SOME ENVIRONMENTAL FACTORS INFLUENCING THE STATE OF THE MEMBRANES ISOLATED BY GRADIENT CENTRIFUGATION FROM CELL-FREE EXTRACTS OF *RPS. VIRIDIS**

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1. Introduction

Most of the studies on the purification of bacterial intracytoplasmic-photosynthetic systems have been carried out in bacteria containing an internal vesicular system. No similar studies have been carried out in photosynthetic microorganisms containing extensive internal lamellar structure.

Chromatophores have been isolated in a variety of states of purity by sucrose density centrifugation of cell-free extracts of *R. rubrum* [1–3] and *Rps. spheroides* [4–8].

As part of our investigation on the morphogenesis of the photosynthetic apparatus in bacteria containing internal lamellar structures, we have studied the effect of different environmental conditions on the state of the membranes isolated by sucrose gradient centrifugation of cell-free extracts of *Rps. viridis*.

2. Methods

2.1. *Culture medium and growth conditions*

Rps. viridis was used throughout these studies. This bacterium was grown in the medium described before [9], under anaerobic conditions at 30° and at approx. 200 ft candles.

2.2. *Membrane purification*

Bacteria grown for three days were centrifuged in the cold at 3,000 *g* for 30 min. The pellet is washed once with sodium phosphate buffer (PBS) 10 mM, pH 7.2, resuspended in the same buffer and sonicated in a Branson sonifier (Model W 185) for 9 min at 70 W output. The suspension is centrifuged twice in the cold at 18,000 *g* to eliminate cellular debris and unbroken cells. The supernatant from this last centrifugation was sometimes used as "crude membrane preparation" (CMP). Alternatively the CMP was centrifuged at 144,000 *g* for 60 min in the no. 40 rotor from a Spinco L2 ultracentrifuge. The pellet was washed three times and finally resuspended in the same buffer. This preparation will be referred to as "washed membrane preparation" (WMP).

2.3. *Gradient centrifugation*

Different discontinuous sucrose gradients (between 18% to 60%) were used for different purposes and they will be described in the Results.

2.4. *Bacteriochlorophyll determination*

The Bchl was determined "*in vivo*" by using a molar extinction coefficient of 10^5 at 1015 nm [10].

2.5. *Protein and nucleic acids*

The presence of proteins and nucleic acids in the gradient were followed by determining the optical density of different samples at 280 and 260 nm.

2.6. *Purity of reagents*

Cytochrome *c* (Sigma type VI from horse heart) and bovine serum albumin (Sigma) were used without further purification.

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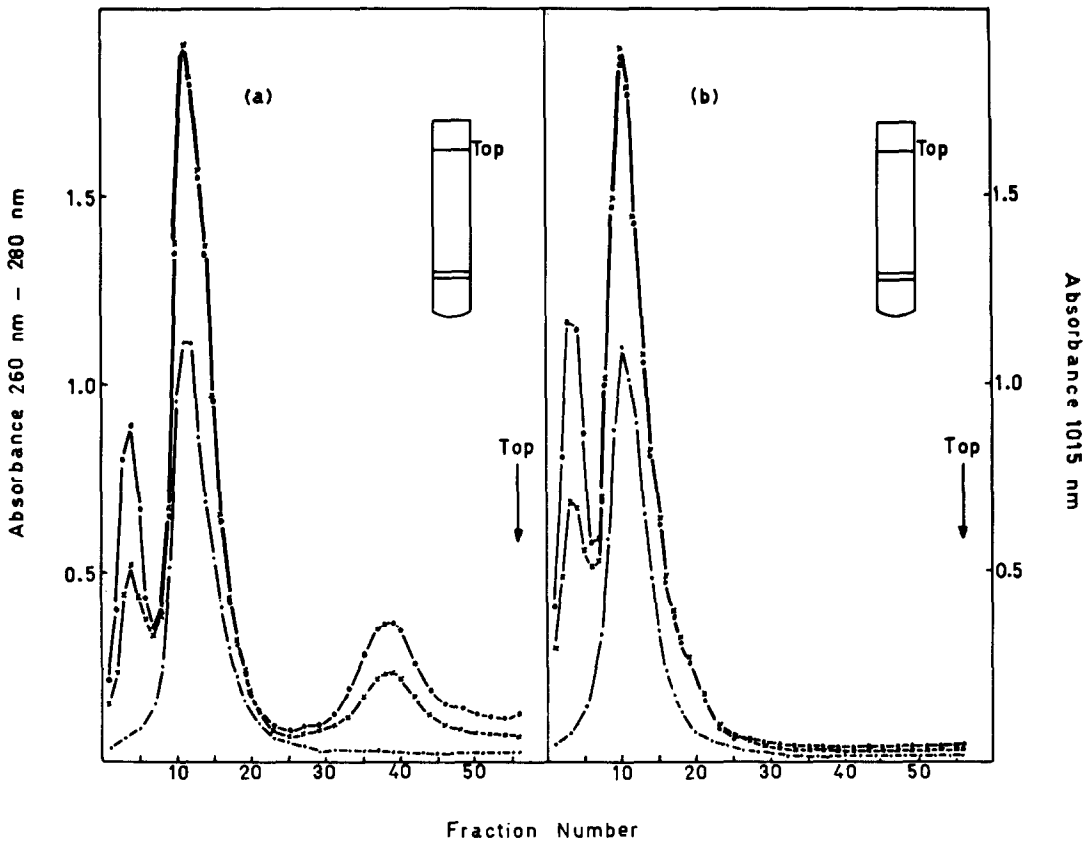
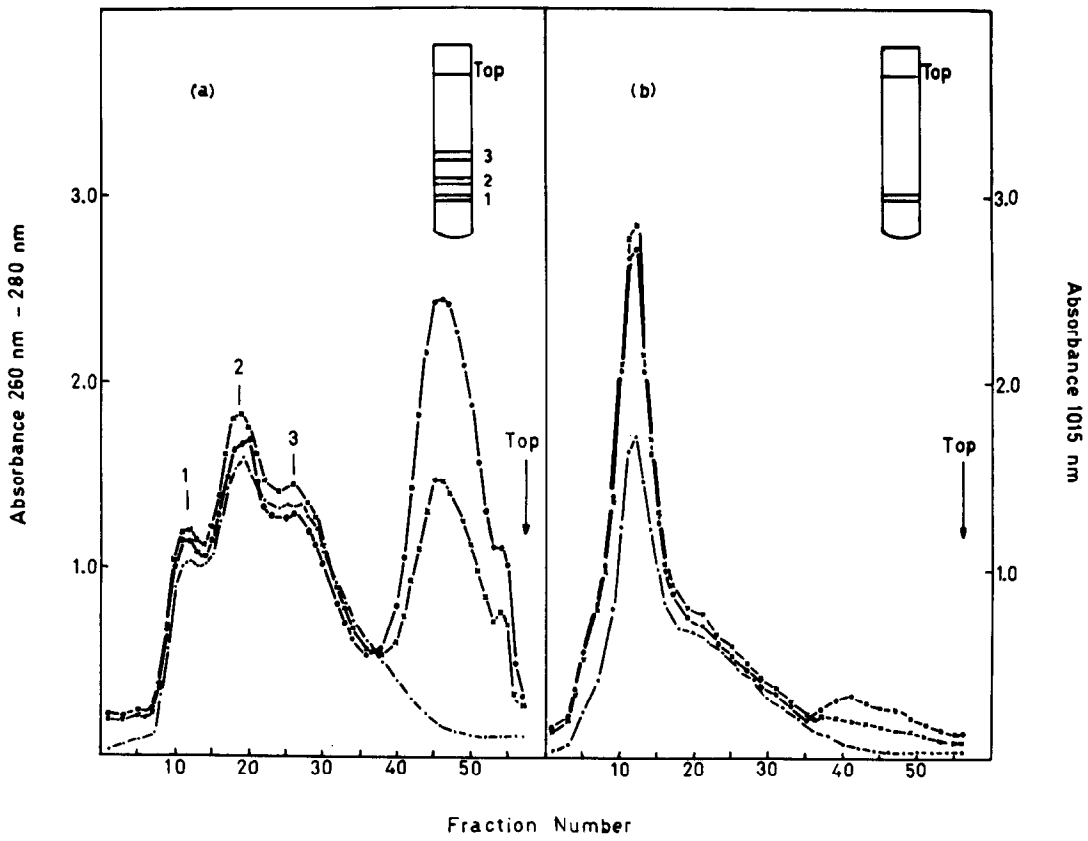


Fig. 1. a) Density gradient centrifugation of CMP in a sucrose gradient consisting of the following different layers of sucrose: 0.3 ml of 60%, 0.7 ml of 45%, 0.8 ml of 36%, 1.0 ml of 27% and 1.2 ml of 18%. 1.0 ml of sample was layered of top of the preceeding gradient and they were run for 12 hr at 35,000 rpm in a SW-39 rotor from the Spinco L2 ultracentrifuge. All dilutions were made with 10 mM PBS, pH 7.2. b) The same as above but using a WMP. (•—•—•) Absorbance at 1015 nm. (●—●—●) Absorbance at 260 nm. (X—X—X) Absorbance at 280 nm.

3. Results

When cells of *Rps. viridis* are broken by sonication and then submitted to a sucrose density gradient centrifugation the results shown in fig. 1a and 1b are obtained. It is possible to detect in the washed membrane (fig. 1b) a single pigmented band. The bottom band probably contains ribosomes as evidenced by a ratio of absorbance 260/280 nm of 1.7. If the membranes are not washed prior to centrifugation a third UV absorbing band is observed containing nucleic acids (260/280 nm = 1.56). When the membranes are centrifuged at high speed for a short time, fractions having different S vlaues are obtained. These results are shown in fig. 2a and 2b. It is possible to see that the washing of the membrane produces an accumulation of the Bchl in a single band (see fig. 2b). Fig. 2a shows the effect of dialyzing the CMP against PBS. In this case three different pigmented bands are detected. It is interesting to point out to the regularity of the 280/1015 nm ratio for all Bchl containing bands. This ratio varies between 1.07 to 1.16, which might indicate a similar Bchl specific content. On the other hand, the ratio 280/1015 nm, for the major band in fig. 2b increases to 1.67. We have further investigated the effect of washing-off the supernatant on the aggregation process. Exhaustive dialysis of CMP against water does not produce aggregation (see fig. 2c). On the con-



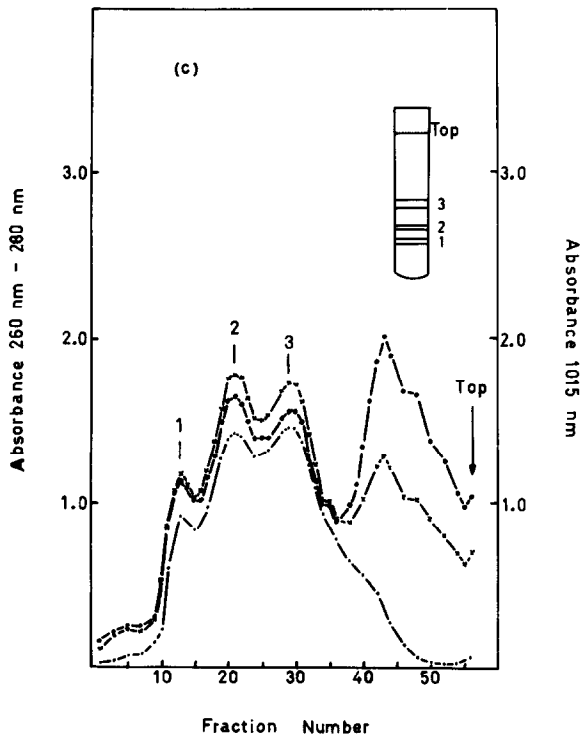


Fig. 2a, b, c: a) Centrifugation of a CMP previously dialyzed against 10 mM PBS, pH 7.2 in a discontinuous sucrose gradient. b) Centrifugation of a CMP previously washed three times with 10 mM PBS pH 7.2, in a discontinuous sucrose gradient. The sucrose gradients were identical to the one described in fig. 1, and they were centrifuged 60 min at 35,000 rpm in a SW-39 rotor from the Spinco L2 ultracentrifuge. c) Centrifugation of a CMP previously dialyzed for 48 hr against distilled water. The sucrose gradient was similar to the one described in fig. 1 except that sucrose dilutions were made with water and centrifuged 60 min at 35,000 rpm in a SW-39 rotor from the Spinco L2 ultracentrifuge.

trary there is a small but reproducible increase in the amount of band 3.

Washing with Tris-HCl buffer containing 10^{-2} M Mg^{2+} also produces aggregation (see fig. 2d). The pigmented band is however in a different position (compare fig. 2d and 2b). Dialysis against Tris-HCl buffer containing 10^{-2} M Mg^{2+} (see fig. 2e) shows an additional UV absorbing zone probably corresponding to ribosomes and at the same time a single IR absorbing band.

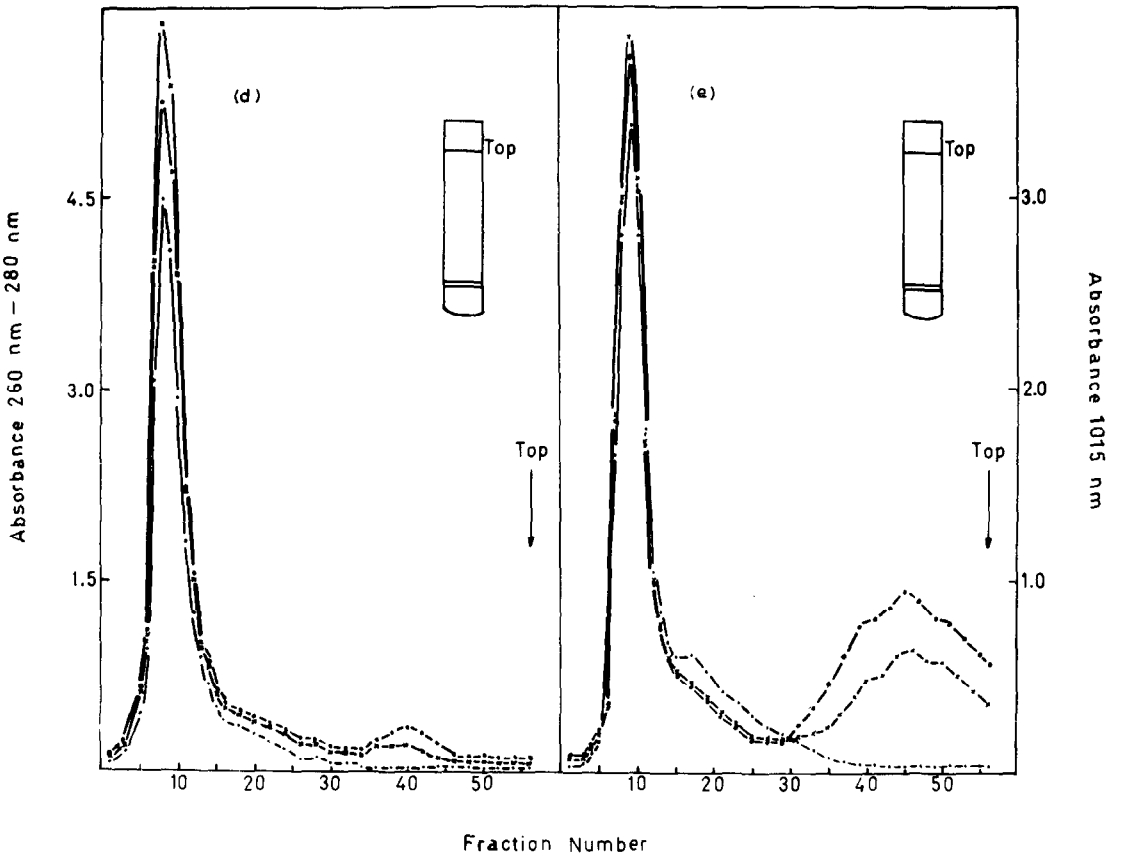


Fig. 2d, e: d) Centrifugation of a CMP previously washed with 50 mM Tris-HCl, pH 8.0 also containing 10 mM Mg^{2+}. e) Centrifugation of a CMP previously dialyzed for 48 hr against 50 mM Tris-HCl pH 8.0 also containing 10 mM Mg^{2+}. The sucrose gradients were similar to the one described in fig. 1 except that sucrose dilutions were made with 50 mM Tris-HCl, pH 8.0 and were centrifuged 60 min at 35,000 rpm in a SW-39 rotor from the Spinco L2 ultracentrifuge. In all cases Mg^{2+} was present at 10 mM final concentration. (•—•—•) Absorbance at 1015 nm. (•—•—•) Absorbance at 260 nm. (X—X—X) Absorbance at 280 nm.

In order to investigate the reason why the proteins from the supernatant affect the aggregation of the membrane fractions, we have tested the effect, on the aggregation process, of washing the membrane with two pure proteins also having different isoelectric points (Ip). We have used horse heart Cyt. *c* (Ip 11.7) and bovine serum albumin (Ip 4.6–4.8). These results are seen in fig. 3a and 3b. Cytochrome *c* produces aggregation having an effect similar to Mg^{2+}. BSA has a completely different effect mantaining the membrane in a more disaggregated state. The aggregation process is also reversed by resuspending the washed membrane back into the supernatant fraction showing approximately the same effect of BSA (not shown here).

4. Discussion

Centrifugation in sucrose gradients of cell-free extracts of *Rps. viridis* for short times show the presence of membrane fractions apparently having different S values, although they present the same density. Aggregation of those membrane fractions is effected by changing the environmental conditions such as: i) washing-off the supernatant or ii) by the presence of Mg^{2+} ions. The aggregation produced by washing is most probably the result of the removal of soluble proteins

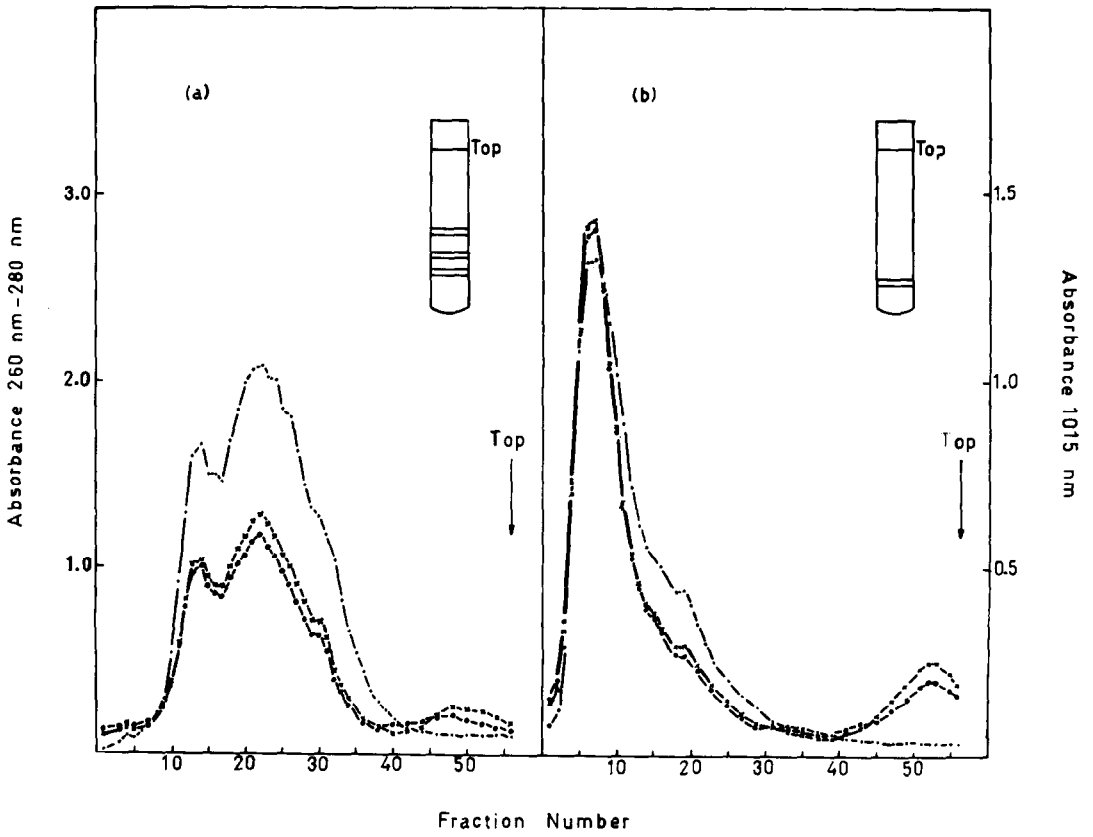


Fig. 3. a) Centrifugation of CMP previously washed three times with a solution of BSA (2.3 mg/ml) in water. b) Centrifugation of CMP previously washed three times with a solution of Cyt. *c* (2.3 mg/ml) in water. The Cyt. *c* was in its oxidized state. In both cases the sucrose gradient was identical to the one described in fig. 1 and it was centrifuged for 60 min at 35,000 rpm in a SW-39 rotor from the Spinco L2 ultracentrifuge. (•—•—•) Absorbance at 1015 nm. (●—●—●) Absorbance at 260 nm. (X—X—X) Absorbance at 280 nm.

since elimination of residual metal ions does not introduce any substantial modification in the pattern of pigment distribution in the gradient. It is known that Mg^{2+} is needed for reaggregation of SDS-solubilized membranes at neutral pH [11]. Razin et al. [12] have also shown that the lower the pH of the system the less amount of Mg^{2+} is required for reconstitution of previously solubilized membranes by facilitating non-ionic interactions between neutralized membranes.

Dialysis of the CMP against Mg^{2+} or washing with Mg^{2+}-containing buffer produce a similar effect. Thus a single pigmented band is detected in the sucrose gradient. In this case we also observe spontaneous aggregation of sonicated membranes at pH's below 4.5. This may indicate that the aggregation process that we observe in the presence of Mg^{2+} may be due to non-ionic interaction between membrane pieces. Besides it is obvious that there is an interaction of Mg^{2+} ions with the membrane which results in structural modifications as evidenced by the change in the S value of the pigmented material.

If the proteins from the supernatant act by increasing the electrical repulsive forces between membrane pieces, the net charge of the soluble proteins are of fundamental importance in bringing about their dissociating effect.

We have tested this possibility by washing the membrane with a solution of two different pure proteins also having very different isoelectric points. Cytochrome

c behaves like Mg^{2+}. This protein molecule is positively charged at the pH used in this experiment. It is then possible to postulate that cytochrome *c* acts as a bridge between negatively charged groups present on the membrane, allowing non-ionic aggregating forces to take place. On the other hand, BSA, negatively charged, somewhat impedes the aggregation phenomenon. The action of BSA may simply be through reinforcing the repulsive forces taking place between membrane fragments.

These results may indicate some functional roles for the interactions: i) membrane negative charged-metal ions and ii) membrane-soluble proteins. It must be borne in mind that the binding of proteins to membranes is a well known phenomenon. From the data presented by Racker et al. [13] and Abrams [14] it seems clear that there is some specificity in the binding of membranes to proteins.

We may also state, as a conclusion, that, in the present case, the term membrane fraction only is an operational definition since the number and structural properties of those fractions simply are a function of the obtention procedure.

Very recently Niederman et al. [8] have shown in *Rps. spheroides* that the photosynthetic apparatus is contained in a single pigmented band separable from the cell envelope by changing the environmental conditions. They attributed the change in the position of the pigmented band, in the gradient, to the permeability of these structures to the ionic species. Our results indicate that the effect of Mg^{2+} is probably due to aggregation rather than to permeability. This would indicate that the photosynthetic membranes isolated from *Rps. viridis*, by sonication, are not topologically closed vesicles. This is further confirmed by the fact that thus far we have been unable to detect any substantial photophosphorylating activity in membranes isolated by these means, from this bacterium [15].

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