The Pigment Complement of the Photosynthetic Reaction Center Isolated from *Rhodospirillum rubrum* *

(Received for publication, June 25, 1973, and in revised form, May 6, 1974)

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**SUMMARY**

Isolated photosynthetic reaction center from the bacterium *Rhodospirillum rubrum* was extracted with acetone-methanol. Its main pigments were identified as bacteriochlorophyll, bacteriopheophytin, and spirilloxanthin. The extinction coefficients of these pigments in acetone-methanol were determined. Quantitative spectroscopic analysis of the dry acetone-methanol extracts indicated a bacteriochlorophyll to bacteriopheophytin mole ratio of 2 and led to an extinction coefficient at 868 nm of 71.3, 142.6, or 214 mm⁻¹ cm⁻¹, depending on whether the photosynthetic center was assumed to contain 2, 4, or 6 bacteriochlorophyll molecules. The extinction coefficient of $P_{870}$ was determined by mixing photo-oxidized photosynthetic center with ferrocytochrome c in a stopped flow spectrophotometer. Complete reaction was observed when the reactants were mixed in equimolar ratio calculated on the basis of an extinction coefficient of 143 mm⁻¹ cm⁻¹ at 868 nm. This photosynthetic center is proposed to contain 4 moles of bacteriochlorophyll, 2 moles of bacteriopheophytin, and 1 mole of spirilloxanthin per equivalent of $P_{870}$.

The quantification of the pigment composition of isolated photosynthetic reaction centers is one of the keys to the elucidation of the primary act of photosynthesis. Clayton (1) was the first to attempt such an analysis on *Rhodopseudomonas spheroides* (carotenoidless strain R-26) chromatophores treated with chlororbidate to destroy their light-harvesting bacteriochlorophyll. Clayton (1) suggested the presence of 3 or a multiple of 3 bacteriochlorophyll molecules per photosynthetic center and proposed the value of 113 mm⁻¹ cm⁻¹ for the extinction coefficient of the 868 nm absorption band.

Clayton's hypothesis was reinforced by circular dichroism spectroscopy performed by Sauer et al. (2) on a photosynthetic reaction center preparation from *R. spheroides* (R-26). According to the interpretation of Sauer et al. (2), this preparation contained a trimer of bacteriochlorophyll with large exciton interaction in the reduced form. No evidence for such strong interaction was found in the oxidized form.

More recently, Mauzerall (3) and Reed and Peters (4) have performed pigment analysis of dodecylidimethylamine N-oxide preparations from the R-26 mutant of *R. spheroides*. The shape of the 535 nm absorption band attributed to bacteriopheophytin led these authors to suggest the presence of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules per unit of reaction center. This proposal was supported by low temperature absorption and circular dichroism spectroscopy carried out by Reed and Ke (5).

While our own manuscript was in preparation, Straley et al. (6) reported very convincing evidence for this model based on the stoichiometry of $P_{870}$ reduction by mammalian ferrocytochrome c. So far, no such thorough analysis has been reported for other photosynthetic center preparations.

The object of this article is a quantitative pigment analysis of the carotenoid-containing center isolated from wild type *Rhodospirillum rubrum* (7). The results are discussed in the light of different possible models.

**EXPERIMENTAL PROCEDURE**

**Materials**

Chemicals

Ammonyx LO (dodecylidimethylamine N-oxide) was a gift from Unyx Chemicals, Jersey City, N. J. Acetone (spectrophotometric grade) was purchased from J. T. Baker Chemical Co., methanol (spectro grade) from American Chemicals Ltd., and ethyl ether (reagent grade) from Mallinckrodt Chemical Workers Ltd. Absolute ethanol was redistilled over zinc powder and KOH pellets. Cellulose powder (MN 300 HR. Macherey, Nagel and Co.) and Silica Gel G (E. Merck AG, Darmstadt) were used for thin layer plate chromatography. Horse heart cytochrome c (type VI) was from Sigma Chemical Co. The other chemicals (reagent grade) were obtained from Fisher Scientific Co.

**Bacteria**

The wild strain of *Rhodospirillum rubrum* was obtained from the American Type Culture Collection (No. 11170). The carotenoidless mutant (strain G9) was a gift from Dr. Germaine Cohen-Bazire.

**Instrumentation**

Kinetic measurements were made by means of a Durrum Instruments stopped flow photometer (model 130) equipped with a logarithmic amplifier (model 131) in series with a Tektronix 5108N storage oscilloscope. A 2-em pathlength cell was used. The analyzing beam was provided by a 650-watt General Electric tungsten-halogen lamp powered by a Kepec JQE75 power supply. Wavelength was selected by means of a 500-mm Bausch and Lomb
monochromator (600 lines per mm of grating, blazed at 500 nm) calibrated at 550 nm against the a-band of reduced cytochrome c. Slit widths of 0.2 mm were used, corresponding to a half-band width of 0.66 nm. Hamamatsu R5374 and R3136 photomultiplier tubes were used. Absorption spectra were recorded with a Cary 14R spectrophotometer. Membrane filters (UM-2) were used in a model 12 ultrafiltration cell (Amicon Corp.).

Methods

Bacterial Cultures and Preparation of Chromatophores

*R. rubrum* was grown semiaerobically in 12-liter bottles at 30°C in the medium described by Cohen-Bazire et al. (8). Illumination was provided by two 150-Watt photoflood lamps. The bacteria were harvested after 4 or 5 days, at the end of their logarithmic growth phase.

Chromatophores were obtained from these cells by alumina grinding followed by a first centrifugation at 3,000 × *g* (5 min) to remove aluminum and cell debris. The supernatant was subjected to differential centrifugation, first at 20,000 × *g* (20 min) and then at 100,000 × *g* (60 min). The pellet of the latter centrifugation was resuspended in 50 mM (pH 7.0) phosphate to a final absorbance of 75 at 880 nm.

Preparation of Photosynthetic Center

Photosynthetic center was isolated by treating chromatophores with Ammonyx LO according to the method of Noël et al. (7), except for the following modifications. The 45% ammonium sulfate precipitate was resuspended and dialyzed for 18 hours in 50 mM (pH 7.0) phosphate for pigment analysis or in 10 mM (pH 7.8) Tris-HCl for kinetic measurements. The buffer was changed twice during dialysis, and subsequently the solution was clarified by centrifugation at 10,000 × *g* for 20 min.

Preparation and Purification of Pigments

The pigments from the G9 (carotenoidless) mutant of *R. rubrum* were extracted in acetone-methanol (7:2, *v*/*v*). The extract was divided into two portions, one of which was totally phosphatized by the addition of concentrated HCl (5% final volume). The two portions were then phase-transferred to ether, and the ether was washed several times with water and dried over anhydrous NaSO₄. Both solutions were separately streaked onto glass plates coated with cellulose and chromatographed in the dark with a mixture of petroleum ether (boiling range, 30 to 60°C) and ethyl ether (9:1, *v*/*v*). The resulting bands were removed from the plate by scraping, collecting the cellulose and then eluting it with ethyl ether stored over Na₂SO₄. All of the spectra reported here were measured within 1 hour of isolation, and all other experiments were carried out within 6 hours after the chromatography.

Spirilloxanthin was purified as follows. A photosynthetic cen ter preparation was extracted with acetone and the pigments were divided into two portions, one of which was totally oxidized as described above by the addition of concentrated HCl (3% final volume). The two portions were then phase-transferred to ether, and the ether was washed several times with water and dried over anhydrous Na₂SO₄. Both solutions were separately streaked onto glass plates coated with cellulose and chromatographed in the dark with a mixture of petroleum ether (boiling range, 30 to 60°C) and ethyl ether (9:1, *v*/*v*). The resulting bands were removed from the plate by scraping, collecting the cellulose and then eluting it with ethyl ether stored over Na₂SO₄. All of the spectra reported here were measured within 1 hour of isolation, and all other experiments were carried out within 6 hours after the chromatography.

Spirilloxanthin was purified as follows. A photosynthetic center preparation was extracted with acetone and the pigments were divided into two portions, one of which was totally oxidized as described above by the addition of concentrated HCl (3% final volume). The two portions were then phase-transferred to ether, and the ether was washed several times with water and dried over anhydrous Na₂SO₄. All of the spectra reported here were measured within 1 hour of isolation, and all other experiments were carried out within 6 hours after the chromatography.

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**Extraction of Pigments from Photosynthetic Center**

In order to avoid the presence of water or detergent in the extract, three different methods were used.

**Method A**—A photosynthetic center preparation (0.15 ml) (*A₄₅₀ = 4.7*) was extracted with acetone-methanol and centrifuged at 2000 × *g* for 10 min; the pellet was washed twice with the same solvent. The volume was brought to 10 ml and the spectrum was measured on the 0 to 0.1 absorbance scale.

**Method B**—Photosynthetic center (2 ml) (*A₄₅₀ = 1.5*) was lyophilized in order to remove water completely. The dry material was extracted with acetone-methanol and the lyophilization process was washed three times with small volumes of acetone-methanol. The pooled extracts were combined and centrifuged for 5 min at 4000 × *g*. The supernatant was transferred to a 10-ml volumetric flask. The pellet and the centrifugation tube were washed twice and the solution was brought to volume.

**Method C**—Photosynthetic center (2 ml) (*A₄₅₀ = 1.5*) was filtered at 4°C on an Amicon UM-2 filter in a model 12 Amicon filtration cell. The filter was then removed from the cell and extracted with acetone-methanol. For a quantitative extraction, the walls of the Amicon cell were carefully washed with a piece of filter paper, which was extracted along with the Amicon filter. This method eliminates most of the water and most of the detergent present in the buffer. In each of the three methods, all steps were carried out in a darkened room, and the spectrum was measured immediately after completion of the extraction.

**Pigment Assay in Acetone-Methanol Extracts**

The presence of spirilloxanthin in our extracts prevented us from using the well-resolved, visible absorption bands of the porphyrins. We used instead their widely overlapping infrared bands. However, independent determinations on known mixtures of bacteriochlorophyll and of bacteriopheophytin in acetone-methanol showed this method to be accurate. The following relationship was applied:

\[
[P] = \frac{A_{747} \times E_{771} - A_{771} \times E_{747}}{E_{771} \times E_{747} - E_{747} \times E_{771}}
\]

\[
[B] = \frac{A_{771} - E_{771} \times [P]}{E_{771}}
\]

where \([B]\) and \([P]\) represent the concentration of bacteriochlorophyll and of bacteriopheophytin, respectively. \(A\) is the absorbance value at wavelength \(\lambda\), and \(E\) is the molar extinction coefficient of species \(z\) at wavelength \(\lambda\).

The amount of spirilloxanthin was determined by the relationship:

\[
[S] = \frac{A_{475} - [B] \times E_{475} - [P] \times E_{475}}{E_{475}}
\]

where \(S\) denotes spirilloxanthin. Values of the extinction coefficients are presented under "Results."

**Stopped Flow Spectrophotometric Analysis**

All solutions were degassed before use by strong stirring at room temperature, in vacuo. All of the experiments were carried out in 10 mM Tris-HCl buffer (pH 7.8) containing no added detergent. Cytochrome c was reduced by treatment with Na₂SO₄ and desalted on Sephadex G-20 in a Pharmacia K15/30 column.

The experimental protocol was as follows: before each measurement, the photosynthetic center preparation contained in the upper mixing syringe of the stopped flow spectrophotometer was illuminated for 60 s with a General Electric D.W.A. 650-watt photoflood at a distance of 25 cm. One second later the pneumatic flow actuator was triggered. Mixing occurred about 10 ms after this.

The absorption change at 650 nm upon reduction of the oxidized photosynthetic center was determined by two different methods: (a) photosynthetic center oxidized as described above was rapidly mixed with 10 mM ascorbate in 10 mM (pH 7.8) Tris-HCl buffer and the total absorption change was measured at 550 and 870 nm; (b) the absorbance changes at 550 and 870 nm induced by a monochromatic actinic beam (800 nm) were measured in a Cary 14R spectrophotometer. With both methods the ratio of Δ*A₅₅₀/Δ*A₈₇₀ was found to be -0.086.

**RESULTS**

The most direct and quantitative procedure consists in extracting the pigments with an organic solvent and measuring spectrophotometrically their concentrations in the extract. This procedure is legitimate if the following three conditions are fulfilled: (a) all of the light-absorbing species are identified and extracted quantitatively; (b) the spectrum and the extinction coefficient are...
known for each species in the extraction solvent; (c) the spectral properties of the pigments in the extract are not modified by the presence of foreign molecules or by strong interaction between the pigment molecules themselves.

In order to extract the pigments quantitatively, we chose the widely used acetone-methanol (7:2, v/v) mixture. The extraction conditions were optimized in order to avoid the possible interference of water and of detergent with the spectroscopic assays. Finally, the extinction coefficients of the pigments in acetone-methanol were determined at various wavelengths.

**Pigment Identification**—Fig. 1 shows the absorption spectrum of the photosynthetic center preparation and of its acetone methanol extract. This extract was chromatographed on thin layer plates in the two systems described (see “Methods”) for the purification of the pigments. One of these systems is designed to separate the porphyrins and the other to separate the carotenoids. Only three main pigments were thus found. There were also traces of other pigments with a total absorbance about 1/5 of the main ones.

The spots were eluted from the plates and the spectra of the porphyrins were measured in ether and that of the carotenoid in benzene. Comparison of these spectra with those of purified pigments showed them to be characteristic of bacteriochlorophyll, bacteriopheophytin, and racemized spirilloxanthin (see below). This composition is to be compared with that of another photosynthetic reaction center prepared from wild type *Rhodospirillum rubrum* (9).

**Determination of Extinction Coefficients in Acetone-Methanol**—The absorption spectra of purified bacteriochlorophyll and bacteriopheophytin in ethyl ether and in acetone-methanol are shown in Figs. 2 and 3. The relative heights of some of their characteristic absorption peaks in ether are compared in Table I with those reported in the literature. By this criterion, these pigment preparations seem to be at least as pure as those previously reported. They can be used legitimately, therefore, as secondary standards for determining extinction coefficients in acetone-methanol.

To this end, the pigments dissolved in ether were transferred to acetone-methanol, and *vice versa*, after evaporation of the solvent in vacuo. A transfer was considered quantitative only when the same extinction value was obtained in either direction.

Bacteriopheophytin was easily transferred, although bacteriochlorophyll was much more difficult to transfer quantitatively from acetone-methanol to ether. This observation is attributed either to the instability of the pigment or to its adsorption on the glass walls of the containers. The difficulty was overcome by using concentrated (0.2 mM) bacteriochlorophyll solutions for the transfers. The results were then reproducible and identical for both directions of transfer. The extinction ratio of the infrared absorption peak in ether over that in acetone-methanol was 1.503 ± 0.009 (average of three experiments) for bacteriopheophytin and 1.396 ± 0.003 (average of five experiments) for bacteriochlorophyll. The extinction coefficients in acetone-methanol were calculated from those reported by three different authors for ether solution (Table I).

We also attempted to assay total porphyrins as bacteriopheophytin by adding HCl (0.007 volume) to the acetone-methanol solution. Several HCl concentrations (12 N, 1 N, and 0.5 N) gave the same value of $\varepsilon_{\text{pp}} / \varepsilon_{\text{ppH}} = 1.558$. However, when the acidified acetone-methanol was neutralized by adding to it equivalent amounts of NaOH, this ratio decreased by about 10%. Because of this dependence of $\varepsilon_{\text{ppH}}$ on the acidity of the solution, phoophytinization was eliminated as a means of quantitative pigment assay.

The extinction coefficient of spirilloxanthin in acetone-
the all-trans isomer and for a racemic mixture of spirilloxanthin near 490 nm in benzene is insensitive to cis-trans isomerization, this is evidenced by a comparison of the spirilloxanthin extracted from the photosynthetic center preparation and purified by chromatography, Spectrum in benzene (---) and in acetone-methanol (7:2, v/v) (--).

methanol was obtained by dissolving equal amounts of the purified pigment in benzene and in acetone-methanol. The spirilloxanthin extracted from the photosynthetic center preparation is spectrally very similar to a racemic mixture of cis and trans isomers. This is evidenced by a comparison of the positions and relative absorbances of the main peaks and troughs of our extract with the results reported by Polgár et al. (13) for the all trans isomer and for a racemic mixture of spirilloxanthin (Table III). Since the extinction coefficient for the trough near 490 nm in benzene is insensitive to cis-trans isomerization, it was chosen as a basis for quantitative determination. The corresponding trough at 475 nm in acetone-methanol (see Fig. 4) was calculated to have an extinction coefficient of 94 mm⁻¹ cm⁻¹.

Table IV summarizes the extinction coefficients used for our assays.

Quantitative Pigment Determination—The amounts of bacteriochlorophyll and of bacteriopheophytin were determined on acetone-methanol extracts from photosynthetic center preparations treated according to Methods A, B, and C described above (see “Methods”). Generally speaking, the three methods yield comparable results (Table V). However, Method A is probably the least reliable of the three because of the presence of water (1.5%) and detergent. In our experience, water is the most harmful foreign substance for such determinations. Method B does not suffer from this drawback. However, small amounts of material are probably lost during the freeze-drying process. Extraction of filtered material (Method C) seems the most reliable method for subsequent quantitative determination, since no material is lost and water and detergent are present in minimal quantities. Freeze-drying of the UM-2 membrane filter, with the photosynthetic center preparation deposited upon

**Table I**

Relative heights of absorption maxima of bacteriochlorophyll and bacteriopheophytin in ether, according to different authors

<table>
<thead>
<tr>
<th>Pigment and Reference</th>
<th>Wavelength</th>
<th>Relative height</th>
<th>Wavelength</th>
<th>Relative height</th>
<th>Wavelength</th>
<th>Relative height</th>
<th>Wavelength</th>
<th>Relative height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriochlorophyll</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weigl (10)</td>
<td>772</td>
<td>1.00</td>
<td>575</td>
<td>0.23</td>
<td>391</td>
<td>0.55</td>
<td>358</td>
<td>0.89</td>
</tr>
<tr>
<td>Smith and Benitez (11)</td>
<td>773</td>
<td>1.00</td>
<td>577</td>
<td>0.229</td>
<td>391.5</td>
<td>0.628</td>
<td>358.5</td>
<td>0.805</td>
</tr>
<tr>
<td>Kim (12)</td>
<td>770</td>
<td>1.00</td>
<td>575</td>
<td>0.225</td>
<td>391</td>
<td>0.584</td>
<td>358</td>
<td>0.761</td>
</tr>
<tr>
<td>van der Rest and Gingras...</td>
<td>771</td>
<td>1.00</td>
<td>573</td>
<td>0.229</td>
<td>391</td>
<td>0.517</td>
<td>357</td>
<td>0.780</td>
</tr>
</tbody>
</table>

| Bacteriopheophytin     |            |                |            |                |            |                |            |                |
| Weigl (10)            | 750        | 1.00           | 680        | 0.15           | 525        | 0.43           | 384.5      | 0.97           | 357        | 1.72           |
| Smith and Benitez (11)| 749        | 1.00           | 680        | 0.158          | 525.5      | 0.420          | 384        | 0.929          | 357.5      | 1.683          |
| Kim (12)              | 750        | 1.00           | 680        | 0.229          | 528        | 0.381          | 387        | 0.835          | 357        | 1.648          |
| van der Rest and Gingras... | 740 | 1.00 | 677 | 0.135 | 524 | 0.396 | 385 | 0.805 | 358 | 1.627 |

**Table II**

Extinction coefficients for bacteriochlorophyll and bacteriopheophytin at their infrared maxima in acetone-methanol (7:2, v/v) based on corresponding values in ether

<table>
<thead>
<tr>
<th>Reference</th>
<th>Reported for ether</th>
<th>Calculated for acetone-methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteriochlorophyll</td>
<td>Bacteriopheophytin</td>
</tr>
<tr>
<td></td>
<td>770</td>
<td>772</td>
</tr>
<tr>
<td>Weigl (10)</td>
<td>749</td>
<td>773</td>
</tr>
<tr>
<td>Smith and Benitez (11)</td>
<td>750</td>
<td>770</td>
</tr>
<tr>
<td>Kim (12)</td>
<td>749</td>
<td>771</td>
</tr>
</tbody>
</table>

**Table III**

Relative absorbance of spirilloxanthin at its maxima (underline) and minima in benzene

<table>
<thead>
<tr>
<th>Reaction center extract</th>
<th>Racemic stereoisomers</th>
<th>All-trans stereoisomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>Relative height</td>
<td>Wavelength</td>
</tr>
<tr>
<td>nm</td>
<td>mm</td>
<td>nm</td>
</tr>
<tr>
<td>543</td>
<td>0.80</td>
<td>541</td>
</tr>
<tr>
<td>528</td>
<td>0.65</td>
<td>528</td>
</tr>
<tr>
<td>507</td>
<td>1.00</td>
<td>505</td>
</tr>
<tr>
<td>487</td>
<td>0.74</td>
<td>485</td>
</tr>
<tr>
<td>479</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>410</td>
<td>0.175</td>
<td>406</td>
</tr>
<tr>
<td>396</td>
<td>0.27</td>
<td>396</td>
</tr>
<tr>
<td>384</td>
<td>0.17</td>
<td>384</td>
</tr>
<tr>
<td>378</td>
<td>0.19</td>
<td>378</td>
</tr>
</tbody>
</table>

* Molar extinction coefficients in benzene (13): all-trans, ε₄₃₇ = 97 mm⁻¹ cm⁻¹; iodine-racemized, ε₄₃₄-₄₄₄ = 85 mm⁻¹ cm⁻¹; heat-racemized, ε₄₃₇ = 94 mm⁻¹ cm⁻¹. The values for the all-trans and heat-racemized stereoisomers are calculated from the data of Polgár et al. (13).

* Point of inflection.
graded porphyrins. In the extract. The higher absorbance of the extract in the 410 nm region can probably be explained by the presence of de-loxanthin.

dominants or of strong interactions between the pigment molecules

equivocal model of the photosynthetic center (see "Discussion").

found to be insignificant except in the 410 nm region (Fig. 5).

The aim of this calculation was to see whether this spectrum could be entirely accounted for by the sum of its known pig-
obvectors during the extraction) —

since, although UM-2 membrane filters performed satis-
ification, did not modify the results. The bacteriochlorophyll to

to bacteriopheophytin ratio does not differ significantly with

Method B or C (Table V). This we take to indicate the absence

technique of Smith and Benitez (11) for heat-racemized spiri-

It should be noted that, although UM-2 membrane filters performed satisfactorily for this purpose, such was not the case for UM-05 (pigment degradation) or for UM-10 filters (pigment inclusion during the extraction).

Table V summarizes the quantitative analysis for bacterio-
chlorophyll, bacteriopheophytin, and spirilloxanthin.

Spectral Reconstitution—We next proceeded to a mathematical reconstitution of the spectrum of the acetone-methanol extract. The aim of this calculation was to see whether this spectrum could be entirely accounted for by the sum of its known pigment components. When the sum of the absorption spectra of bacteriochlorophyll, bacteriopheophytin, and spirilloxanthin in the proportions found by analysis was subtracted from the spectrum of the acetone-methanol extract, the difference was found to be insignificant except in the 410 nm region (Fig. 5).

This excludes the presence of important unnoticed contami-
nants or of strong interactions between the pigment molecules in the extract. The higher absorbance of the extract in the 410 nm range can probably be explained by the presence of de-
graded porphyrins.

Relative Extinction Coefficient—The pigment assays were judged insufficiently accurate to provide a basis for an unequivocal model of the photosynthetic center (see "Discussion").

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriochlorophyll</td>
<td>0.5</td>
</tr>
<tr>
<td>Bacteriopheophytin</td>
<td>0.2</td>
</tr>
<tr>
<td>Spirilloxanthin</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Values based on the coefficients of Smith and Benitez (11) for the porphyrins and of Polgár et al. (13) for heat-racemized spirilloxanthin.

The number of experiments is indicated in parentheses.

Table IV

Extinction coefficients (mM\(^{-1}\) cm\(^{-1}\)) used for the determination of bacteriochlorophyll, bacteriopheophytin, and spirilloxanthin in acetone-methanol (7:2, v/v)

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>771 nm</td>
</tr>
<tr>
<td>Bacteriochlorophyll</td>
<td>0.5</td>
</tr>
<tr>
<td>Bacteriopheophytin</td>
<td>1.2</td>
</tr>
<tr>
<td>Spirilloxanthin</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values based on the coefficients of Smith and Benitez (11) for heat-racemized spirilloxanthin.

Therefore, an independent method was sought for estimating its extinction coefficient. It has been shown (14) that the reduction of \(F_{70}^{\text{red}}\) can be coupled to the oxidation of mammalian ferrocytochrome c. Since the \(\Delta A_{550}^{\text{red-ox}}\) of the latter is well known, this reaction can be used for determining the differential extinction coefficient for oxidation-reduction of \(F_{70}\) (6, 14).

In principle, the method used here is as follows. The photosynthetic center in the mixing syringe of the stopped flow spectrophotometer is exposed to bright white light for 1 min. Under these conditions, bleaching at 870 nm is maximal and recoloration is slow (of the order of minutes). After turning off the actinic source, the preparation is rapidly mixed with ferrocytochrome c and this causes immediate recoloration at 870 nm and bleaching at 550 nm. Both absorbance changes are used to monitor the reaction.

After the mixing of pre-illuminated photosynthetic center with buffer alone, no absorbance change could be detected within 50 ms (Fig. 6a). Mixing with ferrocytochrome c produced fast and kinetically identical absorption changes at 550 and 870 nm (Fig. 6, b and c). A series of six measurements gave an average \(\Delta A_{550}^{\text{red-ox}}\) of 1.012 \(\times 10^{-2}\) \(\pm\) 0.04 \(\times 10^{-4}\) (S.D.), an average \(\Delta A_{870}^{\text{red-ox}}\) of 4.00 \(\times 10^{-2}\) \(\pm\) 2 \(\times 10^{-3}\), and a calculated \(\Delta A_{550}^{\text{red-ox}}\) ratio of 0.253 \(\pm\) 0.017. When the value of \(\Delta A_{550}^{\text{red-ox}}\) (0.065) due to the oxidation-reduction of the center itself is subtracted, one obtains the corrected ratio of 0.167 \(\pm\) 0.017.

By using the generally accepted value of 21 \(\text{nm}^{-1}\) cm\(^{-1}\) for the \(\Delta A_{550}^{\text{red-ox}}\) of cytchrome c (15–17), a \(\Delta A_{550}^{\text{red-ox}}\) of 125.7 \(\pm\) 13.2 \(\text{nm}^{-1}\) cm\(^{-1}\) for photosynthetic center was calculated.

In contrast to the observations made with a preparation of photosynthetic center from \(R.\) spheroides (14), we repeatedly noted complete reduction of \(F_{70}^{\text{red}}\) with approximately equimolecular concentrations of ferrocytochrome c. In order to verify this point, a titration was undertaken by measuring the absorbance increase at 870 nm, 500 ms after mixing a known amount of maximally photo-oxidized center with solutions of increasing ferrocytochrome c concentrations. Assuming the reaction to be bimolecular, the equivalence point should correspond to an equimolar ratio of the reactants. Fig. 7a shows the oscilloscope traces obtained in this experiment. Fig. 7b is a plot of the

![Fig. 5. Reconstitution of the absorption spectrum of the acetone-methanol (7:2, v/v) extract of the photosynthetic center preparation from Rhodospirillum rubrum. Experimental spectrum (-----) and spectra of purified bacteriochlorophyll (----), bacteriopheophytin (-----), and spirilloxanthin (----) in the proportions found by analysis. The difference between the actual spectrum of the extract and the values obtained by reconstitution is shown at top (same scale).](http://www.jbc.org/Download)
extent of recoloration at 870 nm against the molar ratio of ferrocyanochrome c to \( P_{870} \). The amount of \( P_{870} \) was obtained by assuming a \( \Delta A_{870}^{\text{oxid}} \) of 125.7 mm\(^{-1}\) cm\(^{-1}\). The titration curve clearly shows the equivalence point to be situated at an equivalent ratio of the two reactants. Conversely, this experiment provides an independent confirmation of the proposed differential extinction coefficient of \( P_{870} \).

**Discussion**

Our extraction procedure (membrane filtration) and pigment assay are reproducible with a standard deviation of ±0.7% for bacteriochlorophyll, ±3.1% for bacteriopeophytin, and ±1.2% for spirilloxanthin. Among the systematic errors which might affect our results, the foremost is probably due to the extinction coefficients.

Weigl's extinction coefficient for bacteriochlorophyll in ether (10) was derived from the extinction coefficient of bacteriopheophytin determined gravimetrically by French (18). Smith and Benez (11) and Kim (12) directly obtained the extinction coefficient of bacteriopheophytin in ether for spectroscopic determinations of magnesium. In the latter two cases, the extinction coefficient of bacteriopheophytin was obtained by HCl treatment of bacteriochlorophyll.

The methods based on magnesium determinations are likely to be more accurate than gravimetry. On these grounds, we favor the values given by Smith and Benez (11) and by Kim (12). It is more difficult to make a choice between these two sets of values, although fluorescence spectroscopy might be expected to be more accurate than colorimetry when applied to small quantities of magnesium. For clarity's sake, we rather arbitrarily chose the values of Smith and Benez (11) for most of our calculations.

As shown by Table VI, Weigl's values (10) lead to a molar ratio of bacteriochlorophyll to bacteriopheophytin of 1.76. The values of Smith and Benez (11) and of Kim (12) lead to similar values of 2.01 and 1.99.

Assuming the photosynthetic center particles to be all alike and to contain an integral molecular number of each pigment, different models may be proposed, based on the results summarized in Table VI. Any such model must incorporate two experimental findings: the bacteriochlorophyll to bacteriopheophytin molar ratio is 2 and the \( \Delta A_{870}^{\text{oxid}} \) is 125.7 mm\(^{-1}\) cm\(^{-1}\). The accuracy of the first result rests on the internal coherence of the relative extinction coefficients of the two pigments and not on their absolute values. This coherence is good, since bacteriochlorophyll can be converted quantitatively to bacteriopheophytin.

The reduction of \( P_{870}^{\text{ox}} \) by ferrocyanochrome c, first demonstrated by Ke et al. (14), has been used before to measure the \( \Delta A_{870}^{\text{red}} \) of the photosynthetic center isolated from *R. spheroides* (strain R 26) (6, 14). It should be noted that the conditions employed here differed in several respects from those in previous work. Our preparation has a different biological origin and is unstable in solutions containing more than about 0.05% dodecyl dimethylamine N-oxide (7). For this reason, and also to avoid foaming upon mixing, no detergent was added to the reaction mixture. Another, perhaps more important, difference is that,
The extinction coefficients for the porphyrins in acetone-methanol were calculated from their corresponding values in ether according to the references cited.

The number of experiments is indicated in parentheses. Values are means ± standard deviation.

in previous work (6, 14), the photosynthetic center preparations and the ferrocytochrome c were mixed in the dark, the photochemical reaction occurred during a rapid light flash, and the reduction of $P_{700}$ ensued with a half-reaction time of 25 μs (14) or 2.5 ms (6). In the present case, the photosynthetic center was photo-oxidized by a prolonged illumination and was reduced in darkness after having been mixed with ferrocytochrome c.

Under these conditions, a $\Delta A_{570}^{\text{red} - \text{ox}}$ of 125.7 ± 13 mm⁻¹ cm⁻¹ was calculated from the measurement of the absorbance change at 570 nm due to the reduction of $P_{700}$ and the portion of the 550 nm absorbance change due to oxidation of ferrocytochrome c. In a different measurement, $P_{700}$ was titrated with increasing amounts of ferrocytochrome c. Assuming a $\Delta A_{570}^{\text{red} - \text{ox}}$ of 125.7 mm⁻¹ cm⁻¹, the equivalence point was found to correspond to an equimolar ratio of the reactants. This provides an independent verification for the exactness of the adopted value.

Since it avoids the propagation of errors inherent in taking the ratio of absorbances changes, this latter determination is estimated to be accurate within about 5%.

The finding that $P_{700}$ and ferrocytochrome c undergo complete oxidation-reduction at equimolar concentrations is in contrast to the original observation of Ke et al. (14), who reported the reaction to be complete with ferrocytochrome c to $P_{700}$ molar ratios of about 10. It is impossible at present to decide whether this discrepancy should be attributed to the different bacterial origin of the preparations or to other experimental conditions. However, we believe that the greater purity of the preparation from R. rubrum may be a decisive factor.

From the extinction coefficient of Smith and Benitez (11) for bacteriochlorophyll, an extinction coefficient for the 868 nm absorption peak ($e_{565}$) may be calculated, assuming that each mole of photosynthetic center contains 2, 4, or 6 moles of bacteriochlorophyll. The corresponding calculated values for $e_{565}$ are 71.3, 142.6, and 214 mm⁻¹ cm⁻¹. A choice between these can be made by comparison with the $e_{565}$ calculated from $\Delta A_{570}^{\text{red} - \text{ox}}$.

Knowing (from Ref. 7) that $\Delta A_{480}:A_{480}$ is 0.897, where $\Delta A_{480}$ is the absorption difference between the reduced form (in the presence of ascorbate) and the oxidized form (by ferricyanide), and $e_{565}$ of 140 mm⁻¹ cm⁻¹ is obtained. This value is clearly in agreement with the model of 4 bacteriochlorophyll molecules per equivalent of $P_{700}$. Moreover, the close agreement between $e_{565}$ determined by titration with ferrocytochrome c and by pigment analysis lends considerable confidence into the value of 143 mm⁻¹ cm⁻¹ obtained by the latter method. This is 12% higher than the value proposed by Straley et al. (6) for the photosynthetic center of R. spheroides (strain R 26).

Although carotenoids are obviously not essential for the photochemical reaction, they are present in all of the photosynthetic centers extracted so far from wild type Athiorhodaceae (7, 9, 19–21). Moreover, the preparation obtained with docetyltrimethylammonium N-oxide from a carotenoidless mutant (G9) of R. rubrum is much more sensitive to light or salts than its wild type counterpart (22, 23). This indicates a protective role for spirilloxanthin. If the latter is a genuine component of the complex, it would be expected to be present in stoichiometric amounts. The results reported here are reproducibly of the right order of magnitude (1.2 moles of spirilloxanthin for 6 moles of porphyrins) for such a stoichiometric binding. We suggest that the 20% discrepancy with the expected value is explicable in part by experimental error and particularly by absolute error in the extinction coefficients. Because of the propagation of errors, the ratio of the extinction coefficients of the two types of pigments is very sensitive to small deviations from their absolute values.

Recently, Slooten (21) reported that the photosynthetic center of wild type R. spheroides contains about 0.6 mole of spheroidene, calculated on the assumption that the extinction coefficient of the pigment is the same on the particle as in benzene solutions. According to our data, the extinction coefficient of spirilloxanthin is about 30% higher in acetone-methanol (or in benzene) than on the particle. If applicable to the preparation from R. spheroides, this observation would indicate that it also contains about 1 mole of carotenoid.

The available evidence indicates that the photosynthetic center of R. rubrum contains 4 molecules of bacteriochlorophyll a, 2 molecules of bacteriopheophytin, and 1 molecule of spirilloxanthin per equivalent of $P_{700}$.

It is tempting to propose a correlation between these analytical data and the spectroscopic work of others. Low temperature absorption and fluorescence excitation spectroscopy indicates that the Q transition of bacteriopheophytin is split into at least two components (3, 5, 21). As clearly shown by circular dichroism spectroscopy, $P_{700}$ is composed of two transitions with opposite rotational strengths (2, 5, 24). The low temperature circular dichroism spectra of Reed and Ke (5) also indicate $P_{700}$ to be due to at least two components. This last point is of special interest, since removal of 1 electron equivalent leads to the disappearance of the entire 898 nm absorption band. This may mean that $P_{700}$ is due to a dimer with a delocalized hole in its oxidized state. Such an interpretation has also been suggested on the basis of comparative electron paramagnetic resonance spectroscopy of chlorophyll aggregates and of isolated photosynthetic center (25, 26).

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