*PROTEIN CONFORMATIONS IN CELLULAR MEMBRANES**

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The conformations of the proteins of cellular membranes and the interrelationships between membrane lipids and proteins are not well understood but are potentially accessible to at least partial analysis by spectroscopic techniques. Moreover, the recent development of new methods of membrane fractionation[1-4] allows isolation of the plasma membranes of complex mammalian cells in a form suitable for examination by infrared spectroscopy, fluorescence spectroscopy, and ultraviolet optical rotatory dispersion. Therefore, we have applied these methods to the problem of membrane structure and herein report results obtained with plasma membranes of Ehrlich ascites carcinoma.

Experimental.—Materials: Plasma membrane vesicles (PM), about 1500 Å in diameter,[5] were prepared from Ehrlich ascites carcinoma microsomes as previously described.[2, 3] To eliminate the polysucrose used in their preparation, the membranes were washed once with 0.01 *M* $CaCl_2$, 0.02 *M* Tris-HCl (pH 8.2), and once with 0.01 *M* Tris HCl (pH 8.2). Centrifugations were at 50,000 rpm for 30 min at 4°C in a Spinco SW50 rotor. The pellets of the second wash were then dispersed uniformly in appropriate solvents for infrared spectroscopy, fluorescence spectroscopy, and measurement of optical rotation. Protein determinations on the final membrane dispersions or solutions were by both the ninhydrin procedure[6] and the method of Lowry *et al.*,[7] using crystalline bovine serum albumin as reference standard. The ninhydrin procedure gave "protein" values which were 10% higher than that obtained by the Lowry method. One mg of membrane "protein" corresponds to about 1.5 mg dry weight, most of the nonprotein mass consisting of phosphatides and cholesterol. About 1.5% of the dry mass is carbohydrate and 1–2% is contributed by tightly bound RNA. It should be noted that cellular membranes obtained in isoosmotic media (e.g., 0.25 *M* sucrose) ordinarily contain considerable quantities of trapped, soluble proteins,[8] but in the isolation procedure here employed, these contaminants were removed by "osmotic shock."[2-4] The reported optical measurements are thus due to components integral with the membrane structure.

2-Chloroethanol (Eastman White Label) was completely transparent to 260 mμ after fractional redistillation. The apparent pH of 9:1 2-chloroethanol:water was about 1.1. Lysolecithin (Sigma Chemical Co.) was used at a concentration of 0.13 mg/ml which reduced the turbidity of PM suspensions by 50%.

Infrared spectra: A Perkin-Elmer spectrophotometer model 521 was employed. Solid films were prepared by applying about 0.5 mg membrane protein (in aqueous suspension, or chloroethanol or formic acid solution) as a 0.5 × 2-cm band in the center of the silver chloride plate and drying in air at about 25°C. Once dried, the films were strongly adherent to the plate. Quantitative extraction of lipids from the films was achieved by immersing the plates in 2:1 chloroform:methanol (v:v) for 20 min at room temperature. After rinsing with the same solvent, the films were dried in air. To acidify the films, the dried plates were immersed for 20 min in 0.001–0.1 *N* HCl at room temperature, rinsed with distilled water, and air-dried. In order to have the films located reproducibly in the optical path, all manipulations of the films were performed with the plates in their plate-holders.

Fluorescence spectra: A Turner spectrofluorometer model 210 was employed. Fluorescence emission spectra were obtained for excitation at both 270 and 295 mμ. Excitation spectra were obtained measuring emission at 335 mμ and were corrected for the variation of excitation energy with wavelength at 250 mμ and above. The bandwidths for excitation and emission were 15 and 10 mμ, respectively. Spectra were obtained at room temperature.

Optical rotatory dispersion (ORD): Measurements of ORD were on a Cary model 60 spectropolarimeter at room temperature and at an absorbance of <2, using cells with 0.1–10-mm path

length. Base lines were determined using the same cells (with appropriate solvents) immediately after the measurements on the membrane samples. The measured and corrected rotations are presented as specific rotations ([α]) calculated as the rotation in degrees, at wavelength $\lambda m\mu$, for a path length of 1 dm and a concentration of 1 gm of membrane protein per 100 ml. The rotational strength of PM suspensions is rather low relative to their absorbance, in part due to light scattering (the turbidity varies as $\lambda^{-2.38}$).[5] For this reason ORD curves below 240 mμ were obtained at minimal scanning speeds and with a 10-sec pen period. To check quantitative instrumental performance with scattering samples, the ORD curves of PM suspensions and solutions of α-helical poly-L-glutamic acid were measured separately and with the cells containing the two materials in tandem. The rotations of the two samples were found to be strictly additive. (Also see ref. 9 concerning relative insensitivity of ORD to a scattering background.)

Results and Discussion.—(a) *Infrared spectra:* Figure 1 shows the amide I and amide II region of the infrared spectrum of a PM film deposited from aqueous suspension. The amide I band (C=O stretching) is located at 1652 cm^{-1}, the region associated with the α-helical and/or random coil conformation of peptide chains.[10] There is no distinguishable band at 1630 cm^{-1}, the frequency diagnostic of the β-conformation.[10] The amide II band at about 1535 cm^{-1} does not allow distinction between the α and β-conformations. Lipid extraction of the film abolishes the band at 1740 cm^{-1} due to C=O stretching in fatty acid esters, and understandably diminishes the absorption at 1468 cm^{-1} due to CH_2, CH_3 bending. There is also some reduction of the two amide bands, due in part to the extraction of sphingomyelin (amide I—1655 cm^{-1}; amide II—1550 cm^{-1}), which accounts for about 25 per cent of the membrane phosphatide. On the other hand, lipid extraction does not produce detectable transition to β-conformation.

Exposure of lipid-extracted films to pH < 2 (Fig. 2) leads to the appearance of a

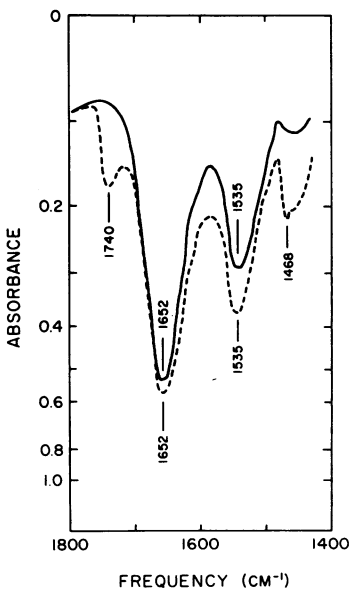

FIG. 1.—Infrared spectrum of a plasma membrane film cast from aqueous suspension: — — —, original film; ————, after extraction with 2:1 chloroform:methanol.

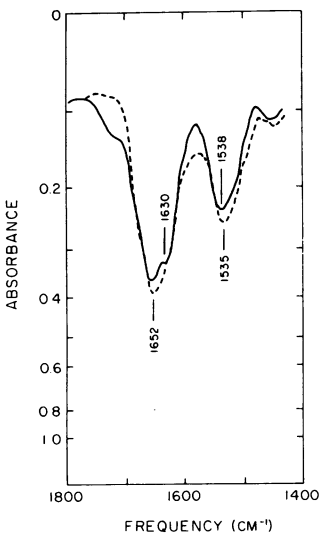

FIG. 2.—Infrared spectrum of a plasma membrane film cast from aqueous suspension: — — —, after extraction with 2:1 chloroform:methanol; ————, same after exposure to 0.1 *M* HCl for 20 min.

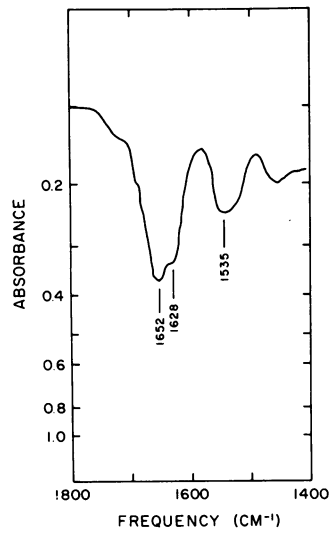

FIG. 3.—Infrared spectrum of plasma membrane cast from solution in 9:1 2-chloroethanol: water.

band at 1630 cm^{-1} and a shoulder at 1690 cm^{-1}, both characteristic of β-conformation.[10] These changes are not reversed by exposure of the films to higher pH. In addition, exposure to pH < 3 produces a shoulder at 1735 cm^{-1}, due to C=O stretching in un-ionized carboxy groups, and decreased absorbance near 1600 cm^{-1}, the region of ionized carboxyl groups. The changes at 1735 cm^{-1} and 1600 cm^{-1} are reversed by washing the film with buffers of pH > 3. Films cast from solutions of PM in 9:1 2-chloroethanol: water (pH < 2) show the same changes at 1690 cm^{-1} and 1630 cm^{-1} (Fig. 3). This is of special interest since ORD measurements in this solvent indicate a large increment in α-helix. Films cast from formic acid solution indicate greater transition to β-conformation than obtained at acid pH (± 2-chloroethanol).

Polarized infrared spectra of oriented films of PM vesicles were identical whether the electrical vibration was perpendicular or parallel to the direction of orientation. This was so also after lipid extraction. It was not possible, therefore, to draw conclusions as to the relative contributions of α and random conformations to the amide I and amide II absorptions. Presumably, orientation of the membrane fragments does not lead to preferential unidirectional alignment of the membrane proteins.

Our data and those on erythrocyte ghosts[11, 12] give no positive evidence for extensive β-structure in cellular membranes. However, studies on model mixtures of α- and β-conformations[13] indicate that small proportions of the latter are not easily detected by IR spectroscopy.

(b) *Fluorescence spectra:* The salient features of the fluorescence spectra of PM are summarized in Table 1. Identical excitation and emission spectra were obtained in 0.02 *M* Tris-HCl (pH 8.2), 0.02 *M* Tris-HCl (pH 8.2) + lysolecithin (130 μg/ml), and 0.1 *M* HCl. The excitation spectra in these solvents were not distinctive from those of many other proteins, and emission spectra were identical whether excitation was at 270 or 295 mμ, indicating that fluorescence emission under these conditions is primarily from tryptophan. The location of the emission maximum at 335 mμ (compare 350 mμ in free Trp) bespeaks a nonpolar environment for the Trp fluorophore.[14] This is not intimately linked to the lipid organization of the membrane, since the emission spectrum is not altered by the action of lysolecithin. However, in 9:1 2-chloroethanol: water, there is a shift of Trp emission to longer wavelength and emergence of a shoulder at 310 mμ due to Ty fluorescence. These changes indicate that the conformations in 2-chloroethanol expose the Trp residues to a more polar environment, and alter the transfer of energy from Ty to Trp.

(c) *Optical rotatory dispersion:* Figure 4 represents the specific rotation [α] as a function of wavelength for PM vesicles in aqueous suspension (pH 8.2). The optical rotation of α-helical poly-L-glutamic acid (pH 4.3)—reduced by a factor of

TABLE 1

FLUORESCENCE EXCITATION AND EMISSION MAXIMA IN VARIOUS SOLVENTS

Solvent	λ_{max} (mμ) Excitation	Emission
0.02 *M* Tris-HCl (pH 8.2)	277–278 277*	335
0.02 *M* Tris-HCl (pH 8.2) + lysolecithin (130 μg/ml)	277–278 277*	335
0.1 *N* HCl	277–278 277*	335
2-Chloroethanol:water (9:1, v:v)	272–277† 277*	335–342‡ 310 (shoulder)

* Not corrected.
† Position of the excitation maximum in this solvent was variable from one preparation to the next.
‡ Maximum fluorescence in 2-chloroethanol:water is about ²/₃ that found in aqueous suspension. Maximum fluorescence with excitation at 270 mμ is about 3 times that obtained when exciting at 295 mμ. The shoulder at 310 mμ is not seen in native plasma membrane or in 2-chloroethanol:water when excitation is at 295 mμ. In the latter solvent the emission maximum is at 335–340 when excited at 270 mμ and at 339–342 when excited at 295 mμ.

4.5—is included for purpose of comparison. The ORD spectrum of PM shows two small inflections between 300 and 250 mμ, one centered near 290 mμ and one at about 255 mμ. Maximal negative rotation is at 236–237 mμ ([α] = −2,640 ± 140°), and zero rotation is at 226 ± 1 mμ. The positive limb of the dispersion curve has an inflection between 220 and 210 mμ, and its maximum is at 202–200 mμ ([α] = 12,900 ± 1,200°).

In the presence of lysolecithin, the irregularities between 300 and 250 mμ diminish;

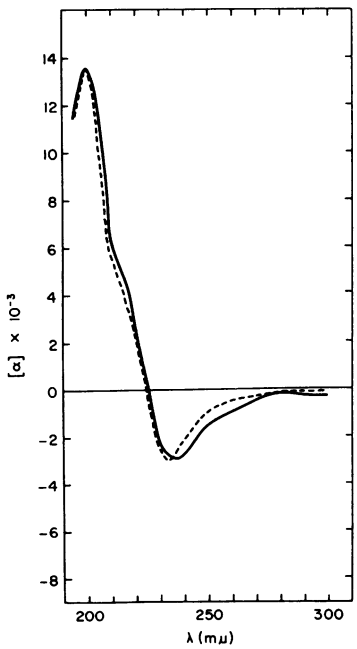

FIG. 4.—Optical rotatory dispersion of plasma membrane in aqueous suspension: ————, plasma membrane; — — —, poly-L-glutamic acid (pH 4.25). The values of poly-L-glutamic acid have been reduced by a factor of 4.5.

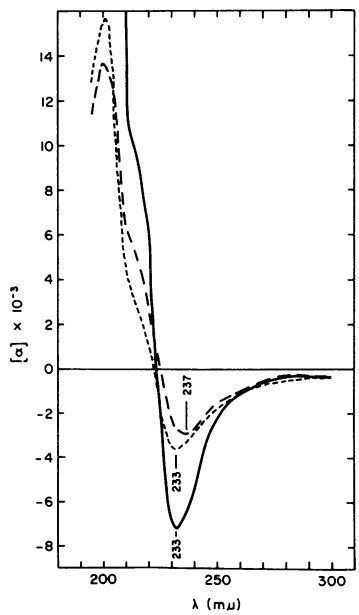

FIG. 5.—Optical rotatory dispersion of plasma membrane: - - -, in aqueous suspension; — — —, in an aqueous solution of lysolecithin (130 μg/ml); ————, in 9:1 2-chloroethanol:water.

the ORD trough shifts to 233 mμ and increases in amplitude ([α] = −3750 + 310°) (Fig. 5). The crossover point is also displaced to shorter wavelength (223 mμ). The positive extremum remains at 200–202 mμ, but is larger than before ([α] = 16,900 ± 1,100°).

In 9:1 2-chloroethanol:water, the rotation in the region of the peptide transitions is markedly augmented (Fig. 5). The ORD curve is devoid of irregularities between 300 and 250 mμ. Maximum negative rotation occurs at 233 mμ ([α] = −7200 ± 150°) and the crossover point lies at 224 mμ. In the positive limb of the curve, there is an inflection between 220 and 215 mμ. Because of the high absorbance of PM solutions in 2-chloroethanol:water, relative to their rotational strength, it was not possible to obtain precise values for the rotation below 210 mμ. However, maximum positive rotation occurs at about 202 mμ.

A major feature of the ORD spectrum of PM in aqueous suspension is the small rotational amplitude throughout the 195–320-mμ region. Similar findings in the cases of erythrocyte ghosts[11] and chloroplast lamellae[15, 16] have been interpreted to indicate very low helix contents on the assumption that the only conformations present are α-helix and random coil. But the shape of the PM ORD patterns are not those of helix-coil combination,[17] and one must therefore attribute the low rotational amplitude to the presence of other conformational combinations with canceling rotations. Most likely we are dealing with a mixture of α-helix, random coil, and small amounts of β-conformation (e.g., 35, 50, and 15%, respectively). This could account for both shape and low rotation,[18] but does not explain the location of the trough at 237 mμ. In view of our IR data, the ORD spectrum in 90 per cent 2-chloroethanol probably represents a mixture of α- and β-conformations.

The second distinctive aspect of the ORD spectrum of native PM is its irregularity in the 250–300-mμ region. The most likely origins of these inflections are aromatic side-chain chromophores held in an asymmetric array.[19–22] Several proteins exhibit such optical activity, although the responsible chromophores are present in low molar proportions.[23, 24] A case in point is carbonic anhydrase (5–6 moles % of tyrosine + tryptophan and 3–4 moles % phenylalanine), which has striking aromatic Cotton effects.[23] Upon denaturation these disappear and the rotation between 300 and 250 mμ becomes more negative. A similar situation exists in the case of egg-white lysozyme.[24] The proportions of Trp, Ty, and Phe in PM, which are about 1.5, 3.5, and 5 moles per cent, respectively,[25] would thus appear to be adequate to produce Cotton effects in the 250–300-mμ region. The reduction of these effects by lysolecithin suggests a more intimate interaction between membrane proteins and lipids than envisaged in current membrane models.[26] There are other possible sources of anomalous dispersion in the 250–300-mμ region, i.e., disulfide bonds,[27] RNA,[28] and amino-sugars,[29] but these are not present in sufficient amounts in PM to be detectable by present means. However, amino-sugars may contribute significantly to the ORD spectra of erythrocyte ghosts.

The third distinctive feature of the ORD spectra of native PM is the location of the ORD trough at 237 mμ. ORD curves of other membrane fragments, such as chloroplasts[15, 16] and erythrocyte ghosts,[11, 30] also show troughs at longer wavelength than expected from the peptide transitions. This might be explained by superposition of peptide and aromatic Cotton effects, as in poly-L-tyrosine,[20, 21] but the published ORD spectra of chloroplasts[15, 16] do not show aromatic Cotton effects and

the inflections in the aromatic region of the ORD spectrum of PM appear too small to effect a shift from 233 to 237 mμ. Moreover, lysozyme has a trough which is located at 233 mμ, whether the aromatic Cotton effects are present or abolished by sodium lauryl sulfate.[24]

Here it should be noted that in cellular membranes the peptide and side-chain chromophores may reside in a quite exceptional environment. Thus, while it is generally thought that membrane lipids exist as ordered bilayers with polar surfaces and hydrocarbon cores, little is known of the protein-lipid relationships, and there is no evidence to exclude penetration of peptide segments into or through the lipid phase. If a substantial proportion of the helical sections of membrane proteins were located in such regions of high refractive index,[5, 31] one might see a red shift of optically active peptide transitions similar to that observed when aromatic chromophores are transferred to media of high polarizability, such as the interior of detergent micelles.[32]

This question also applies to helical homopolypeptides with organophilic side chains, but published ORD spectra of such polymers give no indication of shifted peptide transitions, although the side chains provide a nonpolar environment for the peptide backbone.[33, 34] However, such substances are not adequate models for the proposed situation, if only because the organophilic environment of the peptide chain is itself in regular helical array.

A plausible argument for environmentally induced frequency shifts in peptide transitions has been raised on account of the sensitivity of the ORD parameters of poly-γ-benzyl-α-L-glutamate to solvent refractive index.[35] Moreover, the 233-mμ ORD minimum is due to the 224-mμ n–π^* peptide transition,[36, 37] and such transitions generally shift to longer wavelengths with increasing refractive index.[38] The UV spectra and ORD of a cyclic amide[39] indicate the same for the n–π^* transition of peptide bonds, as do the UV-difference spectra of helical poly-L-glutamic acid.[40] Also, recent theoretical work[36, 37] suggests that the 208-mμ π–π^* peptide transition is quite sensitive to side-chain and solvent effects. Since this transition contributes significant negative rotation to above 240 mμ, changes in its position and/or rotary strength could produce apparent shifts in the position of the 224-mμ Cotton effect.

There is thus considerable reason to suspect that the anomalous position of the ORD trough of PM is due to hydrophobic interactions between membrane lipids and proteins. Such interactions are suggested more directly by the strong, extrinsic Cotton effects arising from lipophilic pigments in chloroplast membranes.[15, 16] Further support comes from the blue shift produced by lysolecithin, which alters the structure of lipid phases but does not effect full dissociation of protein from lipid. The concurrent enhancement of rotation and reduction of the 250–300-mμ Cotton effects indicate, moreover, that reorganization of membrane lipid is accompanied by changes in protein conformation. The blue shift of the ORD spectrum observed in 2-chloroethanol is also consistent with the proposed hypothesis, but this solvent produces both complete dissociation of lipid from protein[41] and major changes in protein conformation.

Conclusion.—Our work and that on erythrocyte ghosts[11, 12, 30] suggest that the proportion of β-conformation in cellular membranes is not extensive. Also, our and other[15, 16, 30] ORD studies suggest that portions of membrane protein reside

within a medium of high refractive index, adding to the increasing evidence[43-46] for extensive hydrophobic interactions between membrane proteins and lipids.

These observations do not fit prevailing membrane models,[26] which envision a primary layer of protein, extended—usually in β-conformation—at the polar surfaces of lipid bilayers (or micelles), i.e., in an arrangement preventing nonpolar protein-lipid interactions.[42] There is, moreover, other new, though indirect, evidence[47] which makes such models unsatisfactory. We therefore propose—as a working hypothesis—a new principle of membrane protein organization which is consistent with established facts of membrane structure, as well as the new data, and which is accessible to experimental test.

We suggest that there are important classes of membrane proteins—both structural and functional—whose unique amino acid sequences impose tertiary and quaternary structures in which two hydrophilic peptide regions are widely separated by a hydrophobic zone. We envision the two hydrophilic sections to lie at the membrane surfaces, connected by hydrophobic rods penetrating the membrane normal to its surface. The length of the hydrophobic units would equal the width of the apolar membrane core. We envision the hydrophobic units to consist of helical peptide segments, packed amidst the hydrocarbon residues of membrane lipids in at least two types of arrangement: (*a*) single units, with only nonpolar side chains; (*b*) aggregates (possibly reversible) in the form of microtubules which, depending on primary sequence, could have a polar interior. (We conjecture that membrane transport occurs largely via such structures, controlled by the conformational state of the protein subunits and/or by subunit aggregation.)

The hydrophilic peptide segments, located at the hydrated membrane surfaces, would be in polar interaction with other (including nonpenetrating) peptide chains, as well as with the headgroups of membrane lipids. We further suggest that the complex structure of such membrane proteins depends upon their association with appropriate lipids, that hydrophobic bonding is critical in these interactions, and that the specificities of the protein-lipid associations depend strongly upon the primary structures of the hydrophobic peptide segments. The role of membrane lipids is thus viewed to be more than maintenance of membrane permeability and plasticity, but the genetic control of membrane structure and the biochemical regulation of membrane function are considered to reside mainly in the membrane proteins.

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