Photochemical Reactions of Purple Bacteria as Revealed by Studies of Three Spectrally Different Carotenobacteriochlorophyll–Protein Complexes Isolated from *Chromatium*, Strain D*

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ABSTRACT: The different spectral forms of bacteriochlorophyll a, B*800, B800, B820, B850, and B890, that occur *in situ* in *Chromatium* chromatophores can be fractionated into three spectrally different pigment–protein complexes by chromatography on hydroxylapatite of chromatophores which have been solubilized in sodium dodecyl sulfate, followed by ammonium sulfate precipitation. One complex, fraction A, contains B890 as the only light-harvesting spectral form; the previously described reaction center complex (P883–P800) is present in this fraction only. Fraction B has approximately equal proportions of B800 and B850, together with a pigment (P836) which undergoes reversible photooxidation with a quantum yield of less than 1%. Fraction C contains B*800 and B820, and is the only complex which does not show any reversible photobleaching. Fraction A (mol wt ~ 500,000) is a solubilized form of the cyclic and noncyclic photoelectron transport systems of *Chromatium*. On exposure of fraction A to light, the reaction center (P883) and a cytochrome (C556) become oxidized, and in the dark they are slowly rereduced; the rate of reduction is greatly accelerated by the addition of phenazine methosulfate or sodium ascorbate, probably indicating that some components of the cycle have been lost during isolation of fraction A. Fraction A contains the cytochrome (C552) of the noncyclic photosystem, and C552 also undergoes light-induced oxidation upon lowering of the redox potential of the complex. There is a second minor spectral form of bacteriochlorophyll (P'890) in fraction A which can undergo an oxidation–reduction reaction; it appears that P'890 supplies electrons to P^{+}883 if both C552 and C556 are in an oxidized state.

The molar proportions of the components in fraction A are P883 (1):P'890 (~4):P800 + B800 (4):B890 (40):spirilloxanthin (12):lycoxanthin (6):lycophyll (1):C556 (2):C552 (~7); fraction B contains P836 (1):B800 + B850 (10):lycoxanthin (2):spirilloxanthin (1). Fraction B has a molecular weight of 100,000 ± 10,000. Fraction C is identical in size and carotenoid composition, and differs only in the spectral forms of bacteriochlorophyll which it contains.

Bacterial photosynthesis has long been thought to be driven by one photosystem (*cf.* Clayton, 1966a). Recently an hypothesis has been developed for the participation of two photoacts in the purple bacteria (*cf.* Hind and Olson, 1968). The evidence for this hypothesis has come from studies on the light-induced oxidation of the chromatophore-bound cytochromes in *Chromatium* and *Rsp. rubrum*. Light-induced oxidation of the cytochromes in a purple bacterium was first observed by Duysens (1954). Olson and Chance (1960a,b) later showed that the cytochromes (C552, C556, and cc')[1] in *Chromatium* are involved in two different electron-transfer pathways. More recently evidence has been presented that the high-potential cytochrome, C555, also called C556, and the low-potential cytochrome, C552, appear to be oxidized by different photochemical reaction centers (Cusanovitch *et al.*, 1968), and that each reaction center is associated with a spectrally different array of light-harvesting chlorophyll (Morita, 1968), *i.e.*, two distinct photosystems are proposed. Sybesma and Fowler (1968) and Sybesma (1969) have reported that in *Rsp. rubrum* the high- and low-potential cytochromes also appear to be oxidized by two different photosystems.

The evidence for two systems is equivocal, and other investigators (Fork and Amesz, 1969; Parson and Case, 1970; Seibert and DeVault, 1970) have argued that both cytochromes are oxidized by the same photosystem. This system has been studied in some detail in *Chromatium* (Clayton, 1962; Duysens, 1952; Ke *et al.*, 1968; Loach, 1966; Parson, 1968, 1969a,b); it is a cyclic electron transport system which is driven by the P883–P800 reaction center

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[1] Abbreviations used in this work are: Bchl-P, bacteriochlorophyll a–protein; Chl, chlorophyll a; B*800, B800, B820, B850, B890, the light-harvesting forms of bacteriochlorophyll which absorb at 800, 807, 823, 850, and 891 nm, respectively (Vredenberg and Amesz, 1967); P883, P836, P'890, pigments whose absorption maxima are at 883, 836, and 890 nm, respectively, and which show light-induced absorbance changes at these wavelengths; F925, F883, fluorescence bands with wavelengths of maximum emission at 925 and 883 nm, respectively; C552, C555, C556, cytochromes whose α-band absorption maxima are at 552, 555, and 556 nm, respectively.

complex. It is undisputed that C556 donates an electron to P883 after the latter's oxidation by light in chromatophores at high redox potentials or under aerobic conditions. What is disputed is whether C552 donates an electron to $P^{+}883$ or to some other reaction center at low redox potentials. One way in which the existence of two photosystems could be proved would be to obtain their physical separation. Since the two photosystems of *Chromatium* are postulated to be composed of different proportions of the several spectral forms of Bchl (Morita, 1968), their separation can be monitored; once homogeneous components have been obtained, they can be examined for the presence of photochemical activity and cytochromes (*cf.* Boardman and Anderson's (1964) resolution of the photosystems of higher plants). This was the approach adopted for the present work. The techniques which were employed to separate the Bchl-containing complexes of *Chromatium* had previously proved successful for the resolution of Chl-Ps in other photosynthetic organisms (Thornber, 1969; Thornber *et al.*, 1969). It was expected that these techniques would give a greater fractionation of the different spectral forms of Bchl in *Chromatium* (B*800, B800, B820, B850 and B890; Vredenberg and Amesz, 1967) than had previously been accomplished by other methods (Bril, 1964; Clayton, 1962; Garcia *et al.*, 1966; Komen, 1956). This was indeed the case. Three Bchl–P complexes were obtained, each of which contained one spectral form which is not present in the other two complexes. Only one complex contained an efficient photochemical reaction center, *i.e.*, a spectral form of Bchl which undergoes reversible photooxidation with a high quantum yield. Both C552 and C556 were associated with this reaction center.

Materials and Methods

Cells. Chromatium, strain D, was grown for 7 days either photoheterotrophically or photoautotrophically at 30–32° in 20-l. flasks which were illuminated by three 60-W incandescent lamps (Bose, 1963). The cells (stationary phase) were harvested by centrifugation, and either used immediately or frozen (−17°).

Fractionation Procedure (Figure 1). The preparation was carried out at room temperature. The infrared spectrum of solutions obtained after various steps of the isolation procedure was monitored and used as an index of the degree of purity; the criterion of purity was that the derived fractions should have the spectra shown in Figures 2 and 3. An additional criterion was that fractions B and C should contain no reversible light-induced bleaching at 883 nm; this property is a function of fraction A only (see below).

Step 1. Harvested cells (7 ml) were suspended in 50 mM Tris-HCl (pH 8.0, 50 ml) and the pH was readjusted if necessary by the addition of solid Tris. The suspension was sonicated in a Raytheon 10-kcps oscillator at 1 A for 10 min, and the sonicate was subsequently centrifuged (15,000*g*, 15 min). Breakage in a French pressure cell also proved to be satisfactory.

Step 2. The spectrum of the supernatant (crude chromatophores) was recorded, and the absorbance at 590 nm was used to estimate the Bchl concentration (ϵ_{590} 20 mM^{-1}). A 1% sodium dodecyl sulfate solution was then added to the supernatant to give a final sodium dodecyl sulfate:Bchl ratio of 12 g/mmole. The final concentration of sodium dodecyl sulfate should be approximately 0.5% (w/v) if good yields are to be obtained from the ensuing chromatographic step.

Step 3. Hydroxylapatite was prepared by the method of Siegelman *et al.* (1965), and a column of this adsorbant (6.5 × 3.7 cm) was equilibrated with 10 mM sodium phosphate–0.2 M NaCl (pH 7.0). The sodium dodecyl sulfate solubilized chromatophores were run into the column, and all of the colored material was adsorbed. The column was then washed with 100 ml of the equilibration buffer followed by the same volume of 100 mM sodium phosphate–0.2 M NaCl (pH 7.0). Elution with 250 mM sodium phosphate–0.2 M NaCl (pH 7.0) removed a large portion of the adsorbed color; the eluate contained a mixture of fractions A and B. Steps 4 and 5 describe the subsequent purification of each fraction. Elution with 350 mM sodium phosphate–0.2 M NaCl (pH 7.0) removed another pink fraction which contained a mixture of fractions A, B, and C; this eluate was discarded as the mixture was difficult to fractionate subsequently. A spectrally pure form of fraction C was removed from the column by 700 mM sodium phosphate–0.2 M NaCl. Addition of ammonium sulfate to this eluate precipitated fraction C, which was isolated by centrifugation, and then redissolved in 50 mM Tris (pH 8.0).

Step 4. Separation of fractions A and B. A 50% (w/v) ammonium sulfate solution was added dropwise to the 250 mM sodium phosphate eluate from the hydroxylapatite column until a cloudiness developed, which occurred at a concentration of 6% (w/v) ammonium sulfate. The mixture was then centrifuged (15,000*g*, 10 min), and the supernatant was separated from the precipitate. The precipitate (fraction A) was redissolved in 50 mM Tris (pH 8.0) and step 4 was repeated if necessary. The final solubilized precipitate was used for studies on fraction A.

Step 5. The supernatant of step 4 was made to 20% (w/v) ammonium sulfate and recentrifuged. The precipitate (mixture of further amounts of fraction A and some of fraction B) was discarded. The pH of the supernatant was lowered to pH 4.5 by addition of 1 M acetic acid, whereupon a cloudiness developed. The suspension was centrifuged, and the precipitate (fraction B) was redissolved in 50 mM Tris (pH 8.0). If the spectrum of the solution did not meet the criteria of purity of fraction B, step 5 was repeated.

Spectrophotometry. Absorption. A Cary Model 14R recording spectrophotometer was used for measurement of absorption spectra at room temperature and at the temperature of liquid nitrogen (77°K). For the determination of the spectra at 77°K, a cryostat described by Cross *et al.* (1968) was used. The path length of the cell was 3 mm. Prior to examination at 77°K samples were mixed with an equal volume of glycerol; the resulting glasses were devoid of cracks in that portion of the cell through which the measuring beam passed.

Emission. Fluorescence spectra were measured in a fluorometer previously described by Sybesma and Olson (1963). Determinations were made at room temperature and at 77°K. A cryostat similar to that described above was used for measurements at both temperatures. The sample thickness was 1 mm. The concentration of samples was such that the absorbance was less than 0.2 cm^{-1} at 590 nm or 1.0 cm^{-1} at the far-red maxima. The spectra obtained were corrected for the spectral characteristics of the monochromator and the

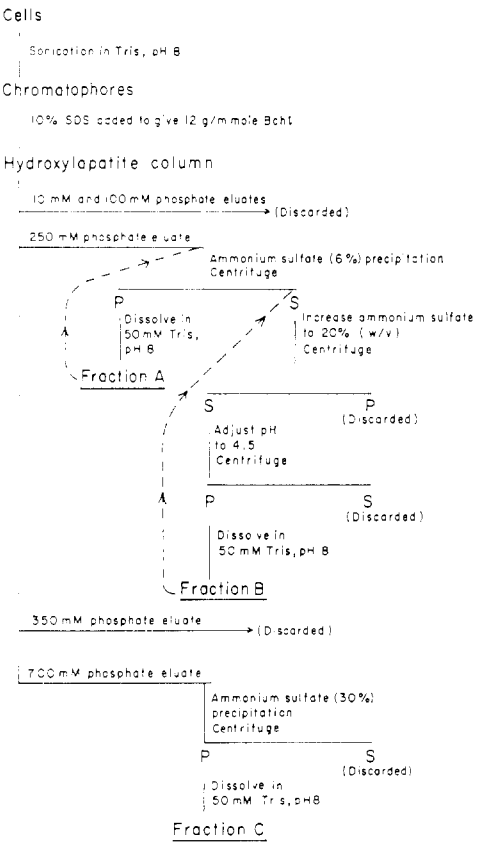

FIGURE 1: Diagrammatic representation of the purification procedure. P and S represent the precipitate and supernatant, respectively, of a centrifugation. The dashed lines represent the manner in which the fractions, if impure, can be recycled through the purification procedure.

phototube. Samples were examined in 50 mM Tris-HCl (pH 8.0).

LIGHT-INDUCED ABSORBANCE CHANGES. A Cary Model 14R recording spectrophotometer was adapted so that an external actinic light source (500-W bulb in a slide projector) could be used for cross-illumination. For more detailed examination of small absorbance changes, a double-beam spectrophotometer previously described by Morita *et al.* (1963) was used. This instrument was also designed so that an external actinic light source could illuminate the sample.

BACTERIOCHLOROPHYLL CONTENT. This was determined from the absorbance at the maxima of the orange band of Bchl (~590 nm) or from the infrared maximum (890 nm) in the case of fraction A using an ϵ value of 20 or 100 mM^{-1}, respectively. The determinations were generally made without extraction of the pigments from the complexes into organic solvents.

Carotenoid Content and Identification. To one volume of sodium dodecyl sulfate treated chromatophores, or fraction A, B, or C, two volumes each of acetone and methanol, and one volume of 10 N NaOH were added. The mixture was allowed to saponify in the dark for 1 hr. An excess of water was then added and the solution was extracted with ether three times. The ether extracts were washed with water until the washings were neutral and then the extract was dried over anhydrous sodium sulfate. The ethereal solution was decanted and the ether was removed by rotary evaporation. The residue was taken up in a small volume of benzene. From the absorbance at the wavelength maximum of this solution, and a knowledge of the $\epsilon_{1\,cm}^{1\%}$ of the major carotenoid (Davies, 1965) in the fraction, the ratio of Bchl:carotenoid was obtained. Afterward the benzene-soluble material was spotted directly onto thin-layer sheets (ChromAR Sheet 500; Mallinckrodt Chemical Works, New York). The chromatograms were developed with a solvent of petroleum ether (bp 20–40°) containing 9% acetone. Immediately after development the chromatogram was placed in a densitometer (Densicord; Photovolt Corp., New York City) and scanned using a blue filter over the phototube. The proportion of each carotenoid was calculated from the area under the derived peak. Carotenoids were identified from their spectra (Davies, 1965) and from their order of movement on chromatography compared with those described by Benedict *et al.* (1961).

Gel Electrophoresis. Electrophoresis on polyacrylamide gel was carried out in an homogeneous buffer system of 50 mM Tris–0.125% sodium dodecyl sulfate (pH 8.0). Stock solutions of 32% acrylamide containing 0.5% *N,N'*-methylenebisacrylamide, 0.3% *N,N,N'N'*-tetramethylethylenediamine, and 1% ammonium persulfate were mixed in the proportion of 2:1:1 (v/v). Gels containing different percentages of acrylamide were obtained by varying the proportion of the three stock solutions to those of buffer and water; the final buffer concentration in the gels was the same as that in the buffer pots. The mixed reagents (5 ml) were pipetted into glass tubing 13 cm in height and 0.8 cm internal diameter, and water was layered on the surface prior to gel formation to ensure a flat loading surface. Gels were equilibrated by electrophoresis for 15 min at 150 V. Samples being examined were mixed with propylene glycol (10% v/v) and layered onto the surface of the gel under the buffer. After electrophoresis the gels were stained if necessary by Amido-Schwarz 10B (0.1% in 10% acetic acid), and destained by leaching in methanol–water–acetic acid (5:5:1, v/v). The molecular size of components was measured by the techniques of Shapiro *et al.* (1967) and by that of Blatter and Reithel (1970).

Results

Fractionation of Chromatium Chromatophores. It was found that if sodium dodecyl sulfate was added to a suspension of chromatophores at a ratio of 12 g/mmole of Bchl, then the suspension clarified to give a clear wine red solution; there was little change in the near-infrared absorption spectrum (*cf.* Suzuki *et al.*, 1969).

The fractionation procedure described in the Methods section (Figure 1) is aimed at providing pure components containing different spectral forms, while paying little attention to yields obtained. Yields can be improved by recycling some of the supernatants and precipitates which are generally discarded through the ammonium sulfate fractionation. For a rapid purification of reasonably pure fraction A in high yields, the hydroxylapatite step may be omitted, and ammonium sulfate fractionation performed directly on the sodium dodecyl sulfate treated chromatophores after they have been diluted threefold (S. J. Kennel, unpublished observation).

Fractions A, B, and C are the major Bchl-carrying com-

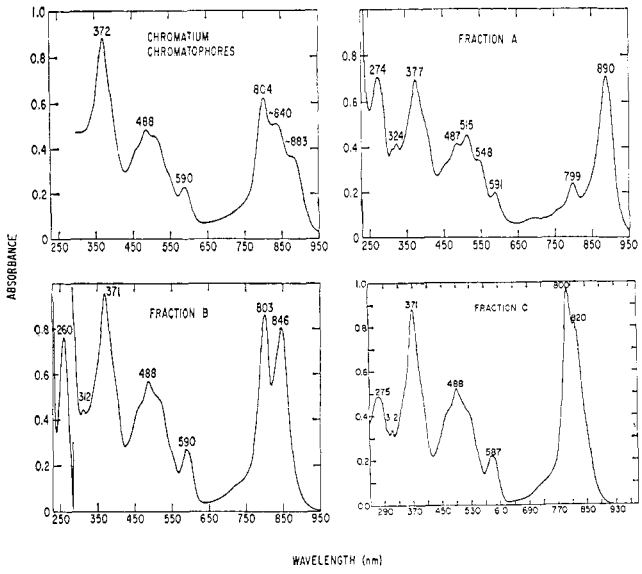

FIGURE 2: Absorption spectra at room temperature of chromatophores and fractions A–C dissolved in 50 mM Tris (pH 8.0). The fractions were derived from photoautotrophically grown cells.

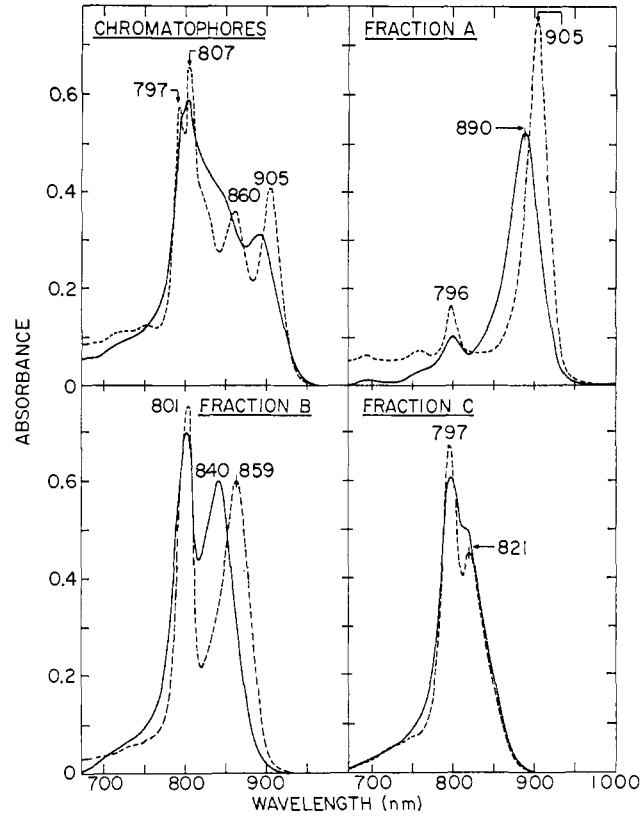

FIGURE 3: Near-infrared absorption spectra of chromatophores and fractions A–C dissolved in 50% glycerol–0.02 M Tris (pH 8.0) at room temperature (solid line) and at 77°K (dashed line). The fractions were derived from photoautotrophically grown cells.

ponents of the organism; little Bchl remains adsorbed to the hydroxylapatite after elution with 0.7 M sodium phosphate. The relative concentrations of each fraction was found to vary in cells grown under different conditions. For example, fraction C occurs in relatively low concentration in the chromatophores whose absorption spectra is shown (Figure 2), and in an even lower concentration in other chromatophore preparations (*e.g.*, Duysens (1952)), but is the most concentrated of the fractions under other growth conditions (*e.g.*, Figure 3; see also Vredenberg and Amesz, 1967). Any published *Chromatium* absorption spectrum can be duplicated by mixing varying proportions of the absorption spectra of fractions A, B and C.

The isolated fractions can be stored in the dark at 4° for several weeks without any noticeable deterioration. Alternatively the fractions can be lyophilized and stored in a dry state for an indefinite period; addition of water to the dried powder results in a rapid dissolution of the complexes to give a clear pink solution; the light-induced spectral changes are still retained.

Absorption Spectra of Fractions A, B, and C. Figure 2 shows the room temperature absorption spectra that are obtained for complexes isolated from photoautotrophically grown cells. There is an obvious fractionation of the Bchl forms. Fraction A contains all the B890 present in the chromatophores, fraction B all the B850, and fraction C all the B820; the 800-nm form is divided between them. The absorbance at ~590 nm represents the second (orange) transition of the Bchl; the precise location of the peak is affected by how far into the near-infrared region the major absorption band is shifted. The peaks around 490 nm and the pink coloration of the isolated components are due to the presence of carotenoids in all the three fractions; the carotenoids in fraction A give rise to a different absorption pattern (max at 515 nm) than those in fractions B and C, whose spectra are identical in this region. The variation in carotenoid composition between fraction A and fractions B and C (see below) accounts for the difference. The Soret peaks of Bchl are located at ~370 nm; the shoulder on the red side of fraction A's 377-nm absorption maximum could be due to the γ peak of cytochrome *c*, which is present in considerable amounts in this fraction. There are minor peaks in fraction A at 690 and 765 nm; the former is probably due to the presence of some oxidized Bchl, whereas the latter may be due to bacteriopheophytin; this latter peak is always present in isolated reaction centers (Gingras and Jolchine, 1969; Reed, 1969; Thornber *et al.*, 1969) and is also observed in some instances (*e.g.*, *Rps. viridis*) in the whole organism. It may be related to the spectral changes observed at this wavelength by Loach (1966). Fraction B is the only one of the three complexes which has a high absorbance at 260 nm in the ultraviolet region (Figure 2); this may be due to a coprecipitation at acid pH of some RNA as well as the fraction itself during the purification procedure.

The spectra of complexes derived from chromatophores with a different near-infrared absorption spectrum (Figure 3) are similar in all respects to those shown in Figure 2, with one exception—the location of the shorter near-infrared peak of fraction B is shifted to a lower wavelength (840 nm).

It could be argued that the 799-nm peak in fraction A is due to contamination by the other fractions; however, as is later demonstrated, the P883–P800 reaction center complex is present in fraction A; thus some of the 799-nm absorption is due to P800. A calculation can be made of the amount of absorbance due to P800 by determining the

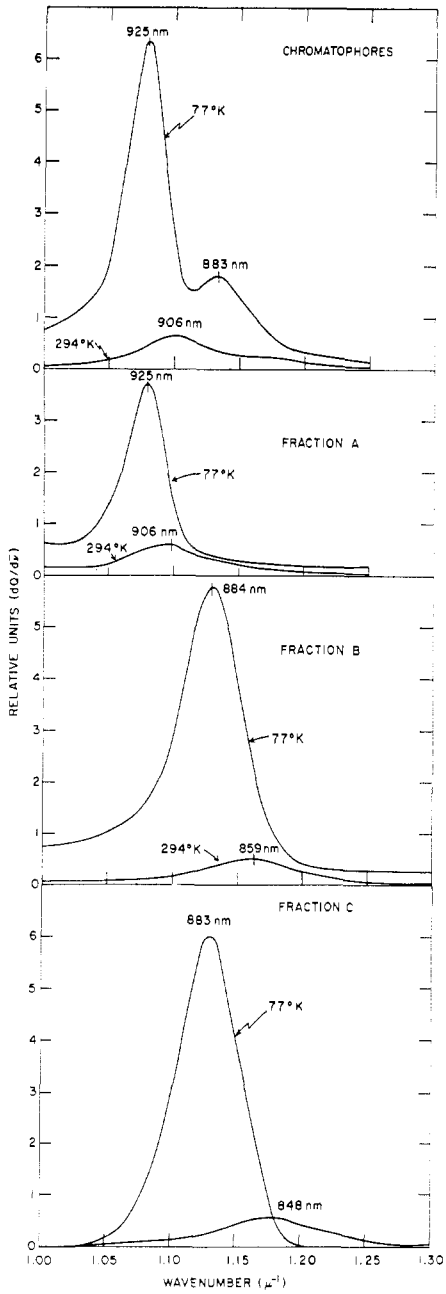

FIGURE 4: Emission spectra of chromatophores and fractions A–C dissolved in 50 mM Tris-HCl (pH 8.0) at room temperature (294°K) and at 77°K. The spectra were recorded of samples having an absorbance at 590 nm of less than 0.2 cm^{-1}. The sample thickness was 1 mm. The spectra in relative quantum flux per unit wave number are corrected for the response of the monochromator and the phototube.

P883 concentration in the fraction, and by assuming the P800:P883 ratio is 2:1, *i.e.*, the same ratio that occurs in other reaction center preparations (Clayton, 1966b; Gingras and Jolchine, 1969; Reed and Clayton, 1968; Thornber *et al.*, 1969; see also Sauer *et al.*, 1968). Using this method, it was found that only half of the absorbance of 799 nm was due to P800; thus P800 + B800 = 1:4.

Light-Harvesting Bchl Forms Associated with Each Fraction as Revealed by Low-Temperature Spectroscopy. Figure 3 shows the sharpening and/or shifting of some absorption bands upon lowering the temperature of the samples studied to 77°K. For these studies complexes which had been isolated from cells grown photoheterotrophically on an acetate medium were used. For chromatophores, the room temperature maximum at 800 nm is split into two peaks at 797 and 807 nm, which represent B*800 and B800, respectively (Vredenberg and Amesz, 1967); there is a shoulder at ~825 nm (B820), and peaks at 860 nm (B850) and 905 nm (B890); the latter two are shifted from their location at room temperature. The low-temperature spectrum reveals more clearly which light-harvesting Bchl forms are associated with which fraction. Fraction A contains B890 and B*800? + P800; fraction B contains B800 and B850, but the location of B800 in the isolated complex has been shifted from 807 nm in chromatophores to 801 nm; fraction C contains B*800 and B820. The presence of the 760-nm absorption band in fraction A shows more clearly in the 77°K spectrum.

Parson (1968) has observed a shift of reaction center bleaching to longer wavelength upon cooling of *Chromatium* chromatophores to 80°K; this is concomitant with the shift of B890 to 905 nm.

Emission Spectra. The fluorescence emission spectra of chromatophores and of the three fractions which had been isolated from photoheterotrophically grown cells are presented in Figure 4. The spectra were recorded at 294 and 77°K. A considerable increase in fluorescence yield as well as an increase in the wavelength of maximum emission occurred as the temperature of the samples was lowered. A narrowing of the band width (from 50 to 30 nm) of the emission spectra also occurred at low temperature.

Chromatophores exhibit a two-banded spectrum (F925 and F883). It appears that these two emissions could arise from different complexes present in the chromatophore, *i.e.*, F925 from fraction A, and F883 from fractions B and/or C. All the spectra obtained are of samples to which some sodium dithionite has been added; this addition ensured that any photobleachable pigments were in their reduced form, since it was observed that upon isolation the reaction center pigment is partially oxidized (see below). Addition of increasing amounts of a reductant gave increased flouresence yields over that of the isolated material, and addition of oxidizing agents decreased the fluorescence yield.

Light-Induced Spectral Changes. The Cary 14R recording spectrophotometer is constructed so that the spectrum of a sample can be recorded by a weak beam of measuring light (IR1 mode), or the spectrum can be recorded while the sample is exposed to a strong beam of white light (IR2 mode). Thus comparison of the spectra of a sample recorded in the two modes can indicate whether any photobleachable pigment molecules are present. If the spectrum is reexamined in the IR1 mode after exposure to the actinic beam, and after a period during which the sample is in darkness, then an indication is obtained of the presence in a sample of any reversible light-induced spectral changes. Submitting the isolated fractions under aerobic conditions to this treatment revealed that (a) fraction A exhibited reversible light-induced adsorbance changes, fraction B showed both reversible and irreversible changes, and fraction C had only irreversible changes, and (b) only a small proportion of the total Bchl molecules in fractions A and B exhibited the reversible absorbance changes.

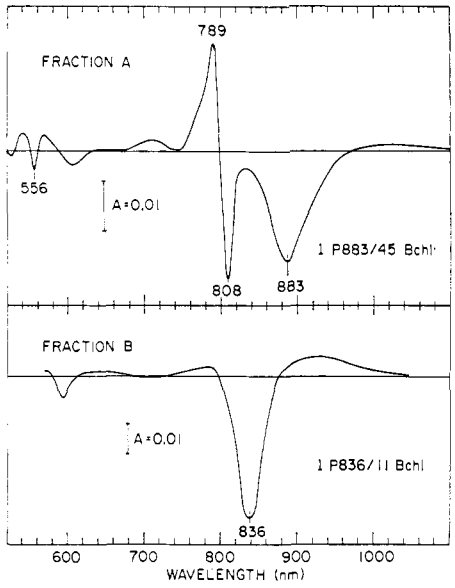

FIGURE 5: Light-induced reversible absorbance changes in fractions A and B. The sample of fraction A contained 9 μM Bchl and 50 μM sodium ascorbate. The sample of fraction B contained 4.9 μM Bchl, 100 μM menadione, and 5 mM sodium nitrite. Light of 375 nm (6.2 nE/cm^2 per sec) was used to oxidize fraction A; broad band blue light (Corning 5-57) was used for fraction B.

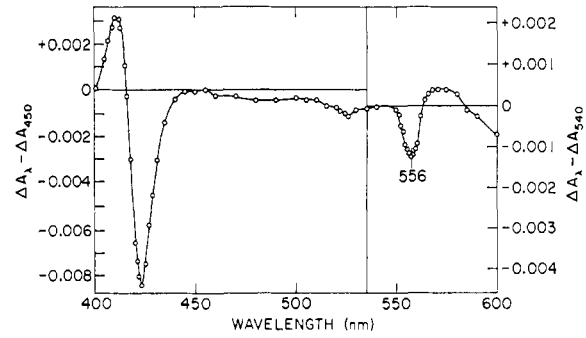

FIGURE 6: Light-induced absorbance changes obtained by examination of fraction A in a double-beam spectrophotometer. The sample contained 2.5 μM phenazine methosulfate, and was illuminated by light of 825 nm (11.3 nE/cm per sec) for 8 sec followed by a dark period of 30 sec during which the light-induced changes recovered.

The proportion of reaction center pigment to light-harvesting Bchl was determined for "crude chromatophores" and for the fractions. The ratio was obtained from a determination of the total Bchl in the sample and from a more sensitive spectrophotometric determination of the ΔA at the wavelength of maximum reversible photobleaching using an ϵ value of 100 mM^{-1}. For chromatophores 50 μM sodium ascorbate was added, and a ratio of 1 P883:190–250 Bchl molecules was obtained; the variability of this value is a reflection of the variation in the ratio obtained for chromatophores which had been isolated from cells grown under different conditions. Autotrophically grown cells have the higher ratio. In the absence of added ascorbate a ratio of one reversibly photobleached pigment molecule to ~50 Bchl molecules was obtained; however, this ratio will later be shown to be a measure of two pigments (P883 and P'890). The wavelength of maximum photobleaching in the absence of ascorbate is 890 nm, whereas in its presence it is 883 nm.

The reversible light-induced spectral changes of fractions A and B are shown in Figure 5. Fraction A demonstrates the changes associated with the known (Duysens, 1952) reaction center pigment of the organism (bleaching at 883 nm, and a blue shift of P800, which gives rise to an increased absorption at 789 nm and to a decreased absorption at 808 nm). Sodium ascorbate (50 μM) or phenazine methosulfate (2.5 μM) (Figures 5 and 6) was added to the fraction to ensure that P883 was fully reduced in the dark; chemical difference spectra had shown that some P883 was present in an oxidized form in the isolated component. A second reason for the additions was that the spectral changes recovered very slowly after illumination even under anaerobic conditions in which electrons will not be lost to oxygen, the presence of phenazine methosulfate and/or ascorbate gave rapid recoveries. Under these conditions a ratio of P883:B890 of 1:45 was determined. Chemical oxidation *vs.* reduction cannot be used for the estimation of the ratio for the reasons outlined below. The light *vs.* dark difference spectra (Figure 5) also shows a broad increased absorption above 1000 nm and a decreased absorption at 600 nm which are due to P883 → P$^+$883 (Arnold and Clayton, 1960). A cytochrome (C556) undergoes reversible bleaching (Figures 5 and 6); it is noteworthy that the ratio of the γ:α peaks (7:1) is much higher than that of most other cytochromes. From Figures 5 and 6 the molar ratio of C556:P883 can be calculated to be 2:1 using an ϵ (α band) value of 20 mM^{-1}. Both hemes of C556 are oxidized by P883 just as *in situ* (Parson, 1969b). Several spectral changes due to changes in unknown compounds are present: there is an increased absorption at ~770 nm (on the blue wavelength side of the 789-nm peak) which may be associated with changes in the 765-nm absorption peak (*cf.* Loach, 1966), an increase in absorption around 720 nm, and an increase around 560 nm. These last three changes are also observed in isolated reaction center preparations (Gingras and Jolchine, 1969; Reed, 1969; Thornber *et al.*, 1969).

When the spectral changes of fraction A are examined without addition of phenazine methosulfate or ascorbate (Figure 7), then an additional reversible bleaching at 890 nm is observed; this bleaching is not accompanied by any associated peak shift as occurs upon oxidation of P883. Thus changes at 808 nm reflect changes in P883, and any change at 890 nm without a change at 808 nm reflects bleaching of pigment termed P'890; P'890 has been used rather than P890 since the latter is used by some investigators for the reaction center species (*i.e.*, P883 in *Chromatium*) of several purple bacteria. Actinic light of 800 nm is only fractionally less efficient than 890-nm light in bringing about the spectral changes of P'890. Figure 7 shows that bleaching of P'890 carries on after P883 oxidation is complete if ascorbate and phenazine methosulfate are absent, but does not occur in their presence. Bleaching at 890 nm occurs more rapidly under aerobic conditions than under anaerobic conditions. The P'890 bleaching is slowly reversed ($T_{1/2} \simeq 5$ min) after illumination, and rapidly reversed if a reducing agent is added at the end of the illumination period; thus P'890 is undergoing oxidation, and not reduction. Oxidation of P'890 is complete after several minutes of illumination under aerobic conditions; thus an estimate can be made of the P883 + P'890 concentration

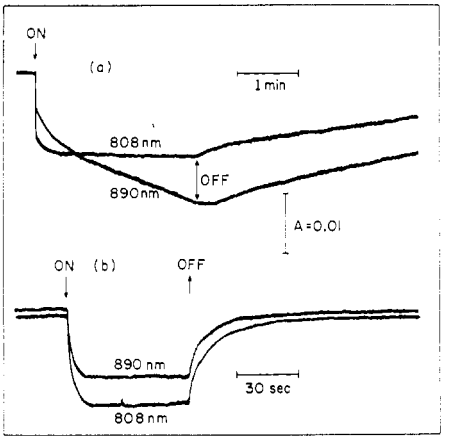

FIGURE 7: Light-induced absorbance changes of fraction A recorded at 808 and 890 nm. (a) In a sample which had been bubbled with nitrogen, and kept in the dark prior to illumination, and (b) in a sample to which sodium ascorbate (final concentration 1 mM) had been added. Light of 890 nm (5.2 nE/cm² per sec) was used to illuminate the samples. The arrows indicate when the light was turned on and off. Both samples contained 5.6 μM Bchl.

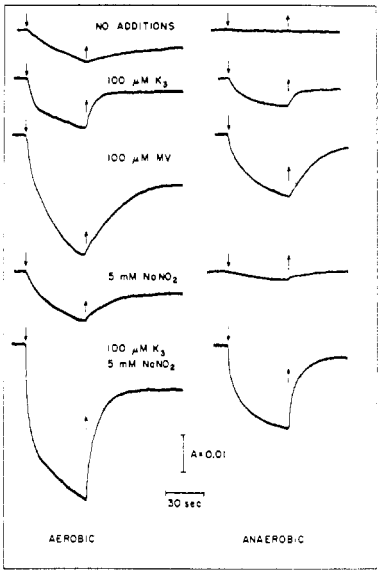

FIGURE 8: Light-induced absorbance changes of fraction B under aerobic and anaerobic conditions; broad band blue light (Corning 5-57) was used to illuminate the sample. The additions to fraction B were as shown; k_3 is menadione and MV is methyl viologen.

from the ΔA 890 nm: a value of (P883 + P'890):B890 of 1:8 is obtained. A similar ratio is obtained if chemical oxidation and reduction are used to determine ΔA at 890 nm. To separate the ΔA of P883 from that of P'890, the concentration of P883 in a sample was determined from a light *vs.* dark difference spectrum measured in the presence of a low concentration (50 μM) of sodium ascorbate; the ratio of P883:P'890 = 1:~4. Hence P883:P'890:B890 is 1:~4:40. P'890 changes were also observed in whole chromatophores using similar conditions.

Fraction B shows a reversible light-induced bleaching of a pigment whose absorption maximum is 836 nm (P836); there are no associated peak shift or cytochrome changes (Figure 5). P836 undergoes oxidation in the light (*cf.* the oxidized and reduced spectra of Bchl *in vitro* of Krasnovskii and Drozdova, 1964). The conditions required for, and the kinetics of, the photooxidation are shown in Figure 8. Under anaerobic conditions, no bleaching of P836 occurs, whereas under aerobic conditions a slow and small reversible oxidation occurs. These observations probably indicate that there is no electron acceptor for P836 in the fraction, and that oxygen can act as such. Examination of the effect of added redox compounds which might be expected to act as a more efficient electron acceptor than oxygen showed that methyl viologen, menadione (vitamin K_3), and ubiquinone-6 permit a more rapid bleaching of P836 (Figure 8). The following redox compounds had no effect on the rate of P836 oxidation: ascorbate, phenazine methosulfate, benzoquinone, FMN, NAD, methyl red, and C552; the first two, in fact, eliminated any bleaching even under aerobic conditions. The most rapid turnover of P836 was obtained in the presence of menadione (100 μM) and sodium nitrite (5 mM); presumably the nitrite acts as an oxidant for any reduced menadione produced by the photoact. Under these conditions a quantum requirement of 400 was determined using 836-, 800-, and 589-nm actinic light. The proportion of P836 to total Bchl in the fraction was determined from the reversible ΔA 836 nm in light *vs.* dark and in chemically oxidized *vs.* reduced difference spectra; a value of 1 mole of P836 to 11 ± 1 moles of total Bchl was obtained in both cases. The traces in Figure 8 were all recorded using broad-band blue actinic light; broad-band near-infrared light was just as effective in bringing about the spectral changes. Preliminary observations indicate that P836 changes also occur in whole chromatophores which have had menadione and nitrite added to them. In all instances when P836 oxidation occurs, an irreversible bleaching of an 848-nm form of Bchl in fraction B is observed; this photolability has been observed previously by Clayton (1962) in deoxycholate-treated *Chromatium* chromatophores. The irreversible change explains why the traces in Figure 8 do not fully reverse.

Composition. FRACTION A. The determination of the proportion of the different Bchl forms present in fraction A is reported above (see also Table I) together with the C556:P883 ratio. The major carotenoids in the fraction were identified as spirilloxanthin (60%) and lycoxanthin (30%); smaller amounts of lycophyll (7%) were present. The carotenoids in chromatophores were found to be mainly represented by lycoxanthin (50%), spirilloxanthin (35%), and anhydroviridin (13%) (*cf.* Goodwin and Land, 1956). It is believed that these estimations of carotenoid proportions were accurate to within 10% of the figures given; however, the main point of the carotenoid estimations was to demonstrate differences in the nature of the carotenoid between the different fractions. Thus spirilloxanthin is the major carotenoid of fraction A, and lycoxanthin is the major carotenoid of fractions B and C. The proportion of carotenoid to total Bchl (Table I) was determined as described in the Methods section.

The cytochrome content was examined by chemical difference spectroscopy. C556 is observed when ferricyanide *vs.* sodium ascorbate difference spectra are studied. The ΔA at 556 nm was used to estimate its concentration using an ϵ value of 20 mM^{-1}; a value of 2.4 ± 0.3 moles/mole of P883 was obtained. It is noteworthy that this is the same value as was obtained from the light *vs.* dark difference spectrum (Figure 5).

TABLE I: Composition[a] of *Chromatium* and *Rps. viridis* Fractions.

	Chromatium			
	Fraction A	Fraction B	Fraction C	*Rps. viridis* Fraction[b]
Reaction Center	P883 (1) P + B800 (4)		0	P958 (1) P830 (2)
Other reversibly bleached pigments	P'890 (~4)	P836 (1)		
Light-harvesting Bchl	B890 (40)	B800 + 850 (10)	B*800 + 820 (10)	0
Carotenoid	Spirilloxanthin (12) Lycoxanthin (6) Lycophyll (1)	Lycoxanthin (2) Spirilloxanthin (1)	Lycoxanthin (2) Spirilloxanthin (1)	Dihydrolycopene[c] (1)
Cytochromes	C556 (2) C552 (~7)	0	0	C558 (2) C553 (~5)

[a] Molar ratio (corrected to the nearest whole number) of constituents of fractions per mole of reaction center or P836; for fraction C the ratio was adjusted so that the light-harvesting Bchl content was the same as fraction B's. [b] From Thornber *et al.* (1969). [c] Lycopene was originally reported as the carotenoid; Malhotra *et al.* (1970) have shown that the carotenoid is most likely 1,2-dihydrolycopene.

Upon further reduction of the sample with sodium dithionite a second cytochrome whose α band is at 552 nm (C552) is observed. The proportion of C552:P883 was determined from a difference spectrum of sodium dithionite *vs.* sodium ascorbate reduced samples using an ϵ value of 20 mM^{-1}; C556 is reduced in both instances and thus does not contribute to the difference spectra; a ratio of 7 ± 1 mole of C552/mole of P883 was obtained.

FRACTION B. This complex contains carotenoid, Bchl, and protein; no cytochromes are present in this component. For every mole of P836 there are 3 moles of carotenoid and 10 moles of B800 + B850; the carotenoid is lycoxanthin (60%) and spirilloxanthin (25%). Anhydroviridin and lycophyll represent the bulk of the remainder (12%); lycopene and demethylated spirilloxanthin occur in traces. No other constituents have so far been identified in this fraction.

FRACTION C. The proportion of carotenoids to each other and to Bchl are indistinguishable from these that occur in fraction B.

The total known composition of all three complexes is given in Table I.

Gel Electrophoresis of the Fractions. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate has been shown by Shapiro *et al.* (1967) to be a useful tool to study the purity and size of sodium dodecyl sulfate treated proteins. Thus the sodium dodecyl sulfate soluble fractions should submit readily to examination by this procedure. Sodium dodecyl sulfate treated chromatophores exhibit three colored bands upon electrophoresis (Figure 9). By comparison of the electrophoretic mobilities of isolated fractions A, B, and C to those of the zones obtained for chromatophores it can be deduced that the slowest running component in chromatophores is fraction A, and that fractions B and C are both contained in the middle zone. The three zones observed in the gel pattern for chromatophores are of quite different colors. The component of lowest electrophoretic mobility is pink, whereas the middle zone is maroon. This difference in color adds confirmation to the identification of zones obtained for chromatophores with the individual fractions since fraction A is pink and fractions B and C are maroon. The fastest moving zone is orange and is analogous to the free pigment zone observed when Chl a containing organisms are examined in an identical manner (*cf.* Thornber *et al.*, 1967; Thornber, 1969); however, in the case of *Chromatium*, this zone contains little free chlorophyll, and is mainly composed of carotenoid. Hence the Bchl-P's of *Chromatium* are more stable to sodium dodecyl sulfate action than those of other photosynthetic organisms studied in this manner.

After ammonium sulfate precipitation the isolated fractions have a tendency to aggregate and to dissolve less easily the more times the precipitation has been performed; this aggregation can be seen in the gel patterns as two faint pink bands which electrophorese more slowly than the major band

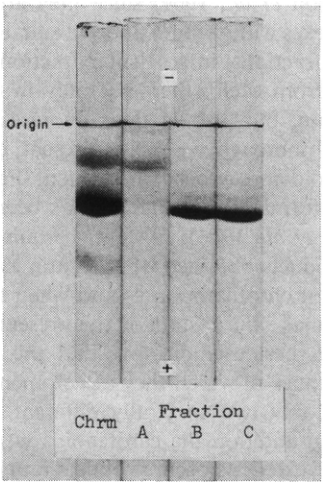

FIGURE 9: Polyacrylamide gel electrophoretic patterns obtained by electrophoresis of sodium dodecyl sulfate treated chromatophores and fractions A, B, and C on 6% acrylamide gels in 50 mM Tris–0.125% sodium dodecyl sulfate (pH 8.0). The gels are not stained.

of fraction A, and which are not seen in the chromatophore pattern. Fraction A contains a trace of fraction B and/or C; this was expected since not all the absorbance at 800 nm could be accounted for by the presence of P800 in the fraction.

Fraction A has a molecular weight greater than 200,000, which is the upper limit that can be used for relating electrophoretic mobility to molecular weight (Shapiro *et al.*, 1967). Hence a different method was used to determine the molecular size of fraction A. Blatter and Reithel (1970) have shown that a good estimate can be made of very high molecular weight molecules by determining the percentage of acrylamide gel which will just prevent the molecule from entering the gel column on electrophoresis. For fraction A an 11% gel will just allow the molecule to enter the column, but a 12% gel will not; this indicates a molecular weight of 500,000 ± 50,000. The fact that fractions B and C are electrophoresed together in any gel percentage indicates that they have the same molecular size. Their molecular size was determined by comparison with markers of known molecular weight (Shapiro *et al.*, 1967), and a value of 100,000 ± 10,000 was obtained.

Discussion

The light-harvesting Bchl in *Chromatium* chromatophores is known to occur in at least five different spectral forms. Vredenberg and Amesz' (1967) terminology for these forms has been used throughout this present study, which has shown that the different spectral forms are contained in different Bchl–P complexes, and that the relative concentration of the complexes in chromatophores varies depending on the growth conditions of the organism. These observations explain the changes that have been observed in the near-infrared absorption spectrum of *Chromatium* cells grown under different light intensities and nutritional conditions (Bril, 1964; Fuller *et al.*, 1963; Garcia *et al.*, 1966; Wassink *et al.*, 1939). A very similar explanation of the phenomenon had been predicted 30 years ago by Wassink *et al.* (1939). The reason for Bchl existing in widely different spectral forms in the complex has not yet been elucidated; however, the material is now available to answer this question. It appears that the Bchl molecules in each complex are buried deep inside a hydrophobic core of protein (*cf.* Olson *et al.*, 1969) since they are not extracted from the complexes with sodium dodecyl sulfate.

The isolated fractions, in particular fractions A and B, are quite different from each other, not only in the Bchl forms that they contain, but also in their fluorescence properties, in the nature of photoactive pigment present, in their size, and in their carotenoid and cytochrome content. Partial resolution of the spectral forms of *Chromatium* has been achieved previously (Garcia *et al.*, 1966); however certain key differences between the products obtained when Triton X-100 is used for the dissolution of chromatophores and when sodium dodecyl sulfate is used make the results of the present study particularly significant, besides indicating that the two detergents differ in the manner in which they attack membranes. Triton X-100 enabled only two spectrally different fractions to be obtained, and in addition the carotenoids were not fractionated to the same extent as occurs with sodium dodecyl sulfate. The B890 fraction obtained by Triton treatment, unlike fraction A, did not exhibit light-induced cytochrome oxidation (Ke *et al.*, 1968), although some C552 and C555 were present in the preparation. Ke *et al.* (1968) reported a higher proportion (1:9) of reaction center pigment to light-harvesting chlorophyll than occurs in fraction A; however, the ratio may be the same in both preparations, since Ke *et al.*, (1968) determined P890 by chemical difference spectroscopy, which would give the P883 + P′890 content, if the latter component were present in their preparation.

Fraction A is a solubilized form of *Chromatium's* photoelectron transport pathways. The isolated component exhibits some of the reactions which have been previously characterized to be associated with the cyclic pathway, *i.e.*, reversible spectral changes of P883 and C556. However, fraction A almost certainly does not contain all of the components of the cycle since the return of electrons to $C^{+}556$ and $P^{+}883$ after illumination of fraction A is much slower than in chromatophores. Addition of phenazine methosulfate and/or ascorbate greatly accelerates the return of electrons; phenazine methosulfate probably creates an artificial cycle, and/or couples electrons from an endogenous electron pool to the reaction center, and ascorbate will donate electrons to $C^{+}556$ and $P^{+}883$. Considerable quantities of the oxidized form of the low-potential cytochrome, C552, are contained in fraction A. This cytochrome can be made to undergo reversible photooxidation upon reducing the redox potential of fraction A (J. M. Olson, unpublished data; W. W. Parson, unpublished data; P. L. Dutton, unpublished data). This observation supports the proponents of the hypothesis that both the high- and low-potential cytochromes are oxidized by one photosystem, *i.e.*, that of fraction A. A possibility did exist, however, that P883 oxidized C556, and P′890 (if indeed it is a reaction center) oxidized C552; this possibility was eliminated by G. D. Case, W. W. Parson, and J. P. Thornber (unpublished data) who found that after exposure of fraction A to a 20-nsec laser flash, the decay kinetics of the spectral change at 785 nm, which is due to changes in the P800 associated with P883, and not in P′890 (see Figures 5 and 7), match the kinetics of the absorbance changes produced by oxidation of C556 at high redox potentials as well as those of C552 at low redox potentials, *i.e.*, the same reaction center species is involved in both cases. The function of P′890 has not yet been fully elucidated, and while the possibility that it is a second reaction center species in fraction A has not been completely eliminated, it appears much more likely that it is an electron carrier pool which donates electrons to $P^{+}883$ should no other donor (C552 or C556) be able to do so. The following observations are concomitant with this latter postulation: the slow rate of P′890 bleaching, the absence of P′890's spectral changes in the presence of added electron donors, the fact that 800-nm light is almost as efficient as 890-nm light in bringing about P′890's oxidation, and the difference in the kinetics of the recovery of the 890-nm bleaching and in those of the 808-nm change (see Figure 7a).

The function of fraction A is analogous to that of the reaction center isolated from *Rps. viridis* (Thornber *et al.*, 1969); however the two components differ in their content of light-harvesting Bchl and carotenoid (Table I). Why *Chromatium* does not give a reaction center preparation devoid of antenna Bchl as occurs with *Rps. viridis* is a matter of conjecture; possibly the greater carotenoid concentration in the photosynthetic unit makes the *Chromatium* complex more stable to sodium dodecyl sulfate action (*cf.* Vernon *et al.*, 1969), or perhaps the antenna Bchl is located differently in the two organisms. So far all the reaction centers which have been

isolated as separate entities (Gingras and Jolchine, 1969; Reed, 1969; Thornber *et al.*, 1969) have been derived from purple bacteria, and all have been observed to contain a trimer of Bchl molecules at the reaction center; thus in all proability the P883–P800 molecules in *Chromatium* are contained in a protein complex analogous to that of the isolated reaction centers. The molecular weight determined by gel electrophoresis of the cytochromeless reaction center of *Rps. spheroides* R26 has been determined to be about 50,000 (J. P. Thornber, J. McElroy, and G. Feher, unpublished data); alternative detergents and preparative procedures to those used by Reed (1969), who first isolated this reaction center, were used for its isolation. Hence in Figure 10, which is a diagrammatic representation of the components that are postulated to be in fraction A, the P883–P800 molecules are shown to be contained in a 50,000 molecular weight component. The B890 form of Bchl in fraction A could quite conceivably be present in such a Bchl–P complex as has been proposed by Thornber and Olson (1968) and Thornber (1969) to be a common entity (*i.e.*, a complex of 150,000 molecular weight containing four identical protein subunits and 20 molecules of chlorophyll) in all photosynthetic organisms. It would require two such Bchl–P molecules to accommodate the 40 molecules of B890 in fraction A. The total molecular weight of the components postulated to be in fraction A (Figure 10) is 450,000 and this size is close to that determined experimentally.

Fraction B has an absorption spectrum very similar to that proposed for the second photosystem of *Chromatium* (Morita, 1968). It does not however have any of the other proposed characteristics; C552 is contained in fraction A only, and the near-infrared absorbance changes observed by Cusanovitch *et al.* (1968) to be associated with C552 photooxidation, and attributed by them to a spectral change of a second reaction center (P905), also occur in fraction A only; the 905-nm change appears to be part of a peak shift of a light-harvesting Bchl form in fraction A. Fraction B contains a reversibly photooxidized pigment, P836. A similar bleaching was observed in whole chromatophores, thereby indicating that P836 is not created by detergent treatment. Furthermore, Duysens (1952), Ke *et al.* (1968), and Cusanovitch *et al.* (1968) have also observed spectral changes at ~835 nm in *Chromatium* or in fractions thereof. The physiological significance of P836 remains to be determined. In this respect it is noteworthy that P836 is quite specific in which added redox compounds will accelerate its oxidation; menadione is closely related to the naturally occurring vitamin K_2 (Takamiya *et al.*, 1967). So far the measured quantum requirement precludes its functioning as a second reaction center. It is concluded that all the known photochemical reactions in *Chromatium* are driven by the same reaction center species, P883.

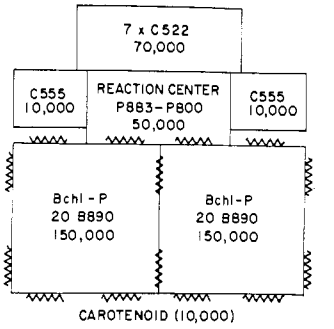

FIGURE 10: Schematic representation showing the hypothetical components of fraction A, and their molecular weight. The short zig-zags represent carotenoid (spirilloxanthin and lycoxanthin) molecules.

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