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Different States of Aggregation for Unbleached and Bleached Rhodopsin after Isolation in Two Different Detergents†

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ABSTRACT: Phospholipid-free rhodopsin has been purified in the detergents sodium cholate and octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$). In both detergents, the native absorption spectrum of the unbleached protein is maintained; however, upon photolysis, the preparation in $C_{12}E_8$ loses its ability to recombine with 11-*cis*-retinal, whereas the preparation in cholate does not. The circular dichroic spectra of the protein in the two detergents are nearly identical, indicating that the secondary structure of the protein is the same in the two detergents. The state of association of the protein in the two detergents is different. In sodium cholate, the smallest species present was found to be a trimer of the rhodopsin polypeptide chain, and this association was unaffected by exposure to light. On the other hand, in $C_{12}E_8$, the protein is monomeric and undergoes a nonspecific aggregation process on exposure to light. These results suggest that protein–protein interactions may play an important role in the stabilization of the native structure of rhodopsin.

Light modulates several enzymatic activities found in rod outer segments which suggests that these activities may play a role in the visual process [see Hubbell & Bownds (1979) for a brief review]. Rhodopsin is the only component of rod outer segments capable of direct interaction with visible light, and therefore, the modulated enzymatic activities must be either directly or indirectly coupled to light absorption by rhodopsin. For a complete understanding of the interactions involved in these processes, it is necessary to isolate each component in a chemically well-defined state. Since rhodopsin and possibly other components are membrane bound, detergents are required in the isolation procedures. Detergent-solubilized preparations are amenable to the study of interactions by classical techniques of physical biochemistry, and furthermore, they provide a vehicle by which the various components can be reconstituted into the more nearly native milieu of a phospholipid vesicle. It is imperative, however, that the native properties of all components be preserved in the presence of the detergent in which one is studying the interactions or that any alteration in a native property be reversible or demonstrably not involved in the particular interaction under study. Since rhodopsin must play a centeral role in any light-modulated response, it is essential that a detergent system be found in which the properties of membrane-bound rhodopsin are fully preserved.

Many detergents can solubilize rhodopsin from disk membranes in the dark without loss of the absorption peak at 500 nm, but when the solubilized protein is bleached by exposure to light, the ability to recover the original spectrum by recombination with 11-*cis*-retinal is usually lost, indicating an inability of the detergent to maintain opsin in a nativelike conformation. Available data for a variety of detergents with respect to this property were summarized in the preceding

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paper (Table I, McCaslin & Tanford, 1981), and experiments were described which demonstrate that recombination with recovery of the 500-nm absorption peak takes place when sodium cholate is used as a solubilizing detergent. In this paper, we compare the molecular properties of rhodopsin and opsin in sodium cholate with the corresponding properties in the nonionic detergent octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$),[1] in which recombination with recovery of the native spectrum has proved to be impossible. The most dramatic difference in the properties of the protein in the two detergent systems is the state of association, and therefore, a major part of this report is a comparison of the states of aggregation in the two detergents.

Experimental Procedures

Materials. Dark-adapted bovine retinas were obtained from George A. Hormel & Co. (Austin, MN). Ficoll-400 and Con A–Sepharose were products of Pharmacia Fine Chemicals Inc. (Piscataway, NJ). $C_{12}E_8$ was a chemically pure product produced by Nikko Chemicals Co. (Tokyo, Japan) and was used as received. Sodium cholate was obtained from Sigma Chemical Co. (St. Louis, MO) and was recrystallized several times from an acetone–water mixture after an initial treatment with activated charcoal followed by filtration through a Celite filter pad. Sodium cholate labeled in the carboxyl group with ^{14}C (59.5 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL) and used without further purification. D_2O was obtained from Aldrich Chemical Co. (Metuchin, NJ). All other reagents were of standard laboratory grade.

The Tes buffer referred to in this paper was 50 mM Tes, pH 7.5, and contained 100 mM NaCl unless otherwise indicated.

Membrane Isolation. The procedure used was essentially that of Smith et al. (1975). The buffer was 0.1 M KH_2PO_4 adjusted to pH 7.0 with NaOH and was saturated with argon. All membrane suspensions were kept at 4 °C under an argon atmosphere. In brief, 50 dark-adapted retinas were thawed and then suspended in sufficient 45% (w/v) sucrose in buffer to give a final volume of 75 mL. This suspension was forced through a disposable plastic syringe (without needle) and subsequently divided into two polyallomer centrifuge tubes. After centrifugation at 25 000 rpm for 1 h in a Beckman SW 27 rotor (Beckman Instruments, Palo Alto, CA), the crude rod outer segments were removed from the air–solution interface. These were again divided into two portions, diluted with buffer (final volume 50 mL in each tube), and pelleted. Each pellet was suspended in sufficient 40% (w/v) sucrose in buffer to fill a polyallomer tube, and the purified rod outer segments floated by centrifugation as described above. The rod outer segments were frozen or used immediately for the isolation of disk membranes.

For the preparation of disk membranes, the purified rod outer segments were divided into two portions, diluted with argon-saturated water to 50 mL, and then pelleted. These pellets were suspended in 34 mL of 5% (w/v) Ficoll in argon-saturated water and allowed to sit for 1–3 h (longer incubation periods were found to decrease the final yield of membranes). The membrane suspensions in polyallomer centrifuge tubes were overlayed with 1.5 mL of water and centrifuged at 25 000 rpm in the Beckman SW 27 rotor for 2–2.5 h. The intact disks floating on the surface of the Ficoll solution were recovered by aspiration. The membranes were stored at −20 °C under argon in the 5% sucrose buffer.

Protein Isolation. For the removal of sucrose prior to solubilization, the disk membranes were washed by centrifugation with Tes buffer which also contained 0.1 mM dithiothreitol. The disk membranes were suspended in a small amount of Tes buffer, and a concentrated detergent solution in Tes buffer was added until turbidity, as viewed through the infrared image converter, disappeared. For cholate, the final concentration was usually 40 mg/mL, and for $C_{12}E_8$, the concentration used was approximately 10 mg/mL. After approximately 1 h, the solubilized sample was slowly run onto a small Con A–Sepharose column containing about 1.5 times more Con A sites than the total amount of rhodopsin being loaded onto the column. The column was washed extensively with detergent in Tes buffer (usually 20 mg/mL for cholate and 5 mg/mL for $C_{12}E_8$) to remove proteins and lipids which were not specificially retained by Con A. The column was next washed with Tes buffer containing the final concentration of detergent needed for the experiment. The protein was eluted from the column matrix in a batchwise procedure using Tes buffer containing detergent and 0.5 M α-methyl mannoside. The eluted protein was dialyzed against buffer containing detergent to remove the mannoside.

Spectroscopic Measurements. Absorption measurements were recorded at room temperature on a Cary 17D recording spectrophotometer equipped with a microcell adapter (Varian Instruments, Palo Alto, CA). The path length in all cases was 1.0 cm. The spectra were digitized by hand at 5-nm intervals, and these data were used for other manipulations, including replotting.

The digitization of the spectra was adequate for most purposes; however, fine structure in the protein spectrum is lost. This fine structure consisted of typical protein spectral features including a tryptophan shoulder on the red side of the 278-nm maximum and a phenylalanine multiplet on the blue side. No attempt was made to reproduce these features in replotted spectra.

Circular dichroic spectra were recorded at room temperature on a Jobin-Yvon Dichrographe III (J-Y Optical Co., Metuchin, NJ). Hand-digitized spectra were used for plotting and calculations. Since samples usually contained variable amounts of opsin, eq 1 was used to extract the molar ellipticity of

$$[\theta]_R = \frac{[\theta]_{OB} - (1 - f_R)[\theta]_{OP}}{f_R} \quad (1)$$

rhodopsin, $[\theta]_R$, at each wavelength, where $[\theta]_{OB}$ is the observed molar ellipticity for the unbleached sample based on total protein. $[\theta]_{OP}$ is the molar ellipticity observed after total bleaching (i.e., we assume that the opsin present initially in the sample has the same ellipticity as the newly formed opsin), and f_R is the fraction of rhodopsin in the sample (see below). The mean residue weight estimated from published amino acid compositions was approximately 110.

Sedimentation Studies. Both sedimentation velocity and equilibrium were performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner (Beckman Instruments, Palo Alto, CA). The distribution of protein in the centrifuge cell was recorded at 500 and/or 280 nm as dictated by the experiment at hand. To minimize bleaching when recording data at 500 nm, we changed the wavelength to 280 nm immediately after scanning past the meniscus of the solution column. When data were not being collected, the window into the vacuum chamber was blocked. Using this method, we have found that five to ten scans can be made

[1] Abbreviations used: $C_{12}E_8$, octaethylene glycol *n*-dodecyl ether; Con A, concanavalin A; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

before there are detectable changes in A_{500} through the cell.

To obtain the values of $M_p(1 - \phi'\rho)$ as a function of concentration, it was necessary to evaluate the slope of the equilibrium distribution at each point in the cell. For this purpose, a portion of the data (ln A vs. r^2) was fit by the method of least squares to a second-degree polynomial. The quality of the fit was assessed visually; slopes and absorbances were calculated in the region fitted by the curve. Another portion of the data overlapping the first set was fit in the same way, and this procedure was repeated until the entire set of data was smoothed (in some instances, an entire data set was adequately smoothed by a single quadratic equation). In regions of overlap, the slopes and absorbances obtained at the same value of r^2 but using different smoothing functions were arithmetically averaged. The least-squares fits were performed by a Gauss-Jordan matrix inversion routine with full pivotal condensation (Dorn & McCracken, 1972).

Plots of $M_p(1 - \phi'\rho)$ as a function of absorbance were visually extrapolated to zero absorbance (i.e., protein concentration) to obtain the value of $M_p(1 - \phi'\rho)$ for the minimum species. On the assumption that the curvature in the ln A vs. r^2 plots is due to the presence of the oligomers of this smallest species, one can rigorously model the equilibrium distribution (Schecter et al., 1976). This modeling does not require any assumptions as to the mechanistic process giving rise to the oligomers. The model takes the form of a polynomial and is easily fit by least-squares methods.

The sedimentation coefficients in $C_{12}E_8$ were corrected to $s_{20,w}$ by assuming that the viscosity of the $C_{12}E_8$ solutions at the concentrations employed was the same as that of water. For cholate at 20 mg/mL, the viscosity at 25 °C was 0.997 cP, and correction to $s_{20,w}$ was made by assuming the temperature dependence to be the same as that of water. No correction for the effect of density on the sedimentation coefficient was included in the calculation of $s_{20,w}$, but this will not significantly alter the reported values.

Protein Assay. For spectral measurements and concentration determinations, 50 mM hydroxylamine was included in the sample to trap the retinal released by bleaching. For the estimation of rhodopsin content, we used 40 600 M^{-1} cm^{-1} for the extinction coefficient at 500 nm (Wald & Brown, 1953). Total protein was estimated by using 1.7 for the minimum value of the spectral ratio (A_{280}/A_{500}) and assuming that all A_{280} was attributable to either rhodopsin or opsin. The fraction of rhodopsin (f_R) in the sample is given by

$$f_R = 1.7\frac{A_{500}}{A_{280}} \quad (2)$$

The minimum value of A_{280}/A_{500} is not absolutely established and may be expected to vary somewhat depending on the nature and the history of the preparation. There is, however, a general clustering of literature values near 1.7, and we have chosen to use this in our calculations. Furthermore, a value of 1.7 was the minimum found for rhodopsin preparations when using the vertical separation between the ln A_{280} and ln A_{500} data (in our centrifuge studies) as an estimate of A_{280}/A_{500}. Values for total protein were occasionally determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard and found to agree with those calculated with eq 2. It is important to realize that none of the calculations in this paper depend on accurate knowledge of the protein concentration.

Other Methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the methods of Weber & Osborn (1975) with modifications described by Nielsen & Reynolds (1978). The gels were stained for protein using Coomassie Brilliant Blue R-250 (0.2% w/v) and diffusion destained. Samples were stained for carbohydrate using the periodic acid–Schiff base technique of Zacharius & Zell (1969). Retinal was covalently attached to the protein moiety by reduction of the Schiff base with $NaBH_4$ (1% $NaBH_4$ was added to the sample which was immediately exposed to light; 1% $NaDodSO_4$ was added and the sample subsequently dialyzed against the electrophoresis buffer). Fluorescent bands were observed visually using a hand-held ultraviolet light.

Solution densities were measured with an Anton Paar DMA 02C densitometer (Mettler Instrument Co., Princeton, NJ). Viscosity measurements were made with Ostwald viscometers in a Neslab viscometer bath (Neslab Instruments, Inc., Portsmouth, NH).

Phospholipid was assayed as total inorganic phosphate following wet ashing by the method of Bartlett (1959). Radioactivity was measured on a Beckman LS 100C scintillation counter (Beckman Instruments, Palo Alto, CA). The counting cocktail was the dioxane-based scintillation fluid of Bray (1960).

Curve fittings were performed on a Tektronix 31 calculator equipped with a Tektronix 4661 digital plotter (Tektronix, Beaverton, OR) using programs written by one of us (D.R.M.).

All procedures involving rhodopsin were performed by using infrared illumination (flashlight with infrared filter from Edmund Scientific Co., Barrington, NJ; less than 5% T for λ <890 nm) and an infrared image converter (FJW Industries, Mt. Prospect, IL). Unless otherwise indicated, all work was at room temperature. Rhodopsin was bleached by exposure to room light and a small high-intensity desk lamp until there was no further change in the absorbance at 500 nm. The high-intensity lamp was kept approximately 1 ft from the sample to avoid significant heating of the sample.

Results

Purification. The method of purification described under Experimental Procedures discriminates between rhodopsin and other proteins of the rod outer segments on the basis of rhodopsin's carbohydrate moiety. There is in principle no distinction between rhodopsin and its apoprotein opsin. Purified samples therefore contain opsin in variable amounts, reflecting for the most part the amount of bleached protein present in the retinas as received from the supplier. The spectral ratio A_{280}/A_{500} can be used to measure the amount of opsin present (Experimental Procedures), and by this criterion, different samples used in this work contained between 0 and 35% opsin.

Purified preparations were analyzed for residual phospholipid, and none was found, the detection limit under the conditions used being about 2 mol of phospholipid per mol of monomeric rhodopsin.

The spectra of unbleached and bleached rhodopsin in both cholate and $C_{12}E_8$ are shown in Figure 1. The spectra of the unbleached protein are nearly identical in the two detergents with the exception of a perturbation near 350 nm. The cause of this perturbation is not known. We have observed that retinal itself when dissolved in these detergents shows a similar difference (McCaslin, 1979), and thus, this spectral perturbation may be the result of a partial exposure of the protein-bound retinal to solvent. The observations of Suzuki et al. (1977) which show the visible circular dichroism spectrum of rhodopsin to be affected by solvent composition would be consistent with such a proposal.

By the criterion of $NaDodSO_4$–polyacrylamide gel electrophoresis, all samples used were free of other rod outer segment proteins after Con A chromatography. The gel

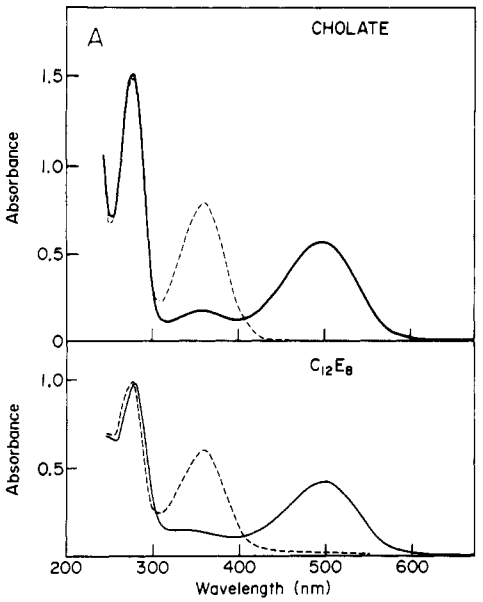

FIGURE 1: Absorption spectra of rhodopsin in 46 mM cholate and in 1.9 mM $C_{12}E_8$, in the presence of 50 mM hydroxylamine. Solid lines represent the unbleached protein, and dashed lines are the spectra recorded after bleaching in white light. The spectral ratios (A_{280}/A_{500}) are 2.6 and 2.3, respectively, in the two samples, and they reflect the opsin contents of the membrane preparations from which the samples were derived.

electrophoretic patterns usually show multiple bands as has been reported by others (Albert & Litman, 1978; Fung & Hubbell, 1978). It is virtually certain that the bands represent oligomers of the opsin polypeptide chain and not contaminating proteins. All the bands stain positively for carbohydrate, and all contain covalently attached retinal when sodium borohydride reduction is used prior to $NaDodSO_4$ solubilization. Apparent molecular weights of the bands (when compared to standard proteins used for calibration) had ratios close to 4:3:2:1. Furthermore, even rhodopsin which was isolated in $C_{12}E_8$ and found to be a homogeneous population of monomeric protein (see below) showed a similar multiple-band pattern.

The mechanism whereby the presumed oligomers in $NaDodSO_4$ are formed is not known. The formation of the oligomers is enhanced by heating the sample in $NaDodSO_4$ prior to electrophoresis. However, neither elimination of the heating step nor the presence of β-mercaptoethanol consistently prevents the formation of oligomers. Furthermore, bleaching rhodopsin in the presence or absence of dithiothreitol in $C_{12}E_8$ initiates an aggregation process for the bleached protein. These observations suggest that factors other than the oxidation of sulfhydryls contribute significantly to the formation of oligomers in $NaDodSO_4$.

State of Association in Sodium Cholate. Sedimentation coefficients of purified rhodopsin in various concentrations of sodium cholate are shown in Figure 2. The sedimentation coefficient is virtually constant at all sodium cholate concentrations above 10 mg/mL, indicating the existence of a particle with constant hydrodynamic properties and presumably constant molecular weight. There is a dramatic increase in the sedimentation coefficient when the detergent concentration falls below 10 mg/mL, presumably the result of aggregation. A careful examination of the absorption profiles in these experiments shows that aggregation occurs at the higher protein concentrations near the bottom of the cell even though the rate of movement of the boundry between pure solvent and the bulk protein solution remains constant. This effect is much more pronounced at 10 mg/mL cholate than at 20 mg/mL cholate.

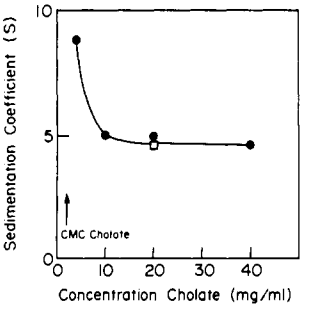

FIGURE 2: Effect of cholate concentration on the sedimentation coefficient. The total protein concentration was approximately constant at 5 μM, and the temperature in all cases was 22.85 °C. The sedimentation coefficient was the same whether monitored at 280 or 500 nm. The filled circles represent samples which were predominately rhodopsin; the square was a measurement determined on a totally bleached sample.

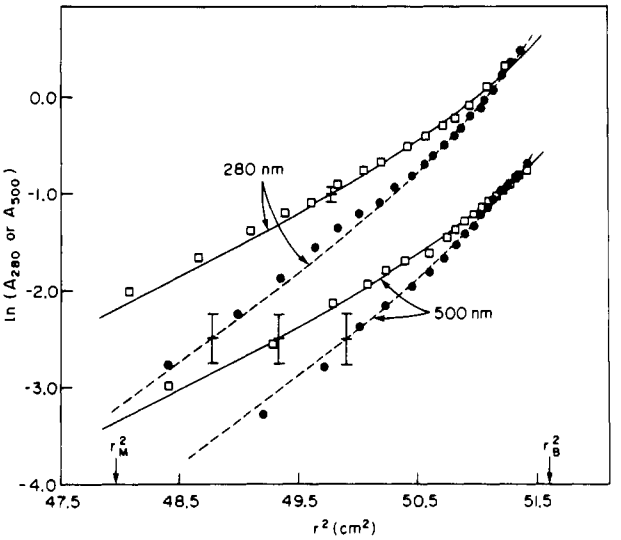

FIGURE 3: Sedimentation equilibrium data for rhodopsin isolated in sodium cholate. The concentration of cholate was 20 mg/mL, and the Tes buffer contained 0.1 mM dithiothreitol. The concentration of rhodopsin was 4.5 μM and had an A_{280}/A_{500} ratio of 2.57. The open squares were determined at 9000 rpm and the filled circles at 11 000 rpm; the temperature was 25.0 °C. The solid and dashed lines are theoretical fits to the data made by assuming that the curvature is the result of the presence of oligomers of a minimum species with $M_p(1 - \phi'\rho) = 33\,000$. Each set of data was fit independently, and the parallelism between the A_{280} and A_{500} fitted curves (at the same speed) supports the applicability of this model to the data. The indicated error bars show the error propagated by taking the logarithm of the absorbance data, assuming an absolute error of 0.02 in the absorbance data. The error bars increase with decreasing A. The positions of the cell bottom and the solution meniscus are located at r_B^2 and r_M^2, respectively.

Sedimentation equilibrium measurements were used to measure absolute molecular weights. The samples used in these experiments had opsin contents of up to 35% based on A_{280}/A_{500}. Both A_{280} and A_{500} were used to monitor the protein concentration as a function of the position in the cell. Plots of ln A vs. r^2 were invariably curved as shown in Figure 3, showing that aggregation was occurring with increasing protein concentration. The vertical separation between the ln A_{280} and ln A_{500} plots is nearly constant for all values of r^2 (hence, the curves are parallel) and is nearly identical with the ln (A_{280}/A_{500}) measured spectrophotometrically on the starting solution. This shows that rhodopsin and opsin must be behaving identically in these experiments, since the data obtained at 280 nm would otherwise have differed from those at 500 nm. Moreover, the sedimentation equilibrium results were unaffected by the presence of 0.1 mM dithiothreitol. This observation in conjunction with the studies of Henselman &

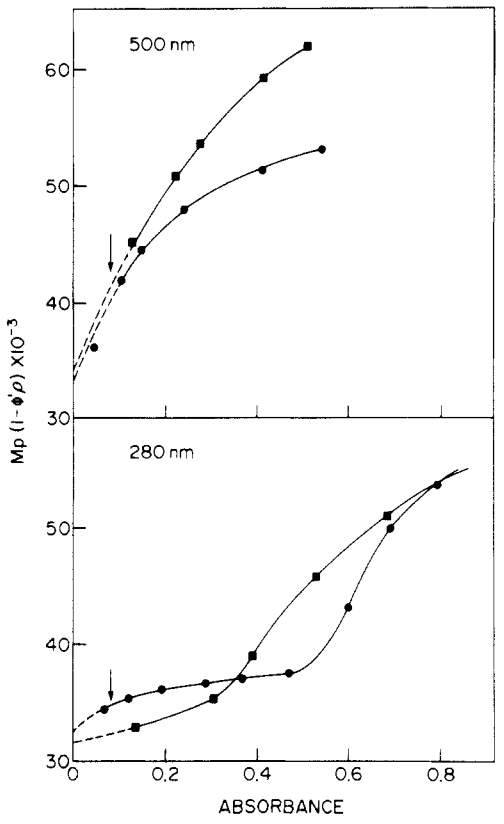

FIGURE 4: $M_p(1 - \phi'\rho)$ as a function of absorbance. These results are to illustrate the typical behavior observed when the ln A vs. r^2 data are smoothed as described in the text. The squares are derived from the 9000 rpm data of Figure 3 and the circles from the 11 000 rpm data. The arrows in the figure are at 0.08 absorbance (ln A = −2.5); due to the large errors propagated by taking the logarithm of the primary absorbance data, values of $M_p(1 - \phi'\rho)$ calculated from the data below this absorbance are of a much lower accuracy than when data above this value are used. Therefore, $M_p(1 - \phi'\rho)$ values calculated by using absorbances less than 0.08 have not been explicitly included in our extrapolation but were in general consistent with the extrapolation lines (dashed portions in figure).

Cusanovitch (1974) on the sulfhydryl chemistry of rhodopsin in cholate is evidence that the oligomeric state of rhodopsin in cholate is not the result of the formation of disulfide-linked polypeptide chains.

The slopes of the equilibrium distributions in these experiments yield the weight-average value of the unsolvated protein molecular weight (M_p) multiplied by the combined buoyancy and interaction factor ($1 - \phi'\rho$), as discussed previously (Tanford et al., 1974). The experimental curves can be analyzed by curve-fitting procedures to give $M_p(1 - \phi'\rho)$ as a function of protein concentration, using the observed absorbance at any point in the cell as a measure of the latter. Figure 4 shows typical data obtained this way. A total of ten independent protein preparations were analyzed in this way, and usually there were several runs for each preparation at different speeds and/or initial protein concentrations. The results always extrapolated to a limiting value of $M_p(1 - \phi'\rho)$ close to 33 000 at zero protein concentration. At 280 nm, where the higher extinction coefficient permits data acquisition to lower protein concentrations than at 500 nm, the values of $M_p(1 - \phi'\rho)$ in several runs leveled off as zero absorbance was approached, indicating that the 33 000 value represents a true minimal value and that species with smaller $M_p(1 - \phi'\rho)$ values are never present in significant amounts.

In the absence of bound lipids, the parameter $M_p(1 - \phi'\rho)$ may be expressed in terms of measurable parameters as (Tanford et al., 1974)

$$M_p(1 - \phi'\rho) = M_p[(1 - \bar{v}_p\rho) + \delta_D(1 - \bar{v}_D\rho)] \quad (3)$$

where ρ is the solvent density, δ_D is the amount of bound detergent (grams) per gram of protein, and $\bar{v}_p$ and $\bar{v}_D$ are the thermodynamic partial specific volumes of protein and detergent, respectively. The value of $\bar{v}_p$ was determined by using published amino acid and sugar compositions for rhodopsin (Sale et al., 1977; Plantner & Kean, 1976; Daeman et al., 1972), and $\bar{v}_D$ (above the critical micelle concentration) was measured by densitometry, both procedures having been previously described (Tanford et al., 1974). The values obtained were $\bar{v}_p$ = 0.735 cm^3/g and $\bar{v}_D$ = 0.771 cm^3/g. If δ_D were known, eq 3 would give an absolute value for the protein molecular weight, M_p. Unfortunately, the high critical micelle concentration of cholate (4.6 mM under the conditions used here) coupled with the requirement that low protein concentrations must be employed to obtain data characteristic of the minimal molecular weight species makes a direct measurement of δ_D impossible unless δ_D is large. A dialysis equilibrium experiment using radioactive cholate showed no significant difference between the protein sample and the dialysate, under conditions where the detection limit was about 0.9 g/g (80 mol per 38 500 g). One can therefore say no more than that $0 \leq \delta_D \leq 0.9$, but even this limits the possible values of M_p if we add the requirement that M_p must be an integral multiple of the polypeptide chain molecular weight. Estimates in the literature based on trustworthy methods [sedimentation equilibrium, Lewis et al. (1974); minimal molecular weight from amino acid composition, see e.g., Daeman et al. (1972) and Sale et al. (1977)] yield values for the polypeptide chain molecular weight of between 35 000 and 42 000, including bound carbohydrate. Our own gel electrophoretic results give apparent molecular weights between 38 000 and 39 000. This and the literature values give a mean of 38 500. With this value and the observed limiting values $M_p(1 - \phi'\rho)$ = 33 000, eq 3 shows that δ_D would have values of 2.71, 0.77, 0.12, and −0.20 g/g (242, 69, 11, and −18 mol/polypeptide), respectively, for M_p to correspond to a monomer, dimer, trimer, or tetramer of the polypeptide chain molecular weight. The possibility of a monomer is clearly eliminated by the upper limit on δ_D and the possibility of a tetramer by the fact that δ_D must be positive.

When all parameters except δ_D are known, it is advantageous to use D_2O–H_2O mixtures to vary the solution density (Reynolds & Tanford, 1976), because the factors $1 - \bar{v}_p\rho$ and $1 - \bar{v}_D\rho$ in eq 3 will be in a different ratio to each other at different values of ρ. This method is most useful when the difference between $\bar{v}_p$ and $\bar{v}_D$ is large, and if $\bar{v}_D > 0.9$ cm^3/g, the $\bar{v}_D$-dependent term can be blanked out altogether at a value of ρ that is experimentally accessible by varying the D_2O/H_2O ratio. Because of the small difference between $\bar{v}_p$ and $\bar{v}_D$ in the present experiments, however, the method is not able to provide a significant discrimination between the possibilities permitted by the data at one solvent density alone, as the following results show.

We substituted D_2O for H_2O by repeated dialysis against a buffer solution prepared with pure D_2O. Sedimentation equilibrium experiments and data analysis were carried out as before, but different $\bar{v}$ values were used because both the protein and the detergent contain exchangeable protons which will affect their mass but will have little effect on the partial *molar* volume (Edelstein & Schachman, 1967). Rhodopsin contains a maximum of about 480 exchangeable protons per polypeptide chain, but not all of these were observed to exchange in a 36–40-h period in hydrogen-isotope exchange experiments (Osborne & Nabedryk-Viala, 1978). Using 300

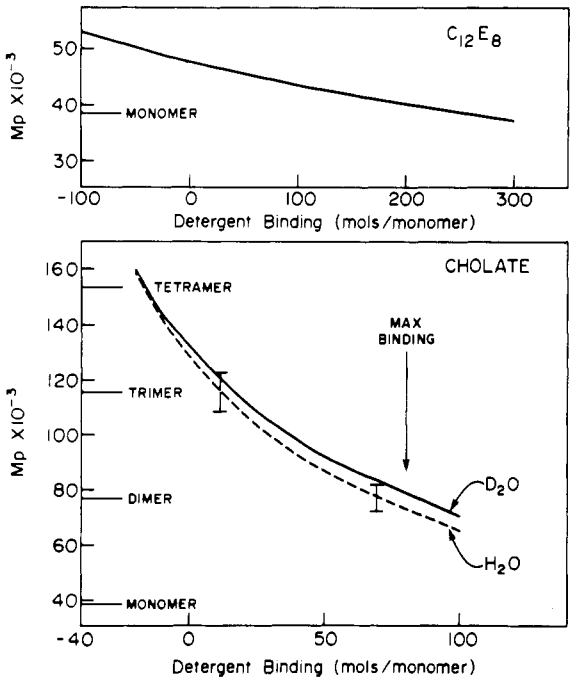

FIGURE 5: Calculated values of M_p and δ_D required to give the experimentally observed values of $M_p(1 - \phi'\rho)$. The upper panel shows the calculations for $C_{12}E_8$ in Tes buffer using $M_p(1 - \phi'\rho) = 12\,300$ and $\rho = 1.01$ g/mL; the other constants needed for eq 3 are in the text. The lower panel presents the same calculations for rhodopsin in cholate with Tes buffer in H_2O ($\rho = 1.01$ g/mL) and Tes buffer in D_2O ($\rho = 1.11$ g/mL). In these calculations, the partial specific volumes are corrected for deuterium incorporation as discussed in the text. Maximum binding refers to the limit set by a dialysis equilibrium experiment described in the text. Monomer, dimer, trimer, and tetramer refer to oligomers of the opsin polypeptide chain (assuming a molecular weight of 38 500). $M_p(1 - \phi'\rho)$ in water is 33 000 ± 2000 and 24 000 ± 2000 in D_2O. The two vertical bars represent the limits to the theoretical curves calculated by using the maximum errors of the value for $M_p(1 - \phi'\rho)$ in water.

for the number of actually exchangeable protons leads to an increase in the polypeptide chain molecular weight of 0.78%, which is of course negligible since we are trying to discriminate between the molecular weights for trimeric and dimeric rhodopsin. However, the change of $\bar{v}_p$ (from 0.735 to 0.728 cmn^3/g) is significant because it is the change in $1 - \bar{v}_p\rho$ that affects the difference in the results between the two solvents. Cholate ions have three exchangeable protons, and $\bar{v}_D$ is expected to change as a result from 0.771 to 0.776 cm^3/g. The expected effect on δ_D is negligibly small.

The limiting value of $M_p(1 - \phi'\rho)$ obtained in D_2O was 24 000, as compared to 33 000 in H_2O. The mutually compatible values of M_p and δ_D for both sets of data are shown in Figure 5. Imposing the condition that both M_p and δ_D must have the same values in the two solvents, we see that the results favor a molecular weight corresponding to a trimer of the polypeptide chain, with δ_D approximately 0.1–0.2 g/g, corresponding to about 10 or 15 bound cholate ions per polypeptide chain or 30–50 bound ions per trimer. The significance of this result depends on the experimental uncertainty in the limiting values of $M_p(1 - \phi'\rho)$ in the two solvents. Because the numbers were obtained by visual extrapolation, a rigorous statistical analysis becomes difficult and was not used. Most of the extrapolations gave limiting values in the range of 33 000 ± 1500 (the experimental error for a single determination in the ultracentrifuge is less than 5%), and none of the values were outside the range 33 000 ± 2000. Even a 5% uncertainty in $M_p(1 - \phi'\rho)$ is, however, sufficient to put the difference between the two curves of Figure 5 at $M_p = 77\,000$ (corresponding to a dimer of the polypeptide chain) within experimental error. The data thus show that the results in D_2O are consistent with those in H_2O, but the extent to which they favor the trimer value of M_p over the dimer value must be taken as slight.

A better discriminant between the two possible values of M_p is provided by the fact that the amount of detergent that binds to a solubilized membrane protein is normally related to the size of the micelle that the detergent forms in the absence of protein. Both cholate and deoxycholate form very small micelles (Small, 1971) and bind to membrane proteins in lower molar ratios than do other detergents. There are no previously reported measurements for cholate, but measurements for deoxycholate have been made for cytochrome b_5 (Robinson & Tanford, 1975) and the sarcoplasmic reticulum CaATPase (LeMaire et al., 1975), under conditions where both are monomeric, and for the dimeric coat protein of bacteriophage (Makino et al., 1975). All values fall within the range of 25–40 mol of bile salt bound per mol of solubilized particle. The binding required if rhodopsin is a trimer, as given above, is within this range. As Figure 5 shows, an M_p value of 77 000, corresponding to the dimeric association state, requires a binding of about 0.75 g/g, corresponding to 135 mol of cholate per solubilized particle which is outside this range. While present data do not allow an unequivocal choice between dimer and trimer as the association state, we will for the purposes of this discussion refer to the cholate particle as a trimer of rhodopsin.

State of Association in $C_{12}E_8$. Sedimentation equilibrium experiments in $C_{12}E_8$ were made by using rhodopsin samples whose opsin content was similar to that of the samples used for the data obtained in sodium cholate (i.e., <35%). In contrast to the data in cholate, equilibrium distributions in $C_{12}E_8$ determined by absorbance measurements at 500 nm differed from those measured at 280 nm. The measurements at 500 nm correspond to a single homogeneous species within the cell, not subject to aggregation, with an $M_p(1 - \phi'\rho)$ value of 12 300 ± 300 for five separate protein preparations each run at at least two speeds. The results at 280 nm were consistent with a minimal $M_p(1 - \phi'\rho)$ value of 12 300, with aggregation occurring as the protein concentration increased. Since this aggregation is seen only at 280 nm, it must reflect a property of the bleached protein alone, and this is confirmed by the partial bleaching experiments described later in this paper.

Because the partial specific volume of $C_{12}E_8$ ($\bar{v} = 0.973$ cm^3/g; Tanford et al., 1977) is close to unity, the detergent makes little contribution to $M_p(1 - \phi'\rho)$, and M_p can be determined unambiguously. As Figure 5 shows, a negative value of δ_D would be required to obtain $M_p > 47\,500$. Thus, rhodopsin in $C_{12}E_8$ is monomeric. Allowing an uncertainty of ±2000 in the polypeptide chain molecular weight (nominally 38 500) leads to a δ_D value of 3.5 ± 1 g/g or 250 ± 60 mol/mol.

Sedimentation velocity measurements in $C_{12}E_8$ were made, using the absorbance at 500 nm to measure the boundary movement arising from unbleached rhodopsin. The data were consistent with sedimentation of a single species with an uncorrected sedimentation coefficient at 21.5 °C of 2.26 S. The corresponding value of $s_{20,w}$ is 2.13 S.

Effect of Bleaching. The sedimentation equilibrium experiments described above show that opsin present in retinas shipped to us exists in the same state of association as that of unbleached rhodopsin after solubilization in sodium cholate, but not after solubilization in $C_{12}E_8$. A similar result was obtained when rhodopsin was partially bleached by exposure

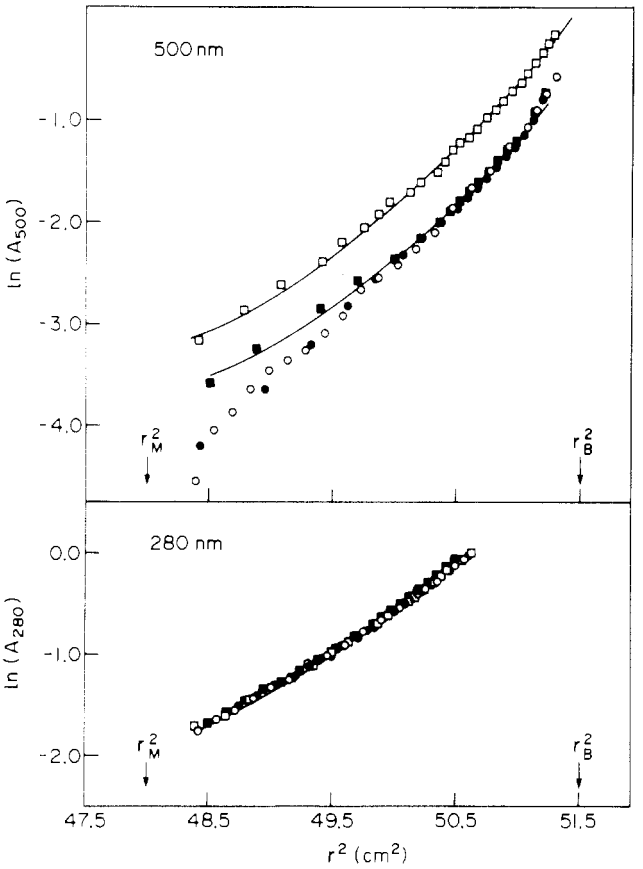

FIGURE 6: Effects of light on aggregation state of rhodopsin in cholate. The samples initially contained 7.5 μM rhodopsin in Tes buffer containing 20 mg/mL cholate, A_{280}/A_{500} = 2.6 initially. The speed was 9000 rpm, and the temperature was 20.02 °C. The details of the experiment are discussed in the text. (□) Sedimentation equilibrium gradient established prior to bleaching; (○) distribution of absorbing materials immediately following in situ bleaching; (●) distribution 24 h after in situ bleaching; (■) distribution at equilibrium after remixing sample.

to light in the untracentrifuge after the equilibrium distribution for the unbleached sample had been established.

The results in sodium cholate are shown in Figure 6. About 20 min were required to obtain a decrease of about 40% in A_{500} at one point in the cell, and the whole cell was scanned immediately after that. Provided no significant redistribution of the protein occurs during the bleaching process, this initial scan is expected to show a decrease in A_{500}, but the plot of ln A_{500} vs. r^2 should remain parallel to that of the original scan. The data showed the expected behavior, except at the very lowest absorbance values near the meniscus of the solution column. A similar deviation from the theoretical result was obtained in $C_{12}E_8$, and it is probably due to small differences in the incident light intensity falling on the sample in different parts of the cell, so that the extent of bleaching is not quite the same everywhere in the cell. The sample was then allowed to spin in the ultracentrifuge for a sufficient time to allow reequilibration to occur (28 h) and was then scanned again. No significant change in the data obtained at 500 nm was expected, because the data at this wavelength monitor the distribution of the remaining unbleached rhodopsin, and as Figure 6 shows, no change was observed. As a final check, the run was terminated, the sample was stirred in the dark, and a new equilibrium was carried out. Again no change was observed, except that the anomalous low absorbance near the meniscus of the cell disappeared.

The important part of this experiment lies in the results at 280 nm, where the freshly bleached opsin will make a major

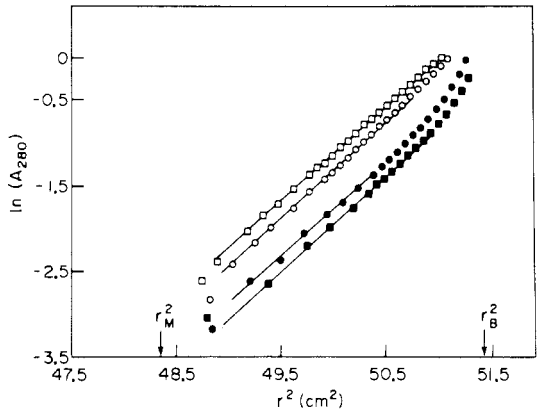

FIGURE 7: Effects of light on aggregation state of rhodopsin in $C_{12}E_8$. The sample was initially 6.2 μM rhodopsin in Tes buffer (the concentration of Tes was 20 mM in this experiment, but otherwise the buffer was as described in the text) containing 1 mg/mL $C_{12}E_8$, and A_{280}/A_{500} = 2.3, initially. The speed was 20 000 rpm, and the temperature was 18.97 °C. Only data collected at 280 nm are shown, and the symbols are as described in Figure 6.

contribution. As the lower panel of Figure 6 shows, all four scans at this wavelength were indistinguishable. Bleaching clearly has no effect on the association state in sodium cholate, and since even the smallest species in this detergent is a trimer, it is likely that many of the oligomeric complexes will be hybrids containing both unbleached and bleached molecules.

Quite different results were obtained when this experiment was repeated with rhodopsin in $C_{12}E_8$. Figure 7 shows the results obtained at 280 nm. A continuous loss of absorbance is observed, reflecting the formation of high aggregates that move rapidly to the bottom of the cell, and formation of intermediate oligomers is reflected in curvature of the plots as the bottom of the cell is approached. The portion of the plots near the meniscus remained linear, with a slope corresponding to the monomer molecular weight, as observed in the original sample.

It is of interest to note that the dramatic difference between the effect of bleaching on rhodopsin in sodium cholate and in $C_{12}E_8$, with respect to both the state of association and the ability to recombine with 11-*cis*-retinal, is not accompanied by a major difference in the secondary structure of the protein. Figure 8 shows that the effect of bleaching on the circular dichroic spectrum of the protein is nearly the same in the two detergents. If one analyzes the results in terms of empirical procedures such as described as by Greenfield & Fasman (1969), complete bleaching gives rise to a 6–8% decrease in α-helical content when in sodium cholate and a 6–9% decrease when in $C_{12}E_8$. Experiments with partially bleached samples (not shown) demonstrated that the change in ellipticity seen in Figure 8 is proportional to the extent of bleaching.

In Figure 1, the spectrum of bleached rhodopsin in $C_{12}E_8$ is seen to have a tailing absorption for wavelengths greater than 400 nm. Our sedimentation studies have clearly shown that opsin in $C_{12}E_8$ aggregates extensively; hence, we attribute the aforementioned absorption tail to light scattering arising from aggregated opsin.

Association States in Sodium Cholate and $C_{12}E_8$ Are Equilibrium States. In order to determine whether the tendency of rhodopsin to associate to an oligomeric state with a defined minimal molecular weight is an inherent thermodynamic property of the molecule, reversibility of the dissociation to monomers in $C_{12}E_8$ was tested. Disk membranes were solubilized in $C_{12}E_8$, and the rhodopsin was bound (in the dark) to a Con A column. Since purified rhodopsin is monomeric at the detergent concentration employed, it is

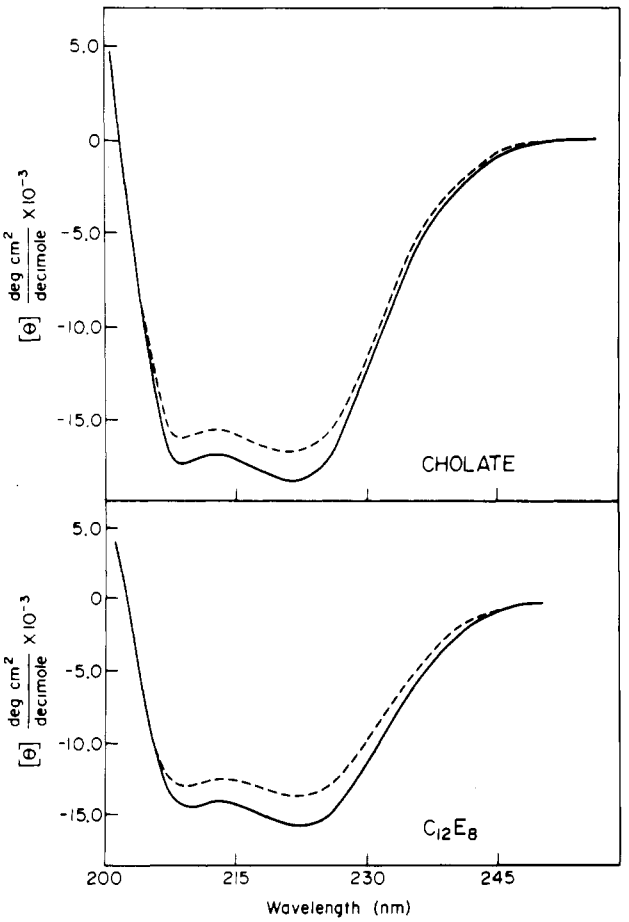

FIGURE 8: Circular dichroic spectra of rhodopsin in cholate and $C_{12}E_8$. The solid lines represent the spectra of unbleached rhodopsin extracted, as described in the text, from the recorded spectrum of the rhodopsin–opsin mixture. The dashed lines are the totally bleached samples. The $C_{12}E_8$ sample was 2.9 μM rhodopsin (A_{280}/A_{500} = 2.4) in Tes buffer (Tes concentration was 20 mM) containing 0.1 mM dithiothreitol and 1 mg/mL $C_{12}E_8$; the path length of the cell was 0.1 cm. The cholate sample was 14 μM rhodopsin (A_{280}/A_{500} = 2.6) in the standard Tes buffer containing 20 mg/mL cholate; the path length was 0.02 cm.

presumably bound to the column as a monomer. The column was washed with $C_{12}E_8$ to remove phospholipid and then with cholate at a concentration of 20 mg/mL. At this point, one presumably has monomeric rhodopsin with cholate bound to it, attached to the column through its carbohydrate moiety. The rhodopsin was then eluted from the column and subjected to sedimentation equilibrium. It was found to behave identically with rhodopsin that had never been exposed to $C_{12}E_8$, with respect to both the state of association of the originally eluted sample (Figure 3) and the effect of bleaching on it (Figure 6). The monomer and trimer states of the protein thus clearly reflect a reversible thermodynamic equilibrium. Moreover, this experiment adds further support to our earlier contention that the oligomers in cholate are not the result of disulfide bond formation since the oligomeric state is induced by merely exchanging $C_{12}E_8$ for cholate.

Discussion

This paper has compared the properties of rhodopsin in esstentially lipid-free form in two detergents. In one of the detergents (sodium cholate), the native state of the protein after bleaching can be recovered by recombination with 11-*cis*-retinal; in the other ($C_{12}E_8$), bleaching is irreversible. We have found that the protein differs in its state of aggregation in the two detergents, being monomeric in $C_{12}E_8$ but oligomeric in sodium cholate, with a well-defined minimal molecular weight corresponding to a trimer of the polypeptide chain. The simplest interpretation of this result in relation to the loss of reversibility upon bleaching in $C_{12}E_8$ is that the oligomeric state is representative of the native state of rhodopsin in the disk membrane. Consistent with this conclusion, the data from freeze–fracture electron microscopy (Chen & Hubbell, 1973; Corless et al., 1976) suggest that the intramembranous particles in the plane of the disk membranes could contain about four monomeric rhodopsin molecules. X-ray diffraction data (Blaurock, 1977) and measurements of rotational diffusion time (Cone, 1972; Baroin et al., 1978) have, however, been interpreted in terms of a monomeric membrane-incorporated molecule. It is perhaps of interest in this connection that the native state of bacteriorhodopsin in the crystalline purple membrane of *Halobacter halobium* consists of an array of trimeric protein molecules (Henderson & Unwin, 1975), and there is evidence (Rehorek & Heyn, 1979) for a relation between trimer formation and retinal binding (in this case, *trans*-retinal). However, a trimer appears not to be required for the biological function of the protein as a light-driven proton pump (Dencher & Heyn, 1979).

If native rhodopsin is in fact a trimer, it is important to note that the dissociation of the unbleached protein to a monomeric state is of itself not an irreversible process, as demonstrated by the reversibility of the dissociation described above. The effect of the association state is manifested only after bleaching has occurred, suggesting that protein–protein interactions in the oligomeric state (in sodium cholate) are sufficient to retain some feature of the protein conformation required for recombination with 11-*cis*-retinal, whereas this feature is lost after the monomer is formed (at least in $C_{12}E_8$).

It is important to mention in this connection that Hubbard (1954) purported to show that rhodopsin is monomeric in digitonin, another detergent in which the ability to recombine with 11-*cis*-retinal is preserved after bleaching. The validity of her conclusion is however questionable: Hubbard measured the molecular weight of the digitonin-solubilized particle as 260 000, with a measured partial specific volume of 0.766 cm^3/g. She then assumed that all of the digitonin in the extracting buffer was bound to the protein, which led to a δ_D value of more than 5 g of digitonin per g of protein, and on this basis concluded that each particle contained only one rhodopsin molecule. In the absence of direct binding measurements, it is impossible to estimate a true value for δ_D, but it should be noted that digitonin contains a fused ring system similar to that of cholate and that a δ_D value closer to the value we observed for cholate (~0.2 g/g) might therefore have been expected.[2]

The sedimentation velocity measurements reported in this paper allow one to make an estimate of the asymmetry of the sedimenting particle (Tanford et al., 1974). The limiting value at high cholate concentration (Figure 2) is 4.7 S under the conditions used, which corresponds to $s_{20,w}$ = 4.57 S, or to a Stokes radius (R_S) of 59 Å. The *minimal* radius of a dry particle containing a trimer of rhodopsin with δ_D = 0.2 g/g

[2] The possibility of lipid retention in the solubilized particle was also ignored. Since rhodopsin ($\bar{v}$ = 0.735 cm^3/g) and digitonin ($\bar{v}$ = 0.738 cm^3/g) have nearly the same partial specific volume, the observed $\bar{v}$ of 0.766 cm^3/g in fact requires the presence of phospholipid, to the extent of about 12%, using a reasonable value for the $\bar{v}$ of the lipid. [The presence of lipid in the particle has been demonstrated experimentally by Krinsky (1958).] The combined mass of protein and digitonin in the Hubbard particle is thus 229 000 rather than 260 000, but one still needs to reduce δ_D to no lower than 1 g/g to obtain a protein mass corresponding to the trimeric association state.

is 34 Å, so that R_S/R_{min} [which is identical with f/f_{min} as defined by Tanford (1961)] has a value of 1.72. Though both hydration and asymmetry contribute to this parameter, hydration alone cannot conceivably account for the large value of R_S/R_{min} observed (it would require more than 4 g of bound water per g of protein), and the result obtained thus indicates that the rhodopsin–cholate *particle* must by highly asymmetric. Because the sedimenting particle is trimeric, the result cannot yield a quantitative measure of the asymmetry of an individual rhodopsin molecule, but the conclusion is in qualitative agreement with other data that indicate rhodopsin to be a highly asymmetric molecule (Wu & Stryer, 1972; Sardet et al., 1976; Osborne et al., 1978; Wright, 1976; Stryer, 1978).

The sedimentation coefficient measured in $C_{12}E_8$ ($s_{20,w}$ = 2.13 S) leads to the much smaller value R_S/R_{min} = 1.24, which suggests that the sedimenting *particle* in this case is fairly symmetric. Because of the high detergent content of the particle ($\delta_D \sim 3.5$ g/g), it is not possible to draw any conclusions about the asymmetry of the rhodopsin molecule within the particle.

Other physical and chemical data, not related to the state of association, suggest that rhodopsin solubilized in cholate has a conformation resembling the conformation of membrane-bound rhodopsin and differing from the conformation of rhodopsin solubilized in detergents where the recombination capacity is lost. For example, Fung & Hubbell (1978) reported that the proteolytic fragments produced from cholate-solubilized rhodopsin were identical with those from membrane-bound rhodopsin. Furthermore, they demonstrated that the proteolytic fragments of cholate-solubilized rhodopsin did not dissociate upon photolysis as is the case for rhodopsin solubilized in detergents in which the recombination reaction has been lost (Fung & Hubbell, 1978; Pober & Stryer, 1975). Since proteolytically cleaved membrane-bound rhodopsin is capable of the recombination reaction (Pober & Stryer, 1975) and since there is no effect of light on the chemical cross-linking behavior of the proteolyzed rhodopsin (Fung & Hubbell, 1978), it is probable that the proteolytic fragments remain associated in the membrane whether bleached or unbleached.

The central role of rhodopsin in visual transduction and its implied interactions with other components (e.g., enzymes) in the rod outer segments provide a great impetus to find a detergent system in which all the properties attributable to native membrane-bound rhodopsin are preserved. As indicated in the introduction, rhodopsin can be isolated in nearly all detergents without loss of its native absorption spectrum, but in most of these detergents, the ability of opsin to recombine with 11-*cis*-retinal is lost. We have begun to systematically examine rhodopsin in various detergents in an attempt to elucidate the molecular properties involved in maintaining the ability of opsin to undergo recombination. For rhodopsin in $C_{12}E_8$ and various other detergents in which recombination is lost, there are data demonstrating or at least suggesting that the unbleached protein is monomeric and undergoes aggregation upon photolysis. Clearly, in these systems, any alteration in a physical or chemical property which occurs upon bleaching should not be interpreted on an a priori basis as playing a role in the visual process.

We have found that in sodium cholate (in which the recombination capacity is intact) the protein is oligomeric and that this association is not affected by bleaching. While the present data base is too small to prove that association is essential to the maintenance of the recombination capacity of rhodopsin, the strong correlation between the loss of recombination and a monomeric rhodopsin in a variety of detergents is very suggestive.

Acknowledgments

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Comparison of Binding Sites for Wheat Germ Agglutinin on Raji Lymphoblastoid Cells and Their Isolated Nuclei and Plasma Membranes†

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ABSTRACT: Raji lymphoblastoid cells and the cell nuclei and plasma membranes isolated by the glycerol-lysis technique [Jett, M., Seed, T., & Jamieson, G. A. (1977) *J. Biol. Chem. 252*, 2134–2142] have been examined for their ability to bind wheat germ agglutinin. Intact cells and isolated nuclei showed similarities (i) in the total number of binding sites (3.38×10^6 and 4.06×10^8, respectively), indicating at least a 2-fold higher receptor density on the nuclei, (ii) in the ratios of the number of high-affinity sites and low-affinity sites (1.05 and 1.07), and (iii) in the apparent association constants at the high-affinity sites (28 nM and 48 nM) and at the low-affinity sites (116 nM and 370 nM). Isolated plasma membranes had a similar number of total binding sites calculated on an equivalent cell basis (2.01×10^6) but showed differences in the ratio of high- to low-affinity sites (1.5) and in their apparent association constants (3 nM and 22 nM). These results suggest similarities in the lectin receptors on the outer surface of lymphoblastoid cells and the cell nuclei. The differences obtained with isolated membranes may be due to inversion of the membrane vesicles or to their decreased rigidity as compared with the intact cell.

The relationship between the plasma and nuclear membranes of cells is not clear but is of obvious importance in understanding membrane biosynthesis, cell growth, and neoplastic transformation. Morphologically, the two membrane systems may be continuous (Grundmann, 1699), and they are known to share enzymes such as 5′-nucleotidase (Crumpton & Snary, 1974; Wallach & Winzler, 1974) and thymidine-5′-phosphodiesterase (Erecinska et al., 1969; Jett et al., 1977).

Lectins have been used extensively to elucidate relationships between the cellular membrane comparments. Fluorescent and ferritin-conjugated lectins such as concanavalin A[1] and wheat germ agglutinin, which bind to intact cells, also bind to bovine liver nuclei (Nicolson et al., 1972) and to rat liver nuclei (Virtanen & Wartiovaara, 1976). Internalization of Con A has been observed with bovine hepatic cells under normal incubation conditions within 1–2 h (Nicolson, 1974). *Ricinus communis* lectin and phytohemagglutinin have been shown to interalize, presumably while bound to their surface receptors, into the GERL of mouse ganglion neurons within 1–3 h upon incubation at 37 °C (Gonatas et al., 1977). The ultimate fate of the internalized lectin is not known in either case. However, quantitative comparisons of the distribution and nature of the lectin binding sites on cells and nuclei are lacking.

WGA binds to surface glycoproteins in a number of different cell types (Nagata & Burger, 1974; Goldstein & Hayes, 1978), and the lectin specifically is directed mainly toward GlcNAc, although AcNeu is about one-half as effective (Peters et al., 1979; Bhavandan & Katlic, 1979). We have now quantitated the binding of wheat germ agglutinin to high- and low-affinity sites on intact Raji lymphoblastoid cells and their isolated nuclei and plasma membranes prepared by this procedure. A preliminary account of some aspects of this work has already appeared (Jett, 1977).

Materials and Methods

Sepharose-bound WGA was obtained from Vector Laboratories (Burlingame, CA). [3H]Glucosamine (173 mCi/mg, uniformly labeled) was obtained from Amersham/Searle (Arlington Heights, IL).

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[1] Abbreviations used: Con A, concanavalin A; WGA, wheat germ agglutinin; PBS, phosphate-buffered saline; GlcNAc, *N*-acetylglucosamine; Cl_3CCOOH, trichloroacetic acid; AcNeu, *N*-acetylneuraminic acid.

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