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DETERMINATION OF THE RETINAL/PROTEIN MOLAR RATIOS FOR THE PURPLE MEMBRANES OF HALOBACTERIUM HALOBIUM AND HALOBACTERIUM CUTIRUBRUM

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SUMMARY: Previously reported values of the retinal/protein molar ratios for the purple membranes from halobacteria are confusing. For H. halobium values of approximately 1.00 and 0.45 and for H. cutirubrum values of 0.45 and 0.43 have been published. A redetermination of these ratios in our hands has yielded identical values of 0.99 ± 0.01 for the membranes of both species. This is in agreement with expectations that the ratio be the same for the two species since the similarity of their membrane structure has been established and that every apoprotein molecule be complexed with a retinal since the uniqueness and equivalence of the membrane protein, bacteriorhodopsin, has been demonstrated.

INTRODUCTION: The halophilic bacteria, Halobacterium halobium and Halobacterium cutirubrum, can synthesize in response to a reduction in the oxygen concentration a specialized membrane patch called "purple membrane" because of its color (1). This membrane under illumination in vivo functions as a proton pump using light energy to transport protons into the surrounding environment against a concentration gradient (1). Subsequently, the proton gradient may be utilized by the bacterium to synthesize ATP (2) or to transport amino acids against a concentration gradient (3).

The purple membrane from either species is composed of 75% protein and 25% lipid by weight (4). The protein moiety consists of a single protein species. Molecular weight determinations of the protein have yielded values ranging from 20,000 to 26,000 daltons depending on the methods employed (4-9).

The characteristic absorption of the purple membrane at about 570 nm has been attributed to the presence of a retinal chromophore covalently bound to an ε-amine lysine group of the protein via a protonated Schiff base linkage. By analogy to the rhodopsins found in vertebrate and invertebrate visual systems

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which contain a similar linkage of retinal to apoprotein, the purple membrane protein has been named bacteriorhodopsin.

Oesterhelt and Stoeckenius (4) first reported the retinal/protein molar ratio for the purple membrane of H. halobium to be approximately one. However, Kushwaha et al. (7) found this ratio to be only 0.45 for the purple membrane of H. cutirubrum which has recently been shown to be similar if not identical in structure to the purple membrane of H. halobium (9-11). Later in a joint publication the two laboratories reported ratios of 0.45 and 0.43 for the purple membranes of H. halobium and H. cutirubrum, respectively (9). Although a molecular weight of 26,000 daltons was assumed for the apoprotein in the first determination of the retinal/protein molar ratio and 20,000 daltons in the last two, this cannot account for the large discrepancies in the reported ratio values. It is expected that this ratio should be the same for the two species in view of the similarity of the purple membrane structure from these two species. Therefore, if the most recently reported values of the ratio are valid this would imply that on the average only about half of the retinal sites are occupied. However, since the incubation of the purple membrane in the presence of retinal does not observably alter the purple membrane spectrum this would further imply that there are more than one species of proteins present or the existence of an ad hoc mechanism which prevents the binding of retinal to all the apoprotein molecules (12).

To resolve the discrepancy of the previous publications, the retinal/protein molar ratios for the purple membranes of both species, H. halobium and H. cutirubrum, have been redetermined. In our hands, the ratios were identically 0.99 $\pm$ 0.01 for the two species. This is in accord with expectations that the ratio be the same for the two species and that the ratio be one (in view of the uniqueness and equivalence of bacteriorhodopsin molecules in the purple membrane).

MATERIALS AND METHODS: Cultures of H. halobium R_1 were grown and the purple membrane was isolated and purified according to the method of Becher and Cassim (5) with minor modifications. Purple membrane from H. cutirubrum cultures was prepared according to the method of Kushwaha et al. (7).

TABLE I

Thin Layer Chromatography of

Purple Membrane Retinal Extracts

Source of Retinal	Rf value
All-trans (commercial)	0.40
H. halobium	0.41
H. cutirubrum	0.40

Pre-coated 0.25 mm thick silica gel plates 60 F_{254} (5x20 cm) from E.M. Labs (Elmsford, New York) were used. The plates were activated at 110°C for 20 minutes, cooled to room temperature, then sprayed with ether containing 50 µg of butylated hydrotoluene and air dried. The developing chambers were lined with Whatman #1 filter paper and allowed to equilibrate for one hour with 25 ml of developing solvent, 18% (v/v) acetone in petroleum ether. The plates were placed in the developing chambers immediately after application of the samples, and developed by the ascending method in the dark at 8°C. Time of development varied from 40 to 45 minutes. (For details see reference (14)).

Purple membrane suspensions were light adapted in 3 ml volumes according to the procedure of Becher and Cassim (5). Retinal was extracted with 8 ml of absolute ethanol several times with vigorous agitation to achieve maximal extraction. Precautions were taken to protect the extracted retinal from light. Retinal concentrations were determined by the extinction coefficients at 318 nm using a molar extinction coefficient of 4.34×10^4 for all-trans retinal in ethanol as given by Dartnall (13) and a protein molecular weight of 24,500 daltons. Thin-layer chromatography of the retinal was doen according to the method of Fung et al. (14). All-trans retinal purchased from Eastman Kodak Company (Rochester, New York) was used as the standard.

Protein concentrations were determined on the retinal-extracted membranes by the micro-Kjeldahl nitrogen procedure assuming a nitrogen factor of 6.25. Since the lipid moiety of the membrane does not contain any nitrogen, no corrections for nonprotein nitrogen were necessary (7).

RESULTS AND DISCUSSION: The absorption spectra of the extracted retinals from the purple membranes of the two species in ethanol are compared with the spectrum for commercial all-trans retinal in Fig. 1. Similarity of the spectra are indicative of minimal contamination of the extracted retinals by protein. In addition, the results of the thin-layer chromotography summarized in Table I are in accord with the retinals being in the all-trans configuration. This is

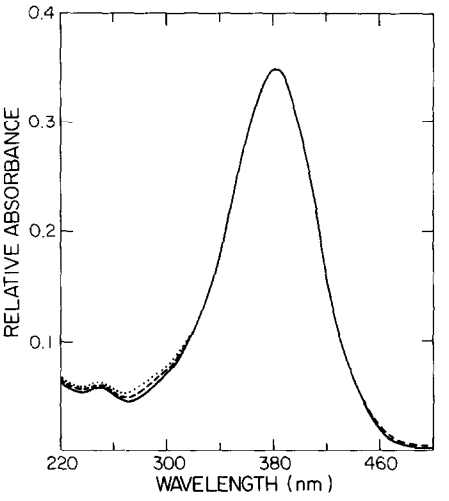

Figure 1. Absorption spectra of retinals extracted from the purple membranes of H. halobium (----) and H. cutirubrum (·····) compared with the spectrum of commercial all-trans retinal (———) in ethanol. All curves were normalized at 381 nm.

in agreement with previous publications identifying the chromophore of the bacteriorhodopsin in the light-adapted state as all-trans retinal (1, 15-17).

The results of retinal and protein determinations on several samples from both species are summarized in Table II. It is readily seen that the retinal/protein ratio is about one regardless of species.

An essential difference between our method of extraction of the retinal from the membrane and that of other laboratories is our use of ethanol as the only extractant with vigorous agitation. Other laboratories have employed much more involved methods requiring solublization of the membrane with cetyltrimethyl ammoniumbromide followed by either hydroxamine or mild acid treatment and extraction with a series of organic solvents (4, 7, 9). The descrepancies previously reported may be due to variability inherent in the multiple step methods used. Our simple method has constantly yielded reproduceable results as long as an essential precaution was taken. Ethanol extraction must be rapid or a complete extraction of the retinal cannot be achieved.

TABLE II

Retinal/Protein Ratios of the Purple Membrane

Species	Preparation Number	Moles x 10^{-7}		Retinal/Protein Ratio
		Retinal*	Protein**	
H. halobium	1	1.990	2.003	0.994
	2	1.996	2.039	0.979
	3	2.007	2.021	0.993
	4	1.990	1.996	0.997
	5	2.004	2.014	0.995
H. cutirubrum	1	1.503	1.511	0.995
	2	1.518	1.536	0.988
	3	1.498	1.507	0.994
	4	1.525	1.539	0.991
	5	1.511	1.521	0.993

* Based on an all-trans extinction coefficient of 4.34×10^4 at 381 nm in ethanol.

** Based on a protein nitrogen factor of 6.25 and protein molecular weight of 24,500 daltons.

It is concluded that the retinal/protein ratio is one regardless of species as expected from the similarity of the membrane structure of the two species and the uniqueness and equivalence of the bacteriorhodopsin molecules.

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