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Photosynthetic Membrane Development in *Rhodopseudomonas spheroides*

II. Correlation of Pigment Incorporation with Morphological Aspects of Thylakoid Formation*

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Abstract

Cells of *Rhodopseudomonas spheroides* were depigmented by aerobic growth in the light and then transferred to 4% oxygen in the dark to induce pigment synthesis. Pigment synthesis and photochemical activity were measured fluorometrically. In conjunction with the fluorescence studies, thylakoid morphogenesis was followed by electron microscopy of thin sections of cells fixed during the repigmentation process.

Both bacteriochlorophyll and the onset of photochemical activity were detected before distinct thylakoids were observed. Subsequent bacteriochlorophyll synthesis was associated with a gradual increase in the thylakoid content throughout the developmental process.

The results obtained strongly indicate that initially the cytoplasmic membrane is modified by pigment incorporation, possibly at specific sites, and that the bacteriochlorophyll is photochemically active in the pigmented cytoplasmic membrane or in the early stages of invagination.

Finally, in a confirmation of previous hypotheses, these studies provide evidence for the origin of the thylakoids as a protrusion and invagination of the cytoplasmic membrane. This is followed by constriction and subsequent proliferation and branching to form a continuous membrane system which gives rise to chromatophores upon cellular disruption.

* Extracted in part from the doctoral thesis of G. A. Peters submitted to the University of Michigan in partial fulfillment of the requirements for the Ph.D. degree. For paper I of this series see reference [3].

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Introduction

Several members of the Athiorhodaceae, including *Rhodospirillum (R.) rubrum* and *Rhodopseudomonas (Rps.) spheroides*, can be grown aerobically in the light or dark and anaerobically in the light. When cells are grown aerobically, bacteriochlorophyll and carotenoid synthesis is inhibited and the existing pigment is diluted out [1-8]. Aerobic growth in the light enhances this depigmentation process [3]. If a culture which has been grown aerobically until it contains little or no pigment is transferred either to low oxygen partial pressures in the light or dark or to anaerobic conditions in the light, pigment synthesis is induced [2-8]. The kinetics of pigment and protein synthesis as well as many other aspects of this developmental process have been studied extensively [2-15].

Additional studies have led to the widely accepted hypothesis that the photosynthetic pigments of these bacteria are located on a tubular membrane system which is connected to the peripheral (or cytoplasmic) membrane and extends into the cell [5, 16-22]. Upon cellular disruption this system gives rise to pigmented vesicles, termed chromatophores, which can be sedimented by high speed centrifugation.* These structures are not found in the depigmented cells grown under high aeration [16, 17, 21, 23-25]. In general these conclusions were based on the results of studies, primarily on *R. rubrum*, in which chromatophores and/or thin sections were obtained from cells grown under steady state conditions at various light intensities, from samples removed at relatively widespread intervals from a photosynthetic culture at a constant light intensity, or from rather undefined "semi-aerobic" cultures.

There have been relatively few investigations on the thylakoids of *Rps. spheroides* [6, 26, 27]. Worden and Sistrom [26] isolated "heavy" and "light" chromatophore fractions from cells grown under a variety of conditions. They suggested that the light fraction was derived from invaginations of the cytoplasmic membrane and the heavy fraction from the cell membrane itself. Gorchein *et al.* [6] reported that during adaptation from high to low oxygen tensions a measurable increase in bacteriochlorophyll was not detectable until 2 h after changing conditions. They found changes in both protein and phospholipid occurred before any pigment or chromatophores were detectable, that pigment and chromatophores were detected simultaneously and that they were then synthesized at an equal pace.

We have shown, in earlier studies, that bacteriochlorophyll can be detected by a very sensitive fluorescence method within 30 min after the

* In this paper the term "thylakoids" is employed to describe the membrane-bound system of internal vesicles and invaginations observed in thin sections of intact cells. The term "chromatophore" is restricted to the particulate, pigmented material isolated after cellular disruption.

transfer of totally depigmented cells of *Rps. spheroides* to semi-aerobic conditions [3, 4]. These studies showed that pigment synthesis, its incorporation into membranes, and photochemical activity could be detected earlier than others had reported for either *R. rubrum* or *Rps. spheroides*, where the onset of pigment synthesis appeared to be simultaneous with the appearance of distinct thylakoids or chromatophores. We report here the morphological changes observed in thin sections of cells fixed at regular intervals during intracellular membrane formation and correlate these findings with the incorporation of bacteriochlorophyll. This work provides additional information on the probable site of pigment incorporation in the initial stages of bacteriochlorophyll synthesis, on the relationship between the peripheral or cytoplasmic membrane and intracellular membranes, and a sequential view of thylakoid morphogenesis in this organism. The results from a number of experiments on *R. rubrum* under static conditions led to the hypothesis that thylakoids arise from the cytoplasmic membrane [16, 17, 19] and this has been supported by a developmental study [5]. The work presented here provides morphological evidence for the origin of thylakoids from the cytoplasmic membrane in *Rps. spheroides* as well.

Materials and Methods

Cells of the green mutant of *Rps. spheroides* (strain 2.4.1/Ga) were grown on modified Hutner's medium [2] using malate as the carbon source. The initial depigmentation under aerobic conditions in light, induction of pigment synthesis by transfer to 4% oxygen (semi-aerobic conditions) in the dark, removal of samples, instrumentation and determination of fluorescence spectra were as described previously [3] with the following modifications: The culture volume was increased to 400 ml and, in addition to the samples removed for fluorescence measurements, a 10 ml sample was removed at each interval for fixation and electron microscopy. The cells to be fixed were centrifuged as described previously and resuspended for 1 h in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8), to which one drop each of 0.1 M $CaCl_2$ and 0.1 M $MgCl_2$ were added to give a concentration of about 6×10^{-5} M. They were then recentrifuged, washed twice with the phosphate buffer, postfixed by resuspending in unbuffered 2% $KMnO_4$ for 2 h, centrifuged and washed with glass-distilled water until the supernatant was clear. The pellet of cells was resuspended in 0.3-0.5 ml of 2% agar solution at 45°, drawn up into the narrow region of a Pasteur pipette, allowed to solidify, and blown out onto a clear glass microscope slide. The agar cylinder thus formed was cut at 2-4 mm intervals and dehydrated by passage through an ethanol series. The ethanol was removed by two washes in propylene oxide and the cylinders were

carried through an infiltration series in a desiccator using ratios of 1 : 3, 1 : 1, and 3 : 1 (v/v) epon mixture [28] : propylene oxide for 1 h each. They were then transferred to pure epon mixture and allowed to remain overnight under desiccation. Finally, individual cylinders from each sample were embedded in fresh epon mixture and heat cured.

Early in these investigations a single photosynthetically grown culture was subjected to several different fixations [29]. The glutaraldehyde-$KMnO_4$ system was employed in subsequent studies since it was found to result in good membrane preservation with a minimum preservation of ribosomal material which, as has been shown previously [16], tends to obscure the thylakoid membranes.

Sections were cut on a Sorvall MT-2 ultramicrotome using a diamond knife, mounted on uncoated 300 or 400 mesh copper grids and stained for 5 min with lead hydroxide [30]. Electron micrographs were obtained with an RCA EMU 4 or a Philips 200 electron microscope operating at 50 and 80 kV respectively.

Results

Figure 1a shows the growth rate and the formation of bacteriochlorophyll when a culture which has been totally depigmented by aerobic growth in the light is transferred to 4% oxygen in the dark. Figure 1b shows the changes in the relative fluorescence yield, *in vivo*, and the changes in the relative effect of dithionite addition during the repigmentation process. We have previously presented this type of study in detail [3]. We have included these data here for purposes of clarity and because they refer specifically to the culture on which the electron microscopic observations were made. They show that the growth rate is unaffected by the change in oxygen partial pressure or light intensity and that bacteriochlorophyll is detectable within 30 min after the change of growth conditions, increases rapidly during the first few hours, and then begins to level off and parallel the growth rate of the culture. There is also a bacteriochlorophyll component which has not yet been incorporated into the membrane [3, 29]. This component appears simultaneously with or slightly precedes the bound bacteriochlorophyll, initially increases at the same rate, but begins to plateau earlier. We have suggested that this unbound bacteriochlorophyll is the final product of the bacteriochlorophyll biosynthetic pathway and is in a "pool" from which it is then incorporated into the membrane [3].

Within 1-1.5 h after the induction of pigment synthesis, the values of the fluorescence yield and dithionite effect, which measure the interaction between the photosynthetic pigment system and the electron transport pathway(s) [3, 29] (Fig. 1b) are equivalent to the values obtained for fully pigmented, photosynthetically grown cells. This

indicates that normal, functional photosynthetic capacity of the bound bacteriochlorophyll present in the cells has been attained [3, 29]. The carotenoids are incorporated into the photosynthetic apparatus simultaneously with the bacteriochlorophyll [3].

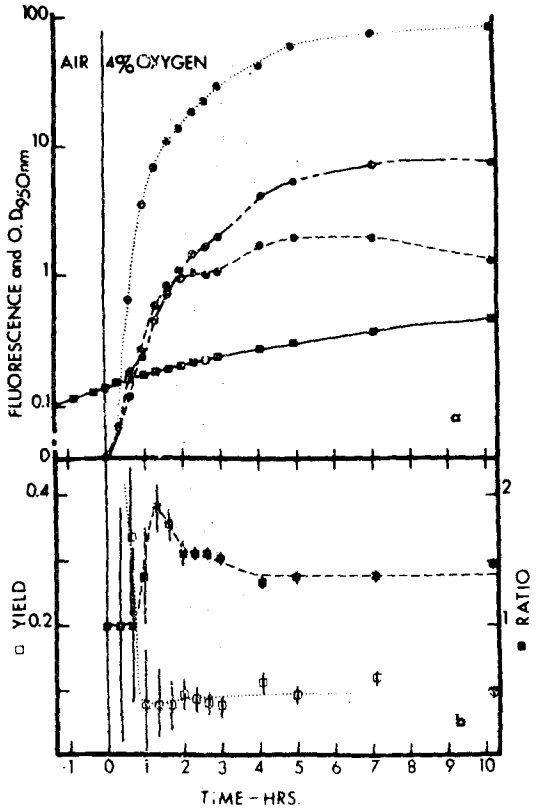

Figure 1. a. The growth rate and increase of fluorescence intensity during bacteriochlorophyll formation in repigmenting cells of *Rps. spheroides.* —— growth rate as determined by $O.D._{950\,nm}$; an O.D. of 0.1 corresponds to approximately 2×10^8 cells/ml. – – – – and intensity of the membrane-bound (885 nm) and unbound (790 nm) bacteriochlorophyll fluorescence *in vivo* respectively from 590 nm excitation, ... intensity of the total bacteriochlorophyll fluorescence emission (785 nm) in a methanol/acetone extract from 595 nm excitation [3, 4]. The values for all fluorescence components have been divided by the $O.D._{950\,nm}$ of the given sample in order to present the increase in bacteriochlorophyll on an approximate per cell basis. b. The increase in the dithionite effect (the ratio of the fluorescence intensity of the bound bacteriochlorophyll after dithionite addition to that before addition) - – – and the decrease in the relative fluorescence yield *in vivo* ..... The vertical bars indicate the uncertainty in the values as determined from the noise level of the photomultiplier tube.

Representative electron micrographs of thin sections of cells at several stages of the repigmentation process are presented in Figs. 2-9. The totally depigmented cells are typically devoid of a thylakoid system (Fig. 2). After 40 min (Fig. 3) and 60 min (Fig. 4) pigment is detectable (cf. Fig. 1a), but the majority of the cells are morphologically indistinguishable from those observed prior to the induction of pigment synthesis. Areas in which the cytoplasmic membrane protruded slightly into the cytoplasm were detected in a few of these cells. Rarely, a small vesicle continuous with the cytoplasmic membrane or poorly defined membranous structures were observed at the end of a cell.

The protrusions seen after 40 and 60 min were observed more frequently after additional semi-aerobic growth. This is quite apparent in the cross section of cells after 100 min (Fig. 5a). We interpret these areas to be an initial stage in the invagination of the cytoplasmic

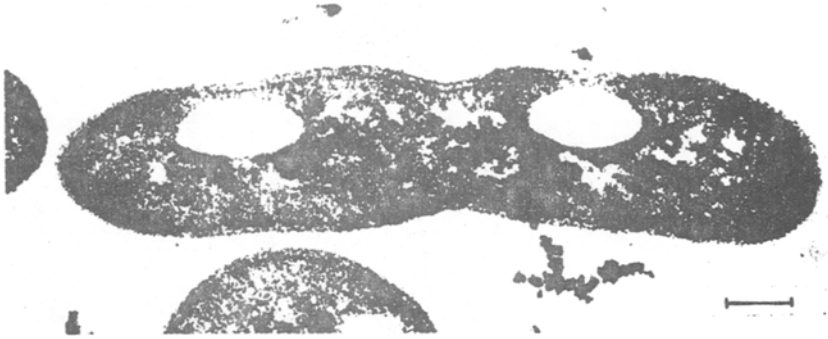

Figure 2. Section of *Rps. spheroides* bleached by exponential growth in air and light for 95 h. Bacteriochlorophyll content less than 0.1 ng/ml culture. The large spherical granules presumably contained poly-β-hydroxybutyrate. Marker lines on this and subsequent figures are equivalent to 0.2 μ.

Figure 3. Section of *Rps. spheroides* 40 min after transfer of culture shown in Fig. 2 to semi-aerobic conditions in the dark. Bacteriochlorophyll content 0.7 ng/ml culture.

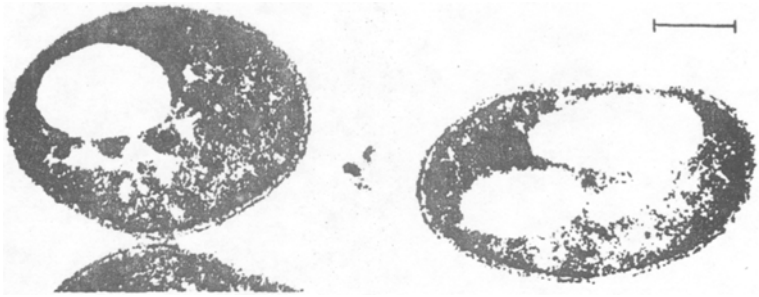

Figure 4. Section of *Rps. spheroides* 60 min after transfer to semi-aerobic conditions in the dark. Bacteriochlorophyll content 3.7 ng/ml culture.

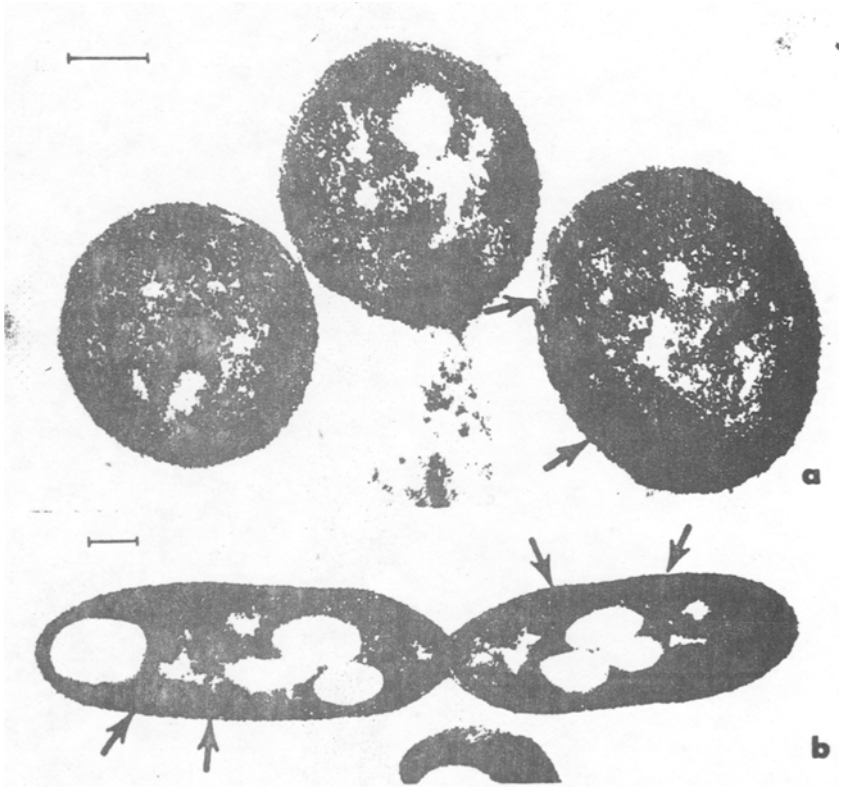

Figure 5. A transverse section (a) and longitudinal section (b) of *Rps. spheroides* 100 min after induction of pigment synthesis. Arrows in (a) indicate some sites at which protrusions are present. The cell in (b) is representative of the higher number of internal vesicles (arrows) observed in any cell at this interval. Bacteriochlorophyll content 13 ng/ml culture.

membrane to form thylakoids. Other than such protrusions, the majority of the sections at this interval revealed only a single peripherally located internal vesicle or were morphologically identical to those observed after 40 and 60 min. Longitudinal sections of a few cells did, however, reveal up to three internal vesicles in close association or clearly continuous with the cytoplasmic membrane (Fig. 5b). At this stage the bacteriochlorophyll in the cells has attained normal photochemical activity, as mentioned earlier. The exceedingly low thylakoid content of these cells, which contain 15 times more bacteriochlorophyll per cell than those after 40 min, suggests that the formation of distinct thylakoids lags behind the increase in cellular pigment content during the initial stages of the repigmentation process.

Cells which have been growing semi-aerobically for 2.3 h (Fig. 6) and 3 h (Fig. 7) exhibit a very gradual increase in the number of internal vesicles but contain approximately 25 and 40 times more extractable

Figure 6. Section of *Rps. spheroides* 140 min after the induction of pigment synthesis. Bacteriochlorophyll content 24 ng/ml of culture.

Figure 7. Section of *Rps. spheroides* 3 h after induction of pigment synthesis. Bacteriochlorophyll content 42 ng/ml culture. Continuity of stalked thylakoid with cytoplasmic membrane is designated by arrow.

bacteriochlorophyll per cell, respectively, than those after 40 min (Fig. 3). The increase in thylakoid content is seen to occur predominantly as a result of an increase in the number of invaginations and internal vesicles around the periphery of the cell rather than as a result of an extended proliferation of a few previously existing vesicles. The continuity of the vesicles and the cytoplasmic membrane is clearly seen in Fig. 7. This cell is representative of the higher level of internal vesicles and protrusions observed at this stage. After 7.2 h of semi-aerobic growth (Fig. 8a, b) the cells contain approximately 100 times more extractable bacteriochlorophyll than the cell shown in Fig. 3. There is a further gradual increase in the thylakoid content, and only a few cross sections of cells, as in Fig. 8a, reveal a plane of a single cell devoid of any internal vesicles. In addition to the gradual increase in the number of internal vesicles at this stage, a second aspect of thylakoid morphogenesis is seen to occur. While new protrusions and invaginations are forming at the cytoplasmic membrane, as indicated by the single arrows in Fig. 8a, and while there are typical internal vesicles showing

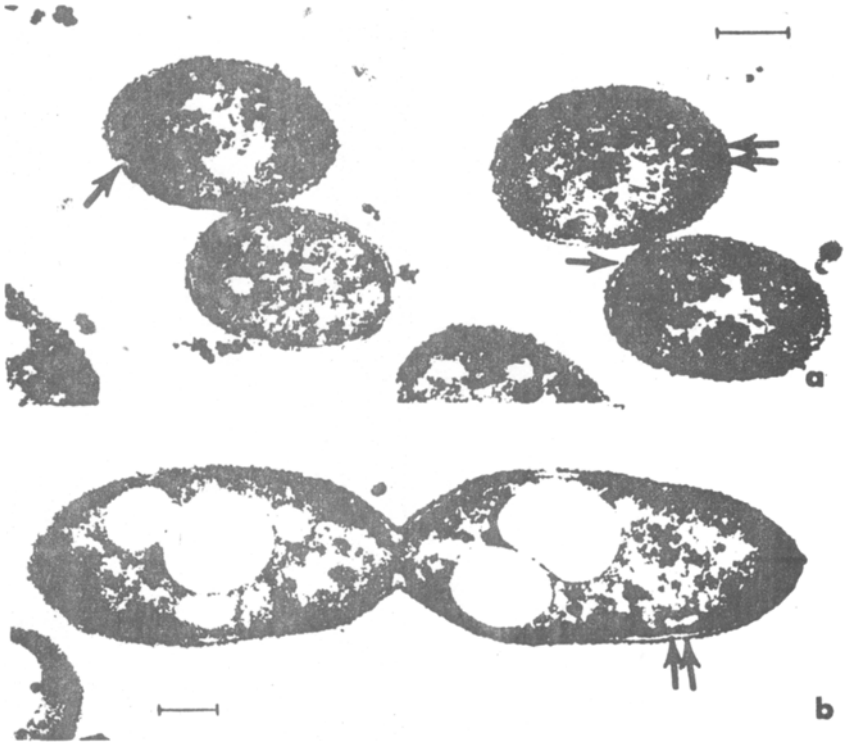

Figure 8(a, b). Sections of *Rps. spheroides* grown semi-aerobically for 7.2 h. Bacteriochlorophyll content 144 ng/ml culture. Arrows explained in text.

continuity with the cytoplasmic membrane, there is also an increase in the penetration of some of the thylakoids into the cytoplasmic area which is accompanied by a proliferation or branching. The double arrow in Fig. 8a illustrates a thylakoid in which this aspect is clearly seen and also shows the continuity via a stalk to the cytoplasmic membrane. The double arrow in Fig. 8b illustrates this phenomenon in another plane in which the thylakoid appears as a dumbbell-shaped structure free in the cytoplasm.

The small number of thylakoids reflects the relatively low pigment content imposed by these experimental conditions in which growth is achieved through oxidative metabolism and the extent of bacteriochlorophyll synthesis is determined by the oxygen partial pressure [2, 8, 9, 16]. Growth under photosynthetic conditions results in a further increase in the pigment content and in the internal membrane system, the amount depending on the light intensity [10, 16, 26, 31]. For example, a thin section of a cell grown under anaerobic conditions at moderate illumination (about 7×10^4 erg/cm^2-sec) and possessing over 200 times more bacteriochlorophyll than any of the cells shown previously is presented in Fig. 9. While the number of internal vesicles,

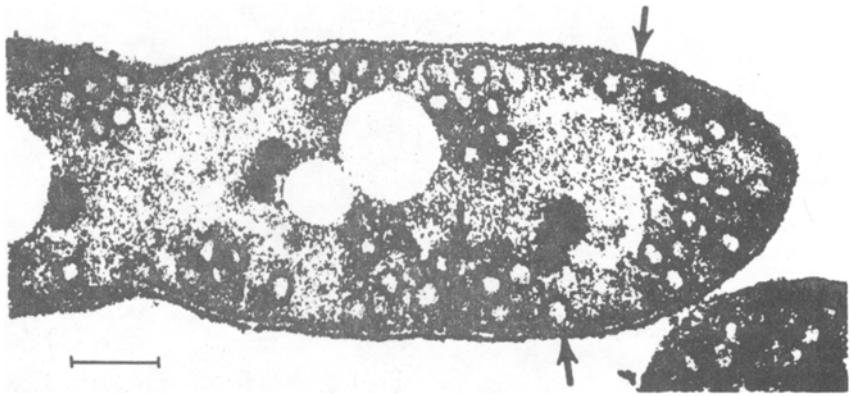

Figure 9. Sections of *Rps. spheroides* grown anaerobically at moderate light intensity. Areas of thylakoid continuity with the cytoplasmic membrane and proliferating vesicles are indicated by arrows. Bacteriochlorophyll content 70.4 μg/ml culture.

most of which appear to be free in the cytoplasm, is much greater, various stages of invagination showing continuity with the cytoplasmic membrane and branching and connections between vesicles, as indicated by arrows, can also be observed.

Discussion

On the basis of these observations the process by which thylakoids are formed can be summarized as follows:

(a) As shown here and by other investigations [6, 16, 17, 21, 23-25], cells which have been totally depigmented are devoid of typical thylakoids.

(b) After transfer to low oxygen tensions in the dark, there is initially a rapid synthesis and incorporation of the photosynthetic pigments into membrane. This initial incorporation of pigment and the onset of photochemical activity are not directly correlated with the formation of distinct thylakoids.

(c) A subsequent increase in pigment is accompanied by a gradual increase in the number of internal vesicles. These thylakoids clearly arise from the cytoplasmic membrane. Initially they appear as simple protrusions, then they invaginate and become constricted, resulting in a stalked more or less spherical vesicle which is open at the cell wall end. Finally these vesicles penetrate more deeply into the cytoplasm, often assuming a tubular appearance and proliferate into a branched network. New protrusions continue to arise at other areas of the cytoplasmic membrane and the process is repeated. This process is illustrated schematically in Fig. 10.

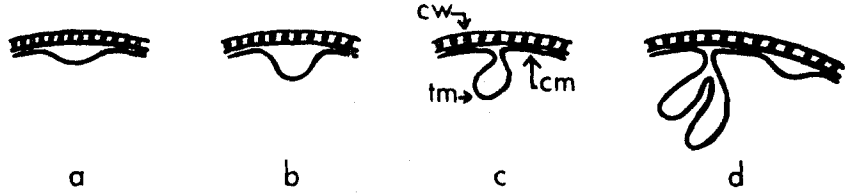

Figure 10. Schematic illustration of invagination of cytoplasmic membrane to form thylakoids. a, Protrusion of cytoplasmic membrane; b, invagination; c, constriction at cell wall end; d, proliferation and new protrusion. cm = cytoplasmic membrane; cw = cell wall; tm = thylakoid membrane.

The data which show that the incorporation of pigment into functional membrane occurs prior to the formation of distinct thylakoids and, by definition, prior to the formation of isolatable chromatophores are of particular interest. They lead to the conclusions that in the initial stage of the repigmentation process pigment is incorporated into the cytoplasmic membrane, possibly at specific sites, and that the thus modified cytoplasmic membrane and/or its protrusions, which appear to be the initial stages of invagination, are capable of energy transfer and photochemical activity. Even after 100 min of semi-aerobic growth, the majority of cells are characterized

by initial stages of invagination at best and possess few or no distinct vesicles (Fig. 5a). Those vesicles which are present in a few cells are located at the periphery of the cell in close association or clearly continuous with the cytoplasmic membrane (Fig. 5b).

The finding that photochemical activity develops simultaneously with the incorporation of bacteriochlorophyll suggests that the other components of the electron transport chain and the enzymes concerned with their synthesis are already present in the cytoplasmic membrane. This is indicated by both the rapidity with which photochemical activity is attained and the fact that one does not observe an immediate increase in the amount of membrane. Under semi-aerobic conditions in the dark the growth of these organisms is dependent upon oxidative metabolism. Therefore, it is possible that, under these conditions at least, the bacteriochlorophyll initially incorporated into the cytoplasmic membrane interacts with the existing oxidative electron transport system.

It is also of interest to note in Fig. 1 that initially the unbound bacteriochlorophyll increases at a rate equivalent to or slightly faster than the bound bacteriochlorophyll but after about 1.5-2 h the unbound form begins to level off while the bound form continues to increase. A possible explanation for this is that at first pigment is synthesized faster than it can be incorporated into membrane at the existing rate of membrane synthesis and that after 1.5-2 h the rate of synthesis of membrane components, possibly specific for thylakoid formation, has increased to parallel more closely that of pigment synthesis. This would be expected to result in a leveling off of the unbound pigment, a continued increase in the bound pigment, and an increase in membrane resulting in definite invaginations of the cytoplasmic membrane and the formation of distinct internal vesicles, all of which we observed.

Although our results cannot resolve whether pigment is incorporated uniformly into the entire cytoplasmic membrane or only into specific sites at which invagination then occurs, there are several factors which indicate that the latter is more probable. First, as presented here and in more detail previously [3], energy transfer and activity of the photosynthetic reaction centers occurs nearly simultaneously with the incorporation of pigment. Therefore, even at extremely low pigment densities the carotenoids, bulk chlorophyll, and reaction center pigment molecules must be incorporated within 50-100 A of one another, the distance required for significant energy transfer [32]. An estimate of the total membrane surface area per cell prior to the formation of internal vesicles can be obtained from the cell dimensions. We used an average cell length of 2 μ and an average diameter of 0.5 μ. From the membrane area, the number of cells per ml of culture and the bacteriochlorophyll concentration, we estimate average values of $(220\ A)^2$, $(127\ A)^2$ and $(96\ A)^2$ of membrane surface area per molecule of bacteriochlorophyll

at the 60, 100 and 140 min stages, respectively. Significant energy transfer would not be expected to occur if the pigments were distributed uniformly throughout the cells of the culture with this spacing. The pigment molecules, therefore, are more likely incorporated at discrete sites and not at random around the total membrane surface. Second and consistent with the above, our data indicate that after 90 min of semi-aerobic growth the relative distribution of reaction centers to the bulk pigments is such that photochemical utilization of the absorbed excitation energy is equivalent to that found in fully pigmented cells. This also implies that even at these very low pigment densities there are "photosynthetic membrane areas" comparable to those in fully pigmented cells. Third, we find that although the formation of distinct vesicles and their increase in number is not initially proportional to the increase in pigment, there is a subsequent gradual increase in the number of internal vesicles. These vesicles were found to arise from invaginations around the total periphery of the cell. Furthermore, in subsequent stages of development, when some vesicles had begun to penetrate more deeply into the cytoplasm and to proliferate, new invaginations continued to occur. This is most easily explained if new sites of pigment synthesis and incorporation are constantly arising around the cytoplasmic membrane. If pigment is incorporated at discrete sites which then invaginate, it raises an interesting question as to the type of control mechanism involved. Are new sites continually being synthesized, is there a controlled activation of previously existing sites, or do both occur?

Pigment synthesis and the attainment of photochemical activity have usually been associated with the formation of an isolatable chromatophore fraction in both *Rps. spheroides* and *R. rubrum*. Our results, using the increased sensitivity of fluorescence measurements, clearly indicate that functional pigment incorporation precedes significant membrane formation in *Rps. spheroides*. These results are not inconsistent with reports that purified chromatophores from both *R. rubrum* [34] and *Rps. spheroides* [33] have a constant composition when isolated from cells of different pigment content, and that the purified chromatophore is a stoichiometric complex of pigment and other membrane constituents. Although we find an initial lack of correlation between pigment and internal vesicle number, we have attributed this to an increase in the number of new sites of incorporation arising within the cell. Once an invagination and the formation of a distinct vesicle has occurred, the subsequent incorporation of pigment may well occur stoichiometrically. Furthermore, Oelze *et al.* [5] have reported that in repigmenting *R. rubrum* pigment is also initially incorporated into membrane without the formation of internal vesicles or isolatable chromatophores. In a second phase, chromatophores were isolated but their specific bacteriochlorophyll content increased with increasing cellular pigment content, and only in the final phase of

development did the specific bacteriochlorophyll content of the chromatophores, which were larger in diameter than those of the second phase, remain constant. A mechanism of pigment incorporation such as this could also occur during repigmentation in *Rps. spheroides* in light of the very similar sequence involved in the initial stages of thylakoid morphogenesis in the two organisms.

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