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Biogenesis of the Purple Membrane of Halobacterium Halobium

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Abstract. A protein closely resembling the purple membrane protein pre-exists in the cell membrane of *H. halobium* prior to the appearance of functional bacteriorhodopsin. It is associated with a differentiated membranous structure which has been isolated on a sucrose gradient and appears to be a precursor of the purple membrane. The identity of the precursor protein as a form of the purple membrane protein was established in different ways: (1) The cell proteins were labelled in vivo with ^{14}C-proline during dark aerobic growth, the label was "chased", and the cells transferred to the illuminated near-anaerobic conditions under which purple membrane is optimally synthesised (induction conditions). Cell lysates were fractionated on sucrose gradients at different times after induction. Label first found in the precursor fraction appeared within 24 h in the purple membrane fraction. (2) SDS-urea-acrylamide[1] gel electrophoresis of the purple membrane protein and the precursor showed only one protein band whose migration coincided with that of the purple membrane band. (3) The amino-acid analysis of the purified precursor was very similar to that of the purple membrane.

The absorption spectrum of the precursor showed little of the characteristic absorption of bacteriorhodopsin at 570 nm. A major band appears at 412 nm, the exact nature of which is not known. The difference spectrum (reduced versus oxidised) of a purified fraction showed only traces of cytochrome. Thin-layer chromatography of an acetone-soluble lipid extract indicated the presence of retinal and β-carotene. Cells grown in the presence of nicotine did not develop purple membrane after induction: the species absorbing at 412 nm was much less abundant than in non-inhibited cells, but a new fraction was present with a sharp peak at 345 nm consisting mainly of lycopene.

Key words: Purple membrane – Bacteriorhodopsin – Biosynthesis – Retinal – Nicotine.

[1] Abbreviations: CTAB = cetyltrimethyl ammonium bromide; SDS = sodium dodecyl sulfate; CAP = chloramphenicol; TLC = thin layer chromatography; CD = circular dichroism

Introduction

The purple membrane of *H. halobium*, first described in detail by Stoeckenius and Rowen (1967), has been the subject of many recent investigations in the fields of bioenergetics and spectroscopy. Indeed this membrane, the main component of which is the pigment bacteriorhodopsin, is a model of choice for energy conversion studies; it functions as a light driven proton pump enabling the cells to photophosphorylate by a mechanism much simpler than that of chlorophyll-mediated photosynthesis. The similarity of bacteriorhodopsin to the visual pigment has in addition provided interesting analogies in the investigation of the corresponding photochemical processes, although in one case the process leads to synthesis of ATP while in the other it leads to excitation of the optic nerve.

The unusual function of the purple membrane has its counterpart in an unusual structure. Chemically it is constituted of a single protein species containing 1 mol of retinal per mol protein bound as a Schiff base to a lysine residue, which comprises 75% of the membrane content (Oesterhelt and Stoeckenius, 1971). Analysis of the X-ray diffraction pattern of the membrane has shown that lipids and protein molecules are packed in a highly ordered structure consisting of an hexagonal array, each protein molecule spanning the entire thickness of the membrane (Henderson, 1975; Blaurock, 1975; Henderson and Unwin, 1975).

Early observations indicated that the purple membrane appears at the end of the exponential growth period of the cells and forms a patchwork on the cell membrane surface (Blaurock and Stoeckenius, 1971). Later it became apparent that the formation of the membrane was highly favored by low oxygen tension and light (Oesterhelt and Stoeckenius, 1973; Danon and Stoeckenius, unpublished results). This finding suggests the existence of a differentiation process initiated by a well defined set of conditions, possibly through an unknown effector which induces the cells to synthesize the membrane protein. A second possibility is that the protein pre-exists in the cell membrane and only the final assembly is induced under the appropriate set of conditions. These two possibilities are investigated in the present study.

Materials and Methods

The *Halobacterium halobium* R_1M_1 used throughout this work was a generous gift from Dr. D. Oesterhelt. This mutant was preferred to the commonly used *H. halobium* R_1 because it lacks the bacterioruberin pigment. However it is able to synthesize the purple membrane under the same conditions as the *H. halobium* R_1 mutant. The culture medium was the same as previously described (Onishi et al., 1965; Danon and Stoeckenius, 1974).

Growth Conditions: All the cultures were started from a slant (2% agar in culture medium), inoculated into a starter culture (100 ml medium in a 500 ml Erlenmayer) kept in the dark at 37° C on a reciprocal shaker at 200 rpm for 3 days. This starter was usually diluted 30-fold for the culture itself. In some experiments, the starter culture was first harvested by centrifugation at 10,000 g for 10 min and then resuspended into fresh medium or into basal salt (a solution of all the salts of the medium without the nutrients).

Dark Aerobic Cultures: These cultures were maintained in the dark as in the case of the starter, with a volume ratio medium/vessel of 1/10.

Light Aerobic Cultures: Grown in the same way as the dark aerobic cultures but illuminated from above by 3 fluorescent tubes (cool white 20 W each).

Light Anaerobic Cultures: Grown for 24–48 h in the dark aerobically, and then transferred into vessels filled to the brim and tightly covered with parafilm. These cultures were kept standing in front of 4 fluorescent tubes for different period of time. The transfer of the cells from the dark aerobic to light anaerobic growth conditions is referred to as "induction" of the cell culture.

Cell Lysis: Cells were harvested and resuspended in basal salt to which 50 μg DNAase and 5 μg RNAase were added. The suspension was dialyzed against water for several hours until complete lysis of the cells.

Spectroscopy: Absorption spectra of the cell lysates were measured with a Cary model 14 or 15. Sometimes a sucrose solution was added in order to reduce the light scattering. In most of the experiments the lysates were spun at low speed to eliminate aggregates and cell debris, and then at 50,000 g to remove the soluble proteins and partially digested polynucleotides. The membrane fragments sedimented into a pellet whose color differed, according to the growth conditions, from brown to purple.

Circular Dichroism: Measurements were performed on a Cary model 60 spectrophotometer. Quartz cells of 10 mm and 1 mm were used. Most of the measurements were carried out with a time constant of 3 min, slit width 0.3 mm and scan rate 5–10 nm/min on a scale range of 0.04 degrees.

Raman Spectra: Obtained with a Spex 1401 double monochromator equipped with an EMI S_{20} photomultiplier. A Spectra Physics argon ion laser provided the exciting light. A rotating cell (about 500 rpm) was used to eliminate photoisomerization products in the spectra. The light intensities ranged from 100–200 mW. A wavelength calibration was carried out with the aid of the plasma lines of the argon laser.

Sucrose Gradients: Fractionation of the membrane fragments was performed on linear sucrose gradients (25–50% sucrose with a 60% solution at the bottom of the tubes). A Beckman SW 27 rotor was used. The gradients were run for 20 h at 25,000 rpm. Fractions were collected by puncturing the tubes. The absorption was read at 280 nm or at 412 and 570 nm with a Zeiss spectrophotometer.

In the experiments where a radioactive amino acid was used, the fractions were counted in a Packard liquid scintillation counter using Bray's solution.

Labelling Experiments: ^{3}H- and ^{14}C-proline were used. The final concentration of the proline was 10^{-6} M (0.2 μCi/ml). ^{3}H-proline was added at the beginning of the dark aerobic growth. Before transferring the cells to the inducing conditions (light anaerobic culture) the label was chased as follows. The cells were spun at 10,000 g

for 10 min, the pellet washed once with fresh medium containing 10^{-5} M "cold" proline, resuspended in this medium and allowed to incubate on the shaker at 37° C for 1 h. The cells were then centrifuged as before, and resuspended in fresh medium either containing cold proline or ^{14}C-proline. This resuspension was induced as described above.

Acrylamide Gel Electrophoresis: This was carried out according to the method of Weber and Osborn (1969) modified by Kushwaha et al. (1975).

Total Lipid Extraction and Acetone-Soluble Lipid Extraction: Carried out according to Kushwaha et al. (1975).

Thin Layer Chromatography (TLC): Carried out on ready made plates (DC-Karten SIF, Riedel de Haen AG, Seelze-Hannover). The following solvents were used: 1% ethyl-ether in chloroform, 0.3% ethyl-ether in hexane, petrol ether-ethyl ether-acetic acid (90 : 10 : 1).

Protein Determinations: Carried out according to the Lowry method (Lowry et al., 1951).

Photophosphorylation: This was measured as described before according to the luciferin-luciferase method (Danon and Stoeckenius, 1974; Stanley and Williams, 1969).

Materials: Nicotine, chloramphenicol, cold amino acids, and luciferin-luciferase were purchased from Sigma. ^{3}H- and ^{14}C-proline were purchased from New England Nuclear.

Results and Discussion

It is useful to start with some preliminary observations on the formation of the purple membrane. Dark aerobic cultures are yellow with a tinge of orange. If such a culture is transferred to light anaerobic conditions as described above, it gradually turns purple. However, this does not occur if (1) the cells are suspended in basal salt instead of the complete medium, even if glycerol, malic acid, and ammonium chloride are added, together or separately; (2) anaerobiosis is established at once by sparging the culture with nitrogen, or by adding an inhibitor of respiration like sodium azide, or by concentrating the cells above 1.5 mg protein/ml; (3) anaerobic cells are kept in the dark, even after a few hours induction in the light (up to 4 h). These observations, which corroborate earlier studies, show that low oxygen tension and the presence of light are necessary conditions for the biosynthesis of the purple membrane.

The fact that the development of purple membrane is inhibited by immediate and complete anaerobiosis as well as by a lack of amino acids (cultures in basal salt) indicates that protein synthesis is required. Indeed, *H. halobium* is unable to grow in the absence of amino acids. Under anaerobic conditions the endogenous ATP of the cells is rapidly depleted (Danon and Stoeckenius, 1974; Danon and Caplan, 1976).

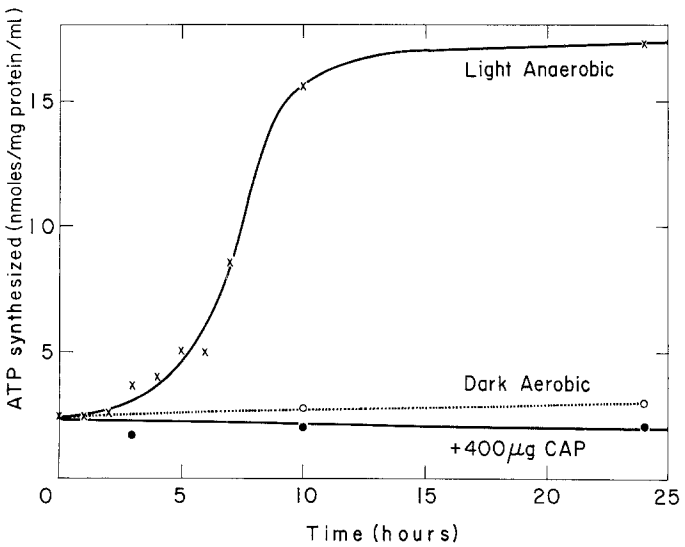


Fig. 1. Photophosphorylation as a function of time after induction. Cells (1 mg protein/ml) were grown aerobically in the dark as described under Materials and Methods, for 36 h, washed, and resuspended in fresh medium. The suspension was divided in 3 portions. To one portion 400 μg/ml chloramphenicol (CAP) were added. The CAP inhibited cells and a second portion were induced in the light as described. The last portion was kept aerobic in the dark. At times aliquots were taken and the cells were spun and washed with basal salt and kept overnight in basal salt under aerobic conditions. Photophosphorylation was determined in these samples as described elsewhere. x—x non-inhibited cells; ●—● + CAP; O---O dark aerobic cells

The purple membrane is not yet present, so photophosphorylation cannot take place. Both ATP and amino acids are necessary to ensure protein synthesis, which suggests that after exposure to the light protein synthesis must take place for at least a certain period of time if the purple membrane is to be formed.

Furthermore, in the presence of the protein synthesis inhibitor chloramphenicol (added before exposure of the cells to the light) the culture did not turn purple under the usual conditions. While the rate of the photophosphorylation following induction increased for the non-inhibited cells, indicating an increase in the amount of purple membrane, no such an increase was observed in the presence of chloramphenicol (Fig. 1). Chloramphenicol added to cells which already contained purple membrane did not inhibit photophosphorylation.

Although protein synthesis is apparently necessary for the development of the purple membrane, this does not exclude the possibility that the purple membrane protein may pre-exist in the cell membrane in a differently organized form. Protein synthesis might be required for the final assembly into the active photoreceptor. This process probably involves the biosynthesis of the chromophore itself.

Labelling Experiments

The following two sets of experiments were carried out in order to decide between the two possibilities: (a) the purple membrane protein is already present before induction, or (b) it is synthesized *de novo* upon induction.

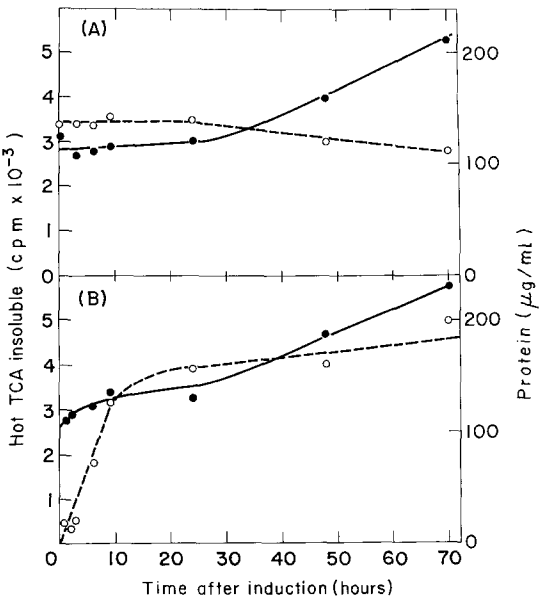


Fig. 2. Incorporation of labelled proline into proteins. (**A**) ^{3}H-proline added during the dark aerobic growth; (**B**) ^{3}H-proline added at the beginning of induction. In both cases 0.2 μCi/ml proline was added (10^{-6} M). 1 ml aliquots of the cell suspension were taken at intervals after induction and 1 ml 10% TCA solution added. The precipitated proteins were heated for 15 min at 90° C. Hot TCA insoluble counts are plotted as a function of time after induction. Total proteins were determined on another aliquot by the Lowry method. ●—● total protein; ○- - -○ hot TCA insoluble counts

(1) ^{3}H-proline was added prior to induction, during the dark aerobic growth (Materials and Methods).

(2) ^{3}H-proline was added at the beginning of the induction.

In both cases samples were taken at intervals and aliquots reserved to determine the amount of proline incorporated into proteins as well as the total amount of protein (Fig. 2). The remaining samples were lysed and fractionated on sucrose gradients. In the experiment described in Figure 3 the whole cell lysate was placed on the gradient and most of the radioactivity was accounted for by the soluble proteins remaining at the top. In Figures 3A and B the label was chased before the induction as described under Materials and Methods. At time 0 after the chase, but before induction, essentially one band was present around the middle of the gradient. This band migrated with time after induction toward the heavier regions of the gradient where the purple membrane band appears (fractions 2 and 3). While the relative amount of radioactivity remained nearly constant during the migration, the absorbance at 280 nm increased with time. This was particularly noticeable after 48 h incubation in the light (Fig. 3B). Cells treated in the same way but kept in the dark under either aerobic or anaerobic conditions also showed one middle labelled band, however here the migration was limited (Fig. 3B). When the label was added at the beginning of the induction, the rate of incorporation into protein was constant for at least 10 h (Fig. 2B) and then levelled off. The middle band in the gradient was labelled within the first hour after induction. Both label and absorbance at 280 nm (A280) migrated as before but now the relative amount of label increased as well as A280 (Fig. 3). The peaks finally reached the position of the purple membrane.

The above experiment shows two main points. Before the appearance of the purple membrane in cells grown aerobically in the dark, a labelled fraction can be separated on a sucrose gradient which bands at about 36% sucrose. This indicates a specific gravity too high for the fraction to be composed of isolated proteins or small

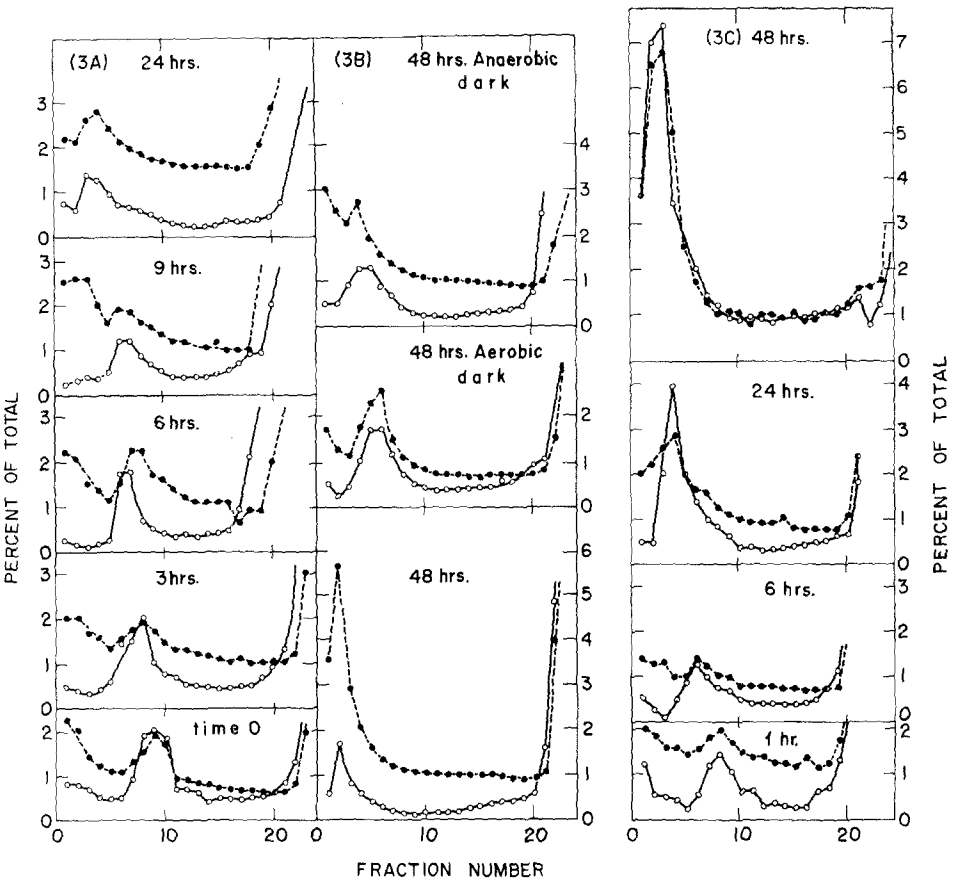


Fig. 3. Fractionation of labelled proteins on sucrose gradients. In **3A** and **3B**, ^{3}H-proline was added during the dark aerobic growth, in **3C**, ^{3}H-proline was added at the beginning of the induction. Results are expressed in percent of the total absorbance at 280 nm or counts/min. ●---● A280; ○—○ cpm. Time 0: fractionation just prior to induction

membrane fragments; ribosomal particles are also unlikely since the ribosomes of extreme halophiles are destroyed at low salt concentration (Bayley and Kushner, 1964), and moreover cell lysis was performed in the presence of RNAase. The protein moiety of this fraction continues to be synthesized after induction and seems to be transferred to the purple membrane band in a gradual process during which its specific gravity increases (as can be seen from the migration of the band).

The experiment was repeated using ^{3}H-proline during dark aerobic growth and ^{14}C-proline after chasing the ^{3}H-proline and transferring the cells to induction conditions. Similar results were obtained: the middle band was labelled with ^{3}H-proline and after induction with ^{14}C-proline. Both labels migrated toward the heavier region where the purple membrane band appears. Although these results indicate that purple membrane protein is probably present in some membranous form before the functional membrane appears, they do not indicate whether the structure to which it is associated is a precursor form or simply smaller fragments of the purple mem-

brane itself. So it seemed appropriate to follow the biogenesis of the bacteriorhodopsin using its spectral properties.

Biosynthesis of the Bacteriorhodopsin as a Function of Time after Induction

Figure 4 shows the spectra of the lysates of cells grown aerobically in the light or in the dark and of aliquots of these cells transferred to light anaerobic conditions 36 h after the beginning of the aerobic growth. The absorption at 570 nm was very strong and characteristic in the anaerobic samples, low and uncharacteristic in the aerobic ones. In the latter, the maximum absorption was at 412 nm. The overall slope of these spectra is largely an effect of light scattering. It is evident that the dark aerobic cells contain predominantly a species absorbing at 412 nm while the light anaerobic cells contain the 570 nm pigment. The spectrum of the dark aerobic cells at 36 h (not shown) was similar to the one at 96 h.

Cells induced for different times were lysed and fractionated on sucrose gradients as described before. Differently colored bands were distinguished during the first 24 h after induction, from yellow at the top to orange brown, reddish brown, and finally purple. About 9 h after induction a light purple band is distinguished below the reddish brown one. After 48 h only one deep purple band appears at the lower part of the gradient. The timing of these transformations differed somewhat from one experiment to another but the overall process was always the same. The

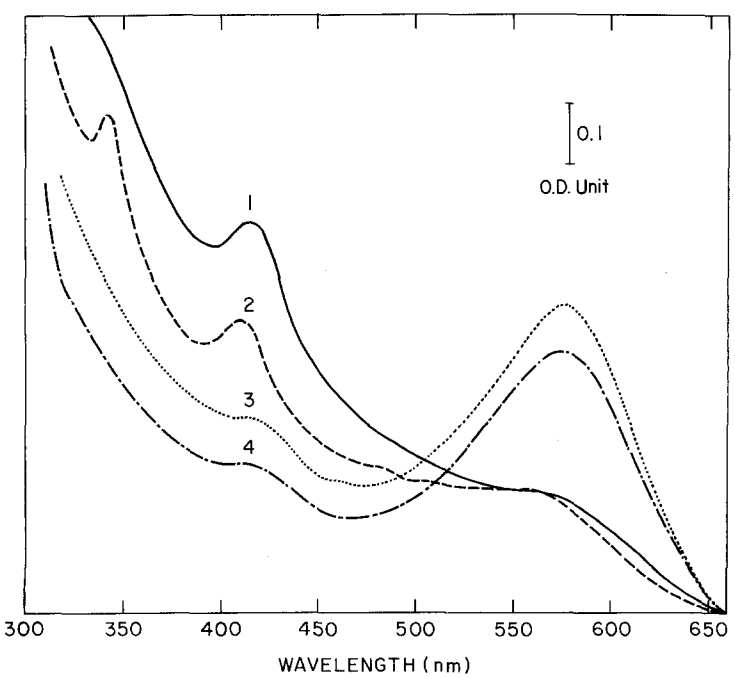


Fig. 4. Spectra of cell lysates: 96 h dark aerobic (1); 96 h light aerobic (2); 36 h dark aerobic, 96 h light anaerobic (3); 36 h light aerobic, 96 h light anaerobic (4)

optical densities at 412 nm and 570 nm of such lysate sucrose gradient fractions were measured. The results obtained at time 0 and after 4, 9, and 48 h induction are shown in Figure 5, together with the dark control. The absorption at 412 nm is predominant during the first hours after induction and in the dark aerobic cells. The absorption at 570 nm increases with time and, as we have seen (in the experiments with labelled amino acid), the band migrates toward the heavier regions of the gradient. The species absorbing at 412 nm (P412) was isolated and purified by repeated fractionation on sucrose gradients. The spectrum of the P412 is shown in Figure 6 together with the spectrum of the purified purple membrane (P570). Preliminary electron microscopic studies have shown that the P412 has a membranous structure apparently different from the purple patches (Danon and Niedermayer, in preparation). The difference spectrum of the reduced versus the oxidised form of the P412 showed very little or no difference; on the other hand, lighter fractions isolated on the sucrose gradient with an absorption band situated at about 415 nm showed,

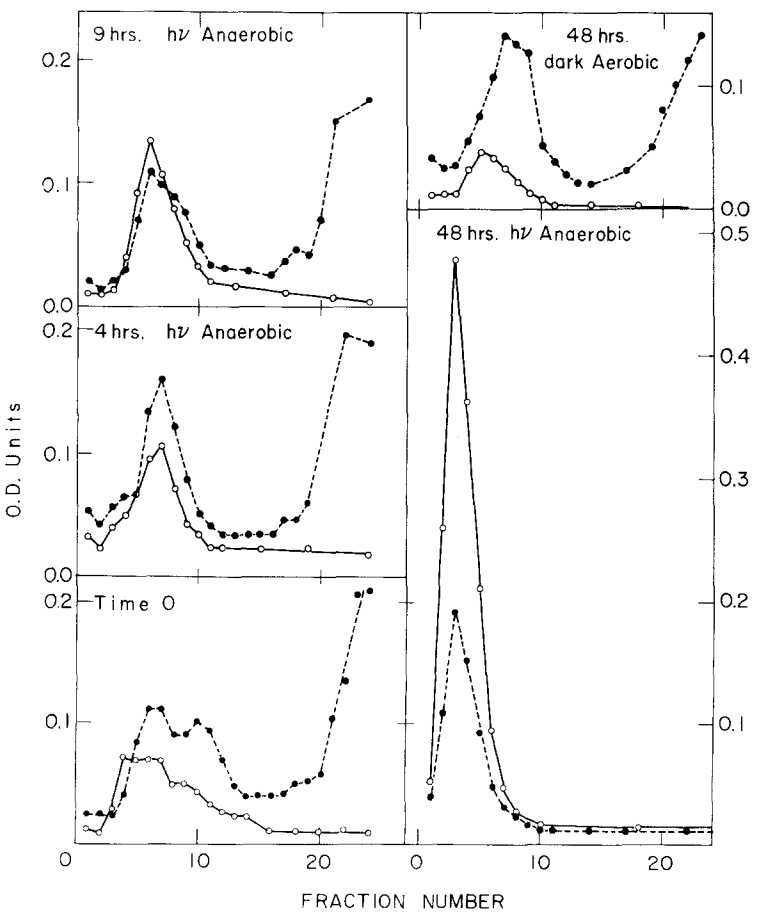


Fig. 5. Fractionation on sucrose gradients of lysates of cells induced for different times; optical densities at 412 nm and 570 nm. ●—●—● A412, O—O A570

under the same conditions, a large difference. The difference spectrum is taken to indicate the presence of cytochromes.

Centrifugation of the P412 over a discontinuous sucrose gradient showed that it bands between 37 and 38% sucrose, which indicates a specific gravity between 1.161 and 1.166. Under the same conditions the purple membrane bands at 42% sucrose (specific gravity 1.187). Analysis of the total lipid content (by weight) and protein content (Lowry method) indicated a composition of 36% lipids and 64% protein. The purple membrane analyzed in the same way gave 74% protein, a value very close to the one given by Stoeckenius and Oesterhelt (1971). Acrylamide gel electrophoresis showed only one band migrating at the same rate as the one obtained from the purple membrane, whether they were run together or in parallel. Table 1 shows the amino acid composition of the P412 compared to the P570, and indicates a strong similarity between the two. Comparison of the CD spectrum in the UV range of the P412 and the P570 indicates that the configurations of the proteins are similar. These data suggest strongly that the P412 is a precursor form of the functional P570.

The CD spectrum in the visible range was also investigated and is shown in Figure 7. In the region of absorption of the P412 there is a CD band composed of a negative and a positive component. At maximum absorption the ellipticity is 0. At longer wavelengths it is negative, at shorter positive. The positive part is broader and more intense. This CD spectrum resembles in shape that which was observed for the purple membrane photointermediate at 412 nm obtained under photostationary conditions at low temperature in a water/glycerol mixture (Heyn et al., 1975). Though the bandwidths of the absorption and CD bands of the P412 are narrower than that of the 412 nm photointermediate the resemblance in shape and position of the bands

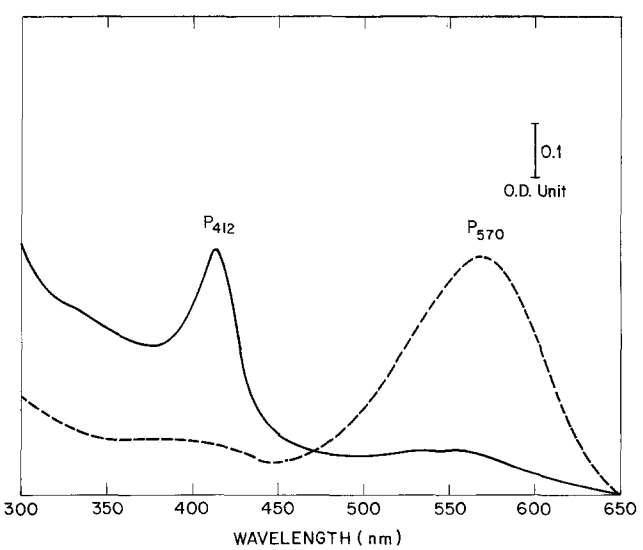


Fig. 6. Spectra of the purple membrane (P570) and of the species absorbing at 412 nm (P412). Both were purified by centrifugation through two successive sucrose gradients. After the first sucrose gradient, the bands corresponding to the P412 and P570 were dialyzed, spun at 50,000 g for 1 h, and resuspended in water

Table 1. Amino acid composition of the species absorbing at 412 nm (P412) and of the purple membrane (P570) of *Halobacterium halobium*. Tryptophane and ½ cystine were not determined. The results are the average of four determinations on two different preparations

	P412	P570
	mol/100 mol	
Lysine	2.8	3.1
Histidine	0.5	0.5
Arginine	3.4	2.8
Aspartic acid	7.8	7.6
Threonine	8.1	8.7
Serine	6.5	6.2
Glutamic acid	8.2	8.5
Proline	5.3	4.8
Glycine	12.0	11.7
Alanine	15.0	13.7
Valine	7.5	7.1
Methionine	1.7	1.7
Isoleucine	4.5	4.6
Leucine	12.0	12.2
Tyrosine	1.7	1.8
Phenylalanine	4.0	4.3

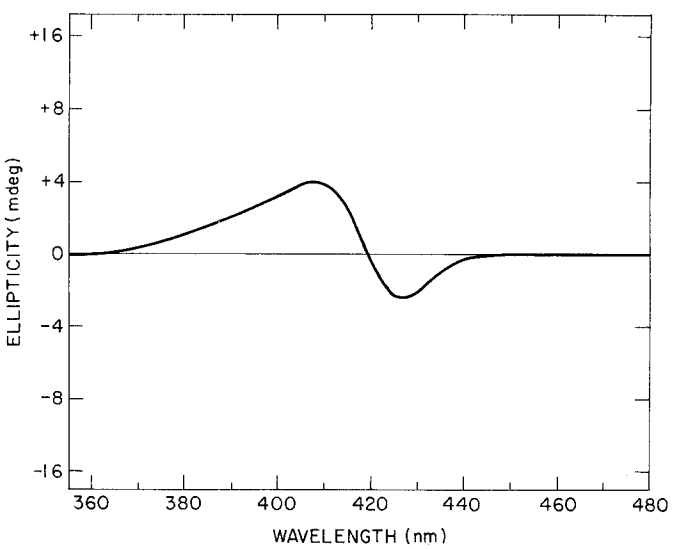


Fig. 7. CD spectrum of the P412 in the visible and near UV range. The P412 was purified as described before and resuspended in 30% sucrose in order to reduce the light scattering

and the fact that the absorption could not be attributed solely to cytochrome led us to look for the presence in the precursor of a retinal form bound to the protein.

We have attempted to isolate the pigment of the P412. The lipids were extracted according to the method of Bligh and Dyer, modified by Kushwaha (1975), either directly or after treatment with cetyltrimethylammoniumbromide (CTAB) at pH 8, followed by hydrolysis with acetic acid (Oesterhelt et al., 1973). Contrary to the

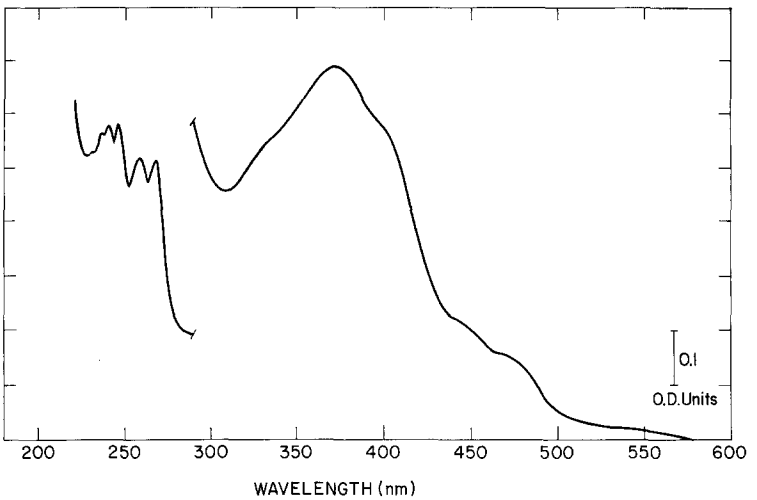


Fig. 8. Absorption of the acetone-soluble lipids of the P412 in hexane

effect of CTAB on the purple membrane, no important bleaching was observed here, only a shift from 412 nm to 405 nm. The absorption spectrum of the acetone-soluble lipids (Kushwaha, 1975) is shown in Figure 8. It is composed of a maximum absorption at 365 nm with shoulders at 450 and 480 nm. The latter may be attributed to β-carotene; traces of this were found by TLC. Furthermore, resonance Raman spectroscopy of the purified P412 showed a typical spectrum for β-carotene (Rimai et al., 1970; Lewis et al., in press) (Fig. 9). (The resonance Raman spectrum was excited with the Ar^+ line at 488 nm, which falls in the vicinity of the maximum absorption of β-carotene.) The main band in the lipid extract absorption spectrum, centered at 365 nm, may be an overlap of several species, but its main constituent is probably all-trans retinal which was detected by TLC. The finely structured bands between 280 and 220 nm (Fig. 6) are probably due to colorless carotenoid compounds which have not yet been identified, but migrate on TLC with the same R_f as squalene, dihydrosqualene, and tetrahydrosqualene as described by Tornabene (1969).

Since β-carotene is known to be a direct precursor of retinal (Singh et al., 1972) we attempted to confirm its presence. The yellow band found at the top of the same gradient of a 48 h dark aerobic cell lysate was isolated (see spectrum in Fig. 10). TLC of the acetone-soluble lipids from this band revealed two spots, one orange-red and one yellow, migrating respectively like lycopene and β-carotene markers in all the solvents used. A direct demonstration of the cyclization of lycopene to β-carotene has been given in chloroplasts and in partially purified green tissues from higher plants (Goodwin, 1972). In *Flavobacterium sp.* Goodwin demonstrated that the cyclization of lycopene to β-carotene requires anaerobic conditions (Goodwin, 1972). In our case β-carotene was found in aerobic cells. In order to see whether lycopene is here too a precursor of the β-carotene, we have used nicotine which is a known inhibitor of the cyclization of lycopene (Howes, 1970; Goodwin, 1972). *H. halobium* was grown in the presence of 5 mM nicotine; the growth of the cells was not considerably impaired. These cells were unable to produce the purple membrane under the

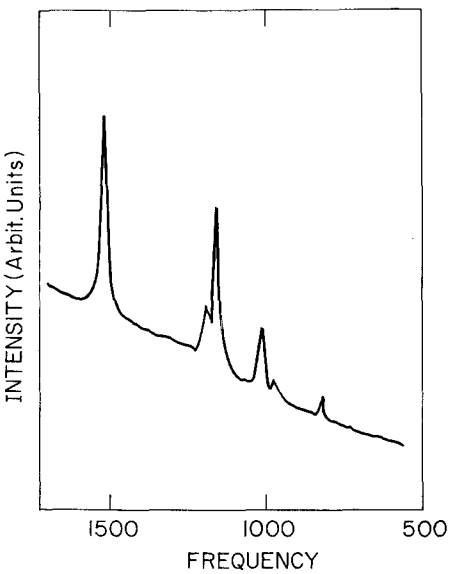
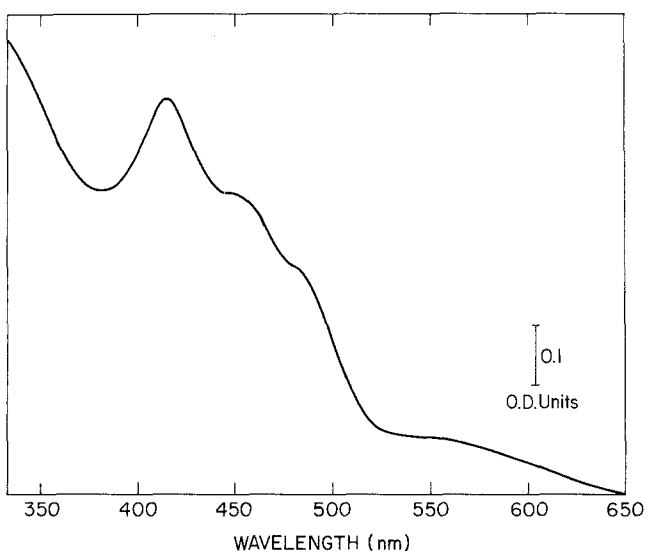


Fig. 9. Resonance Raman spectrum of the purified P412, resuspended in water and excited with the 488 nm line of the Ar^+ ion laser



Fib. 10. Absorption spectrum of the yellow band found at the top of the sucrose gradient of a dark aerobic cell lysate

usual conditions. If the drug was washed out before induction, the purple membrane formed as in the control experiment. Addition of nicotine after the dark aerobic growth, just before induction, was also inhibitory. The spectrum of a nicotine inhibited culture (48 h dark aerobic) is shown in Figure 11. Relatively smaller amounts of the P412 are present as compared to the amount present in the control cells. Diffuse bands at 563 nm, 520 nm, 485 nm, and a sharp and intense peak at 345 nm, were the representative features of this spectrum. Fractionation of the lysate on a

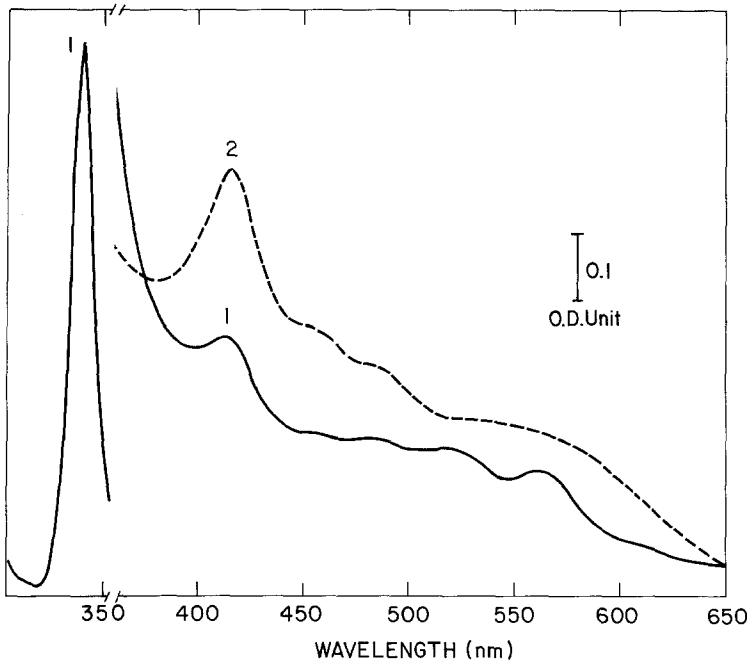


Fig. 11. Absorption spectrum of the lysate of cells grown aerobically in the dark: (1) in the presence of 5 mM nicotine; (2) control without nicotine. In both samples the lysates were diluted 10 fold with 50% sucrose

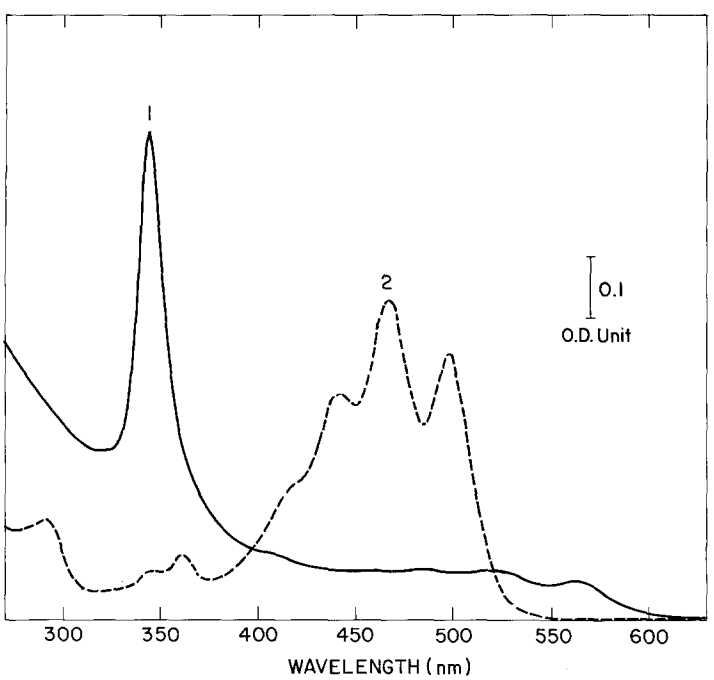


Fig. 12. Absorption spectrum of the red ring found at the top of the sucrose gradient. Fractionation of the lysate of cells grown in the presence of nicotine. The fraction was dialyzed and resuspended in water (1); the spectrum of the lipid extract in hexane of this fraction is also given (2)

sucrose gradient yielded 3 bands. The two lower brownish bands contained the P412 and small amounts of the species absorbing at 345 nm. At the top of the gradient, a bright red ring was present whose spectrum is shown in Figure 12; it contains almost exclusively the 345 nm absorbing species. The lipid extract of this band is also shown in Figure 12; it corresponds closely to the spectrum of "all-trans" lycopene. The 345 nm absorption completely disappeared after extraction of the lipids; it was not found in the water phase either. It is to be noted that a similar absorption was observed sometimes in the lysate of light aerobic cultures (Fig. 4). The CD spectrum of the P345 has an exciton or split band type of structure as found in the case of P570 and P412 with zero ellipticity at the maximum absorption. Contrary to what was observed in the case of the P412 and P570 the ellipticity is positive in the longer wavelength part and negative in the shorter. The dichroism of this band is large. The presence of a lycopene-containing membrane absorbing at 340 nm was also reported by Oesterhelt (1972).

Conclusions

Investigation of the purple membrane biosynthesis has led to the finding of a precursor form which we have tentatively called P412 because of its strong absorption at 412 nm. The P412 present in dark aerobic cells is apparently a membranous structure, already differentiated from the rest of the cell membrane. The results presented here show that the protein of the purple membrane (or a closely related form of it) is also the sole protein in the precursor. Indeed it was shown that the P412 protein labelled during dark aerobic growth appears in the purple membrane fraction after the cells are transferred to light and aerobic growth conditions. Acrylamide gel electrophoresis of the P412 revealed only one band corresponding to the one obtained from the purple membrane. The amino-acid compositions of the P412 and of the purple membrane are very similar (Table 1) and the CD spectra in the UV range indicated that the protein configurations are also similar.

The conditions under which the purple membrane is preferentially formed, that is under low oxygen tension in the presence of light, suggested that it is an inducible process. It requires protein synthesis, as shown by the absence of purple membrane formation when protein synthesis was inhibited either directly by chloramphenicol or indirectly by preventing ATP synthesis. The observation that the P412 is not transformed into purple membrane when protein synthesis is inhibited, in spite of the fact that the purple membrane protein pre-exists in the P412, suggest that an inducible enzyme is required for the final assembly. The formation of the chromophore of the P570 is probably connected with this assembly.

The nature of the chromophore of the P412 has not yet been elucidated. Analysis of the acetone-soluble lipids of the P412 has shown the presence of both retinal and β-carotene together with colorless carotenoid compounds. No quantitative data are available yet and the interaction of these carotenoids with the protein is not clear. The chromophore which confers on the purple membrane its color and function is clearly distinct from the chromophore in the precursor form. The accumulation of lycopene in cells inhibited by nicotine, and the absence of purple membrane formation in these cells, indicates that the carotene probably arises from the cyclization of

lycopene (and the retinal from the splitting of β-carotene) as was shown in other organisms.

The presence of small amounts of purple membrane in dark aerobic cells suggests that the system cannot be completely repressed in the mutant. However, it is only under low oxygen tension and in the light that large quantities of purple membrane are synthesized. It seems that the biosynthesis of the purple membrane involves the transformation of an already differentiated area on the cell membrane. This requires assembly of the chromophore rather than completely *de novo* synthesis of the protein.

After this manuscript was submitted for publication, we became aware of a paper by Sumper et al. (1976) on the biosynthesis of the purple membrane. These authors also found that the purple membrane protein pre-exists in the cells before the appearance of the purple patches. In their work it is associated with a brown membrane whose spectrum displays two main bands: one at 560 nm attributed to bacteriorhodopsin, and one at 419 nm which they showed is due to a b-type cytochrome. In our case, the purple membrane protein was associated with a membrane structure whose main absorption band appeared to be centered at 412 nm. Little absorption was present at 560 nm, and the reduced versus oxidised difference spectrum showed only traces of a Soret band which could not account for the whole absorption at 412 nm. On the other hand, lighter fractions isolated on a sucrose gradient showed a strong absorption band at 420 nm after reduction. It seems that in our case the P412 chromophore cannot be solely attributed to the presence of cytochrome, but must be due to some form of retinal and/or other carotenoid compound.

It is noteworthy that treatment of the P412 with CTAB at pH 8 results in a shift of absorption from 412 nm to 405 nm. This may be an indication that this species is more stable than the purple membrane, which is considerably bleached by such treatment. Further extraction of the chromophore with chloroform-methanol followed by acetone indicated the presence of retinal (Fig. 8).

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References

Bayley, S. T., Kushner, D. J.: The ribosomes of the extreme halophilic bacterium *Halobacterium halobium*. J. molec. Biol. **9**, 654–669 (1964)

Blaurock, A. E.: Bacteriorhodopsin. A trans-membrane pump containing α-helix. J. molec. Biol. **93**, 139–148 (1975)

Blaurock, A. E., Stoeckenius, W.: Structure of the purple membrane. Nature (Lond.) New Biol. **233**, 152–154 (1971)

Danon, A., Stoeckenius, W.: Photophosphorylation in *Halobacterium halobium*. Proc. nat. Acad. Sci. (Wash.) **71**, 1234–1238 (1974)

Danon, A., Caplan, S. R.: Stimulation of ATP synthesis in *Halobacterium halobium*. Biochim. biophys. Acta (Amst.) **423**, 133–140 (1976)

Goodwin, T. W.: Recent developments in the biosynthesis of carotenoids. Biochemical Society Symposia, vol. 35. Current trends in the biochemistry of lipids, pp. 210–231. New York: Academic Press 1972

Henderson, R.: The structure of the purple membrane from *Halobacterium halobium*. Analysis of the X-ray diffraction pattern. J. molec. Biol. **93**, 123–138 (1975)

Henderson, R., Unwin, P. N. T.: A three dimensional model of purple membrane obtained by electron microscopy. Nature (Lond.) **257**, 28–32 (1975)

Heyn, M. P., Bauer, P. J., Dencher, N. A.: A natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. Biochem. biophys. Res. Commun. **67**, 897–903 (1975)

Howes, C. D., Batra, P. P.: Accumulation of lycopene and inhibition of cyclic carotenoids in myobacterium in the presence of nicotine. Biochim. biophys. Acta (Amst.) **222**, 174–179 (1970)

Kushwaha, S. C., Kates, M., Martin, W. G.: Characterization and composition of the purple red membrane of *Halobacterium cutirubrum*. Canad. J. Biochem. **53**, 284–292 (1975)

Lewis, L., Smith, C. D., Kim, M.: Resonance Raman spectroscopic evidence that the pigment responsible for the phototransduction in the Aplysia R-2 neuron is β-carotene. Nature (Lond.) (in press)

Lowry, B. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: J. biol. Chem. **193**, 265–275 (1951)

Oesterhelt, D., Stoeckenius, W.: Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nature (Lond.) New Biol. **233**, 149–152 (1971)

Oesterhelt, D., Stoeckenius, W.: Functions of a new photoreceptor membrane. Proc. nat. Acad. Sci. (Wash.) **70**, 2853–2857 (1973)

Oesterhelt, D., Meentzen, M., Schuhmann, L.: Reversible dissociation of the purple complex in bacteriorhodopsin and identification of 13-cis and all-trans retinal as its chromophore. Europ. J. Biochem. **40**, 453-463 (1973)

Oesterhelt, D.: Isolation of a lycopene containing membrane with unusual spectroscopic properties from *Halobacterium halobium*. Abstr. Comm. Meeting Fed. Europ. Biochem. Soc. **8**, Abstr. No. 125 (1972)

Onishi, H., McChance, M. E., Gibbons, N. E.: A synthetic medium for extremely halophilic bacteria. Canad. J. Microbiol. **11**, 365–373 (1965)

Rimai, L., Kilponen, R. G., Gill, D.: Excitation profiles and laser Raman spectra in the resonance region of two carotenoid pigments in solution. J. Amer. chem. Soc. **92**, 3824–3825 (1970)

Singh, H., Mallia, A. K., Cama, H. R.: Chemistry and metabolism of apocarotenals and their epoxides. Biochemical Society Symposia, vol. 35. Current trends in the biochemistry of lipids, pp. 210–231. New York: Academic Press 1972

Stanley, P. E., Williams, S. G.: Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the Luciferase enzyme. Analyt. Biochem. **29**, 381–392 (1969)

Stoeckenius, W., Rowen, R.: A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. J. Cell Biol. **344**, 365–393 (1967)

Sumper, M., Reitmaier, H., Oesterhelt, D.: Biosynthesis of the purple membrane of halobacteria. Angew. Chem. Int. Ed. Engl. **15**, 187–194 (1976)

Tornabene, T. G., Kates, M., Gelpe, E., Oro, J.: Occurrence of squalene, di- and tetrahydrosqualenes and vitamins MK_8 in an extremely halophilic bacterium, *Halobacterium cutirubrum*. J. Lipid Res. **10**, 294–303 (1969)

Weber, K., Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. biol. Chem. **244**, 4406–4412 (1969)

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