CORRELATION OF LIGHT SCATTERING AND ABSORPTION FLATTENING EFFECTS WITH DISTORTIONS IN THE CIRCULAR DICHROISM PATTERNS OF MITOCHONDRIAL MEMBRANE FRAGMENTS

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The circular dichroism and optical rotatory dispersion patterns of biological membranes resemble, in gross aspect, those of the α-helix but the extrema are red shifted and of low amplitude. Such patterns are not typical for solublized membrane lipoproteins but rather are exhibited by the aggregates of membrane proteins (Steim and Fleishcher, 1968), as well as by aggregated polypeptides and antibiotics (Urry and Ji, 1968 b). Since particulate systems exhibit flattened absorption bands (Duysens, 1956) and light scattering, it is of interest to experimentally relate changes in the circular dichroism patterns with these effects. Absorption flattening results in a decrease of absorbance due to a decrease in the effective concentration of absorbers, while light scattering results in a light intensity loss and thereby an increase in absorbance as measured by the phototube. Light scattering also results in a concentration obscuring effect. The extent of light scattering and absorption flattening can differ slightly for the left-handed and right-handed circularly polarized light. Thus these effects could accentuate distortions in the ORD and CD patterns of particulate systems as compared to the molecularly dispersed samples.

Previously it has been demonstrated that the low amplitude of the membrane type CD curve can be generated from the characteristic α-helix CD pattern by applying correction factors for light scattering and absorption flattening (Urry and Ji, 1968 a & b). In this communication it is shown that the red shift and decrease in amplitude of the CD extrema of particulate systems correlate with changes in the intensity of scattered light and with the extent of absorption flattening as measured, respectively, at wavelengths where significant absorption does not occur and at wavelengths corresponding to major absorption bands.

EXPERIMENTAL: Beef heart mitochondria were prepared as previously and used in 10^{-2} M phosphate buffer at pH 7.6 (Crane et al., 1956). The sodium salt of poly-L-glutamate (PGA) (mw = 68,000) was purchased from Pilot Chemical Co. (Lot No. G-83), Watertown, Mass. Protein concentration was measured by the method of Lowry et al., (1951). Sonication of the mitochondria and PGA was performed at 0°C. In order to fractionate by particle size the fragmented mitochondria were sequentially sedimented at 2,000 x g and 20,000 x g for

30 minutes each. The 2,000 x g pellet was further sonicated to study changes in the CD spectra. The CD of all samples were measured in a 0.1 mm path length cell at protein concentrations of 1 mg or 2 mg per ml on a Cary Model 6001 CD attachment to the Model 60 spectropolarimeter. The above procedure minimized the effects of concentration and path length dependencies on light scattering. The apparent absorbance due to scattered light was measured at 700 mμ, a wavelength outside the range of significant absorption of mitochondria and PGA. Absorption measurements were made on Cary Model 14 and 16 spectrophotometers. For the measurement of PGA at low pH, a reference cell was used which contained the same concentration of HCl as the PGA solution.

TABLE I

AGGREGATION DEPENDENT ABSORBANCE OF PGA

CD Curve	A	B	C	D
pH	3.94	2.43	2.43	2.43
Sonication	None	None	10 Sec	60 Sec
OD 700 (light scattering)	0	0.0025	0.0040	0.0075
OD 190	0.292	0.216	0.146	0.146
CD $n-\pi^*$ extremum (mμ)	222	223	224	225
$(A_L-A_R)\times 10^4$	5.3	4.8	3.5	2.7

Aqueous solution of PGA (1mg/ml in 0.1 mm cell) was acified by adding HCl. Absorbance at 700 mμ represents the intensity loss due to light scattering. Absorbance at 190 mμ is due to the peptide absorption peak which is modified both by absorption flattening and light scattering.

RESULTS AND DISCUSSION: The aqueous solution of PGA at pH 3.94 which has 5% of the carboxyl groups ionized exhibited no apparent absorption at 700 mμ (Table I). At pH 2.4 the aggregation of PGA is observed as an increase in turbidity with an apparent absorption at 700 mμ. Sonication at pH 2.4 enhanced the turbidity and therefore the extent of aggregation. As shown in Figure 1 the CD spectra of PGA changes gradually upon aggregation. The extrema at 190 mμ and 222 mμ, shifted toward longer wavelengths and the amplitude of the extrema decreased gradually as the intensity of the scattered light increased and as the absorbance at the 190 mμ absorption peak decreased (Table I). Also a red shift in the crossover point is observed.

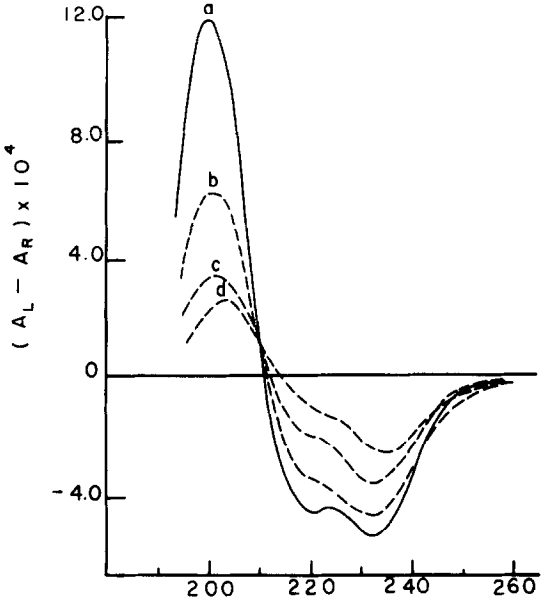

Figure I. pH and aggregation (by sonication) dependent CD curves of PGA. Since the effective concentration of the chromophore is different from the total concentration, (A_L-A_R) rather than ellipticity was plotted vs. wavelength. See the Text for discussion and correlate with values given in Table I.

Mitochondrial membranes fragmented by sonication for 60 sec were fractionated by differential centrifugation. The particle size dependent changes in the CD (Figure 2) show a correlation with changes in light scattering and absorbance of cytochrome peak near 410 mμ (Table II). At these concentra-

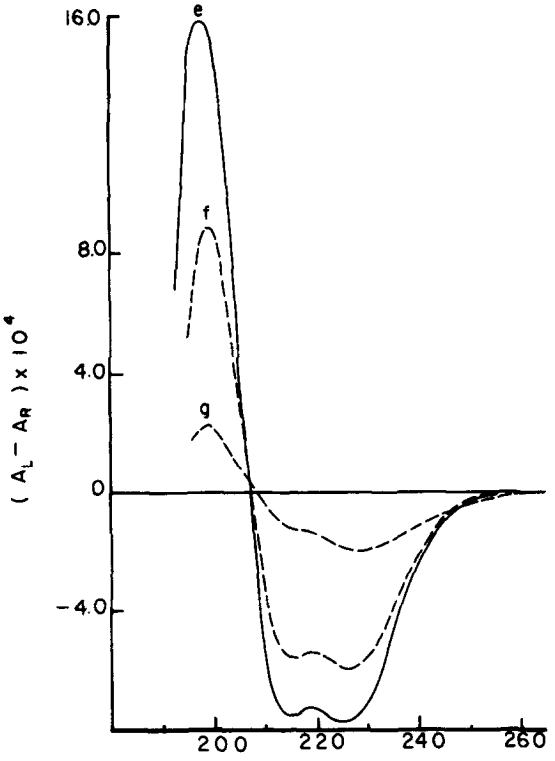

Figure 2. Particle size dependent CD patterns of mitochondrial fragments. See Text for discussion and correlate with information in Table II.

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tions the absorption of the mitochondrial fragments at 190 mμ was greater than 2. The decrease in effective concentration of absorbing units at 190 mμ as the size of particle increases is reflected in the CD pattern of 2,000 x g pellet in Figure 3 and Table III. Light scattering by the mitochondrial particles is seen to be greater than that of the PGA aggregates. In addition to differences in particle size, the enchanced scattering may be due to the shape of the mitochondrial membrane fragments. The flat membranous particles may be expected to scatter light more effectively than more nearly spherical aggregates. The absorption flattening effect, however, may be expected to compare in an inverse manner.

Resonication of the 2,000 x g pellet of the mitochondrial fragment increases the amplitude of the CD extrema and increases absorbance at the

TABLE II

PARTICLE SIZE DEPENDENT ABSORBANCE OF MITOCHONDRIAL FRAGMENTS

CD Curve	E	F	G
Pellet	20,000 x g Supernatant	2,000 x g - 20,000 x g	2,000 x g
OD 700	0	0.022	0.055
OD 410	0.033	0.056	0.080
CD $n-\pi^*$ extremum (mμ)	220	222-223	224
$(A_L-A_R) \times 10^4$	7.5	5.9	2.1

Mitochondria were sonicated at 0° for 60 sec. The particles were fractionated by differential centribugation. The protein concentration was 2 mg/ml and all the optical rotation measurements were carried out in a 0.1 mm cell.

190 mμ peak. Also the $n - \pi^*$ extremum shifts from 225 mμ to 224 mμ.

These results demonstrate changes in the CD patterns which correlate in a regular way with light scattering and absorption flattening. As light scattering is proportional to the difference in the squares of the refractive indices of solute and solvent, it is dependent on the wavelength of the incident light in a manner reflecting the wavelength dependent variation of refractive index. In the region of an absorption band the refractive index is anomolous while it is monotonic in wavelength regions devoid of absorption bands. This anomolous dispersion is reflected in the ORD Cotton effect which derives from the difference in refractive indices of left-

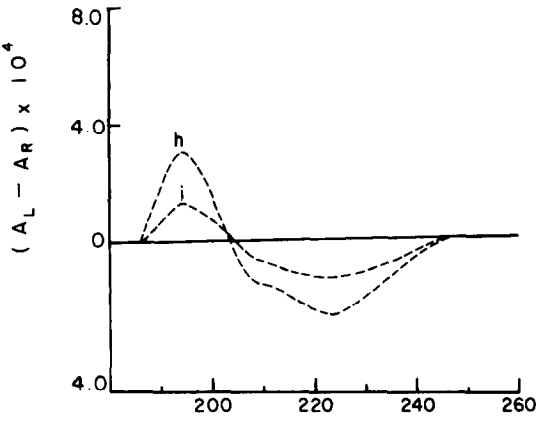

Figure 3. Sonication dependent CD spectra of the insoluble mitochondrial membranes. See Text for discussion and relate to values reproted in Table III.

TABLE III

SONICATION DEPENDENT ABSORBANCE
OF THE INSOLUBLE MITOCHONDRIAL PARTICLES

CD Curve	H	I
Sonication	30 Sec	None
OD 700	0.055	0.064
OD 187	0.96	0.47
CD n-π^* extremum	224	225
$(A_L-A_R) \times 10^4$	2.7	1.6

The 2,000 x g pellet of the fragmented mitochondria was resonicated. The protein concentration was adjusted to 2 mg/ml and the optical properties were measured in a 0.1 mm cell.

handed and right-handed circularly polarized light, i.e, $n_L - n_R$. One may eliminate light scattering in particulate systems by matching the refractive indices of the particle and solvent. This is a useful approach at wavelengths outside an absorption region. In an absorption region, however, due to the above mentioned anomolous dispersion, light scattering can be prevented only when the absorption properties of the solvent are the same as those of the particle.

The light scattering effect on the CD and ORD or particulate membrane preparations is clearly apparent in the data of Lenard & Singer (1966). In Table I of that paper it is reported that when solvents of higher refractive index than water were used the amplitude of the negative extrema at 224 mμ and 210 mμ were enhanced by up to 200% and the 224 mμ peak exhibited a slight blue shift. Since light scattering effects can contribue to the red shift as well as the decreased magnitude of the extrema, such changes in the CD pattern due to changes in the refractive index of the solvent are very relevant and in accord with the calculated ellipticities obtained when applying the appropriate correction factors for light scattering (Urry and Ji, 1968 a & b). Frequently the light scattering effects on CD and ORD have been tested for by changing the concentration of a turbid sample and at the same time changing the path length of the cuvette. As light scattering depends on both the concentration of particles and path length, light scattering due to a higher concentration of particles in a shorter cell may not significantly differ from that of a lower concentration in a longer cell. A properly controlled experiment utilizes the same concentration of absorbers in a given path length and varies only particle size. The Duysens absorption flattening effect (1956) on ORD, CD or absorption spectra is dependent on the absorbance of an individual particle and cannot be eliminated by increasing the refractive index of solvent.

It should be emphasized that the CD pattern of mitochondrial membranes can be changed by further fragmenting the particles (Figure 3); and that,

in addition, there is a limit beyond which further sonication neither alters the CD pattern, nor the measured light scattering and absorption flattening. This continues the correlation of light scattering and absorption flattening with the distortion of the CD curves of particulate systems. (The limit may have its explanation in the expectation that mitochondrial membranes are composed of subunits. The subunit would be the smallest fragment obtained by sonication.) On the bases of these results, there can be little question but that the CD patterns of membranous systems are dependent on particle size and that light scattering and absorption flattening are among the responsible factors.

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