Friedberg, E. C., Ganesan, A. K., & Minton, K. (1975) *J. Virol. 16*, 315–321.
Garrett, E. R., & Mehta, P. J. (1972) *J. Am. Chem. Soc. 94*, 8542–8547.
Haines, J. A., Reese, C. B., & Todd, A. R. (1962) *J. Chem. Soc.*, 5281–5288.
Hems, G. (1958) *Nature (London) 181*, 1721–1722.
Hems, G. (1960) *Radiat. Res. 13*, 777–787.
Heyns, W., & deMoor, P. (1974) *Biochim. Biophys. Acta 358*, 1–13.
Karran, P., & Lindahl, T. (1978) *J. Biol. Chem. 253*, 5877–5879.
Kohn, K. W., & Spears, C. L. (1967) *Biochim. Biophys. Acta 145*, 734–741.
Kriek, E., & Emmelot, P. (1964) *Biochim. Biophys. Acta 91*, 59–66.
Laval, J. (1977) *Nature (London) 269*, 829–832.
Laval, J., Pierre, J., & Laval, F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 852–855.
Lawley, P. D. (1975) in *Molecular Mechanisms of Repair of DNA* (Hanawalt, P. C., & Setlow, R. B., Eds.) pp 25–28, Plenum Press, New York.
Lawley, P., & Brooks, P. (1961) *Nature (London) 192*, 1081–1083.
Lawley, P., & Orr, D. (1970) *Chem.-Biol. Interact. 2*, 154–157.
Lawley, P., & Shah, S. (1972a) *Biochem. J. 128*, 117–132.
Lawley, P., & Shah, S. (1972b) *Chem.-Biol. Interact. 5*, 286–288.
Lawley, P., & Warren, W. (1976) *Chem.-Biol. Interact. 12*, 211–220.
Lindahl, T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3649–3653.
Lindahl, T., Lungquist, S., Siegert, W., Nyberg, B., & Sperens, B. (1977) *J. Biol. Chem. 252*, 3286–3294.
Linsley, W. S., Penhoet, E. E., & Linn, S. (1977) *J. Biol. Chem. 252*, 1235–1242.
Livneh, Z., Elad, D., & Sperling, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1089–1093.
Ludlum, D. (1970) *J. Biol. Chem. 245*, 477–482.
Margison, G. P., & Pegg, A. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 861–865.
Prakash, L., & Strauss, B. (1970) *J. Bacteriol. 102*, 760–766.
Riazuddin, S., & Lindahl, T. (1978) *Biochemistry 17*, 2110–2118.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Singer, B. (1979) *J. Natl. Cancer Inst. 62*, 1329–1339.
Singer, B., & Brent, T. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 856–860.
Sweetman, L., & Nyhan, W. H. (1968) *J. Chromatogr. 32*, 662–675.
Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669–672.
van Hemmen, J. J. (1971) *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 27*, 403–407.
van Hemmen, J. J., & Bleichrodt, J. F. (1971) *Radiat. Res. 46*, 444–456.
Verly, W. G., Paquette, Y., & Thibodeau, L. (1973) *Nature (London), New Biol. 244*, 67–69.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weiss, B. (1976) *J. Biol. Chem. 251*, 1896–1901.
Wilson, J. E. (1976) *Biochem. Biophys. Res. Commun. 72*, 816–820.

Effects of Detergent Micelles on the Recombination Reaction of Opsin and 11-*cis*-Retinal†

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ABSTRACT: When detergent-solubilized proteins interact with hydrophobic or amphiphilic molecules in the presence of detergent micelles, the solubility of the latter species in the micelles must be included in both thermodynamic and kinetic treatments. In this paper, we derive equations which describe the distribution of species present at equilibrium for a system in which a detergent-solubilized protein binds a hydrophobic (or amphiphilic) ligand. We have applied the formalism developed in this paper to the reaction describing the formation of rhodopsin from its apoprotein and 11-*cis*-retinal. Qualitatively, the results demonstrate that a significant portion of the observed decrease in the extent of recombination for rhodopsin solubilized in either sodium cholate or Tween 80 may be attributed to the partition of retinal into detergent micelles and that a detergent-induced protein denaturation need not be invoked to explain the data. We also discuss results for rhodopsin solubilized in a nonionic detergent (octaethylene glycol *n*-dodecyl ether) in which the detergent is clearly causing irreversible loss of the capability to recombine with 11-*cis*-retinal.

Whenever a detergent-solubilized membrane protein reacts with a hydrophobic or amphiphilic molecule, the solubility of the reactants in the detergent influences the thermodynamic and kinetic properties of the system. This paper addresses itself to this problem in terms of the recombination of 11-*cis*-retinal with opsin (bleached rhodopsin) to form the photosensitive protein rhodopsin.

The recombination of bleached rhodopsin and 11-*cis*-retinal occurs readily in intact disk membranes. When recombination is attempted with detergent-solubilized rhodopsin, the results depend on the detergent being used. With some detergents, no recombination is observed at all, and it is generally assumed that opsin has undergone irreversible denaturation in these

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detergents. With other detergents, partial recombination is observed, to an extent depending on the detergent concentration. Does this imply that partial denaturation of the opsin has occurred? The intent of this paper is to show that it is not necessary to invoke partial denaturation in the latter case but that incomplete recombination may simply be the result of partitioning of the retinal into protein-free detergent micelles.

Theoretical Section

This section gives a brief theoretical analysis of the effect of detergent micelles on the extent of recombination at equilibrium, assuming that the detergent does not perturb the structure of the protein. The equation derived is not limited to the rhodhopsin system but may be applied to the combination of a detergent-solubilized protein with any hydrophobic or amphiphilic ligand.

Let M be an empty detergent micelle, MR a micelle containing one retinal molecule, MR_i a micelle containing i retinal molecules, MO a micelle containing opsin, and MOR a micelle containing opsin recombined with retinal, i.e., rhodopsin. We assume that the solubility of opsin or rhodopsin other than in micelles is insignificant. The solubility of retinal in detergent-free aqueous medium is also very low (see below), but we allow for the presence of aqueous retinal (R) so that the equations derived below can be applied to situations other than the specific example of this paper.

The equilibria of interest are

$$M + R \rightleftharpoons MR \quad (1)$$

$$MR_{i-1} + R \rightleftharpoons MR_i \ (i = 2, 3, \ldots) \quad (2)$$

$$MO + R \rightleftharpoons MOR \quad (3)$$

and the corresponding equilibrium constants are

$$K_1 = [MR]/([M][R]) \quad (4)$$

$$K_i = [MR_i]/([MR_{i-1}][R]) \quad (5)$$

$$K_r = [MOR]/([MO][R]) \quad (6)$$

K_r being the constant for the reaction of interest, i.e., the recombination of opsin with retinal. Experimentally, only the *total* concentrations of micelles, protein, and retinal are known, i.e.

$$[O]_T = [MO] + [MOR] \quad (7)$$

$$[R]_T = [R] + \sum_{i=1}^{\infty} i[MR_i] + [MOR] \quad (8)$$

$$[M]_T = [M] + \sum_{i=1}^{\infty} [MR_i] + [O]_T \quad (9)$$

The desired parameter is the fractional extent of recombination

$$f = [MOR]/[O]_T \quad (10)$$

and from eq 6 and 7, we see that

$$f/(1 - f) = K_r[R] \quad (11)$$

To obtain f as a function of the total concentrations, we have assumed that K_i is independent of i, i.e., $K_i = K_1$. This is equivalent to assuming that the solution of retinal in detergent micelles is ideal. Using eq 4 and 5, we have $[M] + \sum_{i=1}^{\infty}[MR_i] = [M](1 + x + x^2 + \ldots)$, where $x = K_1[R]$, and this in turn is equal to $[M]/(1 - x)$ provided that $x < 1$. Similarly, and subject to the same condition, $\sum_{i=1}^{\infty} i[MR_i] = [M](1 + 2x + 3x^2 + \ldots) = [M]x/(1 - x)^2$. Eliminating [M] between eq 8 and 9 then gives

$$[R]_T = [R] + [MOR] + ([M]_T - [O]_T)x/(1 - x) \quad (12)$$

From eq 4, it is readily seen that the condition $x < 1$ has to be satisified whenever $[M]_T > \sim 2[R]_T$, regardless of the value of K_1. The condition may not be satisfied at very low detergent concentrations, where $[R]_T$ may even be greater than $[M]_T$.

Equation 12 can be simplified for the retinal/opsin system because the aqueous solubility of retinal is very small. It has been reported that the solubility of the alcohol, retinol, in water is less than 1 nM (Yoshikami & Noll, 1978). Since aldehydes are generally less soluble in water than their alcohol counterparts (Dean, 1979), the solubility of retinal, which has not been measured, should also be less than 1 nM. The total retinal concentration used in recombination experiments is generally in the micromolar range; therefore, [R] makes a negligible contribution to $[R]_T$ and can be omitted from eq 12.

With this simplification, eq 12 leads to a quadratic equation in f. From eq 11 and the definition of x, we have $x = (K_1/K_r)f/(1 - f)$, and substitution into eq 12 gives

$$\left(1 + \frac{K_1}{K_r}\right)[O]_T f^2 - \left\{\left(1 + \frac{K_1}{K_r}\right)[R]_T + \left(1 - \frac{K_1}{K_r}\right) \times [O]_T + \frac{K_1}{K_r}[M]_T\right\} f + [R]_T = 0 \quad (13)$$

and it is immediately evident that f depends only on the ratio K_1/K_r and not on the individual magnitudes of K_1 and K_r.

In the more general situation, involving a ligand with appreciable solubility in water, where the [R] in eq 12 would not be negligible, a cubic equation for f is obtained, and the solution depends on the separate values of K_1 and K_r, and not merely on their ratio.

A critical assumption in the above derivation is that micelles behave as entities with fixed properties. This assumption also enters into the calculation of $[M]_T$ from analytical detergent concentrations, because the only simple way to do this without adding more adjustable parameters is to assume that the critical micelle concentration (cmc)[1] is unaffected by the presence of retinal. In fact, the presence of retinal may induce the formation of micelles below the normal cmc, and it may also increase micelle size [see Tanford (1980)]. Equation 13 is therefore expected to be least accurate at very low detergent concentrations. A decrease in f may be observed even below the normal cmc, i.e., where $[M]_T$ would be formally = 0, and the decrease may be steeper than the equation predicts because the induced formation of micelles would be highly cooperative with respect to detergent concentration.

The assumption that K_i is independent of i is relatively insignificant. One might expect that K_i should decrease with increasing i, the most extreme case being one retinal molecule per micelle; i.e., we place $K_i = 0$ when $i \neq 1$. This change in the derivation only affects the result at large f, i.e., at low detergent concentration, where the equation has to be considered in any case a weak approximation for the reasons given above.

Kinetics of Recombination. In addition to the equilibrium state of the system being affected, the presence of excess micelles will also affect the rate at which equilibrium is attained, because retinal molecules have to be transferred from protein-free to protein-containing micelles before reaction can occur. This problem has been discussed theoretically, in the context of electron-transfer reactions between solubilized

[1] Abbreviations used: cmc, critical micelle concentration, $C_{12}E_8$, octaethylene glycol *n*-dodecyl ether.

electron-donor and -receptor complexes, by Weiss & Wingfield (1979). In the retinal/opsin system, the transfer becomes the rate-limiting step because recombination is very fast in lipid bilayers, where transfer is not required (Matsumoto et al., 1978).

Materials and Methods

The preparation of detergent-solubilized rhodopsin is described in the following paper (McCaslin & Tanford, 1981). Usually, the solution used for recombination experiments was initially 45 mM cholate and 50 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, pH 7.5, and was adjusted to other detergent concentrations by dialysis and/or dilution with buffer or the addition of cholate. The concentration of rhodopsin in stock solutions was measured by assuming an extinction coefficient of 40 600 M^{-1} cm^{-1} at 500 nm (Wald & Brown, 1953). The 11-*cis*-retinal was a gift from Hoffmann-La Roche, Inc. Stock solutions were in ethanol, and the retinal concentration was determined from an extinction coefficient of 24 900 M^{-1} cm^{-1} at 380 nm (Hubbard et al., 1971).

For the recombination assay, 95-μL samples were prepared in duplicate, containing 4.2 μM rhodopsin and the cholate concentration desired. One sample was bleached by exposure to light for 15–20 min, the other served as a control. A 1-μL aliquot of a concentrated 11-*cis*-retinal stock solution was added to each sample, yielding a final retinal concentration of ~54 μM. The samples were incubated in the dark at room temperature for 1.5–4 h. Both samples were assayed for rhodopsin in terms of the difference in absorbance at 500 nm before and after bleaching (i.e., rebleaching in the case of the recombined rhodopsin) in the presence of 50 mM hydroxylamine. The extent of recombination (f) was taken as the ratio of rhodopsin formed in the recombination assay to that present in the control. The presence of some opsin before bleaching does not affect the recombination assay because each bleached sample was compared in the assay with an unbleached control containing retinal at the same concentration.

No systematic study of the rate of recombination was carried out, but incubation times of 1.5 or 4 h were found to give results identical within experimental error, even at high detergent concentrations. If we assume that f has attained 98% of its equilibrium value in 1.5 h, a half-time for attainment of equilibrium of 15 min is calculated. This is probably a conservative estimate, since the kinetic data of Matsumoto et al. (1978) and Wald & Brown (1956) in excess digitonin (~20 mg/mL) correspond to a half-time of less than 1 min.

All absorbance measurements were made at room temperature on Cary 17D spectrophotometer, equipped with a microcell adapter.

Results

We have studied rhodopsin purified in two detergents, sodium cholate and the nonionic poly(oxyethylene) detergent, $C_{12}E_8$. In cholate, rhodopsin maintains both its native absorption spectrum and the ability to undergo the recombination reaction. In $C_{12}E_8$, the native absorption spectrum is intact, but the ability to undergo recombination is lost. Studies on other molecular properties of rhodopsin in these detergents are presented in the following paper.

Recombination in Cholate. Results of recombination experiments in cholate are shown in Figure 1. This detergent has a high critical micellar concentration and a small aggregation number (Small, 1971); furthermore, the micellar properties are exceptionally sensitive to environmental parameters, such as pH, temperature, and salt concentration. We

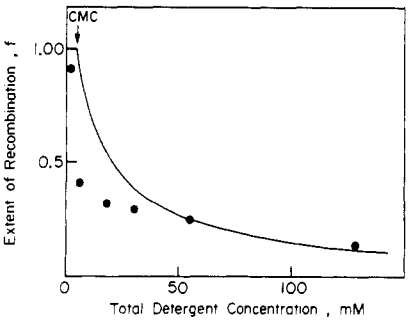

FIGURE 1: Recombination efficiency as a function of cholate concentration. All samples contained 4.2 μM protein and 54 μM 11-*cis*-retinal, and measurements were made at room temperature at pH 7.5. Most of the points represent averages of duplicate determinations. Additional data were obtained at high detergent concentration on several different preparations and were always consistent with the results shown here. The curve shown is a theoretical curve, based on eq 13 with $K_1/K_r = 0.032$.

have not explicitly investigated the effects of retinal on the micellar properties of cholate but can infer from the following observations that the micellar properties of cholate will be altered by retinal. The aggregation number of cholate (10 mg/mL, 0.1 M NaCl and 0.1 M KH_2PO_4 adjusted to pH 7.2 with NaOH, 20 °C) determined by sedimentation equilibrium using interference optics was ~4, in agreement with a measurement by Small (1971) under similar conditions. When we added a small amount of heme (which is incorporated into the micelle) and used its absorption band to determine the equilibrium distribution, the aggregation number was ~9. The presence of heme undoubtedly also decreased the cmc, although this was not measured. Since similar effects can be expected to occur with added retinal, the assumption of fixed micellar properties is likely to be particularly poor in this case, and we have accordingly plotted our results (Figure 1) as a function of total cholate concentration instead of $[M]_T$. The *calculated* curve with which the results are compared is based on eq 13, with $[M]_T$ equal to (total cholate concentration − cmc)/micelle aggregation number. The cmc was assumed to be the same as that in the absence of retinal or protein, which measured in our buffer system is 4.6 mM and is in agreement with values determined under similar conditions reported in Small (1971) and Helenius et al. (1979). Since the shape of curves described by eq 13 is independent of the micelle aggregation number, a choice for the aggregation number used in plotting the *experimental* data becomes arbitrary, with any given plot being describable by eq 13 and a judicious choice of K_1/K_r. On the basis of the study of heme in cholate, we have used a nominal micelle aggregation number of 10. A value of $K_1/K_r = 0.032$ was required to fit the data at high detergent concentration, where the effects of retinal on the average micellar properties are expected to be minimized. The data at low detergent concentration are seen to fall below the calculated curve, which is consistent with the probability that the presence of retinal will induce the formation of micelles in detergent concentrations below the cmc.

Henselman & Cusanovich (1974) have previously studied the recombination reaction as a function of cholate concentration, but their solution contained lower concentrations of 11-*cis*-retinal (15–20 μM vs. 54 μM). Their results are qualitatively similar to ours, but differ quantitatively in two respects. (1) The extent of recombination at high cholate concentration was less than we observed (about 0.15 at 30 mM cholate vs. 0.30 in Figure 2). This difference can be accounted for on the basis of the lower value of $[R]_T$; using eq 13 with the same value of K_1/K_r that was found to fit our data, we

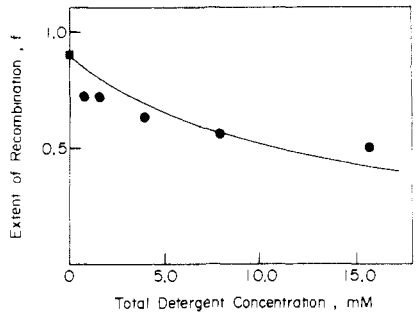

FIGURE 2: Recombination efficiency as a function of Tween 80 concentration. The experimental points are data from Table II of Zorn & Futterman (1973). The point represented by the solid square was obtained by extracting the Tween 80 from the protein and then measuring the extent of recombination. They used 9-*cis*-retinal (instead of 11-*cis*-retinal) at a concentration of 310 μM. The protein concentration was 31 μM, and the pH was 6.4. The curve shown is a theoretical curve, based on eq 13 with $K_1/K_r = 0.12$.

calculate $f = 0.16$ at 30 mM cholate. (2) Henselman and Cusanovich's data at low cholate concentration fall above the theoretical curve instead of below it. We do not know the reason for this discrepancy. The rhodopsin samples used by Henselman and Cusanovich were not completely delipidated, still containing 9–11 mol of organic phosphate per mol of rhodopsin. This would not have affected the results at high detergent concentraton, where the lipid would presumably have been completely dispersed, but it might have promoted the persistance of large protein–lipid–cholate particles at low cholate concentrations, and retinal might partition into such particles in preference to smaller micelles.

Both $[R]_T$ and $[O]_T$ are parameters in eq 13. The dependence of the model on $[O]_T$ is quite small as expected since f itself is normalized by using $[O]_T$. The model is, however, significantly dependent on $[R]_T$. Under our experimental conditions, we have observed that reducing the retinal concentration in half resulted in a decrease in f in qualitative agreement with that predicted by the model. As discussed above, both our data at high cholate concentrations and those of Henselman and Cusanovich which were obtained at a lower $[R]_T$ than we employed can be fit by using the same values for the micellar properties and for K_1/K_r. Thus, the experimental data are, at least qualitatively, in agreement with the predicted dependence of the model on $[R]_T$.

An increase in detergent concentration reduces the rate of recombination as well as the final yield, as was noted in the introduction. The protein therefore remains in the bleached state for a longer time when the recombination assay is carried out in detergent excess. It is possible that a slow structural alteration of the opsin molecule during this period is responsible for the observed change in recombination yield, and Henselman and Cusanovich (1974) in fact ascribed their results to a slow denaturation.

To show that kinetic processes during the recombination assay do not have a significant effect on the results obtained, we allowed one sample at 40 mM cholate to stand in the dark for 50 min after bleaching before recombination was initiated by addition of retinal. The extent of recombination attained in this experiment was $f = 0.23$, which is only slightly lower than the value obtained without this extra incubation period, which was $f = 0.28$. The half-life of bleached protein in the recombination assay is conservatively estimated as 15 min (see Material and Methods), and this experiment therefore indicates that the maximal extent of kinetic inactivation of the protein during the recombination assay was less that 10%. The *unbleached* protein is stable at high concentrations of cholate for at least 2 weeks, and storage of rhodopsin in 46 mM cholate for up to 1 week did not affect the results of recombination experiments.

Another observation indicating that protein denaturation is not a significant factor is the very slow diminution in f seen in Figure 2 as the detergent concentration goes to high values. This is consistent with eq 13. If protein denaturation were a factor, one might have expected f to drop rapidly to 0 since protein denaturation is usually highly cooperative with respect to denaturant concentration. We believe that all results taken together can plausibly by interpreted as reflecting the dispersal of retinal into excess micelles even though the agreement with the theoretical model is not quantitative.

Recombination in Tween 80. We have not studied recombination in Tween 80 but have applied the theoretical equation for partitioning to the data of Zorn & Futterman (1973) shown in Figure 2. The value of $[M]_T$ corresponding to a given total detergent concentration was obtained by using a cmc of 12 μM and a micelle aggregation number of 60 (Helenius et al., 1979). The theoretical curve shown is based on $K_1/K_r = 0.12$, which is larger than the value for cholate, perhaps a reasonable result in view of the fact that they used 9-*cis*-retinal instead of 11-*cis*-retinal in their experiments. The larger value of K_1/K_r would lead to smaller f values than in cholate under otherwise identical conditions, but Zorn and Futterman used a very high retinal concentration, $[R]_T = 310$ μM, which according to eq 13 would shift the equilibrium toward higher values of f. The agreement between the data and the theoretical curve is reasonably good, and the maintenance of a relatively high value of f at the highest detergent concentrations employed is striking.

Recombination in $C_{12}E_8$. No conditions have been found for which delipidated rhodopsin in $C_{12}E_8$ maintains its ability to undergo the recombination reaction. Among the various conditions tried were temperatures of 4 and 20 °C and pH values of 6.5, 7.5, and 8.5 prior to bleaching. No combination of detergent and retinal concentrations has been found for which recombination occurs, including conditions under which there was an average of two retinal molecules per $C_{12}E_8$ micelle. The concentrations in this experiment were $[R]_T = 20$ μM, $[M]_T = 10$ μM, and $[O]_T = 5$ μM, and it would be unreasonable from statistical considerations that most protein-containing micelles would not also contain a retinal molecule. The complete loss of recombination capacity in this experiment must reflect a structural change in the protein *after bleaching*, which cannot be reversed under the conditions used for recombination, even though the protein is spectrally intact *before bleaching*.

Discussion

Zorn & Futterman (1973) observed that the extent of recombination between opsin and retinal in Tween 80 solution decreases with increasing detergent concentration, and Henselman & Cusanovich (1974) obtained a similar result in cholate. In both papers, the decreased recombination yield was ascribed to protein denaturation, i.e., loss of binding capacity due to some structural alteration in the protein. We have shown that these results can be explained at least semiquantitatively in another way, namely, in terms of partitioning of retinal into detergent micelles, which would shift the equilibrium from "protein-bound" to "free" retinal as the concentration of detergent micelles is increased. The same considerations apply to the work of Matsumoto et al. (1978), in which the rate of recombination (instead of the equilibrium yield) in digitonin was observed to decrease with increasing digitonin concentration.

Table I

detergent	spectrum intact	recombination intact
Ionic and Polar Detergents		
sodium dodecyl sulfate[a]	no	no
terdecyltrimethylammonium bromide[d]	yes	no
dodecylamine oxide[c]	yes	no
Fused Ring Detergents		
digitonin[d,e]	yes	yes
cholate[a,f]	yes	yes
deoxycholate[a]	no	no
Sugar-Based Detergents		
octyl glucoside[g]	yes	no
dodecyl maltoside[b]	yes	yes
Poly(oxyethylene)-Based Detergents		
Tween 80[h]	yes	yes
Tween 20[h]	yes	no
Triton X-100[i]	yes	no
Emulphogene BC-720[i]	yes	no
Cemulsol LA-90[j]	yes	no
$C_{12}E_8$[a]	yes	no

[a] This work. [b] Knudson & Hubbell (1978). [c] Ebrey (1971). [d] Matsumoto et al. (1978). [e] Hong & Hubbell (1973). [f] Henselman & Cusanovich (1974). [g] Stubbs & Litman (1978). [h] Zorn & Futterman (1973). [i] Osborne et al. (1974). [j] Osborne et al. (1978).

Our results do not exclude the possibility that a small part of the observed effect is due to denaturation, for the slow rate of recombination in the presence of excess detergent means that some of the protein molecules remain in the bleached state for a long time, and the rate of thermal denaturation will undoubtedly be faster in the bleached state than in native rhodopsin because the latter is stabilized by the binding free energy of the retinal. However, the evidence presented under Results indicates that at most 10% of the observed decrease in recombination efficiency (40 mM cholate, $f \sim 0.25$) can be ascribed to this cause. In the following paper it is shown that the state of aggregation and other conformational parameters for opsin in cholate solution are the same as those for rhodopsin, and remain so for very long time periods, whereas rapid changes occur after rhodopsin is bleached in $C_{12}E_8$.

The values of K_l/K_r required by eq 13 for an approximate fit of the data are 0.032 in cholate and 0.12 in Tween 80. These are reasonable values, for K_l is simply the free energy gained by removing the hydrophobic moiety for retinal from water to the interior of a micelle, whereas K_r reflects *in addition* the contributions arising from the specific interaction of retinal with the protein matrix. It is not unreasonable that the values of K_l/K_r should differ in cholate and in Tween 80, because cholate is a complex molecule that does not provide a fluid hydrocarbon milieu when it forms micelles (Small, 1971). The fact that different isomers of retinal were used in the two studies may also affect the value K_l/K_r.

It may be noted that the limiting value of $f \sim 0.9$, observed in both cholate and Tween 80 at the limit of zero detergent concentration, permits estimation of a minimal value of K_r, the binding constant for association between retinal and opsin. Since [R] cannot be larger than the solubility of retinal in aqueous buffer, i.e., [R] $< 10^{-9}$ M, eq 10 yields $K_r > 10^{10}$ M^{-1}.

Recombination in Disk Membranes and Lipid Bilayers. The principles for recombination in lipid bilayers are the same as those in detergent solution: partitioning of retinal into protein-free regions of the bilayer must compete with binding to opsin. There are, however, no separate particles into which the retinal can partition, and in intact disk membranes, where the protein concentration is high, all dissolved retinal molecules must be in the bilayer, close to a protein molecule. The situation is essentially equivalent to that obtained in detergent solution when there are no excess micelles, i.e., $[M]_T \simeq [O]_T$, and, as we have seen, these are conditions leading close to 100% recombination when an excess of retinal is added.

A decrease in the extent of recombination at equilibrium might be anticipated if the protein in a disk membrane is diluted by fusion with protein-free liposomes or in a reconstituted membrane with a low protein content. Even then, however, the rate at which equilibrium is attained should remain high, since no transfer of retinal through the aqueous medium will be required.

Reversible and Irreversible Conformational Changes. This paper has shown that rhodopsin dissolved in $C_{12}E_8$ retains its native absorption spectrum in the dark but irreversibly loses its ability to recombine with retinal after bleaching. As shown in Table I, most detergents that have been used to solubilize rhodopsin lead to similar behavior. It must be concluded that the unbleached protein in these detergents is in a metastable state, held in that state because nonphotolytic release of retinal from its binding site is kinetically slow.

In these detergents, it is possible that even in the unbleached state portions of the protein molecule not closely linked to the retinal binding site have undergone a conformational change, and the following paper suggests that dissociation of an oligomeric state may be a change in this category. Unbleached rhodopsin originally dissolved in cetyltrimethylammonium bromide (in which recombination is lost upon bleaching) fully recovers its recombination capacity after reincorporation into phospholipid vesicles (Hong & Hubbell, 1972), which suggests that conformational changes occurring in the unbleached state are reversible. It is of interest in this connection that the dissociation of oligomeric rhodopsin described in the following paper proved to be reversible provided the protein remained unbleached.

It is not our intent to imply that reversible conformational changes may not occur in detergents in which the recombination capacity is intact, but the probability can be considered to be lower. For example, it is possible that irreversible denaturation (loss of recombination) may be a two-stage process, with the reversibly denatured unbleached state (X) as an intermediate; i.e., mechanism may be native $\rightleftharpoons$ X $\rightarrow$ denatured. For rhodopsin in detergents in which there is extensive loss of recombination after bleaching, the population of X molecules would be large whereas in detergents in which recombination is largely intact after bleaching the population of X molecules would be small.

From the point of view of obtaining structural information about the *native* protein, any conformational change, whether reversible or not, can lead to misleading results. If detergent-solubilized protein is to be used to obtain structural information, it is clearly desirable to use a detergent in which the ability to recombine with retinal is retained, and results obtained in other detergents [e.g., Sardet et al. (1976) and Osborne et al. (1978)] should be interpreted very cautiously.

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References

Dean, J. A., Ed. (1979) *Lange's Handbook of Chemistry, 12th Ed.*, McGraw-Hill, New York.

Ebrey, T. G. (1971) *Vision Res. 11*, 1007–1009.

Helenius, A., McCaslin, D. R., Fries, E., & Tanford, C. (1979) *Methods Enzymol. 56*, 734–749.
Henselman, R. A., & Cusanovich, M. A. (1974) *Biochemistry 13*, 5199–5203.
Hong, K., & Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2617–2621.
Hong, K., & Hubbell, W. L. (1973) *Biochemistry 12*, 4517–4523.
Hubbard, R., Brown, P. K., & Bownds, M. D. (1971) *Methods Enzymol. 18*, 615–653.
Knudson, P., & Hubbell, W. L. (1978) *Membr. Biochem. 1*, 297–322.
Matsumoto, H., Horiuchi, K., & Yoshizawa, T. (1978) *Biochim. Biophys. Acta 501*, 257–268.
McCaslin, D. R. (1980) Ph.D. Dissertation, Duke University.
McCaslin, D. R., & Tanford, C. (1981) *Biochemistry* (following paper in this issue).
Osborne, H. B., Sardet, C., & Helenius, A. (1974) *Eur. J. Biochem. 44*, 383–390.
Osborne, H. B., Sardet, C., Michel-Villaz, M., & Chabre, M. (1978) *J. Mol. Biol. 123*, 177–206.
Sardet, C., Tardieu, A., & Luzzati, V. (1976) *J. Mol. Biol. 105*, 383–407.
Small, D. M. (1971) in *The Bile Acids* (Nair, P. P., & Kritchevsky, D., Eds.) Vol. 1, pp 249–353, Plenum Press, New York.
Stubbs, G. W., & Litman, B. J. (1978) *Biochemistry 17*, 220–225.
Tanford, C. (1980) *The Hydrophobic Effect*, 2nd ed., Wiley, New York.
Wald, G., & Brown, P. K. (1953) *J. Gen. Physiol. 37*, 189–200.
Wald, C., & Brown, P. K. (1956) *Nature (London) 177*, 174–176.
Weiss, H., & Wingfield, P. (1979) *Eur. J. Biochem. 99*, 151–160.
Yoshikami, S., & Noll, G. N. (1978) *Science (Washington, D.C.) 200*, 1393–1395.
Zorn, M., & Futterman, S. (1973) *Arch. Biochem. Biophys. 157*, 91–99.

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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