

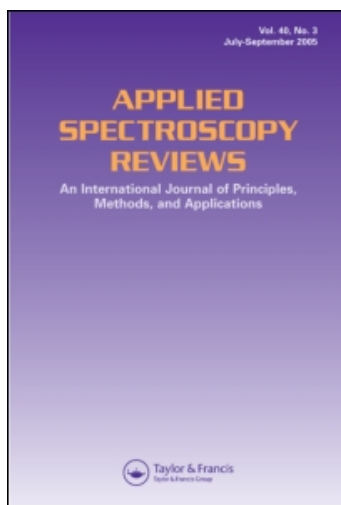
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Recent Studies of the Circular Dichroism and Optical Rotatory Dispersion of Biopolymers

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Recent Studies of the Circular Dichroism and Optical Rotatory Dispersion of Biopolymers

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I. PHENOMENA

When electromagnetic radiation interacts with matter, a number of changes result both in the light and in matter. The intensity of the light at given wavelength may be reduced due to absorption; the velocity of light may change, dependent on the wavelength, upon interaction with the medium due to refractive index dispersion (scattering). If the incident light is plane polarized, a change in the plane of polarization may occur, again dependent on wavelength [optical rotatory dispersion (ORD)]. Also, the radiation may become elliptically polarized at the absorption bands; this phenomenon is termed circular dichroism (CD). While normal absorption and dispersion occur with any molecule, ORD and CD require not only polarized light but also molecular systems with built-in asymmetry or dissymmetry.

Ordinary absorption and dispersion arise due to energy level transitions involving redistribution of charges or electronic distribution in matter. Absorption occurs when the frequency of incident light matches the frequency corresponding to the difference between two energy levels in the system. Redistribution of the electronic structure associated with the reemission process results in scattering of the light and is connected with the dispersion characteristics of the medium (i.e., refractive index). Focusing our attention over an absorption wavelength of the system, one notices the behavior of the refractive index as shown in Fig. 1. The refractive index n_{ij} associated with a transition ij in the molecule increases with decreasing λ . Near λ_{ij} , one notices anomalous dispersion; near $\lambda = \lambda_{ij}$, n_{ij} becomes zero. On the other hand, the absorption curve is gaussian, with a maximum value k_{ij} at $\lambda = \lambda_{ij}$.

The quantities $n_{ij}(\lambda)$ and $k_{ij}(\lambda)$ are interrelated through the Kronig-Kramers transforms:

$$n_{ij}(\lambda) = \frac{1}{2\pi^2} \int_0^\infty k_{ij}(\lambda') \frac{\lambda^2}{\lambda^2 - \lambda'^2} d\lambda' \quad (1)$$

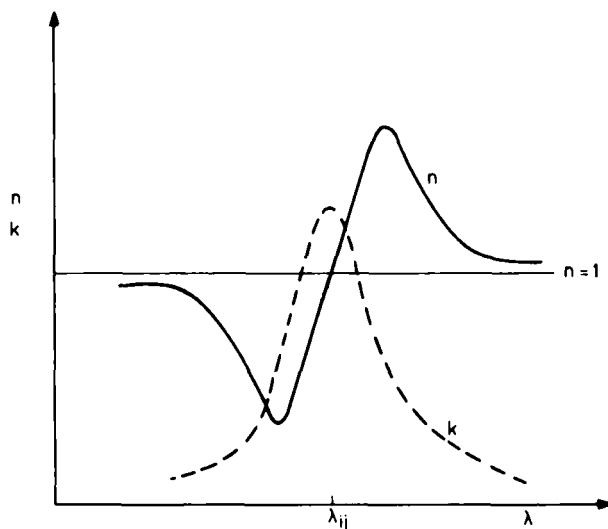


FIG. 1. Behavior of refractive index (n) and absorption coefficient (k) as a function of wavelength (λ). λ_{ij} is the wavelength corresponding to a transition ij .

$$k_{ij}(\lambda) = -8 \int_0^\infty n_{ij}(\lambda') \frac{\lambda'}{\lambda^2 - \lambda'^2} d\lambda' \quad (2)$$

It is seen that knowledge of one quantity can be used for the deduction of the other. The absorption coefficient k_{ij} is related to a spectroscopic quantity termed the dipole strength D_{ij} of the transition by the Mulliken relation

$$D_{ij} = \frac{3hc}{8\pi^3N} \int_0^\infty \frac{k_{ij}(\lambda)}{\lambda} d\lambda \quad (3)$$

and it has been shown by quantum mechanics that the dipole strength $D_{ij} = |\langle \phi_i | \mu | \phi_j \rangle|^2 = |\mu_{ij}|^2$, where μ_{ij} is the electric transition dipole moment for the transition from state i (wavefunction ϕ_i) to state j (ϕ_j). It is therefore necessary that in order for absorption to occur, the transition electric dipole moment be nonzero, and this forms the basis of selection rules for absorption. It can also be seen from Eqs. (1) and (3) that, in principle, it should be possible to deduce the values of μ_{ij} and D_{ij} from the dispersion curve.

Figure 2 schematically depicts the phenomena of optical rotation and CD. Molecules with asymmetric (or dissymmetric) geometric arrangements, termed optically active, rotate the plane of polarization of the incident beam by an angle α . Plane polarized light may

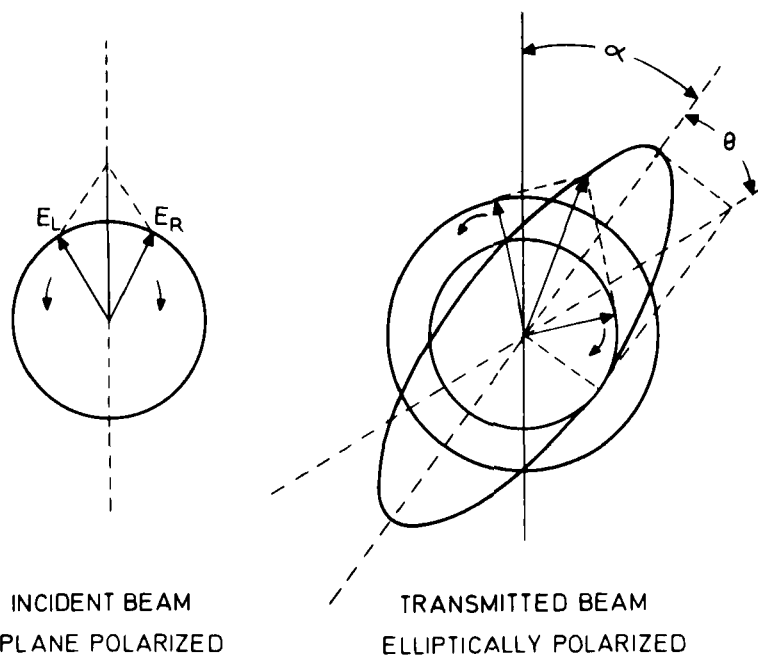


FIG. 2. Generation of optical rotation (α) and ellipticity (θ) by an optically active sample.

be resolved into two circularly polarized components, one right circular and the other left, that are of the same amplitude and in phase. The optically active sample scatters the two components unequally, i.e., the refractive indices n_l and n_r for the left and right circular components, respectively, become unequal. Vector addition of the two scattered components leads to a plane of polarization at an angle α away from the incident plane. This difference ($n_l - n_r$) is termed circular birefringence. Further, the absorption coefficients of the two components k_l and k_r are also unequal, i.e., one component is absorbed more than the other. This results in a difference in the amplitude of the two components in the scattered light, and also a phase difference, resulting in elliptical polarization of the exit beam (see Fig. 2). The angle of ellipticity, θ , is defined as the arctangent of the ratio of the minor to major axes of the ellipse. Notice also that while k_l , in ordinary absorption is always positive or zero, $\Delta k (= k_l - k_r)$ can be zero, positive, or negative, as also ($n_l - n_r$). The angle of rotation and ellipticity are related as follows:

$$\alpha = \frac{\pi}{\lambda} (n_\ell - n_r) \text{ radians/cm} \quad (4)$$

$$\theta = \frac{1}{4} (k_\ell - k_r) \text{ radians/cm} \quad (4a)$$

Expressed in molar terms,

$$\text{molecular rotation } [m]_\lambda = \frac{\alpha 18 M}{\pi C'} \text{ degree/cm}^2 \text{ dmole} \quad (5)$$

$$\text{molecular ellipticity } [\theta]_\lambda = \frac{\theta 18 M}{\pi C'} \text{ degree/cm}^2 \text{ dmole} \quad (5a)$$

where M is the molecular weight of the sample and C' its concentration in g/cm^3 . The subscripts λ refer to the wavelength of measurement. $[m]_\lambda$ and $[\theta]_\lambda$ for a transition ij are interrelated by the Kronig-Kramers transforms as:

$$\{[m]_{ij}(\lambda)\} = \frac{2}{\pi} \int_0^\infty \{[\theta]_{ij}(\lambda')\} \frac{\lambda'}{\lambda^2 - \lambda'^2} d\lambda' \quad (6)$$

$$\{[\theta]_{ij}(\lambda)\} = \frac{-2}{\pi\lambda} \int_0^\infty \{[m]_{ij}(\lambda')\} \frac{\lambda'^2}{\lambda^2 - \lambda'^2} d\lambda' \quad (7)$$

Often $[\theta]_\lambda$ is expressed not as $(k_\ell - k_r)$ but in terms of the decadic molar CD, $[E_\ell - E_r] \equiv \Delta E$ as:

$$[\theta]_\lambda = 3300(E_\ell - E_r) = 3300\Delta E \quad (8)$$

and since the observed differential optical density $\Delta A = (A_\ell - A_r)$ is related by Beer's law to ΔE , one can evaluate ΔE directly from ΔA measurements. ΔE has units of cm^2/mole .

It is important to reiterate that unlike absorption, $[m]$ and $[\theta]$ can take positive or negative signs for a given transition ij . The spectral quantity in ORD or CD that corresponds to the dipole strength in absorption is termed rotational strength, R_{ij} , connected with the transition ij and is expressed as

$$R_{ij} = \frac{3hc}{8\pi^3 N} \int_0^\infty \frac{\theta_{ij}(\lambda)}{\lambda} d\lambda \quad (9)$$

In principle, R_{ij} can be obtained from the CD curve or from the ORD curve through the use of the Transform (6). Quantum mechanically,

the rotational strength R_{ij} is given by the relation

$$R_{ij} = \text{Im}\{(\phi_i | \boldsymbol{\mu} | \phi_j) \cdot (\phi_j | \mathbf{m} | \phi_i)\} \quad (10)$$

where Im stands for the imaginary part. It is seen that the rotational strength is the dot product of the electric transition moment $\boldsymbol{\mu}_{ij}$ and the transition magnetic dipole moment \mathbf{m}_{ij} . In order for a given absorption band to be optically active, i.e., for $R_{ij} \neq 0$, it is required that neither $\boldsymbol{\mu}_{ij}$ nor \mathbf{m}_{ij} vanish, and further that they not be orthogonal. The selection rules for optical activity are also stringent in that, since $\boldsymbol{\mu}$ is an odd operator while \mathbf{m} is even [$\boldsymbol{\mu} = e \cdot \mathbf{r}$ and $\mathbf{m} = e/2mc (\mathbf{r} \times \mathbf{p})$ where \mathbf{r} is the displacement and \mathbf{p} the linear momentum], the molecule should neither have a center nor a plane of symmetry. R_{ij} transforms as a pseudoscalar, and can have zero, positive, or negative values. The relation between optical rotation and rotational strength is given by

$$\alpha_{ij} = \left(\frac{16\pi^2 N}{3hc} \right) \frac{R_{ij} \nu^2}{\nu_{ij}^2 - \nu^2} \quad (11)$$

The dependence of α on λ (i.e., ORD) is shown in Fig. 3, along with the corresponding CD curve. Notice the anomalous dispersion near λ_{ij} . This anomalous dispersion in the vicinity of an optically active absorption band is referred to as a Cotton effect. This term is used interchangeably to denote the ORD behavior or the CD band itself. In a negative Cotton effect, ΔE is negative, and the rotation is negative at $\lambda > \lambda_{ij}$. In a positive Cotton effect, the reverse is true.

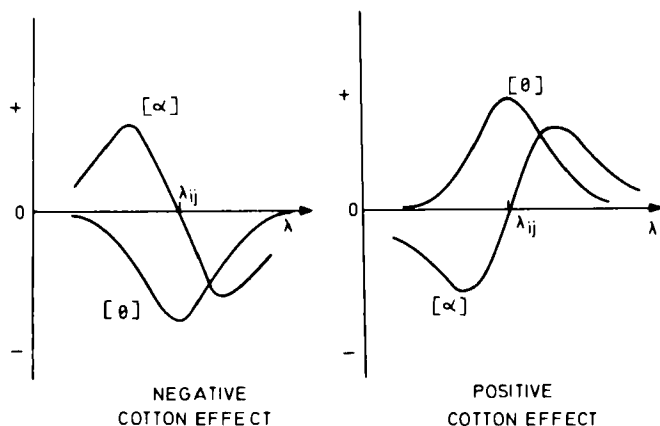


FIG. 3. The Cotton effect corresponding to an absorption band (λ_{ij}) of an optically active sample.

Equation (11) is the famous Rosenfeld equation which gives a quantum-mechanical framework on which further calculations are based. Schellman [1] has comprehensively reviewed the various molecular theories proposed for the generation of cotton effects. (The reader is referred to other excellent reports in this area for detailed discussions of the theory of optical rotation [2-6].) In essence, such calculations involve an evaluation of the rotational strengths of various transitions in a molecule, and predicting their signs, magnitudes, and changes that occur in these as a function of the molecular conformation, interactions between groups, and the absolute configuration. Optical rotatory methods give only the relative orientations of transition moments in its constituents and not the positions and orientations of atoms. Successful theories will attempt to predict R_{ij} values for several molecular conformations (the effect of the other perturbing centers on the R_{ij} of a given chromophore, the so-called regional selection rules), ways in which coupling between two groups in a molecule occurs to generate optical activity, and so on. It is known, for example, that two groups coupled to one another will generate a magnetic moment even if charge displacements within each group are locally linear. Accordingly, one can select as a model of optical activity a molecule that has only two groups and only two electronic transitions. The two groups are allowed to interact through a potential V_{12} that is a sensitive function of the relative dispositions of the groups. The coupling between them may be dipolar (μ_1, μ_2 coupling), electric-magnetic (m_1, μ_2), or alternately, both transitions may be in the same group, one electric (μ_β) and the other magnetic (m_α), and these couple through a potential of the form $V_{\alpha\beta}$ with a static environment. This is referred to as the one-electron mechanism where the environment (i.e., rest of the molecule) perturbs to break down the symmetry of the chromophore and "mixes" the two transitions.

In the one-electron mechanism, developed by Condon, Altar, and Eyring, both transitions are in the same groups. One is magnetic (m_α) and the other electric (μ_β). These two moments couple in an otherwise static environment to yield a rotational strength

$$R_\alpha = -V_{\alpha\beta}(\mu_\beta \cdot m_\alpha)/(E_\beta - E_\alpha)$$

The form of the potential $V_{\alpha\beta}$ that induces optical rotation depends on the symmetry of the chromophore itself. Since R_α is a pseudo-scalar, $V_{\alpha\beta}$ will also belong, or contains a part that belongs, to a pseudoscalar representation. We discuss this point a little later. A second theory, due to Kuhn and to Kirkwood, involves the production of magnetic moments by linear electric transitions, μ_1 from group 1 and μ_2 from group 2. Dipole-dipole coupling of this kind

leads to a rotational strength

$$R_1 = -V_{12}E_1E_2[\mathbf{R}_{21} \cdot \boldsymbol{\mu}_2 \times \boldsymbol{\mu}_1]/hc(E_2^2 - E_1^2)$$

where E_1 and E_2 are the energies of the bands of groups 1 and 2, \mathbf{R}_{21} the vector distance between the groups, and V_{12} the dipolar interaction energy given by

$$V_{12} = \frac{\boldsymbol{\mu}_1 \cdot \boldsymbol{\mu}_2}{R^3} - \frac{3(\boldsymbol{\mu}_1 \cdot \mathbf{R})(\boldsymbol{\mu}_2 \cdot \mathbf{R})}{R^5}$$

If groups 1 and 2 are identical, the excited states are degenerate and the two groups participate equally in the resulting coupled transitions. Such identical group interactions that give rise to a pair are referred to as the exciton couple, and the importance of such exciton interactions has been brought out by Moffitt [7] who used this formalism to analyze the cotton effects of polypeptide helices. Such an identical pair of groups interact by a dipole-dipole coupling mechanism to yield a rotational strength

$$R = \left[\pm \frac{\pi}{2\lambda_1} \right] [\mathbf{R}_{21} \cdot \boldsymbol{\mu}_2 \times \boldsymbol{\mu}_1]$$

We briefly discuss the Moffitt exciton model in the section on the polypeptide α -helix. The third mechanism by which optical activity is generated is by the interaction between group 1 which has a magnetic transition (\mathbf{m}_1) and group 2 with an electric transition ($\boldsymbol{\mu}_2$). Such an electric-magnetic coupling yields optical activity to both transitions:

$$R_1 = -V_{12}[\boldsymbol{\mu}_2 \cdot \mathbf{m}_1]/E_2 - E_1$$

This mechanism is considered important in the optical activity of cyclic peptides and polypeptides.

A. Regional Selection Rules

Schellman [4] has emphasized the point that the potential function must have the symmetry properties of a pseudoscalar in the symmetry group of the unperturbed chromophore in which it generates optical activity. This is necessary since the rotational strength is a pseudoscalar quantity. He has considered several chromophores of different point groups and identified terms in the potential function that correspond to these various point group symmetries. The

potential energy of two arbitrary charge distributions can be written in a series of charge, dipolar, quadrapolar, and higher terms as

$$V = \sum F_{ij}(q_i^1, q_i^2, \mathbf{R})$$

where q_i^k is the i -th moment of charge in group k , and $\mathbf{R} = \mathbf{R}_2 - \mathbf{R}_1$ is the distance between the local origins of groups 1 and 2. F_{ij} are functions that characterize each type of multipolar interactions.

An immediate outcome of this analysis is that it is possible to divide the space surrounding the chromophore into regions or sectors (with nodal planes) and to qualitatively predict the sign of the potential function and hence the sign of the cotton effect in these various sectors. Such a division is termed the regional rule of optical rotation and depends on the chromophore symmetry. This analysis not only explains the empirical rules of predicting cotton effect signs in molecules, i.e., the axial haloketone rule and the octant rule in ketones [8], but extends it to many other chromophores. The importance of this in molecular conformational analysis is evident.

Recently several reviews on ORD/CD have appeared [6, 9-11] and the reader is referred to them for details. We have attempted here to choose representative examples from recent literature that show the utility of these techniques in problems connected with conformations of biopolymers.

II. INSTRUMENTATION

The schematic diagram of a combination ORD/CD spectropolarimeter is given in Fig. 4. Light from an intense xenon (or Xe-Hg or Hg-Ar) lamp is collimated and monochromated by a double prism

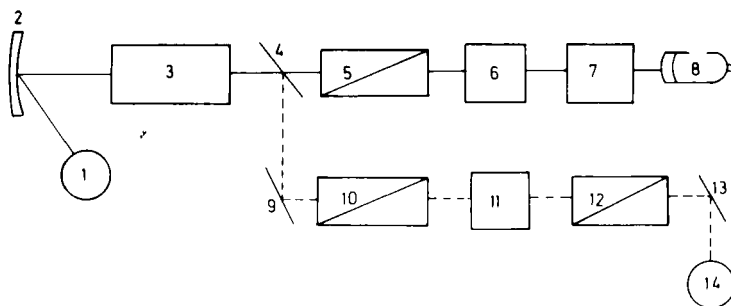


FIG. 4. Schematic diagram of combination ORD/CD spectropolarimeter. 1: Source. 2: Source mirror. 3: Monochromator. 4: Retractable ORD mirror. 5: CD polarizer. 6: Pockels cell. 7: CD cell. 8: CD photomultiplier. 9: Plane mirror. 10: ORD polarizer. 11: ORD cell. 12: ORD analyzer. 13: Plane mirror. 14: ORD photomultiplier.

monochromator and is then plane-polarized by passage through a "polarizer." The polarizer is a Rochon prism of silica or ammonium dihydrogen phosphate. In the ORD mode the plane polarized beam passes through the sample and reaches the phototube through the "analyzer," which is another Rochon prism fixed at a crossed position with respect to the polarizer. The polarizer is continually oscillated through $\pm 1^\circ$ at 10 Hz. When the sample is optically active, the intensities of the successive pulses of light reaching the phototube detector are unequal, and the analyzer is rotated by means of a servodrive till this difference in intensity between successive pulses vanishes. The angle through which the analyzer is rotated is measured and this represents the optical rotation of the sample. In an alternate arrangement, as in the Cary 60 machine, a mirrored Faraday cell, interposed between the sample and the analyzer, is used to cyclically displace the plane of polarization of the beam, rather than the rocking polarizer arrangement.

In the CD recording mode, the plane polarized monochromatic beam passes through a Pockels cell quarter-wave plate. The quarter-wave plates are made of uniaxial crystals which become biaxial when subjected to an electric field, such as ammonium dihydrogen phosphate. By applying a suitably programmed alternating voltage, the quarter-wave plate can be made to give out light that is alternately left and right circularly polarized by retarding one of the components of the plane polarized light selectively. The light then reaches the photomultiplier through the sample cell. When the sample exhibits equal absorption of right and left circularly polarized light, the two components reach the photomultiplier in equal intensity. When the sample shows differential absorption, an ac modulation is caused in the photomultiplier output, the amplitude of which corresponds to the magnitude of CD and the phase to the sign. This ac modulation signal is filtered, amplified, and recorded. Apart from the commercial CD machines, various accessories for the adaptation of UV-visible double beam spectrophotometers for CD measurements are also available (e.g., the Rehovoth model CD-LD-HC accessory).

Many designs have appeared [12-15] that extend the range of CD measurements down to about 130 nm in vacuum UV. In these machines the design is generally similar to the normal CD machines outlined above except that the whole machine is incorporated into an air-tight jacket that can be evacuated, and optical components made of MgF_2 or CaF_2 are used which are transparent through the whole range of the instrument. Vacuum UV measurements are becoming important both to test rigorously theories of CD/ORD and as a method of better characterization of biopolymer spectra due to the large number of bands found in the vacuum UV range.

The measurement of small changes in CD and ORD is important for studies of the effect of perturbants on protein conformation, enzyme-substrate interactions, and so on. This was originally done by simply running the various spectra separately and then subtracting one measurement from the others, or else some other indirect method was adopted. For example, Chignell and Gratzer [16], in an attempt to measure small differences in strong ORD spectra, increased the sensitivity of the measurement by using concentrated sample solutions and placing a solution of known ORD of opposite sign in series with the sample. This, in effect, pushes the instrumental baseline down or up as needed.

Recently, various optical systems have become available which permit the direct measurement of difference CD and ORD. These have been reviewed by Yang and Chau [17].

Difference ORD measurements have been made using a modified (Fig. 5) Cary-60 spectropolarimeter [18]. The sample is contained in cell 1 and the reference is in solution in cell 2. Cell 1 is placed between the polarizer and the Faraday cell while cell 2 is in between the Faraday cell and the analyzer. Due to reflection in the Faraday cell, the beam incident on the reference cell has a plane of polarization opposite to that of the beam when emergent from the sample cell. Thus the effect of the reference is to rotate the reflected beam back toward the mean position, and the analyzer only senses the differences in the rotatory power of reference and sample.

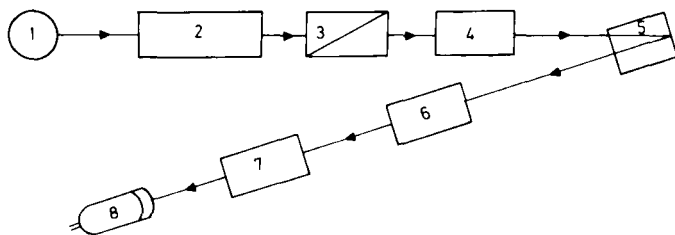


FIG. 5. Schematic diagram of difference ORD setup. 1: Source. 2: Monochromator. 3: Polarizer. 4: Sample cell. 5: Faraday cell. 6: Reference cell. 7: Analyzer. 8: Photomultiplier.

Difference CD measurements are normally made using the assembly in Fig. 6. In this, a half-wave plate and a reference cell are placed between the sample cell and the photomultiplier. The half-wave plate is either a mirror or two Fresnel rhombs, devices that effect a 180° retardation of light. The principle of operation of this setup is simple. The plane polarized light that enters the quarter-

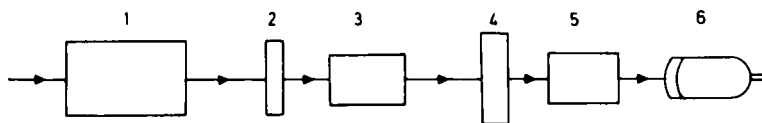


FIG. 6. Schematic diagram of difference CD setup. 1: Polarizer. 2: Quarter wave plate. 3: Sample cell. 4: Halfwave plate. 5: Reference cell. 6: Photomultiplier.

wave plate emerges alternately left and right circularly polarized. The sample may absorb preferentially the left or the right component. Now, the half-wave plate, by retarding the phase of the light beam passing through by 180° , changes the right component into the left circularly polarized component and vice versa. The light now passes through the reference, which acts similar to the sample, except that the reference and samples are out of phase in their action by 180° . If identical (say left component absorbing) substances are placed in the reference and sample cells, the light reaching the photomultiplier will be constant as the two components are alternately absorbed by the sample and the reference equally due to the effect of the half-wave wave plate. Under this condition the photomultiplier will have no CD output and subtraction will be complete. If the reference and sample are not identical, then their difference CD spectrum will be recorded.

A stopped flow circular dichroism system for kinetic studies with a sensitivity of 2×10^{-5} units (ΔA) and time resolution of 2 msec has been described by Bayley and Anson [19]. This system uses high frequency (50 kHz) modulation of the monochromatic light beam (monitored by a beam splitter and photodiode) by a polarizer and a piezo-optical modulator. The emergent beam is passed through the sample chamber of a stopped flow mixing device and is detected by a fast response photodiode. When the sample exhibits CD, the dc signal at the photodiode is modulated at 50 kHz with an amplitude proportional to the transmittance and CD of the sample. By separating the low and high frequency signals, the absorbance and CD of the sample can be monitored over time simultaneously.

The range of the ORD measurements has been expanded in the near-IR region up to 2500 nm in order to detect the effect of the optical activity of the vibrational transitions [20].

Accessories for measurement of ORD and CD at temperatures from 0 to 100°C , as well as at liquid helium or nitrogen temperatures, for studies under magnetic fields up to 50 kG and for studies in the vapor phase, etc., are now readily available. Modern spectropolarimeters also can be interfaced with computers for multiple scanning and signal averaging techniques.

Sample cells from 0.1 mm to 20 cm in length are normally used. Studies in the visible region require a higher concentration of sample and longer path lengths for uncolored substances. Fixed window sample cells are preferable to removable window cells due to inaccuracies in path length and possible stress birefringence in the latter case.

A. Artifacts

Artifacts in measurements arise due to a variety of reasons, due both to the instrument and to the sample. Modern spectropolarimeters have minimized the instrumental artifacts (stray light, impurities in optical components, etc.) by using double monochromators, high-purity components, and sensitive photodetection circuits. Linking the spectropolarimeter with a CAT (computer average of transients) setup, a routine feature in many laboratories now, improves the quality of the spectrum by cancelling random noise. Calibration artifacts can be controlled by periodically checking baselines and by using standard samples, e.g., d-camphor 10-sulfonic acid. Another way to check the compatibility of the ORD and CD modes is to run both spectra of the standard sample and deduce one spectrum from the other via the Kronig-Kramers transforms.

For very accurate work the Lorentz correction for the medium at every wavelength has been suggested. Temperature control is also necessary, though small variations are tolerable if the sample does not show conformational alterations in that range. The choice of the solvent is also a factor since the conformation of a molecule is likely to change with solvents, and even if the molecule is conformationally immobile, solvent shifts both in position and intensity are likely, particularly for $n-\pi$ transitions. The concentration of highly absorbing samples should be adjusted to below 2 OD units to avoid the emerging beam from being too weak for detection.

In many biological specimens such as membranes, chloroplasts, and other particulate systems, artifacts arise due to particle scattering. Urry [21], Ottoway and Wetlaufer [22], and Gordon [23] have considered this problem in some detail and have suggested corrections. The Jasco J-20 machine minimizes this scattering loss by placing the CD sample cell in close proximity to the detection phototube. Artifacts in difference ORD and CD measurements have been discussed by Yang and Chau [17].

B. Data Presentation

Optical rotation data at any wavelength are expressed in various units. If α is the observed rotation in degrees at any given wavelength λ in the polarimeter, then the specific rotation is

$$[\alpha]_{\lambda} = 100\alpha/\ell c \quad (12)$$

where ℓ is the length of the sample-containing cell and c is the concentration of the sample in g/100 ml. Rotations are more often expressed as molar rotation, $[m]_{\lambda} = [\alpha]_{\lambda}(M/100)$, where M is the molecular weight of the sample. In the case of polypeptides, proteins, and other biopolymers, one often expresses $[m]_{\lambda}$ as residue molar rotation, where the mean residue molecular weight (mean monomer weight) M_0 is substituted for M . The mean residue rotation is further corrected for the effect of the polarizability of the medium on the effective field seen by the sample, the Lorentz correction, to yield the reduced mean residue rotation

$$[m']_{\lambda} = [m]_{\lambda} \frac{3}{n_{\lambda}^2 + 2} \quad (13)$$

where n_{λ} is the refractive index of the solvent at wavelength λ . Ideally one should then correct $[m]_{\lambda}$ by the Lorentz factor using n at every λ of measurement; it is, however, chosen as a fixed value n_D or $n_{546 \text{ nm}}$.

The Drude dispersion relation for a single Cotton effect at λ_1 is given in the form

$$[m']_i = \frac{96\pi N}{hc} \frac{R_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \quad (14)$$

from which the rotational strength R_i for the transition i can be deduced from $[m']_i^{\lambda}$ values. If there are multiple Cotton effects for the sample, then

$$[m']^{\lambda} = \frac{96\pi N}{hc} \sum_i \frac{R_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \quad (15)$$

which makes evaluation of individual R_i rather difficult from $[m']_{\lambda}$ values. ORD plots, i.e., plots of $[m']_{\lambda}$ (or $[m]_{\lambda}$) against λ are used essentially to identify Cotton effects or to analyze the dispersion terms semiempirically, e.g., Moffitt-Yang plots (see below).

Circular dichroism measurements involve a measurement of the variation of the differential absorption ($\Delta A = A_2 - A_r$) or directly

the ellipticity θ as a function of wavelength:

$$\Delta A = \Delta E c \ell \quad (16)$$

where ΔE is the dichroism ($E_\ell - E_r$) in molar units, c is the concentration in moles (or mean residue moles) per liter, and ℓ is the path length in centimeters. ΔE thus has the units cm^2/mole . If ellipticity θ is measured, then the molar (or mean residue molar) ellipticity is

$$[\theta]_\lambda = \frac{\theta_{\text{obs}} M}{10 c \ell} \quad (17)$$

where M is the molecular (or mean residue) weight, c is the concentration in g/ml, and ℓ is the path length in centimeters. The units of $[\theta]_\lambda$ then are degrees cm^2/dmole . Strictly, the Lorentz factor should be used here also to yield the reduced ellipticity. The relation between $[\theta]$ and ΔE is

$$[\theta] = 2.303 \frac{4500}{\pi} \Delta E = 3300 \Delta E \quad (18)$$

Since the CD bands are gaussian with the amplitude $[\theta]^0$ (or ΔE^0) and width Δ (usually 10 to 20 nm), rotational strengths are easily obtained for every band by measuring the area under the (gaussian) CD curve for that band. A convenient relation between R_1 and the gaussian parameters ΔE_1^0 , Δ_1 , and λ_1 is

$$R_1 = \frac{1}{2.28} \frac{\Delta E_1^0 \Delta_1}{\lambda_1} \text{ Debye magnetons (DM)} \quad (19)$$

where $1 \text{ DM} = 0.927 \times 10^{-38} \text{ cgs units}$.

III. ORD AND CD STUDIES ON POLYPEPTIDES AND PROTEINS

Perhaps in no other area of biochemistry have the techniques of rotatory dispersion and circular dichroism been used for conformational analysis with greater advantage than in the field of peptides and proteins. In retrospect, it is easy to see the reasons behind this. The great variety and number of peptides and proteins encountered in biochemistry and also the great number of unique conformations that each peptide or protein molecule adopts have been a major reason for this. Compared to the relatively fewer number of conforma-

tions seen in polysaccharides and nucleic acids, those of proteins are large indeed. Due to factors such as hydrogen bonding, charge interactions, hydrophobic associations between side chains, multimeric associations and polymer chain prosthetic group bindings, and also the possibility of monitoring Cotton effects due to side-chain chromophores in proteins, such as the aromatic groups, heterocycles, disulfide cross-links, and also prosthetic groups such as the porphyrins have made the measurement of ORD/CD in proteins and peptide systems attractive and fruitful as reflected by the enormous number of reports in this area. Accordingly, we shall be discussing the applications of ORD/CD in proteins and peptides at greater length than the cases of the other important biopolymers such as polysaccharides and nucleic acids. To some extent this also reflects the bias of the authors. We have, in the following pages, tried not to be exhaustive but rather have presented what we consider as some representative examples where the techniques have been of use and important in detecting or highlighting structural aspects and interactions of proteins and peptides.

The widespread availability and use of homopolypeptides have been of great help in the conformational analysis of proteins, since such homopolymers are easily prepared and serve as models for the various chain conformations encountered in polypeptide chains. Equally important has been the study of small molecular linear and cyclic peptides with well-defined conformations. Theoretical studies on the conformational possibilities of dipeptides, i.e., conformational mapping of peptides in a two-dimensional phase space [the coordinates being the two dihedral angles (ϕ arising from rotation around the N—C $_{\alpha}$ bond) and (ψ rotation around C $_{\alpha}$ —CO bond)], and on the rotatory properties of simple peptides have been of great use in this connection. Indeed, for simple dipeptides, extensive calculations on the signs and magnitudes of rotational strengths have been performed as a function of the two dihedral angles ϕ and ψ , and the results of such calculations are of importance in interpreting and analyzing the ORD/CD profiles of peptides and proteins [24].

In the following several sections, attention is focused on representative examples of the use of ORD/CD in the analysis of conformations, conformational transitions, mobility, and interactions of proteins and peptides. Recent reviews of the various types of chain conformations possible, and seen, in polypeptide and protein chains are to be found in Hopfinger [25] and in Fraser and McRae [26].

A. The α -Helical Structure

The α -helix is a single stranded helical structure in which there are 3.6 amino acid residues per turn and which can be of a right-handed or left-handed twist. This conformation is adopted either

wholly or in part by many synthetic polypeptides, fibrous proteins, globular proteins, and even oligopeptides in the solid and solution states. Accordingly, a great deal of study has been focused on the α -helix. Interpeptide hydrogen bonding between the CO of the i -th and the NH of the $(i + 4)$ -th residue occurs in the α -helix, leading to a 13-membered hydrogen bonded ring; hence the notation 3.6_{13} for the α -helix. The most commonly found ordered structure in synthetic poly L-peptides and in several proteins is the right-handed (α_R) helix. While a poly-L-peptide can also fold in a left-handed spiral way (α_L helix), potential energy considerations based on side chain-side chain interactions generally disfavor this form. A poly-L-peptide that does adopt the α_L helix is poly- β -benzyl-L-aspartate. Interestingly, substitution in the aromatic ring (such as p-Cl) changes the side-chain nonbonded interaction energy and sends the molecule into the α_R fold, as monitored by optical rotation measurements [27].

The intramolecularly hydrogen-bonded helix offers itself as an interesting candidate for dipolar interactions, and hence exciton spectral interactions among the geometrically regularly oriented peptide chromophores as was originally visualized by Moffitt [7]. Moffitt treated this problem along the lines of identical-particle interactions such as occur in linear crystals, i.e., interchromophore dipolar interactions that are sensitive to their mutual spatial orientation, and the banding of levels that obtain as a result. However, the monomeric elements in a helix are related in the sense of an advancing screw rather than of a simple translation, and hence the associated optical properties differ considerably. The main difference in the spectrum of a helix made up of identical chromophores and the associated linear array lies in their respective selection rules: in the latter case only one transition is allowed within a given excitation band, while for a helix there are two allowed, with one of these being doubly degenerate. The asymmetric disposition of identical chromophores in a helix then would lead to exciton interactions with a pair of bands (rather than a single N-fold degenerate band), and the polarization of one of the pair will be parallel to the screw axis of the helix and that of the other member of the pair perpendicular. Moffitt's calculations revealed two things for a α_R helix of homopolypeptides: (1) the peptide $N \rightarrow v_1$ (π - π^*) band that occurs in monomers in the 190 to 200 nm region will be split in a helical polypeptide as one band (parallel polarized) near 205 nm and the other around 192 nm (perpendicularly polarized). Since such exciton split bands are characteristic of the regular disposition of monomers as in a helix, such an effect is expected to vanish in a randomly oriented peptide polymer. If one monitors the optical activity of the polymer, the helical structure will display two π - π^* Cotton effects corresponding to the exciton split pair, one at 206 nm

(negative Cotton effect) and the other a positive at 192 nm. (2) As a result of the exciton effect that gives rise to two overlapping bands, the optical rotatory dispersion of the α_R helix in regions removed from the absorption bands will be complex and obey a multiterm Drude dispersion equation. Moffitt and Yang [28] suggested the following modified empirical equation for the analysis of the ORD curves of polypeptides and proteins.

$$[m']_{\lambda} = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (20)$$

where $[m']_{\lambda}$ is the residue molar rotation at the wavelength λ , a_0 and b_0 are two characteristic constants that reflect the conformational status of the polymer, and λ_0 is an empirical constant usually fixed as 212 nm. Analysis of a large number of polypeptides and proteins has yielded the value of $b_0 = -630$ as the characteristic value for a completely α_R helical polypeptide chain (with $\lambda_0 = 212$ nm). The value of $b_0 = +630$ is indicative of a α_L -helix, while for a chain with randomly coiled segments, b_0 has a value close to zero. The term a_0 usually does not display such a conformation-dependent constancy, and its value depends on several factors such as the solvent, the nature of the side chains, and the primary structure of the polypeptide or proteins.

The Moffitt-Yang dispersion equation offers the inherent advantage of predicting the conformation status of a protein by analyzing the ORD curve of the sample in the visible-near-UV region. Since the second term containing b_0 vanishes for a randomly coiled conformation, the actual value of b_0 is indicative of the amount of a helical structure in a protein. Before the wide availability of commercial spectropolarimeters that yielded accurate ORD data in the deep-UV region (below 250 nm operationally), most workers relied on the b_0 parameter as an index of the helical content of protein and peptide samples with considerable success. A gratifying example is the case of globular proteins such as hemoglobin and myoglobin, where the percentage helicity as estimated by ORD b_0 values agreed well with the helicity obtained from X-ray diffraction studies on the protein crystals. The fraction of α -helix in a given sample is usually estimated as $-b_0(\text{sample})/630$. A discussion of improvements on the treatment of the ORD data and modifications to the estimation of helical content in proteins is given in the review by Yang [29].

It is to be noted here that the other ordered conformation frequently found in peptides, i.e., the β -sheet, has a value of b_0 in the range of zero (with $\lambda_0 = 212$ nm), although values as different as -80 (for poly-O-acetyl-L-serine in dichloroacetic acid) to $+90$ (silk fibroin in 50% dioxan) have been recorded [29]. Despite this, it has

often been assumed that β -forms have vanishing b_0 values, as have random coils, and b_0 values have been directly translated to helix content. It is also to be realized that absorbing side-chain chromophores will contribute to the ORD curve in several instances, and hence it is likely that in these cases b_0 estimates of helix content might be in error, although in many instances this contribution appears negligible.

It is of greater interest to turn our attention to the UV rotatory properties of the α -helix. Since the availability of recording spectropolarimeters and dichrographs from the early 1960s, the emphasis has shifted from visible region ORD to looking directly at the optically active transitions and Cotton effects either in the ORD mode or more directly at the circular dichroic profiles. The ORD curve of a helical polypeptide is shown in Fig. 7. It is characterized by a trough at 232 to 233 nm (with a rotation of $-19,000$) and a cross-over at 224 and a peak at 198-199 nm ($+72000^\circ$). (Another trough has been observed near 184 nm recently.) The CD spectrum, shown in Fig. 8, is characterized by a negative Cotton effect at 222 nm (-3.5×10^4), a second negative band at 206 nm (approximately the same intensity as the 222 nm band), and a positive Cotton effect centered at 191 nm ($+8 \times 10^4$). Transformation of the CD data into an ORD profile using the Kronig-Kramers transform has yielded values in reasonable agreement with the experimental ORD spectrum [30]. The long wavelength Cotton effect observed near 222 nm is ascribed to the peptide $n-\pi^*$ transition, while the pair at 206 and 192 nm is the two exciton-split $\pi-\pi^*$ transitions; the former is parallel polarized while the latter has a perpendicular polarization with respect to the helix axis. The solvent shift of these transitions, particularly the 222 nm one, is surprisingly minimal. This unusual behavior has been ascribed to the relative inaccessibility of the peptide group atoms inside the helix because of the side-chain groups. In helices of rather short lengths, as in oligomers or in short helical sequences in certain proteins, these groups become a little more accessible, and this is reflected in band maxima shifts, albeit small. The critical chain length that seems required to form a stable α -helix and to display the characteristic α -helical ORD/CD spectrum seems to be 7 residues in many oligopeptide cases [31].

In recent years the rotatory properties of the α -helix in the deep-UV has received attention. Johnson and Tinoco [32] have published the deep-UV CD spectra of several polypeptides as measured in a specially constructed dichroimeter, and these spectral features are illustrated in Fig. 8. Young and Pysh [15] have also constructed a deep-UV CD instrument, and their published CD spectrum of helical poly-L-alanine displays the 222, 206, and 192 nm bands discussed above, a shoulder near 180 nm, a negative Cotton effect near 165 nm,

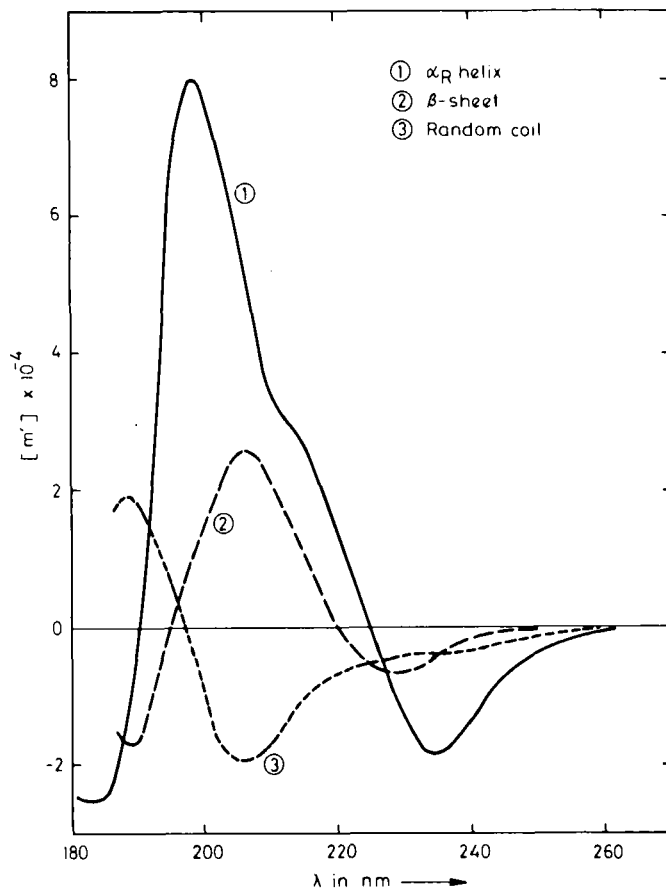


FIG. 7. Ultraviolet ORD curves for (1) right-handed α -helix, (2) antiparallel β -sheets, and (3) randomly coiled polypeptides.

a positive CD band near 140 nm, followed by further negative one below 130 nm. The shoulder near 180 nm is suggested to be part of the 192 nm envelope perturbed by coupling with high-energy monomer or solvent states.

B. The Beta-Pleated Sheet Conformation

Several proteins, e.g., silk proteins such as fibroin, wool keratin, several of the histones, and antibody proteins, have been shown to adopt a planar chain conformation where peptide chains are aligned in a planar form with lateral hydrogen bonding between chains hold-

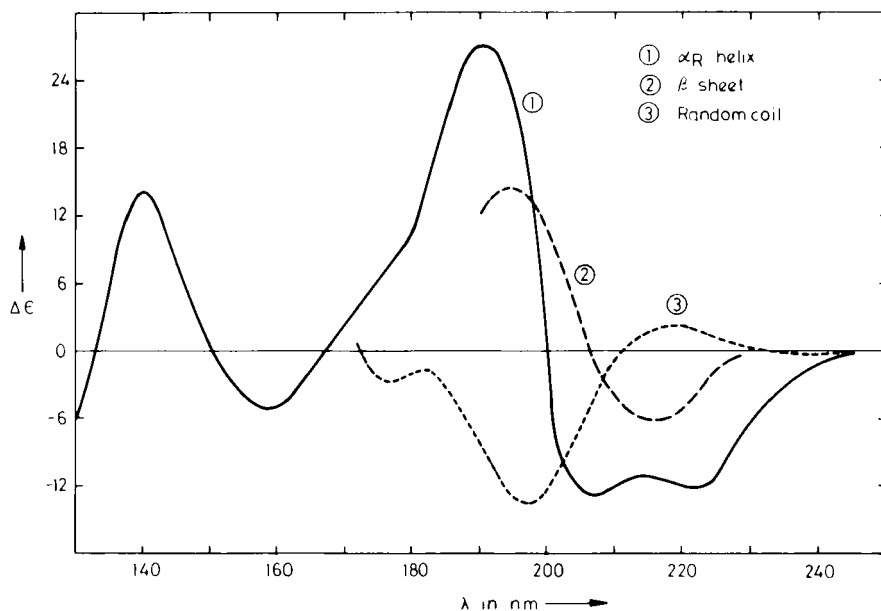


FIG. 8. Far UV CD curves of: (1) right-handed α -helix, (2) β -sheet, and (3) randomly coiled polypeptides. The data have been collected from those reported in Refs. 15, 32, and 34.

ing the sheet together. The synthetic polypeptide poly-L-lysine adopts this pleated sheet form, called the β -structure, under appropriate conditions.

Two kinds of pleated sheets (or β -form) are possible in peptide chains: the parallel β -form in which two peptide chains run parallel and are hydrogen bonded to each other via lateral hydrogen bonds, and the antiparallel β -form where the two peptide chain directions are opposed to each other. It is the latter form that is common in polypeptides and proteins, and hence the optical rotatory properties of this form have received considerable attention both by theory and by experiments.

The ORD curve of the β -form (antiparallel henceforth) is comprised of a trough at 229 to 230 nm (rotation of -5000°), a peak at 205 nm ($+24,000^\circ$), and a second trough near 190 nm ($-17,000^\circ$) (see Fig. 7). The actual magnitudes vary somewhat with the solvent composition. The values quoted above are for silk fibroin in 50% methanol (extrapolated to 100% β -form) [33]. The CD spectrum (Fig. 8) is characterized by a negative Cotton effect centered at 218 nm ($[\theta] = -20,000^\circ$) followed by a positive band at 195 to 197 nm ($+48000^\circ$),

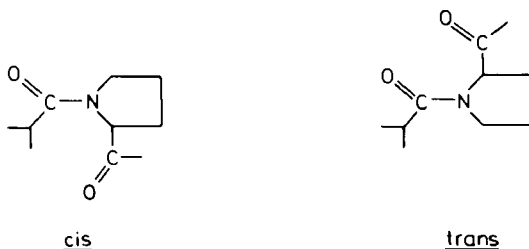
and is thus a simple double dichroic curve, quite different from those of the helix or the random coil. The CD spectrum of the "standard" β -form, i.e., that of poly-L-lysine in water at pH 11.4 heated to 51°C, differs somewhat in magnitude from that of silk fibroin quoted above. The reported ellipticities for poly-L-lysine β -sheets are $-14,300^\circ$ at 217 nm and $+21,500$ at 195 nm [34]. Pysh [35] has calculated the circular dichroism of the antiparallel sheets and has predicted a negative Cotton effect at 218 nm and two contiguous positive Cotton effects at 198 and 195 nm. Calculations for the parallel β -form reveal a negative CD band at 216 nm (rotational strength -4 to -7×10^{-40} cgs) and a positive one at 181 nm (21 to 24×10^{-40} cgs). Several polypeptides and proteins have been found to adopt the antiparallel β -forms either completely or partially. Goodman et al. [36] have recently found that even a poly- β -peptide, poly-L- β -aminobutyric acid, adopts the antiparallel pleated sheet conformation in fluoroalcohol:water solvent mixtures.

The visible ORD spectra of the β -sheet have been analyzed by the Moffitt-Yang equation, but there is no general agreement on the b_0 value that characterizes it. Silk fibroin shows a b_0 value close to zero, and so do γ -globulins and Bence-Jones proteins which exist predominantly in this conformation. On the other hand, the β -form of poly-L-lysine has a b_0 value of -150 . Despite this, the b_0 for a β -sheet has often been assumed to be zero, perhaps more as a matter of convenience.

C. The Polyproline Helices

Besides the α - and β -structures mentioned above, a special set of helical forms occurs in peptides and proteins which have a high composition of the imino acid proline. The homopolymer polyproline is found to occur in two distinct helical forms, one right handed and the other left handed. Proline-rich proteins such as collagen are seen to adopt a triple-stranded helical twist, with individual strands also helical, similar to the one seen in polyproline.

The prolyl residue in peptides is capable of existing in the *cis* and *trans* conformations:



These are interconvertible via a rotation barrier of less than 20 kcal/m around the peptide C—N bond. The polymer poly-L-proline has been found to exist in two distinct helical forms in solution. Each of these forms arises essentially due to the steric restrictions imposed on the chain due to the 5-membered ring. No interamide hydrogen bonding is, of course, possible in this polyimide. In water and in acids, the polymer adopts a left-handed 3_1 helical conformation known as the polyproline II helix with a strong levorotation. The conformation obtained in aliphatic alcohol solutions is the dextro-rotatory right-handed helix polyproline I. Upon the addition of acids (or water) to the alcohol solution of polyproline, a mutarotation or a helix to helix conversion from form I to II occurs over a period of hours. X-ray investigation of the structures reveals that polyproline I is a right-handed helix of axial translation 1.85 Å when the peptide bonds are all in the cis conformation, and thus quite compactly wound [37]. Form II is a left-handed helix with an axial translation of 3.12 Å with 3 residues per turn of the helix and with the peptide bonds in the trans conformation [38]. The naturally occurring connective tissue protein collagen consists of roughly 33% proline and has a formal similarity to the polyproline II helical structure. Collagen is a triple-stranded helix, with each strand taking on the left-handed polyproline II or the 3_1 helical twist.

The ORD and CD spectral details of the polyproline helices are given in Table 1.

TABLE 1

| Form | ORD | | | CD | | | |
|------|-------------------------------|--------------------------------|-------------------------------|------------------------|------------|------------------------|------------|
| | Peak | Trough | Peak | $\lambda_1(\text{nm})$ | ΔE | $\lambda_2(\text{nm})$ | ΔE |
| I | 222 nm (5×10^4) | 208 nm (-9×10^4) | | 215 | +30 | 199 | -10.5 |
| II | | 218 nm (-4×10^4) | 190 nm (3×10^4) | 228 | +0.5 | 206 | -18.0 |

Pysh [39] has calculated the rotatory properties of polyproline I and II helical forms. For form I he predicts a positive Cotton effect at 216 nm and a negative one at 203 nm, while for form II the Cotton effects are positive ones at 216 and 202 nm. The calculations suggest exciton splitting of the $\pi-\pi^*$ bands in both cases. As one goes from form I to II, one sees a marked blue shift and an intensity borrowal from the red to the blue band (from the parallel component to the perpendicular one). In form II there is a further splitting of the blue band. The experimental spectra are thus reproduced quite

well. The $n-\pi^*$ bands for both forms I and II were predicted to be considerably weaker than the $\pi-\pi^*$ band.

Collagen, with its triple-stranded helix, is similar in its CD spectrum to the polyproline II helix. It displays a positive Cotton effect at 223 nm (ellipticity of $+21 \times 10^3$) and a strong negative band at 198 nm (-22×10^3).

Both the polyproline helices can be randomized in strong salt solutions such as 6 M CaCl_2 to yield a singly dichroic CD profile with a Cotton effect at 210 nm ($\Delta E = -9$) [40]. Likewise, collagen when denatured by LiClO_4 gives a single negative cotton band near 200 nm ($\Delta E = -1$), while heat denatured collagen has this band at 200 nm with $\Delta E = -4$ [41].

Several derivatives of proline, such as 4-hydroxy proline, 4-O-acetoxy proline, and also the 6-membered ring analog pipecolic acid, and the 4-membered analog azetidine carboxylic acid, have been polymerized, and the conformations of these polymers have been studied using a variety of techniques, including ORD and CD [42-45]. Most of these polymers are able to take on both the polyproline I type and II type helical orders, and they mutarotate from one form to another upon changing the solvent composition. Recent studies have shown that polytripeptides of (pro-X-Y) structure also adopt the 3_1 helix. Polyglycine is also able to adopt a 3_1 -type of helical fold [46]. Indeed, there has been a recent suggestion that even ionized polyamino acids such as poly-L-glutamate or poly-L-lysine have local regions of extended 3_1 helical fold in them (vide supra) [41, 47].

D. The β -Turn Conformation

Another type of ordered structure that has been found in regions of several globular proteins such as lysozyme and ferricytochrome c is the cross β -structure which has also been variously named as the β -fold, β -turn, or the 3_{10} -bend (type II). This involves a 10-membered hydrogen bonding between the carbonyl oxygen of an i -th residue with the H-N of the $(i + 3)$ -rd residue in which the chain is forced to fold over itself in a bend. There appear to be two types of such a 3_{10} -fold: type I, which is very close to a 3_{10} -helix, except the bend is nonhelical, and the probably more energetically favorable type II, which is obtained from the type I by flipping an amide bond by 180° and making small adjustments of the two dihedral angles ϕ and ψ . The type II bend, referred to as the β -turn (or the cross β occasionally) is seen in six regions in ferricytochrome c and invariably involves a glycyl residue. The characteristics of the β -turn are discussed by Venkatachalam [48] and by Dickerson et al. [49]. Urry and co-workers [50] have recently synthesized a polytetrapeptide (Val-

Pro-gly-gly)_n which, from NMR evidence, seems to adopt the β -turn conformation; the CD spectra of this β -turn polymer model in two solvents have been established. In MeOH the polymer displays a negative Cotton effect band of intensity $-21,000^\circ$ at 216 nm, a positive band at 203 nm ($[\theta] = +52,000^\circ$), and another Cotton effect at 190 nm of magnitude $-42,000^\circ$. In trifluoroethanol these bands are also seen but with slightly different intensities ($[\theta]_{223}$, $-24,000$; $[\theta]_{203}$, $+23,000$; and $[\theta]_{188}$, $-35,000^\circ$). This spectrum is suggested by these authors to characterize the β -turn conformation.

E. The Disordered Chain

The native structure of proteins, as for the ordered structure of polypeptides, can be transformed by a variety of means such as heating, change of pH, and addition of external agents (urea, guanidinium chloride, strong acids). This process, termed denaturation of proteins, involves disruption of all (or most) noncovalent bonds that hold the ordered conformation together. The denatured protein chain approaches a disordered conformation with little or no correlation between residues. Denaturation results in a loss of the biological activity of enzymes and proteins. The nature of the disordered chain has been studied by a variety of techniques, both hydrodynamic and short-range.

The rotatory dispersion of disordered polypeptide chains is generally simple, i.e., obeys a single-term Drude equation, or in other words, the Moffitt b_0 parameter is zero for a random coil polypeptide (with $\lambda_0 = 212$ nm). The UV ORD and CD spectra of ionized poly-L-glutamate, thought to be in the disordered chain conformation, are illustrated in Figs. 7 and 8, respectively. The ORD spectrum consists of a small negative Cotton effect in the 235-nm region, a trough at 205 nm with a rotation of $-15,000^\circ$, and a peak at 189 nm with a value of $+17,000^\circ$. The CD spectrum reveals a very small negative extremum in the 235 to 240 nm region ($[\theta] = -200^\circ$), a positive Cotton effect at 216 nm ($[\theta] = +4700^\circ$), and a large negative band at 198 nm ($[\theta] = -35,000^\circ$). The last-mentioned band is generally agreed to be the peptide π - π^* band, but there has been no unanimity about the assignment of the former two. Several workers, notably Urry [51] and Myer [52], believe the 235-nm band to be an artifact that arises because of either a trace amount of α -helix in the sample or a spectral artifact that occurs due to the contiguity of a weak positive band followed immediately by a much larger negative one. The positive band appearing around 218 nm, while generally thought of as the peptide n - π^* transition has some controversy about it. First of all, the choice of ionized poly-L-glutamate or poly-L-lysine as models for the "random coil" structure was ques-

tioned by Tiffany and Krimm [41] who argued that the interside-chain electrostatic repulsion in these ionized polypeptides would cause a certain amount of chain expansion, and hence the chains would not be truly random. Potential energy calculations on ionized poly-L-glutamate, including side-chain Coulomb interaction terms [47, 53], led them to believe that side-chain charge correlation in the ionized state of polyglutamate sends the polymer to a conformation with local regions of left-handed 3_1 -type extended helical (EH) order, somewhat akin to the polyproline II-type helix. Krimm and Tiffany suggest that the CD spectra of ionized polyglutamate and polylysine resemble those of polyproline II helix and indicate the presence of EH regions. True "random coil" or unordered chain conformations in these polymers were suggested to arise only in the presence of high salt concentrations (such as LiClO_4 or CaCl_2), where the 218-nm positive band is lost, leading to a singly dichroic spectrum with a Cotton effect in the 196-nm region. This posed the question of whether it is proper to consider the CD spectra of ionized polyamino acids as the standard for an "unordered" polypeptide chain.

In recent years there has been considerable controversy generated on this issue. Balasubramanian and Roche [54] showed the presence of the 218-nm positive band in the CD spectra of even nonionizable polypeptides in helix-disrupting "coil-promoting" non-aqueous solvents such as perfluoro gem-diols. Miller and co-workers [55, 56] showed that the incomplete deesterification of polyamino acids cause variations in their CD spectra, and also that under the concentration regions where CD spectra are run, hydrodynamic measurements reveal no extension of the polyglutamate chain. Recalculation of the polyglutamate chain conformations offers little evidence that a regularly ordered electrostatic helix is present. It has also been shown that the CD spectra of poly- ω -peptides, such as poly- β -L-aspartate or poly- γ -L-glutamate in their "unordered" states, display a 218-nm band even though none of these polymers is able to adopt any of the intramolecularly ordered structures of the poly- α -peptides due to their primary structural variations [57]. The question of CaCl_2 and LiClO_4 preferentially binding to the peptide group, which will result in altering the CD spectra of polypeptides, has been raised [57-59]. Thus the suggestion that ionizable polypeptides adopt the EH conformation in local regions does not appear to be well accepted.

Several reports of theoretical calculations on the rotatory properties of the disordered polypeptide chain have appeared recently. Tonelli's [60] calculation suggests the presence of the 218-nm positive band and the low wavelength negative band for random coil poly-L-alanine. Aebersold and Pysh [61] show that the CD spectrum of disordered polypeptides is a sensitive function of local conforma-

tions. Zubkov et al. [62] have calculated the CD spectra of random coil peptide chains with various ϕ and ψ angles, and they find that the curves obtained strongly depend on the angles. Somewhat similar results were also obtained by Ronish and Krimm [63]. It thus appears that the dichroism of an unordered peptide is governed by the ϕ and ψ values that the local segments populate, and that there may not be a unique CD spectrum that characterizes the "unordered" state.

The CD spectra of denatured proteins, however, point out in a different direction. In a definitive paper, Cortijo, Panijpan, and Gratzer [64] have chosen disulfide-free proteins in 6 M guanidine hydrochloride as representing truly disordered chains, as shown by Tanford [65] using hydrodynamic criteria. The CD curves of a variety of proteins in 6 M guanidinium chloride (GuCl) do not show any resolvable features above 210 nm, but only a single negative Cotton effect below this wavelength. Due to solvent absorption problems, the extremum of this Cotton effect could not be realized in experiment. But all the proteins tried showed qualitatively similar curves, while the magnitudes are a function of the amino acid composition. In the absence of large contributions from chromophoric side chains, the variation must be presumed to reflect the optical activity of the peptide bands themselves. Interestingly, there is no correspondence between the CD spectrum of ionized polyglutamate in 4.5 M LiClO₄ [41] and the protein random coil CD, while the CD of poly-L-serine in 8 M LiCl [66] is much nearer to that of proteins in 6 M GuCl. There is therefore justification for the choice of the poly-L-serine CD spectrum in 8 M LiCl as the standard for the coil state [67]. Again, ionized polyglutamate and polylysine take on some α -helical structure in 6 M GuCl, presumably due to charge shielding effects. The notable point from this work [64] is that the qualitative CD profile for random coil proteins is singly dichroic with a negative band below 210 nm, and the qualitative difference seen from protein to protein might arise from the composition dependence of the distribution of the backbone dihedral angles.

Yet another claim has been made about the characteristic CD curve of the "unordered" chain conformation, i.e., a weak negative band near the 225-nm region (ellipticity of about -3000°) followed by a large negative band around 200 nm. Such CD curves have been found for several histones [68, 69], ACTH and its analog [70], and in the case of some homopolypeptides such as poly-L-thialysine [71], and also for several copolypeptides [72-75]. Beychok [76] has suggested this doubly negative dichroic spectrum to be common for several denatured proteins. However, Fasman, Hoving and Timasheff [77] have shown that the CD of polypeptide films in the random conformations are characterized by a negative 225 nm shoulder and

a negative 200 nm band, somewhat similar to those obtained with denatured but not totally unfolded proteins. This raises the possibility that in such systems (where total unfolding into a statistical coil has not been achieved) the steric constraints imposed on the chain (or local regions) are responsible for the 225-nm negative shoulder. Credence to this possibility comes from the CD data on denatured reduced proteins in 6 M GuCl, reported by Gratzer [64] and discussed earlier, which do not show any Cotton effects above 210 nm.

It is our view that there may, therefore, be no one unique CD spectrum that characterizes the random coil polypeptide or protein chain; however, under conditions where the protein chains are otherwise monitored to be structureless, the spectral feature is a single negative Cotton effect below 205 nm. The actual ellipticity magnitudes will be expected to vary depending on the composition-dependent dispositions of the backbone dihedral angles. It is well known from the work of Bayley, Nielsen, and Schellman [24] that the sign (and magnitude) of the $n-\pi^*$ Cotton effects in dipeptides are sensitive functions of the dihedral angles. As has been pointed out by Gratzer [64], there is justification for using the CD spectrum of poly-L-serine in 8 M LiCl as the standard for the random coil [67] since it resembles the CD of totally reduced randomized protein chains.

The deep-UV rotatory properties of the truly "unordered" structure are not reported, but that of ionized poly-L-glutamate has been measured by Johnson and Tinoco [32] (see Fig. 8). Besides the 218-nm positive and the 200-nm negative bands, the polymer displays a further negative band at 175 nm that may owe its origin to the $n-\sigma^*$ band.

F. Conformational Analysis of Proteins in Solution

The two most important and ubiquitous chain conformations encountered in proteins are the α -helix and the antiparallel β -sheet. While several fibrous proteins, e.g., keratin, and the muscle proteins are predominantly α -helical, and the connective tissue protein collagen has a unique triple-stranded helical conformation, most of the globular proteins have local regions that are folded in the α -helical form, some regions where the β -sheet form is prevalent, and the rest of the molecule folded in an "amorphous" fashion mainly governed by side-chain interactions of a hydrophobic nature. Occasionally, stretches of other conformations are also seen in the native structure of globular proteins, such as the β -turn and the 3_{10} -helix, but the predominant chain-folding patterns have been the α -helix and β -sheets.

Conformational analysis of the native structure of proteins by ORD/CD have generally involved analysis of the observed spectrum of the protein as a composite of fractions of helical, β -, and "random coil" conformations. The earliest attempts in this direction involved a measurement of the ORD curve of the protein in the region of 300 to 600 nm, analyzing it using the Moffitt-Yang equation, and evaluating the b_0 parameter. Since the values of b_0 for the "standard" (100%) α_R -helix and disordered coil conformations have been found to be -630 and 0° respectively, the amount of right-handed helical structure in a given protein can be estimated from the observed b_0 . This simple method has been quite successful in a number of cases, and agreement between the helical content as obtained from ORD and from X-ray diffraction of the protein crystals has been good. The inherent difficulties in this method of analysis are the problems associated with strong optical activity of side-chain groups and prosthetic groups, and the simplifying assumption of a two-state model for the conformation, i.e., the helix and the coil. Proteins with considerable β -forms in their structures may be expected to be problem cases, since the b_0 value for the "standard" β -conformation is assumed to be zero; this assumption, as mentioned earlier, is not strictly valid.

When the accessible region of ORD was extended to the UV region, attempts were made to exploit the peptide backbone Cotton effects to estimate the helicity of proteins by explicit introduction of the dispersion terms corresponding to the 222, 206, and 192 nm Cotton effects of the helix and the 198-nm band of the coil. Thus Yamaoka [78] and Carver et al. [79] extended the ORD analysis to introduce four and five Drude terms which expressed the ORD curve of the protein as the summation of the ORD profiles of the α -helix and of the coil for all the backbone transitions. Initial efforts to accommodate the contributions of the β -form dispersion proved of little success.

An alternate approach to the problem was initiated by Greenfield and Fasman [80], who dispensed with the multiterm Drude analysis. Their approach involved an analysis of the Cotton effects observed in proteins in the 260 to 190 nm region by a curve fitting procedure wherein the summation curves representing various combinations of fractions of α -, β -, and random structures are computed. The conformational composition of the computed curve that matches an experimental ORD spectrum should give the secondary structure of the protein in solution. Analysis by this method yielded better fit with the conformations of several proteins whose X-ray crystal structures were known, but it appeared that there was an overestimation of the β -content at the expense of the helicity.

Subsequent to this, Greenfield and Fasman [81] decided to analyze the CD spectra (rather than ORD) to proteins by the curve-fitting method. The "standard" reference states for the α -, β -, and random conformations were those of poly-L-lysine. If f_α , f_β , and f_{RC} are the fractions of the α -helical, β -form, and the random coil, respectively, and $[\theta]_\alpha^\lambda$, $[\theta]_\beta^\lambda$, and $[\theta]_{RC}^\lambda$ are the corresponding ellipticities at a given wavelength λ , then the observed ellipticity $[\theta]_{obs}^\lambda$ of a given sample can be expressed as

$$[\theta]_{obs}^\lambda = f_\alpha [\theta]_\alpha^\lambda + f_\beta [\theta]_\beta^\lambda + f_{RC} [\theta]_{RC}^\lambda \quad (21)$$

and

$$f_\alpha + f_\beta + f_{RC} = 1 \quad (22)$$

the latter equation being the constraint under which the analysis proceeds. Observed CD curves of several proteins in the 200 to 240 nm regions were fitted in the above fashion, and the conformational status so obtained for a given protein was compared with that obtained from X-ray data. The comparison was good for several proteins excepting those cases where f_{RC} was high.

The limitations on this method are by and large due to the following reasons: (1) the choice of the reference "standard" state CD spectra. As mentioned earlier, there does not appear to be unanimity about the standard CD profiles of the β -sheet and the random coil. Others [67] have used the CD spectrum of poly-L-serine in 8 M LiCl to represent the random state and obtained somewhat better results. (2) Contributions of the nonpeptide chromophores, such as —S—S and aromatics, are not totally eliminated. (3) Whether short stretches of an ordered structure such as the helix will have the same band positions and intensities as a long-chain homopolypeptide α -helix. There have been suggestions that this may not be so.

In light of this, the approach taken by Saxena and Wetlaufer [82] seems more "foolproof." These authors use the observed CD spectra of three proteins of known structures (known f_α , f_β , and f_{RC}) to extract the CD profiles of the α -, β -, and RC forms, rather than assume these from polylysine data. In other words:

$$[\theta]_{\text{Protein 1}}^\lambda = f_\alpha^{Pr1} [\theta]_\alpha^\lambda + f_\beta^{Pr1} [\theta]_\beta^\lambda + f_{RC}^{Pr1} [\theta]_{RC}^\lambda$$

$$[\theta]_{\text{Protein 2}}^\lambda = f_\alpha^{Pr2} [\theta]_\alpha^\lambda + f_\beta^{Pr2} [\theta]_\beta^\lambda + f_{RC}^{Pr2} [\theta]_{RC}^\lambda$$

$$[\theta]_{\text{Protein 3}}^\lambda = f_\alpha^{Pr3} [\theta]_\alpha^\lambda + f_\beta^{Pr3} [\theta]_\beta^\lambda + f_{RC}^{Pr3} [\theta]_{RC}^\lambda$$

The values obtained for the α -helical "reference" agree well with

the Fasman value, while those for the β - and RC-form differ slightly. When these values were used to predict the conformational status of other proteins, moderate success was achieved.

Similar improved methods of curve fitting and data analysis have been undertaken by others, notably Chou and Fasman [83], and it appears that the usefulness of this approach to predict the solution state conformations of proteins will improve.

G. Side-Chain Cotton Effects in Proteins

Besides the peptide group transitions, the ORD/CD profiles of proteins will contain information about the Cotton effects of chromophoric side chains. The important side-chain chromophores that will contribute are phenylalanyl, tyrosyl, tryptophan, histidine, and the disulfide groups. Recourse is often taken to the spectra of model compounds in interpreting the ORD/CD spectra of these chromophores in proteins.

The monitoring of side-chain Cotton effects often yields useful information about the tertiary structural aspects of protein chains. As an example, Glazer and Simmons [84] have studied the ORD of the tyr groups in ribonuclease. This enzyme has six tyr (and no try), out of which three are "buried" with the native structural "core" and three are exposed to the solvent milieu. A negative Cotton effect observed at neutral pH at 278 nm shifts to 292 nm at pH 11.5, paralleling the change in the absorption profile of tyr as it ionizes. At higher pH, when the protein unfolds, the buried tyr also are exposed and the negative Cotton effects seen earlier vanish, showing that the interactions that confer optical activity to the exposed tyr groups are destroyed upon unfolding the macromolecule.

Glazer and Simmons have also studied [85] the try group cotton effects in lysozyme. In the native state try displays three positive CD bands in the 280 to 300 nm region. Solvent perturbation of the structure by 50% ethylene glycol, as well as binding of the protein to a substrate N-acetyl-D-glucosamine, intensifies these bands, implicating the involvement of try group in the active site of the enzyme, as confirmed by X-ray crystallography.

Theoretical calculations of the conformational dependence of tyr CD bands have been reported [86, 87]. Strickland [88] has shown that the observed CD band at 275 nm for RNase S accounts for 70% of the calculated tyr CD, using the coordinates of crystalline RNase S. The tyr CD was assumed to arise essentially from a coupling between the 1L_b band of tyr and the π - π^* bands of the other groups. The difference between the observed and theoretical curves was attributed to the disulfide chromophore. The coupling of the bands of tyr 73 and tyr 115 was found to be the most important factor. Coupling between

the tyr bands and the peptide π - π^* bands was also postulated. Upon a small conformational perturbation, the CD band attributed to tyr is increased through a suggested coupling with phe 46 and his 48.

In a study of the binding of the protein bovine neurophysin to the peptide hormones oxytocin and vasopressin, Breslow and Weiss have monitored [89] the CD spectra of aromatic side chains. The tyr of the neurophysin was nitrated, which shifted its λ_{\max} above 300 nm. Upon binding, significant changes were seen in the CD of the hormone in the 280 nm region, attributed to the tyr 2 of the hormone. In an analog of the hormone where phe was substituted for tyr 2, the CD changes in the 280 nm region were lost, establishing the alteration in the region of tyr 2 of the hormone upon binding. The perturbations seen in the CD of the nitrated tyr of neurophysin indicate that this group is close to the tyr 2 of the hormone in the complex.

Conformational similarities between two different proteins of similar homology have been studied using ORD-CD techniques. Bovine α -lactalbumin and chicken lysozyme are two proteins with a great deal of similarity in their primary structural amino acid sequence, Browne et al. [90] have in fact fitted the α -lactalbumin molecule into the lysozyme molecular model structure. Aune [91] reported the ORD spectra of the two proteins to be essentially indistinguishable, suggesting that the structural homology results in conformational near-identity. CD curves of α -lactalbumin in the far-UV region [92] yield roughly 30% helix, 10% β , and 60% un-ordered structure, similar to that of lysozyme (29% helix, 11% β , and 60% coil). Changes seen in the side-chain Cotton effects between lysozyme and α -lactalbumin [93] result from the differences in the aromatic residue contents and tertiary structural variations seen in the two proteins. Low-angle X-ray scattering in a solution of α -lactalbumin suggested that it has a different molecular conformation than lysozyme [94]. However, Achter and Swan [95] think that the variations seen in the small-angle scattering data arise due to the dimerization of lactalbumin.

Interestingly, there is another protein, avidin, which has almost the same amino-acid composition as lysozyme but little similarity in the sequence. CD spectral analysis of this protein [96] reveals almost no α -helix and 50% β -structure. In a similar vein, comparative conformational (and biological activity) studies have been done on two related peptide hormones, human chorionic somatomammotropin (CSM) and pituitary growth hormone (PGH) [97]. These are about 190 residues long and identical in 160 positions, one of them being try at position 85. While the nitrophenyl sulfonylated try 85 derivative of PGH is biologically active, the same derivative of CSM is not. The CD spectra of the two derivatized proteins show no

difference in their secondary structure, but the try side-chain Cotton effects are different. This has been interpreted to mean that the molecular integrity of try 85 is not needed for PGH while it is for CSM, and the effect might be a local conformational one since the secondary structures appear identical.

H. The Disulfide Bridge

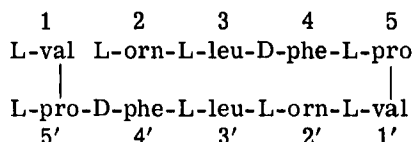
The absorption maxima and the Cotton effect corresponding to the disulfide group (—S—S—) are considered to be in the 240 nm region. There exists the possibility of a screw sense associated with this linkage. The barrier to rotation around the $\text{R}_1\text{—S—S—R}_2$ system (dihedral angle 90°) is about 10 kcal/m. The screw sense of the disulfide in cystine crystals is right handed, while in cystine hydrohalides and glycylcystine it is left handed. There is not much information available about the screw sense of S—S— bonds in proteins. The possibility of screw isomers and also mismatched S—S bridge isomers in a reconstituted protein (i.e., one where the several S—S bonds are broken by reduction and allowed to reoxidize) exists and becomes of interest in enzyme activity and conformational populations of disulfide-enzymes. The position of the S—S Cotton effect depends not only on the screw sense but also on the dihedral angle around the bond. L-Cystine itself exhibits the S—S chromophore Cotton effect at about 250 nm with an intensity of -2×10^{30} . The S—S Cotton effect in oxytocin and vasopressin occurs at a longer wavelength and is positive in sign; that of glycine-oxytocin occurs as a positive band at 250 nm.

One of the problems encountered in the case of disulfide-containing proteins is that if the various S—S— bonds found in a given native protein were reduced and broken, and the reduced (and unfolded) protein were reoxidized, will all the original S—S bonds be formed with the proper register of proper pair screw sense and dihedral angles? This question is of relevance in the structure-function correlation of enzymes. Anfinsen has attacked this problem with ingenuity [98] and has been able to recover essentially all the activity in the reduced-reoxidized ribonuclease system. Interesting results have been obtained by Pflumm and Beychok [99] who have compared the CD spectra of native RNase-A with those of RNase-A which has been reoxidized under various conditions. Reoxidation of the enzyme in the absence of mercaptoethanol leads to a product with 83% of native RNase activity and differing in the CD spectrum in that the positive CD band at 240 nm seen in the native enzyme is absent in the reoxidized material. Partially reduced (only one SS bond out of 4) RNase can be reoxidized to yield a product identical in activity and the 240-nm band to the native enzyme, while extensively reduced protein

lacks this band on reoxidation. Sephadex fractionation of the reoxidized product (in the absence of the thiol) yields an inactive fraction (I) and a fully active product (II) which differs in its 240 nm band from the native form. These suggest that the reoxidized product is perhaps a metastable state that possesses a slightly altered conformation from the native enzyme, but this slight structural alteration is not in a position in the molecule that is critical for activity.

I. Correlation of Conformation with Biological Activity,
by Using CD/ORD: Gramicidin S

The cyclic decapeptide gramicidin S (GS),



is an antibiotic possessing antimicrobial activities. The conformation of the molecule is suggested to be one with intramolecular H bonds suggestive of the β -type in polypeptide chains. Izumiya and associates [100] have synthesized an enormous number of GS analogs over the past 15 years where (1) individual amino acids have been systematically varied in the primary structure, (2) pairs of amino acids have been replaced, (3) the retro-isomer has been prepared, (4) the ring size has been altered, and (5) linear analogs have been synthesized. In each case the inhibitory activity of the peptide synthesized has been studied and compared with the parent GS. Interestingly, the ORD/CD properties of a large number of these analogs have also been recorded. More recently, Sofuku [101] has even synthesized an analog (4,5- δ -aminovaleric acid GS, or δ -ava-GS) wherein one amide group is replaced by a $-\text{CH}_2-\text{CH}_2-$ group which has almost the same length (same ring size) but does not participate in hydrogen bonding and has studied its antimicrobial activity and ORD/CD spectra.

Inspection of Table 2 reveals several features. Replacement of hydrophobic residues such as val^{1,1'}, leu^{3,3'}, D-phe^{4,4'}, and pro^{5,5'} by other hydrophobic residues neither alters the activity significantly nor the conformation of the ring markedly. Glycine in these positions, however, does not seem a desirable choice from both conformational and biological angles. The aromatic ring does not seem too critical for activity; replacing phe and pro does not abolish the

TABLE 2

| Analog | Antimicrobial ^a activity in $\mu\text{g/ml}$ | $[\text{m}]_{232}$ |
|--------------------------------|--|--|
| GS (30-member ring) | 5 | -19,000° |
| 1,1'-digly GS | 100 | — |
| 1,1'-diala GS | 5 | |
| 1,1'-dileu GS | 10 | |
| 2,2'-dilys GS | 10 | -15,000° |
| 2,2'-diaminobutyric GS | 10 | -27,000° |
| 3,3'-digly GS | 100 | — |
| 3,3'-diala GS | 20 | |
| 3N-Me-Leu GS | 3.12 (cf. GS 3.12) | -29,000° |
| 3,3'-di-N-Me-Leu GS | 3.12 | -24,000° |
| 4,4'-di-D-ala GS | 25 | -15,000° |
| 4,4'-digly GS | 50 | -12,000° |
| 4,4'-dicyclohexylala GS | — | -20,000° |
| 4,5- δ -aminovaleryl GS | Half as active as GS | -9,000° |
| 5-gly GS | 5 | -6,000° |
| 5,5'-digly GS | 2.5 | -9,000° |
| 5,5'-disar GS | 5(?) | -19,000° |
| 5,5'-di- β -ala GS | 100 | Monotonic positive ORD profile |
| 1,1',5,5'-tetragly GS | 100 | — |
| Retro GS | 50 | -4,000° |
| Cyclosemi GS (15-ring) | 100 | No trough; plain negative curve |
| Retro-cyclosemi GS | 100 | Similar to cyclosemi GS |
| 5 β -ala-cyclosemi GS | 100 | Quite different but resembling 5,5'-di- β -ala |
| Sesqui GS (45 member ring) | 50 | -6,000° |
| Di GS (60-member ring) | 10 | -7,000° |
| Linear decapep GS | 20 | -10,000° |
| Linear sesqui GS (penta deca) | 20 | -1,500° |
| Linear eicosapeptide GS | 20 | -4,000° |
| Linear pentapeptide (semi GS) | 100 | — |

^aThe amount required to inhibit growth of *B. subtilis*. The lower the amount, the greater the activity, e.g., 1,1'-digly GS is essentially inactive while 1,1'-dileu GS is almost as active as GS.

activity although the Cotton effect is reduced considerably, indicating greater symmetry in the chain conformation. Surprisingly, the conformation restricting tendency of proline (and D-phe) does not appear to play a major role since 4,5- δ -aminovaleryl GS appear to show qualitatively the same ORD profile, and, even more strikingly, replacing the pro by gly (tendency for greater conformational freedom) or by sar does not reduce the antimicrobial activity at all, and neither is the chain conformation vastly changed. It would hence be of interest to study the analog di-(4-5,4'-5')- δ -amino valeryl GS. It appears that changes in the primary structure are tolerated to a degree for activity provided the overall ring conformation is maintained. Clearly, linear chains will not do, neither will smaller ring sizes. Increasing the ring size a little by the introduction of β -alanyl (i.e., β -peptide bonds) results in a loss of activity and a drastic change in the conformation of the molecule, presumably because of the greater conformational flexibility available due to the presence of an extra CH_2 group in the backbone. Similarly the 45-member ring of sesqui-GS causes the activity to be lost, while from the optical rotation value it appears that the original conformation of GS is modified in the expanded cyclic chain. Surprisingly, the 60-membered cyclic "dimer" di-GS is active to a significant extent while its rotation values compare with those of sesqui-GS. The conformation of the di-GS ring and any possible higher order folding of the macroring is worth investigation. Of great interest is the analog retrogramicidin S (retro-GS) which differs from the natural GS only in the direction of the chain progression. It is not only very weak in activity but its conformation also seems to be different from GS. Here is an excellent example to study the conformational aspects that are governed by the reversal of the direction of a polypeptide chain.

A comparison of the antimicrobial activities and the ORD curves shows that compounds with ORD curves that are plain with no extrema in the 230-nm region are biologically inactive cyclopentapeptides and the β -ala analogs. All the peptides with any activity, weak or strong, share the 232-nm trough as a common feature, and may be inferred to be roughly of the same conformational family.

J. Poly- ω -peptides

It is an interesting fact that peptides and proteins occurring in nature are built up predominantly of α -amino acid moieties only; even in multifunctional amino acids such as aspartic and glutamic, or ornithine and lysine, it is the α -amino and the α -carboxyl function that is utilized in chain propagation. The conformational aspects of polymers made by utilizing the ω -functional group for chain propa-

gation have received some recent attention. For example, aspartic acid has been polymerized by using the α -amino and the β -carboxyl groups to yield poly- β -L-aspartic acid ($-\text{NH}-\text{CH}(\text{COOH})-\text{CH}_2-\text{CO}$)_n. Note the introduction of an extra methylene group in the backbone in this polymer. The synthetic and conformational aspects of this polymer have been studied [102]. Likewise, the poly- γ -peptide poly- γ -L-glutamic acid has also been prepared and analyzed conformationally [103]. This polymer is of interest since poly- γ -D-glutamic acid has been isolated as the capsule peptide from *B. subtilis* and *B. anthrax*. Sequential copolymers of (γ -glu-gly), (γ -glu- β -ala) and (γ -glu- γ -aminobutyric) have also been synthesized [104]. Recently the polymer copoly-(L-(γ -glutamyl)- β -L-asp) has also been studied [105].

The poly- β -peptides that have been studied by ORD/CD techniques for conformational analyses are (1) poly- β -L-aspartic acid (PBLA), and its α -benzyl, α -t-butyl, and α -ethyl [102]; and the α -isobutyl esters [106]. " b_0 " measurements for the esters in several solvents show no degree of conformational order. The ORD/CD of PBLA shows a large negative CD band below 190 nm (somewhat similar to that of a disordered chain, though the band extremum is blue-shifted), and a weaker positive Cotton effect that shifts from 203 nm in acid media to 220 nm in alkaline media. Again there seems no order-disorder transition observable in ORD/CD with pH variation, suggesting the lack of chain order. The positive Cotton effect in acid media seems to owe its origin not only to the " $n-\pi^*$ " of the peptide but also to the COOH group transitions. This induction of the Cotton effect of the carboxyl group in PBLA is in contrast to poly- α -peptides such as poly- α -L-glutamic and poly- α -L-aspartic acids. In the latter cases the side-chain carboxyl is one or two methylenes removed from the backbone, while in PBLA it is attached directly to the asymmetric α -carbon atom. It appears on the basis of ORD/CD that poly- β -L-aspartic acid possesses none of the ordered structures seen in polypeptides.

(2) Poly-L- β -aminobutyric acid (PBAB) ($\text{NH}_2-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CO}$)_n has been synthesized by Schmidt [107] and its ORD/CD have been reported by Goodman's group [36]. In neat hexafluoroisopropanol (HFIP), PBAB shows a weak negative Cotton effect near 215 nm and a positive one near 200 nm. Addition of water intensifies the spectrum with a minor shift in positions, but the main effect of water is to promote the β -type sheet conformation as observed in poly- α -peptides. The same β -type CD is seen in a film of PBAB cast from HFIP, and in a solution of 70:30 HFIP:H₂O. The CD spectrum of PBAB in neat HFIP displays a weak positive band near 210 nm and a larger negative one below 190 nm. In demerit to the interpretation of Goodman et al. [36], we feel that the CD spectrum in pure

HFPD is indicative of a disordered polymer chain. Induction of chain order upon the addition of water seen here is not new, since peptides such as PMLG have been shown to adopt the α -helical order upon addition of water to a solution of this peptide in HFPD [108].

It is interesting to compare the conformations of PBLA and PBAB. While the solvents in the two cases are different, it is nevertheless likely that the side-chain COOH hinders chain order in the former. It is also interesting here to note that poly- β -alanine (an optically inactive polymer), which differs from PBAB in that it has no side-chain groups, does not seem to have any chain order [109].

Antoni et al. [110] have synthesized an aspartic acid polymer in which both α - and β -peptide backbone repeat units occur; the polymer is the optically inactive poly(α, β)(hydroxyethyl)-DL-aspartamide, which has the expected disordered chain conformation.

1. Poly- γ -peptides

The γ -peptide poly- γ -L-glutamate ($\text{[NH-CH(COOH)-CH}_2\text{-CH}_2\text{-CO]}_n$) (PGLG) has been synthesized, and its chain conformation has been elucidated by ORD/CD [103]. Here again, the acid form shows a moderately intense broad CD maximum near 205 nm, possibly composed of the peptide and the side-chain carboxyl group transitions, followed by a much larger negative CD band lying below 190 nm. The ionized form ($\text{pH} \geq 5$) displays a CD spectrum similar to what has been seen in disordered poly- α -L-glutamate chains, i.e., a positive band at 215 nm and a negative one below 200 nm. The CD spectrum of PGLG in the acid form, as that of PBLA, has been interpreted as composed of not only the peptide bands but also a carboxylic acid transition which is induced due to its proximity to the backbone. Qualitatively similar CD/ORD curves have been obtained in the copolymers containing γ -glu and other amino acids.

2. Poly- ω -amides

Optically active poly D(-)- β -methyl- ϵ -caprolactam ($\text{[NH-(CH}_2\text{)}_3\text{-CH(CH}_3\text{)-CH}_2\text{-CO]}_n$) was synthesized by Overberger and Jabloner [111], and its ORD was measured in CHCl_3 , cresol, and in mixtures of these two solvents. No chain order was detected and the polymer exists essentially as a solvated coil. Poly- ω -peptides, again optically active, synthesized by the condensation of diamines (e.g., dimethylene diamine, dimethyl piperazine, and piperazine) and diacids (such as 1,2 C-3 cyclohexane dicarboxylic acid and 1,2 cyclopropanedicarboxylic acid) have been investigated by ORD/CD, and in several of these cases the polyamides have been suggested to be of rigid (polyproline-like) chain order as evidenced by exciton-split π - π^* Cotton effects [112]. The polyamide derived from 1,2-cyclo-

hexane—dicarboxylic acid and dimethylene diamine, believed from NMR evidence to possess no order, exhibits rotatory properties similar to those of ionized poly- α -peptides.

K. Interactions of Peptides and Proteins with Other Molecules

The process of binding and complex formation between an optically active compound and others can often be followed and monitored conveniently by the techniques of ORD/CD. The simplest case of association between monomers to form weak multimeric complexes can sometimes be qualitatively followed by simply checking whether "Beer's law," i.e., concentration variation of rotation or ellipticity, is obeyed or not. Interactions between hydrophobic compounds and peptides/proteins have been followed by ORD. The protein β -lactoglobulin was allowed to be in contact with several non-polar gases such as butane, and the alteration in the conformation of the protein upon hydrocarbon binding was monitored by measuring the ORD curve of the protein [113]. While no significant changes were observed either in the values of the Moffitt-Yang b_0 parameter or in the magnitude of the ORD minimum at 233 nm, the values of the specific rotation and the a_0 parameter were altered upon butane binding to the protein. The results were interpreted to mean that the hydrocarbon binds to the "amorphous" regions in the protein reversibly and alters the structure without causing any change in the helical content.

Interaction between detergent molecules such as sodium dodecyl sulfate (SDS) and proteins has been studied in some detail [114]. The binding has been variously suggested to involve an electrostatic interaction between the cationic sites on the protein and SDS, binding of SDS to the peptide bond, and hydrophobic interaction between SDS and hydrophobic parts in the protein. The effect of SDS on cationic peptides such as poly-L-lysine [115] and poly-L-ornithine [116] is to induce a random coil \rightarrow β and random coil \rightarrow α respectively, but it does not affect ionized poly-L-glutamate. In a recent experiment involving both CD and equilibrium dialysis, Igou et al. [117] monitored the interaction between SDS and several water-soluble non-ionic homopolypeptides, i.e., the poly- ω -hydroxyalkyl-L-glutamines. SDS was not found to have any strong interaction with these nonionic peptides, and neither was it found to affect the stability of the conformations of these polypeptides as monitored by changes in the CD values. Equilibrium dialysis showed no significant binding of SDS to the macromolecules. These results provide impressive evidence for the importance of electrostatic interactions between SDS and cationic sites in polypeptides.

The group of cyclic oligopeptides and depsipeptides such as enniatins and valinomycin are able to selectively complex with certain group I metal ions (usually K^+) with high efficiency and transport then through natural or synthetic biological membranes. These cyclic peptides are termed ionophores for this reason. The metal ion is stripped of its hydration sphere and is included inside the cavity in these cyclic peptides where it is held by ion-dipolar forces by the peptide/ester carbonyls. During this process the conformation of the macrocyclic ligand is altered. This has been studied by a number of techniques including ORD and CD (for a comprehensive review of the field, see Ovchinnikov et al. [118]). In Fig. 9 we illustrate the effect of complexation by K^+ on the ORD curve of the ionophore enniatin B in ethanol. The effect on complexation is dramatic in that the sign of the Cotton effect curve changes. There is also a possibility of two and only two equilibrating conformations, one of the free macrocycle and the other of the complex as seen from the "isosbestic" point at 234 nm.

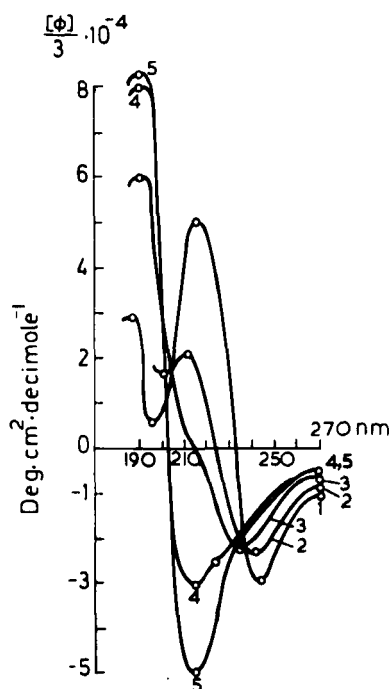


FIG. 9. ORD profile of enniatin B and its K^+ complex in several solvents. 1: Heptane. 2: 96% Ethanol. 3: Acetonitrile. 4: Water:trifluoroethanol (2:1). 5: Enniatin: K^+ complex in 96% ethanol. Reproduced from Ref. 118.

Such ORD data have been used to calculate the stability constants of the complexes. L designates the macrocyclic ligand, M⁺ the cation, and ML the complex. The stability constant K can be calculated from the expression

$$K = \frac{C_{ML}}{C_M C_L} = \frac{\alpha}{(1 - \alpha)(C_M^{tot} - \alpha C_L^{tot})}$$

where $\alpha = (\beta_L - \beta_{obs})/(\beta_L - \beta_{ML})$ = degree of complexation; β_L , β_{ML} , and β_{obs} are the molecular rotations of the free peptide, the complex, and the equilibrium mixture, respectively; and the C's are the concentrations. Figure 10 shows the ORD curve of the cyclododecadepsi-peptide valinomycin and its K⁺ complex in several solvents. The absence of a common crossover point ("isosbestic") here confirms the belief arising from other measurements that valinomycin possesses several (solvent-dependent) conformations in solution. An analysis of the CD/ORD and the nuclear magnetic resonance spectra of the Na⁺ complexing ionophore, antamanide, also shows that this cyclodecapeptide is in the form of a complicated equilibrium mixture of conformers in solution. The CD spectra of the free peptide in water containing solvent mixtures and of the Na⁺ complex were similar in shape. The CD spectrum of the complex is almost independent of the solvent. Interestingly, the Cotton effects arising out of the aromatic side chains (there are four phe residues in the molecule) were very

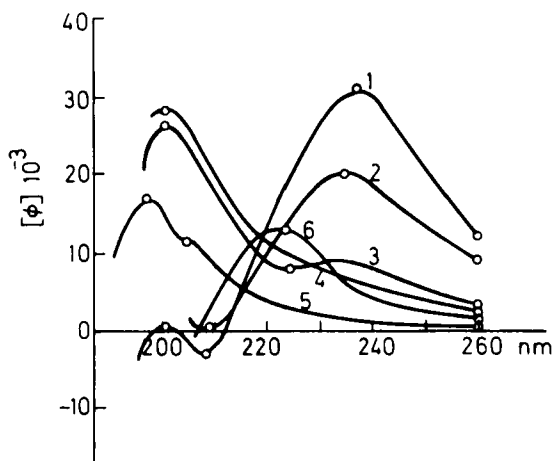
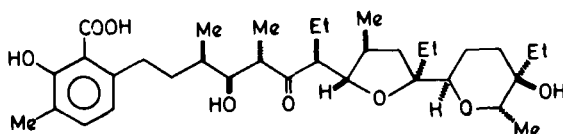


FIG. 10. ORD curves of valinomycin and its K⁺ complex in several solvents. 1: Heptane. 2: Heptane/dioxane (6:1). 3: Ethanol. 4: Acetonitrile. 5: Water/trifluoroethanol (2:1). 6: Valinomycin:K⁺ complex in ethanol. Reproduced from Ref. 118.

sensitive to Na^+ complexation, reflecting the cationic environment on the Cotton effect of the aromatic groups of the macrocycle [119].

Complexones of an open-chain type with no peptide bonds but with ether and keto ligands have been shown to occur in nature, and these bind selectively to metal ions and behave as ionophores as well. An example is the antibiotic X-537A that binds K^+ :



This molecule is thought of as "folding over" and including K^+ within itself. Pressman [120] has shown that upon K^+ binding the CD bands at 245 and 295 nm of the aromatic chromophore are considerably intensified, presumably due to the asymmetric enclosure of the benzene ring. Similarly, an open-chain chiral ether ligand is able to encapsulate Na^+ very efficiently in CH_2Cl_2 , Li^+ weakly, and K^+ not at all. Upon complexing, the ORD curve changes quite dramatically. It has therefore been suggested [121] that this chiral compound should prove useful for spectropolarimetric determinations of alkali cations, especially Na^+ in the presence of Li^+ and K^+ .

Probes into the active site regions of enzymes have been made using the techniques of ORD/CD. This involves monitoring the changes in the spectrum of the enzyme (or substrate) upon binding. Several such studies have been made in the recent past, but we would like to mention an extremely interesting variation of this method that utilizes a photochromic ligand to probe into the active site of acetylcholine esterase [122]. The compound p-phenyl azo-phenyl trimethyl ammonium chloride (PTA) is an efficient inhibitor of the enzyme in its trans form but not in its cis form. It is also possible to effect a reversible photoisomerization of PTA between its cis and trans forms by selective photoflooding (photochromism). It was found that the trans form of PTA binds to the enzyme and exhibits a Cotton effect in its absorption regions ($\pi-\pi^*$ at 320 nm and $n-\pi^*$ at 460 nm, the latter is the red-shifted value when bound to the enzyme, indicating a hydrophobic environment). These induced Cotton effects vanish when PTA is photoisomerized to the cis form. Thus it has been possible to "map" the active site of the esterase; it is roughly as long as the trans-PTA molecule and has two binding sites, one for the N^+Me_3 part and the other the hydrophobic part of PTA, and is not able to bind the cis-PTA isomer since there is only one binding site for the latter.

L. Extrinsic Cotton Effects

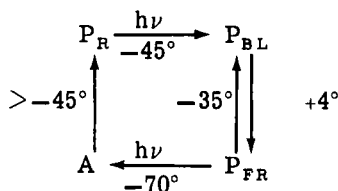
The above example is a situation where the asymmetric environment of the binding site on the macromolecule induces Cotton effects in the optically inactive PTA molecules. Such induced Cotton effects are referred to as extrinsic Cotton effects and occur widely when dyes are bound to peptides and proteins. For example, α -helical poly-L-glutamic acid binds to the dye acridine orange (AO) and induces Cotton effects in the absorption bands of the dye [123]. When the polymer is in its random coil conformation, the magnitude of the extrinsic Cotton effect of AO is reduced (however, see Ref. 124). From an analysis of the signs and number of such Cotton effects, it has been possible to suggest modes of binding of the dye to the peptide.

Enzyme-coenzyme systems often turn out to be good candidates for the induction of extrinsic Cotton effects. For example, the enzyme dihydrofolate (DHF) reductase exhibits three aromatic Cotton effects in the region of 270 to 315 nm. When the coenzyme TPNH is bound to DHF reductase, these aromatic Cotton effects are reduced in intensity and a new extrinsic Cotton effect appears at 340 nm, the absorption maximum of TPNH. Quantitative analysis of the CD spectra leads to a value for the binding constant between TPNH and DHF reductase as 4×10^{-7} M. The absence of any changes in the Cotton effects in the 220-nm region of the protein suggests that TPNH binds to the enzyme with no alteration in the backbone structure of the macromolecule [125]. In a similar study of a chromophoric inhibitor, Henkens and Sturtevant [126] showed that the inhibitor 4-(8-hydroxy-5-quinolylazo)-1-naphthalene sulfonate (HQNS) binds to bovine carbonic anhydrase and displays a negative extrinsic Cotton effect at 520 nm and another positive band at 450 nm. Difference ORD measurements show that the binding is local to the active site, involves the Zn^{2+} ion in the enzyme, and does not alter the secondary structure of the enzyme. Using the Cotton effect at 450 nm, the dissociation constant of the inhibitor-enzyme complex was found to be 5×10^{-4} M. Addition of EDTA removes excess Zn or displaces HQNS from the enzyme-Zn-HQNS complex.

Many proteins contain prosthetic groups, such as porphyrins, which display induced Cotton effects in the bound state. The proteins hemoglobin and myoglobin and cytochromes have been discussed in detail before and hence we do not intend to do so here. The prosthetic groups in these cases are often covalently linked and experience asymmetric environments that generate optical activity. Recent interest has been focused on two other proteins that contain nonheme chromophoric prosthetic groups: The protein rhodopsin in the retinal rod disk membranes has attached to it a long polyene vitamin A aldehyde called 11-cis-retinal. Absorption of a

photon isomerizes the retinal to an all-trans conformation and causes the transformation: rhodopsin \rightarrow metarhodopsin I \rightarrow II \rightarrow opsin plus the release of the trans-retinal. Waggoner and Stryer [127] have followed this sequence by the temperature dependence of the induced CD bands in retinal. The two positive Cotton effects at 500 and 320 nm seen in retinal in the rhodopsin, metarhodopsin I, and metarhodopsin II disappear upon bleaching to yield opsin and retinal. Analysis of the CD spectra leads to the suggestion that a significant portion of the local environment of retinal is conserved in the three stages, and the CD spectra seem to arise due to interactions of retinal with a suitably oriented chromophore on the protein that is in close proximity.

Phytochrome is a plant protein that contains a linear tetrapyrrole prosthetic group and is also responsive to light. Burke, Pratt, and Moscovitz [128] have studied the interconversion of phytochrome to be of the type:



Absorption spectra of P_R and P_{FR} form of the chromoprotein are not significantly dependent on temperature from -70°C to room temperature, though the CD bands of the prosthetic-group-induced Cotton effects are. Calculations and experiments on the induced Cotton effects of the various interconverting forms have revealed that the bound chromophore has an extended conformation in P_R , P_{FR} , and in A, while it is folded in P_{BL} . This interconversion between folded and extended forms is light dependent and thought to involve cis \leftrightarrow trans isomerizations.

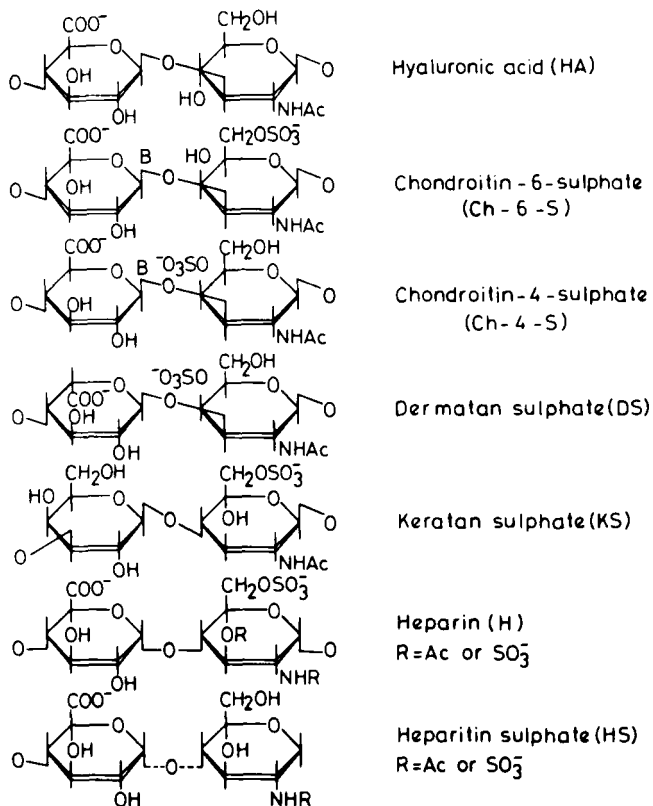
In recent years, use of extrinsic Cotton effects has been made in studies of affinity labeling and hapten-antibody interactions. These have yielded potentially useful information about the number, nature, and strength of these interactions [129, 130].

The question of whether bound solvent molecules contribute to and alter the CD spectra of polypeptides and proteins has been considered by Pysh [131]. He has considered the changes that occur in the ϕ and ψ angles for various peptide conformations upon solvation, and he has suggested that the CD of the β -sheet and the polyproline-I form are not expected to alter upon solvation while the other conformations will. Calculations for polyproline II are in accord with the observation [132] that benzyl alcohol binds to this helix and displays an induced Cotton effect.

IV. OPTICAL ROTATORY PROPERTIES OF POLYSACCHARIDES

The chromophoric groups in naturally occurring sugars are rather limited; the ubiquitous hydroxyl group (and the ring $-O-$) have their longest absorption band in a region barely accessible to commercial spectropolarimeters. As a result, CD studies are somewhat less abundant on these compounds than ORD. Since the transition of the $-OH$ and $-O-$ groups lie below 190 nm, the ORD profiles of sugars are generally monotonic and the Cotton effect extrema in ORD are not seen. However, several empirical correlations of conformations were arrived at in earlier years by the use of $[\alpha]_D$ values of a variety of epimeric sugars (the Hudson rules, etc.).

Naturally occurring polysaccharides are often built up of not only plain polyhydroxy sugars, but also of derivatives of these such as N-acetyl glucosamine (NAG), N-acetyl galactosamine, muramic acid, and 2-acetyl muramic acid, and the 2-acetamido-6- (or 4-) sulfates of hexoses such as glucose. Some of the naturally occurring polysaccharides with these chromophoric monomers are as follows:



In these cases the presence of the acetamide (peptide) chromophore causes the presence of a CD band in the 215-nm region, very likely due to the peptide $n-\pi^*$ transition. The carboxyl group $n-\pi^*$ band is also seen in this region. The OH bands should occur further below in wavelength. The CD spectra of the various mucopolysaccharides mentioned above that have been reported by Stone [133] are shown in Fig. 11.

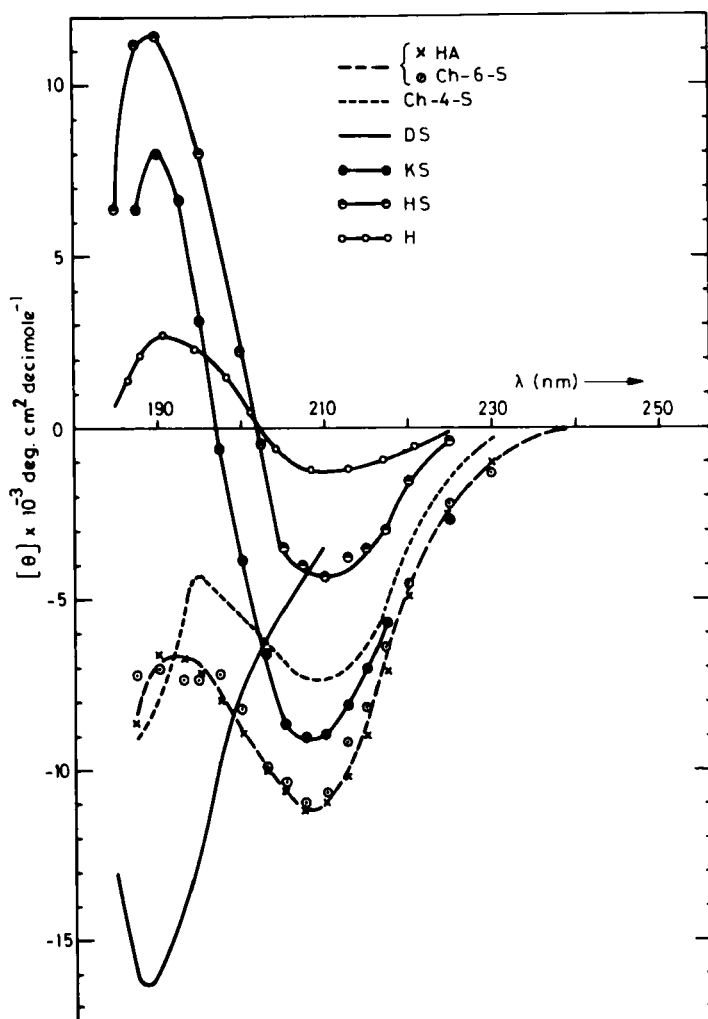


FIG. 11. Ultraviolet Cotton effects of various polysaccharides. Curves drawn from the reported data of Stone [133].

The common feature in all these spectra is the negative CD band at 208 nm. Besides this, however, the 1,3-linked acetamido sugar polymers (HA, ChS, DS) show a second CD minimum around 190 nm, while the 1,4-linked acetamido sugar polymers (KS, H, HS) show a positive CD band around 190 nm. On the basis of the CD profiles, it has been concluded that the 1,3-linked polymers (HA, ChS, DS) possess similar conformations, which are quite different from the common conformation suggested for the family of the 1,4 sugars (KS, H, HS). The band positions, signs, and magnitudes of the CD bands have been used for the identification of the mucopolysaccharides.

Pectic acid is a polymer comprised of β -galactouronic acid moieties linked 1,4. Its CD spectrum is characterized by a positive CD band of $[\theta] = 200^\circ$ centered around 200 nm which can be ascribed to the carboxyl $n-\pi^*$ band [112]. A negative CD band appears, presumably at lower wavelengths. The induction of a carboxyl $n-\pi^*$ CD is interesting; it appears as if in order to induce this Cotton effect the carboxyl group should be proximal to the asymmetric center of the residue (cf. earlier discussion on PBLA and PGLG, Section III-J). Cotton effects of similar signs and roughly the same magnitudes are reported for poly-S-lactic acid [134]. A positive CD band of roughly the same magnitude (though occurring slightly red-shifted) is seen in the case of the fully methoxylated ester of pectic acid, pectin [135]. In general, when the carboxyl group is dissociated, the CD band is red-shifted and reduced considerably in value.

Pectic acid and similar naturally occurring polyhexose-uronic acids, e.g., the alginates, exhibit the tendency of gelating upon the addition of divalent cations, notably Ca^{2+} . Alginates are block copolymers of β -D-mannuronic acid (M) and α -L-guluronic acid (G). The reference CD spectra of poly-G and poly-M are quite different in that while poly-G displays a moderate negative Cotton effect centered around 210 nm, poly-M is characterized by a weak positive ellipticity band slightly blue-shifted to this. The CD spectra of natural alginates are interpreted as a composite mixture of the two reference polymers. The effect of Ca ions on poly-G (or G blocks in alginates) seems more pronounced and is manifested as a sign reversal of the 210-nm negative band, while the effect on poly-M (or M blocks) is much less dramatic (see Fig. 12). It has thus been possible to monitor the Ca ion induced gelation of alginates by CD spectra [135].

It is only in recent years that CD has been used in the polysaccharide field to study conformational effects. In some instances, progress has been similar to the case of peptides, where results of conformational calculations have been used in conjunction with spectral data to pinpoint possible conformations of the molecules. For

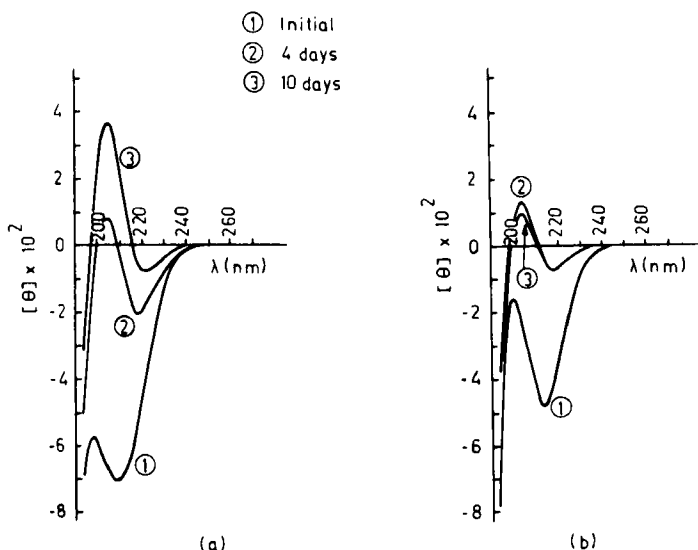


FIG. 12. Effect of Ca^{2+} -induced gelation on the CD spectra of (a) high G alginate and (b) high M alginate. (From Ref. 135).

example, conformational calculations on cellobiose and xylobiose suggest no preferred or fixed conformations [136], and the CD spectra of cellulose and xylon acetates are substantially the same as those of lower oligomers, and devoid of any effects due to interchromophore interactions. On the other hand, α -(1,4)-amylose derivatized by the carbanilate ($-\text{OCONHC}_6\text{H}_5$) chromophore exhibits a closely spaced CD doublet at 240 and 225 nm that has been interpreted as due to exciton coupling between neighboring carbanilates disposed in a helical array of the amylose polymer [137]. Cellulose carbanilate, which is β -1,4-linked, is devoid of this conformational effect as shown in Fig. 13. Here the chain is considered to be a planar sheet conformation stabilized by intramolecular interamide hydrogen bonding.

The ORD/CD behaviors of polysaccharide benzoyl derivatives have been studied by Keilich and Bittiger [138]. They again show conformation-dependent Cotton effects.

The onset of the helical form in the amylose family seems to occur as early as the trimer stage. While glucose acetate and β -maltose acetates give a single CD maximum at 230 nm (300°), with the trimer maltotriose acetate the band splits into a doublet, a positive maximum at 240 nm (100°) and a negative one at 215 nm (-300°), similar to what is observed in amylose acetates and perhaps indica-

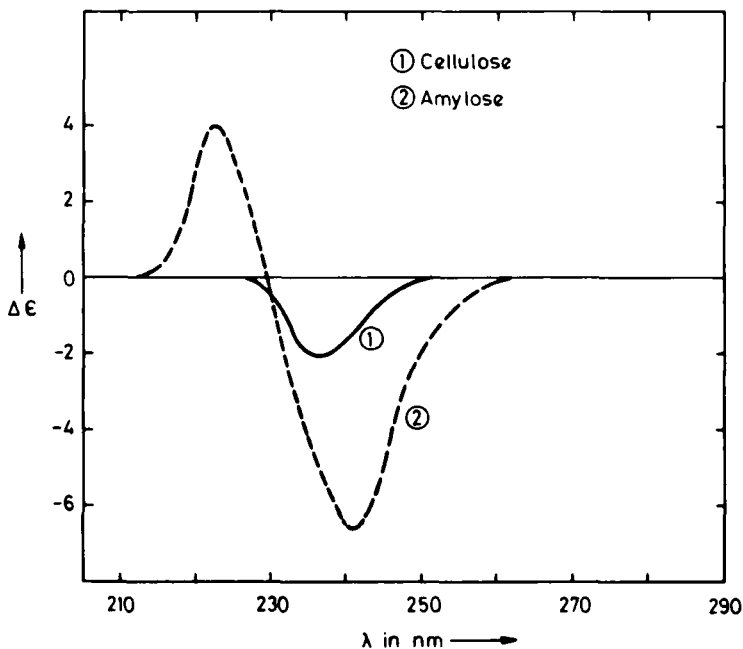


FIG. 13. CD spectra of (1) cellulose and (2) amylose tricarbanilates. (From Ref. 137).

tive of exciton splitting. These results are reminiscent of the work described earlier regarding the onset of the α -helical fold in oligomeric peptides.

Extrinsic Cotton effects or induced Cotton effects have been observed in the absorption bands of dyes and small molecules that are bound to natural polysaccharides. In a thorough study of the I_2 complex of amylose (and of homogeneous fractions of amylose with well-defined chain lengths), Pfannemuller and co-workers [139] observed that the magnitude and position of the extrinsic Cotton effects were chain-length dependent, with the maximum effect occurring with amylose chains of chain length 50 to 80. Here one sees a positive Cotton effect in the complex at 540 nm ($\Delta\epsilon = +3.74$), a smaller positive ($\Delta\epsilon = +1.6$) band at 480 nm, and a third even smaller Cotton effect at 340 nm ($\Delta\epsilon = +0.8$). Chains with smaller or longer lengths than this optimum range bound I_2 less well, and the extrinsic Cotton effects of the I_2 transitions are also smaller in magnitude. It has been suggested that chains of length 50 to 80 are stiff and well ordered, while longer chains are either broken helical rods or worm-like helical chains.

In a study of the binding of the dye acridine orange (AO) to chondroitin-6-sulfate, it was found [140] that the AO displays CD metachromasia, i.e., displays a negative Cotton effect at 470 nm (-5×10^5), a second negative band at 280 nm (-10^6), and two smaller positive CD bands below this. These induced Cotton effects are somewhat formally similar to what has been seen in the case of AO-poly-L-glutamic acid complexes, and it also appears that the carboxyl groups of chondroitin sulfate interact somewhat stronger with AO than do the sulfate groups.

Extrinsic Cotton effects for the dye methylene blue (MB) bound to several mucopolysaccharides have been used to diagnose the intersugar linkage mode in the polymer substrate. Stone [141] has found that the long-wavelength Cotton effect seen in MB is positive when it is bound to 1,3-linked polysaccharides such as chondroitin-4-sulfate and Dermatan sulfate, while the Cotton effect is negative when the polymer to which it is bound is 1,4-linked, e.g., heparin and keratan sulfate. However, this might not be used as a generalization since such differential behavior is not observed when the dye used is AO rather than MB. When we turn to the amylose series, it is found that while glucose peracetate (monomer) and β -maltose acetates give simple positive CD maxima at 220 nm, the trimer maltotriose peracetate reveals a split CD couplet, a smaller positive Cotton effect near 240 nm followed by a much longer negative band below 220 nm. This CD pair has been interpreted to arise due to an exciton split of the acetate π - π^* band in the trimer arising out of the disposition of residues in a helical array. The CD spectrum of amylose triacetate is qualitatively similar to that of the trimer. The onset of the helical conformation seems to be at the trimer level.

Such conformational effects on the CD spectra of polysaccharides have been noted in the case of the carbanilate ($-\text{OCONHC}_6\text{H}_5$) derivatives [137] and benzoyl derivatives of polysaccharides [138]. In β -linked polyglycans such as cellulose tricarbanilate, the CD spectrum is characterized by a single negative Cotton effect at 240 nm ($\Delta E = -2$), while the α -glycans, e.g., amylose tricarbanilate, displays an exciton split couplet with a negative band at 250 nm ($\Delta E = -7$) followed by a positive one near 225 nm ($\Delta E = +4$). The tri-O-benzoyl amylose derivative likewise displays such an exciton pair: at 240 nm ($\Delta E = -25$) and below 230 nm (ΔE positive), besides a positive band at 280 nm (vibrational structuring, $\Delta E = +3$) which is due to the aromatic band of the benzoyl group. This significant difference between the CD profiles of cellulose and amylose derivatives has been suggested to reflect their structural differences: while amylose is a helical chain, the cellulose molecule is considered to have a planar zigzag chain.

V. NUCLEIC ACIDS

Nucleic acids are polymers in which the backbone is made of d-ribose [in ribonucleic acids (RNA)] or 2-deoxy-d-ribose [in deoxyribonucleic acids (DNA)] units linked via phosphodiester bonds. The side-chain groups are any of the following five bases: adenine (A), Guanine (G), cytosine (C), thymine (T) (occurring in DNA), and uracil (U) (in RNA). It is these nitrogenous heterocyclic chromophores that display Cotton effects in the accessible spectral regions, with the sugar essentially offering background rotation. The RNA molecule possesses partial chain order, essentially by a single-chain folding and through complementary hydrogen bonding between A and U, and G and C. The structures of transfer RNA's have been investigated in detail recently. Occasionally double-stranded helical conformation is seen in RNA and polyribonucleotides, but by and large the double-stranded helix (of the Watson-Crick type) is characteristic of the DNA molecule, achieved through A-T and G-C base pair hydrogen bonding between the two strands. Such a double helical structure offers several spectral features such as exciton bands, polarization, and stacking effects which we discuss below.

The spectral characteristics of the constituent bases have been extensively reviewed [142, 143] and so will not be discussed here. The application of CD and ORD techniques to nucleic acids has been reviewed by Yang and Samejima [142], Beychok [143], and T'so [144, 145]. The recent review by Bush [10] covers the field in excellent detail and should be referred to for further details.

A. Polynucleotides

In order to understand the properties of RNA and DNA, the ORD/CD properties of many model homopolynucleotides have been of utility. The ORD of poly-C, poly-U, and poly-A neutral strands were calculated by Cantor et al. [146] from the dinucleotide spectra using the nearest-neighbor effect method. The agreement is good, and discrepancies have been assigned to $n-\pi^*$ transitions [147] and red shifting due to exciton coupling [148, 149].

Inoue et al. [150] have argued that instead of using dinucleotide spectra, if the difference spectra between tetranucleotide AAAC and dinucleotide ApCp is used, better agreement can be achieved because the tetranucleotide possesses some of the polymer characteristics such as nonnear-neighbor interactions. This, though true, is not widely practiced due to the paucity of spectral data on a number of such sequenced oligonucleotides.

Double helices of poly-A have been shown to exist in at least two

structures with differing optical properties. Their conformational differences may involve different amounts of base tilting [151]. CD studies by Brahms et al. [152] show that the oligomer must be at least seven residues long to form these complexes. Poly-C also assumes a double helical conformation on protonation. The differences in CD between the neutral single-stranded and charged double-stranded poly-C are due to both conformational and chromophoric differences. Here again the threshold length for helix formation is seven residues [153]. Poly-U, though little stacked at room temperature, shows intense Cotton effects in ORD at low temperatures with a sharp melting temperature of 10°C [154], which might arise from autocomplexation. Poly-G forms very strong aggregates, but due to the absence of data on the nature of the complexes, interpretation of the CD spectrum here is difficult [155]. Polyinosine shows ORD curves indicative of a double Cotton effect with negative CD at longer wavelength [156]. This is expected for a single-stranded left-handed helix from theory [157]. Since almost all polynucleotides normally form right-handed helices, this becomes very interesting and needs further probing.

Complexing between complementary strands of polynucleotides also has been probed by ORD/CD techniques, but many difficulties are encountered here. For example, the ORD and CD spectra of poly-A:poly-U complex, poly-A:2 poly-U complex, as well as the CD of the random copolymer, poly-AU, all show similar characteristics [158] (Fig. 14). Poly-G:poly-C form double-stranded complexes whose CD-ORD spectra [155, 156] do not agree with calculated spectra for either DNA or RNA geometry [159]. The double-stranded helix poly-I:poly-C shows a double Cotton effect in ORD [155]. Such complex formation normally leads to blue shifts in CD and ORD in comparison with the noncomplexed polynucleotides.

In the case of deoxyoligonucleotides, it is found that the nearest-neighbor effect calculations fail in many cases to reproduce the experimental spectra [160]. Moreover, the CD spectrum of the dinucleotide dApdA is quite different from poly-dA, showing the case to be different from the ribonucleotides where the corresponding spectra are largely similar. Also, normally the polydeoxynucleotides have optical activity of a lower magnitude than the polyribonucleotides (Fig. 15) [161].

Wells et al. have shown that the CD of polydeoxyribonucleotide complexes with all pyrimidines on one strand and all purines on the other strand show specific sequence-dependent CD features even when their total composition is the same, and they are conformationally different from normal DNA with both purines and pyrimidines on each strand (see Ref. 10). Calculations of the CD of the

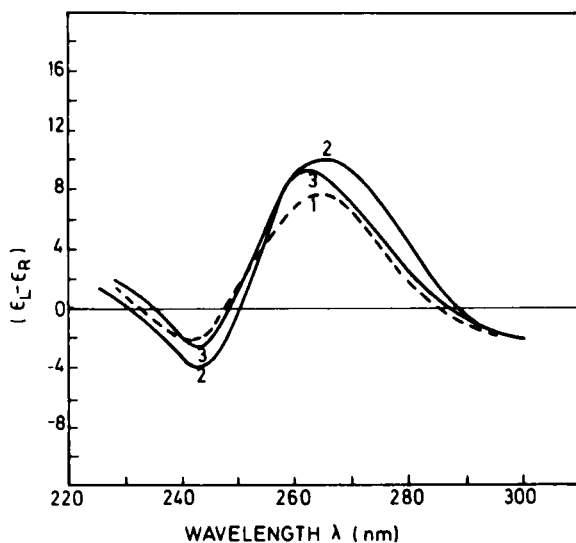


FIG. 14. CD of polynucleotides of A and U. Curve 1, poly A:2poly U. Curve 2, poly A:poly U. Curve 3, poly AU random copolymer [158].

complex poly-dA:poly-dT by the method of Gray and Tinoco [162] fail to reproduce the experimental spectra in this case.

B. Ribonucleic Acid

Single-stranded RNA, free of intrastrand base pairing as found in the absence of salts, retains most of its stacked conformation, and its ORD spectra (Fig. 16) tally with those obtained through nearest-neighbor calculations, based on the assumption of a random occurrence of nearest-neighbor combinations for a given base composition. This method has been applied for a number of cases of viral RNA, mixed t-RNA's, and ribosomal RNA [146, 163]. Formaldehyde-treated tRNA's exhibit CD/ORD spectra similar to salt-free RNA and may have no intrastrand base pairing.

Base pairing, as seen in the difference in spectra between salt-free and 0.1 M KCl solutions of TMV RNA, leads to a blue shift [146] (Fig. 16). These shifts can be arrived at quantitatively as the sum of shifts seen on complexation on the poly A:poly U and poly G:poly C systems per base pair, and in this way the total number of A-U and G-C base pairs formed is arrived at. This method has been applied in a number of systems to choose between alternate conformational possibilities on the basis of CD/ORD data. For example,

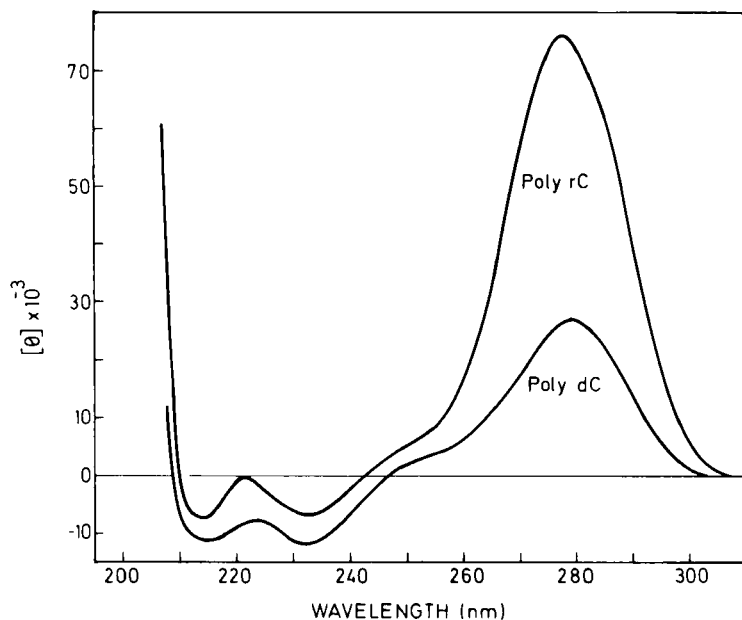


FIG. 15. CD of poly rC and poly dC in 2 M NaCl, pH 10.0 [161].

Blum et al. [164] found in a study of nine transfer nucleic acids that agreement between their calculated CD spectra and experimental spectra improved when they included specific H-bonding between various base pairs in the calculations, and they could estimate the number of H-bonded pairs in each case. In another study, Cantor used the base pairing effects on ORD of 5s ribosomal RNA of *E. coli* to choose between two models, one with 23 base pairs and the other with 49 base pairs [165] (see Fig. 17 for the corresponding CD). It has also been shown that single-stranded, base-paired RNA and its complex with a complementary chain both show very good similarity in many cases [166]. McMullen et al. [167] have shown through rank analysis that only two independent curves, one for the free and one for the base-paired region, are necessary for the synthesis of normal RNA ORD curves.

Certain other problems arise in the interpretation of CD-ORD spectra of RNA's due to the presence of unidentified minor chromophores which may contribute significantly to the optical rotation of the RNA. For example, 4-thiouracil is present in many RNA species as a minor chromophore and gives rise to CD bands in the 340-nm region [168, 169]

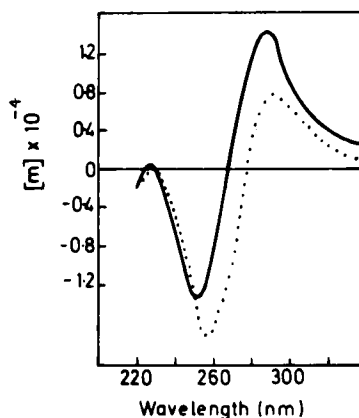


FIG. 16. ORD of TMV RNA at pH7. In presence of 0.15 M salt (—). In absence of salt (· · ·). Note the blue shift on base-pairing in the presence of salt [146].

C. DNA

Studies on single-stranded DNA's are limited in number and only recently have attempts begun to interpret them in terms of nearest-neighbor effects. Cantor et al. [160] have applied the nearest-neighbor method to F-1 DNA in salt-free solution and the calculated spectra are in reasonable agreement with the experimental spectra.

A number of studies have been carried out on double-stranded DNA from various sources. These CD and ORD curves are generally similar (Fig. 18) and are very different from those of RNA.

Mitochondrial DNA's have been shown to differ from nuclear DNA in CD and ORD [170] spectra. Though the gross characteristics are similar, mitochondrial DNA's exhibit a number of shoulders in the larger wavelength region which are not shown by nuclear DNA's.

While normal DNA occurs in the Watson-Crick B-form with its bases perpendicular to the helical axis and gives a double Cotton effect (see Fig. 18), it is possible under different conditions to prepare samples of DNA with changed conformations. For example, in 80% ethanol DNA gives a single positive Cotton effect similar to RNA (Fig. 18). This was interpreted to mean that the bases are tilted in this conformation and the overall geometry is similar to the A-conformation of DNA [171]. Ethylene glycol as well as 6 M CsCl or LiCl induce negative Cotton effects (Fig. 18) in DNA [172, 173] similar to the C-form of DNA. Similarly, RNA-DNA hybrids, which have their bases tilted as in the DNA A-form, produce a posi-

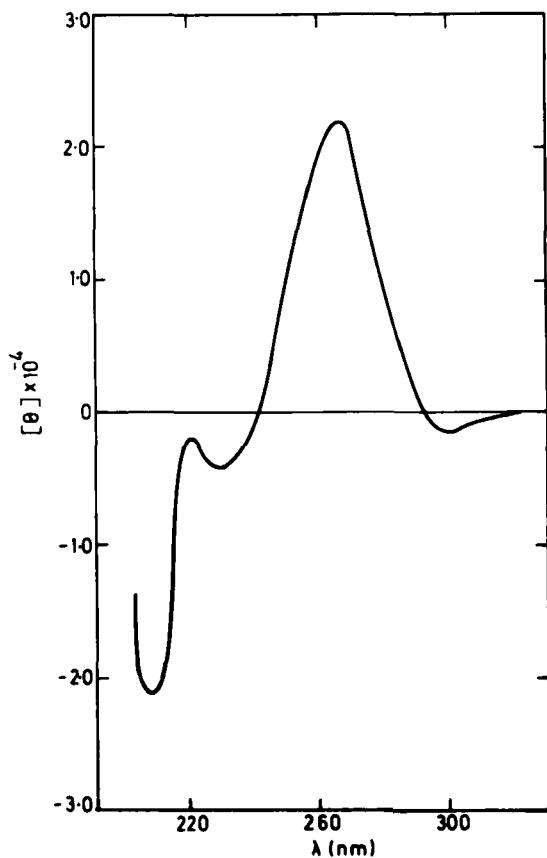


FIG. 17. A representative CD spectrum of RNA:CD of 55 RNA of *E. coli* [166].

tive Cotton effect in high salt solutions [173]. Studies on DNA films under varying conditions also lead to similar results [174].

In summary, normally DNA occurs in the B-form with its bases perpendicular to the helical axis and showing a small double Cotton effect. DNA in A-form, with a positive tilt of its bases, produces a single positive Cotton effect, while the DNA in C-form, with a negative tilt of the bases, produces a single negative Cotton effect.

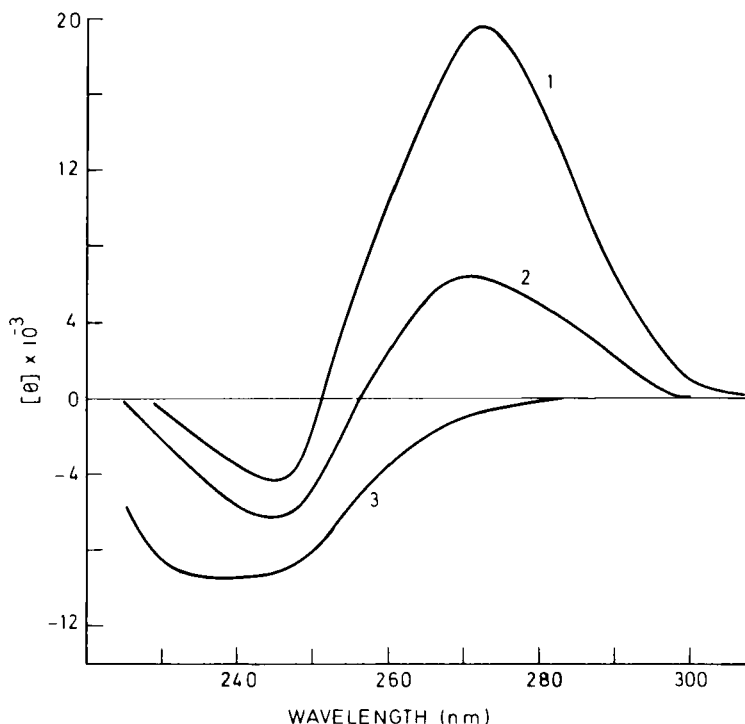


FIG. 18. CD of DNA. Curve 1, DNA in 80% ethanol [171]. Curve 2, DNA in aqueous solution. Curve 3, DNA in ethylene glycol [172].

VI. COMPLEXES BETWEEN BIOPOLYMERS

Interactions between proteins and nucleic acids are important in processes such as replication, transcription, translation, and histone interactions. Hence much effort has been invested to understand this complex formation. CD, in preference to ORD, has been extensively utilized to monitor conformational changes that occur in such complex formation.

Carroll [175, 176] has explored the interaction of polylysine with various polynucleotides and found that complexing of polylysine to double-stranded polynucleotides and DNA leads to enhancement of the CD spectra of the polynucleotides, while no effect of polylysine on single-stranded polynucleotides or RNA was detectable. These enhanced spectra may be due either to changes in DNA conformation or to long-range order in the aggregates.

The interaction of histones with DNA, which is one of the biologically most important interactions, is being probed by CD studies. Permogorov et al. [177] found only very slight differences between the CD of free DNA and of nucleohistones. Sponar et al., from a study of the ORD of native, partially dissociated, and reconstituted nucleohistones, found that the spectrum of DNA in native nucleohistone is substantially retained even when a substantial part of the protein has been removed [178]. Fasman et al. [179] found that a negative Cotton effect is induced on the complexation of pure DNA with F-1 histone. It has also been reported that the positive Cotton effect in DNA is suppressed by the addition of histone [180]. These reports may mean that the DNA, on complexation with histone, changes into the C-form. Wagner and Vandegrift [181] have examined the CD spectra of DNA-histone complexes formed at various concentrations of Ca^{2+} . They found that up to 0.15 M CaCl_2 , the complex and free DNA exhibit similar CD features, while the complex formed at 0.05 M CaCl_2 displays a CD akin to that of DNA in the A-form. Johnson et al. reported differences in the CD of nucleohistones prepared by different methods, and they attribute this to differing amounts of B and C conformation in the DNA [182].

Jordan, Lerman, and Venable [183] have shown that in the presence of polyethylene oxide or polyvinylpyrrolidone above a critical concentration, DNA in salt solution is driven to assume a compact form due to repulsive interactions between the DNA and polymer, accompanied by qualitative changes in the CD spectrum. The changed spectrum somewhat resembles the spectra of F-1 histone or the polylysine complex of DNA. Based on X-ray diffraction studies, as well as the conditions of the study, it has been suggested that DNA in these conditions exists essentially in the B-form, but with long-range interhelix interactions. These interhelix interactions and the consequent ordering may lead to the changes in the CD spectra.

The effect of packing in virions on RNA or DNA has been monitored by comparing the difference between the virus and the ghost with the free viral DNA or RNA. In the case of many phages it was found that the differences between ORD of DNA in phage and free DNA correlate well with the DNA content per unit volume when packed in the phage [184]. This may mean that the changes in CD are due to conformational restrictions on DNA when they are packed in the protein coat. Normal phages are known to exhibit DNA ORD spectra corresponding to all the three standard conformations of DNA, A, B, and C.

In the case of tobacco mosaic virus (TMV), it was found that the difference in ORD between the virus and its RNA-free ghost matches

well with the free RNA ORD, showing the absence of base pairing in TMV RNA in the virus [163]. A similar examination of mengovirus whose ORD, along with that of its free RNA, has been reported becomes difficult due to the absence of protein ghosts [185]. Only a general analysis of the free RNA is possible in this case, and this shows extensive base pairing.

Another interesting example of studies into the nature of interactions of various molecules with DNA is the study of the interaction of some antibiotics with DNA, reported by Dalglish et al. [186]. They found the difference CD spectra of complexes of chromomycin and mithramycin with DNA and free DNA are very similar. This was interpreted to mean that the antibiotic binding sites in the DNA as well as the general conformation in the bound state for these drugs are similar, and the binding process, while altering the CD spectra of the bound drugs, also removes the differences in local conformation that exist in the free drugs. They also found that, based on CD alone, it is not possible to say whether these drugs intercalate or not, which is possible in the case of many other compounds due to the specific CD spectral changes associated with intercalation [187-189].

A. Membranes

The initial uses of ORD and CD in studying membrane systems were for elucidating the gross conformational details of membrane proteins. These studies showed the membrane proteins from various sources to be largely α -helical in the intact membrane [190-192]. Many differences were noticed in these measurements in the form of red shifts of peaks and reduction of intensity of Cotton effects. These effects have been traced to various optical artifacts arising out of the particulate nature of the sample [21, 193-195]. Gordon [23] has calculated the effect of Mie scattering by optically active particles on their CD spectra, and he reached results that explain the experimentally noted artifacts well.

The conformation of proteins and polypeptides in membranes can be easily studied by utilizing liposomes. Such CD studies on alamethicin, a cyclic polypeptide, show spectral characteristics similar to the compound in organic solvents [6]. The obvious interpretation is that alamethicin exists within the nonpolar region of the bilayer membrane. Similarly, valinomycin in lecithin vesicles is conformationally unaffected by the presence of K^+ in the solvent, which again shows valinomycin to be situated in the inaccessible hydrocarbon region of the bilayer membrane.

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