The Characterization of Sodium Cholate Solubilized Rhodopsin†

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ABSTRACT: Rhodopsin was extracted from isolated and lyophilized bovine rod outer segments using solutions of sodium cholate at a concentration of 20 mg/ml. Rhodopsin was partially purified by a 36% ammonium sulfate fractionation which reduced the phosphate (phospholipid) to rhodopsin molar ratio from 45–60 to 9–11. The recombination reaction between photolyzed rhodopsin and 11-*cis*-retinal was dependent upon the sodium cholate concentration, ranging from 90–100% recombination at 1–4 mg/ml to 5–10% at 20 mg/ml of sodium cholate. The pH optimum for the maximum extent of recombination in rhodopsin solutions containing 2–3 mg/ml of sodium cholate was between pH 7.0 and 7.4. The number of reactive sulfhydryl groups in both dark and photolyzed rhodopsin was found to increase with increasing concentrations (2–20 mg/ml) of sodium cholate.

Rhodopsin, the visual pigment protein, has been solubilized with a variety of surfactants to facilitate the study of the chemical and physical properties of the isolated protein. At the present time the only assays for native rhodopsin are its characteristic absorption spectrum and the ability of the light exposed protein to recombine with 11-*cis*-(9-*cis*) retinal to reform rhodopsin (isorhodopsin). With the exception of either digitonin (Wald and Brown, 1956) or Tween-80 (Zorn and Futterman, 1973) solubilized rhodopsin, all other reported detergent solutions of rhodopsin are incapable of undergoing the regeneration or recombination reaction with 11-*cis*-retinal even though the characteristic absorption spectrum is retained.

As pointed out by Hong and Hubbel (1973) a high recombination capacity is characteristic of native rhodopsin and the degree of regeneration is a reflection of the ability of the local environment to maintain the protein in a proper conformation. Their work suggested there is no specific requirement for phospholipids in order that rhodopsin maintain a stable and regenerable conformation. The chief requirement for recombination in detergent solutions of rhodopsin, as suggested by electron paramagnetic resonance studies (Hong and Hubbel, 1973), appears to be one of a local rigid environment for the rhodopsin molecule. These studies yield little information about the actual conformations of rhodopsin in its regenerable form *vs.* its nonregenerable form.

In the course of studies on the recombination reaction we encountered considerable difficulty with digitonin solubilized rhodopsin because of the limited solubility of the surfactant itself. Therefore a search was initiated for a suitable surfactant which would extract rhodopsin from rod outer segment (ROS[1]) membranes in a form capable of regeneration and also in a form which could be studied by physical and chemical methods. Our findings on the characterization of sodium cholate solubilized rhodopsin and properties of the protein in its regenerable and nonregenerable forms are reported here.

Materials and Methods

Preparation of ROS and Extraction of Rhodopsin. Procedures involving rhodopsin were done in total darkness or under dim red light (Kodak Wratten Series 1A filter) at 4° unless otherwise stated. ROS material was prepared from frozen dark adapted bovine retinas (American Stores Packing Co., Lincoln, Neb., and George Hormel Co., Austin, Minn.) essentially as described by Hong and Hubbel (1973). Partially thawed bovine retinas (100) were ground to a paste in a cold mortar and the volume was brought to 100 ml with 1.38 M sucrose in pH 6.5, 67 mM potassium phosphate buffer. The material was centrifuged for 15 min at 2450*g* and the supernatant was collected and diluted with an equal volume of pH 6.5, 67 mM potassium phosphate buffer (buffer A) and recentrifuged at 40,000*g* for 15 min. The resulting pellet was taken up in 40 ml of 1.02 M sucrose in buffer A, homogenized in a glass–Teflon homogenizer, and centrifuged at 40,000*g* for 45 min. The supernatant material was removed, diluted with an equal volume of buffer A, and centrifuged at 40,000*g* for 15 min. The floatation procedure was repeated two more times and the final pellet was washed three times with distilled water. The ROS were lyophilized and stored at −10° in a light proof container.

Rhodopsin was extracted from lyophilized ROS by homogenization with 0.1 M potassium phosphate buffer (pH 7.0) containing 20 mg/ml of sodium cholate. The homogenate was allowed to sit at 4° for 1 hr and was then centrifuged for 45 min at 40,000*g*. The clear supernatant containing rhodopsin was removed and the pellet discarded.

Ammonium Sulfate Fractionation of Rhodopsin. Solutions of rhodopsin in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml of sodium cholate and 1 mM dithioerythritol or mercaptoethanol were brought to 25% saturation by the addition of solid ammonium sulfate. After sitting at 4° for 10 min the mixture was centrifuged for 20 min at 10,000*g*. The supernatant solution was removed, brought to 30% saturation in ammonium sulfate, and recentrifuged as above. The supernatant solution was again collected and brought to 36% saturation in ammonium sulfate

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[1] Abbreviations used are: ROS, rod outer segment; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

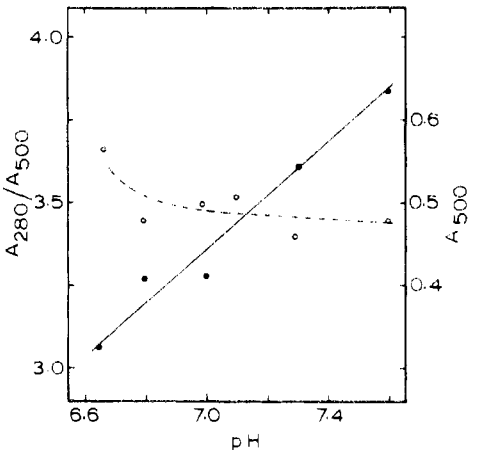

FIGURE 1: The effect of pH on the extraction and A_{280}/A_{500} ratio of rhodopsin using sodium cholate. Equal amounts of lyophilized ROS were extracted with 2.0 ml of 0.10 M potassium phosphate buffer containing 20 mg/ml of sodium cholate as described in the methods section. A_{280}/A_{500} (●—●); A_{500} (○ - - ○).

and after sitting at 4° for 10 min was centrifuged for 20 min at 10,000*g*. The supernatant was poured off and the pellet containing rhodopsin was immediately resolubilized in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml of sodium cholate and 1 mM dithioerythritol. Residual ammonium sulfate was removed by either passing the rhodopsin solution through a Sephadex G-25 column or by dialysis.

Recombination of Rhodopsin. Recombination experiments involving sodium cholate extracted rhodopsin were carried out as follows: 1-ml samples of rhodopsin (5–6 μM) in 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol and at the pH values and sodium cholate concentrations indicated in the results section were bleached by exposure to the white light of a microscope illuminator for approximately 10 min. The absorption spectrum of each sample was recorded before and after bleaching on a Cary 118 spectrophotometer. Recombination was initiated by the addition of exogenous 11-*cis*-retinal (final concentration of 15–20 μM) and each reaction mixture was incubated in the dark for 1 hr at room temperature. The reactions were terminated by the addition of 1 M hydroxylamine to a final concentration of 20 mM. The absorption spectrum of each sample was again recorded before and after a second bleaching. The extent or per cent of recombination was determined by the ratio of the changes in absorbance at 500 nm of the original or "native" rhodopsin and the regenerated rhodopsin both of which were bleached in the presence of hydroxylamine. In the pH dependence experiments a relative or normalized per cent recombination was used and the observed per cent recombination at pH 7.0 was set equal to 100%.

$$\text{per cent recombination} = \frac{\Delta A_{500}(\text{regenerated})}{\Delta A_{500}(\text{native})} \times 100$$

Sulfhydryl Groups of Rhodopsin. The sulfhydryl group reactivity of rhodopsin was determined with Nbs_2 basically as described by Ellman (1959) and the number of reactive sulfhydryl groups was estimated using an extinction coefficient of 13,600 M^{-1} cm^{-1} at 412 nm. Control experiments using cysteine as the reactive sulfhydryl compound indicated the extinction coefficient at 412 nm was not significantly affected (less than a 2% decrease) by carrying out the assay at pH 7.0 in the presence of 20 mg/ml of sodium cholate. All rhodopsin samples used for sulfhydryl assays had an A_{280}/A_{500} ratio of less than 2.6 and had not been in contact with reducing agents such as dithioerythritol or mercaptoethanol. Reaction mixtures were 1 ml in volume and contained pH 7.0, 0.10 M potassium phosphate buffer and were 5–6 μM in rhodopsin. Nbs_2 was added to the samples either in the dark or after 10-min bleaching with white light as indicated in the results section.

Other Methods. Rhodopsin concentrations were calculated using a molar extinction coefficient at 498 nm of 41,400 M^{-1} cm^{-1} for rhodopsin solutions in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml of sodium cholate. This molar extinction coefficient was determined using the thiobarbituric acid assay (Futterman and Saslaw, 1961) and the observed decrease in absorbance at 498 nm of the rhodopsin solution upon bleaching with white light in the presence of hydroxylamine.

Sodium cholate concentrations were determined using the method of Boyd *et al.* (1966). Phosphate analysis was carried out by the method of Bartlett (1959) or Fiske and SubbaRow (1925) after the rhodopsin solutions were extensively dialyzed against distilled water to remove inorganic phosphate.

Chemicals. Cholic acid was obtained from Sigma Chemical Co. and was purified by charcoal treatment and 2–3 recrystallizations from absolute ethanol. The acid was converted to the sodium salt by titration to pH 10 with sodium hydroxide and was then lyophilized. Nbs_2 was obtained from Sigma and used as received. Enzyme grade ammonium sulfate was obtained from Nutritional Biochemicals Corporation. All other chemicals used in this study were of reagent grade quality.

Results

Solubilization, Ammonium Sulfate Fractionation, and Properties of Rhodopsin. Extraction of lyophilized ROS membranes with pH 7.0, 0.1 M potassium phosphate buffer containing 20 mg/ml of sodium cholate resulted in solubilizing from 0.5 to 0.7 μmol of rhodopsin per 100 bovine retinas based upon an extinction coefficient of 41,400 M^{-1} cm^{-1} at 498 nm. These crude extracts of rhodopsin had A_{280}/A_{500} ratios ranging from 2.4 to 3.8 and in most cases were quite concentrated with the absorbance at 500 nm as high as 6. The concentration of sodium cholate used for the extraction was important, when it was less than 15 mg/ml the yields of rhodopsin were poor and the resulting solutions were difficult to clarify. Altering the pH of the extracting solutions, 6.5–7.6, did not appear to significantly affect the amount of rhodopsin extracted but the A_{280}/A_{500} ratio of the resulting rhodopsin solutions increased with increasing pH (Figure 1). Carrying out the extraction at pH 7.0 appears to be the best compromise as at pH values below 7.0 cholic acid tends to crystallize out of the solutions upon storage while at pH values greater than 7.0 the observed A_{280}/A_{500} ratio increases. In preliminary experiments on rhodopsin solutions initially at pH 7.0 which were titrated to higher pH values we have observed increases in the 280-nm absorbance concomitant with small decreases in the 500-nm absorbance. These spectral changes are also characterized by an isosbestic point between 425 and 430 nm. We have not established if the spectral changes are reversible or if some type of pH induced bleaching of the rhodopsin solution is taking place. Phosphate analysis of the initial extracts of sodium cholate solubilized rhodopsin, after ex-

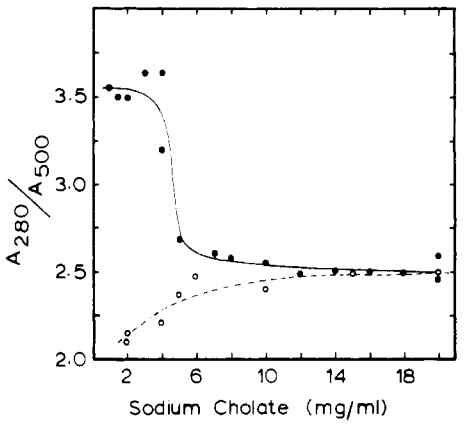

FIGURE 2: Dependence of the A_{280}/A_{500} ratio of rhodospin upon sodium cholate concentration in the presence (O - - - O) or absence (●—●) of 1 mM dithioerythritol. Concentrated solutions of rhodopsin containing 20 mg/ml of sodium cholate and where indicated 1 mM dithioerythritol were adjusted to the desired sodium cholate concentration by the dilution of equal aliquots of the concentrated rhodopsin solution into 0.10 M potassium phosphate buffer (pH 7.0) containing varying amounts of sodium cholate and where indicated 1 mM dithioerythritol. The data presented were determined from three separate rhodopsin preparations.

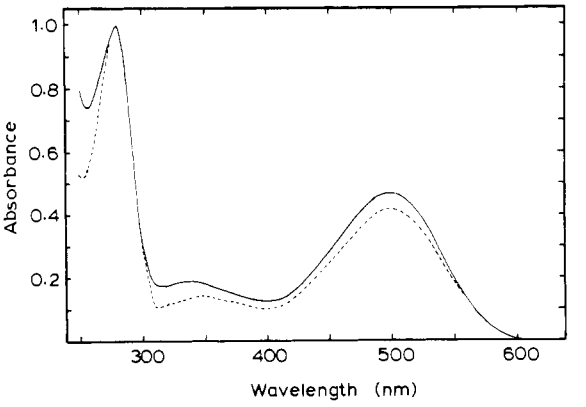

FIGURE 3: Effect of the sodium cholate concentration on the uv-visible absorption spectra of rhodopsin. The rhodopsin solutions were in pH 7.0, 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol and either 20 mg/ml of sodium cholate (- - -) or 3 mg/ml of sodium cholate (—). The rhodopsin samples for this spectral study were obtained from the same concentrated rhodopsin solution and in both cases the spectra presented were normalized to 1 OD unit at 280 nm.

tensive dialysis to remove inorganic phosphate, indicates a maximum of 45–60 mol of phosphate/mol of rhodopsin (range of values for three different preparations). This value represents an upper limit to the amount of phospholipid present. The observed A_{280}/A_{500} ratio of sodium cholate solubilized rhodopsin was found to be dependent upon the sodium cholate concentration and whether or not dithioerythritol was present (Figure 2). In the absence of dithioerythritol the A_{280}/A_{500} ratio increased with decreasing concentrations of sodium cholate due to an apparent increase in turbidity. When dithioerythritol was present in the rhodopsin solutions the A_{280}/A_{500} ratio decreased with decreasing sodium cholate concentration. The uv-visible absorption spectra of rhodopsin solutions containing 1 mM dithioerythritol at high (20 mg/ml) and low (3 mg/ml) sodium cholate concentrations are shown in Figure 3. Even though solutions of rhodopsin containing dithioerythritol and 1–3 mg/ml of sodium cholate appear optically clear the shape of the absorption spectrum between 250 and 350 nm (Figure 3, solid line) suggests some turbidity or aggregation exists in these solutions. It was necessary that the dithioerythritol was present before the rhodopsin solutions were diluted to lower sodium cholate concentrations since the addition of dithioerythritol after dilution did not result in decreasing the A_{280}/A_{500} ratio. The solubility characteristics of sodium cholate–rhodopsin solutions were further assessed by high speed centrifugation studies (105,000g for 1 hr) which revealed the following: (a) solutions of rhodopsin containing 20 mg/ml of sodium cholate at pH 7 were not sedimented by centrifugation in either the presence or absence of 1 mM dithioerythritol; (b) solutions of rhodopsin containing 2 mg/ml of sodium cholate were totally sedimented by the centrifugation; and (c) solutions of rhodopsin containing 2 mg/ml of sodium cholate and 1 mM dithioerythritol were partially sedimented, 50–70%, by the same centrifugation.

The ammonium sulfate fractionation of sodium cholate solubilized rhodopsin carried out as described in the Materials and Methods section results in the purification of rhodopsin with respect to both extraneous protein, as suggested by the lowering of the A_{280}/A_{500} ratio, and phospholipids. Phosphate analysis of ammonium sulfate fractionated rhodopsin, after dialysis to remove inorganic phosphate, indicated these preparations contained from 9 to 11 mol phosphate/mol of rhodopsin (range of values for three separate ammonium sulfate fractionated rhodopsin preparations). Preliminary experiments in this laboratory indicate a second ammonium sulfate fractionation reduces the phosphate/rhodopsin ratio to 4–6. The yield of rhodopsin after ammonium sulfate fractionation ranged from 80 to 95% based upon the absorbance at 500 nm. Failure to include either dithioerythritol or mercaptoethanol in the solutions during the ammonium sulfate fractionation procedure resulted in the low recovery of rhodopsin. Failure to include the fractionation step at 25% ammonium sulfate saturation resulted in the loss of some rhodopsin between 30 and 36% saturation in ammonium sulfate.

Recombination Studies of Sodium Cholate Solubilized Rhodopsin. The recombination of photolyzed sodium cholate solubilized rhodopsin and 11-*cis*-retinal to yield rhodopsin was found to be dependent upon the concentration of sodium cholate present in the reaction mixture (Figure 4) and was maximal at sodium cholate concentrations of less

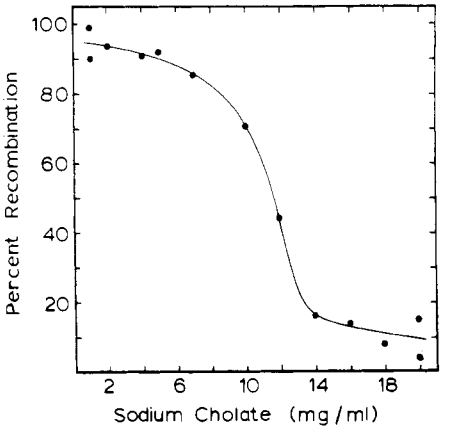

FIGURE 4: Dependence of the recombination of photolyzed rhodopsin on the sodium cholate concentration. Recombination reactions were carried out as described in the text and the per cent recombination at different sodium cholate concentrations was calculated using the formula given in the Materials and Methods section. The data were collected using rhodopsin solutions from two separate ROS preparations.

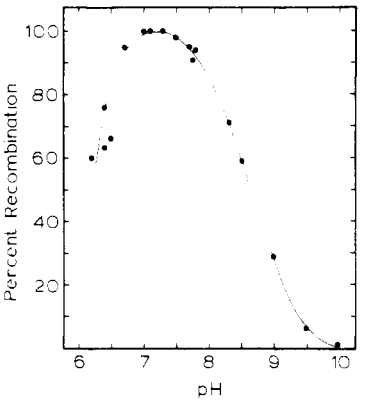

FIGURE 5: The effect of pH on the extent of recombination of sodium cholate solubilized rhodopsin. Recombination reactions were carried out as described in the Materials and Methods section and the concentration of sodium cholate was 2–3 mg/ml. The per cent recombination is presented on a normalized scale where the observed per cent recombination at pH 7.0 was set equal to 100%. These data were collected using rhodopsin solutions from five separate ROS preparations and the observed per cent recombination at pH 7.0 for these preparations varied from 80 to 100% of that theoretically expected.

TABLE I: Sulfhydryl Reactivity of Rhodopsin.[a]

Sodium Cholate (mg/ml)	Nbs_2 Addition[b]	mol of Reactive −SH/mol of Rhodopsin: Dark[c]	Revealed by[d] Bleaching	Total
20	Dark	1.9–2.2	1.9	3.8–4.1
20	Bleached			1.8–1.9
2	Dark	1.0–1.2	0	1.0–1.2
2	Bleached			0.35

[a] Rhodopsin solutions, 1 ml, containing 0.1 M potassium phosphate buffer (pH 7.0) were treated with Nbs_2 under the conditions noted. In all experiments the rhodopsin concentration was approximately 5 μM and the final concentration of Nbs_2 was 0.2 mM. [b] Indicates that Nbs_2 was added to the solution either in the dark or after 10-min bleaching with white light. [c] The number of sulfhydryl groups assayed in the dark. [d] The number of sulfhydryl groups which were revealed upon bleaching when Nbs_2 was present in the solution before bleaching.

than 5 mg/ml. At sodium cholate concentrations of 1–4 mg/ml the per cent recombination typically ranged from 80 to 100% of that theoretically expected. When rhodopsin solutions were used within 24 hr we have observed no significant difference in the per cent recombination in the presence or absence of dithioerythritol. When stored at 4° in the presence of 1 mM dithioerythritol solutions of rhodopsin in sodium cholate retained their high recombination capacity for periods up to a week. The pH optimum for the maximum extent of recombination for rhodopsin solutions containing 2–3 mg/ml of sodium cholate was found to lie between pH 7.0 and 7.4 (Figure 5). Ammonium sulfate fractionation of rhodopsin did not appear to alter either the extent of recombination or its pH dependence.

Sulfhydryl Group Reactivity of Rhodopsin. The assay

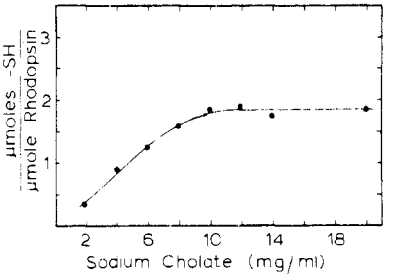

FIGURE 6: Sulfhydryl reactivity of bleached rhodopsin as a function of the sodium cholate concentration. The sulfhydryl assays using Nbs_2 (final concentration of 0.2 mM) were carried out as described in the Materials and Methods section.

for reactive sulfhydryl groups in sodium cholate solubilized rhodopsin with Nbs_2 as described in the Materials and Methods section indicated the number of available sulfhydryl groups was dependent upon both the sodium cholate concentration and whether or not the Nbs_2 reagent was added before or after bleaching the rhodopsin sample. Table I presents the sulfhydryl reactivity of dark or bleached rhodopsin and the dependence of this reactivity upon Nbs_2 addition and sodium cholate concentration. The important point of Table I is the observation that the number of reactive sulfhydryl groups is dependent upon both the surfactant concentration and whether the sulfhydryl reagent (Nbs_2) was added before or after bleaching. Figure 6 illustrates the sulfhydryl reactivity of bleached rhodopsin in the presence of varying concentrations of sodium cholate. From Figure 6 it is apparent that bleached rhodopsin has more titratable sulfhydryl groups at high cholate concentration than at low cholate concentrations.

Discussion

The use of sodium cholate to solubilize rhodopsin in a regenerable form appears in many respects to be superior to either digitonin or Tween-80. The main difficulties with using digitonin have been its insolubility and the lack of good analytical methods to determine the surfactant concentration. Tween-80 also has its disadvantages in that the surfactant has a considerable absorbance in the uv, high concentrations are required to extract rhodopsin from the ROS membranes, and the resulting rhodopsin solutions have low recombination capacities (Zorn and Futterman, 1973). Sodium cholate solubilized rhodopsin does not suffer from any of the above difficulties and moreover it is possible to study both a regenerable and a nonregenerable form of the protein merely by altering the surfactant concentration (Figure 4).

The reported concentration range where sodium cholate solutions form micelles, 5–19 mg/ml (Fontell, 1971), corresponds closely to the sodium cholate concentrations where rhodopsin goes through its greatest transitions with respect to turbidity changes (Figure 2), recombination capacity (Figure 4), and sulfhydryl reactivity (Figure 5). Preliminary experiments on the binding of sodium cholate to rhodopsin indicate the binding is weak since dialysis against distilled water reduces the cholate concentration below 0.05 mg/ml and also results in the precipitation of the rhodopsin.

The sulfhydryl reactivity of sodium cholate solubilized rhodopsin is quite different than that reported for other detergent solutions of rhodopsin. Our results at 20 mg/ml of sodium cholate agree well with determinations made on digitonin solubilized rhodopsin where approximately two sulf-

hydryl groups are available in the dark and upon bleaching two additional sulfhydryl groups are exposed (DeGrip *et al.*, 1973; Kimble and Ostroy, 1973). Data obtained using digitonin solubilized rhodopsin indicated the total number of reactive sulfhydryl groups (dark plus bleached) was the same whether the sulfhydryl reagent was added before or after bleaching (Kimble and Ostroy, 1973). Our experiments demonstrate this is not the case with sodium cholate solubilized rhodopsin where the total number of titratable sulfhydryl groups was dependent upon whether the Nbs_2 was added before or after bleaching. The data in Table I suggest that upon bleaching sodium cholate (20 mg/ml) solubilized rhodopsin goes through a transition where additional sulfhydryl groups are temporarily exposed. At low sodium cholate concentration (2 mg/ml) where the photolyzed rhodopsin solutions undergo their greatest regeneration we have shown that no additional sulfhydryl groups are exposed upon bleaching (Table I). DeGrip *et al.*, (1973) have reported similar results in their study on the sulfhydryl reactivity of isolated ROS membranes where two sulfhydryl groups were available in the dark and upon photolysis no additional sulfhydryl groups became available for reaction with Nbs_2.

The high recombination capacity of sodium cholate solubilized rhodopsin suggests that solutions of rhodopsin containing 1–4 mg/ml of sodium cholate are in a highly native form at least with respect to the recombination activity. The nearly complete lack of recombination capacity of rhodopsin solutions containing 20 mg/ml of sodium cholate is a reflection of a conformational state of rhodopsin which is nonregenerable. Since the availability of specific amino acid residues to chemical modification is dependent upon their particular environment (Means and Feeney, 1971), our results which show that the number of available sulfhydryl groups in rhodopsin solutions, both dark and light exposed, increases with increasing sodium cholate concentration also indicates that conformational differences exist between the regenerable and nonregenerable forms of rhodopsin. It is important to note that these changes in recombination capacity and sulfhydryl reactivity of sodium cholate solubilized rhodopsin were apparently the direct result of changing only the surfactant concentration.

The current experiments on sodium cholate solubilized rhodopsin demonstrate two important factors which have not been reported for other detergent solutions of rhodopsin: (a) surfactant concentration is important in determining the activity of the rhodopsin preparation with respect to its recombination capacity; (b) the conformation of rhodopsin, as reflected both by the number of accessible sulfhydryl groups and its recombination capacity, changes with surfactant concentration.

Acknowledgment

The authors are grateful to Hoffmann-La Roche Inc., Nutley, N.J., for their generous gift of crystalline 11-*cis*-retinal.

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