Journal of Pharmaceutical Sciences

APRIL 1970
VOLUME 59 NUMBER 4

*REVIEW ARTICLE*

# Small Molecule–Macromolecule Interactions as Studied by Optical Rotatory Dispersion–Circular Dichroism

J. H. PERRIN and P. A. HART

**Keyphrases** ☐ Small molecule–macromolecule interactions—review ☐ Cotton effect—molecular binding ☐ Metachromasia—molecular binding ☐ Dye effects—optical rotatory dispersion ☐ Optical rotatory dispersion–circular dichroism—molecular binding determination

This review is intended to summarize the study of specific binding of low molecular weight substances to macromolecular systems by the application of optical rotatory dispersion–circular dichroism (ORD–CD). The authors have specifically not included inorganic ions. In addition, solvent and cosolute effects have not been covered because these phenomena are not necessarily related specifically to binding.

The review includes brief discussions of the phenomena of ORD–CD as well as applications to the proteins, nucleic acids, and polysaccharides. Metachromasia, the color-change phenomenon associated with binding, is discussed from the historical viewpoint and in terms of the more recent spectroscopic studies.

Extrinsic (induced) Cotton effects are discussed for dyes, coenzymes, and related ligands when bound to proteins, nucleic acids, polysaccharides, and membranes. The specific structural information (if any) that can be obtained from these studies is presented.

ORD, or the study of the change of optical rotation with wavelength, goes back to the early work of Biot (1) and Fresnel (2), who showed that the rotation of an optically active medium increases with decreasing wavelength of light. Thirty years later came Pasteur's classical work (3) on the resolution of racemic tartrates. The discovery of the Bunsen burner is frequently considered the major reason that ORD studies did not advance significantly until the 1930's, because measurements were too easily made at the sodium D-line, a wavelength of little significance for most colorless compounds; however, much use was made of these measurements as analytical and characterization tools. In the 1930's, much pioneer work in the field of ORD was done by Lowry (4) and Kuhn (5, 6); they, as well as Mitchell (7), were the first to apply CD to organic chemical problems. Instrumentation still prevented the widespread use of the techniques until the 1950's when commercial ORD instruments became available and, more recently, when CD-measuring devices were introduced. Measurements by both techniques are frequently made down to wavelengths of 185 mμ. For detailed discussions on the theory, instrumentation, and applications of ORD–CD, the reader is referred to books by Crabbé (8), Djerassi (9), and Velluz *et al.* (10), and to reviews by Heller and Fitts (11), Klyne and Parker (12), Moscowitz (13), Tinoco (14), Schellman (15), and Eyring *et al.* (16).

The rotation at any constant wavelength is usually expressed as a specific rotation defined as

$$[\alpha]_\lambda^t = \frac{100\alpha\lambda}{lc} \qquad \text{(Eq. 1)}$$

where $[a]_\lambda^t$ is the specific rotation at a temperature $t$ and a wavelength $\lambda$, $\alpha\lambda$ is the observed rotation, $l$ is the pathlength in decimeters, and $c$ is the concentration in grams per 100 ml. of solution; or as a molecular rota-

tion $[M]_\lambda$ defined as

$$[M]_\lambda = \frac{[\alpha]M}{100} \qquad \text{(Eq. 2)}$$

where $M$ is the molecular weight. The optical rotation of polymeric substances is usually considered a function of individual residues rather than the total molecular weight, and the mean residue rotation is defined as

$$[M]_\lambda = \frac{MRW}{100}[\alpha]_\lambda \qquad \text{(Eq. 3)}$$

where $MRW$ is the mean residue weight which is around 115 for proteins. Optical rotation is dependent on the refractive index of the solvent; the observed rotation can be approximated to vacuum conditions by the Lorentz correction factor, enabling a reduced mean residue rotation:

$$[M']_\lambda = \frac{3}{n^2 + 2}\frac{MRW}{100}[\alpha]_\lambda \qquad \text{(Eq. 4)}$$

to be obtained. For more precise work, the dispersion in the refractive index of the solvent must be considered.

More information concerning the primary or secondary structure of a compound is gained by measuring rotations at a series of wavelengths, and the resultant plot of rotation against wavelength is called the rotatory dispersion curve for the solute.

## ORIGIN OF OPTICAL ACTIVITY

A beam of plane polarized light can be considered to be made up of a right circularly polarized wave $E_R$ and a left circularly polarized wave $E_L$. No optical rotation is observed if these two components are transmitted with equal velocity through the medium. However, if the two vectors pass through the medium with unequal velocity, then the plane of their resultant will have rotated through an angle $\alpha$. If the right circularly polarized component travels faster, then the medium is dextrorotatory; conversely, if the left travels faster, then it is levorotatory. The speed of a lightwave is a function of the refractive index of the medium, and the medium is optically active if it has different indexes of refraction ($n_L$ and $n_R$) for the left and right circularly polarized light. Fresnel (2) has shown that the rotation in radians per unit length is given by

$$\alpha = \frac{\pi}{\lambda}(n_L - n_R) \qquad \text{(Eq. 5)}$$

and, using the more familiar specific rotation in degrees per decimeter,

$$[\alpha] = \frac{1800}{\lambda}\left(\frac{n_L - n_R}{c}\right) \qquad \text{(Eq. 6)}$$

The refractive indexes $n_L$ and $n_R$ vary differently with the wavelength and are approximated by

$$n = 1 + \frac{a\lambda^2}{\lambda^2 - \lambda_v^2} \qquad \text{(Eq. 7)}$$

where $a$ is a constant function of the strength of the oscillator of characteristic wavelength $\lambda_v$. $\lambda_v$ is a constant and is the same as that in London's equation for intermolecular attraction (17). This change in refractive index with wavelength means that the specific rotations also are a function of wavelength; in the regions far from optically active absorption bands, they give rise to plain curves, the equations for which have been given by Drude (18):

$$[\alpha] = \sum_i \frac{K_i}{\lambda^2 - \lambda_i^2} \qquad \text{(Eq. 8)}$$

where $\lambda_i$'s are the wavelengths of the optically active electronic transition and $K_i$'s are constants proportional to the rotatory strength of the $i$th transition. In the simplest case this modifies to the single-term Drude, giving

$$[\alpha] = \frac{K}{\lambda^2 - \lambda_0^2} \qquad \text{(Eq. 9)}$$

where $\lambda_0$ is the wavelength of the closest absorption maximum. The equation clearly indicates an increase in optical rotation as the wavelength is decreased.

Suppose, however, the medium shows unequal absorption of the incident right and left circularly polarized light; then the emerging light is also elliptically polarized and the phenomenon of unequal absorption is known as circular dichroism. To a good approximation (8), it can be shown that the angle of ellipticity $\psi$ is given by

$$\psi = \frac{\pi}{\lambda}(K_L - K_R) \qquad \text{(Eq. 10)}$$

where $K_L$ and $K_R$ are the absorption coefficients of the left and right circularly polarized light. A specific ellipticity $[\psi]$ is analogous to the specific rotation, and a molar ellipticity $[\theta]$ is given by (19)

$$[\theta] = \frac{[\psi]M}{100} = 2.303\frac{4500}{\pi}(\epsilon_L - \epsilon_R) = 3300(\epsilon_L - \epsilon_R) = 3300\Delta\epsilon \qquad \text{(Eq. 11)}$$

where $\epsilon_L$ and $\epsilon_R$ are the molar extinction coefficients for the left and right circularly polarized light. The extinction coefficients also vary with wavelength, and the ellipticity of the medium varies with wavelength, enabling a circular dichroism curve to be obtained. A CD curve is only a function of the unequal absorption of the incident light, but the ORD curve is a function of the unequal transmission as well as the unequal absorption of light, the shape of the ORD curve depending on the proximity of any optically active chromophores (8, 9).

The combination of unequal absorption and unequal transmission of the left and right circularly polarized light in the region in which optically active absorption bands occur is known as the "Cotton effect," after the man who first observed these anomalous rotations (7, 20, 21). Whether single or multiple Cotton effects are noticed depends on the number of optically active absorption bands involved; obviously, the wavelength regions of the Cotton effect are of most interest in ORD studies. The molecular amplitude, $a$, of a Cotton effect is defined as the difference between the molecular rotation at a peak or trough and the molecular rotation of the next extremum of shorter wavelength divided by 100.

This molar amplitude has been shown as a corollary of the Kronig-Kramer theorem (8, 22, 23) to be ap-

proximated to the dichroic absorption ($\Delta\epsilon$) of a CD curve by

$$a = 40.28\Delta\epsilon \qquad \text{(Eq. 12)}$$

or, using molar ellipticity $[\theta]$,

$$a = 0.0122[\theta] \qquad \text{(Eq. 13)}$$

This relationship allows at least semiquantitation between ORD and CD, but it is emphasized that the relationships were obtained for the $n$-$\pi^*$ transition of a saturated carbonyl group and should be used with caution for other chromophores (8).

To transform CD spectra into ORD spectra, one must use the more general form of the Kronig-Kramer equation (9, 24):

$$[M]_\lambda = \frac{2}{\pi}\int_0^\infty [\theta]\left[\frac{\lambda'}{\lambda^2 - \lambda'^2}\right]d\lambda' \qquad \text{(Eq. 14)}$$

This integral can be approximated by summation, but the interval must be small, preferably 1 m$\mu$ or less.

## ORD OF PROTEINS

For a fuller treatment and references, the reader is referred to reviews by Urnes and Doty (25), Yang (26), and Gratzer and Cowburn (27).

**Visible and Near UV Region**—The $[\alpha]_D$ as an oversimplification can be considered to be made up of two contributions, first a levorotatory one from the amino acid residues and second a dextrorotatory one arising from a secondary and tertiary structure. Typically, a protein shows levorotation $[\alpha_D]$, which is lowered to the range $-80$ to $-120°$ upon denaturation (28). It was always realized that because the sodium D-line was so far from the absorbing groups and the rotations measured were small and difficult to quantitate, ORD would give better quantitative data.

Early studies were performed on the synthetic poly-$\gamma$-benzyl-L-glutamate (29) and poly-L-glutamic acid (30, 31) at wavelengths through the visible region down to 300 m$\mu$. These investigations showed that in helix-promoting solvents the polypeptides had anomalous dispersion which was dextrorotatory at long wavelengths, passing through a maximum and then dropping steeply at shorter wavelengths, whereas in the coil-promoting solvents the ORD curve was simple and levorotatory in the visible region. These observations suggested that ORD would be a powerful tool in elucidating the secondary structure of proteins. The $\beta$-forms of polypeptides were also shown to contribute to the optical rotation (29). The oligomers of $\gamma$-benzyl-L-glutamate were known to form $\beta$-aggregates in a poor solvent such as chloroform, and the ORD of the concentrated solution was dextrorotatory throughout the visible region, in strong contrast to the anomalous dispersion of the $\alpha$-helix and the levorotation of the coiled forms. The $\beta$-aggregates dissociated on dilution, and ORD curves similar to the coiled forms were obtained.

**UV Regions**—The laboratories of Blout (32, 33) and Doty (34, 35) in the early 1960's reported ORD and CD measurements on polypeptides in the UV region; Doty's group also measured the CD of polypeptides

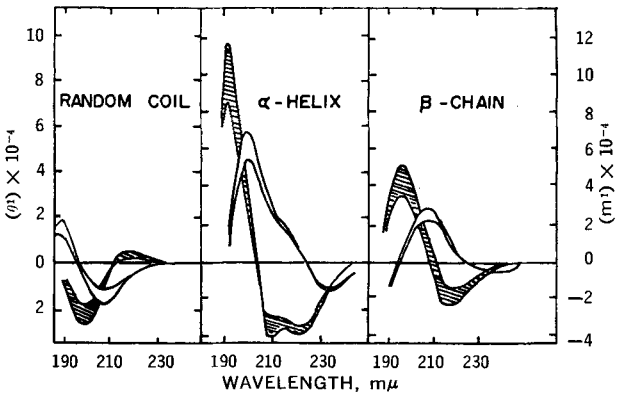


**Figure 1**—*CD (shaded) and ORD of homopolypeptides in the random coil, α-helical, and β-conformations. Taken from* Reference 27 *with permission of MacMillans (Journals) Ltd., London, England.*

down to 190 m$\mu$, and they calculated the ORD curve using the Kronig-Kramer transform and obtained reasonable agreement with the experimental curve. The use of ORD in protein chemistry is mainly to determine conformations; the currently accepted curves for the various forms, taken from *Reference 27*, are shown in Fig. 1. Cotton effects may also arise due to aromatic groups such as tyrosine, tryptophane, and phenylalanine, as well as disulfide bonds (26, 36, 37).

Polypeptides containing less than 50% $\alpha$-helix obey the simple Drude equation, and the constant wavelength term is usually denoted by $\lambda_c$; however, $\lambda_c$ lacks the physical meaning of the wavelength of the nearest optically active absorption band, and it is the result of a mathematical approximation. For denatured or nonglobular proteins, $\lambda_c$ is near 220 m$\mu$ and it is from 230 to 270 m$\mu$ for native proteins. $\lambda_c$, like $[\alpha_D]$, can be used to estimate the helix content if it is assumed that only the helix and a coil are responsible for the changes. These methods are of only historical interest, and the interested reader is referred to the excellent paper of Yang and Doty (29). In 1956, Moffitt (38) used a quantum mechanical approach to the rotatory dispersion of helices; he, together with Yang (39), published a phenomenological equation to explain the ORD of helical macromolecules to enable an estimate of the percentage of the $\alpha$-helix to be made:

$$[M']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad \text{(Eq. 15)}$$

The constants, $\lambda_0$ and $b_0$, are primarily functions of the helical content alone and therefore were regarded as independent of environmental factors such as solvent or side chains; $a_0$, however, represents the intrinsic residue rotations and varies with the environment. Using the suggested value of 212 m$\mu$ for $\lambda_0$ (40, 41), $\lambda_0$ has been set at 212 m$\mu$ and gives a value for $b_0$ for the 100% helical conformation of $-630$; assuming that $b_0$ for the coiled form is close to zero, the fraction in the helical form is given by

$$\frac{-b_0}{630}$$

Numerous modifications of the Moffitt equation have been proposed (see *References 25* and *26* for discussion

and further references), but the method gives reliable results if only random coil and $\alpha$-helical conformations are present. In days when Cotton effects can be directly observed and of readily available CD measurements, the Moffitt equation is really obsolescent. Also, obsolescent are the numerous attempts to use various modification of the multiterm Drude equations to explain the ORD of polypeptides and calculate the percentage $\alpha$-helix (25, 26). For a critical evaluation of Moffitt's equation, the two-term Drude, and a modified two-term Drude, see the work of Blout's group (40–42).

Wada *et al.* (43) have modified the Moffitt equation to include terms for the $\beta$-conformation which had previously been ignored; however, such interpretation is complex, but it appears that the $b_0$ value is only slightly affected by aggregation. It should be pointed out that Cotton effects due to aromatic residues which appear in many proteins probably invalidate the use of Moffitt's and the various Drude equations for quantitative interpretation.

The advent of equipment capable of measuring ORD spectra down to 185 m$\mu$ enables the Cotton effects to be observed directly, and attempts to calculate the percentage helix can be made by obtaining the curve of a model compound in the fully $\alpha$-helical form or in the fully random coiled form and finding the conformation of the protein under investigation by simple proportion. Using poly-L-glutamic acid as a model compound, measurements at 233 and 198 m$\mu$ have been recommended (44); again the presence of $\beta$-aggregates complicates the picture, particularly as the $\beta$-form contribution is variable (45).

## CIRCULAR DICHROISM OF PROTEINS

For a general discussion, see the excellent articles by Beychok (37, 46). CD curves for homopolypeptides in the random coil $\alpha$-helical and $\beta$-conformations are shown in Fig. 1. The percentage of any form in a protein can be estimated from the values of the ellipticities at specific wavelengths of reference synthetic materials known to be in the various configurations. This technique is simplified if the $\beta$-form is again ignored as is often the case (35, 37, 47). The CD of $\beta$-forms has been investigated in detail by Fasman and Potter (45) and Quadrifoglio and Urry (48). Aromatic side chains and disulfides in polypeptides give rise to CD spectra in the 250-m$\mu$ → 350-m$\mu$ region (37, 49). It should also be noted that the amino acids, tyrosine, tryptophane, phenylalanine, and cystine, show intense side-chain optical activity near 220 m$\mu$ at all pH's and whether free or as amides and esters (37). This means that estimating the $\alpha$-helix content and sense for proteins containing a high proportion of these residues is very risky because this side-chain absorption closely overlaps the most important peptide bands. CD has recently been shown to provide a quick criterion of nonidentity between two proteins having similar electrophoretic mobilities and elution profiles (50).

## OPTICAL ACTIVITY OF NUCLEIC ACIDS

The origin of optical activity in the monomeric components of the nucleic acids, the purine and pyrimidine

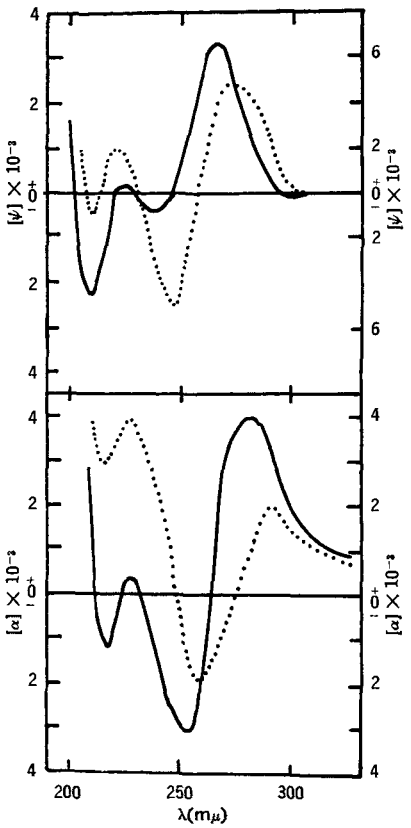


**Figure 2**—*CD (upper) and ORD (lower) of salmon DNA (. . .) and* E. coli *ribosomal RNA* (——). *Taken from* Reference 54 *with permission of Dr. J. T. Yang and Academic Press, New York, N. Y.*

ribosides and 2′-deoxyribosides, at the characteristic UV absorption bands of the aglycones is in the asymmetric perturbation of the aglycone chromophore by the glycosidic portion. The rotation varies as a function of substituent changes in the sugar moiety and as a function of changes in the torsion angle (the angle describing the conformation about the glycosidic bond). It has been generally assumed that the torsion angle for most monomeric nucleosides and nucleotides is in the range corresponding to the *anti*-conformation, but recent work (51, 52) demonstrates that for a number of nucleosides (both purine and pyrimidine ribosides) a significant proportion of the conformer equilibrium is composed of the *syn*-form. It is unknown, at present, whether this new information will significantly alter the current status of the nucleic acid conformation problem to be discussed.

When the monomers are joined by the familiar 3′,5′-phosphodiester linkage to form nucleic acids, one might expect the polymer rotation to be similar to the monomer rotation, differing perhaps by the small perturbation occasioned by the functional group change at C-3′, C-5′. Indeed, that is the observation for unordered polymers (53), but the helical order that can be assumed under appropriate conditions by DNA and some RNA's is characterized by an enhanced optical activity. The complex subject of optical activity of the nucleic acids has been reviewed recently (27, 54) and only the highlights will be discussed here.

Nucleic acid structure can be considered on three different levels, the single-stranded "random" coil, the

single-stranded stacked-base form, and the double-helical form of which there is an A and a B form. The A form of DNA and the double-helical forms of RNA are thought to have similar structural features differing from the B form of DNA in a 15–20° tilt of the base pairs relative to the helix axis compared with virtually no tilt in the B form.

The ORD and CD differences of DNA and RNA may in some cases be rationalized on the basis of the various fundamentally different forms available, but in some cases the observed differences have not been rationalized. Extensive ORD studies of DNA and RNA (55–62) have demonstrated that DNA and RNA from different sources show fairly typical behavior, being characterized by two peaks and a trough in the vicinity of the 260-mμ absorption band (Fig. 2). The relative magnitudes of these characteristic maxima and minima are the distinguishing characteristics that usually allow differentiation between DNA and RNA. The characteristics are best seen in the respective circular dichroism spectra that show a positive ellipticity followed by an intense and a weak negative curve for salmon sperm DNA and a positive effect followed by weak and strong negative effects for double-stranded RNA (63). Extensive prior circular dichroism studies (64–68) recorded similar results.

The first and second ORD peaks of DNA are temperature-dependent as is the first peak of helical RNA. When the variation between rotation at 290 mμ and temperature is studied for DNA, a sharp drop in rotation is seen at the helix–coil transition temperature determined by other means. The same study on helical RNA shows the typical broad melting behavior of that system.

The gross differences in the ORD–CD spectra of DNA and RNA do not arise from double helix–single helix random coil differences, although these forms have typical rotational characteristics. The differences have not been fully rationalized, but some correlations between structure and rotation phenomena do exist. The reader is referred to the review by Yang and Samejima (54) as well as the recent work of Johnson and Tinoco (69 and references therein).

## OPTICAL ACTIVITY OF POLYSACCHARIDES

The study of polysaccharide structure *via* the optical rotation method has historically been carried out at the sodium D-line. The current status of the problem has been reviewed (70). Even when ORD and CD were applied, the non-UV absorbance of most sugars and many polysaccharides made interpretation difficult. It is, therefore, not surprising that less is known about the solution conformation of polysaccharides than about proteins and nucleic acids. Recent reports (71–73) show that the mucopolysaccharides, chondroitin sulfate and heparin, have Cotton effects in the region below 220 mμ arising from $n$-$\pi^*$ and $\pi$-$\pi^*$ amide transitions.

The mucopolysaccharide ORD spectra are of two different types depending on structure. The 3-1-linked glycosamine sugars show a negative Cotton effect (trough at 217–220 mμ), whereas the 4-1-linked glycosamine sugars ($\alpha$- or $\beta$-glycosidic link) show a dominance of the positive Cotton effect in the $\pi$-$\pi^*$ region (~198 mμ) (see Fig. 3). It is thought that the differences may be derived from a preferential association of the 'C-2' nitrogen with the prior C-2 hydroxyl in the first case and of the C-2 nitrogen with the succeeding 2', 3', or 4' hydroxyl in the latter case.

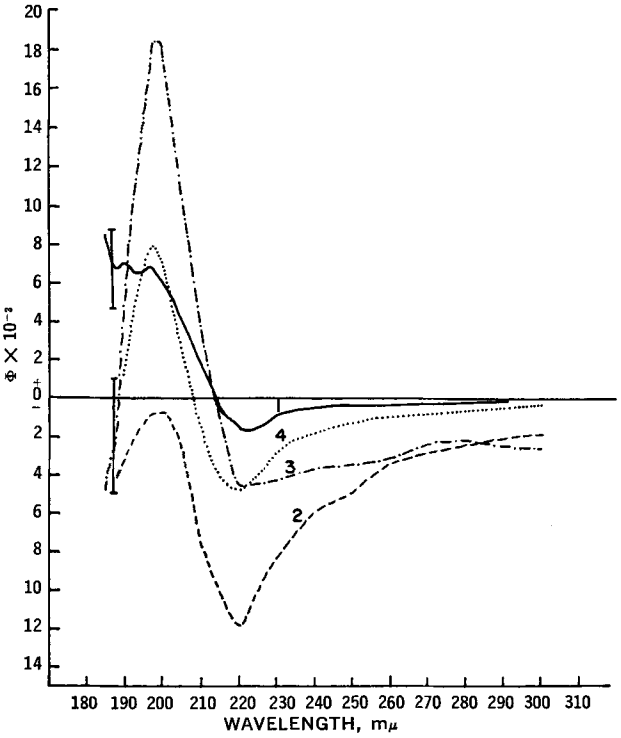


**Figure 3**—*ORD of mucopolysaccharides containing* N-*acetyl glucosamine. Curve 1,* N-*acetyl glucosamine; 2, heparin A; 3, chondroitin sulfate-I; 4,* S-*chondroitin sulfate. Taken from* Reference 70 *with permission of Marcel Dekker, Inc., New York, N. Y.*

That the mucopolysaccharides appear to have optical properties that depend largely on the way the sugars are linked in the polymer regardless of the configuration of the glycosidic bond indicates that rotatory properties derived from the monomer (74) are probably insufficient indexes of the polymer structure. It is concluded that there is a preferred twisting of the polymer strand imposing unique perturbations on the amide chromophores. The observation of these new amide transitions will no doubt allow more informative studies of polysaccharide secondary structure in solution to be made in the future.

## SURVEY OF OTHER METHODS

The reader is referred to the classical review on protein binding by Goldstein (75) for details and references on other experimental methods and to the review by Meyer and Guttman (76) for references concerning specific drugs. These methods, summarized below, can be used for the study of binding of small molecules to macromolecules in general.

**Dialysis**—The equilibrium method is by far the most widely applied and is capable of giving good quantitative data, provided reasonable experimental precautions, such as a careful pretreatment of the dialysis tub-

ing, are followed. Faster kinetic dialysis techniques have been reported by several workers (77–79).

**Ultrafiltration**—This is also a widely practiced equilibrium technique which is quicker than equilibrium dialysis but is quantitatively inferior because of the continually changing macromolecule concentration and the accumulation of the macromolecule on the membrane surface.

**EMF and Conductivity**—Here the incorporation of buffers in the system is an obvious problem and the methods are only useful for studying the binding of ions.

**Electrophoresis**—This method is qualitatively useful in that it can be possible to find to which macromolecule fraction the small molecule is bound. Precise quantitation is difficult.

**Spectroscopy**—The method can be useful if the spectra of either the small molecule or the macromolecule is modified in the presence of the other, but in many the interactions cause little or no spectral changes.

**NMR and ESR**—The use of NMR to study biological interactions has been pioneered by Burgen (80) and Jardetzky (81). The differential changes of the relaxation rates in the high-resolution NMR spectra of both small and large molecules can be used to identify the area of the molecule directly involved in the formation of complexes. Other references can be found in the reviews (82, 83). Electron spin resonance spectroscopy has been applied by studying the ESR spectra of bound free radicals (84). The nature of the ESR spectra is sensitive to the environment, and rapid molecular motion can be detected.

Other methods which have been used include polarography, gel filtration, fluorescence quenching, adsorption, diffusion, the measurement of partition coefficients, and Kerr constant dispersion.

## METACHROMASIA

A recurrent phenomenon that arises in the study of binding processes by ORD–CD is metachromasia (metachromasy). Metachromatic complexes are characterized by a shift to lower wavelength of the absorption maximum of the bound species as well as a decrease in its absorbance (hypochromism). The substance that can induce metachromasia in a dye is called a chromotrope. The usual chromotropic substances of biological origin are high-molecular weight polyanions. Heparin, chondroitin sulfate, hyaluronate, nucleic acids, agar, and alginate are such substances. Some inorganic and synthetic chromotropes are silicates, polyphosphates, chitin sulfate, carboxymethylcellulose, alginate sulfate, pectin sulfate, and polyacrylates. Some relatively low-molecular weight substances are chromotropic such as soaps and anionic detergents, *e.g.*, myristate, surface-active agents (Duponol), and phospholipids (85, 86).

Dyes that stain metachromatically are ordinarily metachromatic by themselves and do not obey Beer's law; it is concluded that metachromasia, whether in the presence or absence of macromolecules, is a function of intermolecular dye–dye association. When chromotropes are present, the initial binding of either the dye aggregate or the dye monomer followed by aggregation at the appropriate concentration is thought to be the sequence of events (87, 88). In fact, when a normally metachromatic dye (methylene blue, toluidine blue) is mixed at low concentration with a substance that normally stains orthochromatically (fibrin, gelatin, and most tissue and cellular structures), the orthochromatic color is seen. Orthochromicity refers to the lack of absorbance maximum shift on binding. Only if the dye concentration is increased does one eventually see metachromasia (89). In addition, ligands that do not ordinarily aggregate in the absence of binding agents (acranil, atebrin, neomonacrin, proflavin, 9-aminoacridine, and some other aminoacridines) show a shift to higher wavelength when bound to nucleic acids, and this bathochromic shift is thought to involve dye–polymer interactions (85, 90, 91). The same observation has recently been made in a study of the binding of proflavin to poly(styrene sulfonic acid) (92).

Elegant studies of the binding of DBTC (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine) to a large variety of polyanions (93–95) revealed that the dye molecule reacts to form at least five distinct complexes depending on the macromolecule, the polymer–dye ratio, and the pH. Four of the complexes are characterized by a shift of the dye absorbance to higher wavelengths and only one is metachromatic. The complexes are thought to involve dye–polymer interactions, resulting in conformational distortion of the dye, dye–counter ion interactions on the polymer, and perhaps in some cases dye–dye interactions, although these are thought to be of minor importance. Dye–counter ion interactions have been invoked as an alternative rationalization for metachromasia by other workers (96).

## PROTEIN INTERACTIONS

**Detergents**—Putnam and Neurath (97–99) pioneered the investigations into the denaturation of proteins by detergents using techniques such as viscosity, electrophoresis, and precipitation. In these investigations of the binding of dodecyl sulfate to horse serum albumin, they concluded that two distinct complexes were formed, the first occurring without detectable changes in the shape of the albumin but the second only occurring with considerable change in asymmetry of the protein. The complexes were said to be due to the binding of detergent anions to cationic protein groups, this erroneous conclusion coming from the similarity between the number of detergent ions bound and the number of binding sites available. Equilibrium dialysis (100–102) was later used to determine accurately the number of detergent anions bound to serum albumin.

Foster (103), among others, suggested that correlation between the number of anions bound and the number of cationic sites was fortuitous, suggesting that the hydrophobic nature of the anion plays an important role in the binding to the proteins. The application of optical rotatory dispersion to the detergent-binding problem has been made mainly by Bruno Jirgensons at the University of Texas. Using a sodium lamp in his earlier work (104, 105), he noticed that detergents produced a negative shift in the specific rotation and this shift was accompanied by an increase in viscosity. Using the same technique (106) to investigate the $[\alpha]_D$ of the plant pro-

teins, tuburin and pea legumin, denatured with sodium dodecyl benzene sulfonate, he again observed an increased negative rotation, and the effect of the detergent became more marked as the pH increased. In this paper he states that optical rotatory power seems to be one of the surest methods of measuring denaturation, a strong statement when one considers he was measuring at a single wavelength far from the absorbing groups in the proteins; one is not surprised at his immediate interest in obtaining dispersion curves when instrumentation became available.

Using the Rudolf instrument (107), he found that all the proteins he investigated obeyed the single-term Drude equation and that on denaturing a metal-binding $\beta$-globulin with AOT and heating to 50°, the dispersion constant of the denatured material was shifted from 243 to 220–230 m$\mu$, whereas the constants for the Bence-Jones protein and $\gamma$-globulin increased under the same conditions from 200–215 to 220 m$\mu$. Realizing the work of Foster (103), Kauzmann (108), and Tanford (109), emphasizing the importance of hydrophobic bonds in the folding of proteins, he proceeded to investigate the possibility that the extreme efficiency of the unfolding of globular proteins by surface-active agents (greater than urea, guanidine salts, and alcohol on a molar basis) might be due to the hydrophobic nature of the tail of the detergent molecule (110). Using the Yang and Doty (29) method of obtaining $\lambda_c$ from the single-term Drude, and suggesting that an increase in $\lambda_c$ above 220 m$\mu$ reflects an $\alpha$-helix formation, he found that detergent-treated $\gamma$-globulins, pepsin, and soybean trypsin inhibitor showed a limited formation of $\alpha$-helices. $b_0$ values from the equation of Moffitt and Yang (39) rose to values close to $-80$, indicating $\alpha$-helix formation.

In the treatment of $\alpha$- and $\beta$-caseins with AOT, the $\lambda_c$ of $\alpha$-casein was enhanced considerably while the value for the $\beta$-form was not affected. It was also observed that the detergents were more active the longer their hydrophobic chain; for example, the 10-carbon chain of sodium caprate caused $\lambda_c$ of $\gamma$-equine to shift to 234 m$\mu$ from the native value of 210, whereas the same concentration of the 6-carbon chain of sodium caproate caused little or no change in $\lambda_c$. He concluded that the hydrocarbon chain of the detergent penetrates into the interior of the proteins, partially unfolding them, these conformational changes being greater the longer the hydrophobic tail of the detergent. Heating the systems to 50° always caused an increased denaturation, presumably due to the fact that at the elevated temperature the micelle-monomer equilibrium of the detergent is shifted toward the free molecules, facilitating the intervention between the protein and the detergent. In an earlier inconclusive paper (111), Jirgensons had found that a bovine albumin, a human albumin, and mercapto albumin had not been denaturated by several long-chain sulfosuccinates at 50 or 70°, using no change in $\lambda_c$ as the criterion. Low reduced viscosities seemed to confirm these observations. Similar observations had been made regarding the effect of detergents on taka-amylase (112), another helical protein. Using improved instrumentation capable of observing the trough of the Cotton effect at 225–240 m$\mu$ due to the $\alpha$-helical conformation, Jirgensons (113) again turned his attention to effects of detergents on the nonhelical proteins. He found that the proteins, reduced and carboxymethylated serum albumin, normal serum $\gamma$-globulin, myeloma globulin, pepsin, trypsin, trypsinogen, $\alpha$-chymotrypsin, chymotrypsinogen, and soybean trypsin inhibitor did not show the Cotton effect, but that the Cotton effect was observed with troughs at 225 to 240 m$\mu$ if the hydrophobic chain of the added detergent was long enough. Sodium dodecyl, sodium decyl, and sodium octyl sulfates, as well as sodium dioctyl sulfosuccinate, were used in the investigations. These observations supported the view that the chains of the detergent disturbed the hydrophobic forces essential for the conformation of the nonhelical proteins.

A paper, again involving the nonhelical proteins and anionic detergents, also published in 1962 (114), contained similar observations. Instrumentation allowing the $\alpha$-helical trough around 230 m$\mu$ to be investigated led Jirgensons (115) to look now at the $\alpha$-helical proteins such as serum albumin, insulin, growth hormone, lysozyme, bacterial $\alpha$-amylase, lactic dehydrogenase, and glutamic dehydrogenase. All these proteins showed a definite Cotton effect at 225–235 m$\mu$, and dodecyl sulfate diminished the levorotation of these helical proteins to a greater or lesser extent. The effects were greater with dodecyl than with decyl sulfate. These effects are opposite to those observed earlier for the nonhelical proteins and suggest that the detergent can promote disorganization of helical proteins. The disulfide-linked macromolecules seemed more resistant to this disorganization than did the cystine-free bacterial amylase. This supported the hypothesis that the conformation of the helical proteins also depends upon hydrophobic bonds. In this paper Jirgensons mentions the existence of the $\beta$-conformation (25) for the first time. Again turning to the nonhelical proteins, human serum $\gamma$-globulin, and myeloma protein (116), treating them with sodium decyl sulfate in alkaline conditions of pH 10.2–11.0 at an elevated temperature of 50°, he found that the $b_0$ values were initially close to zero but became negative on denaturation and fragmentation by the detergent. The levorotation was also increased; although $b_0$ returned almost to zero on removing the detergent by dialysis, the levorotation stayed enhanced. He concluded structural changes in the following steps: native ordered → strongly disorganized and fragmented → partially organized → strong disorganized and aggregated fragments. The $\gamma$-globulin is split into fragments of molecular weight 56,000 to 75,000 by detergent; however, the observations are complicated by the fact that disulfide and ester bonds are sensitive to alkali.

In another paper (117), again looking at detergents and nonhelical proteins, including the plant proteins, $\alpha$-conarachin and edestin, he found $b_0$ to be near zero for the native proteins and negative on treatment with detergent. The specific rotation also became more negative, but he agreed with the statement by Tanford *et al.* (109) that no reliable conclusions concerning conformation can be drawn from the changes of $[\alpha]_D$, especially in the instances when the properties of the solvent are drastically changed. In the same paper it was found that the nonionic surfactant (Brij 35) was less effective than the anionic detergent with the same chain length,

and that lysozyme and pituitary growth hormone are only slightly affected by detergents even on heating. He concluded that the native globular proteins having a $b_0$ value of near zero are not $\alpha$-helical yet possess some order in the folding of the polypeptide chains. So at this time there was a possibility that the structure of these proteins in aqueous solution included the $\beta$-form. Wada *et al.* (43) had expanded the Moffitt equation in 1961 to take into account the $\beta$-configuration; Troitski (118), a Russian worker using a manual spectropolarimeter and a lowest wavelength of 365 m$\mu$, used it to interpret his investigations into the effect of sodium decyl sulfate on $\gamma$-globulin, egg albumin, and serum albumin. He concluded that $\gamma$-globulin loses its $\beta$-structure and retains its $\alpha$-structure on treatment with detergent. On the other hand, egg albumin lost 35% of its $\beta$-form, but the $\alpha$-helix content did not change and there was no change at all in the conformation of serum albumin. The author concluded that the detergent degrades the $\beta$-structure but not the $\alpha$-helix, and he suggested that detergents may be specific reagents for the $\beta$-configuration in the correct conditions. These observations regarding the $\alpha$-helix were contrary to those of Jirgensons, and this, together with the claims for the $\beta$-structure in aqueous solutions of specially treated poly-$\alpha$-L-lysine (119, 120) and silk (121), stimulated Jirgensons (122, 123) to further investigations. The proteins having high $\alpha$-helix content are slightly disorganized, as shown by changes in the amplitudes of the Cotton effect and $b_0$ values. The disordered proteins such as histones are partially disorganized by the detergents, whereas those with considerable $\beta$-conformation like desoxyribonuclease and $\beta$-lactoglobulin seem to lose their $\beta$-form and acquire some $\alpha$-helical form. Several other proteins having some $\beta$-structure as well as the $\alpha$-helical form were also investigated, but no clear interpretation is presented; however, the possibility of a somewhat different $\beta$-structure is mentioned. In other publications concerning the histone F1 (124, 125), he again reported that decyl and dodecyl sulfate convert the disordered histone partially into the $\alpha$-helical form. Decker and Foster (126), investigating the interaction of bovine plasma albumin with alkyl benzenesulfonates at pH 6.5, found an initial binding involving 11 $\pm$ 1 sites and two other complexes involving 38 and 76 detergent molecules. This is in agreement with the complexes of Putnam and Neurath (98) who used dialysis and moving-boundary electrophoresis experiments. The formation of the higher complexes was found by ORD experiments to be accompanied by some loss of $\alpha$-helix content of the protein.

Again looking at the nonhelical proteins in 1967 (127), Jirgensons found that tetradecyl sulfate was more effective than dodecyl sulfate in promoting helical formation, again confirming that the hydrocarbon chain plays a decisive role in protein conformation. In their classical paper on the $\beta$-form of poly-L-lysine in aqueous solution, Sarkar and Doty (119) found that disordered poly-L-lysine in alkaline solution went to the $\beta$-configuration on heating; whereas treatment with sodium dodecyl sulfate in neutral solution seemed to produce a $\beta$-form, forming a peak at 205 m$\mu$ and a trough at 230 m$\mu$. However, the magnitude of the trough was greatly reduced. Supporting evidence for the $\beta$-form was obtained from IR, UV, and CD. These observations stimulated further work on synthetic polypeptides, and Grouke and Gibbs (128) found that sodium dodecyl sulfate shifted the random-coiled form of poly-L-ornithine in neutral solution of the $\alpha$-helical form using ORD and CD methods. This $\alpha$-helix formation is accompanied by the shift of the ORD minimum to 235 m$\mu$. The $\alpha$-helical form of poly-L-ornithine was unchanged on heating; a similar treatment on poly-L-lysine had caused the formation of a $\beta$-structure (119).

Velluz and Legrand (129), using entirely CD techniques, found that treatment of human serum albumin (HSA) with dodecyl sulfate decreases the helical content to 40%. Treatment of HSA with detergent and 2-mercaptoethanol (reducing the S—S bonds) causes disappearance of the dichroism in the 250–300-m$\mu$ region (due to loss of S—S bonds) whereas the dichroism in 200–250 m$\mu$ remains constant, suggesting no change in $\alpha$-helix content. The S—S bonds act as protectors of the rigid structures before denaturation, probably by maintaining a conformation which isolates the sensitive groups from the medium; the detergent seems to prevent the collapse of the rigid structures when the S—S bonds are broken. This is not so on denaturation with urea. The effect of delipidization of lipoproteins has been investigated by ORD and CD (130), but the results are confusing. CD, ORD, and IR show that $\beta$-lipoprotein is disordered and probably has some helical structure; the $\beta$-apoprotein retains its $\beta$-structure, but on treatment with dodecyl sulfate the proteins become more disordered and some $\alpha$-helix formation may be induced. The conformational changes which had been reported earlier on delipidization (131, 132) are probably caused by the presence of dodecyl sulfate. Dodecyl sulfate has no effect on the configuration of $\beta$-lactoglobulin polymorphs A, B, and C as measured by CD (133).

The Cotton effect at near 280 m$\mu$ due to tryptophane residues in egg white lysozyme disappears on exposure to sodium dodecyl sulfate (134) at concentrations which do not affect the $\alpha$-helix content but do completely inhibit the enzyme activity. This suggests that tryptophane is involved in the formation of the enzyme substrate complex. Similarly, dimethyl benzylmyristylammonium chloride did not affect the secondary structure of lysozyme (135) or acetyl lysozyme (136) but did reduce the enzyme activity. The binding of various sulfates and sulfonates to bovine serum albumin has been investigated (137), and it was found that $[\alpha]_{233}$ changes in a linear manner up until 10 moles of detergent are bound per protein molecule; the rotation remains constant as more detergent is added until a large configuration change takes place when between 45 and 60 moles are bound. This may again be due to the alteration of conformation of the tryptophane residues.

As can be seen from this, the interpretation of the ORD and CD spectra of detergent–protein complexes is difficult and is complicated by the fact that detergents may increase $\alpha$-helical content (30), convert helical to $\beta$-structure (27), abolish $\beta$ but not helical structure (26), or act as a denaturating agent (19) with various proteins.

**Dyes**—In 1958, Markus and Karush (138) studied the effect of anionic dyes on the rotatory dispersion of HSA at pH 7.4 from 725 mμ to 550 mμ and found that strongly anomalous dispersion curves were obtained. Using a manual polarimeter, it was found that the $[\alpha]_D$ depended on the substituent in the *p*-position of the *p*-benzene-azo-benzoylamino acetic acid dyes. On removing the dyes, all changes were completely reversible. Correlation with viscosity and dialysis experiments suggested to the authors that the changes in the observed $[\alpha]_D$ were due either to structural changes in the serum albumin involving interaction with the end groups of the dyes or the stabilization of resonance forms of the dyes. However, in a later paper, having obtained improved instrumentation, Winkler and Markus (139) showed that the earlier reported anomalous rotation was due to an artifact (stray light) and that the sudden changes in rotation between 500 and 600 mμ were no longer observed. The curves obtained still showed very strong deviations from the dispersion of HSA in the absence of dye. The earlier conclusions obtained from the sodium D-line measurements were, of course, not affected by these observations.

Blout and Stryer (140) were the first to report a Cotton effect from a polypeptide–dye complex at wavelengths near those of the absorption bands of the dyes. This indicated that the chromophoric group of the dye had acquired asymmetry. L-Polyglutamic acid (141, 142) was used with acriflavine and neutral rhodamine 6G, and it was found that complexing, and the resulting Cotton effects at wavelengths greater than 400 mμ, only resulted when the peptide was in the helical form (below pH 6.0); above pH 6.0 the peptide is in a random configuration, and no anomalous dispersion is observed. The magnitude of the Cotton effect of acriflavine-L-PGA complex as a function of pH showed good agreement with the helix content as determined from $[\alpha]_{546}$. In further investigations with a wider range of dyes, Stryer and Blout (143) again found complexing only in the pH region of high helical content and found molar rotations up to a million for the induced Cotton effects. The signs of the Cotton effects were opposite for the L- and D-polyglutamic acids, leading to the conclusion that helices of opposite screw sense show Cotton effects of opposite sign. Three models were suggested (Fig. 4) for the interaction between the peptide and dye; the first involved an unaggregated dye interacting with the asymmetric α-carbon atom of the peptide residue; the second involved dye end-to-end aggregation giving rise to a super helix around the peptide helix. In the third, the asymmetric polypeptide acts as a sterically determined "seeding center" to favor one of the screw senses of a dye helix which forms tangential to the polypeptide α-helix. None of the models satisfied all the observations (143, 144), although the latter two seem more feasible.

Blout (144) reported that covalent binding of a single dye molecule to a helical macromolecule results in an extrinsic Cotton effect; this does not prove, however, that all dye–polypeptide Cotton effects are the result of a single dye molecule bound to an asymmetric site in the macromolecule. Using the streaming dichroism technique (145), Ballard *et al.* (146) investigated the binding of acridine orange with the α-helical form of poly-α-L-

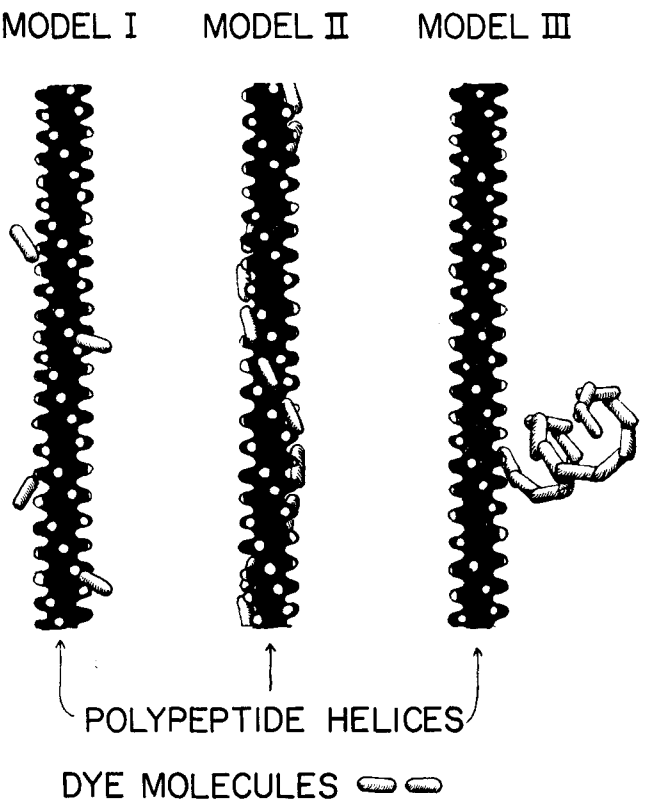


**Figure 4**—*Three suggested modes of interaction between dye and polypeptide. Model I, unaggregated dye interacts near the asymmetric α-carbon atom of the peptide residue; model II, dye end-to-end aggregate giving rise to dye superhelix; model III, tangential dye helix. Taken from* Reference 144 *with permission of the editors and Interscience Publishers, New York, N. Y.*

glutamic acid. They found three circular dichroism bands associated with the long wavelength absorption band of AO at 495 mμ and further CD bands associated with the 270-mμ absorption band of the dye but none associated with the 295-mμ absorption band. The induced activity was relatively insensitive to the glutamate residue-to-dye ratio but was dependent on the ionic strength of the solution. It was concluded that the dye aggregate in the L-PGA complex has the form of a left-handed super helix bound to the core of a right-handed α-helix of L-PGA. AO binds strongly to PGA over the pH region of the transition from a random coil to α-helix, and Myhr and Foss (147) decided to reinvestigate the random coil region of binding. They stress that the method of preparing the complex influences the ORD properties; complexes prepared by changing the PGA conformation in the presence of AO are not the same as those prepared by first changing the PGA conformation and then adding the dye. Their observations suggest that the dye prevents conformational changes in the random coil pH region, and they state that this probably prevented Stryer and Blout from observing optical activity of the random coil complex. Preparing complexes by titrating the L-PGA to the desired pH before adding the dye, they found large Cotton effects in the region of the AO absorption bands for both the helical and random coil pH regions. The curve for the helical form was very similar to that of Stryer and Blout. Yamaoka and Resnik (148) reinvestigated the interaction between helical α-PGA and

acridine orange at several dye-to-polymer ratios (D/P) using ORD. Even at a D/P of 0.0001, a broad positive peak was located at 525 mμ, a steep negative trough with a shoulder around 480 mμ, and a second peak at 450 mμ was also found. The curves were in good agreement with those of Stryer and Blout; however, the complexing at very low D/P ratios agreed with the observations of Mason's group (146), which reported binding of approximately two AO molecules per three polymer molecules. This led to support for Blout's first model, *i.e.*, binding of unaggregated dye near the asymmetric α-carbon atom of the peptide residue. The resultant CD curves from the Kronig-Kramer transform, when compared with the measurements of Mason's group (146), show good agreement with the positions and signs of all three extrema, but there is not good agreement on the magnitude of the rotations. Yamaoka and Resnik (149) also investigated the interaction of helical L-PGA and proflavine and found extrinsic optical activity throughout the absorption bands of the dye, but no new peaks or shoulders were found. The induced Cotton effects were shown to be multiple by the Kronig-Kramer transform. This analysis also shows a weak background rotation which may suggest a slight alteration in the polypeptide conformation when the dye is bound.

Eyring *et al.* (150), reinvestigating the PGA–acridine orange system, found significant differences in the ORD curves depending on whether the pH was adjusted before or after mixing. Heating had little or no effect on the curves except in the case of the helical form above 55°, where a decrease in rotation was observed; this was reversible on cooling. Their complexes with helical PGA are very similar to those of Stryer and Blout (143), and they correctly point out that Stryer and Blout overlooked the Cotton effects of the coiled PGA–AO complex by approximating their data with a smooth curve. Yang's group reported Cotton effects with the coiled PGA, although they were much smaller in magnitude than those found with the helical form. There are considerable differences between their data and that of Myhr and Foss, possibly due to differences in experimental conditions.

**Others**—The binding of coenzymes, substrates, inhibitors, *etc.*, has been investigated by ORD and CD, and particular interest has been shown in extrinsic Cotton effects generated by a chromophoric molecule interacting with an asymmetric site of the protein as in the case of the dyes. Considerable interest has been shown in the interaction of liver alcohol dehydrogenase and nucleotides where a Cotton effect is seen close to the absorption maximum of the complex (151–153). The stoichiometry of this binding has been shown to be two moles of nucleotide per mole of enzyme.

Heme has a plane of symmetry, but the iron atom constitutes a center of asymmetry for a prosthetic group since different ligands may occupy the fifth and sixth coordination sites of the metal atom and because the protein may be linked both to iron and the porphyrin side chains; thus considerable interest has been shown in the optical dispersion of heme proteins (154–157), particularly concerning the Cotton effect in the Soret band. The extrinsic effects of these and other protein-substrate bindings were reviewed by Ulmer and Vallee in 1965 (158) and only the more recent developments, particularly the obvious usage of CD, will be discussed here. Myoglobin and hemoglobin exhibit a positive Cotton effect in the Soret band of the visible spectrum, regardless of the state of iron oxidation or nature of the ligand in the sixth coordination position of the Fe atom (156, 159). As heme is directly bound to the histidine residue which is part of a right-hand helix segment (160), Beychok (37) investigated the poly-L-histidine–hemin complex but found little correlation with the myoglobin spectra, the situation being complicated by the fact that heme aggregates and binding occurs with monomers as well as aggregates. Urry (161), in a theoretical paper concerning the heme–protein interactions, predicts that the rotational strengths of the proteins change on the interaction and that the changes in the peak and trough of the ORD of apomyoglobin upon binding of heme (162, 163) should not be interpreted as changes in helical content. He also proposed that a substantial contribution to the rotational strength of the Soret transition in these heme proteins arises from coupling with the protein transitions. Concentrating on the troublesome region below 300 mμ, Beychok's group (164) concluded that the major contribution to the 260-mμ CD band of hemoglobin was from heme rather than aromatic residues. The difference in ellipticity in the 260-mμ region between the α- and β-chains reflected a considerable difference in the heme environment in the two cases, or a difference in tightness of the attachment, or both. Side-chain chromophores, rather than heme, were found to generate the longer wavelength bands near 280 to 290 mμ. In a later paper, Javaherian and Beychok (165) found that binding of one hemin per apohemoglobulin dimer derived from horse hemoglobin caused restoration of the helix content, not only of the chain having hemin bound but almost all of the helix content in the other chain as well, even without occupation of the heme binding site of that chain. In the same paper they investigated the CD of hemoglobin Gun-Hill (166) and again found that heme is bound only to α-chains. Others (167), investigating the same UV region of human hemoglobin and its subunits, have shown that binding of oxygen and carbon monoxide causes large changes in optical rotation. The changes at 233 mμ were taken to prove a conformational change in the protein but whether both chains are involved was not clear. Similar observations were not made with myoglobulin. Darnall *et al.* (168) have recently investigated the CD spectra of methemerythrin with the iron coordinated to a variety of ligands. They concluded that contributions in the 290-mμ region were from tyrosine residues and from the iron atoms. In the peptide n-$\pi^*$ and $\pi$-$\pi^*$ region, the CD was similar to that of an α-helical conformation, and this did not change with the nature of the iron bound ligand or upon dissociation of the protein into monomers. The extrinsic spectra above 300 mμ due to the heme binding have been well investigated by CD. Myer (169) found that the different oxidation states of the iron in heme of horse heart cytochrome-C caused differences in the Soret region, as well as in the aromatic dichroic bands, and there was an increase of all transitions in the δ absorption region seen in the lower oxida-

tion states. He said that the change in the valence state induces significant alterations in the disymmetrical environments of the prosthetic heme group and the aromatic side chains, possibly due to the increased ligand-binding affinity of the ferrous state. Strickland (170) investigated the CD spectra of horseradish peroxidase complexed with its enzyme substrate compounds and found wavelength shifts in the Soret region similar to those observed by UV absorption spectrophotometry. In the 280-m$\mu$ region the CD of the complex differed from the native enzyme, indicating that heme may contribute to the ultraviolet CD and that the orientation of an aromatic side chain may change. In the 207–222-m$\mu$ region the CD spectrum of the protein is unchanged on complexing and suggests the $\alpha$-helical conformation.

Recently, Japanese workers (171, 172) found that oxidized cytochrome cc′ shows three types of reversible CD spectra in the 300–450-m$\mu$ region depending on pH: type I (neutral), type II (intermediate), type III (alkaline). In the reduced state it has an abnormal spectrum in the pH regions of types I and II, but it has normal spectrum at more alkaline pH's. Alcohols, phenols, and ketones convert type I and type II spectra to type III at fixed pH values, and the efficiency is parallel to the hydrophobicity of the ligand. At pH 7.4, 25% of 2-propanol converts the oxidized form from the neutral to the alkaline type but causes no change in the far UV region, whereas urea at pH 7.0 causes destruction of the $\alpha$-helix but only a slight shift to the alkaline type in the extrinsic regions. These observations show that a shift from neutral to the alkaline type is not necessarily accompanied by a change in the gross helical conformation which might protect the special environment of the heme. Similar observations were made by Tsong and Sturtevant (173) using an apoenzyme from cytochrome $b_2$ free of flavin mononucleotide (FMN). They found a CD spectrum strongly dependent on the oxidation states of heme, and the CD spectrum below 250 m$\mu$ was that of an $\alpha$-helix and independent of the heme. When the enzyme was treated with FMN and *l*-lactate, 80% of the original enzyme activity was recovered and the CD spectrum of the original enzyme was obtained. Denaturing by aging changed the CD spectra below 250 m$\mu$ but not above. The enzyme could be partially reactivated to give the original CD spectra. These results suggest direct heme–FMN interaction is responsible for the catalytic and optical properties of cytochrome $b_2$. Van Holde (174) found that the oxygenated hemocyanin gave two dichroic bands in the visible region; on comparison of this spectrum with some peptide–copper (II) complexes, he saw a similarity between the hemocyanins and histidine-containing peptides, suggesting that perhaps the histidine residues are ligands in hemocyanin. Li and Johnson (175) have recently measured the ORD properties of several fetal and abnormal hemoglobins.

The ORD of rhodopsin, before and after irradiation, has been measured (176) and the $\alpha$-helical content appeared to be diminished after bleaching, and there was a possibility that unbleached rhodopsin also showed a weak Cotton effect in the visible region. Later CD investigations (177) confirmed the extrinsic Cotton effects for rhodopsin and isorhodopsin near the absorption wavelengths of the retinal chromophore. Crescitelli

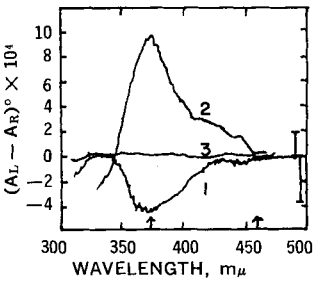


**Figure 5**—*CD spectra of free FAD 1 and the bound FAD 2. Curve 3, base line. Vertical bars indicate scales corresponding to* $\epsilon_L - \epsilon_R = 1$ *for Curves 1 and 2, respectively. Taken from* Reference 180 *with permission of Academic Press, New York, N. Y.*

*et al.* (178) also observed the extrinsic CD bands with rhodopsin and porphyrodopsin, although they differ in behavior in the near UV region; the extrinsic CD bands disappear upon bleaching. The extrinsic CD spectra of squid and cattle rhodopsin (179) have been found to differ, probably due to the difference in protein conformation around the site of attachment of the retinal chromophore. Upon illumination the helical structure of the proteins was converted to a disordered one, and the retinal chromophore lost its optical activity. This change in protein conformation upon illumination is considered to be an important role in initiating the visual process.

One of the more interesting investigations concerning enzymes and their substrates is the binding of flavin adenine dinucleotide to D-amino acid oxidase (180). There is a conformational change of the dinucleotide as a result of the binding to the apoenzyme; this is shown by the inversion of the dinucleotide CD spectrum as seen in Fig. 5 without any appreciable wavelength shift. Apparently the binding between the two rings is preserved, but the mutual steric orientation is markedly changed when the nucleotide is bound to the apoenzyme. Other polypeptides whose interactions have been investigated by ORD or CD include glutamine synthetase (181), ribonuclease (182), bakers yeast *l*-lactate dehydrogenase (183), horse liver alcohol dehydrogenase (184), aspartate transcarbamylase (185), lypolydehydrogenase (186), glutamate dehydrogenase (187–189), pig heart lactate dehydrogenase (190), avidin (191), immunoglobulin (192), muscle phosphorylase B (193), glutamate-aspartate transaminase (194), and lipoproteins (195, 196).

Sonenberg (197) has found that human growth hormone induces changes in the ellipticity of human erythrocyte membranes at 222 m$\mu$, observing a decrease in the negative band of about 30% in a phosphate buffer but not in water. The necessary presence of phosphate suggests that some organization of the membrane is necessary. Similar effects were not observed with bovine growth hormone, bovine serum albumin, bovine insulin, or cortisol. Lenard and Singer (198) had previously investigated the ORD and CD of red blood cells and found approximately one-third in the helical conformation and the remainder in the random coil; however, Urry and Ji (199) have shown that particulate systems can show CD patterns characteristic of an $\alpha$-helix and caution must be exercised in interpreting data from particulate systems.

Hansch *et al.* (200) have correlated protein binding of a variety of organic compounds of miscellaneous structure with their octanol–water partition coefficients, so demonstrating the nonspecific nature of the process. In the same paper this partition coefficient is related to the

protein binding and the rotation at the $Na_D$ line; however, whether the change in rotation is due to a change in configuration and/or to any induced optical activity of the organic molecule is not apparent.

Chignell (201–203) has studied the interaction of phenylbutazone and some of its analogs as well as a number of other anti-inflammatory substances with BSA and HSA and has found that many of these substances exhibit extrinsic Cotton effects. When phenylbutazone was mixed with human serum albumin, a positive ellipticity band was generated at 287 mμ. Similar observations were made for oxyphenbutazone and sulfapyrazone, although the rotational strengths of the latter substances were smaller. More polar derivatives gave greatly reduced rotational strengths, and the reduction was positively correlated with a poorer distribution of the polar derivatives into nonpolar media.

Fifteen *N*-arylanthranilates, including flufenamic and meclofenamic acids as complexes with human serum albumin, were studied (203), and extrinsic Cotton effects were observed for all of them. Metachromasia was not observed. The binding of flufenamic acid to human, porcine, equine, bovine, canine, ovine, and rabbit serum albumins induced optical activity in all cases, but there were significant ellipticity differences observed and, in particular, a reversal of the sign of the long wavelength band was seen for the ovine and rabbit proteins.

## NUCLEIC ACIDS

**Acridines**—Acridine binding to nucleic acids has been actively studied almost from the time that selective staining of nuclear material by dyes was observed. Interest stems from the function of some of these dyes as antibacterial agents (204) and mutagens (205, 206). Studies of the nucleic acid–dye complexes have involved most of the methods mentioned previously, and a portion of those studies has been reviewed, including some of the optical rotatory dispersion–circular dichroism work to be described (54, 207). A large proportion of the more recent work has been devoted to a determination of the stoichiometry of binding as well as to the structure determination of the complexes

The stoichiometry of acridine dye binding to nucleic acids has been studied extensively and can be expressed in terms of the quantity ($r$) of dye (ligand) bound per mole of nucleic acid phosphorus against the concentration of free aminoacridine. The definition of $r$ is

$$r = \sum_{J=1}^{J=P} \frac{n_J k_J c}{1 + k_J c} \qquad \text{(Eq. 16)}$$

where $n_J$ is the number of $P$ classes of binding sites (of intrinsic binding constant $k_J$) for the ligand on the nucleic acid (207). If there are two classes of binding sites, Eq. 16 simplifies to

$$r = \frac{n_I k_I c}{1 + k_I c} + \frac{n_{II} k_{II} c}{1 + k_{II} c} \qquad \text{(Eq. 17)}$$

and in the case of a single type of binding site, Eq. 17 becomes

$$r = \frac{nkc}{1 + kc} \qquad \text{(Eq. 18)}$$

When two or three binding sites are present, it is sometimes possible to select certain linear portions of the *r/c versus r* plots and attribute the linear portions of them to specific binding sites, allowing calculation of individual $n_J$'s and $k_J$'s. However, if the assumption of Langmuir-type binding (implicit in Eq. 18) does not hold because of cooperative phenomena in which a bound ligand promotes the binding of further ligands, it may not be possible to recognize the contribution of each binding site in the *r/c* plot.

The binding of acridine orange to nucleic acids apparently involves two processes as shown by the curved *r/c versus r* plots found by Peacocke (90), a strong binding process (I) involving about 0.2 molecule of dye per nucleic acid monomer and a weaker process (II) with a 1:1 dye–monomer ratio. It has recently been shown (208) that the binding is adenyl or guanyl specific and that the weak complex (II) can be distinguished from the strong (I) by hot dialysis or by fluorescence spectroscopy (209). The weak complex dissociates at room temperature and fluoresces at 620 mμ, whereas the strong complex is stable as high as the denaturation temperature of DNA and fluoresces at 515 mμ.

Efforts to define the structure of the various nucleic acid–dye complexes were abetted when an extrinsic Cotton effect between 450 and 540 mμ (the wavelength region in which DNA-bound acridine orange absorbs) was observed (210) (see Fig. 6). The observed finite rotation must have been due to asymmetric perturbation of the dye chromophore by the DNA, because, by itself, acridine orange is optically inactive. At the same time (210) the observation was made that heat-denatured DNA induced no Cotton effect in bound acridine orange, but later work (211, 212) using denatured calf thymus DNA disclosed an induced Cotton effect in the bound dye. In addition, it has been shown that acridine orange binds to poly A, poly U, and poly C (213).

It was further shown that proflavin displayed an extrinsic Cotton effect when bound to both DNA and RNA (214), followed by similar observations on 1,2,3,9-tetraaminoacridine (215).

Given the observation of induced Cotton effects, it was natural to exploit the observations further as they held a high potential for allowing one to learn more about the nucleic acid–dye complexes. What follows is an historical account of the attempts to establish the structure of the previously mentioned nucleic acid–dye complexes by considerations of the induced optical activity at the absorption maxima of the dye and the dependence of that rotation on temperature, polymer/dye ratios, pH, and ionic strength.

The originally observed induced optical activity in native DNA-bound acridine orange was quite weak at very high polymer-to-dye ratios of around 56. As the P/D ratio decreased from 13 to 1 (maximum dye binding), a strong anomalous dispersion curve developed at 504 mμ, then gave way to a weaker Cotton effect at 465 mμ. The observation corresponds to the metachromatic and hypochromic phenomena that had previously been observed spectroscopically. The observed optical activity was thought, at that time, to arise from neighboring, weakly coupled dyes in the case of the higher P/D ratios and to arise from bound dye-

aggregates (strongly coupled) in the case of the metachromatic and hypochromic spectrum at low P/D ratios.

At about the same time, Lerman (216) studied the binding of several dyes (acridine orange among them) in terms of viscosities and sedimentation coefficients. The studies were done in the concentration range that would correspond to the high polymer/dye ratio (strong binding). Because of increased viscosity, a decreased sedimentation coefficient (reduced mass per unit polymer length) and a loss of helicity, the hypothesis was advanced that the complex involved intrusion (intercalation) of the dye between two adjacent base pairs causing an extension of the sugar–phosphate backbone. The intercalation hypothesis was presumably supported by later polarized fluorescence work (217) showing that the dye was oriented perpendicularly to the helix axis and by chemical studies (218) that showed an inhibition of diazotization of the primary amino groups of some acridine dyes (proflavin but not acridine orange). The latter observation was a unique consequence of binding to DNA because other polyanions had no effect on diazotization rate.

Circular dichroism of the DNA–acridine orange complex (one dye molecule per base pair) under static and streaming conditions (145, 219) showed a strong positive band at higher wavelengths (505 mμ) and a weaker negative band at shorter wavelengths (467 mμ). These bands increased and decreased, respectively, on streaming, indicating that the 505-mμ band is polarized perpendicularly to the helix axis and the 467-mμ band is parallel polarized.

Since the 500-mμ band of the dye is a $\pi$-$\pi^*$ transition requiring a component along the long axis of the dye, the conclusion was reached that the molecular planes of the acridine orange molecules are tipped relative to the helix axis. It was further concluded that the sign of the ellipticity required a left-handed helical arrangement of the dyes and the best such arrangement involves binding of the acridine ring nitrogen (conjugate acid) to the sugar–phosphate backbone.

The concentration range at which these streaming circular dichroism studies were run was probably in the intermediate range between monomer binding (strong) and aggregate binding (weak), however, and Blake and Peacocke (220) have suggested that the streaming measurements were made on a mixed complex, making the results appropriately ambiguous.

More extensive work by Gardner and Mason (221) dealt specifically with the ambiguities. By measuring circular dichroism over a range of ionic strength, pH, and P/D ratios, they were able to detect three ellipticity bands. Two negative bands at 465 and 488 mμ and one positive band at 505 mμ were observed, and they had optimum intensities at P/D ratios of 3, 9, and 4, respectively. Only the 505- and 465-mμ bands were observed at P/D ratios of less than 3 at low ionic strength and low pH, and those bands are diminished at large P/D ratios. The 488-mμ band was optimum at high ionic strength, neutral pH, and a larger P/D ratio. The 505- and 465-mμ bands were assigned to bound dimers, bound in the form of a skewed (dissymmetric) sandwich, and the 488-mμ band was thought to arise from the bound monomer. It was

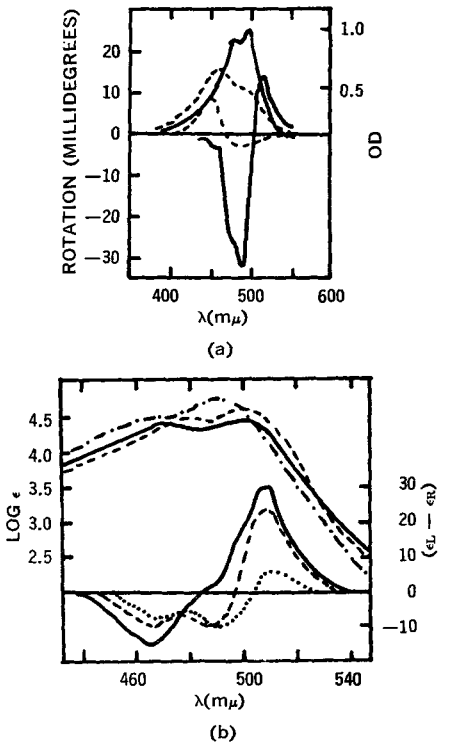


**Figure 6**—(a) *ORD and absorption spectra of acridine orange bound to native DNA at low (- - -), 28.48 to 15.68 μM, and high (——), 85.44 to 15.68 μM, polymer/dye ratios. The upper curve in each pair is the absorption spectrum.* (b) *CD and absorption spectra of acridine orange bound to native DNA at polymer/dye ratios: (——), 3 and (- - -), 9 and (. . .), 15. The upper curves are absorption spectra; (·—·) absorption spectrum of acridine orange in ethanol. Taken from* Reference 54 *with permission of Dr. J. T. Yang and Academic Press, New York, N. Y.*

proposed that the monomer was bound only partly between adjacent base pairs rather than completely occluded (modified intercalation hypothesis) on the basis of the present studies and the previous streaming circular dichroism studies (145).

The binding of proflavin to both DNA and RNA has been studied beginning with observations by Blake and Peacocke (222, 223). These authors studied the optical rotatory dispersion of proflavin bound to DNA and RNA in the 400–500-mμ spectral range. As in the case of acridine orange, no optical activity was observed for the free dye, but a large unsymmetrical Cotton effect (more intense trough than peak) was observed in the presence of DNA and RNA.

The proflavin–DNA complex was studied under conditions of changing ionic strength, DNA denaturation, and P/D ratio. Decreasing the ionic strength increased the extent of binding by a factor of 1.4 but increased the magnitude of the Cotton effect by a factor of 2. At all ionic strengths the magnitude of the Cotton effect increased linearly as the P/D ratio increased (change from type II to type I binding) to a value of about 4, then decreased to noise level with a further increase in the P/D ratio. A statistical treatment of the data indicated that only a small number of proximate dye molecules are required for the observation of optical activity, not an extended helical array as some workers have proposed in polysaccharide studies (224, 225). Denatur-

ation of the DNA did not destroy the optical activity, ruling out the need for long segments of double helix in order to observe optical activity, another requirement that was thought to prevail (140).

Because of the observation of optical activity in the denatured DNA complex, and because RNA is similar in structure to denatured DNA, optical rotation studies were conducted (222) on the RNA–proflavin complex and virtually identical observations were made. However, whereas in the DNA system a reduction of pH to 2.7 obliterated optical activity, which activity returned to higher values when the original pH was restored, the same pH titration in the RNA case was smoothly reversible from pH 7 to pH 2.7 and back to pH 7.

The differences between acridine orange and proflavin probably arise because of the much greater tendency for acridine orange to aggregate (226), and it was concluded that the "monomer" binding of acridine orange is identical with the strong binding of proflavin (type I, high P/D). Because strong binding was observed in denatured systems where double-helical structures are precluded, it was felt that the double-helical structure was not essential for strong binding and that instead a "close contact" between the dye molecules and the nucleic acid bases was the only requirement, leading only incidentally to intercalation in the double-helical structures. A study of the binding of proflavin to poly A and poly U at neutral pH, to poly A at acid pH, and to poly (A + U) led (227) to the conclusion that a relatively rigid conformation of the binding macromolecule is required for the induction of Cotton effects similar to the ones seen in denatured DNA systems. The observations that led to the conclusion were that the homopolymers at neutral pH induced no optical activity in the dye (even though it was bound as shown by other spectroscopic criteria) whereas at lower pH, a Cotton effect was induced. It was further found by a statistical treatment that interaction between as few as two ligands on denatured DNA and RNA was sufficient to generate optical activity.

Further work on the DNA–proflavin complex (149) showed that the unsymmetrical long-wavelength Cotton effect reported by Blake and Peacocke (222) was a composite curve. The latter workers applied the Kronig-Kramers transform to ORD data obtained under conditions similar to those used by Blake and Peacocke, and the calculated CD curves showed the presence of two transitions. The presence of two transitions in the high wavelength region was confirmed by a CD curve of the proflavin–DNA complex determined by Blake and Peacocke (228) which showed an unsymmetrical double-ellipticity curve characteristic of exciton interactions (229–236), confirming the optical activity was a function, in part, of interaction between relatively small numbers of bound chromophores.

Some work has been done (228) on the extent of acridine structure variation tolerated for the induction of Cotton effects by DNA and it has been found that 1,2,3-aminoacridines are optically active at most of the intrinsic ultraviolet absorption bands, but that 9-aminoacridine has optical activity induced only at lower wavelengths and that optical activity is not induced in the 350–500-m$\mu$ region.

Additional conclusions outside the scope of this review have been reached regarding the structure of the DNA–dye complexes (237–239).

**Other Antibiotics**—General studies of the binding of actinomycin D to DNA (240–243) and some related monomeric species have revealed the following characteristics of the complex: (*a*) binding is guanine specific; (*b*) binding occurs adjacent to a guanyl–cytidyl pair; (*c*) binding causes helix distortion; (*d*) several complexes exist in equilibrium; (*e*) the peptide side chains conform to the sugar–phosphate backbone so that there is a high energy barrier to conformational change within the complex; and (*f*) the complexes dissociate slowly. It was concluded that complexation was by intercalation. It has been further shown that actinomycin D is dimeric down to concentrations of $10^{-5}$ *M* and that it binds to nucleoside species in the following order of preference: deoxyguanosine, guanosine, adenosine, deoxyadenosine, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, inosine, and xanthosine.

The circular dichroism of free actinomycin D (211, 244, 245) has been determined in both water (dimer) and ethanol (monomer). The water spectrum shows one strong negative band at 380 m$\mu$ and two weaker negative ellipticity bands between 450 and 440 m$\mu$. The ethanol spectrum is composed largely of one negative band. In the presence of DNA (211, 245, 246) the once weak 440-m$\mu$ band becomes strongly optically active and is shifted to around 460 m$\mu$. There is a concomitant hypochromic effect that appears in the UV spectrum of the complex, indicating that the changes observed in the inherently optically active ligand are induced as a result of the binding process. The question of the structure of the complex has not been settled although Homer (245) proposes that either the actinomycin D dimer is bound or that the monomer is bound and the metachromatic-hypochromic effect is the result of association between the antibiotic and the purine moieties of the nucleic acid.

The cytotoxic antibiotic kanchanomycin, of unknown structure and possessing inherent optical activity, has been studied with respect to its binding to DNA, RNA, and synthetic polynucleotides by spectrophotometric methods (247) and by optical rotatory dispersion–circular dichroism (248). The binding process requires magnesium ion and, apparently, two different complexes are formed, an initial complex (I) that eventually rearranges to a second complex (II). The initial complex dissociates readily when magnesium ion is removed with EDTA and complex II dissociates more slowly. Circular dichroism spectra of the two forms represent significant departures from the spectrum of the free antibiotic. The latter, in the presence of magnesium ion, is composed of a small positive peak at around 405 m$\mu$ and two shallow negative extrema at 365 and 336 m$\mu$. In the presence of magnesium and DNA, two extrema are apparent, a positive one at around 400 m$\mu$ and a negative one at 350 m$\mu$. After 20 hr., a complex, more intense negative band at around 375 m$\mu$ appears, the spectrum of complex II. Binding of kanchanomycin to poly A gives similar results.

**Assorted Ligands**—The interaction of DNA with malouetine [5$\alpha$-pregnan-3$\beta$,20$\alpha$-ylenebis (trimethylammonium iodide)] and irehdiamine (pregn-5-ene-

3β,20α-diamine) has been thoroughly studied (249) by following changes in the rotatory properties of the nucleic acid. At low ionic strength, two different complexes are formed. The first, requiring a steroid/DNA-P ratio of about 0.2, is characterized by enhanced thermal stability, increased hyperchromicity at 260 mμ, and a shift of the long-wavelength circular dichroic band toward longer wavelengths with an increase in the rotational strength. It is thought that the disordering shown by the hyperchromicity is ligand-induced and may involve a transition of the double helical structure away from the β-form (see under *Optical Activity of Nucleic Acids*). The second complex requires a higher steroid/polymer ratio (greater than 0.2) and the nucleic acid rotatory values of this complex resemble disoriented DNA. The two forms are thought to resemble those assumed by DNA in the presence of planar aromatic rings and copper (II) ions, respectively.

The binding of purines as well as purine and pyrimidine nucleosides has been examined by a number of techniques, including rotation at a single wavelength (250, 251); although the rotation studies do not conform exactly with the subject of this review, the work is important and deserves mention. The interaction of poly U with many of the previously mentioned compounds has been studied. A large positive increase of the rotation at 350 mμ was observed when binding occurred and it was found that 1:1 complexes that were temperature sensitive formed between poly U and adenosine, deoxyadenosine, L-adenosine, 3-isoadenosine, 9-(γ-hydroxypropyl) adenine, and 9-(hydroxypentyl) adenine. No interaction was found for *N*-6-methyladenosine, *N*-1-methyladenosine, and the nucleoside antibiotic tubercidin.

A large number of aryl-substituted diammonium compounds have been observed to display extrinsic Cotton effects when bound to native and synthetic nucleic acids and polynucleotides (252–254) and have been adopted as reporter molecules utilizing CD as the spectroscopic probe.

For nitroanilines of the type $Ar(CH_2)_MN^+(CH_3)_2$-$(CH_2)_3N^+(CH_3)_3 \cdot 2Br^-$, a red shift and hypochromism are observed for the complexed (DNA and RNA) state. Of further interest is the induction of a positive and a negative Cotton effect in the aromatic chromophore by RNA and DNA, respectively. The evidence seems to indicate a binding of the diammonium group to the anionic phosphate backbone and that the aryl portion lies in the minor groove of the helix. The origin of the oppositely signed Cotton effects is thought to arise from differential conformational preference of the 2,4-dinitroaniline function, depending on the nucleic acid used.

**Polysaccharides**—The original work of Stone *et al.* (71, 224, 225) on extrinsic Cotton effects in acidic polysaccharide-bound dyes has been reviewed (70). Extrinsic Cotton effects have been observed in dyes bound to λ-carrageenate, heparin, and the chondroitin sulfates (150). The polysaccharide-dye ORD spectra are complex in the region of the dye metachromatic absorption region. Acridine orange, methylene blue, and neutral red all show induced Cotton effects when bound to heparin and the effect disappears at high P/D ratios. At the time, the conclusion was reached that the effect arose from a helical array of dyes along the polysaccharide chain, but subsequent work in the nucleic acid series showed that a long helical array was not necessary, allowing other rationalizations in the polysaccharide area.

More recent work (150) has shown that chondroitin sulfate and acridine orange form two different complexes (neutral pH) depending on the order of mixing. The induced Cotton effect was more intense when the polysaccharide was added to the dye, the apparent reason being related to dye self-association. This order-of-mixing dependence disappeared at low pH.

The binding of a number of azodyes to α-cyclodextrin has been studied (255) by the ORD technique and only congo red displayed reasonably strong extrinsic Cotton effects that appeared outside the rather strong positive background rotation of α-cyclodextrin.

## CONCLUSION

It is apparent from the foregoing that the application of optical rotatory dispersion–circular dichroism to the study of small-molecule binding by macromolecules is able to provide certain specific information and is potentially able to provide much more. It is possible to decide when binding occurs by observing the induction of optical activity in symmetrical chromophores by asymmetrical macromolecules. Of greater interest is the ability to decide whether the chromophore is intimately involved in the binding process and how it is involved. Further, a determination of the effects of binding on the macromolecule is important. Both the latter kinds of information can potentially be obtained from ORD–CD measurements, but greater knowledge of the correlation between macromolecular structure and rotational data is needed as is a more thorough knowledge of the origin of the induced Cotton effect.

A noteworthy recent development that will prove useful in the future, particularly in the study of ligand effects on macromolecular structure, is the difference spectropolarimetric method adopted by Adkins and Yang (256), a method useful in the study of small conformational changes. The method was used to estimate the equilibrium constant for the lysozyme–*N*-acetyl-D-glucosamine interaction.

The interpretation of measurements made on protein systems, if in terms of conformational changes, must take into account recent precautions regarding Cotton effects derived from intrinsic aromatic functional groups as well as recent measurements (257) that reveal how environment alone can change the rotatory properties of cyclic mono- and dipeptides, compounds that are thought to be electronic analogs of polypeptides. The study of membrane-bound small molecules requires that particular attention be paid to the problem of artifacts arising from absorption, flattening, and scattering treated by Urry and Ji (199).

Future studies should concentrate on the careful accumulation of a broader variety of good data and a more rigorous study of the question of optical activity induced by intermolecular interaction. Further, as better correlations between optical activity and conformation in macromolecular systems become available, ligand

effects on that conformation should be studied, perhaps by application of the difference technique mentioned previously.

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## ACKNOWLEDGMENTS AND ADDRESSES

Received from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*