Biochimica et Biophysica Acta, 314 (1973) 15–27

BBA 46579

FLUORESCENCE EXCITATION SPECTRA AND THE RELATIVE NUMBERS OF PIGMENT MOLECULES IN REACTION CENTRES FROM *RHODOPSEUDOMONAS SPHEROIDES*

LUIT SLOOTEN*

Biophysical Laboratory of the State University, P.O. Box 556, Leiden (The Netherlands)

(Received January 31st, 1973)

SUMMARY

1. The excitation spectrum for the bacteriochlorophyll P890 fluorescence in reaction centre preparations was determined at wavelengths ranging from 360 to 890 nm.

2. A fluorescence excitation spectrum corresponding to the absorbance spectrum of bacteriopheophytin was also obtained. This spectrum was used in an analysis of the absorbance spectrum of a reaction centre preparation. Based on this spectrum and on literature data, we estimated that the bacteriopheophytin: bacteriochlorophyll ratio in reaction centre particles is at least 1 : 2.

3. On the basis of literature data, it is shown that bacteriopheophytin occurs probably as such in reaction centres *in vivo*.

INTRODUCTION

It is known that the primary photoreactions in bacterial photosynthesis take place in so-called reaction centres, which (at least in certain *Athiorhodaceae*) can be physically separated from the bulk bacteriochlorophyll which only serves for light absorption. Since the original publication of Reed and Clayton[1] on the isolation of reaction centre particles from a carotenoid-less strain of *Rhodopseudomonas spheroides*, reaction centres have been isolated from a carotenoid-less strain of *Rhodospirillum rubrum*[2] and from wild-type strains of *Rhodopseudomonas viridis*[3], *Rps. spheroides*[4–8] and *Rsp. rubrum*[7,9,10].

In this paper we will describe experiments with reaction centre particles from *Rps. spheroides* (wild type). Fluorescence emission and excitation spectra provided data on the direction and efficiency of the transfer of excitation energy between the various pigments (bacteriochlorophyll, its degradation products, bacteriopheophytin and carotenoids). Absorbance spectra were obtained of bacteriopheophytin and

Abbreviations: bacteriochlorophyll P870, the reaction centre bacteriochlorophyll capable of reversible photooxidation; bacteriochlorophyll P800, the bacteriochlorophyll associated with bacteriochlorophyll P870.

* Present address: Laboratory of Biophysics, Vrije Universiteit Brussel, Adolphe Buyllaan 105, 1050 Brussel (Belgium).

(by analysis) of the bacteriochlorophylls P800 and P870 together. The number of bacteriopheophytin molecules per reaction centre was considered on the basis of the area of the near-infrared absorption band of bacteriopheophytin in various preparations including reaction centres.

MATERIALS AND METHODS

The reaction centre fractions were prepared as described earlier[6]. Treatment of chromatophores with sodium dodecyl sulphate resulted in the isolation of a reaction centre fraction designated as dodecyl sulphate-isolated reaction centre fraction. Treatment of dodecyl sulphate-isolated reaction centre fractions with urea and Triton X-100 at pH 10.0 gave a reaction centre preparation designated as dodecyl sulphate–urea–Triton X-100-isolated reaction centres. The preparations were stored at 4 °C in Tris buffer, containing 0.05 M Tris–HCl, pH 8.0, and 0.01 M $MgCl_2$.

Fluorescence excitation and emission spectra were measured with an apparatus similar to one described earlier[11]. The excitation light, chopped at 50 Hz, was provided by a 900-W xenon lamp or a 600-W tungsten–iodine lamp and passed through a Bausch and Lomb grating monochromator (1200 lines per mm grating). An image of the exit slit of the excitation monochromator was focused upon a 1-mm glass or perspex cuvette. This image was focused upon the entrance slit of a second, analyzing monochromator. The image of the grating of the analyzing monochromator was focused upon the cathode of a photomultiplier with an S1-type of response which was operated at −80 °C. The excitation and analyzing monochromators transmitted pass bands of 7- and 8.5-nm width, respectively. It was usually necessary to place additional filters both in the excitation beam and in the emitted beam, in order to cut off "false" light and stray excitation light, respectively. For measurements at 77 °K the cuvette was immersed in liquid nitrogen in a Dewar vessel with a transparent quartz window. The intensity of the excitation light was measured with an RCA photocell type 925, which was calibrated against a YSI-Kettering Radiometer (Model 65), of which the sensor was put in the place of the cuvette. The radiometer was calibrated against a standard thermopile. The base line for the excitation and emission spectra was provided by the signals obtained with water instead of sample in the cuvette. These signals, due to "false" excitation light, were usually less than 10% of the signals obtained with the sample in the cuvette.

The emission spectra were corrected for the wavelength dependence of the relative sensitivity of the detecting system by putting a surface covered with powdered MgO, instead of the cuvette, in the sample holder. This surface was illuminated with light of a calibrated tungsten band lamp with known intensity distribution[12]. A correction for reabsorption of the emitted light was carried out using an equation as given by Duysens[13], with a modification to account for the different geometry of our apparatus. This correction was carried out only at room temperature. At 77 °K, the increased scattering by ice crystals may have increased the optical path by an unknown factor. The absorbance of the samples at the maximum at 803 nm did not exceed 0.14 in a 1-mm cuvette.

Absorbance spectra were recorded on a Cary Model 14R spectrophotometer.

RESULTS

Excitation spectrum for the fluorescence of bacteriochlorophyll P870

Transfer of light energy from the different pigments present in reaction centre preparations to bacteriochlorophyll P870 was studied by measuring an excitation spectrum for the fluorescence at 920 nm due to reduced bacteriochlorophyll P870 (see below).

Fig. 1 shows the absorbance spectrum of an dodecyl sulphate-isolated reaction centre preparation. Owing to the presence of contaminations, absorbing at 690 nm and in the blue region (see below), this spectrum was more complex than the spectrum of a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation[6].

The preparation shown here was somewhat turbid and the zero line runs probably between the indicated extremes. This approximation is based on the facts that the absorbance is zero at 950 nm and that in clear preparations the absorbance at the 600-nm maximum is 3 times higher than the absorbance at 640 nm and 565 nm. Moreover, the absorbance spectrum of a clear preparation is almost horizontal between 620 and 650 nm.

Emission spectra of the same preparation, plotted on a wave number scale, are shown in Fig. 2. Without additions (dashed line) bacteriochlorophyll P870 was kept photooxidized by the excitation light. The tail which is seen below about 12000 cm^{-1} when bacteriochlorophyll P870 is oxidized, is probably due to a degradation product with an emission maximum at 860 nm. The excitation spectrum for the fluorescence of this impurity has peaks at about 845, 760, 690 and 590 nm, and

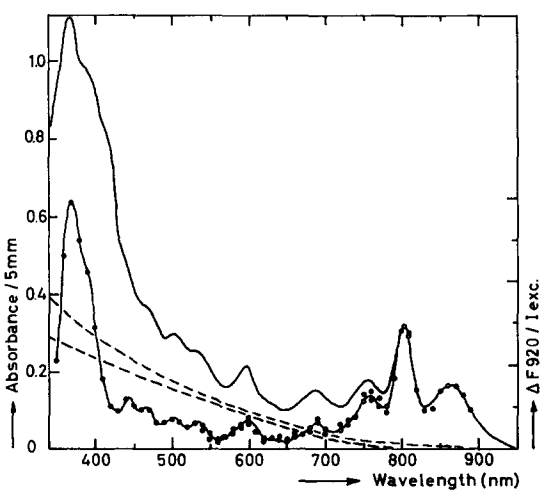

Fig. 1. ———, absorbance spectrum of a dodecyl sulphate-isolated reaction centre preparation. – – – –, extreme values suggested for the base line of the absorbance spectrum (see text). Open symbols, differential excitation spectrum for the fluorescence of bacteriochlorophyll P870, measured with the same preparation, at 920 nm (see text). Solid symbols, same, but with another dodecyl sulphate-isolated reaction centre preparation. The excitation spectra match the absorbance spectrum at 800 nm. The vertical scales in Figs 1–4 (fluorescence intensity divided by the intensity of the excitation light at unit concentration of bacteriochlorophyll P870) are all expressed in the same relative units.

also small carotenoid peaks at 510, 475 and 445 nm (Slooten, L., unpublished). It is suggested that this corresponds to the absorption of reaction centres with degraded pigments. In the presence of 15 mM ascorbate (solid line) both bacteriochlorophyll

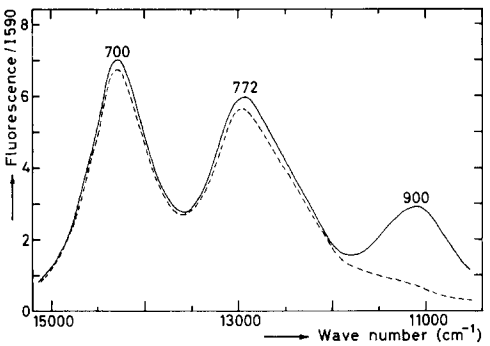

Fig. 2. Fluorescence emission spectra of the preparation shown in the absorbance spectrum of Fig. 1. – – – –, no additions; ———, with 15 mM ascorbate. Excitation with light of 370 nm, intensity 8.4 neinstein·cm^{-2}·s^{-1}.

P870 and the primary acceptor were reduced in the excitation light[7]. Below 12000 cm^{-1} the difference between these two spectra represents the emission due to reduced bacteriochlorophyll P870 in the presence of reduced electron acceptors. The emission peaks with maxima at 700 nm and 772 nm were due to a substance designated as B690 (see below) and to bacteriopheophytin, respectively.

To obtain with this preparation the excitation spectrum for the fluorescence of reduced bacteriochlorophyll P870 in reaction centres in the state PX^-, we took the difference in emission at 920 nm as observed upon irradiation prior to, and after addition of 15 mM ascorbate, respectively (compare Fig. 2). The result is shown in the open and solid circles of Fig. 1. The open symbols refer to measurements with the preparation represented by the absorbance spectrum of Fig. 1. The solid symbols are points measured with another dodecyl sulphate-isolated reaction centre preparation.

Light absorbed by bacteriochlorophyll P800 at 800 nm was transferred to bacteriochlorophyll P870 with an efficiency close to 1, as shown by a comparison of the absorption and excitation spectra of Fig. 1. Light of around 760 nm was transferred to bacteriochlorophyll P870 with an efficiency of 0.8–0.9. This "low" efficiency will be dealt with in the next section. The peak at 690 nm in the excitation spectrum of Fig. 1 suggests the presence of a substance designated as S690, which had an absorption maximum at 690 nm and which transferred energy to bacteriochlorophyll P870. Another product designated as B690 was also present in these preparations. The excitation spectrum for the fluorescence of B690 at 710 nm had a large maximum at 420 nm, which was about 2 times larger than the maximum at 690 nm (Slooten, L., manuscript in preparation). Such a maximum was not present in the excitation spectrum shown in Fig. 1, indicating that B690 was different from S690 and that B690 did not transfer energy to bacteriochlorophyll P870.

Light absorbed by carotenoids was also transferred to bacteriochlorophyll P870, with an efficiency which was estimated to be at least 0.7.

The excitation spectrum shown in Fig. 1 was, apart from the peak at 690 nm, practically identical with the absorbance spectrum of a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation.

Excitation spectra for the fluorescence of bacteriochlorophyll P870 were similar in dodecyl sulphate–urea–Triton X-100-isolated reaction centre and dodecyl sulphate-isolated reaction centre preparations, except that in the former S690 or B690 were practically absent.

Fluorescence of contaminating compounds, including bacteriopheophytin

As indicated above, the rather low efficiency of 760-nm light for the excitation of bacteriochlorophyll P870 fluorescence was due to the presence of contaminations.

From previous work[6] it is known that purified reaction centre particles contain a bound form of bacteriopheophytin (referred hereafter to as "bound" bacteriopheophytin) which has a high efficiency (approximately 1) of energy transfer to bacteriochlorophyll P870, and a low fluorescence yield. In such preparations the absorbance at 756 nm equals 0.9 times the absorbance at 867 nm. Contaminations, if present, could be detected (*cf.* above) firstly, by an increased ratio of $A_{756\ nm}/A_{870\ nm}$; secondly, by a decreased efficiency of 760-nm light, relative to that of 870-nm light, in eliciting bacteriochlorophyll P870 fluorescence; thirdly, by a strong fluorescence emission band with a maximum at about 770 nm; and finally, by the excitation spectrum for this fluorescence. From a comparison of different reaction centre preparations, it appeared that these contaminations were present in varying amounts, corresponding to an absorbance which did not exceed about 1/5 of the absorbance due to "bound" bacteriopheophytin. From the fluorescence excitation spectra the contaminations could be identified as solubilized bacteriochlorophyll (Slooten, L., manuscript in preparation) and bacteriopheophytin. In some of the preparations only one of these two substances was present. B690, if present, transferred in some, but not all cases energy to one or both of these substances (Slooten, L., manuscript in preparation). Carotenoid, which was always present, did not transfer energy to the contaminations. Presumably the carotenoid was bound specifically to the reaction centre particle, transferring energy only to bacteriochlorophyll P870 (*cf.* the bottom and top lines of the previous page). The contaminating form of bacteriopheophytin which does not transfer energy to bacteriochlorophyll P870, will be referred to hereafter as "free" bacteriopheophytin.

Fig. 3 (○—○) shows the excitation spectrum for the fluorescence of bacteriopheophytin at 790 nm, as measured in a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation. The emission was mainly due to "free" bacteriopheophytin, as indicated firstly by the high fluorescence yield relative to the fluorescence yield of bacteriochlorophyll P870, and secondly by the high absorbance at 756 nm, relative to the absorbance at 867 nm (the ratio was 1.1 : 1, as compared to 0.9 : 1 in Fig. 1). Fig. 6 shows that, apart from slight differences in the position of the peaks, the excitation spectrum was virtually the same as the absorbance spectrum of bacteriopheophytin in acetone[14] (solid line). In the region 650–770 nm the excitation spectrum was more similar to the absorbance spectrum of a bacteriopheophytin–protein complex[15,16] (dashed line).

Clayton and co-workers[17] reported that the 535-nm absorption band of bacteriopheophytin in reaction centre preparations split at 77 °K into two bands of equal

height, with maxima at 530 and 542 nm, suggesting that each reaction centre contained two molecules of bacteriopheophytin in slightly different states. In accordance with this we found that the broad 535-nm band of "bound" bacteriopheopheophytin in the excitation spectrum for the fluorescence of bacteriochlorophyll P870 split at 77 °K into two bands of approximately equal height, with maxima at 535 and 543 nm (see Fig. 4A). This indicates that the two molecules of "bound" bacteriopheophytin transferred energy to the reaction centre bacteriochlorophyll with equal efficiency This is consistent with the observation that the efficiency of the energy transfer from "bound" bacteriopheophytin to the reaction centre bacteriochlorophyll was approximately 1.0 (ref. 6).

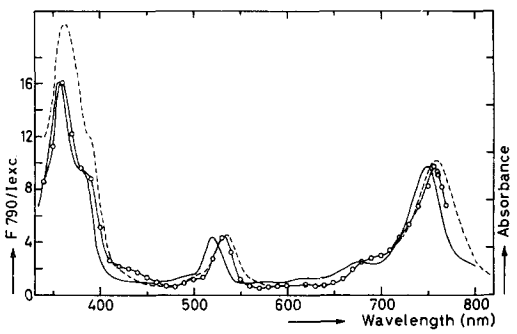

Fig. 3. ○—○, excitation spectrum for the fluorescence of bacteriopheophytin, measured at 790 nm, with a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation (left-hand scale). No addditions. ———, absorbance spectrum of bacteriopheophytin *a* in acetone (from ref. 14). – – – –, absorption spectrum of a bacteriopheophytin–protein complex (from ref. 16). This spectrum coincides with our excitation spectrum between 580 and 710 nm.

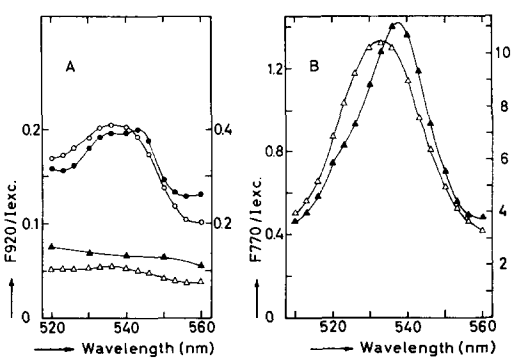

Fig. 4.(A) Excitation spectra for the fluorescence of bacteriochlorophyll P870, measured at 920 nm, with a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation. Open symbols, room temperature (left-hand scale). ○—○, with 15 mM ascorbate; △—△, no additions. Solid symbols: 77 °K (right-hand scale). ●—●, with 15 mM ascorbate; ▲—▲, no additions. In the latter two cases the sample was illuminated first at room temperature for 1 min with light from a 125-W quartz-iodine lamp immediately before the sample was rapidly frozen. This was done in order to ensure either that both bacteriochlorophyll P870 and the primary acceptor were fully reduced (●—●), or that bacteriochlorophyll P870 was completely photooxidized (▲—▲) before freezing. (B) Excitation spectrum for the fluorescence of bacteriopheophytin, measured at 770 nm, with the preparation used in Fig. 4A. △—△, room temperature (left-hand scale); ▲—▲, 77 °K (right-hand scale).

The fact that Fig. 4A shows two maxima approximately 8 nm apart indicates that the half width of each band was, at most, 8 or 9 nm at 77 °K. On the other hand, the excitation spectrum for the bacteriopheophytin emission measured at 770 nm showed at 77 °K a single band with a maximum at 538 nm and a half width of 18 nm (Fig. 4B). This supports the suggestion that most of the bacteriopheophytin fluorescence originates from "free" bacteriopheophytin, which does not transfer energy to bacteriochlorophyll P870.

Analysis of the absorbance spectrum of a reaction centre preparation

The absorbance spectrum of a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation (taken from ref. 6) was analyzed as shown in Fig. 5. For this analysis, first the absorption due to bacteriopheophytin was subtracted from the total absorption, assuming that "bound" bacteriopheophytin has the same absorption spectrum as "free" bacteriopheophytin and that this absorbance spectrum is identical with the excitation spectrum for the 790-nm fluorescence shown in Fig. 3. As follows from a previous paragraph, the first assumption may have introduced an error in the region of the 535-nm absorption band, because of the partially resolved band splitting in the spectrum of "bound" bacteriopheophytin. Next, allowance was made for a small amount of B690, of which the absorbance spectrum was known from the fluorescence excitation spectrum.

Finally, the absorption spectrum of carotenoid was subtracted. This spectrum was obtained from the spectrum of a heptane extract of a lyophilized dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation (Slooten, L., unpublished). It was assumed that the absorption spectrum of this carotenoid was the same *in vivo* as *in vitro*, except that *in vivo* the maxima and minima were shifted along a distance of about 20 nm to longer wavelengths.

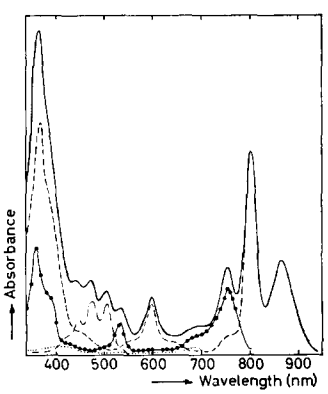

Fig. 5. Analysis of the absorbance spectrum (———) of a dodecyl sulphate–urea–Triton X-100-isolated reaction centre preparation. ○—○, bacteriopheophytin; ·····, B690; –·—·–, carotenoid; – – – –, residual absorption due to bacteriochlorophylls P800 and P870 together. The amount of bacteriopheophytin was taken such that after subtraction there remained a shoulder at about 760 nm, in analogy with the shoulder in the absorption spectrum of bacteriochlorophyll in acetone at about 705 nm (ref. 14). The amount of B690 was taken such that after subtraction the bacteriopheophytin and B690 contribution to the residual absorbance spectrum was approximately horizontal in the region of 675–720 nm, in analogy with the region of 620–660 nm of the bacteriochlorophyll absorption spectrum in acetone. Further details: see text.

The residual absorption (Fig. 5, – – –), which is due to bacteriochlorophylls P800 and P870, was plotted again in Fig. 6, in order to compare it with the absorption spectrum of bacteriochlorophyll *a* in acetone (taken from ref. 14). The similarities between these two spectra indicate that the analysis was reasonably adequate. In both spectra there are shoulders at 385 nm, at about 450 nm and at the short wavelength side of the orange band.

For further comparison the spectra shown in Fig. 6 were plotted on a wave number scale (cm^{-1}). The areas under the Soret bands of bacteriochlorophyll in acetone (from 330 to 420 nm) and of reaction centre bacteriochlorophyll *in situ* (from 340 to 430 nm) had a ratio of 1.10 : 1, indicating that these spectra represented approximately equal amounts of bacteriochlorophyll. In accordance with this, the areas under the near-infrared bands of bacteriochlorophyll in acetone, on the one hand, and of bacteriochlorophylls P800 and P870 together, on the other hand, had a ratio of 1.06 : 1.

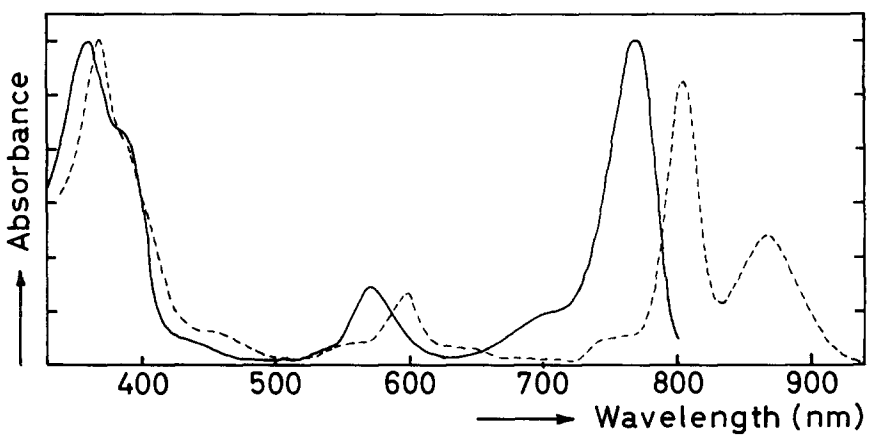

Fig. 6. ———, absorbance spectrum of bacteriochlorophyll in acetone (from ref. 14). – – – – – – absorbance spectrum of bacteriochlorophylls P800 and P870 (from Fig. 5).

DISCUSSION

The occurrence of bacteriopheophytin in reaction centres in vivo

All reaction centre preparations made so far contain bacteriopheophytin. One may ask whether bacteriopheophytin is a degradation product formed as a result of the procedure followed in isolating the reaction centre, or whether it occurs as such in the reaction centre *in vivo*. Evidence in favour of the latter idea can be collected from the literature. Firstly, the absorption spectrum of chromatophores of *Rps. spheroides* strain R-26 exhibits shoulders at about 760 and 800 nm; these shoulders are absent in chromatophores depleted of reaction centres[18]. Secondly, in the same organism, the action spectrum for the photooxidation of bacteriochlorophyll P870 exhibits shoulders at about 760 and 800 nm (ref. 19); these shoulders are absent in the action spectrum for the fluorescence of "light-harvesting" bacteriochlorophyll (refs 19–21). These data indicate that bacteriopheophytin and bacteriochlorophyll P800 occur as such in the reaction centre *in vivo* and that these pigments transfer

energy to bacteriochlorophyll P870 rather than to "light-harvesting" bacteriochlorophyll.

The ratio of bacteriopheophytin to bacteriochlorophyll in reaction centres

In the following discussion it is understood that a reaction centre is a complex in which one electron can be transferred photochemically.

In order to estimate the ratio of bacteriopheophytin to bacteriochlorophyll in reaction centres from *Rps. spheroides*, we will first consider the area under the near-infrared absorption band in different bacteriopheophytin preparations. Table I summarizes the results collected from literature data. It appears that the area under the absorption band, as defined in Table I, has an approximately constant value of $(38 \pm 2) \cdot 10^3$ $mM^{-1} \cdot cm^{-2}$, despite large variations in ε_{max}. For this reason we assume that "bound" and "free" bacteriopheophytin have a near-infrared absorption band with an area (as defined above) of $38 \cdot 10^3$ $mM^{-1} \cdot cm^{-2}$. This enables us to calculate an extinction coefficient of at most 42 $mM^{-1} \cdot cm^{-2}$ for "free" bacteriopheophytin, given a half width of at least 910 cm^{-1} for the near-infrared absorption band (calculated from Fig. 3). Moreover, we assume (as was done in the analysis of Fig. 5) that the shape of the near-infrared absorption is the same in "bound" and "free" bacteriopheophytin.

TABLE I

SPECTRAL CHARACTERISTICS OF THE NEAR-INFRARED ABSORPTION BAND OF DIFFERENT BACTERIOPHEOPHYTIN PREPARATIONS.

The half width of the absorption band was calculated from Figures published in the references mentioned. The area under the absorption band (last column) is defined for our purpose as the product of the extinction coefficient in the absorption maximum (ε_{max}) and the half width. Bph, bacteriopheophytin.

Ref.	*System*	λ_{max}(*nm*)	ε_{max} ($mM^{-1} \cdot cm^{-1}$)	*Half width* (cm^{-1})	*Area* ($mM^{-1} \cdot cm^{-2}$)
22	Bph in chloroform	750	58	700	$40 \cdot 10^3$
23	Bph in ether	749	63	620	$39 \cdot 10^3$
16	Bph–protein	756–760	36	1000	$36 \cdot 10^3$
This report	"Free" Bph	756	≤ 42	≤ 910	

Clayton and co-workers determined the amount of bacteriochlorophyll which can be extracted from an aliquot of a watery suspension of reaction centres with known absorbance at 803 and 867 nm. Knowing that the bacteriochlorophyll corresponded to bacteriochlorophyll P800 and P870, they determined the absorbance coefficients of reaction centres in the absorption maxima of bacteriochlorophyll P800 (at 803 nm) and of bacteriochlorophyll P870 (at 867 nm), assuming that there are 3 or 4 or 5 molecules of bacteriochlorophyll per reaction centre[17]. We extended these calculations to the assumption of 2 molecules of bacteriochlorophyll per reaction centre[24]. The results are shown in the first 3 columns of Table II.

According to the analysis of Fig. 5, the absorption at 756 nm due to "bound"

bacteriopheophytin is 0.71 times the absorption at 867 nm. This enabled us to calculate the absorbance at 756 nm of reaction centres ($mM^{-1} \cdot cm^{-1}$) which was due to "bound" bacteriopheophytin. This is shown in the 4th column of Table II. With the assumption that the specific extinction coefficient of "bound" bacteriopheophytin at 756 nm is 42 $mM^{-1} \cdot cm^{-1}$ (see above), we were able to estimate the best fitting number of bacteriopheophytin molecules per reaction centre (last column of Table II).

TABLE II

CALCULATION OF THE MOLAR RATIO OF BACTERIOPHEOPHYTIN (Bph) PER REACTION CENTER (RC), STARTING FROM A GIVEN NUMBER OF BACTERIOCHLOROPHYLL (Bchl) MOLECULES PER REACTION CENTRE

The middle and bottom row of the first three columns were taken from ref. 17. The top row of the first three columns was obtained in the same way as in ref. 17. Further details: see text.

Assumed molar ratio Bchl/RC	*Absorbance of RC ($mM^{-1} \cdot cm^{-1}$)*		*Absorbance of RC ($mM^{-1} \cdot cm^{-1}$) due to Bph (756 nm)*	*Molar ratio Bph/RC*
	803 nm	*872 nm*		
2	176	76	54	1
3	264	113	80	2
4	352	152	107	2 or 3

It appears from Table II that, whatever the number of bacteriochlorophyll molecules per reaction centre, the molar ratio of bacteriopheophytin: bacteriochlorophyll is at least 1:2.

We favour the assumption of 2 molecules of bacteriopheophytin per reaction centre, firstly, because of the band splitting of the 535-nm absorption band of bacteriopheophytin at low temperatures (see below) and secondly, because the assumption of 1 molecule of bacteriopheophytin is, according to Table II, only compatible with 2 molecules of bacteriochlorophyll per reaction centre, and this leads to rather low specific extinction coefficients of the reaction centre particle in the near-infrared. Using an argument developed earlier[17], this is not a likely possibility because it would mean that the quantum yields measured for the photooxidation of bacteriochlorophyll P870 (number of electrons transported per number of quanta absorbed) are, in reality, higher than reported[6,17,25] (*i.e.* higher than 1) and would approach 1.5. The reported values were based on an extinction coefficient of 113 $mM^{-1} \cdot cm^{-1}$ for the bacteriochlorophyll P870 absorption maximum. (A lower extinction coefficient means that a certain absorbance change represents a larger number of oxidized bacteriochlorophyll P870 molecules.)

The band splitting of bacteriopheophytin at 535 nm

Absorption spectra[17] and fluorescence excitation spectra (this report) of reaction centre preparations measured at 77 °K revealed a splitting in the 535-nm absorption band of bacteriopheophytin. This could be taken as indicating the presence of 2 molecules of bacteriopheophytin per reaction centre. (One might assume that there are 2 kinds of reaction centre, with slight differences in the position of the 535-nm

absorption band of bacteriopheophytin, but then one would have to explain why there are two kinds and why these two kinds always occur in a 1 : 1 ratio.)

One might assume that the band splitting arises because some of the bacteriopheophytin molecules are subject to pigment–solvent interactions, resulting in a shift of the 535-nm absorption band. Such interactions are known to occur with bacteriochlorophyll in polar solvents[26], where a shift of 1 nm of the far-red absorption band (from 772 to 771 nm) is accompanied by a shift of 34 nm of the "yellow" absorption band (from 575 to 609 nm). However, Goedheer[27] showed that the position of the 535-nm absorption band of bacteriopheophytin in polar solvents does not vary more than the position of the near-infrared absorption band as a result of pigment–solvent interactions, and the near-infrared absorption band of bacteriopheophytin in reaction centre preparations does not show a splitting at 77 °K (ref. 28).

A third possibility is that one electronic transition is responsible for two peaks in the 535-nm absorption band of monomeric bacteriopheophytin in reaction centres at low temperatures. However, such a band splitting is not apparent at room temperature in monomeric bacteriopheophytin (refs 22, 23), in detached bacteriopheophytin (Fig. 4B) and in a bacteriopheophytin–protein complex[15,16], whereas even at room temperature the 535-nm absorption band of "bound" bacteriopheophytin has a truncated appearance, suggesting an unresolved band splitting, as shown in absorption spectra (*e.g.* ref. 28) and fluorescence excitation spectra (Fig. 4A) of reaction centres.

Therefore we favour the hypothesis[29] that the band splitting in the 535-nm absorption band of reaction centre preparations is due to dimer interaction between two bacteriopheophytin molecules. Although circular dichroism measurements[30] with reaction centre preparations in the near-infrared region did not indicate a strong interaction between two bacteriopheophytin molecules, one might assume that such an interaction shows up mainly in the electronic transition responsible for the 535-nm absorption band of bacteriopheophytin.

The number of bacteriochlorophyll and carotenoid molecules per reaction centre

The molar ratio of carotenoid and bacteriochlorophyll in reaction centres could be estimated from Fig. 5 as indicated below. However, there is no certainty yet about the number of bacteriochlorophyll molecules per reaction centre. Early evidence suggested the presence of 3 molecules of bacteriochlorophyll, *viz.* 2 molecules of bacteriochlorophyll P800 and 1 molecule of bacteriochlorophyll P870 per reaction centre[31,17]. However, recent results from ESR experiments[32] favour the idea of 4 bacteriochlorophyll molecules, *viz.* 2 bacteriochlorophyll P800 : 2 bacteriochlorophyll P870. A 1:1 ratio of the bacteriochlorophylls P800 and P870 was also suggested by our finding that the ratio of the areas under the near-infrared absorption bands of the bacteriochlorophyll P800 and bacteriochlorophyll P870, respectively, was 1.2:1 (obtained by analyzing spectra as shown in Fig. 6 on a wave number scale).

Assuming a maximum value of 150 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient of carotenoid[33], we obtain from Fig. 5 a value of 0.43 or 0.58 molecules of carotenoid per reaction centre, assuming that there are 3 or 4 molecules of bacteriochlorophyll per reaction centre, respectively. Since both values are significantly less than 1, some reaction centres apparently did not contain carotenoid at all.

ADDENDUM (Received April 24th, 1973)

After submission of the manuscript, two other papers relating to the numbers of pigment molecules in reaction centres appeared. Feher *et al.*[34], on the basis of electron-nuclear double resonance experiments, concluded that there are 2 molecules of bacteriochlorophyll P870 per reaction centre. Reed and Peters[35], on the basis of extraction experiments, found a molar ratio of 1:2 for bacteriopheophytin: bacteriochlorophyll in reaction centres.

ACKNOWLEDGEMENTS

This investigation was supported by the Netherlands Foundation for Chemical Research (SON), financed by the Netherlands Organization for the Advancement of Pure Research (ZWO). The skillful technical assistance of Miss Wil van der Plas is acknowledged.

REFERENCES

1 Reed, D.W. and Clayton, R. K. (1968) *Biochem. Biophys. Res. Commun.* 30, 471–475
2 Gingras, G. and Jolchine, G. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 1, pp. 209–216, Laupp Jr., Tübingen
3 Thornber, J. P., Olson, J. M., Williams, D. M. and Clayton, M. L. (1969) *Biochim. Biophys. Acta* 172, 351–354
4 Jolchine, G., Reiss-Husson, F. and Kamen, M. D. (1969) *Proc. Natl Acad. Sci. U.S.* 64, 650–653
5 Segen, B. J. and Gibson, K. D. (1971) *J. Bacteriol.* 105, 701–709
6 Slooten, L. (1972) *Biochim. Biophys. Acta* 256, 452–466
7 Slooten, L. (1972) *Biochim. Biophys. Acta* 275, 208–218
8 Reiss-Husson, F. and Jolchine, G. (1972) *Biochim. Biophys. Acta* 256, 440–451
9 Smith Jr., W. R., Sybesma, C. and Dus, K. (1972) *Biochim. Biophys. Acta* 267, 609–615
10 Noël, H., Van Der Rest, M. and Gingras, G. (1972) *Biochim. Biophys. Acta* 275, 219–230.
11 Duysens, L. N. M. and Amesz, J. (1957) *Biochim. Biophys. Acta* 24, 19–26
12 Vos, J. C. de (1952) Thesis, Free University of Amsterdam
13 Duysens, L. N. M. (1952) Thesis, University of Utrecht
14 Beugeling, T., Slooten, L. and Barelds-van de Beek, P. G. M. M. (1972) *Biochim. Biophys. Acta* 283, 328–333
15 Kim, Y. D. and Ke, B. (1972) *Arch. Biochem. Biophys.* 140, 341–353
16 Ghosh, A. K. and Olson, J. M. (1968) *Biochim. Biophys. Acta* 162, 135–148
17 Clayton, R. K., Fleming, H. and Szuts, E. Z. (1972) *Biophys. J.* 12, 46–63
18 Reed, D. W., Raveed, D. and Israel, H. W. (1970) *Biochim. Biophys. Acta* 223, 281–291
19 Clayton, R. K. and Sistrom, W. R. (1966) *Photochem. Photobiol.* 5, 661–668
20 Wang, R. T. and Clayton, R. K. (1971) *Photochem. Photobiol.* 13, 215–224
21 Ebrey, T. G. (1971) *Biochim. Biophys. Acta* 253, 385–395
22 French, C. S. (1940) *J. Gen. Physiol.* 23, 483–494
23 Smith, J. H. C. and Benitez, A. (1955) in *Modern Methods in Plant Analysis* (Paech, K. and Tracey, M. V., eds), Vol. 4, pp. 142–196, Springer-Verlag, Berlin
24 Reed, D. W. and Ke, B. (1972) *Biophys. J. Soc. Abstr.* 12, 89a
25 Bolton, J. R., Clayton, R. K. and Reed, D. W. (1969) *Photochem. Photobiol.* 9, 209–218
26 Weigl, J. W. (1953) *J. Am. Chem. Soc.* 75, 999–1000
27 Goedheer, J. C. (1958) *Biochim. Biophys. Acta* 27, 478–490
28 Feher, G. (1971) *Photochem. Photobiol.* 14, 373–387
29 Clayton, R. K. and Straley, S. C. (1972) *Biophys. J.* 12, 1221–1234

30 Sauer K., Dratz, E. A. and Coyne, L. (1968) *Proc. Natl Acad. Sci. U.S.* 61, 17-24
31 Clayton, R. K. (1966) *Photochem. Photobiol.* 5, 669–675
32 McElroy, J. D., Feher, G. and Mauzerall, D. C. (1972) *Biochim. Biophys. Acta* 267, 363–374
33 Goodwin, T. W., Land, D. G. and Sissins, M. E. (1956) *Biochem. J.* 64, 486–492
34 Feher, G., Hoff, A. J., Isaacson, R. A. and McElroy, J. D. (1973) *Biophys. Soc. Abstr.* 13, 61a
35 Reed, D. W. and Peters, G. A. (1972) *J. Biol. Chem.* 247, 7148–7152