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Comparative Studies on the Fine Structure of Purple Membrane from *Halobacterium Cutirubrum* and *Halobacterium Halobium*

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Summary. Direct comparison of the absorption and circular dichroic spectra of dark- and light-adapted purple membrane from *Halobacterium cutirubrum* and *Halobacterium halobium* indicated no apparent species differences. In addition, sequential bleaching and regeneration of the purple membrane with concomitant monitoring of the absorption and circular dichroic spectra showed no species differences as well. Furthermore, perturbation of the structure of the purple membrane from either species with a detergent, Triton X-100, yielded similar spectral changes. It was concluded: (i) no apparent differences exist in the molecular organization and protein fine structure of the two purple membranes, (ii) if exciton interaction among the retinal chromophores is a reasonable possibility in the case of the purple membrane from *Halobacterium halobium*, it must be similarly so for the membrane from *Halobacterium cutirubrum*, (iii) the effects of light adaptation on the membrane structure of both species are essentially the same, and (iv) the underlying molecular mechanisms for the bleaching and regenerative processes must be similar, if not identical, for the purple membranes of the two species.

The genera *Halobacterium* and *Halococci* constitute the family of extremely halophilic bacteria. They are strict aerobes, growing in a medium almost saturated with NaCl. One species, *Halobacterium halobium*, has been extensively studied, particularly concerning the properties of a specialized portion of its membrane called the purple membrane. This membrane is synthesized in response to low oxygen concentration in the growth medium [22]. It contains a single protein species, bacteriorhodopsin, to which a single retinal is bound by means of a Schiff base linkage [21, 23]. This membrane has been shown to act as a light-activated proton pump [22]. A structural model for this membrane has recently evolved from X-ray diffraction, electron microscopy, and electron spin resonance studies [7, 8, 11–14]. Unique features of this model are the extreme rigidity of the molecular organization and the equivalence of

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the local environments of the bacteriorhodopsins. The proteins are arranged in clusters of three with a three-fold axis of symmetry forming a hexagonal lattice. Further support for this model has seemingly emerged from visible absorption and circular dichroic (CD) studies of the purple membrane, the results of which have been interpreted in terms of intermolecular excitonic interaction among the protein chromophores within the clusters [1, 4, 6, 15].

The bacteriorhodopsin exists in two relatively stable states, according to the most recent determination, the dark-adapted form containing a mixture of the 13-*cis* and the all-*trans* stereoisomers of retinal with a ratio of about 1 : 1 and the light-adapted form containing only the all-*trans* stereoisomer [16, 20, 24]. In a recent communication from this laboratory, we have shown that transition between these two membrane states does not result in major structural changes of the membrane [4]. In fact, the only change that was observed could be attributed to alterations in the interaction between the apoprotein and the retinal resulting from the change in the stereoisomeric configuration of the retinal. However, when the membrane is bleached in the presence of hydroxylamine, which results in the loss of the Schiff base bond between the retinal and the apoprotein, major changes in the membrane structure are apparent [5]. Furthermore, these structural changes are approximately reversed upon regeneration of the membrane by incubation with retinal [5].

Recently, a bacteriorhodopsin-containing purple membrane has also been isolated from another species halophilic bacteria, *Halobacterium cutirubrum* [17, 18]. Direct comparison of the purple membrane from this species with that from *H. halobium*, based on chemical and morphological studies, have indicated no species differences in regards to the gross structure of the membranes [19]. In the present communication, we are concerned with the fine structure of the membranes. Since spectral analysis is one of the best methods of probing the fine structure of a membrane, we report the direct comparison of (i) the absorption and CD spectra in the wavelength range of 200–700 nm of both states of the purple membrane, (ii) the absorption and CD spectrum of purple membrane at various stages of bleaching and regeneration, and (iii) the effects of low concentration of the detergent Triton X-100 on the absorption and CD spectra of the purple membrane of both species. No species differences are apparent from these studies which can be interpreted as any detectable differences in the molecular organization and the protein structure of the purple membranes from the two species. Also the effects of light adaptation on the membrane structure of both species

are essentially identical as well as the effects of Triton X-100. Furthermore, the underlying mechanisms of the bleaching and regenerative processes of the membranes of the two species must be similar, if not identical. Therefore, the fine structure of the membrane must be essentially species independent.

Materials and Methods

Purple membrane from *H. halobium* was prepared according to the method of Becher and Cassim [3] with minor modifications. Purple membrane from *H. cutirubrum* was prepared according to the method of Kushwaha *et al.* [18]. The *H. cutirubrum* cultures employed in this study were generously provided by Professor M. Kates and the National Research Council of Canada (NRC numbers 34001, 34013, 34017, 34019). No spectral differences were observed in membranes isolated from the various cultures. Membrane preparations were purified until the ratio of protein aromatic amino acid absorbance at 280 nm to chromophore absorbance at 567 nm was about 1.5 for the light-adapted form. Dark- and light-adapted forms of the membrane were prepared as previously described [4].

Absorption spectra were measured by a Cary Model 118C low ultraviolet double beam recording spectrophotometer with scattered transmission accessory. CD was measured by a Cary Model 60 spectropolarimeter with a model 6003 CD accessory. Spectra were recorded at least twice. A constant 1.5 nm spectral bandwidth was employed for all measurements. The spectra given are the average of results obtained from several membrane preparations. Details of experimental procedures used have been given elsewhere [4].

Bleaching and regeneration of the purple membrane was carried out as previously described with minor modifications [5]. The purple membrane in Triton X-100 (Pierce Co., Rockville, Ill.) was prepared by adding to a light-adapted purple membrane solution, 0.75 M in NaCl, the appropriate amount of 1% (vol/vol) Triton X-100, so that the final detergent concentration would be 0.2% (vol/vol). The solution was allowed to equilibrate for about 8 hr until the visible absorption maximum showed a blue shift (from 568 nm to 555–560 nm), and the two visible CD bands were replaced by a single positive band with a maximum at 535 nm.

Results and Discussion

Spectra of Light- and Dark-Adapted Forms

The absorption and CD spectra of the dark- and light-adapted purple membranes of *H. halobium* in the 350–700 nm region consist of several major bands in addition to several minor ones [4]. The variability in the determination of the positions and intensities of the major bands is given in Table 1. Comparative studies of the spectra of the dark- and light-adapted purple membranes of *H. cutirubrum* were made by normalizing the spectra at 568 nm to the absorption spectrum of light-

Table 1. Variability in the determination of the positions and intensities of the major bands of the absorption and circular dichroic spectra of the purple membrane

State of the membrane	Apparent band position (nm)	Variability	
		wavelength (nm)	intensity (%)
	Absorption		
LA	568	±0.5	0[a]
DA	560	±0.5	<1.0
LA	280	±1.0	<1.0
DA	280	±1.0	<1.0
	Circular dichroism		
LA	600	±2.5	10
DA	595	±2.5	10
LA	535	±2.0	5
DA	525	±2.0	5
LA	317	±1.0	1
DA	317	±1.0	1
LA	290	±1.0	10
DA	290	±1.0	10
LA	264	±2.0	5
DA	264	±2.0	5

LA and DA are light-adapted and dark-adapted membrane, respectively.
[a] All spectra were normalized at 568±0.5 nm.

adapted purple membrane of *H. halobium*. The band positions and intensities of the spectra of the two species were identical within the variabilities given in Table 1. Therefore, the spectra of the two species are indistinguishable in this spectral region within a relatively high degree of reliability.

The bands in the 300–700 nm region (*see* Fig. 4b, curve 1) can be attributed to chromophoric electronic transitions within the apoprotein environment [4]. It has previously been suggested that one interpretation of the two visible CD bands of oppositely signed ellipticities in the spectra of the purple membrane from *H. halobium* could be excitonic interactions among the three retinals within the protein clusters [2]. Results seemingly in accord with this interpretation of the visible spectra have recently been published by Heyn *et al.* [15], Bauer *et al.* [1], and Becher and Ebrey [6]. They have reported that disruption of the membrane structure by a solubilizing agent, Triton X-100, results in a single positive CD band and only 5% loss in absorption and that the low

levels of regeneration of the bleached membrane result in CD spectra dominated by a single positive band.

It is clear that if excitonic interaction is a reasonable explanation for the long wavelength spectra of the membrane from *H. halobium* it must also be so for the membrane from *H. cutirubrum*. Since excitonic interactions of chromophores are extremely sensitive to the spatial arrangement of the interacting chromophores, any change in the relative position and orientations of the bacteriorhodopsins and/or retinals within the protein clusters would strongly affect the CD spectrum [9]. Therefore, a reasonable conclusion would be that the arrangement of the bacteriorhodopsin molecules and the relative positions of the retinals in respect to the apoproteins are essentially the same in the membranes of both species.

The short wavelength negative CD band at 317 nm has been attributed in the case of the membrane from *H. halobium* to strong dissymmetric constraint (s) imposed on the retinal by the environment of the apoprotein [4]. That is, this band may be a sensitive probe of the rigidity of the retinal-apoprotein secondary noncovalent bond. The fact that this band is essentially the same in the spectra of the membranes of the two species indicates that local environments imposed on the retinals by the apoproteins of both species must be very similar.

Several factors can contribute to the spectra in the 250–300 nm region [4]: (i) the π-π^* transitions of Trp, Tyr, and Phe of the apoprotein, (ii) possible dipole coupling between the π-π^* transitions of the retinal and the aromatic amino acids, and (iii) minor π-π^* transitions of retinal in the near ultraviolet wavelength region. In view of this, the similarity in the spectra of the purple membranes from the two species in this wavelength region must arise from a similarity in the local environments and interactions of the aromatic amino acid side chains of the apoproteins of the two species. Furthermore, these results provide additional evidence for the similarity of the local environments and interactions of the retinal chromophore of the two membranes isolated from the two different species.

The far ultraviolet (200–250 nm) CD spectra of the two states of the membrane from the two species are shown in Fig. 1. As has been previously observed for the membrane of *H. halobium*, the state change of the membrane of *H. cutirubrum* from dark-adapted to light-adapted also does not alter the far ultraviolet CD spectrum [4]. A systematic, *albeit* small, difference is observed in the spectra of the two species which cannot be accounted for by the experimental variability of the

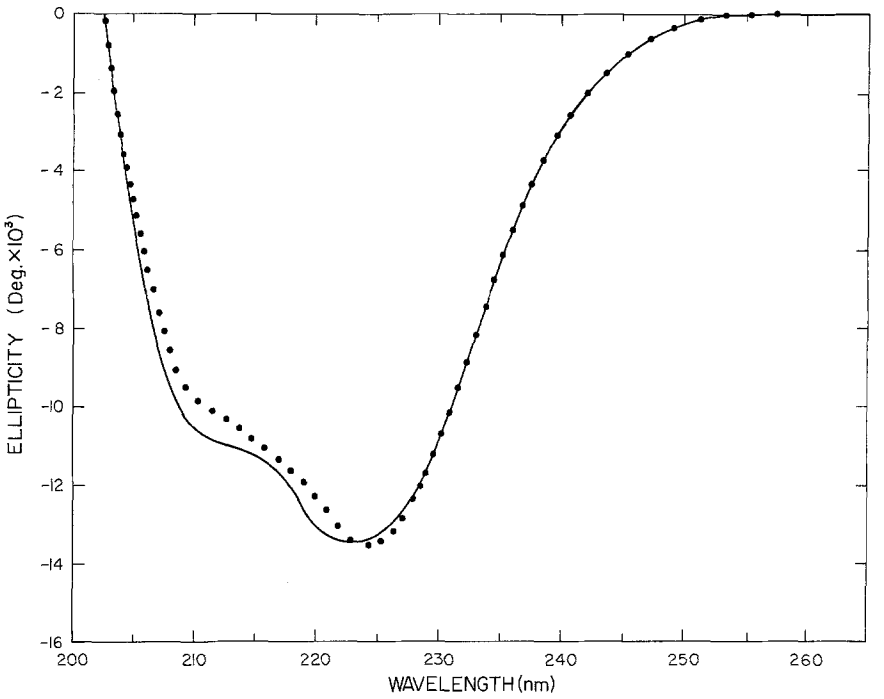


Fig. 1. Far ultraviolet circular dichroic spectra of the purple membrane: *H. halobium*, dark- and light-adapted (—) and *H. cutirubrum*, dark- and light-adapted (●). The optical path was 1.00 nm. Spectra are based on an absorbance of 0.20 at 568 nm for the light-adapted form

± 0.5 nm and 2% in the wavelengths and intensities, respectively, of the apparent band maxima. The ellipticity of the band centered at about 211 nm is slightly depressed, and both the bands are slightly red shifted in the spectrum of the membrane from *H. cutirubrum* relative to those features in the spectrum of the membrane from *H. halobium*. However, the possible variation of the ellipticity of the band centered at about 223–224 nm due to species difference is only about 1% which is well within the experimental variability of the measurements at that wavelength. It has been shown that the particulate nature of membrane suspensions produces such optical artifacts in the far ultraviolet CD spectra of membranes [26]. Therefore, this relative depression of the ellipticity of the 211-nm band and red shift of both bands can be attributed to the difference in the average particle size of the membrane suspensions prepared from the two different species. Spectra in this wavelength region are mainly a consequence of the interactions among the electronic transitions of the amide groups in the peptide chains of the bacteriorhodopsin

molecules [10]. In view of this and the dependency of the near ultraviolet spectra on the local environments and interactions of the aromatic amino acid side chains, the fact that the ultraviolet spectra of the membranes (excluding slight variations due to the differences in average membrane particle size) do not seem to be species dependent can best be interpreted as indicative of a high degree of similarity in the conformation (at every level of protein structure) of the bacteriorhodopsins of the two species. Further support for this conclusion is given by the spectral results discussed previously which are in accord with a very similar local apoprotein environment for the retinals of the two species.

In a previous communication from this laboratory, we showed that the stereoisomerization of the retinal of the *H. halobium* purple membrane from the 13-*cis* to the all-*trans* configuration, which occurs without the loss of the Schiff base bond between the chromophore and the apoprotein, with light-adaptation, results in spectral changes of the retinal transitions within the apoprotein environment [4]. Since the same spectral changes result from the light-adaptation of the *H. cutirubrum* purple membrane as previously observed for the *H. halobium* membrane, clearly, the molecular mechanism(s) of light-adaptation must be the same for the purple membranes from both species.

Bleaching

The sequential bleaching of the purple membranes isolated from the two species, as monitored by absorption and CD in the 300–650 nm spectral region, is demonstrated in Fig. 2. It is apparent that the gross aspects of the curves at various degrees of bleaching are similar for the two species. As bleaching progresses, the absorption at 568 nm decreases with a concomitant increase of absorption at 366 nm. The latter band is attributed to absorption due to the retinaloxime produced by the reaction of retinal with hydroxylamine. There is an isosbestic point at about 414 nm in the absorption spectra and an isoelliptic point at about 570 nm in the CD spectrum indicative of the presence of only two spectral species. The decrease in the ellipticities of the CD bands at 317, 535, and 602 nm is concomitant with the decrease in absorbance at 568 nm and increase in absorbance and ellipticity at 366 nm during bleaching. The rise of a CD band at 366 nm during the bleaching process has been attributed to the main π-π^* transition of the bound retinaloxime in the dissymmetric environment of the apoprotein [16].

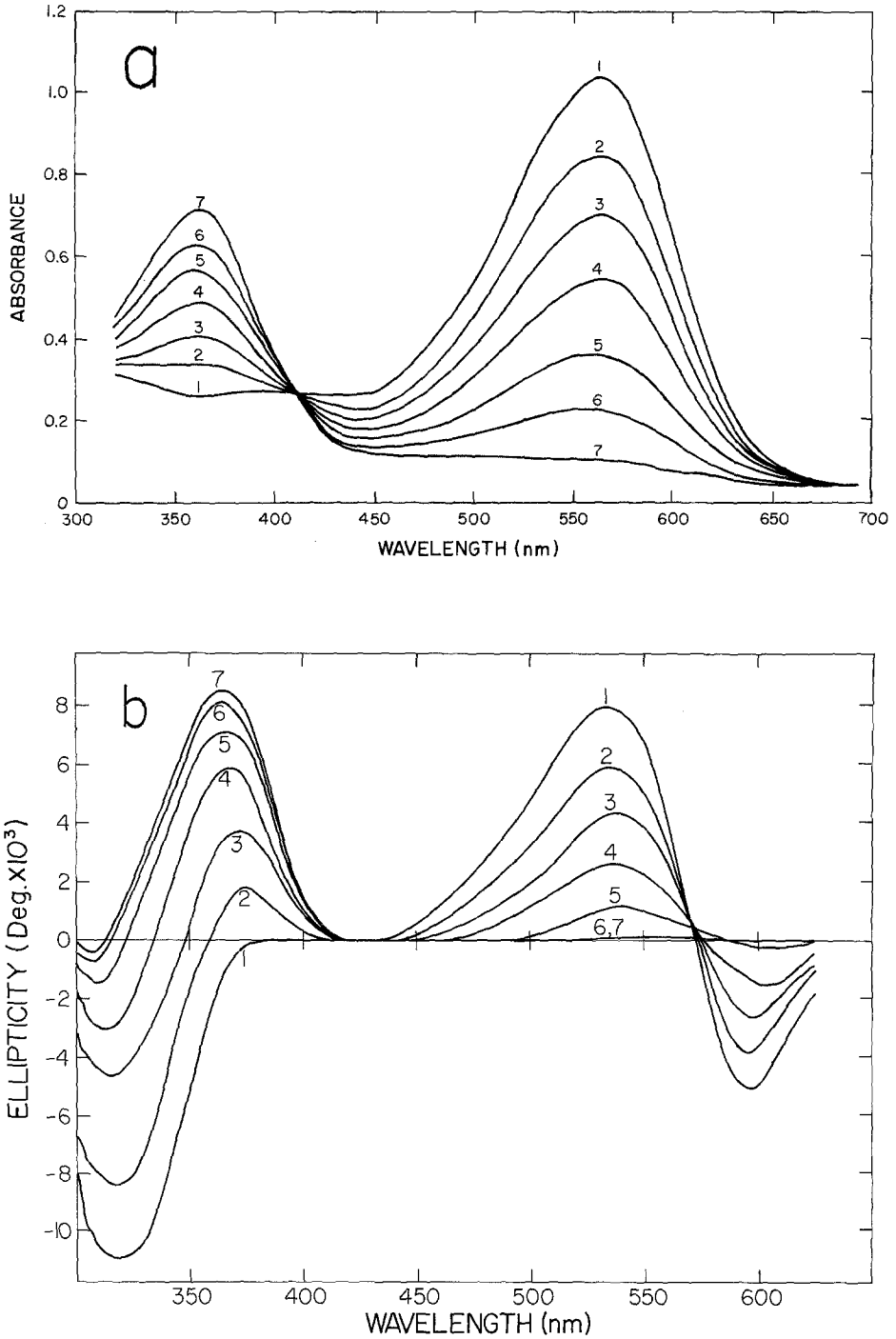
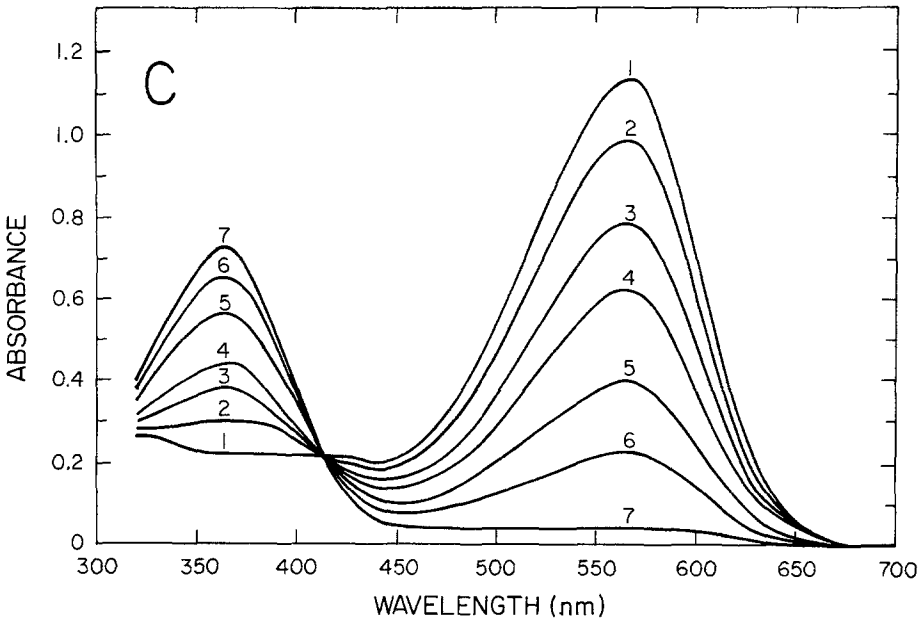


Fig. 2. The effects of sequential bleaching on the spectra of the purple membrane from 300 to 700 nm: (*a*) absorption *H. halobium*, (*b*) circular dichroism *H. halobium*, (*c*) absorption *H. cutirubrum*, and (*d*) circular dichroism *H. cutirubrum*. The optical path was 1.00 cm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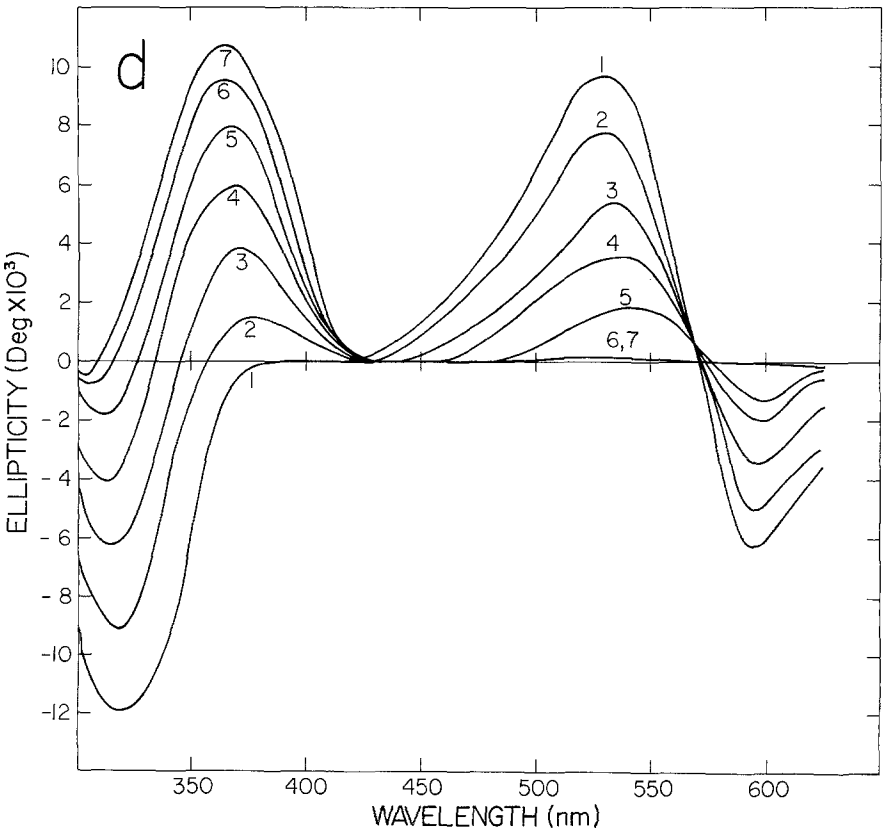
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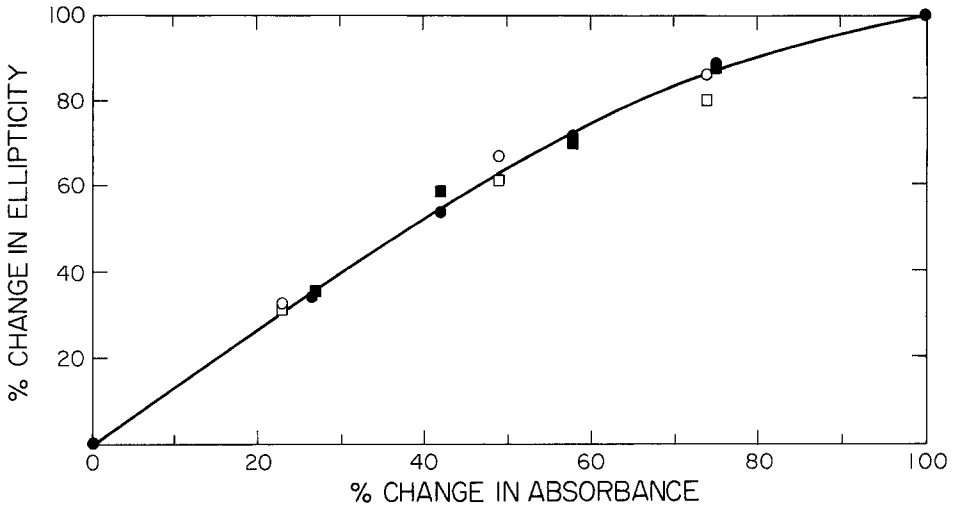


Fig. 3. Change in the ellipticity of the 317-nm band and the peak-to-peak ellipticity of the 535 and 602-nm bands in respect to change in the absorption of the 568-nm band during sequential bleaching of the purple membrane: *H. halobium*, 317 nm (■) and peak-to-peak of 535 and 602 nm (●); *H. cutirubrum*, 317 nm (□) and peak-to-peak of 535 and 602 nm (○) (based on data from Fig. 4.) Native membrane is taken as the reference state

A plot of the changes in the peak-to-peak ellipticity of the 535 and 602-nm bands and the ellipticity of the 317-nm band in respect to the changes in the absorption of the 568-nm band during bleaching is given in Fig. 3. Several interesting findings emerge from this plot: (i) data for both species fall on the same curve and are indistinguishable, (ii) data for the 317-nm band and 535 and 602-nm bands fall on the same curve, and (iii) the ellipticity changes are not linear with absorption changes (possible indication of cooperativity during bleaching). Clearly the results indicate that the mechanism of bleaching of the purple membrane is similar if not identical for the two species. Additional support for this conclusion is obtained from spectral studies in the region below 300 nm. Previously, it was shown that complete bleaching of the purple membrane from *H. halobium* resulted in marked spectral changes in the near ultraviolet CD with lesser changes in the near ultraviolet absorption and no detectable changes in the far ultraviolet CD [5]. Interpretation of these results was that bleaching induces a significant conformation change in the apoprotein involving the tertiary but not the secondary structure of the apoprotein. Changes induced by complete bleaching in the near ultraviolet CD of membranes from both species are compared in Fig. 4. Similar marked changes are apparent for membranes from

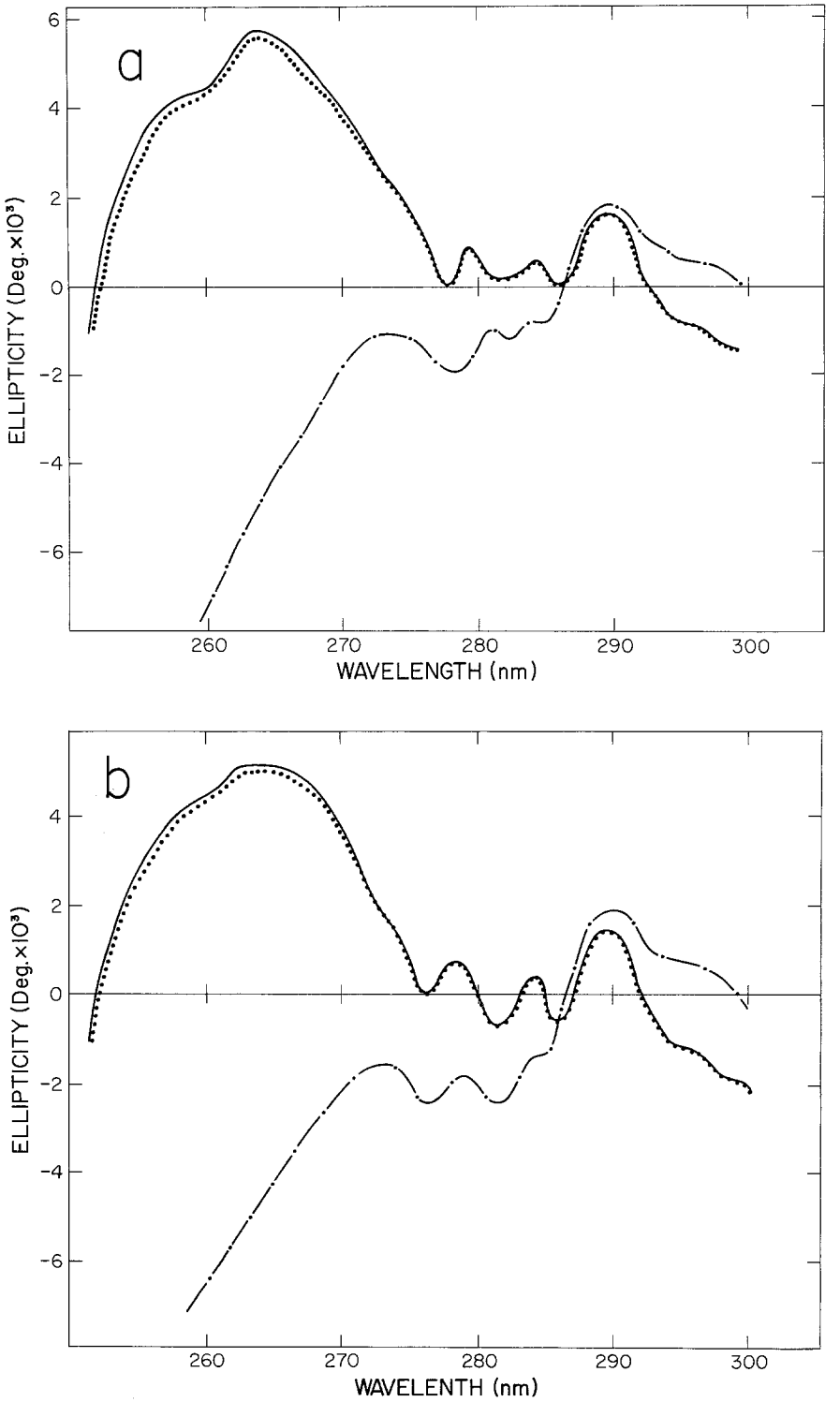


Fig. 4. The effects of bleaching and regeneration on the near ultraviolet circular dichroic spectra of purple membrane from *H. halobium* (*a*) and *H. cutirubrum* (*b*): native membrane (----), bleached membrane (-·-), and regenerated membrane (···). The optical path was 1.00 cm. Spectra are based on an absorbance of 0.50 at 568 nm for the light-adapted form

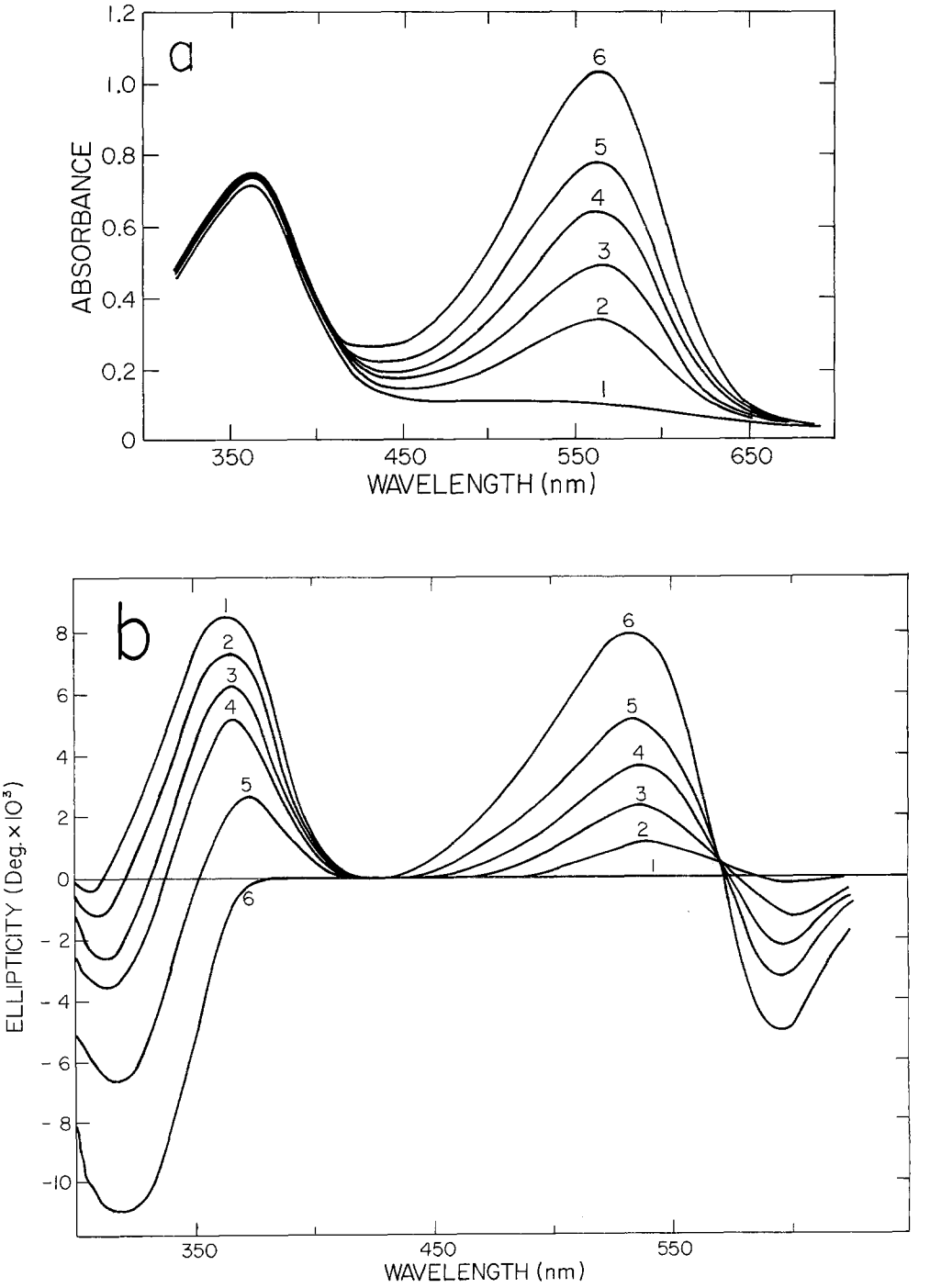
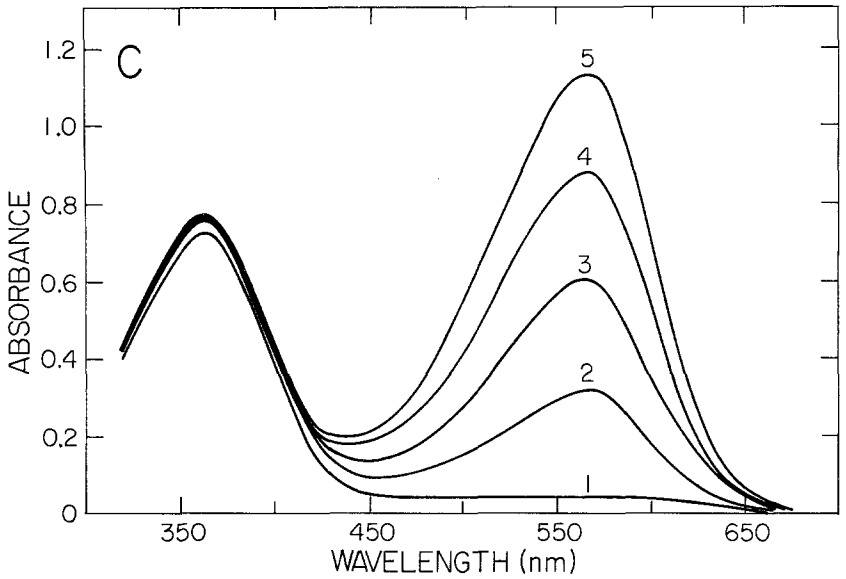


Fig. 5. The effects of sequential regeneration on the spectra of the purple membrane from 300 to 700 nm: (*a*) absorption *H. halobium*, (*b*) circular dichroism *H. halobium*, (*c*) absorption *H. cutirubrum*, and (*d*) circular dichroism *H. cutirubrum*. The optical path was 1.00 cm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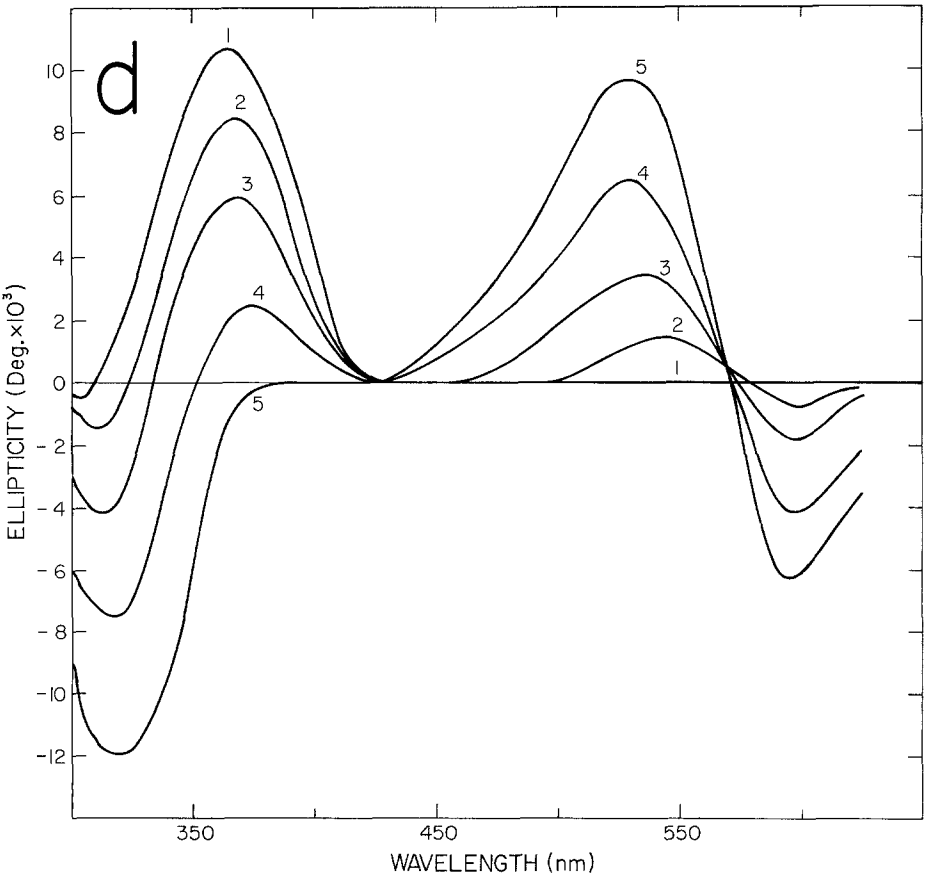
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both species. Comparisons of the near ultraviolet absorption spectra yield the same similarity (spectra not shown). Identical to the results of the far ultraviolet studies of the purple membrane of *H. halobium*, the CD of *H. cutirubrum* is also invariant to bleaching in this spectral region.

Regeneration

The sequential regeneration of the bleached purple membrane as monitored by absorption and CD in the 300 to 650-nm spectral region is demonstrated in Fig. 5 for the two species. Again, it is apparent that there are no species differences. The gross features of the curves at every level of regeneration are the same for the two species including an isoelliptic point at about 570 nm. As previously demonstrated for the membrane of *H. halobium*, regeneration of the bleached membrane results in the loss of the CD band at 366 nm with the persistence of the absorption band at 366 nm [5]. This spectral change has been attributed to the elimination of the dissymmetric interaction between the retinaloxime and the apoprotein during regeneration resulting in the loss of induced optical activity of the retinaloxime. The retinaloxime remains bound to the membrane as evidenced by the persistent absorption band at 366 nm despite several cycles of pelleting and washing of the regenerated purple membrane. The regeneration CD curves for the purple membrane of *H. halobium* shown in Fig. 5 are different in some aspects from previously published curves [1, 6]. In contrast to the previous spectral studies, the present studies indicate the presence of a well-defined isoelliptic point in the CD spectra. In addition, the curves of the present study are not dominated by a single positive band at low levels of regeneration as was the case in the past studies.

A plot of the changes in the ellipticities of the 317, 535, and 602 nm bands in respect to the changes of the absorption band at 568 nm during regeneration (in a similar manner to the plot for bleaching shown in Fig. 3) is shown in Fig. 6. Clearly, the curve in Fig. 6 is identical to the one in Fig. 3. This implies that bleaching and regeneration are spectrally complementary processes. That is, the CD of the membrane with x amount of bleaching is the same as the CD of the membrane with $1\text{-}x$ amount of regeneration.

The complete regeneration of the bleached membrane reverses the effects of bleaching on the near ultraviolet spectra of the purple mem-

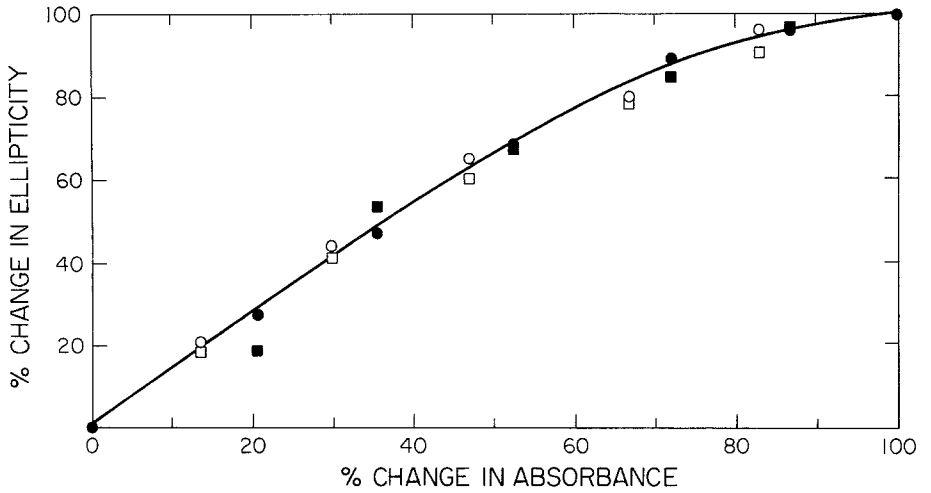


Fig. 6. Change in the ellipticity of the 317-nm band and the peak-to-peak ellipticity of the 535 and 602-nm bands in respect to the change in the absorption of the 568-nm band during sequential regeneration of the bleached purple membrane: *H. halobium*, 317 nm (■) and peak-to-peak of 535 and 602 nm (●); *H. cutirubrum*, 317 nm (□) and peak-to-peak of 535 and 602 nm (○) (based on data from Fig. 7). Native membrane is taken as the reference state

brane, regardless of species. The effects of regeneration on the CD in this spectral region for both species are shown in Fig. 4.

It is apparent from these studies that the molecular mechanism(s) of bleaching and regeneration must be the same for the two species.

Effects of Membrane Solubilization with Triton X-100

Previously published spectra of the purple membrane of *H. halobium* solubilized with the detergent, Triton X-100, have indicated minor changes in the absorption spectrum correlated with major changes in the CD spectrum in the 300 to 700-nm spectral region [8, 12]. Recent hydrodynamic and far ultraviolet CD studies indicated that the bacteriorhodopsin of solubilized purple membrane is monomeric and that there is no evidence for large changes of the protein net secondary structure resulting from solubilization [25]. However, less drastic conformational changes cannot be ruled out by this study. Comparative spectral studies of the solubilized membranes from the two species are demonstrated in Fig.7. The detergent-induced spectral changes are similar. Also, as

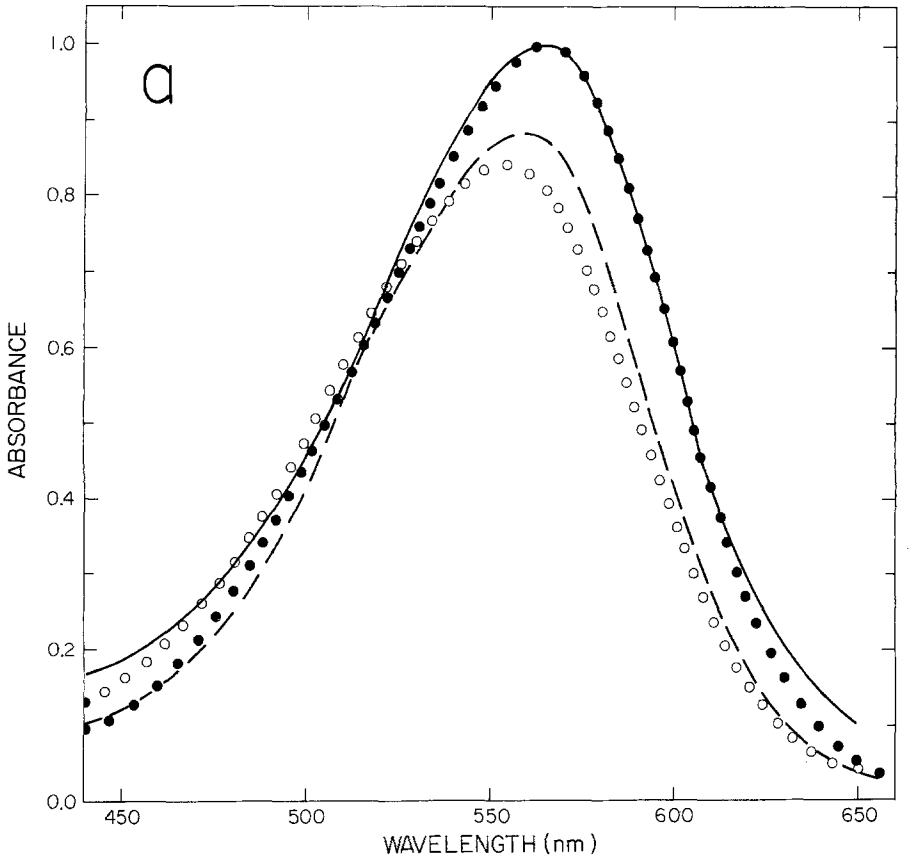
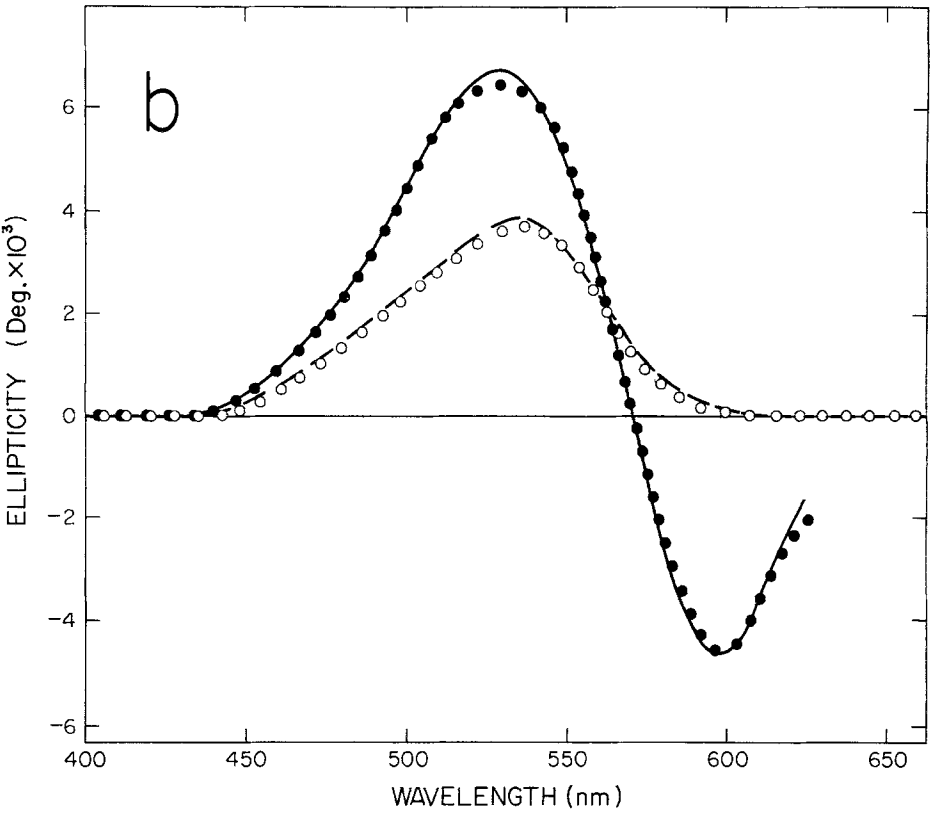


Fig. 7. The effects of solubilization of the purple membrane with 0.2% (vol/vol) Triton X-100 and 0.75 M NaCl on the absorption (*a*) and circular dichroism (*b*) spectra of the purple membrane: *H. halobium, native (—) and solubilized (---); H. cutirubrum*, native (●) and solubilized (○). The optical path was 1.00 mm

previously observed for *H. halobium*, solubilization results in about 70% reduction in the ellipticity of 317-nm band of the *H. cutirubrum* membrane (spectrum not shown).

This study demonstrates that, even when the membrane is subjected to a very strong perturbant such as a detergent which significantly alters the membrane organization, the resulting changes in the membrane spectra are invariant to species differences.

In conclusion, the previous comparative study of the two species of halophilic bacteria established the species invariance in regards to the gross structure of the purple membrane [19]. The present study extends these findings to the fine structure of the membrane to molecular distances no greater the retinal binding site.



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