Biochimica et Biophysica Acta, 440 (1976) 429–447

BBA 47141

MEMBRANES OF *RHODOPSEUDOMONAS SPHAEROIDES*

IV. ASSEMBLY OF CHROMATOPHORES IN LOW-AERATION CELL SUSPENSIONS*

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(Received January 30th, 1976)

SUMMARY

Chromatophore membrane formation was induced in low-aeration suspensions of *Rhodopseudomonas sphaeroides* and highly purified chromatophore preparations were isolated at various intervals between 4 and 18 h. The levels of several functional components associated with the isolated structures were investigated. *B*-875, the light-harvesting bacteriochlorophyll complex associated with the reaction center, was preferentially inserted into the chromatophore membrane during the early stages of induction, and thereafter its levels reached a steady state; *b*- and *c*-type cytochromes were also maintained at essentially constant levels. In contrast, the levels of *B*-850, the accessory light-harvesting bacteriochlorophyll, together with its associated protein, continued to increase throughout the induction process. Increases in the levels of the major carotenoid component followed a similar course. These findings are consistent with a stepwise assembly mechanism for associated bacteriochlorophyll and protein components and suggest that separate regulatory mechanisms control the levels of functionally essential and accessory components within the membrane.

INTRODUCTION

The sequence in which newly formed structural and functional components are inserted into developing organelle membranes is a question that is central to the understanding of the biogenesis of these structures. Is the functional organelle membrane formed in a single step in which all the constituents are simultaneously inserted, or do the functional components enter the membrane in a multi-step assembly process? Cyclic fluctuations in photosynthetic activities during the cell cycle of

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Chlamydomonas reinhardi have been shown to result from the multi-step assembly of components in the chloroplast thylakoid membranes [1]. In contrast, estimates of bacteriochlorophyll *a* and protein levels in the photosynthetic apparatus (chromatophore) of *Rhodopseudomonas sphaeroides* formed after adaptation to photosynthetic growth conditions have suggested that bacterial photosynthetic membranes are assembled in a synchronous manner [2]. The results of measurements of bacteriochlorophyll fluorescence during chromatophore development have been interpreted in terms of a simultaneous assembly of the photosynthetic pigment components [3].

A convenient approach for the study of membrane assembly involves spectral probes of the several bacteriochlorophyll complexes in the chromatophores of *Rps. sphaeroides* [4, 5]. These include two light-harvesting components which comprise more than 95 % of the total bacteriochlorophyll: *B*-850 with absorption bands at 800 and 850 nm, and *B*-875 which absorbs maximally at 875 nm. These antenna components harvest light energy and transfer it to the photochemical reaction center which contains the remaining bacteriochlorophyll. The reaction center, which is the exclusive site for productive photochemistry in the membrane, has absorption bands at 870 and approx. 805 nm that are attributed to reaction center bacteriochlorophyll [4]. On the basis of a linear relation between levels of reaction center bacteriochlorophyll and *B*-875 in cells of different bacteriochlorophyll contents, Aagaard and Sistrom [5] have concluded that a fixed stoichiometry exists between these two components. Since the levels of the light-harvesting bacteriochlorophyll absorption bands at 800 and 850 nm are invariant [6], changes in the ratio of absorption at 875 nm to that at 850 nm reflect overall changes in the levels of each bacteriochlorophyll complex in the membrane.

An additional consideration that provides a basis for the present study is the recent finding that several of the above spectroscopic entities result from complexes between bacteriochlorophyll and specific proteins of the chromatophore membrane in *Rps. sphaeroides* [7–11]. Three polypeptide components are associated with the photochemical reaction center [7–10]; they have molecular weights of 28 000, 24 000, and 21 000 [8], and have been designated as reaction center *a*, *b* and *c*, respectively [9]. The *b* and *c* components have been isolated in a photochemically active form in association with reaction center bacteriochlorophyll [7, 8]. A polypeptide of approx. 10 000 molecular weight [12, 13] has been identified as a component of the *B*-850-light-harvesting bacteriochlorophyll complex [11]. Estimates of the levels of these protein components indicate that the three reaction center polypeptides are present in equimolar quantities [8, 9] and together comprise about 25 % of the total chromatophore protein [9], whereas protein associated with light-harvesting bacteriochlorophyll accounts for approx. 40–50 % [13]. Immunological evidence [2, 13, 14] and studies with various photosynthesis-deficient mutants [10, 15] indicate that these protein components are specific to the chromatophore and are not present in other cellular fractions.

Studies on the levels of these spectral probes and their associated protein components during the development of the photosynthetic apparatus are particularly useful for the elucidation of mechanisms involved in the assembly of a major and specific portion of the chromatophore membrane. Aagaard and Sistrom [5] demonstrated that during reinitiation of bacteriochlorophyll synthesis by reducing the oxygen tension in aerobically growing *Rps. sphaeroides*, the ratios of reaction center bacterio-

chlorophyll to total bacteriochlorophyll and the absorption at 875 nm relative to that at 850 nm were increased over a period of approx. 100 min. Takemoto [16] showed that in concentrated suspensions of *Rps. sphaeroides* a preferential insertion of reaction center bacteriochlorophyll occurs during the first hour after a transfer from high to low aeration. With an unresolved membrane fraction, it was shown that the reaction center polypeptides are also preferentially inserted during this period. In contrast, light-harvesting bacteriochlorophyll-associated protein was nearly undetectable until the end of the first hour; between 1 and 3 h, it represented the majority of the protein synthesized under these conditions [16]. In the present study, the incubation of low-aeration cell suspensions of *Rps. sphaeroides* has been extended to 16–18 h, permitting the isolation of sufficient quantities of highly purified chromatophores to provide a critical test of the possibility that the observed changes occur in the developing photosynthetic apparatus. Levels of the light-harvesting bacteriochlorophyll complexes have been measured over this period in both isolated chromatophores and whole-cell suspensions. Other functional components have also been assessed in the isolated photosynthetic membranes. The results demonstrate that although the *B*-850 and carotenoid levels of the isolated chromatophores continue to increase throughout the course of the incubation, *B*-875 and other functional constituents have reached their steady-state levels within the first 6 h of the induction process. (A preliminary report of some of these studies has appeared: Langan, J. J. and Niederman, R. A. (1975) Abstr. Annu. Meet. Am. Soc. Microbiol. p. 177.)

MATERIALS AND METHODS

Chromatophore induction. Chromatophore formation was induced in concentrated cell suspensions of *Rps. sphaeroides* NCIB 8253 by a modification of the procedure of Takemoto and Lascelles [15]. All growth and cell suspension experiments were performed at 30 °C using the malate/glutamate medium of Lascelles [17] which contained 7.3 μM $MnSO_4$ and 0.1 % yeast extract. Aerobic growth was carried out in a 10-l fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) with vigorous aeration for 7–8 h to a final absorbance of 0.94–1.05 measured at 680 nm on the red-sensitive phototube of a Beckman DU spectrophotometer (1-cm light path). The inoculum for the fermentor was a 500-ml culture grown in a 2.8-l Fernbach flask for 14 h on a gyrotory shaker (New Brunswick Model G-25) at 350 rev./min. The fermentor culture was harvested aseptically at 4 °C in a Sorvall GS-3 rotor at 9000 rev./min (13 700 $\times g$) for 20 min in a Sorvall RC2-B centrifuge. The cells were resuspended in fresh medium to an $A_{680\ nm} = 2.1$–2.3 and dispensed in 400-ml quantities into 500-ml Erlenmeyer flasks that were incubated in the dark at 200 rev./min on the gyrotory shaker. Aliquots of the cell suspension were harvested after 4, 6, 8, 12 and 16–18 h of incubation.

Isolation of chromatophores. All isolation procedures were performed at 0–4 °C with 1 mM Tris · HCl buffer (pH 7.5) [18]. The harvested cells were washed twice in buffer, and resuspended to a final concentration of 0.2–0.4 g wet weight per ml. RNAase A and DNAase I (both obtained from Worthington Biochemical Corp., Freehold, N. J.) were each added to a concentration of 10 μg/ml and the cell suspensions were passed twice through a French pressure cell (American Instrument Co.,

Inc., Silver Spring, Md.) operated at 20 000 lb/inch^2. The resulting extracts were centrifuged at 9000 rev./min (10 000 $\times g$) in a Sorvall SS-34 rotor for 10 min. The supernatant fractions were centrifuged at 60 000 rev./min (254 000 $\times g$) for 75 min in a Beckman type 60 Ti rotor in a Beckman Model L2-65B preparative ultracentrifuge. The precipitates were resuspended by homogenization in approx. 1 ml of buffer for each original g of wet weight. The resuspended precipitates (2.5–3.0 ml) were layered on linear 5–30 weight % sucrose gradients prepared over a 4-ml cushion of 60 weight % sucrose. All sucrose solutions were prepared in buffer. The gradients were centrifuged in a Beckman SW 27 rotor at 27 000 rev./min (96 000 $\times g$) for 230 min ($11.0 \cdot 10^{10}\ \omega^2 t$ units). Fractions of 24 drops were collected by upward flow displacement as described previously [19] except that a polystaltic pump (Buchler Instruments, Inc., Fort Lee, N.J.) was employed. Fractions were diluted in distilled water and absorbance was determined on a Beckman DU spectrophotometer at 850 nm, and at 260 and 280 nm on a Gilford spectrophotometer. Appropriate chromatophore fractions were pooled, diluted in buffer, and sedimented at 254 000 $\times g$ for 75 min. They were resuspended in buffer to a concentration of 6–9 mg of protein per ml.

Analytical procedures. Bacteriochlorophyll and carotenoids were determined by acetone/methanol (7 : 2, v/v) extraction as described by Cohen-Bazire et al. [20]. An extinction coefficient of 82 $mg^{-1} \cdot cm^{-1}$ at 770 nm was used for bacteriochlorophyll which was calculated from the molar extinction coefficient of Clayton [21]. Levels of red carotenoid (spheroidenone) and yellow carotenoid (spheroidene) were calculated from the equations of Cohen-Bazire et al. [20] with the aid of a computer program. Protein was determined by the method of Lowry et al. [22] with crystalline bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.) as a standard. All samples were maintained in 0.5 M NaOH for 60 min at 40 °C prior to the color reaction [23].

Levels of the bacteriochlorophyll complexes in isolated chromatophores and in whole cell suspensions were determined from near-infrared absorption spectra obtained with a Cary Model 14 spectrophotometer using quartz cells with a 1-cm light path. Spectra of whole-cell suspensions were determined by the method of Sojka et al. [24]. Sedimented cells were resuspended in 0.1 ml of distilled water and 0.9 ml of bovine serum albumin (30 %, Sigma Chemical Co., St. Louis, Mo.) was added. A cuvette containing 0.1 ml distilled water plus 0.9 ml of 30 % bovine serum albumin was placed in the reference beam. Levels of the light-harvesting bacteriochlorophyll complexes were calculated with a computer program using the equations of Aagaard and Sistrom [5] for *B*-875, and the approximations of Crounse et al. [25] to correct the absorbancies of the *B*-800, *B*-850, and *B*-875 bands.

Cytochrome difference spectra of the isolated chromatophores were obtained with a Cary Model 118C spectrophotometer equipped with a scattered transmission accessory (Varian Instrument Division, Springfield, N. J.). Chromatophore samples (100 μg of protein per ml in 0.01 M Tris buffer, pH 7.5) in quartz cells with a 1-cm light path were placed in both the sample and reference beams. A few crystals of sodium hydrosulphite were added to the material in the sample beam and spectra were scanned repeatedly until a steady-state reduction was achieved, usually within 20–30 min. An estimate of the total cytochrome content was made from the Soret peak which apparently represents a composite of *b*- and *c*-type cytochromes [18]. For this purpose, the difference between the absorbancies at 427 and 407 nm was calculated.

Dodecyl sulphate-polyacrylamide gel electrophoresis was performed as described by Laemmli [26] with the following modifications: both the stacking gel (3 % acrylamide) and the separation gel (10 % acrylamide) were chemically polymerized with 0.15 % *N,N,N',N'*-tetramethylethylenediamine and 0.06 % $(NH_4)_2S_2O_8$; a 0.1 ml final sample volume which contained 200 μg of protein was employed; the samples were immersed in boiling water for 75 s; and electrophoresis was carried out at 2 mA per gel for the first 60–90 min and at 5 mA per gel for an additional 7 h. The gels were fixed in a solution containing 9.2 % glacial acetic acid and 45.4 % methanol, stained for 4 h in the Coomassie brilliant blue (Schwarz/Mann, Orangeburg, N.Y.) staining solution of Weber and Osborn [27] and destained at 55 °C with 7 % glacial acetic acid. Approximate molecular weights were assigned to the polypeptide species associated with light-harvesting bacteriochlorophyll and the photochemical reaction center by comparison of their relative mobilities in the gels with those of protein standards using the molecular weight values published by Weber and Osborn [27]. For this purpose, the gels were scanned at 550 nm on a Gilford spectrophotometer equipped with a linear transport device. Molecular weights were determined from a plot of R_F vs. log of molecular weight fitted to a straight line by the method of least squares. Standards employed were catalase (EC 1.11.1.6, bovine liver), myoglobin (Type I, equine skeletal muscle), and cytochrome *c* (Type III, equine heart) from Sigma Chemical Co.; bovine serum albumin (Miles Laboratories), albumin (hen's egg) and lysozyme (hen's egg) from Calbiochem, La Jolla, Calif., and γ-globulin (human) from Schwarz/Mann.

RESULTS

Induction of chromatophore formation in concentrated cell suspensions

Chromatophore formation was induced at reduced oxygen tension in concentrated suspensions of *Rps. sphaeroides* that were essentially devoid of bacteriochlorophyll. Under these conditions, an extensive and rapid formation of photosynthetic membranes and their associated photopigment components occurred without appreciable lag (Fig. 1). Bacteriochlorophyll synthesis continued throughout the course of the incubation for periods up to 18 h, as demonstrated by the parallel increases in the $A_{850\ nm}$ of the cell suspensions and the 770-nm absorbance of acetone/methanol extracts. Similarly, the carotenoid levels continued to increase in the suspensions and the accumulation of spheroidenone was far in excess of that for spheroidene. In contrast, the small increases in $A_{680\ nm}$ indicate that under these conditions, only limited division occurred in the concentrated cell suspensions. These data extend and confirm those recently presented by Takemoto [16].

Isolation of chromatophores during induction

As a test of the possibility that changes observed in the levels of bacteriochlorophyll complexes [5, 16] and their associated polypeptide components [16] in crude membrane preparations reflect those which occur in the developing photosynthetic apparatus, highly purified chromatophore preparations were isolated from the low-aeration cell suspensions. In this procedure, crude particulate fractions were prepared at the beginning of induction and at several intervals thereafter and the

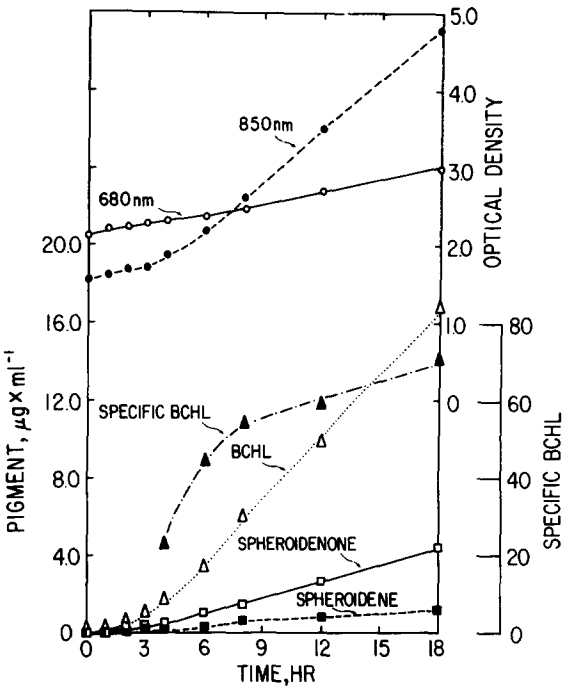

Fig. 1. Kinetics of chromatophore induction in low-aeration cell suspension. Specific bacteriochlorophyll (μg bacteriochlorophyll per mg protein) was determined on highly purified chromatophores isolated as described in Materials and Methods. The remaining values were determined directly on the whole-cell suspensions. Levels of the photosynthetic pigments were determined by the procedures of Cohen-Bazire et al. [20]. The bacteriochlorophyll concentration of the cells at the start of the experiment was 0.015 μg per ml. BCHL, bacteriochlorophyll *a*.

resulting subcellular particles were separated on the basis of sedimentation velocity on discontinuous sucrose gradients (Fig. 2). Unfolded and partially degraded ribosomes banded near the top of the gradient; a peak in their 260-nm absorbance profile was observed in fractions 4–7 (not shown). The absence from the 0 h sample of any appreciable absorbance at 850 nm (the in vivo absorption maximum of bacteriochlorophyll in *Rps. sphaeroides*) confirms previous observations [2, 28] which indicate that cells grown at high aeration contain no chromatophores. In contrast, a detectable amount of material absorbing at 850 nm was observed at 4 h which greatly increased as induction continued. This was accompanied by the appearance of a bacteriochlorophyll-rich fraction banding at approx. 16–20 weight % sucrose. The 260 nm/280 nm absorbance ratio of this fraction approached 1.0, characteristic of pure chromatophores [2, 18, 19, 29]; a marked increase in the 850 nm/280 nm absorbance was also observed which is reflected by specific bacteriochlorophyll values (see below). Despite the rigorous exclusion of divalent cations during the isolation procedure to minimize non-specific chromatophore-cell envelope interactions [18], some 850 nm-absorbing material was also observed in the cell envelope (fractions 19–23) from induced samples. This represented a decreasing proportion of the total bacteriochlorophyll-containing membrane as chromatophore development proceeded; chromatophore

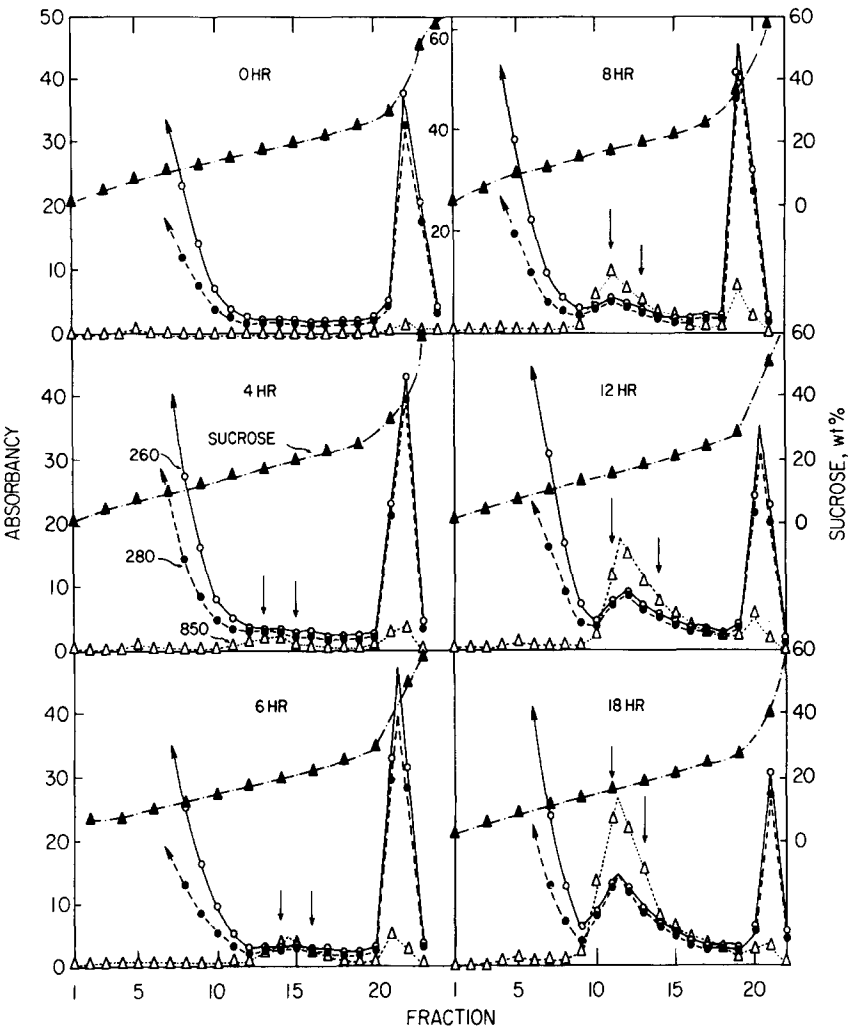

Fig. 2. Isolation of chromatophores at various stages of induction by velocity sedimentation in discontinuous sucrose density gradients. Sucrose concentrations were determined on a refractometer. Absorbance of diluted fractions was determined at 260, 280 and 850 nm. Sedimentation is to the right. Additional experimental details are provided in Materials and Methods. The arrows denote those portions of the chromatophore bands that were pooled for further analysis.

protein never accounted for more than about 13 % of the total protein in the envelope fraction. It is not known whether this represents a casual contamination of the cell envelope with chromatophores or a stage in chromatophore development [30, 31]. In some experiments, a linear 5–60 weight % sucrose gradient [18, 19] was employed. This usually resulted in chromatophore fractions of lower specific bacteriochlorophyll content with the earlier samples, indicating some contamination with protein of non-chromatophore origin.

Assembly of functional components in the chromatophore

Since chromatophores isolated early in induction had a significantly lower specific bacteriochlorophyll content than those isolated at the later stages (Fig. 1), near-infrared absorption spectra were obtained to test if the reduced bacteriochlorophyll levels reflected differences in relative amounts of the light-harvesting bacteriochlorophyll complexes. The results of such a spectral analysis with the isolated chromatophore fractions are shown in Fig. 3. It can be seen that the shoulder at approx. 875 nm is much more pronounced in the 4- and 6-h samples than in samples isolated in the later stages of induction. In contrast, the levels of the 800-nm component of the *B*-850 complex were reduced in the early samples and increased in subsequent stages of the chromatophore induction process. These data are consistent

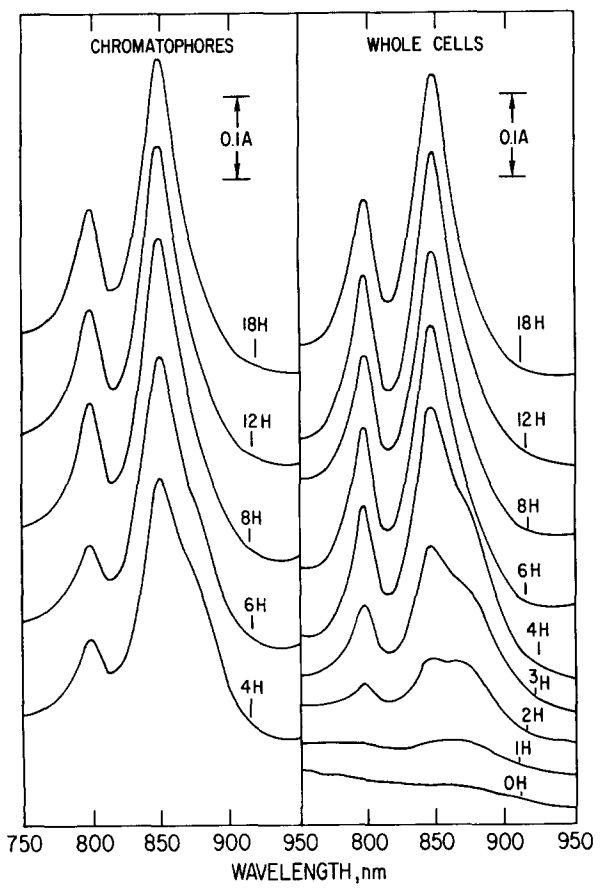

Fig. 3. Near-infrared absorption spectra of isolated chromatophore preparations and whole-cell suspensions. Highly purified chromatophores were isolated as described in Materials and Methods and each sample contained 3.0 μg of bacteriochlorophyll per ml in 0.01 M Tris buffer (pH 7.5). The spectra with the whole-cell suspensions were obtained by the method of Sojka et al. [24]. Whole-cell samples contained the following quantities of bacteriochlorophyll per ml: 0 h, 0.04 μg; 1 h, 0.29 μg; 2 h, 1.0 μg; 3 h, 2.0 μg; 4 h and subsequent samples, 3.0 μg. All cuvettes contained a final volume of 1.0 ml.

with a significantly higher level of *B*-875, relative to *B*-850 in chromatophores isolated early in induction.

To assure that the observed spectral changes reflected alterations in the in vivo photosynthetic apparatus and that they did not result from artifacts caused by the chromatophore isolation procedure, spectra were obtained with whole-cell suspensions. This also permitted observations very early in the induction process when the isolation of sufficient quantities of chromatophores for these measurements was not possible. The results are also shown in Fig. 3. By 60 min, the light-harvesting bacteriochlorophyll complexes can be clearly visualized in the whole cells and during this period, *B*-875 appears as the major species. At 3 h, the absorption maximum has shifted to 850 nm and thereafter, the relative levels of *B*-850 are greatly increased. These spectral results taken together with those from the isolated chromatophores suggest that in low-aeration cell suspensions, the light-harvesting bacteriochlorophyll complexes are inserted into the chromatophore membrane in an asynchronous manner. This was confirmed by a computer-assisted analysis of these spectra; the results are shown in Table I. In the whole cell preparations, it can be seen that the ratio of the reaction center-associated *B*-875 complex to total bacteriochlorophyll was initially high and decreased as induction continued. This was also reflected in the ratio of absorbance at 875 nm to that at 850 nm corrected by an independent technique [25]. A close correlation between these values and those for the isolated chromatophores is also seen in Table I. The 800 nm/850 nm absorbance was essentially constant in whole-cell preparations; this is consistent with the invariant levels of these *B*-850 components demonstrated by Sistrom [6] for cells of different bacteriochloro-

TABLE I

RATIOS OF LIGHT-HARVESTING BACTERIOCHLOROPHYLL COMPLEXES IN WHOLE-CELL AND CHROMATOPHORE PREPARATIONS DURING INDUCTION

Absorbance at 800, 850, and 875 nm corrected by the method of Crounse et al. [25]. Molar ratio of *B*-875 relative to total bacteriochlorophyll calculated by the method of Aagaard and Sistrom [5].

Preparation	Time (h)	$\frac{A_{800\ nm}}{A_{850\ nm}}$	$\frac{A_{875\ nm}}{A_{850\ nm}}$	$\frac{B\text{-}875}{\text{Total bacteriochlorophyll}}$
Whole cells*	0	0.70	1.87	0.61
	1	0.76	1.64	0.61
	2	0.76	0.95	0.46
	3	0.78	0.68	0.36
	4	0.73	0.49	0.24
	6	0.75	0.36	0.16
	8	0.68	0.30	0.10
	12	0.66	0.23	0.05
	16	0.68	0.21	0.04
Chromatophores	4	0.46	0.54	0.30
	6	0.46	0.39	0.19
	8	0.51	0.30	0.12
	12	0.51	0.26	0.07
	18	0.51	0.22	0.03

* The whole cell preparations were from a different experiment than those shown in Fig. 2. Greater amounts of the earlier samples were used to permit more precise estimates of the absorbance ratios.

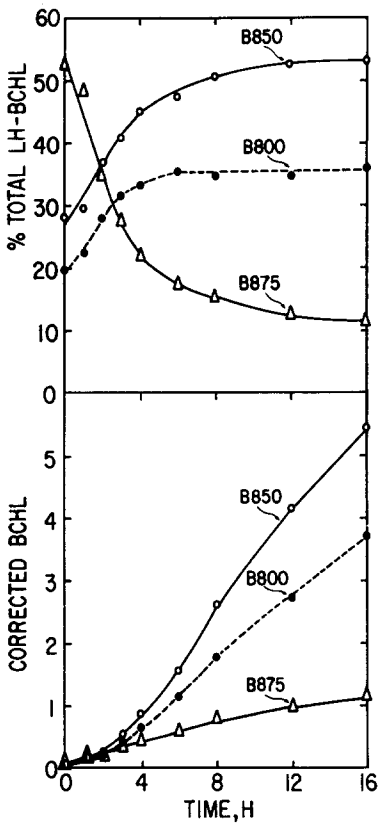

Fig. 4. Relative light-harvesting bacteriochlorophyll concentrations and kinetics of synthesis of the bacteriochlorophyll spectral bands in low-aeration cell suspensions. Although the total light-harvesting bacteriochlorophyll concentration represented by the near-infrared absorption spectrum can be entirely accounted for in the 850 and 875 nm bands [6], total light-harvesting bacteriochlorophyll is expressed here in terms of each of the three absorption bands to facilitate comparison of their relative proportions during induction. In the bottom panel, the relative heights of the absorption bands have been corrected for cell division during induction and bacteriochlorophyll is expressed in arbitrary absorbance units per ml of cells. The data in both panels have been calculated from that presented in Table I.

phyll contents. Although this ratio was also constant in the isolated chromatophores, the absolute absorbance of the 800-nm band was lower; this may reflect a consistent loss of this labile component during purification.

The relative levels of the light-harvesting bacteriochlorophyll components and the kinetics of their synthesis calculated from the whole-cell data are presented in Fig. 4. In the top panel, it is seen that initially, *B*-875 is the major light-harvesting bacteriochlorophyll complex, but after 3 h and throughout the remainder of the induction process, the 800 and 850 nm components of the *B*-850 complex greatly predominate. A coordinate increase in the levels of the 800 and 850 nm bands is also observed. A striking similarity is seen between this plot and that of Sistrom [6] in which the corrected absorbance at 800, 850 and 875 nm was plotted against the specific bacterio-

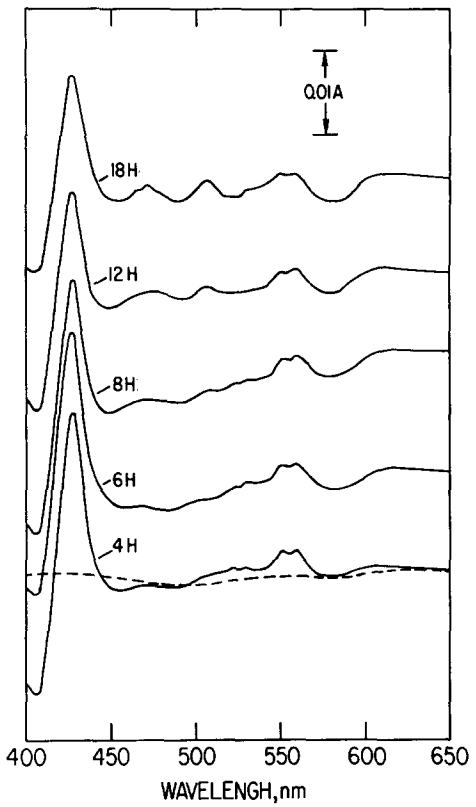

Fig. 5. Reduced-minus-oxidized difference spectra of chromatophore preparations isolated at various stages of induction. Chromatophore samples were isolated as described in the text. The dashed line represents an oxidation baseline for a 4 h chromatophore sample prior to sodium hydrosulphite addition. Other experimental details are described in Materials and Methods.

chlorophyll values for extracts from cells of different bacteriochlorophyll contents. The data presented here for low-aeration cell suspensions support the conclusion [6] that in the near-infrared absorption spectrum of *Rps. sphaeroides*, the total concentration of light-harvesting bacteriochlorophyll can be accounted for in the two independently variable *B*-875 and *B*-850 components.

The bottom panel in Fig. 4 indicates that the synthesis of the *B*-875 and *B*-850 complexes exhibit distinct kinetics. The rate of *B*-875 synthesis was essentially constant over the entire 16 h induction period and there was a 7-fold increase in *B*-875 levels. In contrast, the rate of synthesis of the 800 and 850 nm components is accelerated after 2 h and the final levels at 16 h represent 60- and 63-fold increases, respectively, over those initially present.

In Fig. 5, dithionite-reduced-minus-oxidized difference spectra of the isolated chromatophore preparations are shown. The α bands in the 558 and 551 nm regions and the Soret peak at approx. 427 nm suggest that *b*- and *c*-type cytochromes are present in each sample. These cytochromes represent a tightly bound or entrapped chromatophore increment of the total cytochromes present in the cell [32, 33]. The

TABLE II

LEVELS OF PHOTOSYNTHETIC-PIGMENT COMPONENTS AND CYTOCHROMES IN CHROMATOPHORE PREPARATIONS DURING INDUCTION

Time (h)	Specific bacterio-chlorophyll*	Specific light-harvesting bacteriochlorophyll**			Specific carotenoid*		Specific cyto-chrome***
		B-800	*B*-850	*B*-875	Spheroidenone	Spheroidene	
4	23.0	5.3	11.5	6.2	8.1	3.2	31.8
6	44.5	9.8	25.0	9.7	16.1	6.4	31.1
8	54.5	15.7	29.8	9.1	17.0	6.3	28.6
12	59.1	17.5	32.6	8.9	19.0	6.2	25.5
18	70.6	21.7	40.3	8.6	22.0	6.1	28.0

* Expressed as μg of respective component per mg protein.

** Calculated from absorbance at 800, 850, and 875 nm corrected by the method of Crounse et al. [25]. The absolute bacteriochlorophyll concentration in the *B*-850 complex can be entirely accounted for in the 850 nm band [6]. Thus, the values for each of the spectral bands have been expressed in arbitrary absorbance units on a protein basis to facilitate comparison of their relative proportions during induction.

*** Expressed as 10^2 $A_{427-407\,nm}$ per mg protein.

heights of the α bands of the *b*- and *c*-type cytochromes are essentially equal in each of the samples. Similar ratios of these components have previously been observed in chromatophore and cell envelope fractions from phototrophically grown cells [18] and in membrane fractions from cells grown aerobically [28, 34] and semi-aerobically [34]. The bands which have appeared near 470 and 510 nm in the later samples in Fig. 5 result from changes in the spectrum of the carotenoids upon dithionite reduction [34]. Similarly, the changes near 600 nm in these samples may result from alterations in an in vivo bacteriochlorophyll band absorbing in this region. These changes reflect the increased level of the photopigment components in the later chromatophore samples. The apparently constant ratios of the cytochrome α bands together with estimates of the total cytochrome levels shown in Table II are consistent with the possibility that unlike the light-harvesting bacteriochlorophyll components, the cytochromes become associated with the chromatophore in a coordinated manner and are maintained at steady-state levels throughout the induction process.

The levels of the various photopigment components in the isolated chromatophores are also presented in Table II. Within the first 6 h of induction, the *B*-875 levels have reached plateau values. In contrast, the specific bacteriochlorophyll, *B*-850, and spheroidenone content continue to increase throughout the induction process. By 18 h, the specific bacteriochlorophyll value has approached that observed in steady-state chromatophores from phototrophically grown cells [18]. The increases in the bacteriochlorophyll content of the chromatophores isolated during induction were largely accounted for by the elevated levels of the *B*-850 components (4.1- and 3.5-fold for the 800 and 850 nm bands, respectively). The specific bacteriochlorophyll of the chromatophores increased by 2.4-fold between 4 and 8 h and only 1.3-fold over the remaining 10 h. On the other hand, the increases in cellular bacteriochlorophyll follow an essentially linear course (Fig. 1). These data suggest that the bacterio-chlorophyll and carotenoid components are initially incorporated into photopigment-

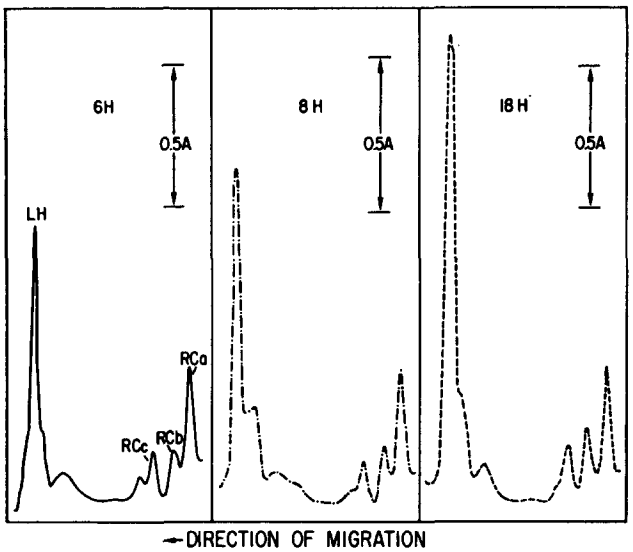

Fig. 6. Dodecyl sulphate-polyacrylamide gel electrophoresis of chromatophore preparations isolated at various stages of induction. Highly purified chromatophores were isolated as described in Materials and Methods. Electrophoresis was performed by the method of Laemmli [26] modified as indicated in the text. The stained gels [27] were scanned at 550 nm on a Gilford spectrophotometer equipped with a linear transport device. LH refers to light-harvesting bacteriochlorophyll-associated protein [11–13] and RC_a, RC_b, and RC_c denote the polypeptides associated with the photochemical reaction center [7–9]. The height of the reaction center a band has been equalized with each gel. Chromatophore polypeptide bands of molecular weights greater than that of reaction center a have been omitted from the figure.

depleted membrane and that as development proceeds, their levels eventually approach a steady state. However, the various components are interspersed asynchronously: the functionally essential *B*-875 and cytochrome components are preferentially inserted during the early stages of induction and are maintained thereafter in an apparent fixed stoichiometry; the levels of the accessory antenna components (*B*-850 and the major carotenoid) continue to increase in a stepwise manner and assume a steady state much later. It is possible that some of the cytochrome present in the chromatophore membrane in its early developmental stages has been conserved from the cytoplasmic membrane from which it had initially arisen [2, 30, 31].

To test if the above multistep assembly mechanism for the bacteriochlorophyll spectral entities also controls the levels of their associated protein components within the chromatophore membrane, the highly purified chromatophore preparations were electrophoresed on dodecyl sulphate-polyacrylamide gels (Fig. 6). The height of the reaction center *a* polypeptide band was equalized in each of the gels. Controls indicated that the levels of the individual protein bands in the gels were essentially proportional to their 550 nm absorbance over the concentration range employed. No differences in staining were detected when lipid and photopigments were removed by acetone/methanol (7 : 2, v/v) extraction. The photopigments ran ahead of the tracking dye and were sufficiently resolved from the low molecular weight polypeptide bands to permit estimates of the latter. It can be seen that as development proceeds,

the level of the low molecular weight polypeptide component associated with the *B*-850-light-harvesting bacteriochlorophyll complex has markedly increased relative to the polypeptide bands of the photochemical reaction center. A similar result was recently reported by Takemoto [16] with unresolved membrane fractions and the results presented here indicate that these changes have occurred in the developing photosynthetic apparatus.

The major polypeptide bands shown in Fig. 6 have been identified as follows: the apparent molecular weights of the bands designated as reaction centers, *c*, *b* and *a* were 22 400, 25 800, and 28 500, respectively, in close agreement with those recently reported by Okamura et al. [8]; the molecular weight of the band designated as light harvesting was estimated at 12 900 which is in agreement with other approximations [10–13]. With respect to the reaction center polypeptides, their 280-nm absorbance and their absorbance ratios at 550 nm after staining are consistent with the 1 : 1 : 1 stoichiometry reported for each of the components [8, 9]. Bacteriochlorophyll synthesis was shown to be coupled to the synthesis of each of the light-harvesting and reaction center protein bands [15] and they have been found to be associated with their respective bacteriochlorophyll complexes [10] in the strain of *Rps. sphaeroides* in the present study. The heterogeneity seen in the light-harvesting band has also been observed by Takemoto and Lascelles [10] and by Hall et al. [35] who resolved it into two components by independent electrophoretic techniques.

An approximation of the levels of the light-harvesting and reaction center protein components is presented in Table III. Since the staining of these proteins has not been shown to reflect their absolute levels within the membrane, the ratio of light-harvesting to reaction center bacteriochlorophyll-associated protein was arbitrarily set at 1.0 for the 6 h sample. It can be seen that between 6 and 18 h, the absorbance of the light-harvesting bacteriochlorophyll-associated band relative to that of the reaction center *a* band has increased by approx. 2-fold. This is in close agreement with

TABLE III

RELATIVE RATIOS OF THE POLYPEPTIDES ASSOCIATED WITH LIGHT-HARVESTING BACTERIOCHLOROPHYLL AND REACTION CENTERS IN CHROMATOPHORE PREPARATIONS DURING INDUCTION

Relative polypeptide levels were estimated in stained polyacrylamide gels from the absorbance of the respective peaks at 550 nm. Light-harvesting protein is the major polypeptide band of approx. 12 900 molecular weight associated with light-harvesting bacteriochlorophyll [11–13]. Reaction center protein represents the heavy polypeptide subunit of the reaction center [8, 9]. All samples were normalized to the 6 h sample in which the ratio was arbitrarily set at 1.0 (see text for details).

Time (h)	Light-harvesting protein / Reaction center protein
4	–*
6	1.00
8	1.25
12	1.60
18	1.90

* Not determined. The 4 h chromatophore preparation apparently contained low molecular weight polypeptide bands [16] which obscured measurements of the light-harvesting protein band.

the increases in the levels of the *B*-850-associated spectral components that has occurred over the same time period (Table II).

DISCUSSION

Much confusion presently exists in the literature with regard to the term "chromatophore". Indeed this designation for the bacterial photosynthetic apparatus was abandoned altogether in the recent review of Oelze and Drews [36] and replaced by "intracytoplasmic membrane." We have previously presented evidence which suggests that the photosynthetic apparatus of *Rps. sphaeroides* is localized on membranes that are separable and largely distinct from the cell wall-cytoplasmic membrane complex [18, 19]. In addition, a single system of chromatophore membranes was unambiguously identified [18]. However, the question of the physical separation of the chromatophore membrane from the peripheral cytoplasmic membrane has not been entirely resolved. Nevertheless, an operational definition for the chromatophore membrane from the standpoint of its biogenesis would seem appropriate at this time. In a previous study [28], and as shown in Fig. 2, no chromatophores were detected by sucrose density-gradient centrifugation of extracts from *Rps. sphaeroides* grown at high aeration. After the transfer of aerobically grown cells to conditions of low aeration, a photopigment-containing membrane band appears in the gradients at the usual position of chromatophores from extracts of phototrophically grown cells. We believe that the new band from the induced cells represents developing chromatophores. In the recent report of Huang and Kaplan [2], the limits for chromatophore formation in *Rps. sphaeroides* were defined; column chromatography on Sepharose 2B revealed no fraction which corresponded to chromatophores in cells grown at oxygen tensions of 5 % or greater, whereas at 2.5 % or below, these specialized photopigmented structures were observed. Thus, the induction and formation of bacterial photosynthetic membranes can be studied in cells that are initially devoid of this structure. Ultrastructural studies [37, 38] are also consistent with the absence of significant quantities of chromatophore membranes in cells grown at high aeration.

In the present study, an extensive and rapid formation of both bacteriochlorophyll and its associated photosynthetic membranes has been demonstrated in low-aeration suspensions of photopigment-bleached cells. Within 4 h after the onset of the induction process, it was possible to isolate much of the newly formed chromatophore membrane as a single component in sucrose density gradients. The conditions used for the induction of chromatophore membrane formation [15] have exploited the inverse relation between oxygen tension and bacteriochlorophyll synthesis [20]. These results, together with those of Takemoto [16] have confirmed that light is unnecessary for either the formation of photopigments or any other component of the photosynthetic apparatus [17, 39]. The lack of a significant lag in chromatophore formation observed here in low-aeration cell suspensions provides a distinct advantage over techniques which rely upon the adaptation of aerobically grown cells to conditions of anaerobic growth in the light. Under the latter conditions of chromatophore induction, a lag of at least 6 h occurs and a considerable turnover of membrane protein was demonstrated during this period [2]. In contrast, the pulse-chase studies of Takemoto [16] revealed no appreciable turnover of membrane proteins between 20 and 120 min after the onset of induction in low-aeration cell suspensions. A lack of significant

turnover of chromatophore proteins has also been reported for *Rps. sphaeroides* during steady-state phototrophic growth [30].

On the basis of results presented in this communication, it has been concluded that during chromatophore induction, bacteriochlorophyll components are interspersed into photopigment-depleted membrane. Thereafter, steady-state levels are approached where a chromatophore membrane of essentially constant composition is formed. A similar result has been reported for *Rps. sphaeroides* [2] and *Rhodospirillum rubrum* [40] after adaptation of aerobically grown cells to photosynthetic growth conditions. It is shown here that in *Rps. sphaeroides* these differences in specific bacteriochlorophyll content largely reflect increases in the *B*-850 antenna bacteriochlorophyll component. Increases in the spheroidenone content of the chromatophore preparations from the low-aeration cell suspensions are for the most part responsible for the increased carotenoid level. In these preparations, the spheroidenone to spheroidene ratio of approx. 3 : 1 is the reverse of that observed in chromatophores obtained from steady-state phototrophic cells, while the overall carotenoid levels of the two preparations are similar (Broglie, R. M. and Niederman, R. A., unpublished experiments). These differences can be accounted for by the effect of oxygen on carotenoid biosynthesis which results in a conversion of spheroidene to spheroidenone [41, 42] by an oxidation reaction in which a conjugated keto-group is formed [43]. The accumulation of elevated spheroidenone levels is apparently responsible for the red color of low-aeration cell suspensions and that of the chromatophores derived from them.

In contrast to the overall increases in the levels of the accessory *B*-850 and spheroidenone components within the chromatophore membrane observed throughout the induction process, other functional membrane constituents were maintained at essentially steady-state levels in the developing chromatophores. This was observed for both the reaction center bacteriochlorophyll-associated *B*-875 complex and *b*- and *c*-type cytochromes. An essential role for the *B*-875 complex is suggested by the fixed stoichiometry of *B*-875 and reaction center bacteriochlorophyll (approx. 30 molecules of *B*-875 per reaction center bacteriochlorophyll molecule) in cells of varying bacteriochlorophyll content [5], and the reduced levels of *B*-875 from mutants of *Rps. sphaeroides* lacking reaction center bacteriochlorophyll [44, 45]; one of these [44] also lacked all three reaction center polypeptides [9]. Both *b*- and *c*-type cytochromes are thought to have essential roles in the energy conservation that accompanies cyclic light-driven electron flow [46]. Cytochrome c_2 is directly photo-oxidized by reaction center bacteriochlorophyll [33], while a *b*-type cytochrome with an α band at 560 nm ultimately accepts electrons from components photoreduced by reaction center bacteriochlorophyll and is oxidized by cytochrome c_2 [46]. Another *b*-type cytochrome (reduced absorption maximum at 558–559 nm) is thought to be involved in substrate-linked non-cyclic electron flow. Functional roles for the multiplicity of other *c*-type [32] and *b*-type [46, 47] cytochromes found in *Rps. sphaeroides* have not been definitively assigned. Although photo-oxidation studies [33] suggest that the major *c*-type cytochrome in *Rps. sphaeroides* chromatophores is cytochrome c_2, at least three *b*-type cytochromes have been resolved potentiometrically [46, 48] and spectrophotometrically [46] in particulate fractions from photosynthetically grown cells. Thus, precise assignments for the stoichiometry of the overall *b*- and *c*-type hemes and the functionally essential cytochrome components during chromatophore

induction will require additional studies.

In accordance with the original suggestion of Aagaard and Sistrom [5], the wide variations observed here in the relative levels of the light-harvesting bacteriochlorophyll complexes in the chromatophore membrane indicate that the ability to synthesize *B*-850 is genetically independent from *B*-875 formation. Thus, a single mechanism may regulate the coordinated increases in the levels of the accessory light-harvesting components (*B*-850 and spheroidenone) observed throughout induction; this is supported by the lack of typical *B*-850 absorption bands in certain mutants which lack colored carotenoids [25]. An independent regulatory mechanism would assure a preferential insertion of *B*-875 (together with the photochemical reaction center, ref. 16) during the initial stages of chromatophore formation, and thereafter maintain them at steady-state levels. The latter mechanism may also regulate the levels of other functionally essential components associated with the developing chromatophore and data presented here is consistent with the possibility that the levels of *b*- and *c*-type cytochromes are maintained in this manner. Separate regulatory control for the synthesis of *B*-850 and *B*-875 would account for their asynchronous appearance in the developing photosynthetic apparatus. Recent evidence has suggested independent regulatory mechanisms for the synthesis of the various light-harvesting bacteriochlorophyll components in *Rhodospirillum molischianum* [49] and in *Rhodopseudomonas capsulata* [50].

Results presented here also support the possibility that bacteriochlorophyll is interspersed into the membrane in bacteriochlorophyll-protein complexes [51]. Estimates of the levels of chromatophore-specific polypeptide bands indicated an approximately 2-fold increase in the amounts of *B*-850-associated protein in the isolated chromatophores over a 12 h period. In contrast, the reaction center polypeptides were maintained at essentially constant levels during this interval. The pulse-labelling studies of Takemoto [16] also suggested a relationship of this type in the relative levels of these polypeptides. When the specific light-harvesting bacteriochlorophyll concentrations were calculated on the basis of a two-component spectrum [6], a 1.8-fold increase was observed for *B*-850 between 6 and 18 h, whereas *B*-875 levels remained unchanged. Although more precise estimates of the polypeptide levels will require further studies, these findings suggest that bacteriochlorophyll and its associated protein components combine in a fixed stoichiometry during their incorporation into the membrane. However, this mechanism may not be operative during the initial stages of chromatophore induction. In the fluorescence studies of Cellarius and Peters [3], an apparently unbound form of bacteriochlorophyll was detected during the first 30 min of a transition from high to low aeration; this may result from a lack of coordination in bacteriochlorophyll and protein synthesis at this stage.

In agreement with results obtained in the present investigation, studies with low-aeration suspensions of *Rps. capsulata* have demonstrated that the rate of bulk bacteriochlorophyll synthesis is not closely correlated to the development of photosynthetic capacity [52] or the formation of chromatophore membranes [53, 54]. In addition, Lien et al. [50] have shown that in *Rps. capsulata*, a close biosynthetic relationship exists between reaction center bacteriochlorophyll and the light-harvesting bacteriochlorophyll component with an absorption maximum at 880 nm; the levels of these components appeared to be coordinately regulated. In contrast, the

synthesis of the accessory light-harvesting bacteriochlorophyll component (absorption maximum located at 855 nm) was independently regulated. It was proposed that the 855 nm component is a primary target for the regulation of bacteriochlorophyll levels by changes in light intensity and oxygen tension. The suggestion was made that these regulatory phenomena may also be exerted upon the synthesis of specific protein components responsible for the expression of the individual molecular properties and membrane environments of the bacteriochlorophyll complexes. With respect to *Rps. sphaeroides* at least, our spectral and electrophoretic evidence is entirely compatible with this possibility.

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service grant GM20367 from the National Institute of General Medical Sciences, National Science Foundation grant BMS74-18151, and the Charles and Johanna Busch Memorial Fund Award to the Rutgers Bureau of Biological Research. The senior author is the recipient of Public Health Service Research Career Development Award GM00093-01 from the National Institute of General Medical Sciences. We thank Dr. David Lester for writing the computer programs and Avice R. Wilson for technical assistance. We are also grateful to Drs. Carl P. Schaffner and William Rouslin for the use of their Cary spectrophotometers and to Dr. Robert L. Hall for useful discussions.

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