptical investigations of phenylalanine containing polymers performed before 1968 have been summarized.²⁰⁹

²⁵⁴ M. Legrand and R. Viennet, *Bull. Soc. Chim. Fr.* **1966**, 2798 (1966).

²⁵⁵ L. Verbit and P. J. Heffron, *Tetrahedron* **23**, 3865 (1967).

²⁵⁶ N. S. Simmons, A. O. Barel, and A. N. Glazer, *Biopolymers* **7**, 275 (1969).

²⁵⁷ I. Weinryb and R. F. Steiner, *Arch. Biochem. Biophys.* **131**, 263 (1969).

²⁵⁸ K. Blahá and I. Fric, *Collect. Czech. Chem. Commun.* **35**, 619 (1970).

²⁵⁹ K. Blahá, I. Fric, and J. Rudinger, *Collect. Czech. Chem. Commun.* **34**, 3497 (1969).

²⁶⁰ E. H. Strickland, E. Kay, L. M. Shannon, and J. Horwitz, *J. Biol. Chem.* **243**, 3560 (1968).

Later CD studies^{32,218,219,261} of such polymers showed positive or weakly negative dichroism in the peptide region, and it was suggested that an ordered structure (possibly α -helical) is formed under certain solvent conditions. Urry³² found that poly-L-phenylalanine forms such a structure in 99% ethylene dichloride plus 1% trifluoroacetic acid (TFA). The CD displays finely structured bands of ~ 100 deg.-cm²/dmole near 260 nm, plus a band at 227 nm ($[\theta]_{227} = -9000$) and a positive shoulder at 215 nm; this spectrum is destroyed by additional TFA. Peggion and co-workers^{218,219} have concluded, from two lines of evidence, that the ordered structure may be a right-handed α -helix, even though its CD properties are unlike a typical α -helix. First,²¹⁸ the CD spectra of copolymers of L-phenylalanine with ϵ -carbobenzoxy-L-lysine in tetrahydrofuran showed a gradual perturbation of the α -helical pattern of poly- ϵ -carbobenzoxy-L-lysine as additional phenylalanine was introduced, but no change in helical sense. Second,²¹⁹ poly-L-phenylalanine undergoes a transition in water-methanesulfonic acid mixtures, in a manner analogous to that of the α -helix-to-coil transition of poly-L-cyclohexylalanine; near UV bands were also observed in this study. Thus, the CD patterns of poly-L-phenylalanine may be attributable to an overlap of conformation-dependent side-chain chromophore bands with normal random-coil and α -helical peptide bands. The same can be said (with somewhat more assurance) for poly-L-tyrosine and poly-L-tryptophan. (A recent CD study by Peggion *et al.*²⁶¹ on copolymers of lysine and phenylalanine in water at various pH values suggests that the presence of phenylalanine in this system induces β -structure formation.)

(ii) *Tyrosine.* The absorption spectrum²⁰⁷ of un-ionized tyrosine (the form present in proteins at neutral pH) contains a weak band at 275 nm (with a shoulder at 282 nm) and two stronger peaks at 224 and 194 nm. ORD studies of this amino acid^{249,250} showed small near-UV Cotton effects. The CD spectrum of un-ionized L-tyrosine^{236,255,262} has positive bands corresponding to the 275 and 225 nm transitions (see Fig. 13); the latter CD band interferes with conformational determination of tyrosine polypeptides. The CD signal is positive also at $\lambda < 200$ nm. Theoretical calculations of tyrosine optical activity have been made.²⁶³ Vibrational fine structure becomes apparent in the CD of tyrosine (and its derivatives) upon cooling.²¹² When the phenolic group is ionized (at pH above about 12) all absorption and CD bands are shifted to longer

²⁶¹ E. Peggion, A. S. Verdini, A. Cosani, and E. Scoffone, *Macromolecules* **3**, 194 (1970).

²⁶² M. Legrand and R. Viennet, *Bull. Soc. Chim. Fr.* **1965**, 679 (1965).

²⁶³ T. M. Hooker, Jr. and J. A. Schellman, *Biopolymers* **9**, 1319 (1970).

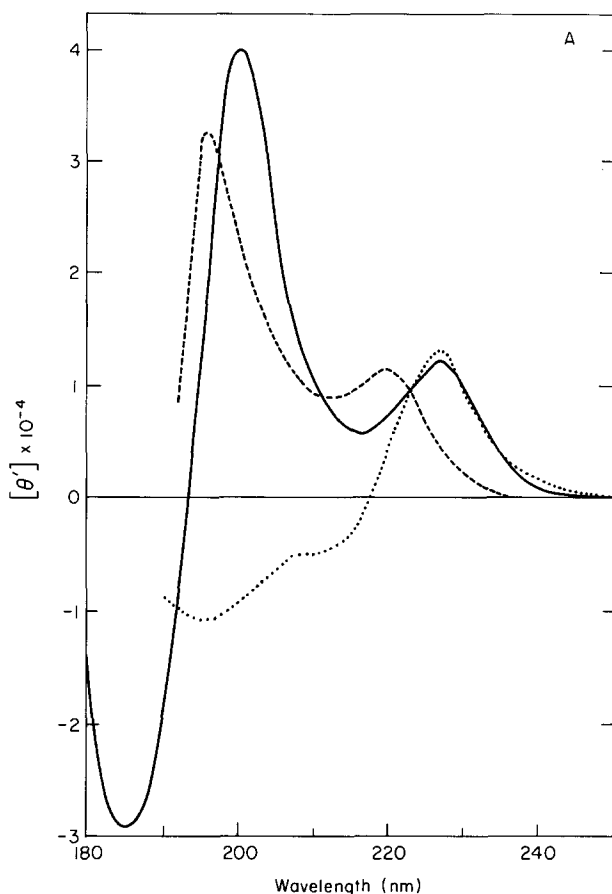


FIG. 12. Circular dichroism spectra of aromatic amino acid derivatives in water in the far ultraviolet (A) and near ultraviolet (B) regions: *N*-acetyl-L-tyrosine amide, —; *N*-acetyl-L-phenylalanine amide, - - - - -; *N*-acetyl-L-tryptophan amide, · · · · · [from M. Shiraki, *Sci. Pop. Coll. Gen. Educ. Univ. Tokyo* **19**, 151 (1969).] Note that molar ellipticities reduced to vacuo, $[\theta']$, are given; these can be multiplied by $(n^2 + 2)/3 \approx 1.3$ to yield $[\theta]$ values.

wavelength.²⁶² (This red-shift is useful to diagnose tyrosine residues in proteins.)

The CD pattern of *N*-acetyl-L-tyrosine amide^{212,253} is shown in Fig. 12; the 275-nm band for this compound in water is negative. L-Tyrosine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester also have been examined at different temperatures²¹² and pH values.³³ The near-UV CD spectra of *N*-acetyl-L-tyrosine amide²⁵³ and ethyl ester²¹² are extremely solvent dependent; the ester 275-nm CD band is positive in dioxane and negative in methanol. Horwitz *et al.*²¹² suggested that it should be possible to

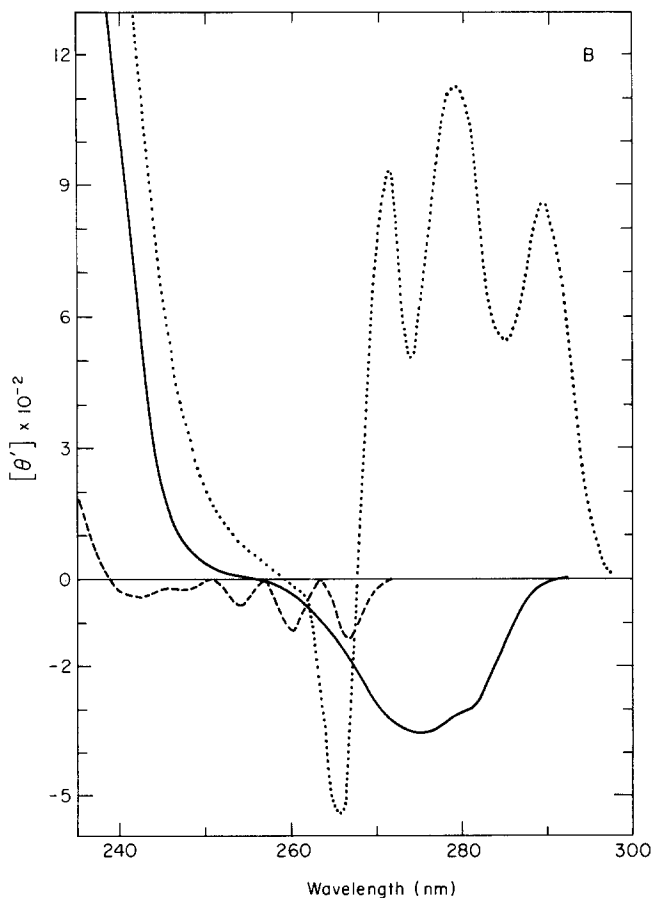


FIG. 12B.

identify the tyrosine residues in proteins from their CD vibrational structure between 275 and 290 nm. The bands between 250 and 270 nm were deemed not as useful due to overlap with phenylalanine residues and disulfide bridge contributions.

The near-UV CD properties of diketopiperazines (cyclic dipeptides) containing aromatic chromophores have been examined at room temperature²⁶⁴ and below.²¹¹ Ellipticity values for L-tyrosine-containing cyclic dipeptides are much larger than those for the analogous linear peptides, apparently because of the rigid conformation of the diketopiperazines.²⁶⁴ Red shifts are caused by tyrosine ionization and by organic solvents.²⁶⁴ Cyclic-L-tyr-L-tyr shows a small exciton contribution in

²⁶⁴ H. Edelhoch, R. E. Lippoldt, and M. Wilchek, *J. Biol. Chem.* **243**, 4799 (1968).

its CD.²¹¹ Ziegler and Bush¹⁹³ compared the CD spectrum of cyclo-(glycine)₅-L-tyrosine with those of the analogous linear hexapeptide and cyclo-(Gly)₅-L-leucine. They concluded, with the help of nuclear magnetic resonance data,²⁶⁵ that the observed enhancement of the amide $\pi - \pi^*$ CD bands is the result of coupling between the tyrosyl and amide chromophores.

As with the cyclic hexapeptides, the main concern of studies on polypeptides containing tyrosine (and other aromatics) is to resolve peptide from side-chain CD bands and to determine conformation. The estimation of secondary structure of these polymers is important if they are to be used as models for protein structural determination, since the contribution of aromatic chromophores to the CD spectra is conformation dependent. The polymer CD spectrum is not the simple sum of that for a monomeric amino acid (e.g., L-tyrosine) derivative and that for a known polypeptide conformation (e.g., α -helix)^{236,241}; side-chain chromophores interact with one another and with peptide chromophores. The problem, as illustrated by poly-L-tyrosine, is a difficult one: the CD curves for this polymer (Fig. 13) obviously reflect a mixture of peptide and tyrosine bands. The resultant spectra do not correspond, even qualitatively, to polypeptide CD data for the known α , β , and random forms. If it can be shown that a given poly-L-tyrosine CD spectrum corresponds to the polymer in any known backbone conformation (for example, α -helix), then the normal CD pattern for this conformation can be subtracted, the side-chain CD contribution can be estimated, and these results can be applied to correct the CD of proteins containing tyrosine residues in segments with this conformation. However, if the poly-L-tyrosine conformation is not known, then backbone and side-chain CD contributions cannot be separated, (because the tyrosine Cotton effects are conformation dependent) and the polymer studies are much less useful for protein applications.

After an enormous number of studies on the ORD,^{235,237,240,266} CD,^{32,65,220,236,238-240} and other physical properties^{220,238,267,268} of poly-L-tyrosine and copolymers^{226,227,241} in various solvents, plus theoretical CD calculations,²⁶⁹⁻²⁷¹ all that can be concluded is that poly-L-tyrosine

²⁰³ K. D. Kopple, M. Ohnishi, and A. Go, *J. Amer. Soc.* **91**, 4264 (1969); *Biochemistry* **8**, 4087 (1969).

²⁰⁴ J. D. Coombes, E. Katchalski, and P. Doty, *Nature (London)* **185**, 534 (1960).

²⁰⁷ J. Applequist and T. G. Mahr, *J. Amer. Chem. Soc.* **88**, 5419 (1966).

²⁰⁸ M. B. Senior, S. L. H. Gorrell, and E. Hamori, *Biopolymers* **10**, 2387 (1971).

²⁰⁹ Y. H. Pao, R. Longworth, and R. L. Kornegay, *Biopolymers* **3**, 537 (1965).

²¹⁰ T. Ooi, R. A. Scott, G. Vanderkooi, and H. A. Scheraga, *J. Chem. Phys.* **46**, 4410 (1967).

²⁷¹ A. K. Chen and R. W. Woody, *J. Amer. Chem. Soc.* **93**, 29 (1971).

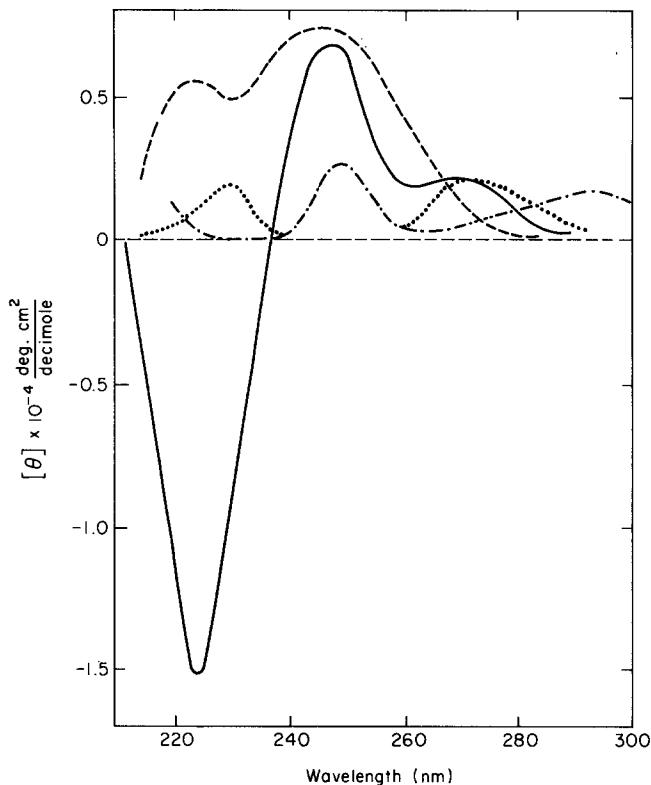


FIG. 13. Circular dichroism of helical poly-L-tyrosine (—), random poly-L-tyrosine (---) (both at pH 11.2), and L-tyrosine at pH 8 (····) and at pH 12 (-·-·-) [from S. Beychok and G. D. Fasman, *Biochemistry* 3, 1675 (1964)]. The helical form of the polymer was prepared by direct dissolution into water at pH 11.2; the random-coil form was first brought to pH > 12 and then back-titrated to pH 11.2.

is probably a right-handed α -helix in aqueous solution under certain conditions. There is also evidence for left-handed helix²⁶⁷ (see Goodman *et al.*²⁶⁹) and β -sheet^{237,268} formation. However, one generalization useful for protein CD interpretation emerges from the chiroptical data: all ORD and CD spectra of poly-L-tyrosine (no matter what the corresponding structure is) have ellipticity values in the 210–240 nm range that are much more positive than those of normal polypeptides in known, ordered conformations. Therefore, tyrosine (and other aromatic) residues in proteins are likely to cause too low an estimation of any ordered conformation from CD data.

The first sign of peculiarity for poly-L-tyrosine was its positive b_0

value.²³⁵ Beychok and Fasman²³⁶ examined its CD and found the spectra shown in Fig. 13. Since poly-L-tyrosine is not soluble in water in its totally un-ionized form, the polymer had to be examined at high pH. In the structured ("helical") form there are CD bands corresponding to the un-ionized tyrosine absorption bands at 275 and 225 nm, plus a CD band at 248 nm attributable to ionized tyrosine. All of these are superimposed on the peptide CD bands. Later work showed the presence of a positive CD band at about 200 nm in trimethyl phosphate^{220,238} and in 0.1 M NaClO₄ at pH 10.8²³⁸; this band, too, is probably a mixture of the 194 nm tyrosine absorption plus $\pi - \pi^*$ peptide absorption bands. Somewhat different CD curves found for poly-L-tyrosine,^{65,220,239} in which the negative 225-nm band is missing, may arise from variations in ionization.

Some recent studies of copolymers containing tyrosine are of interest. Shechter *et al.*²²⁶ found that for a copolymer of sequence (L-Tyr-L-Ala-L-Glu)₂₀₀ the 275-nm CD band is negative in the helical conformation (although the 225-nm band retains the same sign as in the homopolymer). This result indicates the sensitivity of the tyrosine chromophore to its asymmetric environment and shows that its ability to interact with other tyrosines may change its rotatory contribution. Ramachandran *et al.*²²⁷ studied the same copolymer by means of ORD. They compared it to a random sequence copolymer (Tyr, Ala, Glu)_n in both α -helical and random-coil conformations, and gave X-ray diffraction evidence for the helical form of the sequence copolymer. They concluded that, in the α -helical form, the ORD contribution of tyrosine residues in the 200–250 nm region (a peak at 233 nm and a trough below 200 nm) nearly cancels the contribution of the α -helix. The tyrosine contribution of the random copolymer in the α -helical conformation is similar in shape but has only one-third the amplitude.

Polymers of hydroxyethyl-L-glutamine incorporating small amounts of L-tyrosine display negative Cotton effects in the near-UV region²⁴¹ in 80% aqueous methanol (where the polymers are α -helical) as shown in Fig. 14. These CD bands (calculated per mole of tyrosine residue) grow disproportionately as the tyrosine content increases, again showing specific interaction. Furthermore, block copolymers of L-tyrosine with DL-glutamate have positive 275-nm CD bands, similar to poly-L-tyrosine. It is not possible in any of these cases²⁴¹ to calculate the copolymer CD spectra at any wavelength by adding the CD curves of model tyrosine derivatives to those of helical or random polypeptides, even though these copolymers have normal conformations. This result thus shows that aromatic CD contributions are conformation dependent, or that the side chains become immobilized due to the backbone structure and

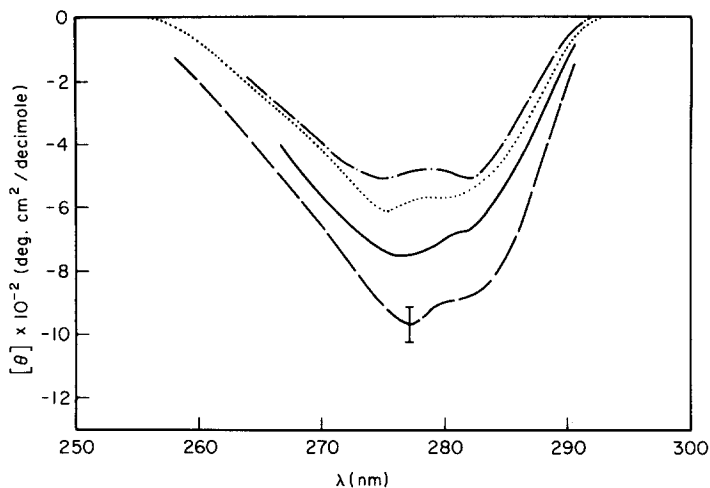


FIG. 14. Near-ultraviolet circular dichroism of copolymers of N^5 -(2-hydroxyethyl)-L-glutamine with various amounts of L-tyrosine (randomly incorporated) (from G. D. Fasman, R. McKinnon, and H. Hoving, unpublished results). The solvent is 80% aqueous methanol, in which the copolymers are α -helical. $[\theta]$ values are given per mole of tyrosine residues. Mole percent tyrosine in the copolymers: 4.5, - - - - -; 8, ———; 17, - - - - -. The CD spectrum of N -acetyl-L-tyrosine amide in methanol, $\cdot \cdot \cdot \cdot \cdot$, is shown for comparison. Error bars indicate agreement of duplicate experiments.

are therefore locked in an asymmetric configuration. Examination of CD curves in the peptide wavelength region for these helical copolymers²⁴¹ shows an apparent decrease in magnitude of the peptide CD bands as the tyrosine content is increased; this finding is very similar to the case of tryptophan copolymers (see Fig. 15, below).

(iii) *Tryptophan*. This amino acid absorbs light at 277 nm (with vibrational structure), 218, and 196 nm.²⁰⁷ The ORD of L-tryptophan,^{250,251} N -acetyl-L-tryptophan amide,²⁵³ and small peptides²⁵¹ plus the CD spectra of the amino acid,^{33,81,83,213,255,262} its esters,^{81,213} and N -acetyl-L-tryptophan amide^{81,253} (see Fig. 12) have been examined. Several positive CD bands are present between 270 and 300 nm for all these compounds. Strickland *et al.* have assigned these vibronic bands^{81,213} at room temperature and 77°K (where they are greatly enhanced) with the assistance of indole absorption spectra.²¹⁴ Two electronic transitions, 1L_a and 1L_b , contribute to the CD of model tryptophan compounds in this region. 1L_b vibronic CD bands are located at 290 nm for the 0-0 transition and at 283 nm for the 0 + 850 cm^{-1} transition; these positions are not shifted by different solvents. Several 1L_a bands, located between 265 and 297 nm are solvent dependent.

Several laboratories^{33,83,255,262} agree that in the low wavelength region L-tryptophan at neutral pH has CD bands at 222 nm (positive) and at about 195 nm (negative), which correspond to its absorption peaks. In addition, small bands at 240 and about 200 nm are noted in some reports. The CD spectrum of L-tryptophan is pH dependent,^{33,255,262} and is very similar to that of *N*-acetyl-L-tryptophan amide.²⁵³ Studies of diketopiperazines^{211,264} show that the near-UV CD bands of cyclic-Gly-L-Trp and cyclic-(L-Trp)₂ are several times as large as those for the corresponding, less rigid, linear dipeptides. Dipeptide solvent effects were demonstrated²⁶⁴: dioxane appears to destroy the interaction between the indole chromophores and the dipeptide ring, permitting greater rotational freedom. The tryptophan CD bands were correlated²⁶⁴ with bands in β -lactoglobulin and carbonic anhydrase.

The chiroptical properties of poly-L-tryptophan and its copolymers show overlap of peptide and side-chain bands, similar to the case of poly-L-tyrosine. Much of the work has been summarized.²⁰⁹ Fasman *et al.*²²⁴ examined the ORD of a poly-L-tryptophan film and found a trough at 233 nm plus small near-UV Cotton effects. They concluded that poly-L-tryptophan, despite its positive b_0 value, is a right-handed α -helix, since a series of copolymers with γ -benzyl-L-glutamate yielded a linear relationship between b_0 and percentage of tryptophan. Stevens *et al.*¹⁶⁰ obtained CD spectra of poly-L-Trp films. Cosani *et al.* studied the ORD^{221,272} and CD²²¹ spectra of a block copolymer of tryptophan with γ -ethyl-DL-glutamate in trifluoroethanol, in which solvent the polymer appears structured. CD bands were found at 290 nm ($\Delta\epsilon = -0.63$), 286 and 280 (positive shoulders), 272 ($\Delta\epsilon = 2.65$), 226 ($\Delta\epsilon = 42.7$), 210 ($\Delta\epsilon = -26.4$), and 190 ($\Delta\epsilon = 14.3$). The two dichroic bands at 210 and 190 nm are of the same sign and position as those observed for polypeptides in a right-handed α -helical conformation. However, the 226 nm band shows that optically active indole transitions are overlapping peptide transitions in this polymer, and the conformational assignment required confirmation.

More evidence from Peggion's group (X-ray analysis of poly-L-Trp films,²⁴² CD spectra in ethylene glycol monomethyl ether of poly-L-Trp and several copolymers with γ -ethyl-L-glutamate,²⁴² and a CD study of poly-2,2-nitrophenyl-sulfenyl-L-tryptophan²⁷³) plus work on a L-Trp, L-Glu copolymer²²⁵ substantiate that poly-L-tryptophan is a right-handed α -helix in some solvents. Therefore, in the case of tryptophan, the polypeptide results can be applied to proteins in a known conforma-

²⁷² A. Cosani, E. Peggion, M. Terbojevich, and A. Portolan, *Chem. Commun.* **1967**, 930 (1967).

²⁷³ E. Peggion, A. Fontana, and A. Cosani, *Biopolymers* **7**, 517 (1969).

tion. Peggion *et al.*²⁴² showed how the CD of an α -helix is distorted by increasing incorporation of L-tryptophan, with the 208 and 222 nm bands of the α -helix being replaced by a band at 220 nm and with a positive band appearing at 230 nm. This change is gradual but not linear with mole fraction tryptophan, perhaps because of exciton interactions between aromatic residues at high tryptophan content.

The effect upon peptide-region CD spectra of incorporation of small amounts (0–15 mole percent) of L-tryptophan into poly-hydroxyethyl-L-glutamine is shown in Fig. 15. These polymers²⁴¹ are α -helical in 80% aqueous methanol (Fig. 15A) and mainly unordered in water (pH 7, Fig. 15B). In Fig. 15A is evidence that the presence of aromatic residues in proteins can result in misleadingly low estimates of the amount of helical structure. It can be seen in Fig. 15B that tryptophan residues may initiate and stabilize helical structures in proteins.

(iv) *Histidine*. The imidazole group of histidine²⁰⁷ has at 210 nm an absorption peak that could interfere with protein CD measurements. CD spectra of the L-amino acid display a positive band at 213 nm^{208,243,255,262} (which is slightly red-shifted in acid²⁵⁵) and a negative band at 193 nm.²⁰⁸ A negative CD band at 217 nm in cyclo-Gly₂-L-Tyr-Gly₂-L-His was shown¹⁹³ to have contributions from $n - \pi^*$ amide and imidazole electronic transitions.

Poly-L-histidine has been studied by means of ORD,^{274,275} CD,^{32,243,275,276} and infrared spectroscopy.^{243,277} There is general agreement that at pH below 4, where the imidazole moiety carries a positive charge and where the CD spectrum is characterized by a positive band at 222 nm,^{32,243,275,276} the polymer is a random coil (or perhaps an extended chain³²). However, in aqueous solutions above the pK of the imidazole transition (pH 5–6), there is controversy about the ordered form (or forms) of poly-L-histidine. At pH ~ 6 the CD contains bands at 221 nm ($[\theta] \sim -5000$)^{32,243,275} and at 203 nm ($[\theta] \sim 16,000$)^{32,243}; very complicated CD changes (involving more than two species) were noticed as the pH was varied from 4 to 6.²⁴³ It is not clear whether poly-L-histidine at neutral pH is a left-handed helix,^{32,274} a right-handed helix (with side-chain contributions confusing the chiroptical properties),²⁷⁵ a β -sheet,²⁴³ or perhaps some other conformation²⁷⁶ or a mixture.²⁴³ In any case (as appears to be the rule for aromatic residues) the

²⁷⁴ K. S. Norland, G. D. Fasman, E. Katchalski, and E. R. Blout, *Biopolymers* **1**, 277 (1963).

²⁷⁵ S. Beychok, M. N. Pflumm, and J. E. Lehmann, *J. Amer. Chem. Soc.* **87**, 3990 (1965).

²⁷⁶ E. Peggion, A. Cosani, M. Terbojevich, and E. Scoffone, *Macromolecules* **4**, 725 (1971).

²⁷⁷ J. Muelinghaus and G. Zundel, *Biopolymers* **10**, 711 (1971).

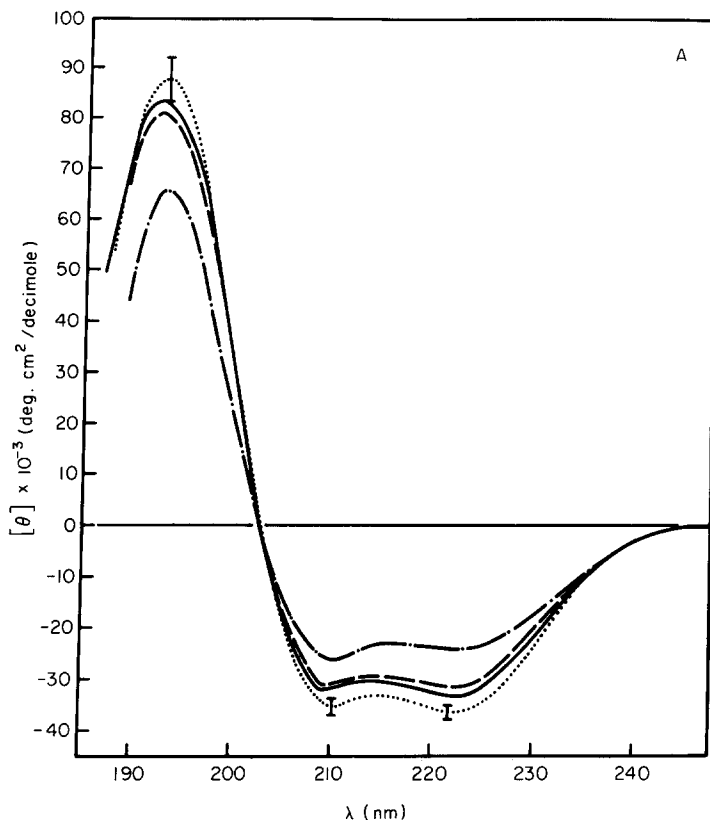


Fig. 15. Far-ultraviolet circular dichroism of copolymers of N^{ϵ} -(2-hydroxyethyl)-L-glutamine with various amounts of L-tryptophan (randomly incorporated) [from G. D. Fasman, R. McKinnon, and R. Hoving, unpublished results]. Mole percent tryptophan in the copolymers: 0, \cdots (poly-hydroxyethyl-L-glutamine); 28, — ; 8.8, $---$; 14.8, $-\cdot-\cdot-$ [θ] values are given per mole of peptide residues. (A) α -helical polypeptides in 80% aqueous methanol. (B) Random-coil conformations in water.

presence of histidine results in a CD spectrum much less negative in the 195–240 nm region than a normal α or β curve.

Peggion *et al.*²⁷⁶ examined the CD spectra of a series of random copolymers of L-histidine with L-lysine, and concluded that the ordered structure assumed by nonprotonated poly-L-histidine in water cannot be an α -helix. A copolymer of sequence L-(His-Ala-Glu)_n, which is water-soluble and a good model for histidine in proteins, has a CD spectrum in water²⁷⁸ which indicates an essentially random-coil conformation at all pH values, with little sign of interference from side-chain optical ac-

²⁷⁸ H. J. Goren, M. Fridkin, and E. Katchalski, personal communication (1971).

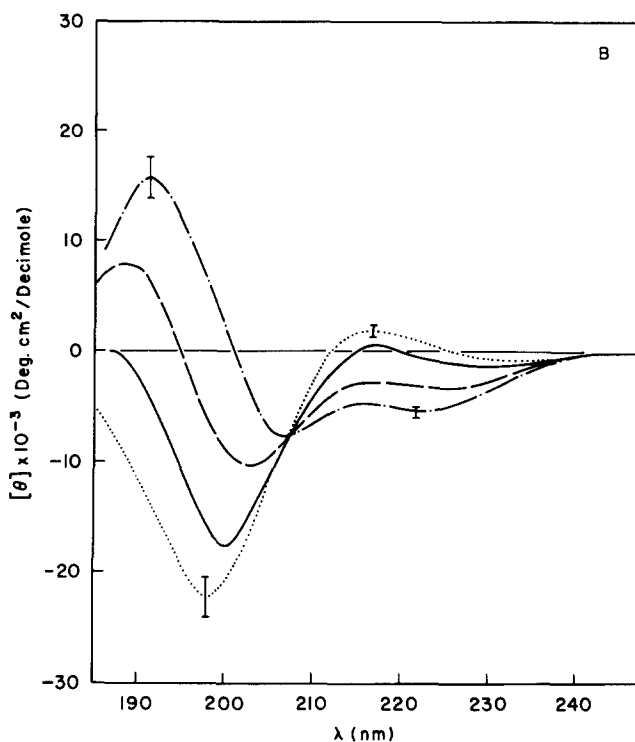


FIG. 15B.

tivity. The ORD²⁷⁴ and CD²⁷⁹ of poly-(1-benzyl)-L-histidine and its copolymers with *N*- ϵ -carbobenzoxyl-L-lysine²⁷⁹ in organic solvents show complexities. The CD spectra of poly-(1-benzyl)-L-histidine show bands similar to those of a right-handed α -helix but with diminished amplitudes.²⁷⁹

(v) *Sulfur-containing residues*. In addition to the aromatic residues the contribution of sulfur containing residues, primarily cystine (S-S), must be considered. Cysteine (SH), methionine (SCH₃), and cystine all have long absorption tails in the 195–230 nm region²⁰⁷; cystine has an additional band at about 250 nm.

The sulfur chromophore itself does not appear to be an important source of optical activity. ORD studies of L-cysteine,^{280,281} its methyl ester,²⁸⁰ and *N*-acetyl-L-cysteine methyl amide²⁸¹ could all be interpreted in terms of known amide and carboxyl Cotton effects.

²⁷⁹ M. Terbojevich, M. Acampora, A. Cosani, E. Peggion, and E. Scoffone, *Macromolecules* **3**, 618 (1970).

²⁸⁰ D. W. Urry, D. Miles, D. J. Caldwell, and H. Eyring, *J. Phys. Chem.* **69**, 1603 (1965).

²⁸¹ D. L. Coleman and E. R. Blout, *J. Amer. Chem. Soc.* **90**, 2405 (1968).

The cystine disulfide chromophore on the other hand, presents a very significant source of optical activity. A disulfide grouping is rigidly fixed; the barrier to rotation about the S-S bond is high, and the disulfide can exist in either of two rotomers having either a right- or left-handed screw sense. Thus, a new center of asymmetry is created in the peptide chain, causing Cotton effects in the near- and far-ultraviolet regions. The dihedral angle (defined by C₁-S-S and S-S-C₂) is close to 90° in cystine itself, but can vary in proteins. The absorption band is significantly red-shifted as the dihedral angle decreases,²³³ so that near-UV S-S CD bands could appear anywhere between 250 and about 270 nm. Furthermore, these bands have no vibrational fine structure²¹²; this fact can be an aid to their diagnosis in proteins.

Beychok^{27(c)} and Coleman and Blout²⁸² found that L-cystine has a broad pH-dependent negative CD band at about 255 nm ($[\theta] \sim -2500$), which is shared by N-acetyl derivatives and by homocystine, and which is unequivocally associated with the disulfide transition. Another pH-dependent band (positive, near 220 nm)²⁸² is also present in these compounds. ORD data²⁸¹ agree well. The optical activity of cystine is modified when the molecules are locked into a fixed configuration within a crystal.²⁴⁵⁻²⁴⁷ This has important implications, as the disulfide groupings in a protein are likely also to be specifically constrained. L-Cystine crystals in mulls²⁴⁵ and in KBr disks²⁴⁶ show a positive CD band near 300 nm and a negative band near 240 nm. Under the same conditions L-cystine dihydrochloride has a negative band at 270 nm and a positive one near 230 nm. These inversions can be correlated with X-ray evidence (see references in Kahn and Beychok²⁴⁵) showing that L-cystine crystallizes with the disulfide as a left-handed screw, while its dihydrochloride crystals have the opposite chirality. Another study²⁴⁷ of crystalline L-cystine in KBr disks considers the possibility of exciton splitting.

Recent experimental²⁸³⁻²⁸⁶ and theoretical²⁸⁷ work has attempted the determination of the sign of the highest wavelength CD band for disulfide derivatives of known screw sense and dihedral angle. A quadrant rule for correlation of CD with geometry was formulated,²⁸⁷ based largely

²⁸² D. L. Coleman and E. R. Blout, in "Conformation of Biopolymers" (G. N. Ramachandran, ed.), Vol. 1, p. 123. Academic Press, New York, 1967.

²⁸³ M. Carmack and L. A. Neubert, *J. Amer. Chem. Soc.* **89**, 7134 (1967).

²⁸⁴ G. Claeson, *Acta Chem. Scand.* **22**, 2429 (1968).

²⁸⁵ R. M. Dodson and V. C. Nelson, *J. Org. Chem.* **33**, 3966 (1968).

²⁸⁶ P. D. Hensen and K. Mislow, *Chem. Commun.* **1969**, 413 (1969).

²⁸⁷ J. Linderberg and J. Michl, *J. Amer. Chem. Soc.* **92**, 2619 (1970).

on studies of dithiane rings,²⁸³⁻²⁸⁵ and confirmed by examination of *S*-allyl-L-cysteine-*S*-oxides²⁸⁶ and [2,7-cystine]-gramicidin *S*.²⁴⁸

Copolymers of L-cystine with L-glutamate, containing inter- and intra-chain S-S bridges, have been studied²⁸² by CD and ORD spectroscopy. All these polymers, charged or uncharged, showed Cotton effects near 260 nm. In only one case did there appear to be a small disulfide contribution at 200 nm. The same authors²⁸² described the cyclic oligopeptides, arginine vasotocin and 8-L-ornithine vasopressin, which have optically active S-S transitions near 200 nm, similar to those of model compounds, in addition to near-UV bands. Beychok²³³ has examined the CD curves of cystine disulfoxide and oxidized glutathione, which are similar to, but less intense than that of cystine. In conclusion, the CD spectra of cystine^{245-247,282} and its peptides^{248,282} contain bands in the 210-240 nm region and near 200 nm²⁸² (which may confuse interpretation of protein peptide CD contributions) as well as a near-UV band of variable sign, wavelength (250-300 nm) and intensity^{37(c),245-248,282-286} (which may yield information about the geometry of disulfide bridges and adjacent residues in proteins).

Nonpeptide Contributions in Proteins

Summary. Examination of near-UV chiroptical properties (mainly circular dichroism) of proteins has recently, become a widespread technique; it can yield useful information about specific aromatic and cystine residues and their environments, for example at the active site of an enzyme. On the other hand, very little is known about side-chain contributions to the CD spectra of proteins in the far-UV "peptide" region, or about general methods of conformational interpretation in the presence of side-chain CD bands. Goodman and Toniolo²⁰⁸ have reviewed work done through 1968 on aromatic optical activity in proteins. A paper by Beychok²³³ constitutes a good introduction to CD of disulfides in proteins. A recent study of insulin⁸⁸ illustrates the problems of overlapping peptide and side-chain bands.

A brief survey of recent CD investigations of side chains in proteins follows. There have been no CD studies specifically on histidine. Few studies have been centered on the role of phenylalanine in proteins because of the weakness of its CD bands; exceptions are investigations of peroxidase,^{215,260} and carboxypeptidase.⁹³ Studies of tyrosine include ribonuclease,^{212,216,288-293} staphylococcal nuclease,⁹⁵ cytochrome *c*,²⁹⁴ lysozyme,^{89,90,92} insulin,⁸⁸ concanavalin,⁹¹ and carboxypeptidase.⁹³ Tryptophan CD or ORD has been examined in cytochrome *c*,^{213,294-296} lyso-

²⁸⁸ A. N. Glazer and N. S. Simmons, *J. Amer. Chem. Soc.* **87**, 3991 (1965).

²⁸⁹ R. T. Simpson and B. Vallee, *Biochemistry* **5**, 2531 (1966).

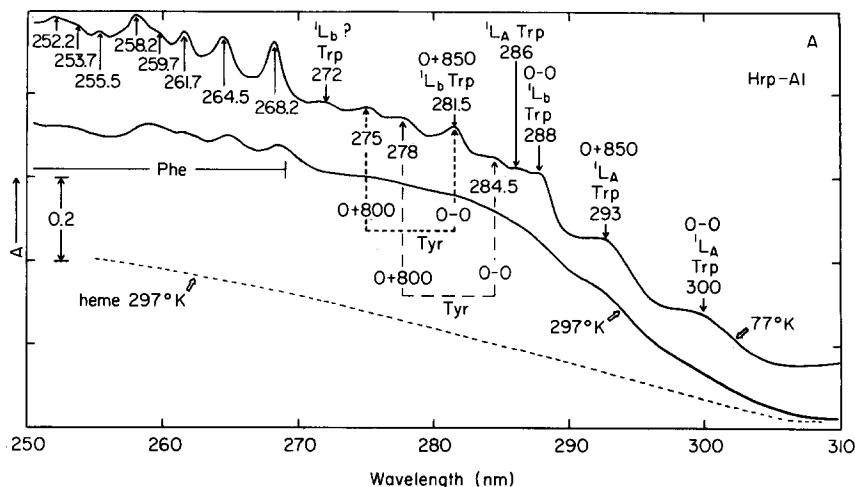


FIG. 16. Near-ultraviolet (A) absorption and (B) circular dichroism spectra of horse radish peroxidase A1 [from E. H. Strickland, J. Horwitz, E. Kay, L. M. Shannon, M. Wilchek, and C. Billups, *Biochemistry* **10**, 2631 (1971)]. Spectra are recorded for 297 and 77°K (—); the 77°K curves have been offset to facilitate viewing. Peroxidase (1.9 mM) was dissolved in water-glycerol (1:1, v/v) containing 50 mM sodium phosphate (pH 7). Notes: (A, absorption): Approximate absorption of heme moiety at 297°K is shown by dashed line. Vibronic assignments are given for aromatic bands at 77°K. B, Circular Dichroism: Dichroism is recorded as ($A_L - A_R$). Cell path length 0.12 mm. Data are not shown at $\lambda < 270$ nm because this investigation was concerned primarily with tryptophan CD.

zyme,^{89,90,92} avidin,²⁴⁴ hemoglobin,²⁹⁷ peroxidase,²¹³ chymotrypsinogen,⁸¹ and carboxypeptidase.²¹⁷ Cystine bridges in ribonuclease,^{212,233,234} lysozyme,^{92,233} insulin,²³³ the neurophysins,⁹⁴ and other proteins (see Beychok^{33,37(c)} for a review) have been investigated.

Strickland's laboratory has been able to differentiate CD bands arising from various side-chain residues by means of their vibronic fine structure. Much of the work was performed at low temperature, where band resolution is better. An example (horseradish peroxidase A1)

²⁹⁰ N. S. Simmons and A. N. Glazer, *J. Amer. Chem. Soc.* **89**, 5040 (1967).

²⁹¹ H. Hashizume, M. Shiraki, and K. Imahori, *J. Biochem. (Tokyo)* **62**, 543 (1967).

²⁹² E. R. Simons, E. G. Schneider, and E. R. Blout, *J. Biol. Chem.* **244**, 4023 (1969).

²⁹³ J. Bello, *Biochemistry* **8**, 4535 (1969).

²⁹⁴ D. D. Ulmer, *Biochemistry* **5**, 1886 (1966).

²⁹⁵ Y. P. Myer, *J. Biol. Chem.* **243**, 2115 (1968).

²⁹⁶ T. Flatmark, *J. Biol. Chem.* **242**, 2454 (1967).

²⁹⁷ A. Wollmer and G. Buse, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **16**, 307 (1971).

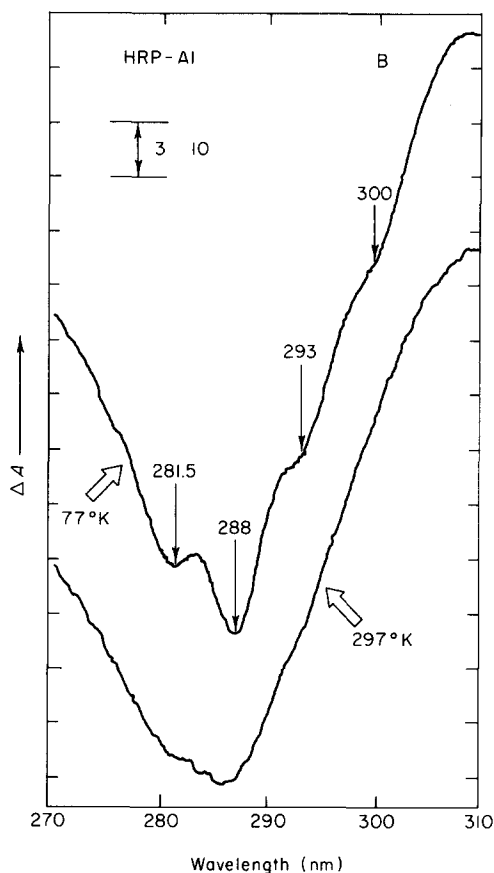


Fig. 16B.

is shown in Fig. 16.²¹³ The CD bands due to a given type of side chain can then in favorable cases, be correlated with specific residues (see discussion of ribonuclease,²¹² which will follow) and yields information about their environment.

Chiroptical properties of prosthetic groups are sometimes studied, for example in hemoglobin. These bands can be resolved from protein bands; this has been done for the heme groups of peroxidase,^{213,260,298} hemoglobin,²⁹⁷ and cytochrome *c*.²¹³ Information about stoichiometry and manner of coenzyme and metal ion binding to enzymes can be obtained

²⁹⁸ K. Hamaguchi, K. Ikeda, C. Yoshida, and Y. Morita, *J. Biochem. (Tokyo)* **66**, 191 (1969).

by monitoring the extrinsic Cotton effects (in the region above 300 nm) formed upon enzyme-cofactor complex formation in the absence or in the presence of substrate. The ORD of several such systems has been reviewed.⁹⁶ An example of a similar CD investigation (of beef liver glutamate dehydrogenase and NADH) can be seen in Koberstein and Sund.⁹⁷

Many investigations employ changes of pH to distinguish between side-chain bands; tyrosine residues show a particularly large red shift upon ionization (see Fig. 13). Protein denaturation [by heat, extremes of pH, or addition of a denaturant (e.g., guanidine hydrochloride)] is another common tool; in this way the protein environment of given residues can be varied, and the effect upon CD or ORD studied. Most of the papers catalogued above measure only near-UV CD ($\lambda > 240$ nm); a few deal also with the shorter wavelength region.

Chemical modification of specific residues shows promise of becoming an important CD technique; tyrosine in insulin⁸⁸ and in lysozyme,⁸⁹ and tryptophan in lysozyme,^{89,90} have been studied in this manner. The effects of substrate or inhibitor binding upon side-chain CD bands have been measured for carboxypeptidase,⁹³ staphylococcal nuclease,⁹⁵ the neurophysins,⁹⁴ concanavalin,⁹¹ lysozyme,^{90,92} and ribonuclease.⁷⁷

Specific Examples. (i) PANCREATIC RIBONUCLEASE. Several types of information can be obtained from careful ORD and CD studies of a protein. Pancreatic ribonuclease has been a favorite enzyme for study because the molecule does not contain tryptophan residues or much α -helix; thus the assignment of Cotton effects arising from tyrosine and cystine are more certain than in most proteins. Some of the RNase investigations have been summarized.²⁹⁹ In 1965, Glazer and Simmons²⁸⁸ first studied the ORD spectrum of ribonuclease. A band was found near 278 nm at pH 6.2 which shifted to 292 nm at pH 11, showing that the band was associated with tyrosine residues exposed to solvent and therefore susceptible to ionization. Sodium dodecyl sulfate removed these bands; thus the asymmetry arose from specific interactions of the tyrosine residues with their native environment. Simpson and Vallee²⁸⁹ found that these external tyrosine bands could be modified without affecting the rest of the ORD spectrum. Simmons and Glazer²⁹⁰ continued their experiments using CD and found negative CD band at 273 nm at neutral pH which shifted to 285 nm at pH 11. Hashizume *et al.*²⁹¹ compared the CD of ribonuclease A with that of poly-L-tyrosine and found that the ellipticity of the tyrosine residues in the protein was much higher than

²⁹⁹ F. M. Richards and H. W. Wyckoff, in "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 4, p. 647. Academic Press, New York, 1971.

would be expected from the ellipticity of poly-L-tyrosine. They felt, therefore, that the residues buried in the protein, because they were constrained, had a greater contribution to the CD than did the external residues. Upon heating of RNase²⁹¹ the ellipticity at 278 nm decreased in two steps, suggesting that the buried tyrosine residues (which stabilize the tertiary structure of the enzyme) were not unfolded simultaneously with the exposed ones. Simons *et al.*²⁹² and Bello²⁹³ also followed the thermal denaturation of ribonuclease and reached similar conclusions.

Horwitz *et al.*²¹² examined the near-UV CD spectrum of ribonuclease at 77°K. Their results are shown in Fig. 17. By correlating the CD with the vibrational fine structure displayed by the protein and by model compounds in absorption spectra, they were able to differentiate the CD contributions of buried and exposed tyrosine residues and of S-S bridges. The same group studied RNase-S,²¹⁶ and found that the environment of a single tyrosine residue was changed upon cleavage.

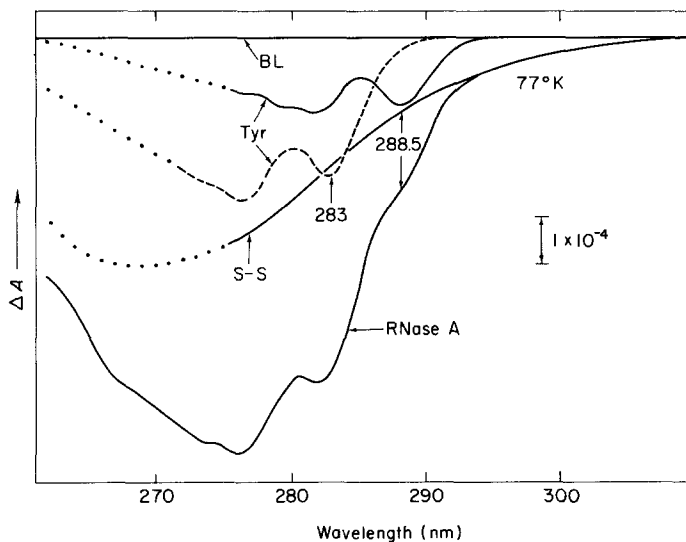


FIG. 17. Near-ultraviolet circular dichroism (CD) spectrum of ribonuclease A at 77°K [from J. Horwitz, E. H. Strickland, and C. Billups, *J. Amer. Chem. Soc.* **92**, 219 (1970)]. Protein concentration 3.6 mM; path length 0.2 mm; solvent water-glycerol (1:1, v:v) with 25 mM sodium phosphate. BL is the baseline. The spectrum has been resolved into components corresponding to CD contributions of various side chains; dotted lines represent regions of extrapolation. S-S designates disulfide contribution; the dashed tyrosine curve results from three exposed tyrosine residues; the small, solid tyrosine curve is caused by one buried tyrosine. Two additional buried tyrosine residues contribute negligibly (<5%) to the total CD spectrum, and were not considered in this analysis.

Pflumm and Beychok²³⁴ studied the reduction and reoxidation of pancreatic ribonuclease and found that the tyrosine CD band at 240 nm appeared to be associated with formation of the correct S-S bridges of the intact enzyme. Simons⁷⁷ examined the effect of binding an inhibitor, 3'-CMP, upon this environmentally sensitive 240-nm tyrosine band. She found that a tyrosine side chain, far removed from the active site of RNase and previously buried, became exposed to solvent as the enzyme-inhibitor complex formed.

The far-ultraviolet CD spectrum of RNase^{21,37(c)} shows little evidence of the characteristic α -helix 222-nm band (see Fig. 8). Since the CD conformational determination²¹ is in good agreement with the X-ray data,³⁰⁰ RNase is probably not a case (like avidin²⁴⁴) in which far-ultraviolet side-chain CD bands seriously obscure the peptide Cotton effects.

(ii) **LYSOZYME.** An aromatic (probably tryptophan) Cotton effect near 280 nm was found in the ORD spectrum³⁰¹ of hen egg-white lysozyme. Exposure of the enzyme to sodium dodecyl sulfate destroys this Cotton effect, but does not change the 233-nm trough characteristic of the α -helical conformation. The near-ultraviolet CD spectrum was resolved³⁰² into three positive bands between 280 and 300 nm and a negative band at 262 nm; the latter band has been attributed to disulfide.²³³ In the presence of the competitive inhibitor, *N*-acetyl-D-glucosamine, the aromatic CD bands are intensified,³⁰² which indicates that exposed aromatic residues at the substrate-binding site are optically active. By studying the effect of pH, Ikeda *et al.*³⁰³ found that some of the optically active tyrosine residues are exposed to solvent, can be ionized at alkaline pH, but become optically inactive when lysozyme is denatured by exposure to pH > 12.

More recently, Halper *et al.*⁹² have distinguished between tyrosine, tryptophan, and disulfide CD bands by means of pH dependence. They compared human with hen egg-white lysozyme and found differences in the near-UV CD region, although the enzymes displayed similar far-UV CD spectra, indicating analogous secondary structure. Two recent articles^{89,90} deal with chemical modification of hen lysozyme: Teichberg and co-workers⁹⁰ selectively oxidized tryptophan-108, and determined its CD contribution by means of CD difference spectra. They found that this residue is responsible for most of the aromatic CD region of

³⁰⁰ G. Kartha, J. Bello, and D. Harker, *Nature (London)* **213**, 862 (1967).

³⁰¹ A. N. Glazer and N. S. Simmons, *J. Amer. Chem. Soc.* **87**, 2287 (1965).

³⁰² A. N. Glazer and N. S. Simmons, *J. Amer. Chem. Soc.* **88**, 2335 (1966).

³⁰³ K. Ikeda, K. Hamaguchi, M. Imanishi, and T. Amano, *J. Biochem. (Tokyo)* **62**, 315 (1967).

lysozyme, that its oxidation does not significantly affect the conformation-dependent ellipticity bands²¹ at 209 and 192 nm, and that there is a change in orientation of Trp-108 when oligosaccharide inhibitors are bound to the active site. Atassi *et al.*⁸⁹ made derivatives of two tyrosines and six tryptophans of lysozyme; examination of ORD and CD data showed various amounts of unfolding.

Conclusion

In conclusion, circular dichroism and optical rotatory dispersion can yield useful estimates of protein secondary structure. In some cases the agreement with X-ray diffraction results is excellent. Furthermore, for synthetic polypeptides CD and ORD are invaluable tools for structural analysis in solution; each basic conformation can be closely correlated with a specific type of CD or ORD spectrum.

However, the mixture of backbone conformations found in proteins can be more complex and varied than those of simple poly- α -amino acids. The model systems used for calculation of protein conformation are far from perfect. Moreover, for proteins there are factors besides the peptide backbone conformation which influence the optical activity. Light scattering in membrane systems is one such case; the contribution of asymmetric side-chain chromophores is another.

The contributions of aromatic residues and disulfide bonds to the optical activity of proteins are complicated, but very interesting. Recent advances in instrumentation and techniques have made it possible to begin to sort out the chiroptical properties of individual amino acid residues and the effects of inhibitor binding at active sites. Such results can be used to gain insight into the location and function of specific residues within a protein. On the other hand, the complexity of the spectra of polypeptides containing aromatic and cystine residues reemphasizes the pitfalls inherent in trying to estimate protein and polypeptide structure from optical activity alone. There is a need to correlate variations in conformation and in side-chain orientation with their contributions to CD and ORD, and to apply this knowledge systematically to proteins.