The kinetics of light-driven electron flow and the nature of redox centers at apparent photosynthetic membrane growth initiation sites in *Rhodopseudomonas sphaeroides* were compared to those of intracytoplasmic photosynthetic membranes. In sucrose gradients, these membrane growth sites sediment more slowly than intracytoplasmic membrane-derived chromatophores and form an upper pigmented band. Cytochromes c1, c2, b6, and b56 were demonstrated in the upper fraction by redox potentiometry; c-type cytochromes were also detected electrophoretically. Signals characteristic of light-induced reaction center bacteriochlorophyll triplet and photooxidized reaction center bacteriochlorophyll dimer states were observed at a 3-fold reduced level on a reaction center basis in comparison to chromatophores. Flash-induced absorbance measurements of the upper pigmented fraction demonstrated reaction center primary and secondary semiquinone anion acceptor signals, but cytochrome b567 was photooxidized and cytochrome c1/c2 re- actions occurred at slow rates. This fraction was enriched approximately 2- and 4-fold in total b- and c-type cytochromes, respectively, per reaction center over chromatophores, but photooxidizable b-type cytochrome was lower. Measurements of respiratory activity indicated a 1.6-fold higher level of succinate-cytochrome c oxidoreductase was present in an upper pigmented band which sediments more slowly than chromatophores during rate-zone sedimentation in sucrose density gradients. Overall, the results suggest that complete cycles of rapid, light-driven electron flow do not occur merely by introduction of newly synthesized reaction centers into respiratory membrane, but that subsequent synthesis and assembly of appropriate components of the ubiquinol-cytochrome c2 oxidoreductase is required.

The facilitative phototrophic bacterium *Rhodopseudomonas sphaeroides* has provided a unique system for studies of the structure, function, and assembly of photosynthetic membranes (1-3). When grown under appropriate phototrophic conditions, *R. sphaeroides* elaborates an extensive system of intracytoplasmic photosynthetic membranes thought to be continuous with the peripherally localized cytoplasmic membrane. All of the components necessary for photosynthetic energy conversion are localized within the intracytoplasmic membrane which functions as the major cellular site for this process. These include a number of redox centers as well as the integral light-harvesting and photochemical reaction center complexes. Radiant energy collected by the extensive arrays of light-harvesting antennae is transferred to reaction center bacteriochlorophyll a where the excitations are rapidly transduced into a transmembrane charge separation (4). This initiates a cycle of electron transfer reactions between the primary iron-quine acceptor (Q₁⁺), the secondary acceptor quinone (Q₂), the ubiquinol-cytochrome c₂ oxidoreductase (bc₃) complex, cytochrome c₅₅₃, and the photooxidized reaction center bacteriochlorophyll a dimer [BChl]₅ (5). This ultimately results in the conservation of the light energy as an electrochemical proton gradient coupled to the synthesis of ATP (6, 7).

Upon cellular disruption, the intracytoplasmic membrane fragments into a uniform population of photosynthetically competent membrane vesicles termed chromatophores (3). Smaller membrane fragments are also produced that are present in an upper pigmented band which sediments more slowly than chromatophores during rate-zone sedimentation of cell-free extracts in sucrose density gradients (8, 9). Pulse-chase studies have suggested that the upper pigmented fraction is enriched in newly synthesized light-harvesting and reaction center bacteriochlorophyll a-protein complexes that are chased into the intracytoplasmic membrane (8, 9). This, together with studies on the sequence in which the pigment-protein complexes are assembled (10) and the results of cellular fractionation (9), fluorescence emission (11), and photochemical (12) studies are all consistent with the possibility that the upper pigmented fraction is derived in part from sites
at which growth and development of the intracytoplasmic membrane is initiated by invagination of the peripheral cytoplasmic membrane.

The fluorescence yield properties of these putative membrane invagination sites have suggested that although excitation energy transfer occurs, extra fluorescence emission from the peripheral 800-850 light harvesting complexes indicates that, in comparison to chromatophores, some of this antenna is not yet connected to the cores of the photosynthetic units (B875 light-harvesting and reaction center complexes) (11). Although previous studies of flash-induced absorbance changes identified photochemically competent reaction centers within the upper pigmented band, they appeared to be associated loosely or even unconnected to the cytochrome components (12). In potentiometric titrations, only a single c-type cytochrome was observed with an $E_m$ = +345 mV which was ascribed to "soluble" cytochrome c$_2$ and only tentative values were reported for oxidation-reduction midpoint potentials of the b-type cytochrome components (12).

Subsequent to these studies, additional components were shown to function in the b$_c$ segment of light-driven cyclic electron flow in R. sphaeroides chromatophores. These include a membrane-bound c-type cytochrome identified as cytochrome c$_1$ (13) and believed to serve as the immediate electron donor to cytochrome c$_2$ (14) and a Rieske-type iron-sulfur center identified in flash-illumination and EPR studies as the apparent donor to cytochrome c$_1$ (15).

In the present investigation, the kinetics of the electron transfer reactions within the R. sphaeroides upper pigmented fraction are studied in much greater detail. Redox centers in this membrane fraction have been characterized by EPR spectroscopy and an improved potentiometric titration technique and the c-type cytochrome components have also been demonstrated by polyacrylamide gel electrophoresis. The results have important implications with regard to the sequence in which photosynthetic competence is developed during the initial stages of intracytoplasmic membrane assembly.

**MATERIALS AND METHODS**

*R. sphaeroides* wild-type strain NCIB 8253 was grown phototrophically in a defined medium (16) essentially as described previously (8). For preparation of the upper pigmented and chromatophore fractions, French pressure cell extracts were applied directly to sucrose density gradients and subjected to rate-zone sedimentation (8). For the kinetic studies, the respective membrane fractions were removed directly from the gradients and not purified further. The upper pigmented and chromatophore fractions used for EPR spectroscopy were washed twice with 10 mM Tris, 20 mM EDTA buffer, pH 7.5, to remove residual manganese derived from the growth medium. The first wash was performed in a Beckman type 50.2 Ti rotor at 50,000 rpm for 4 h and the second in a type 60 Ti rotor at 60,000 rpm for 2 h. The washed pellets were resuspended in 1 mM Tris buffer, pH 7.5, at concentrations of 0.5-1.0 mM bacteriochlorophyll a. Neither flash- nor constant-illumination induced transient absorption changes resulting from a carotenoid spectral response to membrane potential could be demonstrated in the upper pigmented band preparations removed directly from the sucrose gradients. This indicates that these preparations were essentially free of contamination by the more abundant chromatophore vesicles in which a substantial carotenoid spectral response was demonstrated (12). Furthermore, this chromatophore spectral response was not inhibited in the presence of the upper pigmented fraction which suggested that no inhibition of the response was present in the latter. Although during the wash procedure, aggregation and rescaling of the pigmented membrane fragments within the upper band resulted in some reconstitution of an apparent carotenoid spectral response, this was insensitive to valinomycin.

Potentiometric titrations were performed as in Ref. 17, in which reduced-minus-oxidized difference spectra were obtained at each value of the ambient redox potential on a split-beam spectrophotometer. The amounts of b- or c-type cytochromes were calculated from the height of the absorption bands at 560 and 550 nm, respectively, subtracted from a line joining the troughs on either side of these bands. This compensated for base-line drift and provided a more accurate assessment of b- and c-type cytochromes than in an earlier publication in which the titrations were performed at fixed wavelength pairs on a dual-wavelength spectrophotometer (12). The c-type cytochromes were also resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by modifications (18) of the procedures described originally in (13, 19).

Flash-induced absorbance changes at controlled redox potential were measured in a Johnson Foundation dual-wavelength kinetics spectrophotometer using a near saturating flash xenon flash lamp for actinic illumination (20). No attempt was made to resolve redox changes of cytochromes c$_1$ and c$_2$ which are referred to collectively as c/c$_2$. An absorbance increase indicates reduction of the component, except in the case of reduction of the secondary acceptor semiquinone anion Q$_{s2}$ to the quinol, which leads to a decrease in absorbance at 450 nm (21). Valinomycin was routinely added to prevent the build up of a membrane potential and eliminate carotenoid spectral responses. In very fast kinetic measurements on chromatophore membranes, gramicidin was included to eliminate entirely the carotenoid response. This was particularly important when studying cytochrome c$_2$/c$_3$ and reaction center changes. The reduced-minus-oxidized extinction coefficients were 19 mm$^{-1}$ cm$^{-1}$ at 551-540 nm for c-type cytochromes (22), 13.2 mm$^{-1}$ cm$^{-1}$ at 560-570 nm for b-type cytochromes (23), 28.8 mm$^{-1}$ cm$^{-1}$ at 605-540 nm for [BChl] (22), and 24.4 mm$^{-1}$ cm$^{-1}$ at 670 nm for the semiquinone anions of the primary (Q$_E$) and secondary (Q$_{G}$) acceptor qanones (24). UHDBT was kindly provided by Dr. B. L. Trumpower (Department of Biochemistry, Dartmouth Medical School) and added as a solution in dimethyl sulfoxide.

EPR measurements were made using a Varian E-109 X band spectrometer. Low temperatures were achieved with a variable temperature flowing helium cryostat (Air Products, LTD-3-110) and the temperature (below 50 K) was monitored with an Allen-Bradley carbon resistor directly below the sample. The magnetic field was calibrated using weak pitch. Spectra were accumulated using a Nicolet 1024 signal averaging computer. Light excitation of samples in the EPR cavity was achieved using a high intensity illuminator (Model 170D, Dolan-Jenner Industries, Inc.) set at maximum and focused on to the cavity. A heat filter (~3 cm water) was included in the light path. The light was not saturating. Samples for EPR spectroscopy were rapidly frozen in the dark by immersion into methylcyclohexane:isopentane (3:1) to ~81 K. Detailed conditions are described individually in the figure legends.

**RESULTS**

**Identification of Cytochromes in the Membrane Fractions**—Fig. 1 shows the results of a dark equilibrium potentiometric titration of the upper pigmented membrane fraction in which an improved procedure (17) was employed. At 550 nm, two components of $E_m$ = +280 and +555 mV were resolved at oxidation-reduction potentials in the region from +220 to +400 mV. The latter value is consistent with the $E_m$ of +340 mV reported for cytochrome c$_2$ in solution (22). The former is very close to the $E_m$ of +290 mV reported for a membrane-bound cytochrome component identified as cytochrome c$_1$ in ruptured R. sphaeroides spheroplast preparations (13, 14); this component is thought to function as the immediate electron donor to cytochrome c$_2$. Similar $E_m$ values near +270 and +320 mV at 550 nm were obtained for the chromatophore fraction purified in the present study (data not shown) which further corroborates that cytochromes c$_1$ and c$_2$, respectively, can be resolved potentiometrically in situ (26). The titrations at 560 nm demonstrate the presence in the upper pigmented fraction of cytochromes b$_{560}$ (b$_{560}$) and b$_{500}$ (b$_{500}$), the b-type cytochromes thought to participate in cyclic electron flow (26-28). In addition, about 5% of the total 560 nm absorbance was accounted for by the component of $E_m$ = +370 mV that was absent from chromatophores; this high potential cyto-
Development of Redox Components of *R. sphaeroides*

![Graph](image)

**Fig. 1.** Potentiometric titrations of cytochrome components in the upper pigmented fraction. The membranes were suspended in 20 mM MOPS, 100 mM KCl, pH 7.0, at a concentration of approximately 2.1 mg of protein/ml determined as in Ref. 25. Mediators consisted of 25 μM hydroquinone, 49 μM 2,3,5,6-tetramethyl-p-phenylene diamine, 25 μM N-methyl phenazonium methosulfate, 25 μM N-ethyl phenazonium ethosulfate, 6 μM pyocyanine, and 20 μM 2-hydroxyl-1,4-naphthoquinone. A, plot of oxidation-reduction potential versus log of concentration of oxidized per reduced c-type cytochrome determined at 561 nm (●) and b-type cytochrome at 560 nm (○). B, resolution of potentiometric titration curves into individual cytochrome components. The points were fitted visually about theoretical n = 1 lines derived from the Nernst equation as described in Ref. 20. ○, 551 nm; ●, 560 nm. E_m values were (i) +370 mV, (ii) +353 mV, (iii) +278 mV, (iv) +161 mV, (v) +56 mV, (vi) −110 mV.

Chromophore may provide further evidence for the presence of respiratory cytoplasmic membrane (8, 29) in the upper pigmented fraction. Cytochrome *b*150 was also found to be present in the upper pigmented and chromatophore fractions.

A polyacrylamide gel electrophoresis procedure described in Ref. 18 was used to identify further the c-type cytochrome components present in the purified membrane fraction. During this procedure, the covalent linkage between the mesoheme and the c-type cytochrome apoproteins is retained, whereas the protoheme of the b-type components is lost. Because the iron is displaced from the mesoheme, the remaining porphyrin is easily identified by red fluorescence upon excitation in the UV. The scans of the gels shown in Fig. 2 confirm the presence of cytochromes *c*1 and *c*2 in both the upper pigmented and chromatophore fractions subjected to these conditions but the relative level of cytochrome *c*2 was considerably diminished in the former. Their apparent *M*ₘ values were 34,000 and 13,000, close to the respective reported values (13, 30). Two unidentified fluorescent components with apparent *M*ₘ of 28,000 and 20,000 were present in each preparation. The presence of cytochrome *c*′, a major soluble *R. sphaeroides* cytochrome (30) was suggested in potentiometric titrations which revealed a c-type component with an *E*_ₘ of +32 mV (average of three determinations). Although this is close to the *E*_ₘ of +30 mV reported for *R. sphaeroides* cytochrome *c* (30), this dimeric protein (*M*ₘ = 28,000, Ref. 31) migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent *M*ₘ near 13,000 and it was noted by Wood (13) that purified cytochrome *c*′ was not separated from cytochrome *c*₂ by this procedure. It is possible that the minor band migrating at 20 kDa could represent an undisassociated form of cytochrome *c*₂₃₅.

It is noteworthy that a 20-kDa component is such gels was observed as a major c-type cytochrome in cells grown in the dark with dimethyl sulfoxide as an electron acceptor (18).

The cytochrome contents of the membrane fractions were analyzed further by lithium dodecyl sulfate-polyacrylamide gel electrophoresis as in Ref. 18. Heme staining of the resolved components revealed no qualitative differences between the staining patterns of the b- and c-type cytochromes present in the upper pigmented and chromatophore fractions (not shown).

**EPR Signals in the Membrane Fractions**—On illumination of ascorbate-reduced chromatophore or upper pigmented fractions in the microwave cavity, a radical signal with *g* = 2 and peak to peak derivative line width of ~1 mT, characteristic of [BChl]⁺ (the oxidized primary donor of the reaction center) was observed (not shown). This indicates that the bacteriochlorophyll a dimer of the reaction center is present in the upper pigmented band (see Refs. 32 and 33 for reviews on the structure and function of photochemical reaction centers). The “*g* = 1.82” signal of *Q*ₐ could not be detected in either upper pigmented or chromatophore membranes, presumably owing to the low reaction center concentration in the samples. The flash-induced absorbance changes presented below as well as the requirement for electron transfer in order to generate the stable [BChl]⁺ EPR signal suggest that *Q*ₐ is present in the upper pigmented membranes.

On illumination of dithionite-reduced chromatophore and upper pigmented fractions, a spectrum attributable to the spin-polarized bacteriochlorophyll a triplet state was observed (Fig. 3). The triplet state is generated from the radical pair [BChl]⁺ BPh⁻ when electron transfer from BPh⁻ to *Q*ₐ is inhibited by reduction of the latter, and electron transfer from

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cytochrome $c_5$ is prevented by the low temperature (34). Bacteriopheophytin functions as an intermediate electron acceptor from $[\text{BChl}]^\pm$. The positions of the peaks in the triplet spectrum can be used to calculate the zero field splitting parameters which are sensitive indicators of the geometry and environment of the triplet species. The values were $D = 188 \times 10^{-4}$ cm$^{-1}$ and $E = 31 \times 10^{-4}$ cm$^{-1}$ for both chromatophore and upper pigmented membranes. These values are identical to those reported by Leigh and Dutton (35) for R. sphaeroides R-26, but are quite different from the reported values for R. sphaeroides Ga (36). Although the field positions of all the “lines” are identical in both membrane preparations, the relative magnitudes (peak/trough) differ slightly. The amplitudes of both the triplet and $[\text{BChl}]^\pm$ signals were greater in chromatophore membranes than in the upper pigmented band at the same optically determined reaction center concentration, presumably because of the non-saturating light and the greater efficiency of the light-harvesting pigments in chromatophores (11). Taken together, these results indicate that the photochemical reaction centers in the upper pigmented membranes are functional at least up to the primary acceptor quinone $Q_A$.

Fig. 4 shows spectra of the $g_Y = 1.90$ resonance of the Rieske iron-sulfur cluster (36, 37) in chromatophore and upper pigmented membranes. The Rieske cluster is thought to act as the direct electron donor to cytochrome $c_5$ (15, 38). The amount of Rieske cluster present in the upper pigmented band was significantly less than that in chromatophores; about three and five times less on reaction center and cytochrome $b$ bases, respectively. UHDBT, an inhibitor of electron transfer from the Rieske cluster to cytochrome $c_5$ (15, 39) shifted the $g_Y$ resonance from 1.90 to 1.89 in both preparations. This shift was seen in R. sphaeroides Ga (15), and indicates that the Rieske cluster in the upper pigmented band interacts with UHDBT. It is also noteworthy that UHDBT caused a narrowing of the peak to peak derivative line width from 5.3 to 3.5 mT.

Flash-induced Electron Transfer Reactions in the Membrane Fractions—The preceding results have suggested that most of the components of cyclic electron flow are present in the upper pigmented band. The kinetics of the electron transfer reactions were examined in an attempt to further define the extent of functional development of membranes within the upper pigmented band in comparison to the mature intracytoplasmic membrane.

Fig. 5 illustrates kinetic traces of changes due to reaction center $[\text{BChl}]^\pm$, $b$-type cytochrome and semiquinone anion $Q_S$ (or $Q_A$) following two flashes at high ambient redox potential with all the cytochromes oxidized before the flash. The time between the two flashes was chosen to enable a complete relaxation of $[\text{BChl}]^\pm$ before the second flash. It is noteworthy that $[\text{BChl}]^\pm$ relaxes considerably more rapidly after both flashes in the upper pigmented than in the chromatophore fraction. It is generally considered that the reductant for $[\text{BChl}]^\pm$ under these conditions is potassium ferrocyanide competing with a back reaction from $Q_S$ ($t_q \sim 1$ s). The faster re-reduction of $[\text{BChl}]^\pm$ in the upper pigmented band might reflect a greater accessibility of the reduction site.
Chromatophores were suspended in a cluster. UHDBT was added where indicated to contain pigmented centers were recorded using the same gain. Spectra of the upper pigmented portion of the Rieske iron-sulfur cluster in the two membrane fractions was estimated from the peak to trough amplitude of the signal; low signal to noise ratios precluded spin quantitation by double integration.

to ferrocyanide in these membranes. The absorption changes measured at 450 nm are primarily due to changes in redox state of [BChl] and the formation of QX (or QX) (21, 23, 27, 40). In the upper pigmented band, following the first flash, a portion of the 450 nm change relaxes with essentially the same kinetics as [BChl] leaving a residual long-lived absorption change due to QX, which remains after the second flash. The extent of this residual absorption change indicates that approximately one Qb was formed per [BChl] oxidized on the first flash. o-Phenanthroline inhibits electron transfer from light-generated QX to Qb (41, 42) and the back reaction from QX to [BChl] is considerably faster than reduction by the exogenous donor (Fig. 5, t<sub>4</sub> = 60 ms). At 450 nm, the increased rate of reduction of [BChl] in the presence of o-phenanthroline is observed, but in addition, the change relaxes due to the concomitant re-oxidation of QX and is repeated on the second flash. Overall, these observations indicate that the gating function of Qa has been established within membranes of the upper pigmented band and will be discussed in detail below.

Although in an earlier publication (12), no cytochrome b photoreduction was detected in the upper pigmented fraction poised at approximately +80 mV using a succinate/fumarate couple, the measurements presented in Fig. 5 at 560-570 nm indicate that photoreduction of cytochrome b<sub>561</sub> occurred in this fraction at +430 mV. This was confirmed by measuring the change at a number of wavelengths in the a-band region of the cytochrome (not shown). The pattern of cytochrome b reduction is similar to that reported for chromatophores of Rhodopseudomonas capsulata Alapho<sup>+</sup> (23) and R. sphaeroides GQ (43), with more cytochrome b reduction on the second flash than on the first. This has been taken to indicate that the electron on Qb is not available to reduce cytochrome b<sub>561</sub> until Qb is reduced following a second flash to QbH<sub>2</sub> (see "Discussion"). Cytochrome b<sub>561</sub> reduction is inhibited by o-phenanthroline, as would be expected if Qa is the source of reductant.

The behavior of the R. sphaeroides NCIB 8253 chromatophores (Fig. 5) is rather different from that reported elsewhere for chromatophores, mainly with respect to the large extent of cytochrome b reduction on the first flash. The factors which affect the ability of QbH<sub>2</sub> to reduce cytochrome b<sub>561</sub> (directly or indirectly) are not yet understood, but it is clear that the acceptor quinine complex of reaction centers within the upper pigmented band can transfer electrons to cytochrome b<sub>561</sub>.
The kinetics of cytochrome b photoreduction were studied at high (approximately +430 mV) and low (approximately +150 mV) redox potentials (Fig. 6). The half-time of the reaction was similar (~10 ms) in the upper pigmented and chromatophore fractions at high potential, but the reaction rate accelerated approximately 10-fold in chromatophores as the redox potential was lowered, with an apparent midpoint potential around +120 mV. This acceleration was not observed in the upper pigmented fraction. In chromatophores, it has been attributed to reduction of the ubiquinone pool, which is thought to undergo a second order oxidation reaction linked to reduction of the Rieske cluster and cytochrome b55/330.

The reduction of b-type cytochrome in both the upper pigmented and chromatophore fractions was sensitive to UHDBT at +150 but not at +430 mV (not shown); this was consistent with the behavior in *R. sphaeroides* Ga and *R. capsulata* Ala pho*+* chromatophore preparations reported previously (46).

Fig. 7 illustrates the kinetics of oxidation and re-reduction of the c-type cytochromes following a single flash excitation. There are pronounced differences in the reactions of the upper pigmented membranes when compared with chromatophores. It was noted earlier (12) that c-type cytochrome photooxidation in the upper pigmented band was monophasic and slow (tν = 13 ms), and that exogenously added equine ferrocytochrome c could be photooxidized, which indicated that the appropriate sites on the reaction center are exposed to the medium, unlike the case with chromatophores. These observations are confirmed here (see Table I, which lists the half-times for several of the reactions). The faster re-reduction of [BChl]5 in the upper pigmented fraction at high redox potential around +120 mV. This acceleration was not observed in the upper pigmented fraction. In chromatophores, it has been attributed to reduction of the ubiquinone pool, which is thought to undergo a second order oxidation reaction linked to reduction of the Rieske cluster and cytochrome b55/330.

Fig. 7. Kinetics of redox changes in c-type cytochromes. Absorbance changes were measured at 550–540 nm. Upper pigmented and chromatophore preparations were suspended as described in the legend to Fig. 6 at an ambient redox potential of about +150 mV. A. kinetics of c-type cytochrome photooxidation. Antimycin and UHDBT were added as indicated in the figure. The traces for the upper pigmented band were not averaged; the chromatophore traces were an average of 16 (no UHDBT) and 32 (UHDBT added) flashes. B. kinetics of c-type cytochrome re-reduction. Antimycin and UHDBT were added as indicated in the figure. The traces for the upper pigmented band were not averaged. The chromatophore traces were an average of eight flashes.

The half-reduction of cytochrome c1/c2 at a redox potential of +150 mV, pH 7, in the upper pigmented band was extremely slow (τν = 255 ms); under the same conditions, the half-time in chromatophores was 7 ms. In the upper pigmented fraction, antimycin had no effect on the kinetics of cytochrome c1/c2 oxidation but slightly diminished the reduction rate. UHDBT caused a small increase in the extent of cytochrome c1/c2 oxidation, but did not further slow the reduction rate over that seen in the presence of antimycin. These effects contrast with those seen in the chromatophores, which are typical of those reported elsewhere (15). A detailed discussion of the kinetics observed here for the bc1 complex is presented below.

The results in Table II show that there is a molar excess of b- and c-type cytochromes over the reaction center in the upper pigmented fraction. Despite this, only about 7% of the total b-type cytochrome is photoreducible in the presence of...
train of four flashes at an ambient potential of 150 mV in the presence
from the maximal absorbance decrease at 551-540 nm following a
cytochromes and reaction center content were determined similarly
onite-minus-ascorbate) in the presence of carbonyl cyanide p-
oxidized difference spectra (ascorbate-minus-ferricyanide and dithi-
length mode. Cytochromes of antimycin and valinomycin (see Fig. 7). Photoreducible b-type
obtained using a Perkin-Elmer spectrophotometer in the two-wave-
the total. Thus, although the fraction of the cytochrome bX1
compared to about 75% in chromatophores. At least seven
mintum-reduced cytochrome relative to the total cytochrome content
is reduced upon flash illumination. It is suggested that it was cyto-
50% of the cytochrome bX1 is the major b-type component
in both membrane fractions and can account for 33-67% of the
total. Thus, although the fraction of the cytochrome bX1 which is photoreducible is very small in both membrane fractions, it represented a much smaller fraction in the upper pigmented membranes because of the higher total b-type cytochrome in these membranes and the lower amount which is reduced upon flash illumination.

It is also shown in Table II that the upper pigmented fraction had a considerably higher total c-type cytochrome content/reaction center than the chromatophores; approximately 50% was photoreducible in the upper fraction as compared to about 75% in chromatophores. At least seven cytochromes c/reaction center were photoreduced upon addition of excess ferrocyanochrome c to the upper pigmented band; presumably the ubiquinone pool acted as an electron sink for this purpose. It is possible that some of the non-
photooxidizable c-type cytochrome can be accounted for by the variety of other soluble c-type cytochromes present in R.
sphaeroides of unknown function (30)2 that have become entrapped or otherwise associated with the membranes.

**Respiratory Activity of Membranes Fractions**—In the form in which it is isolated, the upper pigmented fraction contains both relatively unpigmented cytoplasmic membrane (47) and pigmented membrane (8); the latter is thought to be derived from photosynthetic membrane growth initiation sites. The cytoplasmic membrane fragments have a polypeptide profile in sodium dodecyl sulfate-polyacrylamide gel electrophoresis that is essentially identical to that of the respiratory cytoplasmic membrane of aerobically grown cells (47) and they may be a source of much of the non-photoreducible cytochromes in the upper pigmented band. Although the upper pigmented fraction was shown to be enriched in succinate dehydrogenase activity relative to that of chromatophores (8), their respiratory activities have not been previously compared. These are especially pertinent because they should provide direct activity measurements of levels of the functional bc1 complex in the two fractions.

In Table III, levels of succinate-cytochrome c reductase and succinate dehydrogenase activities are shown for the upper

<table>
<thead>
<tr>
<th>Cytochrome components</th>
<th>Upper pigmented</th>
<th>Chromatophores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>molar ratio</td>
<td></td>
</tr>
<tr>
<td>c-type/reaction center</td>
<td>Total 3.3 (1.00)*</td>
<td>0.90 (1.00)</td>
</tr>
<tr>
<td></td>
<td>Photoreducible 1.81 (0.55)</td>
<td>0.68 (0.76)</td>
</tr>
<tr>
<td>b-type/reaction center</td>
<td>Total 4.8 (1.00)</td>
<td>2.6 (1.00)</td>
</tr>
<tr>
<td></td>
<td>Photoreducible 0.32 (0.07)</td>
<td>0.46 (0.17)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses give the fraction of photooxidizable or photoreducible cytochrome relative to the total cytochrome content taken as 1.00.

<table>
<thead>
<tr>
<th>Cytochrome content/reaction center</th>
<th>Upper pigmented</th>
<th>Chromatophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>[BChl] re-reduction (605-540 nm)</td>
<td>+430 241.0</td>
<td>2310 ms</td>
</tr>
<tr>
<td>+ o-phenanthline</td>
<td>43.0</td>
<td></td>
</tr>
<tr>
<td>450 nm decay + o-phenanthline</td>
<td>+430 11.0</td>
<td>10 ms</td>
</tr>
<tr>
<td>b-type cytochrome photoreduction</td>
<td>+150 18.0</td>
<td>1 ms</td>
</tr>
<tr>
<td>(560-570 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-type cytochrome photooxidation</td>
<td>+150 4.0</td>
<td>(i) 30 µs</td>
</tr>
<tr>
<td>(551-540 nm)</td>
<td></td>
<td>(ii) 320 µs</td>
</tr>
<tr>
<td>+ UHDBT</td>
<td>8.0</td>
<td>(ii) 30 µs</td>
</tr>
<tr>
<td>+ 5 µM equine ferrocyanochrome c</td>
<td>0.8</td>
<td>420 µs</td>
</tr>
<tr>
<td>Re-reduction</td>
<td>+150 255.0</td>
<td>7 ms</td>
</tr>
</tbody>
</table>

*1 and ii represent first and second phases of reaction, respectively.

### TABLE I

**Reactant half-times of redox components in upper pigmented and chromatophore fractions**

The reaction conditions are described in the legends of Figs. 5-7.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Apparent t1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper pigmented band</td>
<td>Chromatophores</td>
</tr>
<tr>
<td>E&lt;sub&gt;a&lt;/sub&gt;</td>
<td>ms</td>
</tr>
<tr>
<td>[BChl] re-reduction (605-540 nm)</td>
<td>+430 241.0 2310 ms</td>
</tr>
<tr>
<td>+ o-phenanthline</td>
<td>43.0</td>
</tr>
<tr>
<td>450 nm decay + o-phenanthline</td>
<td>+430 11.0 10 ms</td>
</tr>
<tr>
<td>b-type cytochrome photoreduction (560-570 nm)</td>
<td>+150 18.0 1 ms</td>
</tr>
<tr>
<td>c-type cytochrome photooxidation (551-540 nm)</td>
<td>+150 4.0 (i) 30 µs (ii) 320 µs</td>
</tr>
<tr>
<td>+ UHDBT</td>
<td>8.0 (ii) 30 µs (ii) 420 µs</td>
</tr>
<tr>
<td>+ 5 µM equine ferrocyanochrome c</td>
<td>0.8</td>
</tr>
<tr>
<td>Re-reduction</td>
<td>+150 255.0 7 ms</td>
</tr>
</tbody>
</table>

### TABLE II

**Molar ratios of b- and c-type cytochromes in membrane fractions**

Total cytochrome contents were determined from reduced-minus-oxidized difference spectra (ascorbate-minus-ferricyanide and dithionite-minus-ascorbate) in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrozone as uncoupler. The spectra were obtained using a Perkin-Elmer spectrophotometer in the two-wavelength mode. Cytochromes c were measured at 551-540 nm and b-type cytochrome at 560-570 nm.

Photooxidizable c-type cytochrome reaction center was calculated from the maximal absorbance decrease at 551-540 nm following a train of four flashes at an ambient potential of 150 mV in the presence of antimycin and valinomycin (see Fig. 7). Photoreducible b-type cytochromes and reaction center content were determined similarly from measurements made at 560-570 nm and 605-540 nm, respectively. Extinction coefficients are listed in the text.

<table>
<thead>
<tr>
<th>Cytochrome components</th>
<th>Fraction</th>
<th>Upper pigmented</th>
<th>Chromatophores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>molar ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-type/reaction center</td>
<td>Total 3.3 (1.00)*</td>
<td>0.90 (1.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Photoreducible 1.81 (0.55)</td>
<td>0.68 (0.76)</td>
<td></td>
</tr>
<tr>
<td>b-type/reaction center</td>
<td>Total 4.8 (1.00)</td>
<td>2.6 (1.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Photoreducible 0.32 (0.07)</td>
<td>0.46 (0.17)</td>
<td></td>
</tr>
</tbody>
</table>

* The numbers in parentheses give the fraction of photooxidizable or photoreducible cytochrome relative to the total cytochrome content taken as 1.00.

Variable concentrations of phenazine methosulfate were employed in the succinate dehydrogenase assays (6) and the rate of 2,6-dichlorophenolindophenol reduction presented for the upper pigmented fraction was calculated by extrapolation to infinite phenazine methosulfate concentration. The rate for chromatophores was obtained at 1.8 mM phenazine methosulfate at which 2,6-dichlorophenolindophenol reduction appeared to be maximal. The assay mixture for measurements of succinate-cytochrome c reductase activity consisted of 50 mM potassium phosphate, 1.5 mM potassium cyanide, 50 µM horse heart cytochrome c, and 20 mM sodium succinate, pH 7.8; 0.25% sodium cholate was present for assays with chromatophores. Rates of cytochrome c reduction were calculated from the initial rate of absorbance change at 550 nm with a reduced-minus-oxidized extinction coefficient of 18.5 mM<sup>-1</sup> cm<sup>-1</sup> (49).

### TABLE III

**Succinate oxidation reactions in membrane fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate oxidation reactions in membrane fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c reductase</td>
<td>µmol h&lt;sup&gt;-1&lt;/sup&gt; nmol reaction center&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Upper pigmented</td>
<td>16.1 ± 1.0</td>
</tr>
<tr>
<td>Chromatophores (± 0.25%)</td>
<td>10.1 ± 0.9</td>
</tr>
</tbody>
</table>

* Activities in the presence of 1 µM antimycin were inhibited by 93 and 96% in the upper pigmented and chromatophore fractions, respectively.
pigmented and chromatophore fractions. The reductase assay with chromatophores was determined in the presence of 0.25% cholate which stimulated the activity about 4-fold. This is thought to be due to cholate-induced disruption of the chromatophore membrane enabling cytochrome c to associate with its reduction site which is mostly inaccessible in untreated chromatophores. No stimulation was observed with the upper band in which the membranes probably exist as open fragments with accessible reduction sites (9, 12, 48).

The respiratory activity of the upper fraction as measured by the succinate-cytochrome c reductase assay was about 1.6-fold higher than that in chromatophores on a reaction center basis. However, the rates in both preparations were low, corresponding to turnover numbers of 4.5 s\(^{-1}\) for the upper pigmented fraction and 2.8 s\(^{-1}\) for the chromatophores, assuming one cytochrome c\(_R\)/reaction center. This can be compared with a value of 44 s\(^{-1}\) for bovine succinate-cytochrome c reductase complex (50).

The activity of the chromatophore membranes could be limited by the low level of succinate dehydrogenase in these membranes (Table III). Furthermore, succinate dehydrogenase is also involved in photosynthetic reduction of NAD\(^{+}\) by succinate via energy-dependent reverse electron flow and is not necessarily unique as a respiratory marker. Nevertheless, Gabellini et al. (51) have reported a turnover of only 4.2 s\(^{-1}\) for ubiquinol-9 cytochrome c\(_0\) oxidoreductase in chromatophores, even though single turnover flash kinetic studies indicate turnover rates of \(\geq 500\) s\(^{-1}\) under optimal conditions. Results from steady state cytochrome c reductase assays would, therefore, appear to be less diagnostic than the single turnover flash studies. It is also possible that measurement of the activity of the bc\(_1\) complex with ubiquinol-9 is limited by availability of electrons from this substance when incorporated into Triton X-100 micelles (51).

**DISCUSSION**

In this study a number of the redox components of light-driven electron flow have been identified in a membrane fraction which forms an upper pigmented band upon rate-zone sedimentation of cell-free R. sphaeroides extracts. This fraction was previously shown to be enriched in newly synthesized bacteriochlorophyll \(a\)-protein complexes (8, 9) and is believed to contain membrane invagination sites destined to give rise to the R. sphaeroides intracytoplasmic membranes. These redox components include cytochromes b\(_{561}\) (b\(_{50}\)) and b\(_{594}\) (b\(_{586}\)) and cytochromes c\(_1\) and c\(_2\), which were resolved in dark, equilibrium potentiometric titrations; the identity of the c\(_{1}\)-type cytochromes was confirmed from the fluorescence of the porphyrins which remained associated with their apoproteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, EPR spectroscopy permitted the identification of the light-induced reaction center \([\text{BChl}^{2}]_{n}\) and bacteriochlorophyll \(a\) triplet signals and the Rieske iron-sulfur center; the latter component, which is the apparent electron donor to cytochrome c\(_1\) during light-driven electron flow (15), was present in reduced amounts in comparison with chromatophores.

From flash-induced absorbance changes, the upper pigmented band was also shown to contain Q\(_{A}\) and Q\(_{B}\), the primary and secondary ubiquinone acceptors, respectively. It was also possible to demonstrate the gating function of Q\(_{B}\) within this membrane fraction at high redox potential (approximately 430 mV). This is explained in Scheme 1, where \(P\) represents \([\text{BChl}]_{n}\), the protonation is ignored. The net result is that the same amount of Q\(_{B}\) is present following the first and second flashes, and this is essentially what was observed in Fig. 5. Scheme 2 demonstrates the effect of o-phenanthroline on these reactions. In this case, o-phenanthroline has blocked electron transfer from Q\(_{B}\) to Q\(_{A}\) and the absorbance change due to Q\(_{A}\) reduction is quickly obliterated by the back reaction from Q\(_{A}\) to Q\(_{B}\) via the ambient potential.

This behavior is consistent with that of isolated reaction centers of R. sphaeroides R-26 (21, 33, 42), and confirmed that Q\(_{A}\) and Q\(_{B}\) are both present in reaction centers of the upper pigmented band. The observed behavior is also similar to that seen in chromatophores (23, 43, 52), except that O’Keefe et al. (43) have reported that photooxidized \([\text{BChl}]_{2}\) is selectively re-reduced over chemically oxidized \([\text{BChl}]_{2}\).

With regard to the flash-induced cytochrome changes observed here, the rate of oxidation of cytochrome c\(_1/c_2\) in the upper pigmented band was more than 100-times slower than that in chromatophores. This slow rate of oxidation is similar to that reported by Overfield and Wraight (53) for the oxidation of cytochrome c\(_2\) in the bulk phase by reaction centers incorporated into phosphatidylcholine vesicles at similar reactant concentrations and ionic strength. Membranes of the upper band, therefore, appear to differ from chromatophores in that the diminished content of cytochrome c\(_2\) is in solution in the bulk aqueous phase, rather than being sealed into vesicles. Presumably, cytochrome c\(_2\) remains membrane associated during preparation of the upper pigmented band but becomes solubilized on dilution into high ionic strength buffer. The rate of cytochrome c oxidation could be increased about 2.5-fold by addition of exogenous ferrocyanochrome, demonstrating that the oxidation rate was not limited by a property of the interaction site on the reaction center. The increase in the rate of oxidation of ferrocyanochrome c was not accompanied by an enhanced re-reduction rate. The absence of any flash or constant illumination induced transient absorption change resulting from a carotenoid spectral response to membrane potential (12) further indicates that the membranes of the upper pigmented band are not vesicular. Proteolytic diges-
with pronase (9) as well as membrane surface labeling studies (48) have also suggested that the upper pigmented band consists largely of open membrane fragments and it is possible that pigmented membrane within this fraction is not sufficiently invaginated to reseal when the cells are disrupted in the French press.

Comparison of the extent of cytochrome $c_1/c_2$ oxidation in the upper pigmented fraction in the presence and absence of UHDBT indicated that some electron transfer from the Rieske iron-sulfur cluster occurred, but to a lesser extent than that seen in chromatophores. This would be consistent with the diminished content of Rieske cluster in this fraction. Furthermore, inability of the diminished Rieske protein pool to serve cytochrome $b_{561}$ complexes lacking this protein could account for the small fraction of cytochrome $b_{561}$ reducible in the presence of antimycin, and for the small extent of UHDBT-sensitive electron transfer to cytochrome $c_1/c_2$. The kinetics of oxidation of cytochrome $c_1/c_2$ in the presence of UHDBT, which inhibits electron transfer from the Rieske iron-sulfur center to cytochrome $c_1/c_2$ (15), suggest that electron transfer from the Rieske cluster in the absence of inhibitor occurs at a similar rate to that of cytochrome $c_2$ oxidation, and probably reflects a second-order reaction between membrane bound Rieske cluster and soluble cytochrome $c_2$ (via cytochrome $c_1$).

Cytochrome $b_{561}$ photoreduction was observed in the upper pigmented fraction at high redox potential with kinetics which indicate that the transfer of ubiquinol reducing equivalents from the reaction centre $Q_b$ sites to at least a fraction of the quinol oxidase sites on the $bc_1$ complex occurred as in chromatophores (44). However, the rate of reduction did not accelerate as the ubiquinone pool was reduced. According to the Q-cycle model of electron transfer, one potential rate-limiting step in the reduction of cytochrome $b_{561}$ in the presence of antimycin is the oxidation of the Rieske cluster (44). Comparison of the cytochrome $c_1/c_2$ kinetics in the upper pigmented fraction in the presence and absence of UHDBT suggests that a slow rate of oxidation of the Rieske iron-sulfur cluster could be responsible for the failure to observe an increased rate of cytochrome $b_{561}$ reduction as the potential was lowered. This could be related in part to the low concentration of cytochrome $c_2$ relative to $c_1$ in the upper pigmented membranes which would be insufficient to oxidize the Rieske cluster at a significant rate. It did not result from the absence of an ubiquinone pool, since the ubiquinone/bacteriochlorophyll $a$ ratios were similar in the upper pigmented and chromatophore fractions (12).

This hypothesis cannot explain the abnormally slow re-reduction time of a large fraction of the photooxidized cytochrome $c_1/c_2$ in the upper pigmented membranes under near-optimal conditions for electron transport. The re-reduction rate is already so low ($t_{1/2} 255$ ms) that the decay time is of the same order of magnitude as that for the UHDBT- and antimycin-inhibited reactions in chromatophores. The relatively diminished content of Rieske cluster in the upper pigmented fraction means that if there is a pool of active $bc_1$ complexes, these would have to undergo multiple turnovers to achieve rapid re-reduction of all the photooxidized cytochrome $c_2$. That this does not occur would suggest that the putative active $bc_1$ complexes have limited access to their reaction partners. Alternatively, the cytochrome $b_{561}$ re-oxidation pathway through the antimycin-sensitive site (44), a prerequisite for complete turnover of the $bc_1$ complex, may be non-functional in the upper fraction even in $bc_1$ complexes containing an iron-sulfur cluster.

The fact that many of the putative $bc_1$ complexes in the upper pigmented fraction appear to be non-functional raises the possibility that the $bc_1$ complex is not an essential component of the respiratory pathway (cf. the antimycin-insensitive pathway from ubiquinol to the cytochrome $b_{561}$ oxidase in $R. capsulata$ (54)), or simply that the intracytoplasmic precursor membrane has an intrinsically low respiratory activity. In support of the former possibility, it has been observed that the respiratory activity of photosynthetically grown $R. sphaeroides$ is partially resistant to high ($> 2$ mm) levels of cyanide and that an oxidase is present which is linked to a cyanide- and antimycin-resistant pathway associated with a $b$-type cytochrome.\(^3\)

The low activity of the putative $bc_1$ complexes in the upper pigmented fraction appears to be at least partially due to a deficiency in the Rieske iron-sulfur cluster, but other components, including phospholipids, may also be required. One possibility that we cannot at present exclude is that the upper pigmented fraction is more susceptible to loss of the Rieske iron-sulfur cluster than the chromatophore fraction. In this respect it would be similar to the purified $bc_1$ complex (51).

The possibility has already been noted that pigmented membrane within the upper pigmented band arises from peripheral cytoplasmic membrane invagination sites at which growth of the intracytoplasmic membrane is initiated. This is supported by: (i) pulse-chase studies with several radiolabeled amino acids in both $R. sphaeroides$ (8, 9) and $Rhodospirillum rubrum$ (55) which indicate that light-harvesting and reaction center complexes are inserted preferentially into these sites which mature to intracytoplasmic membrane having the isolation characteristics of chromatophores; (ii) the demonstration in $R. sphaeroides$ that these regions represent sites at which bacteriochlorophyll $a$ is synthesized preferentially and in which the completed pigment-proteins exist transiently (10); (iii) the apparent peripheral localization of these sites as indicated by membrane surface labeling (48) and subcellular fractionation (9) studies; (iv) observations that this fraction comprises more of the pigmented membrane during early stages of intracytoplasmic membrane induction and in cells in which intracytoplasmic membrane content is significantly reduced (9); (v) the lower B800-850 levels (12) and the fluorescence rise kinetics (11) which are characteristic of early stages of photosynthetic membrane development (25, 56); and (vi) partial separation of the isolated fraction into pigmented and essentially unpigmented membrane by gel filtration or ultracentrifugation procedures (48). Further evidence for the respiratory origin of membranes within the upper pigmented band has been provided here by the succinate dehydrogenase and succinate-cytochrome $c$ reductase activities, and demonstration of a high potential $b$-type cytochrome. Thus, pigmented membranes within the upper fraction can be considered as an intermediate in the process of development of intracytoplasmic membrane from the respiratory cytoplasmic membrane.

The results presented here suggest that at this stage in the development sequence, the presence of newly synthesized reaction centers and respiratory membrane is insufficient to produce a fully competent photosynthetic system. To achieve photosynthetic competence, the necessary components may presumably be synthesized or assembled subsequent to reaction center insertion as the growth initiation sites further invaginate to become fully functional intracytoplasmic membranes. There are precedents for such a stepwise mode of assembly, notably in the case of the B800-850 light-harvesting complex, much of which is inserted directly into the mature membrane.

\(^3\) C. N. Hunter, and O. T. G. Jones, unpublished results.
intracytoplasmic membrane rather than into membranes isolated with the upper pigmented band (9, 10).

The reconstitution of a competent cyclic photosynthetic electron transfer system has been attempted by mixing membranes from a R. sphaeroides mutant unable to synthesize bacteriochlorophyll a with reaction centers in the presence of the detergent sodium cholate (57, 58). Removal of this detergent by dialysis yielded a pigmented membrane which exhibited cytochrome b photoreduction ($t_{\text{on}} = 27$ ms) and cytochrome c photooxidation. Cytochrome c re-reduction was slow ($t_{\text{on}}$ of the order of several hundred milliseconds) and only partially sensitive to antimycin. There is, therefore, an interesting parallel between the reconstituted system and the membranes of the upper pigmented band in terms of these kinetics. This provides support for the hypothesis that although the components of a respiratory membrane are able to accept a newly synthesized B875 light-harvesting complex/reaction center entity, they cannot fully participate in efficient cyclic electron flow because other components are not synthesized or appropriately organized into the growing membrane until a later stage in the developmental sequence. Thus, the process of the development of fully competent photosynthetic membranes has proved much more complex than originally envisaged.

Acknowledgments—We are grateful to P. L. Dutton for the use of the dual-wavelength kinetics spectrophotometer, O. T. G. Jones for providing the facilities for the redox potentiometry, B. L. Trumpower for providing the UHDBT, and M. A. Cusanovich for advice in identification of cytochrome components. S. V. Kolaczkowski and D. Farrelly assisted in the preparation of membranes.

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