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CIRCULAR DICHROISM OF BIOLOGICAL MEMBRANES

II. PLASMA MEMBRANES AND SARCOTUBULAR VESICLES

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SUMMARY

Circular dichroism (CD) spectra of fresh, frozen and sonicated samples of plasma membranes from rat liver and of sarcotubular vesicles from rabbit muscle were obtained. Using the pseudo reference state approach, the distorted spectra were corrected for absorption flattening and dispersion distortion arising from the particulate nature of the samples. The results show interesting differences between the two membranous systems: while the values of the absorbances and ellipticities of plasma membranes are similar to those of mitochondria and red blood cell ghosts, the values for sarcotubular vesicles show lower absorbances and ellipticities. Furthermore, the difference in behavior with respect to solvents and detergents used to achieve the pseudo reference state indicates differences in the structural forces of the two membranous systems.

INTRODUCTION

During the past few years numerous attempts have been made in employing CD and ORD techniques to gather more information on the structure of biological membranes. All of the systems investigated to date have shown two remarkable features in their CD and ORD spectra when compared to those of soluble α-helical proteins: the red shift of maxima, minima and inversion points, and a progressive reduction in amplitude of peaks and troughs with decreasing wavelength.

Such results have been interpreted as arising from interactions between α helical sections of the protein in the membrane[1,2], hydrophobic interactions between lipids and proteins[3,4], solvent polarizability[5] or contributions of the lipids to the optical activity of the membranes[4]. Subsequently, however, Urry and Ji[6] proposed, and experiments carried out in this laboratory[7–9] and more recently confirmed by other investigators[10,11] showed that the distinguishing spectral features of biological membranes when compared to those of model α-helical polypeptides are primarily artifacts due to the particulate nature of the systems. Studies to apply corrections for absorption flattening and dispersion distortions were made on suspensions and

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solutions of poly-L-glutamic acid[7]. Urry and Krivacic[12] also showed that the CD of optically active particulate systems is further complicated by differential scatter of the left and right circularly polarized beams. An additional feature of differential absorption flattening has also been utilized in calculating distorted ellipticity curves of particulate samples[13]. Gordon and Holzwarth[14] have argued that the flattening quotient for absorption, Q_A, is different from that for circular dichroism. This difference has been shown to be equivalent to differential absorption flattening[15] and it has been shown that, while Q_A is the quotient for absorption, the quantity Q_A^2 is a good approximation for the flattening quotient in circular dichroism, *i.e.* the differential absorption flattening quotient[15].

In the preceding paper on optical rotation data of biological membranes[8] it has been shown that different approaches can be used to improve the distorted spectra. The present work deals with plasma membranes and sarcotubular vesicles whose corrected CD spectra are calculated applying the pseudo reference state approach. Also this paper utilizes a more general form of the corrections outlined in a recent review on protein conformation in biomembranes[15].

MATERIALS AND METHODS

Plasma membranes

Rat liver plasma membranes were prepared according to Ray[16] with slight modifications. The purity of the preparations and the size of the vesicles were verified with electron micrographs.

To obtain systems of particles in order of decreasing size, plasma membranes were sonicated in aliquots of 2 ml. A precooled test tube 1 inch in diameter and 2 inches deep purchased from Heat Systems Company was used and the sample was kept in ice. Sonication was performed using an MSE sonicator at an output of 1.1 A. The effectiveness of sonication in improving the amplitudes of CD extrema was checked and the best sonication time was found to be 15 sec. The pseudo reference state was obtained by stirring the sample with 0.2 % sodium dodecyl sulfate for 2 h at room temperature. No difference, as far as CD patterns are concerned, was found when the sample was stirred either at room temperature or in ice. The sample was then diluted with trifluoroethanol such that the sample to trifluoroethanol ratio was 1:4 and subsequently sonicated 15 sec.

Sarcotubular vesicles

The sarcotubular vesicles from rabbit muscle were prepared following the method of Seraydarian and Mommaerts[17]. By differential centrifugation the large microsomal fraction (41000 $\times$ *g*) was collected and resolved by zonal centrifugation in the sucrose density gradient labelled by the authors as System I[17]. The sucrose gradient tubes without samples were pre-equilibrated in the cold room overnight. In such a system the large microsomal fraction is separated into two zones at about 40 % and 65 % sucrose, respectively. We chose the 40 % sucrose fraction shown to contain the sarcotubular vesicles with very stable biological activities. When the purity was checked with the electron microscope, only membranous vesicles and tubular fragments were observed. We further checked the ATPase activity of the 40 % sucrose fraction and found it to be stimulated by Ca^{2+}. The sarcotubular vesicles'

ATPase was assayed under the following conditions: 2 mM $MgCl_2$, 2 mM potassium oxalate, 2 mM ATP (disodium salt) (with prior adjustment to pH 7.2 with NaOH), 50 mM Tris acetate (pH 7.2) and approx. 1 mM Ca^{2+} (as calcium acetate), with the total volume being 1 ml. The incubation time was 8 min at 25 °C. Liberated phosphate was determined with the method of Fiske and SubbaRow[18]. For the CD studies the fraction was isolated, washed 4 times and finally resuspended in 20 mM Tris buffer (pH 7.4) containing 80 mM KCl and 5 mM potassium oxalate.

The sarcotubular vesicles, suspended in KCl–potassium oxalate buffer, were sonicated at ice temperature for 5 sec in aliquots of 2 ml using an MSE sonicator at an output intensity of 1.1 A. Further step-wise sonication up to a total time of 30 sec did not result in improvement of CD pattern.

Treatment with 0.2 % sodium dodecyl sulfate for 1.5 h was used to achieve the pseudo reference state. The following 5-fold dilution with trifluoroethanol caused aggregation and precipitation. As a result, the spectra showed a very low signal to noise ratio and the patterns were significantly dampened.

The spectra were recorded at room temperature using a Cary Model 6001 spectropolarimeter modified to measure absorption and CD simultaneously[7,19]. The solvent was run immediately before the corresponding sample. A 0.2-mm cell was used for the samples at the original concentration and 1.0 mm cell for the pseudo reference state. Protein concentration, determined according to a biuret method[20] and Lowry method[21], was 2.11 mg/ml for sarcotubular vesicles and 1.75 mg/ml for plasma membranes.

RESULTS AND DISCUSSION

The suspension ellipticity can be related to a corrected ellipticity—an ellipticity to be expected if the molecules were molecularly dispersed but in the same conformation—by an ellipticity distorting quotient, Q_E, *i.e.*

$$[\theta]_{susp} = [\theta]_{corr} Q_E \tag{1}$$

It has been shown[7], under defined experimental conditions of similar particle size, concentration and path length and at wavelengths where differential scatter of left and right circularly polarized is zero, that Q_E may be approximated by the simplified expression (Q_A—A_S) where Q_A is the absorption flattening quotient of Duysens[22] and A_S is that part of the absorption measured by the phototube which arises due to the scattering of photons in directions away from the phototube. More exact expressions for Q_E are much more complex[13,15]. However, a more generally applicable, approximate expression has been derived[15] which again applies when differential scatter is zero (*i.e.* $A_{SL} = A_{SR}$) and that is

$$Q_E^{A_{SL}=A_{SR}} = Q_A^2 Q_\sigma \tag{2}$$

The ellipticity distorting quotient is seen as the product of the square of the Duysens absorption flattening quotient and a light scattering quotient, Q_σ which is defined as

$$Q_\sigma = e^{-\sigma} \tag{3}$$

where

$$\sigma = \frac{A_{obsc}}{A_{soln}} \tag{4}$$

The obscurred absorption, A_{obsc}, which is a result of light scattering is

$$A_{obsc} = -\log(1 - X'_A \cdot X_S) \tag{5}$$

where

$$X'_A = I_A/I_0 = (1 - 10^{-Q_A A_{soln}}) \tag{6}$$

and

$$X_S = I_S/I_0 = (1 - 10^{-A_S}) \tag{7}$$

with the intensities being the intensity loss due to absorption, I_A, the intensity loss due to scattering, I_S, and the initial beam intensity, I_0. By this analysis when studying suspensions the absorption measured by the phototube has three components

$$A_{susp} = Q_A A_{soln} + A_S - A_{obsc} \tag{8}$$

By using the same phototube to measure both absorption and circular dichroism of the suspension and a satisfactory molecularly dispersed state, it is possible to

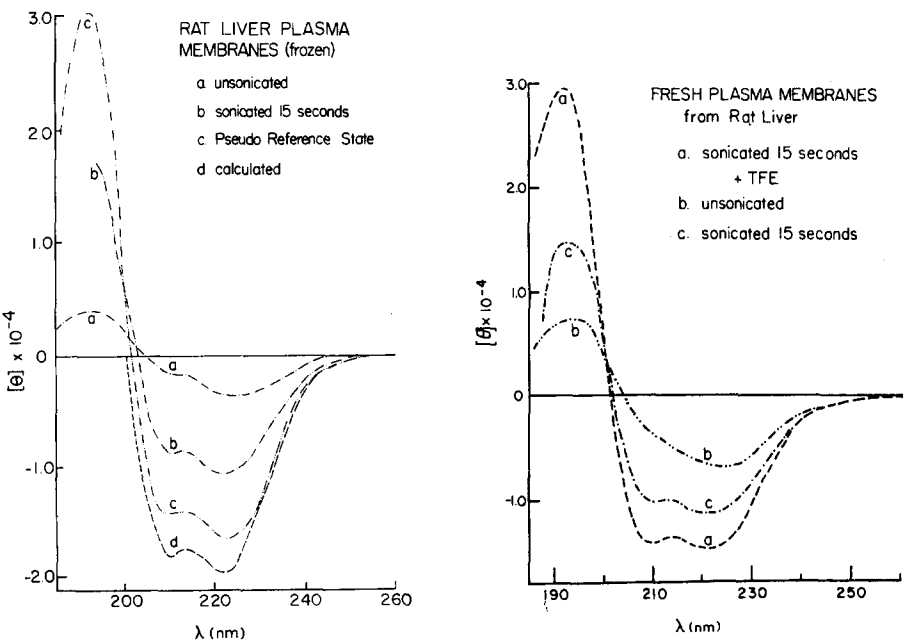

Fig. 1. Circular dichroism spectra of frozen rat liver plasma membranes. The concentrations of protein was 1.75 mg/ml and the pathlength used was 0.218 mm. Note the progressive red shift of the long wavelength extrema as one approaches the intact plasma membrane. The crossover of Curve a is red shifted when compared to the crossover of Curves b and c.

Fig. 2. Circular dichroism spectra of fresh plasma membranes from rat liver. The protein concentration was 1.5 mg/ml; the pathlength used was 0.218 mm. Sonication and addition of 4 parts trifluoroethanol (TFE) result in a curve which has an ellipticity at 192 nm of $2.94 \cdot 10^4$.

approximate Q_A and Q_σ to correct the circular dichroism spectra on suspensions and thereby to obtain approximate spectra of molecularly dispersed molecules retaining their membrane conformations.

As seen in Figs. 1, 2 and 4, sonication results in enhancement of the CD patterns. This is due to less absorption flattening and light scattering by the smaller particles. Sonication is used to bring the particle size and light scattering characteristics to the range obtained on the model poly-L-glutamic acid system where confidence in the corrections is optimal. Marked enhancement of ellipticity can be obtained using trifluoroethanol. It is important to note that when the value of Q_σ^{224}, initially approximated using the membranous systems treated with trifluoroethanol, was used in Eqn. 2 with $Q_A^{224} \simeq 1$, the corrected ellipticities were higher than those obtained when dissolved in trifluoroethanol. This initial approximation to $[\theta]_{corr}^{224}$ can be used to seek out a solvent system exhibiting ellipticities close to the calculated value. This system is called the pseudo reference state. In Table I the absorbance values relative to Curve b (the sonicated suspension) and Curve c (the pseudo reference state) of Fig. 1 are shown.

At 224 nm, as well as at 192 nm, where optical rotatory dispersion curves are zero, there is no differential scatter. The absorption of the particles at 224 nm is very small, and there is little hyper- or hypochromism. We can, as noted above,

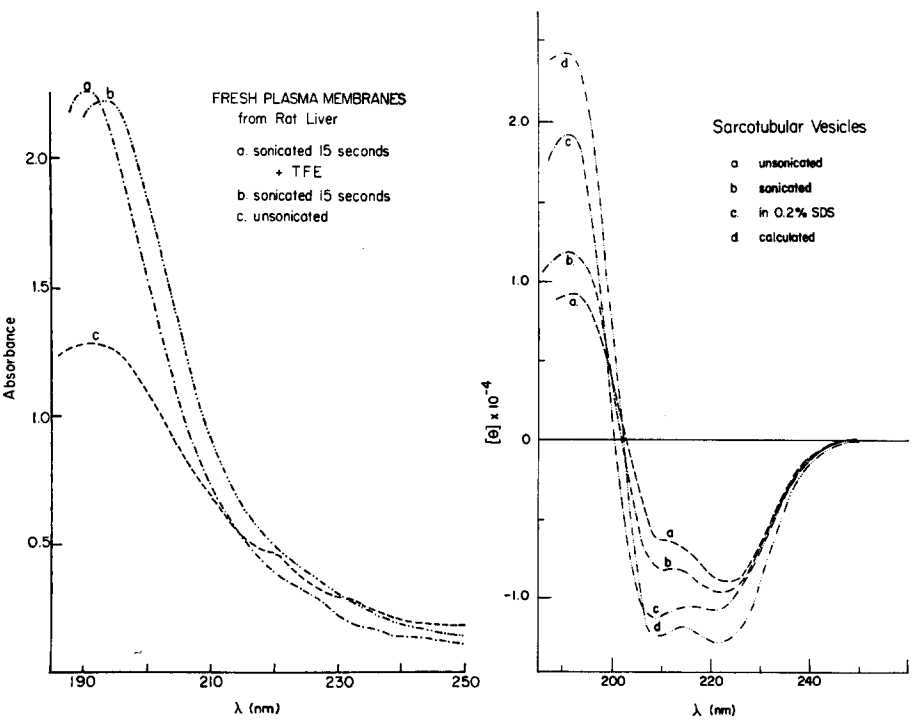

Fig. 3. Absorption curves obtained simultaneously with circular dichroism spectra of Fig. 2.

Fig. 4. Circular dichroism spectra of sarcotubular vesicles. The protein concentration was 2.11 mg/ml; the pathlength used was 0.218 mm. Note that the ellipticities are lower than those of Fig. 1. SDS = sodium dodecyl sulphate.

initially approximate $Q_{A_1}^{224} = 1$ and take the absorption of the pseudo reference state as the absorption of the molecularly dispersed system. An initial value called ${}^{\circ}A_S$ (= A_{susp}—A_{PR}) for frozen plasma membranes (see Table I) is

$$ {}^{\circ}A_S = 0.77 - 0.44 = 0.33 \tag{9} $$

and from the relationships in Eqns. 3–7 and using an iterative approach Q_σ is approximated as 0.52 (see Fig. 6 of ref. 15). The first corrected value for the ellipticity at 224 nm becomes

$$ [\theta]_{C1}^{224} = \frac{-9.4 \cdot 10^3}{0.52} = 1.8 \cdot 10^4 \tag{10} $$

The subscript C1 stands for the corrected value obtained on the first approximation. Then, assuming that absorbance and CD of the membranes are related in the same manner as those of α-helical model systems with their CD patterns being very similar, it is possible to calculate a fractional change, Δf, from the CD at 224 nm

$$ \Delta f = \frac{[\theta]_{PR}^{224} - [\theta]_{C1}^{224}}{-3.8 \cdot 10^4} \tag{11} $$

TABLE I

ABSORPTION VALUES FOR FROZEN PLASMA MEMBRANE SUSPENSION SONICATED 15 sec AND TREATED WITH DETERGENT (PSEUDO REFERENCE STATE)

Wavelength (nm)	A_{Susp}	A_{PR}
250	0.29	0.07
248	0.30	0.07
246	0.30	0.05
244	0.31	0.07
242	0.22	0.07
240	0.34	0.09
238	0.36	0.11
236	0.41	0.16
234	0.45	0.16
232	0.51	0.20
230	0.56	0.26
228	0.63	0.35
226	0.70	0.41
224	0.77	0.44
222	0.85	0.50
220	0.93	0.57
218	1.01	0.64
216	1.12	0.75
214	1.26	0.84
212	1.41	1.00
210	1.58	1.17
208	1.78	1.36
206	2.00	1.63
204	2.26	1.89
202	2.53	2.23
200	2.78	2.61
198	2.99	3.02
196	3.08	3.42
194	3.24	3.77
192	3.29	3.96

and relate the Δf to an absorption change at 192 nm (ref. 8)

$$\Delta A^{192} = \Delta f \cdot 10^3 Cl \tag{12}$$

Consequently,

$$A_{C1}^{192} = A_{PR}^{192} + \Delta A^{192} \tag{13}$$

Next, the initial corrected ellipticity at 192 nm is calculated:

$$[\theta]_{C1}^{192} = [\theta]_{PR}^{192} - 8.8 \cdot 10^4 \Delta f \tag{14}$$

where the coefficient is the difference in ellipticities at 192 nm between α-helix and random coil conformations for poly-L-glutamic acid. Since the obscured absorption, A_{obsc}, is approximately equal to A_S when the actual absorbance, $Q_A A_{soln}$, is greater than one, an initial ${}^{\circ}Q_A$ becomes

$${}^{\circ}Q_{A1}^{192} = \frac{A_{susp}^{192}}{A_{C1}^{192}} \tag{15}$$

and Q_σ^{192} can be obtained

$$Q_\sigma^{192} = \frac{[\theta]_{susp}^{192}}{[\theta]_{C1}^{192}(Q_A^{192})^2} \tag{16}$$

The Duysens relationship between A_P and Q_A for spheres, or that of Gordon and Holzwarth[14] for vesicles, is then used where A_P, the absorption along the diameter of the particle, is the dependent variable, for the Duysens case

$$Q_{A1}^{192} = \frac{3}{2A_{P1}^{192}} \left\{ 1 - \frac{2[1 - (1 + A_{P1}^{192}) \exp(-A_{P1}^{192})}{(A_{P1}^{192})^2} \right\} \tag{17}$$

With $A_{P_I}^{192}$ and the pseudo reference state absorption curve, $A_{P_I}^{224}$ can be calculated. Specifically, at 224 nm,

$$A_{P1}^{224} = \frac{A_{P1}^{192} \cdot A_{PR}^{224}}{A_{C1}^{192}} \tag{18}$$

Having the $Q_{A_I}^{224}$ by Eqn. 17 it is now possible to calculate an improved $[\theta]_{corr}^{224}$ and the process is repeated in an iterative manner until no more improvement is observed.

The values of the ellipticities of membranes are given in Figs. 1 and 2, with a corrected curve given in Fig. 1, and in Table II values are reported. The absorption data corresponding to Fig. 1 is given in Table I and that for Fig. 2 is given in Fig. 3. For the frozen, unsonicated plasma membranes, the negative extremum due to the n–π^* transition is at 224 nm (Fig. 1), while for sonicated and detergent treated membranes it is at 222 nm. The same results were achieved after corrections.

The shoulder at 212 nm, arising from the || band of α-helices, is barely evident in the unsonicated plasma membranes (see Figs. 1 and 2) due to high dampening of the curve; however, in Curves b and c it is properly positioned at 208 nm. The broadened $\perp$ band of Curve a results in a well defined peak which exhibits the maximum at 192 nm in Curves b and c. Sonicated membranes and the pseudo reference

TABLE II

ELLIPTICITIES OF PLASMA MEMBRANES AND SARCOTUBULAR VESICLES REPORTED IN $[\theta] \times 10^{-4}$

Membrane system	*224 nm*			*192 nm*			*With 2 % sodium dodecyl sulfate, sonication and trifluoroethanol*			*Solvent system with maximal values*		
	As prepared	*Sonicated*	*Corrected*	*As prepared*	*Sonicated*	*Corrected*	*224 nm*	*208 nm*	*192 nm*	*224 nm*	*208 nm*	*192 nm*
Plasma membranes												
Frozen	—0.33	—1.1	—1.9	0.4	1.7	3.1	—1.6	—1.4	3.1	—1.6	—1.4	3.1
Fresh	—0.7	—1.1		0.7	1.4					—1.5	—1.4	2.9*
Sarcotubular vesicles												
Fresh	—0.6	—0.8	—1.2	—0.9	1.2	2.4	—0.9	—0.6	0.9	—1.0	—1.1	1.9**

* In 0.2 % sodium dodecyl sulfate, buffer, KCl–potassium oxalate.
** In 80 % trifluoroethanol.

state show the same crossover at 202 nm which, however, is red shifted for the unsonicated plasma membranes.

Sonication, effective in improving the CD shape of plasma membranes, mitochondria and red blood cell ghosts, does not as readily improve the CD patterns of sarcotubular vesicles (Fig. 4), nor is there a large increase in absorption near 192 nm (Fig. 5). However, the n–π^* transition band originally located at 224 nm for unsonicated samples is blue shifted to 222 nm after sonication and to 220 nm after treatment with 0.2 % sodium dodecyl sulfate. Sonication somewhat increases the magnitude of the 208 nm-band which is greatly enhanced by treatment with sodium dodecyl sulfate in agreement with what has previously been found[23]. When compared with the crossover shown by the pseudo reference state of sarcotubular vesicles (see Fig. 4), the calculated value is slightly red shifted. This could be due to solvent interactions affecting the π–π^* transition split in the two components at 208 and 192 nm, but more likely, it is due to neglect of the differential scatter effect.

Furthermore, the sarcotubular vesicles exhibit a particular behavior when treated with trifluoroethanol as shown in Fig. 6: their patterns are not enhanced as with heavy beef heart mitochondria, plasma membranes and red blood cell ghosts, but are significantly attenuated because of the formation of large particles. This difference in behavior along with lower ellipticities likely reflects difference in protein conformation and in the membrane structure.

The CD patterns of the two kinds of membranes presented in this paper re-

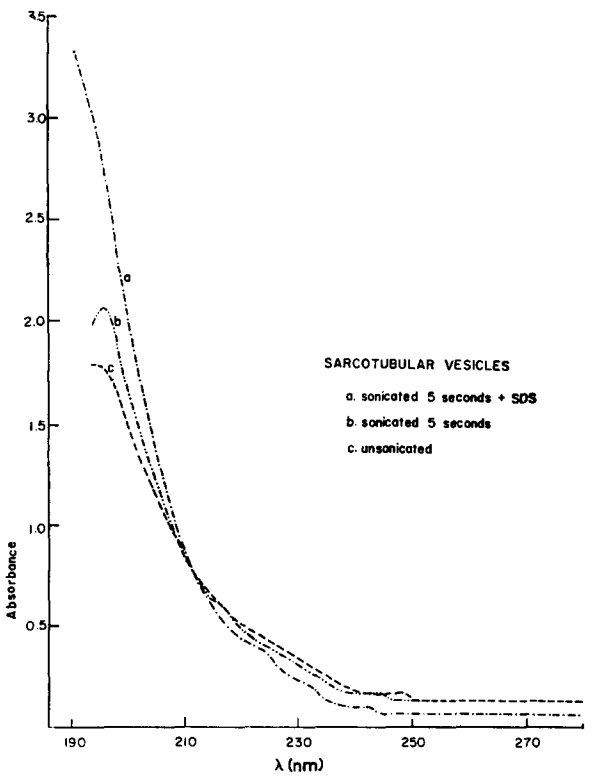

Fig. 5. Absorption curves relative to Fig. 4.

semble those of polypeptides in the α-helical conformation. Estimates of helical content can be meaningful when dealing with systems showing high ellipticities and may be applicable to the plasma membranes, but such calculations are more dubious for sarcotubular vesicles which show substantially lower ellipticities.

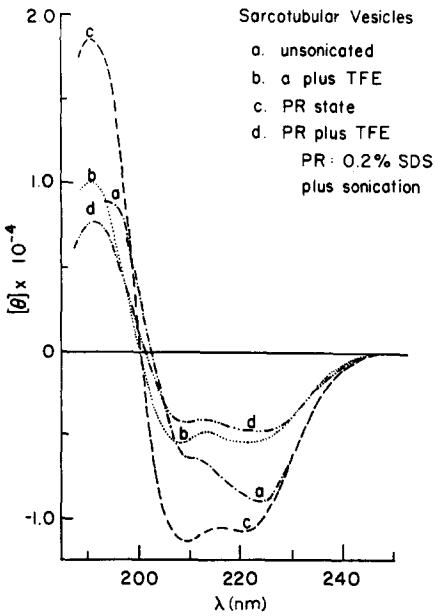

Fig. 6. Effect of solvents on sarcotubular vesicles. Addition of trifluoroethanol (TFE) results in particle aggregation and, therefore, causes the circular dichroism curves (b and d) to be distorted. SDS, sodium dodecyl sulphate.

Recently[24] it has been stated that systems like plasma membranes composed of water filled lipoprotein shells exhibit negligible light scattering and absorption flattening effects in absorption regions. The experimental results in Figs. 1–3 show that this is not the case. It can be seen also, comparing Fig. 1 and Fig. 2, that the plasma membranes, freshly prepared and sonicated, exhibit larger ellipticities than do the frozen membranes. This is due to the fact, as shown by microscopy, that freezing causes the membranes to aggregate in clusters with resulting increased absorption flattening and dispersion distortion effects.

The experimental data unequivocally show that light scattering and absorption flattening artifacts are responsible for the circular dichroism patterns typical of biological membranes. We maintain, of course, that the optical rotation properties of biological membrane particles cannot be meaningfully related to aspects of membrane structure until the distortions are corrected. In the first paper of this series the different approaches to the correction of the distortions have been discussed. Each of them leads to satisfactory and similar results. It is to be expected that the applied corrections are minimal. When the ellipticities are high and resemble those of the α-helix, the pseudo reference state approach as outlined is quite straight forward with perhaps the most limiting factor being the finding of a suitable solubilized state. What is needed are the ellipticities and absorbances of an appropriate pseudo

reference state and the suspension. The appropriateness of a given molecularly dispersed state as the pseudo reference state is judged by $[\theta]^{224}_{Cr}$. Sarcotubular vesicles represent a kind of membrane characterized by ellipticities lower than those of mitochondrial membranes, red blood cell ghosts, and plasma membranes. Accordingly they seem to represent a transition to a class of membranes, *e.g.* axonal membranes[15,25], which exhibit very low ellipticities and high absorbances. The CD patterns of biological membranes, which not so long ago were regarded as indicative of similar structural features, instead reflect, once the distortions have been corrected, differences in the membrane structure.

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