it may be a circulating protease. The fact that native BPA is completely resistant to this enzyme suggests the possibility that it might serve the role of a scavenger by degrading only denatured or otherwise damaged protein molecules. Alternatively, it is conceivable that it occurs as a zymogen and that the active form appears only as a result of activation in some stage of the commercial fractionation process. It might arise from one of the known zymogens of the blood clotting system. Studies in progress in our laboratories may lead to a clarification of the properties and possible physiological role of this interesting enzyme.

Acknowledgments

The authors wish to pay a special note of thanks to both Professor Michael Laskowski, Jr., and Dr. Robert Hagenmaier for many interesting discussions and helpful suggestions concerning this research. We are also indebted to Harvey J. Nikkel for supplying the recrystallized ^{14}C-labeled iodoacetamide and for help in its use, and to William J. Kohr for performing the N-terminal amino acid determinations.

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Circular Dichroism and the Conformations of Membrane Proteins. Studies with Red Blood Cell Membranes*

Michael Glaser and S. J. Singer†

ABSTRACT: Previous studies of the circular dichroism spectra in the region of 190–230 nm of suspensions of red blood cell membranes and of other membrane preparations have shown some of the features characteristic of proteins in a partially α-helical conformation, with, certain anomalous features however, such as low values of $[\theta]$ near 190 nm and a "red shift" in the circular dichroism spectra above 220 nm. In this investigation, a careful comparison has been made of the circular dichroism and ultraviolet absorption spectra of suspensions of red blood cell membranes (1) before and after fragmentation in a French press; and (2) before and after treatment with preparations of phospholipase C. The magnitudes of the anomalies in the circular dichroism spectra were found to be correlated with increases in ultraviolet absorbance, probably due to light scattering from the suspensions, supporting the suggestions of Urry and coworkers that the circular dichroism anomalies may be explained as optical artifacts. With the aid of theoretical treatments of (1) the effects of light scattering on circular dichroism spectra from suspensions of large particles, and (2) the absorption flattening from suspensions of spherical shells, the circular dichroism spectra of suspensions of intact red blood cell membranes have been analyzed in detail. In particular it has been shown that the value of $[\theta]_{222}$ is not significantly influenced by optical artifacts, and that in the intact membranes, the protein is on the average about 40% in the right-handed α-helical conformation.

Considerable interest has been generated in recent years in the optical properties of the proteins of intact biological membranes. Optical rotatory dispersion and circular dichroism measurements in the peptide-bond absorption band around 200 nm have yielded the interesting information that a large fraction of the protein in a variety of membranes is in

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the α-helical conformation (Ke, 1965; Wallach and Zahler, 1966; Lenard and Singer, 1966; Urry *et al.*, 1967; Mommaerts, 1967). The circular dichroism spectra for different membranes were not only generally similar, but they showed certain unique features in common. These included a "red shift" of about 1–3 nm in the ellipticity minimum near 222 nm, and a reduced value of the ellipticity at the maximum near 190 nm.

The red shift has attracted attention, and has been variously attributed to a number of different structural features of the membrane, including protein helix–helix interactions (Cassim and Yang, 1967; Lenard and Singer, 1966), and an apolar environment for the protein helices (Wallach and Zahler, 1966). Urry and coworkers, after having first concluded that the red shift was due to optical rotatory contributions by lipid chromophores (Urry *et al.*, 1967), have more recently attributed the unique features of these circular dichroism spectra to optical artifacts (Urry and Ji, 1968; Urry and Krivacic, 1970). Urry and coworkers have furthermore suggested that these artifacts have produced gross distortions of the circular dichroism spectra obtained with membrane preparations, rendering them uninterpretable without major corrections.

Over the past several years we have extended our initial circular dichroism investigations of membrane systems in several directions. Better circular dichroism spectra have been obtained with a more sensitive instrument, and absorbance measurements were usually made on the same samples to correlate with possible optical artifacts. In this paper, we report calculations and experimental studies with preparations of red blood cell membranes which support the view that the optical artifacts proposed by Urry and coworkers do indeed alter the circular dichroism spectra, but that the magnitudes of the artifacts do not significantly affect the estimates of the average helicity of the membrane proteins made previously (Lenard and Singer, 1966).

Theory

The optical artifacts suggested by Urry and coworkers have recently been discussed several times (Urry and Krivacic, 1970; Urry *et al.*, 1970; Urry, 1970). For suspensions of particles which are sufficiently large, at least three kinds of optical artifacts are considered to affect the circular dichroism spectra. (1) *Absorption flattening:* absorption by the chromophores in the large particles depletes the light available for absorption by other chromophores which are in the "shadows" of these particles (Duysens, 1956). The total absorption is thus less than would be observed for the same concentration of chromophores dispersed in molecular solution. This is therefore called a "concentration obscuring" effect. Since the difference between the absorption of the left and right circularly polarized light is measured directly in circular dichroism instruments, absorption flattening diminishes the magnitude of the circular dichroism signal proportionately. (2) *Light scattering:* some of the light which would have been absorbed by the chromophores dispersed in molecular solution is instead scattered. This effect is also a concentration obscuring effect and diminishes the magnitude of the circular dichroism signal. (3) *Differential light scattering:* light scattering depends on the difference between the refractive index of the solvent and of the particle, but for a particle which is optically asymmetric the refractive indices for left and right circularly polarized light (n_L and n_R, respectively) are slightly different. The apparent absorbance, A_{SL}, due to light scattering of the left circularly polarized light and the apparent absorbance, A_{SR}, due to light scattering of the right circularly polarized light, can then be different, and the difference $A_{SL} - A_{SR}$ could contribute to the measured circular dichroism signal; increasing it if $A_{SL} - A_{SR} > 0$, and decreasing it if $A_{SL} - A_{SR} < 0$. An equation for the effects of these three factors on the ellipticity of a suspension, $[\theta]_{susp}$, was given by Urry and Krivacic (1970) as

$$[\theta]_{susp} = \frac{3300}{C_0 l}[Q_A (A_L - A_R) - (A_{SL}A_L - A_{SR}A_R) + (A_{SL} - A_{SR})] \quad (1)$$

in which C_0 is the concentration of chromophore in moles per liter; l is the path length in centimeters; Q_A is the flattening coefficient, which is defined as

$$Q_A = A_{susp}/A_{soln} \quad (2)$$

the ratio of the absorption exhibited by the suspension to that of the solution of molecularly dispersed chromophores at the same concentration; and A_L and A_R are the absorbances of the left and right circularly polarized beams for the molecularly dispersed chromophore solution. The three separate terms on the right side of eq 1, reading from left to right, account for the three kinds of optical artifacts discussed above, respectively.

Upon examining the theoretical basis for eq 1, we have obtained a relation (eq 3) which is similar, but contains several significant differences

$$[\theta]_{susp} = \frac{3300}{C_0 l}[Q_A(A_L - A_R) - Q_A(A_L S_L - A_R S_R) + (A_{SL} - A_{SR})] \quad (3)$$

In this equation S_L and S_R, the fractions of the left and right circularly polarized light incident on a single particle which are scattered (Appendix A), replace A_{SL} and A_{SR} in the second term on the right side of eq 1; and this term also contains Q_A. Because of these differences, our derivation of eq 3 is given in Appendix A. That eq 3 is more satisfactory than eq 1 is suggested by the following argument. Under conditions where $A_{SL} - A_{SR} = 0$ and $S_L - S_R = 0$ (*e.g.*, at wavelengths where $n_L = n_R$ and the optical rotation is zero), eq 1 and 3 reduce to eq 4 and 5, respectively

$$[\theta]_{susp} = \frac{3300}{C_0 l}[(Q_A - A_S)(A_L - A_R)] \quad (4)$$

$$[\theta]_{susp} = \frac{3300}{C_0 l}[Q_A(A_L - A_R)(1 - S)] \quad (5)$$

in which $A_S = A_{SL} = A_{SR}$ and $S = S_L = S_R$. Equation 4 predicts that whenever $A_S > Q_A$, $[\theta]_{susp}$ will change sign. ($A_S > Q_A$, for example, for large particles with a highly absorbing chromophore ($Q_A \sim 0$) exhibiting substantial light scattering.) On the contrary, however, it is in the nature of the concentration obscuring effects due to absorption flattening and light scattering that in the limit they may reduce the effective concentration to zero, and hence may reduce $[\theta]_{susp}$ to zero, but they cannot cause a reversal of sign. Equation 5 is consistent with this condition, but eq 4 is not.

Let us now consider the problem of absorption flattening. In an earlier treatment of this problem, Urry and Ji (1968) used the equations developed by Duysens (1956) to calculate

TABLE I: Calculated Absorption Flattening for Red Blood Cell Membranes.[a]

Wavelength (nm)	ϵ[b]	T_p[c]	Q_A[d]
190	6100	0.682	0.728
200	4200	0.763	0.790
210	1500	0.900	0.910
220	540	0.961	0.968
230	175	0.987	0.990

[a] Calculations using eq B2 and B3 of Appendix B, with $a_i = 4.0000\ \mu$, $a_o = 4.0075\ \mu$, and with $\gamma = 0.00236\ \epsilon$ in units of μ^{-1}. The density of peptide chromophores was taken as the density of the membrane, 1.17 g/cm^3, and the average molecular weight of peptide unit as 114. [b] Extinction coefficient, M^{-1} cm^{-1}, of the peptide chromophore 40% in the α-helical and 60% in the random coil conformations (Gratzer, 1967). [c] Transmittance of single red blood cell membrane. [d] Absorption flattening coefficient, eq 2.

values of Q_A appropriate to suspensions of aggregated helical polyglutamic acid at low pH. The Duysens' equation that was used was for the case of a suspension of spherical particles, and the calculated values of Q_A were substantially less than 1.0 at wavelengths in the peptide-bond absorption band around 200 nm. However, in the case of a suspension of intact red blood cell membranes or other vesicular membrane particles, the protein is not aggregated into a solid sphere but is rather distributed in a thin shell. To estimate more properly the magnitude of absorption flattening effects in membrane suspensions, we therefore extended the theory of Duysens to the case of a suspension of uniform spherical shells. The theory and calculations are given in Appendix B. To apply these theoretical considerations to red blood cell membranes, the following parameters were used. The radius of the spherical shell was taken as 4 μ and the thickness 75 Å; the shell was assumed to be entirely made up of protein (which overcorrects the absorption flattening effect since the membrane is about 40% lipid) whose extinction coefficients at different wavelengths were taken as those of a mixture of 40% α-helical and 60% random coil polypeptide conformations (Gratzer, 1967). The computed values of T_p and Q_A (Appendix B) are given in Table I. The calculations show that Q_A attains values significantly smaller than 1.0 only for wavelengths below 210 nm. According to these calculations, absorption flattening therefore makes only a negligible contribution to $[\theta]$ for a suspension of red blood cell membranes at 222 nm, a point to which we return in the Discussion.

Materials and Methods

Human red blood cell membranes were prepared from freshly drawn blood by the method of Dodge *et al.* (1963). The human hemoglobin was a twice-crystallized preparation obtained from Pentex, Kankakee, Ill. The phospholipase C experiments that are described in this paper were carried out with a partially purified preparation of the enzyme from *Clostridium perfringens* obtained from Worthington Co., Freehold, N. J. The conditions for the treatment of the membranes with this enzyme were the same as used by Glaser *et al.* (1970). The protein concentration of the membrane suspensions was determined by Lowry analyses (Lowry *et al.*, 1951) which were calibrated by Kjeldahl nitrogen analyses of membrane suspensions. The protein was assumed to contain 16% nitrogen. A correction was made for the nitrogen contained in the lipids from phosphorus analyses (Bartlett, 1959) on the membrane suspensions; the average atom ratio of nitrogen to phosphorus in the lipids was taken as 1.2.

Absorption and circular dichroism spectra shown in this paper were taken with the J-10 modification of the Durrum-Jasco ORD/UV/CD-5 instrument. The J-10 modification results in an improvement of the signal-to-noise ratio of the instrument by a factor of about 5, and an increase in circular dichroism scale sensitivity by a factor of 6.6. All measurements were made in 0.007 M phosphate buffer (pH 7.4) near 25°. The absorbance was below 2.0 for all circular dichroism measurements. A careful comparison of two similar circular dichroism spectra was made by measuring the two spectra successively in the same 0.5-mm cell and superimposing them on the same experimental record, after first adjusting the concentrations of the two samples so that they gave the same circular dichroism signal at the minimum near 222 nm. Lowry analyses were then carried out on the solutions which were used for the circular dichroism spectra. Ultraviolet absorbance measurements were carried out with the same samples.

For some of the experiments, the red blood cell membranes were fragmented in an Aminco-French pressure cell mounted on a Carver laboratory press. The suspensions at a concentration of about 2.5 mg/ml were passed through the press at a pressure of 10,000–11,000-lbs load.

Results

A careful comparison of the circular dichroism spectra of solutions of hemoglobin and of red blood cell membranes is shown in Figure 1. The hemoglobin has the larger value of $[\theta]_{222}$, so the scales were adjusted to superimpose at the minima near 222 nm. It is clear that the two circular dichroism spectra are quite similar, but that certain significant differences exist. Whereas the ratio $[\theta]_{192}/[\theta]_{222}$ is about -1.9 for hemoglobin, as is typical for proteins and polypeptides which are largely in the α-helical conformation, it is only -1.1 for the membranes. Furthermore, the circular dichroism spectrum above 220 nm appears to be shifted toward the red for the membranes compared with hemoglobin; the minimum is at 223 rather than 222 nm, and at 250 nm the spectrum has not returned to the base line.

After the French press treatment, the membranes were largely fragmented. In the analytical ultracentrifuge, the apparent sedimentation coefficient decreased from 2600 to 34 S at a concentration of about 2.5 mg/ml. Correspondingly, the light scattered by the suspension was markedly reduced, as was reflected by an apparent *decrease* in ultraviolet absorbance (Figure 2). The circular dichroism spectrum of the fragmented membranes, although still similar to that of the original membrane suspension (Figure 2), more closely resembles that of a soluble protein with considerable helical content. The values of $[\theta]_{192}$ and of $[\theta]_{192}/[\theta]_{222}$ are both substantially increased, and the red shift above 220 nm is largely reversed by fragmentation.

In another set of experiments, some of which have been reported elsewhere (Glaser *et al.*, 1970), the effects of phospholipase C treatment of red cell membranes were examined. This enzyme catalyzes the hydrolysis of the phosphorylated

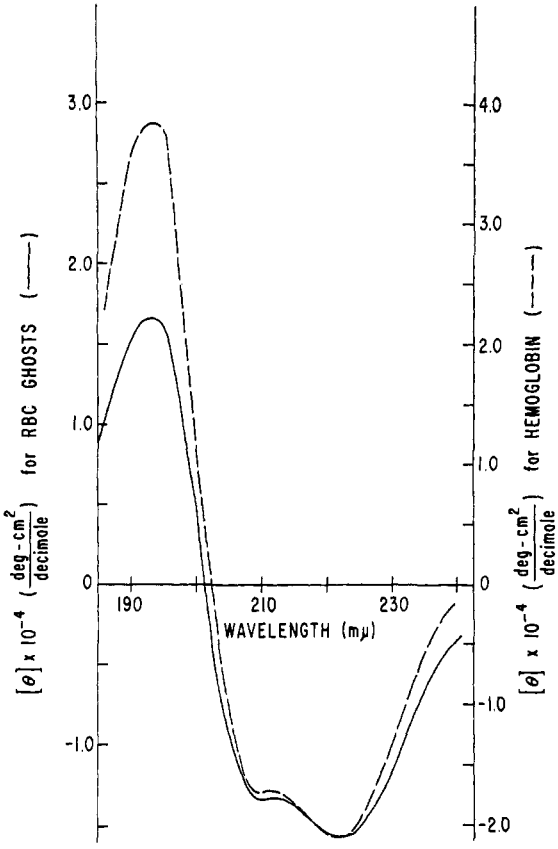

FIGURE 1: The ultraviolet circular dichroism spectra of a solution of hemoglobin (dashed line) and of a suspension of red blood cell membranes (solid line), both in 0.007 M phosphate buffer (pH 7.4) at about 25°. The ellipticity scales have been adjusted to give coincidence of the spectra at the minima near 222 nm.

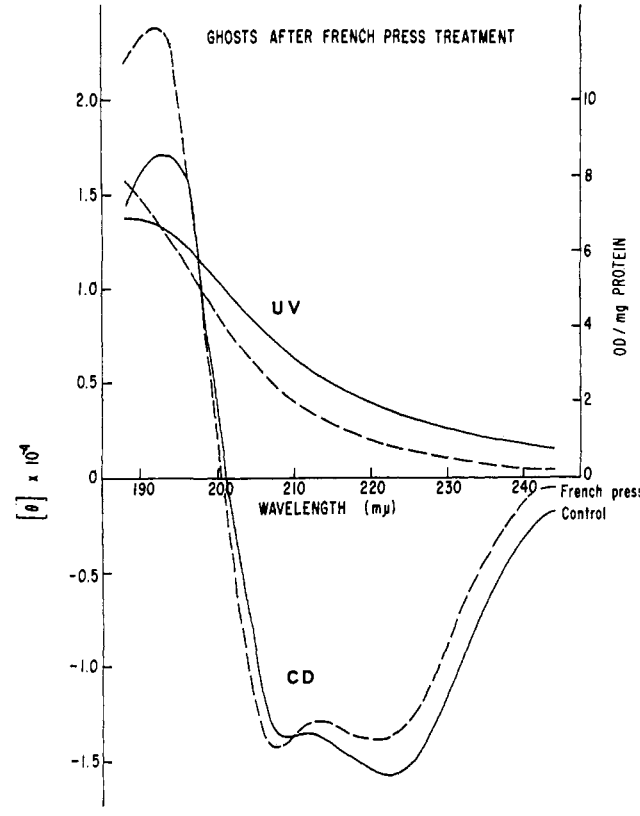

FIGURE 2: Ultraviolet absorption (0.5-mm path length) and circular dichroism spectra for red blood cell membranes before (solid lines) and after (dashed lines) fragmentation in a French press.

amines from phospholipids, and releases about 70% of the membrane phosphorus without disruption of the red blood cell membrane (Lenard and Singer, 1968; Glaser *et al.*, 1970). When a highly purified enzyme from *Bacillus cereus* (Ottolenghi and Bowman, 1970) was used, no significant changes in either the circular dichroism or the ultraviolet absorption spectrum of the red blood cell membranes were detected (Glaser *et al.*, 1970). When preparations of only partially purified enzyme were used, however, the circular dichroism spectrum was altered (Figure 3). The value of $[\theta]_{192}$ was decreased and the spectrum above 220 nm was shifted even further towards the red. Correlated with these circular dichroism changes, however, there was always found an *increase* in the apparent ultraviolet absorbance of the treated samples (Figure 3), probably due to an increase in light scattering from the suspension of treated membranes.

Discussion

The first reported ultraviolet circular dichroism measurements made with membrane preparations were those of Lenard and Singer (1966). These relatively crude circular dichroism spectra already showed double minima near 208 and 222 nm, and this was interpreted to indicate the presence of a substantial amount of right-handed α-helical conformation in the proteins of several kinds of intact membranes. The minima, however, exhibited the "red shift" mentioned earlier. In the present study, the close similarity between the more accurate circular dichroism spectra of hemoglobin solutions and of suspensions of intact red blood cell membranes (Figure 1) reinforces the conclusion that the membrane protein contains a substantial fraction of α helix. The membrane spectra, however, do appear to be influenced by optical anomalies as suggested by Urry and coworkers.

This is supported by the results shown in Figures 2 and 3. Fragmentation of the red blood cell membranes by French press treatment markedly reduced the average particle size, as shown by the sedimentation results. It is, however, unlikely that any significant changes in the conformations of the membrane proteins occurred in this mechanical treatment. This decrease in particle size should have decreased any absorption flattening and light-scattering artifacts in the membrane circular dichroism spectra. The suspension of fragmented membranes did indeed show an increase of $[\theta]_{192}/[\theta]_{222}$, and a reversal of the red shift above 220 nm. After phospholipase C treatment of the intact red blood cell membranes, the ultraviolet absorbance increased, presumably due to an increase in light scattering of the suspension; and circular dichroism spectral changes were observed opposite to those occurring after French press treatment (Figure 3). There was a decrease in $[\theta]_{192}$ and a further increase in the red shift above 220 nm. On the other hand, in other experiments with phospholipase C (Glaser *et al.*, 1970) where there was no change in ultraviolet absorbance after treatment of red blood cell membranes with the enzyme, no detectable changes in the circular dichroism spectrum were observed.

These experiments therefore show a strong correlation between the presence of anomalies in the circular dichroism spectra and the magnitude of light scattering from the suspen-

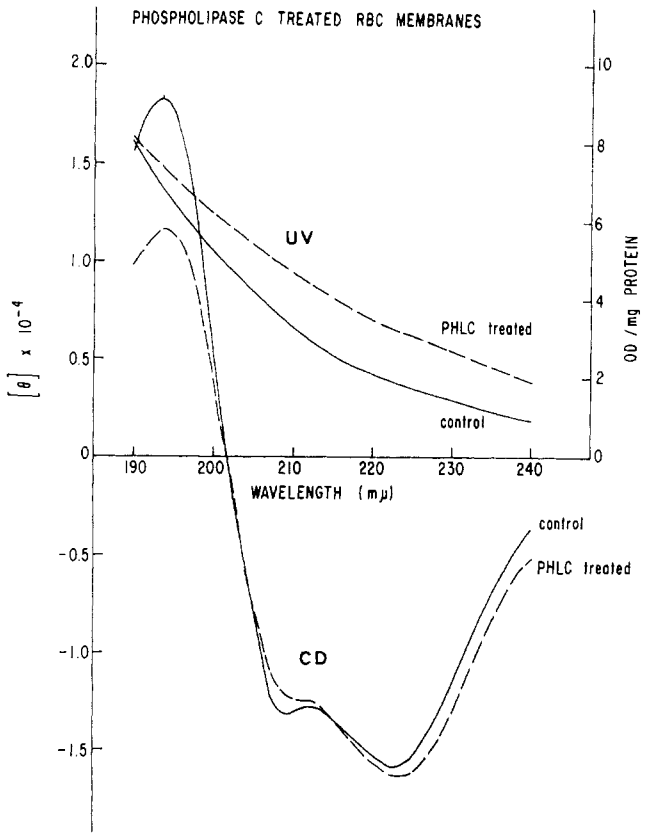

FIGURE 3: Ultraviolet absorption (0.5-mm path length) and circular dichroism spectra for red blood cell membranes before (solid lines) and after (dashed lines) treatment with a crude phospholipase C enzyme preparation (see text for details).

sions. While it cannot be ruled out that subtle conformational changes occurred in these experiments, the correlation strongly suggests that the circular dichroism anomalies are largely attributable to optical artifacts.

Circular dichroism spectral changes similar to those of Figure 3 for phospholipase C treated membranes were observed by Gordon *et al.* (1969) but these authors did not report the ultraviolet absorbance of the preparations, and ignored the possibility that optical artifacts were responsible for the observed circular dichroism changes.

After these experiments were completed, Schneider *et al.* (1970) reported experiments with sonicated red blood cell membranes which led these authors to a similar conclusion about the qualitative features of these anomalies.

In order to discuss these anomalies more quantitatively, however, and in particular, to get a reliable estimate of the helicity of the membrane proteins, direct light-scattering measurements on the membrane suspensions are needed.[1] These we have not performed, and therefore our analysis must be qualitative and semiquantitative for the present. In making estimates of the magnitudes of the anomalies expected it is useful to consider separately three wavelength regions: (a) below 210 nm; (b) above 230 nm; and (c) around 220 nm.

(a) In the region below 210 nm, contributions from absorption flattening (Q_A, Table I) and also from the concentration obscuring effect of light scattering, may be significant.[2] Both effects act to reduce the magnitude of $[\theta]_{susp}$ below the true $[\theta]$ (eq 3). To estimate the effect of differential light scattering, we note that the optical rotation (which is proportional to $n_L - n_R$) is positive below 210 nm (Wallach and Zahler, 1966; Lenard and Singer, 1966); therefore $A_{SL} - A_{SR} > 0$, and this term acts to *increase* the positive value of $[\theta]_{susp}$. In view of the experimental indications that $[\theta]_{192}$ is anomalously small for the suspension of intact red blood cell membranes (Figure 2), the concentration obscuring factors appear to outweigh the differential light-scattering term below 210 nm in this case.

(b) Above 230 nm, absorption flattening is negligible (Table I). At these wavelengths the optical rotation is negative, $A_{SL} - A_{SR} < 0$, and differential light scattering therefore makes a *negative* contribution to the already negative $[\theta]$. If the negative contribution of this last factor is more important than the positive contributions of the two concentration obscuring factors above 230 nm, the red shift above 220 nm and the negative values of $[\theta]$ at wavelengths out to 250 nm can be reasonably accounted for. It is therefore not necessary to invoke an unusual conformational property of membrane proteins to account for the red shift in the circular dichroism spectrum, although it is not entirely ruled out by the present analysis.

(c) Around 220 nm, absorption flattening for the red blood cell membrane suspension is negligible (Table I). The optical rotatory dispersion curve shows a crossover point close to 220 nm, and differential light scattering is therefore also a negligible factor. The only one of the three factors that might be significant is concentration obscuring due to light scattering. The experiment fact is, however, that $[\theta]_{222}$ was only slightly affected by the French press fragmentation of the red blood cell membranes (Figure 2), although the apparent absorbance at this wavelength was substantially decreased. This suggests that the light scattering from the suspension of *intact* membranes did not contribute significantly to $[\theta]_{222}$; at 222 nm S ($=S_L = S_R$ under these circumstances, eq 5) must be much smaller than unity for a suspension of intact red blood membranes. The overall conclusion, therefore, is that for *suspensions of intact red blood cell membranes, $[\theta]_{222}$ is not significantly altered by optical artifacts.*

The magnitude of $[\theta]_{222}$ can be used as a measure of the helicity of proteins (Beychok, 1966), and is particularly significant if, as in the case of the red blood cell and other plasma membranes (Maddy and Malcolm, 1966; Wallach and Zahler, 1966), the protein has little or no β structure, as shown by infrared spectra. Under these circumstances, f_H, the fraction of the protein in the right-handed α-helical conformation, is given by

$$f_H = \frac{[\theta]_{222,obsd} - [\theta]_{222,RC}}{[\theta]_{222,H} - [\theta]_{222,RC}} \quad (6)$$

where $[\theta]_{222,obsd}$ is the observed ellipticity at 222 nm of the

[1] A further complication is that part of the light scattered in the forward direction is collected by the phototube of the circular dichroism instrument. For suspensions of red blood cell membranes, with particle dimensions exceeding the wavelength of the light, the dissymmetry of light scattering should be large, and accordingly the light scattered in the forward direction is likely to be disproportionately large. The net effect of light scattering on the circular dichroism signal is therefore not readily calculated. In particular, the recent analysis by Ottaway and Wetlaufer (1970) of the effect of light scattering on circular dichroism spectra, which assumes Rayleigh scattering, is of uncertain relevance to membrane suspensions.

[2] In Figure 2, the absorbances of the suspensions of French press treated and untreated membranes appear to cross over below 195 nm. It is not clear whether this is real or an experimental artifact.

specimen in question and $[\theta]_{222,RC}$ and $[\theta]_{222,H}$ are the ellipticities for a protein entirely in the random coil conformation and the right-handed α-helical conformation, respectively. There is some uncertainty about the latter two quantities; we have used the values -0.3×10^4 and -3.6×10^4, respectively (Fasman *et al.*, 1970). The best average value of $[\theta]_{222,obsd}$ for red blood cell membranes is -1.6×10^4. Application of eq 6 then yields $f_H = 0.40$. On the average, the protein of intact red blood cell membranes is about 40% in the right-handed α-helical conformation, the remainder presumably in the random-coil form.

The presence of such a large proportion of α helix in a wide variety of different membranes has suggested that the proteins of membranes are predominantly globular rather than spread out over the membrane surfaces, and has been important in the development of the lipid–globular protein–mosaic model of membrane structure (Lenard and Singer, 1966; Glaser *et al.*, 1970; Singer, 1971). In this model, the globular proteins are largely intercalated in the membrane, alternating with lipid bilayer in the plane of the membrane.

Finally, although we have shown in the case of red blood cell membranes, and for other membranes of similar thickness, that the optical anomalies of Urry and coworkers do not grossly distort the protein circular dichroism spectra, particularly near 222 nm, it is clear that in other cases the artifacts may be very pronounced. They probably are responsible, for example, for the circular dichroism spectral differences observed with whole mitochondria in different configurations (Wrigglesworth and Packer, 1968), since these are known to be correlated with pronounced changes in light scattering. On the other hand, in another case where perturbations of membrane systems were made and were accompanied by circular dichroism spectral changes (*cf.* Sonenberg, 1969) no ultraviolet absorbance changes were observed (M. Sonenberg, personal communication), and the circular dichroism changes therefore most probably reflect conformational changes in the membrane proteins.

Appendix A

By Michael Glaser, Bruno H. Zimm, and S. J. Singer

Effect of Light Scattering on Circular Dichroism Spectra from Suspensions of Large Particles. Consider a suspension of uniform particles, of arbitrary shape, which are assumed to be large in at least two dimensions compared to the wavelength of the incident light, as in the case, for example, for intact red blood cell membranes. In such cases, the scattered light can be thought of as made up of three parts (van de Hulst, 1957): a reflected part, a refracted part, and a part that produces a diffraction pattern. Only the first two parts are scattered at large angles; the diffracted part is confined to small angles when the particles are large. In the usual circular dichroism instrument, the diffracted part will be received by the photodetector and will be recorded as part of the transmitted beam. Since the reflection and refraction occurs at the surfaces of the particle, their interaction with absorption, which occurs in the interior of the particle, is uncomplicated, and can be described in the manner discussed below.

Consider a thin layer of depth, δl, in the suspension. Let B be the area covered by the incident light beam of intensity, I_0, B_p be the area per particle projected on a plane perpendicular to the light beam; N the number of particles in the volume $B\delta l$, and n the number of particles per cubic centimeters. Of the light incident on a particle a fraction S_1 will be scattered at the front surface of the particle so that it does not reach the instrument detector; of the remaining light, a fraction η will be absorbed; and finally, of the remainder, a fraction S_2 will be scattered at the rear surface of the particle so that it does not reach the detector. Part of the incident light beam will strike particles in the thin layer, the rest will pass through the solvent. The quantity of light passing through the solvent is $I_0(B - NB_p)$. The quantity of light scattered at the front surface of a single particle is $I_0B_pS_1$. The quantity of light absorbed by a single particle is $(I_0B_p - I_0B_pS_1)\eta$. (It should be noted that $\eta = T_p$, and includes the absorption flattening term, Appendix B.) Finally, the quantity of light scattered at the rear surface of the particle is

$$[I_0B_p - I_0B_pS_1 - (I_0B_p - I_0B_pS_1)\eta]S_2$$

The intensity of light, I, emerging from the thin layer is then the sum of that passing through the solvent plus that passing through the N particles

$$I = I_0(B - NB_p) + N\{I_0B_p - I_0B_pS_1 - (I_0B_p - I_0B_pS_1)\eta - [I_0B_p - I_0B_pS_1 - (I_0B_p - I_0B_pS_1)\eta]S_2\} \quad \text{(A1)}$$

Collecting terms, and letting $S = S_1 + S_2 - S_1S_2$, we obtain

$$I = I_0(B - NB_p) + N[I_0B_p - I_0B_pS - (I_0B_p - I_0B_pS)\eta] = I_0B\left[1 - \frac{NB_p}{B}(\eta - \eta S + S)\right] \quad \text{(A2)}$$

Since $N = nB\delta l$, the transmittance, $T_{\delta l}$, through the thin layer is

$$T_{\delta l} = 1 - nB_p\delta l(\eta - \eta S + S) \cong \exp[-nB_p\delta l(\eta - \eta S + S)] \quad \text{(A3)}$$

For a cuvet of path length, l, the transmittance, T, is the product of the $T_{\delta l}$

$$T = \exp[-nB_pl(\eta - \eta S + S)] \quad \text{(A4)}$$

The absorbance of the suspension $A_{susp} = \log(1/T) = 0.434 \ln(1/T)$; hence

$$A_{susp} = 0.434nB_pl(\eta - \eta S + S) \quad \text{(A5)}$$

In the absence of scattering ($S = 0$), $A_{susp} = AQ_A$ (eq 2), where Q_A is the Duysen's flattening coefficient (Appendix B). If we let $0.434nB_plS = A_S$, the apparent absorbance due to light scattering, eq A5 becomes

$$A_{susp} = Q_AA - Q_AAS + A_S \quad \text{(A6)}$$

The molar ellipticity, $[\theta]$, is given by

$$[\theta] = \frac{3300}{C_0l}(A_L - A_R) \quad \text{(A7)}$$

where A_L and A_R are the absorbances of the left and right

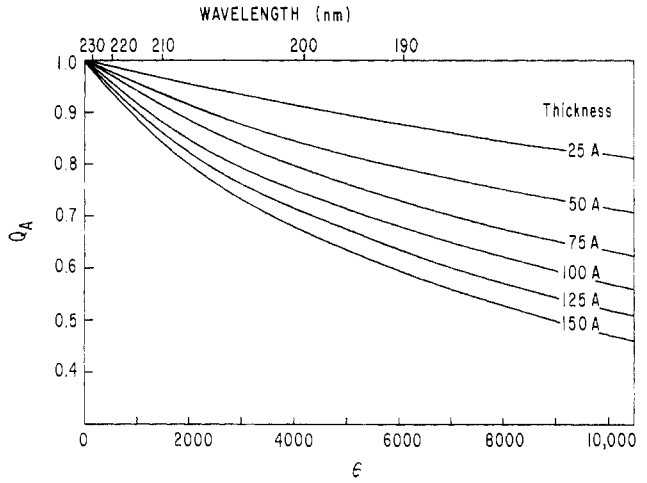

FIGURE 4: Calculations of the absorption flattening coefficient for spherical shells all of inner radius 4.00 μ and of the different thicknesses indicated, as a function of the molar extinction coefficient of the chromophore making up the shell. If the chromophore is the peptide bond 40% in the α-helical and 60% in the random coil conformations, these extinction coefficients correspond to the wavelengths shown on the upper scale.

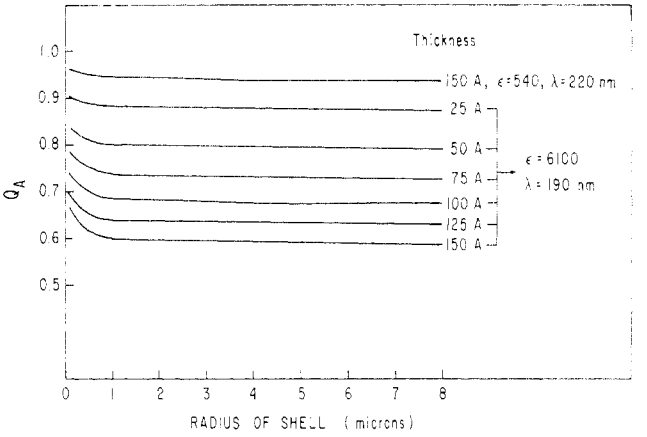

FIGURE 5: Calculations of the absorption flattening coefficient for spherical shells of the different thicknesses indicated as a function of the inner radius of the shell. The six lower curves are all for a chromophore with ϵ 6100; the uppermost curve is for a chromophore with ϵ 540. These extinction coefficients correspond to wavelengths of 190 and 220 nm, respectively, for the peptide bond 40% in the α-helical and 60% in the random coil conformations.

circularly polarized beams, respectively. It follows that

$$[\theta]_{susp} = \frac{3300}{C_0 l}[Q_A(A_L - A_R) - Q_A(A_L S_L - A_R S_R) + (A_{SL} - A_{SR})] \quad (A8)$$

which is eq 3 in the text.

Appendix B

Absorption Flattening for Spherical Shells. Duysens (1956) gives the following expression for a suspension of uniform particles of arbitrary shape

$$Q_A = \frac{B_p(1 - T_p)}{V_p \gamma} \quad (B1)$$

where Q_A is the absorption flattening coefficient (eq 2), T_p is the average transmission of a single particle, B_p is the area per particle projected on the plane perpendicular to the light beam; V_p is the volume occupied by the particle mass; and γ is the absorption per unit length inside the particle. For a spherical shell, $V_p = (4\Pi/3)(a_o^3 - a_i^3)$, and $B_p = \Pi a_o^2$, and a_i and a_o are the radii of the inner and outer surfaces of the shell, respectively.

Then

$$Q_A = \frac{3a_o^2(1 - T_p)}{4\gamma(a_o^3 - a_i^3)} \quad (B2)$$

To evaluate T_p, consider an origin at the center of the spherical shell. In cylindrical coordinates, with the light beam traveling in the z direction

$$T_p = \frac{1}{\Pi a_o^2}\left\{\int_0^{a_i}\int_0^{2\Pi} e^{-2\gamma(z_o - z_i)} r dr d\theta + \int_{a_i}^{a_o}\int_0^{2\Pi} e^{-2\gamma z_o} r dr d\theta\right\} \quad (B3)$$

where $x^2 + y^2 + z_i^2 = a_i^2$ and $x^2 + y^2 + z_o^2 = a_o^2$. The first term in the parentheses on the right side of eq B3 represents the transmission through the spherical shell for beams passing through the plane $z = 0$ within the region between the origin to the radibs of the inner sphere, and the second term for beams passing through the plane $z = 0$ within the annular region between the inner and outer radii of the spherical shell. The second term has the value 2Π $(1 - \alpha e^{-\alpha} - e^{-\alpha})/4\gamma^2$, where $\alpha = 2\gamma(a_o^2 - a_i^2)^{1/2}$. The first term cannot be solved in closed form, and was evaluated by computer.

In Figure 4 Q_A is plotted for spherical shells of the same internal radius but different thicknesses as a function of the molar extinction coefficient of the chromophore in homogeneous solution. As expected, Q_A decreases with increasing extinction coefficient and increasing shell thickness. For the peptide chromophore in a 40% α-helical–60% random coil conformation, the wavelengths corresponding to the extinction coefficients shown (Gratzer, 1967) are also plotted on the abscissa. Increasing the radius of the spherical shell at constant thickness and constant extinction coefficient (Figure 5) does not have much effect on Q_A.

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Enzymatic Acetylation of Aminoglycoside Antibiotics by *Escherichia coli* Carrying an R Factor*

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ABSTRACT: Strains of *Escherichia coli* carrying an R factor which inactivate the aminoglycoside antibiotic kanamycin A by N acetylation have been found to acetylate a wide variety of other aminoglycosides. These include kanamycin B, neomycins B and C, some of the components of the gentamicin and nebramycin complexes, and the hybrimycins. The smallest antibiotic moiety required for recognition as a substrate by the acetylating enzyme is a 6-amino-6-deoxyhexose glycosidically linked to a streptamine or deoxystreptamine ring. Isolation of the purified acetylated antibiotics has revealed that acetylation does not necessarily result in inactivation of the drug. *N*-Acetylkanamycin A is not an antibiotic, whereas *N*-acetylkanamycin B, *N*-acetylneomycin B, and *N*-acetylgentamicin C_{1a} retain substantial antibiotic activity—although they are not as potent as the unacetylated parent compounds.

There are four enzymes found in strains carrying resistance (R) factors which can inactivate many of the aminoglycoside antibiotics. One enzyme adenylylates streptomycin and spectinomycin on the D-*threo*-methylamino alcohol moiety of their amino sugar and aminocyclitol rings, respectively (Yamada *et al.*, 1968; Benveniste *et al.*, 1970; Smith *et al.*, 1970). Streptomycin, but not spectinomycin, can also be phosphorylated at that same hydroxyl group by a phosphorylating enzyme (Ozanne *et al.*, 1969). Another enzyme phosphorylates neomycin, kanamycin, paromomycin, and some of the components of the gentamicin and nebramycin complexes on a hydroxyl group of the amino sugar moiety which is linked to 2-deoxystreptamine (Kondo *et al.*, 1968; Ozanne *et al.*, 1969).[1]

A fourth enzyme, first reported by Okamoto and Suzuki (1965), inactivates kanamycin by acetylation, since a crude extract of an *Escherichia coli* strain carrying R factor R-5 required acetyl coenzyme A to inactivate the drug. Subsequently, Umezawa *et al.* (1967) isolated the product of the enzymatic acetylation of kanamycin A and showed that the 6-amino group of its 6-amino-6-deoxy-D-glucose moiety was acetylated. Okanishi *et al.* (1967) compared several structurally related antibiotics as substrates for the acetylation reaction and concluded that whereas kanamycin A was inactivated, kanamycin C, paromomycin, and neomycin were not inactivated. Their results were obtained by use of a microbiological assay which measures the residual potency of these drugs after incubation with a crude cell extract and acetyl coenzyme A.

A more detailed study of this acetylating activity is the subject of this communication. We have modified our cation-exchange paper binding assay (Benveniste *et al.*, 1970) to provide a simple method of testing for the enzymatic acetylation of the aminoglycosides. In the presence of a partially purified acetylating enzyme obtained from a strain of *E. coli* carrying either R factor R-5 or NR79, we have found that, in addition to kanamycin A, the antibiotics kanamycin B,

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