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THE OPTICAL ACTIVITY OF PLASMA MEMBRANES AND ITS MODIFICATION BY LYSOLECITHIN, PHOSPHOLIPASE A AND PHOSPHOLIPASE C

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SUMMARY

1. The circular dichroism and optical rotatory dispersion of the plasma membranes of human erythrocytes and of Ehrlich ascites carcinoma cells for the spectral range 190–250 mμ are reported.

2. The spectra indicate the presence of some peptide in an α-helical conformation but also suggest that the helical π°–π^{-} transitions of membrane proteins are of a lower rotational strength and are located at a longer wavelength than the corresponding bands in synthetic polypeptides. This view is supported by the analysis of the spectra by curve-fitting using the Gauss nonlinear least-squares method.

3. The helical n–π^{-} transitions of these membranes have the same spectral position as those of synthetic α-helical polypeptides but are of low intensity and of a large bandwidth, of which the latter accounts for the red displacement of the optical rotatory dispersion trough observed in many membranes.

4. Except for the width of the n–π^{-} bands, the optical activity parameters of these membranes differ from those of synthetic polypeptide standards in the same manner, although more so, as do certain globular proteins of which the structure is known from X-ray analysis. These deviations are attributed to the presence of short helices, distorted helices and location of some helical peptide chromophores in a highly polarizable apolar environment.

5. We present the effects upon the membrane optical activity of phospholipase A, phospholipase C, lysolecithin and digitonin and conclude that the protein architecture of these membranes depends upon lipid–protein interactions and/or protein–protein interactions which are sensitive to lipid.

INTRODUCTION

The optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of various cellular membranes have shapes suggesting a considerable helical conformation in membrane proteins. These spectra differ from those of synthetic α-helical

Abbreviations: ORD, optical rotatory dispersion; CD, circular dichroism.

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polypeptides in that they are situated at a longer wavelength and are of a low amplitude[1–7]. The CD and ORD spectra between 190 and 250 mμ of the plasma membrane of Ehrlich ascites carcinoma cells and of erythrocyte ghosts are reported in detail here. We conclude that the optical activity of membrane proteins is basically similar to that of other natural polypeptides having a globular tertiary structure except for one distinctive feature; the ORD trough of membrane proteins is red shifted from that of other natural proteins due to an increase in the helical n–π^* bandwidth. This phenomenon appears to be related to lipid–protein interactions or protein–protein interactions which are lipid sensitive.

Finally, we report the changes in the optical activity of these cell membranes upon perturbation with phospholipase A, phospholipase C, lysolecithin and digitonin.

MATERIALS AND METHODS

Optical activity measurements

A Cary model 6001 spectropolarimeter was used with calibrated 1.00-, 0.5- and 0.1-cm cells (Optical Cell Co., Inc., Beltsville, Md.). The absorbance was held below 2.0, and the resulting signal-to-noise ratio was 16.6 at 220 mμ and 2.28 at 195 mμ. All measurements were made at 26° and at a scan speed of 10 sec/Å. We also obtained several spectra with a Jasco UV/5 spectropolarimeter and found that in the accessible spectral region (205 mμ and above), the results were identical to those measured with the Cary spectropolarimeter. The instruments were calibrated with (+)-10-camphor sulphonic acid at 290 mμ. Base lines were run immediately after each spectrum employing the same cell. ORD and CD spectra were run on the same samples.

We plot the CD spectra as mean residue ellipticity, $[\theta]$ (degrees·cm²·decimole^{-1}), *vs.* wavelength. The mean residue ellipticity is given by

$$[\theta] = 3300\,(\varepsilon_L - \varepsilon_R) \qquad (1)$$

where ε_L and ε_R are the mean residue absorptivities of left and right circularly polarized light, respectively. ORD spectra are plotted as $[m]$, the mean residue rotation, in degrees·cm²·decimole^{-1}. Mean residue ellipticities and rotations are not corrected for the refractive index of the solvent (Lorentz correction) because the effective refractive index in the vicinity of the optically active chromophore is not known.

Preparation of native membranes

Ehrlich ascites carcinoma plasma membrane. Plasma membrane fragments of Ehrlich ascites carcinoma cells were prepared as before[9] and were stored in 0.25 M sucrose at —28°. Prior to measuring the optical activity, sucrose was removed either by passing the membrane sample through a calibrated column of Sephadex G-25 or by washing it twice in 1 mM Tris (pH 8.2) in a Spinco SW-50 rotor at 50000 rev./min for 10 min. The solvent for all the measurements on plasma membrane fragments was 1 mM Tris–HCl (pH 8.2). Membrane concentrations were between 50 and 100 μg/ml, determined by the ninhydrin method[10], using crystalline serum albumin as a standard, with the fat-free dry weight of the membrane as an absolute reference.

A mean residue weight of 130 (see ref. 11) was used to calculate ellipticities and rotations.

Erythrocyte ghosts. Erythrocyte ghosts were prepared by lysis of washed, freshly collected erythrocytes in 7 mM (20 mosM) phosphate buffer (pH 7.4), employing the methods of DODGE *et al.*[12]. The membranes were essentially free of hemoglobin; there was no measurable Soret band. Identical spectra were obtained with freshly prepared ghosts and membranes stored for several weeks in 0.1 M glycerol at —28° and subsequently were washed twice in 7 mM phosphate (pH 7.4) in a Spinco SW-50 rotor at 50000 rev./min for 10 min.

The solvent for CD and ORD measurements of intact erythrocyte ghosts was 7 mM phosphate (pH 7.4). Protein concentrations were between 50 and 100 μg/ml. Ghost protein was determined by the method of LOWRY *et al.*[13] using the fat-free dry weight of the membranes, corrected for 7.5% carbohydrate[14], as absolute reference. A mean residue weight of 130, calculated from the data of ROSENBERG AND GUIDOTTI[15], was used to compute ellipticities and rotations.

Preparation of modified membranes

Reagents were added in concentrated form to solutions of membranes in which the optical activity had just been measured. Base lines were measured with equivalent concentrations of reagent.

Lysolecithin (Sigma Chemical Co., St. Louis, Mo.) shown to be pure using thin-layer chromatography, was used at a final concentration of 0.07 mg/ml. Digitonin (Merck and Co., Rahway, N.J.) was dissolved in distilled water by briefly heating an aqueous suspension of 5 mg/ml at 100°. It was used at a final concentration of 0.1 mg/ml.

Phospholipase A was obtained from the venom of *Naja Naja* (Sigma Chemical Co., St. Louis, Mo.). The enzyme was further purified[16] by boiling a solution of 2 mg/ml in 0.01 M citrate (pH 5.5) for 5–10 min. Denatured protein was centrifuged at 50000 rev./min for 30 min in a Spinco SW-50 rotor, and the supernatant was adjusted to pH 7.4 with 50 mM Tris–HCl buffer. The resulting protein concentration was approx. 1 mg/ml. 10 μg/ml of enzyme were incubated per 100 μg/ml of membrane for 1 h at 25° (pH 8.2). No Ca^{2+} was added, as the Ca^{2+} associated with the membranes was adequate for enzyme action. The enzyme was inactive in the presence of $5 \cdot 10^{-5}$ M EDTA. Under the conditions of incubation, essentially all membrane phosphatidyl ethanolamine and phosphatidyl choline were converted to the lyso-form, but the lysophosphatides, sphingomyelin, fatty acids and cholesterol remained bound to the membrane protein which could be sedimented after centrifuging at 50000 rev./min for 30 min in a Spinco SW-50 rotor. However, with phospholipase A, (Na^+-K^+)-dependent ATPase was abolished, and Mg^{2+}-dependent ATPase activity was reduced by 66%[17].

Phospholipase C (partially purified, Mann Research Lab., N.Y.) was found to exhibit no peptidase activity under the conditions employed here (no increase in α-NH_2). 7 μg/ml of the enzyme per 100 μg/ml membrane protein were incubated for 1 h at 25° in 1 mM Tris–HCl (pH 8.2) containing 1 mM $CaCl_2$; the enzyme was inactive without added Ca^{2+}. Under these conditions, 40–50% of the lipid phosphorus was found to be released, but all the protein could be sedimented by centrifuging at 50000 rev./min for 30 min; the diglyceride and ceramide resulting from the enzyme

action, as well as cholesterol and uncleaved phosphatide, remained associated with the membrane protein. With phospholipase C, the ATPase activity is only partially destroyed; 85 % of the Mg^{2+}-dependent and 54 % of the (Na^+- K^+)-dependent ATPase remains[17].

Analysis of spectra

We have attempted to estimate the conformational content of membrane proteins mathematically by fitting their CD and ORD spectra using the Gauss least-squares method[8]. An IBM 7094 computer and a Calcomp plotter were employed, as in our previous study of globular proteins[8]. However, since the helical content of membrane proteins is not known (in contrast to the globular proteins studied in which the content is known from X-ray crystallography), a number of solutions could be obtained by varying the rotational strengths of the transitions. Many of these solutions could be eliminated by comparison of the Kronig-Kramers' transformation of the CD fit to the experimental ORD spectrum. Thus the solutions we will discuss represent the "best fit" for the CD and ORD spectra taken together.

RESULTS

Native plasma membrane

Fig. 1 shows a representative CD spectrum of native plasma membrane fragments with that of a 25 % helical poly-L-glutamic acid. The negative band at 223.2 $\pm$ 0.2 mμ (average deviation) with $[\theta] = -7619 \pm 776$ arises from the n-π^- tran-

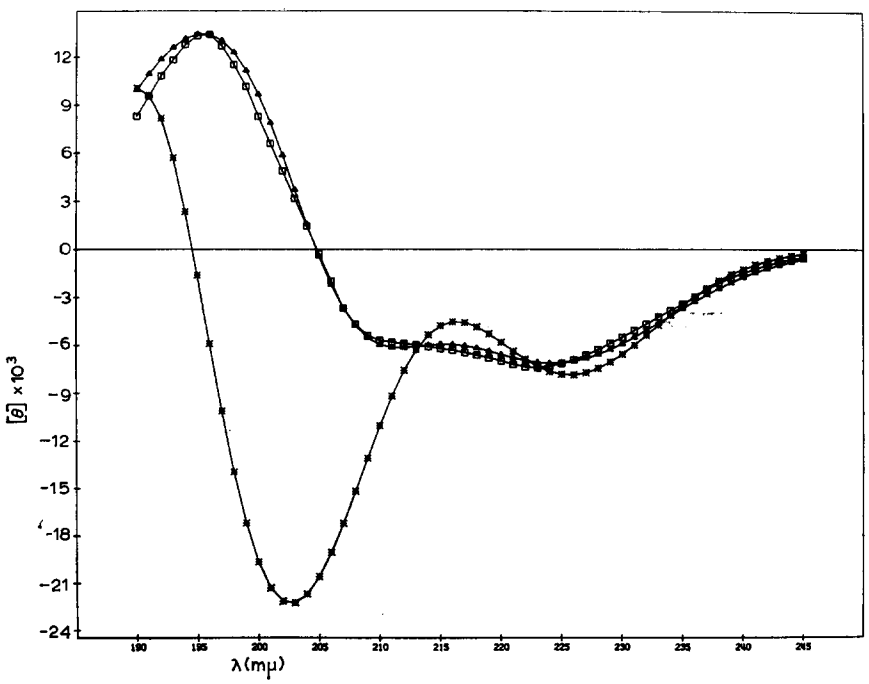


Fig. 1. Observed (□—□) and calculated (▲—▲) CD spectra of plasma membrane fragments along with that of 25 % helical poly-L-glutamic acid (✱—✱).

sition of the helix conformation. The shoulder at 212 mμ represents the parallel π°–π^{-} transition of the helical peptide with a small contribution from the π°–π^{-} band of the "unordered" peptide. The crossover to positive ellipticity at 204.4 ± 0.3 mμ is far to the red of that of poly-L-glutamic acid as is the positive band at 194.9 ± 0.6 mμ ($[\theta] = +12970 \pm 985$) the resultant of the perpendicularly polarized component of the helical π°–π^{-} transition and to the π°–π^{-} transition of the "unordered" conformation. The error estimates presented are representative of both the CD and ORD spectra for poly-L-glutamic acid and erythrocyte ghosts and indicate both the experimental error and variability in the sample preparation.

The width of the 223 mμ band is measured to be 13.6 ± 0.2 mμ, *i.e.*, considerably larger than that of helical poly-L-glutamic acid (10.8 mμ). However, it is difficult to determine this bandwidth accurately from CD spectra, since the wavelength at which the bandwidth is measured, *i.e.* where θ equals $1/e$ of θ_{max}, is in a region of low ellipticity on one side and is obscured by other transitions on the other. It is therefore best to calculate this parameter from the ORD trough position by using the Kronig–Kramers' transformation.

Fig. 1 also compares the experimentally obtained CD spectrum of plasma membrane fragments with a solution computed using the Gauss least-squares method; Table I summarizes the CD parameters used to generate this curve. Mathematically there is no single "best fit" solution, but most solutions can be eliminated because they involve unreasonably low rotational intensities of the "unordered" π°–π^{-} tran-

TABLE I

PARAMETERS FOR BEST FIT OF PLASMA MEMBRANE

Standard deviation between experimental curve and curve computed from parameters is 1.93%.

Parameter	*Transition**		
	I	*II*	*III*
Bandwidth (mμ)			
Helix	11.2	7.3	13.8
"Unordered"	10.0	10.3	8.5
Wavelength of transition (mμ)			
Helix	197.5	206.9	222.7
"Unordered"	198.0	217.0	235.0
Maximum ellipticity (degrees·cm²·decimole⁻¹)			
Helix	34 119.3	−16 234.5	−13 913.0
"Unordered"	−12 000.0	3 240.0	−219.0
Rotational strength (ergs·cm³ × 10⁴⁰)			
Helix	23.8	−7.1	−10.6
"Unordered"	−7.5	1.9	− 0.1
Conformation (%)			
Helix	58.0	58.0	58.0
"Unordered"	42.0	42.0	42.0

* For α-helix, I, II and III refer to the n–π^{-}, $\| \pi^{0}$–π^{-}, and $\perp \pi^{0}$–π^{-} transitions, respectively. For "unordered" conformations, the numbers refer to the three bands specified in CARVER *et al.*[39].

sition or of the long wavelength helical π°–π^{-} transition. In the solution presented here the rotational strengths of the various peptide transitions are in the same relative proportions found in poly-L-glutamic acid, myoglobin, hemoglobin and lysozyme[8], but the absolute values are 60% less than those of poly-L-glutamic acid or 15% less than those computed for lysozyme[8].

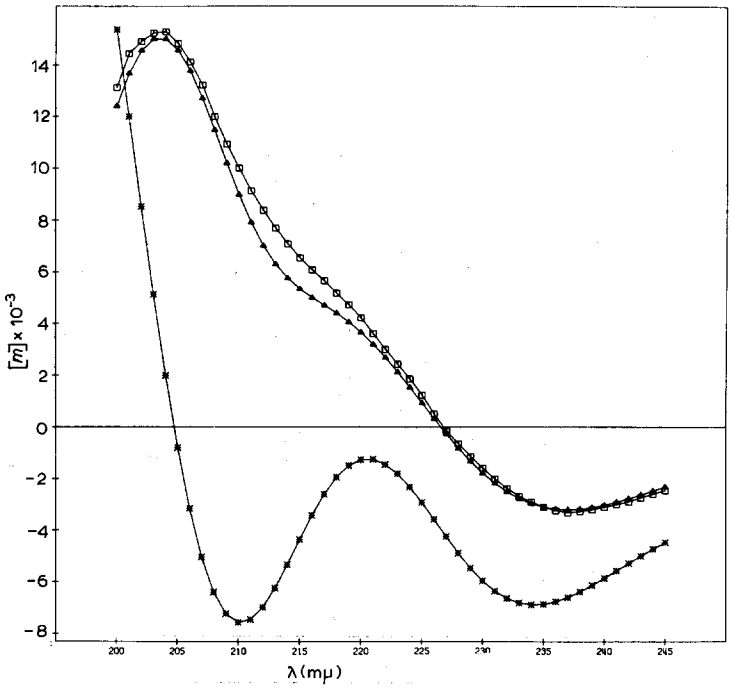


Fig. 2. Observed (□—□) and calculated (▲—▲) ORD spectra of plasma membrane fragments along with that of 25% helical poly-L-glutamic acid (*—*).

In Fig. 2 the Kronig-Kramers' transformation of the computed CD spectrum of plasma membrane fragments (Fig. 2) is compared with the experimentally obtained ORD spectrum which has the following mean residue rotations: $[m]_{236\ m\mu} = -3300$; $[m]_{203\ m\mu} = 14280$. The crossover is at 226.5 mμ, the trough at 236 mμ and the peak at 203 mμ. The most notable feature of the ORD spectrum is the location of the trough at 236 mμ rather than at 233 mμ, as is typical of other proteins.

Erythrocyte ghosts

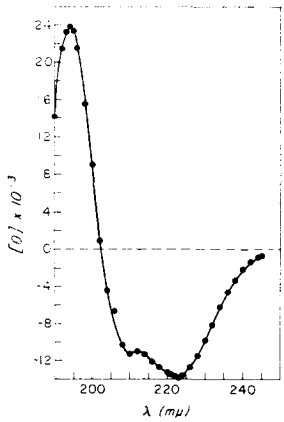
The CD spectrum of erythrocyte ghosts (Fig. 3) resembles that of plasma membrane fragments in that the n–π^{-} bandwidth is large and the positive π°–π^{-} band is near 195 mμ. However, the CD crossover in the ghost spectrum is at 202.5 mμ, similar to hemoglobin[8] but at distinctly shorter wavelengths than that of plasma membrane fragments. The ellipticity ($\theta_{223\ m\mu} = -13800$) is greater than that of plasma membrane fragments. The corresponding ORD spectrum is shown in Fig. 4.

Modified membranes

Effects of lysolecithin, digitonin and phospholipase A. The effects of lysolecithin on the CD and ORD spectra of plasma membrane fragments (Figs. 5 and 6) are as

follows: (a) decrease to 10.8 mμ in the width of the n–π^- band accounting for the shift of the ORD trough to 233 mμ; (b) increased ellipticity at 212 mμ; (c) shift of the CD crossover to 201 mμ and the ORD crossover to 224 mμ; and (d) shift of the short wavelength π°–π^- band to 193 mμ and the ORD peak to 198 mμ.

The effects of phospholipase A are quite similar (Figs. 5 and 6), as are those of digitonin. The effects of all these lipid-perturbing agents on erythrocyte ghosts resemble those on plasma membrane fragments.



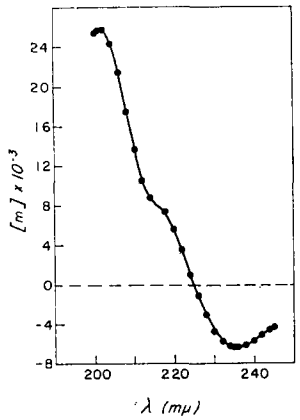


Fig. 3. Observed CD spectrum (●—●) of erythrocyte ghosts.

Fig. 4. Observed ORD spectrum (●—●) of erythrocyte ghosts.

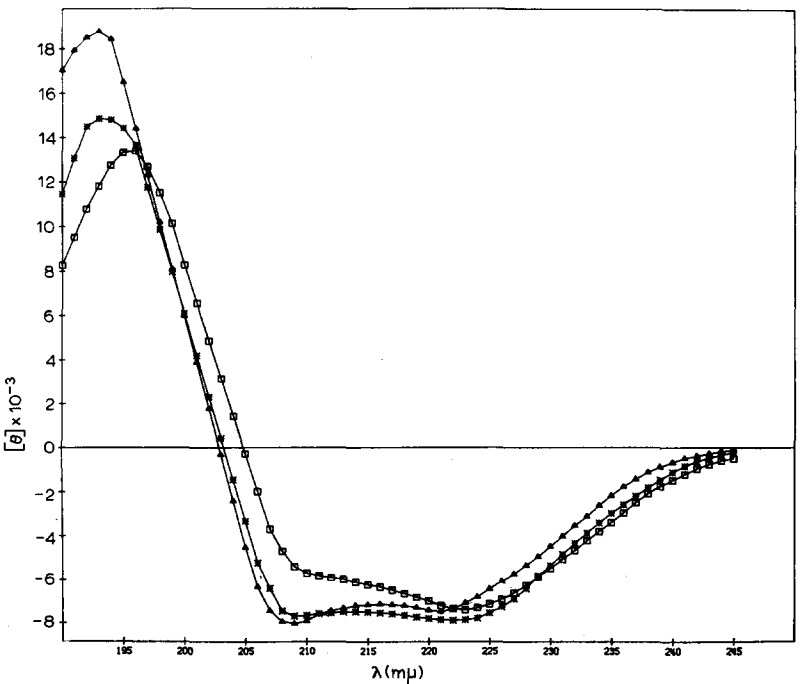


Fig. 5. Observed CD spectra of plasma membrane fragments (□—□), plasma membrane fragments *plus* lysolecithin (▲—▲), and plasma membrane fragments *plus* phospholipase A (✱—✱).

The effect of phospholipase C. Fig. 7 shows the effect on the CD spectrum of plasma membrane fragments of incubation with phospholipase C. The distinguishing features of this spectrum are: (a) diminished amplitude of the minimum at 223.2 mμ; (b) a marked decrease in the shoulder at 210 mμ; (c) a red shift in the crossover to 205 mμ; and (d) a red shift in the peak to 196 mμ. The corresponding ORD spec-

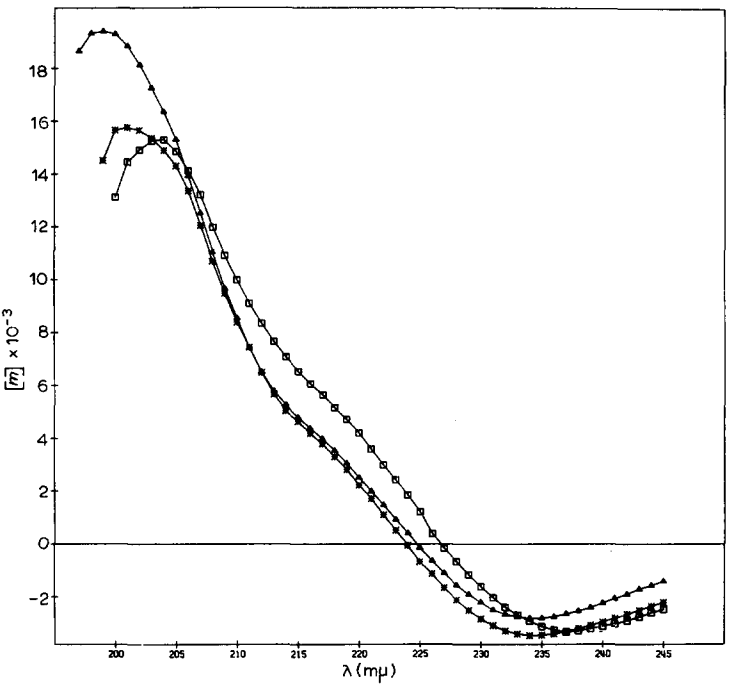


Fig. 6. Observed ORD spectra of plasma membrane fragments (□—□), plasma membrane fragments *plus* lysolecithin (▲—▲), and plasma membrane fragments *plus* phospholipase A (✱—✱).

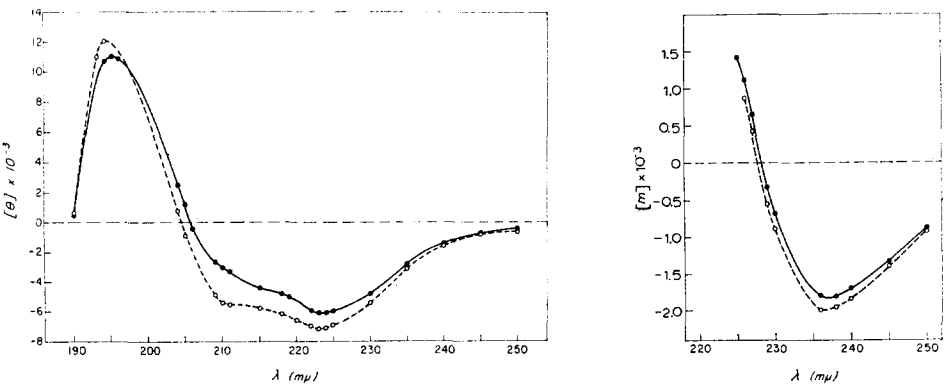


Fig. 7. Observed CD spectra of plasma membrane fragments (O---O) and plasma membrane fragments *plus* phospholipase C (●—●). (Solutions contain 1 mM Ca^{2+}.)

Fig. 8. Observed ORD spectra of plasma membrane fragments (O---O) and plasma membrane fragments *plus* phospholipase C (●—●). (Solutions contain 1 mM Ca^{2+}.)

trum (Fig. 8) shows a decrease in and a red shift to 237 mμ of the trough (due to an increase in the n–π^- bandwidth to >14 mμ), and a red shift of the crossover to 228 mμ. The action of phospholipase C on erythrocyte ghosts also displaces the ORD trough and the crossover 1 mμ to the red.

DISCUSSION

Light-scattering effects

Several authors have concerned themselves with the effects of the particulate nature of membranes upon their optical activity. Indeed URRY AND JI[18] have suggested that all of the unusual features of membrane optical activity might arise from two turbidity artifacts, namely "absorption flattening"[19] and "dispersion distortion", due to anomalous light scattering in regions of light absorption. They use as a theoretical model a suspension of solid 0.3 μ polypeptide spheres and contend that in this system the above artifacts could account for the low amplitude of membrane optical activity and the location of the CD minimum of mitochondria at >223 mμ under some circumstances. However, the light-scattering properties of this model differ from those of plasma membranes which are water-filled lipoprotein shells (about 100 Å thick)[20,21]; in such systems scattering and absorption distortions are small and cannot explain the observed amplitudes and the displacement of the CD crossovers and maxima to the red. The location of the CD minimum of mitochondria is most reasonably attributed to the presence of β-conformations[22–24]. Finally, present and other control studies[3,24,25] show that turbidity artifacts cannot adequately explain the optical activity of cellular membranes.

Interpretation of the spectra

Between 190 mμ and 250 mμ the optical activity of proteins arises primarily from the various peptide chromophores in several conformations. Since infrared spectroscopy has shown that the plasma membranes of erythrocytes and of Ehrlich ascites carcinoma contain negligible proportions of β-conformation under the conditions used[11,26], their spectra can be considered to represent mixtures of "unordered" and helical conformations*. However, these spectra differ from those of synthetic polypeptides in the positions of the helical π°–π^- bands, in bandwidths and in band intensities.

Band positions. We find the principal negative CD band of plasma membrane fragments and of erythrocyte ghosts at 223.2 ± 0.2 mμ, as is the case for predominantly helical proteins and polypeptides**. The red shifts seen in the ORD troughs of these membranes thus cannot be attributed to displaced helical n–π^- transitions. However, the helical π°–π^- bands are clearly shifted to wavelengths higher than those found in synthetic polypeptides. Red shifts of helical π°–π^- bands also occur in globular proteins and have been attributed to the highly polarizable apolar environment of many of the peptide chromophores in these substances[8].

None of the membrane modifiers displace the 223 mμ band appreciably (see also ref. 28), although they do influence the ORD trough.

* Certain cellular membranes, *e.g.* "inner" membranes of rat liver mitochondria, contain considerable amounts of peptide in β-conformation, judging by infrared spectroscopy. This severely complicates the analysis of optical activity data[22].

** Unlike the membranes under discussion, mitochondrial membranes have a CD minimum at 224–226 mμ.

Bandwidths. The fundamentals of optical activity[29] indicate that the ORD trough of the 223 mμ CD band will occur approximately at the wavelength where the ellipticity equals 1/*e* of the maximal ellipticity, *i.e.* the trough position depends on the bandwidth. In helical polypeptides and in highly helical proteins, including high density serum lipoproteins, the ORD troughs of the 223 mμ CD bands lie at 233 mμ. However, in the membranes under study, the ORD troughs lie at 236–237 mμ, signaling an unusual width of the 223 mμ band in these structures. We attribute this to lipid–protein and/or protein–protein interactions which are sensitive to lipid because phospholipase A, lysolecithin and digitonin all shift the ORD trough to 233 mμ (band narrowing), while phospholipase C shifts the ORD trough further to the red (band broadening).

Rotational strength and helical content. From considerations of shape and magnitude of the CD spectra, the rotational strengths of the peptide transitions of membrane proteins appear much lower than those of synthetic polypeptides. As in the case of globular proteins[8], we attribute this either to the possible presence of short helical segments or helix other than α-helix, or to possible effects of the local environment on peptide optical activity or to a combination of these factors.

Because of the probable operation of one or more of these mechanisms, neither the helical content nor the rotational strengths of the peptide transitions can be specified[8]. Within the observed limits of band positions and bandwidths a number of mathematically generated spectra fit the observed ones very well. We illustrate one solution in Figs. 2 and 3 and in Table I in which the rotational strengths of the various peptide transitions are in the same relative ratios as in poly-L-glutamic acid but are reduced by 60%. This gives a "helix content" of 58%. Other ratios of rotational strengths also yield well-fitting curves but with "helix contents" ranging from 35 to 80%. The above ambiguities are similar to, but more extreme than those encountered in soluble globular proteins and present a serious obstacle for the estimation of the conformational content using CD and ORD analyses.

Nonpeptide chromophores

It has been suggested that the unusual CD and ORD features of membranes might arise from the optical activity of ester linkages in membrane phosphatides. This is unlikely since membrane lipids in aqueous dispersion or inorganic solvents at concentrations near to or greater than those in membrane suspensions do not exhibit significant optical activity in the peptide region[2] (D. F. H. WALLACH AND A. S. GORDON, unpublished observations), and since the membranes of halophile bacteria which lack ester phosphatides exhibit the same ORD anomalies as do other membranes[31]. In erythrocyte ghosts, one might expect an optical activity due to carbohydrate which accounts for 7.5% of the ghost weight. Evaluation of the data of LLOYD *et al.*[33] suggests that this might be the reason why the CD crossover of erythrocyte ghosts lies at a shorter wavelength than that of Ehrlich ascites carcinoma plasma membrane fragments.

LENARD AND SINGER[2] have suggested that the red displacement of the ORD trough arises when α-helices are packed parallel but at a slight twist[34]. This concept has also been invoked to explain the small red shift which accompanies aggregation of α-helical poly-L-glutamic acid[35] and the large red displacement of the ORD spectra of aggregated "mitochondrial structural protein"[36]. However, the infrared spectra of

mitochondrial structural protein preparations indicate the presence of considerable β-structure, and their CD spectra can be explained as reflecting this conformation[22]. Mitochondrial structural protein is thus not an adequate model for membranes in general, whatever its relevance to mitochondria.

Lipid perturbants

The data on lipid perturbants indicate that the properties of the membrane proteins are affected by the presence of lipid. There are three possible modes of interaction: (1) the hydrocarbon portions of membrane lipids create an environment of high polarizability and of low polarity for portions of the membrane protein; recent studies of membranes by nuclear magnetic resonance and infrared spectroscopy[37,38] are consonant with this view; (2) the quaternary structure of membrane proteins depends on the membrane lipids; and (3) the secondary structure of membrane proteins is dependent on the membrane lipids. Present data do not differentiate between these modes.

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