

Biosynthesis of the Purple Membrane of Halobacteria

By Manfred Sumper, Heribert Reitmeier, and Dieter Oesterhelt^[*]

Dedicated to Professor Feodor Lynen on the occasion of his 65th birthday

Halobacteria are extremely specialized organisms. They live exclusively in saturated solutions of common salt. The cell membrane of these bacteria exhibit insular regions which can be isolated by membrane fractionation. These regions consist of a lipid matrix containing bacteriorhodopsin molecules in a hexagonal crystalline arrangement. Bacteriorhodopsin is a deep purple retinal-protein complex ("purple membrane"). The purple membrane functions as a light energy converter.—How can such a differentiated membrane region arise? *In vivo* studies on the biosynthesis of the purple membrane showed another cell membrane fraction, the so-called brown membrane, to be a biosynthetic precursor. Bacterioopsin (the retinal-free protein) is initially incorporated into the brown membrane and can only form the purple membrane by crystallization in an energy-dependent reaction after prior reaction with retinal. This reaction is reversible. Removal of the retinal by formation of retinal oxime causes the purple membrane regions to disappear. Reconstitution of the bacteriorhodopsin by addition of retinal regenerates the purple membrane.

1. Introduction

During the past decade, studies on biological membrane systems have led to a generally accepted model of membrane structure known as the fluid mosaic model^[1]. In essence, this model describes a membrane as a solution of oriented protein molecules in a two-dimensional lipid matrix. Such a concept allows for essentially free diffusion of both lipid molecules and membrane proteins in the plane of the membrane.

This normal case contrasts with special cases in which the membrane protein forms a two-dimensional crystalline network. Such a case is encountered in the cell membrane of halobacteria where the retinal-protein complex bacteriorhodopsin forms a crystal lattice detectable in the intact cell^[2,3]. These crystalline regions can be liberated by membrane fragmentation and isolated. Owing to their intense color these membrane fragments are termed the purple membrane^[4-6]. In contrast to the visual pigments (*e.g.* rhodopsin) which act as light sensors, bacteriorhodopsin functions as a light energy

converter^[7]. Absorption of light triggers a photochemical cycle, to which is coupled the transport of a proton through the membrane. The resulting electrochemical gradient of the proton is utilized for ATP synthesis in the cell (reviewed in ref. [8]).

The composition and structure of the purple membrane were found to be unusually simple. Knowledge of both the function and the structure makes the purple membrane a suitable model for studies on the biosynthesis of a membrane and its regulation.

2. Regulation Phenomena in the Synthesis of the Purple Membrane

2.1. Chemical Composition of the Purple Membrane

Isolated purple membrane consists of *ca.* 75% (pigment-containing) protein and 25% lipid, corresponding to about ten

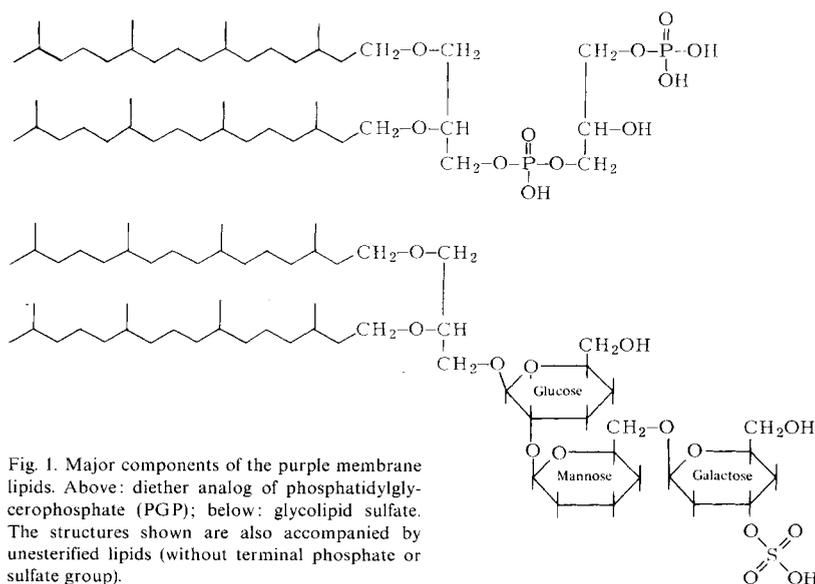


Fig. 1. Major components of the purple membrane lipids. Above: diether analog of phosphatidylglycerophosphate (PGP); below: glycolipid sulfate. The structures shown are also accompanied by unesterified lipids (without terminal phosphate or sulfate group).

[*] M. Sumper, H. Reitmaier, and D. Oesterhelt
Institut für Biochemie der Universität
Röntgenring 11, 8700 Würzburg (Germany)

lipid molecules for every protein molecule. The sole protein species present is bacteriorhodopsin, a protein molecule having a molecular weight of about 26000 daltons and containing one retinal molecule^[21]. The retinal-free protein is called bacterioopsin. More than 50% of the lipids comprise a diether analog of phosphatidylglycerophosphate, and a further 30% two glycolipids (Fig. 1). A special feature of halobacterial lipids is the absence of fatty acids; the invariable side chain of the isoprenoid alcohol dihydrophytol bound by an ether-type linkage appears in their place^[91].

2.2. Influence of Oxygen and Light on Purple Membrane Synthesis

Halobacteria whose growth is not limited by restricted oxygen supply do not synthesize the purple membrane^[20]. Falling oxygen partial pressure in the medium triggers the synthesis of the purple membrane, directly or indirectly. Thus, since this membrane does not belong to the constitutive equipment of the bacteria but can be utilized as a light energy

converter, its function clearly appears to be that of an "emergency power generator" for the cell. When the rate of ATP synthesis *via* oxidative phosphorylation becomes inadequate the purple membrane system is brought into operation. Light does not have a similar effect on purple membrane synthesis (cf. Section 3.2).

Studies on this inducible membrane synthesis must have the ultimate aim of elucidating the time-based control of synthesis of all membrane components, *i.e.* of pigment (retinal), protein (bacterioopsin), and lipid, and in particular of answering the question of the correlation of the three synthetic pathways.

3. Synthesis of the Components of the Purple Membrane

3.1. Carotenoid Synthesis in Halobacteria

Formation of bacteriorhodopsin and thus of purple membrane is necessarily dependent on the presence of vitamin

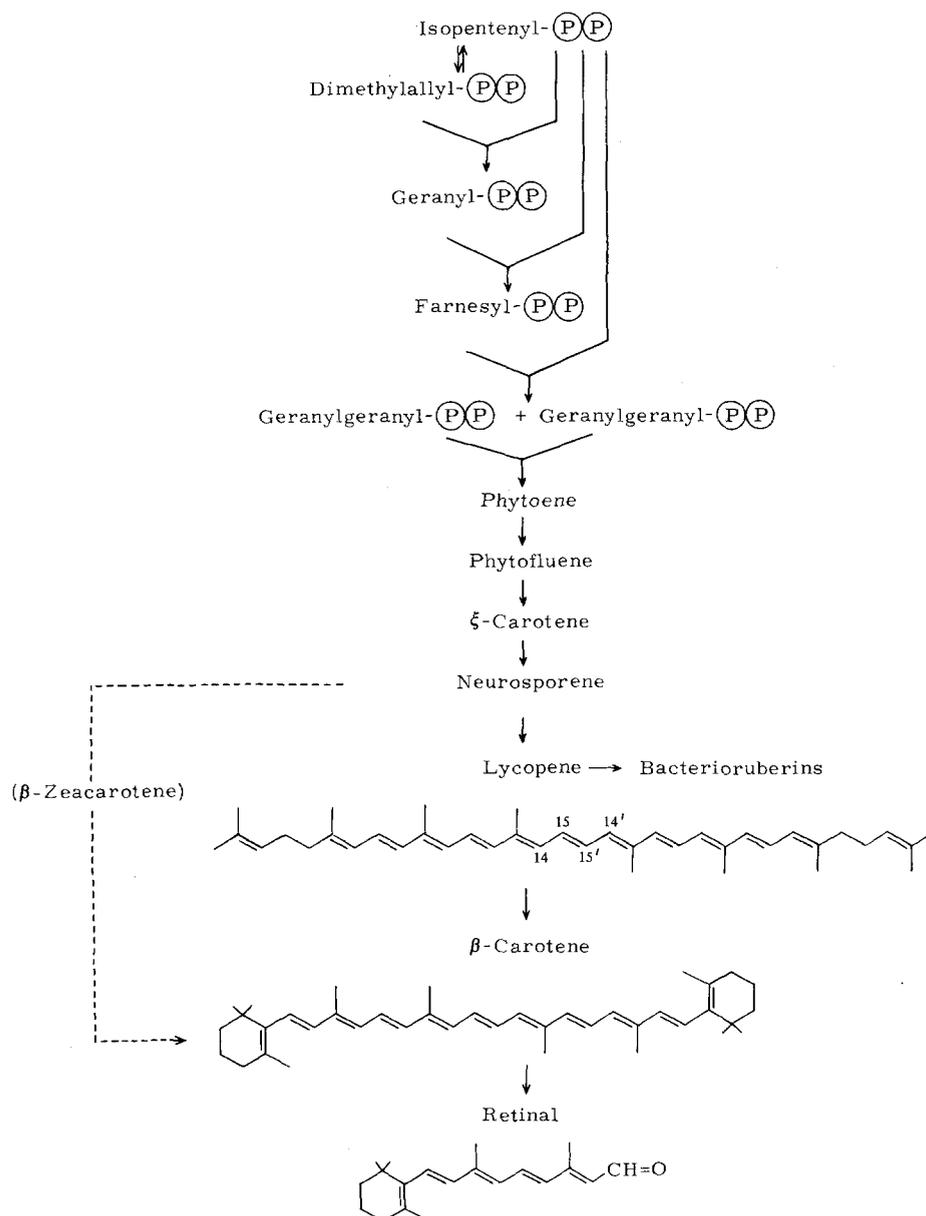


Fig. 2. Schematic representation of carotenoid biosynthesis. PP = pyrophosphate.

A aldehyde (retinal). In animals, retinal is formed by oxidative cleavage of the central double bond of β -carotene. β -Carotene itself is formed in plant systems from isopentenyl pyrophosphate *via* the pathway sketched out in Figure 2 and consumed by animals as provitamin A^[10-15].

The two syntheses mentioned above are most probably also involved in *de novo* retinal synthesis in halobacteria, as shown by numerous recent experiments. On the one hand, the intermediates postulated in Figure 2 could be directly detected in some cases, and on the other the synthesis of β -carotene from isopentenyl pyrophosphate has recently been demonstrated in an *in vitro* system from *H. cutirubrum*^[16, 17]. A special feature of carotenoid synthesis in halobacteria is the appearance of C₅₀-carotenoids, particularly bacterioruberins, as major products. They are formed by linkage of two C₅ isoprene building blocks to the ends of a C₄₀-carotenoid^[18]. It remains an open question whether lycopene is a joint intermediate of β -carotene (retinal) and bacterioruberin synthesis. A conceivable alternative route is the pathway leading to β -carotene *via* β -zeacarotene as formulated in Figure 2^[19].

3.2. Influence of Oxygen and Light on the Pigment Synthesis

In order to minimize interference in the analysis of biosynthetic precursors of retinal, all studies were performed with a mutant of *H. halobium* which does not synthesize bacterioruberins (*H. halobium* R₁M₁). Figure 3 compares the spectra of wild-type bacterial cells and the derived mutant cells R₁M₁. While the absorption bands of the bacterioruberins mask those of all other components in the wild-type cells, the individual absorption bands of bacteriorhodopsin, cytochromes, and lycopene can be recognized in the case of R₁M₁ cells.

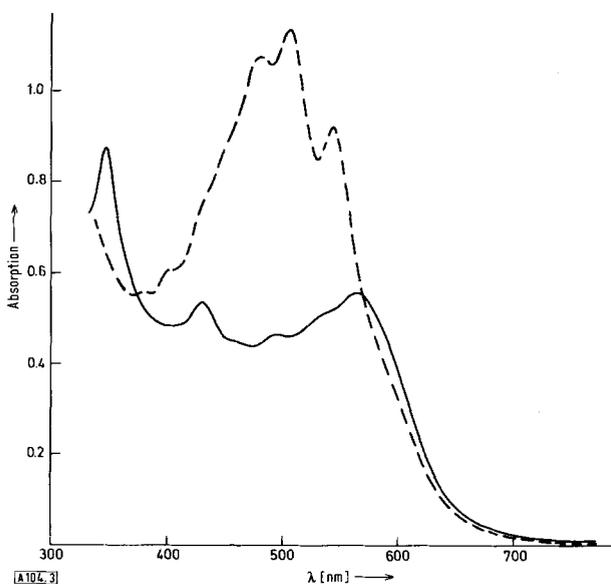


Fig. 3. Absorption spectra of halobacteria cells: wild type cells (----) and R₁M₁ mutants (—).

Determination of the pigments lycopene, β -carotene, and retinal was accomplished by spectroscopy after acetone extraction of cell lysates. The retinal bound in bacteriorhodopsin is liberated under these conditions and can therefore be determined by the specific color reaction due to *Futterman*^[21].

Figure 4 shows the change of concentration of the individual pigments with time in a growing halobacteria culture. The pigment pattern for adequate oxygen supply is shown in Figure 4a, and that for insufficient aeration is seen in Figure 4b. Cells having a sufficient supply of oxygen attain only 10 to 20% of the normal retinal content. The occurrence of retinal coincides with the appearance of the purple membrane in the cells. Optimal synthesis of retinal leads to low concentrations of lycopene, and conversely a low retinal concentration results in a high lycopene level.

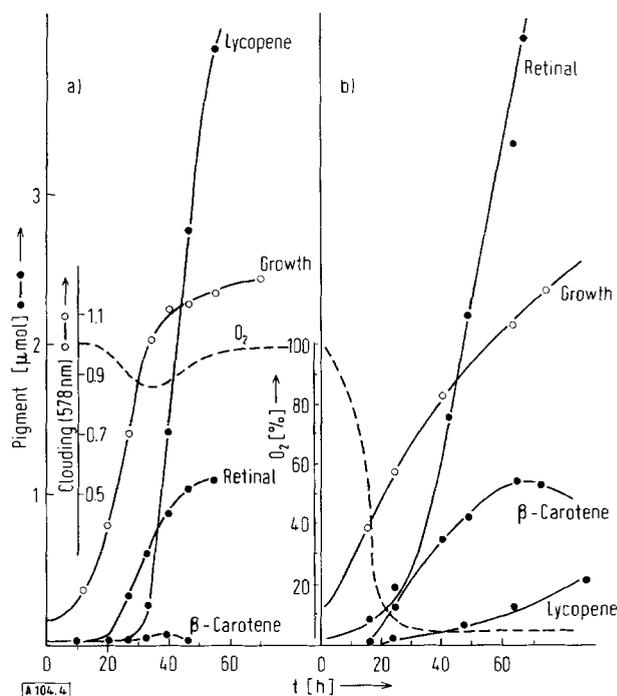


Fig. 4. Carotenoid synthesis in halobacteria under various growth conditions: a, optimal aeration; b, growth-limiting aeration.

The cells were cultured in a 14-liter Magnaferm (New Brunswick) fermenter on a complex medium (250 g NaCl, 20 g MgSO₄ · 7 H₂O, 2 g KCl, 3 g trisodium citrate · 2 H₂O, and 10 g Oxoid Pepton L 37 per liter) with illumination

rpm; aeration in case b, 120 liter/h, 300 rpm.

Samples (100 ml) were taken at the times given. The pigments were extracted from the centrifuged cells with acetone/H₂O (9:1) and, after transferring to light petroleum (boiling range 50 to 70°C), applied to an alumina column (16 g, Activity Grade II-III). Elution of β -carotene: 60 ml 20% ether in light petroleum. Elution of retinal: further 140 ml of the same mixture. Elution of lycopene: 40 ml ether. β -Carotene and lycopene were determined by spectroscopy in hexane solution, and retinal according to ref. [21]. The amounts of the pigments are given for 10 l of cell culture.

In order to examine the influence of light, the experiment depicted in Figure 4 was repeated with dark cultures. The same qualitative result was obtained; however, the β -carotene concentration is about five-fold higher. Part of the β -carotene may be destroyed on illumination; there is no compelling reason to assume intervention of a regulatory phenomenon between the synthesis of β -carotene and that of retinal.

3.3. Synthesis of Bacteriorhodopsin and of Lipids

An interesting question concerns the course of bacterioopsin synthesis with time. Conceivably, bacterioopsin could belong to the constitutive proteins of the cell; that being so it would account for a constant proportion of the protein population

of the cell in each growth phase. Figure 5 shows the protein pattern of whole cells in four consecutive growth phases and correlates it with the occurrence of the purple membrane. While almost all detectable protein species appear qualitatively unchanged the proportion of bacterioopsin increases synchronously with the appearance of the purple membrane even in the stationary growth phase of the cells.

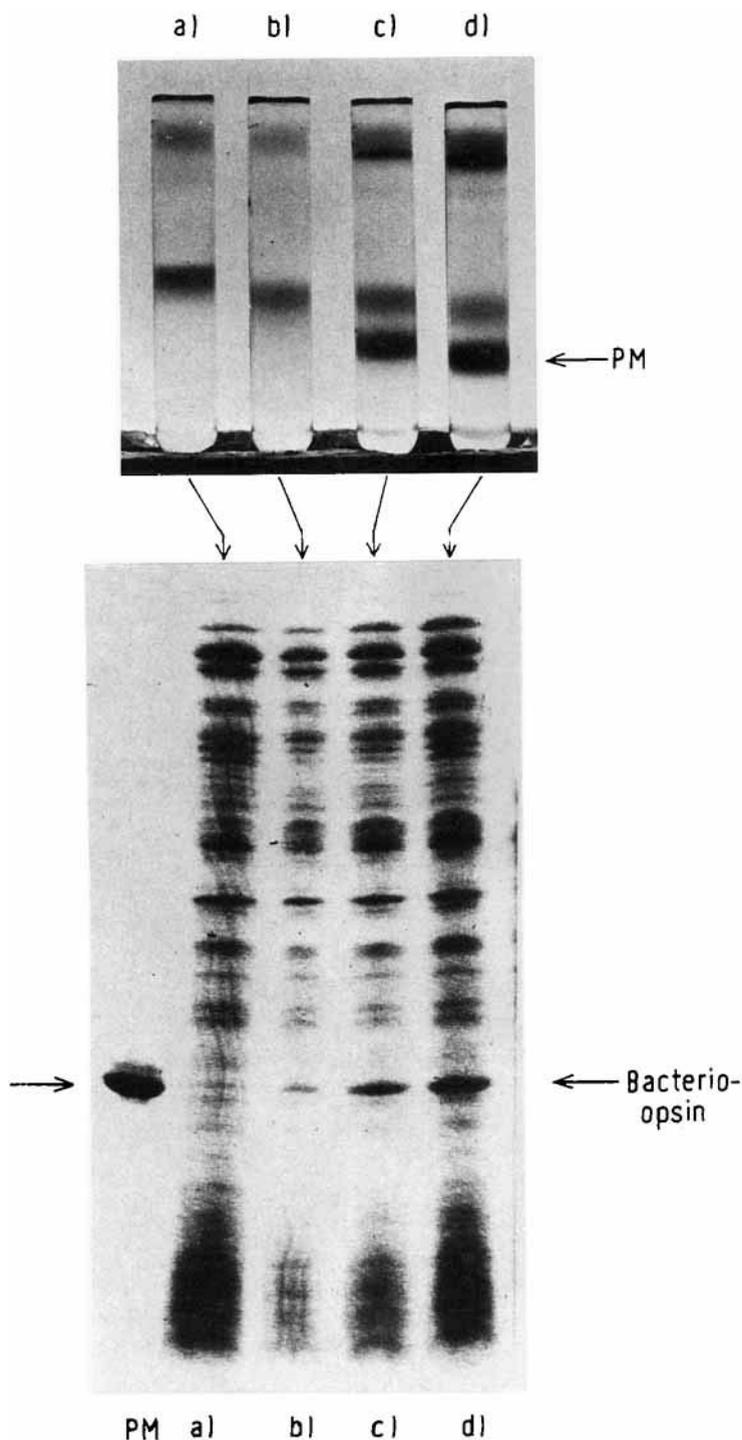


Fig. 5. Analysis of the content of purple membrane (above) and bacterioopsin (below) of halobacteria cells in four consecutive phases of the growth curve (for limiting aeration). a, logarithmic phase (47 h); b, late logarithmic phase (55 h); c and d, early (72 h) and late (101 h) stationary phase. Equal amounts of cells were lysed with water, placed on a sucrose density gradient (20–45%), and centrifuged at 200000 g overnight. Aliquots of the lysate were separated by electrophoresis on polyacrylamide gels (gradient gels: 10–25%) in the presence of 0.1% sodium dodecyl sulfate. Isolated purple membrane (PM) served as bacterioopsin reference.

^{32}P and ^{35}S labeling experiments^[22] were undertaken to examine the time-dependent control of synthesis of the purple membrane lipids. It was found that the purple membrane lipids are formed long before the start of retinal and bacterioopsin synthesis, *i.e.* the lipids required for formation of the purple membrane are taken from the lipid reservoir of the cell.

4. Membrane Fractions from Halobacteria

4.1. The Purple Membrane as a Differentiated Membrane Region

The isolated purple membrane contains bacteriorhodopsin molecules in a two-dimensional hexagonal crystalline arrangement^[3]. Electron micrographs of intact cells obtained by the freeze fracturing technique demonstrated the same arrangement of bacteriorhodopsin in the cell membrane. Thus according to these studies, the purple membrane also exists *in vivo* as a differentiated insular region of the cell membrane^[3]. The genesis of such a differentiated region is an interesting problem. Direct crystallization of bacteriorhodopsin immediately after its incorporation into the cell membrane is conceivable. Alternatively, biosynthetic precursors of the purple membrane might also exist in the form of differentiated regions in the cell membrane. Fragmentation experiments on the cell membrane and subsequent analysis of the fragments may serve to answer these questions.

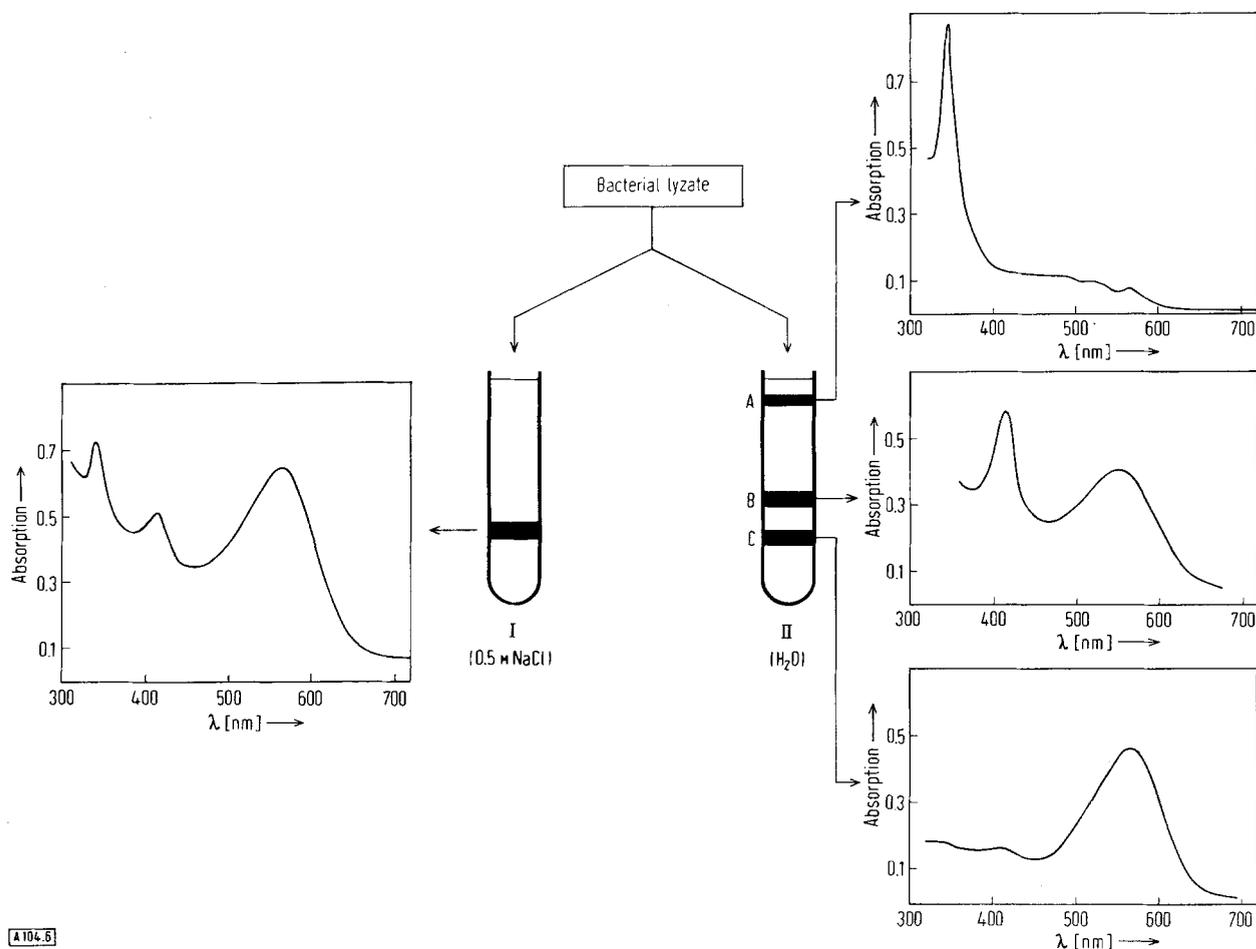
Those membrane fractions which contain bacteriorhodopsin, bacterioopsin, or one of the biosynthetic precursors of retinal will be of interest in investigations on the biosynthesis of the purple membrane.

4.2. Fragmentation of the Cell Membrane

A drop in salt concentration of the medium leads to lysis of halobacteria, *i.e.* the cytoplasm of the cell is liberated^[4]. Membranes are usually fractionated by density gradient centrifugation in sucrose. Density gradient centrifugation of the membrane component of halobacterial cell lysates in the presence of 0.5 M NaCl affords a principal membrane fraction whose absorption spectrum is depicted in Figure 6. The absorption bands at 340, 419 and 560 nm are due to lycopene, cytochrome, and bacteriorhodopsin. Centrifugation of the same lysate in the absence of NaCl gives rise to three membrane fractions A, B, C having different absorption properties. Fraction A contains all the lycopene, fraction B all the cytochrome and part of the bacteriorhodopsin, and fraction C (purple membrane) only bacteriorhodopsin. This separation suggests that the cell membrane is made up of differentiated regions.

Table 1 lists some properties of the membrane fractions A, B, and C. Owing to their characteristic colors they are known as red membrane (membrane fraction RM 340), as brown membrane, and as purple membrane.

Membrane fraction RM 340 is characterized by several unusual properties: its protein content lies below 5% of its dry weight; this is the reason for its low buoyant density. A strong absorption at 340 nm is unusual for lycopene. Addition of organic solvents (*e.g.* acetone) drastically



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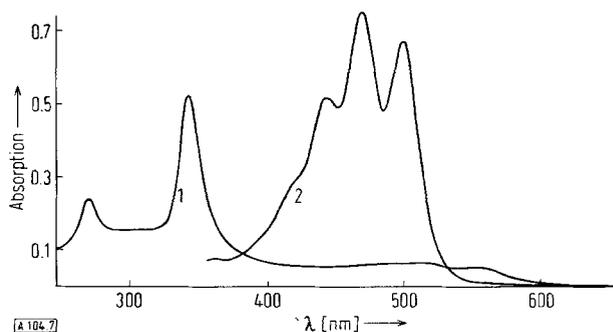
Fig. 6. Fragmentation of the cell membrane at low ionic strength. A cell lyzate was placed on a sucrose density gradient (20 to 45%) with (I) and without (II) NaCl (0.5 M) and centrifuged at 200000 g overnight. The spectra of the individual fractions were recorded with an Aminico DW2 instrument. A, red membrane; B, brown membrane; C, purple membrane (see text).

Table 1. Some properties of the various membrane fractions A, B, and C (see Fig. 6).

Fraction	Absorption maxima [nm]	Density [g/cm ³]	Protein content [%]	Pigment
A: red membrane RM 340	340 (492, 523, 565)	ca. 1.06	5	lycopene
B: brown membrane	419, 560	ca. 1.14	?	heme, retinal
C: purple membrane	560	1.18	ca. 75	retinal

changes the absorption spectrum (Fig. 7); it then corresponds to that of all-*trans*-lycopene. Identity of the pigment of RM 340 and lycopene was further substantiated by its melting point and mass spectrum. The all-*trans* configuration was demonstrated by iodine-catalyzed isomerization to a mixture of the *cis* and *trans* isomers^[23].

The unusual absorption behavior of lycopene in the RM 340 membrane fraction is not easy to interpret. The existence of a *cis* isomer in the membrane which transforms into all-*trans* lycopene upon addition of organic solvents appears unlikely: isomers displaying such high absorption in the "cis region" and so little in the visible are unknown^[23]. It is more reasonable to assume that the lycopene molecule is twisted around its 14-15 and 14'-15' single bonds (cf. Fig. 2). An *s-cis* conformer of this kind has a rod-like shape in which the π electrons of the central double bond are perpendicular to the π -electron system of the rest of the molecule. This interrupts the conjugated π -electron system containing eleven double bonds: two partial systems result, each containing five conjugated double



[A104.7]

Fig. 7. Detection of lycopene as pigment of the red membrane RM 340. 1, absorption spectrum of RM 340 in water; 2, in acetone.

bonds. Thus the absorption at 340 nm and the high extinction coefficient ($\epsilon_M = 120000 \text{ M}^{-1} \text{ cm}^{-1}$) can be rationalized.

The brown membrane exhibits two characteristic absorptions. That at 419 nm must be assigned to a cytochrome

b type because the heme group can be extracted and as a pyridinium complex shows characteristic absorption bands at 418, 524, and 554 nm in the difference spectrum (reduced/oxidized). All the criteria tested (*e.g.* photochemical properties) indicate that the absorption band at 560 nm should be assigned to bacteriorhodopsin. The bacteriorhodopsin content of the brown membrane is also confirmed by electrophoretic analysis of the proteins present in this membrane. Two major components are obtained, one of which migrates in a manner identical to bacterioopsin and the other probably being the protein component of the heme chromophore.

5. *In vivo* Experiments on Purple Membrane Biosynthesis

All the experiments considered so far show a close correlation to exist between the synthesis of retinal and that of the purple membrane. A strategy designed to detect possible intermediates of membrane formation utilizes specific inhibitors for the synthetic pathways leading to the individual membrane component, *e.g.* inhibitors of protein biosynthesis, carotenoid synthesis, or lipid synthesis.

Table 2. Influence of nicotine on the carotenoid content of halobacteria. Halobacteria were cultured with or without nicotine in the medium. (No change in the growth rate takes place up to 1 mM nicotine.) Concerning the analysis of the pigments in the stationary phase, cf. legend of Fig. 4.

Pigment	Nicotine [mM]			
	0	0.1	0.5	1.0
Lycopene	100%	enhancement	100%–500%	
β -Carotene	100%	<1%	<1%	<1%
Retinal	100%	8%	2%	<1%

Let us now consider the results obtained so far with nicotine, an inhibitor of β -carotene formation. As shown by Table 2, formation of β -carotene in halobacteria is inhibited between 0.1 and 1 mM nicotine in the medium, with a concomitant increase in the lycopene content of the cells (nicotine cells). This result is to be expected from the known fact that nicotine inhibits cyclization of lycopene to β -carotene^[24]. If the synthesis of bacterioopsin is regulated by retinal then nicotine must inhibit bacterioopsin synthesis at the same time as that of retinal. However, this is not the case. Bacterioopsin is formed in cells in the presence of nicotine (*i.e.* in the absence of retinal).

The question thus arises as to the localization of the bacterioopsin in the cell membranes of the "nicotine cells". If bacterioopsin behaves like bacteriorhodopsin, then a colorless membrane fraction of equal density and composition has to be expected in place of the purple membrane. The simplest test for bacterioopsin consists in addition of retinal, whereupon bacteriorhodopsin is reconstituted^[26]. This method was applied to the membrane fractions of "nicotine cells" after density gradient centrifugation. Surprisingly, the entire bacterioopsin is found in the brown membrane (Fig. 8).

Apparently the brown membrane is a precursor of the purple membrane. Newly formed bacterioopsin occurs initially in the brown membrane and can only "crystallize to form the purple membrane" after reaction with retinal, affording bacteriorhodopsin. This working hypothesis is illustrated in Fig. 9.

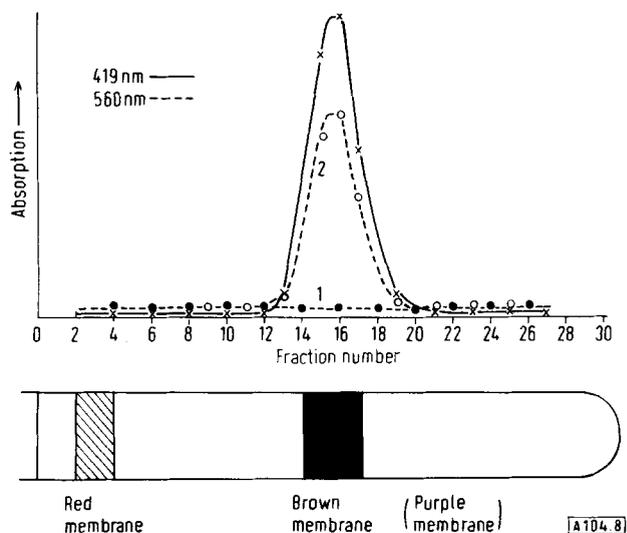


Fig. 8. Detection and localization of bacterioopsin in "retinal-free" cells. The cells were cultured in the presence of 1 mM nicotine with limiting aeration. The membrane fractions were separated by density gradient centrifugation (*cf.* Fig. 6). The absorption of the fractions were measured at 419 and at 560 nm before (curve 1) and after addition of retinal (curve 2).

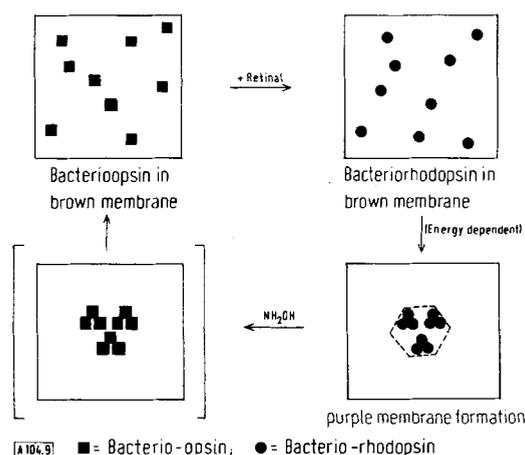


Fig. 9. Schematic representation of a working hypothesis of purple membrane formation.

Addition of retinal to "nicotine cells" should accordingly give rise to purple membrane formation, provided retinal can diffuse into the cell membrane. The feasibility of such diffusion is apparent from Figure 10. In this experiment "nicotine cells" were merely suspended in saturated salt solution (basal salt), *i.e.* without any source of energy, and treated with retinal. Once bacteriorhodopsin has formed the purple membrane should be formed by crystallization. Figure 11a shows that this does not happen, all the bacteriorhodopsin formed remaining localized in the brown membrane. Bacteriorhodopsin clearly does not crystallize spontaneously.

If the experiment of Figure 11a is repeated in the presence of a source of energy, *e.g.* L-alanine, the purple membrane is formed. The energy dependence of purple membrane formation from the brown membrane also followed from the ability of light to serve as energy source for this process in place of alanine. Since light absorbed by bacteriorhodopsin raises

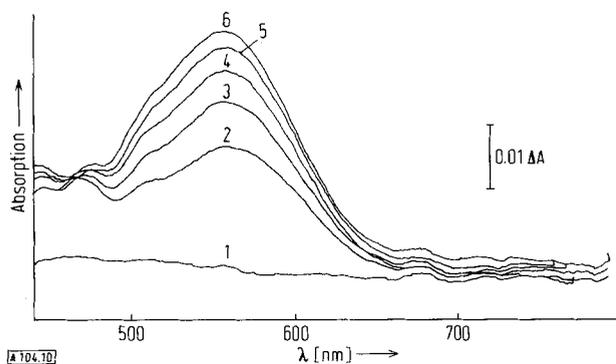


Fig. 10. Reconstitution of bacteriorhodopsin in "retinal-free" cells by addition of retinal.

Cells cultured in the presence of nicotine (1 mM) were resuspended in basal salt containing 1 mM nicotine (250 g NaCl, 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$ per liter). After treatment of the cell suspension with retinal the difference spectrum was recorded (Aminco DW 2) at various times: 1, before addition of retinal; 2-6, after 11, 18, 31, 77, and 122 min.

the ATP level of the cells it seems an obvious step to assume the occurrence of some ATP-dependent modification of one of the purple membrane components.

on abstraction of retinal from the purple membrane by reaction with hydroxylamine (cf. Fig. 9). X-Ray diffraction experiments indicate that the purple membrane loses its lattice structure on bleaching with NH_2OH and regains it on addition of retinal^[25]. Isolated patches of purple membrane necessarily constrain the bacterioopsin to adopt a dense packing so that no change in density is to be expected for the bleached purple membrane (apomembrane); nor has any such change been detected experimentally. In contrast, a lateral diffusion of bacterioopsin in the cell membrane should be feasible in intact cells leading to dissolution of the purple membrane regions (cf. Fig. 9). Confirmation is provided by density gradient centrifugation of the lysates obtained from cells treated with NH_2OH and analysis of the membrane fractions: the entire bacterioopsin is now located in the brown membrane.

6. Conclusion

The studies described in this article merely represent the first steps toward an understanding of the synthesis and association of membrane building blocks and the regulation of

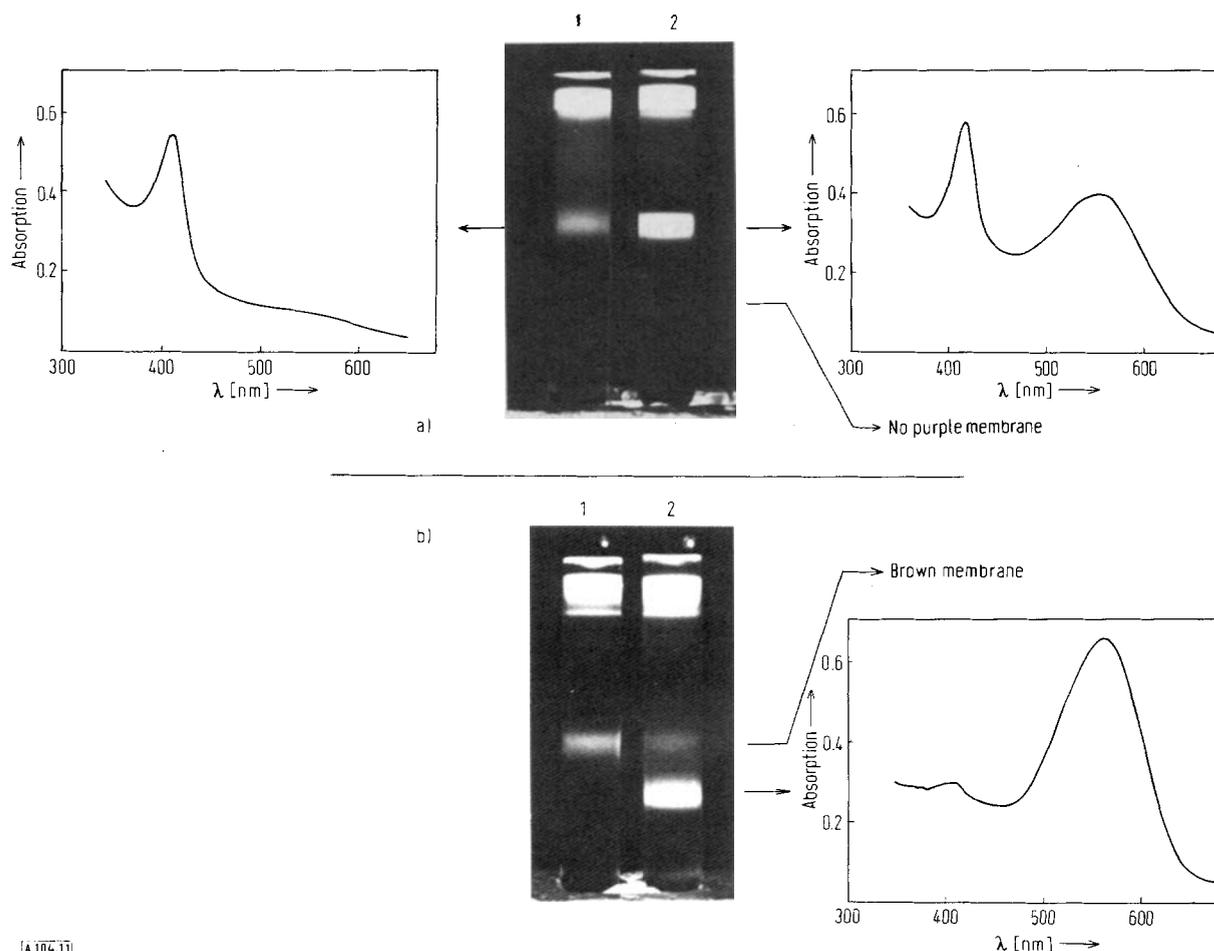


Fig. 11. "Crystallization" of bacteriorhodopsin in the brown membrane to from the purple membrane. a) "retinal-free" cells were suspended in basal salt (containing 1 mM nicotine); one half was treated with retinal and incubated at $40^\circ C$ for 8 h. (For analysis of the membrane fractions see Fig. 6.) 1, without retinal; 2, with retinal. b) as for a) except for addition of 0.5% L-alanine to the incubation medium. 1, without retinal; 2, with retinal.

As shown by the above experiments, bacterioopsin is not able to form the lattice structure of the purple membrane. Such a property of bacterioopsin should also be detectable

such processes. However, they have already clearly demonstrated the suitability of the halobacterial system for experimental work in this area.

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Neuron Models

By Bernd Hamprecht^[*]

Dedicated to Professor Feodor Lynen on the occasion of his 65th birthday

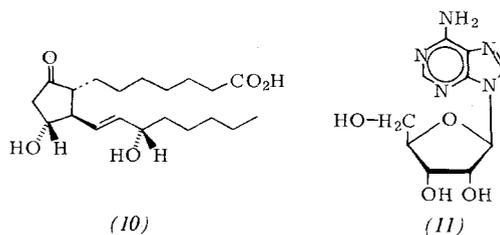
The nervous system is made up of several kinds of cells which should ideally be examined separately in biochemical studies. Since we are not yet able to isolate the different kinds of cells, use is made of cell lines derived from a single cell which are isolated from tumors of the nervous system and which can be reproduced at will in the same way as bacteria. They include cell lines isolated from a mouse neuroblastoma which exhibit some of the properties of nerve cells. Hybrid cells obtained by fusion of mouse neuroblastoma cells with rat glioma cells display even more characteristics of nerve cells. This article considers properties which justify our regarding the hybrid cells as neuron models. Application of the cells to problems of the nervous system is then demonstrated: 1) processing of information arriving in the form of mutually opposing hormonal signals; 2) mode of action of morphine.

1. Introduction

The brain is a complex structure consisting of various cell types. If the cells of the blood vessel system are left out of account, the most important cells are the neurons (nerve cells) and the glial cells; each type comprises about 50% of the cell mass in the brain.

The neurons are joined together by connections called synapses and possess a highly specialized and characteristic structure that can be seen in an electron microscope (Fig. 1). At the synapses information is transferred from one (presynaptic) nerve cell to another (postsynaptic) nerve cell. The membranes of the presynaptic and the postsynaptic cells are separated by a gap. Information is transferred by means of

a small molecule (the neurotransmitter) which passes from the presynaptic cell into the synaptic gap when the presynaptic cell is excited. The neurotransmitter diffuses from the presynap-



tic membrane through the synaptic gap onto the membrane of the postsynaptic target cell. There it becomes bound to highly specific receptors and in that process discharges electric signals into the target cell or suppresses signals that have been evoked by other neurotransmitters in other synapses

[*] Dr. B. Hamprecht
Max-Planck-Institut für Biochemie, Abteilung Lynen
Am Klopferspitz 1, 8033 Martinsried (Germany)