BBA 46227

ANOMALOUS ENERGY TRANSFER BEHAVIOUR OF LIGHT ABSORBED BY BACTERIOCHLOROPHYLL IN SEVERAL PHOTOSYNTHETIC BACTERIA

THOMAS G. EBREY

Department of Biological Sciences, 757 Schermerhorn Hall, Columbia University, New York, N.Y. 10027 (U.S.A.)

(Received June 14th, 1971)

SUMMARY

Energy transfer has been investigated in chromatophores of the blue-green mutant of *Rhodopseudomonas spheroides*, wildtype *Rps. spheroides* and *Rhodospirillum rubrum*. Measurements of the fluorescence polarization and fluorescence excitation spectra suggest anomalies in the energy transfer behavior of light absorbed by bacteriochlorophyll. Light absorbed either on the red edge of the first excited state or by the second excited state is not transferred with as high an efficiency as light absorbed by the main part of the first excited state. Furthermore, fluorescence action spectrum measurements suggest that light absorbed by the second excited state is less effective in exciting fluorescence than light absorbed by the main part of the first excited state.

INTRODUCTION

Bacteriochlorophyll serves at least two different functions in converting light to chemical energy in photosynthetic bacteria. A small fraction of the bacteriochlorophyll, from 0.5 to 5 %, participates actively in the photochemistry of photosynthesis. The bulk of the remaining bacteriochlorophyll acts as an antenna to absorb light quanta for the photochemical reaction center. These absorbed quanta are delivered with high efficiency to the reaction center, presumably by a Förster-type energy transfer mechanism[1,2].

In photosynthetic bacteria, the visible and infrared absorption spectrum can be attributed to (1) the bulk bacteriochlorophyll's lowest excited state, (2) its second excited state, (3) accessory pigments such as carotenoids and (4) reaction center bacteriochlorophyll. Bulk light-harvesting bacteriochlorophyll molecules can exist in several forms marked by the shifting by different amounts of their longest wavelength electronic transition. For example, in wild type *Rhodopseudomonas spheroides*, there are at least three types of light harvesting bacteriochlorophyll, called B800, B850 and B870. The second excited state of all three forms of bacteriochlorophyll is at 590 nm. Most photosynthetic bacteria also contain carotenoids which can act as light harvesting molecules. DUYSENS and others[1,3–6] found that light absorbed by the carotenoids and the shorter wavelength bands of bacteriochlorophyll can sensitize the fluorescence from the longest wavelength band. This demonstrated that

energy can be transferred to the long-wavelength band from these other bands. If the efficiency of energy transfer were constant regardless of the wavelength of light used, then the action spectrum for either bacteriochlorophyll fluorescence or some reaction center event such as a cytochrome oxidation would match the absorption spectrum of the bacterium. Where this has not been observed, several different explanations have been offered[1,3,4]. It has been proposed that there are two or more pools of light harvesting bacteriochlorophyll, each pool serving the reaction centers with different efficiencies or having different fluorescent yields. It has also been suggested that there is more than one kind of reaction center connected to different pools of light harvesting bacteriochlorophyll. Most often it is assumed that such an action spectrum deviates from the absorption spectrum because the efficiency of energy transfer from any other molecule to the lowest excited state of the bulk bacteriochlorophyll is less than 100 %. However, some phenomena may require even more complicated explanations. WEBER AND SHINITZKY[7] have recently challenged the assumption that the efficiency of energy transfer between molecules in a homogeneous solution is independent of wavelength. In their studies of fluorescence depolarization of pure solutions of aromatic molecules at high concentrations, they found that light which is normally absorbed and transferred from one molecule to another in solution is transferred with a lesser efficiency if the light is absorbed on the red edge of the absorption band of the molecule. If this phenomenon held for bacteriochlorophyll in its *in vivo* environment, then one would expect the efficiency of energy transfer for light absorbed by the longest wavelenth absorption band to fall off on the red edge of that band.

We have therefore examined energy transfer in chromatophores of photosynthetic bacteria looking especially for discrepancies in energy transfer between like bacteriochlorophyll molecules. The principle technique available to study energy transfer between like molecules is the depolarization of the fluorescence from the molecules. The second method we used is to compare the fluorescence action spectrum and the absorption spectrum of the chromatophores of the bacteria studied. This method not only can show the relative efficiency of energy transfer between different kinds of molecules (*e.g.* carotenoids to B870 and B800 to B870), but also can show whether light absorbed by higher excited states of a molecule is transferred to the lowest excited state of the same molecule with the expected 100 % efficiency.

METHODS AND MATERIALS

All measurements were made with chromatophores of either *Rps. spheroides* strain 2.4.1/L_2-26 (the carotenoid-less, blue-green mutant) *Rps. spheroides* strain 2.4.1 (wild type) and *Rhodospirillum rubrum* strain 1.1.1 (wild type). Chromatophores were prepared according to the method of SISTROM AND CLAYTON[8]. All absorption spectra were recorded on a Cary-14R spectrophotometer. The absorption spectra of the chromatophores differed slightly from culture to culture, especially with respect to the size of the B800 band and the slight amounts of pheophytin (bands at 535 and 760 nm) and a bacteriochlorophyll degradation product (band at 680 nm) present. Most of our effort was concentrated on L_2-26 because it has a simpler absorption spectrum than the wild type, having only one principle light harvesting pigment, B860.

Fluorescence polarization spectra

The depolarization of B860 fluorescence is the best method for measuring energy transfer between indentical B860 molecules. The technique has been discussed in several places[7,9,10]. The polarization of the fluorescence is defined as $p = I_v^{\parallel} - I_v^{\perp}/I_v^{\parallel} + I_v^{\perp}$, where $I_v^{\parallel}$ is the component of the fluorescence polarized parallel to the polarization of a vertically polarized exciting light and $I_v^{\perp}$ is the component of the fluorescence perpendicular to it. Both $I_v^{\parallel}$ and $I_v^{\perp}$ were measured at right angles to the exciting light. All theories predict that the extreme values of the polarization of the fluorescence p, which ranges in bacteriochlorophyll from 0.43 for the infrared band to −0.23 for the 590-nm band wil be proportionally reduced as the number of energy transfers increases.

The experimental arrangement for exciting and detecting the fluorescence was similar to one used previously[9]. Exciting light from a 650-W Sylvania Sun Gun was first focused on the slit of a 500-nm Bausch and Lomb monochromator blazed at 750 nm. The light from the exit slit of the monochromator was rendered parallel and passed through either a Baird–Atomic or Jena interference filter so that it was doubly monochromatic and then was focused on the sample cuvette. The exciting light was vertically polarized with a type HR (Polaroid Land) polarizer for wavelengths greater than 750 nm and a type HN-32 polarizer for wavelengths less than 750 nm. The band width of the monochromator was 6.6 nm and the band widths of the interference filters were about 12 nm. The fluorescence was detected at right angles to the incident beam with a cooled RCA 7102 photomultiplier. The axis of polarization of the fluorescence was selected by a type HR polarizer which could be rotated so that its direction of polarization was either perpendicular or parallel to the electric vector of the exciting light. For exciting wavelengths above 845 nm, a 1-cm thick solution of *R. rubrum* chromatophores (absorbance greater than 4) and a Baird Atomic 952 nm interference filter were used between the cuvette and the photomultiplier tube to isolate the fluorescence from any scattered exciting light. This combination of filters blocked all scattered light with exciting wavelengths up to 907 nm when a dilutal milk blank was used. This procedure was checked carefully in every experimental run because any scattered light getting through the filters could be polarized, thus invalidating a fluorescence polarization measurement. For wavelengths below 875 nm two Kodak Wratten 87C cut off filters and either a solution of *Rps. spheroides* 2.4.1 chromatophores (A approx. 2.5) or a Baird–Atomic 887-nm interference filter were used. Checks made with several interference filters indicated that the polarization of fluorescence was approximately constant across the emission band.

The fluorescence polarization measurements depend only on the ratio of fluorescence intensities. Therefore to obtain sufficient fluorescence intensity, especially when exciting on the red edge of the absorption band, it was possible to use dense samples of L_2-26 (A about 1.2 at 860 nm). Of course on the red edge of the 860 nm band, the absorbances will be much less than this. For wavelengths other than those on the red edge, the fluorescence polarization spectrum was found to be identical with samples of lower absorbances at 860 nm. Since horizontally polarized exciting light should not give polarized fluorescence[11], with each of the bacteria the ratio of $I_H^{\parallel}$ to $I_H^{\perp}$ was measured at every wavelength to correct the fluorescence intensity measurement for any bias in the detecting system. A very small correction, which was found to be independent of exciting wavelength, was applied to all the data.

Fluorescence action spectra

The fluorescence excitation spectra were measured with the same apparatus used to measure the fluorescence polarization spectra. A special problem complicates the determination of fluorescence action spectra of rigid fluorescening systems. Polarizational biases in the exciting and detecting systems become important when fluorescent molecules cannot rotate to depolarize the fluorescence between the absorption of the light and its emission. Errors in the fluorescence action spectrum can then result because with a polarized exciting light, the ratio of the intensity of any polarized component of the emission to the intensity of the total fluorescence depends on which absorption band is excited. Thus, when using an exciting and detection system with a polarizational bias, the percent of the total fluorescence intensity measured will vary from one absorption band to another. These errors are largest when the fluorescence is strongly polarized and when a fluorescence action spectrum is being recorded which involves two perpendicular transitions such as those at 590 nm and 860 nm in bacteriochlorophyll. These difficulties, almost impossible to estimate or correct for, can be eliminated by purposely exciting with totally polarized light and by measuring both polarization components of the emitted light. In our experiments, the exciting light intensity at each wavelength was adjusted until the amount of fluorescence was approximately equal to some constant value. With this light intensity set, the polarizer was rotated to measure $I_v^{\parallel}$ and $I_v^{\perp}$. WEBER[12] has shown that $I^{\text{total}} = 2I_v^{\perp} + I_v^{\parallel}$. This value of I^{total} was then divided by the (relative) number of incident quanta at that wavelength to give the fluorescence per incident quanta. Since, at the exciting light intensities used, the fluorescence was very close to linear with respect to the incident light intensity, this method determined 1/(the number of quanta needed for a constant amount of fluorescence). This number *versus* the exciting wavelength is the action spectrum.

In our action spectra measurements, the absolute exciting light intensity at 860 nm was about 100 ergs/cm^2 per sec. The relative number of quanta incident at each wavelength was measured with either an EG and G Model 580/585 Radiometer or a calibrated RCA 7102 photomultiplier. The values at some wavelengths were checked at high light intensities with an ESCO spectroradiometer.

The action spectra obtained by the above procedure was compared with the absorption spectrum plotted as percent absorption. All samples used in determining action spectra had absorbances at the infrared maximum of less than 0.6. Checks made using samples with $A_{860\text{ nm}}$ of approx. 0.3 gave the same results. The amount of nonspecific absorbance at 1000 nm (usually 0.0 to 0.01 A units) was substracted from all recorded absorption spectra. Measurements in the visible part of the absorption spectrum using the opal glass technique gave essentially identical absorption spectra. Hence, in these chromatophore preparations, scattering made only a very minor contribution to the measured absorption spectrum.

RESULTS

Fig. 1 shows the absorption spectrum and the fluorescence polarization spectrum in the region from 450 to 907 nm of chromatophores of L_2-26. The fluorescence polarization spectrum in the region 500–850 nm had been reported in a previous paper[9], and the data here agree with the previous results which were determined

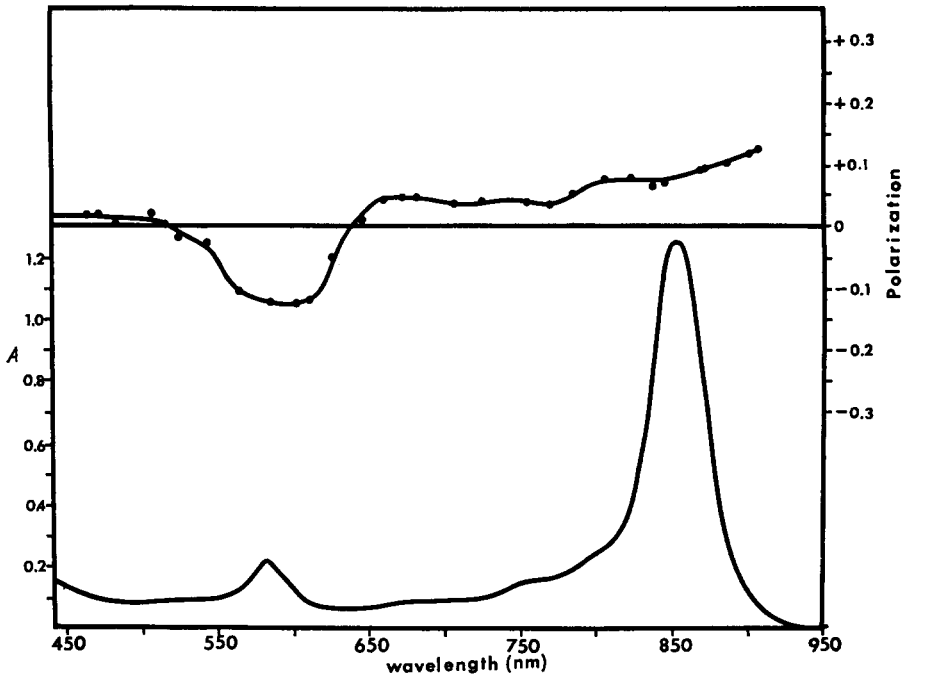

Fig. 1. Fluorescence polarization spectrum of chromatophores of *Rps. spheroides* (2.4.1/$L_2$26). At 590 nm, the absolute value of p, the fluorescence polarization, is approximately 3 times larger than would be predicted. The predicted value of p is from a comparison with the reduction of p for the longest wavelength transition of bacteriochlorophyll in solution to the value of p at 800–850 nm. Past 850 nm, the value of p begins to rise.

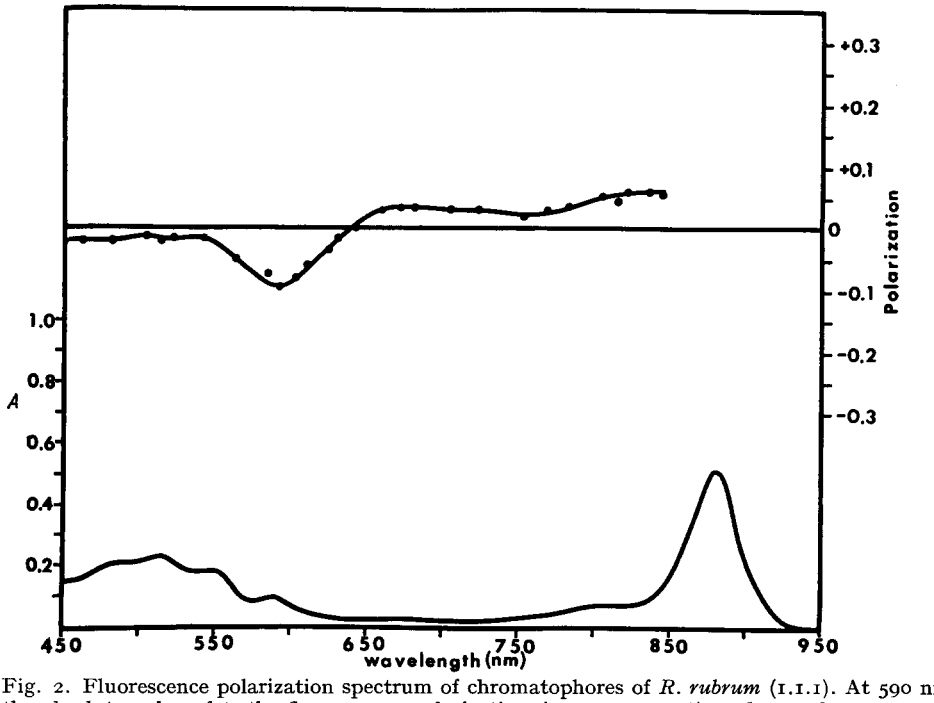

Fig. 2. Fluorescence polarization spectrum of chromatophores of *R. rubrum* (1.1.1). At 590 nm, the absolute value of p, the fluorescence polarization, is approx. 2.5 times larger than expected.

with a different instrument. A peculiar feature of the fluorescence polarization spectrum is the large amount of polarized fluorescence from light absorbed by the second excited state of bacteriochlorophyll at 590 nm. Although the value of p at 590 nm is somewhat reduced from the value of p for this transition of bacteriochlorophyll in solution ($p = -0.23$) (ref. 9), nevertheless, it is much greater than the value expected from a proportional reduction of p (or $g(p) = (1/p - 1/3)^{-1}$ see ref. 10) at all wavelengths. This would seem to indicate that light absorbed by the second excited state of B860 is not transferred with high efficiency between B860 molecules.

Figs. 2 and 3 show the fluorescence polarization spectra of *R. rubrum* and wild type *Rps. spheroides*. As can be seen, our results for *R. rubrum* are similar to those for L_2-26. This contradicts the fluorescence polarization spectrum given for *R. rubrum* by GOEDHEER[13] who found less polarization when exciting at 590 nm than was found here. Although at 590 nm wild type *Rps. spheroides* also emits more polarized fluorescence than expected, the discrepancy is not as marked as in the other two bacteria.

The fluorescence polarization data from 845 to 907 nm are displayed in both Fig. 1 and Table I. The amount by which the fluorescence is polarized rises from $p = 0.071 \pm 0.008$ at 845 nm to $p = 0.125 \pm 0.019$ at 907 nm. It was technically impossible to extend the fluorescence measurements beyond this last wavelength because of low fluorescence intensities and scattered light. Although it is not possible to know if p would attain its theoretical maximum value of 0.5, there is some evidence that it does not. Our values for the rate of rise of p *versus* wavelength do not increase as quickly as those of WEBER AND SHINITZKY[7].

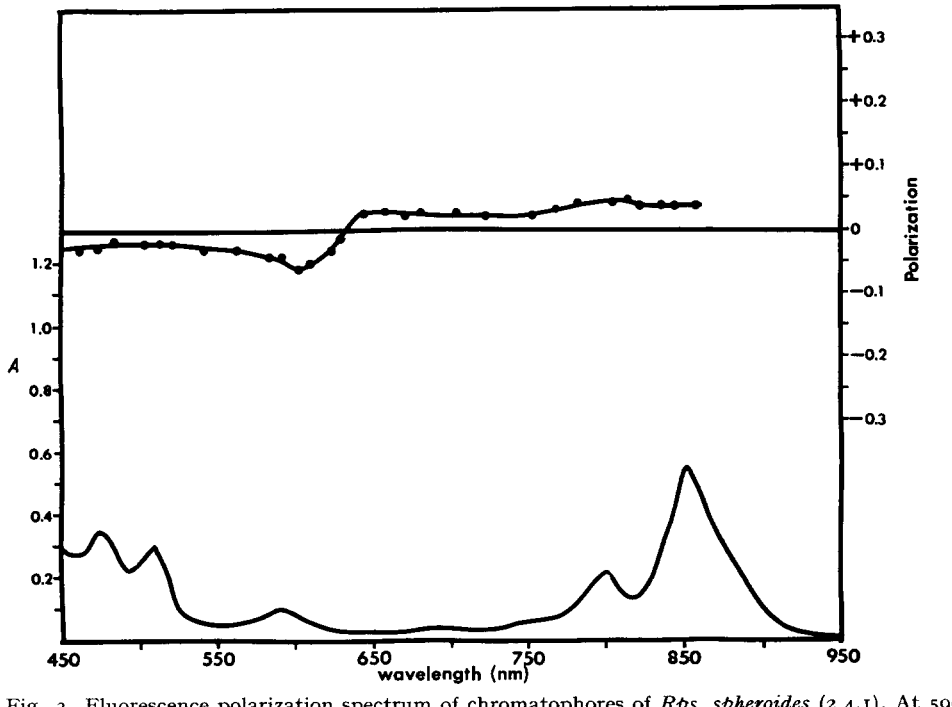

Fig. 3. Fluorescence polarization spectrum of chromatophores of *Rps. spheroides* (2.4.1). At 590 nm, the absolute value of p, the fluorescence polarization, is approx. 2 times larger than expected.

Fig. 4 shows the action spectrum for the excitation of B860 fluorescence measured as the total amount of fluorescence greater than about 900 nm. As can be seen, light absorbed by the second excited state of bacteriochlorophyll, at 590 nm, is less effective in eliciting bacteriochlorophyll fluorescence than light absorbed by the first excited state. Figs. 5 and 6 show that a similar effect can be seen in chromatophores of *R. rubrum* and *Rps. spheroides* strain 2.4.1.

One question which might be asked here is, could the chromatophores contain some inert absorbing substance which raises the absorption in the visible region without being able to transfer energy to the bacteriochlorophyll? Such an explanation is

TABLE I

FLUORESCENCE POLARIZATION OF CHROMATOPHORES OF *Rps. spheroides* (2.4.1/L_2-26)

Wavelength (nm)	*Polarization* $\pm$ *S.D.*
845	0.071 $\pm$ 0.008
869	0.091 $\pm$ 0.013
871	0.097 $\pm$ 0.014
886	0.106 $\pm$ 0.012
901	0.119 $\pm$ 0.016
907	0.125 $\pm$ 0.017

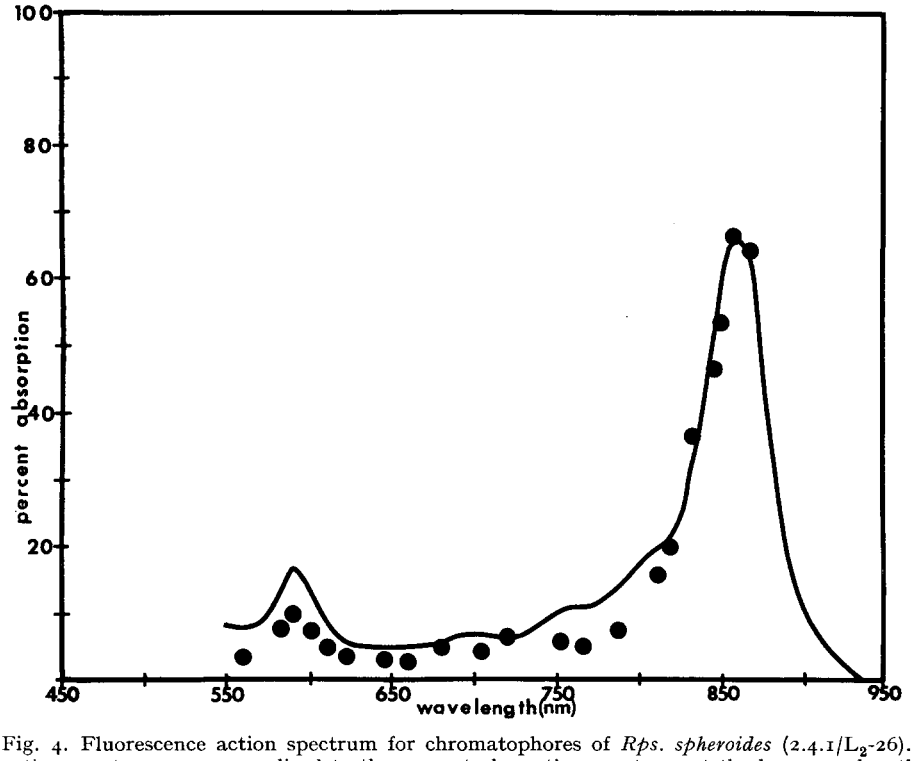

Fig. 4. Fluorescence action spectrum for chromatophores of *Rps. spheroides* (2.4.1/L_2-26). The action spectrum was normalized to the percent absorption spectrum at the long-wavelength absorption band. The ratio of action to percent absorption at 590 nm varied from 0.5 to 0.65 in different action spectrum determinations.

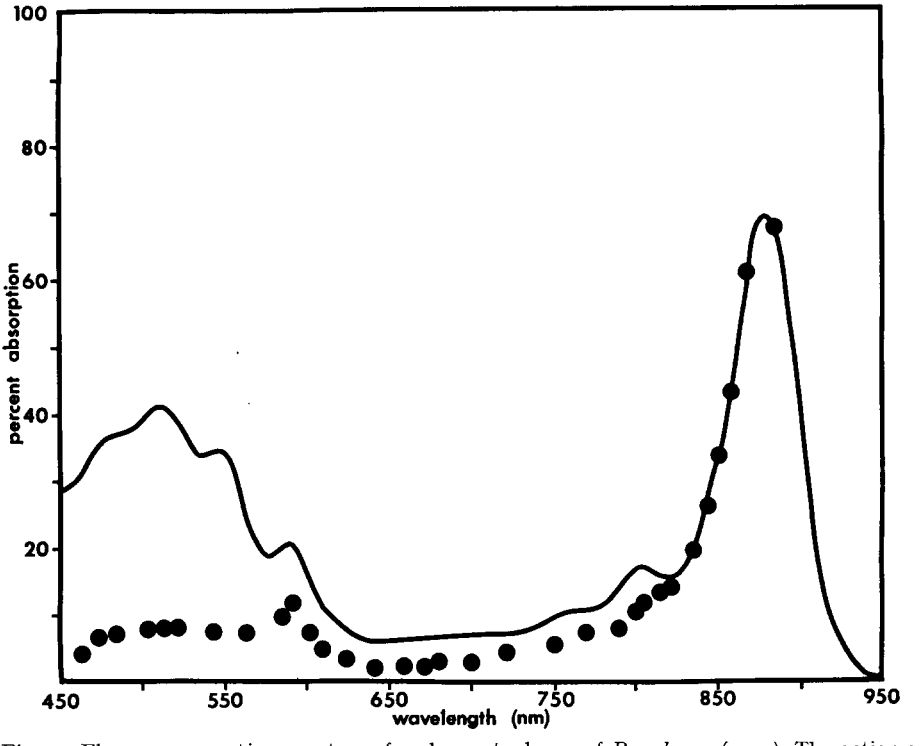

Fig. 5. Fluorescence action spectrum for chromatophores of *R. rubrum* (1.1.1). The action spectrum was normalized to the percent absorption spectrum at the long-wavelength absorption band. The ratio of action to percent absorption varied from 0.5 to 0.6 in different action spectrum determinations.

unlikely unless this hypothetical substance had an absorption peak just at 590 nm rather than at some other place in this general region of the absorption spectrum. Otherwise, the discrepancy between the absorption spectrum and the action spectrum at the 590-nm peak would still be large.

A second question is, could some of these effects be due to absorption of 590-nm light by the carotenoids? While the use of the carotenoidless mutant L_2-26 partially excludes this, it is possible that carotenoids might effect the results seen in the 590-nm region in the wild-type bacteria. However, even in these bacteria, a computer reconstruction of the absorption spectrum indicates little contribution of the carotenoids to the absorption at 590 nm.

DISCUSSION

These measurements of intra- and intermolecular energy transfer in chromatophores of photosynthetic bacteria tentatively suggest that (1) energy absorbed on the red edge of the first excited state's absorption band is not transferred with as high efficiency as energy absorbed in the main part of the absorption band and (2) some portion of the light energy absorbed by the second excited state is not transferred from one bacteriochlorophyll molecule to another and (3) light absorbed by the second

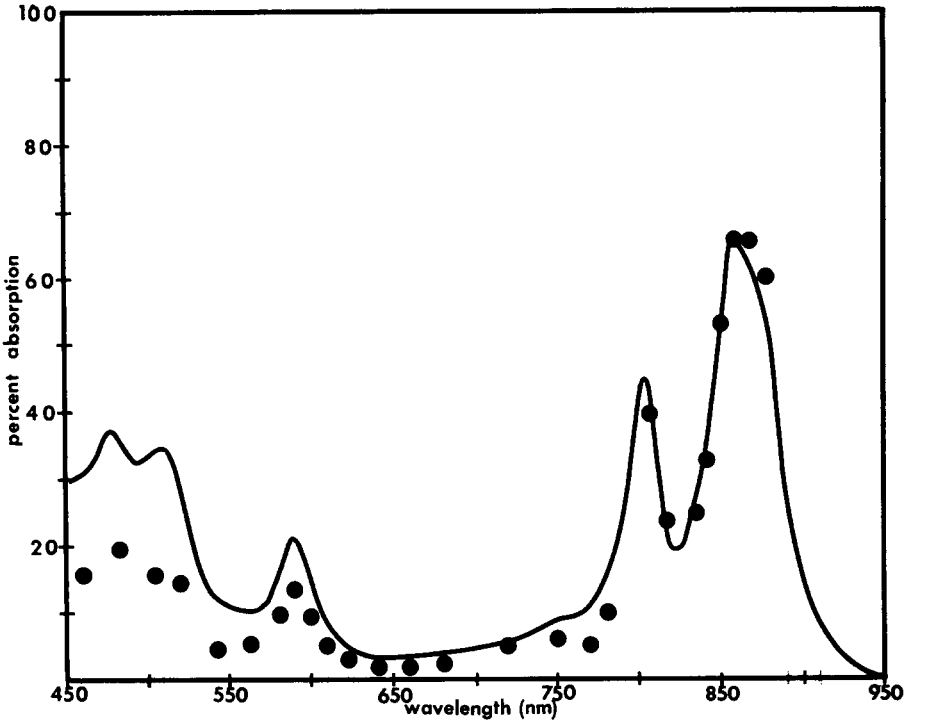

Fig. 6. Fluorescence action spectrum for chromatophores of *Rps. spheroides* (2.4.1). The action spectrum was normalized to the percent absorption spectrum at the long-wavelength absorption bands. The ratio of action to percent absorption at 590 nm varied from 0.6 to 0.7 in different action spectrum determinations.

excited state of bacteriochlorophyll is less effective in exciting bacteriochlorophyll fluorescence than light absorbed by the first excited state.

Other investigators have published several kinds of action spectra for the bacteria studied here. These suggest that, in general, the fluorescence action spectrum matches the action spectrum for such reaction center events as P870 and cytochrome oxidation[3,4,13]. However, many previous action spectra are difficult to compare with our fluorescence excitation spectra because they do not cover both the visible and infrared.

Concerning L_2-26 chromatophores, the fluorescence action spectrum for 550–850 nm published by CLAYTON AND SISTROM[14] is similar to that reported here. However, the authors attributed the difference between the absorption and the fluorescence excitation spectrum to the effect of light scattering on the absorption spectrum. Here we do not believe that our absorption spectrum is significantly distorted by scattering because the chromatophores were of good optical quality and the absorption spectrum did not change when measured by the opal glass technique. These authors also proposed a special hypothesis to explain the extreme lack of agreement between their fluorescence action spectrum and the absorption spectrum at 800 nm. This explanation, based on a close coupling of the 800-nm state to the P860 state of the reaction center, may perhaps also explain the fluorescence action spectrum data at 800 nm in *R. rubrum*.

For wild type *Rps. spheroides*, a fluorescence excitation spectrum has been reported by AMESZ[6]. However, it is difficult to compare results since he gave only a few data points and his absorption spectrum differed from ours, probably because of culture conditions.

Several investigators have studied *R. rubrum*. Both CLAYTON[15] and MANTEN[16] have measured the action spectrum for phototaxis, while FORK AND GOEDHEER[17] have measured the action spectrum for the inhibition of photorespiration. In none of these measurements did the amount of action at 590 nm equal the absorption spectrum when the action spectra were normalized at 880 nm. However, these measurements are inconsistent among themselves on the actual shape of the action spectrum. Recently KIHARA AND MCCRAY[19] reported a red edge effect for cytochrome oxidation in *R. rubrum*.

There seems to be no straightforward explanation for the results reported in the first paragraph of this section. It might be thought that the fluorescence polarization data at 590 nm results from the existence of approximately equal amounts of two types of bulk bacteriochlorophyll molecules (*e.g* B860 and B'860 in L_2-26), one type emitting polarized fluorescence when excited at 590 nm and the other type not. However, such an explanation would require that there be a proportional reduction in the amount of polarized fluorescence at both the 590 and 860 bands. The data shows that the reduction in the amount of fluorescence polarization at these wavelengths is not proportional to the values of p for bacteriochlorophyll in solution.

Likewise it might be thought that the difference between the fluorescence action spectrum and absorption spectrum is due to two types of bacteriochlorophyl present in approximately equal amounts, one type fluorescent and the other not. Again however, this cannot explain the relative difference in the fluorescence excitation spectrum for light absorbed by the 590 and 860 bands. These hypotheses are inadequate whether or not the two pigment systems referred to serve the same or different reaction centers.

In a few organic molecules it has been found that, contrary to expectation, light absorbed by the second excited state is less effective in producing fluorescence than light absorbed by the lowest excited state[18]. However, the explanation usually given for this phenomenon, *i.e.* other processes such as photochemistry competing with radiationless transistions to the first excited state, while it may be offered as an explanation for the action spectra results, cannot explain the fluorescence polarization results. This is because the fluorescence polarization technique measures only that light absorbed by the second excited state which does go to the first excited state (and then fluoresces).

It appears that only an unconventional mechanism can explain our results. WEBER AND SHINITZKY[7] have hypothesized the existence of two closely lying excited states contributing to the long-wavelength absorption band, with a fluorescent, non-transferring state absorbing principally at the long-wavelength edge of the band. A possible model, which has some similarity to their line of thinking, is one which postulates the division of each excited state of bacteriochlorophyll (590 and 860) into two closely lying states. The lower 860 state is non-transferring but the upper state, responsible for most of the absorption in this region is transferring. Radiationless de-excitation between the two closely lying states of the 590 band is (weakly) allowed, but radiationless de-excitation between the lowest state of 590 and the upper state

of B860 and between the upper and lower states of B860 are forbidden. If the lower 860 state had a smaller fluorescence yield than the upper state, all the fluorescence action and polarization data could be explained. A second type of hypothesis involves two classes of bacteriochlorophyll molecules with different ratios of extinction at 590 nm to extinction at 860 nm. From these two populations, one would be responsible for a large percentage of the absorption at 590 nm, would emit highly polarized fluorescence, and if slightly red-shifted in absorption, would be responsible for the large amount of polarized fluorescence on the red-edge of the absorption bands. The other population would have little fluorescence action at 590 nm but would be responsible for most of the fluorescence action at 860 nm. Its fluorescence would be highly depolarized. Although there is no evidence for these postulated bacteriochlorophyll classes *in vivo*, monomers and dimers of bacteriochlorophyll as seen in solution might have the required absorption spectra[20]. It is certainly possible that other models may explain our data.

The data presented in this paper suggest that light absorbed by the second excited state of bacteriochlorophyll and the red edge of the first excited state are not involved with the same energy transfer processes as light absorbed by the main part of the first excited state. It is unclear whether the energy which does not go to the B860 state is involved in a second photochemical process or whether this energy is just lost via radiationless de-excitation. In either case, the widely held assumption that light absorbed by different excited states of the same bacteriochlorophyll molecule have identical energy transferring properties is probably incorrect.

REFERENCES

1 L. M. N. DUYSENS, *Prog. Biophys. Mol. Biol.*, 14 (1964) 1.
2 R. K. CLAYTON, *Brookhaven Symp. Biol.*, 19 (1967) 62.
3 L. M. N. DUYSENS, Thesis, Utrecht, 1952.
4 J. AMESZ AND W. J. VREDENBERG, *Biochim. Biophys. Acta*, 126 (1966) 254.
5 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 35 (1959) 1.
6 J. AMESZ, *Biochim. Biophys. Acta*, 66 (1963) 22
7 G. WEBER AND M. SHINITZKY, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 823.
8 W. R. SISTROM AND R. K. CLAYTON, *Biochim. Biophys. Acta*, 88 (1964) 61.
9 T. G. EBREY AND R. K. CLAYTON, *Photochem. Photobiol.*, 10 (1969) 109.
10 R. S. KNOX, *Physica*, 39 (1968) 381.
11 G. WEBER, *J. Opt. Soc. Am.*, 46 (1956) 962.
12 G. WEBER, *Biochemistry*, 51 (1952) 145.
13 J. C. GOEDHEER, Thesis, Utrecht, 1957.
14 R. K. CLAYTON AND W. R. SISTROM, *Photochem. Photobiol.*, 5 (1966) 661.
15 R. K. CLAYTON, *Arch. Mikrobiol.*, 19 (1957) 107.
16 A. MANTEN, *Antonie van Leeuwenhoek*, 14 (1948) 65.
17 D. FORK AND J. C. GOEDHEER, *Biochim. Biophys. Acta*, 79 (1964) 249.
18 W. A. NOYES, W. A. MULAC AND D. A. HARTER, *J. Chem. Phys.*, 44 (1966) 2100.
19 T. KIHARA AND J. MCCRAY, *Abstr. Biophys. Soc.*, 11 (1971) 33.
20 K. SAUER, J. SMITH AND A. SCHULTZ, *J. Am. Chem. Soc.*, 88 (1966) 12