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Circular Dichroism, Optical Rotatory Dispersion, and Absorption Studies on the Conformation of Bovine Rhodopsin in situ and Solubilized with Detergent*

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Abstract. Circular dichroism, optical rotatory dispersion and absorption of rhodopsin, the visual pigment of bovine rod outer segment membranes, were studied in situ and in membranes solubilized with various detergents. The α-helical content of the membrane protein is approximately 30%. The membrane protein possesses little β-structure. Solubilization of the membrane by the detergents, Emulphogene BC-720 and cetyltrimethylammonium salts, results in loss of protein helical structure and perturbation of aromatic residues. These effects are not observed on digitonin solubilization.

In regard to the structural stability of the membrane during bleaching, the following conclusions were reached: (1) Delocalized conformational changes of rhodopsin in situ involving secondary and/or tertiary structure are very unlikely. (2) Localized conformational changes of rhodopsin in situ involving secondary structure must be limited to the involvement of no more than three amino acid residues and localized conformational changes involving tertiary structure must be limited to very short segments of the protein chain containing, at the most, only a few aromatic residues. (3) Large changes in the interaction of lipid and protein moieties of the membrane are unlikely. (4) The detergents, Emulphogene, cetyltrimethylammonium salts, and digitonin, significantly decrease the conformational stability of rhodopsin as compared to the in situ conditions. The effect is smaller with digitonin.

Evidence is presented against a proposed mechanism by which optical activity of the prosthetic group, retinal, is induced by resonance coupling of the transition dipoles of retinal and the lowest energy transitions of the aromatic groups of the apoprotein, opsin. A mechanism in which atropisomers of retinal are preferentially bound by opsin is consistent with the present results. The optical activity of the prosthetic group is markedly changed upon solubilization of the mem-

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brane by detergent. This change in optical activity is probably coupled to changes in conformation of the protein moiety induced by solubilization.

Key words: Conformation – Rhodopsin – Membranes – Optical activity – Absorption.

Introduction

A central problem in the chemistry of visual systems is understanding how the absorption of visible light by photoreceptor pigments is coupled to the production of neural signals. In vertebrates, hyperpolarization of the photoreceptor plasma membrane is the first detectable neural event and is probably initiated by changes in ionic conductance of the membrane composing the photoreceptor structure (Hagins, 1972). Presently, the origin of conductance changes is not understood in terms of changes in the molecular structure of the membrane components. Since the visual pigment, rhodopsin, constitutes a large and integral part of vertebrate rod outer segment (ROS) membranes, it is generally thought that conformational changes in the structure of rhodopsin would strongly influence membrane conductance and thus play a central role in visual excitation (e.g., Abrahamson and Wiesenfeld, 1972; Hagins, 1972; Heller, 1972; Kropf, 1972; Morton, 1972; Poo and Cone, 1973; Wald, 1973; Williams, 1975).

In the present study, systematic spectral measurements were made to unify and extend what is currently known about the conformation of rhodopsin and the effect of illumination on conformation. Circular dichroism (CD), optical rotatory disperson (ORD), and absorption spectra were recorded throughout the accessible ultraviolet and visible regions. Sonicated bovine ROS membranes were used to preserve the structural integrity of the membrane at the molecular level while minimizing light scattering artifacts. The effects of various detergents were also investigated. In each wavelength region, the spectra of detergent solubilized ROS were compared to the spectra of sonicated ROS. Spectra recorded between 185 nm and 250 nm were used, in part, to probe the protein secondary structure. Extension of the spectra to the lowest possible experimental wavelength, 185 nm, permitted a more reliable description of the secondary structure and of any light-induced changes which might occur. The Kronig-Kramers transform was applied to CD data obtained between 185 nm and 250 nm and the resulting calculated ORD compared to ORD spectra recorded in the same region. Information about the existence of optically active transitions with maxima below 185 nm and associated with membrane lipid and/or protein aromatic and cystine residues was thus obtained (Cassim and Yang, 1970; Imahori and Nicola, 1973). Spectra recorded between 250 nm and 300 nm were used to probe the interaction of local protein environments with aromatic residues and disulfide bonds. The reproducibility of recorded spectra was also investigated so that detection limits for possible light-induced changes in secondary structure and aromatic-cystine residue environment could be clearly specified. Spectra recorded between 300 nm and 650 nm were used to probe interactions between the prosthetic group, 11-cis-retinal, and the apoprotein, opsin. Possible mechanisms for the induction of optical activity in the retinal prosthetic group were evaluated by comparison of the ultraviolet and visible CD spectra before and after illumination.

Experimental Procedure

Isolation of Bovine ROS Membranes

Bovine ROS membranes were prepared by modification of the method according to McConnell (1965). This method results in a suspension of ROS membranes substantially devoid of mitochondrial and microsomal contamination. A typical procedure is described as follows. 100 fresh bovine eyes were obtained from a local slaughter house. The excised retinas were immersed in 100 ml of 45% (w/v) sucrose, 0.01 M tris-acetate (pH 7.5) and either frozen for later use or immediately processed. The retinas were then briefly homogenized in the sucrose, tris-acetate medium and repeatedly centrifuged at 1100 g for 15 min until no cell debris was sedimented. The supernatant was then diluted with two volumes of 0.01 M tris-acetate and centrifuged at 3000 g for 15 min. The pellet was resuspended in 1.10 density sucrose, 0.01 M tris-acetate, homogenized vigorously, and layered on top of three 34 ml capacity tubes containing a linear 1.12–1.14 sucrose density gradient. The tubes were then centrifuged at 90,000 g for 60 min in a Beckman Spinco model L centrifuge using a type SW 25.1 swinging bucket rotor. The red band at the top of the gradient was retained. Electron micrographs show that this material consists primarily of truncated rod and individual disc fragments (McConnell et al., 1968). The ROS suspension was then diluted by approximately 30 volumes of 0.01 M potassium phosphate buffer (pH 7.00) in which rod fragments undergo osmotic swelling and further disruption of lamellar structure (Brierley et al., 1968). The ROS membranes were finally sedimented at 35,000 g for 30 min. The pellet was then rinsed three times and taken up in 20–30 ml of 0.01 M potassium phosphate buffer (pH 7.00). The yield from 100 retinas was 80–120 mg protein and 1.0–1.5 micromoles rhodopsin chromophore. All operations were carried out in darkness or in dim red light. Solution temperatures were maintained at 0–2° C. The ROS preparations were normally frozen and maintained at −20° C until use.

Assay for Protein and Rhodopsin

Protein was determined using the Biuret reagent (Gornall et al., 1949) with crystalline bovine serum albumen as standard. Rhodopsin concentration was determined by illumination of ROS samples in the presence of 0.1 M hydroxylamine (pH 7.00). Rhodopsin concentration was then calculated from the change in absorbance at 498 nm using the molecular extinction coefficient of 40,600 determined by Wald and Brown (1953).

Purity of Isolated ROS Membranes

The ratio of absorbance at 279 nm and 498 nm (A_{279}/A_{498}) of ROS membranes solubilized in detergent solution is a measure of the relative contamination by proteins with no prosthetic group (including opsin). The A_{279}/A_{498} ratio of 17 preparations used in this study ranged from 2.0–3.2 (ROS in 1% Emulphogene). A smaller

range of ratios (2.0–2.5) is reported by de Grip et al. (1972) and was obtained only after residual opsin protein was converted to the chromophore form (rhodopsin) by incubation with excess 11-cis-retinal. Lower ratios are reported only for chromatographically purified detergent extracts of ROS membranes (e.g., Heller, 1968; Shichi, 1970; Daemen et al., 1972).

The rhodopsin protein content of each of the ROS preparations used in this study was calculated from the assayed concentrations of the rhodopsin chromophore and the total protein, assuming a molecular weight of 38,850 for the protein moiety of bovine rhodopsin (Daemen et al., 1972). For preparations in which frozen retinas were used, the estimated rhodopsin protein content was 36.9% ± 4.6% of the total protein (average of 9 preparations). For preparations in which fresh retinas were used, the estimated rhodopsin protein content was 54.7% ± 5.6% of the total protein (average of 8 preparations). Recent investigations indicate that 80–90% of the total protein present in bovine rods is either opsin or rhodopsin (Daemen et al., 1972; Heitzmann, 1972; Papermaster and Dreyer, 1974). Rhodopsin, alone, usually constitutes less than this maximum amount in bovine ROS preparations not incubated with 11-cis-retinal (de Grip et al., 1973). A sample of one preparation used in this study and made from fresh retinas was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (kindly undertaken by Hermann Kühn of the Institut für Neurobiologie der KFA, Jülich, West Germany). The electrophoretic pattern indicated that rhodopsin and opsin together comprised greater than 80% of the total protein. Thus the non-rhodopsin protein component of ROS preparations used in this study probably consisted primarily of opsin.

The A_{400}/A_{498} ratio, an indicator of contaminants absorbing in the visible spectral region, varied in the range 0.20–0.26 for preparations used in this study. Lower ratios are reported only for chromatographically purified detergent extracts of ROS membranes (e.g., Heller, 1968; Shichi, 1970; Daemen et al., 1972).

Preparation of Samples for Spectroscopy

ROS membrane suspensions in 0.01 M phosphate buffer (pH 7.00) were first diluted to approximately two times the final concentration and then sonicated for 60 s at maximum power with either an Artek Sonic Dismembrator Model 150 or a Fisher Ultrasonic Probe Model SS-2. Solutions were kept on ice during the sonication procedure. Sonication in excess of 60 s did not result in further decrease in apparent absorbance at 650 nm (the rhodopsin chromophore does not absorb at 650 nm), indicating that constant particle size was attained. The sonicated ROS membrane suspensions were then diluted to the desired final concentration with 0.01 M phosphate buffer (pH 7.00) and other reagents when required (e.g., detergents, hydroxylamine). In this way, a series of samples was prepared with identical concentrations of membrane components (lipid, rhodopsin, opsin, and remaining minor proteins). The final concentration of phosphate buffer was always 0.01 M. When the pH was adjusted to values other than 7.00, the samples were resonicated for 60 s to reverse as much as possible any tendency of the membrane particles to reaggregate. pH of samples was adjusted with microliter quantities of HCl or KOH to avoid significant (greater than 1%) changes in concentration. All samples were handled in dim red light or darkness.

Illumination of Samples in CD, ORD, and Absorption Studies

Samples were illuminated at room temperature (20–25° C) with intense monochromatic light obtained by passing the beam of a 150 W xenon lamp (Osram) through a high intensity Bausch and Lomb monochrometer with the grating adjusted to pass wavelengths near 500 nm. Bleaching of ROS samples (determined by measurement of the absorbance at 498 nm) was normally complete within 10 s. However, to insure maximum bleaching, samples were illuminated 5 min under these conditions. Sample temperature was constant during the illumination period.

Measurement of CD, ORD, and Absorption Spectra

CD and ORD spectra were recorded with a Cary Model 60 spectropolarimeter equipped with a Model 6003 circular dichroism attachment. The CD mode was calibrated by use of standard solutions of d-10-camphorsulfonic acid in water. The molar ellipticity of d-10-camphorsulfonic acid in water was taken to be +7260 at 290.5, the value established by Cassim and Yang (1969). In recording CD and ORD spectra, the dynode voltage was always less than 0.5 kV. Recording speeds for both CD and ORD spectra were 10 Å/min to 50 Å/min between 185 nm and 300 nm and 50 Å/min to 250 Å/min between 300 nm and 650 nm.

Absorption spectra were measured with either a Cary 14 spectrophotometer or a Cary 118 spectrophotometer equipped with the far ultraviolet modification and the scattered transmission accessory. In the measurement of far ultraviolet spectra with the Cary 118 spectrophotometer, samples were positioned in the normal compartment (sample distant from photomultiplier) rather than in the accessory compartment (sample close to photomultiplier). This spectrophotometer geometry resulted in a much closer correspondence to Beer's Law of a set of absorbance data obtained by measuring the absorbance of the same sonicated ROS membrane sample in a series of cells with different pathlengths. In the normal compartment, the deviation from Beer's Law at 192 nm, was less than 3% at absorbance values near one. In the accessory compartment, the measured absorbance at 192 nm was 14% less than that predicted by Beer's Law at absorbance values near one.

Spectrophotometer cells (Pyrocell Manufacturing Company and Hellma) with pathlengths of approximately 0.1 mm, 0.2 mm, and 1.0 mm were calibrated against a standard 1.000 cm pathlength cell by comparison of either the measured ellipticity of solutions of camphorsulphonic acid at 290.5 nm or the measured absorbance of solutions of alkaline potassium chromate at 372.0 nm.

Recording of CD, ORD, and absorption spectra was normally initiated within 30 min of final dilution of the sample. Preincubation of ROS membranes in the selected detergent did not normally exceed this period with the exception of samples prepared with digitonin. Following mixing of ROS membranes in digitonin solution, the absorbance was observed to slowly decrease at wavelengths shorter than 250 nm and at a temperature of 25° C until an apparent equilibrium was achieved after 2–4 h. Consequently, spectra of samples containing digitonin were recorded after a preincubation of 4 h at 25° C. All other samples achieved spectral stability within the 30 min time period as judged by consecutive scans of the spectrophotometer or

spectropolarimeter. Following sample illumination, recording of spectra was normally initiated within 5 min. As a standard procedure, the spectrum of each sample, both before and after illumination, was recorded two times in order to increase precision and to monitor sample stability.

The recorded CD spectra of sonicated ROS samples in the visible region (300–650 nm) usually demonstrated positive ellipticity at wavelengths longer than 620 nm (where the rhodopsin chromophore does not absorb) perhaps arising from differential light scattering of the membrane particles. For this case only (spectra of sonicated ROS between 300 nm and 650 nm), the recorded spectra were linearly adjusted so that the measured ellipticity between 620 nm and 650 nm was zero.

All samples were maintained at 25° C during the recording of spectra.

Presentation of CD, ORD, and Absorption Data

All experimental measurements were made on suspensions of ROS membranes or detergent solubilized ROS membranes in which the concentration of the predominant molecular species, rhodopsin,varied from preparation to preparation and never exceeded an estimated 64% of the total membrane protein. Furthermore, substantial disagreement exists concerning the molecular weight of rhodopsin. [Values range from 26,400 (Heller, 1968) to 40,000 (Hubbard, 1954)]. Consequently, the CD spectra are presented in units of degrees ellipticity and are not corrected for molecular weight. In the section of this study in which ORD is calculated from CD data and compared to experimental ORD, the measured ellipticity and rotation have been corrected for pathlength of the spectrophotometer cell used. The units expressed are then degrees per decimeter in both the CD and ORD spectra. Tabulated CD data, obtained in the visible spectral region and exclusively associated with rhodopsin chromophore transitions, are expressed as molecular ellipticity $[\theta]$, in units of deg · cm^2/decimole.

$[\theta] = \dfrac{\theta^\circ \mathrm{M}}{10\,LC}$ where θ° is the

measured ellipticity in degrees, M is the gram molecular weight, L is the pathlength in cm, and C is the concentration in grams per cm^3. (The molar concentration of the rhodopsin chromophore can be obtained with high precision for each ROS preparation, in contrast to the case for the membrane protein molecules.)

The rotational strength R of CD bands associated with chromophore transitions in the visible region was calculated from $R = 1.33 \times 10^{-4}\ [\theta]_{max}\ \Delta/\lambda_{max}$ where $[\theta]_{max}$ is the maximum molecular ellipticity, Δ is the half band width at $1/e[\theta]_{max}$, and λ_{max} is the corresponding ellipticity maximum (Moscowitz, 1960). R is expressed in units of Debye magnetons (one Debye magneton equals 0.927×10^{-38} erg-cm^3).

Transformation of CD to ORD

ORD spectra were calculated from experimental CD spectra obtained in the region, 185–260 nm, by application of the Kronig-Kramers transform equation.

$$\alpha_{\lambda}^{*\text{calc}} = 2/\pi \int_{\lambda_1'}^{\lambda_2'} [\theta^*(\lambda')]\,[\lambda'/(\lambda^2 - \lambda'^2)]\,d\lambda'$$

In this form of the equation, molar concentration factors have been cancelled out. In addition, the theoretical limits of integration, 0 and ∞, are approximated by λ_1' and λ_2', which are the limits of the wavelength interval in which CD was measured.

The experimental CD at different wavelengths, $\theta^*(\lambda')$, is expressed in units of degrees per decimeter, obtained by dividing the measured ellipticity data by the cell pathlength. Consequently, the calculated rotation, $\alpha_{\lambda}^{*\text{calc}}$, is also expressed in units of degrees per decimeter and can be compared only to experimental ORD obtained for a sample identical to that used in the CD measurements. Data points were taken at intervals of 0.5 nm. Evaluation of the Kronig-Kramers equation was accomplished with a revised version of a computer program written by Thiery (1969). Further discussion of the assumptions involved in this calculation and details of the computational procedure are given by Cassim and Yang (1969, 1970).

Reagents

Several detergents were used in this study: Triton X-100 (polyoxyethylene p-t-octyl phenol, Rohm and Hass), Emulphogene BC-720 (polyoxyethylene isoalcohol, General Aniline and Film Corporation), CTAB (cetyltrimethylammonium bromide, Eastman), CTAF (cetyltrimethylammonium fluoride, prepared from cetyltrimethylammonium hydroxide, Eastman), and digitonin (Nutritional Biochemical Company). Triton X-100, Emulphogene, and CTAB were used without further purification. Digitonin was recrystallized repeatedly from ethanol until absorbance in the far ultraviolet (185–220 nm) was reduced to the minimum attainable value. CTAF was prepared by adding excess HF to an aqueous solution of cetyltrimethylammonium hydroxide. The solution was then concentrated by evaporation at 60° C and CTAF recovered by crystallization at 0° C. CTAF was then recrystallized from ethanol several times until minimal absorbance in the far ultraviolet was attained.

In 1% solution, CTAF precipitated at pH values above 5.0. Consequently, samples which contained 1% CTAF were always adjusted to pH 5.0. Samples containing either Emulphogene or digitonin were stable over the pH range, 5.0–9.0.

Results

The absorption spectrum of ROS membranes solubilized in 1% Emulphogene is shown in Figure 1. This spectrum identifies the regions associated with different electronic transitions of rhodopsin. The visible region, 300–650 nm, shows band maxima at 498 nm and 340 nm which are attributed to π-π^* transitions of the prosthetic group, retinal (Abrahamson and Wiesenfeld, 1972). The far ultraviolet region, 185–250 nm, is characterized by an intense band at 191.0 nm and a weaker incompletely resolved band near 227 nm. These bands arise, respectively, from the π-π^* and n-π^* transitions associated with the peptide bonds of the membrane protein (Gratzer, 1967). The near ultraviolet region, 250–300 nm, shows a band maximum at 279 nm. Some fine structure is evident on the short wavelength side of the

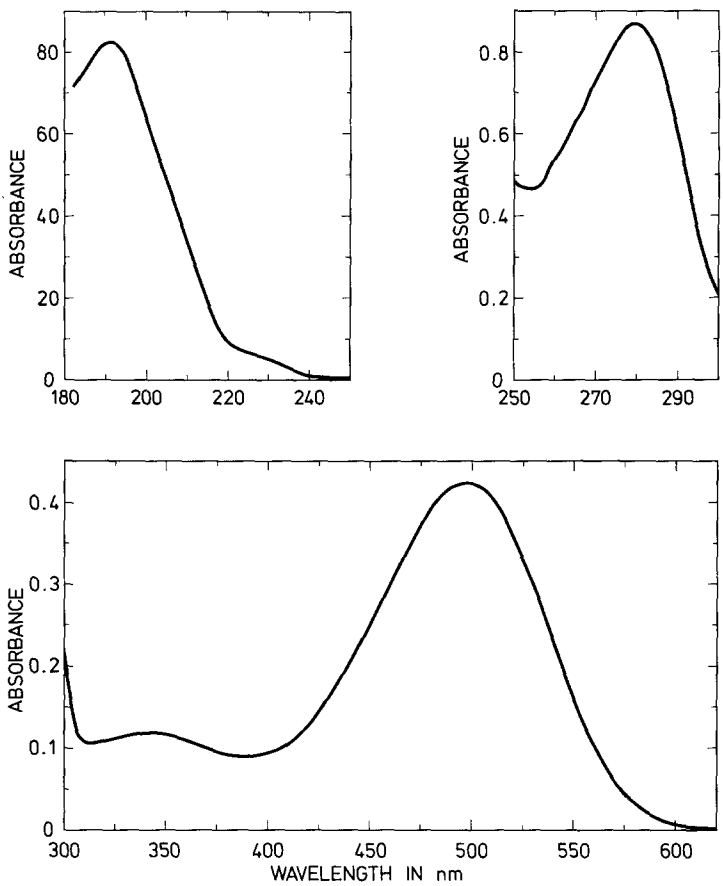

Fig. 1. Absorption of ROS in 1% Emulphogene. All experimental absorbance data were transformed to correspond to absorbance measured with a spectrophotometer cell of 1.00 cm pathlength. Rhodopsin concentration 10.44 nmoles/ml. Protein concentration 0.632 mg/ml. pH 7.00

maximum in high resolution recordings. Absorption in this region is primarily attributed to π-π^* transitions of the aromatic protein residues, tryptophan, tyrosine, and phenylalanine, and to n-σ^* transitions of cystine. Of these residues, tryptophan and tyrosine make by far the strongest contributions (Wetlaufer, 1963). Although obscured by intense peptide bond absorption, higher energy transitions of the aromatic residues and cystine undoubtedly also contribute to absorption in the far ultraviolet (Wetlaufer, 1963). Similarly, a small but significant contribution to both the near and far ultraviolet is very likely made by higher energy transitions of retinal (Erickson and Blatz, 1968; Ebrey and Honig, 1972).

CD and Absorption in the Visible Region

In the present experiments, ROS membranes were either sonicated or directly solubilized by various detergents. To determine if these treatments result in loss of rho-

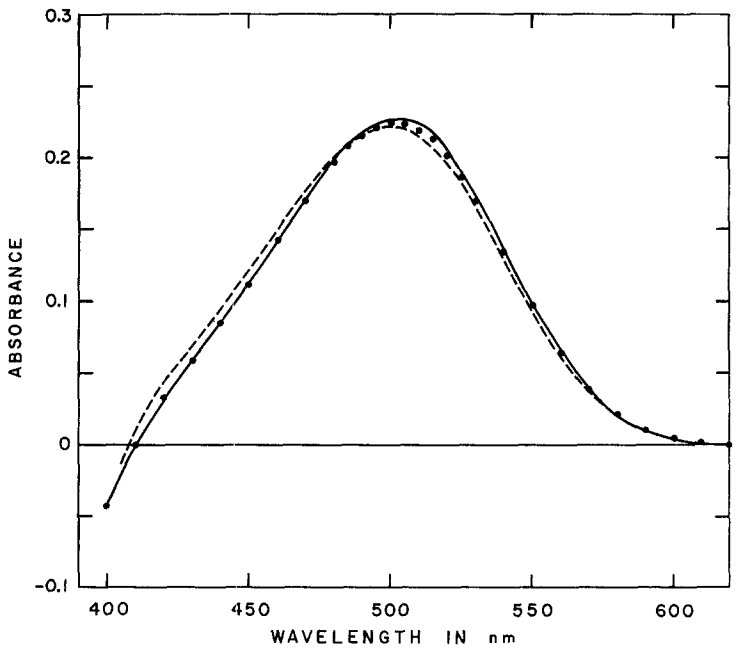

Fig. 2. Difference absorption spectra of untreated, sonicated and detergent solubilized ROS in the visible. Spectra were obtained by subtraction of sample absorbance before and after illumination in the presence of 0.1 M hydroxylamine. All samples were prepared by identical dilution of one stock ROS preparation. Sonication or solubilization in 1% Emulphogene does not result in major decrease in the concentration of rhodopsin chromophore. Untreated ROS (——). Sonicated ROS (●, closed circles indicate data points). ROS in 1% Emulphogene (– – –). Rhodopsin concentration 5.52 nmoles/ml. Protein concentration 0.632 mg/ml. pH 7.00. Optical pathlength 1.00 cm

dopsin, difference absorption spectra of ROS samples, before and after illumination, were obtained (Fig. 2). Samples were prepared in which ROS membranes were untreated, sonicated, and solubilized in 1% Emulphogene. The use of difference spectra cancels out the effect of light scattering on the recorded absorbance and allows the relative chromophore concentrations to be directly measured. (The product of illumination, all-trans-retinaloxime, does not absorb appreciably at the absorption maximum of rhodopsin.) The decrease in maximum absorbance in the spectrum of ROS membranes is 1.3% upon sonication and 2.2% upon detergent solubilization, indicating that the respective treatment results in little loss of rhodopsin. Difference spectra of ROS solubilized in 1% Triton X-100, 1% digitonin and 1% CTAF were identical to the difference spectrum in 1% Emulphogene. This analysis assumes that the molar extinction coefficient of the chromophore is not significantly affected by the treatments. Changes in light scattering in the particulate samples before and after illumination were negligible since changes in apparent absorbance at 650 nm (where rhodopsin does not absorb) were small (less than 1%) when compared to the change at 498 nm.

Figure 3 presents the CD spectra of sonicated and of detergent solubilized ROS membranes. Corresponding spectral parameters are given in Table 1. In contrast to

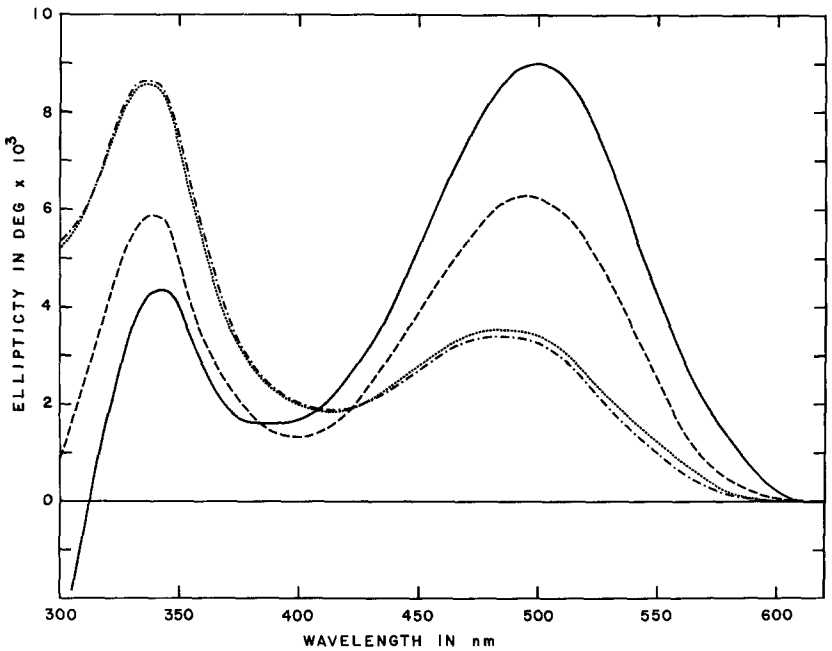

Fig. 3. CD of sonicated and detergent solubilized ROS in the visible, before illumination. All samples were prepared by identical dilution of one stock ROS preparation. Solubilization of ROS results in decrease in intensity of the long wavelength band near 498 nm and an increase in intensity of the short wavelength band near 340 nm. Sonicated ROS, pH 7.00 (——). ROS in 1% digitonin, pH 7.00 (– – –); in 1% Emulphogene, pH 7.00 (· · · · ·); in 1% CTAF, pH 5.00 (– · – · –). Rhodopsin concentration 13.2 nmoles/ml. Protein concentration 0.922 mg/ml. Optical pathlength 1.00 cm

Table 1. Circular dichroism data in the visible spectral region of sonicated ROS membranes before illumination at pH 7.00 and 25° C

Composition of media	Wavelength maxima in nm	Molecular ellipticity[a] in deg · cm^2/decimole × 10^{-4}	Rotational strength in Debye magnetons	
			Individual bands	Sum of two bands
Aqueous buffer	499	6.80[b]	1.15	1.47
	342	3.30	0.32	
1% Emulphogene, aqueous buffer	483	2.67	0.50	1.54
	337	6.48	1.04	
1% CTAF, aqueous buffer[c]	483	2.58	0.46	1.53
	337	6.53	1.07	
1% Digitonin, aqueous buffer	494	4.76	0.79	1.35
	339	4.45	0.56	

[a] The computation of molecular ellipticity assumed a molar extinction coefficient for the rhodopsin chromophore of 40,600 at 498 nm

[b] The calculated standard error for this datum was $\pm$ 0.20 × 10^4 based on the measured ellipticity of samples obtained from three different ROS preparations

[c] pH 5.00

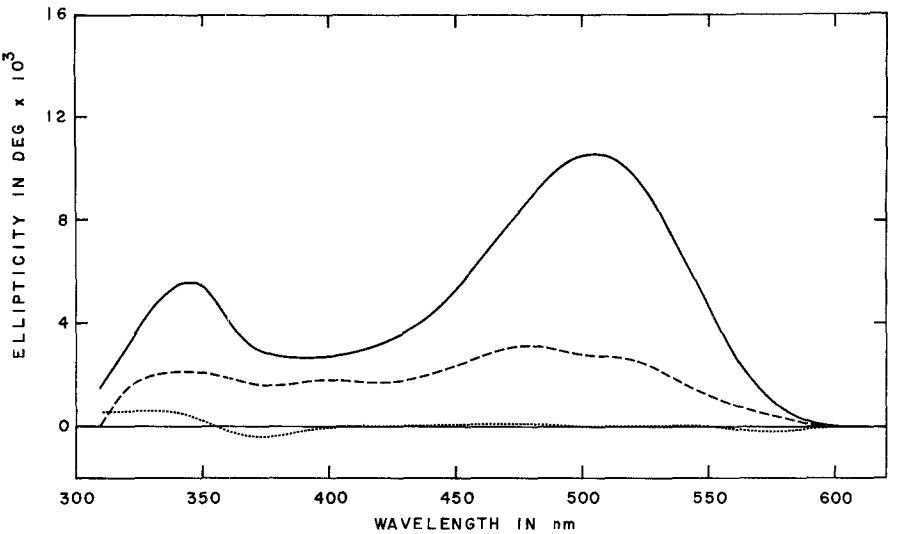

Fig. 4. CD of sonicated ROS in the visible, before and after illumination. Illumination in the absence of hydroxylamine results in diminished CD with non-specific band shape. Illumination in the presence of 0.1 M hydroxylamine results in essential loss of CD. Sonicated ROS before illumination (—); after illumination (– – –); after illumination in the presence of 0.1 M hydroxylamine (· · · ·). Rhodopsin concentration 14.8 nmoles/ml. Protein concentration 1.27 mg/ml. Optical pathlength 1.00 cm. pH 7.00

the absorption spectra of detergent solubilized ROS, the CD spectra of both sonicated and detergent solubilized ROS exhibit relatively intense bands at about 340 nm. The effect of detergent solubilization is to decrease the relative magnitude of the longer wavelength band (maximum between 483 nm and 499 nm) and to simultaneously increase the magnitude of the shorter wavelength band. The influence of 1% Emulphogene and 1% CTAF on the relative magnitudes of the two bands is most pronounced. 1% digitonin has an intermediate effect. The sum of the rotational strengths of the longer wavelength band and the shorter wavelength band is essentially independent of detergent solubilization and choice of detergent. The CD spectra of ROS membranes solubilized in 1% CTAB and 1% Triton X-100, have also been measured and are identical to the spectrum of ROS solubilized in 1% Emulphogene. In the CD spectra, detergent solubilization promotes a blue shift in the maximum of the longer wavelength band (499 nm for sonicated ROS membranes), the magnitude of which depends on the detergent used. This result contrasts with that found for the corresponding absorption spectra. The absorption maximum of the longer wavelength band of sonicated ROS membranes is 498 nm when the spectrum is recorded with the sample positioned close to the photomultiplier. The absorption maximum of this band is not significantly different (within 1 nm) for ROS membranes solubilized in any of the detergents used in this study.

When ROS membranes solubilized in detergent are illuminated with intense monochromatic light (500 nm) at 25° C, CD in the visible region vanishes. This loss of induced optical activity indicates that there are no dissymmetric interactions of the illumination products (primarily hydrolysed all-trans-retinal) with the ROS protein.

When measured about 20–30 min after illumination, the CD of sonicated ROS membranes shows a non-specific band shape and a lower intensity compared to that before illumination (Fig. 4). Absorption spectra of a similar sample, recorded in this time interval, indicate the presence of a mixture of photo-products, consisting primarily of metarhodopsin, pararhodopsin, and hydrolyzed retinal. In addition, a difference absorption spectrum of the illuminated sample, before and after the addition of 0.1 M hydroxylamine shows that approximately 10% of the original rhodopsin remains, in the illuminated sample, as a mixture of photo-regenerated rhodopsin and isorhodopsin. Since pararhodopsin (Yohizawa and Horiuchi, 1973) and free retinal are not optically active, any residual CD probably derives from metarhodopsin II, photo-regenerated rhodopsin and isorhodopsin, and unspecified binding products of previously hydrolyzed retinal and the membrane protein. When sonicated ROS membranes are illuminated in the presence of 0.1 M hydroxylamine, the CD in the visible region is not significantly different from the solvent baseline indicating complete loss of optically active products (Fig. 4). Absorption spectra show that virtually 100% of the original rhodopsin is lost under these conditions.

CD and Absorption in the Far Ultraviolet Region

CD and absorption spectra were recorded for both suspensions of sonicated ROS membranes and detergent solubilized ROS membranes. Spectral data are collected in Table 2.

The CD and absorption spectra of sonicated ROS membranes before and after illumination are presented in Figures 5 and 6, respectively. These spectra are characteristic of membrane suspensions which possess a protein component with partial α-helical secondary structure (Glaser and Singer, 1971; Schneider et al., 1970; Urry et al., 1970; Urry, 1972). The CD spectrum consists of three bands; one positive with a maximum at 194.2 nm and two negative with minima at 211.5 nm and 223.5 nm. In the absorption spectrum, bands corresponding to the 211.5 nm and 223.5 nm CD bands are not distinctly resolved and appear as shoulders on the long wavelength side of the 193.0 nm band. The CD band extrema at 194.2 nm and 223.5 nm are slightly shifted to longer wavelengths compared to the extrema of α-helical polyglutamic acid (191 nm and 222 nm, respectively; Cassim and Yang, 1970). The ratio of absolute magnitude of the 194 nm band to the 223.5 nm band is 1.8, indicating a relative depression of the 194 nm band compared to helical polyglutamic acid (band ratio is 2.2; Cassim and Yang, 1970). The origin of CD and absorption bands are considered to be the same both for particulate systems containing helical protein and for soluble helical polypeptides (Schneider et al., 1970; Urry, 1972). For helical polypeptides, bands near 191 nm and 210 nm are thought to arise by exciton splitting of the amide π-π^* (NV_1) transition (Gratzer, 1967; Sears and Beychok, 1973). Thus, the origin of the two shorter wavelength bands in the CD and absorption spectra of sonicated ROS is probably the same and is a consequence of the partial helical structure of the ROS membrane protein. The origin of the CD band near 222 nm (absorption band near 227 nm) is probably the amide n-π^* transition (Gratzer, 1967; Sears and Beychok, 1973). The relative intensity of the n-π^* transition is clearly less in the absorption spectrum of sonicated ROS than in the corre-

Table 2. Far ultraviolet spectral data for sonicated ROS membranes at pH 7.00 and 25° C[a]

Composition of media	Wavelength extrema in nm		Crossover wavelength in nm (CD)	Ellipticity[d] in deg × 10^3	% Decrease in absolute ellipticity on illumination	Absorbance[d]	% Increase in absorbance on illumination
	CD	Absorption					
	223.5	227[c]		− 6.91	0.4[e]		
Aqueous buffer	211.5[c]	210[c]		− 5.52			
			202.8	0			
	194.2	193.0		12.41	0.9[e]	0.830	0.04[e]
	221.5	227[c]		− 6.97	20.9		
1% Emulphogene,	211.5	210[c]		− 6.40			
aqueous buffer			202.7	0			
	193.5	191.0		12.47	19.9	0.894	2.40
	222.5			− 6.71	15.9		
1% CTAF,	209.5			− 6.48			
aqueous buffer[c]			202.2	0			
	194.2			12.36	13.8		
	221.5	227[c]		− 7.61	0.0[e]		
1% Digitonin,	210.5	210[c]		− 7.05			
aqueous buffer			202.3	0			
	192.8	191.5		14.32	0.9[e]	0.866	0.66

[a] Samples for which CD data were collected were made by identical dilution of one stock ROS preparation. Final rhodopsin concentration 8.92 nmoles/ml. Final protein concentration 0.605 mg/ml. Optical pathlength 0.116 mm. Samples for which absorption data were collected were made by identical dilution of a different stock ROS preparation. Final rhodopsin concentration 4.94 nmoles/ml. Final protein concentration 0.345 mg/ml. Optical pathlength 0.205 nm

[b] pH 5.00

[c] Shoulder

[d] Ellipticity and absorbance values are for samples before illumination

[e] These data do not significantly differ from zero. Under conditions in which the CD and absorption spectra from 185–260 nm were recorded, the absolute deviation of ellipticity or absorbance was determined from the difference of spectra obtained by two consecutive scans on the same sample. The absolute deviation of ellipticity near 223 nm and 193 nm was typically 2% and 3%, respectively. The absolute deviation of absorbance near 193 nm was typically 0.1%

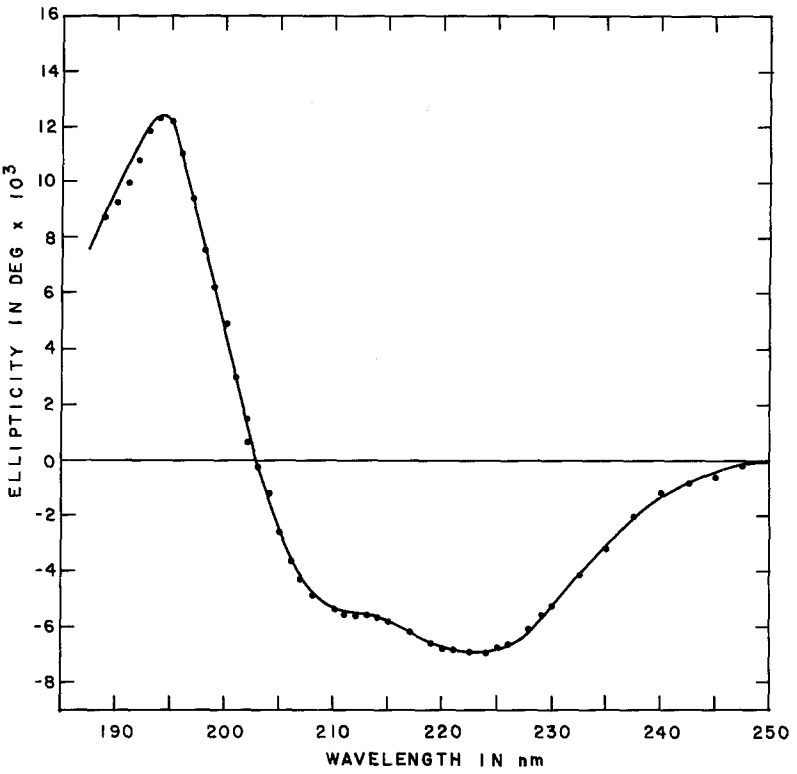

Fig. 5. CD of sonicated ROS in the far ultraviolet, before and after illumination. The spectrum is insensitive to illumination indicating stability of protein secondary structure. Sonicated ROS before illumination (—); after illumination (●, closed circles indicate data points). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 0.116 mm. pH 7.00

sponding CD spectrum. This pattern is also typical of proteins with partial helical structure (Gratzer, 1967; Adler et al., 1972).

The mean residue molecular ellipticity at 223.5 nm for sonicated ROS membranes was calculated to be $-11{,}000 \pm 250$ deg · cm^2/decimole from the known ellipticity, total protein content, and pathlength of three samples, each obtained from different ROS preparations, and from an estimate of the mean residue molecular weight of the ROS protein. [The assumed mean residue molecular weight was 113.6 g/mole · residue and was calculated from the amino acid analysis reported by de Grip et al. (1973) for total ROS protein.] Assuming that the extremum mean residue ellipticity is $-32{,}600$ deg · cm^2/decimole for the helix and $-2{,}340$ deg · cm^2/decimole for both the β- and unordered form (Chen and Yang, 1971) and that the secondary structure of the ROS protein consists only of helical, β-, and unordered forms, then the helical content of the ROS protein is estimated to be 29%.

The result of bleaching 80–90% of the rhodopsin on the CD spectrum of sonicated ROS is also shown in Figure 5. No reproducible changes were detected over the entire recorded CD spectrum. The evident stability of the secondary structure of the ROS protein to illumination was examined under experimental conditions which

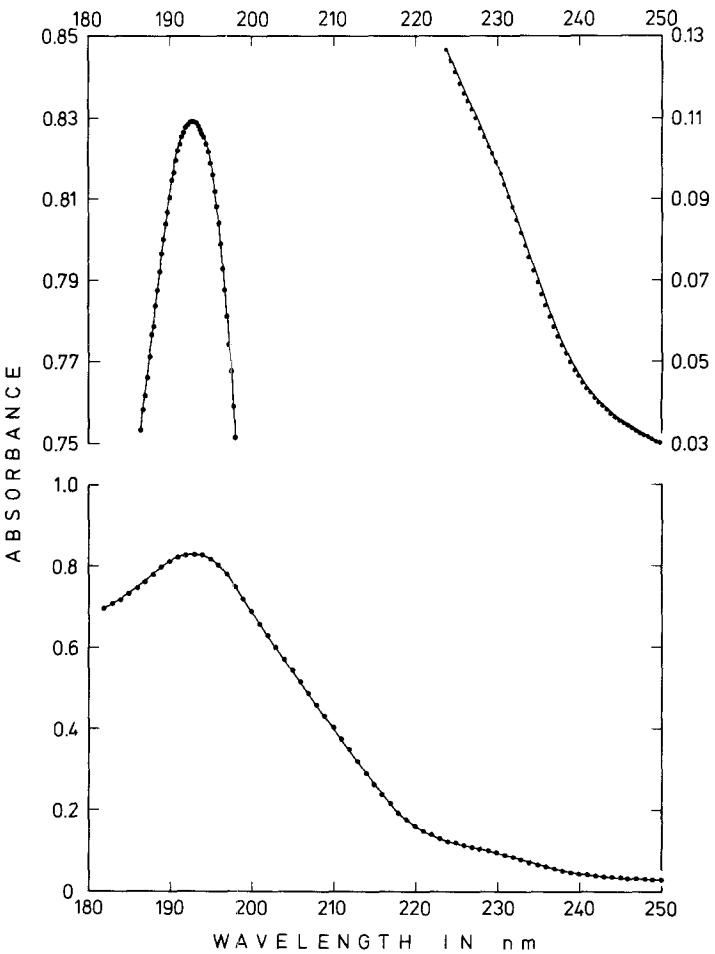

Fig. 6. Absorption of sonicated ROS in the far ultraviolet, before and after illumination. Sections of the spectrum shown in the lower half of the figure are reproduced in a ten fold expanded absorbance scale in the upper half of the figure. In the region of the 193.0 nm maximum, the spectrum is insensitive to illumination indicating stability of protein secondary structure. A small light-induced absorbance change occurs near 235 nm. Sonicated ROS before illumination (—); after illumination (●, closed circles indicate data points). Rhodopsin concentration 4.94 nmoles/ml. Protein concentration 0.345 mg/ml. Optical pathlength 0.205 nm. pH 7.00

provided the highest instrument signal to noise ratio (Fig. 7). Because of sample absorbance, only the 223.5 nm band could be recorded. 0.1 M hydroxylamine was present in the sample to insure maximum bleaching of rhodopsin. (The use of hydroxylamine is not possible in recordings at wavelengths less then 200 nm because of high absorbance of the compound.) The absolute percent deviation of the spectra of the illuminated and unilluminated samples was determined at 1 nm intervals in the wavelength range, 218–250 nm (Fig. 8). As a measure of the reproducibility of CD spectra recorded under these experimental conditions, data points for the absolute percent deviation between two consecutively recorded spectra of the unilluminated

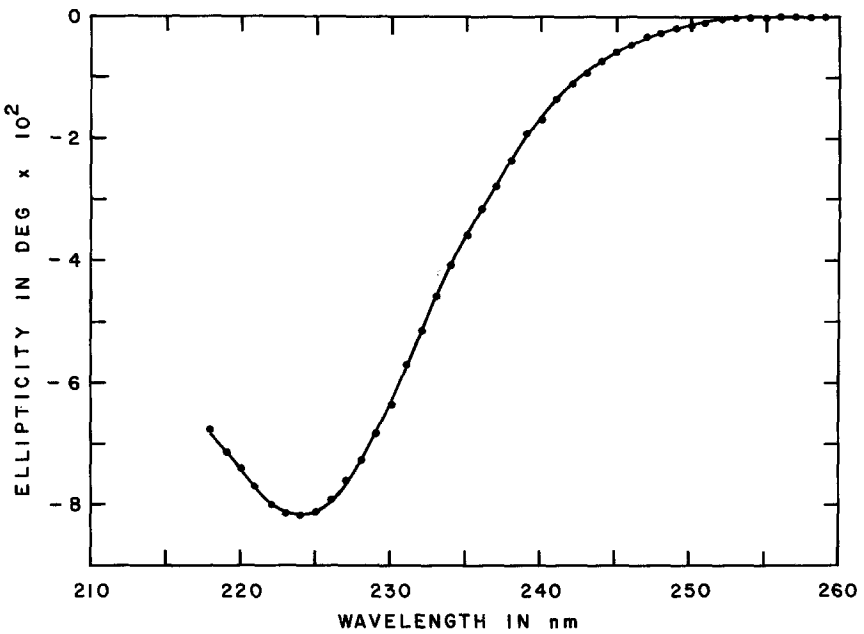

Fig. 7. CD of sonicated ROS in the far ultraviolet, before and after illumination. Spectropolarimeter operating parameters and sample concentration were adjusted to provide maximum reproducibility of the CD of the longest wavelength band shown in Figure 5. Illumination results in no change in the recorded CD indicating stability of protein secondary structure. 0.1 M hydroxylamine was present in the sample to insure maximum bleaching of rhodopsin. Sonicated ROS before illumination (—); after illumination (●, closed circles indicate data points). Rhodopsin concentration 7.36 nmoles/ml. Protein concentration 0.843 mg/ml. Optical pathlength 1.01 mm. pH 7.00

sample are also plotted. The distribution of the data points is indistinguishable for the two cases, showing that within specified limits of experimental reproducibility no light induced changes occur in the recorded CD spectrum. In the neighborhood of the 223.5 nm extremum, the absolute deviation of the recorded spectra before and after illumination was less than 1%. Consequently, the minimum detectible change (if one occurred) would have had to exceed 1% of the measured ellipticity at 223.5 nm. The reproducibility of recorded CD spectra over the extended ultraviolet, 185–250 nm, (Fig. 5) was typically 3% of the measured ellipticity at 193 nm (2% at 223 nm). The effect of illumination on the far ultraviolet CD spectrum of sonicated ROS membranes has been repeated for at least 10 different ROS preparations. No change has ever been observed which could not be attributed to instrument error. When the pH is adjusted to 5.00, the CD spectrum of sonicated ROS membranes still retains its insensitivity to illumination.

In the neighborhood of the 193 nm maximum, the far ultraviolet absorption spectrum of sonicated ROS membranes (Fig. 6) is also insensitive to illumination which resulted in bleaching of 80–90% of the original rhodopsin. The reproducibility of consecutive recordings of the spectrum of the sample before illumination was within 0.0008 absorbance units near 193 nm, corresponding to 0.1% of the maximum absorbance value. A small light-induced decrease in absorbance with a maxi-

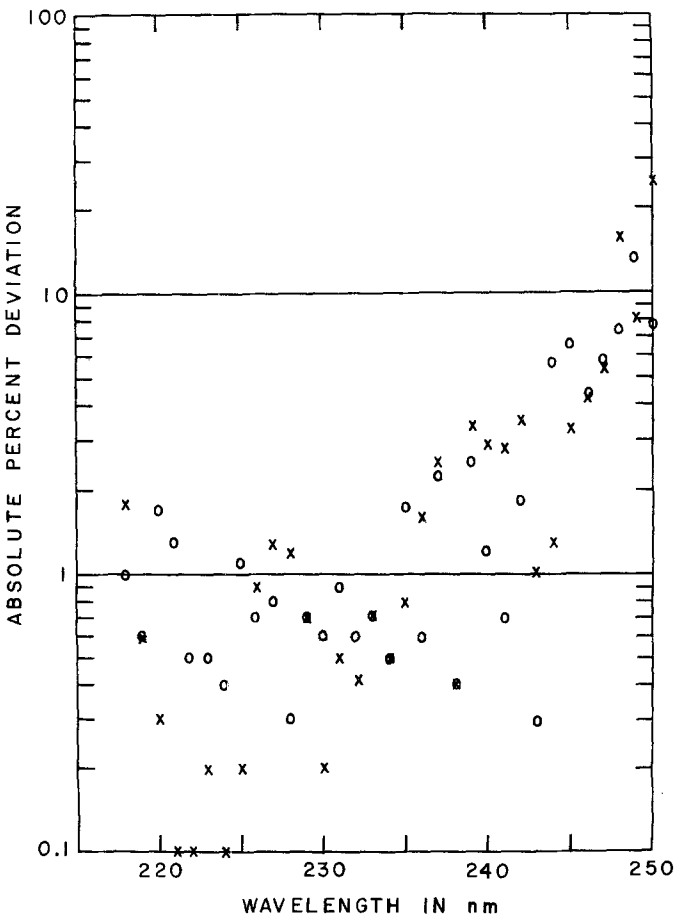

Fig. 8. Reproducibility of the CD of sonicated ROS shown in Figure 7. Open circles (O) indicate data points for the absolute percent deviation of the CD of two consecutive spectropolarimeter scans on the unilluminated sample. Crosses (×) indicate data points for the absolute percent deviation of the CD of the unilluminated sample compared to the CD of the sample after illumination. Data points were taken at 1 nm intervals between 218 nm and 250 nm

mum change near 235 nm was observed in the long wavelength tail of the spectrum.

The CD spectra of ROS membranes solubilized in 1% Emulphogene and 1% digitonin are shown in Figures 9 and 10, respectively. The corresponding absorption spectra are shown in Figure 11. These spectra are characteristic of right-handed α-helical proteins in solution. The observed CD extrema near 194 nm, 210 nm, and 222 nm correspond well with extrema reported for helical polyglutamic acid (191 nm, 209 nm, and 222 nm; Cassim and Yang, 1970). The shortest wavelength band is still slightly red shifted in detergent solubilized ROS as it was for sonicated ROS. The ratio of the absolute magnitudes of the 194 nm band to the 222 nm band is also depressed (1.8) compared to helical polyglutamic acid (2.2). The CD spectrum of ROS membranes in 1% CTAF has also been recorded before illumination

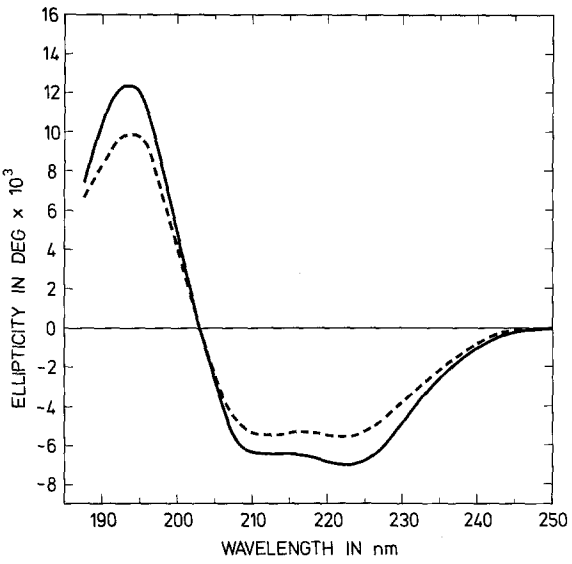

Fig. 9. CD of ROS in 1% Emulphogene in the far ultraviolet, before and after illumination. Sample illumination results in spectral changes characteristic of loss of protein helical structure. ROS in 1% Emulphogene before illumination (—); after illumination (– – –). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 0.116 mm. pH 7.00

and is essentially identical to the CD spectrum of unilluminated ROS membranes in 1% Emulphogene (Fig. 12).

Upon illumination, the CD spectrum of ROS solubilized in 1% Emulphogene undergoes large changes. The intensity of each of the 194 nm, 210 nm, and 222 nm bands is decreased. The per cent decrease in absolute ellipticity at 222 nm is similar to the per cent decrease at 194 nm (Table 2). These spectral changes are indicative of a loss in helical protein structure (Adler et al., 1972; Sears and Beychok, 1973). The CD spectrum of ROS membranes in 1% CTAF shows similar light-induced changes. Conformational stability of the protein secondary structure of ROS in 1% digitonin is variable, apparently dependent on the particular ROS preparation used. Insensitivity to illumination as shown in Figure 10 is the result found for most ROS preparations and is considered to be typical. An occasional preparation (one out of ten) demonstrates light-induced spectral changes characteristic of a loss in helical structure. Incubation of a sample identical to the one used in recording the spectrum of Figure 10 (spectrum insensitive to illumination) for 24 h at 25° C also did not result in detectable changes in the 185–250 nm region on illumination.

In the absorption spectrum of ROS in Emulphogene, an increase in intensity of the 191 nm band and a smaller decrease at wavelengths above 228 nm is observed following illumination, similar to changes characteristic of loss in helical structure in synthetic polypeptides (Gratzer, 1967). Small light-induced changes in the absorption spectrum of ROS in 1% digitonin (decreases maximal near 205 nm and 231 nm and an increase maximal near 191 nm) are not characteristic of loss in helical structure but rather suggest a perturbation of one or more aromatic residues (Donovan, 1969).

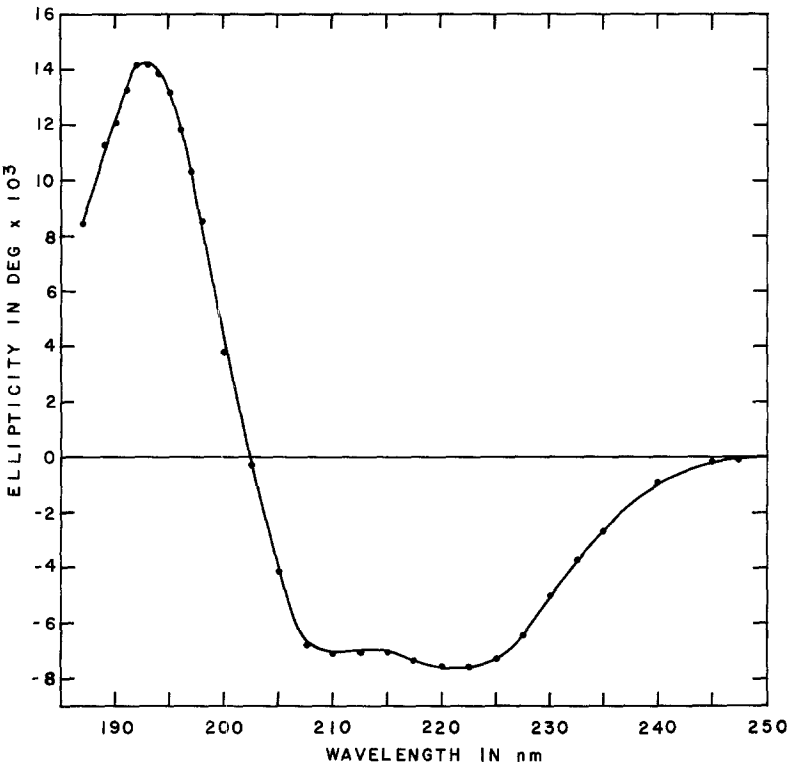

Fig. 10. CD of ROS in 1% digitonin in the far ultraviolet, before and after illumination. The spectrum is insensitive to illumination indicating stability of protein secondary structure. ROS in 1% digitonin before illumination (—); after illumination (●, closed circles indicate data points). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 0.116 mm. pH 7.00

A series of samples was prepared by identical dilution of one stock ROS preparation to permit the direct comparison of the CD spectrum of unilluminated sonicated ROS to the CD spectra of unilluminated ROS solubilized in detergents. The results are given in Figure 12. The relative depression of the 210 nm band of sonicated ROS compared to the 210 nm band of ROS dispersed by detergent is evident. The spectrum of ROS solubilized in 1% digitonin is enhanced compared to the spectra of ROS solubilized in 1% Emulphogene and in 1% CTAF. This enhancement is usually considered as characteristic of greater protein helical content. A comparison of the CD spectrum of digitonin solubilized ROS to the CD spectrum of sonicated ROS shows that the 194 nm and 210 nm bands of sonicated ROS are markedly depressed and the 222 nm band depressed to a lesser extent. Conversely, the long wavelength tail of the CD spectrum of digitonin solubilized ROS is somewhat depressed compared to the tail of the sonicated ROS spectrum. These spectral differences between sonicated ROS and digitonin solubilized ROS are similar to the differences observed between aggregated and dispersed polyglutamic acid (Urry et al., 1970) and intact and fragmented red blood cell membranes (Glaser and Singer, 1971), both of which occur without accompanying helical conformational change.

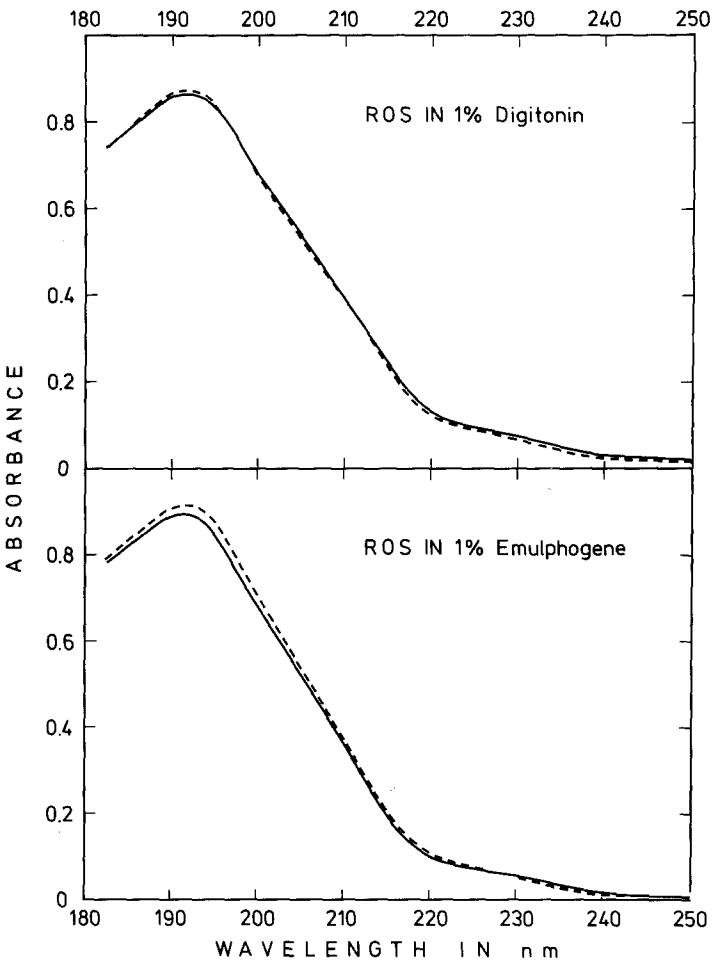

Fig. 11. Absorption of ROS in 1% digitonin and in 1% Emulphogene in the far ultraviolet, before and after illumination. Illumination of the digitonin sample results in small spectral changes characteristic of aromatic residue perturbation. Illumination of the Emulphogene sample results in spectral changes characteristic of loss of protein helical structure. Before illumination (——); after illumination (– – –). Rhodopsin concentration 4.94 nmoles/ml. Protein concentration 0.345 mg/ml. Optical pathlength 0.205 mm. pH 7.00

These observations are essentially confirmed in high signal to noise ratio CD spectra obtained for a similar series of samples in the region of the 222 nm band.

The absorption spectra (before illumination) of a similar series of samples made from a different stock ROS preparation are presented in Figure 13. The differences between these spectra arise primarily from the particulate properties of the samples. Even for samples with 1% Emulphogene, light scattering probably makes a small contribution to the apparent absorbance at wavelengths between 180 nm and 250 nm since a slight non-colored precipitate is formed on centrifugation (30 min at 48,000 g maximum). The presence of 1% digitonin results in only partial solvation of

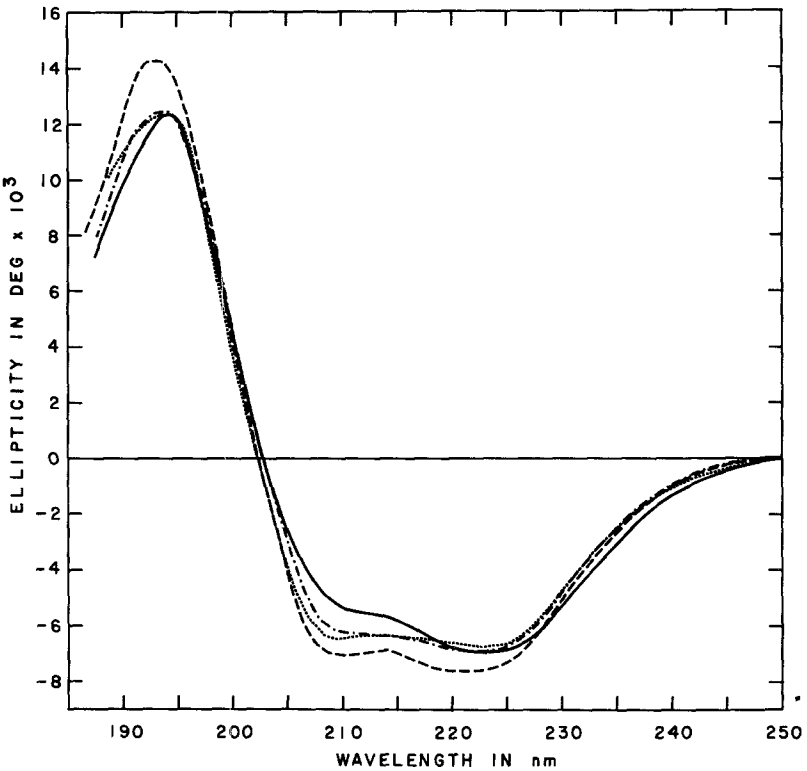

Fig. 12. CD of sonicated and detergent solubilized ROS in the far ultraviolet, before illumination. All samples were prepared by identical dilution of one stock ROS preparation. Sonicated ROS, pH 7.00 (——). ROS in 1% digitonin, pH 7.00 (– – –); in 1% CTAF, pH 4.80 (· · · ·); in 1% Emulphogene, pH 7.00 (– · – · –). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 0.116 mm

the membrane and on centrifugation a red pellet is formed which represents approximately 10–20% of the total rhodopsin in the sample. If the sample with 1% Emulphogene is used as a reference, then the sample with 1% digitonin and the sample without detergent (sonicated ROS) show an increase in apparent absorbance at wavelengths longer than 198 nm and a decrease in apparent absorbance between 180 nm and 198 nm. As expected, the effects are more pronounced for the sonicated ROS suspension. Similar distortions in absorption spectra are seen for aggregated versus dispersed polyglutamic acid (Urry et al., 1970) and for intact versus fragmented red blood cell membranes (Glaser and Singer, 1971).

CD in the Near Ultraviolet Region

The CD spectra of natural proteins in the 250–300 nm region are attributed in part to interaction of local protein environments with tryptophan, tyrosine, phenylalanine, and cystine residues. Phenylalanine and cystine absorb rather weakly in this

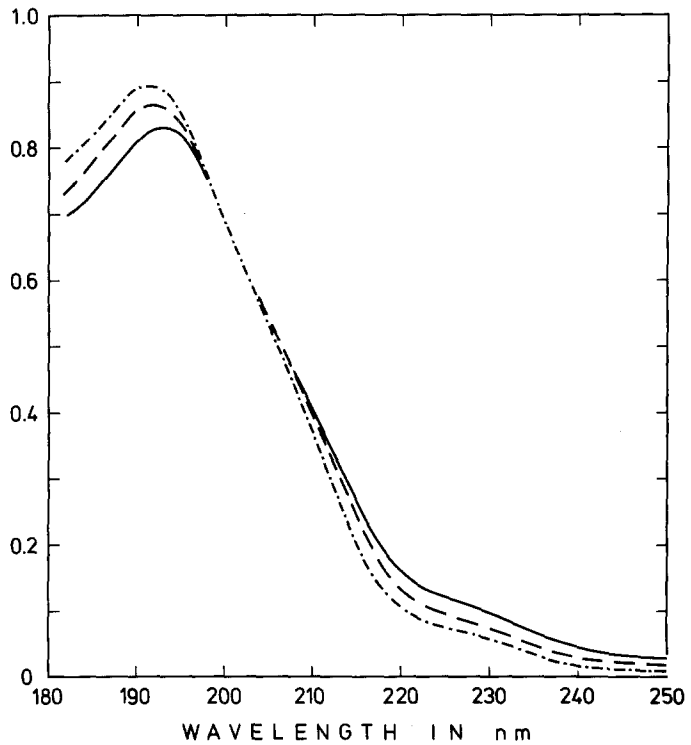

Fig. 13. Absorption of sonicated and detergent solubilized ROS in the far ultraviolet, before illumination. All samples were prepared by identical dilution of one stock ROS preparation. Sonicated ROS (——). ROS in 1% digitonin (– – –); in 1% Emulphogene (– · – · –). Rhodopsin concentration 4.94 nmoles/ml. Protein concentration 0.345 mg/ml. Optical pathlength 0.205 mm. pH 7.00

region; however, they may contribute to a greater extent to the CD spectra of proteins (Timasheff, 1970; Sears and Beychok, 1973).

The spectra of sonicated ROS fragments before and after illumination in the 250–300 nm region are presented in Figure 14. Some fine structure is evident (unilluminated sample 260, 286, 292 nm; illuminated sample 260, 277, 292 nm). Within the limits of spectropolarimeter reproducibility, the CD spectrum is not observed to change on illumination at wavelengths shorter than 280 nm. At wavelengths longer than 280 nm, the CD spectrum is sensitive to illumination. Because of probable overlap of the 340 nm CD band (associated with π-π^* transitions of the prosthetic group, retinal), changes in the CD spectrum in the interval, 280–300 nm, can be attributed, at least in part, to changes in intensity of the 340 nm band observed upon bleaching of the rhodopsin chromophore (see Figs. 3 and 4).

Due to sample absorbance, the signal to noise ratio in the near ultraviolet region is substantially lower than in the far ultraviolet region. Consequently, the coincidence of the spectra before and after illumination at wavelengths shorter than 280 nm is subject to less certainty than in the far ultraviolet region. Reproducibility of the CD spectra of sonicated membranes in the near ultraviolet region is given in Figure 15. The per cent deviation of the spectra of the illuminated and unilluminated

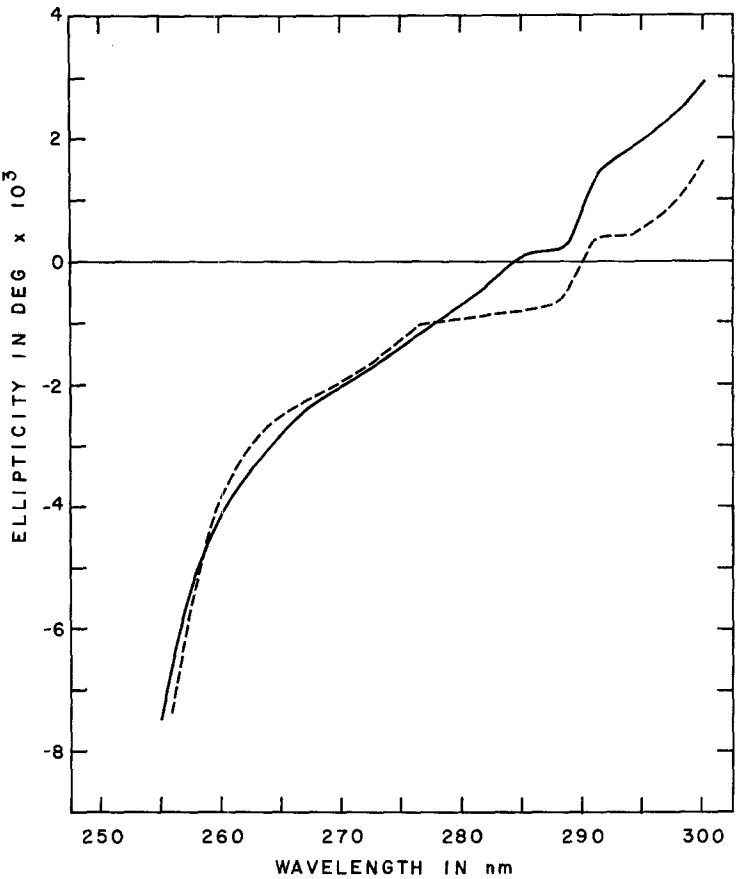

Fig. 14. CD of sonicated ROS in the near ultraviolet, before and after illumination. No reproducible sensitivity to illumination is detected at wavelengths shorter than 280 nm indicating stability of aromatic-cystine residue environment. Sonicated ROS before illumination (—); after illumination (- - -). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 1.00 cm. pH 7.00

sample at wavelengths shorter than 280 nm is similar to that obtained between two consecutive recordings of the spectrum of the unilluminated sample (approximately 10% of the measured ellipticity). This analysis indicates that the small differences between the spectra of the sample before and after illumination at wavelengths shorter than 280 nm are of the same magnitude as the differences produced by instrument error. This anlysis also establishes the magnitude of possible spectral changes, indicative of alteration in the electronic environments of the aromatic/cystine residues, which can be detected under the extant experimental conditions. If the difference in the measured ellipticity of the spectra of Figure 14 is calculated in the wavelength interval, 280–300 nm, slight irregularities (maxima and shoulders in the difference spectrum) are revealed. This fine structure in the CD difference spectrum is of the same magnitude as that produced by instrument error and is not reproducible from sample to sample.

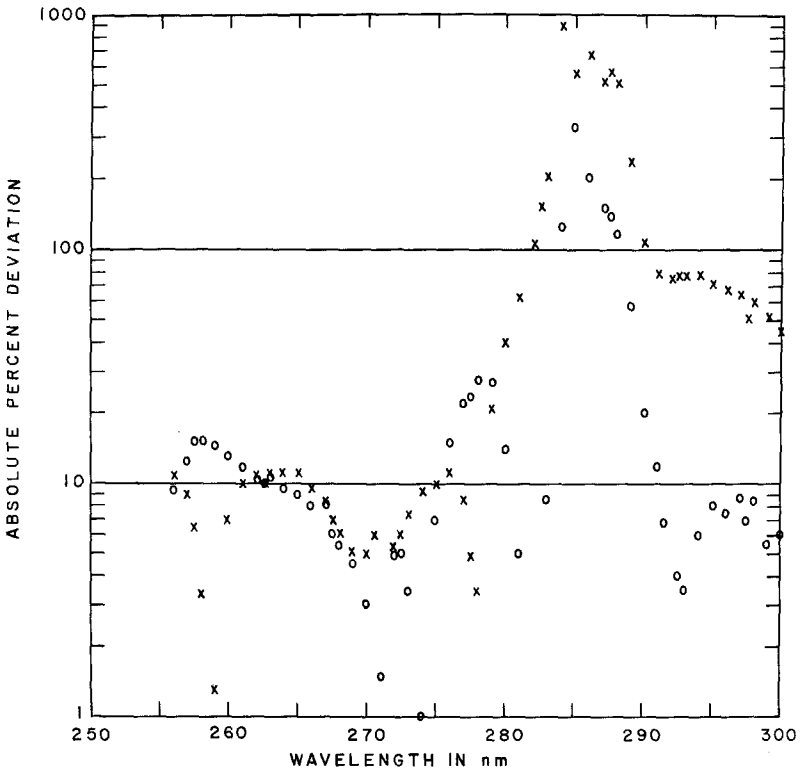

Fig. 15. Reproducibility of the CD of sonicated ROS shown in Figure 14. Open circles (O) indicate data points for the absolute percent deviation of the CD of two consecutive spectropolarimeter scans of the unilluminated sample. Crosses (×) indicate data points for the absolute percent deviation of the CD of the unilluminated sample compared to the CD of the sample after illumination. Data points were taken at 1 nm intervals between 256 nm and 300 nm

After sonication, most ROS preparations yield near ultraviolet CD spectra similar to those in Figure 14. An occasional preparation yields a spectrum in which the predominant feature is a strong positive CD band at 258 nm. At present, the reason for this apparent sample variation is not clear. However, regardless of this variation, illumination has not resulted in significant changes below 280 nm, in any case studied.

The spectra before and after illumination of ROS solubilized in 1% Emulphogene and 1% digitonin are shown in Figures 16 and 17, respectively. A composite of spectra before illumination of sonicated ROS and ROS solubilized in 1% Emulphogene, 1% CTAF, and 1% digitonin is shown in Figure 18. (These samples were obtained by identical dilution of one stock ROS preparation.) The signal to noise ratio was substantially higher in the recording of detergent solubilized ROS spectra than sonicated ROS spectra because of reduction of apparent absorbance due to particulate light scattering. Considerable fine structure is evident in the spectra of the Emulphogene and CTAF samples. The less complex fine structure observed for sonicated ROS samples is due in part to the superimposition of instrument noise and

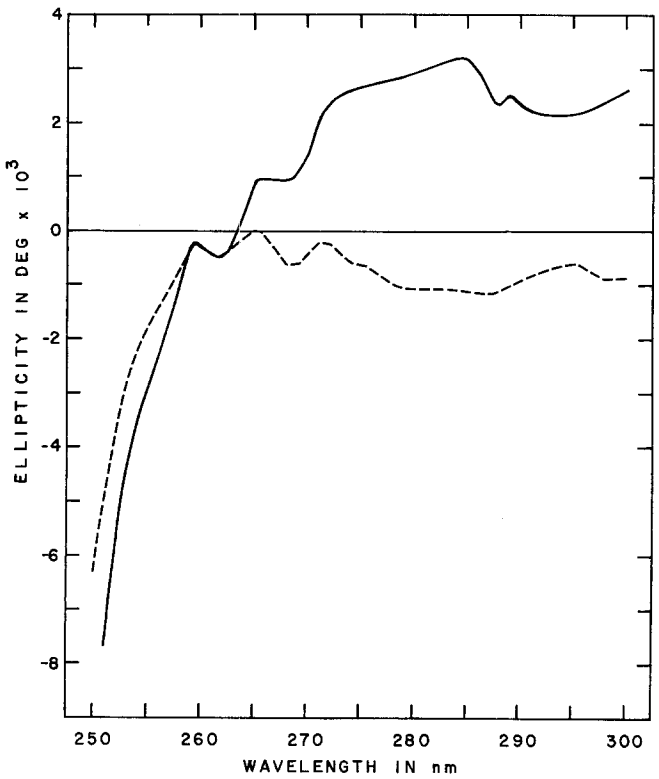

Fig. 16. CD of ROS in 1% Emulphogene in the near ultraviolet, before and after illumination. Illumination results in major spectral changes characeristic of perturbation of aromatic residues. ROS in 1% Emulphogene before illumination (——); after illumination (– – –). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 1.00 cm. pH 7.00

consequent lower resolution. However, most digitonin samples (in which particulate effects are markedly reduced) also show lesser fine structure. At wavelengths less than 285 nm, the spectrum of the unilluminated digitonin sample is basically similar to the spectrum of sonicated ROS membranes. The spectra of the unilluminated Emulphogene and CTAF samples not only show the development of considerable fine structure but the development of a broad positive CD band centered at 280 nm, the observed maximum in the absorption spectrum of detergent solubilized ROS. Since the residues primarily contributing to the near ultraviolet absorption are tyrosine and tryptophan, solubilization of the ROS membrane by Emulphogene and CTAF probably results in significant alteration in the local electronic environment of several of these residues.

Upon illumination, the spectra of ROS solubilized in 1% Emulphogene, 1% CTAF, and 1% CTAB undergo major changes over the entire spectral region, the measured ellipticity falling to near zero between 260 nm and 300 nm and the maximum change occurring near 280 nm. (The spectra of the CTAF sample after illumination and of the CTAB sample before and after illumination are not shown but are

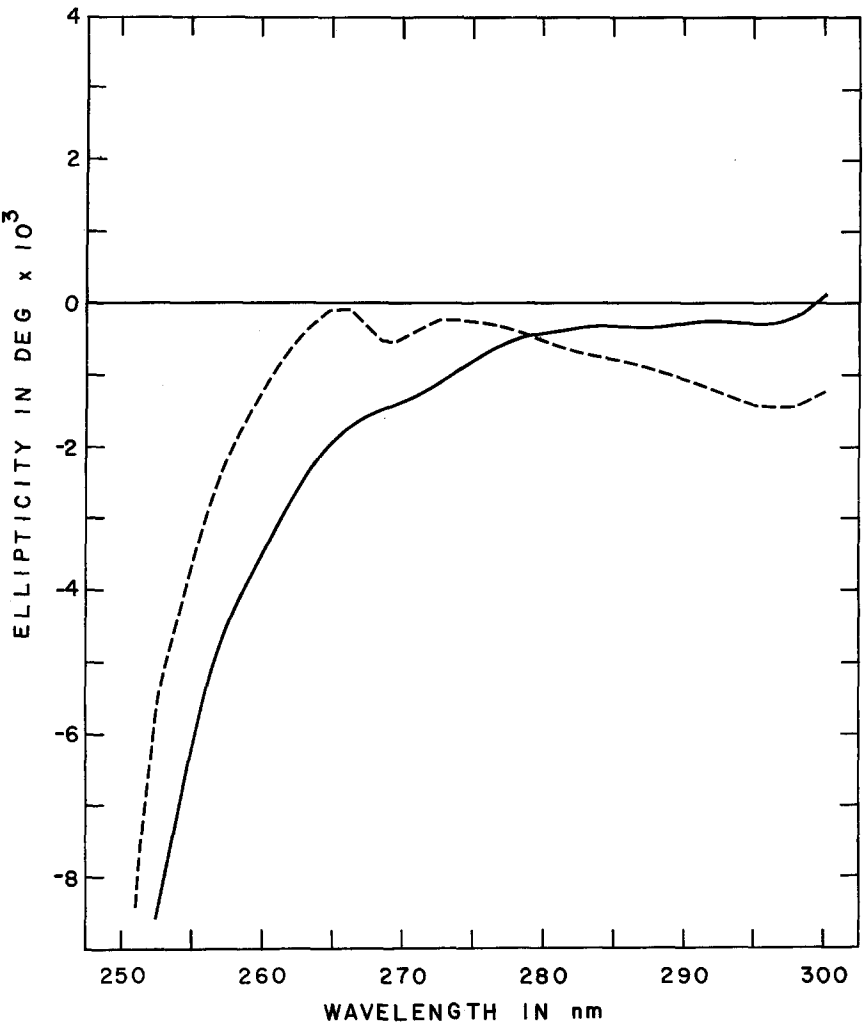

Fig. 17. CD of ROS in 1% digitonin in the near ultraviolet, before and after illumination. Illumination results in spectral changes indicating perturbation of aromatic and/or cystine residues. ROS in 1% digitonin before illumination (—); after illumination (– – –). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 1.00 cm. pH 7.00

similar to those of the Emulphogene sample shown in Figure 16.) Thus illumination appears to result in a second perturbation of tyrosine and tryptophan residues following the initial perturbation associated with membrane solubilization. (Between 260 nm and 290 nm, the measured ellipticity increases on detergent solubilization and decreases on illumination.) The fact that the largest changes in ellipticity are observed in the 270–290 nm region and not in the 290–300 nm region indicates that changes in magnitude of the 340 nm band on bleaching of the rhodopsin chromophore can account for only part of the observed changes in the near ultraviolet spectra.

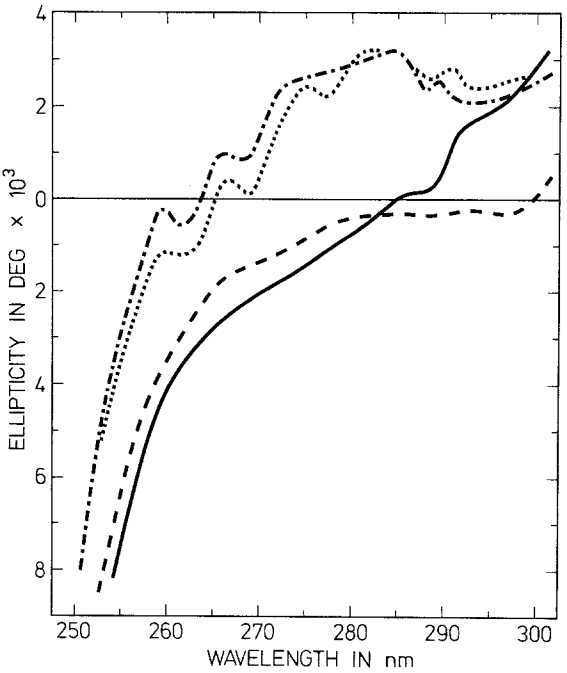

Fig. 18. CD of sonicated and detergent solubilized ROS in the near ultraviolet, before illumination. All samples were prepared by identical dilution of one stock ROS preparation. Sonicated ROS, pH 7.00 (—). ROS in 1% digitonin, pH 7.00 (– – –); in 1% CTAF, pH 4.80 (· · · ·); in 1% Emulphogene, pH 7.00 (– · – · –). Rhodopsin concentration 8.92 nmoles/ml. Protein concentration 0.605 mg/ml. Optical pathlength 1.00 cm

Major changes are observed in the near ultraviolet region of ROS in 1% digitonin upon illumination (Fig. 17), even though the far ultraviolet spectrum (Fig.10) is insensitive to illumination. (The spectra of Figure 7 were obtained from the same stock ROS preparation used in recording the spectra of Figure 10.) The near ultraviolet spectral properties of ROS solubilized in 1% digitonin also apparently depend on the particular ROS preparation used. The near ultraviolet spectra of ROS in 1% digitonin, obtained from a different stock ROS preparation, which showed sensitivity to illumination in the far ultraviolet region, have also been recorded. These spectra show positive CD band structure at wavelengths longer than 257 nm and undergo changes on illumination similar to the spectra of Emulphogene, CTAF, and CTAB samples.

ORD in the Far Ultraviolet Region

The experimental ORD spectra of sonicated ROS membranes are presented in Figure 19. Illumination of the sample results in no reproducible spectral changes between 190 nm and 260 nm. This observation is important since ORD is a dispersive phenomenon and consequently may reflect contributions from optically active transitions with band maxima outside of the experimentally accessible region. The fact that the experimental ORD of sonicated ROS does not change on illumination suggests that hypothetical CD bands of comparable intensity to CD bands in the 185—250 nm region also do not undergo appreciable changes on illumination.

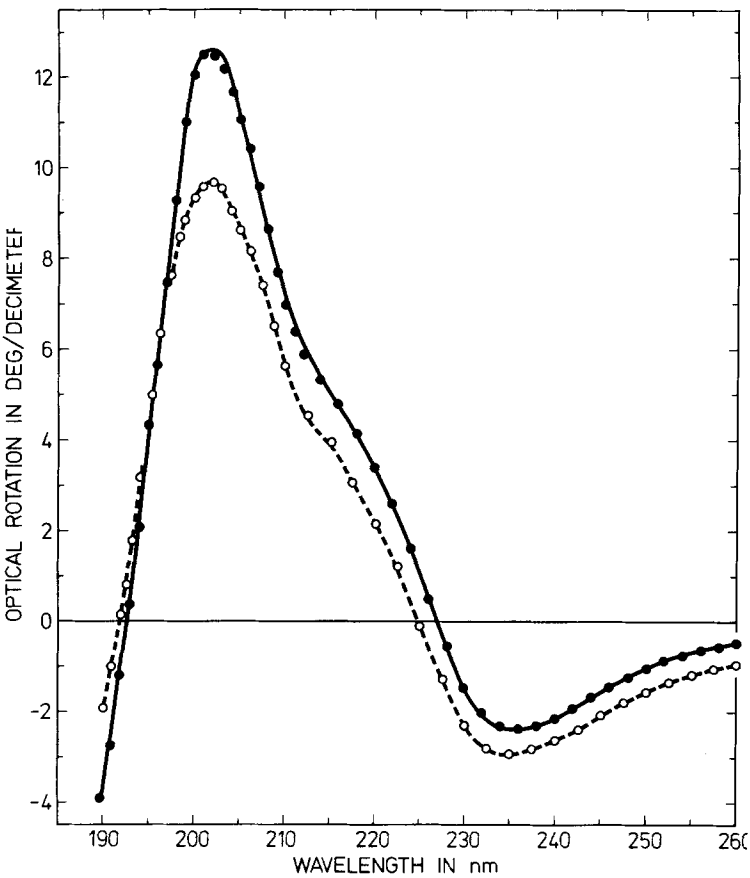

Fig. 19. Experimental and calculated ORD of sonicated ROS in the far ultraviolet, before and after illumination. The calculated ORD was obtained by application of the Kronig-Kramers transform equation to the CD data of Figure 20. The experimental ORD data of this figure and the experimental CD data of Figure 20 were obtained for samples prepared by identical dilution of one stock ROS preparation. Illumination does not result in significant changes in the experimental ORD spectrum. The calculated ORD spectra differ in the intensity of extrema from the experimental ORD spectra. Experimental ORD of sonicated ROS before illumination (——); after illumination (●, closed circles indicate data points). Calculated ORD of sonicated ROS before illumination (– – –); after illumination (○, open circles indicate data points). Rhodopsin concentration 7.41 nmoles/ml. Protein concentration 0.635 mg/ml. pH 7.00

Studies were also undertaken to determine if the experimental ORD of sonicated ROS entirely originates from electronic transitions which give rise to the CD spectrum in the 185–260 nm region. The Kronig-Kramers transform equation was applied to the CD spectra of unilluminated and illuminated sonicated ROS shown in Figure 20. These CD spectra were obtained for samples which were identical to samples used in recording the experimental ORD spectra of Figure 19. The extrapolated segment of the 194 nm CD band was constructed (below 185 nm) to approximate a nearly gaussian band shape. Slight deviations in the shape of this extrapo-

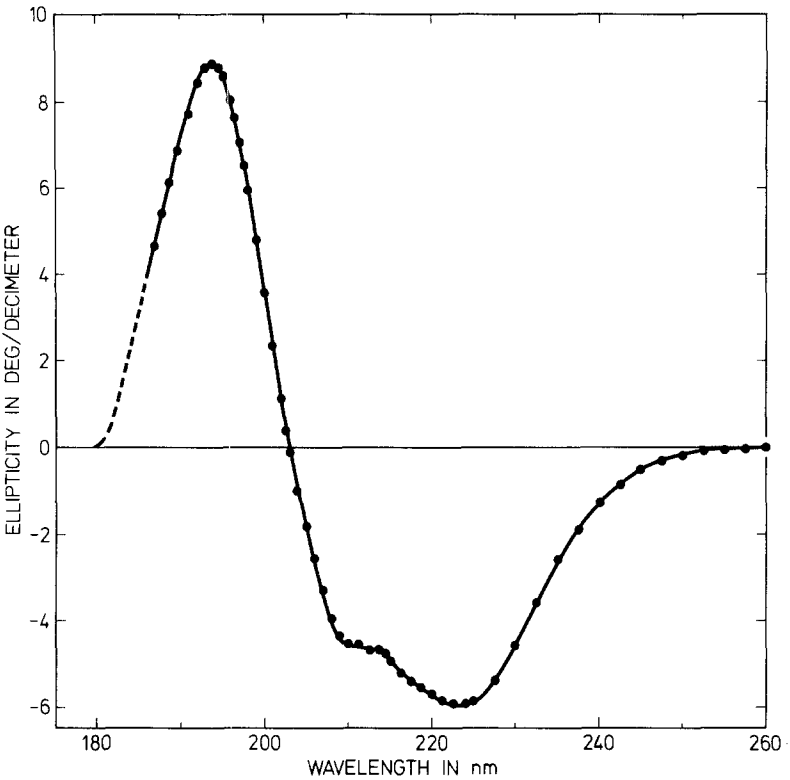

Fig. 20. The CD of sonicated ROS in the far ultraviolet from which the calculated ORD of Figure 19 was obtained. The experimental CD was recorded between 186 nm and 260 nm. The CD was extrapolated to zero at wavelengths shorter than 186 nm. Experimental CD of sonicated ROS before illumination (—); after illumination (●, closed circles indicate data points). Extrapolated CD (– – –). Rhodopsin concentration 7.41 nmoles/ml. Protein concentration 0.635 mg/ml. pH 7.00

lated tail do not result in significant changes in calculated ORD below 260 nm (Cassim and Yang, 1970). The absolute intensity of the calculated positive ORD bands is decreased and the absolute intensity of the calculated negative ORD band is increased relative to the corresponding experimental ORD bands. This noncorrespondence suggests the existence of strong electronic transitions below 185 nm which contribute to the experimental ORD spectrum as a tail of a positive Cotton effect which can be approximated by the Drude equation.

The wavelengths of the ORD extrema of Figure 19 are 201.8 nm, 235.8 nm (experimental spectra); 202.0 nm, 235.0 nm (calculated spectra). The crossover wavelengths are 226.8 nm (experimental spectra) and 224.8 nm (calculated spectra).

An identical study was undertaken on ROS membranes solubilized in 1% Emulphogene to determine the extent to which the observed differences in the calculated and experimental ORD of sonicated ROS membranes might arise from differences in contribution of particulate artifacts to the experimental ORD and CD. Figure 21 presents the experimental and calculated ORD spectra of ROS membranes solubi-

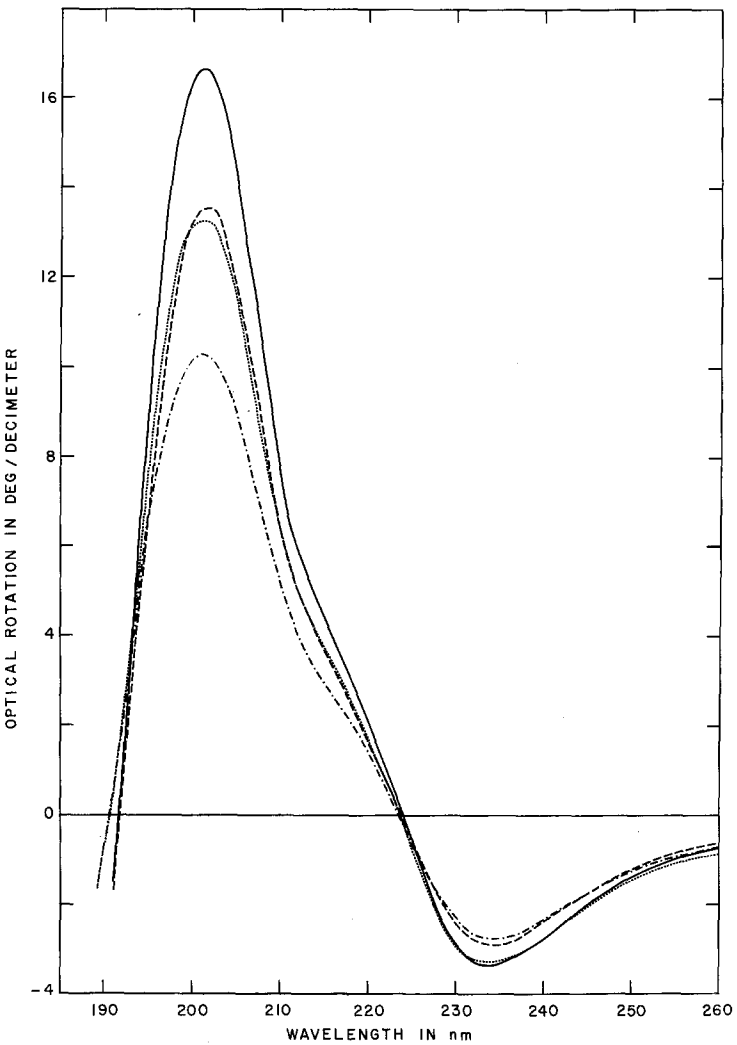

Fig. 21. Experimental and calculated ORD of ROS in 1% Emulphogene before and after illumination. The calculated ORD was obtained by application of the Kronig-Kramers transform equation to the CD data of Figure 22. The experimental ORD data of this figure and the experimental CD data of Figure 22 were obtained for samples prepared by identical dilution of one stock ROS preparation. Illumination results in changes in the experimental ORD spectrum characteristic of loss in protein helical structure. The experimental ORD spectra differ in the intensity of the 201 nm extremum compared to the calculated ORD spectra. Experimental ORD of ROS in 1% Emulphogene before illumination (——); after illumination (– – –). Calculated ORD of ROS in 1% Emulphogene before illumination (· · · ·); after illumination (– · – · –). Rhodopsin concentration 9.08 nmoles/ml. Protein concentration 0.738 mg/ml. pH 7.00

lized in 1% Emulphogene. On illumination, the experimental ORD spectrum undergoes changes indicative of loss in protein helical content (Imahori and Nicola, 1973), consistent with the changes observed in the corresponding experimental CD spectrum (Fig. 22). Comparison of the experimental ORD spectra to the ORD spectra

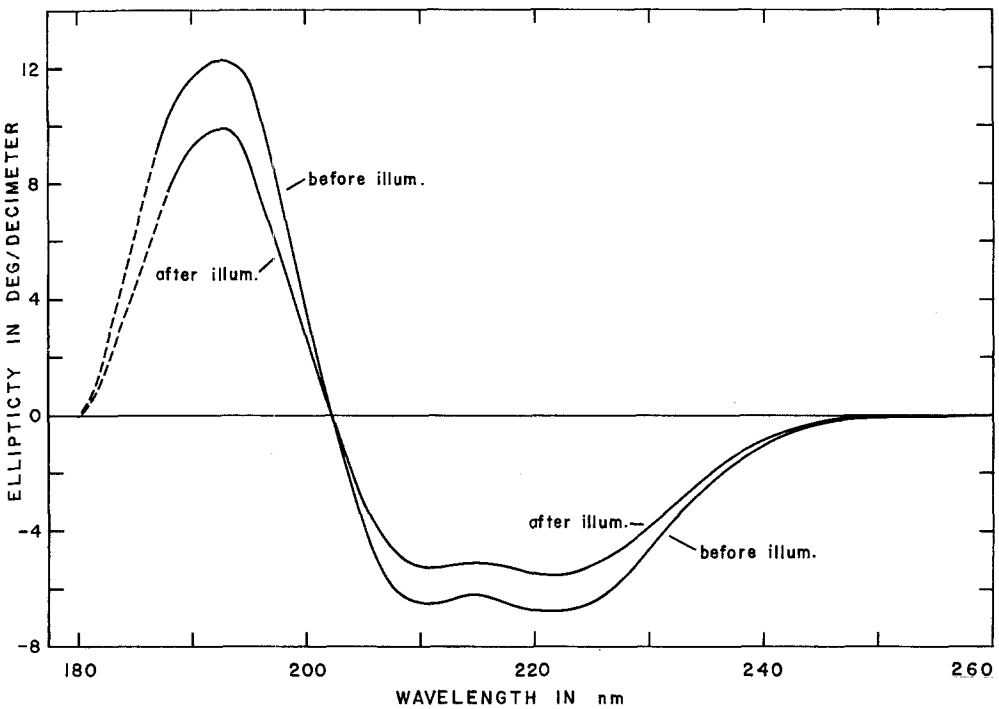

Fig. 22. The CD of ROS in 1% Emulphogene from which the calculated ORD of Figure 21 was obtained. The experimental CD was recorded between 187 nm and 260 nm. The CD was extrapolated to zero at wavelengths shorter than 187 nm. Experimental CD of ROS in 1% Emulphogene (—). Extrapolated CD (– – –). Rhodopsin concentration 9.08 nmoles/ml. Protein concentration 0.738 mg/ml. pH 7.00

calculated by application of the Kronig-Kramers transform to the experimental CD spectra of Figure 22 gives results for the positive ORD bands which are similar to those obtained for sonicated ROS. However, in the case of Emulphogene samples, the calculated ORD curves converge near the crossover point and become virtually the same as the experimental ORD curves in the region of the negative ORD band.

The wavelengths of the ORD extrema of Figure 21 are:
201.3 nm, 234.0 nm (experimental spectrum before illumination);
201.5 nm, 234.5 nm (experimental spectrum after illumination);
201.0 nm, 233.7 nm (calculated spectrum before illumination);
201.3 nm, 234.0 nm (calculated spectrum after illumination).

The crossover wavelengths are:
224.0 nm (experimental spectrum before illumination);
223.7 nm (experimental spectrum after illumination);
223.5 nm (calculated spectrum before illumination);
223.3 nm (calculated spectrum after illumination).

Discussion

The far ultraviolet CD and absorption spectra of ROS membranes are clearly indicative of partial right-handed α-helical structure in the membrane protein. Comparison of the far ultraviolet CD spectra of ROS membranes to the CD spectra of synthetic polypeptides with known random, α-helical, and β-form secondary structures suggests that the ROS membrane protein does not possess appreciable β-structure. The observed depression of the 210 nm CD band and the red shift in the 224 nm CD band of sonicated ROS membranes relative to the band structure of partially helical polyglutamic acid can probably be attributed to the particulate nature of the membrane suspensions. The observed distortion of the CD spectra of particulate ROS membrane suspensions probably arises from a complex contribution of several optical artifacts (Urry, 1972). The contribution of particulate effects is clearly discerned in the absorption spectrum of sonicated ROS and to a lesser extent in the absorption spectrum of ROS solubilized in 1% digitonin. The observed increase in apparent absorbance (compared to the Emulphogene sample) is probably primarily due to scattering of incident light away from the phototube. However, in the region of the 193 nm band maximum, absorption flattening (Duysens, 1956) probably plays the dominant role since the observed absorbance is actually decreased in the particulate case, opposite to the expected contribution of light scattering alone. The far ultraviolet CD spectra of detergent solubilized ROS (in which particulate effects are essentially abolished) still show a slight red shift and depression of the 194 nm band compared to the CD spectrum of helical polyglutamic acid. However, proteins which possess appreciable non helical, non β-structure content normally show depression of the CD band near 191 nm relative to the CD band near 221 nm because of the negative contribution of the "random coil" conformation to the ellipticity at wavelengths shorter than 200 nm (Timasheff, 1970; Sears and Beychok, 1973). The slight red shift of the 194 nm band of ROS membrane samples may be associated with the presence of a very small β-structure content (Timasheff, 1970; Adler et al., 1972; Sears and Beychok, 1973) or possibly with a contribution from aromatic residues to the far ultraviolet CD spectrum. (Aromatic residues are found abundantly in the ROS membranes [de Grip et al., 1973] and are known to possess intense absorption bands in the 185–250 nm region [Wetlaufer, 1962; Timasheff, 1970].) A slight red shift of the positive CD band relative to the band position in partially helical polypeptides has been observed in the spectra of other proteins. The reason for this shift is still not well understood.

Because of the dispersive nature of ORD, optically active transitions with band maxima outside of the experimentally accessible spectral region (if they exist) make contributions to experimental ORD spectra. However, the experimental ORD spectra of synthetic helical polypeptides in the far ultraviolet region can be attributed, within the limits of experimental error, to peptide bond transitions which generate CD band structure entirely within the 180–260 nm region (Cassim and Yang, 1970). Similar results have also been obtained for polypeptides with random-coil or unordered secondary structure (Cassim and Yang, 1968). Also, analogous studies of several globular and fibrous proteins have indicated a difference between experimental and calculated ORD of no greater than 7% at the 198 nm ORD peak, with the majority of the proteins exhibiting no detectable differences (Cassim, unpublished

results). The ORD spectra of sonicated and detergent solubilized ROS can primarily be attributed to the same peptide bond transitions. However, an additional contribution from bands with appreciable intensity at wavelengths below 185 nm is suggested by the Kronig-Kramers transform calculations. The difference between the experimental and calculated ORD is about 20% at the 201 nm ORD peak in the case of the ROS membranes. Potential far ultraviolet chromophores other than the peptide bond are a constituent part of ROS membranes. Phosphoglycerides comprise approximately 40% of the dry weight of ROS membranes (de Grip et al., 1973). The parent compound of the phosphoglyceride series, L-glycerol-3-phosphate, has a dissymmetric carbon atom and consequently would be expected to contribute to the vacuum ultraviolet optical activity of ROS membranes.The phosphoglyceride component of ROS membranes is also known to consist partly of highly unsaturated fatty acid side chains composed primarily of non-conjugated double bonds which are not intrinsically optically active (Borggreven et al., 1970; Nielsen et al., 1970; Poincelot and Abrahamson, 1970). However, optical activity may be induced by a dipole-dipole coupling mechanism or by interaction with static dissymmetric electric fields associated with local protein environments (Sears and Beychok, 1973). Also, aromatic residues, of which approximately 18 tyrosines, 8 tryptophans, and 30 phenylalanines per mole of rhodopsin are found in ROS membranes (de Grip et al., 1973; Daemen et al., 1972), are also known to absorb strongly below 190 nm and are expected to be optically active as they are in the near ultraviolet region (Wetlaufer, 1962; Timasheff, 1970). However, in view of the high lipid content of the ROS membrane and of the relatively weak optical activity in the near ultraviolet, much of the difference between the experimental and calculated ORD can be attributed to optically active transitions of the lipid moiety of the ROS membrane.

Assignment of the CD band structure observed in the near ultraviolet spectra of ROS membranes to particular aromatic amino acids and cystine transitions is difficult at the present time. Further studies are clearly required involving perturbation of intact and detergent solubilized systems by pH, temperature, chemical denaturants, and chemical modification of aromatic residues and cystine. Tryptophan, tyrosine, and cystine can each make appreciable contributions to the ellipticity at any wavelength between 250 nm and 300 nm, depending on the polypeptide or natural protein examined (Timasheff, 1970; Adler et al., 1972; Sears and Beychok, 1973). Beychok (1965) has suggested that cystine is more likely to give rise to ellipticity bands in the 250–270 nm region in most proteins. If valid, bands observed in the 250–270 nm spectral region of ROS samples (these bands are more predominant in occasional sonicated membrane samples) may partially be associated with the two cystine (disulfide) residues per mole rhodopsin found in ROS membranes (Bonting et al., 1974). Both tyrosine and tryptophan are likely to contribute to the ellipticity of bands in the 270–300 nm region as indicated by the probable contribution of tyrosine and tryptophans to the 279 nm absorption band of ROS samples. Phenylalanine is a weak chromophore and in general is not thought to make a large contribution to the optical activity of most proteins (Beychok, 1965; Sears and Beychok, 1973). However, because of the relatively high concentration of phenylalanine residues in ROS membranes (30 per mole rhodopsin), some of the detailed fine structure observed in the near ultraviolet CD spectra of detergent solubilized ROS may be

attributable to this amino acid. The absorption spectrum of phenylalanine possesses considerable fine structure compared to the absorption spectra of tyrosine and tryptophan (Wetlaufer, 1962; Timasheff, 1970).

The estimated α-helical content of ROS membrane protein, based on the mean residue ellipticity of sonicated ROS suspensions at 223.5 nm, is about 29%. This value is similar to those reported by Azuma and Kito (1967) for detergent solubilized bovine and frog ROS and sonicated bovine ROS but is substantially less than the values reported by Shichi et al. (1969) for chromatographically purified detergent solubilized bovine rhodopsin, 52–62%, by Shichi and Shelton (1974) for sonicated bovine ROS, 47%, and by Stubbs et al. (1976) also for chromatographically purified detergent solubilized bovine rhodopsin, 58%. These discrepancies can probably be attributed to several factors: different methods of evaluating helix content, different reference values for the helical, β-, and unordered forms, different analytic techniques for measuring protein content, and different degrees of purity in regard to protein composition of the samples studied. In this study, helical content was evaluated at one wavelength in the ultraviolet CD spectra. The standard values used for the helix ($-32{,}600$ deg $\cdot$ cm^2/decimole) and β- and unordered forms (-2340 deg $\cdot$ cm^2/decimole) near 222 nm are those of Chen and Yang (1971) and are preferred because they were derived from the CD spectra of a series of natural proteins using structural information obtained by x-ray diffraction. In this study, protein concentration was determined using the Biuret reagent. This is probably the most reliable method other than micro-Kjeldahl nitrogen analysis (Layne, 1957). The micro-Kjeldahl method was not applied because of large quantities of nitrogen-containing lipid in ROS membranes. The protein composition of the samples used in this study was quite uniform as shown above. Rhodopsin and opsin together accounted for more than 80% of the total membrane protein. Since the secondary structure of rhodopsin remains unchanged upon bleaching, rhodopsin and opsin are indistinguishable in regard to helical content. A final point to consider is the influence of particulate effects on the calculated helical content. The value of 29% was obtained for sonicated ROS samples. Particulate effects probably result in some diminution of the absolute ellipticity values at 223.5 nm. However detergent solubilization results in sufficiently small changes in the measured ellipticity that the value of the helical content would at most be altered by a few percent (in the case of digitonin solubilization from 29 to 32%).

Errors inherent in the estimation of helical content of natural proteins based on experimental CD and ORD studies have been extensively discussed (e.g.: Adler et al., 1972; Imahori and Nicola, 1973; Sears and Beyhock, 1973; Rosenkranz, 1974). In general, calculated values of helical content should be taken only as approximate. Regardless of any uncertainty in the value of helical content for natural proteins, changes in secondary structure can be detected with a high degree of precision (Rosenkranz, 1974).

To the extent that the present methodology gives a correct measure of protein helical content, a quantitative limit can be established for the possible existence of light-induced net changes in protein helical structure in ROS membranes which have not been solubilized by detergent. The reproducibility of far ultraviolet CD spectra of sonicated ROS samples indicated that light-induced net changes in protein helical content as small as 1.3% could be detected. One of the ROS preparations used in

recording several of these high accuracy spectra consisted of protein, 57% of which was rhodopsin. Assuming a molecular weight of 38,850 for rhodopsin and a mean residue molecular weight of 113.6 for the ROS membrane protein, a preparation consisting of 57% rhodopsin protein is calculated to have 690 amino acid residues per rhodopsin molecule. A 29% helical content for ROS protein indicates that approximately 200 amino acids per rhodopsin molecule are located in helical segments of the ROS membrane protein. Figure 8 shows that the absolute percent deviation of ellipticity is less than 1% at 223.5 nm upon illumination. The mean residue ellipticity is about $-11{,}000$ deg $\cdot$ cm^2/decimole. Thus a change of about 110 deg $\cdot$ cm^2/decimole could be detected which corresponds to a 1.3% change in helical content when a 1% change in ellipticity is observed, assuming the standard values of Chen and Yang (1971) for the helical, β-, and unordered forms. Therefore, the observed insensitivity to illumination of the far ultraviolet CD spectra of sonicated ROS membranes indicates that if light-induced net helical conformational changes indeed occur, these changes are equivalent to a loss or gain in participation of less than three amino acid residues in the helical structure of ROS protein per molecule of rhodopsin bleached.

The absorption spectrum of sonicated ROS membranes also clearly indicates stability of the protein helix to illumination since the absorbance near 193 nm is insensitive to illumination under conditions of high spectral reproducibility. Compared to the CD spectra of proteins with considerable helix, the corresponding absorption spectra are less easily calibrated in terms of helical content (Gratzer, 1967). However, helix-coil transitions are specifically characterized by a marked increase in absorbance of the 193 nm band and a lesser decrease in the long wavelength tail with a crossover between 215 nm and 220 nm (Gratzer, 1967), similar to that observed for ROS solubilized in Emulphogene. The very small light-induced decrease in absorbance near 235 nm observed for sonicated ROS [previously reported by Takagi (1963) for bovine ROS suspensions] is not typical of a helix-coil transition because such a transition should be accompanied by a much larger absorbance increase at 193 nm. The origin of the 235 nm absorbance change is not presently understood. It may be due to perturbation of one or more aromatic residues (Donovan, 1969) which are not strongly optically active. It may represent the higher energy π-π^* transitions of the prosthetic group, retinal, which would undergo expected changes on bleaching. Finally, it may represent a spectrophotometer artifact in which fluorescent radiation associated with strong aromatic residue absorption (strong compared to absorption at 280 nm) is captured by the phototube and thus decreases the true absorbance at 230 nm, i.e., it represents a fluorescence action spectrum. Experiments are planned to distinguish between these possibilities.

The ORD spectra of sonicated ROS not only provide information about peptide bond transitions in the far ultraviolet but also about hypothetical optically active transitions below 185 nm in the vacuum ultraviolet. The insensitivity to illumination of the experimental ORD spectra confirm the results of the CD spectra concerning stability of the helix in membrane suspensions. Additionally, the ORD spectra indicate that major changes in the intensity of hypothetical transitions below 185 nm also do not occur on bleaching of rhodopsin. If lipid is the membrane component which is the source of these vacuum ultraviolet transitions, then this result is evi-

dence for the constancy of interaction of lipid and protein components before and after bleaching.

Studies in the near ultraviolet also provide important information about the conformation of proteins. The intensity and distribution of CD bands are considered to arise from dissymmetric interaction of the side chains of the aromatic residues and cystine with local protein environments (Timasheff, 1970; Adler et al., 1972; Sears and Beychok, 1973). In consequence, the near ultraviolet CD spectra of proteins which contain these residues are highly sensitive to conformational changes in the protein which alter these local environments. In general, detectable changes in the conformation of proteins involving helix-coil transitions (secondary structure changes) or reordering of segments of the polypeptide chain(s) relative to each other (tertiary structure changes) result in major changes over the entire near ultraviolet spectral region. This has been demonstrated for a variety of proteins which contain aromatic amino acids and/or cystine residues by examination of both the near and far ultraviolet spectra on perturbation by pH, temperature, and chemical denaturants: carbonic anhydrases b and c (Beychok et al., 1966); chymotrypsinogen (Fasman et al., 1966); insulin (Ettinger and Timasheff, 1971); peroxidase a (Hamaguchi, 1969); ribonuclease (Simmons and Glazer, 1967). The near ultraviolet spectral region of some proteins has also been shown to be sensitive to conformational changes involving tertiary and/or quaternary structure which occur without associated major changes in secondary structure (as indicated by far ultraviolet CD spectra). Some examples are: cytochrome C_1, oxidation-reduction (Yu et al., 1971); lamprey and human hemoglobin, oxygenation-deoxygenation (Sugita et al., 1968); concanavalin A, before and after binding of alpha-methyl-D-mannoside (Pflumm et al., 1971); staphylococcal nuclease, before and after binding of deoxythymidine 3′,5′-diphosphate (Omenn et al., 1969); lysozyme, before and after binding of N-acetyl-D-glucosamine (Glazer and Simmons, 1966; Imota et al., 1972).

By comparison to spectroscopic studies on other proteins, the present result, concerning the lack of light-induced sensitivity of the near ultraviolet CD spectrum of sonicated ROS at wavelengths shorter than 280 nm, strongly suggests that major changes in secondary and tertiary protein structure do not occur upon illumination. This result is consistent with the properties of the far ultraviolet absorption, CD, and ORD spectra of ROS membranes but gives, in addition, information about the constancy of tertiary structure. In other protein systems, an analogous process to bleaching of rhodopsin (and the consequent change of interaction of the prosthetic group, retinal, with opsin) is the release and binding of substrate as cited above for several specific cases. The present result contrasts strongly with that usually observed in the near ultraviolet CD spectra of other proteins. Since near ultraviolet changes on substrate release and binding are generally ascribed to conformational changes of a subtle nature, similar changes in the ROS membrane protein are not evident. However, because of the large numbers of aromatic residues in the ROS membrane (56 per molecule of rhodopsin), the sensitivity of the present CD measurements is very likely insufficient to detect local light-induced conformational changes involving tertiary structure which affect the electronic environments of only a few residues. It is perhaps noteworthy that the CD of the purple membrane from *Halobacterium halobium* which contains bacteriorhodopsin (a retinal containing chromoprotein), while being insensitive to the bleaching process in the far ultraviolet region

of the spectrum, is very sensitive to this process in the near ultraviolet region (Becher and Cassim, 1975).

The conclusions concerning conformation, based on the observed lack of light-induced sensitivity of the near ultraviolet CD spectrum of sonicated ROS membranes below 280 nm, are invalid if hypothetical changes in CD due to perturbation of aromatic residue transitions are exactly compensated by hypothetical changes in CD due to perturbation of ultraviolet transitions of the prosthetic group, retinal. However, such a mechanism must be considered very unlikely since it requires the coincidence of similar magnitude, similar wavelength position, and opposite direction of these hypothetical CD changes.

The lack of light-induced sensitivity of the near ultraviolet CD spectrum of sonicated ROS membranes below 280 nm is also not inconsistent with the observation by Ebrey (1972) that the intensity of tryptophan fluorescence in bovine ROS fragments suspensions increases on illumination. It is known that intramolecular energy transfer by means of the Förster mechanism probably occurs between the aromatic residues of rhodopsin and the prosthetic group, retinal (Ebrey, 1971; Kropf, 1967). The efficiency of energy transfer depends on the overlap of the fluorescence emission spectra of the aromatic residues and the absorption spectrum of the prosthetic group as well as the distance between and the orientation of the corresponding transition dipole moments (Förster, 1965). Upon illumination, the visible absorption spectrum of rhodopsin and the location and orientation of retinal with respect to the protein are all known to change (Abrahamson and Wiesenfeld, 1972). The resultant decrease in efficiency of energy transfer would consequently be expected to result in an increase in intensity of tryptophan fluorescence as observed by Ebrey (1972). In addition, changes in local environment and/or reorientation of perhaps one or two tryptophan residues may also occur which result in only small undetectable alterations in the near ultraviolet CD spectrum of sonicated ROS membranes.

A possible objection to the conclusion that major conformational changes involving secondary and/or tertiary structure do not occur upon illumination lies in the fact that the present CD, ORD, and absorption measurements are made on a time scale (minutes) which does not resolve possible conformational changes on a time scale similar to that involved in neural excitation (milliseconds). In principle, it is possible that several intermediate spectra, perhaps associated with the appearance of different thermal intermediates of rhodopsin, cancel each other and result in final spectra identical to initial spectra. However, if delocalized conformational changes are involved in at least one stage in bleaching, it is highly unlikely that the final protein conformation is identical to the initial protein conformation. It follows that spectra sensitive to changes in both secondary structure and aromatic/cystine residue environment also should not be identical. Therefore, the examination of both the far and near ultraviolet spectra suggests that such compensating spectral changes are not probable. In addition, compensating secondary structure changes of the protein during illumination are also unlikely because of the low probability that changes in both secondary structure and aromatic/cystine residue environment can simultaneously cause CD changes that cancel each other. Therefore, the lack of far ultraviolet CD spectral changes during illumination can be interpreted not only as being indicative of no net secondary structure change but also as being indicative of

no conformation change involving secondary structure. However, the existence of transient localized changes in conformation perhaps associated with strained intermediate conformations of retinal cannot be excluded on the basis of the present experiments. Additional experiments are clearly required in which the near and far ultraviolet CD and absorption spectra of ROS membrane suspensions are recorded at reduced temperatures (where the thermal intermediates are stabilized).

Current models of how absorption of visible light by photoreceptor pigments is coupled to the production of neural signals invariably assume a conformational change of the rhodopsin molecule. In most models proposed, there has been a requirement for a delocalized conformational change in contrast to a localized one. It is essential to put the distinction between delocalized and localized conformational changes into perspective (Cassim and Lin, 1975). By the term "delocalized conformational change", we mean any change in the molecular geometry of the protein which has resulted in the transmission of mechanical free energy in the form of conformational distortion over great distances in the protein from the point of inception. Such conformational changes may or may not involve large changes in the secondary structure and shape of the protein. In contrast, the term "localized conformational change" is reserved for any change in which conformational distortion is restricted to the point of inception, such as an enzyme's active site. Delocalized conformational change is of great importance in mechanisms of biological function since it provides the means by which information as mechanical free energy can be transferred through and between macromolecules. On the other hand, localized conformational changes are of importance in mechanisms of chemical reaction where there is a need for localization of energy. Clearly any perturbation of a protein must involve some kind of conformational change. Therefore, local conformational changes are inherent in every protein perturbation, whereas delocalized conformational changes are unique. The emerging picture for rhodopsin as a major component of the ROS membrane is that light-induced conformational changes must be restricted to only small regions of the protein in which only a few residues participate, ruling out the possibilities of a unique delocalized conformational change. It is suggested that models requiring delocalized conformational change as an essential part of the mechanism for light induction of neural signals be reexamined.

The strongest evidence for the presence of large delocalized light-induced conformational changes in the rhodopsin protein has been obtained from analysis of the activation parameters associated with the decay of the thermal intermediates of rhodopsin (e.g., Abrahamson and Wiesenfeld, 1972). The results of the present study suggest that the observed large values of entropy and enthalpy of activation are associated instead with activated states of the rhodopsin chromophore.

Solubilization of ROS membranes in detergent evidently results in both conformational alteration of the membrane protein and loss of conformational stability to illumination. Of all the detergents used in this study digitonin results in the least destabilization of the membrane protein conformation. The far ultraviolet CD spectra indicate that the spectral changes observed on digitonin solubilization are primarily associated with reduction or elimination of artifacts arising from the particulate nature of membrane suspensions rather than a change in protein secondary structure. Dispersal of ROS membranes in Emulphogene, CTAF, and CTAB probably results in a slight loss in helical structure as seen by comparison of the far ultraviolet

CD spectra of Emulphogene, CTAF, and CTAB samples to the spectra of digitonin samples. This information is obscured in the corresponding absorption spectra because of the magnitude and direction of the particulate effects. In the CD spectra of digitonin samples, particulate effects are largely eliminated. In addition, the predicted direction of change in the intensity of CD bands is opposite upon detergent solubilization (bands increase in magnitude) to that expected from helix loss (bands decrease in magnitude). In contrast, particulate effects are still prominent in the absorption spectra of digitonin samples. The predicted direction of change in intensity of the 193 nm absorption band is the same for both detergent solubilization and helix loss (band increases in magnitude) which makes the resolution of the origin of absorption changes in the 193 nm band more difficult. Detergent solubilization always alters the CD spectral region associated with aromatic/cystine residue environment, again excepting the digitonin case. In general, illumination of detergent solubilized ROS results in evident protein conformational changes associated with both secondary structure and aromatic/cystine residue environment. The only exception is found for digitonin solubilized ROS in which the protein net secondary structure is stable to illumination for most ROS preparations. However, digitonin samples always undergo light-induced conformational changes associated with aromatic-cystine residue environment. This is shown in both the near ultraviolet CD spectra and the far ultraviolet absorption spectra. The influence of detergent on rhodopsin protein conformation shown in this study is confirmed by spin label studies on an analogous series of samples in which the spin probe was reacted with protein sulfhydryl groups (Pontus and Delmelle, 1975).

The results of the present study suggest that chemical and spectroscopic experiments on detergent solubilized ROS membranes may yield results which are unrelated to the physiological function of the intact membrane system.

Two different mechanisms have been proposed to account for the observed optical activity of rhodopsin in the visible spectral region. One possible mechanism is that atropisomers of the prosthetic group, 11-cis-retinal, distinguished by non-planarity of the conjugated polyene chain are isolated by preferential binding to the apoprotein, opsin (Mommaerts, 1969; Sperling and Rafferty, 1969; Burke et al., 1973; Ebrey and Yoshizawa, 1973). A second mechanism is that the electronic transitions of retinal undergo dipole-dipole coupling with optically active transitions associated with the protein aromatic residues (Johnston and Zand, 1972; Kropf et al., 1973) or the peptide bond (Waggoner and Stryer, 1971). The dipole-dipole coupling mechanism suggests that the observed loss in rotatory strength of the retinal transitions on rhodopsin chromophore bleaching would in turn perturb the rotatory strength of the electronic transitions associated with the protein. According to the Kuhn sum rule (Kuhn 1930), the sum of the rotatory strengths of all optically active transitions of a molecule is zero. Consequently, a loss in optical activity in one spectral region must be compensated by a gain in optical activity in another region in such a manner that the net rotatory strength is conserved. The observed stability of both the far and near ultraviolet CD spectra of sonicated ROS membranes upon illumination indicates that peptide bond transitions and aromatic residue transitions are not perturbed to a *measurable* extent by interaction with retinal. Thus, a mechanism for induction of optical activity in retinal by dipole-dipole coupling between π-π^* retinal transitions and lowest energy π-π^* transitions of the aromatic residues

can evidently be disregarded. A similar conclusion is not possible concerning dipole-dipole coupling of retinal-peptide bond transitions because the intensity of the far ultraviolet CD bands is much stronger than the visible CD bands (on the order of 200 times more intense). Consequently, measurement of small changes in rotatory strength in the far ultraviolet CD spectra corresponding to changes in rotatory strength in the visible CD spectra is outside the sensitivity limits of present instrumentation. Interest in a dipole-dipole coupling mechanism has been stimulated because of the difficulty in explaining the induction of retinal optical activity in pigment forms in which the retinal prosthetic group is presumed to be sterically unhindered and therefore essentially planar; e.g., in isorhodopsin (Takezaki and Kito, 1967) and the metarhodopsins (Waggoner and Stryer, 1971). However, the 9-cis and all-trans isomers (the retinal isomers found in isorhodopsin and the metarhodopsins, respectively) are also strongly sterically hindered in the planar representation because of overlap of the β-ionone ring methyl groups and the C_7 and C_8 hydrogens of the linear conjugated chain. Consequently, the β-ionone ring should be rotated so that the plane of the ring (and thus the C_5—C_6 double bond) is different than that defined by conjugation of the linear segment of the molecule. This mechanism for relief of steric hindrance has been predicted by theoretical studies (Pullman et al., 1969; Honig et al., 1971). In the crystal structure of all-trans-retinal, the plane of the β-ionone ring is rotated 59 degrees out of a molecular plane defined by a s-cis configuration of the C_6—C_7 bond and an all-trans configuration of the remaining single and double bonds in the linear conjugated segment (Gilardi et al., 1971). As a consequence, atropisomers of all-trans-retinal should also exist and be preferentially bound by opsin. Thus, a dipole-dipole mechanism need not be invoked to explain the induced optical activity of retinal in either rhodopsin or other visual pigment forms.

Selective binding of atropisomers of retinal by opsin is a sufficient mechanism to explain the phenomenon of optical activity of rhodopsin in the visible spectral region. If this is the predominant mechanism, the present result concerning the effect of detergent solubilization of ROS membranes on the visible CD clearly requires some perturbation of the electronic structure of the selected atropisomer which is, in turn, coupled to solubilization. The origin of this perturbation could very likely be a conformational change in the protein moiety induced by detergent solubilization, as was demonstrated to occur by the effect of solubilization on both the near and far ultraviolet spectra of unilluminated ROS membranes. This protein conformational change must, at least, be localized near the binding site of retinal, although simultaneous spectral changes characteristic of a loss in helical structure and perturbation of aromatic residues suggest a delocalized conformational change when stronger detergents such as CTAF and Emulphogene are used. The relative order of the magnitude of spectral effects produced by different detergents is similar in both the ultraviolet and visible region, i.e., digitonin has the least effect whereas CTAF and Emulphogene have a more pronounced effect in both spectral regions. This supports a direct relationship between perturbation of retinal and protein conformational changes as induced by detergent solubilization. In a theoretical study, Honig et al. (1973) have shown that the rotatory strengths of the π-π^* transitions of 11-cis-retinal should be strongly dependent on the exact angles of rotation around various single bonds characteristic of the selected atropisomer of 11-cis-retinal in rhodopsin. Therefore, a consistent interpretation of detergent effects on CD spectra is that a

protein conformational change, induced by detergent solubilization, changes the ground state conformation of bound retinal, probably by altering the tortional angles around one or more single bonds, so that the rotatory strengths of the π-π^* transitions are also changed.

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