Evidence of β Structure in *Mycoplasma* Membranes. Circular Dichroism, Optical Rotatory Dispersion, and Infrared Studies*

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ABSTRACT: By use of a technique (Timasheff, S. N., Susi, H., Townend, R., Stevens, L., Gorgunoff, M. J., and Kumosinski, T. F. (1967), *Conform. Biopolym., Pap Int. Symp.*, 173.) we combined circular dichroism, optical rotatory dispersion, and infrared data to give evidence for more β (pleated sheet) structure than α structure in whole membranes of *Mycoplasma laidlawii*, strain A. The circular dichroism data exhibit a typical α-helical pattern. However, when this pattern is subjected to computer analysis, using a curve-fitting program, the best fit to the membrane experimental curve is obtained with 56.0% β, 30.7% α, and only 13.2% coil. Calculation of the Moffit–Yang a_0 and b_0 parameters from optical rotatory dispersion data yields 30–45% β, 26% α, and 30–45% coil. Infrared data also indicate a higher percentage of β than α structure. Each of the standard poly-L-lysine circular dichroism curves is reduced to a set of near Gaussian curves by simple graphical technique. The computer was fed Gaussian parameters from the poly-L-lysine data and given the estimated percentages of the respective structures. The computer then calculated true Gaussian curves and totaled these to give a surprisingly good fit to the original circular dichroism data.

An additional computation was made by the method of Greenfield, N., and Fasman, G. D. [(1969), *Biochemistry 8*, 4108]. This method yielded 23% α, 20–40% coil, and 37–57% β by difference.

Circular dichroism and optical rotatory dispersion spectra of various cellular membranes have generally been interpreted to have shapes indicating substantial amounts of α helix and showing no evidence of β structure. While circular dichroism spectra may not give evidence for β structure, it would be difficult to disprove the presence of substantial amounts of beta structure on the basis of such spectra. Gratzer *et al.* (1968), Hammes and Schullery (1968), and Stevens *et al.* (1968) have shown that the circular dichroism bands and optical rotatory dispersion maxima of certain β structures can have much lower intensity and be displaced to the red of the commonly used reference poly-L-lysine in the β conformation. Thus it is possible that the circular dichroism minimum of the β structure could actually be placed at a position normally assigned to the negative ($n-\pi^-$) transition of α-helical peptides (222–225 mμ).

Wallach *et al.* (1969) and Graham and Wallach (1969) have recently used this argument to account for evidence of β structure in their infrared spectra of mitochondrial membranes in the dry-film state. They do this in spite of the fact that several authors (Urry *et al.*, 1967; Stein and Fleisher, 1967; Wrigglesworth and Packer, 1968; Urry and Ji, 1968) have assumed that β structure is lacking on the basis of optical rotatory dispersion and circular dichroism measurements.

In this paper we will show that, despite the fact that the circular dichroism and optical rotatory dispersion spectra are suggestive of α helix, the circular dichroism data are so ambiguous there may be as much as 40% β structure in the plasma membranes of *Mycoplasma laidlawii*, strain A. We conclude this on the basis of spectra of the α, β, and coil forms of poly-L-lysine without any assumptions about amplitude changes. We do, however, make wavelength adjustments to match the red-shifted circular dichroism bands of the membranes with the bluer bands of poly-L-lysine. Yet, we do not assume that β bands have been shifted any more than similarly positioned bands of α helix or random coil. Circular dichroism, optical rotatory dispersion, and infrared data are analyzed by a method published by Timasheff *et al.* (1967) to give a self-consistent analysis of membrane structure. A similar analysis on circular dichroism, optical rotatory dispersion, and infrared spectra of cytochrome *c* shows a predominance of random coil with smaller amounts of β and α structure despite the fact that the circular dichroism spectrum resembles that of a low-amplitude α helix (Urry and Doty, 1965; Ulmer, 1965). This is a good qualitative agreement with the X-ray analysis of Dickerson *et al.* (1967) at 4-Å resolution.

Materials and Methods

Materials. Practical grade 2-chloroethanol from Matheson, Coleman and Bell was purified by redistillation and had a pH of 2.7 when diluted with 9 volumes of water. Cytochrome *c* (salt-free equine heart) A grade, assay 92.9%, was obtained from Calbiochem, Los Angeles, Calif. Portions were lyophilized to constant weight and dissolved in appropriate amounts of water. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid was also obtained from Calbiochem. [U-^{14}C]-amino acid mix was obtained from New England Nuclear Corp., Boston, Mass.

Poly-L-lysine·HBr (62,000 mol wt) was dialyzed overnight against a large volume of 0.01 M HCl, again overnight against demineralized water, and then freeze-dried to constant weight. Weighed samples were dissolved in water, the pH was adjusted with 1 M NaOH, and each sample was finally diluted

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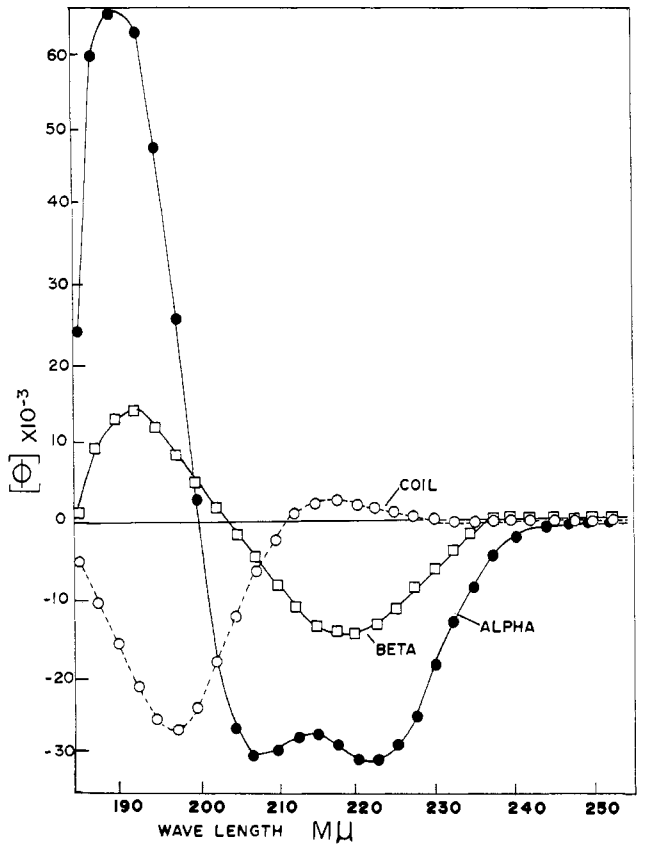

FIGURE 1: Circular dichroism spectra of the three helical forms of poly-L-lysine in distilled water.

to 2.00 mg/ml with demineralized water. The pH was checked again and small adjustment made with 1 M NaOH. Random coil was obtained by adjusting the pH to 7.0. Another sample was converted into α helix by adjusting its pH to 11.0. Finally, half of the α-helix sample was converted into β structure by heating at 50° for 10 min. Circular dichroism measurements were performed in a 0.05-mm path-length cell. These methods are similar to those of Sarkar and Doty (1966).

Membrane Preparation. Mycoplasma laidlawii, strain A, is grown in a medium (SP-YE, Pollock *et al.*, 1963) composed of 2% soy peptone (Sheffield Chemical, U.S.P. grade), 1% yeast extract (Difco), and 0.5% sodium chloride in distilled water, pH 7.8. Cultures were harvested after about 12 hr at 35° and commonly reached a density of nearly 10^{10} at harvest time.

The following operations were done at 0°. The organisms were recovered by centrifugation at 7900*g* for 20 min. They were washed twice in 0.9% sodium chloride to remove the culture medium and then lysed in 10^{-2} M EDTA, pH 7.4. The lysed organisms were spun at 40,000*g* for 30 min to bring down the membranes and then washed twice more in deionized, distilled water, apparent pH 7.5–8.0. In some experiments, nucleic acid was removed by digestion for 30 min at room temperature in a mixture containing 1 μg/ml of RNase and 1 μg/ml of DNase with 2×10^{-2} M TES[1] buffer, pH 7.5, and 10^{-3} M magnesium. The membranes were then washed twice more with water. Typically, 1 l. of culture medium yields 1.2 g of wet organisms which, in turn, yield 75 mg of membrane protein.

Optical Activity Measurements. A Cary Model 60 spectropolarimeter was used with circular dichroism attachments. Measurements were made at room temperature using 1.0-cm path-length cells (Bausch and Lomb, Rochester, N. Y.). Each sample was scanned three times and a base line taken. Pencil lines were drawn through the center of the noise on each scan and the three resulting pencil curves were averaged. During circular dichroism measurements, signal to noise ratio varied from 10:1 at 220 mμ to about 3:1 or less at 190 mμ. Membrane suspensions containing 0.025–0.030 mg of protein per ml were used and these exhibited no visible turbidity. For the experiment in which circular dichroism and optical rotatory dispersion measurements were compared, both types of measurements were performed on the same sample and the protein concentration was obtained by acid hydrolysis followed by amino acid analysis on a Telicon-TSM amino acid analyzer. The number of mmoles of amino acids were summed and multiplied by the mean residue weight of 121 to yield the protein concentration. Another portion of the sample was dissolved in 9 volumes of 2-chloroethanol, and, by comparison with the amino acid analysis the coefficient for DNase–RNase digested membranes in 90% 2-chloroethanol was found to be 0.58 mg/ml of protein per optical density unit at 280 mμ in a 1-cm path cell. This value was used for subsequent preparations.

Amino Acid Analysis. Amino acid analysis was performed on delipidized membrane protein. Each sample was suspended in 6 N HCl, flushed with nitrogen, and sealed. Digestion was for 22, 36, and 48 hr, respectively, at 110°. Analysis was performed on a Beckman, Spinco 120C, amino acid analyzer. Tryptophan was assayed by the method of Spies and Chambers (1949) and also by the 294:280 mμ ratio method of Beaven and Holliday (1952). Good agreement was obtained between the two methods. Cystine was determined as cysteic acid after performate oxidation (Moore, 1963).

Infrared Measurements. All infrared measurements were carried out with a Perkin-Elmer 421 spectrophotometer. Instrumental accuracy and precision were better than ± 0.5 cm^{-1} based on the 1601.8-cm^{-1} peak of a standard polystyrene film. Matched Perkin-Elmer Model 127-1690 cells, with 0.1-mm path length and CaF_2 windows, were used for these measurements in D_2O solution. Whale myoglobin and cytochrome *c* were deuterated by dissolving in 0.9% NaCl in D_2O and then lyophilized to dryness. Enough D_2O was then added to bring the final concentration of protein to 10 mg/ml. Membranes were deuterated by spinning at 40,000*g* for 30 min and resuspending three times in D_2O. Enough dry NaCl was finally added to bring the concentration to 0.9%.

Mathematical Methods. It was conjectured that the circular dichroism spectrum of a protein of unknown structure could be represented (approximately) as the sum of contributions from parts of the molecule having α, β, and coil conformations. We wrote a curve-fitting computer code that determined the optimum fit (in the least-squares sense) to the circular dichroism spectrum of a protein by a linear combination of the α, β, and coil spectra of poly-L-lysine. The coefficients of the terms in the linear combination were taken to be estimates of the proportions of α, β, and coil structure in the protein. The code used the ellipticities of the three poly-L-lysine forms and the experimental protein at 2.5-mμ intervals as input

[1] Abbreviation used is: TES = *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

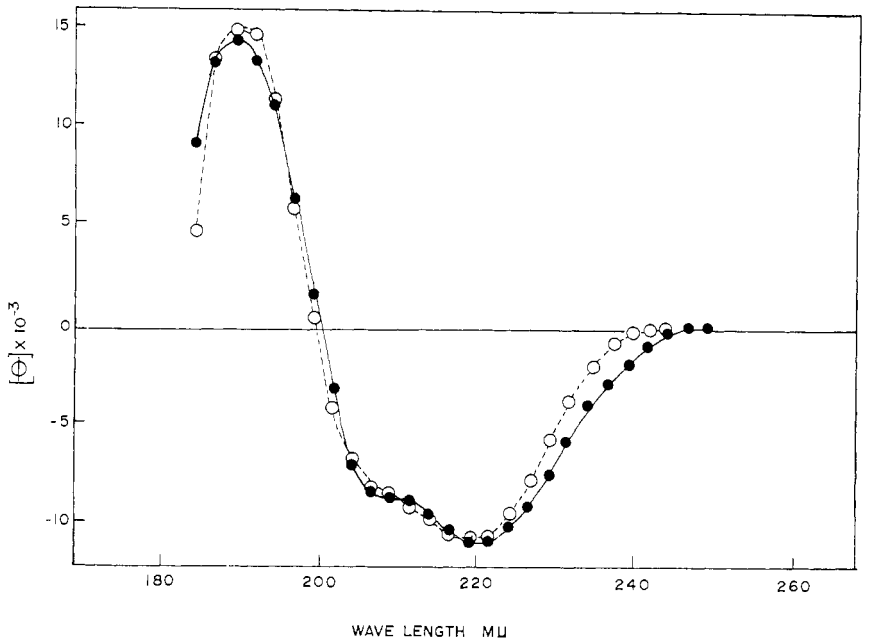

FIGURE 2: Comparison between the experimental (O---O) and the fitted (●—●) circular dichroism curves for *Mycoplasma* membranes. The fitted curve was calculated by computer from the poly-L-lysine curves shown in Figure 1. This curve represents the least-squares best fit to the experimental data and the calculated component values were 56.0% β, 30.7% α, and only 13.2% coil.

data and provided the three coefficients of the optimum fitted curve, the ordinates of the fitted curve, and the sum of squares of deviations between the fitted and experimental curves at the data points as output. It required no assumptions regarding the analytical form (*e.g.*, approximation by Gaussians) of the curves.

Circular dichroism and optical rotatory dispersion spectra are not independent—it is possible to predict either one from the other by the use of the Kronig–Kramers transform. If the circular dichroism spectrum of a molecule is represented as a sum of Gaussian peaks and troughs, then an explicit analytic expression for the corresponding optical rotatory dispersion spectrum is available (Moscowitz, 1961). In order to use this expression it is necessary to evaluate integrals of the form:

$$I(y) \equiv \exp(-y^2) \int_0^y \exp(x^2)\mathrm{d}x$$

Because the integrand is a rapidly increasing function of the upper limit, accurate results are obtained more conveniently by numerical integration of the differential equation, $\mathrm{d}I/\mathrm{d}y = 1 - 2yI$, satisfied by the function I than by direct numerical evaluation of the integral. A function subroutine that integrated this differential equation using the "one-half" variant of the Milne and Adams–Bashforth predictor–corrector methods gave solutions accurate to 7 significant figures over the entire range of interest. This subroutine was incorporated into a code that predicted the optical rotatory dispersion spectrum corresponding to a circular dichroism spectrum consisting of an arbitrary number of Gaussian peaks and troughs.

Results

Washing Methods. Our membranes are pure by the following criteria. (a) Electron micrographs showed that no intact organisms were present and there was no evidence of residual cytoplasm adhering to the membranes. (b) Nonradioactive membranes were mixed with [^{14}C]amino acid labeled cytoplasm. After 1 hr in the cold the membranes were subjected to the washing procedure and counted. Only 0.07% of the 573,000 counts present in the cytoplasmic fraction remained with the membranes. A control experiment showed that 92.9% of the cytoplasmic counts were precipitable with *hot* perchloric acid solution and resisted extraction with lipid solvents. The counts were therefore predominantly in the protein fraction of the cytoplasm. The conclusion of the mixing experiment was that only negligible amounts of cytoplasmic proteins (those extracted by the osmotic lysis procedure) are exchangeable with membrane proteins or absorbed by reexposure to membranes.

Our operational definition of membrane proteins are those proteins which are not extracted from the membranes by the lysis and washing procedures.

Circular Dichroism Data. The circular dichroism spectra for the α, β, and random coil forms of poly-L-lysine are shown in Figure 1. The effect of adding the random coil curve to the α curve would be to increase the relative depth of the negative peak at 208 mμ and broaden it toward the blue. The effect of added β structure, on the other hand, would be to add to the 222-mμ trough and make it deeper and broader.

Figure 2 shows the circular dichroism spectrum of *Mycoplasma* membranes and the optimum fitted curve using the poly-L-lysine spectra. The sum of the squares of the deviation between the experimental curve and the fitted curve provides a convenient measure of the amount of misfit. It was found that the misfit of the optimum curve could be reduced to a minimum by shifting each point in the membrane curve toward the blue by progressive increments. Other kinds of shifts were attempted but greater amounts of misfit resulted in each case. The shifts were undertaken to optimize the positions of

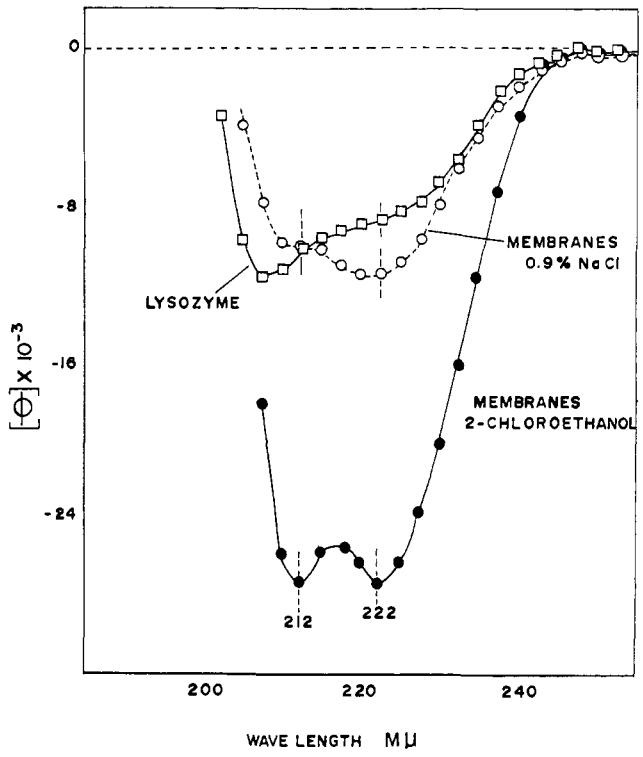

FIGURE 3: *Mycoplasma* membranes and lysozyme circular dichroism spectra in 0.9% NaCl are compared with the spectrum of membranes in 2-chloroethanol. Note the broadening out of the 222-mμ negative peak region of both the membranes and lysozyme in 0.9% NaCl but not of membranes in 2-chloroethanol where the maximum amount of helical coiling is present. Note also that the effect of a large percentage of random coil in the lysozyme deepens the lower wavelength negative peak and shifts it toward the blue. Based on past high estimates of random coil in membranes it is puzzling that the same effects are not seen with membranes in 0.9% NaCl or in water (as shown in Figure 2).

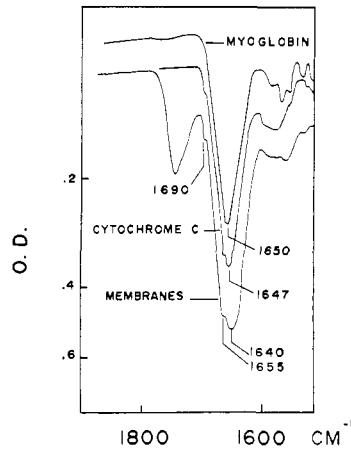

FIGURE 4: Infrared spectra of membranes, with cytochrome *c* and myoglobin shown for comparison. The 1640-cm^{-1} and 1690-cm^{-1} negative peaks are attributed to β structure. The 1647-cm^{-1} peak is due to random coil and the 1650–1655-cm^{-1} peaks are believed to represent α helix.

the α peaks in the membrane spectrum. The surprising result was that the best fit to the membrane experimental curve is obtained with 56.0% β, 30.7% α, and only 13.2% coil.

Figure 3 shows a comparison of the near circular dichroism spectrum for membranes in 0.9% NaCl *vs.* membranes in 2-chloroethanol. The membranes exhibit a maximum amount of helical coiling in 2-chloroethanol (Lenard and Singer, 1966) and it is interesting to note that the negative peak positions remain much the same at 212 and 222 mμ, respectively, although the amplitudes are much diminished in aqueous solution. There is, however, a pronounced broadening of the 222-mμ peak toward the blue. Lysozyme is also shown for comparison. The prominence of the negative peak toward the blue clearly illustrates the point that this is an indication of a predominance of random coil structure. The broadening of the remaining red portion is a reflection of the presence of appreciable amounts of both α and β structures in this protein.

Alternative Analysis of Circular Dichroism Data. A semigraphical method of circular dichroism data analysis has been suggested by Greenfield and Fasman (1969) based on a method previously applied to optical rotatory dispersion data (Greenfield *et al.*, 1967). This provides an interesting alternative means of calculation.

The first step is to calculate the amount of α structure by following approximate relation: % α helix = $[[\theta]_{208} - 4000)/(33{,}000 - 4000)] \times 100$. The −4000 corrects for the small β and coil contributions to the α extremum at 208 mμ. It makes very little difference whether we choose 208 or 212 mμ for this analysis. But the simplest thing is to assume that the 208-mμ α negative peak is shifted to 212 due to a red shift in membrane spectra that is discussed later. The calculation yields, at most, 23% α helix when the data shown in Figure 5 are used. Greenfield and Fasman have published families of circular dichroism curves with varying percentages of the three types of coiling. The next step is to choose one among their curves for mixtures that all contain on the order of 20–25% α helix. When this is done we note that shapes reasonably similar to our membrane circular dichroism curves are obtained when between 20 and 40% random coil is present. β structure is then obtained by difference to be 37–57% of the total. This result is in surprisingly good qualitative agreement with the curve-fitting analysis above and the optical rotatory dispersion data analysis below.

Infrared Data. Membranes in D_2O suspension exhibit a major infrared peak at 1640 cm^{-1} and a minor one at 1690 cm^{-1} (Figure 4). These are the positions that Wallach *et al.* (1969) attribute to β structure in dry mitochondrial membrane films. Susi *et al.* (1967), on the other hand, review data that suggest a major peak position at 1632 cm^{-1} is the most probable position for antiparallel β structure in D_2O–protein solutions. Nevertheless, both authors agree that α helix and random coil should exhibit peaks above 1640 cm^{-1}. The random coil peak of cytochrome *c* comes at 1647 cm^{-1}. The shoulder at 1655 cm^{-1}, seen both with membranes and cytochrome *c*, is attributable to α helix and the 1650-cm^{-1} peak of myoglobin is almost surely due to α helix and little else. The broadness of the 1640-cm^{-1} peak of the membranes is consistent with the idea that substantial amounts of both random coil and β structure are present.

Optical Rotatory Dispersion Data. The curves for both optical rotatory dispersion and circular dichroism derived from the same sample are shown in Figure 5. The circular dichroism curve is not typical in that the positive peak is un-

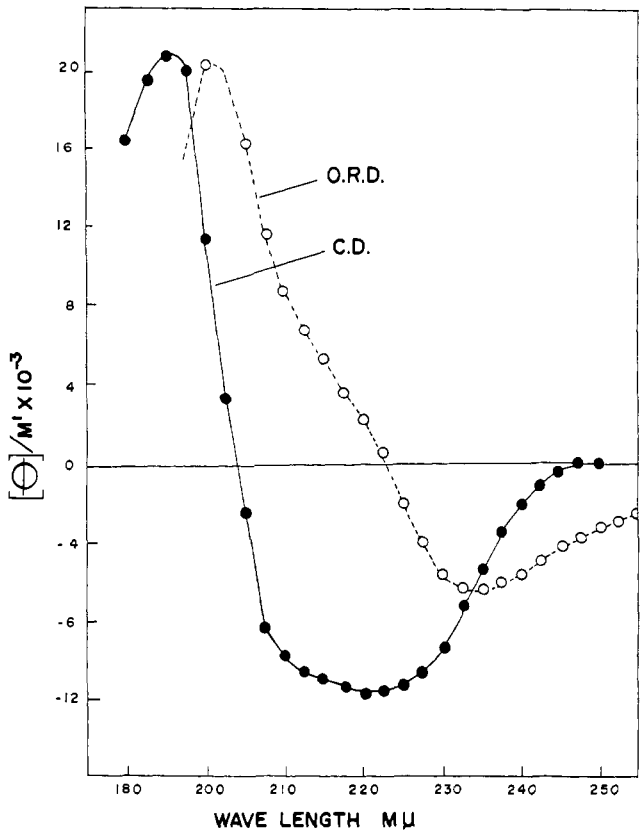

FIGURE 5: Circular dichroism and optical rotatory dispersion spectra of the same preparation of *Mycoplasma* membranes at the same concentration.

usually high, indicating perhaps more α structure for some reason in this preparation. These data are nonetheless used for optical rotatory dispersion analysis, in order to give α structure a fair chance to show up maximally.

Figure 6 shows a Moffit-Yang plot of optical rotatory dispersion data between 250 and 380 mμ. A value for λ_0 of 217 mμ was chosen because it yields a straighter line than the usual 212 mμ. If 212 mμ is chosen, the percentage of α helix comes out somewhat higher than the 26% indicated. The striking thing is that the a_0 parameter is very near zero (only 13.4 out of a possible ±500–700). This could mean that there is nearly 100% α structure present, but it is more likely in view of the b_0 value of −162 that the low a_0 value means that we have nearly equal amounts of β and coil in membranes, each amounting to 30–45% using a_0 values given by Timasheff *et al.* (1967). Refer to the cytochrome *c* data below.

Gaussian Fit. Figure 7 shows the fitting of seven Gaussians to the circular dichroism experimental data. The amount of β and coil was arbitrarily fixed at 40% and 30%, respectively. Data points for the respective poly-L-lysine curves were multiplied by 0.4 and 0.3 and these were drawn in graphically. In accordance with our previous experience in curve fitting (Figure 2), the β trough (represented by Gaussian 5 on Figure 7) was shifted 2.5 mμ to the red and the two blue bands (2) and (3) were shifted 5.0 mμ to the red to compensate for the red shifts in the membrane bands. The amount of α helix was taken by difference using a simple graphical technique and came out to be 20% based on the depth of Gaussian 6. Gaussian parameters (θ_{max}, half-width, and γ_{max}) were derived from the fitted poly-L-lysine curves by direct measurement with calipers and these data were fed to the computer.

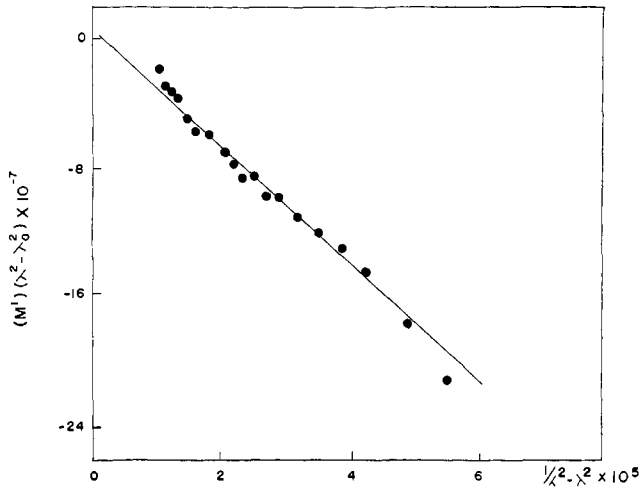

FIGURE 6: Moffit-Yang plot of the optical rotatory dispersion data shown in Figure 5. Parameters are given in the text.

The computer calculated true Gaussians from the parameters given it and then took the total of all seven Gaussians represented by the solid line in Figure 7. The fit with the data

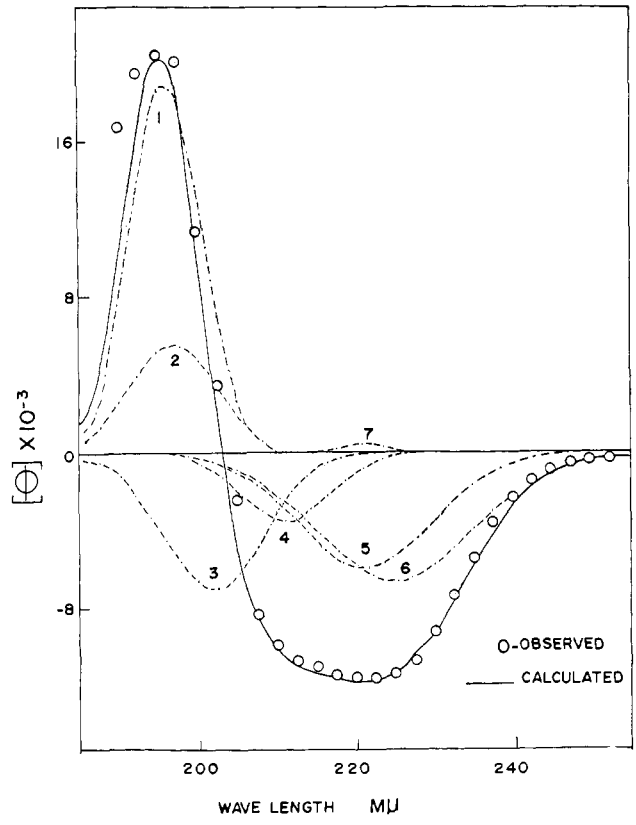

FIGURE 7: Component Gaussian curves (1–6) were calculated on the basis of estimated amounts of α, β, and coil structure in membranes. The sum of the Gaussians is shown as the solid line in the figure and this is compared with the observed points.

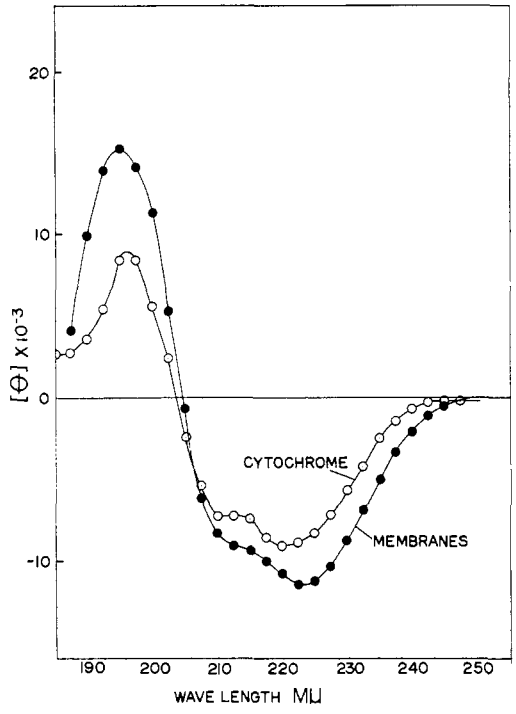

FIGURE 8: The circular dichroism spectrum of cytochrome *c* is compared with that of *Mycoplasma* membranes. Note the great similarity between them. On the basis of X-ray studies cytochrome *c* has very little α helix.

points is gratifying. The Gaussian parameters are given in Table I.

This analysis does not prove that β structure is present in membranes but it does point out that the circular dichroism data are ambiguous since we can also get a good fit with just three Gaussians. Unfortunately, no unique set of component Gaussians exists for the circular dichroism curve; however, if one applies the restriction that the difference curve must resemble an α helix, the range of possible Gaussian parameters becomes quite narrow.

Amino Acid Analysis of Membranes. The following analysis was performed to find the hydrophobicity of the amino acid residues. Results are given in Table II.

Calculations by a method suggested by Tanford (1962) give an average hydrophobicity of 1031 cal/residue (average hydrophobicity is the average unitary free energy required to transfer the residue side chains of a given protein from alcohol to water). A calculation of the polarity according to the method of Fisher (Bigelow, 1967) gives a ratio of polar to nonpolar residues of 1.05 to 1. Comparison of these figures with those given by Bigelow (1967) for a large number of proteins shows that these membrane proteins are average for globular proteins and quite different from values found for most structural proteins such as fibroin, flagellin, tropomyosin, etc. These values are also similar to those given for a variety of other membrane proteins (Wallach and Gordon, 1968).

TABLE I: Gaussian Parameters for Figure 7.

Gaussian No.	θ_{max}	Half-Width (mμ)	λ_{max} (mμ)
1	19.0×10^3	6.5	196.0
2	5.7	8.0	197.0
3	−7.0	8.5	202.0
4	−3.4	7.7	211.1
5	−5.8	12.2	221.5
6	−6.4	14.2	225.0
7	0.5	8.0	220.0

TABLE II: Amino Acid Composition of *Mycoplasma* Membranes.

Nonpolar Amino Acid	Mole %	Polar Amino Acid	Mole %
Tryptophan	2.3	Tyrosine	4.1
Isoleucine	7.0	Lysine	7.1
Phenylalanine	4.8	Arginine	3.0
Proline	3.9	Threonine	7.5
Leucine	9.5	Serine	6.4
Valine	7.0	Histidine	1.4
Methionine	2.3	Aspartic acid	12.5
Alanine	8.1	Glutamic acid	9.4
Glycine	8.1	Amide	6.4
Half-cystine	Trace		

Cytochrome c Data. Figure 8 shows the similarity between the spectrum of cytochrome *c* and that of *Mycoplasma* membranes. There is a broad negative peak, centered at 220 mμ, a negative shoulder at about 210 mμ, and a positive peak at 197 mμ. The cytochrome *c* spectrum is of still lower amplitude than that of membranes. This is particularly true of the positive part of the spectrum.

Further analysis was performed by first subjecting the circular dichroism data to the Kronig–Kramers transform (Moscowitz, 1961) by computer to obtain the optical rotatory dispersion spectrum. Moffit–Yang parameters were then derived from the computed optical rotatory dispersion points. These resulted in an a_0 value of −347, b_0 value of −85.6, and λ_0 212 mμ. Timasheff *et al.* (1967) point out that while the value of a_0 is complicated by solvent effects, *etc.*, it is still possible to assign limited ranges for the contributions of α, β, and coil structures to the a_0 value. They suggest the following ranges: 0 to +360 for α, −500 to −690 for coil, and +400 to +700 for β structure.

The b_0 value was used to obtain percentage of α helix equal to 13.6. This, together with the value obtained for a_0, yielded the following values for cytochrome *c*, by the method of Timasheff *et al.*: α 4–20%, β 11–15%, and coil 68–81%.

It is interesting that a calculation by the method of Greenfield and Fasman (1969) yields only 11% α helix. It is evident though, from their families of curves that a suitably shaped circular dichroism curve would have no more than about 40% random coil. The figure of about 50% β structure derived by difference is significantly higher than that derived by the Timasheff method, but it is likely with the Greenfield and Fasman method that when the percentage of α structure is low the other structures are less determined. This is because differences in random coil circular dichroism spectra (Tiffany

and Krimm, 1969; Dearborn and Wetlaufer, 1970) become important when the relatively large ellipticities of the α helix are missing.

Discussion

Circular Dichroism and Optical Rotatory Dispersion Data. The circular dichroism and optical rotatory dispersion spectra presented here for *Mycoplasma laidlawii* membranes are very similar to data derived from a variety of other biological membranes. Reference is made to the optical rotatory dispersion spectra of chloroplast membranes (Ke, 1965), optical rotatory dispersion and circular dichroism of Ehrlich ascites tumor cell membranes (Wallach and Zahler, 1968; Wallach and Gordon, 1968; Gordon *et al.*, 1969), optical rotatory dispersion and circular dichroism of human red cell membranes and *Bacillus subtilis* membranes (Lenard and Singer, 1966), and optical rotatory dispersion and circular dichroism of mitochondrial fragments (Urry *et al.*, 1967; Stein and Fleisher, 1967; Wrigglesworth and Packer, 1968; Urry and Ji, 1968).

For synthetic α-helical polypeptides, negative circular dichroism bands appear at 208 and 222 mμ corresponding to the π^0–π^-, and n_1–π^- electronic transitions of the peptide band, respectively (Holzwarth and Doty, 1965). A positive band appears at 190 mμ corresponding to the π–π^- transition. Membranes exhibit two peaks that are shifted to the red and a third, the n–π^- transition, which is not shifted in position but appears to be much broader than for α-helical polypeptides (Wallach and Zahler, 1968). The so-called "broadening" is debatable and this point is taken up in feature 2 below. For now it is recorded that most workers have tried to find other ways of explaining the anomalies in the circular dichroism spectra of membranes.

These spectral features have been interpreted as a result of lipid–protein interaction (Wallach and Zahler, 1968), the interaction between protein and protein (Lenard and Singer, 1966), and the presence of phospholipids (Urry *et al.*, 1967). The red shift and, to some extent, the amplitudes of the bands can be altered by phospholipase A and sodium dodecyl sulfate (Lenard and Singer, 1968) and by phospholipase A, phospholipase C, lysolecithin, and digitonin (Gordon *et al.*, 1969) so there is little question that lipid–protein or lipid-sensitive protein–protein interactions are involved in the red shift.

Urry and Ji (1968) have suggested that all of the unusual features of membrane optical activity might arise from turbidity artifacts. They use, as a theoretical model, a suspension of solid 0.3-μ polypeptide spheres. This point is still controversial (Ji and Urry, 1969; Ottaway and Wetlaufer, 1970) but, as pointed out by Gordon *et al.* (1969), plasma membranes are water-filled lipoprotein *shells* (about 100 Å thick) and in such systems scattering and absorption distortions are small. We agree with this because *Mycoplasma* membrane vessicles are smaller than plasma membranes such as red cell ghosts and show less light scattering, yet display about the same spectra. We can also show that light absorption, due to scattering, falls off rapidly with concentration. As *Mycoplasma* membranes are diluted they disaggregate and, at the concentrations used in these experiments, they exhibit little light scattering.

We do not disagree with any of these conjectured reasons for shifts in the bands attributable to α helix in membranes, but it should be pointed out that contributions from other helical structures have been ignored. Consider the following two features of the circular dichroism spectra of *Mycoplasma* membranes and of all other membranes that have been studied so far.

(1) The shoulder that appears at about 212 mμ is smaller and shifted more to the red than in most synthetic α-helical peptides. Inspection of Figure 1 shows that the effect of the presence of random coil would be to increase the amplitude of this shoulder and extend it toward the blue. The spectrum of lysozyme shown in Figure 3 shows this effect very well, as does the spectrum of 40% α-helical polyglutamate published by Wallach and Gordon (1968). The families of circular dichroism curves of peptide mixtures published by Greenfield and Fasman (1969) also emphasize this point. Tiffany and Krimm (1969) and Dearborn and Wetlaufer (1970) point out that the circular dichroism spectrum of random coil in proteins is different from that of poly-L-lysine in the random coil configuration. However, two features that apply here seem to be true of all random coils. There is a large negative transition at about 200 mμ and only very small transitions exist at longer wavelengths. Any theory about the circular dichroism spectrum of membranes must therefore account for the apparent lack of a random coil contribution to the 212-mμ minimum.

We suggest that the random coil contribution is cancelled by the presence of comparable amounts of β structure. Inspection of Figure 1 shows how this is possible.

(2) The negative circular dichroism peak at about 222 mμ is broader than a comparable α-helical band with the same amplitude. This may be due to helix–helix interactions which reduce the amplitude of this transition (T. H. Ji, 1970, personal communication). If this were somehow proven, we could assume that membranes were predominantly α helical and this could explain all of our data including the low value of b_0. However, until such proof is at hand, we can point out that the possibility remains that the broadness is due to the presence of a β transition at very nearly the same position as the α.

Infrared Analysis. FIGURE 4. Based on the values of 1632 cm^{-1} for antiparallel β structure in D_2O solution and 1643 cm^{-1} for unordered structures (Susi *et al.*, 1967), our values of 1640 ± 1 cm^{-1} and a small shoulder at 1690 cm^{-1} are consistent with the idea that a mixture of coil and antiparallel β structure exists in *Mycoplasma* membranes because the 1640-cm^{-1} peak is broad and it is probable that spectra of the two structures were close enough together that they were not resolved. Maddy and Malcom (1966) found no evidence for β structures in dry films of red cell membranes, but Wallach *et al.* (1969) believe that the 1640-cm^{-1} and 1690-cm^{-1} shoulders present in their infrared spectra of dry films of mitochondrial membranes do give evidence of β structure. They suggest that red cell and mitochondrial membranes differ in β content.

These dry-film spectra should be repeated in D_2O solution because of the possibility that the helical content of lipoproteins is altered during or after the drying procedure due to the fluidity of the lipid component. On the other hand, much more data is needed on the amide I spectrum of various proteins in D_2O solution before we can be certain that the band positions given by Susi *et al.* (1967) will apply to all proteins.

Cytochrome c. There is an apparent conflict between the circular dichroism curves of Urry and Doty (1965), Ulmer (1965), and Flatmark *et al.* (1970), which have a low-amplitude

α shape similar to membranes, and the X-ray analysis of Dickerson *et al.* (1967) who detected no α helix at 4-Å resolution. Because of this, we applied the analytical method of Timasheff *et al.* (1967), used here with membranes, to cytochrome *c*. The infrared data showed simply that the spectrum was heterogeneous but predominantly random coil in form. The Moffit–Yang parameters also indicated a predominance of coil. It is likely that a small percentage of α helix, 10% or so, contributes to the α shape of the circular dichroism spectrum and the broadness of the 220-mμ trough. This could be due to short helices that are not detectable by the low-resolution X-ray analysis. A somewhat larger amount of β structure could contribute strongly to the 220-mμ trough and the 197-mμ peak and the shoulder at 210 mμ probably results mainly from the distortion of the β spectrum due to the presence of random coil.

Structure of Membranes. Benson and Singer (1965) have postulated that lipids are associated with globular proteins in membranes *via* hydrophobic interactions between hydrocarbon chains of lipids and the nonpolar interior of the globular proteins. [For a discussion of this lipoprotein monolayer model, refer to Lenard and Singer (1966), Benson (1966), and Ji *et al.* (1968).] Our data on the average hydrophobicity of membrane amino acid residues are consistent with the Benson–Singer model, but their globular protein concept would not predict the high percentage of β structure suggested by our circular dichroism, optical rotatory dispersion, and infrared data. There are, however, fibrous proteins in red cell membranes (Marchesi and Steers, 1968) and contractile proteins in mitochondria. It may be that some fibrous β proteins exist in all membranes or that some globular proteins may also have a β-structural component as does lysozyme.

Summary

We have shown, in agreement with Wallach *et al.* (1969), that the circular dichroism data derived from membranes are ambiguous with respect to the presence or absence of β structure. Whether or not the infrared data and the Moffit–Yang parameters are definitive or not remains to be seen. Too little data are now available. We can only say that self-consistent evidence presented here points to the presence of as much as 40% β structure in *Mycoplasma laidlawii* membranes and that there is nothing to disprove the possibility that 10% or more β structure exists in all membranes.

The results of our analyses of *Mycoplasma* membranes are summarized as follows: (1) curve-fitting method, 30.7% α, 56.0% β, and 13.2% coil, (2) method of Timasheff *et al.* (1967), 26% α, 30–45% β, and 30–45% coil; (3) method of Greenfield and Fasman (1969), 23% α, 37–57% β, and 20–40% coil.

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Fate of Phospholipids in Liposomal Model Membranes Damaged by Antibody and Complement*

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ABSTRACT: Liposomes, containing either Forssman antigen or globoside I, have been prepared with radioactive [^{32}P]-sphingomyelin and lecithin isolated from rat liver. These liposomes release 50 to 80% of their trapped glucose marker when incubated with the appropriate antiserum and a source of complement. Analysis of the reaction mixtures did not reveal the appearance of any phospholipid degradation product from liposomes which had undergone "immune damage," and approximately 98% of the total radioactivity recovered from thin-layer plates was in the form in which it had been originally incorporated into the liposomal membrane (*i.e.*, as either lecithin or sphingomyelin). The methods employed would have detected a 1% degradation of phospholipid if the radioactive products had behaved chromatographically similar to phosphatidic acid, phosphorylcholine, lysolecithin, glycerylphosphorylcholine, or sphingosylphosphorylcholine. In control experiments, lecithin liposomes were incubated with phospholipase C. Approximately 40–50% of the lipid was degraded when 50% of the trapped marker had been released indicating that measurable amounts of radioactive product (phosphorylcholine) should have been formed if activation of the terminal complement components had led to the generation of an enzymatic activity with properties analogous to exogenous phospholipase C. Subject to the limitations characteristic of all "negative" experiments, the available data are consistent with the hypothesis that complement-dependent membrane damage may not occur by the enzymatic rupture of covalent bonds in phospholipids.

Previous papers have described the preparation of liposomes from either the chloroform-soluble material of sheep erythrocytes (Haxby *et al.*, 1968; Alving *et al.*, 1969) or from simple artificial lipid mixtures containing pure Forssman antigen (Kinsky *et al.*, 1969). These liposomes release trapped glucose marker when incubated with rabbit antisheep erythrocyte serum and a source of complement. The available data suggest that all of the complement components required for maximum damage to natural membranes are also essential for maximum damage to the model membrane (Haxby *et al.*, 1969). Because lipids thus appear to be the "substrate" for complement action, the present investigation was undertaken to determine whether functional impairment of the liposomal membrane is a consequence of phospholipid degradation. Subject to certain experimental limitations, our results indicate that cleavage of covalent bonds in phospholipids does not occur.

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Experimental Design

In these experiments, guinea pig serum and rabbit immune serum were used as the source of complement and antibody, respectively. Because whole serum may be contaminated by some compounds which conceivably could be products of an enzymatic reaction by the terminal complement components (see Figure 8), it was necessary to employ liposomes prepared with radioactive [^{32}P]phospholipids. If release of glucose occurs as a consequence of phospholipid degradation, then it was anticipated that a new radioactive compound should be formed. Under ideal circumstances, appearance of this compound should be dependent on the presence of both native (*i.e.*, unheated) complement and the immune serum. However, further consideration revealed at least 2 reasons for the inadequacy of controls which contained either heat inactivated complement or normal rabbit serum. First, human or guinea pig serum may contain various phospholipase activities (*cf.* legend to Figure 8) and it would be difficult to exclude the possibility that the decomplementation procedure (incubation at 56° for 30 min) would not also inactivate these enzymes. Second, there was no guarantee that normal and immune serum were identical with regard to the absolute activity of phospholipid degradative enzymes.

An alternative experimental approach was, however,