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Effect of Light Scattering on the Circular Dichroism of Biological Membranes†

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ABSTRACT: In order to establish the origin of the anomalies observed in the circular dichroism spectrum of biological membranes, we studied this property in *Streptococcus faecalis* plasma membranes, under conditions of varying particle size. This membrane exhibits the anomalies generally associated with membrane circular dichroism spectra. Reduction in particle size, effected by sonication for varying lengths of time, led to reduction in turbidity and an elimination of the anomalies, whereas Ca^{2+} induced aggregation of sonicated particles led to increased turbidity and a reappearance of the membrane associated anomalies. $[\theta]_{222}$ appears to be less sensitive to these optical artifacts than shorter wavelength ellipticities and reliable estimates of helicity in unsonicated preparations can be obtained using this value. $[\theta]_{208}$ is found to be very dependent on the turbidity of the membrane suspension and only yields reliable estimates of α-helical content in sonicated preparations under which conditions both $[\theta]_{222}$ and $[\theta]_{208}$ yield values of 27% helicity. In addition, infrared measurements performed on dried membrane films show a shoulder at 1630 cm^{-1}, suggesting the presence of β structure in the protein of this membrane. ATPase activity measurements and density gradient experiments indicate that neither extensive denaturation nor liberation of protein from the membrane surface has occurred during the sonication procedure.

The application of physical techniques to the problem of structural analysis in biological membranes has brought varied success. Circular dichroism has been used successfully to determine structural parameters in globular proteins (Timasheff *et al.*, 1967); obtaining similar data for membrane associated proteins would provide valuable information for formulating and confirming various models of membrane structure. The application of this technique to membrane systems has produced ambiguous results due to certain anomalies which are observed in the circular dichroism spectra; these anomalies are characterized by a red shift in the position of Cotton effects associated with the n–π and π–π^* transitions of the amide group and a loss of intensity of the π–π^* transitions at 208 and 190 nm (Lenard and Singer, 1966; Wallach and Zahler, 1966; Urry *et al.*, 1967). A variety of explanations have been put forward to explain these observations; they have been attributed by various investigators to be the result of hydrophobic protein–lipid interactions (Wallach and Zahler, 1966), protein–protein interactions (Lenard and Singer, 1966), and optical artifacts caused by the particulate nature of the membrane system (Urry and Ji, 1968; Urry and Krivacic, 1970).

In order to make use of the information available from circular dichroism measurements, the origin of these anomalies must be clarified. Several attempts have been made to treat scattering corrections for membrane systems quantitatively (Gordon and Holzwarth, 1971; Schneider, 1971; Glaser and Singer, 1971; Gordon, 1972; Urry, 1972); in this paper we assess the effect of the particulate nature of membrane systems on the circular dichroism spectrum of the plasma membrane of *Streptococcus faecalis* bacterium under conditions where the membrane particle size was varied. Our results lead us to conclude that light scattering, which results from the particulate nature of membrane suspensions, is largely responsible for the anomalies observed in the membrane circular dichroism spectra. In addition, it appears as though reasonable estimates of helicity can be obtained using $[\theta]_{222}$, since this region of the spectrum shows very little dependence on particle size.

Experimental Section

Membrane Preparation. ATPase-depleted membranes of *S. faecalis* (ATCC No. 9790) were prepared by the procedure of Schnebli and Abrams (1970). After release of ATPase the membranes were further treated with 0.01 mg/ml of RNase and 8 mM EDTA to assure removal of rRNA, then washed. Samples were spun down at 100,000*g* for 30 min, then resuspended by homogenization in fresh Tris buffer for the next wash. The initial wash showed a burst of 260-nm absorbing material. Subsequent washing was continued until no discernible 260-nm peak was observed in the wash supernatant and the absorbance in the 280-nm region remained constant in two successive washes, generally at about 5% of the original absorbance in the first wash solution. Membrane samples in 0.02 M Tris buffer (pH 7.5) were sonicated for varying lengths of time in a jacketed, glass sonication vessel thermostated at 2°, in a nitrogen atmosphere. Sonication was accomplished with a Branson S-12 automatic tuning sonifier set for a power output of 90–100 W. Turbidity readings at 325 nm were employed to establish the length of time required to reach minimum particle size. All samples were stored under nitro-

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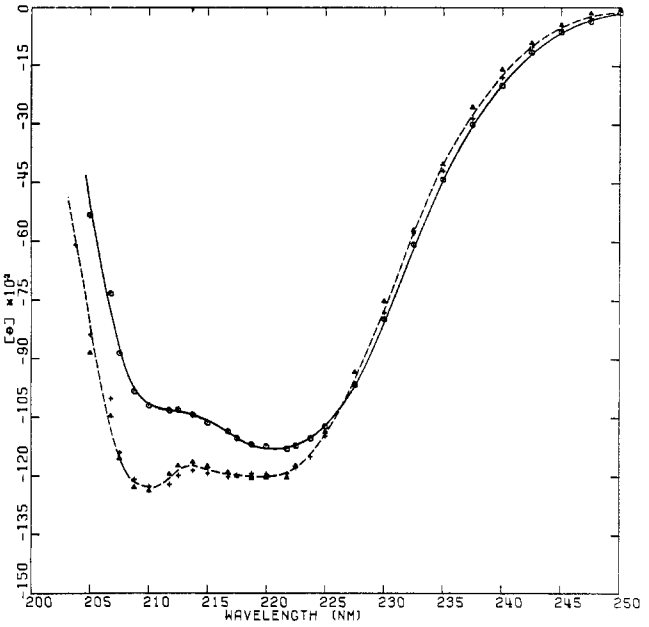

FIGURE 1: Circular dichroism spectra of *S. faecalis* plasma membranes sonicated for 0 (○), 1 (+) and 5 (▲) min, respectively.

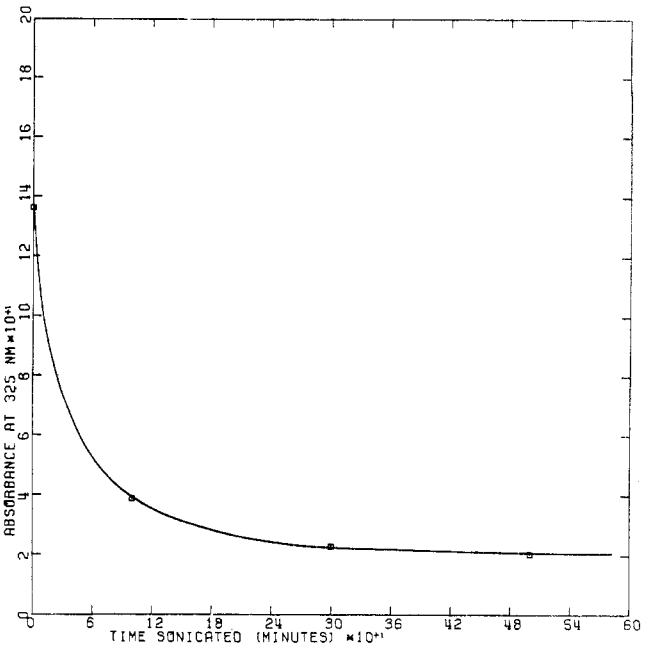

FIGURE 2: Reduction in the absorbance at 325 nm with increasing sonication time.

gen at 4°. Membrane samples were all in 0.02 M Tris buffer (pH 7.5) at room temperature unless otherwise specified. Protein determination was carried out by the method of Lowry *et al.* (1951). Phosphate determination was carried out by a modified Bartlett (Bartlett, 1959) procedure. Chemicals employed were all analytical reagent grade.

Enzyme Assays. Enzyme activity was assayed by measuring the release of inorganic phosphate resulting from the hydrolysis of ATP (Abrams, 1965). Inorganic phosphate was determined using the elon method (Gomori, 1942).

Circular Dichroism. Circular dichroism measurements were carried out on a Cary 60 spectropolarimeter equipped with a 6001 circular dichroism attachment. All circular dichroism measurements were made in cylindrical cells of path length ranging from 0.1 to 0.5 cm. Spectra of samples used in circular dichroism measurements were taken both prior to and after polarimetric scans to check on any changes in turbidity during the run. All samples employed had absorbances less than two in the range in which polarimetric data was collected. In all cases circular dichroism spectra were retraced to ensure reproducibility and to rule out time-dependent effects.

Absorption Spectra. All absorption spectra were recorded on a Cary Model 15 recording spectrophotometer. Turbidity measurements were made using a Gilford Model 240 spectrophotometer.

Infrared Measurements. Aliquots (150 μl) of *S. faecalis* plasma membranes, 1.56 mg/ml of protein, suspended in 0.02 M phosphate buffer (pH 7.5), were deposited on a 9 × 19 mm portion of a 25-mm diameter, 4-mm thick AgCl disk. The solution was allowed to dry to a film in a vacuum desiccator for at least 24 hr over potassium hydroxide. Infrared measurements were made on a Perkin-Elmer 521 infrared spectrophotometer. A scan speed of 0.3 cm^{-1}/sec was employed on all runs. In each case the spectrum was the result of at least three scans through the region of interest. An AgCl disk was used to establish a baseline and the instrument calibration was found to be in error, shifted to the red, by one wave number at 1600 cm^{-1}. The reported spectra were not corrected for this shift. The instrument was equipped with a Puregas Heatless Dryer, Model/HF-211-112-0, purging accessory and was swept with dry air during all measurements.

Density Gradient Centrifugation. Linear sucrose gradients, from 20 to 60%, were formed in 5-cm^3 tubes. Membrane sample (200 μl) was layered at the top of the gradient in 20% sucrose. Centrifuge runs were made in a Beckman Model L3-40 centrifuge, employing a 39,000 rpm swinging-bucket rotor. Samples were spun at 39,000 rpm for 16 hr at 4°. Sampling was accomplished by inserting a syringe needle to the bottom of the tube and pumping out the gradient solution from the bottom of the tube with a peristaltic pump. Fractions were collected as a function of time; both protein and phosphate determinations were carried out on each fraction.

Results

Circular Dichroism. The circular dichroism spectrum of an α helix is characterized in the ultraviolet region of the spectrum by negative troughs at 208 and 222 nm; in addition, the ratio of the amplitude of the long-wavelength trough to the short-wavelength trough is approximately 1 (Greenfield and Fasman, 1969). Circular dichroism spectra of biological membranes generally display a modified helical profile, in that the two negative troughs are shifted and their intensity ratio is greater than 1. The circular dichroism spectrum observed for the plasma membrane of *S. faecalis* bacterium (Figure 1) exhibits the latter characteristic, in that the intensity ratio measured for this membrane is 1.28 as opposed to a value of 1.09 found for the helical form of poly-L-lysine. In addition, the 208-nm trough is red shifted to 212 nm. The observed spectrum is atypical of that observed for most membranes in that the long-wavelength trough position does not appear to be substantially red shifted, suggesting that some structure other than an α helix may be present.

Effect of Sonication. In order to evaluate the particle size dependence of the circular dichroism spectrum, membrane suspensions which were subjected to ultrasonic irradiation for varying lengths of time to induce a progressive decrease

TABLE I: Dependence of the Circular Dichroism Parameters and Estimated α-Helical Content of *S. faecalis* Plasma Membrane on the Time of Sonication.

Sonication Time (min)	% of Total Reduction of A_{325}	$[\theta]_{222}/[\theta]_{208}$	$[\theta]_{208}$	% α Helix Based on $[\theta]_{208}$	$[\theta]_{222}$	% α Helix Based on $[\theta]_{222}$
0	0	1.27	−8,900	17	−11,300	25
1	84	1.04	−11,400	27	−11,900	27
3	98	1.03	−11,800	28	−12,200	27
5	100	1.03	−11,600	28	−12,000	26

in particle size were studied. The reduction of particle size brought about by sonication is demonstrated by the diminished turbidity, as measured by the absorbance at 325 nm, with increasing sonication time (Figure 2). A region of constant turbidity is reached after 3-min time. The effect of sonication time on the circular dichroism curve of *S. faecalis* plasma membrane is shown in Figure 1. After 1-min sonication, 84% of the total reduction in turbidity has been obtained; membrane suspensions sonicated for 3- and 5-min intervals show only minor alteration of the curve observed after 1-min sonication. As a result of sonication, both trough positions have shifted toward the blue, the two bands appearing at 209 and 219 nm. The intensity change observed for the 219-nm trough is much less marked than that observed for the 209-nm trough. $[\theta]_{222}$ shows a slight intensification from −11,300 to −12,000 while $[\theta]_{208}$ increased from −8900 to −11,400, yielding a final intensity ratio of 1.03. Using $[\theta]_{208}$ to estimate the α-helical content yields values of 17, 27, 28, and 28% for sonication times of 0, 1, 3, and 5 min, respectively; this is in contrast to the essentially constant estimate of 27% α helix obtained using $[\theta]_{222}$. These results, as summarized in Table I, demonstate that the long-wavelength circular dichroism has less ambiguity associated with its interpretation than the shorter wavelength circular dichroism.

Since the changes in the circular dichroism spectrum could be attributed to the denaturation of membrane protein by sonication, the activity of a membrane-bound ATPase was monitored as a function of sonication time. No difference in activity was observed between an unsonicated membrane sample and membrane samples which were sonicated for 5, 10, and 30 min. Thus it would appear that no gross protein denaturation has occurred during the sonication process.

To assess the possibility that the changes in the circular dichroism curves were due to protein liberated from the membrane surface during the sonication process, sucrose density gradient runs were performed on samples sonicated for 0, 5, 10, and 20 min. Any gross separation of protein from the membrane surface occurring as a result of sonication will result in both a protein-rich and a lipid-rich component; these components, due to their large density difference, would be easily separable on a density gradient. Hence, if liberation of protein occurs, one expects to find that the protein concentration profile down the density gradient tube differs greatly from that observed for the lipid concentration profile. The result of the density gradient runs for unsonicated and 5-min sonicated samples are shown in Figure 3. Protein concentration is represented as absorbance in a Lowry determination and lipid concentration as absorbance recorded in a

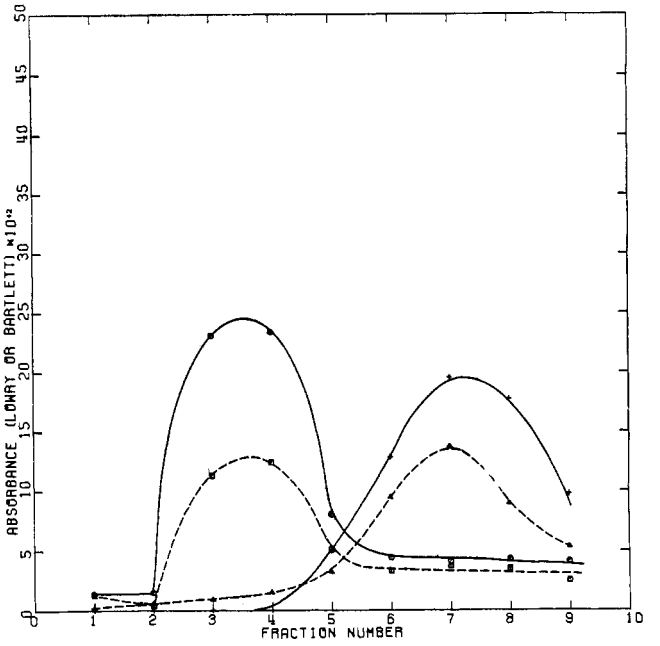

FIGURE 3: Sucrose density gradient distribution: unsonicated membranes, protein (⊙) and lipid (⊡); 5-min sonicated membranes, protein (+) and lipid (▲).

Bartlett test. These two concentration profiles parallel one another as well for the 5-min sonicated preparation as they do for the unsonicated membrane sample, demonstrating that little or no liberation of protein from the membrane surface has occurred during sonication.

Effect of Induced Aggregation. It was noted in the preparation of sonicated membrane suspensions containing Mg^{2+} that sonication times of up to 30 min were required to reach a region of constant turbidity. It was also observed that addi-

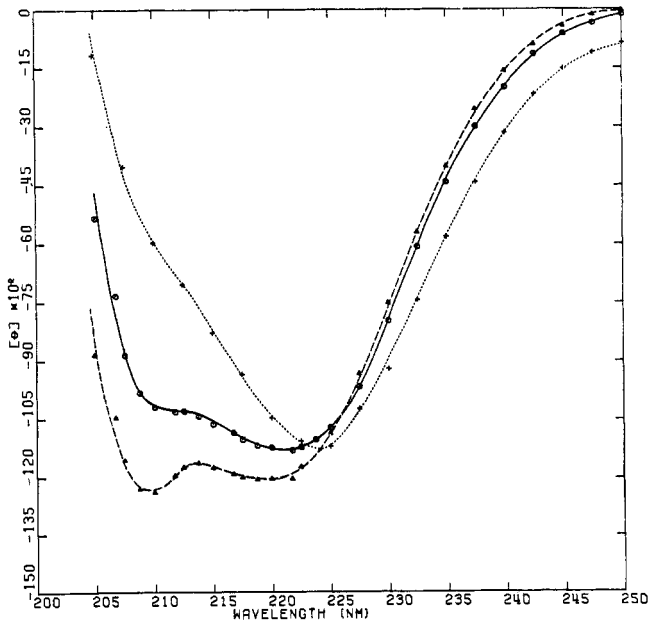

FIGURE 4: Effect of Ca^{2+} on the circular dichroism spectrum of *S. faecalis* plasma membranes; unsonicated membranes (⊙), membranes sonicated for 5 min (▲), membranes sonicated for 5 min plus 5 mM Ca^{2+} (+).

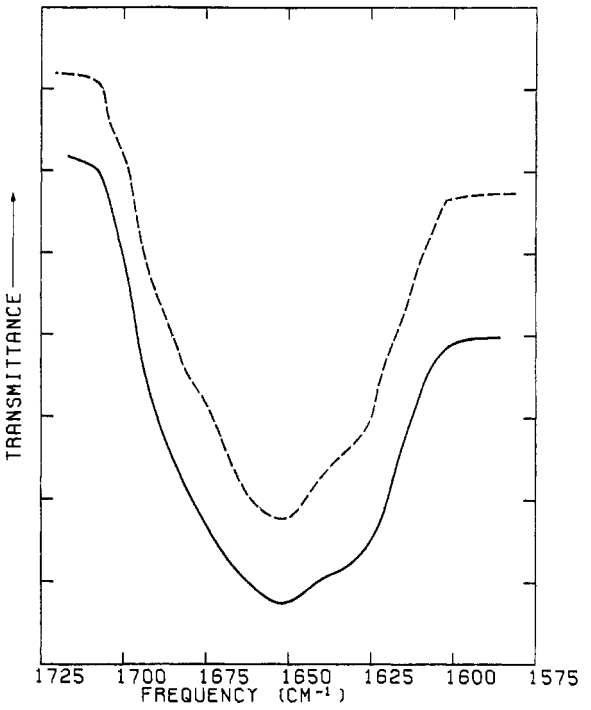

FIGURE 5: Infrared absorption spectra of dried films of *S. faecalis* plasma membranes: unsonicated membranes (——), 5-min sonicated membranes (---).

tion of Mg^{2+} to suspensions of membranes which were orig inally sonicated in the absence of divalent cations led to induced aggregation of the membrane suspension. Ca^{2+} was found to have a similar effect at lower concentrations. The effect of this increased turbidity on the circular dichroism spectrum of a 5-min sonicated membrane preparation is shown in Figure 4; the circular dichroism spectrum in the presence of 5 mM Ca^{2+} is seen to shift so as to be even more distorted with respect to trough position and intensity ratio than was the unsonicated preparation. Solutions containing more than 10 mM Ca^{2+} formed precipitates immediately, whereas those containing lower levels of Ca^{2+} were stable for several hours.

Infrared Spectrum. The infrared spectra of polypeptides in dry films have proved useful in determining the presence of β structure. A β conformation exhibits an amide I band in the region of 1630 cm^{-1}, whereas random-coil and α-helical conformations have an amide I band around 1650 cm^{-1}. Figure 5 shows infrared spectra of dry films formed from both an unsonicated and a 5-min sonicated membrane preparation; both these spectra show peaks at 1650 cm^{-1} and a strong shoulder at 1630 cm^{-1}. The presence of the 1630 cm^{-1} shoulder is strong evidence for the existence of β structure in the plasma membrane protein (Timasheff *et al.*, 1967).

Discussion

The circular dichroism spectrum of *S. faecalis* plasma membranes shows several of the anomalies generally associated with membrane curves. In order to evaluate the effect of particle size in causing these anomalies, we studied membrane suspensions in which the particles size could be reduced by sonication or increased through Ca^{2+} induced aggregation. The reduction of particle size, as indicated by the diminished turbidity of the membrane suspension, leads to a marked decrease of the anomalies generally associated with membrane circular dichroism spectra. If one attempts to estimate the per cent of α-helical structure in the membrane protein using $[\theta]_{208}$ one finds that the value obtained is a strong function of the turbidity of the solution. However, as little as a minute of sonication, which effects 84% of the total reduction in turbidity, leads to a sharp rise in estimated helicity, which does not change with further sonication. In addition, it is seen that $[\theta]_{222}$ is almost unaffected by the optical artifacts and hence can yield a reliable estimate of helicity in the unsonicated membrane system. While these investigations were in progress Glaser and Singer (1971) and Schneider *et al.* (1970) reported on the effect of fragmentation of red blood cell ghosts, by use of a French press and sonication respectively, on the circular dichroism spectrum; their results are in agreement with those reported here. We have been able to extend this type of investigation to demonstrate that the anomalies in the circular dichroism spectrum can be reintroduced by addition of Ca^{2+} to a sonicated membrane suspension; this leads to aggregation of the membrane particles with concomitant increase in turbidity. The aggregating ability of Ca^{2+} in this system may be related to the lipid composition of the membrane; *S. faecalis* plasma membrane contains phosphatidylglycerol, which bears a net negative charge (Vorbeck and Marinetti, 1965). Ca^{2+} may reduce the electrostatic repulsion between membrane particles allowing them to form larger aggregates.

We have also carried out density gradient centrifugation experiments on both sonicated and unsonicated membrane suspensions to demonstrate that the observations made on sonicated samples are not related to mechanical liberation of protein from the surface of the membrane by sonication. The observed concentration profiles for protein and lipid show that no such separation has occurred. To assess the degree of denaturation induced by the sonication process, the activity of a membrane bound ATPase was followed as a function of sonication time. Since the activity was found to remain essentially constant up to sonication times of 30 min, we conclude that no gross denaturation of the membrane protein has occurred.

The long-wavelength trough in the circular dichroism spectrum in the unsonicated membrane sample appears at 222 nm; sonication shifts this trough position to 219 nm. The infrared spectra offer an explanation as to why this trough appears to be blue shifted relative to a pure α-helical spectrum. The infrared spectra show a distinct shoulder at 1630 cm^{-1}, indicating the presence of some degree of β structure in the protein of this membrane. The ultraviolet circular dichroism spectrum of a β conformation is characterized by a negative trough at 217 nm (Greenfield and Fasman, 1969). Therefore, if there was a contribution to the circular dichroism spectrum due to the presence of β structure, one would expect to find the long-wavelength trough blue shifted and the short-wavelength trough red shifted relative to the usual positions observed for an α-helical conformation; this is in agreement with our observations.

Several attempts have been made to quantitatively evaluate the effect of the particulate nature of suspensions of biological membranes on the observed optical properties of these systems. Gordon (1972) and Gordon and Holzwarth (1971) have applied Mie scattering theory to the problem of optical artifacts in membrane circular dichroism spectra; this theory treats absorption and refraction as the real and imaginary parts of a single wavelength-dependent function, as opposed to dealing with them as independent phenomena. Their cal-

culations indicate that for particles whose shape is either a solid sphere or a spherical shell anomalies of the type observed in membrane circular dichroism spectra are generated by contributions due to both differential scattering and absorption flattening effects. Mie theory calculations also predict that the size dependent contribution due to absorption flattening is small above 200 nm and that the major contribution to the observed anomalies comes from the differential scattering of left-handed and right-handed circularly polarized light by the optically active membrane particles. Gordon's plot of the calculated wavelength dependence of the differential scattering contribution shows an intersection with the base line. Such a crossover point is expected at the wavelength where the difference in refractive index for right-handed and left-handed circularly polarized light equals zero and should therefore coincide with the position of the crossover point in the optical rotatory dispersion curve. If in fact the contribution due to absorption flattening is small above 200 nm, then the crossover point in the optical rotatory dispersion curve, which occurs at 227 nm for *S. faecalis* membranes (Litman, 1971),[1] ought to coincide with an isodichroic point in the circular dichroism spectra. The circular dichroism curves in Figure 1 do indicate such an isodichroic point near 227 nm, confirming that the major contribution to the circular dichroism anomalies in this wavelength region results from differential scattering. The small variation in $[\theta]_{222}$ is explained by its close proximity to the crossover point. Gordon's Mie scattering calculations also indicate increasing distortion of the circular dichroism spectrum with increasing particle size, in good qualitative agreement with our observations.

Electron micrographs (Greenawalt and Litman, 1971)[1] of negatively stained ATPase depleted membranes, both sonicated and unsonicated, show the presence of randomly shaped membrane fragments with a large size variation. A qualitative survey of particle size shows unsonicated membrane preparations have a size distribution ranging from 600 to 3000 Å with an average value of 1500 ± 375 Å; occasional fragments as large as 4000 to 5000 Å were observed. Membrane preparations falling in the region of the sonication plateau had a particle size range of 200 to 1200 Å with an average size of 477 ± 180 Å. A comparison of our circular dichroism spectra for unsonicated and sonicated membranes to Gordon's calculated circular dichroism curves for 1000 and 300 Å spheres shows that the calculated curves predict more distortion due to scattering than is shown in our observed curves. This lack of quantitative agreement may be due to an overestimate of the scattering contribution in the theoretical calculation, or may be a reflection of the random shape and broad size distribution of the membrane fragments employed in this study.

The results presented here demonstrate a direct correlation between the presence of certain anomalies in the circular dichroism spectra of membrane associated protein and the degree of turbidity or light scattering in the system. Systematic reduction in turbidity by sonication leads to an apparent elimination of these anomalies, whereas increased turbidity, resulting from the addition of Ca^{2+} to the membrane solution, causes the anomalies to return in a form that is even more exaggerated than in the unsonicated system. It is apparent from these studies that the anomalies are associated primarily with differential scattering due to the particulate nature of the membrane system and that reduction below some certain particle size reduces this artifact below the level of detection. In addition, $[\theta]_{222}$ is only slightly influenced by the size-dependent artifact, and consequently yields a reliable estimate of the helical content in the unsonicated membrane samples. It would appear that in any experiments aimed at demonstrating conformation changes in particulate systems as a function of some type of perturbation, controls will have to be carried out to demonstrate that the observed effects are not a result of changes in the turbidity of the solution and hence artifactual in nature.

[1] Unpublished results.

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