OPTICAL ROTATION OF MITOCHONDRIAL MEMBRANES

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Several papers have appeared recently in which optical rotation data were used to study the structure of membranes.[1-3] The optical rotatory dispersion curves for erythrocyte membranes[2] and for plasma membranes of Ehrlich ascites carcinoma[1] are reported to be of the general form corresponding to an α-helix with minor but significant differences. The trough, normally found at 233 mμ for model polypeptides, is shifted to 237 mμ. This long wavelength shift was interpreted by Lenard and Singer[2] to reflect parallel alignment of helical segments, whereas Wallach and Zahler[1] invoked a model of hydrophobic (lipid) interactions with helical segments to explain the red shift. Another aspect recognized by Wallach and Zahler was the low amplitude of the Cotton effect. The usual considerations of α-helix random coil combinations could not account for all the protein. On the basis of the work of Iizuka and Yang,[4] it was proposed that the optical activity of a small amount of β-structure cancel the rotation of substantial amounts of random coil.

This communication presents optical rotatory dispersion (ORD) and circular dichroism (CD) data for electron transport particles and for whole mitochondria under conditions in which the enzymatic activities of these systems are commonly studied. Mitochondrial membranes exhibit Cotton effects of the α-helix form with the differences noted previously for other membranes. The problem of low amplitude is most acute in mitochondria, where a larger portion of the protein's optical activity is masked. In interpreting these data we discuss the solvent Stark effect, the suggestion of interacting helices,[2] the possibility of other types of helices, and possible contributions due to phospholipids. An argument is presented which shows that both the long wavelength shift of the trough and the low amplitude of the Cotton effect may be due to contributions from phospholipids, e.g., L-α-lecithin.

Experimental.—Beef heart mitochondria and electron transport particles were obtained by the method of Green and Ziegler[5] and by a modification of the method of Low and Vallin.[6] KCl mitochondria were prepared according to the procedure of Crane *et al.*[7] Protein concentration was determined by the biuret method.[8] Beef lecithin, obtained from General Biochemicals as an ethanol solution, was dried under nitrogen and redissolved in trifluoroethanol. The intact mitochondria and submitochondrial particles were studied in a 0.25 *M* sucrose solution buffered with tris-HCl at pH 7. A thermostatted, differential ORD cell block was used with the suspended particles placed in the initial beam and the suspending medium in the return beam of a Cary model 60. The circular dichroism curves were run on a prototype instrument built by Cary Instruments for the above spectropolarimeter.

Results.—A direct ORD curve of electron transport particles is almost totally dominated by the plain curve of sucrose, with perhaps a slight deviation due to

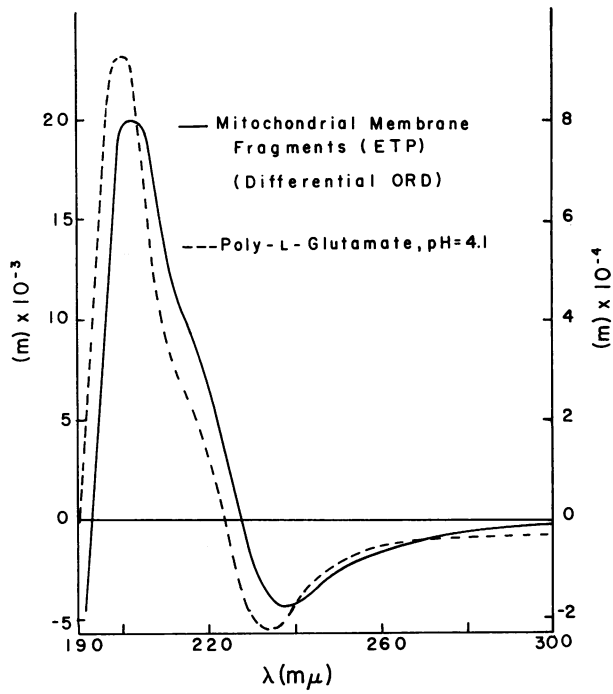

FIG. 1.—Differential ORD curve of electron transport particles from heavy beef heart mitochondria (*left ordinate*). For calculation of the mean residue rotation, [*m*], it was assumed that all the optical activity was due to biuret protein. A mean residue weight of 115 was used. Also included in the figure is poly-L-glutamic acid (*right ordinate*).

mitochondrial fragments. When the differential ORD technique is used with the suspended electron transport particles (ETP) in the initial beam and the suspending medium in the return beam, a complex Cotton effect is clearly resolved (Fig. 1) which, except for a few mμ red shift, contains all the characteristics of an α-helix curve, i.e., trough, shoulder, and peak in the proper relationship. It should be noted that the entire complex Cotton effect is shifted to longer wavelengths. Particles from both light and heavy beef heart mitochondria give identical curves when compared on the basis of protein content. The ORD curve for the organelle is given in Figure 2. The curve for KCl mitochondria exhibits the same characteristic pattern although it appears to be riding on a positive background. The positive background is more pronounced and variable for sucrose mitochondria and may be due to the concentrating of metabolites within the organelle or it may reflect an ordered array of lipid.

Standard calculations of helical content assuming a mixture of α-helix and random coil[9] and using the ORD peak suggest a value of 20–25 per cent for the ETP and a considerably lower value (5%) for the whole mitochondrion. Therefore much of the optical activity of the protein is masked while there is retention of the basic features characterizing the α-helix. Adequate interpretation of the optical rotation data of mitochondrial membranes should include explanations for both the long wavelength shifts and the low amplitudes.

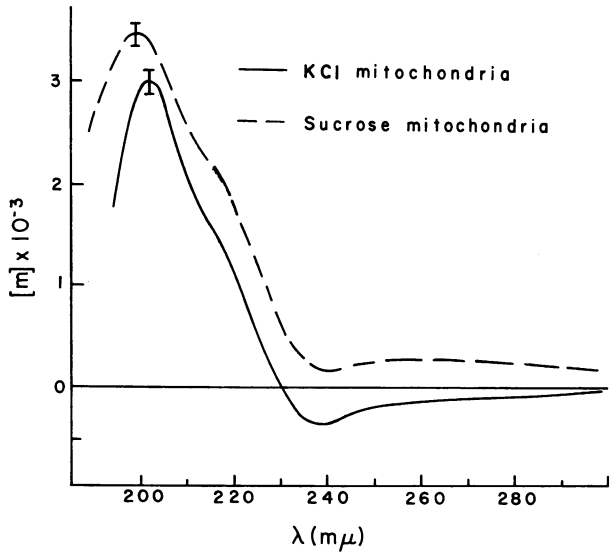

FIG. 2.—Differential ORD curves of KCl mitochondria and sucrose mitochondria. For calculation of the mean residue rotation, $[m]$, it was assumed that all the optical activity was due to biuret protein. A mean residue weight of 115 was used. Note the low amplitude of the Cotton effect when compared to Fig. 1.

Discussion.—The characteristic values in the ORD of an α-helix are a trough at 233 mμ, a crossover at 223 mμ, a shoulder between 220 and 210 mμ, a peak at 198–199 mμ, and a second crossover at 190–191 mμ. The characteristic CD values for an α-helix are negative extrema at 222 mμ and 208 mμ, a crossover at 201 mμ, and a positive peak at 191 mμ. The relationship between ORD and CD for a simple Cotton effect is such that an ORD extremum occurs at a wavelength where the ellipticity equals the ellipticity of the peak divided by e, that is,

$$[\theta]_\lambda = [\theta^0]/e.$$

This relationship may be applied directly to the characteristic negative CD extremum at 222 mμ to place the ORD trough at 233 mμ. Figure 3 contains a circular dichroism curve for electron transport particles. The long wavelength, negative extremum, is at 225 mμ, and the wavelength at which the ellipticity is equal to the ellipticity at 225 mμ divided by e is 237 mμ. Those factors which result in a CD peak at 225 mμ in the ETP curves are therefore responsible for the red shift of the trough to 237 mμ. The position of a particular CD (or ORD) extremum may be shifted by effects which change the energy of the electronic transition, or the position may be shifted by rotations of other electronic transitions with slightly different energies and/or band shapes. We will first discuss the effects of solvent on changing the energy of electronic transitions and then consider the effects of extraneous optically active transitions on altering positions of extrema. The peptide chromophore in an α-helical array exhibits two types of electronic transitions, an $n \rightarrow \pi^*$ transition resulting in the 222 mμ CD extremum and two $\pi \rightarrow \pi^*$ transitions giving rise to the CD extrema at 208 mμ and 191 mμ.[10] Hy-

drogen bonding by polar solvents of the nonbonding electrons on the oxygen heteroatom lowers the energy of the nonbonding, n, orbital and thereby increases the energy for an electronic transition from the nonbonding ground state to the excited, π^*, state.[11] Removal of the hydrogen bonding, as in going from a polar to a nonpolar medium, raises the energy of the nonbonding orbital with a corresponding decrease in the energy of the $n \rightarrow \pi^*$ transition. The latter is seen as a long wavelength shift in the position of an absorption or CD extremum. These effects are usually large. In helical polypeptides and helical proteins, however, hydrogen bonding is maintained whether the helix is found in a hydrophilic or hydrophobic environment. The effects of solvent on the energy of the nonbonding orbital are minimized. It then becomes necessary to consider changes in the dipole moment of the peptide chromophore which attend the optical transition.[12]

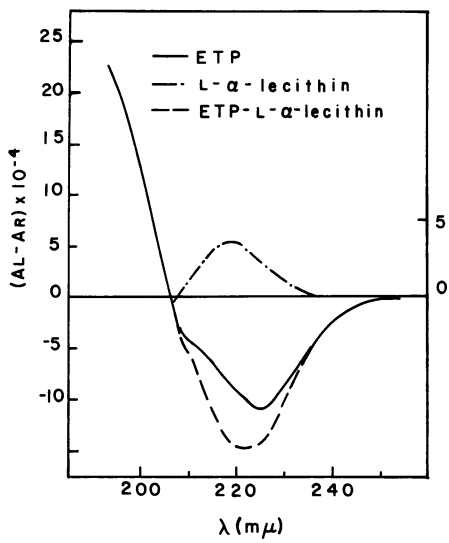

FIG. 3.—Circular dichroism curve of electron transport particles from heavy beef heart mitochondria (————) and L-α-lecithin (—·—·—·—). The ordinate is the difference in absorbance at left, A_L, and right, A_R, circularly polarized light. The left ordinate is for ETP and the right ordinate for L-α-lecithin. The dashed curve results from the subtraction of the L-α-lecithin curve from the curve for ETP. Note the negative extremum is shifted from 225 mμ to 222 mμ.

The excited state for $\pi \rightarrow \pi^*$ transitions generally has a larger dipole moment than the ground state, whereas the reverse is true for $n \rightarrow \pi^*$ transitions. On the basis of these changes in dipole moments the transfer of a molecule from a polar to a nonpolar medium would cause the $\pi \rightarrow \pi^*$ transitions to shift to shorter wavelengths and the $n \rightarrow \pi^*$ to shift to longer wavelengths. Accordingly one expects the 222 mμ CD minimum to be red shifted in a nonpolar or hydrophobic environment, and one might expect the negative CD extremum at 208 mμ and the positive extremum at 191 mμ to be blue shifted in media of low dielectric. If, on the contrary, the dipole moment of the peptide is less in the excited state for the $\pi \rightarrow \pi^*$ transitions, then a nonpolar environment would effect a bathochromic or red shift (see below). As hydrogen bonding is constant for the α-helix whether it exists in a polar or nonpolar environment and as the transition dipole moment of the $n \rightarrow \pi^*$ transition is small,[10] the 222 mμ CD extremum is not expected to be very solvent sensitive. Studies on model helical polypeptides in solvents varying from water with a dielectric of 78 through trifluoroethanol to dioxane with a dielectric constant of 2.2 show no substantial shift in the 222 mμ minimum.[13] Thus it would appear that hydrophobic interactions cannot explain the shift from 222 mμ to 225 mμ in

the CD minimum or the shift from 233 mμ to 237 mμ in the ORD trough of electron transport particles or other membrane systems.[1, 2]

As the peptide chromophore is heteroatomic, the general rules for solvent shifts of $\pi - \pi^*$ transitions may not apply. Indeed the calculations of Yomosa[14] show the permanent dipole moment of the monomeric peptide chromophore to be in a direction opposite to the 190-mμ transition dipole moment. The dipole moment of the peptide group decreases during this transition. On the basis of Yomosa's calculations[14] and Bayliss and McRae's analysis of solvent effects,[12] the 190-mμ $\pi - \pi^*$ (or NV_1) transition of the monomeric peptide group should exhibit a long wavelength shift when transferred from an aqueous or polar environment to a lipid or nonpolar environment. On helix formation the monomer $\pi - \pi^*$ transition moment is resolved into moments parallel and perpendicular to the helix axis.[15, 10] Since the calculated monomer transition dipole and permanent dipole moments are nearly diametrically opposed,[14] resolution into parallel and perpendicular moments is such that there remains a decrease in the dipole moment for the excited state of both transitions. Therefore, both the parallel and the perpendicular $\pi - \pi^*$ transitions would red shift on changing from a polar to a nonpolar environment. Evidence in support of this view may be found in studies on model polypeptides. Poly-L-alanine in 12 per cent trifluoroacetic acid and 88 per cent trifluoroethanol exhibits blue shifts on both $\pi - \pi^*$ transitions when compared to poly-γ-methyl glutamate in trifluoroethanol.[13] Poly-γ-benzyl glutamate and poly-γ-methyl glutamate in dioxane show a small red shift of the accessible parallel band.[13]

It has been proposed that long wavelength shifts seen in the ORD and CD curves of red blood cell membranes reflect the presence of aligned helical segments in the membrane.[2] This proposal was based on aggregation studies of helical poly-L-glutamic acid.[16] The aggregation of poly glutamic acid is an extreme case in which the aggregate consists of ten or more helical rods;[17] yet there is a red shift of only 1 mμ in the position of the ORD trough.[16] A red shift of 4 mμ is observed in the mitochondrial membranes. Alignment of helical segments would seem to hold neither the explanation for the red shift of the ORD trough nor a unique explanation for the red shift of the $\pi - \pi^*$ transitions.

Wallach and Zahler[1] have suggested that the presence of some β-structure may cause the Cotton effects to be of low amplitude; yet it is only when the lipid has been extracted and the films have been treated with acid, pH <2, that their infrared measurements provide evidence for β-structure. They further report that addition of lysolecithin or of 2-chloroethanol effects an increase of the amplitude of the Cotton effects and a return of the ORD trough to 233 mμ. Treatment of membranes with substances which have a direct effect on lipid results in a return to the typical α-helix curve. It would seem that a direct contribution by lipid to the observed rotation should be considered (see below).

The rotational contributions of electronic transitions other than the peptide $n - \pi^*$ transition may be added with positive or negative sign to shift the 222 mμ minimum to 225 mμ. Addition of a negative rotational band at wavelengths greater than 222 mμ would shift the extremum to longer wavelengths and increase the magnitude of the extremum. Addition of a positive rotational band at wavelengths just short of 222 mμ would shift the negative extremum to longer wave-

lengths and decrease its magnitude. Since low amplitude is an accompanying feature, the latter possibility would seem most interesting. As the ORD peak was used to calculate the low helical contents, it would also seem necessary to introduce a negative circular dichroism peak in the vicinity of 191 mμ. One source of differing rotational strengths would be in protein structure other than α, β, or random coil conformations. The most reliable evidence for such structures is found in the X-ray work on lysozyme;[18] however, the CD curves of lysozyme, while containing interesting and distinguishing features,[19] could not be used to explain the membrane data. Another source of optically active electronic transitions may be found in the lipids, particularly in the ester groups of phospholipids. Phospholipid represents over 90 per cent of the membrane lipid of mitochondria.[20] Lecithin and phosphatidyl ethanolamine comprise 75 per cent of the total phosphorus in beef heart mitochondria.[21] Accordingly, we have determined the ORD and CD curves for lecithin and phosphatidyl ethanolamine in trifluoroethanol in order to determine the presence or absence of optically active transitions near 220 mμ and 191 mμ. It was found (Fig. 3) that lecithin contained a positive CD peak at 218 mμ and a negative peak at 192 ± 2 mμ. Phosphatidyl ethanolamine exhibits a similar CD pattern. In Figure 3 it is seen that subtraction of the positive 218-mμ CD peak of lecithin can result in a return of the 225-mμ minimum of submitochondrial particles to the 222-mμ peak characteristic of α-helix. It should be recognized that the conformation of these phospholipids in trifluoroethanol is not necessarily the conformation of lecithin in the membrane and, in fact, the rotational strengths of the transitions must be larger for membrane phospholipid in order to be significant enough to substantially shift the 222-mμ minimum. An increased rotational strength is to be expected for an ordered array of molecules in fixed conformation. The negative CD peak at about 192 mμ for lecithin and phosphatidyl ethanolamine would result in a decreased amplitude of the positive 191-mμ peak for α-helix. Consequently, calculations of helical content based on the positive ORD extremum would be low. The possibility that the low amplitude and trough shift are due to rotations of the phospholipid is consistent with the data of Wallach and Zahler, noted previously. Information on the structure of lipid also appears to be obtainable from the optical rotation data of membranes. If, as is likely, the 218-mμ CD extremum of lecithin is due to an $n - \pi^*$ transition in the ester groups, then an octant rule may be applied to limit the possible lipid conformations within the membrane.[22, 23]

Summary.—The optical rotation curves of mitochondria and submitochondrial particles exhibit features qualitatively similar to those reported for red blood cell membranes[2] and Ehrlich ascites carcinoma membranes,[1] namely, a characteristic α-helix curve which is shifted to longer wavelengths. In the case of mitochondria the Cotton effect is displaced on a positive background and the amplitude of the Cotton effect is extremely low. The low amplitude implies that much of the optical activity of membrane protein is masked.

The central problems in interpreting optical rotation data of membranes are the red shift of the critical values and the low amplitude of the Cotton effects. Considerations of solvent effects lead to the conclusion that the trough shift is too great to be explained by changing the environment of helical segments. An argument based on the calculations of Yomosa and the Bayliss and McRae treatment of

solvent effects shows that the $\pi - \pi^*$ transitions would be expected to red shift on transfer to a less polar environment. Accordingly the red shift of the ORD peak and of the short wavelength crossover could reflect a hydrophobic environment.

The long wavelength shift in the ORD trough and low amplitude of the Cotton effect may be explained by the optical activity of membrane phospholipids. Should the optical activity of phospholipid prove to be in large part responsible for the atypical α-helix curve, then conformation of membrane phospholipid may also be deduced.

[1] Wallach, D. F. H., and P. H. Zahler, these PROCEEDINGS, **56**, 1552 (1966).
[2] Lenard, J., and S. J. Singer, these PROCEEDINGS, **56**, 1828 (1966).
[3] Maddy, A. H., and B. R. Malcolm, *Science*, **150**, 1616 (1965).
[4] Iizuka, E., and J. T. Yang, these PROCEEDINGS, **55**, 1175 (1966).
[5] Green, D. E., and D. M. Ziegler, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 416.
[6] Low, H., and I. Vallin, *Biochim. Biophys. Acta*, **69**, 361 (1963).
[7] Crane, F. L., J. L. Glenn, and D. E. Green, *Biochim. Biophys. Acta*, **22**, 475 (1956).
[8] Mokrasch, L. C., and R. W. McGilvery, *J. Biol. Chem.*, **221**, 909 (1956).
[9] Urnes, P., and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).
[10] Holzwarth, G. M., and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).
[11] Kasha, M., in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore, Md.: Johns Hopkins Press, 1961), p. 31.
[12] Bayliss, N. S., and E. G. McRae, *J. Phys. Chem.*, **58**, 1002 (1954).
[13] Quadrifoglio, F., and D. W. Urry, unpublished data.
[14] Yomosa, S., *Biopolymers Symp.*, **1**, 1 (1964).
[15] Moffitt, W., *J. Chem. Phys.*, **25**, 467 (1956).
[16] Cassim, J. Y., and J. T. Yang, *Biochem. Biophys. Res. Commun.*, **26**, 58 (1967).
[17] Tomimatsu, Y., L. Vitello, and W. Gaffield, *Biopolymers*, **4**, 653 (1966).
[18] Phillips, D. C., *Sci. Am.*, **215**, 78 (1966).
[19] Urry, D. W., and F. Quadrifoglio, unpublished data.
[20] Lehninger, A. L., *The Mitochondrion* (New York: W. A. Benjamin Inc., 1965), p. 206.
[21] Fleischer, S., and G. Rouser, *J. Am. Oil Chemists' Soc.*, **42**, 588 (1965).
[22] Urry, D. W., and H. Eyring, *J. Am. Chem. Soc.*, **86**, 4574 (1964).
[23] Urry, D. W., D. Miles, D. J. Caldwell and H. Eyring, *J. Phys. Chem.*, **69**, 1603 (1965).